Mathematical Modeling of Cell Proliferation:

Stem Cell Regulation in Hemopoiesis

Volume I

Model Description, Irradiation, Erythropoietic Stimulation

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Dedicated to
Rudolf Gross,
Hematologist and Admirer
of Mathematics

It is a bold venture to attempt to marry mathematics, the purest of sciences, with biology, which has all too many "unknowns". Nevertheless, such an attempt is well justified since it brings much desired clarity into biological thinking, and it underlines the need for hardening biological data. Model building is an essential part of scientific thinking, as long as the experiments can provide the tools with which to open the iridescent shells of ideas, to look for the pearls of truth inside. This book is a worthy step in the right direction.

L. G. Lajtha

PREFACE

The experimental investigation of hemopoietic stem cells experienced its decisive stimulation in 1961 when Till and McCulloch developed their assay for colony forming units in the spleens of mice (CFU-S). Within the next decade further assays became available to measure morphologically unidentifiable erythropoietic (ERC, BFU-E, CFU-E) and granulopoietic (CFU-GM) progenitor cells. Since then, the properties of hemopoietic stem cells and their progeny have been studied extensively and their reaction measured to various types of stimulation and suppression.

However, until now, a generally accepted interpretation of all these data is missing. Given this situation it seems challenging to look for the "common structure" which should — theoretically — be inherent in the experimental findings. What are the regulatory rules and laws that govern hemopoiesis? Certainly one cannot be successful if one only looks at a very few experiments. To obtain a complete picture of the capabilities of the system, one has to consider many different experimental situations. To analyze all these data is, however, a time-consuming task which has additional problems: the available measurements are quite heterogeneous and in part contradictory and different strains of animals and different experimental protocols have to be compared.

To provide a practicable basis for the analysis, we decided to restrict our attention to the most extensively investigated hemopoietic stresses. For each type of experiment we asked a competent experimentalist to review the knowledge and the data for that special area. Complementary to these reviews we wrote theoretical chapters in which we tried to interpret these findings using a mathematical model of stem cell regulation.

Thus, this volume combines three distinct contributions: summaries of the "state of the art" of experimental knowledge on stem cell regulation; an extensive survey of the available data; and finally a unique theoretical concept (paradigma) for the interpretation of these data. The last point, of course, is, in many ways, the most critical one. The proposed model does not solve the questions on hemopoietic regulation. It is designed as a preliminary attempt to a comprehensive way of thinking. If the model serves as a rational basis for the discussion of hemopoietic stem cell regulation then we feel our effort will have been worthwhile.

One of us (H.-Erich Wichmann) started mathematical modeling of hemopoiesis in 1974. The other (Markus Loeffler) joined-in in 1977, when we began our common work on stem cells, out of which Loeffler's dissertation thesis evolved. Discussing our first considerations with Laszlo Lajtha (Manchester, England) in 1979 we received harsh criticism ("Modelling is like sitting in a chair and smoking pipes") but also stimulation to continue. At that time we had a model which was restricted to stem cells and erythropoietic progenitor cells. Martin Murphy (Dayton, Ohio) and Francis Monette (Boston, Mass.) made us aware of the fact, that the model inadequately described erythropoietic suppression. They convinced us with a lot of material (in part unpublished). Finally we found that the next "natural" step towards generalization of the model, namely the inclusion of granulopoiesis, could resolve most of the problems in a surprisingly simple way.

The idea to write and edit this book was initially suggested in 1980 at a breakfast talk with Jim Okunewick (Pittsburgh, Pa.). Its realization kept us busy for 3 years. During this time the model product derived through a common set of principles. We feel incapable to separate the origin of the ideas between us. This may become visible in the rather arbitrary arrangement of authorships in the model chapters.

The preliminary form of this work was presented in Baltimore (ISEH-Meeting, August 1982), in Manchester (Paterson Laboratories, December 1982), and in Lausanne (European Stem Cell Club, April 1983). The participants of these sessions supported us with valuable suggestions as to how we might improve the model description. Their comments entered into this volume.

Many persons have been involved in the completion of these two volumes. Most of all we have to thank the authors of the chapters dealing with experimental data. They have not only reviewed the available literature but also incorporated new measurements from their laboratories. Here especially Hans Seidel and Ludwika Kreja (Ulm), Francis Monette, Roy Ziegelstein, and Michael Hunter (Boston) must be mentioned. They performed multiple experiments on the combination of irradiation with anemia and hypertransfusion especially for publication in this book. Martin Murphy (Dayton) and Brian Lord (Manchester) also contributed a lot of unpublished material on hypoxia and ex-hypoxia.

We are especially indebted to Chris Dunn (Houston) who read all our chapters patiently. He made numerous helpful suggestions, made us aware of many mistakes and weak points, and he tried his best to improve our often ineloquent English. We could not have found a better adviser. Nevertheless, we are of course exclusively responsible for all mistakes, inconsistencies, and items which might be somewhat less than clear.

We were lucky to have had the help of eight enthusiastic collaborators. First of all, Peter Herkenrath has to be mentioned who worked with us for 2 years and contributed a lot of essential improvements. Christopher Wesselborg and Alexander Jarczyk spent many efforts to enhance our computer software for the model calculations and their graphical presentation; the curves and figures in the model chapters are their work. Erich Backes and Jochen Schmidt checked and completed the data collection and the references. They also helped in typing the manuscripts, together with Stephan Gontard. Wilfried Weiss and Guenther Michel implemented in the computer our data base of more than 800 published and unpublished experimental time curves. Finally we are thankful to the directors of our clinic for internal medicine, Rudolf Gross and Volker Diehl. They gave us an "ecological niche" to develop theory in an environment which is completely clinical and experimental. Whether this niche was successfully seeded by a "scientific stem cell" remains to be seen.

H.-Erich Wichmann
Markus Loeffler
Cologne, March 1984

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Chapter 1

SOLVED AND UNSOLVED PROBLEMS IN THE REGULATION OF HEMOPOIESIS

Brian I. Lord

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I. ABSTRACT

A number of the current problems in the basic understanding of the maintenance and performance of the hemopoietic stem cell are considered in this preface. Hemopoiesis is described as a population of stem cells — taken by most experimentalists to be synonymous with the cells which form colonies in the spleens of irradiated mice, CFU-S — which gives rise to a series of transit populations. While feedback controls on the transit populations are outlined (and used to illustrate questions of interpretation) the emphasis is restricted to the pluripotent CFU-S.

The stem cell is defined simply as a cell with self-maintenance capacity. The CFU-S, as a population, has this capacity. Individually, however, CFU-S are heterogenous in their self-renewal capacities. Alternative explanations are considered, but it seems most probable that most of the CFU-S population is also a transit population of aging cells with declining self-renewal capacity. Just a few of them exist in environmental niches which confer on them the degree of immortality required to maintain the system. The developing field of stem cell regulation (proliferation and differentiation) is discussed, as is the question of the generation of cellular diversity from the stem cell; the continuing argument between the microenvironmentalists and the protagonists of stochastic regulation: the application of the quantal cell cycle model. Microenvironmental influences are favored, supported by the spatial distributions of CFU-S and regulator-producing cells within the marrow spaces. The current analyses of the adherent layer in long-term marrow cultures are now resolving the structure of this microenvironment.

II. INTRODUCTION

In principle, hemopoiesis can be described as a multitiered, hierarchical development of functionally mature blood cells, primarily in adult animals, in the bone marrow. It originates in a self-maintaining population of pluripotential stem cells from which a diverse range of committed precursor cells with more restricted developmental potentials is generated. These cells are generally considered (though no evidence exists) to have little or no self-renewal potential. They are, therefore, thought to be transit cells which have embarked on a suicide maturation pathway and which, without a supply from the stem cell pool, become extinct by giving rise to the morphologically recognizable maturing cells. These cells, too, form a transit population and progress along a similar suicide maturation route eventually becoming nonproliferative, mature, functional blood cells. Throughout the transit compartments, the population is expanded by cell proliferation in order to give the required output of mature cells.

Much is known about the behavior of hemopoietic tissue in response to a wide variety of stimuli and abnormal conditions as the pages of this book will testify. The sequence of events and the proliferative behavior of both stem and transit lines following such perturbations as irradiation (acute and chronic), hemorrhage (acute and chronic), hypoxia, hypertransfusion, and drug treatments (hemolytic), together with combinations of these insults, have been studied and documented in detail. Since this information basically comes into the category of descriptive (albeit kinetic) and functional biology, some of the principal problems remaining relate to the nature of the regulation of these kinetic behavior patterns.

In 1968, an endogenously occurring feedback proliferation inhibitor, specific for granulopoiesis, was described.[1] Although it met with a hostile reception and is still not fully accepted, subsequent work has shown that an extract made from mature granulocytes can, both in vitro and in vivo, limit granulocyte production by modulation of the length of the cell cycle in the maturing precursor cell compartment.[2] Similar factors were also described for erythropoiesis[3,4] and for the lymphopoietic system.[5,6] Erythropoietin (Epo) has long been

recognized as a major factor in the development of the erythron beyond the committed stage[7-9] and an erythropoietic Burst Promoting Activity (BPA), obtained from normal marrow, has been found to be important for the development of the very early erythropoietic precursor cell in vitro.[10] Similarly, a family of colony-stimulating factors (CSF) is important for the development of the granulocyte/macrophage committed precursor in vitro.[11]

Since the subject of this book is "Stem Cell Regulation in Hemopoiesis", consideration of the regulatory processes in the committed and maturing cell populations is largely outside its scope. Consequently, the above remarks, incomplete as they are, serve mainly to illustrate that regulation is not limited to stem cell behavior and to point the interested reader to a few appropriate references. The role of Epo and its effect on erythropoiesis, however, illustrate a problem in the formulation of a model system and a word of caution that must be borne in mind. The brief for writing this preface was the "*solved and unsolved problems*" in regulation. This poses something of a difficulty: When is a problem solved? Whether a problem is solved because it meets with general acceptance is a moot point. The mechanism of action of Epo is an appropriate example.

It is well known that if an animal is made polycythemic by hypertransfusion of red blood cells, then the ouput of Epo by the kidney is reduced. Cells already synthesizing hemoglobin continue their maturation process, but the generation of fresh hemoglobin synthesizing cells from the Epo-responsive committed precursor appears to stop — for as long as the red blood cell count is elevated and the Epo level reduced. However, an injection of exogenous Epo leads rapidly to a wave of stimulated erythropoiesis. Erythropoietic development through the committed precursor stage is, thus, shown to continue unaffected in the absence of Epo until the cell reaches that vital stage of initiating hemoglobin synthesis.[12] Exogenous Epo appears to switch the system on, giving rise to large numbers of hemoglobin-synthesizing cells and an eventually measurable output of new erythrocytes. Thus, it is generally accepted (and frequently stated) that Epo affects the differentiation step which switches on hemoglobin synthesis in the late stages of the committed precursor development, allowing the cell to progress through to its final maturation sequence. There is, however, an alternative explanation. If, by the time an erythropoietic committed cell reaches the stage at which hemoglobin synthesis starts, population amplification divisions are complete, then each cell may mature to an erythrocyte without further division. Epo may merely provide a proliferation stimulus which forces the cell through a further sequence of five or more amplification divisions during maturation. It is only this amplification which renders the population of maturing cells measurable. Without it, i.e., without Epo, production would be below the level of resolution in any measurement. In fact, this may, perhaps, be a more appropriate explanation since it is known that Epo promotes the rate of proliferation and amplification within the maturing cell compartment.[13-15] Furthermore, such a function would make any parallel with CSF as a granulopoietin more valid, since this certainly acts to promote proliferation (in vitro) but has no known function associated with a specific differentiation step.

One must be careful, therefore, to appreciate that any modeling, mathematical or otherwise, can at best only reflect our current understanding of the experimental data and hope to point the way forward to ways of destroying or reinforcing the interpretation.

III. THE STEM CELL

The only necessary characteristic of a stem cell is that it shall be capable of self-maintenance. The hemopoietic stem cell is such a cell with extensive self-replicative capacity for at least the duration of the animal's life span and which, as a bonus, is capable, simultaneously, of supporting the continuous production of a wide diversity of maturing hemopoietic cell lines.

What cell has this capacity? By common consent, the CFU-S[16] has become the obvious

candidate and most workers use "stem cell" and CFU-S as synonymous. These cells can be assayed only by the mouse spleen colony-forming assay, whereby when they are injected into an irradiated mouse, a proportion settles in the spleen and proliferates to form macroscopic colonies containing differentiated transit cells as well as a growing population of further spleen colony-forming cells, CFC-S. It should be remembered that a colony-forming unit, CFU-S, is a colony-forming cell, CFC-S, which has lodged in the spleen and grown to form a colony. CFU-S are related to CFC-S by the formula CFU-S = f × CFC-S, where f is the CFC-S spleen-seeding efficiency.

Measured as a population of cells, the CFU-S are at least a good approximation to stem cells. In terms of the above definition, they certainly have extensive self-replication and CFU-S from an old animal are just as good as cells from a young animal.[17] In fact, CFU-S from an old animal can be used to repopulate a radiation-depleted animal or to cure the congenital anemia of the W/W^v mouse, and this latter experiment has been carried through several sequential transfers of old, cured W/W^v mice to young, anemic W/W^v mice. At the same time they are capable of generating a large diversity of differentiated cell lines, including erythrocytes, macrophages, eosinophils, basophils, megakaryocytes, lymphocytes (B and T), osteoclasts, mast cells, and Langerhans cells.

Do these observations hold true for every single CFU-S, however? Since each spleen colony is derived from a single, seeded CFC-S,[18,19] then by transplanting single colonies into secondary irradiated recipients it is possible to measure the degree of self-replication undergone by each one of a large collection of individual CFC-S. Schofield et al.[20] assayed some 4600 individual spleen colonies (including 400 derived originally from normal marrow) and found that CFU-S may generate anything from zero to about 2000 further CFC-S in 11 days of colony growth. Clearly, therefore, not all CFC-S express the same degree of "stemness" at least when an intense proliferative and differentiative demand is imposed on them.

The literature, in fact, reveals a whole series of publications demonstrating a heterogeneity in the capacity of the CFU-S population for self-renewal proliferation.[20-27] Furthermore and, perhaps, most significantly, CFU-S at different locations in the marrow are found to have different self-renewal potentials.[28] This apparent, qualitative heterogeneity in the CFU-S population, thus represents one of the major questions regarding the "stem cell". When is a stem cell not a stem cell? The answer appears to be: when it is a CFU-S (at least sometimes).

IV. RELATIONSHIP BETWEEN COLONY FORMING CELLS AND THE STEM CELL

On the basis of this heterogeneity of function, Schofield[29] suggested that the CFC-S population is essentially another transit population, and that as a CFC-S proliferates, it ages and as a consequence has less self-renewal capacity remaining. This leaves a problem: an aging population would rapidly become extinct, especially if, for example, just a few of them are expected to repopulate a lethally irradiated mouse. Schofield overcame this problem by proposing that a true stem cell retains its identity and immortality by virtue of its residence in an environmental niche.[29] The progeny of this stem cell, emerging from the regulatory influences of the niche, then represents the transit CFC-S population. A CFC-S (perhaps even an "older" CFC-S) entering an appropriate environment, e.g., on transplantation, might have the stem cell immortality bestowed on it, i.e., its maturation arrested. Indeed, there is some evidence that later cells, e.g., the committed granulocyte/macrophage colony-forming cell, may become a fully self-maintaining population on the application of appropriate (niche type?) factors.[30,31]

On the other hand, this concept has been criticized on the grounds that in order to accommodate such a structure, the number of real stem cells would have to be vanishingly and unrealistically small.[32] The alternative explanation for the apparent heterogeneity in

CFC-S self-replication put forward by Lajtha,[33] therefore, is that the performance of a CFC-S may depend entirely on its G_0 history. He suggested that this is a period required for "genetic housekeeping". In a cell population with a life-long self-maintenance capacity, any error in the genome, if not corrected, would be perpetuated and amplified. The value of the G_0 period would thus be as a time to repair such errors in order to maintain the genetic integrity of the stem cell. In a normal CFC-S population the average G_0 period is long, although with random removal for differentiation there is clearly a spectrum of G_0 residence. When stressed, CFC-S proliferation increases, decreasing the average duration of G_0; housekeeping time is reduced. If cells in cycle, or with a recent history of cycling, are more susceptible to differentiation stimuli, then there is an efficient and economical way of eliminating cells at risk. Thus, cells with a short G_0 history may well have a more limited capacity to function as self-maintaining stem cells, i.e., they will be detected as a population with a reduced self-renewal probability.

Both these explanations remain hypothetical and more experimental data will be necessary before a clearer picture can emerge.

V. REGULATION OF CFU-S PROLIFERATION

The question of how CFU-S proliferation is regulated is now slowly moving into the category of "problems solved." From partial-body irradiation studies[34,35] it became clear that CFU-S proliferation is controlled locally, i.e., in the vicinity of the cells themselves, rather than humorally. From there it was a relatively short step to demonstrate that normal marrow with its very low rate of CFU-S proliferation contains an inhibitor, specific for CFU-S, which can be used to switch off the proliferation of regenerating CFU-S.[36] Although Riches et al.[37] confirmed this observation, Guignon and Frindel[38] described an endogenous inhibitor which presents certain differences from the preparation of Lord et al.[40] (almost certainly technical as a result of the method of preparation) but appears to have substantially the same function. Conversely, Frindel et al.[39] showed that radiation or drug damaged marrow can stimulate CFU-S proliferation in normal marrow, while Lord et al. showed that hemopoietic tissue containing proliferating CFU-S contains a stimulator which can switch proliferation on in the quiescent normal CFU-S population.

Although considerable progress has been made in understanding the way in which these factors interact to maintain CFU-S proliferation at the level required by the animal,[41] a major outstanding question remains untouched. What is the CFC-S recognition mechanism in vivo that governs the appropriate production of inhibitor or stimulator? This must almost certainly stem from the CFC-S population itself, either as a whole or, perhaps more likely, within local environmental pockets (see below). In order to comply with normal cybernetic principles, the strength of any such signal must be proportional to the size of the CFC-S population and must, therefore, be a signal requiring inhibitor production.

Recently, Bazill et al.[42] characterized a growth factor obtained from a myelomonocytic leukemia cell line, WEHI-3. This factor stimulates the development of a variety of hemopoietic progenitor cells, including the pluripotent CFU-S in vitro. Any role it may have in vivo, however, remains to be clarified.

VI. REGULATION OF CFU-S DIFFERENTIATION

The inhibitory and stimulatory factors thus act to control proliferative activity, but they may have little bearing on the overall regulation of the size of the CFU-S population, i.e., they do not govern the degree of self-renewal undergone by CFU-S. This depends on the degree of loss for differentiation purposes, a field of work notable only for its multitude of unsolved problems.

Under normal steady-state conditions, the rate of differentiation and, therefore, the net probability of CFU-S self-renewal is clearly 0.5. Under stressed conditions, such as in spleen colony growth, where there is a strong demand both for CFU-S growth and the production of differentiated cells, the net probability of self-renewal increases to about 0.65.[20,43-45] In order for the population to grow, the probability of self-renewal must be greater than 0.5, so although the need of the animal is for more differentiated cells, the differentiation probability must fall. How this is brought about is not known. If the growth of the CFU-S population and its differentiated progeny is a stochastic process such as was proposed by Till et al.,[44] there must also be an overall control which limits the average differentiation rate and requires an intercommunion within the CFU-S population which recognizes the extent of differentiation from other parts of the population. The same result could more simply develop if the differentiation and proliferation rates of the CFU-S proceed as independent functions. Then by increasing the proliferation rate (by means of the CFU-S proliferation stimulator) to a greater extent than the differentiation rate (by means of unknown factors), the CFU-S population will grow. This is completely analogous to the situation in the erythron where the maturation process is independent of the number of amplifying divisions occurring during maturation.[46] It is also more compatible with the transit CFU-S model proposed by Schofield[29] in which a CFU-S may be required to reach a certain degree of maturity before differentiating. In attaining that maturity it may well have completed extra amplification divisions. These possibilities, however, remain hypotheses and require experimental investigation. Factors involved in the renewal and differentiation processes need to be isolated, and the new and developing application of cell surface biochemistry to hemopoietic tissue promises suitable inroads in this field.

A further aspect of differentiation is the generation of cellular diversity. CFC-S are the source of at least eight different functional cell types, and several theories have been put forward to explain this differentiation capacity.

The microenvironmental model[47] requires that a CFC-S will be influenced by the particular cellular environment in which it finds itself. This principle is based primarily on the observation of relatively homogenous regions of erythropoietic or granulopoietic development in spleen colonies. By contrast, the finding that "pure" erythropoietic colonies contain significant numbers of committed granulocyte/macrophage precursors led to the stochastic model which requires that any CFU-S (and its daughter CFC-S) is always subject to the same chance of developing along a given differentiation pathway.[44,48] Both models, however, are subject to criticism. Environment clearly plays a part since the erythropoietic-to-granulopoietic cell ratio is different in spleen and bone marrow. This might indicate that the restrictive influence of the inductive microenvironment is aimed, therefore, not at the stem cell but at a later stage of development. David and MacWilliams,[49] on the other hand, showed, mathematically, that the variation in marrow clones is much larger than could be generated by a population responding in a stochastic manner.

The quantal cell cycle model[50,51] offers an entirely different concept. The microenvironment and stochastic models both depend on external stimuli (factors) to bring about the development of the differentiated cell lines. In this model, diversification is a programmed sequence of events which are tied to proliferation cycles. At each stage, a cell has only two potential types of daughter, each of which has its own two, though well-defined, choices.

There is some evidence in the hemopoietic system to support this concept. The granulocyte/macrophage committed precursor cell produces only granulocytes and macrophages. Similarly, some of the erythropoietic burst-forming units have the potential to produce both erythropoietic and megakaryopoietic cells,[52] others the potential to produce erythropoietic and eosinophilic progeny.[53] Johnson[54] has suggested that there is a sequential development of the differentiation options available. There is no evidence to link the diversification points to a quantal cell cycle or asymmetrical division process, but it may imply the need for some degree of maturity (age structure) in the precursor population before specific differentiation choices become available.

Clearly, the structure of the stem cell population, its relationship with the generation of diversity, and the manner of diversification are questions of extreme importance. The current stem cell techniques, both in vitro and in vivo, are now beginning to shed some light on these problems, but for the time being they remain unsolved.

VII. MICROENVIRONMENTAL INFLUENCES ON STEM CELL BEHAVIOR

The different erythropoietic-to-granulopoietic ratios in bone marrow and spleen, the differential recovery rates of CFU-S and other marrow cell populations in the shielded and unshielded regions of a partial-body irradiated mouse, and the differential spleen and marrow CFU-S kinetics in induced-hemolytic anemias all point to an influence of the microenvironment on cellular (particularly CFU-S) behavior. How far, therefore, does the microanatomy of hemopoietic tissue influence this behavior? Measurement of the spatial distribution of CFU-S (and other cell types) has demonstrated that hemopoietic cells are not randomly distributed throughout the normal marrow spaces.[55-58] Furthermore, while the proliferative activity of CFU-S in normal bone marrow is low, it is high in the immediate vicinity of the bone,[56] and this fits with the finding that there is a greater capacity for production of proliferation stimulator from cells close to the bone.[72] By contrast, inhibitor-producing cells appear to be more uniformly distributed across the marrow spaces. Our current understanding of the control of CFU-S proliferation requires that the CFU-S be in an appropriate spatial relationship with the inhibitor and stimulator-producing cells.[41] The fact that recovery of the CFU-S population following lethal irradiation plus normal marrow sublethal irradiation,[61-63] protracted irradiation,[64] or serial transplantation[17] levels off at a suboptimal plateau while following certain drug treatments, e.g., isopropylmethane sulfonate,[22] CFU-S recover to normal levels implies a change in this environmental organization.

A number of techniques is now available and being exploited to define, dissect, and manipulate the role of the microenvironment in hemopoietic tissue. The long-term marrow culture system[65] is based on the establishment of an adherent monolayer which is thought to approximate to the in vivo microenvironment. The genetically anemic W/W^v and S1/S1^d mice reproduce their defects in these cultures.[66] The S1/S1^d marrow (defective microenvironment) fails to produce an adherent layer which can support the culture growth and, the W/W^v marrow (defective stem cells) produces a fully competent adherent layer. Analysis of the adherent layer at the functional, microscopic, ultrastructural, and biochemical levels now beginning to produce insights into the nature of the microenvironment. To a similar end, the development of a bone marrow environment in which active marrow growth is eventually established following an implant of hemopoietic tissue under the kidney capsule[67] is now being exploited, as are collagen gel matrices, in vitro where foci of marrow stromal cells are produced and CFU-S proliferation maintained.[68] Some clues to the cellular make-up of the environment are already available. For example, distinctive cell types are recognizable in the long-term culture-adherent layer,[63] and several reports have indicated the cooperation of a thymus-derived (or similar marrow) cell with the development of some CFU-S.[69-71]

Little over 20 years ago, the hemopoietic stem cell was simply a morphologically unrecognizable cell which could repopulate an irradiated animal with differentiated blood cells. Within a few years it was found that a wide range of intermediate committed precursor cells existed, thus reducing drastically the size of the stem cell population which had to be accounted for. Now arguments rage over fine structure within that reduced stem cell population. Thus, while much has been learned, the complexity of the stem cell and the problems associated with it have proliferated. Whether the next 20 years will witness further amplification of this complexity remains to be seen.

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Chapter 2

A BIOLOGIST'S VIEW OF MATHEMATICAL MODELS IN HEMOPOIESIS

C. D. R. Dunn

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There cannot be a language more universal and more simple, more free from errors and obscurities... more worthy to express the invariable relations of natural things (than mathematics). It interprets (all phenomena) by the same language, as if to attest the unity and simplicity of the plan of the universe, and to make still more evident that unchangeable order which presides over all natural causes.

Joseph Fourier
"Analytic Theory of Heat"
1822

Some scientists question the validity of the reductionist approach when applied to the endlessly complex human body. Indeed, some scientists are reluctant to accept the description of the wonderfully mysterious mechanisms of biological reactions in the rational language of mathematical formulations. Many, it appears, simply do not wish to see the mechanisms of life described by formulas. The mathematical problems involved may someday prove tractable and more easily handled than the reluctance of researchers to accept the equations.

J. P. Green and H. Weinstein
"Recognition, Response"
1981

I. ABSTRACT

In this chapter an attempt is made to narrow the philosophical gap between those scientists who use mathematical models almost exclusively and those who limit their work to the biological mechanisms. To that end, the basic mathematics are summarized which describe the behavior of a mechanical (water tank) system. These are then put into the context of a general hemopoietic model. "Bridging the gap" between scientists who model and those who do not is addressed by a discussion of various points often raised by these two groups. It is concluded that mathematical models can contribute much to our understanding of hemopoiesis and several examples of these contributions are cited. However, for this goal to be fully realized it is suggested that it will be necessary for the biologists and the modelers to work more directly together than they have in the past.

II. INTRODUCTION

I believe that there is general agreement that mathematical models of biological processes have their uses. The question, it seems to me, is whether these uses are restricted to elegant mathematical exercises (which are no doubt very gratifying to the composers) or whether the models are permitted to have a more general utility in, for example, assisting in the design of biological experiments, hypothesis testing, or in predicting hard-to-measure variables. The difficulty in answering this question is compounded by the fact that biologists often find the mathematics overwhelming (and little attempt is generally made to make them understandable to the nonspecialist reader), and the modelers all too often fail to appreciate how difficult it might be to measure a certain variable in order to close a feedback loop, even if a biologist could be found who would be interested in such a study. There are a few groups who combine an active biological research program with sufficient available expertise to fully evaluate their results in mathematical terms and vice versa. The object of this paper is threefold: (1) to demonstrate to the biologists that the mathematical concepts involved in modeling are not too complex, (2) to demonstrate to the mathematicians that the onus is on them to accumulate data necessary for the complete evaluation of their elegant models, and (3) to merge some biological results with the mathematical predictions to illustrate how rewarding such an interaction can be. The terms "mathematicians" and "biologists" will be used for convenience, although it should be appreciated that this distinction is somewhat

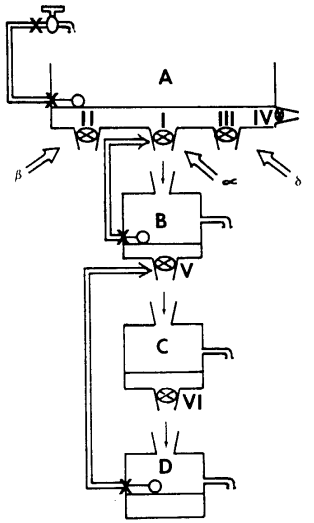

FIGURE 1. A water-tank model as an example of a system whose various characteristics can be expressed in mathematical concepts. (Reprinted by courtesy of The Immunology Research Foundation, Inc.)

artificial and, if it exists, should really be between "biologists who model" and "biologists who do not."

III. A SYSTEM TO MODEL OR A MODEL SYSTEM

Consider the "water-tank" system represented in Figure 1. The water level in tank A is controlled by a valve which, when the water drops below some certain critical depth, activates a tap to bring in more water. When the desired water depth is reached, the tap is shut off. This system, therefore, is similar to the standard toilet cistern where tap IV might be considered the overflow pipe. The outflow of "useful" water from tank A is through three controlled taps (I to III) which can be influenced by their own external but independent forces α, β, δ, respectively. In addition, these taps are controlled by some feedback system from the other tanks. For simplicity we will consider only the outflow through tap I.

Water flows from tank A to tank B through tap I. Tap I is controlled by an external (i.e., "manual") force as well as by the level of water in tank B by the cistern mechanism. In addition, tank B is equipped with an overflow pipe which also acts as a crude controller of water level. Also controlling the level of water in tank B is the aperture of tap V which is, in turn, controlled by the level of water in the last tank (D). Water flows from tank B through tank C (which is under no direct controlling influence) into tank D. The whole object of the system is to control the level of water in tank D by a combination of inflow and outflow (i.e., waste). The rate of outflow from D is governed by the level of the waste pipe, while inflow is directly controlled by the valve in tank D and indirectly by the "dynamics" of the water in tank B.

Let us now consider this system in very simple mathematical terms. The flow of water

through these tanks can be described by a variety of "primary" and "secondary" equations composed of various characteristics (or functions, F) of the tanks and taps. For example, the flow from A = F_1 (quantity of water in A, aperture of tap I) will be termed a "primary" equation since it describes the overall flow of water from tank A. However, it is made up of several "secondary" equations which describe the individual functions in more detail. Thus, the quantity of water in A = F_2 (valve in tank A, tap II, tap III, tap IV, β, δ); the aperture of tap I = F_3 (α, quantity of water in B); the flow from B = F_4 (flow from A, aperture of tap V, quantity of water in B); the quantity of water in B = F_5 (flow from A, valve in tank D); the aperture of tap V = F_6 (quantity of water in tank D); the flow from C = F_7 (flow from B, aperture of tap VI); the water level in D = F_8 (flow from C, level of waste pipe, valve in tank D).

The steady state in D occurs when the rate of change of the water level with time (dR/dt) is zero. That is, inflow = outflow, or flow from C = flow out through the waste pipe in tank D.

The above series of equations describes the flow of water through the series of tanks. The mathematics become considerably more complex when these equations are "embellished" to more realistically simulate the real situation. This embellishment occurs by defining the functions in more detail either mathematically or in general descriptive terms. For example, it might be beneficial to describe the flow of water from A (B = F_5) as an exponential function where flow from A = log (amount of water in A). Other embellishments of a more specific biological meaning might be the addition of sensitivity functions (for example, how far would the water in tank D have to drop before tap V was activated), of time delays (how long after the valve in tank D is activated before it passes the message to tap V), and a mechanism for controlling the actual flow of water through the system. An example of this latter approach would be a "random" vs. "nonrandom" exit of water from, say, tank B. That is, is the first molecule of water into the tank the first one out (nonrandom) or is the exit order independent of the inflow order (random)? The simulations, therefore, are not complex to formulate, and although biologists may not be able to fully appreciate the intricacies of all the functions it should surely be possible to subjectively evaluate them. For example, no particular mathematical powers are necessary to appreciate that a large time delay in the information flow between the valve in tank B and changes in tap I will result in gross fluctuations in the water level in tank B, which will reverberate throughout the system according to the equations delineated above. Likewise, if the valve in tank D were not suitably sensitive to changes in water depth, large variations in level would occur throughout the system because of the feedback. It is even possible to imagine some of the more complex functions. The water level in tank A will only decrease with time if the tap is closed. Therefore, the slope of the relationship of quantity of water vs. time will have to be negative. Likewise, as the water level decreases and the hydrostatic pressure consequently declines, the rate at which the water flows out will also fall. This is a negative exponential function generally represented as

$$h = h_0 e^{-kt}$$

where h_0 = height of water at time t = 0, h = height at time t, and k = constant, which includes functions of surface area, water density, and resistance generated by tap I.

Let us now move from this entirely physical system to the biological. In mathematical terms this is easy because the preceding equations can be equally well applied to a mathematical model of any system represented by the same working diagram. If the scheme outlined in Figure 1 were to be considered hemopoietic, tank A would reflect the pluripotential stem cells where taps I, II, and III represent the three lines of differentiation. External influences, designated as α, β, and δ, might be used to simulate microenvironmental influences. Switching on the tap to prevent the water dropping below a certain critical level might be equated with self-maintenance in the stem cell pool. Tap IV might be considered a mechanism to account for a death function. If tanks B through D are taken to represent the erythropoietic series, then B can be equated with the erythropoietin-responsive cells, tank C with the morphologically recognizable precursor cells in the bone marrow, and tank D the mature, circulating red blood cells. The feedback loop from tank D to tank B would then represent erythropoietin. However, it is not essential that any particular function has a direct biological corollary for this similarity to be obvious, nor is Figure 1 meant to represent a close analogy with any particular model for hemopoietic regulation. Obviously, it is a relatively easy mathematical job to add more compartments (e.g., BFU-E, CFU-E) or more feedback loops (e.g., chalones) once the basic concepts have been grasped.

With this somewhat simple view of mathematical models it is the intention now to take a hopefully objective (and somewhat personal) view of some of the models available, to consider the assumptions on which they are based, to attempt to evaluate what these assumptions indicate about the real system, and to outline how the application of specific models would help in our understanding of the biological system. Possibly the most informative way to accomplish these goals is to take some of the statements made concerning mathematical models and evaluate them in some depth.

IV. THE MATHEMATICAL FORMULATIONS ARE SO COMPLEX I CAN'T UNDERSTAND THE SYSTEM

To most biologists this statement is probably true. The responsibility for changing this situation lies on both sides of the fence: with those mathematicians who make little effort to make their analyses understandable to any but the specialist reader and who do not always ensure that their models are physiologically plausible, and with the biologists who often make little effort to grasp the concepts involved in constructing a computer model. It is the author's experience that the information that can be gleaned from models is well worth the extra time involved in unraveling some of the features of the system. The attitude "I don't understand it, therefore it does not exist" reflects poorly on all concerned.

V. THE MODEL IS ONLY AS GOOD AS THE DATA ON WHICH IT WAS BASED

The obvious answer to this statement is "correct". Or is it? Certainly it seems unjustifiable to use average data when there may be considerable differences in results between different laboratories.[1] A more useful approach would be to use one set of data and employ the mathematical model in an effort to explain the discrepant results. In this way some explanation for the discordance might be obtained. For example, in the model constructed by Loeffler and Wichmann,[2] stem cell recovery after irradiation was predicted to be very sensitive to the initial cell kill, i.e., the irradiation dose, and it is conceivable that minor differences in recovery patterns might be related to inadequate techniques in monitoring the dose. To accept the above statement uncritically implies that an accurate simulation of the experimental data suggests that the hypotheses on which that model is based are correct. This could be as fallacious as the opposite scenario, i.e., if the model does not fit the data, therefore, it too is wrong. Agreement between data and simulation "offers certain postulates"[3] to explain the observed effects. Disagreement suggests that the hypothesis is incorrect *given the other constraints in the model.* In fact, there is some advantage to testing a mathematical model to the point where it is no longer capable of simulating the available data and then addressing the question "what do I have to do mathematically to bring the simulations and data back into agreement?" This should then lead to the design of biological experiments to further delineate what is happening in the "real" system.

VI. THE SIMULATIONS HAVE TO MAKE PHYSIOLOGICAL SENSE

The above is another of those statements to which the obvious answer "of course" oversimplifies the situation. "Sense" in this context is really a subjective word. Most would probably agree that a simulation which predicted decreasing erythropoietin production as the hematocrit decreased would make no "sense" whatsoever. But what about some of those predictions of data which have historically been much more difficult to measure with any degree of certainty? For example, the mathematical model constructed by Necas et al.[4] suggests that to account for the delay in recovery of CFU-S after 150 rad whole-body irradiation, the proportional loss of stem cells through differentiation has to increase from less than 0.1/hr to 0.6/hr. This seems a very high figure given that self-maintenance of the stem cell population (turning on the tap in tank A) might be expected to take preference over differentiation (outflow through tap I), so it could be said that this simulation makes no "sense". However, that reaction is somewhat subjective as the rate of stem cell differentiation and how it might be influenced by irradiation or other perturbations is still an area about which we know very little. At some time in the future these simulations might make excellent "sense". Likewise, it is hard to rationalize that in a mathematical model aimed at explaining spleen colony differentials in terms of the stochastic model of stem cell differentiation,[1] a basic assumption is that granulopoiesis is favored 10:1 over erythropoiesis. (This is comparable to ten units of water leaving tank A through tap II in a given time compared to one unit through tap I.) It is well established that erythropoietic colonies outnumber granulopoietic 2 or 3:1. The fact that despite this assumption the mathematical model can very realistically simulate spleen colony development is of little help — the mathematical formulations which have apparently reconciled these diametrically opposed views need to be put in a physiological context and the appropriate biological experiments performed.

VII. COMPUTERS CAN HELP THE BIOLOGISTS BY PREDICTING HARD-TO-MEASURE PARAMETERS

Perhaps they can, but if we cannot measure the parameter in question we are no better off experimentally and the mathematical model is no nearer being verified for this situation. What the computer can conceivably do is suggest those parameters which it might be particularly rewarding to measure. Consider, for example, a situation where two hypotheses have evolved from experimental studies. A computer simulation might suggest that there would be a 100-fold change in one parameter according to one theory and a 10% change in the other parameter according to the alternative theory. Most assay systems have difficulty detecting anything much less than a twofold difference with any degree of certainty,[5,6] so it would probably be realistic to assess the former hypothesis first and measure the parameter with the greatest predicted change.

VIII. MATHEMATICAL MODELS ARE USEFUL FOR HYPOTHESIS TESTING

Mathematical models can be used very efficiently for hypothesis testing. What must be clear, however, is whether the mathematical formulation of the program will allow the model to simulate the hypothesis under consideration in a realistic way. For example, in the mathematical model with which we have been working,[7] erythropoietin production is under the control of the "hematocrit", so the simulated results after an increase in hematocrit are the same whether that parameter is increased by a transfusion of red blood cells or by a reduction of the plasma volume (i.e., erythropoiesis is independent of blood volume). This

is a feature of the mathematical model such that it is only capable of responding in this particular way (normally this may be perfectly reasonable, but the simulations are of limited validity in a study designed to test whether plasma volume decreases suppress red blood cell production by the same mechanism as an absolute increase in red cell mass). On the other hand, this particular model limitation makes it clear that we do not really understand the way in which blood volume effects erythropoiesis. In order to correct this deficiency the model has suggested particular experiments (some of which have been completed[8]) which have not only improved the model, but added to our knowledge of how the hemopoietic system behaves at least within a fairly clearly defined set of circumstances.

A further example of how mathematical models can assist in hypothesis testing is found in the discussion of "regulatory cells" which have been proposed to modulate CFU-S proliferation and differentiation. In 1981, Blackett and Botnick[9,10] proposed that a population of cells, tentatively identified as being intermediate granulocytes, regulated CFU-S cycling activity by what was essentially a positive feedback mechanism, i.e., a reduction in the population size of the regulatory cells led to a decrease in the proportion of CFU-S which differentiated. Lord[11,12] proposed an alternative explanation based on a negative feedback, whereby a reduced number of regulatory cells resulted in an increase in the proportion of CFU-S which differentiated. Mathematical simulations[13] of these two hypotheses with the underlying assumptions showed that given the constraints in the particular model employed, the hypothesis of Lord resulted in a better fit to the experimental data without the large undamped oscillations predicted from the theory of Blackett and Botnick. These simulations have, therefore, allowed two hypotheses to be tested and suggest, but do *not* prove, that the conceptually more satisfying negative feedback mechanism is more tenable than one based on positive feedback.

IX. THE MODEL SHOULD CONTRIBUTE TO OUR UNDERSTANDING OF THE SYSTEM AND/OR SUGGEST NEW APPROACHES

Provided this statement is viewed as being equally applicable to the mathematical modelers and to the biologists, it provides the most significant justification for the investment of time and money for the use of computers in hematology. As will be discussed below, some significant contributions to the biologists view of hematology have arisen from mathematical models. However, by being "equally applicable" to both sides, models should contribute to the understanding of actually how the simulations are derived. This can be done by delineating the particular mathematical manipulation necessary to obtain the desired simulation (and noting a possible biological corollary), and by using other simulations which fit the observed data equally well to propose alternative hypotheses.

X. COMPUTER PREDICTIONS

Let us now take a look at some of the predictions at least partially derived from mathematical modeling and see how valid they have proved to be biologically. It will be obvious going through this section that the most benefit from mathematical models is obtained when their predictions are tested in vivo — a simulation without an attempt at verification is of limited value, although mathematicians might argue that "plausibility" is more important than "experimental validation" per se. As a step towards a biological validation some efforts need to be made to understand why the mathematical model is giving the result that it is. Only by an extension of the realm of mathematical modeling into these areas of consideration can the mathematicians gain access to "respectability" among the biologists.

In this section little attempt is made to directly compare the various models except from the point of view of the validity of some of the assumptions that went into their design and

of the predictions they make. This is necessarily a rather narrow approach. No account is taken of how well individual models fit their design objectives. Furthermore, while models constructed, say, 15 years ago are probably now regarded as incomplete, the basic assumptions that went into them may be valid and, consequentially, they may remain informative to use.

A. The Number of Stem Cells

How many type A tanks are required to maintain a certain water level in tanks of type D?

OKunewick and Kretchmar[3] made certain assumptions regarding marrow transit times, red blood cell survival times, and the number of cell divisions between stem cells and reticulocytes in the rat, and calculated that 3×10^8 stem cells were required to maintain normal erythropoiesis. From the number of CFU-S per rat femur it is possible to calculate the total number of stem cells after certain assumptions are made concerning seeding efficiencies, the loss of CFU-S through differentiation,[14] and estimating one femur to represent 10% of the total marrow. Such a calculation results in a figure of approximately 10^7 stem cells per 250-g rat. There is, therefore, a discrepancy of a factor of 30 between a mathematical prediction and a figure calculated from a biological result. It is somewhat hard to reconcile these figures. It should be noted that since the CFU-S are capable of generating granulocytes and platelets as well as erythrocytes, the figure of 10^7 cells represents an estimate of the total stem cell population size. In contrast, the mathematically predicted figure of 3×10^8 cells is to provide erythrocytes alone and makes no allowance for stem cell requirements to produce other hemopoietic cell lines. On the other hand, the assumptions of certain values for seeding efficiencies and extinction coefficients can only be considered approximate, so we have no way of knowing whether the "mathematical" or "biological" estimate should be considered "correct". Consideration of the number of stem cells in the human (calculated at 5×10^9) is even more problematical[15,16] because of the absence of a stem cell assay in this species.

B. The Number of Cell Divisions between Stem Cell and Reticulocyte

How many tanks of types B and C?

A basic assumption in the mathematical derivation of the number of stem cells has been the assumption of the number of cell divisions between the stem cells and the reticulocyte. The numbers used have been 5 to 6,[15] 4,[3,16] 20,[17] and 12 to 15[18,19] Some of the discordance in these assumptions arises because, at least in the earlier models, no cell populations between the stem cells and the proerythroblasts were considered. Given that there are cells between the CFU-S and the earliest morphologically recognizable cells which are capable of the production of colonies of 10^5 cells or greater,[20] and that there are four to six divisions from the proerythroblasts to the reticulocyte,[21] the higher numbers quoted above would seem more appropriate. Careful analysis during the development of erythropoietic colonies in vitro should provide data to substantiate this conclusion.

C. Local Control of the Stem Cell Population

What controls the various taps and valves in tank A?

Although a biological basis for the local control of the stem cell population (either by size or proliferation rate) has only relatively recently been suggested,[22,25] mathematical models have incorporated such a system since at least 1966.[26] In some models the idea of feedback control through locally active factors has some quite specific meanings. Thus, Kretchmar[26] envisaged the control system as acting to modulate the probability of cells moving from G_1 to S and that this, in turn, was controlled by population size. Lajtha et al.[27] also considered a feedback control to the stem cells, while Aarnaes[18,19] postulated the

presence of a "feed forward" control mechanism. In this model some product of the stem cells is postulated to act on a more mature cell population to control the rate of cell division (comparable to an information flow from tank A to tank B). A feedback loop from this more mature cell population to the stem cells was envisaged to control the rate of stem cell differentiation. In the model of OKunewick and Kretchmar,[3] the feedback was considered to be by two cell populations (the stem cells and a less clearly defined population) competing for a limited amount of nutrients. These and other local control mechanisms were found necessary in order to be able to realistically simulate the post irradiation decline and recovery of CFU-S. Since this is still a controversial area it is worth considering some of these postulated mechanisms in more detail.

Following whole-body irradiation, CFU-S continue to decline for some hours, which is a little like a continued loss of water from tank A after a "leak" has been sealed. Lajtha et al.[16,27] and Vacha and Znojil[28] considered that this resulted from an increased rate of loss of stem cells to the "triggered" cell population, i.e., a loss due to an increase in the proportion of stem cells which differentiate out of that population. More explicity, OKunewick and Kretchmar[3] consider that the stem cells have two quite different genetic sites: one that controls differentiation (outflow through tap I) and one that regulates self-maintenance (activation of the valve to refill tank A). By postulating that the "self-maintenance" gene is more sensitive to irradiation than the gene modulating differentiation, the post irradiation "dip" was explicable — a certain dose of irradiation will remove the capacity for self-maintenance but differentiation will go on unabated so the end result will be a protracted fall in the number of CFU-S. Loeffler and Wichmann[2] also considered that the CFU-S are under the competing influences of differentiation and self-maintenance and that it is a change in this balance which accounts for the post irradiation dip. Differences in radiation sensitivity were not, however, specifically proposed as a mechanism. Necas et al.[4] simulated the post irradiation dip by a protracted and variable "death function" which is of limited benefit in trying to explain why cell death actually continues to occur after the cause of the cell death (i.e., the X-ray machine) is switched off.

What is known biologically about the post irradiation dip? Changes in the histological type of spleen colony when the marrow is taken from irradiated donors seem well established,[29-31] (i.e., the relative flow rates of water through taps I and II have been changed). This has been variously ascribed to two populations of CFU-S with different levels of commitment and different radiosensitivities,[29,30] or to an increase in the proportion of "transient" colonies due to greater CFU-S migration within the recipient spleen.[31] Whatever the reason, all these data suggest is a change in differentiation patterns rather than a change in the balance of differentiation to self-maintenance, which is what the models are predicting. The technical problems associated with measuring "extinction coefficients"[32] have so far precluded a test of this hypothesis. However, the concept that the stem cells may, at least partly, be influenced by another population of cells in the bone marrow (tank C, for example, influencing water level in tank A) has recently received some support from the studies of Blackett and Botnick.[9] In searching for an explanation for why pretreatment of mice with agents that are themselves cytotoxic enhances the rate of recovery of CFU-S after subsequent irradiation, these workers provided evidence that the number of CFU-S is under the control of a more mature cell population tentatively identified as "intermediate granulocytes". Blackett and Botnick speculate that when this cell population is intact it acts as a feedback on increasing CFU-S population size no matter what is the actual size of that population. After CFU-S reduction by irradiation, differentiation continues, the stem cell proliferation rate increases, but no increase in population size occurs until the controlling population decreases as a result of the initial stem cell decrease. The CFU-S are entirely directed towards differentiation until the controlling population is decreased and "frees" the CFU-S for population recovery. These observations, therefore, provide some support for a change in

the balance of stem cell differentiation to self-maintenance as an explanation for the post irradiation dip.

Another facet of the hemopoietic effects of irradiation which has received considerable attention from the modelers is the so-called "abortive" recovery seen in various of the mature cell populations.[33-36] This is characterized by a transient and sometimes complete recovery in the cells prior to a secondary decline before total recovery is evident. The general explanation for this observation is a perhaps natural extension of the "increase in stem cell differentiation during the acute post irradiation phase" theory. This increase in differentiation produces an increased production of, say, red blood cells, but in the process, stem cell numbers are further compromised. When this is detected by some hypothetical mechanism, stem cells are directed back towards self-maintenance, the flow of differentiated cells is interrupted, and the second dip occurs. Experimental testing of this hypothesis is hindered by the same technical reasons as the lack of firm evidence for the CFU-S post irradiation dip. Although convenient to do so, we should perhaps not close our minds to the possibility that the CFU-S dip and the abortive recovery in the more mature cells are not due to two entirely different mechanisms; the computer models offer "certain postulates" but do not prove the hypothesis (see above). The data provided by Blackett and Botnick[9] however, provided circumstantial evidence in support of changes in the balance of self-maintenance to differentiation as an explanation for the abortive recovery in the more mature cells.[36]

D. Cyclic Hemopoiesis

In the belief that normal hemopoiesis represents a cyclical system with greatly dampened oscillations, much attention has been directed at those situations where the dampening appears to be defective and large oscillations in the number of peripheral blood cells occur. The *a priori* example of such a situation is found in the gray collie dog where cycling of all peripheral blood elements has been observed.[37] As a complement to the extensive biological studies, cyclic hemopoiesis (CH) has received some attention from the mathematicians. In an extensive investigation, Mackey[38] has presented a unified hypothesis which predicts a sequence of events from pancytopenia through CH to aplastic anemia. Basically, in this model the stem cell population is controlled through five interrelating factors: the rate of cell "death" (outflow through tap IV in tank A), the rate of differentiation (outflow through taps I to III), the rate of proliferation (how rapidly water flows in), a function related to population size (how much water), and a sensitivity function relating proliferation rate to changes in population size (the operation of the valve). Alterations in any one of these parameters result in oscillations if the others are held constant. (To push this observation to the limit might be to suggest that the underlying defect in CH will be found in the parameter that does not cycle; so far all that has been found is cycling of progenitor cells and humoral factors.) Another suggestion from these simulations is that a gradually increasing loss of stem cells will result, sequentially, in pancytopenia, CH, then aplastic anemia. In clinical situations and in some dogs the order of the first two diseases seems to be reversed.[39] Interestingly, Kirk et al.[17] predicted from their mathematical model that in order for oscillations in peripheral red blood cell counts to occur, defects in both stimulatory (e.g., erythropoietin) and inhibitory (e.g., chalone) feedback loops (which must be of comparable frequencies) were required. Unfortunately, this model was based on the in vivo response to erythropoietin as a measure of stem cells. In the paper following that of Kirk et al. the problems with interpretation of the results from this method are discussed at some length.[40] MacDonald[41] has made the point that if the defect in CH resides in the stem cells, then cycling of all blood elements would be expected as, indeed, is the case at least in canine CH. However, the periodicity need not occur in the same phase in all the cell populations because of potential differences in transit times through the marrow. We have considered that differences in marrow transit times are insufficient to explain the asynchronous cycling of granulocytes and reticulocytes in canine CH, although we were unable to offer an alternative explanation.[42]

E. The Erythropoietin Response to Hypoxia

One of the facets of erythropoietic regulation which is difficult to explain solely in terms of erythropoietin (Epo) responding to some change in the ratio of oxygen supply to oxygen demand is the fluctuation in hormone titers in response to hypoxia. After the expected increase, elevated Epo titers are maintained for only a short period and then decrease to near baseline levels despite continuation of the hypoxic stress and before the hematocrit shows any dramatic change.[43-45] The inability of various mathematical models to simulate this response has, in fact, been termed a "major deficiency" in the computer simulations.[43] At least two groups[43,46] have attempted to provide an explanation for the transient elevation of Epo titers using mathematical models. These explanations derive, in part, from changes that were found necessary to the models to bring the simulations into agreement with the biological results.

Mylrea and Abbrecht[43] considered that Epo declines during hypoxia because of changes in sensitivity functions of the "controller" (i.e., kidney — or valve in tank D) or of the "plant" (i.e., the bone marrow — tap V). Kretchmar's hypothesis.[26] originally proposed to explain the greater erythropoietic response to divided doses of Epo compared to the same total dose given as one treatment, was invoked to explain the sustained erythropoiesis when Epo is back to near baseline. Decreases in plasma volume during hypoxia, with a resulting increase in hematocrit, were thought to be the major influences in bringing Epo titers down to near normal levels. In their second paper, Mylrea and Abbrecht[47] produced a superficially acceptable response to hypoxia in that the predicted levels did fall significantly from the high values seen during the acute phase. However, closer examination of the data reveals that Epo titers are still many orders of magnitude above the prehypoxic levels.

Like Mylrea and Abbrecht, we[46] have combined a mathematical and a biological approach in attempts to explain the Epo response to hypoxia. The idea that plasma volume decreases were sufficiently large to seriously impinge on Epo titers was not supported by our simulations. Likewise, the observed changes in P_{50} values were predicted to have a minimal effect. Dramatic but transient increases in all three in vitro clonogenic erythropoietic precursor cell populations, perhaps, provide support for Mylrea and Abbrecht's concept of changes in the bone marrow sensitivity to Epo. In our simulations, however, the in vivo Epo response to hypoxia was best simulated by changes in the function which describes the sensitivity of the Epo-producing mechanism. If renal sensitivity was decreased, to complement adjustments to reflect the measured changes in the bone marrow sensitivity function such that the overall "gain" of the renal to bone marrow axis remained constant, then an excellent simulation of the Epo response to hypoxia was obtained. (The best water tank corollary to this situation would be a reduced sensitivity to changes in water level of the valve in tank D linked to an increase in sensitivity of somewhat greater magnitude of tap V to the information flow.) In support of this hypothesis it was noted that mice do not eat normally during hypoxia (which is also associated with more subtle metabolic changes[48]), and that the production of Epo in response to an acute hypoxic exposure is severely impaired if the animals are previously starved.[49-51] There are, therefore, some data which can be interpreted as supporting the concept of changes in what might mathematically be equated with a sensitivity function, but proof of this hypothesis probably awaits the recognition of Epo-producing sites in the kidney and their controlling influences.

Wichmann et al.,[52] using their mathematical model for the regulation of erythropoiesis, have recently proposed an alternative explanation for the declining Epo titers during hypoxia. They have assumed that the hyperactive erythroid marrow might "consume" the hormone. When the equations for Epo clearance are modified to, at least partially, relate Epo half-

life to the number of proerythroblasts in the marrow, an excellent simulation of the hormone response to chronic hypoxia is observed. Such simulations involve a reduction in the "effective" half-life of Epo to approximately 30% of normal. In the same study Wichmann confirmed predictions that the plasma volume decreases and P_{50} shifts are probably of minimal importance in bringing Epo titers down during hypoxia.

Attempts to mathematically model the Epo response to chronic hypoxia have, therefore, led to two new theories to explain the Epo decline: one related to decreased production and one related to an increase in the rate of clearance. At this stage in formulating hypotheses it is not too important which one, if either, ultimately turns out to be correct, although recent data[53] suggest a change in Epo production may be more important than a change in clearance rate. The important point is that the mathematical models have identified two approaches to a problem; both are based on, but represent a considerable extension of, available information on erythropoietic regulation, and both are experimentally testable to varying degrees. For example, Jelkmann[53] has shown temporal changes in Epo extractable from the kidneys of hypoxic rats which are similar to, but precede, changes in serum Epo titers. These observations, perhaps, add support to the concept that the decline of serum Epo titers despite continuation of the hypoxic stress is an adaptational phenomenon (either reduced production[46] and/or reduced reabsorption[45]) and not due to increased marrow consumption of the hormone.

F. Miscellaneous

The mathematical model of stem cell kinetics employed by Newton[54] predicts that decreased granulopoiesis will result in increased erythropoiesis. Although this author can accept the concept of stem cell competition (despite the lack of evidence from spleen colony studies[55]) where stimulation of one cell line impairs production of another cell line, there is something very uncontrollable about the opposite situation which predicts that if one "tap" is turned off (tap I), another (tap II) must automatically be turned on even if there is no extra demand for those cells.

The model described by Korn et al.[1] has been used to support the concept that the differentiation of CFU-S is explicable by stochastic mechanisms with minimal involvement of the microenvironment (α,β, and δ). Like other reports,[18,19,56] Korn et al. assume that the proportion of stem cells differentiating into nonerythropoietic cells exceeds the proportion being committed to erythropoiesis by 10:1. At least in the later papers, data from Iscove and Sieber[20] on the relative numbers of CFU-GM and BFU-E were used to substantiate this assumption — the tenuousness of such a conclusion has already been pointed out.[57] The interesting part of the paper of Korn et al., however, is that despite this assumption of a 10:1 nonerythropoietic bias, the development of spleen colonies, including the increasing proportion of mixed colonies with time to the 3:1 erythropoietic-to-granulopoietic colony ratio on day 7, is realistically simulated. It is unfortunate that the authors made little attempt to translate the mathematical manipulations which produced this result into biological terms and to assess them experimentally. Mackey[38] and OKunewick and Kretchmar[3] assume a more-or-less equal probability of a stem cell differentiating into the erythropoietic or granulopoietic series during normal steady state.

The concept of an age structure within the Epo-responsive cell compartment was proposed by Lajtha[16] to explain changes in the number of erythropoietic cells in developing spleen colonies as a function of time of colony development. The hypothesis, perhaps, finds support in the recognition of several classes of committed erythropoietic precursors between the CFU-S and the proerythroblasts.[58]

A testable prediction from mathematical models which is probably feasible today, even though our concepts of the regulation of platelet production are somewhat vague, is that the plasma half-life of "thrombopoietin" should be less than 24 hr.[59]

Several groups[2,60] have made the plea that in constructing mathematical models consideration should be given to introducing formulas to reflect the range of cell cycle times rather than just using an average value. This seems an eminently reasonable suggestion. It would, perhaps, help us to get away from the concept of "boxes" joined only by arrows to represent cell populations when what we are probably dealing with, at least within the progenitor cells, is a gradual and continual change of properties as a cell moves towards a morphologically recognizable entity.

In a recent study[8] aimed at understanding the mechanism for the erythropoietic suppression in mice deprived of water as a model for the fluid shifts of space flight, a computer model was used as an integral part of the research program. A computer prediction was experimentally tested, the result incorporated into further simulations, and this iterative procedure repeated until an optimal simulation of the animal data was obtained. By omitting, in turn, the various components of this optimal simulation, their relative importance could be assessed. As a result the somewhat subjective conclusion from the biological studies, that the primary cause of the erythropoietic suppression in dehydrated mice was due to the reduced food intake, was confirmed. This powerful interaction of animal and computer "models" led to a reevaluation of actual space flight data where support for the previously unconsidered, dietary involvement in the "anemia" of space flight was obtained.

XI. CONCLUSIONS

Mathematical models have their place in hemopoietic research. The lack of general acceptance of such procedures by the biological community generally is multifactorial, but the major problems seem to be due to the fact that the mathematicians have worked somewhat in isolation of the biologists. To obtain the maximum benefit to all concerned a much greater collaboration between the two groups is essential. Only in this way can the full potential of mathematical models be realized. The problems encountered by biologists, such as validity of the data and adequacy of the programming and of the assumptions, should hopefully fade as we learn to appreciate the intricacies of mathematical modeling though a greater level of involvement. The computer cannot, and indeed should not, be expected to compensate for deficiencies in the data. The benefit derives from a translation of the mathematical procedures necessary to obtain a good simulation of the biological response, into biological terms and their assessment, where feasible, in the animal. In this way the computer does not necessarily have to produce an optimal response, but it can assist in understanding biological processes if the interaction of mathematicians and biologists is pursued to its fullest extent.

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Mathematical Model of Stem Cell Regulation

Chapter 3

BIOLOGICAL DESCRIPTION OF THE MODEL ASSUMPTIONS*

H.-Erich Wichmann and Markus Loeffler

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I. ABSTRACT

A mathematical model of stem cell regulation in murine hemopoiesis is described in four parts. In Part A, which is presented here, the biological description of the model assumptions is given. This part may serve as introduction for the biologist and physician.

Three interrelated feedback loops are considered, one for stem cell autoregulation, one for intramedullary feedback, and one for the regulatory influences from peripheral (erythropoietic) blood cells on bone marrow proliferation. However, the model focuses on the first two processes taking place in the bone marrow and it is restricted to stem cells, progenitors, and precursor cells. Two main regulatory mechanisms are considered: the regulation of the self-renewal probability and the fraction of stem cells in the active cell cycle. The basic model characteristics and parameters are described including the properties of these two mechanisms. Furthermore, the model is compared with a number of different concepts of stem cell regulation and it is shown which concepts are covered by the model and which are not.

II. INTRODUCTION

Mathematical modeling of hemopoiesis has become popular during the last 20 years and a large number of models has been constructed. These models, which have been reviewed by Dunn[1] in the preceding chapter or by other authors,[2] use quite different mathematical and biological concepts and many of them are restricted on very special topics.

In the following, a general model on stem cell regulation will be presented. It needs only a few assumptions and has a broad spectrum of applications. With this model, we are looking for a unique structure to the plethora of data available on early hemopoiesis in mice. Of course, we are not able to explain all experimental results, simply because some of these are contradictory themselves. However, we want to give a consistent interpretation of the typical experimental findings.

The model as presented here is the advanced form of an earlier version.[3-5] It has become more sophisticated and a number of additional applications[6-9] and theoretical analyses[10-12] have been performed. The model now has reached a certain degree of complexity which is reflected in a more complicated mathematical description. It has to be realized, however, and this is important with respect to the model point of view, that this complexity has been introduced stepwise, where each step has been tested separately.[4,5,7,13] This complexity is necessary if one wants to compare the model results with the available data for a large number of different experiments. However, the model can be formulated in a much simpler form, if one is only interested in the general type of model reaction.[5,10]

We will restrict ourselves mainly to the presentation of the ideas in our model and their consequences. Alternative approaches will only be discussed in comparison with these. It is not possible to review the large variety of hypotheses on the physiology of hemopoiesis here, and, therefore, this restriction is necessary. Since our readership probably will be heterogenous, the model description has been separated in four parts: this Part A describes

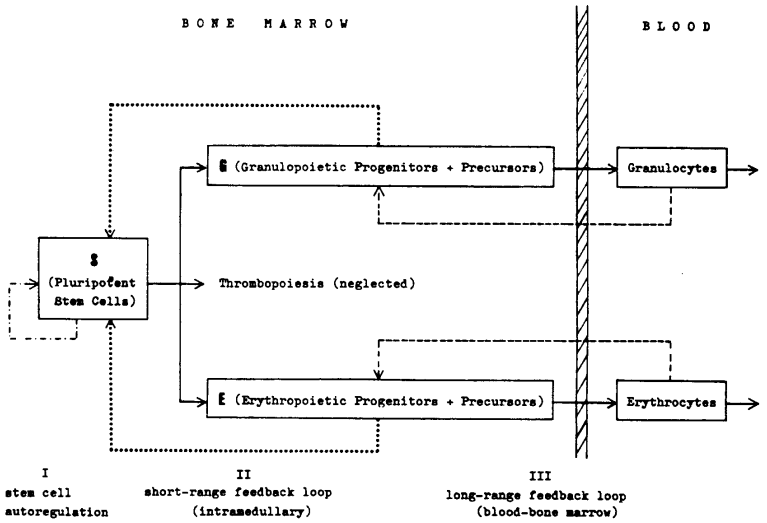

FIGURE 1. General concept of hemopoietic regulation. Three types of interrelated feedback loops are identified (for autoregulation of stem cells, for short-range intramedullary feedback, and for long-range feedback from blood to bone marrow).

the biological interpretation of the assumptions and is mainly written for biologists and physicians. Part B (Chapter 4)[13] gives the mathematical description of the model and will be of special interest for biomathematicians. In Part C (Chapter 5)[14] the reaction of the model system to typical perturbations is analyzed. This part should be of interest to both groups. The same holds true for Part D (Chapter 13, Volume II),[15] which summarizes the model simulations of the investigated experiments.

III. CONCEPT OF REGULATION

In hemopoiesis three types of interrelated feedback loops can be identified:[16] one for the autoregulation of stem cells, another for the intramedullary influence of differentiated bone marrow cells on early hemopoiesis, and a third for the stimulation of bone marrow cells by peripheral demand in the circulating blood (Figure 1).

A. Autoregulation of Stem Cells

All theoretical analyses of hemopoietic stem cell behavior come to the conclusion that there exists a negative feedback of the stem cells controlling their own population size (see reviews[1,2]). This autoregulation is responsible for the return of the stem cell number to normal after severe depletion or after elevation as soon as the perturbation has disappeared.

Although in many experiments the stem cells may also be directly or indirectly influenced by other hemopoietic cells, there exist situations where they behave autonomously.[17] This, for example, is the case in the bone marrow and spleen during the early recovery phase from exposure to acute irradiation or cytotoxic drugs. This is also the case in spleen colony growth in lethally irradiated recipients which received marrow transplants. In these exper-

iments stem cells grow exponentially, independently of the situation in the differentiated cell pools. In addition, there exists experimental evidence that besides autoregulation of the number, there is also autoregulation of the cycling activity of stem cells, mediated by humoral factors.[18-21] This is compatible with observations from several different experiments, that the rate of stem cell cycling is inversely related to the number of stem cells.[5,22-26]

In the following, autoregulation of both the stem cell number and their cycling activity will be called feedback loop I.

B. Intramedullary Feedback Loop

Originally one thought that there was a direct influence from the blood (via erythropoietin or colony stimulating factor [CSF]). After it had been shown that these factors do not directly influence stem cells[27] but act on more differentiated cell stages,[28-31] a gap in our knowledge became apparent. How could changes in the stem cell number and their cyclical activity, e.g., in suppressed erythropoiesis,[32-34] be explained if no direct message from the blood to the stem cells could be found?

The simplest solution would be an indirect message. Instead of being regulated by blood parameters, the stem cell behavior could be under the control of differentiated bone marrow cells which, themselves, depend on blood parameters. Such an intramedullary regulation shall be assumed in the following and shall be referred to as feedback loop II. Although not very much is known about this loop, it seems to become important in experiments where the numbers of differentiated progenitors and precursors are severely changed. This is the case in sustained hypo- or hyperproliferative situations and it is reflected in subsequent alternations in stem cell behavior.

Mathematically, different assumptions about the intramedullary feedback have previously been discussed.[4,11,35-37] However, except for some general features, no clear answer has been given to the nature of this feedback loop and it will be one of the aims of this book to partially clarify this phenomenon.

C. Feedback Loop Blood — Bone Marrow

After bleeding, additional mitoses of the erythropoietic bone marrow cells are induced which start to compensate for the peripheral demand.[38] After hypertransfusion, in the same cell compartments some mitoses are omitted and bone marrow production declines rapidly.[39-41] This type of regulation from the blood cells back to their ancestors in the bone marrow was identified many years ago. Especially for erythropoiesis, much information has been collected about the regulatory hormone erythropoietin since the days of Carnot and Deflandre (1906). The kidney has been identified as the site of its production (or activation) and CFU-E and erythroblasts as its target cells.[2] Similar feedback loops have tentatively been identified for granulopoiesis and thrombopoiesis. They all will be denoted as feedback loops of type III.

Many mathematical models of erythropoietic, granulopoietic, or thrombopoietic feedback have been constructed. Although quite different in details, they all consider a negative feedback from the blood cells to the precursors (see reviews[1,2]).

After acute changes of the number of blood cells (bleeding, hypertransfusion) or during changed environmental conditions (hypoxia, ex-hypoxia, hyperoxia), only the feedback from the blood to the differentiated bone marrow cells becomes effective. For sustained perturbations feedback loops II and I also become involved, but even then the influence of the peripheral stimuli is the most important.

Summarizing the above considerations, one may say that all three feedbacks are involved during perturbations of hemopoietic equilibrium, but to different degrees. Feedback loop I is important if the stem cell number is altered; loop II becomes involved if the differentiated progenitors and precursors in the bone marrow are severely changed; and loop III must be considered if the number of mature cells in the blood (erythropoiesis) or in bone marrow and blood (granulopoiesis) is significantly modified.

IV. OUR WAY TO MATHEMATICALLY MODEL HEMOPOIETIC REGULATION

The feedback loops described above form a quite complicated network. Fortunately, there exist experimental situations in which the regulatory loops can be investigated separately. This property allows the stepwise construction of a complex model and the ability of testing for the correctness of each step separately by simulating specific types of stresses. In the first step, autonomous proliferation of stem cells (feedback loop I) can be modeled and tested using data from severe irradiation. In a second step, feedback loop III can be modeled separately for erythropoiesis, granulopoiesis, or thrombopoiesis and tested using data on increased or reduced blood cell numbers. In a third step, feedback loop II can be introduced linking I and III. This stepwise procedure has been applied by our group (loop I and II,[3-9] loop III[2,42-45]). The model presented here is not the final but an intermediate step. It focuses mainly on feedback loops I and II and considers peripheral stresses only in a simplified way and only for erythropoiesis. Thrombopoiesis is totally neglected, and for granulopoiesis only the bone marrow cells are taken into account.

A. Model Compartments

In the model, six cell compartments are considered (Figure 2). They have been chosen in such a way that a direct comparison with experimental cell types is possible. The model parameters refer to mice. They will be presented here and justified later.[13]

For simplicity, the cellular compartments and the number of cells in these compartments are denoted by the same symbols. Throughout this volume relative cell numbers will be considered, which are normalized to 1. They correspond to the ratio of the absolute cell numbers of the whole animal after treatment to the absolute cell numbers in normal control.

B. S: Pluripotent Stem Cells

In the model, the stem cells are denoted by S. They are characterized by two regulated variables (namely, the self-renewal probability "p" and the fraction of cells in active cell cycle, "a_S") which are described in more detail below.

For the fraction of stem cells in active cycle, $a_S = 0.15$ is assumed for the normal steady state. This means that 85% of the stem cells are in a resting phase. By stimulation, all resting cells may be activated ($a_S = 1.0$) and by inhibition 99% of the stem cells may become inactivated ($a_S = 0.01$). In the following we will talk about the "proliferative fraction" when referring to "a_S".

A variable, quite distinct from the proliferative fraction "a_S", is the self-renewal probability "p". Normally "p" equals 0.5. This means that on average 50% of the newly formed cells (after mitosis of stem cells) remain in the stem cell compartment, while 50% are lost through differentiation. If "p" is greater than 0.5, more than 50% of the daughter cells remain stem cells and S increases. The opposite happens if "p" is smaller than 0.5.

It is important to emphasize that "p" and "a_S" are not dependent on each other. Their values depend on the number of stem cells and differentiated bone marrow cells, respectively (see below).

Of those stem cells differentiating, the fraction α_E goes into the erythropoietic cell lineage while the fraction α_G goes into granulopoiesis. Thrombopoiesis is neglected; α_E and α_G are constant in the model. Therefore, they need not to be specified as long as only relative cell numbers are used.

In the model, the stem cell compartment is homogeneous and an age structure is not

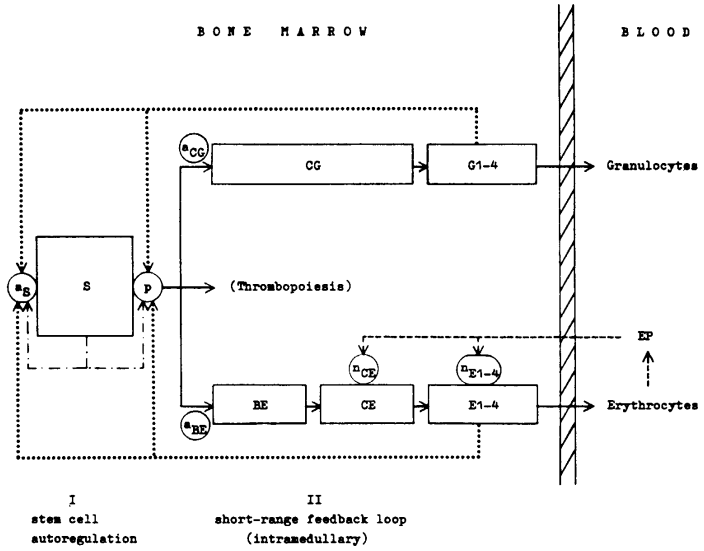

FIGURE 2. Regulatory influences as considered in the model. The model is mainly constructed around feedback loops I and II. Long-range feedback (from blood to bone marrow) is only considered for erythropoiesis and only in a very simplified way. S — pluripotent stem cells (CFU-S); BE — EP-independent erythropoietic progenitors (BFU-E); CE — EP-dependent erythropoietic progenitors (CFU-E); E1-4 — erythropoietic precursors; CG — granulopoietic progenitors (CFU-GM); G1-4 — granulopoietic precursors; EP — erythropoietin; p — self-renewal probability in S; a_S, a_{BE}, a_{CG} — proliferative fraction in S, BE, CG; n_{CE}, n_{E1-4} — number of divisions in CE, E1-4. p, a_S, a_{BE}, and a_{CG} are regulated by S, E, and G. Thrombopoiesis is neglected.

considered. The number of potential divisions of the stem cells is not limited during the period of observation. The stem cells have a long resting phase, a large variation of the individual generation times, and a cell cycle time of 8 hr.

No details are assumed about the mechanism of how stem cells differentiate. Specifically, we do not address the question whether after mitosis of an individual stem cell one daughter cell may leave and the second may stay, whether both daughter cells may leave (and both daughters of another stem cell may stay), or whether all daughter cells may stay, some of them leaving later (e.g., from the resting phase). In the model, the self-renewal probability "p" gives only the average fraction of daughter cells which keep the stem cell property of their mother cell for at least one complete cell cycle. In other words, the daughters of stem cells are counted as stem cells only if they divide as stem cells.

The stem cells S are self-reproductive and pluripotent. The CFU-S (colony forming units of the spleen) are considered as their experimental counterpart.

C. BE: Early Erythropoietic Progenitors (Not EP-Sensitive)

These cells are the first population of differentiated erythropoietic cells. Each cell which enters BE performs five divisions before it leaves the compartment. The cell cycle time is 8 hr. Normally, only 33% of the BE cells are in active cycle ($a_{BE} = 0.33$). Under stimulation, this fraction may increase to $a_{BE} = 1$; during suppression it may reduce to $a_{BE} = 0.3$. This corresponds to a normal generation time of 24 hr which can be reduced to 8 hr and prolonged to 27 hr.

The BE cells are functionally comparable with the BFU-E (burst forming units — erythropoietic) which are measured after 7 days of incubation (BFU-E-7d). They are not influenced by erythropoietin (EP).

D. CE: Late Erythropoietic Progenitors (EP-Sensitive)

After leaving BE, the erythropoietic cells enter the CE population. The cells in this population normally perform five divisions before they leave the compartment. Stimulated by EP, two additional mitoses are possible, while for a missing EP-stimulus all five mitoses may be omitted. The cells in the population are equally sensitive to EP. In this population all cells are actively cycling ($a_{CE} = 1$). Therefore, the generation time equals the cell cycle time which is 8 hr.

The CE cells are comparable to the CFU-E (colony forming unit — erythropoietic). In some examples, the sum of BE and CE cells (the major contribution of which is CE) is compared with ERC (Erythropoietin responsive compartment).

E. E1-4: Erythropoietic Precursor Cells (EP-Sensitive)

This compartment represents the morphologically identifiable erythropoietic cells in the bone marrow. For this population, normally six mitoses (with a generation time equal to the cell cycle time of 8 hr) and an additional postmitotic maturation time of 24 hr are assumed. This corresponds to a marrow transit time of 72 hr (3 days). Stimulated by EP, one additional mitosis may occur, and for a missing stimulus, all six mitoses may be omitted.

The population E1-4 represents the majority of erythropoietic bone marrow cells and is comparable to the total erythropoietic bone marrow cellularity. Only in one case (55Fe-experiments) will E1-4 be divided into its subcompartments E1, E2, E3, E4 which compare with the proerythroblasts, basophilic, polychromatic, and orthochromatic erythroblasts.

F. E: All Erythropoietic Bone Marrow Cells

Compartment E represents the population of all erythropoietic progenitor and precursor cells in BE, CE, and E1-4. Since the later cells are much more numerous than the earlier cells, due to mitotic amplification, the behavior of E reflects mainly the behavior of E1-4. Therefore, E and E1-4 often will be used synonymously.

G. CG: Granulopoietic Progenitors

The first granulopoietic cell population shall be denoted as CG. Ten mitoses with a cell cycle time of 8 hr and a generation time depending on the active fraction of cells, "a_{CG}", are assumed. As for "a_{BE}", normally 33% of the cells are in active cell cycle ($a_{CG} = 0.33$). This fraction can increase to 100% ($a_{CG} = 1$) or decrease to 30% ($a_{CG} = 0.3$) depending on stimulation. This corresponds to a normal generation time of 24 hr which may be shortened to 8 hr or enlarged to 27 hr. Action of peripheral stimuli (like CSF) on these cells is neglected since in the present stage of the model only erythropoietic stimuli shall be considered. The CG cells are comparable to the experimental population of CFU-GM (colony forming units of granulocytes/macrophages).

H. G1-4: Granulopoietic Precursor Cells

For this population four mitoses are assumed with a generation time of 8 hr (equal to the cell cycle time) and a postmitotic transit time of 40 hr. This corresponds to a marrow transit time of 72 hr (3 days). Stimulatory influences on these cells (by CSF) also are neglected. G1-4 is either comparable to the granulocytic bone marrow cellularity or the sum of myeloblasts, promyelocytes, myelocytes, and metamyelocytes, banded and segmented granulocytes of the bone marrow.

I. G: All Granulopoietic Bone Marrow Cells

The population of all granulopoietic progenitor and precursor cells in CG and G1-4 shall

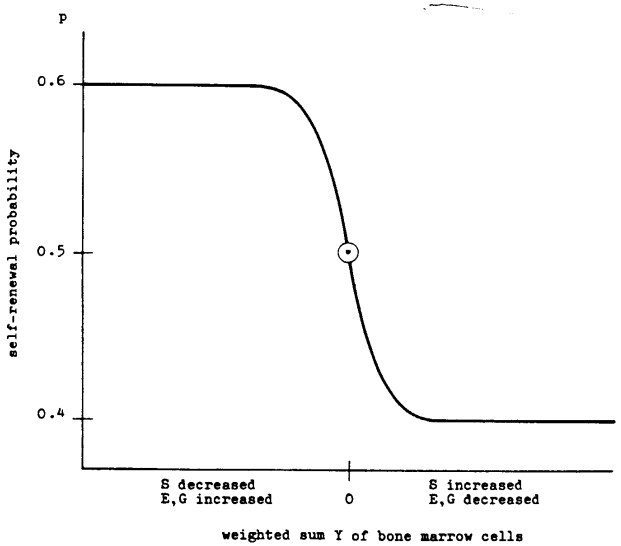

FIGURE 3. Dose-response curve for the self-renewal probability "p" of stem cells which depends on the weighted sum, Y, of bone marrow cells. "p" may vary between 0.6 (maximum need for stem cells) and 0.4 (maximum need for differentiated cells) and has a normal value of p = 0.5.

be denoted by G. Similar to E, G represents mainly G1-4, since this is the biggest subpopulation. Therefore, G and G1-4 are approximately identical and both notations will be used synonymously.

J. Regulatory Dependencies

1. "p": Self-Renewal Probability

The probability of a daughter cell to remain, for the next cell cycle, of the same cell type as the mother cell shall be defined as self-renewal probability "p". If all daughter cells keep the cellular properties of the mother cells, then p = 1; if all daughter cells change to a different cell type, p = 0.

In the model version used in this book, the property of self-renewal is restricted to stem cells. However, as will be discussed below, the concept of self-renewal can also be applied on other cell types.

We make the basic assumption that "p" is a function of the three variables S, E, and G. We found that a sigmoid dose-response curve for "p" guarantees an optimal behavior of the regulatory system.[13] This curve is shown in Figure 3; "p" is restricted to values between 0.4 and 0.6 and depends on the weighted sum

$$Y = Y(S,E,G) = p^S * (S - 1) + p^E * (E - 1) + p^G * (G - 1)$$

where S, E, and G represent the (normalized) numbers of stem cells, erythropoietic and granulopoietic bone marrow cells and p^S, p^E, p^G are the corresponding weighting factors.

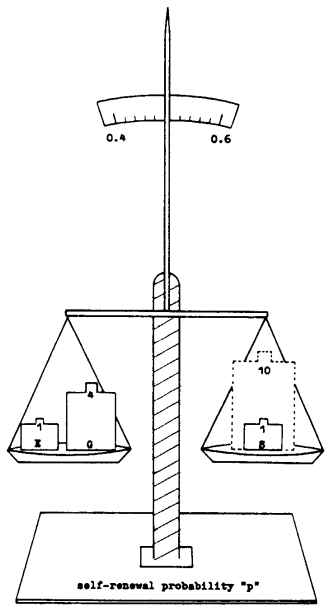

FIGURE 4. The regulation of the self-renewal probability "p" can be symbolized by a balance. "p" depends on the competing demand for stem cells (S), erythropoietic (E), and granulopoietic (G) bone marrow cells. The relative influence of S, E, and G corresponds to 1:1:4 for increased or slightly decreased cell numbers and to 10:1:4 for a severe demand of stem cells.

If Y is negative, "p" is greater than 0.5. If Y is positive, "p" is smaller than 0.5. In the normal steady state (S = E = G = 1), Y equals 0. The mathematical form for the dependency of "p" on Y will be discussed in Chapter 4[13] as well as the weighting factors. This dependency has the following consequences:

- The stem cell pool on one side and the differentaited cell pools on the other side are in competition for the newly formed stem cells. This can be symbolized by a balance (Figure 4).
- If only the stem cell number is reduced (higher demand for S), "p" increases.
- If only the number of differentiated cells (either erythropoietic or granulopoietic) is reduced (demand for E or G), "p" decreases.
- Changes in granulopoietic cell numbers influence "p" four times as strongly as equivalent changes in erythropoietic cell numbers.
- If the stem cells are severely reduced (below 1%,) "p" becomes maximum irrespectively of the differentiated cell numbers. In this situation, the stem cells recover autonomously, i.e., independently of changes in demand for E and G.

The self-renewal probability influences mainly the stem cells: if "p" is above 0.5, the

stem cell number increases; for "p" below 0.5 the stem cell number decreases. Therefore, only slight changes of "p" from below to above 0.5 (or vice versa) will influence S dramatically. On the other hand, even strong changes of "p" have only small effects on the flux into the differentiated cell lineages.

The self-renewal probability cannot be measured directly, and only for some cases (maximum value, equilibrium) is indirect information available. Therefore, no comparison of the theoretical curves for "p" with experimental points is possible.

2. "a": Fraction of Cells in Active Cell Cycle (Proliferative Fraction)

The cell cycle time of actively cycling cells shall be denoted by τ and the generation time, which is the average cycle time of all cells, cycling and noncycling, shall be called T^g. If $\tau = T^g$, all cells are in the "active" part of the cell cycle (i.e., G_1, S, G_2, or M phase). This shall be denoted by $a = 1$. If not all cells are actively cycling, but some of them are in a resting phase (G_0 phase or prolonged G_1 phase), the generation time T^g will be longer than the cell cycle time τ. This follows because the intermitotic phase of the resting cells is prolonged with the consequence that the average intermitotic time (which is the generation time) is also prolonged. In this case, the fraction of actively cycling cells in that population is smaller than 1.

In general, the proliferative fraction "a" can be defined as the ratio of cell cycle time, τ, to the generation time, T^g:

$$a = \tau/T^g$$

It is a basic model assumption that "a" depends on S, E, and G. Technically it proved useful to choose a quantity X for which "a" has a sigmoid dose-response curve as shown in Figure 5. X may be interpreted as a weighted sum correlating the different impacts of S, E, and G:

$$X = X(S,E,G) = a^S * \ln S + a^E * \ln E + a^G * \ln G$$

Here, again, S, E, and G are the (normalized) numbers of stem cells, erythropoietic, and granulopoietic bone marrow cells, and a^S, a^E, and a^G are the weighting factors (quantifying the impact of S, E, and G on "a"). In normal steady state $S = E = G = 1$) we find $X = 0$. X is negative for reduced cell numbers and positive for enlarged cell numbers.

As already stated, the active fraction of the stem cells, denoted by "a_S", normally equals 0.15. It can increase to $a_S = 1$ and can be reduced to $a_S = 0.01$ (Figure 5). For the granulopoietic progenitors, CG, and the early erythropoietic progenitors, BE, the active fractions are "a_{CG}" and "a_{BE}", respectively. For both, a normal value of $a_{CG} = a_{BE} = 0.33$ is assumed which can increase to 1 and decrease to 0.3. For the more mature cells $a_{CE} = a_{E1\text{-}4} = a_{G1\text{-}4} = 1$ is assumed, i.e., these cells cannot be activated because they are already in active cell cycle (see Figure 5).

The dependency of "a" on the sum X of bone marrow cells has the following consequences:

- Since the fraction of active cells corresponds to the ratio of cell cycle time to generation time, an increase of "a" is equivalent to a decrease of the generation time. For a constant cell cycle time of $\tau = 8$ hr, the generation time is related to "a_S" as shown in Figure 5 (right ordinate).
- Each cell reduction (erythropoietic, granulopoietic, or stem cells) leads to an activation of resting cells in the compartments S, BE, and CG. This can be symbolized by a letter scale as shown for "a_S" in Figure 6. Smaller cell counts correspond to a higher demand (weight) and, therefore, to higher "a" values.
- Changes of the stem cells have the strongest influence on "a", followed by the erythropoietic cells and the granulopoietic cells. The ratio is 10:3:1

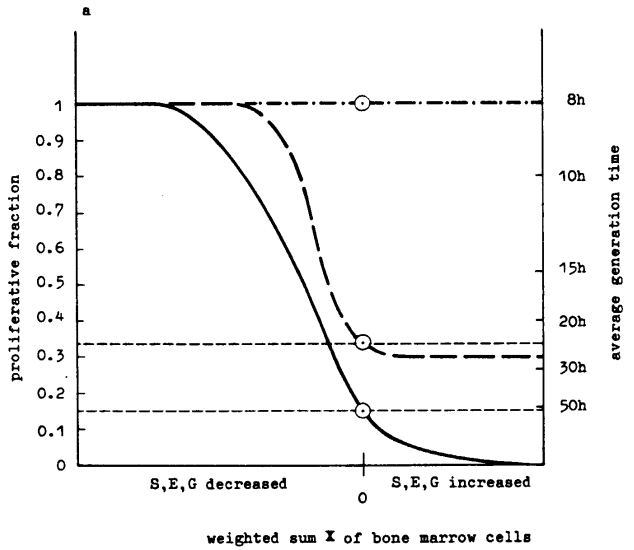

FIGURE 5. Dose-response curve for the proliferative fraction "a" of actively cycling cells. "a" depends on the weighted sum X of bone marrow cells. In the model different proliferative fractions are assumed for stem cells (— , normal value $a_S = 0.15$), early erythropoietic and granulopoietic progenitors (— — , normal value $a_{BE} = a_{CG} = 0.33$), and all other progenitor and precursor cells (— · — , normal value $a_{CE} = a_{E1\text{-}4} = a_{G1\text{-}4} = 1$). Equivalently to "a" one may consider the average generation time. For a cell cycle time of 8 hr, the right scale is valid.

The active fraction of cells can be approximated with the proportion of cells killed experimentally by ^{3}HTdR. Since this kill measures the fraction of cells in S phase, only a qualitative comparison with theoretical time courses for "a" is possible.

3. How "p" and "a_S" Determine the Way of Regulation

The influence of the self-renewal probability "p" and the proliferative fraction "a_S" of stem cells is shown in Figure 7 for the normal steady state. In this situation, 15% of the cells in S are in the active part of the cell cycle ($a_S = 0.15$), while 85% are in a resting phase. After mitosis, 50% of the cells remain in the stem cell compartment ($p = 0.5$) and 50% differentiate. Of these, the fraction α_E and α_G go into the erythropoietic and granulopoietic cell lineages, respectively, where additional amplification takes place. Thrombopoiesis is neglected in this scheme.

If the proliferative fraction: "a_S" is enlarged, more cells are actively cycling (Figure 8). This increases the average "velocity" of the cells (number of cells passing a particular stage per unit time) on their way through the cell phases. However, faster cycling has no influence

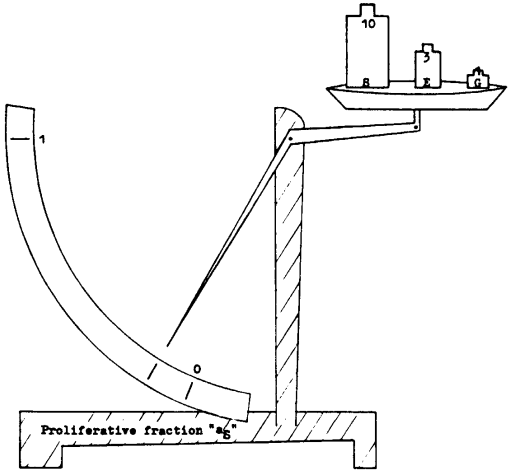

FIGURE 6. The activation of resting stem cells (as represented by "a_S") may be symbolized by a letter scale. "a_S" may vary between 0.01 and 1 with a normal value of 0.15. It depends on the demand for stem cells (S), erythropoietic (E), and granulopoietic (G) bone marrow cells. For reduced cell numbers their relative influence corresponds to 10:3:1.

on the cell number in S as long as p = 0.5, but it leads to enlarged rates of differentiating cells.

If the self-renewal probability "p" is different from 0.5, the stem cell number S changes with time. This is shown for maximum self-renewal (p = 0.6) and normal activation (a_S = 0.15) in Figure 9. In this situation, S will increase because the cell flux is, in part, diverted in favor of the stem cell pool.

K. n_{CE}, $n_{E1\text{-}4}$: Amplifying Erythropoietic Divisons, Stimulated by Erythropoietin (EP)

To investigate peripheral erythropoietic influences on the bone marrow (feedback loop III), the red blood cells must be taken into account. This has been done in a separate model of mature erythropoiesis,[2,45] which considers late progenitors, dividing and maturing precursors, reticulocytes, and erythrocytes. The number of red cells in the blood (reticulocytes and erythrocytes) is responsible for the oxygen supply of the body. It is assumed that the partial oxygen pressure in the tissue of the kidney regulates the production (or activation) of the feedback hormone EP. For a reduced red cell number, EP stimulates the amplification of late progenitors and precursors and shortens the marrow transit time; for a missing stimulus some amplifying mitoses are omitted and erythropoiesis is severely reduced.

The best way to mathematically simulate the (indirect) influence of the red blood cells on stem cell regulation would be to unify the model of mature erythropoiesis with the stem cell model. However, we hesitate to do this here, since then we would have to discuss the details of mature erythropoiesis, which are not the topic of this volume. Therefore, we prefer a more artificial presentation: we open feedback loop III and consider only the influence of EP on erythropoietic amplification. For this purpose time courses of EP as derived from the model of Wulff and Wichmann[2,45] will be used as input to the stem cell model.

EP influences the numbers of mitoses, n_{CE} (EP) and $n_{E1\text{-}4}$ (EP), in the compartments CE

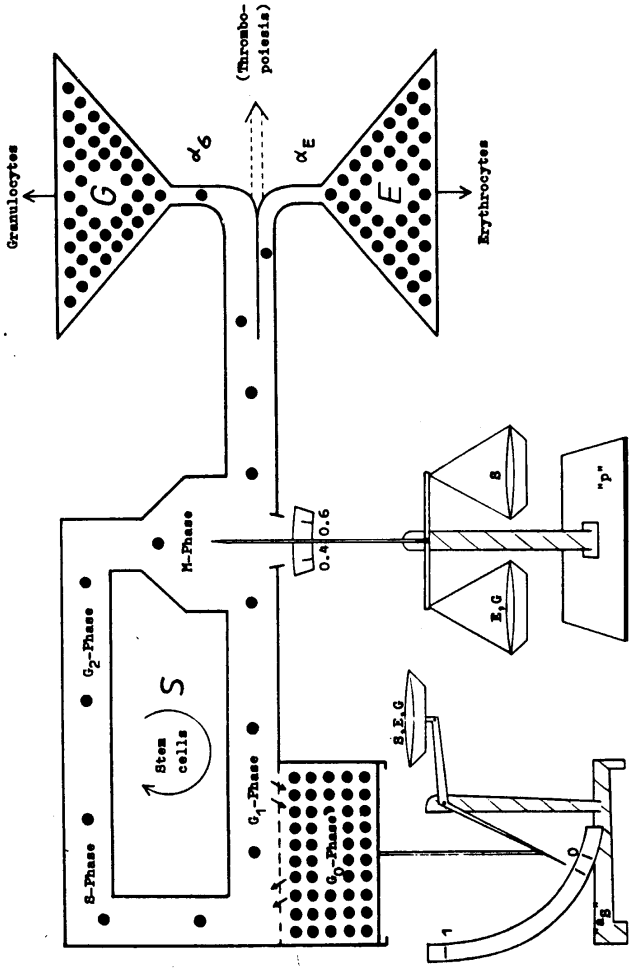

FIGURE 7. Stem cell proliferation in normal steady state. The fraction 1-a_S of the stem cells S is in a resting phase (G_0), while the fraction "a_S" is in active cell cycle. The self-renewal probability "p" equals 0.5, e.g., after mitoses of the newly formed cells 50% remains stem cells and 50% differentiate. Of the differentiating cells, the fractions α_E and α_G develop into the erythropoietic (E) and granulopoietic (G) cell lineages. Thrombopoiesis is neglected.

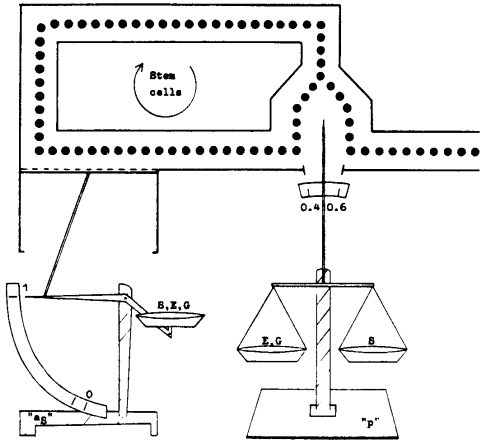

FIGURE 8. Activation of stem cells with normal self-renewal probability. If all stem cells are in active cell cycle (a_S = 1), more cells are formed per unit time. This leads to an increased flux of cells into differentiation and thus to an increase of the number of differentiated cells. However, the number of stem cells does not increase but is constant as long as the self-renewal probability is normal (p = 0.5).

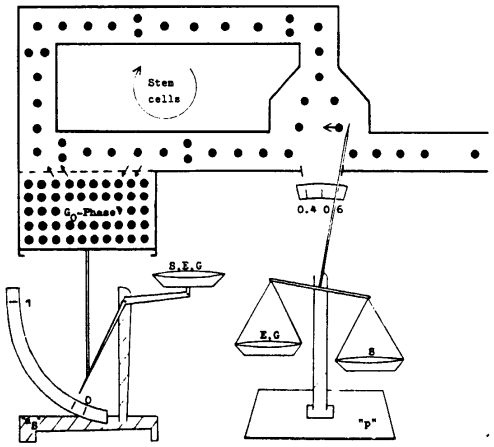

FIGURE 9. Increased self-renewal probability with normal fraction of active cells. If the self-renewal probability is increased (p = 0.6) the number of stem cells increases because more than 50% of the postmitotic cells remains stem cells.

and E1-4. For high values of EP, additional mitoses take place; for reduced EP some mitoses are omitted. The dose-response relations between the amplification and EP are described in Chapter 4.[13]

For the late erythropoietic progenitors, CE, normally n_{CE} = 5 is assumed. Maximum EP induces two additional mitoses and for missing stimulus, all five mitoses may be omitted.

The precursors E1-4 normally have $n_{E1\text{-}4}$ = 6 amplifying divisions. During stimulation, one additional mitoses may take place, but since the transit time through this compartment is approximately halved, the compartment size is not influenced. These two antagonistic influences of EP are, therefore, neglected and six mitoses are also assumed for increased stimulation. For low levels of EP, all six mitoses may be omitted.

As a consequence of these assumptions, total erythropoiesis (BE, CE, and E1-4) is characterized by 5 + 5 + 6 = 16 mitoses in normal steady state. Of these, 5 are EP-independent (in BE) and 11 are EP-dependent (5 in CE and 6 in E1-4). The 11 EP-dependent mitoses may be omitted if the stimulus is missing. During increased EP stimulation, two additional mitoses are possible.

V. DISCUSSION

The model should be analyzed from many different points of view. What are the mathematical properties? How does it react to perturbations of the steady state? What are the underlying biological assumptions? Which simplifications have entered into the model? The first two questions will be investigated extensively in Chapter 4[13] and 5[14] and only the biological implications and the simplifications will be discussed here.

A. Role of the Spleen

In a detailed analysis of the published data[46] we could show that under normal steady-state conditions for most strains, the spleen of mice contains not more than 5% of the CFU-S, not more than 10% of the BFU-E, CFU-E, CFU-GM, and granulopoietic precursors, and approximately 15% of erythropoietic precursors. Therefore, in the normal steady state, splenic hemopoiesis can be neglected in these animals.

During stresses, hemopoiesis in the spleen may increase dramatically. However, it has to be kept in mind that the starting values are low. Thus, for example, if the CFU-S numbers in the spleen reach ten times normal, this does only mean an increase of the spleen's contribution from 5 to 33% of the total production if the bone marrow CFU-S number is not increased. If the bone marrow count is doubled, the tenfold increase of splenic proliferation leads to an increase from 5 to 20% of the total hemopoietic production.

As will be shown in detail the spleen may be neglected except in anemia and hypoxia.[46] In these two situations, the splenic cell numbers has to be taken into account.

B. Asymmetric Divisions

As already stated, the self-renewal probability "p" of the model is not concerned with the fate of individual cells, but refers to large cell numbers. Therefore, no assumptions are made whether after mitosis of a stem cell the two daughter cells are identical or different (the "individual cells" in Figures 7 to 9 are only used symbolically). For example, p = 0.5 can follow by asymmetric divisions (one daughter differentiates and one daughter remains a stem cell) as well as by symmetric divisions (two daughters of one stem cell differentiate and two daughters of a different stem cell remain stem cells).

C. G_0 Phase or Prolonged G_1 Phase

In terms of the model it also makes no difference whether the cell cycle of stem cells and early progenitors has a separate quiescent phase (G_0 phase) or a prolonged G_1 phase. It is only necessary that the time spent in this phase is variable and is under regulatory influence. In the model, we use random compartments which reflect a large variance of the G_0 or prolonged G_1 phase duration. A description by a random compartment (for the G_0 phase)

and a subsequent first-in first-out compartment (for the G_1, S, G_2, and M phase), as, for example, used by Smith and Martin,[47] would lead to similar results because the time scale for the observations is much longer than the cell cycle or generation times.

D. Biochemical Interpretation of Regulation

Often a misunderstanding between experimental biologists and model builders is found, on how regulation is realized. The biologist is interested in identifying and isolating feedback "factors" or hormones. He wants to know where they are produced, what the target cells are, and how these are influenced (inhibition, depression, sensitivity, etc.) This leads to complex knowledge about identified regulators and their action and a lot of missing links and gaps in the chain of substances and reactions involved. The problem with this knowledge is — at least in many cases — that it is often not known which of the identified substances are the important ones for in vivo regulation.

Mathematical modeling as investigated in this volume has a totally different approach. We are not interested in a complete list of regulators and their properties. On the contrary, we want to consider as few regulatory processes as possible and as simple a mode of production and action as possible. Therefore, we typically start with the assumption of one regulatory mechanism, which effectively encompasses the net effects of all biochemical processes involved. We then want to learn in which experimental situations such an assumption is sufficient to interpret the (in vivo) measurements. We are very reluctant to introduce more complex dependencies even if there is a lot of experimental evidence for their existence. The reason for this reductionist attitude is that the consideration of complicated influences in the model makes additional parameters necessary which cannot be directly measured and thus makes the model more arbitrary.

Thus, the simplified way of describing regulation in a model, which sometimes is criticized as "unrealistic" by experimentalists, is an essential feature of a "realistic" model. It is not ignorance, when we assume only one regulatory mechanism for stem cell proliferation, although the existence of at least one stimulator and one inhibitor has been shown by Lord and Wright[19,48,49] In terms of a model, the action of an increased inhibitor concentration cannot be distinguished from the action of a reduced stimulator concentration, and, therefore, stimulator and inhibitor may be comprehended as one virtual substance which, as a whole, is responsible for the regulation.

To give another example, we are not interested to include into the model the complicated and mostly unclear way of erythropoietin action on sensible cells, its receptor binding, breakup, RNA-action induced, etc. We want to know how many cells will be produced as the result of this action if a particular amount of erythropoietin is administered.

In summary, at the actual stage of modeling, we are not interested in the biochemical or cellular details of regulation. We only want to understand the main net effects in terms of a demand-reaction model. The details will have to come into this scheme later on, once they are better known.

E. Self-Renewal of Nonstem Cells

In the model, only for the stem cell population a self-renewal probability "p" is considered. For the transient proliferative cell types, an age structure is considered with a certain number of mitoses through which each cell has to pass. However, especially for the progenitor populations, one can use a different concept which is preferred by some authors[50] (Figure 10, top). Instead of considering the CFU-GM population as a pipeline of ten mitoses (as assumed for the CG compartment of our present model), one may consider these cells as a homogeneous population with a certain self-renewal probability "p". From a model point of view, both concepts are essentially equivalent and can be transformed into each other. As the number of mitoses in a transient population is finite, "p" remains below 0.5 in

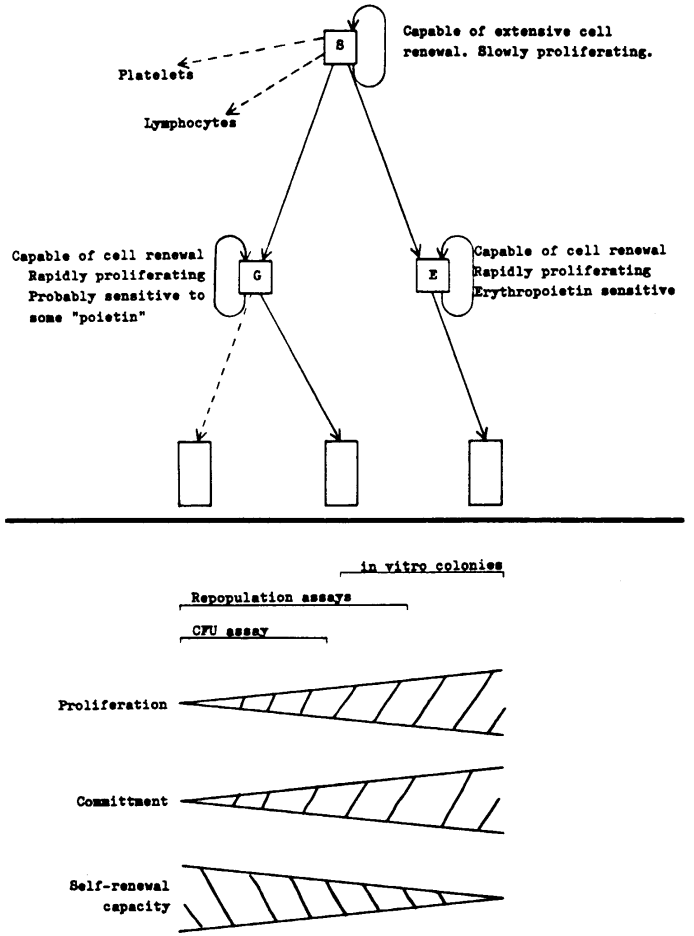

FIGURE 10. Different concepts for cell development in hemopoiesis. (Top) Distinct populations with self-renewal capacities in early hemopoiesis;[50] (bottom) early hemopoiesis, considered as a continuum.[51] The mathematical model as presented in this volume can be transformed such that it is equivalent to both concepts.

steady state; "p" equals zero if no division takes place and approaches 0.5 for an indefinite number of mitoses. The efflux from a population with ten subsequent mitoses is identical with that of a homogeneous population with a self-renewal probability of $p = 0.4995$ for each cell (five mitoses correspond to $p = 0.4839$). Therefore, the model could also be formulated in an equivalent way using self-renewal probabilities for the progenitor and precursor compartments instead of fixed numbers of subsequent divisions. The concepts are two equivalent expressions of the same fact.

F. Age Structure of Stem Cells

Let us, for a moment, forget the stem cell concept of our model. Let us assume that the stem cells and the progenitor cells are not distinct populations (as drawn in Figure 2 or Figure 10, top), but are sections of a continuum of cells[51] (as shown in Figure 10, bottom). This continuum would represent an age structure where the cells have an inherent clock which counts the generations. Then the cells which are identified experimentally as CFU-S may correspond to the first n generations, the subsequent transient population (e.g., BFU-E) to the next generations, etc. In fact, there would probably not be clear borderlines between the measurable cell types, but there might be overlap and gaps.

Let us now restrict our attention to those first n generations which are measured as CFU-S. At least the first generation must be self-replicative with a self-renewal probability p = 0.5 in equilibrium. Otherwise the cell number cannot be maintained. The other n-1 generations can then be interpreted as belonging to a transient pipeline. As shown above, this is equivalent to one or more homogeneous subpopulations with a normal "p" somewhat below 0.5. Thus, an age structure within the stem cell population can be equivalently described by a series of stem cell subpopulations with different self-renewal probabilities.

If regulatory influences are considered, it is quite reasonable that the boundaries in which "p" may vary are different for the different subpopulations. There may be one group of stem cells (the early ones?) which have a maximum "p" of 0.7 or more and a different subgroup (the late ones?) with a maximum "p" of 0.51. This may be compatible with an average maximum value for "p" of about 0.6, as has been found in growing spleen colonies.[52]

Now let us come back to the model. In the model, only the average behavior is considered. Thus, the boundaries of 0.6 and 0.4, as assumed for "p" in the model, represent only average boundaries and do not exclude subpopulations with higher or lower values. Therefore, there is no conflict of the model assumptions and the continuum hypothesis in Figure 10 (bottom).

In a similar way, the proliferative fraction of stem cells "a_S" also may be different for different stem cell subpopulations. It might be that (in normal equilibrium) in one subgroup (the early stem cells?) only 5% of the cells are cycling while in a different subgroup (the late stem cells?) 30% are in active cycle. This may be compatible with an average of 15% for the total population, as assumed in the model. In that case, the reserve of activation would be big for early stem cells and only small for later stem cells.

This interpretation would fit with the sequence as assumed in the model for the degree of active cycling. The normal values for "a" increase from stem cells ($a_S = 0.15$) to progenitor cells ($a_{BE} = a_{CG} = 0.33$) and precursor cells $a_{CE} = a_{E1\text{-}4} = a_{G1\text{-}4} = 1$). In other words, the presented mathematical model, although considering distinct populations with different properties, is, nevertheless, equivalent in all its important features to a model where the cell characteristics change gradually and where the experimentally defined populations (CFU-S, CFU-GM, BFU-E, CFU-E) represent a section of a continuum rather than distinct cell types.

These reflections, together with the statements about the equivalent description of transient populations by subsequent mitoses or a self-renewal probability, show that the mathematical model as presented here and as symbolized by Figure 2 is not an alternative to the concepts shown in Figure 10, but can be interpreted as including them.

G. Hayflick's Hypothesis

Some biologists believe to have evidence that stem cells have a limited self-renewal capacity (e.g., of about 50 to 100 divisions).[53] Others believe that there is no such limitation and that the stem cell may have an unlimited capacity to divide for the life span of the animal. What is the position in our model?

In the model, no limitation of the self-replicative properties of the stem cells is assumed. However, the model itself is restricted to situations in which the system returns to the normal steady state after a perturbation. Thus, if Hayflick's hypothesis is true, the model is only applicable as long as the limit of divisions is not reached and no severe manipulations of the age structure in the stem cell pool are performed.

H. Role of Microenvironment

The hemopoietic microenvironment plays an important role in blood formation. Its integrity is necessary for a normal physiological response. The model deals only with situations in which the microenvironment is intact. Experiments in which it is destroyed by physical or chemical manipulations, or in which the stresses lead to residual damage, cannot be analyzed. It is an essential property of the model that the cell numbers return to normal if the perturbations are over. A displacement of steady-state values will not be considered.

I. Schofield's Niches

Schofield[54] has proposed a concept where fixed stem cells exist which occupy niches (in the bone or close to the bone), and others which are free (in the bone marrow). The fixed stem cells are "immortal" and can replicate indefinitely while the free stem cells undergo a limited number of divisions before they differentiate. However, if a free stem cell finds a vacant niche, it changes its character and becomes a fixed cell with unlimited self-renewal ability.

This hypothesis corresponds to the concept of an age-structured stem cell population which is compatible with our model as stated above.

J. Interaction of Erythropoiesis and Granulopoiesis

In our model we consider both erythropoietic and granulopoietic progenitors and precursors and their regulatory influences on the common mother stem cells. This implies interactions of both cell lineages, because an activation of resting stem cells due to an erythropoietic stimulus automatically changes the cell flux into differentiation. Many speculations are possible about how the efflux from the stem cell compartment is channeled. The simplest assumption (which has been adopted in the model) is that the influx into the erythropoietic and granulopoietic lineage occurs at a constant ratio. However, the concept of variable determination also exists. A popular hypothesis is that the need for one cell type determines the direction of stem cell differentiation. So it has been assumed that the cell flux is directed towards erythropoiesis at the expense of granulopoiesis after irradiation or bleeding in cell transplantation experiments,[55,56] or that it is directed towards granulopoiesis at the expense of erythropoiesis during hypertransfusion.[57] However, the arguments for these assumptions follow from changes of the ratio of erythropoietic and granulopoietic bone marrow cellularity (E/G ratio), since the direction of stem cell determination itself cannot be measured. It is obvious that the E/G ratio depends not only on the influx into the two lineages, but also on many other variables (stimulation by erythropoietin or CSF, different amplification, or different transit times of erythropoietic and granulopoietic precursors). As the model analysis will show,[14] the changes of the E/G ratio can also be explained for a fixed partitioning of the stem cell efflux. Therefore, we abandon regulation of the direction of cell flux although we cannot exclude its existence principally.

K. Thrombopoiesis

Thrombopoiesis is neglected at this stage of the model. However, the long-range feedback loop (blood-bone marrow) has already been analyzed separately[42,44] and will be incorporated in the present model as a next step.

L. Negative Feedback — Positive Feedback

Although the model is valid for numerous interpretations of cellular development, it is quite restrictive as far as the negative feedback structure is concerned. Negative feedback means that a decreased number of controlling cells influences the regulatory system such that this cell number will increase. Correspondingly, an enlarged cell number influences the system such that the cell number will decrease. Negative feedback exists for the autoregulation of the stem cells (feedback loop I) as well as for the long-range regulation from blood to bone marrow (feedback loop III). In addition it can be shown that negative feedback must also be effective for the intramedullary short-range regulation (feedback loop II). Positive feedback in this loop (where an enlarged number would lead to further increase and vice versa) leads to unstable oscillations even after small perturbations.[12]

M. Relative Cell Numbers

Until now we do not know exactly the absolute cell numbers of any hemopoietic cell stage present in an animal, not even in the normal steady state. The early populations, in particular, are only approximately quantitative in terms of the number of generations and cell cycle times, and the measured population sizes may differ by a factor of 10 from one study to the next. How can one be so optimistic to believe that one can find reliable results in the even more complex situation of nonsteady-state situations?

The answer lies in the use of relative cell numbers. The model does not compare absolute numbers of different cell types (e.g., erythropoietic and granulopoietic progenitor cells), but only numbers of the same cell type in different situations. Thereby, it is possible to refer the cell numbers in each compartment to the normal steady-state values. Thus, statements like "in hypoxia the late erythropoietic progenitors increase to three times the normal value on day 3" are possible without knowing the normal value in absolute cell numbers. Even if one uses different absolute values in different simulations, the relative effects of a perturbation may, nevertheless, be very similar. This corresponds to the experience that the outcome of the same experiment performed in different laboratories or with different animal strains may differ quite significantly in absolute cell numbers, but may be very similar if expressed in relative terms (e.g., in percent of control values). The same holds true in model calculations and is, in fact, one important basis for the model analysis of regulated systems.

N. Influence of the Mathematical Description

A mathematical model is the translated version of biological knowledge or biological hypotheses, formulated in the language of mathematics. However, this translation is not unique and different mathematical formulations of the same biological phenomena are possible. How does the specific formulation influence the results?

A complete answer to this question cannot be given here. Concerning the model's sensitivity towards parameter variation we find a relatively great robustness. Changes of individual parameters by 10 to 20% can be tolerated.[13] Concerning structural stability, the answer is more difficult. We have tried a number of different mathematical descriptions[4,5] (e.g., differential equations with time delay, different formulas for "a_s" and "p", different description of EP effects) and always come to similar model curves. This may indicate that the basic model results are independent of the particular mathematical description as long as the regulatory assumptions are not changed.

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Chapter 4

STRUCTURE OF THE MODEL*

Markus Loeffler and H.-Erich Wichmann

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I. ABSTRACT

A detailed theoretical description of the hemopoietic stem cell model is presented. It includes the mathematics of the model, the parameter estimation, and the computation techniques. Furthermore, a sensitivity analysis of the important critical parameters is performed.

It is vain to do with much, what can be done with fewer.

William Occam
Medieval Philosopher

It is the theory that decides what can be observed.

Albert Einstein

II. INTRODUCTION

The model description, as presented here, gives mathematical and technical details. This chapter is primarily designed for the biomathematician and theoretical biologist.

The chapter is subdivided into six sections: the mathematics of the standard model are described in Section III (Table 1). In Section IV, parameters of the standard model are derived and problems of relating them to actual measurements are discussed. The results are summarized in Table 2. Section V deals with the techniques to simulate specific experiments, while Section VI gives some information regarding the computational procedures. In Section VII we describe what would happen if some critical model parameters were given different values. This sensitivity analysis will motivate the choice of important but unknown model parameters and demonstrates their influence on the regulatory system considered in the model. Finally, Section VIII gives a short historical review of the development of the model, indicating its stepwise evolution.

III. THE STANDARD MODEL

The standard model (Figure 1) considers stem cells, erythropoietic and granulopoietic progenitors and precursors, and two essential regulatory functions (self-renewal probability, "p", and fraction, "a_S", of stem cells in active cell cycle). In addition, the influence of erythropoietin (EP) on erythropoietic amplification is considered. The term "standard model" refers to the mathematical form as used throughout this volume. For the simulation of experiments, however, specific modifications of the standard model are necessary. These do not alter any of the assumptions or parameters but are needed to characterize the perturbations of hemopoiesis which are experimentally induced. We will illustrate this facet in more detail in Section V.

A. Model Compartments

The mathematical description of the hemopoietic stem cell model is based on the compartment concept. We distingush six different compartments: one for hemopoietic stem cells

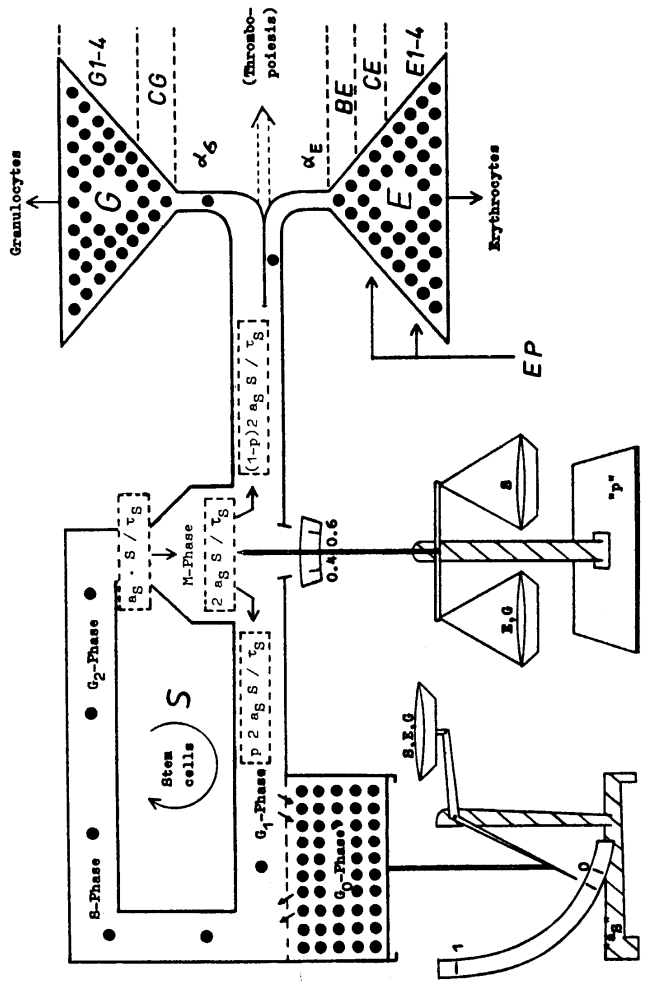


FIGURE 1. Scheme of the model, displaying the model compartments and the main regulatory processes (see, also, Reference 7). The formulas refer to the cell fluxes.

Table 1
MODEL EQUATIONS AND NORMAL STEADY STATE VALUES

Compartments (absolute numbers)	Differential equations	Normal values					Equation number
	$\dot{C}^* = \dot{C}^{*in} - C^*/T_C$, $\dot{C}^{*out} \equiv \dot{Z}_C^{out}$ C^*/T_C	T_C[a] (hr)	n_C	a_C	T_C^m (hr)	C^*_{norm}[b]	
$S^* = S$	$\dot{S} = (2p - 1)\, S/T_S$; $\dot{S}^{out} = (1 - p)\, S/T_S$[c]	53.3	—	0.15	0	1.00	1
BE^*	$\dot{BE}^* = \alpha_E * \dot{Z}_{BE}^{in} * \dot{S}^{out} - BE^*/T_{BE}$	120	5	0.33	0	2.11	2
CE^*	$\dot{CE}^* = \dot{Z}_{CE}^{in} * \dot{BE}^{out} - CE^*/T_{CE}$	40	5	1	0	22.32	3
$E1\text{-}4^*$	$\dot{E1\text{-}4}^* = \dot{Z}_{E1-4}^{in} * \dot{CE}^{out} - E1\text{-}4^*/T_{E1-4}$	72	6	1	24	2177.18	4
CG^*	$\dot{CG}^* = \alpha_G * \dot{Z}_{CG}^{in} * \dot{S}^{out} - CG^*/T_{CG}$	240	10	0.33	0	372.00	5
$G1\text{-}4^*$	$\dot{G1\text{-}4}^* = \dot{Z}_{G1-4}^{in} * \dot{CG}^{out} - G1\text{-}4^*/T_{G1-4}$	72	4	1	40	4147.20	6
	$E^* = BE^* + CE^* + E1\text{-}4^*$	232	16	—	24	2201.71	7
	$G^* = CG^* + G1\text{-}4^*$	312	14	—	40	4519.20	

[a] The numerical values for T_C are obtained by Formulas 9 and 10 (see below) under the assumption that all cell cycle times are 8 hr (τ_C = 8 hr for all compartments).

[b] To achieve these values $\dot{C}^* = 0$, $p = 0.5$, $\alpha_E = 0.15$, $\alpha_G = 0.8$ was assumed. The latter two parameters are only relevant for the absolute numbers shown here.

[c] Stem cells do not properly fit into the scheme of influx and efflux because they generate a cell efflux $\dot{S}^{out}$ without a real influx. The derivation of these equations will be discussed below.

(S); three for the erythropoietic cells: progenitors (BE, CE) and precursors (E1-4); and two for the granulopoietic cells: progenitors (CG) and precursors (G1-4). The sum of erythropoietic progenitors and precursors is denoted by E, the corresponding sum of the granulopoietic cells by G. However, due to the amplification in both lineages, E and G can be identified with the precursors E1-4 and G1-4 (see below).

In the following the absolute cell count in any compartment C will be symbolized by C*. C may stand for S, BE, CE, E1-4, CG, or G1-4. For the normal absolute cell count, the additional subscript "norm" will be used: C^*_{norm}. The relative compartment size will be denoted by C ($= C^*/C^*_{norm}$) without any super- or subscript (e.g., BE = BE^*/BE^*_{norm}). These relative numbers as derived from the model will, in later chapters, be compared to experimental data which are also expressed as fraction of normal. The use of relative values has an advantage which will become more obvious later: it allows us to leave several model parameters unspecified because they only affect the absolute cell count.

Table 1 summarizes the model Equations 1 to 7, which will now be discussed in more detail.

Each model compartment (S, BE, CE, E1-4, CG, G1-4) is described by one first-order differential equation with random transition of the following type:

$$\dot{C}^* = \dot{C}^{*in} - C^*/T_C \tag{8}$$

$\dot{C}^{*in}$ denotes the influx rate (per hour) into compartment C. T_C can be interpreted as the average transit time through compartment C. It may have two components, one for the proliferating part, denoted by T_C^p, and one for the maturing (postmitotic) part, denoted by T_C^m:

$$T_C = T_C^p + T_C^m \tag{9}$$

T_C^p is identical to the product of the number of amplifying divisions, n_C, and the average generation time for each division, T_C^g. The latter can be expressed as the ratio of the cell cycle time, τ_C, and the proliferative fraction, a_C. In total we find

$$T_C = n_C * T_C^g + T_C^m = n_C * \tau_C/a_C + T_C^m \tag{10}$$

T_C^m is the maturation time of nondividing cells which only are found in the precursor compartments G1-4 and E1-4.

The obvious way to describe the flux of cells through concatenated pools would be the use of "first in first out" transitions. For this approach, one would find the steady-state relation

$$C^* = [(2^{nC} - 1) * T_C^g + 2^{nC} * T_C^m] * \dot{C}^{*out}_{-1} \tag{11a}$$

Here C* is the cell number in compartment C and $\dot{C}^{*out}_{-1}$ denotes the efflux from the preceding compartment. Formula 11a is valid if n_C consecutive mitoses occur, with the generation time T_C^g, and if the cells spend thereafter the postmitotic time T_C^m in the compartment. In this situation, the efflux from compartment C, $\dot{C}^{*out}$, is larger than the efflux from the preceding compartment, $\dot{C}^{*out}_{-1}$, by a factor of 2^{nc}:

$$\dot{C}^{*out} = 2^{nC} * \dot{C}^{*out}_{-1} \qquad (11b)$$

Mathematically, this approach may be realized by differential equations with time delay.

Unfortunately, this obvious description does not consider adequately the large variance of the cellular parameters experimentally found. Therefore, in the following, the cell compartments are characterized by ordinary differential equations (Equation 8), although this method provides some mathematical problems.[1,2] In our approach the amplification 2^{nc}, which in reality is distributed over the whole compartment, will be artificially concentrated at the "entrance" and at the "exit" of the compartment. The appropriate input and output amplification correction factors Z_c^{in} and Z_c^{out} are linked together in the following way:

$$\dot{C}^{*in} = Z_C^{in} * \dot{C}^{*out}_{-1} \qquad (11c)$$

$$\dot{C}^{*out} = Z_C^{out} * C^*/T_C \qquad (11d)$$

$$Z_C = Z_C^{in} * Z_C^{out} = 2^{nC} \qquad (11e)$$

These formulas indicate that the efflux from the preceding compartment, $\dot{C}^{*out}_{-1}$, is amplified by Z_C^{in} as soon as the cells enter C. Immediately before they leave compartment C, they are amplified by Z_C^{out}. By this subdivision of the total amplification Z_C the equilibrium conditions (Formulas 11a and 11b) are fulfilled if one chooses

$$Z_C^{in} = (2^{nC} - 1) * T_C^g/T_C + 2^{nC} * T_C^m/T_C \qquad (11f)$$

In an earlier version of the model[3] the "first in first out" transition of Formula 11a was used instead of the random description using Formulas 8 and 11c to 11f. A more general derivation of both concepts from partial differential equations is possible.[3-6]

When hemopoietic stem cells lose their ability of self-renewal they become differentiated cells. Of those stem cells which differentiate, the fraction α_E develops erythropoietic and fraction α_G granulopoietic progeny. This decision for a unipotent fate will be called determination, and the parameters α_E and α_G will be called determination fractions. Throughout all calculations they will be kept at constant values. The determination fractions are not regulated. Therefore, these parameters only play a role for the calculation of the absolute compartment contents. Their values are irrelevant as long as relative cell numbers are used.

The normal absolute numbers C^*_{norm} in Table 1 follow from the steady-state condition $\dot{C}^* = 0$.

B. S: Pluripotent Stem Cells

Figure 1 indicates how the differential equation for S can be derived. Proliferatively active are a_S^* S cells. The cycle time of proliferating cells is τ_S. Therefore, a_S^* s/τ_S cells enter mitosis per unit time. Doubling gives a factor of 2. Of these cells the fraction (1 − p) leaves the stem cell compartment and differentiates, while the fraction p remains. Thus, the $2p^*a_S^*$ S/τ_S cells replace the a_S^* S/τ_S stem cells which have entered mitosis, per unit time. This yields the equation:

$$\dot{S} = (2p - 1) * S * a_S/\tau_S = (2p - 1) * S/T_S$$

with $T_S = \tau_S/a_S$. The rate of cells differentiating then equals

$$\dot{S}^{out} = 2(1 - p) * S * a_S/\tau_S = 2(1 - p) * S/T_S$$

As described in Chapter 3[7] the normal steady-state values for the model are chosen as τ_S = 8 hr, a_S = 0.15, p = 0.5. The normal absolute compartment value is arbitrarily set to be S_{norm} = 1. Therefore, relative and absolute values are identical (S* = S). This will be different for the following compartments. As will be discussed below, "a_S" and "p" are not constant but may vary between 0.01 and 1 and 0.4 and 0.6, respectively; "a_S" and "p" are independent of each other. This is an important feature showing that in the model the process of cell cycling is distinct from the process of self-renewal.

C. BE: Early Erythropoietic Progenitors

BE is the most immature erythropoietic population. Of all stem cells differentiating, the fraction α_E = 0.15 enters BE. With mitoses taking place every 8 hr for a whole of five mitoses, the minimum transit time is 40 hr. We assume that normally only one third of the cells is in cycle (a_{BE} = 0.33) so that the average transit time equals T_{BE} = 40 hr/0.33 = 120 hr. We will discuss below that "a_{BE}" is variable between 0.30 and 1.

D. CE: Late Erythropoietic Progenitors

CE is the successor of BE and represents a more mature erythropoietic progenitor compartment. It is fed by BE and normally has five mitoses with a cell cycle time of 8 hr. So the minimum transit time is again 40 hr. In contrast to BE, however, it is assumed that all cells are actively cycling (a_{CE} = 1), so that the average transit time T_{CE} is 40 hr. As will be discussed below in more detail, the number of mitoses in CE may vary between zero and seven depending on the level of Epo.

E. E1-4: Erythopoietic Precursors

E1-4 represent the morphologically identifiable erythropoietic precursors. They are fed by CE, normally have six mitoses with a cycle time of 8 hr, a proliferative fraction of $a_{E1\text{-}4}$ = 1, and a maturation time of 24 hr. In total the average transit time equals $T_{E1\text{-}4}$ = 48 hr + 24 hr = 72 hr. Again the number of mitoses is dependent on EP. As will be shown below it may vary between zero and six.

F. E: All Erythropoietic Bone Marrow Cells

Here, all erythropoietic progenitor and precursor cells, as defined above, are added. Using the absolute cell numbers, we define

$$E = (BE^* + CE^* + E1-4^*)/(BE^*_{norm} + CE^*_{norm} + E1-4^*_{norm}) \qquad (12a)$$

Since in E all individual cells have the same influence and since the later cells in E1-4 are much more numerous than the earlier cells, due to mitotic amplification, the behavior of E reflects mainly the behavior of E1-4.

The total erythropoietic pathway in the model includes 16 divisions and a total transit time of 232 hr (almost 10 days).

G. CG: Granulopoietic progenitors

CG represents the population of granulopoietic progenitors. It is constructed similarly to BE with a proliferative fraction "a_{CG}" that varies between 0.3 and 1 and has the normal value 0.33. The number of mitoses is assumed to be 10. Consequently, the average transit

time is T_{CG} = 80 hr/0.33 = 240 hr. The fraction of granulopoietic determination, α_G, equals 0.8. (The rest enters erythropoiesis (α_E = 0.15) or thrombopoiesis.)

H. G1-4: Granulopoietic Precursors

G1-4 represent the morphologically identifiable granulopoietic precursors. They are fed by CG, normally have mitoses every 8 hr for a total of four mitoses, and a subsequent maturation time of 40 hr. In total the average transit time is $T_{G1\text{-}4}$ = 32 hr + 40 hr = 72 hr. In contrast to E1-4 peripheral influences on the number of mitoses in G1-4 are not considered in the model. Where it was necessary to simulate granulopoietic stimulation another approach was chosen (see Section V).

I. G: All Granulopoietic Bone Marrow Cells

Here all granulopoietic progenitor and precursor cells are added:

$$G = (CG^* + G1-4^*)/(CG^*_{norm} + G1-4^*_{norm}) \tag{12b}$$

Similar to E, G represents mainly G1-4 since they are the largest subpopulation. Total granulopoiesis includes 14 mitoses and a total transit time of 312 hr (13 days).

J. Regulatory Dependencies

1. "p": Self-Renewal Probability

As already discussed in Chapter 3[7] the self-renewal probability "p" of stem cell is a regulatory function which depends on S, E, and G. Mathematically, we denote this dependency in the following way:

$$p = p(Y) \text{ and } Y = Y(S,E,G) \tag{13a}$$

with

$$p(Y) = p_1 * \frac{\exp(-Y) - \exp(Y)}{\exp(-Y) + \exp(Y)} + 0.5$$

$$p_1 = 0.1 \tag{13b}$$

and

$$Y(S,E,G) = YS(S) + YE(E) + YG(G) \tag{13c}$$

$$YS(S) = p^S * (S - 1) \tag{13d}$$

$$YE(E) = p^E * (E - 1) \tag{13e}$$

$$YG(G) = p^G * (G - 1) \tag{13f}$$

p^S, p^E, and p^G are called "weighting factors" and indicate the relative influence of S, E, and G on "p". While p^S is a function:

$$p^S = \begin{cases} \dfrac{+2}{S^{0.6}} & \text{for } S \leq 1 \\ +2 & \text{else} \end{cases} \tag{13g}$$

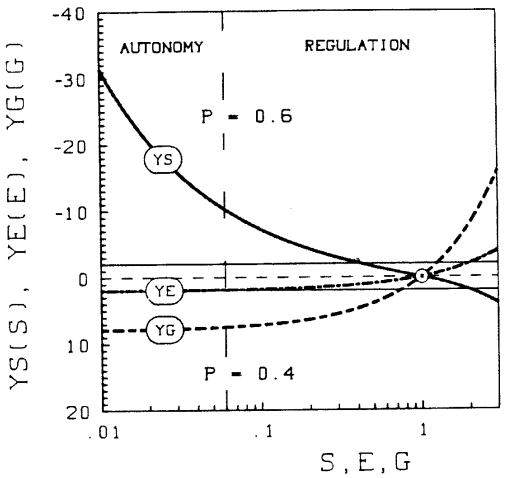


FIGURE 2. Auxiliary functions YS(S), YE(E), YG(G) of the self-renewal probability "p" (see Equations 13d to 13f). For reduced values of S, E, or G the functions YE and YG reach positive plateaus while YS shows a continuous decrease (note inverted scale). For any triple of S, E, and G values, the corresponding values of YS, YE, and YG can be derived from the figure. By adding them graphically, one can estimate the value for "p". It can be seen that the "regulatory window" of "p" (values between 0.6 and 0.4) is relatively small. The borderline between autonomy and regulation is determined by the condition YS(S) = −max (YE + YG). The critical value is S = 0.06. For lower values of S, p = 0.6, independent of E and G.

$$p^E = -2 \text{ and } p^G = -8 \tag{13h}$$

are constants.

The shape of p(Y) is sigmoidal, asymptotically reaching p = 0.6 for negative Y and p = 0.4 for positive Y. The Equation (13b) was chosen because it is symmetric in its asymptotic behavior. However, any other function with a similar characteristic could also be used (Figure 3, Chapter 3) shows the function p(Y). In normal steady state we find S = E = G = 1, Y = 0, and p(Y) = 0.5.

The idea for introducing the function Y(S, E, G) is to correlate the influences of S, E, and G in an additive way. For this purpose we multiply the deviations from the normal (relative) value (e.g., [S − 1]) with weighting factors p^S, p^E, and p^G and add these expressions.

First it is important to comment on the signs of the weighting factors; p^S is positive, p^E and p^G are both negative. Therefore, a reduction of S acts opposite to a reduction of E or G. In Figure 1 this is symbolized by a balance with S on one side and E and G on the other. While p^E and p^G are constants, p^S is a function (Equation 13g): for values of S above normal p^S equals 2. For subnormal values of S its value increases (e.g., for S = 0.06 p^S = 10). If one reduces S, E, and G in parallel the functions YS, YE, and YG change monotonously as shown in Figure 2. YS becomes increasingly negative for diminished S, while YE and YG asymptotically reach their maximum of 2 and 8, respectively.

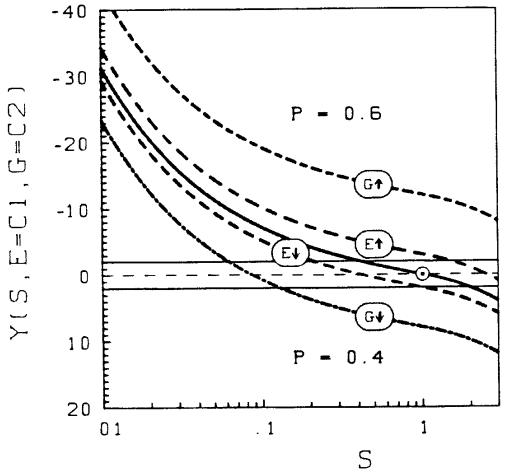


FIGURE 3. Auxiliary function Y(S,E,G) of the self-renewal probability "p" (see Equation 13c). For constant values of E and G the dependence of the function Y(S, E = C1, G = C2) on S is illustrated. The following constants are used: C1 = C2 = 1: full line; C1 = 2.5, C2 = 1: E ↑; C1 = 1, C2 = 2.5: G ↑; C1 = 0.01, C2 = 1: E ↓; C1 = 1, C2 = 0.01: G ↓.

If the stem cells are reduced below 0.06, the absolute value of YS becomes larger than the sum of the maximum values of YE and YG. In this domain the regulatory influence of the stem cells on the self-renewal probability dominates all feedback influences from differentiated cells. We call it the "domain of autonomy" because the stem cells determine "p" and, thus, their own recovery irrespective of the need for differentiated cells.

For any combination of S, E, and G one can construct the sum Y = YS + YE + YG. A negative value of Y means that p(Y) exceeds 0.5 so that the stem cell number grows. For positive Y, p is below 0.5 and S will decrease. It is interesting to see that for Y larger than 2 or smaller than −2, p remains constant at 0.4 or 0.6, respectively (shaded areas).

In Figure 3 the function Y(S,E,G) [= YS(S) + YE(E) + YG(G)] is evaluated as depending only on S considering constant values for E and G (either 0.01, 1.0, or 2.5). An increase in E or G shifts the curves upwards and the point of steady state (Y = 0) moves to higher values of S. A reduced number of E or G provokes the opposite. Here the steady state is reduced. The curves are shifted more by alterations in G than in E. This is understandable since $p^G:p^E$ = 4:1.

2. "a": Fraction of Cells in Active Cell Cycle (Proliferative Fraction)

The proliferative fractions "a_S", "a_{BE}", and "a_{CG}" depend on S, E, and G. Mathematically, we denote this dependency in the following way:

$$a = a(X) \text{ and } X = X(S,E,G) \tag{14a}$$

with

$$a(X) = \frac{a_1 * \exp(-X') + a_2 * \exp(X')}{\exp(-X') + \exp(X')} \tag{14b}$$

$$X' = a_3 * X + a_4 \tag{14c}$$

$$X(S,E,G) = XS(S) + XE(E) + XG(G) \tag{14d}$$

$$XS(S) = a^S * \begin{cases} \ln S & \text{for } S \leq 1 \\ (S - 1) & \text{else} \end{cases} \tag{14e}$$

$$XE(E) = a^E * \ln E \tag{14f}$$

$$XG(G) = a^G * \ln G \tag{14g}$$

a^S, a^E, and a^G indicate the relative influence of S, E, and G on "a". They are constant:

$$a^S = 1, \quad a^E = 0.3, \quad a^G = 0.1 \tag{14h}$$

Formulas 14a to 14h are valid for "a_S", "a_{BE}", and "a_{CG}". Only the coefficients a_1, a_2, a_3, a_4 are different:

$$a_1 = 1, \quad a_2 = 0.01, \quad a_3 = 1.106, \quad a_4 = 0.867 \quad \text{for "}a_S\text{"} \tag{14i}$$

$$a_1 = 1, \quad a_2 = 0.3, \quad a_3 = 0.489, \quad a_4 = 1.553 \quad \text{for "}a_{BE}\text{" and "}a_{CG}\text{"} \tag{14j}$$

The motivation for these formulas is similar to that of "p"; "a" reaches maximum and minimum values asymptotically for negative and positive X, respectively. X is the sum of the three influences from S, E, and G (Equation 14d). While for "p" the difference of the relative cell number from the normal value is used [e.g., YE = $a^E * (E - 1)$], here the difference of the logarithm of the relative cell number from the logarithm of the normal value is used [e.g., XE = $a^E * (\ln E - \ln 1) = a^E \ln E$]. As S, E, or G approach zero the logarithm may become very large. To avoid artificial problems of this type, we replace S, E, and G in Equations 14e to 14g by 0.001 if they decrease below this value. (Note that Equations 14a to h, if inserted into each other, can be written as a rational function. This was not presented in order to keep the structural similarity between Equations 3 and 14 visible.)

Only for S > 1.0 was a different approach retained in order to provide an effective suppression of cell cycling if the stem cell number is enlarged. The factors a^S, a^E, a^G are called weighting factors because they quantify the relative importance of the different feedback influences. The superscripts indicate that S, E, and G have impact on the a-functions, while subscripts as in "a_S", "a_{BE}" indicate that the a-functions become effective in the compartments S and BE. For "a_S" the constants a_1, a_2, a_3, a_4 can be determined if one assumes that "a_S" has a maximum of 1, a minimum of 0.01, a normal value of 0.15, and an intermediate value a_S (0.5, 1, 1) = 0.45. Similarly, a_1, a_2, a_3, a_4 are determined for a_{BE} and a_{CG} from 1, 0.3, 0.33, and 0.66.

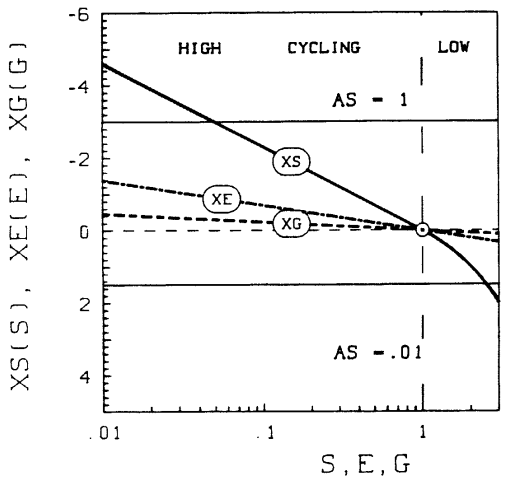


FIGURE 4. Auxiliary functions XS(S), XE(E), XG(G) of the proliferative fraction "a_s" (see Equations 14e to 14h). For reduced values of S, E, or G the functions XS, XE, and XG decrease monotonously. For any triple of S, E, and G values the corresponding values of XS, XE, and XG can be derived from the figure. By adding them graphically, one can find the corresponding "a_s". The "regulatory window" of "a_s" is much larger than of "p".

Figures 4 and 5 show how X depends on S, E, and G. Figure 4 shows the behavior of XS, XE, XG separately if S, E, G are reduced. Apparently the curves differ in amplitude but not in sign. Any reduction of S, E, or G leads to a decrease of X and, thus, to an acceleration in cycling.[7,8] Maximum cycling is reached for $X < -3$; minimum cycling is reached for $X > 1.5$. Figure 5 illustrates this in a different way. Here X depends on S with fixed values for E and G (either 0.01, 1.0, or 2.5). If E or G are smaller than 1 the curves are shifted upwards and vice versa. Figure 5 demonstrates the influence of the differentiated cell stages on the activity of stem cell cycling. High numbers depress, reduced numbers activate. The influence of E is greater than the influence of G.

3. How "p" and "a_s" Determine the Way of Regulation

The importance of "p" and "a_s" can be illustrated in two further figures which give some deeper insight into the model mechanisms. For this purpose we examine two stem cell quantities which depend on both parameters "p" and "a_s": the time derivative $\dot{S}$ and the differentiation rate $\dot{S}^{out}$.

Figure 6 shows how the time derivative $\dot{S}$ depends on SE, and G. E and G are fixed and it is demonstrated how $\dot{S}$ varies with S. Positive values for $\dot{S}$ indicate that the stem cell number is growing; negative values characterize a decrease.

Similarly, Figure 7 shows the efflux rate $\dot{S}^{out}$ of cells leaving the stem cell compartment and entering differentiation. Again $\dot{S}^{out}$ is related to a change in S with E and G being fixed to either 0.01, 1.0, or 2.5.

Figures 6 and 7 shall be discussed in parallel. The full lines represent normal values of E and G. If the stem cell number is slightly reduced, two effects occur: the differentiation

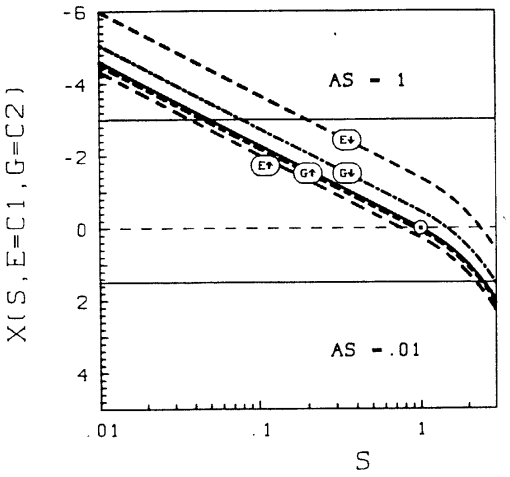


FIGURE 5. Auxiliary function X(S,E,G) of the cycling fraction "a_s" (see Equation 14d). For constant values of E and G the dependence of X(S, C1, C2) on S is shown. Symbols as in Figure 3.

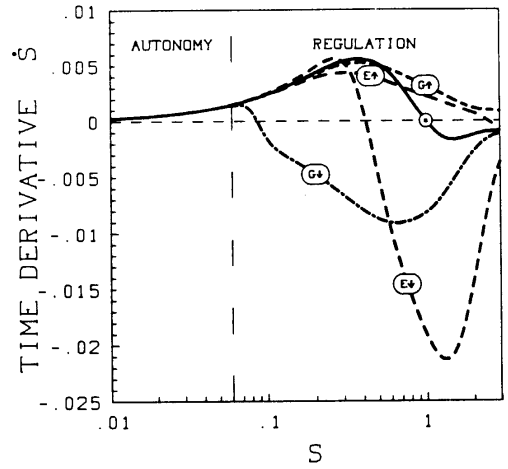


FIGURE 6. Time derivative $\dot{S}(S,E,G)$. The time derivative of the stem cells depends on S, E, and G [$\dot{S} = (2p[S,E,G] - 1) * S * a_s(S,E,G)/\tau_s$]. For constant values C1 and C2 inserted in E and G it is shown how $\dot{S}(S,C1,C2)$ behaves as a function of S (C1 and C2 are chosen as in Figure 3). Positive values for $\dot{S}$ indicate that the number of stem cells is growing. This is always the case in the autonomous domain. Decreasing stem cell counts (negative $\dot{S}$) can be found either if E or G are reduced (differentiation pressure) or if S is enlarged (no need for stem cells).

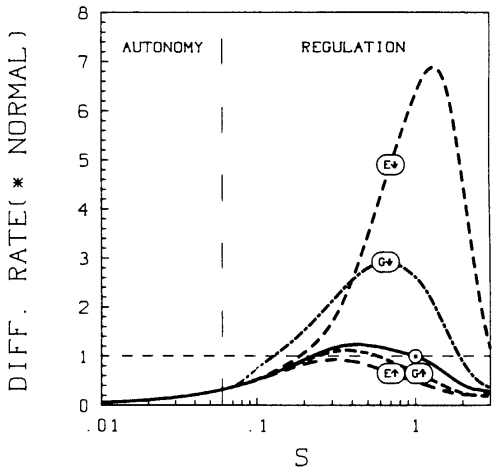


FIGURE 7. Differentiation rate $\dot{S}^{out}(S,E,G)$ (see Equation 16). The differentiation rate of stem cells, $\dot{S}^{out}$, is a function of S, E, and G[$\dot{S}^{out} = 2(1 - p[S,E,G] * S * a_S(S,E,G)/\tau_S$]. For constant values C1 and C2 inserted in E and G it is shown how $\dot{S}^{out}(S,C1,C2)$ behaves as a function of S. (C1 and C2 are chosen as in Figure 3.) Low E and G increase the differentiation rate and vice versa.

rate $\dot{S}^{out}$ increases so that more cells than normal enter into differentiation. In addition, the derivative $\dot{S}$ adopts positive values indicating that the stem cell number grows and recovers. If S is more severely reduced the differentiation rate and $\dot{S}$ approach zero.

If E or G are kept at low values the pattern changes: self-renewal of stem cells is reduced and a larger rate of differentiation is induced. Elevated values of E or G, in contrast, lead to a higher self-renewal ($\dot{S}$ positive, Figure 6) and a suppression of differentiation (Figure 7.)

For S between 0.06 and 3, Figures 6 and 7 illustrate that a broad spectrum of model reactions is possible. In contrast to this, for values of S below 0.06 the picture becomes very uniform. All five curves show the same behavior with positive $\dot{S}$ and a differentiation rate $\dot{S}^{out}$ below normal. In this domain of autonomy the stem cells recover independent of E or G.

The behavior of S for numbers larger than 3 is not considered in this volume. For completeness it should, however, be stated that $\dot{S}$ will be asymptotically negative indefinite for increasing S so that large stem cell counts are forced to drop towards normal values. In general, the whole model is only valid for $S \in [0.001,3]$, $G \in [0.001,2.5]$, and $E \in [0.001,5]$.

4. Erythropoietic Amplification, Stimulation by EP

Two compartments, CE and E1-4, are assumed to be EP-sensitive. In CE high EP levels may induce two additional mitoses. If EP is missing all five normal mitoses are omitted and only a maturation compartment without amplification remains. For E1-4 no additional mitoses are considered. This is a simplification of the biological situation (compare Chapter 3). It is known (e.g., analyzed by Wulff[9]) that high EP levels induce one additional mitosis in the erythroblast stage but reduce the transit time to about half of normal. Taking both effects together the number of erythroblasts does not change. This provides the rationale to assume

that the normal and maximum values for the amplication factor $Z_{E1\text{-}4}$ are almost identical. This simplification was introduced because incorporation of subsequent compartments (reticulocytes, etc.) was not intended. Normally six mitoses take place in E1-4 which may be omitted if EP is missing.

In total 5 + 6 = 11 mitoses may be omitted in the erythropoietic pathway so that erythropoiesis may be reduced by over a factor of 1000. However, even during suppression erythropietic maturation still goes on but at a very low level.

The number of mitoses increases to maximum values for high EP and drops to minimum values for low EP. As only normal and extreme values are known there is no direct way to extract the EP dose response curve from available data. However, a good interpolation can be found by exponential functions of the following type:

$$Z(EP) = Z_{max} - (Z_{max} - Z_{min}) * \exp(-Z_1 * EP^b) \tag{15a}$$

The parameters Z_{min}, Z_{max}, Z_1 can be derived from the minimum, maximum, and normal number of mitoses n_C in CE and E1-4:

$$Z_{min} = 2^{\min(nC)} \tag{15b}$$

$$Z_{norm} = 2^{\text{norm}(nC)} \tag{15c}$$

$$Z_{max} = 2^{\max(nC)} \tag{15d}$$

$$Z_1 = \ln\left[\frac{Z_{max} - Z_{min}}{Z_{max} - Z_{norm}}\right] \tag{15e}$$

Parameter b influences the asymptotic behavior. It is chosen to be 0.7, a figure which was proposed by Wulff[9] from models of the erythropoietic pathway.

As one knows the "theoretical" minimum, normal, and maximum numbers of n_{CE}, (0, 5, 7), and $n_{E1\text{-}4}$, (0, 6, 6.02), one can deduce Z^{in}_{CE}, $Z^{in}_{E1\text{-}4}$, Z^{out}_{CE}, $Z^{out}_{E1\text{-}4}$ according to Equations 11e and 11f and 15a.

Figure 8 gives an illustration of the total amplifications Z_{CE} and $Z_{E1\text{-}4}$ calculated according to Equation 11f as products of $Z^{in} * Z^{out}$. Since in the model calculations EP is restricted to values 0.01 and 100 times normal, Z_{CE} may practically vary between 1.5 and 128 while $Z_{E1\text{-}4}$ may decrease to 11 or increase to 65 which corresponds to "practical" minimum numbers of n_{CE} and $n_{E1\text{-}4}$ of 0.5 and 3.5, respectively.

IV. PARAMETER DETERMINATION FOR THE STANDARD MODEL

It is necessary to outline which of the parameters in Equations 1 to 15 are based on biological data, which are "reasonable" *a priori* or practically irrelevant, and which are speculative.

Parameters necessary for modeling of hemopoiesis of stem cells, progenitors, and precursors have been collected and reviewed by Loeffler[3] and Wulff.[9] The essentials of these reviews are summarized in Table 2.

In general, the median of the data is chosen as the model value. However, sometimes this value has been rounded and, wherever it was possible, identical values were chosen for analogous quantities (e. g., identical cell cycle times of 8 hr in all compartments). We do

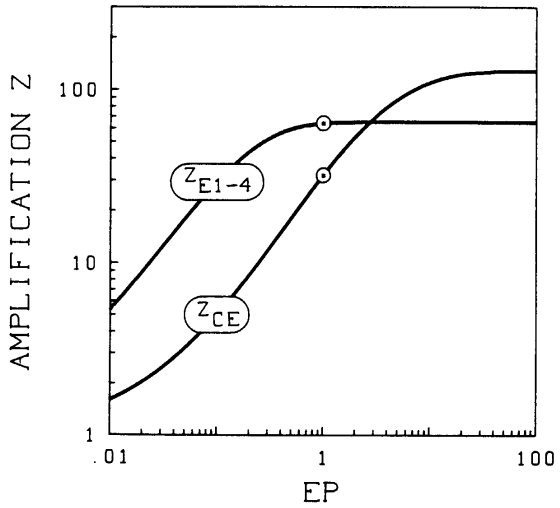


FIGURE 8. Dose-response curves for erythropoietic amplification Z(EP) (see Equations 15a to 15e). The amplification of erythropoietic progenitors, $Z_{CE}(EP)$, and precursors $Z_{E1-4}(EP)$ depends on EP. In the normal steady state (EP = 1) $Z_{CE}(1) = 32$ and $Z_{E1-4}(1) = 64$, which corresponds to five and six amplifying mitoses, respectively. As can be seen, the "proliferative reserve" (for enlarged EP) is compared to the number of mitoses which may be omitted for low values of EP. An amplification of Z = 1 means that cells may leave the compartment without having experienced a mitoses during their passage. For practical purposes EP is restricted to the interval [0.01, 100].

Table 2
MODEL PARAMETERS AND AUXILIARY QUANTITIES (ONLY DATA FROM MICE ARE TAKEN INTO ACCOUNT)

Cell stage quantity	Model symbol	Model value	Median (range) of data	Ref.
S/CFU-S				
Cell cycle time	τ_S	8 hr	8 hr (6—10 hr)	9—14
"a_S" Proliferative fraction				
Under normal conditions	Norm (a_S)	0.15	0.08[a] (0.01—0.22)	14—27
In regenerating marrow (maximum)	Max (a_S)	1.0	0.50[a] (0.45—0.60)	14, 16, 18, 20, 23, 24, 26, 28—31
In the spleen of PHZ-treated animals (minimum)	Min (a_S)	0.01	0.04[a] (0.0—0.05)	30, 31
Intermediate (50% reduction of CFU-S)	$a_S(0.5, 1, 1)$	0.45	0.27[a] (0.07—0.50)	23, 28—31[b]

Table 2 (continued)
MODEL PARAMETERS AND AUXILIARY QUANTITIES (ONLY DATA FROM MICE ARE TAKEN INTO ACCOUNT)

Cell stage quantity	Model symbol	Model value	Median (range) of data	Ref.
Coefficients of "a_S"	a_1, a_2, a_3, a_4	1.0, 0.01, 1.106, 0.867	—	Derived (see Equation 14i)
Weighting factors	a^S	1.	—	Speculative
	a^E	0.3	—	Speculative
	a^G	0.1	—	Speculative
"p" Self-renewal probability				
Normal steady state	Norm (p)	0.5		By definition of steady state
During maximum growth of stem cell numbers	Max (p)	0.60	0.61 (0.55—0.75)	10, 12, 22, 32—37[c]
During maximum decline of stem cell numbers	min (p)	0.40	0.35	38, 39[b]
Coefficients of "p"	exponent in p^S	$\begin{cases}0.6 \text{ if } S \leq 1\\0.0 \text{ else}\end{cases}$	0.7[d]	33
	p1	0.1		Derived (see Equation 13b)
	p^S	−2	—	Speculative
Weighting factors	p^E	2	—	Speculative
	p^G	8	—	Speculative
BE/BFU-E				
Cell cycle time	τ_{BE}	8 hr	—	Not available
"a_{BE}" Proliferative fraction				
Normal	norm (a_{BE})	0.33	0.30 (0.2—0.5)[a]	15, 40—43
Under maximum stimulation	max (a_{BE})	1.0	0.6 (0.4—0.8)[a]	15, 40, 41
Under maximum suppression	min (a_{BE})	0.30	0.25 (0.05—0.35)[a]	40—43
Intermediate	$a_{BE}(0.5, 1, 1)$	0.66	—	Interpolated
Coefficients of "a_{BE}"	a_1, a_2, a_3, a_4	1.0, 0.3, 0.489, 1.553		Derived (see Equation 14k)
Weighting factors	a^S	1.0	—	See above
	a^E	0.3	—	See above
	a^G	0.1	—	See above
Number of mitoses[b]	n_{BE}	5	6	12, 17, 44—47[c]
Normal transit time	T_{BE}	120 hr	—	Derived (see Equation 10)
CE/CFU-E				
Cell cycle time	τ_{CE}	8 hr	9.2 hr (6—16 hr)	12, 17, 48—52
"a_{CE}" Proliferative fraction				
Under normal conditions	norm (a_{CE})	1.0	0.73 (0.7—0.9)[a]	15, 27, 40—42, 53—55
Under stimulation	max (a_{CE})	1.0	0.78 (0.73—0.80)[a]	40, 41, 56

Table 2 (continued)
MODEL PARAMETERS AND AUXILIARY QUANTITIES (ONLY DATA FROM MICE ARE TAKEN INTO ACCOUNT)

Cell stage quantity	Model symbol	Model value	Median (range) of data	Ref.
Under suppression	min (a_{CE})	1.0	0.61 (0.50—0.69)[a]	40, 41, 43
Number of mitoses[b]				
Under normal conditions	norm (n_{CE})	5	4 (2.0—6.0)	15, 41, 42, 46, 57
Under erythropoietic stimulation	max (n_{CE}) − norm (n_{CE})	2[f]	1.6 (1.6—3.0)	15, 40, 45, 46, 58
Under erythropoietic suppression	min (n_{CE}) − norm (n_{CE})	−4.5[g]	− 2.5 − (1.5—3.5)	46, 59—64
Transit time	T_{CE}	40 hr	—	Derived (see Equation 10)
E1-4/ ERYPREC				
Cell cycle time	$\tau_{E1\text{-}4}$	8 hr	9 hr (7—24 hr)	48, 65—71
"$a_{E1\text{-}4}$"-Proliferative fraction	$a_{E1\text{-}4}$	1.0	—	Not available (see a_{CE})
Number of mitoses				
Under normal condition	norm ($n_{E1\text{-}4}$)	6	6 (6—7)	70—73
Under maximum erythropoietic stimulation	max (n_{CE} + $n_{E1\text{-}4}$) − norm (n_{CE} + $n_{E1\text{-}4}$)	2	1.6 (0.5—2.5)	15, 40, 41, 58, 60, 62, 74—80
→	max ($n_{E1\text{-}4}$) − norm ($n_{E1\text{-}4}$)	0.02[f]	—	Derived (see n_{CE})
Under minimum stimulation	min (n_{CE} + $n_{E1\text{-}4}$) − norm (n_{CE} + $n_{E1\text{-}4}$)	−7	−7.6 (−10—−5.6)	60, 62, 81, 82
→	min ($n_{E1\text{-}4}$) − norm ($n_{E1\text{-}4}$)	−2.5[g]	—	Derived (see n_{CE})
Transit time	$T_{E1\text{-}4}$	72 hr	82 hr (72—99 hr)	71—73, 83
CG/CFU-GM				
Cell cycle time	τ_{CG}	8 hr	≤12 hr	84—86
"a_{CG}" Proliferative fraction				
Under normal conditions	norm (a_{CG})	0.33	0.35 (0.20—0.50)[a]	87—91
Under stimulated conditions	max (a_{CG})	1.0	0.75 (0.70—0.80)[a]	90, 91[h]
Minimum	min (a_{CG})	0.30	—	Not available see a_{BE}
Intermediate	a_{CG}(0.5, 1, 1)	0.66	—	See a_{BE}
Coefficients	a_1, a_2, a_3, a_4	See a_{BE}		Derived (see Equation 14k)
Number of mitoses	n_{CG}	10	≤ 10—16	84, 86, 92
Weighting factors	a^S	1.0	—	See above
	a^E	0.3	—	See above
	a^G	0.1	—	See above
Normal transit time	T_{CG}	240 hr		Derived (see Equation 10)

Table 2 (continued)
MODEL PARAMETERS AND AUXILIARY QUANTITIES (ONLY DATA FROM MICE ARE TAKEN INTO ACCOUNT)

Cell stage quantity	Model symbol	Model value	Median (range) of data	Ref.
G1-4/Granulopoietic precursors				
Cell cycle time	$\tau_{G1\text{-}4}$	8 hr	12 hr (6—24)	92—94
"$a_{G1\text{-}4}$" Proliferative fraction	$a_{G1\text{-}4}$	1.0	0.50[a]	94
Number of mitoses	$n_{G1\text{-}4}$	4	4 ≥2[i]	93, 94
Maturation time of G4	T^m_{G4}	40 hr	23 hr	93
Transit time	$T_{G1\text{-}4}$	72 hr	65 hr	94
Determination rates				
Erythropoietic	α_E	0.15	≤0.15	46, 85
Granulopoietic	α_G	0.8	—	85

Note: Only data from mice are taken into account.

[a] Thymidine suicide results. They can only roughly be compared with the model quantity "a" according to a = min (2 * ^{3}HTdR-Kill, 1.).
[b] Values are estimated from data according to Loeffler.[3]
[c] Some data are determined from spleen colony growth.[10,22,32,36,37]
[d] Chervenick and Boggs[33] hypothesized a 10% autonomy threshold. In the model description this would roughly correspond to an exponent of 0.7 in function p^S.
[e] The total number of mitoses of erythropoietic progenitors n_{BE} + n_{CE} can be obtained from this reference. If one substracts the number of mitoses in CFU-E (n_{CE}), known from different sources (see below), one obtains n_{BE}.
[f] In the model, EP does not exceed 100 times normal. However, "practical" (EP = 100) and "theoretical" (EP = ∞) maxima of n_{CE} and $n_{E1\text{-}4}$ hardly differ (see Figure 8).
[g] In the model, EP does not decrease below 0.01 times normal. Therefore, the "practical" minima for n_{CE} and $n_{E1\text{-}4}$ are 0.5 (=5-4.5) and =6-2.5), respectively. Both values are above the "theoretical" minima (EP=0) of O (see Figure 8).
[h] High cycling rates were observed in regenerating marrow[91] and near the bone surface.[90]
[i] Similar data (four mitoses) exist for human marrow.[97,98]

this in order to retain a greater clarity and simplicity of the model and to reduce the 45 model parameters to 26 relevant ones, of which 20 can directly or indirectly be derived from actual data (see below).

The remaining six parameters are the weighting factors p^S, p^E, p^G, a^S, a^E, and a^G which determine how stem cells, erythropoietic, and granulopoietic cells act on "p" or "a". As will be shown throughout this volume, the speculative choice of these six parameters leads to a unique understanding of several hundred recovery curves of different origin.

Table 2 shows that maximum and minimum values of the self-renewal probability "p" can be derived from experiments. The value of 0.6 in the power function of p^S (Equation 13g) can be obtained under the assumption that a stem cell reduction below 6% leads to an autonomous stem cell growth. This is close to the 10% threshold hypothesized by Chervenick and Boggs.[33]

For the proliferative fractions "a_S", "a_{BE}", "a_{CG}" only thymidine suicide data are available. It is difficult to relate these data, which measure the fraction of cells in S-phase

Table 3
VALIDITY OF THE PARAMETERS

Parameters with good data base	Transit times: T_S, T_{CE}, $T_{E1\text{-}4}$, $T_{G1\text{-}4}$ "p": p_i (Amplitude of p) "a_S": All coefficients (a_1, a_2, a_3, a_4) "a_{BE}": One coefficient (maximum) "a_{CG}": One coefficient (maximum) Mitoses: n_{BE}, n_{CG}, $n_{G1\text{-}4}$ (constants); n_{CE}, $n_{E1\text{-}4}$ (maximum, normal, minimum)
Poorer data base but plausible choice possible (by analogy or other model results)	Transit times: T_{BE}, T_{CG} "p": Exponent autonomy threshold "a_{BE}": Three coefficients (normal, intermediate, minimum) "a_{CG}": Three coefficients (normal, intermediate, minimum) Mitoses: b factor in Z functions
Speculative	"p" p^S, p^E, p^G "a" a^S, a^E, a^G
Parameters without importance in model considering only relative compartment size	α_E, α_G Normal values of n_{BE}, n_{CE}, $n_{E1\text{-}4}$, n_{CG}, $n_{G1\text{-}4}$
Number of Parameters	Total: 6 (transit times) + 5 (p) + 3 * 7 (a_S, a_{BE}, a_{CG}) + 3 (n_{CE}, n_{CG}, $n_{G1\text{-}4}$) + 2 * 4 (n_{CE}, $n_{E1\text{-}4}$) + 2 (α_E, α_G) = 45 Redundant: 7 (a_{BE} = a_{CG}) + 4 (a_{BE} parameters: max (a_{BE}), a^S, a^E, a^G chosen as in a_S) + 1 (b in Z_{CE} as in $Z_{E1\text{-}4}$) = 12 Irrelevant in relative size model: 2 (α_E, α_G) + 5 (normal values n_{BE}, n_{CE}, $n_{E1\text{-}4}$, n_{CG}, $n_{G1\text{-}4}$) = 7 → Relevant parameters: 26 (known by data or estimate: 20; speculative: 6)

(with a large experimental error), directly to the fraction of cells in cell cycle. For a rough semiquantitative comparison we use the following rule:[3] if the thymidine kill is below 0.5 multiply it by two to obtain the fraction "a". Values above 0.5 indicate complete cycling (a = 1) (in formula: a = min [2 * ^{3}HTdR-Kill; 1]). The data base for "a_S" is sufficient. For "a_{BE}" and "a_{CG}" it is rather scanty, just enough to establish a maximum of 1.0 and a minimum considerably smaller. Therefore, the factors a_1, a_2, a_3, a_4 can be estimated for "a_S", but only one of them (maximum) can be obtained for "a_{BE}" and "a_{CG}". Choosing a_{BE} = a_{CG} reduces the six remaining factors to only three unknown values. One remaining value is chosen by the argument that minimum and normal value are almost equal in the data, one is fixed by the fact that the normal cycling is much slower than the maximum, and the last value is fixed as an interpolation with a_{BE} (0.5, 1, 1) = 0.66.

The cycling of CFU-E and the erythropoietic and granulopoietic precursors is maximum independent of erythropoietic stimulation or suppression. Therefore, the proliferative fractions of CE, E1-4, G1-4 do not change in the model.

The number of mitoses in the erythropoietic pathway is not constant for CFU-E and erythroblasts. We conclude that 2 mitoses can be added in CE and that up to 4.5 (of 5) mitoses can be omitted in CE, while 2.5 (of 6) can be omitted in E1-4. The resulting minima (0.5 and 3.5) refer to the "practical" minimum of EP = 0.01 as used for the model calculations. They are larger than the "theoretical" minima of no additional mitosis used in Equation 15b.

The determination rates α_E and α_G are listed for completeness. Their numerical values are of minor importance because only relative compartment sizes are considered in the following.

Table 3 gives an attempt to validate the model parameters. In total we have: six transit times for the compartments, five parameters for "p", seven for each "a" function, three for the Z-functions in compartments with constant numbers of mitoses (BE, CG, G1-4),

eight for the Z-functions in CE and E1-4, and two for the determination rates. Together these are 45 parameters. Twelve of these parameters are redundant because they are chosen identical with other parameters. Another seven are redundant because "a_{BE}" and "a_{CG}" are made numerically equal and yet another four by choosing a^S, a^E, a^G and the maximum values identical in "a_S", "a_{BE}", and "a_{CG}", and the last one by the choice of the same values for b in all Z-functions. A further seven parameters are only relevant if absolute compartment sizes are considered, which is not generally done. So only 26 relevant parameters remain to be determined. Thirteen of them can be fixed on the basis of the available data. For seven further parameters the data base is poor but the choice is reasonable and not contradictory to date. For example, T_{BE} and T_{CG} are not known but one knows the number of mitoses in BE or CG. Together with an estimated cycle time of 8 hr their values can be derived. The exponent in p^S is a parameter chosen so that the stem cell autonomy starts for stem cell counts below 6%. Two "a_{BE}" ("a_{CG}") parameters are estimated or interpolated from data as far as possible (see Table 2). The factor b was taken from a different model analysis.[9] So only the six speculative parameters, a^S, a^E, a^G, p^S, p^E, p^G remain which comprise the essential hypotheses of the model.

V. SPECIAL MODEL MODIFICATIONS

In this section some modifications of the standard model are described as they will be used in different chapters of this volume. We describe how the following situations are simulated:

1. Acute cell loss (e.g., in acute irradiation)
2. Continuous cell loss (e.g., during continuous irradiation)
3. EP time courses (e.g., after bleeding)
4. Increased EP sensitivity (e.g., after phenylhydrazine)
5. Nonrandom age structures (e.g., during hypertransfusion)
6. Granulopoietic stimulation (e.g., during continuous irradiation)
7. Migration of cells (e.g., after phenylhydrazine)

A. Acute Cell Loss

Acute cell loss is simulated by a reduction of the initial values of the differential Equations 1 to 7. If available, the experimental minima measured between day 0 and 3 are taken as initial values. Missing values are estimated by a special interpolation procedure (see References 99 to 101).

B. Continuous Cell Loss

Continuous cell loss is simulated by subtracting a constant loss term from the standard compartment equation. So, the equation for stem cells (Equation 1 in Table 1) reads

$$\dot{S} = (2p - 1)\, S * a_S/\tau_S - K_S * S \qquad (16)$$

K_S is the "loss coefficient" for S. It describes the loss of cells per hour. Similar loss terms can be introduced for all other model compartments (e.g., $-K_{CE} * CE$, $-K_{BE} * BE$), as shown in other chapters.[102,103]

C. Time Course of Erythropoietin

It is possible to calculate the theoretical time course of EP during erythropoietic stimulation or suppression using a model of mature erythropoiesis.[9] Such an EP curve is taken as external

input into the calculations with our model, where it influences the number of mitoses in CE and E1-4. The EP curves used are, therefore, an external trigger to the model. Mathematically speaking we use EP curves as boundary conditions to the model. This approach is used in many chapters in this volume.[100-102,104-109]

D. Increased Sensitivity to Erythropoietin

The analysis of the effect of phenylhydrazine (PHZ) on erythropoiesis reveals that PHZ increases the sensitivity of CFU-E to EP.[104] The effect is simulated by modifications of two parameters in the function $Z_{CE}:Z_{max}$, is increased to 320 (standard value is 128), and the parameter b is changed from 0.7 to 1.0. Both modifications lead to a steeper EP dose response curve so that the compartment (CE) becomes more reactive to EP. This is illustrated in greater detail in Chapter 12, Volume II.[104]

E. Nonrandom Age Structures

In the standard Equations 1 to 6 the efflux terms are designed as random transition terms. As a consequence, the age distribution of cells in the compartments is exponential. This description proves to be sufficient in most situations, except during erythropoietic suppression. In the model the problem may arise that the numbers in the sensitive compartments do decline only slowly, although the EP level drops very rapidly. The reaction of the cell numbers on EP becomes quicker by introducing a subcompartment structure for CE and E1-4 in the following way (shown for a model compartment C without postmitotic cells in which n_C mitoses take place, and which is subdivided into N similar subcompartments):

$$C^* = \sum_{i=1}^{N} C_i^* \tag{17a}$$

$$\dot{C}_1^* = Z_1^{in} * \dot{C}_{-1}^{*out} - C_1^*/(T_C/N);\ \dot{C}_i^{*out} = Z_i^{out} * C_i^*/(T_C/N) \tag{17b}$$

$$\dot{C}_i^* = Z_i^{in} * \dot{C}_{i-1}^{*out} - C_i^*/(T_C/N) \quad \text{for } i = 2,\ldots,N \tag{17c}$$

$$n_C = n_C\,(EP) \tag{17d}$$

$$Z_i^{in} = \frac{2^{nC/N} - 1}{n_C/N};\quad Z_i = 2^{nC/N} \quad \text{for } i = 1,\ldots,N \tag{17e}$$

These formulas are a generalization of Equations 11b to 11f where now N structurally identical subcompartments C_i^* are considered. Their sum gives the total number C* of cells. Each subcompartment is described as a random compartment. The time constant for each subcompartment is defined by T_C/N, so that the total transit time of all subcompartments remains T_C. The amplification factors Z_C^{in}, Z_C^{out} are distributed over all subcompartments so that Z_i^{in}, Z_i^{out} must be introduced. For convenience we chose Z_i^{in} and Z_i^{out} identical for all subcompartments. In each subcompartment the amplification (efflux/influx) amounts to $Z_i = 2^{nC/N}$, so that the total amplification of all subcompartments in C is $(2^{nC/N})^N = 2^{nC}$) as it was before. In total, no additional parameters (except N) enter into the model. For practical purposes we use for N the normal number of mitoses, N = norm (n_C).

In steady state the age distribution of such a multirandom compartment is a gamma distribution.[1,2] For N > 1 it has a smaller variance of the compartment transit time than the exponential distribution which corresponds to one random compartment (N = 1). The advantage of combining this multirandom description of C with a multilocus action of EP in C (expressed by EP-dependent functions Z_i) is that the compartment and the efflux out

of it react very quickly to changes in EP. This subcompartment approach is employed for the simulation of exhypoxia[105] and hypertransfusion.[106]

In a similar way, by subcompartments, we substructure compartment E1-4 into four individual compartments: E1 (= proerythroblasts), E2 (= basophilic erythroblast), E3 (= polychromatic erythroblast), and E4 (= orthochromatic erythroblast). This is necessary if one analyzes the action of Iron-55.[103] The four compartments are calculated with different transit times and numbers of mitoses

$$n_{E1} = n_{E2} = n_{E3} = 2,\ n_{E4} = 0 \tag{18a}$$

$$T_{E1} = T_{E2} = T_{E3} = 16\text{ hr},\ T_{E4}^m = 24\text{ hr} \tag{18b}$$

F. Granulopoietic Stimulation

The standard model ignores granulopoietic stimulation. Problems only arise if mature granulopoiesis is depleted for a long period of time. This is true throughout chronic irradiation where severe neutropenia may develop if the daily dosage is high enough. However, neutropenia goes in parallel with development of anemia. Under the assumption of similar dose-response curves one can expect that the granulopoietic and erythropoietic effects on their precursors are comparable. Then it is possible to substitute G by E in the regulatory functions:

$$p = p(S, E, E) \tag{19a}$$

$$a_S = a_S(S, E, E) \tag{19b}$$

and similarly for "a_{BE}" and "a_{CG}".

This way of indirect inclusion of granulopoietic stimulation into the model is used for the simulation of chronic irradiation [102] and the ensuing recovery.[107]

G. Migration of Cells

Migration of bone marrow cells to the spleen or vice versa does not change the total cell count if no migrating cells are lost. If a loss of cells has to be assumed, this is simulated by a loss term according to Section V.B. An application is shown in Chapter 12, Volume II.[104]

VI. COMPUTATION TECHNIQUES

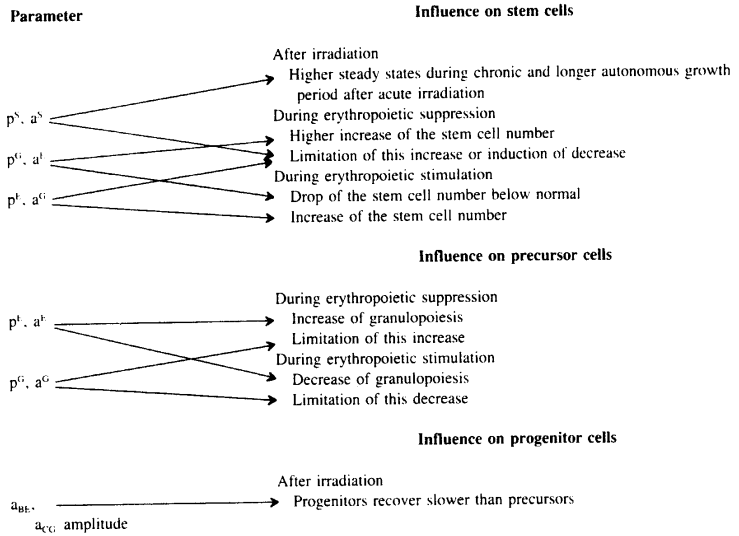
For the numerical integration of the model equations the program RESYS[110] has been used. It is based on the method of extrapolation and has a procedure to adjust the steps of integration. The calculations have been performed on a CYBER 72/76M computer at Cologne University. All relevant software has been written in FORTRAN IV. For the plots the Erlanger-Graphic-System (EGS) has been used.

VII. SENSITIVITY ANALYSIS

In this section we briefly discuss how a change of the standard model parameters would affect the model's behavior. This sensitivity analysis is performed only for parameters which are connected with the regulatory functions.

Table 4 summarizes the results. The arrows indicate which model effects are influenced

Table 4
SENSITIVITY ANALYSIS. QUALITATIVE EFFECT OF AN INCREASE OF THE PARAMETER VALUES (LEFT) ON THE CELL NUMBERS (RIGHT)



Note: The arrows indicate which effects become pronounced if the numerical values of the parameters are increased.

most if the parameter values are increased. Table 4 illustrates that any parameter change results in a number of different consequences.

A. The Self-Regulatory Parameters (p^S, a^S)

We may call p^S and a^S self-regulatory parameters because they characterize the effect of stem cells on their own self-renewal probability, "p", and their own proliferative activity, "a_S", (see Formulas 13 and 14).

Parameter p^S plays an important role for autonomous stem cell recovery from small initial values. For stem cell numbers below normal p^S is a power function ($p^S = 2/S^{0.6}$, Formula 13g. Thus, for small values of S, p^S increases. Below S = 0.06 this term overrules the influence of E and G on the self-renewal "p" (see above). The choice of p^S in the standard form guarantees:

- Global stability of the normal value
- A narrow domain of autonomy (0 to 6%) and a broad regulatory domain (above 6%)

A larger value of p^S (by increasing the constants 2 and/or 0.6) leads to a larger domain of autonomy, at the expense of the domain of regulation. For acute irradiation it prolongs the period of autonomous recovery. For chronic irradiation it elevates the plateau values of the stem cells. For S above normal, a greater p^S (by enlarging the constant 2) would reduce the maximum stem cell counts found, e.g., after hypertransfusion.

Parameter a^S plays a similar role for the cycling fraction "a_S" as p^S for the self-renewal probability "p". For reduced stem cell counts, a greater a^S accelerates cycling and recovery of stem cells. For elevated stem cell numbers, a greater a^S decelerates stem cell cycling and prevents further growth. During chronic irradiation an enlarged a^S reduces the pressure for stem cells to differentiate and leads to higher plateau values of all cells.

Obviously, reductions of p^S and a^S have the opposite effects to those just described. If the reduction is large enough a bifurcation may appear as a new phenomenon with two stable positive steady states at normal and subnormal levels. This phenomenon is presently under investigation.

B. E-G Competition Parameters (p^E, p^G, a^E, a^G)

Most important are the parameters p^G and a^E. a^E leads to an accelerated stem cell cycling if the erythropoietic precursors are diminished (e.g., hypertransfusion). As a consequence, more cells enter differentiation per unit time so that the granulopoietic cell counts increase. Due to p^G the increase in G leads to an increased self-renewal "p" and the stem cell count rises. Any increase in the weighting factors, p^G and a^E, will enhance the stem cell rise during erythropoietic suppression. It is apparent that the effect will be reversed in the opposite situation of erythropoietic stimulation. Here larger a^E and p^G would induce a stronger suppression of cycling, a steeper drop of G values, and a drop of stem cells numbers.

With respect to the stem cells the impact of p^G and a^E is counteracted by the weighting factors p^E and a^G. The latter tends to increase the number of stem cells during erythropoietic stimulation and to keep them at lower levels during suppression. Any increase of p^E and a^G would primarily lead to stem cell elevation under erythropoietic stimulation and to a fall under suppression.

While the pairs (p^G, a^E) and (p^E, a^G) act antagonistically with respect to stem cells, with respect to the precursors the opposing pairs (p^E, a^E) and (p^G, a^G) are different. An increase in p^E or a^E would lead to a more enhanced granulopoiesis during a red cell polycythemia. This effect would be limited by an increase in p^G or a^G and vice versa.

The different antagonistic pairs of parameters for stem cells and precursors are characteristics of the model. They produce a system whereby changes in the granulopoietic pathway are direct reactions to changes in erythropoiesis, and where stem cell changes are secondary as a result of the combined impact of E and G. This special "grammar" of the model allows, simultaneously, competition of granulopoiesis vs. erythropoiesis and of differentiated cells vs. stem cells.

C. Cycling Rates of Progenitors ("a_{BE}", "a_{CG}")

The behavior of the progenitor cells depends on the amplitude of the cycling fractions "a_{BE}" and "a_{CG}" (normal value = 0.33, maximum = 1.0). If this amplitude is reduced the recovery of progenitors from small initial values becomes faster and vice versa. The model compartments BE and CG are similar to S as they also have a variable proliferative fraction. They are different from S with respect to their self-renewal capacity which is limited.

D. Summary of the Sensitivity Analysis

The model is still compatible with the data if individual parameter values of p^S, p^E, p^G, a^S, a^E, a^G are changed by not more than 20 to 30%. If several parameters are changed simultaneously this rule must be considered with more caution. Effects may add or cancel so that the individual parameter may eventually not be changed by more than 5%.

Evidently many alternative sets of model parameters could in principle be tested and discussed. Table 4 summarizes only the most important effects. The six parameters (p^S, p^E, p^G, a^S, a^E, a^G) were obtained by "trial and error". All other model parameters can in

principle also be modified and tested for their sensitivity. One finds that the results are qualitatively identical if the parameter variation does not exceed 20%. This shows that the standard model is a good representative of a larger class of similar models.

VIII. THE MODEL'S EVOLUTION

The presentation of the model in a deductive way has the disadvantage that it shows the final product without the logic of its stepwise development. Historically, the basic steps are connected with the development of the regulatory functions and the compartments (Table 5). In the first two steps we assumed that the cycling of stem cells was self-regulated [$a_S = a_S(S)$] and the self-renewal probability "p" depended only on the stem cells S and erythropoietic progenitors CE [$p = p(S,CE)$]. With this minimum assumption it was possible to understand some characteristics of stem cell behavior after irradiation (step 1). If one allows for an EP-dependent amplification of the CE compartment by introducing an EP-dependent Z function, it is possible to reproduce many features of erythropoietic stimulation (anemia, hypoxia, Epo-injection) (step 2). However, stem cell reactions following erythropoietic suppression cannot be understood. Investigation of this "defect" showed that the problem could not be satisfactorily explained if only the erythropoietic series was considered.[3]

The essence of step 3 is that granulopoietic progenitors are also considered. The regulatory functions are expanded without destroying the properties of the model derived in the previous two steps. Granulopoiesis had to be given a strong impact on the self-renewal probability, "p", but only a minor one on "a_S" (step 3a). Furthermore, in step 3b a variable proliferation rate was introduced in BE and CG. This allowed us to understand that BFU-E and CFU-GM recover almost as slowly as CFU-S after irradiation, while the successor stages (e.g., CFU-E) showed a more rapid recovery.

In step 4, the model is expanded to include, also, granulopoietic (G1-4) and erythropoietic (E1-4) precursors. Now the regulatory functions are considered as being dependent on the whole number of granulopoietic (G) and erythropoietic (E) cells in the bone marrow instead of CG and CE.

Table 5 illustrates how the model structure evolved over the last 5 years. We always tried to pursue the concept of minimum assumptions: in each developmental step we introduced a minimum of speculative assumptions and determined how many effects could be explained by them. If contradictions remained we expanded the model by an additional simple assumption so that the explicative spectrum could also cover these cases. This philosophy lead to an iterative process in which the model became more complex, but in which each small step (i.e., each new parameter) was carefully examined. This "dialectic" process — in our opinion — provides insight into the necessary conditions nature fulfills in order to show its fascinating diversity.

Table 5
HISTORY OF THE PRESENT STEM CELL MODEL

Step	Cell stages considered	Regulatory functions: a	p	Z	Provides explanation for behavior	Ref.
1	S, BE, CE	$a_S(S)$	p(S,CE)	$Z = 1$	After acute and during chronic irradiation	3,111,112
2	S, BE, CE	$a_S(S)$	p(S,CE)	$Z_{CE}(EP)$	And during erythropoietic stimulation	3,113
3a	S, BE, CE,CG	$a_S(S, CE,CG)$	p(S, CE, CG) with p^S, p^E, p^G all constant	$Z_{CE}(EP)$	And during erythropoietic suppression	114,115
3b	S, BE, CE, CG	$a_S(S, CE, CG)$	p(S, CE, CG) with p^S, p^E, p^G all constant	$Z_{CE}(EP)$	And of combinations of irradiation and erythropoietic stresses	116
4	S, BE, CE, E1-4, CG, G1-4	$a_S(S, E, G)$ $a_{BE}(S, E, G)$ $a_{CG}(S, E, G)$	p(S, E, G) with power law for p^S	$Z_{CE}(EP)$ $Z_{E1-4}(EP)$	In 12 different experimental situations	Present model

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Chapter 5

FUNDAMENTAL SYSTEM BEHAVIOR*

H.-Erich Wichmann, Markus Loeffler, and Peter Herkenrath

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I. ABSTRACT

The mathematical model of stem cell regulation, as described in Chapters 3 and 4, is now applied to typical stresses of the hemopoietic system. To make the essential behavior more explicit, "artificial" stresses are simulated which consider the characteristics of "real" stress experiments in a simplified way. We have investigated how stem cells, erythropoietic, and granulopoietic bone marrow cells influence the self-renewal probability and the fraction of actively cycling stem cells and vice versa. The model analysis offers an explanation for the variety of recovery curves after stem cell depletion. It shows that the phenomenon of stem cell competition can be understood without assuming regulation of determination rates.

II. INTRODUCTION

The basic concept of the model of stem cell regulation and its translation into the mathematical language has been described in parts A and B.[1,2] Now the reaction of the model to perturbations will be investigated. The term "perturbation" is used in a general way including acute or permanent modification of cell numbers or parameters. However, no systematic mathematical analysis of the model properties will be given, as this has already been published, at least in part.[3-7] Here only those perturbations shall be analyzed which are of "biological relevance" in respect to the experiments described in the following chapters.

To make the process of regulation clear, it is useful to consider first some artificial situations which are simpler than the "real" ones. The reaction of the model to these artificial stresses provides insight into the details of regulation. The simulation of "real" stress experiments can then be composed using these artificial elements.

III. GENERAL INFLUENCE OF THE CELL NUMBERS

A. Influence on the Self-Renewal Probability "p"

In the model, the self-renewal probability "p" of stem cells is regulated by the weighted sum Y of bone marrow cells [$Y = Y(S, E, G) = p^S(S - 1) + p^E(E - 1) + p^G(G - 1)$, see References 1 and 2] (Figure 1). The dose-response curve has a sigmoid shape with "p" varying between 0.4 and 0.6 (Figure 1). The normal value is $p = 0.5$. The dependency of "p" on the different types of bone marrow cells can be symbolized by a balance where the influence of the stem cells is antagonized by the influence of the differentiated precursors (Figure 4 in Chapter 3). If the stem cell number (S) is reduced, "p" increases above normal; if the number of erythropoietic (E) or granulopoietic (G) precursors (or both) is reduced, "p" decreases below 0.5.

To make the influence of the individual cell types more explicit, on the abscissa of Figure 1 separate scales for S, E, and G are given. From these follows $p = 0.5$ for the normal steady state ($S = E = G = 1$ which is equivalent to $Y = 0$). For an isolated reduction of the stem cell number, "p" increases above 0.5 and the maximum value $p = 0.6$ is reached for S below 0.4. On the other hand, an isolated reduction of the erythropoietic or granulopoietic precursors leads to a decrease of "p". However, the relative influence of E and G is quite different. While "p" reaches its minimum value ($p = 0.4$) for G below 0.6, a reduction of E has a significantly smaller influence. This is reflected by the ratio $p^E:p^G = 1:4$ of the weighting factors p^E and p^G for E and G.

More complex is the influence of simultaneous changes of all cell numbers. The combined effect can be derived from Figure 1 using the scales for S, E, and G on the abscissa. One only has to subtract the corresponding distances geometrically, e.g., if one wants to find the value of "p" for a reduction of all cell numbers to 50% of normal ($S = E = G = 0.5$), one has to take the distance between $S = 1$ and $S = 0.5$ (Figure 1, ①) and to subtract from it the distances between $E = 1$ and $E = 0.5$ (Figure 1, ②) and between $G = 1$ and $G = 0.5$ (Figure 1, ③). Doing this, one arrives at $p = 0.4$ (Figure 1, ④). By the same method, one finds $p = 0.6$ for a simultaneous reduction of all bone marrow cells to 2.5% ($S = E = G = 0.025$, Figure 1, ⑤).

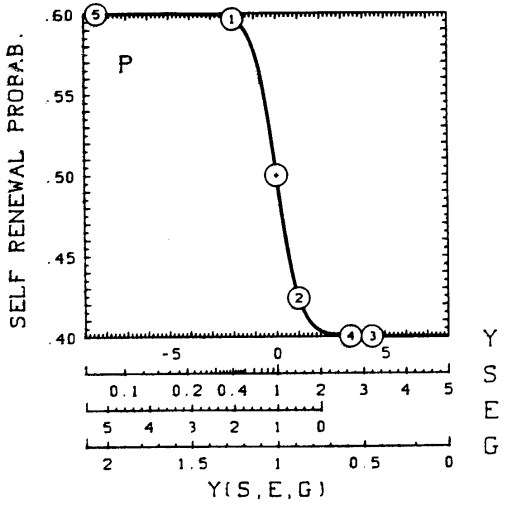

FIGURE 1. Influence of the stem cells (S), erythropoietic (E), and granulopoietic (G) precursors on the self-renewal probability "p". ①, ②, ③: Isolated reduction of one cell type (①: S = 0.5, ②: E = 0.5, ③: G = 0.5); the other cell numbers are normal. ④, ⑤: Simultaneous reduction of all cell types (④: moderate reduction to S = E = G = 0.5, ⑤: severe reduction to S = E = G = 0.025). The value of the weighted sum Y can be found by geometric subtraction of E and G from S.

The biological meaning of this result is as follows: if all cell numbers are reduced, competition takes place and the need for stem cells is set off against the need for differentiated cells. For a moderate reduction, the need for differentiated cells has the higher priority, while for a severe reduction self-renewal of the stem cells is more important. It has to be kept in mind, however, that "p" cannot increase above 0.6, even for maximum stimulation. This is an important limitation, which is not trivial but is necessary to guarantee an optimum behavior of the regulatory system as a whole.[4,5]

Mathematically, the influence of S, E, and G is reflected by the weighting factors p^S, p^E, p^G in the weighted sum Y. For a moderate reduction of all cell numbers ($S = E = G = 0.5$) the weighting factors are [2] p^S: $(p^E + p^G) = -1.4{:}5$. Therefore, the balance for "p" falls in favor of differentiation leading to $p = 0.4$. For a severe reduction ($S = E = G = 0.025$), the weighting factors are p^S: $(p^E + p^G) = -9.1{:}5$ and the balance sinks in favor of self-renewal. In the latter case the influence of E and G is less important and "p" depends intrinsically on S.

For increased cell numbers or combinations of increased and reduced numbers "p" can be derived using Figure 1 in the same way, as will be shown by many examples in the following.

B. Influence on the Proliferative Fraction "a_s"

If one considers the proliferative fraction of stem cells or progenitor cells in active cell

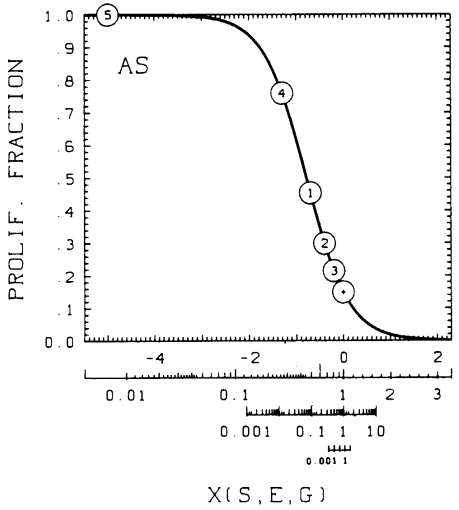

FIGURE 2. Influence of S, E, and G on the proliferative fraction "a_S" of stem cells. The value of the weighted sum X can be found by geometric additional of S, E, and G (see text). Symbols as in Figure 1.

cycle, the situation becomes simpler. The discussion shall be restricted on the proliferative fraction "a_S" of stem cells as shown in Figure 2. Qualitatively, the same holds true for the proliferative fractions "a_{BE}" and "a_{CG}" of the early erythropoietic and granulopoietic progenitor cells.

The proliferative fraction "a_S" depends on the weighted sum X of bone marrow cells (Figure 2) [$X = X(S, E, G) = a^S \ln S + a^E \ln E + a^G \ln G$; for $S > 1$ the formula is slightly different,[2] but this shall not be considered here]. Unlike "p", the stem cells (S), erythropoietic (E), and granulopoietic (G) precursors act synergistically, like weights on a letter scale (Figure 6 in Chapter 3). The dose-response curve for "a_S" also has a sigmoid shape, with the normal value 0.15, a maximum of 1, and a minimum of 0.01. As discussed earlier,[1] one may use a variable generation time instead of "a_S". The variability of "a_S" due to regulation corresponds to an average generation time in S between 8 and 800 hr with a normal value of 53 hr.

By separate abscissas for S, E, and G one may investigate the influence of the different cell types on "a_S" in more detail. As the scale for S in Figure 2 shows, a reduction of the stem cell number below 10% leads to maximum activation ($a_S = 1$). For E, only very small cell numbers have the same effect and a reduction of G cannot increase "a_S" to its maximum value. The corresponding holds true for the inactivation of "a_S" due to enlarged cell numbers.

The different influences of S, E, and G are reflected mathematically by the weighting factors a^S, a^E, and a^G. For decreased cell numbers, their ratio is $a^S:a^E:a^G = 10:3:1$, showing that the stem cells have the major effect, followed by the erythropoietic precursors.[2] Compared with these, the influence of the granulopoietic cells on "a_S" is negligible.

If S, E, and G are reduced separately to 50%, "a_S" remains below 0.5 (Figure 2, ①, ②, ③). If all three are simultaneously reduced to 50%, their stimulatory influence adds up to $a_S = 0.6$ (Figure 2, ④). In this case, the value of "a_S" can also be determined geometrically from Figure 2 using the scales for S, E, and G. Here the distances from the normal values (S = 1 to S = 0.5, E = 1 to E = 0.5, G = 1 to G = 0.5) have to be added, and the value of "a_S" can be read off from the curve.

For a more severe reduction "a_S" reaches 1. This is shown in Figure 2, ⑤ for a simultaneous depletion of S, E, and G to 2.5%. However, the same degree of activation is found if only S is reduced to 2.5% but E and G are normal or even above normal. In other words, for small stem cell numbers "a_S", similar to "p", is intrinsically regulated by S.

IV. KINETIC BEHAVIOR AFTER TYPICAL STRESSES

To understand the time courses of the cell numbers after perturbations of the steady state, it is necessary to appreciate not only the influence of the cell numbers on the regulatory mechanisms for activation and self-renewal, but also the reverse, i.e., the influence of "p" and "a_S" on the cell numbers. This mutual influence of S, E, and G on "p" and "a_S" and vice versa shall be studied in some typical situations.

Since the interactions are sometimes complex, the time course during or after a stress shall be subdivided into phases. For each phase, the influence of the cell numbers on "p" and "a_S" is described and then the influence of "p" and "a_S" on the cell numbers is discussed. Thus, we end up with the following scheme: phase of the time course

1. Influence of the cell numbers S, E, and G on the self-renewal probability "p" and the proliferative fraction of stem cells "a_S"
2. Influences of "p" and "a_S" on the cell numbers S, E, and G

In Figures 3 to 9 the phases are indicated by encircled numbers. The figures are composed of six curves: the curves on the top show the average values of "p" and "a_S" in the different phases as they follow from S, E, and G (or the weighted sums Y and X). The middle curves show the time courses of "p" and "a_S", and the bottom curves those of S, E, and G. In the time courses, the phases are seperated by dashed vertical lines.

A. Stem Cell Depletion

1. Severe Reduction of Stem Cells and Precursors

If all bone marrow cells are reduced to 1%, in the model the following regulatory influences are observed (Figure 3).

a. First Phase (Autonomous Stem Cell Recovery, Figure 3, ①)

1. The influence of stem cell reduction on the regulatory mechanisms plays the major role. Thus, the self-renewal probability becomes maximum (p = 0.6) and all stem cells are activated ($a_S = 1$). This phase can be interpreted as phase of autonomous stem cell recovery in which the regulatory influences of the differentiated cells are negligible.
2. Maximum "p" and maximum "a_S" lead to a steep exponential increase of S. The increase of the differentiated precursors is also exponential but slower.

b. Second Phase (Preference of Differentiation, Figure 3, ②)

1. After S has reached more than 6% the requirement for differentiated cells on the mechanism of self-renewal becomes effective. This leads to a decrease of "p" from 0.6 to 0.52. However, the cell numbers are, yet, too small to change "a_S".
2. The smaller "p" (p = 0.52) reduces the increase of S, while the increase of E and G becomes a little steeper. The main characteristics of the second phase is that S

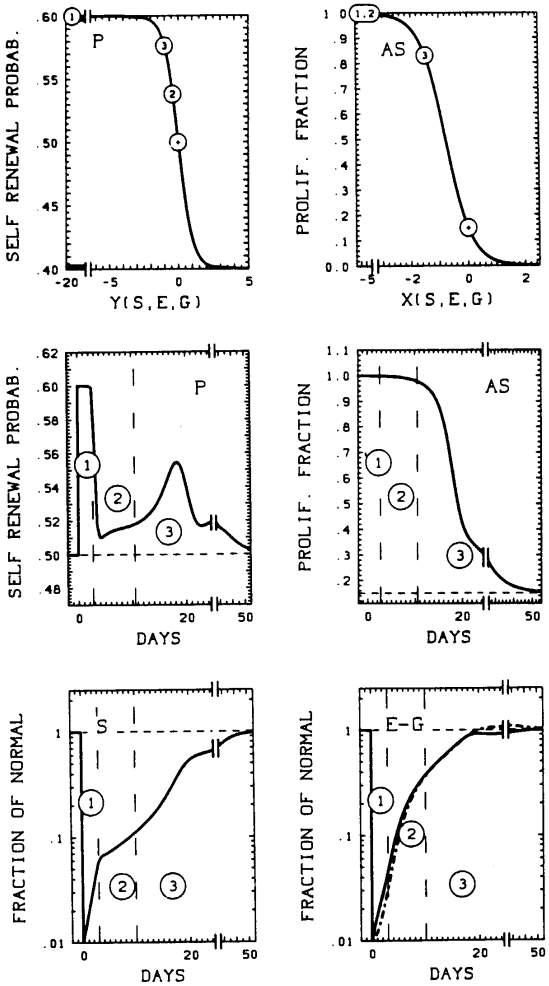

FIGURE 3. Initial reduction of S, E, and G to 1% of normal. ①: Phase of autonomous stem cell recovery ("p" and "a_s" maximum); ②: preference for differentiation ("p" slightly above normal, "a_s" maximum); ③: normalization ("p" and "a_s" return to normal). Symbols: top — self-renewal probability "p" and active fraction of stem cells "a_s" depending on the weighted sums Y,X of bone marrow cells; middle — time course of "p" and "a_s"; bottom — time course of S, E (——), and G (— · — · —).

remains in the region of 10% while the differentiated cells increase to about 50%: the differentiated cells recover at the expense of the stem cells.

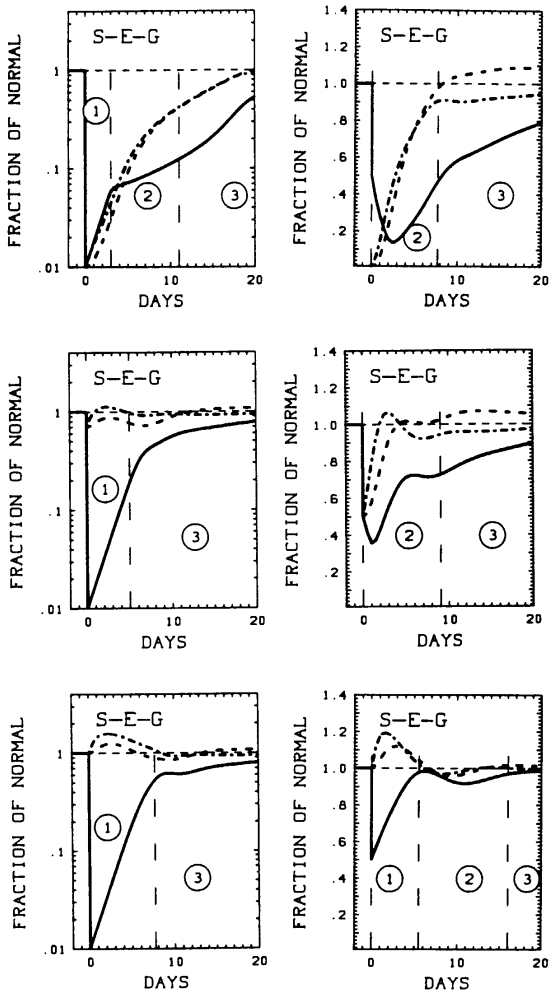

FIGURE 4. Different degrees of acute cell loss. Time course of S (——), E (— · — · —), and G (— — — —). ①, ②, ③: phases as in Figure 3. Left panel: after severe stem cell reduction (S = 0.01) a phase of autonomous stem cell recovery always occurs (logarithmic scale); right panel: after moderate stem cell reduction (S = 0.5) S may initially increase or decrease, depending on the differentiated cells E and G (linear scale).

c. Third Phase (Normalization, Figure 3, ③)

1. Since E and G now are much higher than S, self-renewal of the stem cells is favored until S also has recovered. This leads to an intermediate increase of "p" to 0.55 from

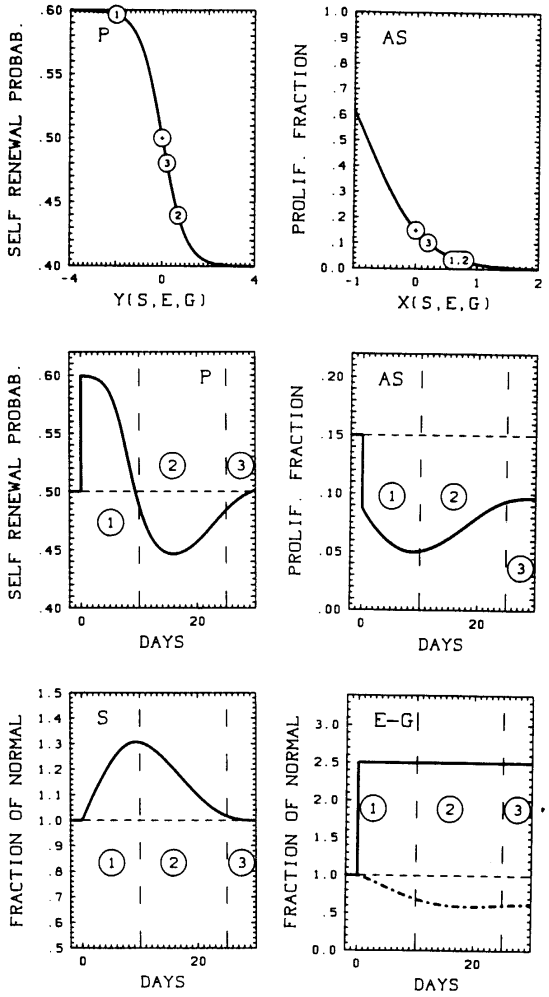

FIGURE 5. Permanently enlarged pool of erythropoietic precursor cells (E = 2.5). ①: Initial phase ("p" and S enlarged, "a_s" and G reduced); ②: intermediate phase ("p" reduced and S enlarged, "a_s" and G reduced); ③: plateau phase (p = 0.5 and S about normal, "a_s" and G reduced). Symbols as in Figure 3.

which it returns to normal. During this time, the highly activated fraction "a_s" decreases in parallel with the recovery of the cell numbers.

2. The intermediate maximum of "p" leads to an intermediate acceleration in the recovery of S. Then S, E, and G normalize slowly until day 50.

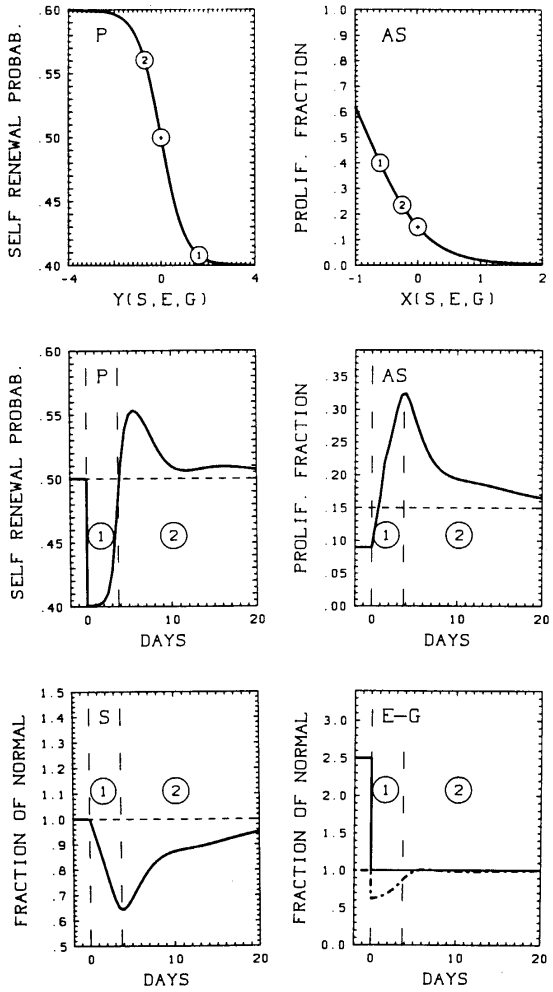

FIGURE 6. Normalization of an enlarged pool of erythropoietic precursors (E = 2.5 → 1). ①: Initial phase ("p" and S reduced, "a_s" enlarged G reduced); ②: final normalization ("p" enlarged and S reduced, "a_s" enlarged and G normal). Symbols as in Figure 3.

2. *Different Degrees of Stem Cell Reduction*

The patterns of recovery after bone marrow depletion depend markedly on the degree of the initial reduction. This will be demonstrated for different initial values of S, E, and G (Figure 4).

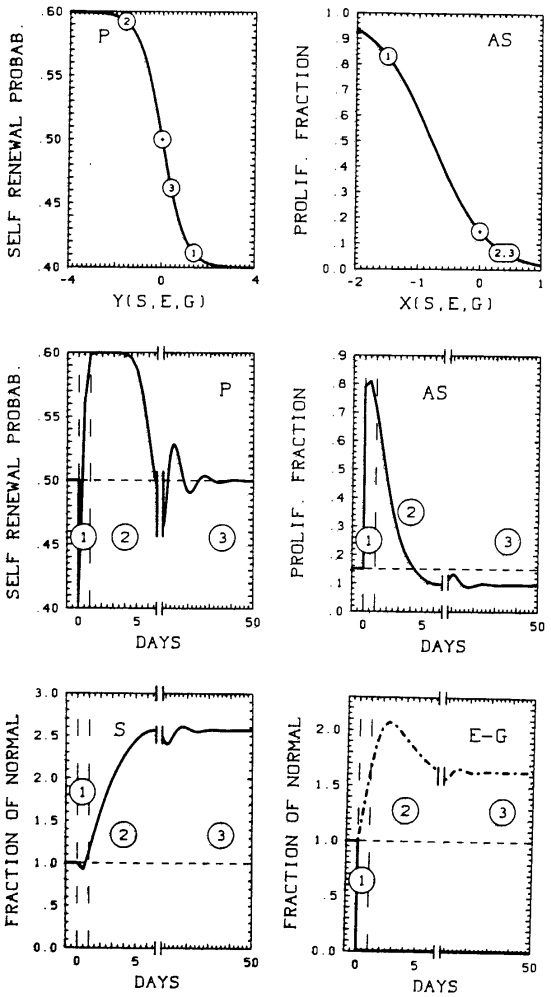

FIGURE 7. Permanently reduced pool of erythropoietic precursor cells (E = 0.01). ①: Initial phase ("p" reduced, "a_s" and G enlarged); ②: intermediate phase ("p" and S enlarged, "a_s" and G enlarged); ③: plateau phase ("p" normal, S and G enlarged, "a_s" reduced). Symbols as in Figure 3.

a. Severe Reduction of S (S = 0.01)

In all situations where the stem cells are severely reduced, we find an initial phase of autonomous stem cell recovery. During this phase, self-renewal is maximum (p = 0.6) and all stem cells are activated (a_s = 1), independent of the precursor cells. However, the duration of this phase depends on the cell numbers in E and G.

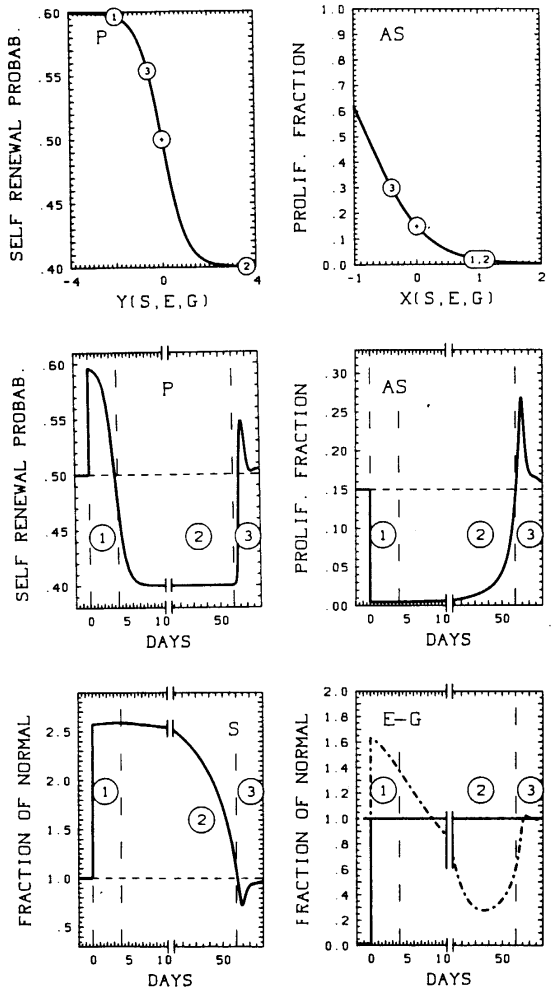

FIGURE 8. Normalization of a reduced pool of erythropoietic precursors (E = 0.01 → 1). ①: Initial phase ("p" and S enlarged, "a_s" reduced, G enlarged); ②: intermediate phase ("p" reduced, S enlarged, "a_s" and G reduced); ③: final normalization ("p" enlarged and S reduced, "a_s" enlarged and G reduced). Symbols as in Figure 3.

E = G = 0.01 (Figure 4 left, top) — This situation has already been shown in Figure 3. The autonomous phase of maximum exponential stem cell growth lasts until S has reached 6%. Then a second phase begins in which differentiation has higher priority and in which E and G increase much higher than S. In the third phase, the stem cells increase more rapidly again and all cell numbers normalize.

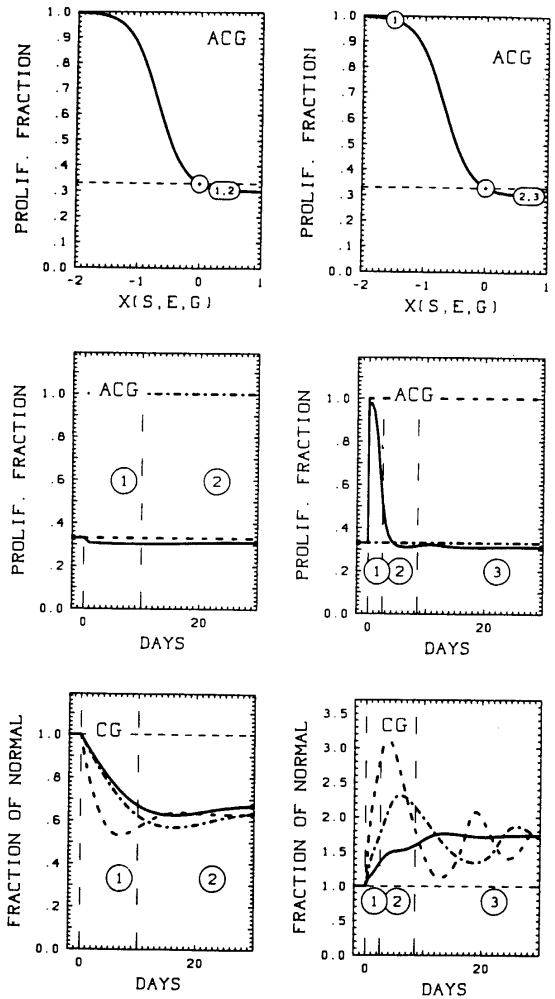

FIGURE 9. Influence of the proliferative fraction "a_{CG}" of the granulopoietic progenitors CG.——: "a_{CG}" regulated by the weighted sum X of bone marrow cells; — —: a_{CG} = 0.33 (no regulation); — · —: a_{CG} = 1 (no regulation). Reaction of "a_{CG}" and CG on sustained enlargement (E = 2.5, left panel) or reduction (E = 0.01, right panel) of erythropoietic precursor cells. The corresponding curves for "p", "a_S", S, E, and G are shown in Figures 5 and 7.

E = G = 0.5 (Figure 4 left, middle) — The stem cells are severely depressed but the differentiated cells are only moderately reduced. This leads to a prolonged autonomous phase of self-renewal for S. During this phase, the differentiated cells also increase slightly, since "a_S" is maximum. After S has reached 20%, the shape of its recovery curve becomes

gradually flatter until the cell numbers have normalized. Here no intermediate phase can be identified in which the differentiated cells grow at the expense of the stem cells. The reason is that the demand for stem cells is always higher than the need for differentiated cells.

E = G = 1 (Figure 4 left, bottom) — Since only the stem cells are reduced and no need for forced differentiation exists, we have a very long autonomous phase. Only after S has reached 50%, the rate of increase is reduced. The differentiated cells E and G show an initial overshoot due to the maximum activation of stem cells (a_S = 1).

b. Moderate Reduction of S (S = 0.5)

If the stem cell number is only moderately reduced, an autonomous phase is not necessarily found.

E = G = 0.01 (Figure 4 right, top) — Here the differentiated cells are depleted to a greater extent than the stem cells. Therefore, from the beginning differentiation has a higher priority than self-renewal of stem cells. This is reflected in an exponential increase of E and G while S decreases. Only after the differentiated cells have nearly normalized does recovery of the stem cells ensue. As the figure shows, we can only identify two phases, namely, preferential differentiation and normalization. No initial phase of autonomous stem cell recovery exists.

E = G = 0.5 (Figure 4 right, middle) — If stem cells and differentiated precursors are reduced moderately, differentiation, nevertheless, has the higher priority. As before, E and G increase rapidly while S decreases. After E and G are nearly normal, S also recovers. Here we find a long and oscillatory second phase, reflecting the competition between self-renewal and differentiation.

E = G = 1 (Figure 4 right, bottom) — Only if the differentiated cells are normal (or above normal), a moderate reduction of S to 50% leads to autonomous recovery. This phase is followed by a phase of competition between S, E, and G with an intermediate minimum of S, before all cells normalize. Again we find an initial overshoot of E and G, due to "a_S".

In summary, the examples in Figures 3 and 4 show the variety of patterns of cellular behavior after stem cell depletion.

For the stem cells, one finds three phases:

1. Autonomous recovery with maximum exponential growth
2. Slower growth, dip, or intermediate plateau due to effective regulatory influences from differentiated cells
3. Normalization

The duration of these phases depends on both the depletion of the stem cell pool and the available differentiated precursors:

- If the stem cells are not reduced below 6% the phase of exponential growth may be missing.
- If stem cells and precursors are severely reduced, the phase of steep exponential growth of S ends early (below 10%).
- If only S is reduced the exponential growth ends late (above 50%).

For the differentiated cells one finds

- An exponential increase and normalization if their number is severely reduced.
- An initial peak, if their number is not reduced or only slightly decreased.

B. Enlarged Pool of Erythropoietic Precursor Cells

If only the differentiated precursors and not the stem cells are affected by an external

stress, the regulatory behavior of the model is totally different, compared with the situations described above. Two examples will be considered in which the cell number in the erythropoietic lineage is modified. In the first example, an external stimulus keeps the number of erythropoietic precursors permanently at a high level (Figure 5). In the second example, the normalization after cessation of this external stimulus is considered (Figure 6). In both situations the reaction of stem cells and the granulopoietic cells, neither of which are directly influenced by the external stimulus, will be of special interest.

1. Permanently Enlarged Erythropoietic Precursor Pool

A permanent elevation of the erythropoietic precursors E to 250% of normal will be simulated (Figure 5 bottom, right). It leads to the following reactions.

a. First Phase (Initial Reaction of S and G, Figure 5, ①)

1. The enlarged number of differentiated precursors acts, through the feedback loop, to reduce further differentiation. Therefore, "p" increases to 0.6 and "a_s" decreases to subnormal levels.
2. S increases due to $p > 0.5$, G decreases because "a_s" has dropped, and E remains permanently elevated due to the permanent external stimulation.

b. Second Phase (Intermediate Reaction, Figure 5, ②)

1. The self-renewal mechanism becomes sensitive to the enlarged S and the reduced G. Therefore, "p" decreases below 0.5. On the other hand, the active fraction "a_s" is suppressed by the high S and E values and, therefore remains at its subnormal plateau.
2. The reduced "p" leads to a decrease of S. G remains below normal due to the constantly subnormal value of "a_s".

c. Third Phase (Plateau Phase, Figure 5, ③)

1. The self-renewal probability returns to $p = 0.5$ since the influence of the enlarged E (2.5 times normal) on "p" is compensated by the influence of the reduced G (0.65 times normal). This follows because the influence of G on "p" is much higher than the influence of E ($p^E:p^G = 1:4$). On the other hand, the active fraction "a_s" remains subnormal due to the enlarged E which is not compensated by the reduced G (relative influence $a^E:a^G = 3:1$).
2. The system reaches a steady state with a (nearly) normal stem cell number, a reduced number of granulopoietic precursors, and a sustained enlargement of the erythropoietic precursor pool due to external influences.

2. Normalization of an Enlarged Erythropoietic Precursor Pool

Here the artificial situation will be investigated in which E is immediately reduced from $E = 2.5$ to normal and kept constant at the normal value (Figure 6, bottom right). The initial values on day 0 are taken from the final steady state in Figure 5. We find the following influences on the stem cell and the granulopoietic precursors.

a. First Phase (Initial Reaction, Figure 6, ①)

1. The sudden return of E to normal now leads to a drop of "p" and an acceleration of cycling since now more differentiated cells are needed.
2. Due to the subnormal "p" the stem cell number decreases while the enlarged "a_s" leads to an increase of G. E is artificially kept at normal levels.

b. Second Phase (Final Normalization, Figure 6, ②)

1. The subnormal S leads to a temporary increase of "p" to 0.55 which returns to 0.5 as soon as the cell numbers tend to normalize; "a_s" decreases steadily to normal.
2. S and G return to normal without oscillations.

Summarizing the results from Figures 5 and 6, one finds the following influences of an enlarged pool of erythropoietic precursors:

- The stem cells show an intermediate peak as long as E is elevated and an intermediate dip during nomalization of E.
- As long as E is elevated, the granulopoietic precursors are reduced.

The most interesting pattern is the inverse behavior of erythropoietic and granulopoietic precursors (Figure 5). It is important to note that this does not follow by a variable diversion of the cell flux into the two lineages. For an enlarged E the efflux from S into the erythropoietic and granulopoietic lineage is reduced by the same amount. This leads to subnormal numbers of G, but the numbers of E remain elevated due to the external influence.

The number of erythropoietic precursors is enlarged in hemolytic anemia, hypoxia, after bleeding, or after the administration of erythropoietin (Epo). However, in these realistic situations the phases of enlargement and normalization cannot be as clearly separated as in the artificial cases shown in Figures 5 and 6.

C. Reduced Pool of Erythropoietic Precursor Cells

Two situations will be considered where the formation of erythropoietic bone marrow cells is permanently suppressed (Figure 7) and normalized afterwards (Figure 8). As before, the erythropoietic cell numbers are kept fixed and the reaction of stem cells and granulopoiesis is considered.

1. Permanently Reduced Erythropoietic Precursor Pool

The erythropoietic precursors will be reduced to 1% and kept fixed at this level (Figure 7, bottom right).

a. First Phase (Initial Reaction, Figure 7, ①)

1. The self-renewal mechanism interprets the reduction of E as need for differentiated cells. Therefore, "p" decreases to 0.4. For the mechanism of cell cycle activation, the reduction of E leads to an increase of "a_s" to 0.8.
2. The consequence of the reduced "p" is a transient minimum of S. More important is the enlarged "a_s" which leads to an increased influx into differentiation and an increase of G.

b. Second Phase (Intermediate Behavior, Figure 7, ②)

1. The increased G is now responsible for a switch of the self-renewal probability "p" to 0.6. The enlarged S reduces "a_s" to a subnormal level.
2. The enlarged "p" leads to a significant increase of S to more than twice the normal value. Due to the drop of "a_s", the granulopoietic precursors decrease.

c. Third Phase (Plateau Phase, Figure 7, ③)

1. The decrease of S and G leads to a reduction of the self-renewal probability. In this

phase, we find some damped oscillations of "p" which induce small oscillations in S.
2. The normalization of "p" demonstrates that a new steady state is achieved with high S and G. In total, the severe and sustained reduction of the erythropoietic precursors (E = 0.01) leads to increased values of the stem cells and the granulopoietic precursors.

2. Normalization of Reduced Erythropoietic Precursors

If the permanent reduction of E is stopped and E is returned to normal (Figure 8, bottom right), the stem cells and the granulopoietic cells also will normalize, starting at the plateau values of Figure 7.

a. First Phase (Initial Reaction, Figure 8, ①)

1. The sudden increase of E from 0.01 to 1 leads to an enlargement of the number of differentiated cells. Therefore, "p" increases and "a_S" drops.
2. The subnormal "a_S" reduces the flux into differentiation and G decreases. Since "p" is enlarged for a short period, S shows a transient increase.

b. Second Phase (Intermediate Behavior, Figure 8, ②)

1. The decline of G leads to a subsequent reduction of "p" to 0.4; "a_S" remains at its low level and is almost unaffected from the behavior of G as long as S is elevated.
2. The subnormal plateau of "a_S" is responsible for the decline of G to subnormal values. S decreases due to the low value of "p". However, this decrease is very slow because "a_S" is so small (i.e., nearly all stem cells are in the resting phase).

c. Third Phase (Final Normalization, Figure 8, ③)

1. As soon as S normalizes "a_S" and "p" increase and show an intermediate peak.
2. "a_S" is responsible for an increased influx into differentiation and, thus, for the normalization of G; "p" is responsible for the normalization of S.

In total, a reduced pool of erythropoietic precursors has the following consequences:

- The stem cells increase to high numbers for sustained reduction of E and return to normal only very slowly after normalization of E.
- The number of the granulopoietic cells significantly increases above normal and normalizes after an intermediate dip.

In the model the reduced number of erythropoietic precursors obviously has a "beneficial effect" on granulopoiesis. Again this follows without a special diversion of the cell flux into the granulopoietic lineage. Rather it occurs only because of the increased efflux from the stem cell compartment. This increased cell flux becomes effective in G but not in E, since E is suppressed by external influences.

Experimental situations in which the erythropoietic precursors are reduced are hypertransfusion, hyperoxia, and ex-hypoxia. In these realistic situations the phases of continuous suppression and subsequent recovery of E often cannot be as clearly separated as in the artificial situations shown in Figures 7 and 8.

D. Proliferative Fraction of Progenitor Cells

Until now, only the activation or inactivation of stem cells (described by the proliferative fraction "a_S") has been discussed. What is the relevance of a similar mechanism for the activation of (early) progenitor cells?

In the model, the actively cycling fraction of early erythropoietic (BE) and granulopoietic (CG) progenitors is described by "a_{BE}" and "a_{CG}", respectively. For simplicity, both depend on the same weighted sum X of bone marrow cells as "a_S" (Figure 9 in Chapter 3). The normal value for "a_{BE}" and "a_{CG}" is 0.33, the minimum is 0.30, and the maximum is 1.

As an example, Figure 9 gives a more detailed analysis of the relation between CG and "a_{CG}" which similarly holds true for BE and "a_{BE}". The situations already discussed in Figures 5 and 7 serve as illustrations. For sustained enlargement (E = 2.5, left panel of Figure 9) and reduction (E = 0.01, right panel of Figure 9) of erythropoietic cells three different hypotheses for "a_{CG}" are compared:

1. "a_{CG}" is a regulated variable as assumed in the standard model with value between 0.3 and 1.0 (full lines).
2. "a_{CG}" is constant at 0.33 (dashed lines).
3. "a_{CG}" is constant at 1.0 (dashed-dotted lines).

Since "a_{CG}" is inverse proportional to the average transit time through the CG compartment, these three cases correspond to either a variable transit time (between 8 and 27 hr with a normal value of 24 hr) or a constant transit time of 24 or 8 hr, respectively.

During sustained enlargement of E the proliferative fraction of stem cells "a_S" is below normal (Figure 5). This leads to a reduced cell flux into differentiation and to a decrease of the granulopoietic progenitors and precursors. As shown in Figure 9, left, the decrease of CG is quite independent of the choice of "a_{CG}". If "a_{CG}"is reduced, the final plateau is CG = 0.65; if not, the plateau is CG = 0.6.

For sustained reduction of E (Figure 9, right panel) "a_{CG}" influences the cell number in CG more severely. While a constant "a_{CG}" (0.33 or 1) leads to oscillations of CG a variable "a_{CG}" dampens the oscillations.

In total a regulated proliferative fraction "a_{CG}" of granulopoietic progenitors has the following consequences compared with the situation where "a_{CG}" is constant:

- For increased cell numbers in S, E, and G, a variable "a_{CG}" has only a minor influence on the number of cells in CG (Figure 9, left).
- For reduced cell numbers in S, E, and G, "a_{CG}" increases significantly during the first days if it is under regulatory control. This corresponds to a shortening of the transit time, and thus, to a shift of cells from the progenitor (CG) to the precursor (G) stage. As a consequence, CG is smaller as it would be for constant "a_{CG}" (Figure 9, right). This effect is only transient and disappears after the combined stimulus of S, E, and G has normalized.
- The variability of "a_{CG}" has no serious consequences on the regulation of the whole stem cell system.

What has been said about CG and "a_{CG}" is also valid for the early erythropoietic progenitors BE and their proliferative fraction "a_{BE}", since these are described in the same way.

V. DISCUSSION

In the model, we essentially consider three quantities (the numbers of stem cells, S, erythropoietic, E, and granulopoietic, G, cells) and two regulatory mechanisms (the self-renewal probability, "p", and the proliferative fraction, "a_S", both of stem cells); "p" and "a_S" are influenced by S, E, and G, but not to the same degree. The self-renewal

probability is under competitive control where demand for stem cells tends to increase self-renewal and demand for differentiated cells tends to decrease it. For small cell numbers, the influence of S on "p" dominates; for nearly normal cell numbers, G has the prior influence. The proliferative fraction "a_s", i. e., the fraction of stem cells in active cell cycle, is under additive control of the bone marrow cells, and a demand for either stem cells, erythropoietic, or granulopoietic cells increases "a_s". Its value is mainly influenced by S, and, of the differentiated cells, E is more important than G.

The cell numbers themselves react to modifications of "p" and "a_s". The stem cells are mainly influenced by the self-renewal probability, since "p" determines whether S increases or decreases. On the other hand, the differentiated cells are mainly influenced by the proliferative fraction: if "a_s" is high, the stem cells divide at a high rate and the flux into the differentiated lineages is enlarged (and vice versa).

In different stress situations, this general characterization may lead to quite different reactions of the hemopoietic system. Since in "realistic" stresses some of the underlying essentials may be masked, "artificial" stresses have been simulated first.

The reduction of the number of stem cells leads to quite different recovery curves, depending on the degree of depletion as well as on the reduction in the differentiated cell lineages. In general, three phases can be identified: during the initial phase of exponential growth stem cell recovery has priority. In the second phase, competition for the newly formed stem cells takes place and the differentiated cells recover at the expense of stem cells. Finally, in the third phase, stem cells and precursors normalize in parallel. However, the first or the second phase may be missing.

If the number of erythropoietic precursors is enlarged due to peripheral demand, the indirect reaction of stem cells and granulopoietic precursors can be calculated. For S one finds an intermediate peak while G decreases to subnormal values. This decrease follows because the enlarged E inactivates the stem cells and, thus, suppresses the efflux from the stem cell pool. After cessation of the peripheral stimulation the cells normalize, where S shows a temporary dip.

The opposite situation, namely, a reduced number of erythropoietic precursors due to reduced need for mature cells, results in a significant elevation of S and G. Here the strong influence of G on the self-renewal probability is primarily responsible. After cessation of the peripheral suppression of E, S and G normalize where G shows a clear intermediate dip.

Both situations, erythropoietic stimulation and suppression, lead to the opposite behavior of the granulopoietic cells. In the model this follows from indirect stimulation or suppression of the stem cell efflux by E. This leads to an increase or decrease of G. However, E does not show similar changes because it is under the control of peripheral influences. Thus, the different effect of granulopoiesis on erythropoietic influences (compared to erythropoiesis on granulopoietic influences) can be understood without a diversion of the stem cell efflux to or from one cell lineage.

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Experiments and Model Analysis

Chapter 6

ACUTE IRRADIATION — EXPERIMENTAL RESULTS: SURVIVAL AND RECOVERY OF HEMOPOIETIC CELLS

James P. OKunewick

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I. ABSTRACT

The experimental data for the response of hemopoietic cells to acute irradiation reveal both a marked sensitivity and a pronounced recovery ability. As measured by several different investigators, with each Gy of radiation there is approximately a one-log-unit decrease in the number of surviving hemopoietic stem cells. Nevertheless, even at fairly high doses recovery of the stem cells can be rapid and virtually complete by 2 weeks. Recovery of the more differentiated cell compartments are dependent upon the stem cell recovery, but these may also experience other perturbations arising out of their own unique characteristics or out of the varying stimuli for differentiation along the different cell lines. At the tissue level, if one compares hemopoietic recovery in the spleen vs. the marrow, different patterns are evident. Recovery of total nucleated cell numbers to normal is, in general, faster in the marrow than in the spleen, and may precede recovery of the stem cells. This reflects the fact that the tissue taken as a whole consists of a variety of different types of cells, some of which may have a greater recovery potential than do the hemopoietic cells. Also, the different tissues have different types of hemopoietic functions, which would lead to variability in recovery. Therefore, in the construction of models for the mechanism of hemopoiesis it is important that one be cognizant of these variabilities in tissue and cell responses and how they may be reflected in the overall picture of hemopoiesis in the animal taken as a whole.

II. INTRODUCTION

The response of blood-forming cells to acute irradiation exposure reflects the complexity of the hemopoietic system, and not all aspects of it can be measured satisfactorily. Two of the aspects of hemopoiesis whose response to irradiation can be measured with a fair degree of reliability are the pluripotent stem cell population and erythropoietic activity. Both of these have been studied by numerous investigators, and we will focus on some of their results in this chapter.

III. RESPONSE OF STEM CELLS TO IRRADIATION

The animal systems in which the most extensive work has been done on the effects of acute irradiation on hemopoietic cells are mice [1-4] and rats.[5-7] Work in these animal models was greatly facilitated by the discovery of Till and McCulloch[3,8] in 1961 of a method for directly measuring the survival of pluripotent hemopoietic stem cells after radiation exposure.

This method, referred to as the colony-forming unit (CFU-S) technique, involves irradiating the subject mouse or rat in vivo, or its marrow or spleen in vitro, with whatever dose or doses of irradiation are of concern and transferring by i.v. injection a given number (usually between 10^5 and 10^7) of marrow or spleen cells to another recipient mouse that has also been given total body irradiation (TBI) at a level of exposure sufficient to destroy all of its own pluripotent hemopoietic stem cells. At a fixed time later (usually 8 days) the recipient mice are sacrificed, their spleens removed and fixed in Bouin's solution, and the number of hemopoietic colonies, visible as nodules on the surface of the spleen, are counted. Each colony represents a clone of cells from a single CFU-S or pluripotent hemopoietic stem cell.[9] With this technique the stem cell killing effect of the radiation exposure can be directly measured for a variety of doses and the percent of stem cells killed can be determined by comparing the surviving number of CFU-S to the CFU-S number found in unirradiated control mice.

Applying this technique to C_3H and C57BL mice, Till and McCulloch[3] were able to determine that the initial radiation survival curve for CFU-S in these mouse strains was of an exponential nature with the log of the surviving fraction of stem cells being a function

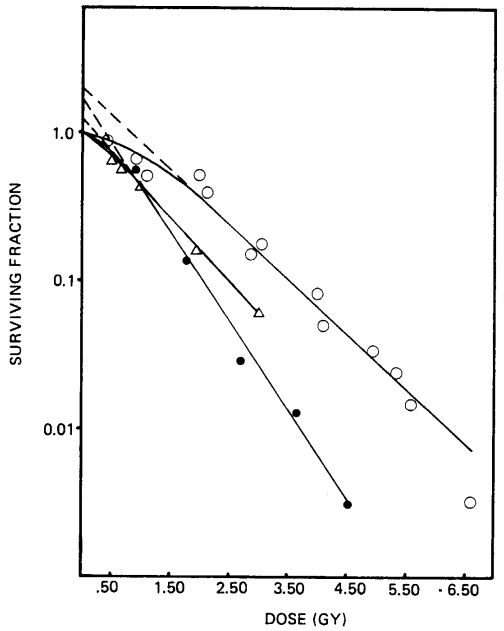

FIGURE 1. Comparative estimates of the radiosensitivity of mouse hemopoietic stem cells obtained by three different techniques, as described in the text. Data are taken from Gurney et al.[1,14] △, Do = 1.1 Gy (110 rad); Hodgson,[13] ●, Do = 0.71 Gy (71 rad); Till and McCulloch,[3] ○, Do = 1.15 Gy (115 rad).

of the dose of gamma-radiation received by the animal (see Figure 1). From this they were able to obtain a D_0 dose of 1.15 Gy (i.e., the dose on the linear portion of the curve at which survival is reduced to 37%) and a zero-dose Y-intercept extrapolation (or hit) number of 2.*

A separate, but related, method for measuring stem cell survival is the endogeneous colony-forming (ECFU-S) technique. This method measures the identical type of stem cell but differs from the CFU-S technique in that it does not involve transplantation of the cells from one animal to another recipient animal. Instead, survival of stem cells is measured *in situ* in the lethally irradiated mouse. It is more limited than the CFU-S technique in that: (1) only a narrow range of radiation doses can be investigated; (2) neither extremely small nor extremely large numbers of CFU-S can be measured; and (3) the number of surviving CFU-S in the irradiated mouse cannot be related back to an unirradiated control. Nevertheless, the comparative effects of total-body irradiation doses within the limited range 5 to 8 Gy (500 to 800 rad) on several different mouse strains can be measured and relative D_0 values can be calculated from the results. Yuhas and Storer[4] have measured these for a number of

* To achieve values in Gray (Gy) multiply rad values with 0.01. The factor for conversion of Röntgen (R) into Gray depends on the source of irradiation (γ-ray or X-ray) and the tissue considered, varying between 0.009 and 0.012. For practical reasons an average factor of 0.01 will be used in the following.

Table 1
COMPARATIVE D_0 VALUES FOR PLURIPOTENT HEMOPOIETIC STEM CELLS OBTAINED FOR VARIOUS MOUSE STRAINS BY DIFFERENT TECHNIQUES

Technique	D_0[a]	Strain	Ref.
CFU-S	1.15 ± 0.08 Gy	C57	3
CFU-S	1.15 ± 0.08 Gy	C_3H	3
ECFU-S	0.794 ± 0.058 Gy	BALB/cJ	4
ECFU-S	0.761 ± 0.036 Gy	CBA	4
ECFU-S	0.650 ± 0.044 Gy	C57BL/6	4
ECFU-S	0.838 ± 0.073 Gy	C57BR/cdJ	4
ECFU-S	0.571 ± 0.040 Gy	SWR/J	4
Unstimulated erythropoiesis after marrow transplant	0.71 Gy	$BC3F_1$	13
Epo stimulated erythropoiesis *in situ*	1.10 Gy	CF-1	14

[a] Values have been converted to Gray according to 16 Gy = 0.01 R.

different strains of mice and found them to range between 0.57 and 0.84 Gy (57.1 and 83.8 rad) (see Table 1). All of these values are considerably lower than those reported by Till and McCulloch using the CFU-S technique, as is particularly emphasized by the discrepancies for the C57 mouse as indicated in Table 1.

Yuhas and Storer[4] offer no explanation for the differences between their ECFU-S-derived D_0 values and those of Till and McCulloch, obtained using the CFU-S technique, but do suggest that the differences observed within their own studies for the various mouse strains may reflect a strain dependency, and hence, they could also reflect the influence of different strain-dependent physiological factors affecting the accuracy of the ECFU-S measurement. One such physiological factor could be a pressure for differentiation among the surviving stem cells which would compete, in the manner described by Osgood,[10] with the ability of the cell to reproduce as an undifferentiated stem cell. Although on first appraisal one would expect that the same level of differentiation pressures would apply equally to both the assay mice used for the CFU-S and those used for the ECFU-S techniques, such might not be the case. Relative to this, Chervenick and Boggs[11] have studied the effect of the size of the surviving stem cell population on postirradiation differentiation. They observed that if the number of pluripotent stem cells was reduced to less than 10% of normal, self-replication of stem cells took priority over differentiation. In contrast, when survival was more than 10% a significant proportion of the stem cells would proceed along the path of differentiation instead of self-replication. This loss of cells from the self-replicating pool would result in a further postirradiation depletion of stem cells occurring as a direct result of the physiological pressure of differentiation.

Considering the differences in the results of the ECFU-S vs. the CFU-S techniques in light of this, the number of stem cells transplanted with the CFU-S technique is, in most cases, much smaller than the numbers surviving the *in situ* radiation of the ECFU-S technique. Therefore, with the CFU-S technique it is possible that there is little early shift among transplanted stem cells to a differentiated compartment until after the clones are first established. Whereas, in the case of the untransplanted stem cells of the ECFU-S technique, there could be a large-enough early shift to differentiation before self-replication to significantly lower the resultant number of clones established, thus giving a lower value for the D_0 dose following TBI and resulting in an apparently higher level in radiosensitivity.

Additional evidence in support of the theory of the effect of differentiation pressure on recovery lies in the "postirradiation dip" that has been observed in both the mouse [1,12] and rat[6] following exposures of 3.0 Gy (300 rad) or less. This is illustrated in Figure 2. In both

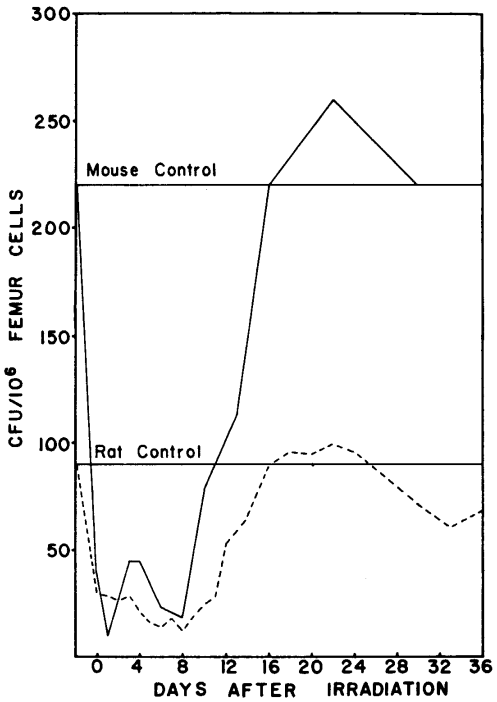

FIGURE 2. Recovery curves for femoral stem cells in the mouse and the rat following sublethal total body irradiation. The curves illustrate the delay in recovery of the stem cell compartment and the additional loss of stem cells that occurs in vivo following TBI at these dose levels, known as the "postirradiation dip". Data are taken from OKunewick et al.,[6] rats, dashed line; Beran,[12] mice, full line.

animal species the initial decrease in stem cell number due to irradiation damage is also followed by a further smaller reduction and a delay in recovery, which does not begin until about 10 days after radiation. Thereafter, recovery occurs rapidly and reaches a maximum at about 22 days, following which there may be another fall to lower levels and an oscillatory return to near-normal values. This postirradiation dip is observable not only in the direct evaluation of stem cell survival using the CFU-S technique, but also in earlier applied indirect techniques which employed the postirradiation recovery of erythropoiesis with[5] or without transplantation[6] to estimate stem cell loss and recovery.

IV. RESPONSE OF ERYTHROPOIESIS TO IRRADIATION

Besides the direct CFU-S estimates of the effect of radiation on stem cells, recovery of erythropoietic response in marrow-transplanted animals or in erythropoietin (Epo)-stimulated animals has also been used to estimate stem cell damage by irradiation exposure. However, these methods are also less direct and less accurate than the CFU-S technique, in that they

are dependent upon selective differentiation after irradiation along the erythropoietic line. Moreover, differing demands on the pluripotent stem cell for differentiation in alternative directions (e.g., granulopoietic vs. erythropoietic) can affect the values obtained by this estimate.

In both of the erythropoiesis techniques the end points measured are erythrocyte production as estimated from ^{59}Fe incorporation into the hemoglobin of the red blood cells produced after irradiation. The two principal contributors to these studies have been Hodgson[13] and Gurney et al.[14]

Hodgson's approach to the problem was to irradiate a set of animals with a dose of TBI sufficient to eliminate all erythropoietic activity. These then received a marrow transplant of a fixed number of cells from other donor mice of the same strain. Prior to transplant the donors were irradiated with sublethal TBI ranging from 0.9 to 4.5 Gy (90 to 450 rad). After transplant (9 days), the recipients were given ^{59}Fe intravenously, and 24 hr later blood samples were taken and measured for uptake of the radioisotope. This approach assumed that the relationship between erythropoietic recovery of the transplanted animals and the surviving stem cells transplanted would be a linear one, which assumption was also tested by Hodgson[13,15] and found to be valid.

Gurney's approach to the problem involved the use of mice which were first rendered polycythemic by hypertransfusion and then given varying doses of TBI, followed by injection of Epo. The purpose of hypertransfusion before irradiation was to cease all normal erythropoiesis and thus allow for an artificially controlled level of erythropoiesis in response to the Epo. The assumption was that the extent of Epo response, as measured by ^{59}Fe uptake 72 hr later, would reflect the proportion of surviving stem cells. In contrast to Hodgson's approach, Gurney's technique involved no transplantation of hemopoietic tissue, but procedures that were performed *in situ*. This could be considered advantageous in that the results obtained via the Gurney technique would be more reflective of the relative stem cell radiosensitivity on the basis of the whole animal.

The D_0 values and extrapolation numbers obtained by Hodgson[13] and Gurney et al.[14] are compared in Figure 1 and Table 1 to those of Till and McCulloch.[3] In both instances the erythropoiesis-dependent techniques yielded a lower extrapolation number (Y-intercept) than the CFU-S technique. However, the D_0 value obtained by Gurney et al.[14] nearly matched that of Till and McCulloch.[1] Since the estimates of stem cell radiosensitivity that are obtainable by measurements of red cell production are dependent upon erythropoietic differentiation, then the conditions most likely to achieve the more accurate D_0 values are those in which the majority of the differentiating cells are forced to proceed along the erythropoietic lines after the establishment of the clones for hemopoietic renewal by the surviving stem cells. On the other hand, the D_0 values estimated from levels of red cell production in the Epo-unstimulated assay animal may be less accurate, due to loss of cells to the other differentiation pathways. Relative to this it is interesting that the D_0 value of 0.71 Gy (71 rad) obtained by Hodgson[13] is in the lower range of the values reported by Yuhas and Storer[4] with the ECFU-S technique.

V. RESPONSE OF GRANULOPOIESIS TO IRRADIATION

A separate method for estimating the radiation sensitivity of hemopoietic stem cells is the in vitro agar colony-forming technique.[16] This technique does not measure the pluripotent stem cells, but rather a committed stem cell derived from them, which is the CFU-GM. This committed stem cell, when cultured in vitro under the proper conditions, is capable of producing mixed colonies of differentiating megakaryocytes, macrophages, and granulocytes, but not erythropoietic or lymphopoietic cells. Estimates of radiation sensitivity drawn from the in vitro colony technique must be considered valid, therefore, for only the myeloid-

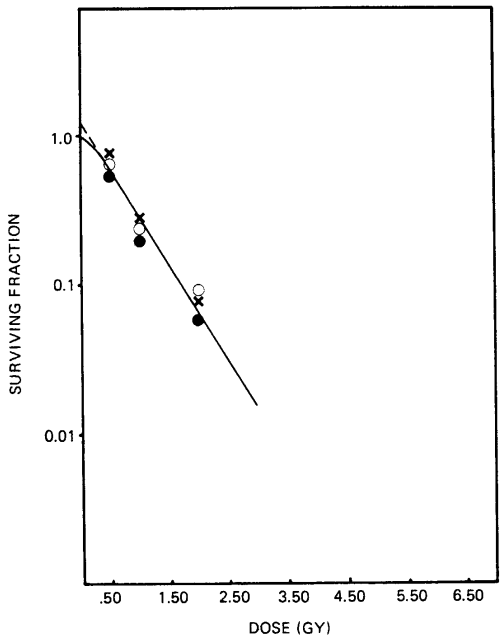

FIGURE 3. Estimate of radiosensitivity of granulopoietic-megakaryocyte committed stem cells obtained with the use of the in vitro CFU-GM technique. Data taken from reference 16.

megakaryocyte progenitors. Nevertheless, D_0 and extrapolation values that have been obtained are quite similar to those reported for the other techniques we have described, especially that of Hodgson[15] for erythropoiesis by transplanted marrow cells. Robinson et al.[16] examined the radiosensitivity of the CFU-GM in C57BL mice irradiated in vivo and found that the ratio of CFU-GM colonies formed in culture when compared to the irradiation dose received by the donor mice in vivo yielded a D_0 value of 0.85 Gy (85 rad) (see Figure 3). This value was consistent over a wide range of cell numbers plated, from 6.25×10^4 to 1×10^6, but lower than that for the CFU-S technique[3] or the Epo-stimulation technique of Gurney.[1,14] However, it must be again emphasized that, like the erythropoietic techniques, the CFU-GM technique measures a defined subpopulation of hemopoietic cells and the values obtained, therefore, only reflect those stem cells which differentiate into that particular subpopulation.

VI. RECOVERY OF HEMOPOIESIS IN THE BONE MARROW

The recovery of hemopoiesis in vivo following irradiation has been studied by several groups of workers, and the recovery patterns observed have been much more consistent than the estimates of stem cell radiosensitivity. We have already compared in Figure 2 the recovery patterns for pluripotent stem cells in rats and mice as reported by OKunewick et al.[6] and by Beran.[12] Similar patterns have been reported by Blackett et al.[5] and by Porteous and

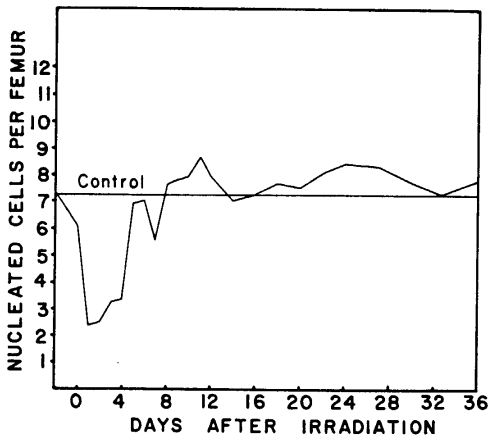

FIGURE 4. Recovery curve for total nucleated cells per femur in the rat following 2.0 Gy (200 rad) TBI. Comparison with the data of Figure 2, which was obtained from the same animals, illustrates the more rapid recovery in numbers of total nucleated cells as compared to stem cells alone. Data taken from OKunewick et al.[6]

Lajtha.[2] Recovery in vivo of the stem cell compartment in all cases was observed to be delayed.[2,5,6,13,17] In contrast, recovery of total nucleated cells per femur has been observed to begin much earlier than stem cell recovery and, as shown in Figure 4 for the rat, reaches normal levels at about 5 days or roughly half the time required for stem cell recovery.[2,6,13] Similar data have been presented for the recovery of femur cellularity in the irradiated mouse.[17] This suggests, as has already been discussed above, that recovery of the differentiated cell compartment in the sublethally irradiated animal may proceed more rapidly than stem cell recovery and to some extent at the expense of the stem cell compartment.

Not only does the total nucleated cell compartment of the femur recover more rapidly than the stem cell compartment, but all of the individual functioning compartments of the femur recover more rapidly as well. Beran[12] has measured erythropoietic, granulopoietic, and lymphopoietic cells per femur in NMRI mice given 3.0 Gy (300 rad). His data for these are presented in Figure 5 and may be compared to his data for mouse stem cell recovery as given in Figure 2. As can be seen each cell type also reflected its own pattern of variability, and as has also been reported by Beran,[12] these could be further affected by eliminating the normal stimulus for erythropoiesis in the mice, either by hypertransfusion or preirradiation hypoxia. This will be discussed in more detail below.

VII. RECOVERY OF HEMOPOIESIS IN THE SPLEEN

Recovery of hemopoiesis in the spleen follows different patterns than recovery in the femur. Figure 6 illustrates this point with regard to the rat. Recovery of spleen cellularity in this species is delayed, similar to the delay in femoral stem cell recovery, and does not return to normal ranges until about the third week following exposure to 2.0 Gy (200 rad) of X-ray. In contrast, in the mouse recovery of splenic activity and cellularity proceeds at a more rapid rate,[17] and in the case of erythropoietic function may also significantly overshoot the normal values.

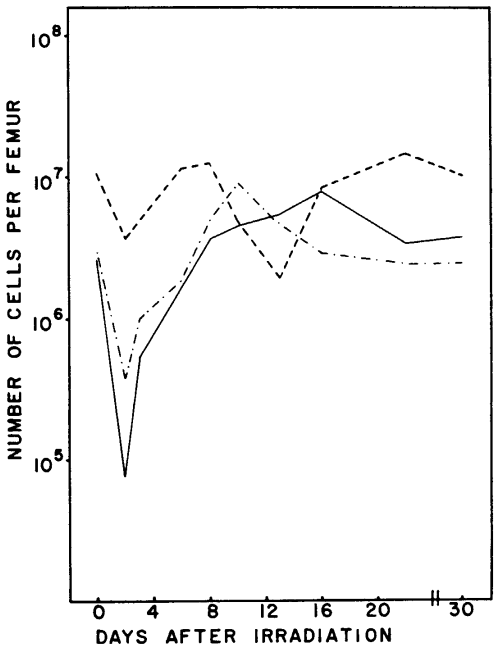

FIGURE 5. Recovery patterns for femoral cells in the mouse after 3.0 Gy (300 rad) TBI. The data illustrate the contrasting behavior of the granulopoietic (— — —), erythropoietic (——), and lymphopoietic (— •—•—) compartments and the more rapid recovery of all three differentiated compartments, as compared to recovery of pluripotent stems as given in Figure 2. Data taken from Beran.[12]

In addition to the femur data presented above, Beran[12] has also studied recovery of the splenic erythropoietic, granulopoietic, and lymphopoietic compartments in mice following exposure to 3.0 Gy (300 rad). These data are presented in Figure 7. By 4 days the erythropoietic cells have been regenerated to a level twice normal, following which there was a return to normal values and a second rise to a point ten times the normal levels within 2 weeks. In contrast, the granulopoietic cells did not recover and instead showed a continuous fall as the number of erythropoietic cells increased. Lymphopoietic cells showed a decrease until about 5 days when recovery began once again, paralleling the second recovery rise of the erythropoietic compartment, but at lesser orders of magnitude. These data suggest that in the mouse the spleen may be an important organ for erythropoietic recovery, which recovery overshadows that of other hemopoietic components. Thus, the complexity and interactions involved in postirradiation hemopoietic recovery indicate that for the purposes of mathematical modeling, unless a careful analysis is made, excessive compartmentalization on the basis of cells per spleen or femur may lead to difficult and not always valid interpretations. Conversely, studies at the level of the whole animal may be more easily interpretable and valid as they represent the end result of an overall control mechanism rather than individual effects in various different tissues which may have overlapping properties and functions.

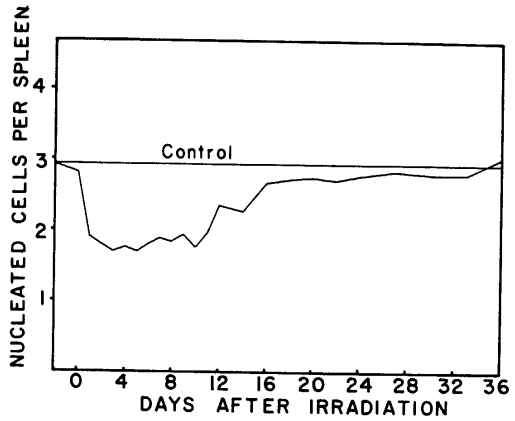

FIGURE 6. Recovery pattern of rat spleen cells following exposure to 2.0 Gy (200 rad) TBI, illustrating the slower recovery of cellularity in the spleen as compared to that of the femur as shown in Figure 4 for the same experimental animals. Data taken from OKunewick et al.[6]

VIII. ABORTIVE RISE

Among the most reliable and reproducible measurements of postirradiation hemopoietic recovery are those which have been obtained for erythropoiesis as measured by the appearance of ^{59}Fe-labeled cells in the whole blood of the nontransplanted and nonstimulated animal. Figure 8 compares the data obtained by several different workers[17-21] for rats which received total body exposures of between 1.0 and 4.5 Gy (100 and 450 rad). In all instances there was an initial rapid recovery, peaking at about 6 days, followed by a second drop and recovery which reached its maximum at about 3 weeks. The early recovery peak of erythropoietic activity in the rat has been referred to as the "abortive rise" in a previous mathematical model,[18] which model assumed that it reflected passage into the erythropoietic compartment of damaged cells which had lost their ability for continous reproduction as stem cells, but still retained a differentiation capability. The data of Figure 8 suggest that the height of the abortive rise is a dose-dependent phenomenon and that it becomes less as the radiation dose increases. This would be consistent with the interpretation of OKunewick and Kretchmar[18] that the process of differentiation is less sensitive to irradiation than stem cell reproduction, but that with increasingly higher doses of radiation the injured stem cells also progressively lose their differentiation capabilities as well as their capacity for indefinite clonal reproduction. On the other hand, the stem cells responsible for the later (and more permanent) recovery of erythropoietic function are in all probability those which escaped serious injury from the radiation and retained their full reproductive and differentiation potentials.

Confirmation of an abortive rise in the mechanism of postirradiation hemopoietic recovery has been provided by Boggs et al.[22] from studies done in mice. These workers demonstrated the appearance in the spleen of small erythropoietic colonies after 6.0 Gy (600 rad) of X-ray, which colonies had a limited life span of only between 4 and 6 days as opposed to the longer-lived colonies from which true recovery develops. They also observed that the number of these abortive erythropoietic colonies could be increased if the animals were bled within 1 to 12 hr after X-ray exposure, with the greatest effect being observed when bleeding

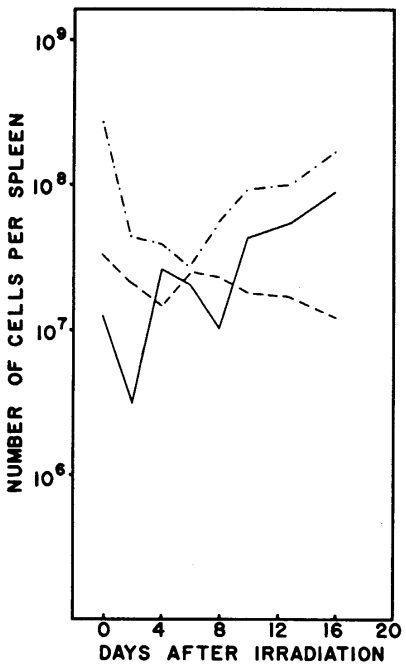

FIGURE 7. Recovery patterns of the erythropoietic (——), granulopoietic (— —), and lymphopoietic (—•—) cells in the mouse spleen following 3.0 Gy (300 rad) TBI, illustrating the variations in behavior of the three compartments within the spleen and the further contrast with their behavior in the femur as shown in Figure 5. Data taken from Beran.[12]

occurred in less than 4 hr after irradiation. Later bleeding, initiated at 24 and at 48 hr after irradiation, induced progressively lower numbers of abortive splenic colonies in the irradiated mouse. This indicated that the demonstration of these colonies was not only influenced by the bleeding stimulus, but was also dependent on the response to that stimulus of progenitor cells with a relatively short postirradiation survival time. Thus, the cells responsible for the abortive rise were a different population than those uninjured surviving pluripotent stem cells that are responsible for the later restoration of normal erythropoiesis. Studies to confirm that the colonies were erythropoietic were also carried out. These consisted of determining the extent of ^{59}Fe uptake in the spleens of the above irradiated and bled mice. The results of these iron incorporation studies paralled the increases noted in numbers of abortive colonies, thus confirming that the abortive colonies contained significant amounts of erythropoietic cells.

IX. COMBINATION OF IRRADIATION AND ERYTHROPOIETIC STIMULATION OR SUPPRESSION

Bleeding exerts its stimulating effect by inducing the production of Epo, and studies with

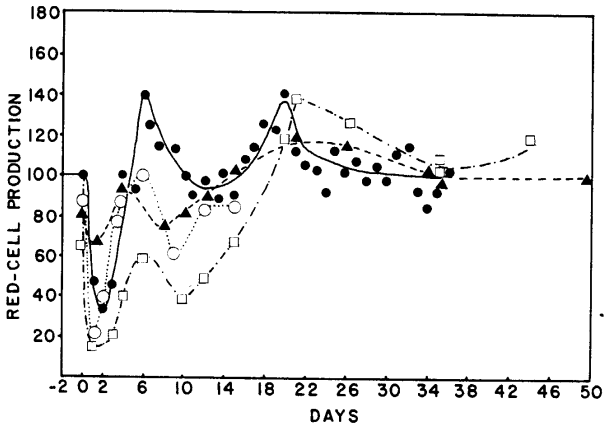

FIGURE 8. Postirradiation recovery of erythropoiesis at the level of the whole animal, as indicated by the appearance of radio-iron-labeled reticulocytes as a function of time after TBI exposure. The data for three different doses as obtained by three different groups using similar techniques are compared. The results indicate the similarity of recovery patterns obtained. The 6-day "abortive rise" is apparent in all curves and its dose dependency is illustrated, with maximum height following 1.0 Gy (100 rad) and progressively lesser magnitudes at 2.0 Gy (200 rad) and 4.5 Gy (450 rad). Data taken from Hennessy and OKunewick,[19] ●, 3.0 Gy; Harriss,[20] ○, 2.0 Gy; Blackett and Roylance,[21] ▲, □, 2.0 Gy, 4.5 Gy.

bled or hypoxic animals have shown that significant increases in Epo levels can be demonstrated within an hour after the initiation of the stimulus. The effect of irradiation on Epo production is also stimulatory but the effect is less immediate. O'Grady et al.,[23] Schooley,[24] and OKunewick et al.[6] have reported increased levels of Epo in irradiated mice and rats beginning between 1 and 7 days after exposure.[6]

However, the amounts of Epo detected have been quite variable[6] and it is doubtful that the observed Epo levels are a direct effect of the radiation. Rather, they are more likely an indirect result of the anemic condition that develops in the irradiated animal. These increased levels of postirradiation Epo are sufficient to induce increased erythropoiesis in the irradiated animals, but the added stimulus of bleeding immediately after irradiation could produce still earlier and higher levels, consistent with the demonstration of the abortive rise in spleen colonies, as described above.

Further evidence for the influence of erythropoietic stimulus on recovery of hemopoietic stem cells and progenitors comes from work in which erythropoiesis is suppressed, such as posthypoxic and hypertransfused mice.[12,25,26] OKunewick et al.[25] and Tribukait and Forssberg[26] observed that the posthypoxic mouse was significantly more capable of surviving a lethal dose of irradiation than the normal mouse, and that this increased radioresistance was reflected in changes in ECFU-S of the irradiated posthypoxic mouse and their response to Epo,[25] suggesting that reduction in the stress for erythropoiesis may have had a favorable effect on postirradiation recovery of the stem cells and on other types of differentiated hemopoietic progenitors. The work of Beran[12] also extends into this area. In the irradiation of mice that had been subjected to extensive hypoxia before irradiation, he observed a more rapid recovery of the CFU-S in both the femur and spleen. Granulopoietic recovery was also accelerated. In contrast to this, erythropoietic recovery was depressed, while lymphopoietic recovery appeared essentially unchanged. The results suggest that under conditions of lowered stress

for erythropoietic differentiation, a greater number of the surviving stem cells are available for differentiation along other lines, or for self-replication to more rapidly restore the radiation-depleted stem cell compartment. An improved recovery of the stem cell compartment would, on the basis of the Till and McCulloch[3,8] studies, predict a greater probability of survival for posthypoxic mice. Thus, the results obtained by Beran[12] are consistent with the improved survival of posthypoxic mice that had been observed by the earlier workers.[25,26]

X. RESIDUAL DAMAGE

A final element to be considered in the evalulation of effects of acute irradiation on the hemopoietic system is the matter of long-term unrepaired injury. This has been studied by Baum,[7] who found that following 3.0 Gy (300 rad) of whole-body irradiation, the erythropoietic recovery ability, as reflected in response to Epo was still significantly impaired as late as 3 months after the radiation. A second irradiation at that time produced still further impairment. As reported by him, the impairment was not in the maximum levels of erythropoietic recovery that were reached, but in the rate of recovery of erythropoietic function. Baum's interpretation of these results is that each additional irradiation lowers the stem cell reserve, such that fewer cells from this reserve are available for initial repopulation of the erythropoietic compartment after irradiation. However, at the same time the total reserve in the recovered animal would be still greater than the minimum needed to maintain normal steady-state erythropoiesis, and, therefore, the unrepaired radiation injury would only be detectable in recovery from severe conditions of stress such as another large dose of radiation.

The work of Braunschweiger et al.[27] offers a confirmation of this thesis in a related mouse model involving the chemotherapeutic drug, adriamycin, rather than irradiation. These workers observed that at 45 days following exposure to the drug the steady-state erythropoietic parameters of adriamycin-treated mice had returned to what were apparently normal control values. However, the recovery response of the drug-treated mice to a stress of severe bleeding was markedly impaired in contrast to that of the nontreated controls. Reduced erythropoietic response was detectable in both the marrow and spleen of the drug-treated mice. Further, the extent of residual injury was a dose-dependent function of the amount of drug initially administered. Finally, comparison with other animals evaluated at later time points of 120 days after drug treatment indicated that the level of residual injury was the same. Thus, no additional recovery had occurred in the period between 45 and 120 days after exposure to the drug. Therefore, the damage to the hemopoietic reserve by the drug appeared to be permanent, similar to the damage caused by radiation.

XI. CONCLUSION

In summary, evaluation of the effects of acute irradiation on hemopoiesis must take a variety of factors into consideration. The first of these is the sensitivity of the pluripotent stem cells and committed hemopoietic progenitors to the radiation dose employed. Accurate information for the radiosensitivity of the CFU-S is obtainable. Likewise, sensitivity estimates for CFU-GM and erythropoietic progenitors have been determined, but since these represent compartments into which cells are both feeding into from the pluripotent stem cell compartment and out of differentiation, their radiosensitivity estimates may be more variable and less accurate than that for the CFU-S. Second, the pressure within the hemopoietic system for the cells to differentiate into many competing directions must be taken into consideration, as differentiation may further complicate the system, especially under those conditions where a particular differentiation stimulus may be increased or decreased. Third, the sites of hemopoiesis are multiple and naturally redundant in the normal animal, but each site does not necessarily carry out the same level of activity either with respect to specific

functions (e.g., granulopoietic vs. erythropoietic) or total output of cells. As such, their activity should be viewed as a whole, otherwise projections based primarily on one organ or tissue could lead to erroneous interpretation. Lastly, the possibility of long-term residual injury which would be expressed primarily in what normally constitutes the reserve component of stem cells in the animal should be considered. Such long-term injury, as the data indicate, may be demonstrable only in the event of later hemopoietic stress, and detectable only in variation in rate of recovery, and may not be otherwise demonstrable under normal steady-state conditions.

ACKNOWLEDGMENT

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Chapter 7

ACUTE IRRADIATION — A MODEL ANALYSIS*

Markus Loeffler and H.-Erich Wichmann

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I. ABSTRACT

The recovery of hemopoiesis after acute irradiation exhibits a variety of phenomena. Stem cells and progenitor cells recover slower than precursors. Depending on the irradiation dose, stem cells grow exponentially or have an initial plateau. In some reports precursors show oscillations, in others oscillating behavior has not been noted. These phenomena are analyzed by a mathematical model of stem cell regulation. The characteristics of the experimental data can be reproduced by the model. The analysis suggests that an intramedullary regulation occurs at the level of stem cell self-renewal and cell cycling. There is a competition between stem cell self-maintenance and differentiation for the newly formed cells. For very low stem cell numbers, their recovery is favored, while for only slightly reduced stem cell numbers differentiation is preferred. This alternate play of the two influences explains most of the experimental findings. The radiosensitivity of differentiated cells varies between 8 and 100%, if compared with stem cells. Its consequences on the recovery curves are demonstrated.

II. INTRODUCTION

The behavior of hemopoietic stem cells after a single dose of irradiation belongs to the most intensively examined subjects in the field of murine hemopoiesis. Many investigators have studied CFU-S after X-ray or gamma-ray doses between 1.0 and 6.0 Gy.[1-15] Further data are available for CFU-GM,[4,7,9,13,15,16] BFU-E,[10,13] CFU-E,[10,13,15,17] and ERC.[11] The experiments on iron-incorporating cells in rats have been reviewed by OKunewick.[18] For mice, data on 59Fe-incorporation,[19,20] erythropoietic and granulopoietic precursors,[1,16] and total nucleated cell count[5,16,21,22] have been recorded.

After acute irradiation the hematocrit remains normal over 20 days if not more than 4 Gy are administered.[16,20,23] For higher doses it drops in a dose-dependent way.[23-25] In contrast to this, the granulocyte blood count seems to be more sensitive to irradiation in mice.[16,20] Rats[27] and rabbits,[28] however, may exhibit elevated levels of granulocytes for 20 days if the doses remained below 2 Gy.

Dose response curves have only been reported for CFU-S[5,9,29,32,33,45] and CFU-GM.[9,32,33]

As data on rats are limited,[27,35-40] the following analysis will refer to data from mice. It can be shown that the spleen never contributes more than 10% of hemopoietic cells during irradiation recovery.[41] Thus, the bone marrow is a good representative for the whole blood-forming system.

OKunewick[40] has postulated four criteria which should be taken into account for mathematically modeling the effect of irradiation. The model should consider

1. The consequences of different cellular sensitivities to radiation
2. Competition between granulopoiesis and erythropoiesis for differentiating stem cells
3. The whole animal's hemopoiesis rather than that in special organs
4. Long-term effects of residual injury

To these criteria we add the following ones:

1. The characteristics of CFU-S recovery which often show an initial phase with exponential growth,[1,3,5,6,8,14] sometimes followed by a temporary plateau,[1,3,5,11,12] before the cell number returns to (nearly) normal values
2. The possibility of an early "abortive rise" in precursor cells[1,10,18,25,27]
3. The (mostly) slow and not overshooting recovery of the immature erythropoietic and granulopoietic progenitors[4,7,9,10,13,15,16]

It is the aim of this study to satisfy most of these criteria.

Table 1
SURVIVAL OF EARLY HEMOPOIETIC CELLS AFTER SINGLE DOSE IRRADIATION

Dose (Gy)	Range of survival (fraction of normal)		
	CFU-S	Progenitors	Precursors
0—0.99	n.e.d.[a]	0.25—0.75	n.e.d.
1—1.99	0.09—0.2	0.05—0.50	0.1—0.7
2—2.99	0.01—0.07	0.10—0.25	0.35—0.6
3—3.99	0.002—0.02	0.01—0.20	0.02—0.45
4—4.99	0.002—0.005	n.e.d.	0.10—0.20
5—8	n.e.d.	0.005—0.04	0.003—0.06

Note: Due to the heterogeneity of the data, only the range of survival is given. Data entering into this table are taken from minimum values of recovery studies for CFU-S,[1-14] erythropoietic and granulopoietic progenitors,[4,7,9-11,13,15-17] and precursors.[1,5,16,21,22] In addition, dose-response curves for CFU-S[5,9,29,32,33,45] and CFU-GM[9,32,33] were taken into account.

[a] n.e.d.: not enough data.

III. MATHEMATICAL METHODS

The experiments on acute irradiation are simulated by the mathematical model of stem cell regulation as characterized earlier.[42-44] For the simulation and for the comparison with the measurements one has to find out

1. How to describe the acute destruction of cells mathematically and how to relate this description to the experimental dosage
2. How to derive the relative radiosensitivity of differentiated cells compared with stem cells

These problems are solved as follows: (1) the usual way to describe the destruction of cells by acute irradiation are dose-response curves which relate the surviving fraction to the dose. These curves could, in principle, be obtained for stem cells and all differentiated cells. For the purpose of a subsequent model simulation of postirradiation recovery, it would be good to consider the surviving fraction not immediately after irradiation, but to take the minimum cell numbers which are reached within the first 3 days after irradiation. Recovery after a certain dose could then be simulated by simply reducing the initial values in the model compartments according to these dose-response curves. In practice, dose-response curves are not available for all compartments. If one tries to overcome this lack by pooling the data from different experiments, one also does not yield satisfactory dose-response relations (Table 1). The data show an enormous scatter (up to a factor of 20) which reflects the different types of radiation sources, radiation regimes, animal strains, and evaluation procedures. The problem with the missing dose-response curves could be ignored if simultaneous measurements of the postirradiation minima were available for all cell compartments. Then, the initial values could be taken directly from these measurements. However, such complete sets of data also are not available. In total, it will not be possible in this study to relate model calculations directly to a certain dose. Only qualitative correlations to the dosage can

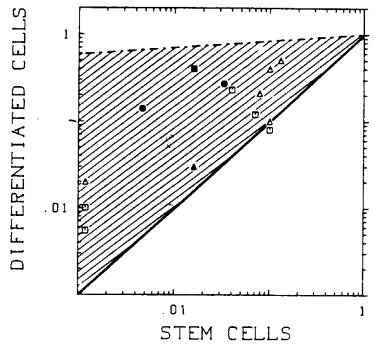


FIGURE 1. Survival fraction of stem cells (CFU-S) vs. differentiated cells (progenitors and precursors) determined in the same experiment. The full line indicates equal radiosensitivity of stem cells and differentiated cells. The dashed line represents a 12 × smaller radiosensitivity of the differentiated cells. The hatched segment between the lines covers the range in which the experimental points lie. The data points indicate measurements of CFU-S paired with erythropoietic progenitors (BFU-E,[10,13] CFU-E,[10,13,15] or ERC[11]): △; granulopoietic progenitors (CFU-GM[4,7,9,15]): □; erythroblasts:[1] ▲; myeloblasts:[1] ■; total nucleated cells:[5] ●.

be given; (2) while the absolute radiosensitivity, in terms of dose-response relations, cannot be obtained, information about the relative radiosensitivity is available. For this purpose survival data of simultaneously measured cell stages are compared with each other irrespective of the dose applied. This is shown in Figure 1 where the surviving fraction of differentiated cells is plotted against the survival of CFU-S. The data are analyzed below.

Two types of recovery experiments will be simulated. The first type represents equal relative radiosensitivity of all cells. Here five sets of initial values are considered. The second type represents a reduced radiosensitivity of the differentiated cells, for which three calculations are performed.

IV. RESULTS

A. Initial Value Problem

Before recovery from acute irradiation can be simulated with the model, one has to determine the initial values. From the minima (day 0 to 3) of the available cell numbers the following information can be derived.

1. Dose-Dependent Survival of Cells

In Table 1 dose responses are collected for CFU-S, progenitor (BFU-E, CFU-GM, CFU-E), and precursor cells. Apparently, the data are so heterogeneous that no "pooled" dose-response curves can be drawn. The table shows qualitatively that the same dose stem cells, on average, are reduced more than progenitors or precursors. However, the scatter of the data is so large that no quantitative conclusions can be made nor can the results be used to estimate initial values for the model simulation of irradiation recovery.

2. Relative Radiosensitivity

The survival of differentiated cells (erythropoietic and granulopoietic progenitors and precursors) can be plotted against the survival of CFU-S if they have been measured simultaneously. This kind of plot eliminates the dose of irradiation and compares the relative effects on the differentiated cells vs. stem cells (Figure 1). If the radiosensitivity of both cell types is the same, the survival is reduced to the same degree. This corresponds to the full diagonal line in Figure 1. A case in which the differentiated cells are less radiosensitive than the stem cells is shown by the dashed line. The data points lie between these two lines. Thus, the area between the full and the dashed line covers the experimental range for the relative radiosensitivity. The two extremes can be characterized by the relation

$$\ln D = r * \ln S \qquad (1)$$

where D represents the differentiated cells (BE, CE, E, CG, and G) and S the stem cells. The coefficient r = 1 corresponds to the full line (same relative radiosensitivity) and r = 0.08 to the dashed line (the radiosensitivity of D is 8% that of S).

B. Recovery Behavior

Since it is not possible to relate the model calculations to certain doses, a more qualitative comparison of theoretical and experimental recovery curves will be presented.

1. Model Calculations

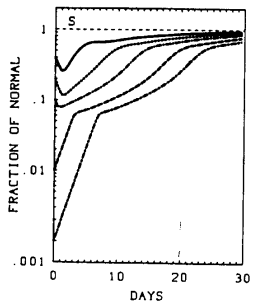
a. Same Radiosensitivity of All Hemopoietic Cells

Figures 2 to 10 show five examples simulating different doses of irradiation where the cell numbers were reduced to values between 0.001 and 0.4 times normal. In these figures a homogeneous reduction is assumed in all compartments so that the initial values chosen are identical for S, BE, CE, E, CG, and G.

As can be seen in Figure 2 the behavior of stem cells depends very much on the initial values. It is possible to discriminate three phases of recovery of which the first or second may be missing.

Phase I (stem cell autonomy) — For very small initial values S increases exponentially (Figure 2, lower curves). The slope is determined by the maximum values of the proliferative fraction (a_s = 1.0, Figure 9) and the self-renewal probability (p = 0.60, Figure 8). According to Equation 1 in Chapter 4, this is equivalent to a doubling time of 27.7 hr. Stem cell recovery is autonomous here, i.e., independent of the number of available precursors. In other words, a strong demand for stem cells overrules any demand for differentiated cells. The term "autonomy" is chosen to indicate that self-renewal becomes insensitive to signals from the differentiated cells.[43] It must be emphasized, however, that even during autonomous growth a considerable number of stem cells will differentiate (40% of the newly formed cells). Consequently, the differentiated cells also show an exponential increase (Figure 4), which is nearly parallel to the exponential increase of S. Exponential growth of S continues up to a threshold which is at about 6% in Figure 2, but may be higher under different circumstances (see References 43 and 44).

Phase II (regulation) — For values of S above 6% regulatory processes gain impact and the slope of the recovery curve flattens (Figure 2, lower curves). During this phase all precursor cells are below normal (Figures 5 and 7, lower curves) representing an increased need for differentiated cells. This demand existed already during phase I, but now the stem cell number has recovered so that the need for stem cells is no longer dominating. The result is shown in Figure 8 (upper curves); "p", which has been maximum during the first phase, now drops to values only slightly above normal. The recovery of S slows down and a "plateau" follows after the exponential growth phase (Figure 2, lower curves), while the differentiating hemopoietic elements receive an increased supply. If the initial values are



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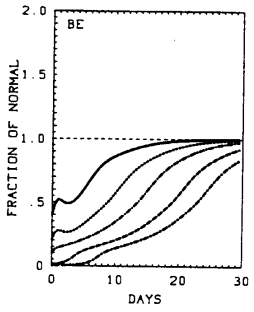

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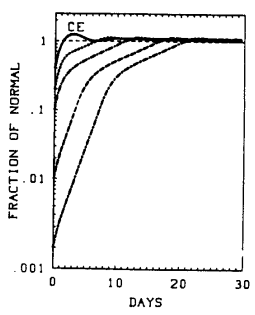

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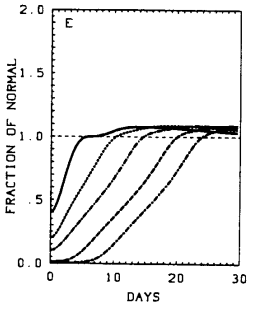

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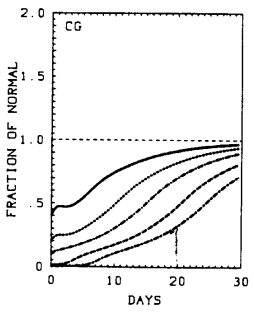

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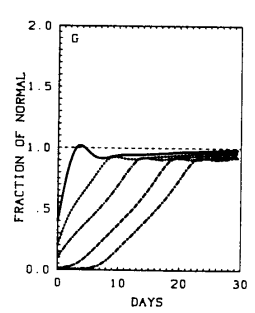

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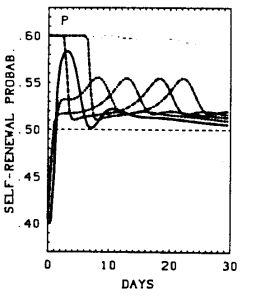

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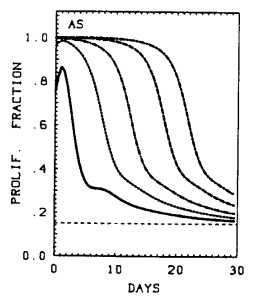


FIGURE 9

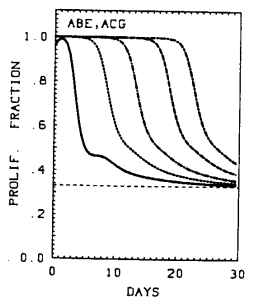


FIGURE 10

FIGURES 2 to 10. Acute irradiation — equal radiosensitivity. Five model calculations are performed where all compartments were reduced to the same initial values of 0.001 (— - —), 0.01 (— —), 0.1 (— • —), 0.2 (- - - -), 0.4 (—).

only moderately reduced the behavior of S is characterized by a temporary dip instead of exponential growth (Figure 2, upper curves). This follows because phase I does not occur for such high initial values. As the system starts above the 6% threshold it is immediately placed in the regulated region.[43] The dip shows that for several days differentiation actually exceeds self-renewal ($p < 0.5$, Figure 8 lower curves). In summary, phase II is characterized by S plateaus or dips and rapid normalization of CE, E, and G.

Phase III (normalization) — The last phase is triggered as soon as the precursor cells (Figures 5 and 7) reach about normal values. The pressure on stem cells to differentiate vanishes and self-renewal is favored again (Figure 8). Phase III is characterized by the normalization of stem cells while E and G are already normal.

Two aspects of this recovery process deserve extra mentioning: (1) Figure 9 displays the behavior of the stem cell cycling. The curves for "a_S" indicate that the duration of increased cycling depends on the initial values. It lasts longer if the initial reduction was more severe. (2) Figures 3 to 7 demonstrate that the early progenitors (BE, CG) recover slower than the late progenitors (CE) and the precursor cells (G, E). What is the reason for this difference? To elucidate this, a look at the proliferative fractions "a_S", "a_{BE}", and "a_{CG}" is helpful (Figures 9 and 10). All are increased because the cycling of these cells is activated. The higher cycling rate of stem cells ($a_S = 1$) leads to an increased rate of stem cells entering differentiation. The higher cycling of progenitors ($a_{BE} = a_{CG} = 1$) leads to an accelerated flux of progenitor cells into the precursor compartments. In total, the wave of cells going into the progenitor compartments is (in part) shifted through these into the precursor compartments. Therefore, one finds a more pronounced recovery of precursors than of progenitors.

One can formulate this in a different way: the increase of "a_{BE}" and "a_{CG}" to 1 corresponds to a shortening of the compartment transit time from 120 to 40 hr for BE and from 240 to 80 hr for CG. The stem cells, which have differentiated, pass through BE and CG three times faster than normal. Therefore, the chance to find cells within these compartments is three times smaller than in the case of normal transit times. The cells return to the normal maturation velocity after having arrived in CE, E, and G, because the proliferative fractions (a_{CE}, a_E, and a_G) of these cells are already maximum under normal conditions and cannot be further increased (see References 42 and 43).

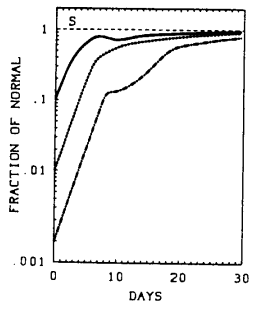
b. Higher Radiosensitivity of Stem Cells

In Figures 11 to 16 the initial values of the differentiated cells are chosen to be larger than for the stem cells. This corresponds to a lower radiosensitivity of the differentiating cells. Three model calculations are shown. Since the characteristics of the regulatory functions "a_S", "p", "a_{BE}", and "a_{CG}" are similar to those in Figures 8 to 10, only the cell compartments are drawn.

The stem cells (Figure 11) show the same type of behavior as detailed in Figure 2, except that the autonomous phase lasts longer. This follows because the need for differentiated cells is less pronounced, due to their higher initial values. The initial decrease of BE and CG (Figures 12 and 15) is caused by the shift of these cells to the subsequent precursor compartments. Characteristically, a pronounced early peak is found which is followed by a nadir after 5 to 10 days (Figures 13, 14, and 16). The peak is the more pronounced the higher are the initial values for progenitors and precursors and the smaller they are for the stem cells.

2. Comparison with Data

As has already been stated the calculations presented in Figures 2 to 16 have not been designed to reproduce particular sets of data. They rather show the broad spectrum of possible model behavior during recovery after acute irradiation. It is evident that a comparison with data cannot be strictly quantitative.



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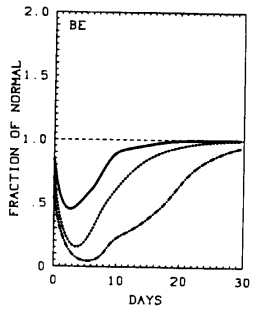

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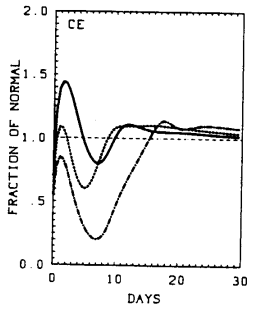

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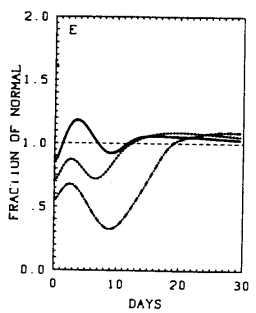
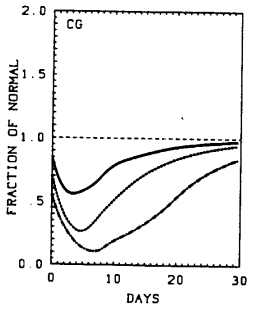


FIGURE 14



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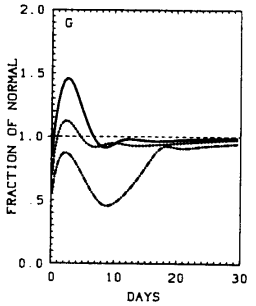


FIGURE 16

FIGURES 11 to 16. Acute irradiation-heterogeneous radiosensitivity. In these model calculations, the stem cells are reduced to initial values of 0.1 (—), 0.01 (- - -), 0.001 (— • —), and the other compartments to 0.85, 0.7, and 0.55, respectively. This corresponds to a 12 × smaller radiosensitivity of the differentiated cells.

The data are derived from mouse bone marrow which represents the total hemopoiesis of these animals.[41] To make them comparable with the model calculations the data curves do not start with the first measured value but with the minimum value found between day 0 and 3. This corresponds to the model description of radiation damage by reduced initial values. For all cell stages a large number of recovery curves has been measured (and will be quoted) of which only a small collection can be shown (Figures 17 to 22).

The experimental error of the CFU-S assay is too large and the experimental conditions are too heterogeneous to allow a clear identification of different recovery phases (Figure 17) as was possible for the theoretical curves of S (Figures 2 and 11). Nevertheless, for low starting values one finds a steep exponential recovery of CFU-S [2,5,6] which is missing for higher starting values.[4,7,10,13] Furthermore, in some curves[1,3,5] an intermediate plateau also can be identified.

The early progenitors (BFU-E, CFU-GM) recover slowly after acute irradiation[4,7,9,10,13,15,16] (Figures 18 and 21), if compared with CFU-E or precursor cells, and resemble the CFU-S behavior.

If only few CFU-E survive irradiation an exponential growth phase is observed for these cells (Figure 19). Typically, normalization is faster than for any of their ancestors.

Erythropoietic precursors[16] and the iron uptake in mice[19,20] show an overshoot 5 to 15 days after irradiation[16] (Figure 20). This result seems to be fairly comparable with the first type of calculations, i.e., similar radiosensitivity of stem cells, progenitors and precursors (Figure 5).

The granulopoietic precursors (Figure 22) normalize with overshoots and oscillations. Measurements exhibit a broad initial peak between day 5 and 10 followed by a deep nadir and a second overshoot after day 20.[1,16,46] These findings fit better with the second type of calculations, i.e., greater radiosensitivity of stem cells compared with progenitors and precursors (Figure 16).

It is well known that cycling of stem cells is accelerated after irradiation. Some authors have investigated the temporal behavior of this effect.[5,8,14,47,48] The thymidine suicide data recently published by Necas[47] represent the most extensive studies. Due to the difficulties in relating quantitative thymidine suicide data to "a_s", such data are not presented here. Qualitatively, they show that after irradiation stem cells cycle faster as long as their number is below 50%. Then, the cycling rate returns to normal within a few days. This is in qualitative agreement with the behavior of "a_s". Data on cycling behavior of BFU-E and CFU-GM after irradiation have not been found.

In total, the main characteristics of the data for mice and the model calculations are the same: exponential recovery of stem cells for severe initial reduction, slow recovery of early progenitors, fast recovery of late progenitors and precursors, and enhanced cell cycling of stem cells.

For rats, only few data are available and these are not presented here. In part they are discussed by OKunewick[40] in the preceding chapter. Generally speaking, rats and mice show a similar pattern of irradiation recovery. This is especially found for ^{59}Fe uptake[40] but is not so clear for granulopoietic precursors.[27,36] Comparing the ^{59}Fe data[40] (Figure 8) with the model calculations from Figure 14 reveals good agreement.

V. DISCUSSION

It is now possible to discuss the criteria demanded in the introduction.

There exists a straightforward method to quantify the radiosensitivity of early hemopoietic cells. One has to measure dose-response curves for all cellular compartments. Unfortunately, such measurements are not available and the recovery data[1-20] are too heterogeneous to find precise "pooled" dose-response relations. Therefore, model simulations of irradiation recovery can only loosely be correlated to the doses.

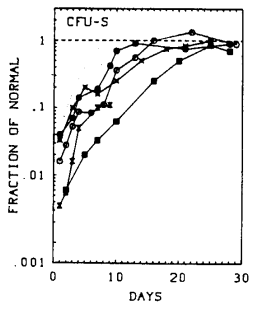
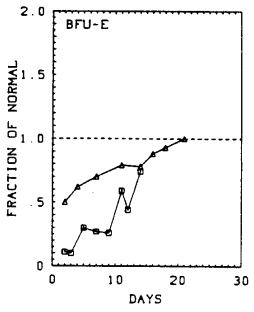


FIGURE 17



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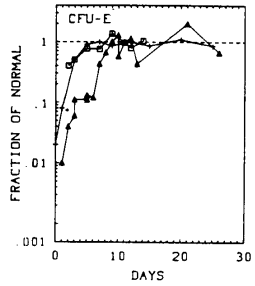

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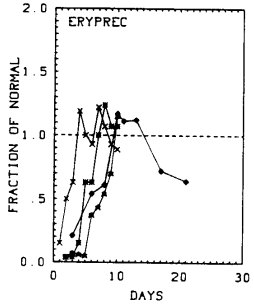

FIGURE 20

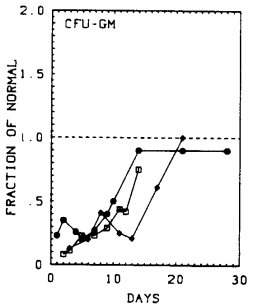

FIGURE 21

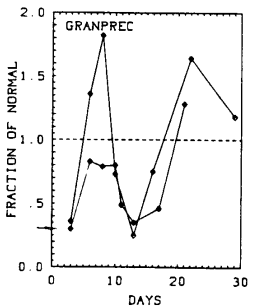

FIGURE 22

FIGURES 17 to 22. Acute irradiation-experimental data from the bone marrow of mice. CFU-S: 3.0 Gy (○),[1] 5.0 Gy (■),[2] 4.0 Gy (⧗),[3] 3.0 Gy (◖),[5] 2.0 Gy (●);[7] BFU-E: 1.5 Gy (△),[10] 1.5 Gy (□);[13] CFU-E: 3.5 Gy (▲),[17] 1.5 Gy (□),[13] 5.0 Gy (+);[15] erythropoietic precursors: 2.0, 4.0, 6.0 Gy (×,*,★ [iron uptake]),[19] 3.5 Gy (♦);[16] CFU-GM: 2.0 Gy (●),[7] 1.5 Gy (□),[13] 3.5 Gy (♦);[16] granulopoietic precursors: 3.0 Gy (◇),[46] 3.5 Gy (♦).[16]

A different approach was chosen, in that we have dealt with relative radiosensitivities. Here the survival data (minima taken on days 0 to 3) of stem cells are related to those of precursor cells. Since the measurements can be taken from the same experiment, this "intraindividual" comparison has a smaller scatter than pooled data from different experiments. One finds that the relative radiosensitivity of stem cells to differentiated cells varies between 0.08 and 1. This means that the differentiated cells are between a factor of 1 to 12 more radioresistant than stem cells. In none of the experiments was it suggested that the precursors were more radiosensitive than the stem cells. The two extreme sets of model calculations clearly show the importance of a precise knowledge of radiosensitivities and initial cell survival.

There is no need to alter the concept of constant determination rates in the model. The irradiation data we consider can be consistently explained by the above calculations, in which no variable rate of determination into the erythropoietic and granulopoietic lineage is assumed.[42] This is in contrast to a hypothesis discussed by Hellman et al.[49] who assume that, during recovery from acute irradiation, granulopoietic differentiation is preferred at the expense of erythropoiesis for the first days.

The model considers total hemopoiesis. As the spleen does not contribute more than 10% during irradiation recovery,[41] the model curves can be compared to bone marrow data.

Residual injury by irradiation cannot be interpreted within the framework of our model.[42-44] This is a limitation, which is necessary as long as only regulatory effects are considered. In the model, the cell numbers return to normal after the perturbation is over. For residual damage this is obviously not the case. On the other hand, residual damage has been reported only for doses above 5 Gy[6,30,50] which are not considered here.

The characteristic of stem cell recovery can be understood. The model curves show even clearer than the actual CFU-S data that a triphasic pattern seems to exist: during the first phase, steep exponential (autonomous) recovery of stem cells is found. This growth is characterized by a maximum of 0.6 for the self-renewal probability and by a maximum proliferation rate. In contrast to a proposal of Chervenick and Boggs,[19] there is no complete stop of differentiation; 40% of all stem cells still differentiate. This phase guarantees stem cell survival and has a security function for the stem cells. This phase always is found for stem cell numbers below 6%. In the second phase, above this threshold, regulated (nonexponential) recovery takes place. Here the demand for differentiation achieves the higher priority. Differentiation may take place at the expense of stem cells. The reserves of stem cells are already so large that the system can switch to maintain the supply of functional cells. The third phase starts as soon as the demand for differentiated cells is satisfied. Now the recovery of stem cells becomes accelerated. The "differentiation pressure" is small during this phase and stem cells make up for the lead of the differentiated cells in normalization.

The recovery pattern measured for the erythropoietic and granulopoietic precursors is also found in the model curves. The initial peak, often called the "abortive rise",[27,41] can be explained as a regulatory phenomenon. This statement does not contradict the fact that experimentally a delayed death of cells is found.[37] If some of the stem cells do not die immediately at the time of irradiation but perform a few mitoses along the differentiation pathway before they die, one would also expect a decrease of CFU-S and an initial peak of precursors. The result of the model analysis is that even if no delayed cell death occurs, one would find an early peak in CE, E, and G, as shown in the calculations of Figures 13, 14, and 16. The model explanation involves an accelerated proliferation of immature progenitor cells (BFU-E, CFU-GM) which are shifted rapidly to the precursor compartments. This interpretation of the "abortive rise" is consistent with OKunewick's reasoning[40] that a cohort of selectively activated cells is capable of proliferation. He assumes that irradiation itself has a selective activating effect on stem cells, while the model suggests a regulatory process. We, therefore, consider the peak basically as a regulatory event on top of which

a small fraction of cells with delayed death might be found. In summary, the "abortive rise" can be interpreted as an essential feature of normal stem cell regulations. Its appearance is not obligatory and depends on the surviving fraction of progenitors and precursors. This interpretation completes our earlier explanation[51] where the "abortive rise" was attributed to a regulatory impact of differentiated cells on stem self-renewal. Both effects act synergistically.

The difference between slow recovery of the progenitors and rapid recovery of the precursors is also explicable within the model. It can be understood as a result of the variable proliferative fraction (a_{BE}, a_{CG}) of the early progenitors. The cells pass quicker through BE and CG and, therefore, one finds fewer cells in these compartments. Thus, BE and CG numbers recover slower than E and G numbers, and the same is found if one compares BFU-E and CFU-GM with erythropoietic and granulopoietic precursor cells. It should be emphasized that up to now no reliable data are available about cycling kinetics of progenitor cells after acute irradiation. From our model calculations we would suspect that cycling of BFU-E and CFU-GM is two- to threefold accelerated following irradiation.

The mathematical model provides consistent explanations for the phenomena observed after various acute, single-dose irradiations in mice.

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Chapter 8

CHRONIC IRRADIATION — EXPERIMENTAL RESULTS

Ivan Kalina

TABLE OF CONTENTS

I. ABSTRACT

Based on our experimental observations and those of others, permanent changes in bone marrow CFU-S following chronic irradiation with doses of 0.03 to 0.06 Gy/day seem to occur after a total dose of 5 Gy.

A gradual reduction of CFU-S was noted with doses up to 0.5 Gy/day until approximately day 20 to 30 of irradiation. The total decrease depended on the dose rate and on the accumulated dose. During subsequent irradiation periods (until day 130) CFU-S values achieved a new steady state at a decreased number, reflecting a new equilibrium between their damage and neogenesis.

Steady-state CFU-S values depended on the dose rate in an exponential way which can be expressed by the equation

$$\log y = a + bx + cx^2$$

where y denotes the CFU-S value as a fraction of normal, x the dose rate in Gy/day, and where a, b, and c are constants.

This equation can be applied to predict the CFU-S values between days 30 to 100 of chronic irradiation with doses ranging from 0 to 0.5 Gy/day.

For doses higher than 0.765 Gy/day a permanent decrease in CFU-S was observed from the onset of irradiation up to the death of the animals.

II. INTRODUCTION

The hemopoietic tissue consists mainly of dividing and continuously maturing cell populations classified according to their morphological and functional properties. The reaction of the various hemopoietic series to chronic irradiation may differ considerably. This can be observed partly in bone marrow tissue itself and partly by the changes in the final hemopoietic products in the peripheral blood.

The radiosensitivity of hemopoietic pluripotent stem cells, with their ability to generate hemopoietic elements, is particularly important to the organism. Knowledge about the effects of chronic irradiation upon pluripotent hemopoietic stem cells (CFU-S) demonstrates that the radiation damage of these cells depends on the dose rate and on the total accumulated dose.

The response of the CFU-S to single-dose irradiation is relatively well known. On the other hand, only a few publications describe the effect of chronic irradiation upon these cells, although more attention has been paid to this question of experimental hematology in the last few years. However, taking into account the many possibilities of choice in duration and dose rate for chronic irradiation our knowledge is still not sufficient.

In this paper the effects of chronic irradiation upon CFU-S are discussed with an emphasis on doses ranging from 0.01 to 10 Gy/day. We have also attempted to understand some of the mechanisms contributing to the steady-state formation of CFU-S at a subnormal level.

III. MATERIAL AND METHODS

Male, "H" strain mice weighing 24 to 26 g at the time of initiation of irradiation were used. The donor animals were chronically irradiated by a ^{60}Co source on an open experimental gamma field with dose rates of 0.01, 0.03, 0.06, 0.1, 0.25, and 0.5 Gy/day during 130 days.

The colony-forming activity of hemopoietic stem cells in the bone marrow of control

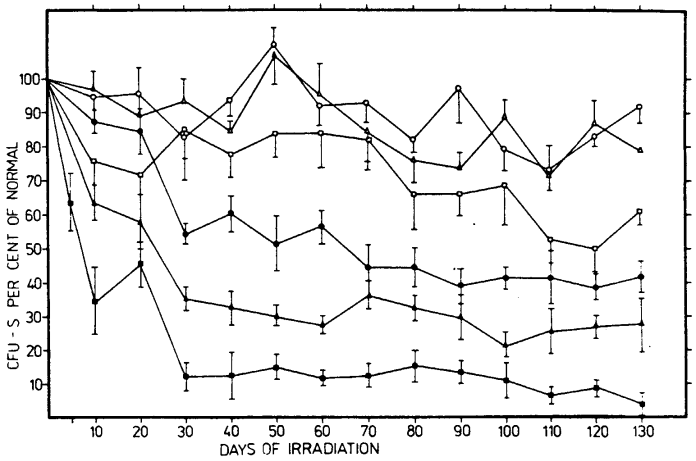

FIGURE 1. Bone marrow CFU-S in mice chronically irradiated with doses of 0.01 to 0.5 Gy/day. ○: 0.01 Gy/day, △: 0.03 Gy/day, □: 0.06 Gy/day, ●: 0.1 Gy/day, ▲: 0.25 Gy/day, ■: 0.5 Gy/day.

animals or of mice chronically irradiated with various dose rates was studied according to the spleen colony technique of Till and McCulloch.[1] Within comparable periods after the onset of chronic irradiation, the mice were decapitated and the tibial cells were suspended in Hanks' solution; 10^5 nucleated bone marrow cells suspended in 0.2 mℓ were injected into recipient mice.

The recipient mice were irradiated before injection with X-rays to a total dose of 6.5 Gy delivered by a TUR-T-250 RTG apparatus (180 kV, 15 mA, filter of 0.5 mm Cu, 0.5 mm Al, Ok = 47 cm, 0.5 Gy/min). The recipients were killed 9 days later and the colonies were counted on the facies parietalis surface of the spleen.

In addition, five groups were irradiated with doses of 0.8, 1.0, 3.0, 6.0, or 10.0 Gy/day up to the death of the last animal, and their bone marrow was also assayed with the same procedure.

Control animals were kept under the same conditions as the irradiated ones, but the area of the gamma field was shielded against irradiation.

CFU-S numbers from tibial bone marrow were related to the 10^5 injected nucleated cells and expressed as percent of the control concentration measured at the same time.

IV. RESULTS

Tibial marrow concentrations of CFU-S in control mice living in the shielded area of the experimental gamma field were found to range from 14.8 ± 0.88 to 17.4 ± 0.48 per 10^5 nucleated cells over a 130-day period.

CFU-S concentrations of the groups irradiated with doses of 0.01, 0.03, 0.06, 0.1, 0.25, and 0.5 Gy/day during 130 days are depicted in Figure 1. Chronic irradiation with a dose of 0.01 Gy/day caused no significant changes in CFU-S concentration with the exception of day 110 of irradiation (accumulated dose 1.10 Gy) when they had decreased to 72.2% of control. A dose of 0.03 Gy/day caused a permanent decrease in CFU-S which was statistically significant on days 80, 90, and 110, i.e., with total accumulated doses of 2.4,

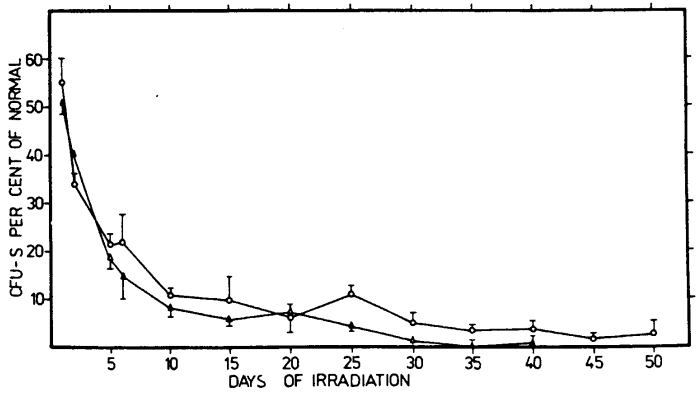

FIGURE 2. Bone marrow CFU-S in mice chronically irradiated with doses of 0.8 Gy/day (○) and 1.0 Gy/day (△).

2.7, and 3.3 Gy. During irradiation with doses of 0.06 Gy/day, CFU-S values were reduced to 70 to 75% at days 10 and 20 followed by a transient increase during days 30 to 70. At day 80 a second decrease was observed which persisted until day 130. A dose of 0.1 Gy/day caused a decrease in CFU-S of approximately 40 to 50% of normal reached at day 30. Then a new lower steady state of CFU-S values appeared to occur. This phenomenon could also be observed after doses of 0.25 and 0.5 Gy/day. Following 30 days of 0.25 Gy/day, steady-state values of CFU-S at the 30% level was observed. After 0.5 Gy/day CFU-S concentration was maintained at 13% of control. The steady state was perturbed and a new decrease in CFU-S occurred after 100 days of irradiation with doses of 0.5 Gy/day, i.e., when a total accumulated dose of 50 Gy was reached.

Increasing irradiation doses up to 0.8 and 1.0 Gy/day caused rapid reductions in CFU-S concentrations. Thus, CFU-S were reduced to 50% of control by day 1, and by day 40 only 1.8% (1.0 Gy/day) and 2.6% (0.8 Gy/day) survived (Figure 2).

The changes in CFU-S concentrations after chronic irradiation with doses of 3, 6, and 10 Gy/day are illustrated in Figure 3. Under these conditions, significant reductions in CFU-S were seen. Within the final irradiation phases ending with the death of the animals, CFU-S values numbered 2% of control in all groups.

The relative importance of time and dose during the chronic irradiation is depicted on Figure 4. As shown, the accumulated dose effect is dependent on the dose rate, whereas the time factor plays an insignificant role, e.g., for the total dose of 6 Gy neither differences between the effect of doses of 0.1 to 0.25 Gy/day were observed nor between the effects of doses of 1 to 6 Gy/day.

V. DISCUSSION

The effect of 0.01 Gy/day on CFU-S was equivocal even when a total dose of 1.3 Gy was administered. Therefore, we are unable to comment on the damage to the CFU-S compartment of irradiation administered under these conditions.

The experimental results presented in this paper and previously[2,3] allow us to discuss, to some extent, the more important changes in pluripotent hemopoietic stem cells exposed to varying total doses of irradiation administered at different rates. We feel able to conclude,

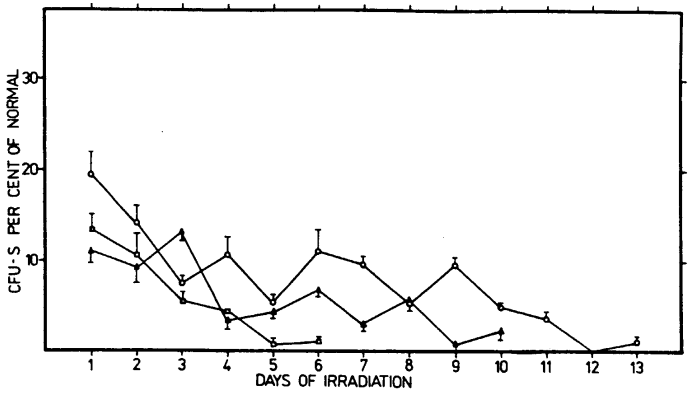

FIGURE 3. Bone marrow CFU-S in mice chronically irradiated with doses of 3 to 10 Gy/day. ○: 3 Gy/day, △: 6 Gy/day, □: 10 Gy/day.

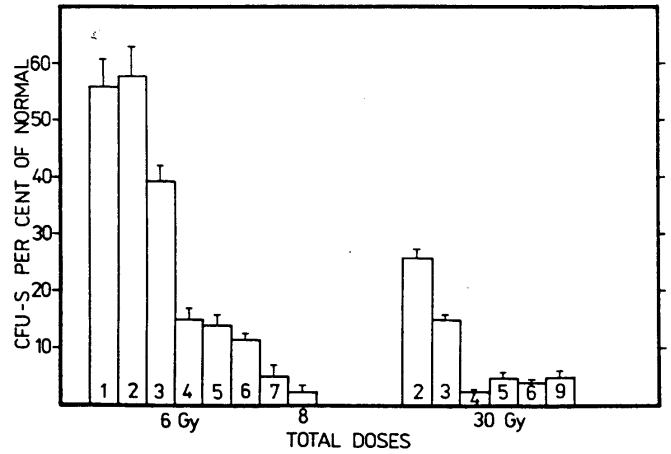

FIGURE 4. CFU-S values corresponding to particular dose rates and to accumulated doses of 6 and 30 Gy. 1: 0.1 Gy/day, 2: 0.25 Gy/day, 3: 0.5 Gy/day, 4: 1 Gy/day, 5: 3 Gy/day, 6: 6 Gy/day, 7: 6 × 1 Gy/day, 8: 1 × 6 Gy/day, 9: 10 Gy/day.

based on these experimental findings, that for doses of 0.03 Gy/day, CFU-S values fluctuate below values for the control group, although the decrease was not statistically significant before day 90.

However, a dose of 0.06 Gy/day only results in a significant, long-term reduction in CFU-S when the accumulated dose exceeds 4.6 Gy. Our results are supported by those of other authors. Drasil et al.[4] observed a decrease in CFU-S concentrations to a minimum of 60% of normal at days 50 and 150 in mice treated with chronic irradiation of 0.026 Gy/day. Gidali et al.[5] noted a decrease to 80% of normal for similar irradiation conditions. Following

an increase in dose rate to 0.0755 Gy/day[4] or to 0.153 Gy/day,[5] significant changes of CFU-S concentrations in bone marrow during all periods of chronic irradiation were observed.

For continuous irradiation with daily doses ranging from 0.1 to 0.5 Gy/day, the responses of the CFU-S provide additional information. In the first phase, during the initial 20 to 30 days, characterized by a gradual decrease in CFU-S values, the rate of decline was determined by the dose rate as well as by the accumulated dose. During the second phase, when CFU-S were maintained at a new steady state, the level depended on the dose rate and only marginally on the total dose. These biphasic survival patterns of CFU-S during chronic irradiation under the above-mentioned conditions may be explained by the fact that during the first phase more radiosensitive stem cells are eliminated accompanied by a slower entry into the cell cycle of the survivors and a more intensive loss through differentiation.[6] During the second phase the equilibrium may be attributed to the remaining CFU-S being able to compensate by neogenesis for the damage, i.e., the steady-state level is then proportional to the dose rate and is not influenced significantly by the total accumulated dose during this phase. During the steady-state phase at diminished CFU-S values, an increase has been reported in proliferative activity,[6-8] suggesting an intensive entry into the cell cycle from the G_0 stage and a shortening of the cell cycle time. The breakdown of the new CFU-S steady state observed with doses of 0.5 Gy/day after an accumulated dose of 50 Gy (i.e., after 100 irradiation days) probably occurred due to the loss of the capacity to maintain this increased proliferation due to the accumulation of various cytological and cytogenetical damages.

Similar changes in bone marrow CFU-S of chronically irradiated animals with doses up to 0.5 Gy/day have also been reported. Juraskova[9] observed that the onset of the steady-state phase during irradiation at a dose rate of 0.23 Gy/day occurred by day 10 at 60% of normal values, 10 days earlier than we found. Blackett[10] showed, using ^{59}FE-incorporation, that in rats treated with a dose of 0.43 Gy/day the repopulating ability of the bone marrow decreased during the first 5 weeks to 10% of normal with a subsequent stabilization. For similar conditions, Twentyman and Blackett[11] noted a decrease in the repopulating ability after 3 irradiation weeks to 5% at which level CFU-S was maintained for 20 irradiation weeks. Some differences between our results and those of the other authors may be explained to a certain extent by the application of different methods and of other animal strains.

The close connection between the changes in hemopoietic stem cells (CFU-S) during chronic irradiation and the dose rates suggest a correlation between these dose rates and the steady-state values. Pooling the CFU-S data from irradiation days 30, 40, 50, 60, 70, 80, 90, and 100 for each dose rate, a relationship between the average CFU-S steady-state values and the daily dose rates can be derived which is expressed by the equation

$$\log y = a + bx + cx^2$$

where y denotes the CFU-S value as a fraction of normal, x the dose rate in Gy/day, and the coefficients are $a = 1.9883625$, $b = -0.02612943$, $c = 0.0001854$.

This equation, which correlates CFU-S steady-state values with the daily doses of chronic irradiation, was derived from the experimental data found within the periods between day 30 and 100, and is only suitable up to a dose rate of 0.5 Gy/day. Above this rate the steady state breaks down and a new decrease in CFU-S occurred followed by the death of the animals (the average survival time of the irradiated mice exposed to a dose rate of 0.5 Gy/day is 133 days.[12] According to data for irradiation with a dose of 0.5 Gy/day[13] the steady state of various hematological parameters can persist for a long time at a reduced level and the breakdown only occurs a few days before the death of the animals. We would like to call attention to the fact that for dose rates up to 0.5 Gy/day, in the initial survival phase, the CFU-S changes are dependent on the total accumulated dose as well as on the dose rates. For dose rates higher than 0.5 Gy/day no steady state occurs. Here it is necessary to deduce the mathematical relation for each dose rate and for each accumulated dose.

Evaluating the significance of irradiation duration and doses for the overall radiosensitivity of the hemopoietic stem cells, we can note the intensive time dependence of the irradiation. So, after an "accumulated" single dose of 0.5 Gy CFU-S values in bone marrow were reduced to approximately 50% of normal, but when this accumulated dose is applied chronically with doses up to 0.03 Gy/day no significant changes in CFU-S values were noticeable.[2,5] This different reaction of CFU-S to single vs. continuous irradiation can be explained by the data on CFU-S proliferative activity in bone marrow of control and chronically irradiated animals. Gidali et al.[5] noted a significant increase in CFU-S cycling in chronically irradiated mice even with doses lower than 0.15 Gy/day. While about 5% of CFU-S are in S-phase in controls, the proportion increased to 12.8% in mice irradiated with 0.01 Gy/day. With a dose rate of 0.0135 Gy/day, 20.9% of CFU-S were in S-phase increasing to 25.0% with 0.025 Gy/day and to 27.2% with 0.15 Gy/day. These experimental data confirm that the basis for the compensation of CFU-S loss in the bone marrow during chronic irradiation is an increase in the proliferative activity of the surviving cells.

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Chapter 9

CHRONIC IRRADIATION — A MODEL ANALYSIS*

Markus Loeffler, H.-Erich Wichmann, and A. J. Jarczyk

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I. ABSTRACT

During chronic irradiation the following effects on early hemopoiesis are observed:

- A biphasic pattern of CFU-S behavior
- The ability of hemopoiesis to stabilize at a reduced steady state if the doses do not exceed 0.6 to 1 Gy/day
- A more severe reduction of CFU-S than of erythropoietic and granulopoietic precursors

These observations are analyzed with a mathematical model of stem cell regulation. The analysis suggests that in the available data the relative radiosensitivity of stem cells under continuous irradiation is 50% higher than that of differentiated cells. For doses above 0.25 Gy/day, the presence of a peripheral demand for erythrocytes and granulocytes has an important radioprotective effect on the marrow cell compartments. The results can be summarized in dose-response curves which relate the cell numbers during the plateau phase with the daily radiation dose.

II. INTRODUCTION

The influence of chronic irradiation on hemopoietic stem cells has been extensively examined.[1-18] Animals were given daily doses between 0.01 and 10 Gy/day and the changes in number of CFU-S were investigated.[9]

Typically found is a biphasic pattern of CFU-S behavior. After an initial decrease, lasting several days, the CFU-S level off at an equilibrium cell number significantly below the normal value. Both the steepness of the decrease and the plateau of the steady state are dose-dependent. For doses above 0.6 to 1 Gy/day the system continuously declines and finally dies out.

Less is known about the behavior of more mature cell stages during chronic irradiation. Data for BFU-E are not available and those for CFU-GM,[10,16] CFU-E,[16] and ERC[11] are rare. Iron uptake was only measured once in mice[14] and once in rats.[1] Total nucleated cell counts, however, have been recorded several times.[7,13,15,17,19] Information on granulopoietic and erythropoietic precursors is obtainable in only one reference.[3] In general, the differentiated bone marrow cells show a higher plateau than has been observed for stem cells at the same dose. This can even amount to the "paradoxon" that the precursors remain almost normal while the CFU-S are already reduced to 20%.[1,7,12,14]

During chronic irradiation peripheral blood counts have been repeatedly examined.[7,10,14,18-20] Up to a dose of 0.25 Gy/day the erythrocyte number will not drop below 85% of its normal value.[10,18-20] For higher doses the red cell count may drop to lower values in a dose-dependent way.[7,10,14,19] Blood granulocyte counts seem to be more sensitive to continuous irradiation. They show a clear reduction by at least 30% with doses as low as 0.1 Gy/day.[18-20] Values below 25% of normal are reached if daily doses exceed 0.25 Gy/day.[7,10]

III. MATHEMATICAL METHODS

The experiments on chronic irradiation are simulated by the mathematical model of stem cell regulation as described.[21-23]

For this simulation and for the comparison with experimental doses one has to determine

1. How to describe the chronic destruction of cells in mathematical terms
2. How to consider the different radiosensitivities of different cells types

Table 1
THEORETICAL DOSE-RESPONSE RELATIONS FOR HEMATOCRIT AND EP DURING THE PLATEAU PHASE OF CHRONIC IRRADIATION (DAY 40)

Dose (Gy/day)	0.03	0.1	0.25	0.5	0.7
Loss coefficient K_S (h^{-1})	0.0011	0.0035	0.0088	0.0175	0.0245
Hematocrit (Hct) (times normal)	0.99	0.98	0.92	0.72	0.38
EP[a] (times normal)	1.03	1.12	1.43	4.75	200.

[a] EP values have been determined from Hct using a model of mature erythropoiesis.[27,28]

3. How to relate the mathematical parameters with experimental doses
4. How to consider the peripheral stimuli which are due to the anemia and neutropenia
5. How to identify the "limiting dosage" beyond which hemopoiesis fails to maintain a steady state.

These points are considered in the following way:

1. For the specific effect of continuous destruction due to radiation damage it is assumed that the cells in each compartment are destroyed "at random", i.e., irrespective of the cell cycle position of individual cells. The compartment contents are reduced as a proportion of their numbers and the loss coefficients which represent the destruction are chosen to be constant with time. Mathematically, this leads to the following equations:[22]

$$\dot{S} = (2p - 1)\, S * a_S/\tau_S - K_S * S \tag{1}$$

$$\dot{BE}^* = \dot{BE}^{*in} - BE^*/T_{BE} - K_D * BE^* \tag{2}$$

$$\dot{CE}^* = \dot{CE}^{*in} - CE^*/T_{CE} - K_D * CE^* \tag{3}$$

$$\dot{E1-4}^* = \dot{E1-4}^{*in} - E1-4^*/T_E - K_D * E1-4^* \tag{4}$$

$$\dot{CG}^* = \dot{CG}^{*in} - CG^*/T_{CG} - K_D * CG^* \tag{5}$$

$$\dot{G1-4}^* = \dot{G1-4}^{*in} - G1-4^*/T_G - K_D * G1-4^* \tag{6}$$

where K_S and K_D are the fractional loss coefficients for stem cells and differentiated cells. If they are equal to zero, the formulas are identical with those of Loeffler and Wichmann[22] (Table 1). The definition of total erythropoietic and granulopoietic precursors remains the same as in the standard model:

$$E^* = BE^* + CE^* + E1-4^* \tag{7a}$$

$$G^* = CG^* + G1-4^* \tag{7b}$$

2. Since not enough data are available to estimate the loss coefficients for the different cell compartments separately, only two loss coefficients are considered, one for the stem cells (K_S) and one for the differentiated cells (K_D). Thus, it is assumed that all differentiated cells have a similar radiosensitivity which is different from that of the stem cells. The mathematical relationship between K_D and K_S has to be determined from the data.
3. The relation between the experimental dose and the loss coefficients of the model has to be identified. For this purpose, measurements between day 30 and 45 of continuous irradiation are taken as steady-state values. They are related to the experimental doses. In a similar way the calculated steady-state values (taken on day 40) are related to the loss coefficients. It is then possible to match the experimental dose-response curve with the model dose-response curve by a proper choice of scaling factors, so that they can be drawn in the same diagram. These scaling factors represent the relation between the experimental doses and the loss coefficients of the model.
4. Chronic destruction of immature hemopoietic cells leads to anemia and neutropenia in the peripheral blood. The need for mature cells stimulates the amplification of erythropoietic and granulopoietic precursors (via EP and CSF) and thus indirectly influences the intramedullary feedback. The blood granulocytes are more severely reduced than the erythrocytes, but for granulopoiesis it is the total pool of granulocytes (in the bone marrow and the blood) rather than blood granulocytes, which is responsible for feedback.[24,25] Total granulocytes show a similar behavior compared with erythrocytes under chronic irradiation.[24,25] Therefore, in the model calculations, it is assumed that the demand for erythropoietic and granulopoietic cells increases in parallel. Consequently, E and G, as well as BE and CG, will behave very similarly. Technically, the parallelism of granulopoietic and erythropoietic stimulation is simulated as follows: only erythropoiesis is calculated and it is assumed that the granulopoietic progenitors and precursors show the same behavior as their erythropoietic counterparts (CG = BE, G = E). Consequently, in the regulatory functions "p", "a_s", "a_{BE}", and "a_{CG}", G is replaced by E:

$$p(S,E,E),\ a_S(S,E,E),\ a_{BE}(S,E,E) = a_{CE}(S,E,E) \tag{8}$$

As usual in this volume mature erythropoiesis (erythrocytes, hematocrit) is not considered explicitly but indirectly by theoretical curves for erythropoietin (EP). They have been derived by combining the present stem cell model[21-23] with a model of mature erythropoiesis.[27,28] The EP increase is a consequence of the hampered production of red blood cells resulting from the irradiation and, therefore, demonstrates the anemic stimulation that develops.[28] Table 1 gives some examples for the dose-dependence of EP levels. Minor changes of hematocrit and EP are found up to 0.25 Gy/day. For higher doses the anemic stimulus increases dramatically. By the above simplification (parallelism between granulopoiesis and erythropoiesis), the EP time curves may also be interpreted as time curves of CSF as they would have to be expected in a more realistic model.
5. The chronic destruction of cells leads to a subnormal plateau of the cell numbers both in experiment and in the model. The plateau depends on the dosage and will be lower for higher doses. However, there exists a threshold beyond which no plateau can be obtained and the animals die. Experimentally, this "limiting dosage" can be measured. In the model, it can be calculated from the condition $\dot{S} = 0$. With this condition Equation 1 gives the relation

$$(2p - 1)\, a_S/\tau_S = K_S \tag{9}$$

The maximum values for "a_s" and "p" are 1.0 and 0.6.[22] With the cell cycle time τ_S = 8 hr one finds the limit loss coefficient $K_S = 0.025\ h^{-1}$. Higher values lead to the death of the system.

In the following, first a steady-state analysis of the experimental data in the plateau phase of chronic irradiation will be performed. This analysis leads to an estimate of the scaling factors between the experimental dosage and the loss coefficients for stem cells (K_S) and differentiated cells (K_D). From these factors the relative radiosensitivity of the cells can be derived and the theoretical dose limit can be expressed in Gy/day. Furthermore, the contribution of amplifying divisions (stimulated by EP) can be quantified.

With the knowledge obtained from steady-state analysis the system dynamics in nonsteady state will then be investigated. A number of experimentally relevant doses will be simulated and the critical region close to the "limit-dosage" will be analyzed in more detail.

IV. RESULTS

A. Steady-State Analysis

From the plateau values of the available data[2,3,7,9-11,13,14,17-20] between days 30 and 45 the following information can be derived.

1. Dose-Response Relation of Stem Cells

In the model the severity of continuous irradiation is quantified by loss coefficients. K_S is the parameter for fractional loss of stem cells per hour. The experimental dose is usually measured in Gy/day. A good matching of model dose-response (for S) with experimental dose-response (for CFU-S) can be achieved with a scaling factor of 0.035 [day/(Gy * h)] as shown in Figure 1:

$$K_S\ (h^{-1}) = 0.035 * \text{dose (Gy/day)} \tag{10}$$

This relation defines how a certain value of K_S can be derived for a given dose. In addition we use Formula 10 in all three dose-response relations (Figures 1 to 3) as the gauge for the axes.

2. Dose-Response Relation of Differentiated Cells

Similarly, one can match the plateau values for progenitors and precursors of the model with the experimental ones (Figure 2). K_D is the parameter for the fractional loss of differentiated cells per hour. The same K_D is taken for all progenitors and precursors. A good matching is obtained if K_D is evaluated for a given dose with a scaling factor of 0.023 [day/(Gy * h)]:

$$K_D\ (h^{-1}) = 0.023 * \text{dose (Gy/day)} \tag{11}$$

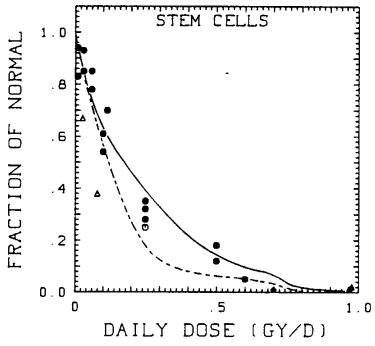


FIGURE 1. Relation between the daily dose of continuous irradiation and the plateau values of stem cells. Experimental points (CFU-S[2,7,9-11]) have been derived between days 30 and 45 of irradiation. Model curves correspond to day 40 of simulated continuous irradiation. The full line considers peripheral stimulation and represents the dose-response curve of the model. The dashed line is only shown for comparison: here the hypothetical situation has been calculated that peripheral stimulation is missing. On the abscissa 1 Gy/day corresponds to a loss coefficient of K_S = 0.035 h^{-1}. Data are taken from: ●, Kalina;[9] ▲, Kalina et al.;[7] △, Drasil et al.;[2] ◆, Lajtha et al.;[11] ○, Knospe et al.[10]

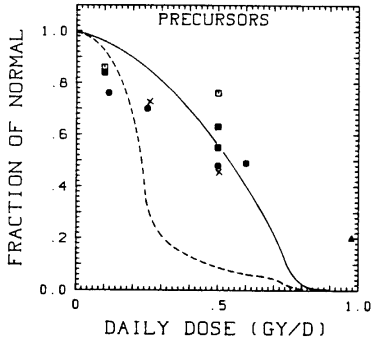


FIGURE 2. Relation between the daily dose and the plateau values of precursor cells. Model curves as in Figure 1. The data are taken from: total erythroblasts — □, Fedotova and Belousova;[3] total myeloblasts — ■, Fedotova and Belousova;[3] total nucleated cells — ●, Kalina;[9] ▲, Kalina et al.;[7] ×, Muskinova.[17]

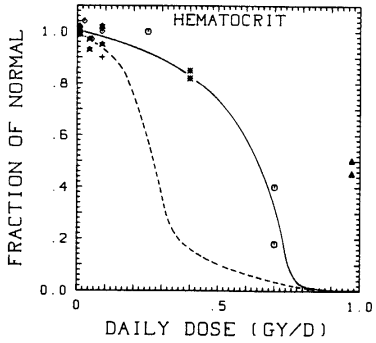


FIGURE 3. Relation between the daily dose and the plateau values of the hematocrit. Model curves as in Figure 1. The data are taken from: +, Lorenz et al.;[19] ○, Knospe et al.;[10] ▲, Kalina et al.;[7] ◇, Praslicka and Kalina;[18] ★, Spargo et al.;[20] *, Twentyman and Blackett.[14]

3. Radiosensitivity

The scaling factors in Formulas 10 and 11 have been obtained by matching the model results in steady state with the corresponding data points (Figures 1 and 2). Obviously, this is a crude method which is based on the simplification of a linear relationship between loss coefficients and dosage and an identical radiosensitivity of all differentiated cells. The formulas suggest that for an increase in dosage by 0.1 Gy/day an additional 0.35% of stem cells and 0.23% of differentiated cells are destroyed per hour in each compartment.

The ratio of the scaling factors (and thus the ratio of K_D to K_S for a fixed dosage) can be interpreted as relative radiosensitivity of differentiated cells compared with stem cells and shall be denoted by

$$r \doteq K_D/K_S \tag{12}$$

Here one finds r = 0.66, suggesting that the differentiated cells are two thirds radiosensitive to chronic irradiation as stem cells.

4. Influence of Peripheral Stimuli

During continuous irradiation an anemia develops. The data points in Figure 3 indicate to which degree the hematocrit has fallen between days 30 and 45.[7,10,14,18-20] In the model a similar reduction is found (full line); the curve is derived from values at day 40 of simulated chronic irradiation. The (full) dose-response curve in Figure 3 corresponds to the (full) curves in Figures 1 and 2. In these curves peripheral stimulation is considered. It is an interesting question to ask what the dose-response curves would look like if peripheral stimulation would not be effective. This is shown in the dashed lines of Figures 1 to 3. The comparison of full and dashed lines shows that the amplifying divisions, which are induced by peripheral stimulation, increase the number of precursors (Figure 2) and, subsequently, the hematocrit (Figure 3). The higher number of precursors has a beneficial effect on stem cell numbers which are also kept at a higher level (Figure 1). The latter is an indirect consequence of intramedullary feedback and it is not so pronounced as the direct stimulatory effect on the precursors.

5. "Limiting Dosage"

From Formula 9 it can be concluded that the hemopoietic system, as simulated by the model, dies when the loss coefficient exceeds K_S = 0.025 h^{-1}. Using Formula 10, this leads to a "limiting dosage" of 0.71 Gy/day. For higher doses in the model no steady state can be formed. Experimentally, one finds the "limiting dosage" between 0.6 and 1 Gy/day.[6,11,15,16]

B. Dynamic Behavior

1. Model Calculations

With the relations between loss coefficients and dosage, the time course of the changes in cell number during chronic irradiation can now be simulated. This has been done for five different doses (0.03, 0.1, 0.25, 0.5, 0.7 Gy/day) and is shown in Figures 4 to 10. The results have the following characteristics:

- Depending on the dose, applied anemia (and neutropenia) of increasing severity develops. Accordingly, EP increases in a dose-dependent way (Figure 4). These EP curves are used as input to the model. They are obtained by making use of a model of mature erythropoiesis (see methods).
- Starting from normal, S decreases for several days and, after some minor fluctuations, tends towards a new steady state. Both the steepness of the decrease and the steady-state level are dose-dependent (Figure 5).
- A similar behavior is found for BE and CG. However, the curves are slightly higher than for S (Figure 6).

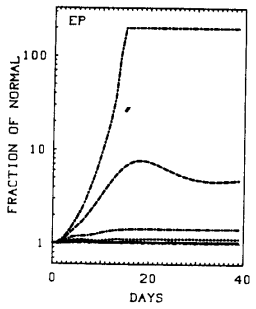
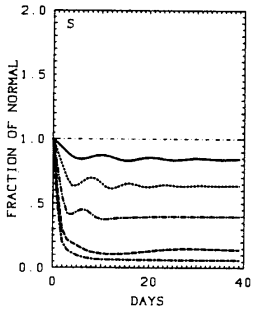


FIGURE 4



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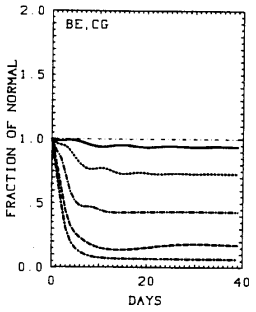

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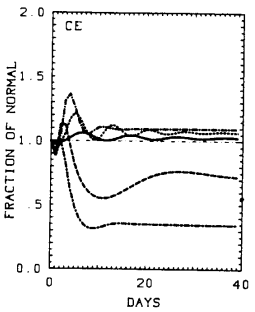

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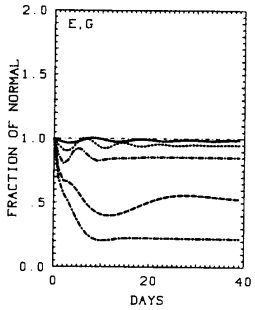

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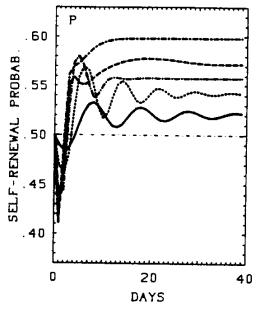

FIGURE 9

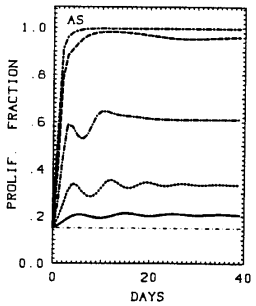

FIGURE 10

FIGURES 4 to 10. Model simulation of the time course of continuous irradiation. Five different doses are chosen: 0.03 Gy/day (———), 0.1 Gy/day (- - - -), 0.25 Gy/day (— • —), 0.5 Gy/day (———), 0.7 Gy/day (— - — -). The model curves for BE and CG as well as for E and G are identical. The anemia which develops during chronic irradiation is considered by the theoretical curves for EP shown in Figure 4. These EP curves are used as input for the model.

- CE, E, and G exhibit a more interesting pattern (Figures 7 and 8). For small doses below 0.4 Gy/day these compartments show steady-state values close to normal. For higher doses, their steady-state values are reduced but remain above those of S.

The behavior of the stem cells can be understood as follows: due to the constant removal of cells, S is reduced and the self-renewal probability "p" (Figure 9) as well as the proliferative fraction "a_S" (Figure 10) increase. So more new stem cells are produced. In the first days this gain is insufficient to compensate for the loss by irradiation. Only after several weeks does the system adapt and a balance between loss and gain is achieved.

The behavior of BE, CG, and CE is mainly determined by increases in the proliferative fractions "a_S", "a_{BE}", and "a_{CG}". The increase in "a_{BE}" (not shown) leads to a shift of cells from BE to CE, thereby giving rise to the initial overshoot in CE. The increase in "a_S" (Figure 10) maintains Ce at almost normal levels for low doses where EP is hardly elevated. For higher doses (above 0.3 Gy/day) the increased cell production of stem cells alone can no longer maintain these high precursor cell counts. Up to two additional amplifying divisions at the precursor stage are then activated by peripheral stimulation. This keeps the precursor cells and, consequently, erythrocytes and granulocytes, at relatively high levels.

While CE, E, and G profit from exploitation of stem cell activation, almost the contrary is true for BE and CG. An activation of "a_{BE}" shortens the transit time and fewer cells are found in BE. This explains why BE is always reduced to a lower level than CE. On the other hand, E is more reduced than CE because some of the cells passing from CE to E are removed by irradiation.

2. Comparison with Data

Figures 11 to 16 provide data for chronic irradiation with doses between 0.03 and 0.7 Gy/day. They can be compared with the model curves in Figures 5 to 8.

Many measurements of CFU-S are available of which only some are reproduced here (Figure 11). CFU-S show the same biphasic behavior as the S-curves. For comparable doses the steady-state values for CFU-S and for S are similar. However, during the initial phase do the calculated S-curves drop faster than the corresponding CFU-S curves.

Information is scarce concerning the behavior of the progenitors CFU-GM and CFU-E (Figures 12 and 14). CFU-GM fit the model curves well, while CFU-E remain below the corresponding calculations. For BFU-E no measurements are available.

The data on erythropoietic, granulopoietic, and total nucleated precursors[1,3,7,11,13,15-18] show that the cell numbers do not differ very much from normal for doses below 0.5 Gy/day (Figures 13, 15, and 16). Characteristically, all numbers are higher than corresponding stem cell numbers for the same dose.

3. "Limiting Dosage"

In Figures 4 to 10, loss coefficients have been considered which correspond to doses between 0.03 and 0.7 Gy/day. In these figures, higher doses lead to lower plateaus. However, there is a critical dose for which no subnormal steady state exists and where the cell numbers decrease to zero. In the model, this critical dose corresponds to 0.71 Gy/day or, in model terms, to $K_S = 0.025\ h^{-1}$. For higher doses a steady state becomes impossible because the loss of stem cells is permanently greater than the maximum gain of new stem cells. Figures 17 to 19 show the behavior of the system near this threshold value. For 0.7 Gy/day a steady state is still achieved in S, while for 0.75 Gy/day a monotonous decline is found, which is more pronounced for 0.8 Gy/day.

A similar behavior is found for the experimental cell numbers, although here the border between survival and death cannot be drawn so precisely as in the model. Nevertheless, for doses between 0.6 and 1.0 Gy/day CFU-S continuously decline[2,3,6,-9,11,12] and eventually die out.

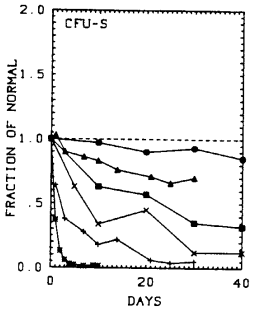

FIGURE 11

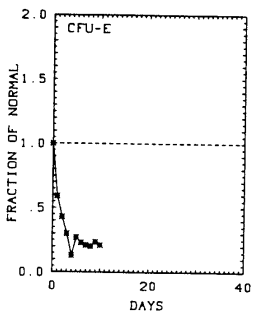

FIGURE 12

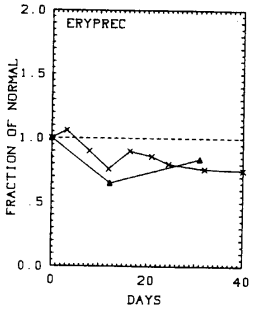

FIGURE 13

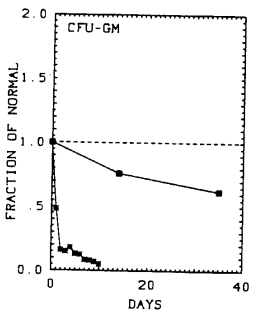

FIGURE 14

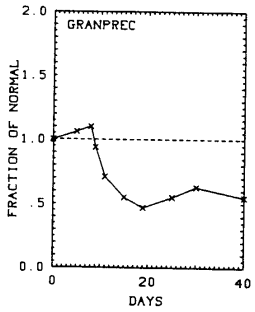

FIGURE 15

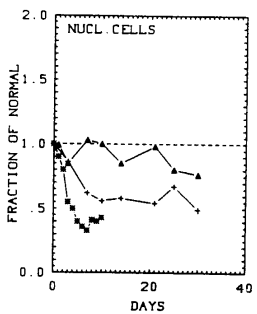

FIGURE 16

FIGURES 11 to 16. Cell numbers measured in the bone marrow of mice during continuous radiation. The symbols are chosen according to the daily dose range: ● — 0 to 0.05 Gy/day, ▲ — 0.06 to 0.15 Gy/day, ■ — 0.16 to 0.45 Gy/day, × — 0.46 to 0.55 Gy/day, + — 0.56 to 0.65 Gy/day, * — 0.66 to 0.75 Gy/day. Data are taken from: CFU-S: Kalina[9] — 0.03 Gy/day, 0.11 Gy/day, 0.25 Gy/day, 0.5 Gy/day, 0.6 Gy/day; Wu et al.[16] — 0.7 Gy/day; CFU-E: Wu et al.[16] — 0.7 Gy/day; erythropoietic precursors: Fedotova and Belousova[3] — 0.1 Gy/day, 0.5 Gy/day; CFU-GM: Knospe et al.[10] — 0.25 Gy/day; Wu et al.[16] — 0.7 Gy/day; granulopoietic precursors: Fedotova and Belousova[3] — 0.5 Gy/day; total nucleated cells: Kalina[9] — 0.11 Gy/day, 0.6 Gy/day, 1.0 Gy/day; Wu and Lajtha[15] — 0.7 Gy/day.

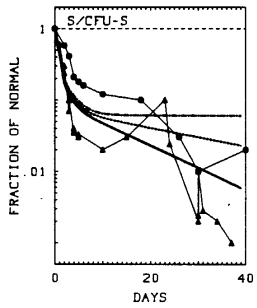

FIGURE 17

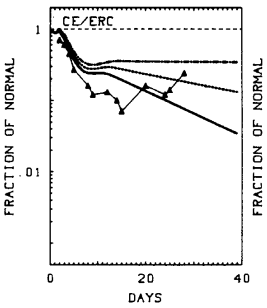

FIGURE 18

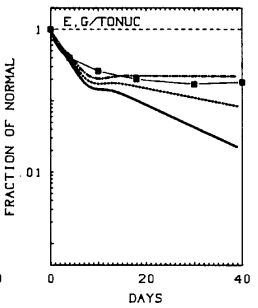

FIGURE 19

FIGURES 17 to 19. Model behavior during continuous irradiation near the "limiting dosage" of 0.71 Gy/day (for higher doses the system dies out). The figures show three model calculations with 0.7 Gy/day (- - -), 0.75 Gy/day (— ● —), and 0.8 Gy/day (———). Only in the first case is steady state achieved. In the other two cases stem cell numbers decrease steadily. For comparison CFU-S and ERC data from Lajtha et al.[11] (0.7 Gy/day, ▲), CFU-S data from Kalina et al.[7] (1.0 Gy/day, ●), and total nucleated cell counts from Kalina[9] (1.0 Gy/day, ■) are shown.

V. DISCUSSION

As has been demonstrated, the mathematical model of stem cell regulation is able to provide an explanation for the behavior of hemopoietic bone marrow cells during continuous irradiation. The model helps to establish a rough estimate for radiosensitivity of cells showing that in the analyzed experiments stem cells are about 50% more radiosensitive to chronic irradiation than differentiated cells. This result is surprising because the dose-response curves (Figures 1 and 2) would suggest a much bigger difference. It can be understood if one considers the regulatory influence of functional cells on granulopoietic and erythropoietic precursors. The peripheral demand induces additional mitoses in the precursor stages. The higher number of precursors reduces the differentiation pressure on the stem cells and the stem cell pool is less depleted. In total, one finds a direct "radioprotective" effect of peripheral stimulation on both stem cells and precursor cells.

Technically, the influence of blood cells has been simulated in a simplified way. As the stem cell model discussed here lacked a description of mature granulocyte and erythrocyte formation, information about erythropoietic and granulopoietic stimulation during continuous irradiation had to be taken from different sources. Applying an established model of mature erythropoiesis[26,27] helps to calculate the EP values from the observed degree of anemia. These values are then introduced into the stem cell model. For granulopoiesis we have assumed that it follows the same characteristics as erythropoiesis. This is the simplest assumption without introducing additional model parameters.

The finding that anemia and neutropenia have a "protective" effect on stem cell numbers during continuous irradiation is perhaps surprising. However, it is biologically reasonable. From the model point of view it is important to note that this "self-protection" is not an additional assumption, but is a consequence of our description of intramedullary feedback, in which an increase of the precursors indirectly leads to an increase of the stem cells.

It would be interesting to look at the opposite hypothesis, namely, that EP stimulation

would lead to a decline of stem cells by inducing an increased differentiation, as has been proposed by Blackett and Botnick.[29] Under this hypothesis, in the short run the peripheral demand could in part be satisfied. But the decline of stem cells would continue as long as the anemia persists. This would lead to a further depletion of the stem cells, which finally would result in a more severe anemia. This cascade would make the situation worse for the animal, in the long run.[30]

It is obvious that the relation between loss coefficients and experimental dosages can only be a rough average, because the use of constant coefficients is a simplification which neglects important biological mechanisms, e.g., the consideration of repair mechanisms would lead to lower loss coefficients in the beginning (repair) and higher values in later phases (residual damage). Such a model description would be biologically more reasonable, but there is not enough information available to quantify these effects. Furthermore, quantitative differences for the relation between loss coefficients and dosages may occur for mouse strains and radiation sources different from those used in the experiments which have have been analyzed here.

A special comment on dose-response curves for chronic irradiation is necessary. Some authors prefer cumulated dose diagrams.[2,3] Such diagrams may be useful in describing residual damage. However, for the description of effects during the first weeks of irradiation they are not very helpful. This statement may be explained by a quantitative example: looking at the steady-state values shown in Figure 5, one finds after 40 days of irradiation with 0.1-Gy/day a CFU-S plateau at 65%. Thus, the accumulated dosage of 4 Gy corresponds to a CFU-S cell number of 65% of normal. Taking, from the same diagram, the data for 6-day irradiation with 0.7 Gy/day (i.e., an accumulated dose of 4.2 Gy), CFU-S is lower than 10%. Thus, approximately the same cumulated dosage leads in one case to CFU-S of 65%, but in the second to less than 10%. Many examples of this type can be calculated from the data in Figure 5 showing that cumulated dose is a misleading quantity to describe early irradiation effects on CFU-S. Instead we propose dose-response curves relating the daily dosage with the plateau values of CFU-S or precursors (e.g., taken on day 40), as shown in Figures 1 and 2. After more than 20 days of chronic irradiation, the cell numbers are quite stable and such dose-response curves can be drawn.

Mackey[31] recently predicted that chronic irradiation might lead to an onset of sustained oscillations for a certain dose range. In the stem cell model presented here oscillations do not occur for any dose rate. This corresponds to the observations in normal mice. However, in $S1/S1^d$ and W/W^v mice, both having genetic defects in stem cell regulation, oscillations may well occur as shown following the administration of Strontium-89, which after incorporation into the bone acts as a permanent irradiation source.[32,33]

It is interesting to compare the model analysis of chronic and acute irradiation:

1. In both situations, the differentiated cells appear less radiosensitive than the stem cells. In chronic irradiation, the ratio of the radiosensitivity of differentiated cells to that of the stem cells is found to be $r = 0.66$. In acute[34] irradiation this ratio varies between 0.08 and 1. The ratios for acute and for chronic irradiation are comparable if one considers acute irradiation as a special case of chronic irradiation with extreme dose rates applied for a few minutes. Then regulatory effects are negligible, and an exponential decrease of stem cells [$S(t) = \exp(-K_S * t)$] and differentiated cells [$D(t) = (-K_D * t)$], where t is the duration of irradiation and S(t) and D(t) are the relative cell numbers of stem cells and differentiated cells, can be assumed. If one calculates the ratio lnD/lnS with this approach one obtains: $\ln D/\ln S = K_D/K_S = r$. This equation is equivalent with Formula 1 in Loeffler and Wichmann[34] showing that our definitions of relative radiosensitivity for acute and chronic irradiation (see Formula 12) are equivalent.

2. After acute irradiation, the peripheral stimulation is of minor importance because it is not very pronounced and becomes effective only when the recovery is almost finished. During chronic irradiation, however, the permanent cell loss may lead to severe anemia or neutropenia which stimulates the amplification of precursors and thus influences intramedullary feedback.
3. As long as the stem cell number is not too small (below 6%), the precursor cells recover quicker (in acute irradiation) or maintain higher plateau values (in chronic irradiation).
4. Finally, the spectrum of reactions seems to be broader for acute irradiation, compared with the uniform behavior after chronic irradiation. In acute irradiation, it was not possible to derive dose-response curves using pooled data from different experiments. In chronic irradiation this was possible. It remains open whether this finding corresponds to a general difference between acute and chronic irradiation or whether it results by chance from the special data under consideration. For the analysis of acute irradiation[34] data from 15 experimental groups have been used, while for the analysis of chronic irradiation only data from 7 groups were available, the majority coming from only one group.[9]

In conclusion, it is our impression that the response of the hemopoietic system to chronic irradiation can still not be completely explained. A number of interesting questions (radiosensitivity, radioprotection) requires further experimental and theoretical consideration.

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Chapter 10

POSTCHRONIC IRRADIATION — EXPERIMENTAL RESULTS

Ivan Kalina

TABLE OF CONTENTS

I. ABSTRACT

Chronically applied ionizing radiation at doses of 0.25 and 0.5 Gy/day in mice and 0.115 and 0.6 Gy/day in rats induced significant decreases in concentrations of CFU-S and bone marrow cellularity in all irradiated groups.

The rate of recovery of CFU-S as well as that of the cellularity was found to be dependent on the dose rate and on the total accumulated dose of the preceding irradiation.

Based on these experimental results on the recovery rate of CFU-S in mice previously chronically irradiated, a formula was derived expressing these processes:

$$B_t = B_s + (B_o - B_s) * \left(1 - \exp\left[-\frac{t}{T}\right]\right)$$

Repair coefficients were calculated using this formula for individual dose rates and accumulated doses.

II. INTRODUCTION

The recovery of hemopoietic tissue injured by ionizing radiation depends primarily on the repopulating ability of pluripotent hemopoietic stem cells. The study of hemopoietic tissue injury and recovery processes in irradiated animals is of great importance as this tissue is highly radiosensitive.

Hemopoietic tissue injury and repair (including that of hemopoietic stem cells) has been investigated in much more detail after single-dose irradiation than after chronic irradiation schedules. However, Blackett[1] studied the rates of decrease and recovery of bone marrow hemopoietic cells in continuously irradiated rats using a dose of 0.45 Gy/day. He found the marrow erythropoietic repopulating ability increased from 10 to 50% of normal within 14 days after the irradiation. Shvetz and Gorlov[2] found that the colony-forming ability of bone marrow cells in mice chronically irradiated with a dose of 1 Gy/day up to an accumulated dose of 18 Gy could recover from 8% of normal to normal values within 28 days. This problem was discussed also in our studies published earlier.[3,4]

III. MATERIAL AND METHODS

Male, "H"-strain mice with an average body mass of 24 g and male Wistar rats with a mass of 80 g (recipients) and 160 g (donors) were used in our experiments.

Donor mice were chronically irradiated with a ^{60} Co source. Two dose rates (0.25 Gy/day, 0.5 Gy/day) were used for a duration of either 30 or 100 days. Similarly, donor rats were treated with two different dose rates (0.115 Gy/day, 0.6 Gy/day) but only for 30 days.

Animals were sacrificed by decapitation in intervals of 7, 14, 21, and 28 days after termination of the irradiation. Tibial bone marrow cellularity was estimated using Burker chambers. CFU-S was determined according to the exogenous colony assay of Till and McCulloch[5] for mice and in a version modified by Vacek et al.[6] for rats.

Animals of both species used as recipients were irradiated with a single dose of 6.5 Gy using the TUR T-250 X-ray apparatus (180 kV, 15 mA, filtration 0.5 mm Cu + 0.5 mm Al, focus distance 50 cm, dose rate 0.5 Gy/min).

The suspension of bone marrow cells was yielded by washing the tibia diaphyses with Hanks' solution; 10^5 nucleated cells were injected into mice, 10^6 nucleated cells into rats.

IV. RESULTS

The course of CFU-S recovery in mice chronically irradiated with a dose rate of 0.25 Gy/day up to an accumulated dose of 7.5 Gy (30 days) is shown in Figure 1a. CFU-S

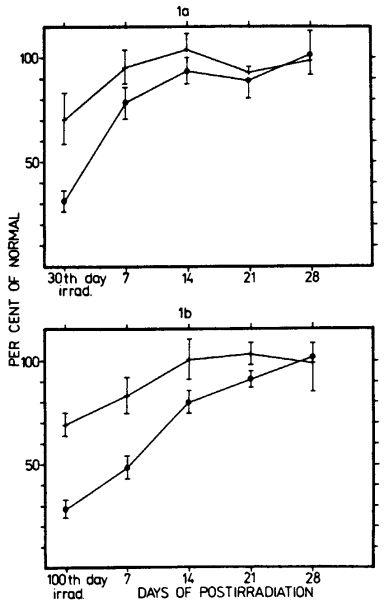

FIGURE 1. Recovery of CFU-S (●) and bone marrow cellularity (+) in mice chronically irradiated for 30 and 100 days at 0.25 Gy/day.

concentration recovered from 30% of control to control values at day 14 after irradiation. Marrow cellularity in mice decreased during irradiation to 70% of normal recovering to their normal level on day 7 after irradiation. In the case of mice irradiated with 0.25 Gy/day up to an accumulated dose of 25 Gy (100 days), Figure 1b, marrow CFU-S concentration recovered to control level on Day 21 and the cellularity was normal on day 14 after irradiation.

The recovery of CFU-S and cellularity in chronically irradiated mice following 0.5 Gy/day is shown in Figure 2. CFU-S in the group irradiated for 30 days (i.e., to the accumulated dose of 15 Gy) had recovered by day 21 after irradiation (Figure 2a). In the case of mice irradiated with doses of 0.5 Gy/day up to an accumulated dose of 50 Gy (100 days), Figure 2b, normal CFU-S values were not found until day 28 postirradiation. The course of CFU-S recovery can be expressed by the following formula:

$$B_t = B_s + (B_o - B_s) * \left(1 - \exp\left[-\frac{t}{T}\right]\right) \quad (1)$$

where B_t = CFU-S concentration at time t, B_o = normal CFU-S concentration before irradiation, B_s = CFU-S concentration at steady state during irradiation, T = repair coefficient [days], and t = time in days.

Values for repair coefficients calculated for individual dose rates and accumulated doses are shown in Table 1.

In rats chronically irradiated with doses of 0.115 Gy/day (Figure 3) bone marrow CFU-

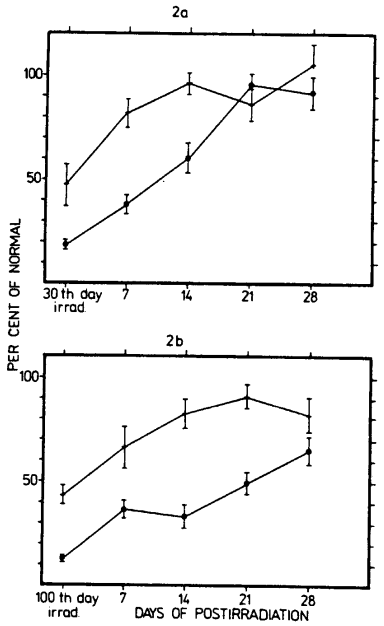

FIGURE 2. Recovery of CFU-S (●) and bone marrow cellularity (+) in mice chronically irradiated for 30 and 100 days at 0.5 Gy/day.

Table 1
VALUES OF REPAIR COEFFICIENT T

Dose rates (Gy/day)	Time of irradiation (days)	Repair coefficient (T)
0.25	30	6.0
0.25	100	12.0
0.5	30	14.5
0.5	100	24.1

S concentrations decreased to 65% of normal after 25 days of irradiation. Bone marrow cellularity had decreases by day 25 of irradiation but had recovered to the control level by day 7 postirradiation.

The decline in CFU-S and total cellularity in rats chronically irradiated with 0.6 Gy/day is shown in Figure 4. Under these conditions CFU-S concentrations reached 5% of control after 21 to 30 days, while the cellularity count dropped to only 50% of normal between 7 and 30 days of irradiation. Cellularity recovered to normal level within 14 days after chronic irradiation, whereas CFU-S had not normalized by day 28.

V. DISCUSSION

The question of the ability of hemopoietic tissue to recover after ionizing radiation rep-

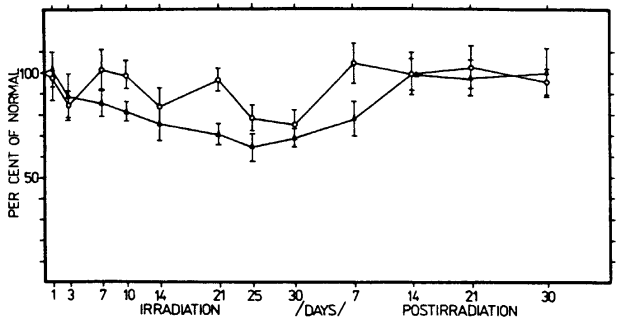

FIGURE 3. CFU-S (○) and bone marrow cellularity (▲) of rats during and after chronic irradiation with 0.115 Gy/day.

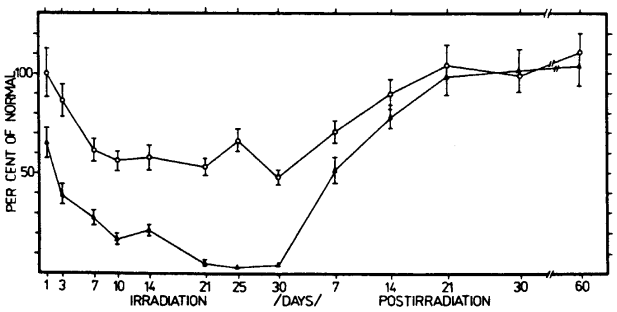

FIGURE 4. CFU-S (○) and bone marrow cellularity (▲) of rats during and after chronic irradiation with 0.6 Gy/day.

resents an important problem in radiation hematology and radiobiology. The recovery of hemopoietic tissue is a complex process involving recovery of different hemopoietic compartments. Hemopoietic tissue functions under the control of feedback mechanisms that regulate the proliferation of stem cells according to demand.

It has been shown[7] that CFU-S are able to maintain the steady state at a decreased level when irradiated with doses less than 0.5 Gy/day for relatively long times. The question arose whether the CFU-S ability to recover from decreased levels is the same after a short steady-state period as compared with a prolonged duration. The present results show that the recovery of bone marrow CFU-S in mice chronically irradiated with doses of 0.25 and 0.5 Gy/day depends on whether the irradiation is continued for 30 or 100 days. This difference occurred in spite of nearly equal CFU-S concentrations at the end of all exposure periods. Thus, after 0.25 Gy/day in mice irradiated for 100 days, CFU-S recovered to normal 1 week later than in animals irradiated for only 30 days. Even greater differences were found in groups irradiated with 0.5 Gy/day, where in the group irradiated for 30 days CFU-S reached the control level at day 21, while in the group irradiated for 100 days CFU-S had recovered to only 65% of control by day 28.

The pattern how CFU-S recover in mice after chronic irradiation can be expressed by

Formula 1. In this formula, the coefficient T describes the repair of stem cells and can be used to estimate the pattern of repair until day 28 after chronic irradiation with dose rates of 0.25 or 0.5 Gy/day.

Differences in the recovery of CFU-S and bone marrow cellularity from approximately equally decreased levels after chronic irradiation cannot be explained by the dose rate. Obviously, the duration of the continuous irradiation, in other, the accumulated dose, must also be taken into account. This is markedly demonstrated in the group receiving 0.5 Gy/day where recovery is unimpaired after 15 Gy but delayed after 50 Gy.

On this basis it might be speculated that chronic irradiation, even with low dose rates but lasting long enough, impairs the mechanisms which are normally responsible for regulation of proliferation processes. Arguments in this direction are also supported by Akoev.[8]

A further possibility is that the mechanisms which help to keep the CFU-S population in chronically irradiated animals at the new low steady state are exhausted. These mechanisms are, for example, the increase of the proliferation rate by activation of cells from the G_0-phase, the shortening of the cell cycle,[9,10] and also, the increase of the number of CFU-S in the cell cycle.[11] They may exhaust the proliferation ability of CFU-S to such a degree that in the repair phase they are not able to return to their normal level. It is also possible that prolonged irradiation causes such injuries in stem cells that their seeding ability in the spleens of recipients decreases.

If one compares the repair ability of CFU-S after irradiation under similar conditions for mice and rats (see Figures 2a and 4) one finds that the control levels are reached in both species at the same time, in spite of accelerated repair in rats during the first 14 days.

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Chapter 11

POSTCHRONIC IRRADIATION — A MODEL ANALYSIS*

Markus Loeffler and H.-Erich Wichmann

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I. ABSTRACT

The recovery of hemopoiesis after chronic irradiation is terminated is characterized by monotonous recovery of stem cells and precursor cells. The time of normalization depends on the daily dose applied during the preceding irradiation phase. In total the system seems to recover in a way very similar to that after acute irradiation. These characteristics can be explained with a mathematical model of stem cell regulation. The model analysis suggests that residual damage following irradiation is not very important if the daily dose does not exceed 0.5 Gy/day and if the duration is less than 30 days.

II. INTRODUCTION

Although the behavior of murine stem and precursor cells has often been studied during chronic irradiation, measurements on their recovery after terminated chronic irradiation are rare.[1-7] CFU-S have been measured in all of these experiments, but about BFU-E nothing is known. CFU-GM,[7] CFU-E,[7] and all nucleated precursor a cells[3] have also been investigated by only one author. In addition, some data on stem cells in rats (repopulating ability) and iron uptake are available.[5] In the following, marrow hemopoiesis after chronic irradiation is investigated mathematically.

III. MATHEMATICAL METHODS

The experimental situation following the end of a chronic irradiation period is simulated by the mathematical model of stem cell regulation as described earlier.[8-10] The preceding phase of chronic irradiation will not be considered explicitly. Instead the values measured at the end of the irradiation period are taken as starting values for the model calculations. In compartments where no starting values have been measured, these are estimated from the appropriate plateau values of chronic irradiation as calculated by Loeffler et al.[11] Three experiments will be considered in which preceding doses of (1) 0.25 Gy/day (30 days), (2) 0.5 Gy/day (30 days), and (3) 0.7 Gy/day (49 days) were employed. The corresponding initial values are

1. S = BE = CG = 0.3, CE = 0.9, E = G = 0.70 (full lines in Figures 1 to 6)
2. S = BE = CG = 0.15, CE = 0.60, E = G = 0.45 (dashed lines in Figures 1 to 6)
3. S = BE = CG = 0.0045, CE = E = G = 0.02 (Figures 7 to 10)

During chronic irradiation, anemia develops. As can be derived from the model of Wulff[12] and Wichmann[13] for mature erythropoiesis, the red blood cells are reduced to approximately (1) 90%, (2) 75%, and (3) 50% of normal in the experiments considered here (see Loeffler et al.[11]). With the same model,[12,13] the corresponding theoretical value for erythropoietin (EP) and the recovery of EP after termination of irradiation can be calculated. One finds

1. EP = 1.5 which normalizes within 1.5 days.
2. EP = 3 which normalizes within 3 days.
3. EP = 200 which normalizes within 13 days.

Since the influence of EP in cases (1) and (2) is of minor importance, it is neglected in the presented curves and EP is kept at normal values throughout the simulation. Calculations for (1) and (2) are performed with the standard model.[8-10] For case (3), however, the

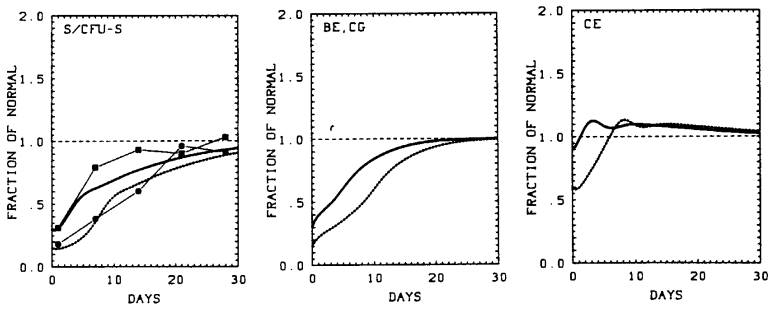


FIGURE 1 FIGURE 2 FIGURE 3

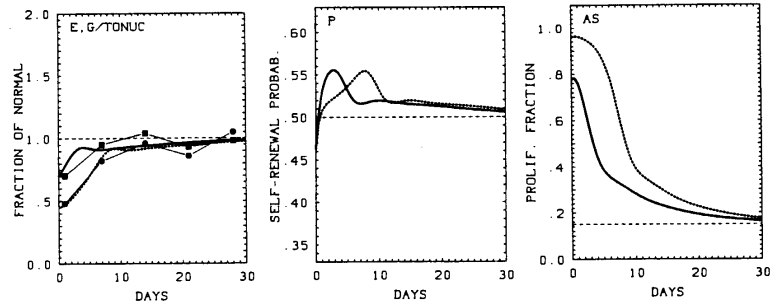


FIGURE 4 FIGURE 5 FIGURE 6

FIGURES 1 to 6. Recovery after moderate chronic irradiation. Two calculations are presented for two different doses (——, - - -). The calculations for the recovery phase are compared with data following a 30-day irradiation with 0.25 Gy/day (■, CFU-S, TONUC = total nucleated cells) or 0.5 Gy/day (●, CFU-S, TONUC = total nucleated cells) as presented by Kalina.[3] In these calculations no peripheral stimulation is considered.

stimulation by erythropoietin is considered explicitly. Here the theoretical EP-curve in Figure 7 is used as input for the stem cell model.

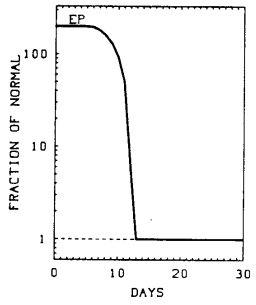
During the preceding irradiation not only anemia but also neutropenia develops. As the model does not explicitly include the effect of granulopoietic stimulation on the granulopoietic precursors, we proceed as described for chronic irradiation:[11] granulopoiesis is neglected and erythropoiesis is considered as representing the total peripheral stimulus. Mathematically, G is replaced by E in the regulatory functions: $p(S,E,E)$, $a_S(S,E,E)$, $a_{BE}(S,E,E)$, $a_{CG}(S,E,E)$.

In total, postchronic irradiation is simulated either like the recovery from acute irradiation[14] (cases 1 and 2) or its combination with anemia[15] (case 3).

IV. RESULTS

A. Model Calculations

Figures 1 to 6 show two model calculations representing the recovery after termination of 30 days of chronic irradiation with two different doses (0.25 Gy/day, 0.5 Gy/day). As E and G, on one hand, and BE and CG, on the other hand, behave very similarly they are drawn in the same figures.



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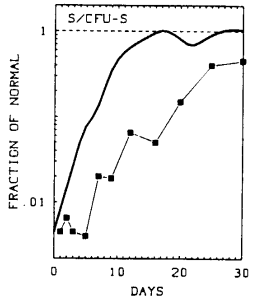

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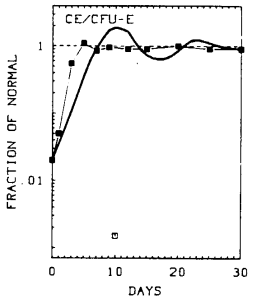

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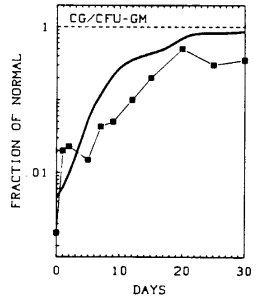

FIGURE 10

FIGURES 7 to 10. Recovery after severe chronic irradiation. The model curves (———) are compared with data following 49 days of irradiation with 0.7 Gy/day (■, CFU-S, CFU-E, CFU-GM) measured by Wu et al.[7] In the calculations stimulation by EP is used as input to the model (Figure 7). The delayed and incomplete recovery of CFU-S and CFU-GM can be interpreted by residual damage.

The model calculations exhibit the following characteristics:

- The initial values at the beginning of recovery are small for the stem cells (S), and progenitors (BE, CG) and are considerably higher for the more mature progenitors (CE) and the precursors (E, G).
- The recovery of S starts immediately after the termination of irradiation. No dip or plateau phase is found (Figure 1). S increases monotonously and normalizes without overshoot.
- In all other cell compartments normalization always occurs faster and earlier than in S. Slight overshoots may be possible for CE, E, and G (Figures 3 and 4).
- During recovery the increased stem cell proliferation rapidly slows down (Figure 6).

In a second set of calculations recovery from a 49-day period of irradiation with 0.7 Gy/day is simulated (Figures 7 to 10). The above characteristics are also found here. The initial increase in CE is, in part, due to the extreme peripheral stimulation (mediated by EP) (Figure 7) which leads to a temporary overshoot.

In total, the model curves on postirradiation recovery show three effects. First, stem cells profit from an immediate elevation of the self-renewal probability "p" (Figure 5). Second, the compartments of differentiated cells benefit from the high stem cell proliferation "a_S" which is responsible for an increased flux of cells into the differentiated lineages. In addition, they profit from the fact that many of these cells would have been destroyed during the preceding irradiation phase but remain alive now. A third effect, which is only found for high doses, is that EP stimulates the amplification of precursors. The last two effects lead to a recovery of the differentiated cell stages which might show an overshoot.

B. Comparison with Data

The model curves in Figures 1 and 4 can be compared with CFU-S and total nucleated cells from mice following a 30-day treatment with 0.25 and 0.50 Gy/day as collected by Kalina.[3] Since after chronic irradiation the nucleated cells are approximately proportional to granulopoietic and erythropoietic precursors, they may be compared with E or G (which are identical here). Shape and time behavior of S, E, and G agree well with the measurements of CFU-S and the nucleated cells. Data from mice for other cell stages under this dose regime are not available. However, some data on repopulating ability and iron uptake from Blackett[5] for rats (0.45 Gy/day, several weeks) show a similar behavior as shown in the curves for S and E.

Comparison of the third calculation with data from Wu et al.[7] (after 49 days with 0.7 Gy/day) shows that CFU-S and CFU-GM recover slower than S and CG and do not normalize within 30 days. This indicates a residual damage in the experiment which is not taken into account in the model.

V. DISCUSSION

As could be demonstrated, the model reproduces the measurements available for the recovery of hemopoietic stem and precursor cells after chronic irradiation if the dose rate is not too high and the application not too long. The choice of the initial values for the calculations is difficult for compartments for which no measurements are available (BE, CG, CE). These missing initial values were approximately estimated by using model calculations for chronic irradiation.[11]

If the preceding phase of chronic irradiation does not exceed a dose of 0.5 Gy/day and a duration of 30 days, the model is in quantitative agreement with the data. This has been shown using the data reported by Kalina.[3] A dose of 0.7 Gy/day for 49 days, as applied by Wu et al.[7] leads to a delayed increase of CFU-S and CFU-GM, if compared with the corresponding model curves. This may be interpreted as evidence for residual damage which occurs in the experiment but is not considered in the model. A similar pattern is found for a dosage of 0.5 Gy/day for 100 days. CFU-S and nucleated cells from this experiment[3] show a delayed recovery if compared with corresponding model curves (not shown). In this situation, residual damage also might be the reason for the delayed recovery of the measured cell numbers.

A similar explanation may be valid for the observation that the stem cells number after fractionated irradiation (which might be considered as an odd chronic irradiation) does not recover to normal values. After 4.5 Gy on four successive days reducing CFU-S below 0.1%, an imperfect recovery of CFU-S to only 10% of normal within 4 weeks is observed.[16,17]

Residual damage is not considered in the present model, because we imply that regulation brings the system back to normal. In the model, changes of the normal steady state are not included in the present volume, which is restricted to the analysis of "normal" regulatory phenomena.

Although anemia and granulocytopenia seem to have an important radioprotective effect

during chronic irradiation,[11] they are of less importance after chronic irradiation. So one can show that the effect of peripheral erythropoietic and granulopoietic stimulation in the first two cases (1 and 2) is negligible. Taking it into account would hardly affect S, BE, and CG, and would elevate CE, E, and G by not more than 10%. Only after termination of severe irradiation the peripheral stimulation is relevant, as shown in the third case. The relatively small influence of anemia during the recovery period from irradiation damage has also been shown for the combination of acute irradiation and bleeding anemia.[15]

In conclusion, the model simulations reproduce the basic characteristics of postirradiation recovery and a first estimation can be obtained under which dose and time regiment of chronic irradiation residual damage can be expected.

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Chapter 12

BLEEDING ANEMIA — EXPERIMENTAL RESULTS

Hiroshi Hara

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I. ABSTRACT

Since the 1950s, bleeding anemia is commonly used as an erythropoietic stimulation in investigation of erythropoiesis. The anemia can induce the decrement in number of BFU-E, CFU-GM, and CFU-S in bone marrow and the increment in number of CFU-E in bone marrow and spleen. The changes in number of the hemopoietic progenitors can be explained by their migration from marrow to spleen and differentiation from BFU-E to CFU-E. In the response to the anemia, large reticulocytes with abnormally short survival period are produced by early denucleation of erythroblasts which have differentiated from BFU-E under the influence of the anemia. The changes in the anemia are generally considered to be due to the elevation of erythropoietin (Epo) produced in response to the anemia.

II. INTRODUCTION

Since Reissman[1] demonstrated the presence of a humoral erythropoietic regulator named Epo in blood using parabiotic rat experiments in which one rat exposed to hypoxia induced an erythropoietic response in another parabiotic animal breathing normal air, many papers have appeared on the regulation of hemopoiesis.[2-4] These papers revealed that Epo activity in serum increases in parallel with the severity of hypoxic stimulation, and Epo is mainly produced in the kidney in both animals and man. Changes in erythroblasts in bone marrow[5-13] and reticulocytes in blood[14-18] induced by erythropoietic stimulation have also been investigated in order to analyze erythropoiesis in animals and man.

Many techniques have been developed for studying hemopoietic progenitors and stem cells. In 1961, Till and McCulloch[19] were the first to develop a method to assay pluripotent stem cells (CFU-S) which give rise to colonies in the spleens of irradiated mice. Bradley and Metcalf[20] devised soft agar culture techniques to detect macrophage-granulocyte progenitors (CFU-GM), which have the ability to form macrophage-granulocyte colonies. At present, the techniques improved by many researchers make it possible to assay macrophage-granulocyte progenitors (CFU-GM),[20] eosinophile progenitors (CFU-Eo),[21] megakaryocyte progenitors (CFU-M),[22] mature erythropoietic progenitors (CFU-E),[23] relatively immature erythropoietic progenitors (BFU-E),[24,25] and pluripotent progenitors (CFU-MIX) from animals.[26,27] Using these techniques, studies have been conducted on the differentiation of pluripotent stem cells along the erythropoietic pathway[28] and on the kinetics of erythropoietic progenitors (BFU-E and CFU-E)[29-33] and macrophage-granulocyte progenitors (CFU-GM),[34,35] after erythropoietic stimulation.

This chapter will cover studies on kinetics of erythropoietic progenitors and precursors in response to bleeding anemia.

III. MATERIALS AND METHODS

Various animal species, such as mice, rabbits, and dogs, have been used to study hemopoiesis after bleeding. Mice were bled once with approximately one third of the blood and then the numbers of the various colony-forming units in bone marrow were assessed using the in vivo and in vitro colony assay techniques by Adamson et al.[32] By double cardiac puncture, 0.5 mℓ of blood was removed by Hara and Ogawa[30] from BDF_1 mice on day 0 and 3, and the population of erythropoietic progenitors, CFU-E and BFU-E, in the marrow, the spleen, and circulating blood were assayed using methylcellulose cell culture techniques. By single cardiac puncture, 1.1 to 1.4 mℓ of blood from BDF_1 mice was withdrawn and the population of erythropoietic progenitors and granulopoietic progenitors in bone marrow was measured by Iscove,[31] using methylcellulose cell culture techniques. Dogs were bled (25 $\sim$ 30 mℓ/Kg) three times daily and assays were done on marrow cellularity, erythroblast

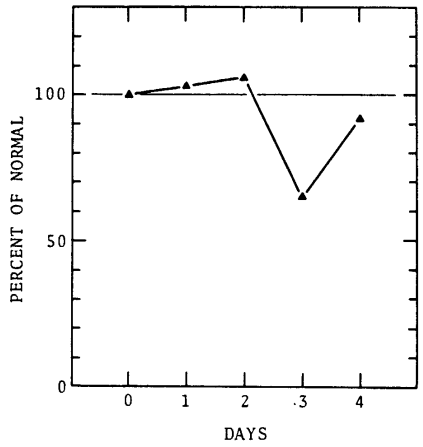


FIGURE 1. Changes of CFU-S in murine bone marrow after massive bleeding, reported by Adamson et al.[32]

generation time using ^{59}Fe autoradiography and the appearance rate of radioactive iron in peripheral red cells by Alpen et al.[5,6] Rabbits were bled once (20 mℓ/kg) from the ear lobe vein by Nagai and Hara[10,12] and then the marrow cellularity in the animals was measured with ^{59}Fe according to the method of Donohue et al.[37] C57BL and BALB/C mice were bled (0.3 mℓ) from the tail vessels using a suction apparatus and CFU-GM in bone marrow were assayed by Metcalf[34] using agar culture techniques developed by his group.

IV. RESULTS: EFFECTS OF BLEEDING

A. On CFU-S

Several articles have described changes of CFU-S in response to erythropoietic stimulation.[36] Though they revealed a decrement in the number of CFU-S after the stimulation, few have described the effects of bleeding on CFU-S.

1. On the Number of CFU-S in Bone Marrow

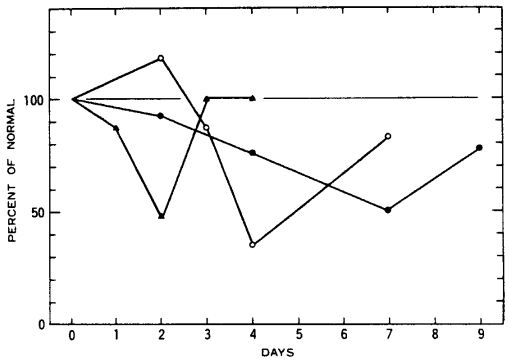
Changes in the numbers of CFU-S following massive hemorrhage were recently described by Adamson et al.[32] The numbers in bone marrow from mice do not change until day 2, decrease on day 3, and then increase towards the control value as presented in Figure 1. The serial changes are essentially similar to those in animals with other erythropoietic stimulation.[36]

2. On the Proliferative State of CFU-S

Following the bleeding, the proportion of CFU-S in active DNA synthesis remains unchanged until day 4 when it increases to a value 50% above the simultaneously determined controls.[32]

B. On BFU-E

Changes in erythropoietic progenitors CFU-E and BFU-E in response to anemia have been investigated by many researchers[29-33] in order to clarify the stage at which erythropoietic progenitors are the target of Epo.



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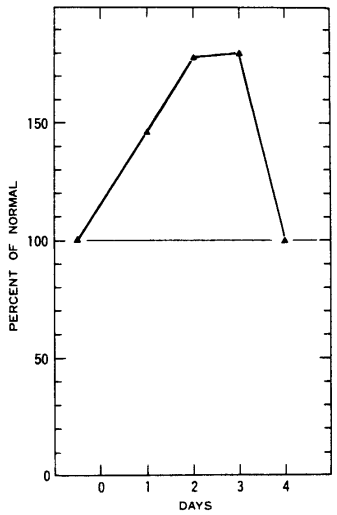


B

FIGURE 2. (A) Changes of BFU-E in murine bone marrow after bleeding. Closed circles (●—●), open circles (○—○), and triangles (▲—▲) are data from Hara and Ogawa,[30] Iscove,[31] and Adamson et al.,[32] respectively; (B) changes of BFU-E-4d in murine bone marrow after bleeding found by Adamson et al.[32]

1. On the Number of BFU-E in Bone Marrow

As presented in Figure 2A, marrow BFU-E after bleeding decreased transiently, with the grade and duration of the decrement depending on the method of bleeding and the volume of blood loss. After double cardiac puncture, marrow BFU-E continuously decreased, reached a nadir at 51.2% of the control on day 7, and started returning towards normal by day 9.[30] Following single bleeding, BFU-E initially declined, reached a nadir on day 2, and returned to normal on day 3.[32] In contrast, marrow BFU-E initially increased to day 2 after single massive bleeding, then decreased until day 4, and started returning towards the normal level by day 7.[31]

2. On the Proliferative State of BFU-E

When assessed by the sensitivity to killing by brief exposure to tritiated thymidine (^{3}H-TdR) in vitro, the portion of BFU-E in the DNA synthetic phase was normally 30% and neither increased nor decreased after single massive bleeding according to Iscove.[31] Similar results with mice with hemolytic anemia induced by phenylhydrazine were reported by Hara and Ogawa.[30] Adamson et al.,[32] however, reported that the cell cycle state of BFU-E is initially unchanged but their proportion in the S-phase subsequently increases.

3. On Relatively Mature BFU-E

Adamson et al.[32] described the influence of a single hemorrhage on the relatively mature BFU-E-4d.

The sequential changes of BFU-E-4d in bone marrow due to an acute hemorrhage are more similar to those of CFU-E than BFU-E as presented in Figure 2B, though the magnitude of the changes of BFU-E-4d is quite different from that of CFU-E. The proportion of BFU-E-4d in active DNA synthesis increased by 40% within 24 hr, was 158% by day 2, increased to 183% on day 3, and then fell to 132% on day 4, according to Adamson et al.[32]

C. On CFU-E

The serial changes of mature erythropoietic progenitors, termed CFU-E, in response to erythropoietic stimulation such as hemolytic anemia,[29] administration of Epo,[30,31,33] and acute blood loss,[30-32] are quite different from those of BFU-E.

1. On the Number of CFU-E

The numbers of CFU-E in bone marrow after a massive loss of blood rapidly increased as shown in Figure 3A, peaked between day 2 and 4, and then decreased towards the control level.[30-32] After double cardiac puncture, the numbers of progenitors in bone marrow on day 9 were still higher than the control value. After the treatment, the number of CFU-E in spleen dramatically increased 25-fold on day 4, and then fell towards the control values by day 9 as presented in Figure 3B.

2. On the Proliferative State of CFU-E

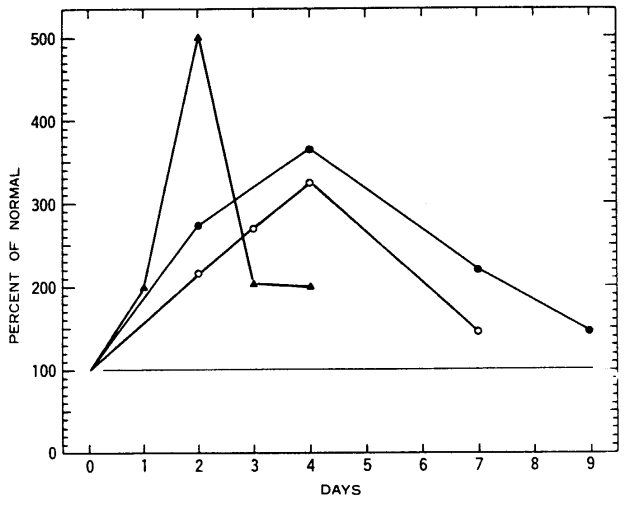
The loss of blood had no influence on the proliferative state of CFU-E in bone marrow.[30-32]

D. On Erythroblasts

A few reports[6,10,34] described serial changes in the numbers of erythropoietic precursors after bleeding, although changes in the kinetics of erythroblasts after treatment have been reported in many papers.[5-14]

1. On the Number of Erythroblasts

Nagai et al.[10] investigated serial changes in the number of erythroblasts in bone marrow from bled rabbits, as shown in Figure 4. The number of erythroblasts in bone marrow increased continuously until day 3 and then decreased towards the base line level again. Although nucleated cells in bone marrow from mice gradually decreased until day 3 after bleeding, erythroblasts increased and reached a plateau level on day 1.[34]



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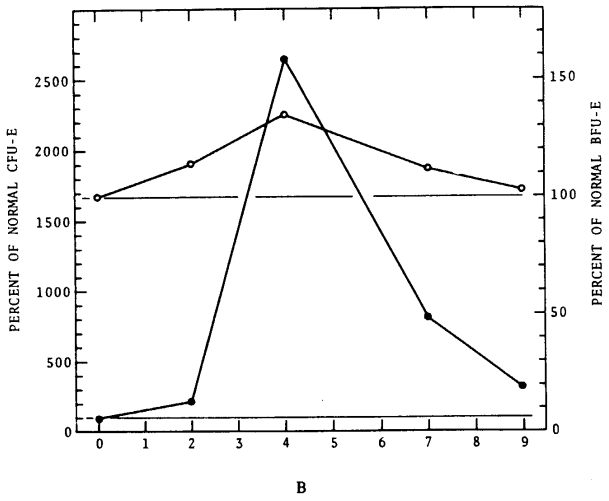

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FIGURE 3. (A) Changes of CFU-E in murine bone marrow after bleeding. Closed circles (●—●), open circles (○—○), and triangles (▲—▲) are data from Hara and Ogawa,[30] Iscove,[31] and Adamson et al.,[32] respectively; (B) changes of CFU-E and BFU-E in murine spleen after bleeding. Open circles (○—○) and closed circles (●—●) give data for BFU-E and CFU-E, respectively, found by Hara and Ogawa.[30]

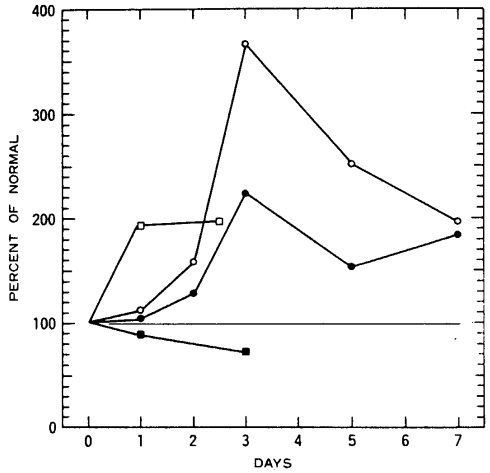

FIGURE 4. Changes of nucleated cells and erythroblasts in mouse and rabbit bone marrow after bleeding. Closed squares (■—■) and open squares (□—□), respectively, indicate the total numbers of nucleated cells and erythroblasts in bone marrow from mice found by Metcalf.[34] Closed circles (●—●) and open circles (○—○), respectively, indicate total numbers of nucleated cells and erythroblasts in rabbit found by Nagai and Hara.[10]

2. On the Number of Erythroblasts in Each Stage

Prominent increment in numbers of relatively immature erythroblasts, pronormoblasts, and basophilic normoblasts in bled dogs, which had been subjected to massive daily bleeding, was found by Alpen et al.[6] Relatively matured erythroblasts also increased but to a much smaller percentage as shown in Figure 5A. In bled rabbits, sequential changes in the numbers of erythroblasts in bone marrow were investigated by Nagai and Hara[10] based on the nuclear diameter of the cells,[10] with the results shown in Figure 5B. The numbers of relatively immature erythroblasts, E_1 and E_2, rapidly increased until day 3 and then started to decrease continuously towards the normal value. Meanwhile, the numbers of relatively mature erythroblasts, E_3 and E_4, showed no increment on day 1, then increased until day 3 after which they began to decrease towards normal values. A large gap existed between magnitudes of changes in E_2 and E_3.

3. On Erythroblast Generation Time

Alpen and Cranmore[5] revealed that no changes occurred in the cycle time of erythroblasts in severely anemic dogs using ^{59}Fe grain count halving methods.

However, several investigators[7,10,14] have presented data showing shortening of the generation time of cells in animals under erythropoietic stimulation based on observations that mitotic indices of erythroblasts[7] and rates of labeled erythroblasts with ^{3}H-thymidine after flash labeling[9,12,11] were elevated under erythropoietic stimulation. On the other hand, Nagai and Hara[10,13] disclosed that the elevation in mitotic and labeling indices of erythroblasts in bled animals results from a decrement in the number of mature nonmitotable erythropoietic precursors due to shortening of the compartment transit time of the erythroblasts.

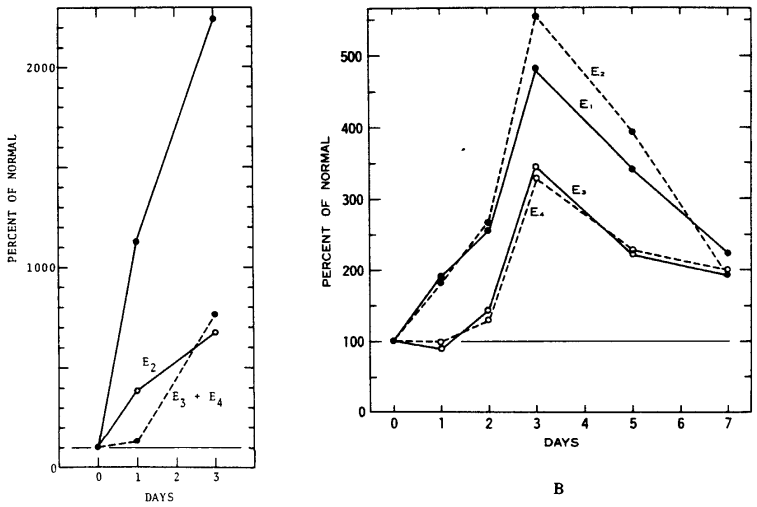

FIGURE 5. (A) Changes of erythroblasts in each maturation stage in canine bone marrow after bleeding. Closed circles with a solid line (●—●), open circles with a solid line (○—○), and closed circles with a dotted line (● — — ●), respectively, indicate populations of erythroblasts in the first (E_1), second (E_2), and third and fourth (E_3 + E_4) generations, as reported by Alpen et al.;[6] (B) changes of erythroblasts in rabbit bone marow. Closed circles with a solid line (●—●), closed circles with a dotted line (● — — ●), open circles with a solid line (○—○), and open circles with a dotted line (○ — — ○), respectively, indicate the percentages of erythroblasts in the first (E_1), second (E_2), third (E_3), and fourth (a nonmitotable generation, E_4) observed by Hara and Nagai.[16]

4. On Denucleation of Erythroblasts

Alpen and Cranmore[5] demonstrated early denucleation of relatively immature erythroblasts after bleeding with ^{59}Fe autoradiography. They found heavily labeled red cells comparable to pronormoblasts after hemorrhage. Later, they confirmed the phenomenon by quantitative analysis of erythroblasts following the treatment.[6] Similar experimental results were reported by several investigators.[10]

E. On Red Cells

Although bleeding directly reduces the number of red cells in animals as presented in Figure 6, the numbers of red cells in circulating blood influence the production of red cells, as previously described. The characteristics of red cells newly formed after hemorrhage are described here.

1. On Reticulocytes

After massive blood loss, macrocytic cells are produced and the degree of macrocytosis is proportional to the degree of erythroid stimulation.[7,8] Clinically and experimentally, the cells in bled animals produced during the initial phase of erythropoietic response are more macrocytic than subsequently produced red cells. When humans are bled, Nagai et al.[14] found an increased number of erythroblasts containing fetal hemoglobin (HbF) using anti-HbF antibody labeled with fluorescein isothiocyanate (FITC). The labeled erythroblasts containing HbF differentiate into red cells containing HbF (HbF-RBC). The numbers of

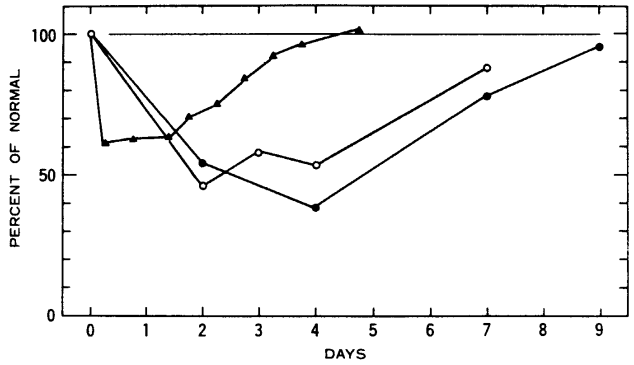

FIGURE 6. Changes of packed red cell volume in mice after bleeding. Closed circles (●—●), open circles (○—○), and triangles (▲—▲) represent the packed red cell volume data reported by Hara and Ogawa,[30] Iscove,[31] and Adamson et al.,[32] respectively.

HbF-RBC also increased at the early stage of erythropoietic response to bleeding and decreased until day 10.

2. On the Life Span of Red Cells

Berlin and Lotz[15] reported a shortened life span of red cells following acute hemorrhage. Later, similar results were reported by several investigators.[16,18] Nagai et al.[14] described the shortened survival of HbF-RBC following massive hemorrhage in man. They also revealed that some of the reticulocytes formed in early erythropoietic response to severe bleeding were fragile and were destroyed several days after their production by in vitro ^{59}Fe-labeling techniques. Approximately 10% of the reticulocytes on day 2 were destroyed in a few days.[18]

F. On CFU-GM

The effects of anemia on CFU-GM were mainly investigated in animals with hemolytic anemia induced by administration of phenylhydrazine hydrochloride.[35] The population of CFU-GM in animals after bleeding has been reported only by Metcalf[34] and Iscove.[31]

Metcalf revealed a marked reduction of CFU-GM in bone marrow as shown in Figure 7, and an increment of CFU-GM up to 238% of the normal control in spleen on day 1 and up to 929% on day 3.[34] As presented in Figure 7, the decrement of CFU-GM in bone marrow was also reported by Iscove.[31]

V. DISCUSSION

The model of hemopoiesis recently presented indicates that pluripotent stem cells provide erythrocytic (BFU-E), macrophage-granulocytic (CFU-GM), and megakaryocytic progenitors (CFU-M), and these progenitors feed the corresponding differentiated cells along each hemopoietic pathway.[38,39]

Massive bleeding is a commonly used method of erythropoietic stimulation in studies on erythropoiesis.[5-18] Generally, anemia by hemorrhage is thought to accelerate Epo production, and the magnitude and duration of which depends on the size and duration of the stimulation.[3,4]

BFU-E[30-32] in bone marrow decrease after massive hemorrhage, as do CFU-GM[31,34] and CFU-S[32] in bone marrow. The reduction of the progenitors and stem cells in bone marrow can be explained by their migration from marrow to spleen through blood, because their

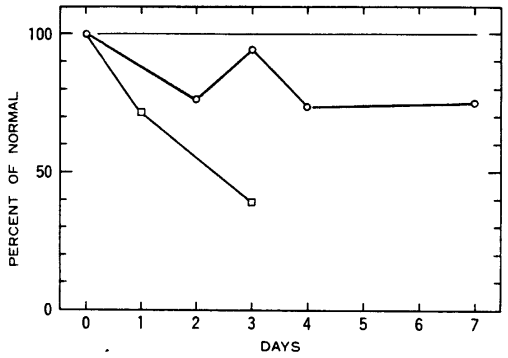

FIGURE 7. Changes of CFU-GM in murine bone marrow after bleeding. Open circles (○—○) and squares (□—□) indicate the data from Iscove[31] and Metcalf,[34] respectively.

amounts increase in the blood and the spleen.[30,34] The slower kinetics of BFU-E compared to that of CFU-E found by Hara and Ogawa,[30] Iscove,[31] and Adamson et al.[32] also supports this idea. In contrast with BFU-E, CFU-E in both bone marrow and spleen increase after the stimulation and the administration of Epo.[30] The portion of CFU-E in the DNA synthetic phase is approximately 75%[30-32] and different from that of BFU-E. Based on these two facts, that is, these findings on the kinetics and the proliferative state of both erythropoietic progenitors after bleeding, Iscove[31] proposed that the Epo responsiveness is located in the progenitors, which are intermediate in maturity between BFU-E and CFU-E. This hypothesis agrees with the results found by Adamson et al.[32] on the kinetics of BFU-E-d4 after bleeding and the different requirements of BFU-E and CFU-E for Epo in culture.[32]

Furthermore, early denucleation of erythroblasts, especially in immature erythroblasts after massive bleeding, is followed by the production of large reticulocytes with abnormally short survival periods. The magnitude of the denucleation from immature erythroblasts (E_1 and E_2) depends on the quantity of removed blood, according to the results of Alpen and Cranmore[5] and Hara and Nagai.[13]

The work of the investigators summarized in this chapter has shown that bleeding is a useful technique in studies on erythropoiesis and the studies provide a lot of data for mathematical models of erythropoiesis.

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Chapter 13

BLEEDING ANEMIA AND STIMULATION BY ERYTHROPOIETIN — A MODEL ANALYSIS*

Markus Loeffler and H.-Erich Wichman

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I. ABSTRACT

Stimulation of erythropoiesis either by blood loss or by the injection of erythropoietin leads to an increase of the number of late erythropoietic progenitors (CFU-E) and precursors (erythroblasts). This behavior is accompanied by a diminution in size of the other progenitor compartments (BFU-E, CFU-GM) which are not sensitive to erythropoietin. The experimental pattern can be reproduced by a mathematical model of stem cell regulation. The analysis suggests that the enlarged number of erythropoietic precursors reduces the cycling of stem cells and thereby the number of cells entering differentiation.

II. INTRODUCTION

The behavior of hemopoietic stem and progenitor cells after bleeding has been studied repeatedly.[1-9] As has been summarized by Hara,[10] the following pattern is observed in the bone marrow:

- CFU-S fluctuate around normal values.[1,9,10]
- BFU-E show a decrease to minimum values of about 50% followed by a recovery.[1,3,4,9]
- CFU-E and erythropoietic precursors increase to several times normal.[1,3-5,7,9]
- CFU-GM show a reduction by about one third.[4,6,9]

These experimental findings are quite consistent, except in one case.[8]

It is a well-known fact that during erythropoietic stimulation not only the bone marrow but also the spleen contributes significantly to erythropoiesis.[2,3,5,8,9]

In the model,[11-13] total hemopoiesis is considered. Therefore, the total proliferation has to be calculated from the measurements in the bone marrow and in the spleen. This is possible by using the marrow-spleen ratios (as described elsewhere[14]) and has been performed for those data where marrow and spleen measurements are available.[3,5,9] This analysis has demonstrated that the total cell numbers behave very similar to the cell numbers in the bone marrow. Therefore, further data[1,4,6] can also be compared with the model results, although only bone marrow cells have been measured.

Since the hemopoietic stimulus after bleeding is mediated by erythropoietin (Epo), the direct administration of Epo has a quite similar influence. Therefore, data on cellular behavior after Epo injections[3,15,16] will also be compared with model calculations.

It should be kept in mind that the concentration of the "real" hormone will be denoted by "Epo". In contrast to that, the theoretical values of eythropoietin, as they are used in the model calculations, will be denoted by "EP".

III. MATHEMATICAL METHODS

The experiments on bleeding anemia and the administration of erythropoietin are simulated by the mathematical model of stem cell regulation as described.[11-13] Since in the model the peripheral blood is not considered, anemia has to be simulated indirectly. Two theoretical EP curves are used as "input" for the model, to simulate a severe and a moderate blood loss (Figure 1). For the severe blood loss (removal of about 35% of the red cell mass) EP increases to ten times normal and normalizes within 8 days; for moderate blood loss (removal of about 25% of the red cell mass) EP increases to five times normal and normalizes within 4 days.

As described earlier in detail,[11] the theoretical EP curves have been generated by a mathematical model of the mature murine erythropoiesis[17,18] and are consistent with the available measurements of Epo, reticulocytes, and hematocrit.

In a similar way as for bleeding, a theoretical curve for EP can be derived for the administration of Epo. The injection of the hormone could be simulated by a huge increase of EP, with a dose-dependent peak, from which it would return to normal exponentially. However, in the model erythropoietic amplification is maximum for EP above 80 times normal, and to simulate maximum stimulation it is sufficient to use this value. Therefore, in the calculations EP is elevated to 80 times normal for 1 day and drops back to normal after 48 hr (Figure 10). This corresponds to an injection of a single dose of about 3.5 units of Epo (diluted in 0.5 mℓ medium) in mice.[17]

IV. RESULTS

A. Model Calculations

1. Bleeding Anemia

Figure 1 shows the theoretical EP curves simulating bleeding anemia of 25 and 35% blood loss. They are used as input to the model. Figures 2 to 9 demonstrate how the model reacts upon these changes of EP.

Day 0 to 3 — The elevated EP results in additional mitoses in CE and E which lead to an increased size of both compartments (Figures 4 and 5). All other compartments are relatively unaffected.

Day 3 to 7 — The acutely (day 0 to 3) elevated numbers in CE and E induce counteracting regulation. This regulation is through two mechanisms: the proliferative fraction of stem cells, "a_s", becomes reduced (Figure 9) and the self-renewal probability "p" increases (Figure 8). Together both reduce the number of stem cells differentiating per unit time. This leads to a drop in the BE, CG, and G numbers (Figures 3, 6, and 7). During this phase the S values (Figure 2) show an increase because "p" is above 0.5, but it remains small because "a_s" is small.

Day 7 to 12 — The Ep levels have now normalized which leads to a rapid drop of CE and E (Figures 4 and 5). This results in an increase of "a_s" and a decrease of "p" and inverts the situation between days 3 and 7: now the flux of cells into differentiation increases so that BE, CG, and G recover (Figures 3, 6, and 7). Simultaneously, the stem cell number, S, decreases (Figure 2).

Day 12 to 20 — The subnormal S stimulates an increase of "p" and "a_s" (Figures 8 and 9). Together, both lead to the final normalization in all cell numbers.

2. Administration of Erythropoietin

A single injection of erythropoietin is simulated by an elevation of EP to 80 times normal for 1 day (Figure 10). The consequences for early hemopoiesis are similar to those in bleeding anemia. Only those theoretical curves are shown which can be directly compared with experimental results (Figures 11 to 14).

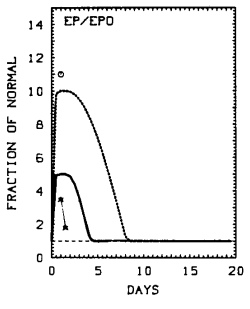
Day 0 to 5 — The high EP induces additional mitoses in CE and E, followed by a twofold increase of their compartment sizes (Figure 13). This leads to a reduction of "a_s" and an increase of "p". Both together result in a reduced rate of differentiation from the stem cell compartment which is reflected by the decrease of BE and CG (Figures 12 and 14) and a slight increase in S (Figure 11).

Day 5 to 10 — After EP has normalized, the cell numbers return to normal in the same way as in bleeding anemia.

B. Comparison with the Data

1. Bleeding Anemia

Measurements of Epo[19,20] are rare after bleeding anemia (Figure 1). They indicate that the range of the hormone's concentration is large (between 4 and 11 times normal) if measured directly after bleeding.



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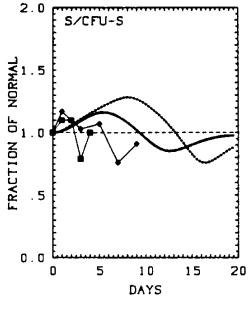

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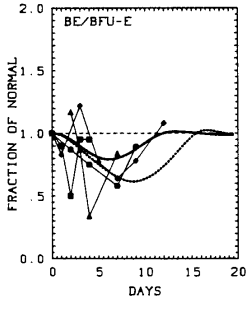

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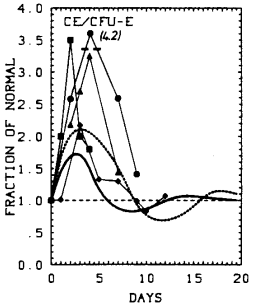

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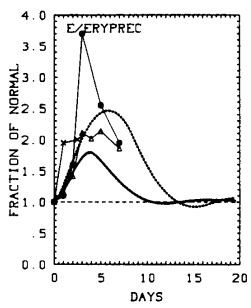

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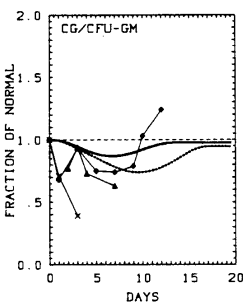

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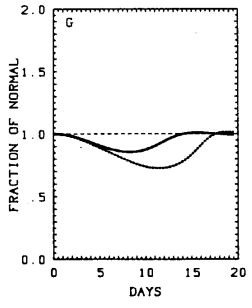

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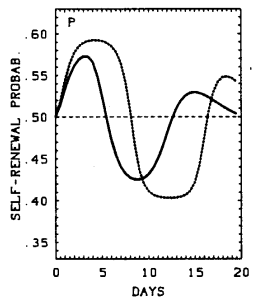

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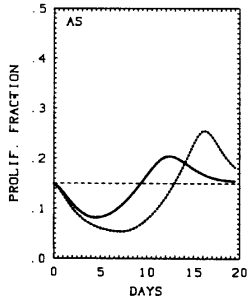

FIGURE 9

FIGURE 1 to 9. Bleeding anemia. Comparison between model results and data. Bone marrow data are taken from Adamson et al.[1] (CFU-S, BFU-E, mice ■); Iscove[4] (BFU-E, CFU-E, CFU-GM, mice ▲); Metcalf[6] (CFU-GM, erythropoietic precursors, mice ×); Nagai and Hara[7] (erythropoietic precursors, rabbits ●). Total cell numbers (bone marrow + spleen) are taken from Hara and Ogawa[3] (BFU-E, CFU-E, mice ●); Lord[5] (BFU-E, erythropoietic precursors, rats △); Seidel and Kreja[8] (CFU-S, BFU-E, CFU-E, CFU-GM, mice ♦). Measurements of erythropoietin (Epo) have been performed by Miller et al.[19] (○) and Reincke and Cronkite[20] (★). In the model the stimulation of erythropoiesis by moderate (—) and severe (- - - -) anemia is simulated by the theoretical EP curves shown in Figure 1. The EP curves correspond to a 25 and 35% blood loss. They are used as input to the model while Figures 2 to 9 show the model reaction.

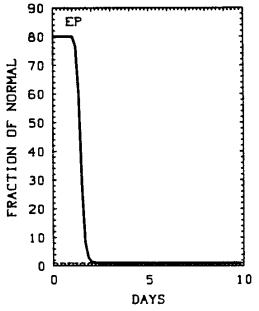

FIGURE 10

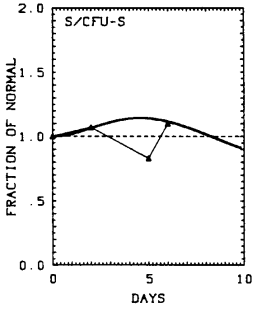

FIGURE 11

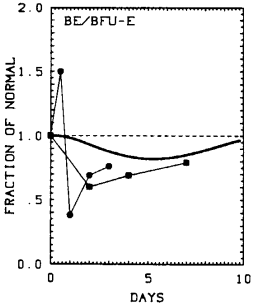

FIGURE 12

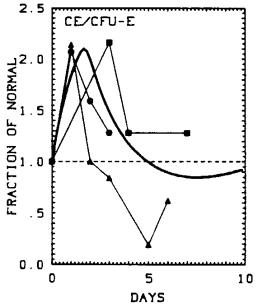

FIGURE 13

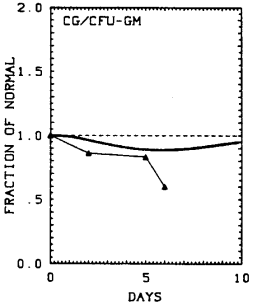

FIGURE 14

FIGURE 10 to 14. Injection of erythropoietin. Comparison of model results and data. Bone marrow data of mice are taken from Gregory et al.[15] (CFU-S, CFU-E, ▲); Hara and Ogawa[3] (BFU-E, CFU-E, ■); Peschle et al.[16] (BFU-E, CFU-E, ●). In the model (—) the injection of erythropoietin is simulated by an elevation of EP to 80 times normal and a subsequent drop to normal within 2 days, as shown in Figure 10. Again the EP curve is used as input to the model, and Figures 11 to 14 show the model reaction.

Most of the cell numbers presented in Figures 2 to 6 were obtained from mice.[1,3,4,6,9] Only the numbers of erythroblasts were derived from rats[5] and rabbits[7] since no mouse data were available. From the measurements in bone marrow and spleen total cell counts have been estimated.[3,5,9] Where spleen data were not available only bone marrow data[1,4,6] are presented. Consequently, the data are relatively heterogeneous. Taking this heterogeneity into account the model calculations reproduce the major experimental characteristics. Agreement can be found in the following points:

- The peaking behavior of CFU-E and erythroblasts is reproduced in CE and E (although the theroretical peaks are too small).
- BE shows a drop below normal as do the BFU-E data.
- In accordance with CFU-GM, CG remains below normal for several days before recovery takes place.

- The S curves are close to normal in accordance with in CFU-S data.
- Experimentally, the cycling of stem cells does not change markedly from normal.[1] Taking into account the limited sensitivity of the available suicide techniques one would not expect to detect a variation of the fraction of cycling stem cells between 5 and 25% as is suggested for "a_S" from the model calculations (Figure 9). The proliferation rates of BE and CG in the model remain close to normal throughout the whole calculation (not shown), which also is in agreement with measurements.[1,3,4] The proliferation rate of CFU-E is also unchanged.[3,4]

Some discrepancies should also be mentioned:

- The data reported by Pannaciulli et al.[8] significantly differ from other observations and from the model results. In their C57BL/C3H mice, CFU-E fall below normal after bleeding. This effect cannot be understood within the model where erythropoietic stimulation leads to amplification of CE and E.
- Similarly, Pannaciulli et al.[8] find, for Swiss mice, a more than twofold increase of CFU-GM during the first 3 days of anemia. This behavior has not been observed by other authors nor can it be reproduced with the model.

2. Administration of Erythropoietin

The data available for the administration of erythropoietin (Figures 11 to 14) are taken from studies of the bone marrow of mice.[3,15,16] The amount of Epo given was 2.4 units[16] or 10 units.[3,15] Considering an average plasma volume of 1.5 mℓ per mouse, the injected dose should result in initial Epo concentrations of 1.2 and 5 units/mℓ plasma,[17] followed by an exponential decrease. In the model this peak is simulated by a constant EP value of 80 times normal which is sufficient for maximum stimulation of erythropoiesis. Higher EP values would lead to identical reactions of the cell numbers. The model reaction of S, BE, and CG on this stimulus is in good agreement with the measurements of CFU-S, BFU-E, CFU-E, and CFU-GM.

V. DISCUSSION

As is demonstrated in Figures 1 to 14 the model gives a consistent interpretation of stem and progenitor cell reactions following bleeding anemia or administration of erythropoietin. It demonstrates that erythropoietic stimulation leads to a wave of erythropoiesis seen in elevated numbers of CFU-E and erythropoietic precursors. On the other hand, intramedullary feedback mechanisms induce a reduction of stem cell proliferation by at least one third. Consequently, the flux of cells entering BFU-E and CFU-GM is diminished and the cell numbers in both compartments decrease. The changes of CFU-S are explicable as a balance between increased erythropoiesis and decreased granulopoiesis on the self-renewal probability.

As far as possible the calculations were compared with total cell counts which were pooled from bone marrow and spleen measurements. For CFU-S, BFU-E, CFU-E, and CFU-GM the spleen contains not more than 20% of the whole animal's cells and the bone marrow behavior is a good representative of the total hemopoiesis. However, up to 30% of the erythropoietic precursors are found in the spleen of mice during the phase of maximum proliferation (days 3 to 5 after bleeding) (see Figures 8 to 13 in Reference 14). The cell counts in the bone marrow may, therefore, be an underestimate of the total number of erythroblasts.

Often bleeding anemia and PHZ-induced hemolytic anemia are considered as equivalent forms of anemia. However, as shown separately PHZ anemia differs from bleeding anemia in a dramatic migration of stem and progenitor cells to the spleen during which, apparently,

a considerable number of migrating cells is lost.[21] A second difference is an increased sensitivity of erythropoietic precursors to Epo after the administration of PHZ. Both effects influence feedback.

If one compares bleeding anemia (erythropoietic stimulation) and hypertransfusion (suppression)[22,23] one finds that BFU-E, CFU-E, and CFU-GM behave symmetrically. So, for example, CFU-E increase in anemia but decrease in hypertransfusion, and CFU-GM decrease in anemia but increase in hypertransfusion. This symmetry, however, is not found for the CFU-S. They dramatically increase after hypertransfusion but are nearly unchanged in anemia. This asymmetry has already been discussed[12,13] and can be interpreted as expression of an heterogeneous influence of erythropoiesis and granulopoiesis on self-renewal and cycling of stem cells. It can be understood if granulopoietic precursors have a higher influence on "p" than erythropoietic precursors, while the reverse applies for the influence on "a_S". What has been said about anemia and hypertransfusion also holds true for hypoxia[24] (erythropoietic stimulation) and ex-hypoxia[25] (suppression).

In addition, it should be mentioned that the reaction of mature erythropoiesis to bleeding anemia (shortened generation time of erythroblasts, reticulocytosis, shortened life span of red cells) as reviewed by Hara[10] is satisfactorily understood by the model of Wulff and Wichmann.[17,18]

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Chapter 14

HYPOXIA — EXPERIMENTAL RESULTS: THE RESPONSE OF HEMOPOIETIC STEM CELLS TO HYPOXIA

Brian I. Lord and Martin J. Murphy, Jr.

TABLE OF CONTENTS

I. ABSTRACT

A hypoxic environment generally results in increased production of erythropoietin (Epo) which stimulates excess erythropoiesis, thus enabling the restoration of normal tissue oxygenation. Parallel responses in both the erythropoietic and the pluripotent progenitors are apparent. Since much of the erythropoietic response arises in the spleen, a relatively minor hemopoietic organ, the behavior of the pluripotent spleen colony-forming cell (CFU-S) population was followed in both bone marrow and spleen. Over 14 days of exposure to 0.4 atm pressure (equivalent to an altitude of 6.7 km or 22,000 ft) CFU-S establish a somewhat unstable plateau about 30% above normal, with a tendency for their number and proliferation to oscillate about this plateau. The overall behavior of the CFU-S population is highly dependent on the level of Epo production. Some strains of mouse respond poorly to hypoxia but, although not producing the required amount of Epo, are not unresponsive to it. Exogenously supplied Epo raises the level of erythropoiesis to that seen in the responsive mice. This change is also associated with a massive increase in the splenic CFU-S population.

II. INTRODUCTION

A hypoxic environment such as is encountered on transferring from a low to a high altitude results in the red blood cell mass being incapable of supplying the body tissues with their immediate optimum oxygen requirements. If the hypoxic conditions are maintained for any length of time, most animals, including the human, respond with an increased production of Epo. This aspect of the process of acclimatization to high altitude results in a severe erythrocytosis and in increase in hematocrit, or packed red cell volume, which continues until the normal level of tissue oxygenation is restored. Figure 1 illustrates the change in hematocrit of the peripheral blood in BDF_1 (C57B1/6 × DBA/2) mice after being placed in a decompression chamber and the pressure lowered to 0.4 atm (a stimulated altitude of 6.7 km or 22,000 ft). During the initial shock of lowering the pressure, a degree of hemoconcentration and possibly an early mobilization of erythrocytes occurred which increased the hematocrit from 45 to 50% within 3 hr. The total red cell mass, however, did not start to increase for about 2 days.[1] Thereafter, the hematocrit increased uniformly to about 70% after 14 days and then stabilized. Returning the mice to normal atmospheric pressure after 9 or 14 days left them polycythemic. The maturation phase of erythropoiesis (though not the progenitor phase) shut down completely and the hematocrit returned slowly to normal as the circulating erythrocytes gradually died off.

Thus, elevated erythropoiesis is a major response to hypoxia and this holds true whether the hypoxia is physically induced by altitude changes, whether it is pathologically induced by a low oxygen tension from cardiac or pulmonary disease, or whether it is mechanically induced by severe hemorrhage. In fact, it appears that any alteration which brings about arterial hypoxia results in elevated erythropoiesis. Changes in the hemopoietic stem cell compartment are mainly secondary to, and dependent upon, the demands for erythropoiesis, which itself is regulated by the production of the hormone Epo.

The most dramatic response to a demand for increased erythropoiesis is frequently seen in the spleen whose mass may increase three- to fivefold, swollen by the volume of erythropoietic differentiation, maturation, and proliferation taking place. For example, the spleen weight in mice increased from 80 to 180 mg during 5-day exposure to a relatively moderate hypoxic stress of 0.5 atm (5.2 km or 17,000 ft).[2] Although in most animals the spleen is hemopoietically quiescent, it clearly presents a favorable environment for erythropoiesis. Hemopoietic tissue regenerating in the mouse spleen is predominatly erythropoietic (compared to bone marrow which is predominantly granulopoietic) and under a variety of conditions (hemorrhage,[3] starvation, estrus, and infection[4-8]) erythropoiesis is shunted from bone

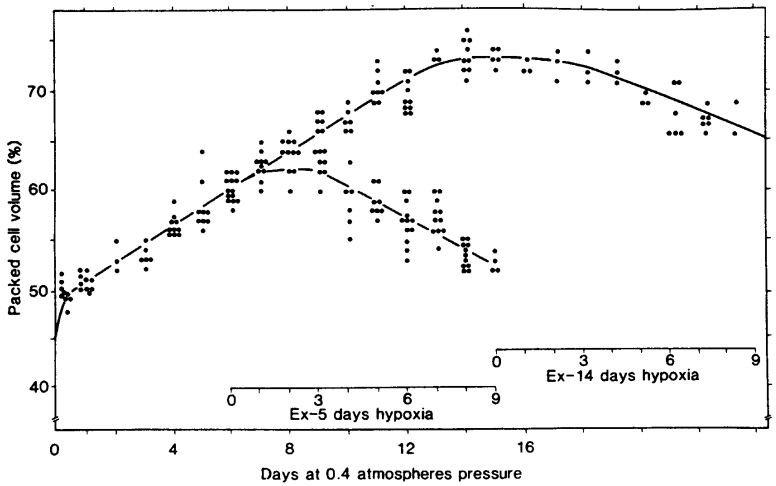

FIGURE 1. Changes in the packed red cell volume during and after exposure to hypoxic conditions in a decompression chamber at a pressure of 0.4 atm, a simulated altitude of 6.7 km or 22,000 ft.

marrow to spleen. It is interesting to note that even in the rat where splenic erythropoiesis is normally nonexistent, it has been calculated that in response to acute hemorrhage the spleen contributes at least as much to the extra erythropoiesis as, and probably up to three times more than, the whole of the marrow tissue.[3]

In studying the effect of hypoxia on the hemopoietic stem cell, taken in this context as the spleen colony-forming unit, CFU-S, it is necessary, therefore, to follow changes both in the bone marrow and in the spleen. For the purposes of this overview we will first describe the results of our observations,[9,10] and then discuss them in the light of other observations reported in the literature.

III. MATERIALS AND METHODS

BDF_1 ♂ mice aged 9 to 12 weeks were subjected to hypobaric conditions (0.4 atm, equal to 6.7 km or 22,000 ft of simulated altitude) in a decompression chamber for intervals up to 15 days. Changes in the spleen and femoral cellularity and CFU-S content were measured together with an estimate of the kinetic status (percent in DNA-synthesis) of the CFU-S. CFU-S were measured by the spleen colony technique.[11] Recipient groups of ten syngeneic mice were irradiated with 8 Gy X-rays (300 kVp) and then injected with 3.3×10^4 bone marrow or 5×10^5 spleen cells from the donor test mice. These hemopoietically reconstituted recipients were killed 9 days later. Spleens were excised and fixed in Bouin's solution and the resultant spleen colonies counted under a dissection microscope.

The proportion of CFU-S in DNA synthesis was measured by the tritiated thymidine (^{3}HTdR) suicide technique.[12] Bone marrow or spleen cell suspensions from the donor test mice were prepared in Fischer's medium and adjusted to give 5×10^6 cells per milliliter. Two 1-mℓ aliquots were then incubated at 37°C for 10 min in ventilated 5-mℓ Bijou bottles. One of the paired samples then received 200 μCi ^{3}HTdR (Sp. Act. 25 Ci/m*M* in 0.2 mℓ) while the other received 0.2 mℓ Fischer's medium and the incubation continued for a further 30 min. The suspensions were then adjusted to give the appropriate cell concentrations and

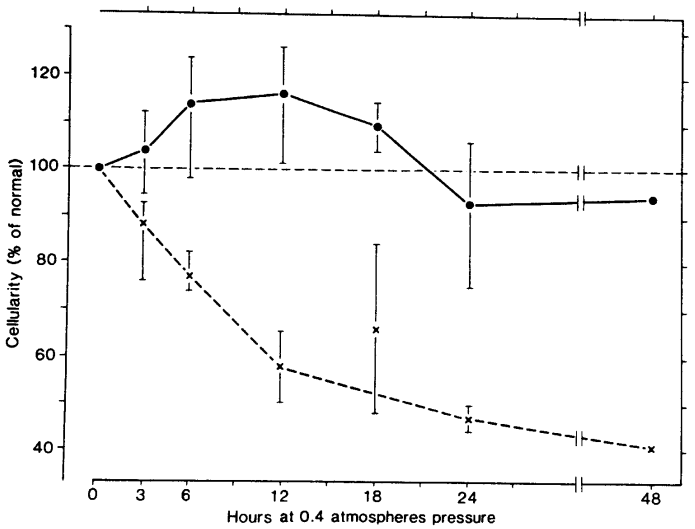

FIGURE 2. Changes in total cellularity of bone marrow (●) and spleen (X) during 48-hr exposure to hypobaric oxygen. Results show mean ± standard deviation for several series of experiments.

assayed for CFU-S as before. The proportion in DNA synthesis was calculated as the proportion killed by ^{3}HTdR = $\frac{C-T}{C}$ where C = number of spleen colonies in the control group and T = of spleen colonies in the ^{3}HTdR-treated group.

IV. RESULTS

A. Effects of Hypoxia on Spleen Colony-Forming Units

1. Acute Hypoxia

Figure 2 shows the gross cellular response to acute hypoxia. Most notable is the rapid decline in spleen cellularity. More than half the nucleated cells (~10^8 cells) disappeared within 48 hr. By contrast, the femoral cellularity showed little change, if anything slightly increasing between 6 and 12 hr of hypoxia. The cell loss in the spleen, however, is not a random one because the concentration of CFU-S increased and thus resulted in a slow decline in the total number of CFU-S (Figure 3). Femoral CFU-S increase rapidly over the first 12 hr of hypoxia but then return to normal levels by 24 hr. Since the cellularity remained constant, this was a real increase in CFU-S numbers, which could be the result of migration from the spleen to the marrow or an increase due to proliferation of CFU-S in the marrow. The former seems unlikely since splenic CFU-S numbers decreased only slowly, and in other examples requiring excess erythropoiesis there is a clear migration of CFU-S in the opposite direction. i.e., marrow to spleen.[13,14]

Increased stem cell proliferation is shown by direct measurements of the ^{3}HTdR kill (Figure 4), which indicated that at 3 hr there is a marked kill of femoral CFU-S. Increased proliferation of CFU-S also occurs in the spleen over this same early period (Figure 4), a property which may act to counter stem cell loss in this organ.

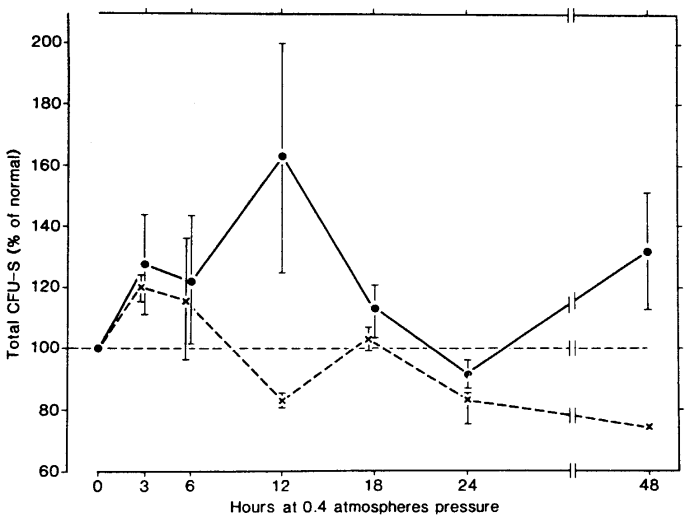

FIGURE 3. Changes in total number of CFU-S in femur (●) and spleen (X) during 48-hr exposure to hypobaric oxygen. Results show mean ± standard deviation for several series of experiments.

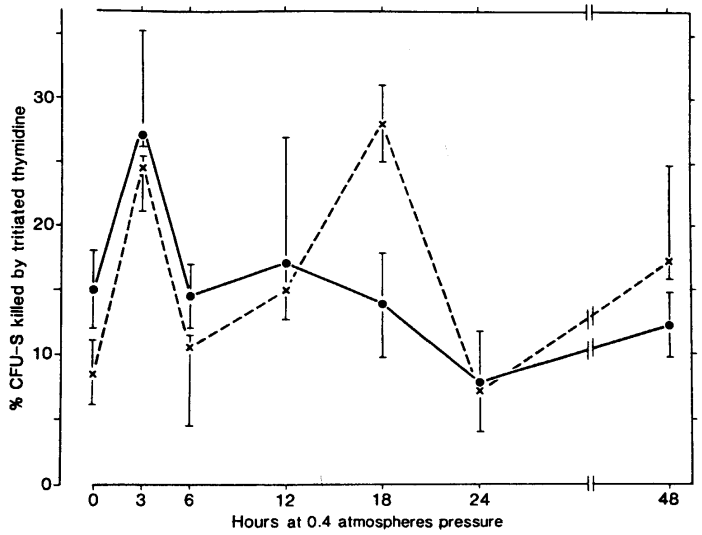

FIGURE 4. Proportion of CFU-S in DNA-synthesis during 48-hr exposure to hypobaric oxygen; bone marrow (●) and spleen (X). Results show mean ± standard deviation for several series of experiments.

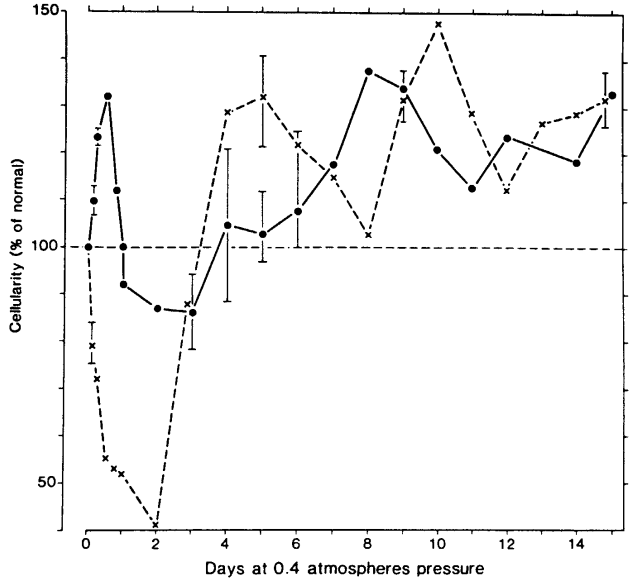

FIGURE 5. Changes in total cellularity of bone marrow (●) and spleen (X) during 15-day exposure to hypobaric oxygen. Results show mean ± standard deviation for several series of experiments.

2. Prolonged Hypoxia

Extending the period of hypoxia to 15 days resulted in a rapid recovery in the cellularity of both spleen and femur, reaching normal levels by 4 days and thereafter continuing to rise to a somewhat unstable plateau about 20 to 30% above normal (Figure 5). The lines joining the means for each day's observations tend to oscillate about the plateau. These oscillations are particularly evident when looking at the total numbers of CFU-S (Figure 6). CFU-S numbers in the femur vary around a mean value slightly above normal and in the spleen about 30% above normal. These observations are confirmed by measurements of endogenous spleen colonies in mice subjected to sublethal irradiation. Phillips and Hanks,[15] using isobaric 5% O_2, and OKunewick et al.[16] at hypobaric 0.5 atm, both reported increases in endogenous colony formation. (It should be noted here that there was no tendency for CFU-S numbers in the spleen to fall — see later.)

An analysis of the kinetic behavior of the CFU-S (Figure 7) lends support to this apparent oscillatory behavior of the CFU-S populations. In view of the large error margin inherent in the technique, one cannot place too much reliance on the absolute values nor on the phase relationships between the oscillation in kill and total numbers of CFU-S. Hence, we regard a kill of more than 20% of the total CFU-S as very significant and signaling fairly rapid proliferation. Using this as our criterion of proliferation, it is clear that the CFU-S in the femur frequently swing into the proliferative phase in a manner which may well be linked to the total number of femoral CFU-S. Fewer observations are available for splenic CFU-S. It appears, however, that the ^{3}HTdR kill hovers around the 20% gray area, indicating that while there is some proliferation of CFU-S in the spleen it is not very significant.

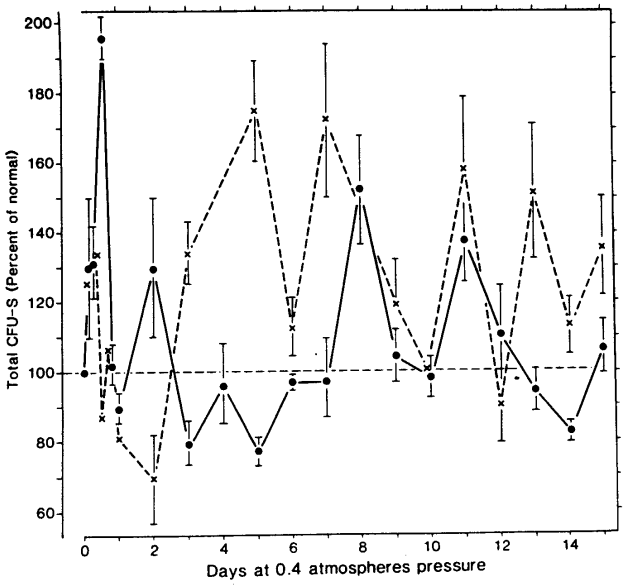

FIGURE 6. Changes in total numbers of CFU-S in femur (●) and spleen (X) during 15-day exposure to hypobaric oxygen. Results show mean ± standard deviation for several series of experiments.

A similar observation has been made for phenylhydrazine-induced hemolytic anemia. This is another condition in which a large volume of splenic erythropoiesis is required. The result is an influx of CFU-S from the bone marrow simply to supply sufficient stem cells for erythropoietic differentiation. CFU-S do not themselves proliferate in the spleen although they are proliferating rapidly in the marrow.[13,14]

Nevertheless, several of the spleen data points correlate well with the marrow data (Figure 7, and also see Figure 4 for the early points). This feature is, perhaps, indicative of a correlation in the cycling properties of CFU-S proliferation in bone marrow and spleen.

3. Proliferation Capacity of CFU-S under Conditions of Hypoxia

Beran and Tribukait[17] found that CFU-S (normal and hypoxic) demonstrated a significantly greater self-renewal capacity when grown in irradiated mice which had been exposed for 10 days to a hypobaric pressure of 434 mmHg (i.e., 0.47 atm or 5.9 km or 19,500 ft simulated altitude). They showed the number of CFU-S from hypoxic mice grown in irradiated hypoxic hosts increased from 54 to 609 per spleen colony between days 11 and 13 of growth compared to an increase from 6 to about 55 CFU-S per spleen colony for normal CFU-S in normal recipients. Since the proportional increase was similar in both cases, they concluded that the hypoxia had not directly affected the capacity of the CFU-S to proliferate, but that the hypoxic environment provided more favorable conditions for earlier CFU-S self-renewal than the normoxic environment: the hypoxic CFU-S did not have any significant proliferative advantage. What environmental changes took place were not defined, but it was clear that the earlier regeneration was not related to the cellularity of hemopoietic organs at the time of transplantation.

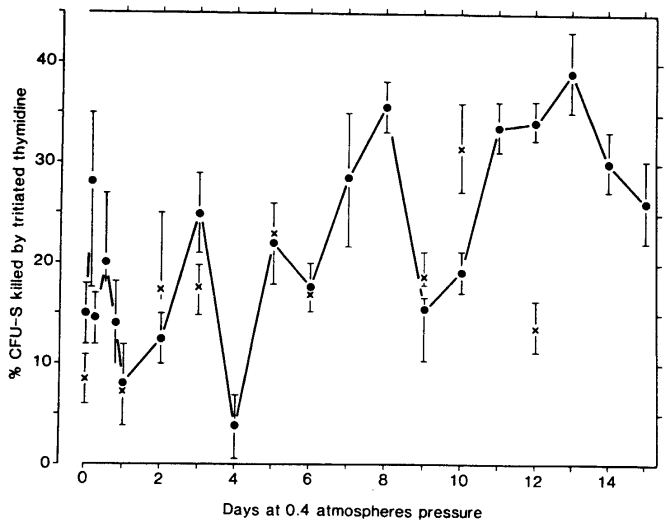

FIGURE 7. Proportion of CFU-S in DNA-synthesis during 15-day exposure to hypobaric oxygen; bone marrow (●) and spleen (X). Results show mean ± standard deviation for several series of experiments.

V. DISCUSSION

It seems most probably that the overall behavior of the stem cell population under conditions of acute hypoxia is highly dependent on fluctuations in the production of Epo. Gordon et al.[18,19] showed that the plasma levels of this hormone increase from just detectable levels (0.05 IU/mℓ) at 1 hr of hypoxia to a peak at 8 to 10 hr (0.9 IU/mℓ) and then decline to normal at 16 hr. Whether Epo has any direct effect on CFU-S proliferation is unclear. Certainly, Guzman and Lajtha[20] demonstrated that exogenously administered Epo results in an increased turnover of both femoral and splenic CFU-S and an increase in the absolute number of CFU-S in the femur, though, interestingly, not in the spleen, an observation in accord with our results. Fogh[21] too, demonstrated that an increase in erythropoietic activity is associated with an increase in the total number of stem cells. The reverse situation, however, is not necessarily true. The absence of Epo in irradiated, hypertransfused polycythemic mice does not prevent the growth of grafted CFU-S, for example,[22] nor does it prevent the proliferation of the erythropoietic progenitor cells.[23] It seems most probable, therefore, that the erythropoietic differentiation demand is met by fluctuations in the Epo levels and CFU-S variations are secondary to that demand.

A. The Unresponsive Mouse

The above results illustrate a clear-cut response of the CFU-S population to prolonged hypoxia with a continuous increase in the circulating red blood cell mass (Figure 1) to a point where the hematocrit is>70% — probably about the physical limit in terms of blood viscosity. It has been assumed that throughout this period the tissues have remained relatively hypoxic. According to the classical concept, a reduced oxygen tension in the kidney leads to an increase in the circulating Epo levels. It is this hormone which stimulates the continued

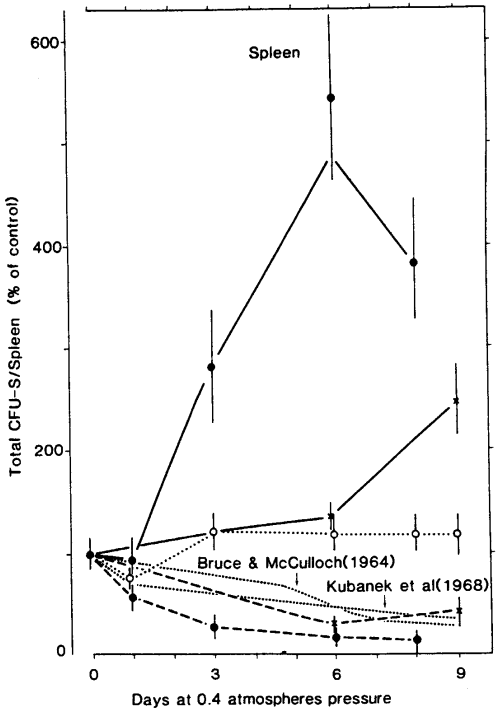

FIGURE 8. Changes in the total splenic CFU-S content during exposure to hypoxic conditions in various strains of mice and their response to exogenous erythropoietin. (○) BDF_1 (control), (—x—) CAF_1 + 5u Epo, (–x–) CAF_1 + neuraminidase inactivated Epo, (—●—) BALB/c + 5u Epo, (–●–) BALB/c + neuraminidase inactivated Epo. The results of Bruce and McCulloch[24] for C57B1 mice and Kubanek et al.[1] for CAF_1 mice are shown for comparision.

erythropoiesis and the consequent CFU-S replacement of erythropoietic progenitor cells. However, it is clear that some strains of mice do not have the same capacity to respond to hypoxia as do BDF_1 mice.

Bruce and McCulloch,[24] placing C57B1 and (C3H × C57B1)F_1 mice in air with reduced oxygen content (10.5%) at normal pressure (a simulated altitude of 5.2 km or 17,000 ft), found that the red cell mass increased only marginally (equivalent to a hematocrit increase from 46 to 57%) and remained steady between 4 and 28 days. Kubanek et al.,[1] working at 0.4 atm (7 km or 23,000 ft), made essentially the same observation for CAF_1 mice. Measuring CFU-S, both groups of investigators observed that while there was a marginal increase in the femur, there was a dramatic loss of CFU-S in the spleen — to 10% of control in 15 days for C57B1 mice and to 50% of control at 8 days for CAF_1 mice (see Figure 8). By contrast, when Kubanek et al.[1] repeated their experiments with CF_1 mice they found, as we did, that there was no decrease in splenic CFU-S. In fact, as with the BDF_1 mice (Figure 6), splenic CFU-S went through a shallow trough at about 2 days and then settled down at 20 to 30% above control for the duration of the hypoxia.

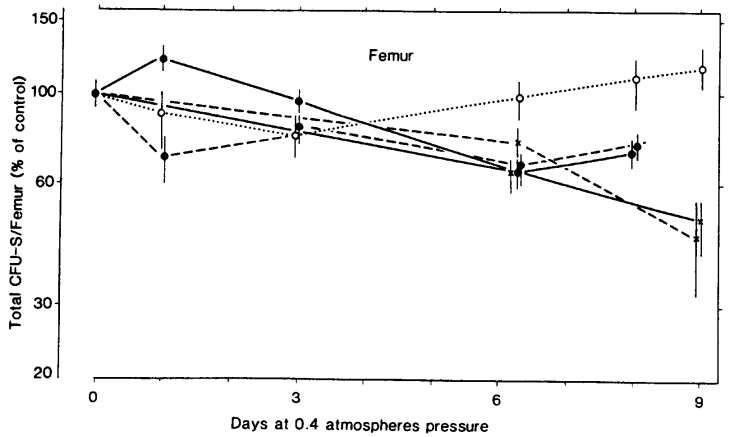

FIGURE 9. Changes in the total femur CFU-S content during exposure to hypoxic conditions in various strains of mouse and their response to exogenous Epo (○) BDF_1 (control), (—X—) CAF_1 + 5u Epo (--X--) CAF_1 + neuraminidase inactivated Epo, (—●—) BALB/c + 5u Epo, (--●--) BALB/c neuraminidase inactivated Epo.

Fried et al.,[25] working with rats at 0.5 atm (5.2 km or 17,000 ft), found the circulating Epo titers went through a peak at 8 hr after making the animals hypoxic and returned to normal by 48 hr. Radio-iron measurements, however, showed that the rate of release of new erythrocytes increased over this 48-hr period and then remained steady at this high rate for at least a week. They concluded, therefore, that the initiation of increased erythropoiesis requires a higher level of Epo than does its maintenance during continued hypoxia. It merely requires a surge of Epo such as might result from an exogenous injection of it.

Is the unresponsive mouse, therefore, insensitive to Epo under conditions of hypoxia or is it not capable of producing that initial surge of Epo? Kubanek et al.[1] reported that the unresponsive CAF_1 mouse could not respond to exogenous EPO at normal atmospheric pressure with a large surge of erythropoiesis, and this was associated with more than fivefold increase in splenic CFU-S. We tested the response of both CAF_1 and BALB/c (another unresponsive strain, Nohr[26]) mice to exogenous Epo under hypoxic conditions. As both Bruce and McCulloch[24] and Kubanek et al.[1] found, splenic CFU-S declined under hypoxic conditions (Figure 8). A dose of 4 U Epo injected intraperitoneally caused a fivefold increase in CAF_1 and threefold increase in BALB/c splenic CFU-S (Figure 8). There was relatively little effect on CFU-S numbers in the marrow (Figure 9). Clearly, therefore, hypoxia does not render the mice unresponsive to Epo. The defect must, instead, lie in an inability of these mice to produce the appropriate wave of Epo. It must be stressed that since Epo production and the erythropoietic response to anemia, post hemorrhagic or hemolytic, is similar in both hypoxia-responsive and unresponsive mice,[27] there is a fundamental difference in the mechanism by which Epo production is triggered by anemia as opposed by hypoxia.[1] Epo production does not depend simply on a lowered oxygen tension in the kidney. Other mechanisms must, therefore, be sought, and although it is outside the scope of this chapter, it is worthwhile pointing out that Miller and colleagues[28] have presented evidence that the important factor concerned in the production of Epo is the acid-base balance, a feature which can be highly dependent on the nature of the process requiring the extra erythropoiesis. Nevertheless, the most appropriate acclimatization response to hypoxia is one of increased

erythropoiesis. This requires Epo so that from the point of view of the mathematical modeler it seems important to take results from those animals which do respond to hypoxia with adequate Epo production.

B. Effects of Hypoxia on the Progenitor Cells

The literature is largely devoid of information on the effects of hypoxia on the granulopoietic progenitors, CFU-GM, as it is also for the erythropoietic progenitors. However, having established Epo production as a major requirement of the hypoxic response, it is permissible to speculate that in common with other anemic situations, proliferation of both the erythropoietic burst-forming units, BFU-E, and the erythropoietic colony-forming cell, CFU-E and, for that matter, the whole maturing erythropoietic population, is stimulated to proceed through extra amplification divisions. This, in itself, is responsible for the greater output of mature red blood cells.

ACKNOWLEDGMENT

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Chapter 15

HYPOXIA — A MODEL ANALYSIS*

H.-Erich Wichmann, Markus Loeffler, and Peter Herkenrath

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I. ABSTRACT

The influence of hypoxia on erythropoietic progenitors and precursors and on the non-erythropoietin-sensitive hemopoietic cells is analyzed by a mathematical model of stem cell regulation. The analysis suggests that the erythropoietic stimulus leads to additional mitoses of CFU-E and erythroblasts and that their elevated numbers indirectly lead to a reduced differentiation rate of CFU-S. By this mechanism the increase of erythropoiesis and the decrease of granulopoiesis during hypoxia can be explained. The model results are consistent with the experimental findings.

II. INTRODUCTION

Lowering of the atmospheric pressure either during ascent to altitude or in a hypobaric chamber leads to hypoxia. The available red cell mass is too small to meet the necessary oxygen supply of the tissues and, therefore, erythropoiesis is stimulated.[1] Erythropoietin (Epo) increases to many times normal.[2,3] In the bone marrow the Epo-sensitive progenitors (CFU-E) increase up to twice normal[4,5] and the erythroblasts to three times normal.[6,7]

To analyze the influence of intramedullary feedback on these reponses it is necessary to look also at the cells which are not directly affected by the hypoxic stimulus. As has been reviewed by Lord and Murphy,[1] CFU-S remain nearly normal and their proliferative fraction (measured by the ^{3}HTdR kill rate) is slightly elevated but close to normal. The nonerythropoietin-responsive progenitors[4,5] (BFU-E, CFU-GM) as well as the granulopoietic precursors[6,7] are reduced.

These data have been measured in the bone marrow of mice, but during hypoxia a significant fraction of hemopoietic, and especially erythropoietic, proliferation takes place in the spleen.[1] To take the splenic contribution into account, total hemopoiesis has been calculated from the cell numbers in bone marrow and spleen (see Figures 8 to 13 in Reference 8). These calculations show that the spleen contributes less than 10% to the total cell count of CFU-S, BFU-E, and CFU-GM, less than 25% of CFU-E and granulopoietic precursors, and more than 50% of erythropoietic precursors. (Only in C_3H mice the spleen contains a higher amount of BFU-E [40%] and CFU-E [70%],[8] but these data will not be considered in the following for reasons discussed below.)

As has been stated by Lord and Murphy,[1] there are some strains of mice which do not respond to hypoxia with adequate Epo production. The reason for this behavior remains obscure. These animals produce an appropriate quantity of Epo in anemia and their bone marrow proliferation increases after the administration of exogeneous Epo.

However, the essential physiological effect of hypoxia is the stimulation of erythropoiesis. Therefore, we follow the advice of Lord and Murphy[1] and consider, for the model analysis, only data from those mice which show a pronounced increase in erythropoiesis. More precisely, we only consider experiments which fulfill two criteria: (1) Epo increases to more than ten times normal and (2) the total number of CFU-E and erythroblasts increases to more than twice normal. Only in these situations can the influence of the erythropoietic cells be expected to be strong enough to influence intramedullary feedback significantly.

The following analysis will be restricted to hypoxia corresponding to 5 to 7 km altitude for the mouse strains BDF_1, CD_1, BCBA-F_1, CF_{15}, $C_3H \times AKR$, NMRI, albino. Data for the strains C57BL, CAF_1, BALB/c are not considered since these animals do not show an adequate stimulation of erythropoiesis. The data from Dunn et al.[9] (C_3H-mice) also are excluded since no a stimulation of the total erythropoiesis was measured.

III. MATHEMATICAL METHODS

Hypoxia is simulated using the mathematical model of stem cell regulation as described.[10-12] The oxygen demand, which is responsible for the erythropoietic stimulus, cannot be considered directly in the model. Therefore, it is taken into account indirectly via a theoretical curve for erythropoietin (EP). This curve has been derived from the mathematical analysis of oxygen supply in hypoxia as described by Wulff[13] and Wichmann[14] (see References 10 and 11). An altitude of 6 km is simulated, corresponding to a maximum EP of 80 times normal at day 1, which decreases exponentially to a plateau value of five times normal. Similar to the simulation of anemia,[15] the theoretical EP curve is used as "input" for the model, in order to test the response of intramedullary feedback on isolated erythropoietic stimulation. Again, the abbreviation "EP" refers to the theoretical values while "Epo" denotes the measured concentration of erythropoietin.

IV. RESULTS

A. Model Calculations

During hypoxia of approximately 6 km altitude Epo levels are known to rise enormously in the initial phase and drop to still elevated values later on (symbols in Figure 1). The experimental behavior is adapted by the theoretical curve of EP (full line in Figure 1). This curve is used as input to the model. Figures 2 to 9 demonstrate how the model reacts on the elevation of EP.

Day 0 to 5 — EP induces additional mitoses in CE and E which leads to an increase in these compartments (Figures 4 and 5). The elevated numbers have a twofold effect on intramedullary feedback: the proliferative fraction of stem cells, "a_S", becomes reduced (Figure 9) and their self-renewal probability, "p", increases (Figure 8). This leads to an elevated number of stem cells (Figure 2) and a lowered rate of differentiation. The latter is followed by a drop to subnormal in BE, CG, and G (Figures 3, 6, and 7) and a slight reduction of the elevated numbers in CE and E (Figures 4 and 5).

Day 5 to 15 — The EP level now has returned from 80 to approximately 5 times normal (Figure 1) and the strong initial stimulation becomes moderate. This leads to a further reduction in CE and E (Figures 4 and 5). Subsequently, "a_S" recovers and "p" becomes subnormal since more differentiated cells are needed. As a consequence of the latter, S normalizes (Figure 2).

Day 15 to 30 — In total, for sustained hypoxia the hemopoietic system approaches a new steady state where the stem cell number is close to normal, the erythropoietin-sensitive compartments CE and E are elevated, and the non-EP-sensitive compartments BE, CG, and G are reduced.

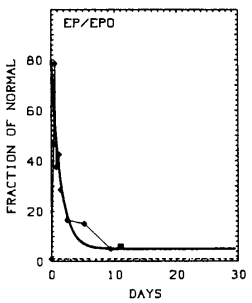
B. Comparison with the Data

Severe hypoxia leads to a dramatic increase of plasma Epo during the first days (Figure 1). The response of the CFU-S numbers to this erythropoietic stimulation is not very pronounced.[1,3,16] However, most of the data points are slightly below normal during the first 2 weeks while the theoretical S values are slightly elevated. The few data on BFU-E[3,5] (Figure 3) are subnormal and correspond to the reduced numbers for BE. CFU-E[2,3] and the erythropoietic precursors[6,17] (Figures 4 and 5) are elevated as are CE and E. Additional data on Fe-incorporation[4,16,17] demonstrate a similar increase (not shown). CFU-GM and the granulopoietic precursors[6,7] (Figures 5 and 6) are below normal but show a deeper nadir than the theoretical curves for CG and G.

The model suggests reduced values of "a_S" while, experimentally, the cycling of stem cells is slightly elevated.[1] However, considering the large experimental error of the suicide technique, these measurements cannot be distinguished from normal or subnormal values.

V. DISCUSSION

For hypoxic environment the stem cell model predicts changes in early hemopoietic cells consistent with the available data. It demonstrates that the hypoxic stimulus activates eryth-



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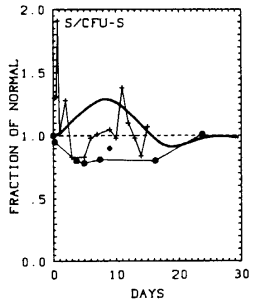

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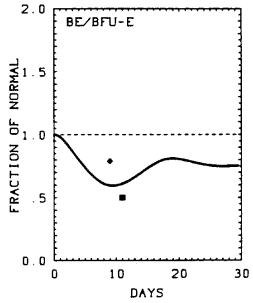

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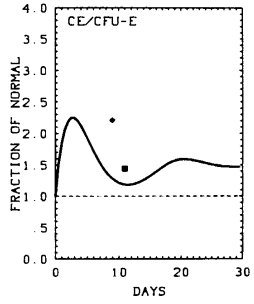

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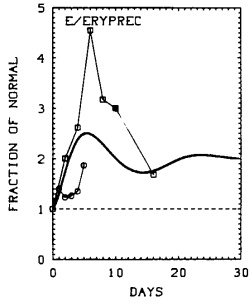

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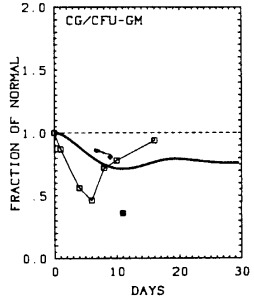

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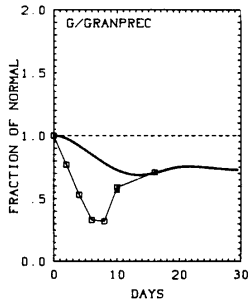

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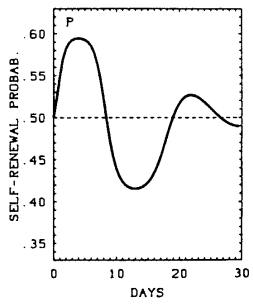

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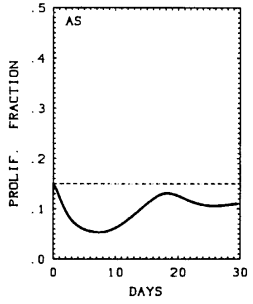

FIGURE 9

FIGURES 1 to 9. Hypoxia. Comparison between model calculations (6 km simulated altitude, —) and data (5 to 7 km simulated altitude). The data represent total hemopoiesis (bone marrow plus spleen)[8] for mice and are taken from Lord and Murphy[1] (CFU-S, BDF_1-mice: +); Abbrecht and Littell[3] (erythropoietin, albino mice: ♦); Wagemaker et al.[4] (CFU-S, BFU-E, CFU-E, CFU-GM, BCBA-F_1- mice, only bone marrow data: ♦); Peschle et al.[5] (BFU-E, CFU-E, CFU-GM, erythropoietin, CD_1-mice: ■); Rickard et al.[6] (erythropoietic and granulopoietic precursors, CFU-GM, CF_{1S}-mice: □); Beran and Tribukait[7] (erythropoietic and granulopoietic precursors, NMRI-mice: ▲); Kubanek et al.[16] (CFU-S, CF_{1S}-mice: ●); Turner et al.[17] (erythropoietic precursors, $C_3H \times AKR$-mice: ○). In the model, the hypoxic stimulus is simulated by the theoretical EP curve shown in Figure 1. It is used as input to the model. Figures 2 to 9 show how the model responds.

ropoiesis via erythropoietin and enlarges the numbers of CFU-E and erythroblasts. This perturbation of the intramedullary equilibrium leads to indirect reactions. Stem cell proliferation is reduced and, consequently, the flux of cells entering BFU-E and CFU-GM is diminished. Thus, the number of these cells as well as the number of granulopoietic precursors decreases. Only the erythropoietin-sensitive cells remain above normal. CFU-S show some fluctuations which are a consequence of the balancing influences of increased erythropoiesis and decreased granulopoiesis. This interpretation is very similar to that given for bleeding anemia.[15] However, after bleeding the Epo stimulus disappears within a few days and the cell numbers return to normal. During sustained hypoxia Epo persists at an elevated level and a new steady state is assumed in which erythropoiesis is enforced and granulopoiesis is reduced.

The reduced formation of granulopoietic cells is not only found for the progenitors and precursors (Figures 6 and 7), but also for the blood granulocytes.[18] An even more pronounced reduction is observed for thrombocytes[18-20] and their precursors.[19] In the model, the reduction of non-Epo-sensitive cells is explained as an indirect effect due to suppressive influence of the enlarged number of erythroblasts on stem cell cycling. In this approach, no "competition" mechanism between the cell lineages for differentiating stem cells (as discussed, e.g., by Rickard et al.[6]) is needed to explain the experimental findings.

The data from Lord and Murphy[21] have already been analyzed with an earlier version of the stem cell model.[22] That analysis assumed that the erythropoietic and granulopoietic progenitors (CE and CG) are responsible for the feedback and not the precursors (E and G) as assumed in the present model version. The results of both model versions are very similar, indicating that the analysis of hypoxia cannot clearly discriminate whether only progenitors, or precursors, or both participate in the regulation.

The contribution of the spleen in the response to hypoxia is similar to that in bleeding anemia: for CFU-S, BFU-E, CFU-E, and CFU-GM the spleen contains not more than 25% of the whole animal's cells (except for C_3H-mice). However, for erythropoietic precursors the spleen may contribute more than 50% of total. Therefore, for quantitative comparison the total number of erythroblasts in both organs has to be considered.

The most dramatic effect of severe hypoxia is the enormous increase of Epo within 1 day and rapid decrease from this level to a supranormal plateau. This initial peak is much higher than observed in anemia and its origin is not clear. As has been discussed by Dunn et al.,[9] Wulff,[13] and Wichmann,[14] the rapid decrease might either be explained by adaptation or by consumption of Epo by erythropoietic progenitors and precursors.

Obviously there are some strains of mice which do not respond adequately to hypoxia. As has been discussed by Lord and Murphy,[1] these animals either have insufficient production of Epo and/or the erythropoietic progenitors and precursors do not respond sufficiently to Epo. Experiments with these animals have been omitted here.

In summary, the above analysis provides a consistent interpretation of the data in animals, where hypoxia is followed by a distinct stimulation of erythropoiesis. The feedback influences of the elevated erythropoietic precursors as assumed in the model are sufficient to explain the indirect reaction of stem cells and granulopoietic cells.

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ABBREVIATIONS

Experiment		Model	
CFU-S	(colony forming unit in the spleen)	S	(common stem cell for erythropoiesis, granulopoiesis, and thrombopoiesis)
CFU-Mix	(colony forming unit with mixed colonies—in vitro)		
BFU-E	(burst forming unit-erythropoietic, obtained between day 7 and 9 in culture)	BE	(primitive, not erythropoietin dependent erythropoietic progenitor cells)
CFU-E	(colony forming unit-erythropoietic)	CE	(erythropoietin dependent erythropoietic progenitor cells)
ERC	(erythropoietin responsive cell)		
PRO	(pronormoblasts)	E1	(first subpopulation of erythropoietic precursors, proliferating)
BASO	(basophilic normoblasts)	E2	(second subpopulation, proliferating)
POLY	(polychromatic normoblasts)	E3	(third subpopulation, proliferating)
ORTHO	(orthochromatic normoblasts)	E4	(fourth subpopulation, postmitotic)
ERYPREC	(all erythropoietic precursors)	E1-4	(all erythropoietic precursor cells)
		E	(sum of cells in BE, CE, and E1-4; practically identical with E1-4)
CFU-GM	(colony forming unit-granulocyte/macrophage)	CG	(granulopoietic progenitors)
GRANPREC	(all granulopoietic precursors)	G1-4	(all granulopoietic precursor cells)
		G	(sum of cells in CG and G1-4; practically identical with G1-4)
TONUC	(total nucleated cells)	—	
Epo	(erythropoietin)	EP	(model erythropoietin)
BPA	(burst promoting activity)		(Not explicitly included)
CSA	(colony stimulating activity, synonymous: CSF)		(Not explicitly included)

MODEL SYMBOLS

Symbol	Explanation
p	Self-renewal probability of stem cells
a, a_S, a_{BE}, etc.	Proliferative fraction (= actively cycling fraction) of stem cells (S), progenitors (BE, CG), precursors (E1-4, G1-4)
Z_{CE}, $Z_{E1\text{-}4}$	Dose response relations for EP effects on erythropoietic amplification
τ, τ_S, etc.	Cell cycle times
T^g, T^g_S, etc.	Generation times
T, T_S, T_{BE}, etc.	Compartment transit times
n, n_{BE}, n_{CE}, etc.	Number of mitoses
α_E, α_G	Fraction of stem cells undergoing determination into erythropoiesis or granulopoiesis
X	Weighted sum of all cell types, regulating a_S, a_{BE} and a_{CG}
Y	Weighted sum of all cell types, regulating p
S^*, BE^*, etc.	Absolute cell number in the model compartment
S^*_{norm}, BE^*_{norm}, etc.	Normal cell numbers
S, BE, etc.	Relative cell numbers (e.g., $BE = BE^*/BE^*_{norm}$)

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Mathematical Modeling of Cell Proliferation:

Stem Cell Regulation in Hemopoiesis

Volume II

Erythropoietic Suppression, Combined Stresses, Drug Effects

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Dedicated to
Rudolf Gross,
Hematologist and Admirer
of Mathematics

It is a bold venture to attempt to marry mathematics, the purest of sciences, with biology, which has all too many "unknowns". Nevertheless, such an attempt is well justified since it brings much desired clarity into biological thinking, and it underlines the need for hardening biological data. Model building is an essential part of scientific thinking, as long as the experiments can provide the tools with which to open the iridescent shells of ideas, to look for the pearls of truth inside. This book is a worthy step in the right direction.

L. G. Lajtha

PREFACE

The experimental investigation of hemopoietic stem cells experienced its decisive stimulation in 1961 when Till and McCulloch developed their assay for colony forming units in the spleens of mice (CFU-S). Within the next decade further assays became available to measure morphologically unidentifiable erythropoietic (ERC, BFU-E, CFU-E) and granulopoietic (CFU-GM) progenitor cells. Since then, the properties of hemopoietic stem cells and their progeny have been studied extensively and their reaction measured to various types of stimulation and suppression.

However, until now, a generally accepted interpretation of all these data is missing. Given this situation it seems challenging to look for the "common structure" which should — theoretically — be inherent in the experimental findings. What are the regulatory rules and laws that govern hemopoiesis? Certainly one cannot be successful if one only looks at a very few experiments. To obtain a complete picture of the capabilities of the system, one has to consider many different experimental situations. To analyze all these data is, however, a time-consuming task which has additional problems: the available measurements are quite heterogeneous and in part contradictory and different strains of animals and different experimental protocols have to be compared.

To provide a practicable basis for the analysis, we decided to restrict our attention to the most extensively investigated hemopoietic stresses. For each type of experiment we asked a competent experimentalist to review the knowledge and the data for that special area. Complementary to these reviews we wrote theoretical chapters in which we tried to interpret these findings using a mathematical model of stem cell regulation.

Thus, this volume combines three distinct contributions: summaries of the "state of the art" of experimental knowledge on stem cell regulation; an extensive survey of the available data; and finally a unique theoretical concept (paradigma) for the interpretation of these data. The last point, of course, is, in many ways, the most critical one. The proposed model does not solve the questions on hemopoietic regulation. It is designed as a preliminary attempt to a comprehensive way of thinking. If the model serves as a rational basis for the discussion of hemopoietic stem cell regulation then we feel our effort will have been worthwhile.

One of us (H.-Erich Wichmann) started mathematical modeling of hemopoiesis in 1974. The other (Markus Loeffler) joined-in in 1977, when we began our common work on stem cells, out of which Loeffler's dissertation thesis evolved. Discussing our first considerations with Laszlo Lajtha (Manchester, England) in 1979 we received harsh criticism ("Modelling is like sitting in a chair and smoking pipes") but also stimulation to continue. At that time we had a model which was restricted to stem cells and erythropoietic progenitor cells. Martin Murphy (Dayton, Ohio) and Francis Monette (Boston, Mass.) made us aware of the fact, that the model inadequately described erythropoietic suppression. They convinced us with a lot of material (in part unpublished). Finally we found that the next "natural" step towards generalization of the model, namely the inclusion of granulopoiesis, could resolve most of the problems in a surprisingly simple way.

The idea to write and edit this book was initially suggested in 1980 at a breakfast talk with Jim Okunewick (Pittsburgh, Pa.). Its realization kept us busy for 3 years. During this time the model product derived through a common set of principles. We feel incapable to separate the origin of the ideas between us. This may become visible in the rather arbitrary arrangement of authorships in the model chapters.

The preliminary form of this work was presented in Baltimore (ISEH-Meeting, August 1982), in Manchester (Paterson Laboratories, December 1982), and in Lausanne (European Stem Cell Club, April 1983). The participants of these sessions supported us with valuable suggestions as to how we might improve the model description. Their comments entered into this volume.

Many persons have been involved in the completion of these two volumes. Most of all we have to thank the authors of the chapters dealing with experimental data. They have not only reviewed the available literature but also incorporated new measurements from their laboratories. Here especially Hans Seidel and Ludwika Kreja (Ulm), Francis Monette, Roy Ziegelstein, and Michael Hunter (Boston) must be mentioned. They performed multiple experiments on the combination of irradiation with anemia and hypertransfusion especially for publication in this book. Martin Murphy (Dayton) and Brian Lord (Manchester) also contributed a lot of unpublished material on hypoxia and ex-hypoxia.

We are especially indebted to Chris Dunn (Houston) who read all our chapters patiently. He made numerous helpful suggestions, made us aware of many mistakes and weak points, and he tried his best to improve our often ineloquent English. We could not have found a better adviser. Nevertheless, we are of course exclusively responsible for all mistakes, inconsistencies, and items which might be somewhat less than clear.

We were lucky to have had the help of eight enthusiastic collaborators. First of all, Peter Herkenrath has to be mentioned who worked with us for 2 years and contributed a lot of essential improvements. Christopher Wesselborg and Alexander Jarczyk spent many efforts to enhance our computer software for the model calculations and their graphical presentation; the curves and figures in the model chapters are their work. Erich Backes and Jochen Schmidt checked and completed the data collection and the references. They also helped in typing the manuscripts, together with Stephan Gontard. Wilfried Weiss and Guenther Michel implemented in the computer our data base of more than 800 published and unpublished experimental time curves. Finally we are thankful to the directors of our clinic for internal medicine, Rudolf Gross and Volker Diehl. They gave us an "ecological niche" to develop theory in an environment which is completely clinical and experimental. Whether this niche was successfully seeded by a "scientific stem cell" remains to be seen.

H.-Erich Wichmann
Markus Loeffler
Cologne, March 1984

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Experiments and Model Analysis — Continued

Chapter 1

HYPERTRANSFUSION — EXPERIMENTAL RESULTS: EFFECT ON ERYTHROPOIETIC PROGENITORS

Francis C. Monette

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I. ABSTRACT

The effect of erythrocyte hypertransfusion, sufficient to elevate the blood hematocrit by at least 40 to 50% and to ablate all peripheral indices of erythropoiesis, on marrow and splenic levels of hemopoietic stem cells is reviewed. Although some inconsistencies between laboratories exist, hypertransfusion usually results in the expansion of the compartment size of primitive, erythropoietin-independent cell progenitors (i.e., CFU-S, primitive BFU-E, CFU-GM). In contrast, erythropoietin-dependent cell progenitors (mature BFU-E, CFU-E, erythropoietic clusters) are substantially reduced in number in plethoric animals. The cycling of primitive, but not mature, progenitors may be increased somewhat by hypertransfusion. The enhancement of primitive hemopoietic progenitor cell numbers in plethoric animals may be best explained by the accumulation of cells which precede erythropoietin dependence, secondary to the reduction in the demand for erythropoietic differentiation. Other explanations may be equally applicable, however.

II. INTRODUCTION

For well over 20 years the transfusion of blood erythrocytes has been one of the preferred methods for suppressing erythropoiesis in animals for the bioassay of erythropoietin (Epo). Other murine bioassay methods have also been employed for the detection of Epo which all rely on the suppression of endogenous erythropoiesis by plethora. These have included prior exposure to either hypoxia[1] or carbon monoxide.[2] These two methods produce a state of plethora by first stimulating endogenous erythropoiesis prior to the return to ambient conditions. Thus, these two procedures are thought to be a less physiologic means of inducing a plethoric state than is the hypertransfusion of erythrocytes.[3] In hypertransfusion, one does not first create a period of intense erythropoietic activity prior to the suppression of erythropoiesis. However, the common denominator of all three bioassays is the suppression of erythropoiesis by plethora and, as such, all three methods provide suitable test animals for the detection of Epo.[3]

III. PHYSIOLOGICAL EFFECTS OF HYPERTRANSFUSION

A primary reason for choosing hypertransfusion (HT) over other methods is its (presumed) physiological mode of inducing the plethoric state. Oxygen delivery to the tissues may be augmented and Epo levels decline with an increased number of circulating red cells, thus, diminishing red cell production. Three critically important erythropoietic parameters provide direct evidence that erythropoiesis is rapidly and substantially depressed following HT. These include: (1) circulating reticulocyte levels, (2) marrow erythropoietic precursor numbers, and (3) radioiron incorporation into blood erythrocytes. Jacobson et al.[4] demonstrated that three successive 0.5 mℓ intraperitoneal injections of washed homologous red cells were effective in virtually abolishing blood reticulocytes within 5 to 6 days of the onset of the plethoric state as well as being effective in substantially reducing ^{59}Fe-RBC-incorporation in the peripheral blood. These authors also demonstrated the severe reduction in morphologically recognizable erythropoietic precursors in both medullary and splenic tissues following HT. The hypertransfused mouse was soon shown to be the most sensitive assay animal available,[5] insofar as the degree of erythropoietic reduction is greater in the hypertransfused mouse than by other means of inducing plethora. The administration of exogenous Epo to the plethoric animal, therefore, elicits a rapid and substantial wave of erythropoiesis which is readily discernable from that of uninjected controls regardless of the parameter studied (Figure 1).

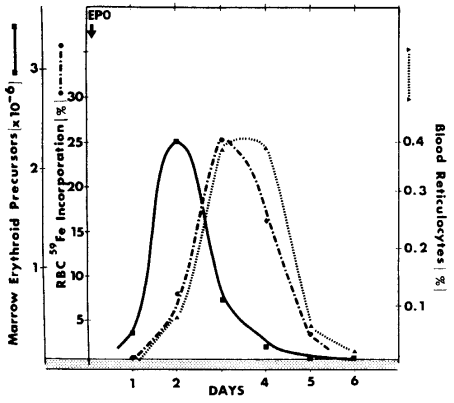

FIGURE 1. The erythropoietic response in hypertransfused CD_1 mice to a challenging dose of three units erythropoietin (Epo) administered intravenously. (■), Tibial marrow erythropoietic precursors which stain positively with benzidine; (●), 48-hr ^{59}Fe-incorporation (%) into peripheral blood erythrocytes; (▲), relative blood reticulocyte levels. Stippled area represents baseline levels for all three parameters observed for saline-treated plethoric or normal controls. Epo was administered on the 6th day following the first erythrocyte injection.

The mechanism by which plethora suppresses erythropoiesis has been attributed to an increase in the ratio of oxygen supply vs. requirement, but a number of independent observations suggest that this explanation is perhaps too limiting. Kilbridge et al.[6] injected mice with erythrocytes which were defective in their oxygen-carrying capacity, yet they still observed a reduction in erythropoiesis in these animals. Another observation which tends to reduce the importance of oxygen delivery as a primary mechanism for the plethoric suppression of red cell production was made by Miller.[7] She observed that alterations in the affinity of hemoglobin for oxygen followed changes in the plasma acid-base balance (i.e., pH and pCO_2) and consistently preceded changes in the elaboration of Epo. Although it has long been assumed that plethora results in the elimination of plasma Epo levels, Moccia et al.[8] recently showed with a radioimmunoassay for Epo that plasma Epo levels are not abolished by plethora even when the hematocrit is increased 1.75-fold. Although they observed a nearly 50% drop in circulating Epo levels within 1 day of an erythrocyte injection, the plasma Epo level was not reduced below 38% of the control value of 11.2 mU/mℓ even with an additional erythrocyte injection and over an 8-day period. Whether the Epo detected by this assay is functional remains to be determined, but it is clear that plethora does not abolish circulating Epo although it greatly reduces erythropoietic cell parameters.

Although an elevated blood hematocrit may prove deleterious with regard to the efficiency of blood flow,[9] prolonged polycythemia (for periods of up to 3 months in the mouse) has no apparent detrimental effect on the capacity of the hemopoietic tissues to reestablish erythropoiesis.[10-12] Thus, a challenging dose of Epo elicits a similar peripheral response both qualitatively and quantitatively, whether administered 7 or 47 or more days following the induction of plethora.[11,12] In addition, the beneficial effects of plethora on postirradiation granulopoietic[13-15] and megakaryocytopoietic platelet[16] recovery strongly implies a close ancestral lineage between erythropoietic and granulopoietic-megakaryocytopoietic precur-

sors.[17,18] These observations have been taken as supporting evidence for "competition" at the stem cell level[19] and raise the question of the nature of the effect of plethora on hemopoietic stem cells and their progeny. It will be the intent of this chapter to review the observed effects of plethora as induced by hypertransfusion on pluripotent hemopoietic stem cells and their progeny.

IV. MATERIALS AND METHODS

The hemopoietic effects of consecutive erythrocyte hypertransfusions will be limited to observations in mice, since this method is less effective in other rodents in suppressing red blood cell production.[20] Results with both inbred (BDF_1, C_3H, $C_{57}Bl$, Balb/c, CBA/H, La/F_1, CDF_1) and random-bred (CF_1, CD_1) strains of either sex will be described since no strain differences are readily apparent in the overall erythropoietic effects of HT (see Reference 21 for a possible exception).

Animals are usually rendered plethoric by the intraperitoneal injection of 0.5 to 1.0 mℓ of saline-washed, homologous erythrocytes with an average hematocrit of about 70%. This procedure is usually repeated either once (1.0 mℓ), or two or more times (0.5 mℓ) on consecutive days, and weekly thereafter if the plethoric state is to be maintained for >10 days. Some authors choose to administer the blood intravenously, but this method does not appear to offer any advantages over the intraperitoneal route. Either method appears equally effective in increasing the peripheral hematocrit by ~50% within 2 days of the first erythrocyte injection (Figure 2). It is a common practice to remove the "buffy coat" of leukocytes prior to transfusion; alternatively, some authors irradiate (>1000 rad) the whole blood prior to injection. Either procedure ensures a substantial reduction in the number of transplantable stem cells transferred to the recipient animal.

The methods for assaying pluripotent stem cells and erythropoietic progenitors are considered in detail in the Appendix and are only briefly outlined here. Marrow cells are obtained by flushing the femurs and tibias of three or more donor mice with culture medium. Single cell suspensions are then prepared by gentle pipetting and the cells are then centrifuged and resuspended in fresh medium before enumeration by hemacytometer or electronic counter. The exogenous CFU-S assay of Till and McCulloch[22] is employed to enumerate pluripotent hemopoietic stem cells. Recipient mice receive a lethal dose of radiation (usually 800 to 950 rad, depending on the strain employed). Within about 2 to 4 hr of animal irradiation, groups of mice receive cells via the tail vein and are sacrificed 9 to 10 days later for spleen colonies. Quantitation of stem cells is accomplished by first determining the average total femoral or tibial nucleated cellularity for each group of experimental animals. The frequency of marrow stem cells, determined independently, is then applied to this value and the total number per femur or tibia calculated. All data are expressed relative to the corresponding control value given for animals evaluated either (preferably) at each time point studied or sacrificed randomly throughout a given experiment. Erythropoietic progenitors are assayed by the in vitro clonal cell assay methods previously described[23,24] in semisolid medium. Essentially, this involves plating marrow cells at concentrations of 1 to 10 × 10^5/mℓ in medium containing 20 to 30% fetal calf serum, L-asparagine, bovine serum albumin (1%), 2-mercaptoethanol (10^{-4} *M*), and partially purified erythropoietin at concentrations ranging from 0.1 to 3.0 units per milliliter. Either clotted bovine plasma[23] or methyl cellulose[24] is used as the semisolid medium. Cultures are incubated in a humidified CO_2 incubator at 37°C, 5% CO_2 for variable intervals of time depending on the cell type assayed as follows: BFU-E (primitive), 7 to 14 days; BFU-E (mature), 3 to 4 days; CFU-E, 2 days; and erythropoietic clusters, 1 day. The primitive BFU-E correspond to what is denoted by BFU-E by many authors. An up-to-date review of the biologic characteristics of each of these erythropoietic progenitors will be found in many of the chapters in this volume as well as elsewhere.[25-27]

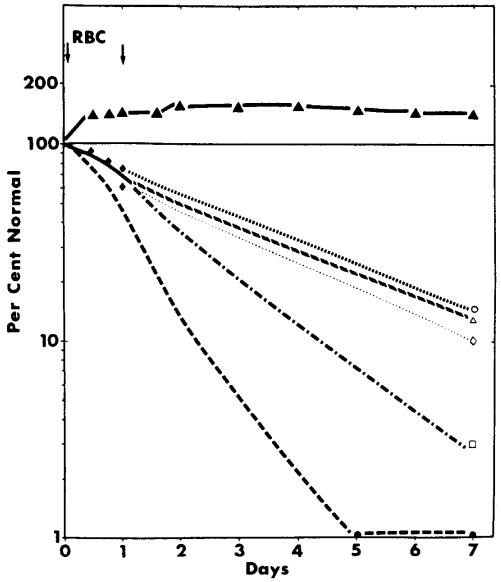

FIGURE 2. Traditional erythropoietic parameters following the injection of erythrocytes (RBC) administered 24 hr apart. (▲), The blood hematocrit;[46] (○), 4-hr splenic ^{59}Fe-uptake;[29] (△), splenic erythropoietic precursors staining with benzidine;[29] (◇), 4-hr marrow ^{59}Fe-uptake;[29] (□), marrow erythropoietic precursors staining with benzidine;[29] (●), blood reticulocytes;[4,28,29] (◆), marrow proerythroblasts.[29,30]

Peripheral erythropoietic parameters (hematocrit, reticulocytes, ^{59}Fe-incorporation into RBC) are performed by standard methods (e.g., see Reference 28) as were marrow differentials.[28,29] Marrow granulocyte-macrophage colony-forming cells (CFU-GM) are assayed essentially by methods originally detailed by Bradley and Metcalf,[30] employing either agar or methyl cellulose as the growth medium. Sources of colony stimulating activity (CSA) include: L-cell conditioned medium,[15,31,32] postendotoxin mouse serum,[14,33] or either mouse kidney cell-conditioned medium[14,34] or feeder layers.[35]

Classically, in vivo responsiveness to Epo is measured by determining the 1- to 3-day incorporation of radioiron into blood erythrocytes 2 days following the intraveneous injection of a challenging Epo dose of 1 to 3 (or more) units per assay animal. The overall peripheral radioiron response is taken as a measure of Epo-responsive cells (ERC).[36] Erythropoietic repopulating ability (ERA) is a measure of the integrity of the cellular compartments responsible for repopulating the marrow erythropoietic compartments following lethal irradiation.[37] One variation of the assay involves the transplantation of ~15 × 10^6 normal marrow cells to lethally (or sublethally) irradiated mice. About 1 week later radioiron is administered and the uptake into circulating RBC is determined.[38]

V. RESULTS

A. General Erythropoietic Parameters

Within 2 days of erythrocyte hypertransfusion, the hematocrit is elevated by about 50%

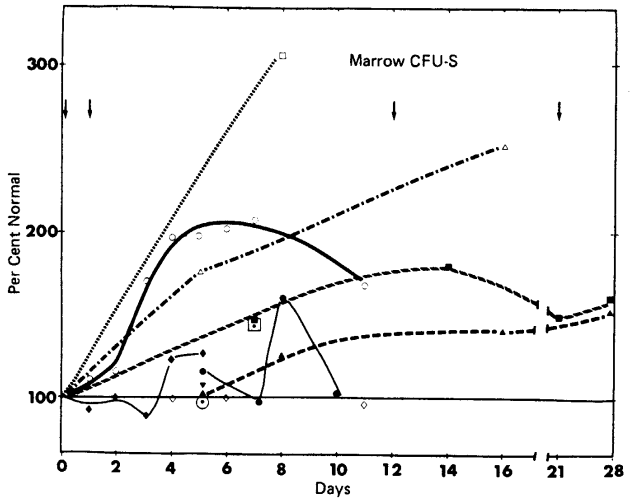

FIGURE 3. Marrow CFU-S levels at various intervals following hypertransfusion. Mice were injected with erythrocytes initially, and at approximately weekly intervals thereafter (arrows). CFU-S are uncorrected for splenic seeding. (□),[21] (△),[44] (○),[43,46] (■),[11] (◆),[33] (●),[38] (▼),[45] (▲),[42] (◇),[40] (⊙),[41] (□),[29]

and is maintained at or near this level for nearly a week without an additional injection of blood cells (Figure 2). For example, two consecutive injections of 1.0 mℓ of packed (70%) erythrocytes raise the hematocrit from the normal level of ~45% to an average of ~67%, although individual hematocrits will range from ~63% to >70%. Animals with hematocrits of >60% are usually considered polycythemic since little marrow erythropoiesis is evident 5 to 7 days following cell injection. Although the "standard" erythropoietic parameters show an ~85% reduction within 1 week of such treatment (Figure 2), a more rapid decline is likely, however, few reports exist in the literature. For example, proerythroblasts have been reported to fall by 25 to 40% within 24 hr of a single erythrocyte transfusion.[29,39] Both marrow and splenic erythropoiesis appear to decline following HT although the splenic reduction tends to be somewhat less (Figure 2). Blood reticulocytes virtually disappear from the circulation within 5 days of HT. As previously discussed, a marked increase in all erythropoietic parameters follows the injection of Epo with peripheral indices following the peak in marrow erythropoiesis by 1 to 1.5 days (Figure 1).

B. The Stem Cell Response to Hypertransfusion

1. Pluripotent Stem Cells (CFU-S; CFU-Mix)

The effect of transfused erythrocytes on pluripotent stem cells (CFU-S) has been studied by numerous groups over a time interval of nearly 15 years with, unfortunately, conflicting results (Figure 3). Although significant augmentation in marrow CFU-S, up to three times control levels, has been reported, some laboratories report no net change.[40,41] However, most groups report at least a 25% increase within 7 days of HT.[11,21,29,33,38,39,42,43]

In addition to the work with CFU-S, Hara[44] reports that marrow pluripotent hemopoietic colony-forming cells which grow in vitro (i.e., CFU-Mix) and may be analogous to CFU-S in their state of differentiation, are also increased, from 1.8 to 2.5-fold, by HT. Since it

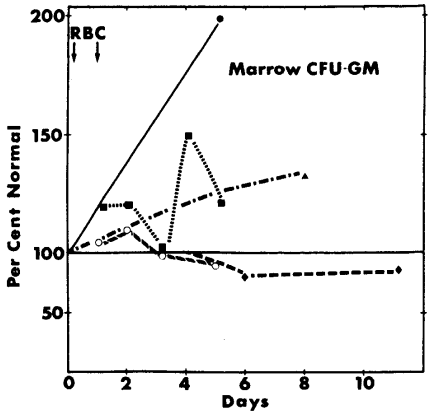

FIGURE 4. Marrow granulocyte/macrophage progenitors (CFU-GM) following hypertransfusion. (●),[35] (■),[33] (▲),[34] (○),[32] (◆),[40]

seems very likely that the observed reduced splenic seeding efficiency of plethoric marrow-derived colonies is a generally applicable phenomenon,[21,43] it would appear that the actual number of stem cells in plethoric bone marrow is underestimated by a factor of *at least* two or more.[21] Those workers who have examined splenic CFU-S levels in plethoric animals have generally found a significant increase over normal controls (range: 1.7 to 3.6-fold, References 11, 21, 29, 42, 45). Therefore, the conclusion that red cell transfusion significantly and substantially augments the stem cell level in hemopoietic tissue is inescapable. In addition, although we observed the cycling of marrow stem cells to be slightly augmented by HT,[43] others have not observed a significant increase in CFU-S proliferation following this procedure.[38] Nevertheless, at least in our hands, the proportion of CFU-S in S-phase of cycle appears to stabilize following the second transfusion of erythrocytes at ~28%, and remains fairly constant throughout the subsequent 10-day period of polycythemia.[46]

2. Granulopoietic Progenitors (CFU-GM)

Primitive marrow granulocyte-macrophage progenitors (CFU-GM) are known to be closely related in differentiation to CFU-S.[40,47] Following HT these progenitors are also observed by some groups to increase by at least 25% in marrow,[33-35] although others observe no significant change[32] or even a slight decline[40] (Figure 4).

3. In Vivo Erythropoietic Progenitors (ERC: ERA)

The cellular compartments which precede the morphologically recognizable erythropoietic precursors have been assayed in vivo by the response to Epo (i.e., ERC) and by the erythropoietic repopulating ability of marrow grafts (i.e., ERA). Following HT, both parameters are reduced by approximately half (Figure 5). For example, Milenkovic and Pavlovic-Kentera[38] observed a reduction in the ERA of marrow grafts following HT by ~55%. The reduction of the Epo-induced radioiron uptake by blood erythrocytes following HT reflects the ablation of Epo-dependent cells within the hemopoietic tissues. HT may reduce the Epo response by as much as 45 to 50% within 5 to 7 days of erythrocyte injection.[46] Schooley and Lin[11] showed that the response to Epo was maintained at or near this level as

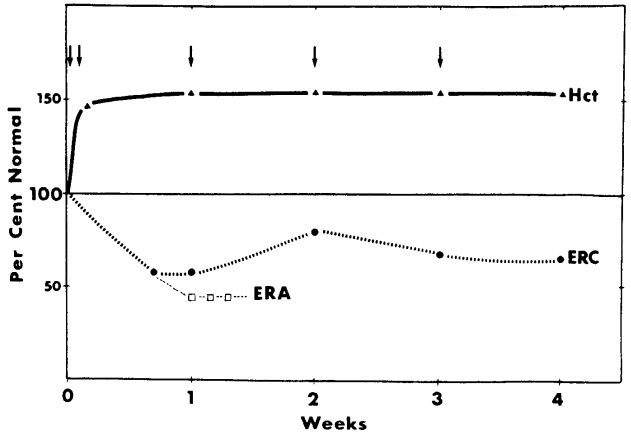

FIGURE 5. The response of erythropoietin-responsive cells (ERC) and erythropoietic repopulating ability (ERA) of marrow preparations following erythrocyte hypertransfusion. Erythrocyte injection was performed initially and at weekly intervals thereafter to maintain the blood hematocrit (Hct) at a level of about 150% of normal. Data on ERC obtained from References 11 and 46; ERA from Reference 38.

long as the plethoric state was maintained (Figure 5). Periods of continuous polycythemia as long as 47 days do not appear to adversely affect the Epo response.[10,12] Von Wangenheim et al.[12] also showed that ERC maintained their high cycling rate throughout the period of greatly reduced demand for erythrocytes, thus providing additional support for the notion of a considerable death function for these cells.[12,48] The reduction of both ERA and ERC following HT would also suggest the possibility that at least some of the erythropoietic progenitors forming colonies in vitro are reduced in number by HT.

4. In Vitro Erythropoietic Progenitors (BFU-E; CFU-E)

Erythropoietic progenitors which are assayed in vitro comprise a continuum of cell types with diminishing proliferative capacity, but increasing sensitivity to the hormone Epo as cell differentiation progresses from the most primitive to a cellular stage immediately preceding the proerythroblast (for recent reviews see References 25 to 27). As it is presently understood, the developmental sequence is as follows: primitive BFU-E → mature BFU-E → CFU-E → cluster forming cell/proerythroblast. Primitive BFU-E require 7 or more days in culture to manifest themselves and are thought to be closely related developmentally to CFU-S and CFU-GM.[47] As such, one might predict a similar response to HT for primitive BFU-E as has been observed for CFU-S and CFU-GM. This, in fact, seems to be the case insofar as four separate groups report an increase of 25% or more in the number of primitive marrow BFU-E following HT[34,46,49,50] (Figure 6). Other laboratories, however, report no change[32,51] or only a slight increase[41] following HT. Interestingly, both we[46] and Peschle et al.[52] have observed a marked decline in primitive BFU-E in the marrow of mice within 16 to 24 hr of a single erythrocyte injection (Figure 6). This observation is highly reproducible and the net reduction averages 35 to 50% of the normal marrow control value. Peschle et al.[46] attribute this decline to a marked diminution in the BFU-E cycling rate, but we as well as others,[34,41,49,53] have failed to see any substantial effect of HT on BFU-E cycling even at these early time intervals (Table 1). In contrast to the overall augmentation of marrow BFU-

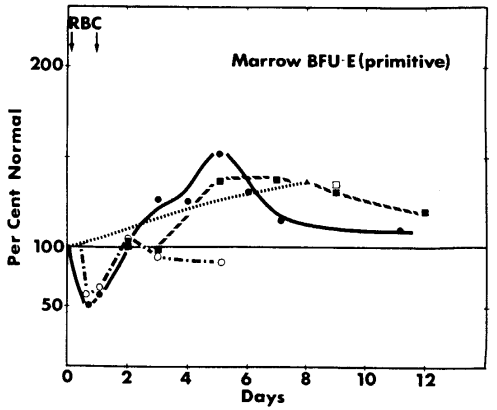

FIGURE 6. The response of primitive marrow BFU-E to erythrocyte hypertransfusion. (○), Data from Reference 32, (●),[46] (▲),[34] (■),[49] (□).[50]

Table 1
CYCLING OF PRIMITIVE MARROW BFU-E FOLLOWING HYPERTRANSFUSION[a]

Time following hypertransfusion	Reduction with hydroxyurea (%)[b]
Normal marrow control[c]	20.8 ± 11.5
18 hr	32.8 ± 14.2
1 day	34.5 ± 11.2
2 days	41.7 ± 14.6
3 days	54.8 ± 21.9
5 days	38.2 ± 7.0
6 days	37.6 ± 9.7
7 days	24.6 ± 6.0

[a] Performed in CD_1 female mice. Two to four separate determinations for each time point.[46]
[b] Hydroxyurea administered at a dose of 0.9 mg/g body weight 1 hr prior to cell collection.
[c] Normal bone marrow control level was 2119 ± 505 BFU-E per tibia.

E by HT, both splenic and blood BFU-E decline by as much as 65% following erythrocyte injections.[49] The behavior of primitive BFU-E following HT is, therefore, not a simple reflection of what occurs in the pluripotent stem cell compartment (i.e., CFU-S).

Primitive BFU-E eventually differentiate into what are termed mature-type (or day 3 to 4) BFU-E. These colony-forming cells are principally characterized by a substantial reduction in their proliferative capacity when compared to primitive BFU-E. Some authors have indicated that these cells are equivalent, in whole or in part, to ERC.[34,41,49,50] Within 5 days following erythrocyte transfusion both we[39,46] and Adamson et al.[41] have observed a marked decline in mature BFU-E to about half the normal marrow level (Figure 7). Slightly lower levels are observed at later time intervals following HT. Interestingly, we also observe what

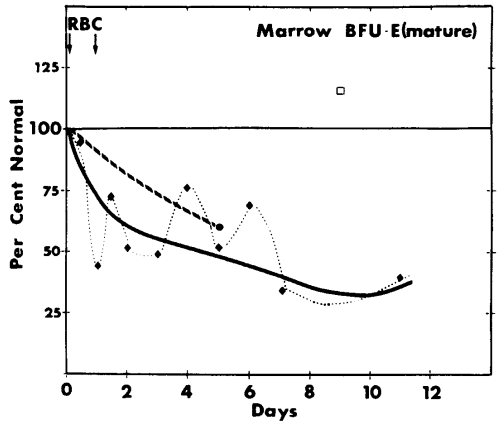

FIGURE 7. The response of mature-type marrow BFU-E to erythrocyte hypertransfusion. (●), Data from Reference 41, (♦),[39,46] (□).[50] The colony data from References 39 and 46 are drawn literally (dashed line) and by eye (solid line).

may be an oscillatory response to HT as mature BFU-E decline (dashed line in Figure 7). However, the significance of this variable response is uncertain even though it appears to be somewhat reproducible in our hands. On the other hand, Gregory and Eaves[50] reported a slight increase in marrow mature BFU-E following three consecutive blood transfusions. The reason for this discrepancy between their work and that of Adamson et al.[41] and ours is uncertain. Little information exists as to the effects of HT on splenic mature-type BFU-E.[54]

C. Mature Erythropoietic Progenitors

It has been well documented that CFU-E, which form small, eight or more celled colonies in a 2-day incubation period, are severely reduced by HT. Gregory et al.[40] showed in 1973 that marrow CFU-E are reduced by ~95% by this procedure. However, more recent work from this laboratory suggests a reduction of ~73% relative to normal marrow controls.[50] The difference in marrow levels following HT, presumably, is due to the better in vitro growth conditions in the latter report. No fewer than eight separate laboratories observe a similar reduction in marrow CFU-E following HT ranging from ~70 to 87% by the 5th to 8th day posttransfusion[32-34,39,41,46,49,50,53] (Figure 8). Although few groups have examined splenic CFU-E, the effect of HT on the CFU-E in this organ appears to be similar to that of marrow.[49] Normal marrow CFU-E are characterized by an S-phase distribution of ~75%. Following HT, CFU-E cycling is still high,[41,49,55] although some groups report a slight but significant decline in the cycling level after HT.[34,52]

CFU-E may not be the last erythropoietic progenitor capable of colony formation in vitro. Small, two- to seven-celled erythropoietic clusters form during a 1-day growth interval which are distinguished from other erythropoietic progenitors by their very high Epo sensitivity in vitro.[56] This is also observed in vivo when one follows their marrow levels following HT (Figure 8). The rate of decline of erythropoietic clusters is most rapid when compared to other colony types and approaches a nadir of 10% relative to normal controls by the 6th posttransfusion day. In contrast to other erythropoietic progenitors, the cycling rate of erythropoietic clusters is significantly reduced from control levels by HT (64.3 ± 1.3 vs. 77.9 ± 2.2% in S-phase, respectively[57]).

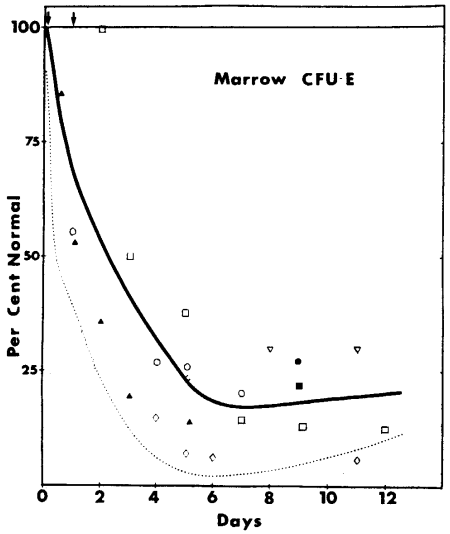

FIGURE 8. The response of marrow CFU-E to erythrocyte hypertransfusion (arrows). (□), Data from Reference 49, (▲),[32] (○),[39,46] (◇),[41] (■),[33] (●),[50] (▽),[34] (△).[41] Collective data are approximated by the solid line. The dashed line represents the response of erythropoietic cluster-forming cells.[39,46]

VI. DISCUSSION

The overall effect of erythrocyte hypertransfusion on marrow hemopoiesis is summarized in Figure 9. The cellular response to HT is clearly bimodal. The number of primitive progenitor cells (including CFU-S, CFU-GM, and 7-day BFU-E) is augmented, whereas more mature progenitor cells (4-day BFU-E, CFU-E, and erythropoietic clusters, along with marrow erythropoiesis, in general) are reduced by erythrocyte injection. This cellular response to HT would appear to transcend a number of important variables including the murine strain employed, culture techniques, and the work of individual laboratories and may, therefore, be considered a basic, physiological consequence of erythropoietic suppression.[42] The dichotomy in the hemopoietic cellular response to HT would appear to rely directly on the Epo dependence of the cells, and the decline in both in vivo Epo responsiveness and mature erythropoietic progenitor cells can readily be explained on this basis. Although the above findings are also consistent with the Epo independence of primitive hemopoietic progenitors, they cannot be readily explained solely along these lines. If, indeed, the behavior of primitive progenitor cells in vivo is independent of Epo, as is generally assumed,[25,26,34] then another explanation must be sought to account for their augmentation in vivo following HT. Perhaps the simplest model would be one which suggests that these cells increase (i.e., accumulate) due to the reduction in demand for erythropoietic cell differentiation.[29] This explanation may also be consistent with the stem cell "competition" model of Hellman and others.[13,14,19,58] One might, therefore, envision a differentiation step sensitive to Epo levels in vivo between primitive and mature BFU-E (Figure 10). Since it appears well established that early progenitors continue to cycle in plethoric animals, such a blockage could result

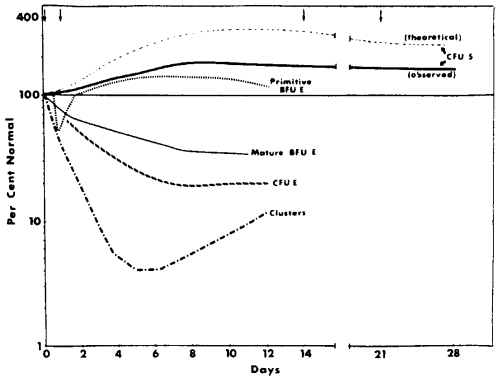

FIGURE 9. The overall response of marrow erythropoietic cell progenitors to erythrocyte hypertransfusion (arrows) averaged from the data given in Figures 3 and 4, and 6 to 8. The data for theoretical CFU-S were obtained by correcting the observed CFU-S for an average splenic seeding ("f") of 6.8% (see References 21 and 43). The response of CFU-GM is not shown but is similar to that of primitive BFU-E.

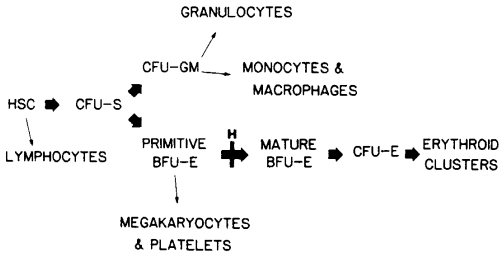

FIGURE 10. A schematic summary of murine erythropoietic differentiation. H, the locus of action of erythrocyte hypertransfusion mediated by the appearance of erythropoietin dependence.

in an accumulation of cell types which precede it in differentiation. This could explain the increase in both primitive marrow BFU-E as well as CFU-S (i.e., continued proliferation in the absence of differentiation). In addition, one might expect such an augmentation in stem cell numbers to benefit other cell differentiation pathways. The increase subsequent to HT of both granulopoietic progenitors and their progeny[15] as well as platelets[16] may simply reflect the reduced demand for erythropoietic cell differentiation. However, other explanations for the increase in stem cells following HT must also be considered.[65] A correction for splenic seeding of CFU-S derived from hypertransfused animals leads to the conclusion that the augmentation of these cells in marrow and spleen is underestimated by a factor of at least 2 to 5.[21,43] It is, therefore, unlikely that the magnitude of this cell increase following HT can be explained solely by the "accumulation" model described above. This is particularly evident when other stem cell properties are studied subsequent to HT. For example, we have reported substantial changes not only in CFU-S seeding, but also in their radiosensitivity and modal cell volume following HT as well as a change in their cell-cycle distribution.[43] Additional explanations for the HT-induced augmentation of primitive cell progenitors include the effect on other hemopoietic regulatory factors[59] as well as on the microenvironment.[21] In addition, a more direct effect of the transfused erythrocytes (or their breakdown products[66]) on progenitor cells[60-62] cannot be excluded. Thus, a more detailed mechanistic explanation must await the results of further experimental work. Of particular importance in these studies is the ruling out of possible endotoxin contamination of the erythrocyte preparations.

Table 2
THE S-PHASE DISTRIBUTION OF HEMOPOIETIC PROGENITORS DERIVED FROM PLETHORIC MURINE BONE MARROW[a]

Progenitor cell type	Number in S-phase[b] (%)	Net change from normal marrow (%)
CFU-S	25.6 ± 8.9	+10.6
Primitive BFU-E	33.4 ± 7.8	+12.6
Mature BFU-E	55.0 ± 16.2	−11.1
CFU-E	69.1 ± 2.4	−7.4
Cluster-forming cells	64.3 ± 1.3	−16.8

[a] Determined on the 5th to 8th day following two consecutive erythrocyte transfusions in CD_1 mice.[46]
[b] Determined by the hydroxyurea method (see Table 1).

One additional point should be kept in mind when comparison is made between the levels of different cell progenitors in normal vs. experimental animals. Although the substantial change in cell plating efficiency following HT has been well documented for CFU-S,[21,43] no similar measurement has been made for any other progenitor cell. Thus, the level of cell progenitors in plethoric mice may actually vary from those summarized in Figure 9. Nevertheless, the results suggest that the augmentation in the number of early marrow progenitor cells tends to *follow* the decline in Epo-dependent cell progenitors by 1 to 2 days. Notwithstanding a more direct effect by the transfused erythrocytes,[60-62,66] this time lag may reflect the temporal relationship between CFU-S and mature BFU-E, and the indirect nature of the effect of HT on early hemopoietic progenitors.

The continued proliferation of cells responsive to Epo in vivo following the induction of plethora has long been inferred from indirect measures.[12,28,63,64] By employing clonal cell assays, it is now possible to approach the question of the proliferative capacity of specific Epo-dependent cell populations more directly. The average cycle distribution of five separate cell progenitors following HT is summarized in Table 2. Although some slight changes relative to normal marrow may be apparent, all cell types demonstrate a significant level of proliferation in plethoric animals. This is especially true for the more mature progenitors whose marrow levels are substantially reduced by HT. This observation of extensive progenitor cycling in plethoric animals permits two important conclusions regarding erythropoietic regulation in vivo. First, Epo is primarily responsible for regulating the compartment size of late erythropoietic progenitors and not their proliferation per se, otherwise a more substantial effect on the cycling of Epo-dependent cells would have been observed. Second, in the face of the continued proliferation of late erythropoietic progenitors in plethoric animals, the existence of a cellular death function or an arrest of maturation is strongly implied. At which stage(s) of erythropoietic differentiation this loss or blockage of cells occurs is uncertain, however. Since the marrow frequency of late erythropoietic progenitors in plethoric animals is usually less than the number of morphologically recognizable pro-

erythroblasts,[28,39] it is possible that this cell loss or maturational arrest occurs very late in erythropoiesis (i.e., near the proerythroblast stage).

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Chapter 2

HYPERTRANSFUSION — A MODEL ANALYSIS*

H.-Erich Wichmann and Markus Loeffler

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I. ABSTRACT

After hypertransfusion of mice with erythrocytes the formation of erythropoietin (Epo) is stopped and a steep decrease of erythropoietin-sensitive bone marrow cells follows. Simultaneously, the number of stem cells and granulopoietic cells increases although they are not directly affected by Epo. The mathematical analysis suggests that an intramedullary feedback is responsible for this phenomenon: the low number of erythropoietic precursors stimulates cell cycling of the stem cells which, as a consequence, increases both the stem cell number and the cell flux into the differentiated lineages. The enlarged cell flux is followed by enlarged cell numbers in the granulopoietic compartments and the first erythropoietic compartment (BFU-E). The Epo-sensitive compartments remain depleted because their amplification is suppressed. The available data are reproduced by the model.

II. INTRODUCTION

The behavior of hemopoietic bone marrow cells in mice after red cell transfusion has been extensively studied. As has been summarized by Monette,[1] the following pattern can be observed:

- The CFU-S count rises up to 200%.
- The granulopoietic progenitors and precursors show an increase up to about 150% of normal in most of the data.
- After an initial drop the BFU-E increase to 150% of normal.
- The erythropoietin-sensitive progenitors show a clear decrease to a quarter or less of the normal values.
- The erythropoietic precursors decrease to less than 10% of normal.
- The plasma concentration of erythropoietin decreases to 50% of normal.

Two mechanisms have been discussed to explain these observations.[1] They may be called the "accumulation" model and the "competition" model. The "accumulation" model assumes that the step from BFU-E to CFU-E is blocked because the hematocrit is high. This block acts like closing a floodgate. On one side (CFU-E) the system is drying out. On the other side (BFU-E) the steady cell influx from CFU-S leads to an accumulation. The arguments are not totally clear as far as CFU-S and CFU-GM are concerned.[1] It appears as if the damming up of cells sweeps back into the CFU-S compartment and finally should pour into other pathways giving rise to granulopoietic progenitors.

The "competition" model assumes that during diminished demand for erythropoiesis the erythropoietic determination rate is reduced in favor of other pathways. This concept easily explains an increase of granulopoiesis as a response to hypertransfusion, but additional assumptions are necessary to account for the increases in CFU-S and BFU-E. As will be demonstrated in the following, there exists a completely different possibility to explain the data on hypertransfusion without using the concepts of accumulation or competition.

III. MATHEMATICAL METHODS

The experiments on hypertransfusion are simulated using the mathematical model as described.[2-4] Since the elevated red cell mass cannot be directly considered in the model, the corresponding erythropoietin values are taken into account. According to the mathematical model of mature erythropoiesis of Wulff[5] and Wichmann,[6] a theoretical EP curve (Figure 1) is used as input into the model with values close to zero for 10 days and a return to normal by day 25. This corresponds to an initial hematocrit of 1.5 times normal according

to the majority of data which are used for comparison. The EP curve has been used as input for the stem cell model (as before, "EP" denotes the theoretical model value while "Epo" is used for the measured concentration of erythropoietin).

For normal or stimulated erythropoiesis it is sufficient to characterize the erythropoietic precursors (El-4) by one differential equation. For suppressed erythropoiesis this simplification is not adequate, because it cannot describe the quick reduction of the cell number after cessation of the EP stimulus. Therefore, in hypertransfusion and other situations of severe erythropoietic suppression, the model compartment El-4 is described by six identical differential equations (see Reference 3, Section V.D). As discussed earlier,[2,3] for practical purposes El-4 is identical with the sum of all erythropoietic progenitors and precursors, E. Therefore, we will refer to E as "erythropoietic precursors" as it has been done throughout this volume.

IV. RESULTS

A. Model Calculations

The theoretical curves which are generated by the simulation of hypertransfusion are shown in Figures 1 to 9. Figure 1 gives the time course of erythropoietin (EP) used as external model input. Figures 2 to 9 show the model reactions to this boundary condition. The results can be interpreted as follows.

Day 0 to 1 — Due to the suppression of EP the number of cells in CE and E starts to decrease (Figures 4 and 5). The cell numbers of the other compartments are unaffected.

Day 1 to 3 — The reduced number of erythropoietic precursors induces counteracting regulation. The proliferative activity "a_S" of the stem cells increases and the self-renewal probability "p" is below normal (Figures 8 and 9).The consequences are twofold. S is slightly reduced (Figure 2) while BE, CG, and G increase (Figures 3, 6, and 7) as a result of the enlarged cell flux from S.

Day 3 to 12 — The elevated number of G brings "p" temporarily to almost maximum values. Therefore, a steep growth of stem cells is observed in this phase. BE, CG, and G continue their increases as long as "a_S" is elevated.

Day 12 to 30 — EP returns to normal (Figure 1). The erythropoietin-sensitive cells increase and the reverse pattern, compared to the beginning of hypertransfusion, can be observed: the proliferative fraction "a_S" and the efflux from S is reduced. The granulopoietic cells normalize, "p" reaches the minimum of 0.4, and S decreases. At the end of the simulation (day 30), the system has not yet reached the normal steady state again.

B. Comparision with the Data

Of the data reviewed by Monette[1] for the stem cells and progenitor cells, only some are presented in the figures, in the interest of clarity. For precursor cells, additional data have been collected.[7-18] In most experiments two injections of erythrocytes were administered during the first 2 days. These increased the hematocrit to 150% of normal or more. In some experiments the injection was repeated weekly thereafter.[11,16,18]

Most of the available data on CFU-S show an increase similar to the theoretical curve for S. Only in few experiments is the increase missing. The BFU-E data are constant for some days or have an initial dip before they become elevated. The initial behavior is not reproduced by the model and the theoretical maximum is higher than in the data. CFU-E and the erythropoietic precursors decrease during hypertransfusion which is reproduced by the theoretical cell numbers CE and E. However, the subnormal plateau of CE is somewhat too high. CE and E start to normalize after 12 days, but in some experiments[11,16,18] the Epo-sensitive cells remain suppressed longer because hypertransfusion is sustained for weeks. The granulopoietic progenitors and precursors increase quite similarly in experiment and

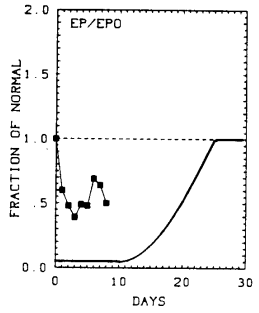
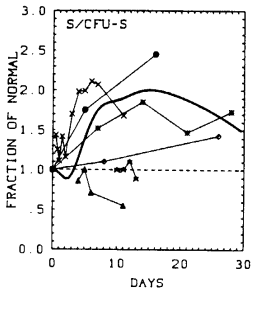


FIGURE 1



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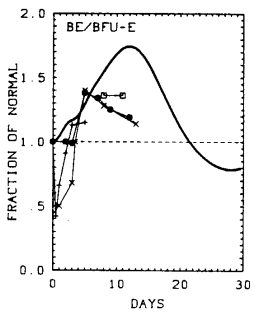

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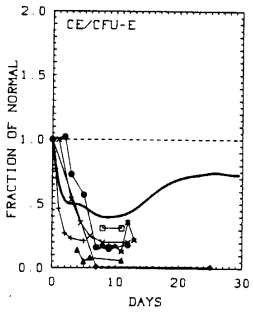

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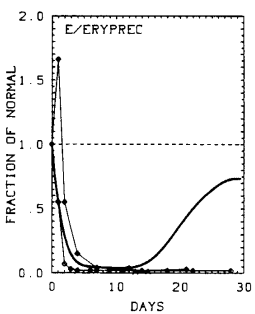

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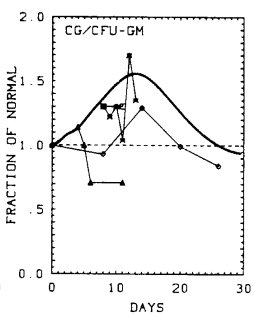

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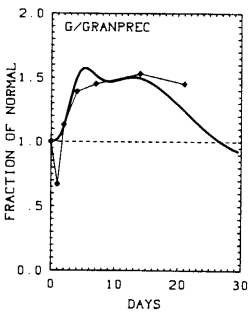

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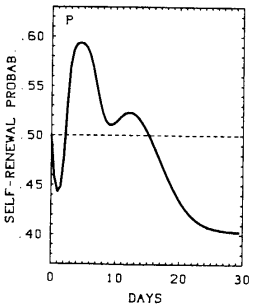

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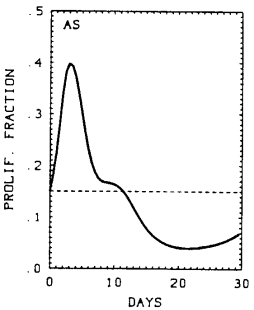

FIGURE 9

FIGURES 1 to 9. Hypertransfusion. Comparison of model results (—) and data. The model simulations correspond to an initial hematocrit of 150% of normal which normalizes within 25 days. The EP curve (Figure 1) is used as input to the model. Figures 2 to 9 show the model reaction. The data are from the bone marrow of mice: (BFU-E, CFU-E, CFU-GM, □);[7] (CFU-S, BFU-E, CFU-E,●);[8-14](CFU-S, CFU-E, CFU-GM, ▲);[12] (BFU-E, CFU-E, +);[9] (CFU-S, BFU-E, CFU-E, ×) (CFU-GM, ◇);[1-13](CFU-S, BFU-E, ERYPREC [original data: ^{59}Fe-uptake]);[16] (CFU-S, CFU-E, CFU-GM, erythropoietic precursors, ★);[10-17](CFU-S, *);[11] (erythropoietic and granulopoietic precursors, ◆);[18] (erythropoietin, ■).[15]

model. The theoretical and the measured values of erythropoietin are subnormal although the theoretical plateau is significantly lower.

In the model, cycling of stem cells (Figure 9) and early progenitor cells is activated during the first week after hypertransfusion. This also has been measured for CFU-S and BFU-E in some experiments, where the fraction of cells in S-phase is more than doubled.[1]

V. DISCUSSION

Hypertransfusion suppresses the formation of erythropoietin (Epo) and as a consequence the Epo-sensitive cells decrease dramatically. This direct effect can be understood since for the normal amplification in the erythron the presence of Epo is necessary. During hypertransfusion most of the 12 mitoses which normally take place in the CFU-E and the erythroblast compartment are omitted. However, the non-Epo-sensitive cells also react to hypertransfusion of erythrocytes. In our stem cell model the increase of CFU-S and granulopoietic cells is understood by intramedullary feedback. The low number of erythropoietic cells activates the stem cells. Their higher cycling rate enlarges the cell flux into differentiation and the number of granulopoietic progenitors and precursors increases. The elevated number of granulopoietic bone marrow cells provides the signal that no further cell supply is needed. This leads to a higher self-renewal probability and thus to an increase of the stem cell number.

The above interpretation is self-consistent and allows a quantitative understanding of the measurements although there remain minor discrepancies: (1) the decrease of BFU-E[1,9] and granulopoietic precursors[18] during the first 1 or 2 days cannot be reproduced by the model; (2) in one experiment[12] CFU-S and CFU-GM show no increase but a decrease; (3) the experimental erythropoietin values[15] are much higher than the theoretical curve. The last discrepancy may, in part, depend on the methodological difficulties in measuring subnormal Epo concentrations, even with radioimmune assays.

Despite these limitations it seems obvious that additional mechanisms like "accumulation" or "competition" are not needed. These hypothetical mechanisms often are stressed because the decrease of erythropoietic and the increase of granulopoietic cells seem to suggest a canalization from one cell lineage to the other. However, one has to realize that an enlarged flux of cells from the stem cell pool into both lineages may have the same result. The enlarged flux into granulopoiesis is multiplied by the normal number of amplifying divisions and leads, therefore, to an enlarged number of granulopoietic cells. On the other hand, the enlarged flux into erythropoiesis is not multiplied by the normal number of amplifying divisions. Since erythropoietin is very low, the Epo-sensitive cells perform (nearly) no amplifying divisions and, despite the enlarged influx, the number of erythropoietic precursors and the efflux from this compartment are reduced.

If one looks carefully at the data, one finds that in hypertransfusion all cells (including BFU-E) are enlarged except those which are Epo-sensitive. If a canalization to granulopoiesis occurred, BFU-E should be diminished, but the opposite is found. The parallel behavior of BFU-E and CFU-GM may be considered as an argument against the concept of "competition".

The increase of cycling of CFU-S and BFU-E,[1] although not reproduced in all experiments, also supports our hypothesis that stem cells and early progenitors are activated in hypertransfusion. In our interpretation, this activation rather than a canalization or an accumulation would be responsible for the enlarged flux of cell into the differentiated lineages.

It should be noted that hypertransfusion is not the exact mirror-image of anemia,[19] although the erythropoietic and granulopoietic cell numbers behave oppositely in both situations (and opposite to each other). In the model, this is understood by an asymmetric influence of E and G on the self-renewal probability and the activation of stem cells: the enlarged number of erythropoietic precursors in anemia has only a minor influence on the self-renewal prob-

ability. Therefore, the stem cell number remains close to normal. In hypertransfusion, the reduced number of erythropoietic precursors leads to an increase of the granulopoietic cells. These have a severe influence on the self-renewal probability and S increases.

A second asymmetry between anemia and hypertransfusion concerns the role of the spleen. While splenic proliferation, at least of erythropoietic cells, has to be considered in anemia, it might be neglected in hypertransfusion. Here less than 20% of hemopoiesis takes place in the spleen, and eventually this contribution is diminished further.[20]

Erythropoiesis is similarly affected by hypertransfusion and by the termination of hypoxia. As will be discussed later[21] erythropoietic suppression is more pronounced in plethoric than in exhypoxia mice. This has some theoretical implications for the bioassay of erythropoietin.

In total, the behavior of early hemopoietic cell numbers as measured in the majority of experiments on hypertransfusion is reproduced by the stem cell model. A consistent interpretation of the data is possible without additional assumptions on the "accumulation" or "competition" of stem cells.

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Chapter 3

EX-HYPOXIA — EXPERIMENTAL RESULTS: THE RESPONSE OF HEMOPOIETIC STEM CELLS TO EX-HYPOXIA

Martin J. Murphy, Jr. and Brian I. Lord

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I. ABSTRACT

The sequelae of cellular and kinetic events which attend the return of mice from hypobaric hypoxia to 1 atm of ambient pressure with the resultant ex-hypoxic polycythemia were investigated. Splenic and, to a lesser extent, femoral pluripotent hemopoietic stem cells (CFU-S) responded to these physiological perturbations and with a biological pattern of oscillation. The BDF_1 mice used in these experiments were able to respond to an hypoxic stimulus as evidenced by their elevated hematocrits. Their stem cells also clearly responded to polycythemia but with complex patterns indicative of the multiple controls which regulate this vital cell population.

II. INTRODUCTION

A classical approach in experimental physiology is the controlled perturbation of an organ followed by the sequential examination of the establishment of a new equilibrium. Conversely, the removal of the original perturbation affords an opportunity to analyze the return of the physiological system to its proemial state. The hemopoietic system, containing a pluripotential compartment of cells endowed with the properties of self-perpetuation as well as differentiation, offers a wide latitude for studies of the physiology of blood cell formation. We will restrict our attention to but one of the cellular facets of hemopoiesis, namely, the pluripotential cells from whence hemopoietic elements stem (i.e., the spleen colony-forming unit, CFU-S).

It is clearly established in both animals[1,2] and man[3-5] that the ambient partial pressure of oxygen is a prime mover in the regulation of erythropoiesis mediated by erythropoietin (Epo).[6,7] We have observed that hypobaric-induced hypoxia elicits dramatic alterations within the hemopoietic stem cell complex.[26] Not only were BDF_1 mice capable of an acute (i.e., within 3 hr) hypoxic response,[8] but also the process of acclimatization to chronic hypoxic exposure was accompanied by oscillations of stem cell kinetics.[9] This present chapter describes the sequelae of cellular and kinetic events attending the return of BDF_1 mice from hypoxia (i.e., 0.4 atm) to 1 atm of ambient pressure ("ex-hypoxia").

III. MATERIALS AND METHODS

BDF_1 (C_{57} female × DBA_2 male) male mice, 9 to 12 weeks of age, were used throughout. At frequent intervals throughout these studies, groups of four control donor mice were assayed to obtain normal data for all the parameters measured.

A. Hypoxia

Those mice serving as hemopoietic donors were maintained in an altitude chamber at a reduced atmospheric pressure of 320 mmHg (i.e., 0.42 atm or a simulated altitude of 6.7 km or 22,000 ft) for 5 days. The hypoxic environment was continuous except that at 48 hourly intervals (i.e., twice during the hypoxic regime) the animals were returned to ambient pressure (i.e., 1 atm) for about 1 hr during which time food and water were replenished and fresh bedding supplied. Groups of not less than four mice were returned to ambient pressure after the 5 days of hypoxia. To avoid potential problems associated with any diurnal variation, all mice were originally exposed to hypoxia between 0830 and 0930 hr, and were returned to ambient pressure 5 days later during the same time interval.

B. Irradiation

The recipient assay mice were irradiated with a Siemens X-ray machine operating at 300 kVp, 12 mA, HVL 2.0 mmCu. The total dose of 800 rad (30 rad/min) represents an $LD_{100/15}$ and reduces endogenous spleen colony (CFU-S) formation to less than 0.2 colonies per spleen.

C. Cell Suspensions

The animals were killed, and their femoral bone marrow and spleen cell suspensions were prepared in ice-cold Fischer's medium, according to the techniques of Schofield and Cole.[10] The cell concentration, determined by hemocytometric counting, was adjusted to render countable spleen colonies in recipient assay mice at 9 days.[11] Bone marrow cells, 3.3×10^4, and 5×10^5 spleen cells in 0.2 mℓ of medium were intravenously inoculated into groups of not less than ten assay mice. The cell suspensions were kept for no more than 1/2 hr at ice-bath temperature before incubation and were injected within an hour after incubation.

D. Incubation

To assess the turnover state of the stem cell pool (i.e., the percent of the CFU-S undergoing DNA synthesis), the "^{3}H-thymidine-killing" technique[12,13] was modified as follows. Two aliquots of the cell suspension under test, containing 5×10^6 cells per milliliter, were prewarmed for 10 min in ventilated 5-mℓ bijou bottles at 37°C in a constantly shaking water bath; 0.2 mℓ high specific activity ^{3}H-thymidine (1 mCi/mℓ, ~25 Ci/mmol) in Fischer's medium was added to one of the paired samples. The paired control received the same volume of Fischer's medium. After 30 min of incubation, the samples were suitably diluted with ice-cold Fischer's medium and injected via a lateral tail vein into groups of not less than ten irradiated recipient assay mice. They were housed, two or three to a cage, and had free access to food and water.

E. Spleen Colony (CFU-S) Counting

Nine days after inoculation, the recipients were killed; their spleens were removed, freed of fatty adventitia, and placed flat on filter paper in a plastic petri dish containing Bouin's solution. After at least 24 hr of fixation, the spleen colonies were counted using a stereoscopic dissecting microscope at 10 × magnification. The ^{3}H-thymidine killing was calculated from the difference in colony yields from the paired samples of cell suspensions.

Blood was collected from the retroorbital sinus with a heparinized 75-mm microcapillary tube (Clay-Adams, Inc., New York) and heat sealed. After centrifugation at 12,000 rpm for 3 min, the percent packed red cell volume (PCV) was measured.

Statistical analyses were carried out using standard analysis of variance technique.[14]

IV. RESULTS

During exposure to 0.42 atm of hypoxic-hypoxia, the packed red blood cell volume rose from a control level of 46 ± 2% at 1 atm to 58 ± 2% by day 5 of hypoxia (Figure 1). It remained at or above 58% for the first 3 days ex-hypoxia, and thereafter declined to near normal levels by day 9 ex-hypoxia.

Upon the return of hypoxic mice to normal oxygen tension, the femoral (Figure 2) and, more pronounced, the splenic (Figure 3) complement of nucleated cells were elevated. Both these waned in total cell numbers, ex-hypoxia reaching a nadir at 3 days ex-hypoxia. The femoral cellularity declined to almost one half of its cellularity observed on day 5 of hypoxia, some 33% of control, nonmanipulated mice, while splenic cellularity underwent a marked reduction of over 60% during the first 72 hr ex-hypoxia, or approximately to 30% below that of normal mice.

From 3 days ex-hypoxia, the femoral nucleated cellularity returned to normal or hypercellular levels and remained elevated for the rest of the period of observation (Figure 2). In

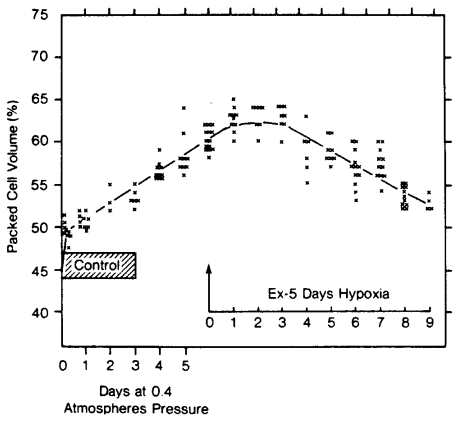


FIGURE 1

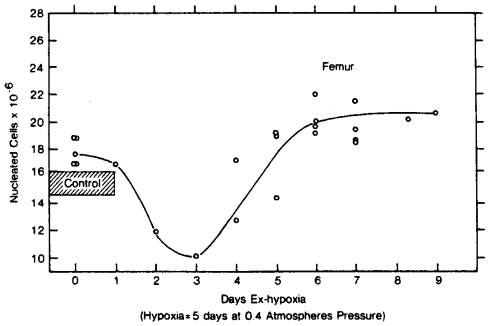
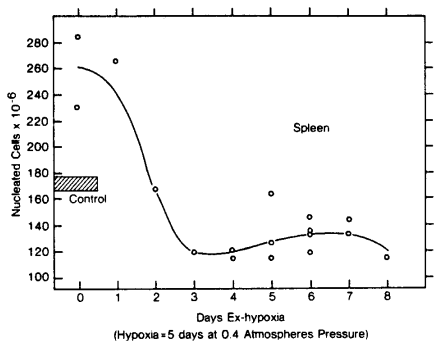


FIGURE 2

FIGURES 1 to 9. The control values on the ordinate refer to normal, nonmanipulated mice.

contrast, the number of nucleated cells in the spleen remained substantially below both the 5-day hypoxic level as well as below normal nonmanipulated controls.

The relative concentrations of CFU-S in femoral marrow and spleen are given in Figures 4 and 5. The femoral concentration of CFU-S was subnormal after 5 days of hypoxia, but rose to supranormal levels by 3 days ex-hypoxia (Figure 4). Thereafter, the CFU-S concentration returned to normal, or slightly elevated levels. The concentration of CFU-S in the spleen presents a different response pattern ex-hypoxia (Figure 5). For the initial 4 days ex-hypoxia, the splenic concentration of CFU-S was normal or marginally below control values. By 5 days ex-hypoxia, the splenic CFU-S concentration increased and peaked at 6 days ex-hypoxia, resulting in a twofold enhancement of splenic CFU-S concentration. This was followed by a gradual decline in the concentration of splenic stem cells, which again approached normal levels by 9 days ex-hypoxia.



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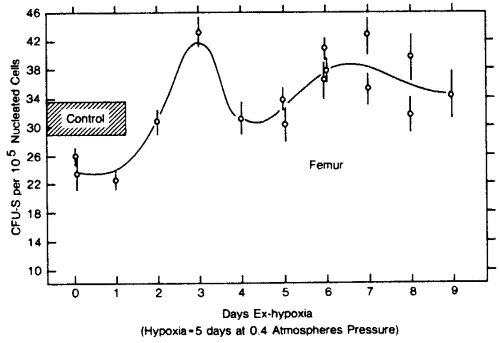

FIGURE 4

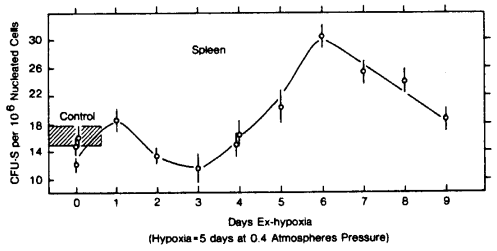

FIGURE 5

When the relative concentrations of CFU-S are converted to the absolute numbers of CFU-S per femoral shaft (Figure 6) or whole spleen (Figure 7), these previous data take on a different complexion. The total femoral CFU-S complement on the fifth day of hypoxia is not different from control values (Figure 6). After a modest increase at 24 hr ex-hypoxia,

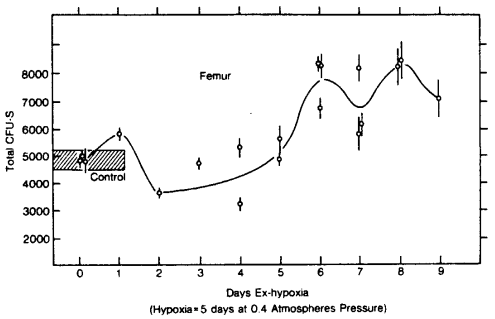

FIGURE 6

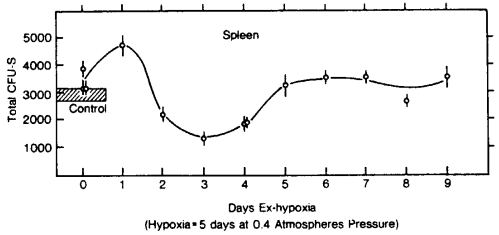

FIGURE 7

the femoral CFU-S remained at or slightly below control values for the first 5 days ex-hypoxia. By 6 days ex-hypoxia, however, the absolute numbers of femoral CFU-S had increased to 140 to 160% of control and thereafter remained elevated for the 9 days of ex-hypoxic observation.

The total number of splenic CFU-S during the 9 days ex-hypoxia is presented in Figure 7. Similar to total femoral CFU-S, the number of splenic CFU-S during the first 5 days ex-hypoxia was very nearly that of normal, nonmanipulated control mice. After an ephemeral rise at 24 hr ex-hypoxia, the splenic CFU-S numbers declined and remained moderately depressed until 5 days ex-hypoxia, when their numbers had returned to control levels. In contrast to femoral CFU-S, the total numbers of splenic CFU-S did not "overshoot" and remained within the normal range throughout the rest of the period of observation.

An assessment of the cell proliferation of CFU-S in femur and spleen is given in Figures 8 and 9. The limitations of the ^{3}H-thymidine "killing" assay can but afford one an approximation of the percent of CFU-S in DNA synthesis. We have, therefore, arbitrarily ascribed a "marked killing" to values above 20% and a "moderate killing" to values below 20%. Even within these parameters, the fluctuations between the percent of stem cells actively in cell cycle and those with repressed proliferative potential are readily apparent.

It is clear that both femoral (Figure 8) and splenic (Figure 9) CFU-S are actively proliferative after 5 days of hypoxia. While femoral CFU-S retained an accelerated turnover rate for 48 hr ex-hypoxia, splenic CFU-S reverted to a quiescent state over the same interval. Femoral CFU-S continued to proliferate at a normal or marginally elevated rate from 3 to 6 days ex-hypoxia. This trend abruptly ended at day 7 ex-hypoxia when marrow CFU-S virtually withdrew from DNA synthesis. Femoral CFU-S then returned to normal rates of proliferation by day 9 ex-hypoxia.

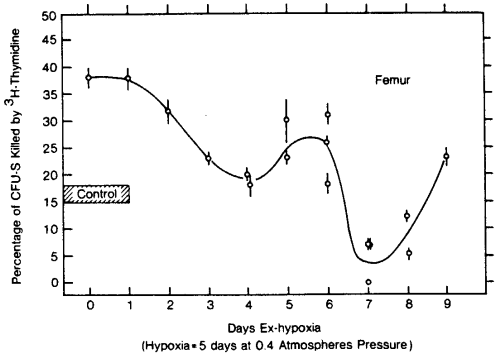

FIGURE 8

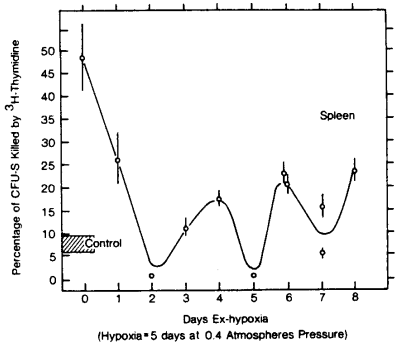

FIGURE 9

The percent of splenic stem cells in DNA synthesis oscillated during the 9 days ex-hypoxia (Figure 9). Following a precipitous reduction in the proportion of cycling splenic CFU-S which occurred during the first 48 hr of ex-hypoxia, the rate of proliferation of splenic stem cells returned to a more normal profile on days 3 and 4 ex-hypoxia. Again, at 5 days ex-hypoxia, the splenic CFU-S reverted to a noncycling status, which was followed by a further proliferative burst at 6 days ex-hypoxia. During the remaining days ex-hypoxia (i.e., days 6 through 8), the rate of splenic CFU-S proliferation vacillated between proliferation (i.e., on days 6 and 8 ex-hypoxia) and a moderate decline in CFU-S turnover (i.e., on day 7 ex-hypoxia).

V. DISCUSSION

Hypobaric-hypoxia (e.g., 0.4 atm pressure), an established physiological stimulator of erythropoiesis, was used to perturb normal steady-state hematopoiesis, thereby affording us an opportunity to analyze some kinetic parameters of hematopoietic stem cells (CFU-S). Previously we have shown that continuous, chronic hypoxic-hypoxia induced oscillations

in the absolute numbers of femoral and splenic CFU-S as well as in estimations made on the state of CFU-S turnover.[8,9] The current studies were designed to investigate in detail the events which attend ex-hypoxic polycythemia. In this instance, erythropoiesis is suppressed through an inhibition of erythropoietin (Epo) formation and/or release.[7] This is brought about by the excess tissue oxygenation which results from the polycythemia caused by hypoxic exposure. Since Epo initiates the erythropoietic differentiation of erythropoietin responsive cells (ERC) which are progeny of the pluripotent CFU-S, then an inhibition of erythropoiesis might be expected to have a "knock on" effect on CFU-S.[15-21] Clearly, CFU-S show a rapid response to their return to normal atmospheric pressure after having been maintained under hypoxic conditions for 5 days. Although the upward trend of the hematocrit continues for 1 to 2 days, this is the result of maturation of the rapid erythropoiesis already in the pipeline. The lack of erythropoietin due to the subsequent polycythemia reduces erythropoietic differentiation and leads to a transient build-up of the CFU-S population which passes with a rapid shutdown of CFU-S proliferation. Although these changes are seen in both femur and spleen, they are particularly dramatic in the spleen which appears to act as the major emergency erythropoietic organ in the mouse. Due to the dynamic nature of these changes, some oscillation of the CFU-S kinetics ensues before they settle back to their normal relatively quiescent rate of proliferation in about 9 days.

The BDF_1 male mice used in these present studies clearly responded to an hypoxic challenge of 0.4 atm pressure by elevating the numbers of circulating erythrocytes (i.e., PCV) from a normal control value of 46% at ambient pressure to 58% after 5 days of hypoxia. The PCV remained at that elevated level during the first 3 days ex-hypoxia and thereafter smoothly declined to a value of 50% by the ninth ex-hypoxic day. This predicted elevation in hematocrit is of considerable consequence in the subsequent evaluation of CFU-S kinetics. It is quite important that the strain of mouse chosen for such studies be competent in its response to hypoxia. The BDF_1 mouse fulfills that criterion. Other mouse strains (e.g., BALB/c and CAF_1) which are unable to produce optimal titers of Epo in response to hypoxic-hypoxia are thus unable to elevate their hematocrit sufficiently and, therefore, may be compromised in such experimental circumstances.[15,22-26]

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Chapter 4

EX-HYPOXIA — A MODEL ANALYSIS*

H.-Erich Wichmann, Peter Herkenrath, and Markus Loeffler

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I. ABSTRACT

After termination of hypoxia the formation of erythropoietin (Epo) is suppressed. This leads to a reduction of Epo-sensitive progenitor and precursor cells. As an indirect effect, granulopoietic cells and stem cells increase. These findings are analyzed by a mathematical model of stem cell proliferation. The analysis suggests that the low number of erythropoietic precursors activates the cycling of stem cells. More cells are formed per unit time, and they enlarge the cell flux into the differentiated cell lineages, as well as the stem cell pool.

II. INTRODUCTION

Hypoxia stimulates erythropoietic amplification in the bone marrow and the spleen and an enlarged number of red blood cells is formed. After termination of hypoxia, the body is left with excess erythrocytes which then suppress erythropoiesis. In mice one observes the following features: erythropoietin (Epo) decreases from an elevated level to values below normal.[1,2] Subsequently, CFU-E numbers are reduced to one third or less,[1,3] erythroblasts to 10% or less,[4-6] and ^{59}Fe-incorporation may drop very close to zero.[3,7] The indirect influence of ex-hypoxia leads to an increase of CFU-S,[1,4,7,8] BFU-E,[1,3] and CFU-GM.[1,3,9]

These findings from the bone marrow of mice may be considered as representative for the whole animal, since during suppressed erythropoiesis the spleen contributes less than 10% to the total hemopoiesis.[10]

Historically, the analysis of ex-hypoxia has had a severe impact on the development of our stem cell model. In the first version,[11] only erythropoietic influences on stem cell regulation have been considered. However, the intensive work with a large base of unpublished data measured by Murphy and Lord taught us that granulopoiesis also has to be included.[12,13] We are very obliged to Dr. Murphy and Dr. Lord for providing the data which resulted in this analysis.

In the following only experiments are considered in which the preceding hypoxic phase lasted for 5 to 11 days and in which hypoxia corresponded to a simulated altitude of 5 to 7 km.

III. MATHEMATICAL METHODS

Ex-hypoxia is simulated using the mathematical model of stem cell regulation as described.[14-16] Since the duration of the hypoxic phase varies in the available experiments, hypoxia is not considered explicitly in the model, but the initial values for the calculation are taken from the average values of the measurements on day 0 of ex-hypoxia. These values are S = 0.8, BE = 0.6, CE = 1.5, E = 2.2, CG = 0.6, G = 0.65, and EP = 6.0.

The suppression of mature erythropoiesis during ex-hypoxia (after prior erythropoietic exposure to 6 km altitude) has been calculated by a mathematical model of Wulff[17] and Wichmann.[18] It leads to a theoretical EP curve which is close to zero for 7 days and normalizes within 20 days (Figure 1). This curve is used as input for the stem cell model. Again "EP" denotes the theoretical values of erythropoietin while "Epo" is used for the measured concentration of the hormone.

As discussed earlier, the suppression of erythropoiesis can be more adequately simulated by a set of differential equations for the erythropoietic progenitors E than by one equation. Therefore, in ex-hypoxia as in hypertransfusion[19] six identical differential equations are used for E (see also Reference 15).

IV. RESULTS

A. Model Calculations

In Figures 2 to 9 it is shown how termination of hypoxia influences the stem cell system.

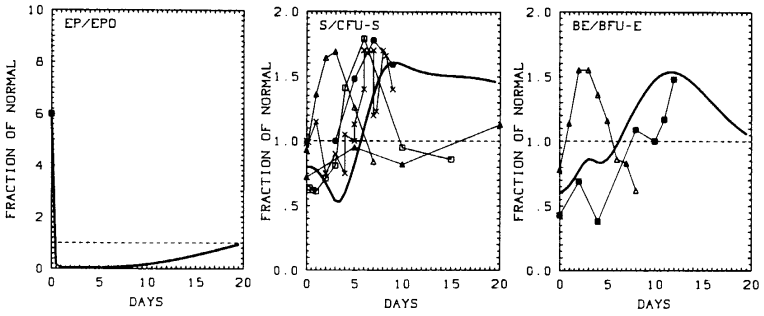
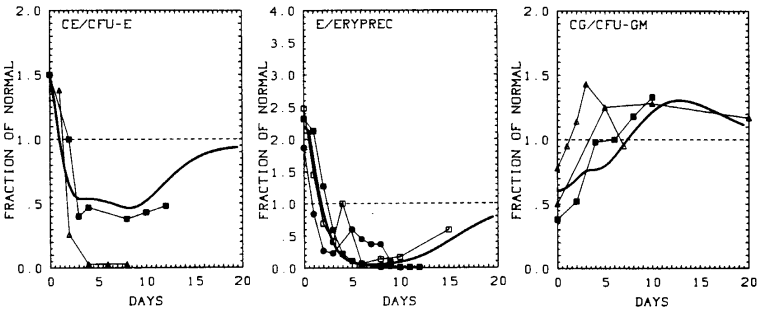


FIGURE 1

FIGURE 2

FIGURE 3



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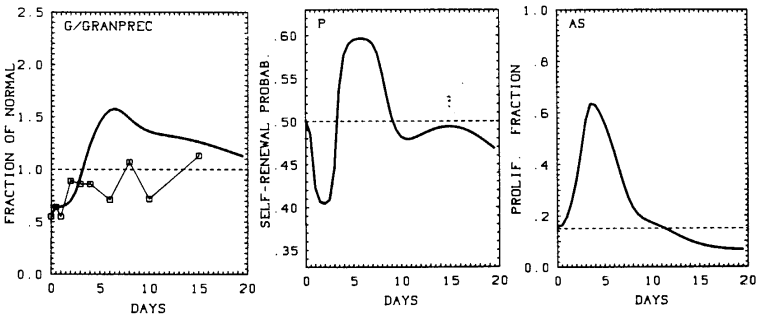

FIGURE 7

FIGURE 8

FIGURE 9

FIGURES 1 to 9. Comparison between model calculations (—) and data. The cell numbers have been measured in the bone marrow of mice after a preceding hypoxia of 5 to 11 days at a simulated altitude of 5 to 7 km. (CFU-S, ●);[4] (erythropoietic precursors, ●);[5] (erythropoietic and granulopoietic precursors, □);[6,7] (BFU-E, CFU-E, erythropoietic precursors, CFU-GM, erythropoietin, ■);[1] (CFU-S, BFU-E, CFU-E, CFU-GM, △);[3] (CFU-S, ×);[8] (CFU-S, CFU-GM, ▲).[9] The model curves (—) correspond to a preceding hypoxia of 6-km altitude for 5 to 11 days. Suppression of erythropoietin is simulated by the theoretical EP curve in Figure 1, which is used as input to the model.

Day 0 to 3 — Due to the missing EP stimulus some mitoses in CE and E are omitted. Consequently, CE and E decrease from initially elevated values to very low numbers (Figures 4 and 5). The reduced erythropoietic pool in E activates stem cell cycling ("a_s") and leads to an intermediate decrease of "p" (Figures 8 and 9).

Day 5 to 10 — The elevated "a_s" is responsible for an enlarged cell flux into the progenitor compartments. BE, CG, and subsequently G increase from subnormal to supranormal levels (Figures 3, 6, and 7). The enlarged G leads to an elevated "p" and thus to a steep increase of S (Figure 2).

Day 10 to 20 — EP returns to normal, followed by the normalization of CE and E (Figures 4 and 5). Subsequently, the other cell types also normalize.

B. Comparison with the Data

In most experiments, CFU-S respond to ex-hypoxia by an increase from subnormal values to approximately 150%, similar to the theoretical curve for S. However, the maximum occurred some days earlier in the experiments (between days 3 and 7) than in the calculations (day 9).

BFU-E, CFU-GM, and granulopoietic precursors recover from values below normal and increase to approximately 150%. The same behavior is found for BE, CG, and G. The Epo-sensitive progenitors (CFU-E) drop to subnormal numbers below 40% while CE is only reduced to 50%. The erythropoietic precursors adopt very small values both in the experiments and in the mathematical simulation.

In addition to the data in Figures 1 to 7, the cycling activity of stem cells has been measured[8] (percentage of CFU-S killed by ^{3}HTdR). The kill rate shows that cycling of CFU-S is enhanced during the first week of ex-hypoxia and normalizes thereafter. This behavior corresponds to what is found for "a_s".

V. DISCUSSION

After termination of an hypoxic stimulus the Epo-sensitive cells decrease dramatically and the granulopoietic cell counts increase. This situation is the mirror image of hypoxia, where erythropoiesis is elevated and granulopoiesis is below normal.[20] On the basis of the mathematical model, the same intramedullary feedback is responsible for both findings: during hypoxia the erythropoietic precursors reduce the cell flux into the progenitor compartments; after hypoxia their low number enlarges the cell flux into differentiation. In both cases, the behavior of granulopoiesis is not due to a variable canalization of the cell flux but it is a mere consequence of the stem cell turnover.

However, there is one important fact that does not show this symmetry in hypoxia and ex-hypoxia, and that is the behavior of the stem cells. In hypoxia, the CFU-S remain close to normal; in ex-hypoxia they increase significantly. This observation, which is even more pronounced in anemia[21] and hypertransfusion,[19] is reflected in the model by an asymmetric impact of granulopoiesis and erythropoiesis on "p" and "a_s". While the granulopoietic impact is dominating on "p" it is erythropoiesis which predominates on "a_s". The enlarged erythropoiesis in hypoxia is not able to increase "p", but the enlarged granulopoiesis in ex-hypoxia is able to do this.

Ex-hypoxic as well as hypertransfused mice are used as bioassay for exogeneous erythropoietin because their own erythropoiesis is suppressed. One might ask whether after hypertransfusion or after the termination of severe hypoxia the suppression is more pronounced. From the model analysis one finds that the degree of suppression is similar in both situations but the duration is different. After both treatments, the theoretical number of erythropoietic precursors, E, is reduced to plateau values of 5 to 10% of normal. After hypertransfusion (to a hematocrit of 1.5 times normal) E is below 10% for 12 days.[19] In ex-hypoxia (after a simulated altitude of 6 km for 5 to 11 days) E is below 10% only for 6 days (Figure 5). This difference is also found in the data, where not only the duration but also the degree of suppression is higher in plethoric than in ex-hypoxic mice.

Thus, from the theoretical point of view hypertransfused animals should provide a better bioassay for Epo. However, for us it remains open whether the expected theoretical difference is relevant or whether it is compensated by practical advantages of the technique of ex-hypoxia.

In ex-hypoxia, the spleen contributes less than 10% to hemopoiesis[10] and can, therefore, be neglected. This is different from hypoxia where more than 50% of the erythropoietic cells are formed in the spleen.

Due to the heterogeneity of the data, the measured values on day 0 have been taken as initial values for the calculations. However, if the preceding hypoxic phase also is simulated mathematically, the above results remain still valid. Model behavior similar to that described can also be found, if feedback does not depend on the precursors (E, G) but on the progenitors (CE, CG). This has been calculated in an earlier version of the model.[12,13]

In total, the characteristics of the experimental findings in ex-hypoxia are reproduced by the model. The increase of the granulopoietic cells is identified as an indirect consequence of enhanced stem cell cycling. On the other hand, the subsequent increase of the stem cell number is interpreted as a consequence of the enlarged number of granulopoietic precursors. This interpretation makes it clear that ex-hypoxia cannot be understood without considering granulopoiesis, although only erythropoiesis is directly influenced by this stress.

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Chapter 5

COMBINATION OF IRRADIATION AND BLEEDING — EXPERIMENTAL RESULTS

Hans J. Seidel and Ludwika Kreja*

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I. ABSTRACT

Three groups of CBA/Ca mice were studied. One group was irradiated with 1.5 Gy total body irradiation, a second was bled, and a third was irradiated and bled 48 hr later. Two days after irradiation the numbers of CFU-S were 8 and 5%, respectively, in the bone marrow and the spleen, and BFU-E and CFU-GM were between 2 and 20% of controls. The CFU-E numbers were 40% in the femur and 30% in the spleen. The recovery of the erythropoietic cells after bleeding, measured as reticulocyte numbers in the peripheral blood, was not influenced by the irradiation. At the stem cell level (CFU-S, CFU-GM, BFU-E) the recovery from irradiation-induced depression in the femur was somewhat slower in the group which received additional bleeding. In the spleen an overshoot in the concentration of all stem cell types was seen after bleeding. This shift was delayed in animals irradiated before bleeding and was not seen after irradiation only.

II. INTRODUCTION

Whole body irradiation was used in this study in order to reduce the compartment sizes of the hemopoietic stem cells in the bone marrow and the spleen of CBA/Ca mice. The question was how a reduced level of, e.g., CFU-S, BFU-E, and CFU-E would influence the recovery of the erythropoietic cell system after bleeding. At the same time it could be asked how the regeneration of the stem cell compartments after the radiation-induced depression would be influenced by the increased demand of erythropoietic cells after bleeding. This indicates that the combination of the two manipulations (whole body irradiation and bleeding) creates a rather complex situation.

III. MATERIAL AND METHODS

Mice — CBA/Ca mice, bred at the Zentrale Tierversuchsanlage of the University of Ulm with a body weight of 18 to 22 g, were used. Female mice served as experimental animals (donors), female and male mice as recipients in the assay for pluripotent stem cells (CFU-S). They were kept ten to a cage in artificial light 12 hr daily and fed commercial pellets and water ad libitum.

Experimental procedures — For each experiment at least four mice were used. Bleeding was performed with a Pasteur pipette from the retroorbital sinus (0.5 mℓ per mouse). Total body irradiation consisted of 1.5 Gy exposure; 280 kV, 12 mA, a filter of 1.4 mmCu, and 1 mm Al, focal distance 50 cm, dose rate 30 R/min. For hematological studies standard procedures were used. Reticulocytes were stained with new methylen blue and counted. The mice were killed by cervical dislocation, the spleen weight was determined, and a single cell suspension of pooled spleens and bone marrow cells from one femur was prepared in α-medium (Flow Laboratories, Rockville) containing 2% fetal calf serum (Seromed, Munich). Appropriate cell concentrations were obtained by dilution and the cells were counted in a Coulter counter. The total spleen cellularity was calculated from the spleen weight. Correlations of the spleen weight and the cellularity were determined in separate runs for all experimental points.

CFU-S — The procedure described by Till and McCulloch[1] was used to assay pluripotent hemopoietic stem cells. The pooled marrow or spleen cells were injected i.v. (6 to 20 × 10^4 marrow cells and 5 to 20 × 10^5 spleen cells in 0.25 mℓ) into irradiated CBA/Ca recipients (ten animals per group). Irradiation consisted of an 8-Gy exposure. The recipient animals were killed 9 days after injection of cells, and their spleens were removed and placed in Bouin's solution in preparation for counting macroscopic colonies after 24 hr in the fixative. From these data, the mean CFU-S content per femur or spleen was calculated without consideration of an "f" factor. No endogenously formed colonies were found in mice exposed to radiation and not engrafted with cells.

BFU-E — The cultures were carried out according to the method of Iscove and Sieber[2] with modifications. The cell concentration was 1 to 2 × 10^5 bone marrow and 1 × 10^6 spleen cells per milliliter. The cultures contained 0.7% methylcellulose, 4% fetal calf serum (Seromed), 1 to 3 U/mℓ Epo step III (Connaught), 10^{-4} *M* α-thioglycerol (Sigma), 378 μg/mℓ transferrin (Behring Werke), 8 mg/mℓ bovine serum albumin (Behring Werke), a sonicated mixture of oleic acid 5.6 μg/mℓ (Serva), dipalmitoyl lecithin 8.0 μg/mℓ (Serva), and cholesterol 7.8 μg/mℓ (Serva) prepared according to the method of Iscove et al.[3] Transferrin was fully iron saturated and bovine serum albumin was delipidated and deionized exactly as described by Iscove et al.[3] Thirty percent of medium conditioned by a mixed lymphocyte culture was added to the culture as a source for BPA (burst-promoting activity). For each experiment three parallel petri dishes 35 mm with 1 mℓ BFU-E culture components and cells were set up and incubated for 9 days at 37°C in a humidified atmosphere containing 5% CO_2. Colonies of more than 50 cells with CFU-E-like aggregates were scored at a magnification of 40 or 80. To check the cell types within the colonies, they were transferred to slides and stained by the Pappenheim method.

CFU-E — The method described by Iscove and Sieber[2] was used. Mixed in a final volume of 2.5 mℓ were 0.8% methylcellulose; 30% fetal calf serum; Epo step III (Connaught Medical Research Laboratories, Toronto), 0.2 or 0.3 U/mℓ, depending on the batch; α-thioglycerol at an end concentration of 10^{-4} *M;* and bone marrow or spleen cells in α-medium. Then 1 mℓ was plated into a 3.5-cm plastic petri dish (Greiner and Co., Nürtingen, West Germany). For each experiment, four parallel cultures were set up. Colonies were counted after 48-hr incubation at 37°C in a humidified atmosphere containing 5% CO_2. Erythropoietic colonies with more than eight small cells (erythroblasts) were scored without staining at an 80-fold magnification. The variation in the number of colonies per plate filled with the same cell suspension did not exceed 10%.

CFU-GM — Cells from bone marrow or spleen were incubated in 3.5-cm plastic dishes containing 1 mℓ of α-medium (Flow Laboratories) with 20% horse serum (Seromed) and 0.3% agar. Colony growth was stimulated by the use of heat-inactivated serum obtained from NMRI mice 3 hr after they received i.v. injections of 50 μg endotoxin (*Salmonella abortus equi;* Difco Laboratories, Detroit). Optimal stimulation was obtained by the addition of 12.5 or 25 μℓ of endotoxin-activated serum. The cell number per plate was 1 or 2 × 10^5 for bone marrow cells and 5 to 10 × 10^5 for spleen cells. The cultures were done in triplicate and incubated in 5% CO_2 at 37°C in a humidified atmosphere. After 7 days of incubation, the colonies (>50 cells being a colony) were counted with the use of a 40-fold magnification. In each experiment, a control culture with normal bone marrow and spleen cells was included. The number of colonies per dish varied about 10% of the mean.

IV. RESULTS

A. Control Values for Hemopoietic Stem Cells

The study has been performed with CBA/Ca mice which, in our hands, are characterized by the absolute stem cell numbers per femur and per spleen presented in Table 1. For each stem cell compartment of these control animals the absolute numbers are given as 1 (= 100%) in the figures presenting the experimental results. A daily control group was included in all experiments in order to correct for the daily variations which were seen in the different assay systems.

B. Hemopoiesis 2 Days after 1.5 Gy

At day 2 after 1.5 Gy total body irradiation the stem cells in the femur and the spleen were depressed to the level presented in Table 2. Bleeding was performed at that stage.

Table 1
HEMOPOIETIC STEM CELLS PER FEMUR AND SPLEEN OF YOUNG CBA/CA MICE[a]

	Femur	Spleen
CFU-S	1,750—2,610	100—180
CFU-GM	6,600—12,000	100—180
BFU-E	5,400—10,200	1,200—2,800
CFU-E	33,000—45,000	380—1,000

Note: Femur cell count = 16×10^6 cells; spleen cell count = 14×10^7 cells.

[a] As observed in this laboratory in many control groups during the performance of the experiments presented here and in other studies.

Table 2
HEMOPOIETIC STEM CELLS PER FEMUR AND SPLEEN OF YOUNG CBA/CA MICE 2 DAYS AFTER 1.5 GY TOTAL BODY IRRADIATION

Unirradiated Controls (%)

	Femur	Spleen
CFU-S	8	5
CFU-GM	5	20
BFU-E	14	2
CFU-E	40	30

Obviously, the more immature stem cell types were more affected than the erythropoietic progenitors CFU-E.

C. Hemopoiesis after Irradiation and Bleeding

1. CFU-S

Bleeding had a small influence on the number of CFU-S per femur; after 1 day an increase to 117% was seen, and at day 9 there was a decrease to 70% of controls with subsequent regeneration. In the spleen the number of CFU-S rose four- to fivefold on days 5 and 7 with normalization at days 11 and 14 (Figures 1 and 2).

After *radiation* exposure the regeneration of CFU-S in the femur and in the spleen was rather slow. In the marrow values above 50% were seen after more than 9 days, in the spleen at day 14.

The *combination* of irradiation and bleeding (bleeding 2 days after irradiation) resulted in a slower regeneration, at least at days 9, 11, and 12, of CFU-S in the femur. In the spleen the CFU-S increase was much smaller and delayed to day 7; the relative increase, compared to the "irradiated only" group, is about 20-fold, however.

2. CFU-GM

Bleeding was followed by a decrease in CFU-GM numbers per femur to 67 to 90% of controls between days 3 and 11. In the spleen they rose to 150% 1 day after bleeding,

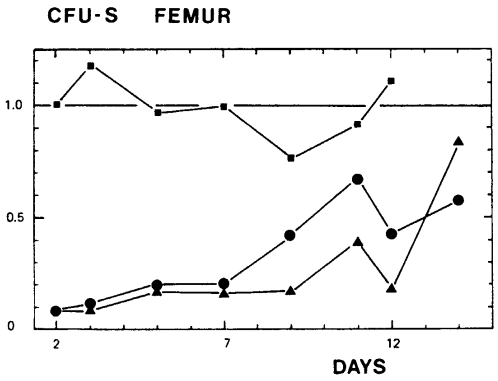

FIGURE 1

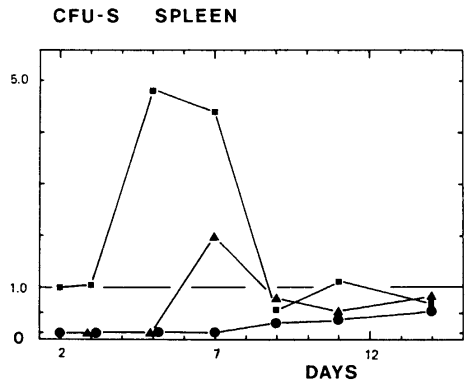

FIGURE 2

FIGURES 1 to 9. Hemopoietic cells per femur and per spleen; 1.0 represents 100%, the control value as determined at each experimental point. ■—Mice bled at day 2; ●—mice irradiated with 1.5 Gy at day 0; ▲—mice irradiated with 1.5 Gy at day 0 and bled at day 2.

increased to 650% at day 7, and the wave ended between days 11 and 12 (Figures 3 and 4).

Irradiation caused a depression of CFU-GM below 10% in both organs. In the femur there was a steady increase to about 75% at day 14; in the spleen control levels were reached at day 9 but later lower numbers were seen.

Bleeding following the radiation caused a small but consistent delay in the CFU-GM regeneration in the femur. The CFU-GM elevation seen in the spleen after bleeding alone was delayed 4 to 5 days; the regenerative wave had about the same height but seemed to be shorter.

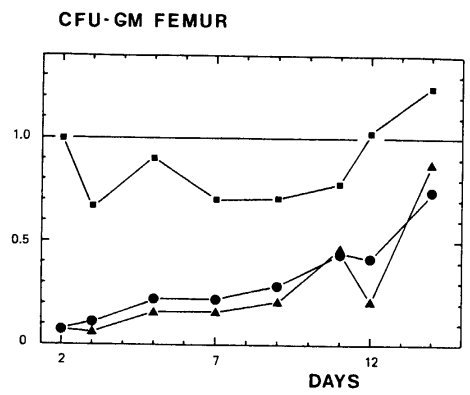

FIGURE 3

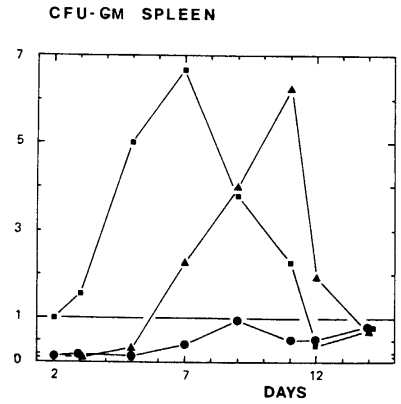

FIGURE 4

3. Erythropoietic Progenitor Cells BFU-E and CFU-E

BFU-E were influenced by *bleeding*. In the marrow there was an initial dip at day 3 and about normal values were seen at day 4; at days 7 to 11, however, was a decrease to 50 to 75%. In the spleen bleeding caused a six- to sevenfold increase at day 5, and normal numbers were present at day 9 (Figures 5 and 6).

After 1.5 Gy there was an initial depression in the femur to about 10%, at days 5 to 9 the regeneration reached a first plateau of 25 to 30%, and even at day 14 normalization had not yet occurred. The BFU-E numbers in the spleen recovered slower than those in the marrow; about 50% of controls were seen at days 11 and 14.

In *irradiated and bled* mice the recovery of BFU-E in the femur was slightly reduced throughout compared to the "irradiation only" group. In the spleen the bleeding caused a rise in BFU-E numbers above the control level at day 9; about fivefold values were seen in comparison to the "irradiated only" group; the rise, however, was delayed 4 days compared to the "bled only" group.

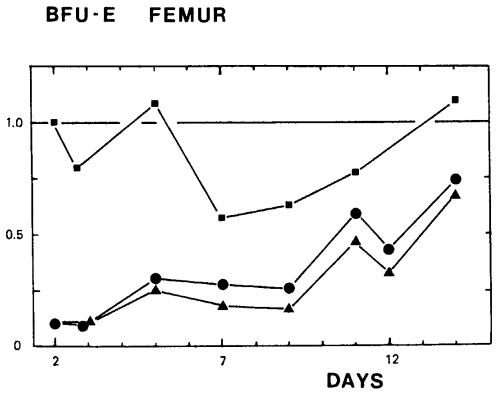

FIGURE 5

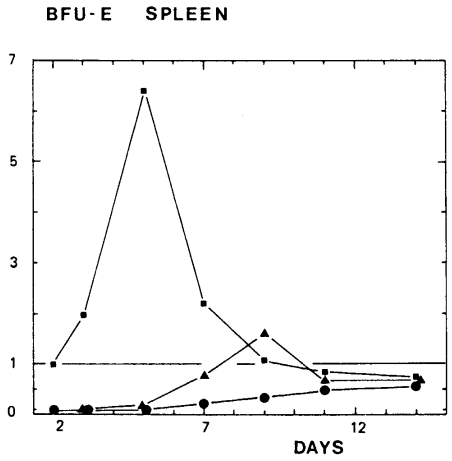

FIGURE 6

The CFU-E numbers in the femur increased to 175% at day 5 after *bleeding* and remained above control levels up to day 9. In the spleen a tremendous CFU-E rise was seen, reaching 65-fold values at day 5 and control levels not before day 12 (Figures 7 and 8).

In the *irradiated group* the CFU-E regeneration in the femur showed an overshoot at day 9 and after a dip at day 12 control levels were seen at day 14. In the spleen control levels were already seen at days 7 and 9 with a slight overshoot at day 11.

Bleeding after irradiation caused a further dip of the CFU-E number in the marrow at day 3, but later there was no difference to the "irradiated only" group. The rise in the CFU-E numbers in the spleen was not quite as high as in the mice "bled only"; the onset was delayed for about 2 days, the maximum for about 4 days. Control levels were seen at day 14.

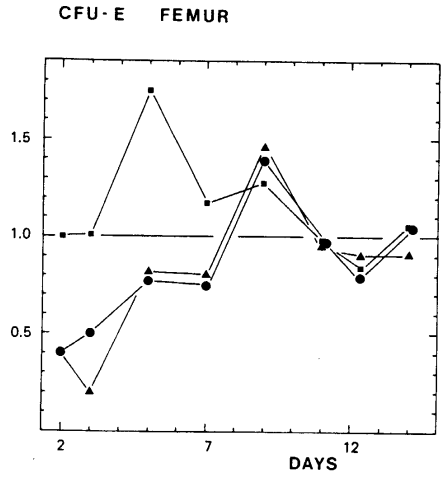

FIGURE 7

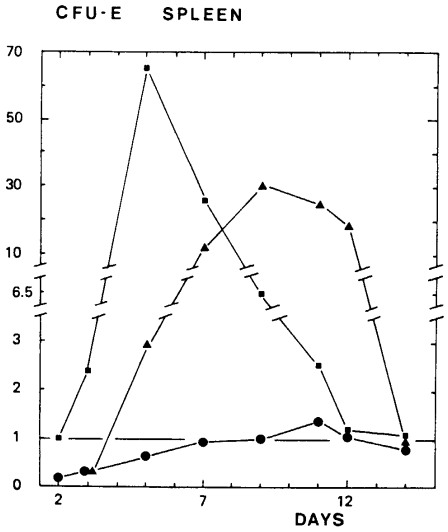

FIGURE 8

4. Peripheral Blood

Finally, the erythropoietic recovery of normal and 1.5-Gy irradiated mice after bleeding can be seen by the study of the reticulocytes in the peripheral blood, where these newly formed cells appear independent from the site of their production. As seen in Figure 9, control mice responded to bleeding with a peak reticulocytosis at day 5, and normal levels

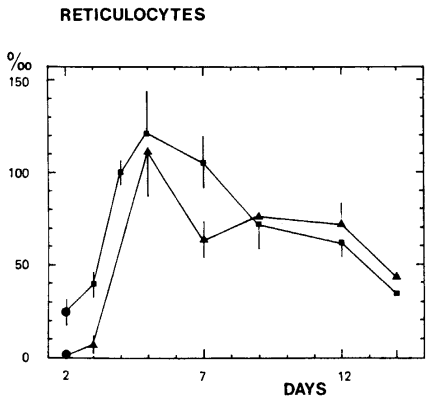

FIGURE 9

were not seen during the observation period. The mice bled after irradiation started with a very low level of reticulocytes which was also seen at day 3, but then they also had their peak values at day 5 and were not different from "bled only" mice at day 9 and 12. The initial rise seemed to be somewhat lower but the difference to the nonirradiated group was very small.

V. DISCUSSION

The influence of a single bleeding on the different hemopoietic stem cell pools has been studied by several authors[4-6] and is also presented in separate chapters.[15,16] The data of our experiments seem to be in good agreement with the previous findings: CFU-S numbers fell slightly in the bone marrow and increased in the spleen, BFU-E and CFU-GM had the same kinetics, and CFU-E showed a slight increase in the marrow and a tremendous increase in the spleen. In the peripheral blood a reticulocytosis was seen from day 3 to day 14 with a peak value at day 5.

The massive involvement of the spleen in the regeneration of the erythropoietic cell system is wellknown; a similar phenomenon is seen after injection of Epo,[7,8,16] but also after injection of endotoxin in the granulopoietic and the erythropoietic cell system.[9,10] In all these circumstances there is a shift of stem cells to the spleen, not restricted to the differentiation pathway under stress, but also involving the CFU-S and the CFU-GM compartment after bleeding[11] or the BFU-E and the CFU-E compartment after endotoxin injection. The granulopoietic cell system shows an increase in neutrophil production after bleeding.[12]

When preirradiated mice were bled, the reticulocytosis with a peak value at day 5 was not much different from the unirradiated group. This production of erythrocytes is performed by progenitor cells in the bone marow and the spleen, which are very much reduced in numbers by the irradiation exposure and have to regenerate their own compartment sizes. The reduction, however, is less pronounced in the CFU-E compartment than in more immature stem cell types. At the CFU-S level in the bone marrow the recovery seems to be slower in the "irradiated and bled" group compared to the "irradiated only" group, and this could indicate that the increased demand for erythropoietic cells is responsible for the delay in the self-regeneration of the CFU-S. The BFU-E showed the same phenomenon: bled animals had less BFU-E numbers in the femur throughout the observation period. At the CFU-E level there was no difference between the groups with the exception of 1 day after bleeding.

The data from the spleen seem to be more impressive than those from the bone marrow. The spleen, however, contains only 1/6 to 1/10 of the hemopoietic stem cell CFU-S[13] and, as seen in our studies using incorporation of ^{59}Fe in these CBA mice, about 10% of the erythropoietic cell system under steady-state conditions.[14] Furthermore, as seen in Table 1, especially, the CFU-E concentration is very low in the spleen and, therefore, any shift of the erythropoietic cell production to this organ causes a high relative increase. Three days after bleeding, however, it can be calculated from the CFU-E kinetics that the majority of the erythropoietic cell production is located in the spleen.

The discussion of the stem cell numbers in the spleen of mice bled after irradiation is complicated by the high probability of an influx of at least CFU-S, but possibly, also, BFU-E and CFU-GM from the bone marrow. It should be calculated if such a migration to the spleen could be responsible for the lower CFU-S and BFU-E numbers in the marrow as discussed above for the irradiated and the nonirradiated group. The rise in CFU-S and BFU-E numbers in the spleen is delayed and much smaller in the "irradiated and bled" group, and, also, the CFU-E numbers rise and fall later in the spleens of these mice, in contrast to the bone marrow where no difference was seen. This, however, is not seen at the reticulocyte level. A separate problem concerns the CFU-GM compartment. Although there is only a limited need for granulopoietic cells after bleeding, the CFU-GM showed a similar rise in the spleen like CFU-S and BFU-E and this rise is delayed, but of the same magnitude, in preirradiated mice.

In summary, the data indicate that the hemopoietic system regenerates preferentially cells of the functional cell pool — the erythrocytes in this study. Even after the reduction of CFU-S and BFU-E to less than 10% of controls the hemopoietic stem cells are able to produce enough erythropoietic cells for the recovery from an acute anemia with the same speed as under normal conditions. The data give no information on the mechanisms by which this is achieved; furthermore, one should be aware that the assay systems give information about the number of cells in a specific cell compartment, but so far there is no way to measure cell fluxes.

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Chapter 6

COMBINATION OF IRRADIATION AND BLEEDING — A MODEL ANALYSIS*

H.-Erich Wichmann and Markus Loeffler

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I. ABSTRACT

The effect of bleeding was studied on the recovery of hemopoiesis in irradiated mice. This situation was analyzed by a mathematical model of stem cell regulation. From the analysis only a stimulation of the erythropoietin-sensitive cells by bleeding can be expected, while stem cells and granulopoiesis are nearly unaffected. These results are different to the findings after hypertransfusion which enhances the recovery of stem cells and granulopoiesis after irradiation.

II. INTRODUCTION

The separate influence of anemia and irradiation on hemopoietic stem cell regulation has been investigated intensively. For the combination of both stresses, however, only data from Seidel and Kreja[1] are available. Two days after total body irradiation with 1.5 Gy, their CBA mice were bled.

In the bone marrow the blood loss did not modify significantly the recovery after irradiation. The authors found that CFU-S, BFU-E, and CFU-GM were reduced to about 10% by irradiation and did not normalize within 14 days, while CFU-E recovered within 8 days. The only effect of the additional bleeding was a somewhat delayed increase of CFU-S, compared with the irradiated controls.

In contrast to this, the cell numbers in the spleen show a clear stimulation of all progenitors by the additional anemia which increased up to 70 times normal. However, the spleen does not contribute very much to total hemopoiesis in the investigated animals.[2] In normal steady state less than 2% of the CFU-S and less than 1% of the CFU-E are found in the spleen of CBA mice. In irradiation plus anemia, the splenic fraction of the total hemopoietic system does not exceed 3%[1,2] CFU-S and 4% for the progenitor cells.[2]

In the following, the pooled data of bone marrow and spleen will be compared with the model calculations on irradiation and on the combination of irradiation and bleeding anemia. Data on blood loss without irradiation, which also have been measured by Seidel and Kreja[1] in the same experiment, are considered separately.[3] It should be mentioned that this experiment (together with a similar experiment on the combination of irradiation and hypertransfusion) has been stimulated by a theoretical prediction in 1980.[4] The experiment has been designed and the protocol has been optimized using an earlier version of the stem cell model.[4,5] We are very obliged to Hans Seidel and Ludwika Kreja for this fruitful cooperation.

III. MATHEMATICAL METHODS

The combination of irradiation and anemia is simulated by the mathematical model of stem cell regulation as described.[6-8] Since in the experiment the animals were bled 2 days after irradiation, the model simulation starts not on day 0 but on day 2. The effect of irradiation is considered by the reduction of the initial values in the cellular compartments. For S, BE, CE, and CG these have been taken from the measurements of CFU-S, BFU-E, CFU-E, and CFU-GM: S = BE = CG = 0.1, CE = 0.4 (irradiation) and S = BE = CG = CE = 0.1 (irradiation + bleeding). For other compartments no data are available, and the initial values are chosen within the limits known for acute irradiation,[9] being equal in both cases (E = G = 0.3). The additional anemia is simulated by a theoretical curve for erythropoietin (EP) which corresponds to a blood loss of 35%.[3]

IV. RESULTS

A. Model Calculations

Figures 1 to 9 show the model calculations for irradiation and the combination of irradiation and bleeding.

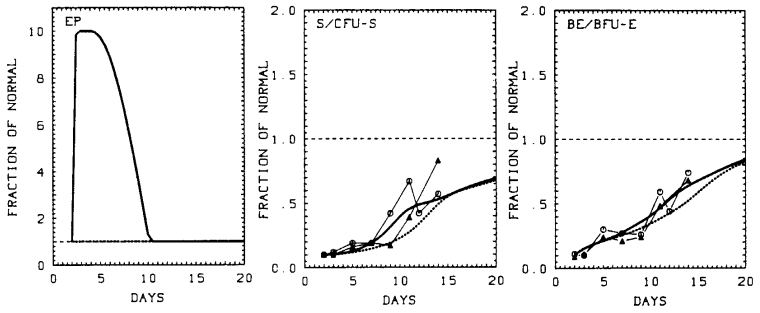

FIGURE 1 FIGURE 2 FIGURE 3

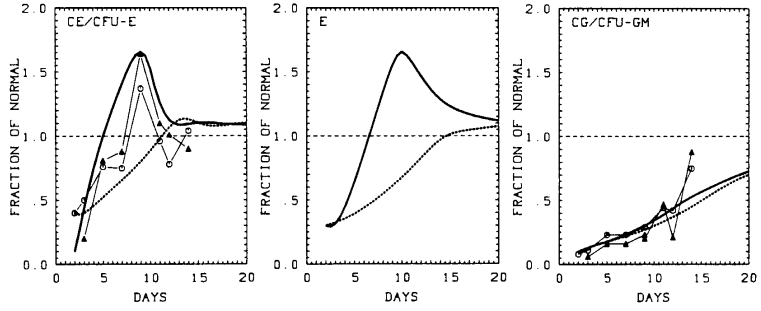

FIGURE 4 FIGURE 5 FIGURE 6

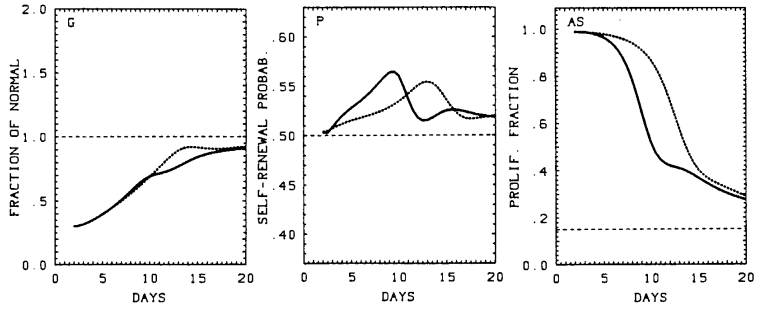

FIGURE 7 FIGURE 8 FIGURE 9

FIGURES 1 to 9. Combination of irradiation and bleeding. Comparison of model results (curves) and experimental data (symbols). The data from Seidel and Kreja represent total hemopoiesis (bone marrow plus spleen) in CBA-mice. The animals were irradiated on day 0 and bled on day 2. ○, - - -: irradiation of 1.5 Gy; ▲, —: irradiation of 1.5 Gy and bleeding. The stimulation of erythropoiesis by anemia is simulated by a theoretical curve for erythropoietin (EP) in Figure 1 (—) which is used as input for the model.

The curves for irradiation (dashed lines) exhibit the characteristics already discussed:[9] S, BE, and CG recover slowly, reaching only 70 to 80% of normal within 20 days (Figures 2, 3, and 6). The more mature cells (CE, E, G) return to (or close to) normal within 15 days (Figures 4, 5, and 7). This difference between early and late cells is caused by the accelerated cycling rate of the early progenitors during irradiation recovery.

The additional anemia (full lines) stimulates the erythropoietic amplification in CE and E (Figures 4 and 5). Therefore, these cells recover significantly quicker than in the irradiated controls (within 4 to 6 days) and show a clear overshoot. CE and E decrease as soon as the erythropoietic stimulus disappears. The recovery of the other cells (S, BE, CG, and G, Figues 2, 3, 5, and 7) is nearly not influenced by the anemia.

B. Comparison with Data

CFU-S, BFU-E, and CFU-GM show nearly the same recovery as their theoretical counterparts S, BE, and CG: they increase slowly and no clear difference can be found between irradiation and irradiation plus anemia. In contrast to these cells the CFU-E recover much earlier and show an overshoot. This corresponds to the findings for CE after the additional anemia. Surprisingly, the overshoot of CFU-E is also found in the irradiated controls which is not reproduced by CE.

V. DISCUSSION

For stem cells and erythropoietin-insensitive cells no severe influence of bleeding on hemopoietic recovery after irradiation can be demonstrated either in experimental data or in theoretical calculations. The numbers of these cells increase slowly and the recovery curves are not modulated by the anemic stimulation.

For erythropoietin-sensitive cells, however, a significant acceleration of recovery due to bleeding should be theoretically expected. This is demonstrated by the curves for CE and E which show a slow normalization after irradiation (within 10 to 15 days) but a steep recovery (within 4 to 6 days) with a significant overshoot on day 10 if anemia is added.

In the experimental curves for CFU-E, the early recovery with an overshoot on day 10 is already found for irradiation alone, and bleeding leads only to a slightly higher maximum. This discrepancy between theory and experiment remains obscure.

One possible explanation may come from most recent investigations[10,11] on erythropoiesis in CBA mice (which have been used in the experiment discussed here). The red cell life span in these animals is shorter, the hematocrit is lower, and the serum erythropoietin titer is significantly higher in CBA mice than in C57Bl mice. CBA mice can almost be considered to have a "compensated hemolytic anemia" with reference to C57Bl animals.[10]

If the progenitors and precursors of CBA mice are destroyed by irradiation, they may develop an anemia earlier than other strains. The unexpected peak of CFU-E after irradiation may be the consequence of this peripheral stimulus. On the other hand, the additional bleeding may not allow a further increase of erythropoietic amplification since the normal operating point of erythropoietin may be already elevated in CBA mice.

Additional data for the combination of irradiation and anemia are only available following bone marrow transplantation.[12,13] They are not directly comparable with the curves discussed above. However, they also seem to indicate that anemia has only small effects.

In summary, the addition of bleeding to an acute irradiation does not markedly influence the recovery of stem cells or granulopoiesis. This result is different from the addition of hypertransfusion which ameliorates the recovery of stem cells and granulopoietic cells in the irradiated animals.[14,15]

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Chapter 7

COMBINATION OF IRRADIATION AND HYPERTRANSFUSION — EXPERIMENTAL RESULTS

Francis C. Monette, Roy C. Ziegelstein, and Michael J. Hunter

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I. ABSTRACT

The present work was undertaken to test whether a reduced demand for erythropoietic cell differentiation affects the growth kinetics of hemopoietic stem cells when applied following exposure to low levels of radiation (1.5 Gy). Mice were first irradiated and then hypertransfused with erythrocytes 2 to 4 days later. During the subsequent 3 weeks, tibial marrow CFU-S, 7-day BFU-E, and CFU-E were determined by established assays. The results indicate that pluripotent stem cell (CFU-S) recovery following 1.5 Gy is essentially unaffected by erythrocyte hypertransfusion. In contrast, marrow recovery of erythropoietic cell progenitors was affected by hypertransfusion, but in a differential manner. Recovery of BFU-E was substantially depressed by hypertransfusion throughout the observation period and remained well below plethoric control levels. In contrast, CFU-E generally remained well above plethoric control levels, although CFU-E recovery was also depressed by the hypertransfusion. We, therefore, conclude that erythrocyte hypertransfusion exerts no beneficial effect on pluripotent stem cell and erythropoietic progenitor cell recovery following low levels of gamma radiation.

II. INTRODUCTION

The hemopoietic system has long been known to be highly radiosensitive. Following exposure to medium-to-low levels of gamma radiation, cellular parameters in both marrow and spleen undergo a precipitous decline. This is followed by a rapid, dose-dependent regeneration.[1-3] In the 1960s, several laboratories documented the ability of pluripotent hemopoietic stem cells (HSC) to restore hemopoiesis in lethally irradiated mice injected with normal bone marrow cells. Although the growth of HSC, as determined by the spleen colony assay (i.e., CFU-S[4]), was shown not to be directly influenced by exogenous factors such as erythropoietin,[5,6] a variety of agents or experimental procedures were shown to indirectly affect the growth kinetics of HSC. For example, Boggs and co-workers[7,8] demonstrated that exposure to bacterial endotoxin or loss of blood could alter the observable levels of splenic colonies when administered *prior* to but not immediately following irradiation. In addition, both procedures substantially increased radiation survival and the HSC response to blood loss was observed to be spleen-dependent. Later studies[9,10] showed that the loss of blood could enhance the splenic regeneration of marrow-derived HSC as determined by both the endogenous[9] and exogenous[10] colony assay methods. In the latter system, although the growth of *splenic* colonies was enhanced by bleeding, *marrow* CFU-S were reduced by this procedure.[7] Interestingly, the effect of bleeding appeared temporally related to the irradiation since the greatest splenic enhancement of CFU-S was observed when animals were bled within a few hours of radiation exposure.

In most of the above studies, experimental animals were exposed to high levels of radiation including lethal doses followed by a marrow cell transplant. Since such high levels of radiation (e.g., >5 Gy) may have seriously compromised the compensatory mechanisms normally responsible for reestablishing hemopoiesis, it may be more meaningful to examine the effects of erythropoietic manipulations following *low* levels of gamma radiation. Exposure to 1.5 to 2 Gy of total body radiation has been shown to quickly reduce the marrow levels of early hemopoietic progenitors, including HSC, while permitting a rapid and complete recovery of all hemopoietic cell compartments.[2,3,11] When radiation is combined with manipulations of the erythron (i.e., bleeding or plethora), feedback upon the HSC compartment might be expected to occur in one of several possible ways. If "competition" is operative at the level of the HSC, either increasing or decreasing a single pathway of cellular differentiation might impart a reciprocal effect on other cell lines.[12-15] Recently, Smith et al.[16,17] showed that erythrocyte hypertransfusion of sublethally irradiated animals substantially augmented marrow granulopoietic and megakaryocytopoietic recoveries when compared to irradiated controls. These and other [18,19] studies suggest the possibility that hypertransfusion may augment the number of HSC available for differentiation into other cellular pathways. However, a mechanistic explanation for the stem cell effect is still lacking. Factors other than a direct competition at the level of the pluripotent stem cell may be operative in the regulation of HSC. For example, regulatory feedback may occur via more-mature cells in a cell differentiation pathway.[20-22] The recently developed mathematical model of Loeffler and Wichmann,[21] for example, suggests a regulatory feedback upon HSC via mature erythropoietic progenitors (e.g., CFU-E).

The present work was undertaken to test whether a reduced demand for erythropoietic cell differentiation affect the growth kinetics of HSC when applied *following* an exposure to low level radiation (1.5 Gy). The marrow frequencies of CFU-S, primitive erythropoietic cell progenitors (7-day BFU-E), and mature erythropoietic progenitors (CFU-E) were followed over a 3-week recovery period. The results of these preliminary investigations suggest that HSC recovery in irradiated hypertransfused animals is *not* enhanced by the reduced demand for red cell production and, indeed, may actually be reduced in these animals relative to irradiated controls. In contrast to CFU-S, the regeneration of primitive erythropoietic progenitors (7-day BFU-E) was substantially decreased in plethoric, irradiated animals, suggesting possible feedback upon these cells.

III. MATERIALS AND METHODS

Both CD_1 and BDF_1 strain female mice (Charles River Labs, Wilmington, Mass.) were used with an age range of 10 to 14 weeks. All animals were maintained on antibiotic (neomycin sulfate/tetracycline) acidified water for at least 7 days prior to experimentation.

A. Experimental Procedures

Three to five mice were used for each data point. Total body irradiation was performed via exposure to a ^{137}Cesium gamma source. Mice were irradiated in groups of 16 at a dose rate of 0.87 Gy/min. Preliminary studies were conducted in each mouse strain to determine the optimal radiation dose needed to reduce medullary CFUs by ~90% within 2 days but permitting ~50% recovery within 12 days of irradiation. In five separate studies an optimal dose of 1.5 Gy was obtained. Thus, mice of either strain were exposed to 1.5 Gy and groups of four sacrificed at daily intervals thereafter. In two separate studies, additional groups of 1.5 Gy-irradiated mice were given two consecutive erythrocyte hypertransfusions on either days 2/3 or 3/4 following irradiation. Normal mice served as RBC recipients for the plethoric controls. Erythrocyte hypertransfusion was conducted as follows. Three-month-old syngeneic mice were exsanguinated via cardiac puncture with heparin and the blood from 75 to 125 donors pooled. Blood plasma was removed via centrifugation and the packed cells washed in five volumes of sterile, endotoxin-free saline (Difco Co., Detroit) three consecutive times. Following the last saline wash, peripheral blood cells were depleted of visible buffy-coat cells by aspiration. The erythrocytes were then diluted to a hematocrit of ~70% with saline and 1 mℓ was injected (i.p.) per recipient animal. The entire procedure was again repeated 24 hr later. As indicated elsewhere in this volume,[23] this procedure results in an elevation of the blood hematocrit by ~50% within 5 days of the last erythrocyte injection.

B. Cell Preparation

Marrow cells from three to five donor animals were collected by flushing the contents of both femurs and pooling. Cells were suspended in ice-cold Alpha medium (Flow Laboratories, Rockville, Md), supplemented with 2% fetal calf serum, nonessential amino acids, sodium pyruvate, L-glutamine, and penicillin/streptomycin as previously described.[24] The

total tibial cellularity for each cell donor was determined by flushing the tibial contents and counting either electronically or via hemacytometer.

C. CFU-S Assay

The method of Till and McCulloch[4] was employed. Briefly, the pooled marrow cells were washed in culture medium, enumerated by hemacytometer, and diluted prior to injection (i.v.) into lethally irradiated syngeneic recipients (^{137}Cesium: 8.5 Gy, CD_1 mice; 8.1 Gy, BDF_1 mice). This radiation level was sufficient to reduce endogenous colony formation in uninjected controls to <0.05 colonies per spleen. A minimum of 12 irradiated mice per group served as cell recipients. Nine days following cell injection spleens were removed and fixed in Bouin's solution prior to enumeration of macroscopic surface colonies. The colony counts of at least two independent observers were averaged and from these data the average CFU-S content per tibia was calculated without consideration of the seeding factor.[25]

D. In Vitro Assay of Erythropoietic Progenitor Cells

The pooled, washed marrow cells were also assayed for primitive (7-day BFU-E) and mature (2-day CFU-E) erythropoietic progenitors utilizing the microplasmaclot method developed by McLeod et al.[26] as recently described by us.[24] The culture system consisted of 10.0% bovine serum albumin, 2.0% beef embryo extract, 10% citrated bovine plasma, 0.2 mg L-asparagine per milliliter, and either 20 or 25% fetal calf serum for CFU-E and BFU-E, respectively. A concentration of 10^{-4} *M* 2-mercaptoethanol was employed for both cell assays. Pretested tissue culture water (Difco Laboratories) was employed as the diluent throughout. Sheep plasma erythropoietin (step III, Connaught Laboratories, Willowdale, Can.; 5 to 17 IRP units per milligram protein) was used at a final concentration of either 0.25 units (CFU-E) or 2.0 units (BFU-E) per milliliter. Normal marrow cells were cultured in microtiter wells (Cooke Engineering Co., Alexandria, Va.) at final concentrations of 25 $\times$ 10^4 and 10^6/mℓ for CFU-E and BFU-E, respectively. Slightly higher cell concentrations were used following irradiation. For normal marrow samples, colony efficiency usually ranged between 300 and 400 CFU-E and 8 to 15 BFU-E per 10^5 nucleated cells. No conditioned media (as a source of burst-promoting activity) was added to the cultures. Cultures were harvested following either a 2-day (CFU-E) or 7-day (BFU-E) incubation at 37°C in a mixture of 3 to 4% CO_2 in humidified room air. Colony-scoring criteria involved a minimal threshold of eight cells for CFU-E and 50 cells for BFU-E as previously established.[26,27] Eight separate cultures were scored per data point for CFU-E and six to eight for BFU-E.

E. Statistics

The Student's *t*-test on nonpaired independent variables was used to compare control groups to experimental groups. Results are expressed as probability (*p*).

IV. RESULTS

The range of marrow values observed in this series of studies for three different colony-forming cell types in two separate strains of mice is summarized in Table 1. CFU-S values were not corrected for "f", the seeding factor. In addition, BFU-E were grown without added growth factors (i.e., BPA). Since both murine strains responded to irradiation and hypertransfusion in a similar fashion, the experimental results with either strain will be described. Within a few days following exposure to 1.5 Gy total body radiation, tibial cellularity declined slightly from the control level of 10.0 $\pm$ 0.2 $\times$ 10^6 cells but thereafter quickly recovered (Table 2). A similar cellular response was observed in irradiated animals hypertransfused several days following exposure to 1.5 Gy. In this latter group, the blood

Table 1
MARROW STEM CELL VALUES IN TWO STRAINS OF NORMAL MICE[a]

	Number per tibia		
	Observed range	(N)[b]	Mean ± SEM
BDF_1 mice			
CFU-S[c]	1,770—3,215	(7)	2,437 ± 186
7-day BFU-E[d]	227—1,036	(9)	581 ± 107
CFU-E	21,432—38,115	(9)	29,603 ± 2,440
CD_1 mice			
CFU-S	1,695—4,076	(7)	2,737 ± 518
7-Day BFU-E	809—1,189	(6)	949 ± 54
CFU-E	20,633—43,568	(14)	29,904 ± 2,870

[a] Age range of 10—14 weeks.
[b] N = number of separate determinations (experiments).
[c] Numbers are not corrected for splenic seeding.
[d] Grown without added BPA.

Table 2
RECOVERY OF TIBIAL NUCLEATED CELLULARITY FOLLOWING 1.5 Gy TOTAL BODY RADIATION WITH AND WITHOUT ERYTHROCYTE HYPERTRANSFUSION[a]

	Total nucleated cells per tibia ($\times$ 10^{-6})			
Time following irradiation (days)	Normal	1.5 Gy	1.5 Gy + RBC	Hematocrit[b] (1.5 Gy + RBC)
4	10.2	6.9	8.0	150
7	10.1	10.0	8.1	176
11	9.9	9.6	9.1	142
14	9.5	9.4	8.8	155
18	9.4	9.3	7.6	154
21	10.6	8.8	10.9	136
25	10.1	—	8.5	128
Mean ± SEM:	10.0 ± 0.2			

[a] Performed in female BDF_1 mice, 10—14 weeks of age, 3 to 5 mice per data point. Mice were hypertransfused with RBC on days 3 and 4 following exposure to 1.5 Gy. Similar responses were obtained in three additional studies with radiation alone and in an additional study where radiation was combined with erythrocyte hypertransfusion.
[b] Expressed relative to normal controls.

hematocrit remained well above normal for the duration of the study (Table 2). The marrow pluripotent stem cell (CFU-S) recovery pattern following 1.5 Gy is shown in Figure 1. This level of radiation reduced marrow CFU-S by an average value of 87% within 2 days. This stem cell response was observed in both strains of mice examined. Normal marrow CFU-S values were not observed until ~18 days following 1.5 Gy after which they again declined. As previously noted (see Chapter 1), the injection of erythrocytes alone produces a significant expansion in the size of the marrow stem cell compartment (Figure 1). However, when erythrocyte hypertransfusion (either days 2/3 or 3/4 following irradiation) is combined with an exposure to 1.5 Gy, marrow CFU-S are reduced by the radiation and recover at a rate

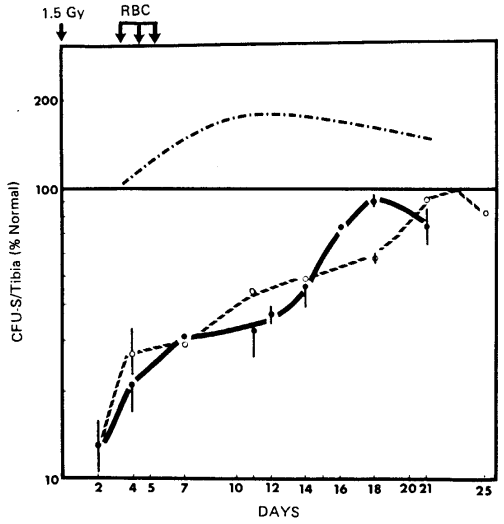

FIGURE 1. Recovery of marrow CFU-S following exposure to either 1.5 Gy (150 R) alone (●—●) or in combination with erythrocyte hypertransfusion (RBC) (○- - -○) expressed relative to CFU-S levels in normal marrow. The tibial CFU-S response to hypertransfusion alone is shown for comparison (■-■). The difference between irradiated control and irradiated plus hypertransfused group at day 18 was significant (p) ≦ 0.01). Data represent the average of a total of five 1.5-Gy control studies with CD_1 mice and two with BDF_1 mice. Two separate hypertransfusion studies are summarized with BDF_1 mice. Standard errors are shown for data representing the mean of at least two separate determinations.

similar to the irradiated controls only during the first 14-day interval. Although CFU-S levels in irradiated controls appeared to return to normal by day 18, full recovery was not observed in hypertransfused irradiated mice until sometime later, perhaps as late as day 22 to 23 following the irradiation (Figure 1). The difference between the 1.5 Gy control and hypertransfused groups was significant (p ≦ 0.01) at this point in time (18 days).

In contrast to pluripotent stem cells, primitive marrow erythropoietic progenitors (7-day BFU-E) were reduced by only ~40% when compared to normal marrow control levels within 4 days of the irradiation (Figure 2), following which they gradually returned to normal. Although it has been previously shown that 7-day BFU-E are not dependent upon erythropoietin in vivo and, indeed, their numbers actually rise following the induction of plethora[23] (see also Figure 2), 7-day BFU-E were substantially reduced in number when hypertransfusion was combined with a 1.5-Gy radiation (Figure 2). Indeed, irradiated plethoric marrow BFU-E remained well below the 50% level for 2 weeks following 1.5 Gy in marked contrast to both the 1.5-Gy and erythrocyte controls. Full recovery of BFU-E was not observed until days 21 to 25 following the irradiation. The difference in the marrow levels of 7-day BFU-E between the 1.5-Gy group and the hypertransfused, irradiated groups was significant (e.g., p ≦ 0.05 on days 11 and 18).

More-mature erythropoietic progenitors (CFU-E) require erythropoietin in vivo so when mice are hypertransfused with erythrocytes, marrow CFU-E levels fall rapidly to a nadir of

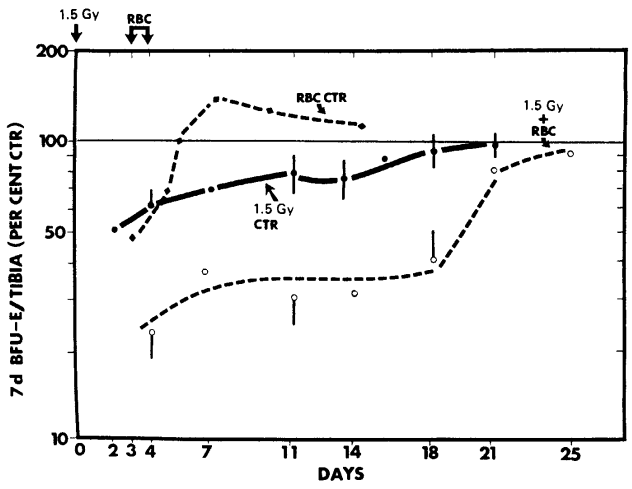

FIGURE 2. Recovery of marrow BFU-E following exposure to either 1.5 Gy (150 R) alone (●—●) or in combination with erythrocyte hypertransfusion (RBC) (○- -○) expressed relative to BFU-E levels in normal marrow. The tibial BFU-E response to hypertransfusion alone is shown for comparison (◆-◆). The difference between irradiated control and irradiated plus hypertransfused groups at days 11 and 18 was significant (p ≦ 0.05). Data represent the average of a total of two 1.5-Gy control studies with CD_1 mice and two with BDF_1 mice. Two separate hypertransfusion studies are summarized, one with each strain of mouse. Standard errors are shown for data representing the mean of at least two separate determinations.

~25 to 30% of the normal control value (Figure 3) (see also, Reference 23). Although CFU-E are substantially reduced by an acute exposure to 1.5-Gy total body radiation (our unpublished data indicates an ~88% reduction within 1 hr of a 1.5-Gy irradiation), marrow CFU-E levels recover very rapidly to overshoot control levels within 2 days (data not shown). At day 4 following 1.5 Gy, marrow CFU-E levels still exceed controls but decline thereafter only to again return to control levels between days 14 and 21 (Figure 3). However, when irradiated mice were hypertransfused on either days 2/3 or 3/4, marrow CFU-E declined and remained at a level between 30 and 60% of normal for the duration of the study. This CFU-E population size was substantially lower than that observed in the irradiated control group but well above the marrow level observed in the plethoric controls (Figure 3).

V. DISCUSSION

During the early time intervals following a sub-lethal irradiation, surviving stem cells are called upon to repopulate principally the erythropoietic and granulopoietic lines of cellular differentiation. By eliminating a major pathway of cellular differentiation (e.g., with erythrocyte hypertransfusion), one could anticipate one of several possible effects at the stem cell level. First, if stem cell competition is a major facet of hemopoietic cell regulation, enhancement of HSC regeneration in hypertransfused irradiated animals might occur, reflecting the reduced proliferative demand on the HSC and thus their enhanced self-renewal. A second possible HSC response to hypertransfusion would give growth curves identical to the irradiated controls. In this case, one might envision a situation characterized by a maximal

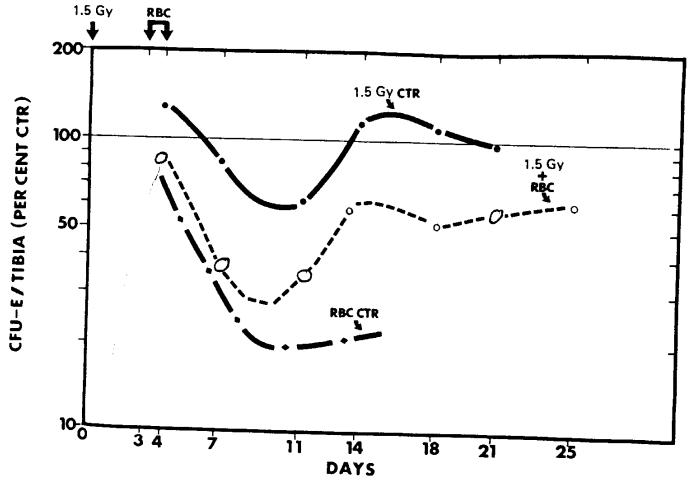

FIGURE 3. Recovery of marrow CFU-E following exposure to either 1.5 Gy (150 R) alone (●—●) or in combination with erythrocyte hypertransfusion (RBC) (○- - -○) expressed relative to CFU-E levels in normal marrow. The tibial CFU-E response to hypertransfusion alone is shown for comparison (◆–◆). Data are derived from a single experiment with BDF_1 mice but is typical of many others in both CD_1 and BDF_1 mice.

demand for both HSC self-renewal and differentiation regardless of the erythropoietic manipulation. A third HSC response might be one in which stem cell regeneration actually lags in hypertransfused, irradiated animals in contrast to irradiated controls.

The results of this investigation clearly eliminate the first of these three possible HSC responses: the data do not provide any evidence for an enhanced HSC recovery in irradiated mice whose demands for erythrocyte production have been greatly reduced by hypertransfusion. Although HSC regenerated at a similar rate during the first 2 weeks following 1.5 Gy, hypertransfused irradiated animals exhibited a significantly reduced HSC recovery rate at later time intervals (day 18) when compared to that of irradiated controls. Although this late-phase reduced HSC recovery was observed in two separate studies it should, perhaps, be further verified. In any case, the data are consistent with the interpretation that hypertransfusion does not *enhance* HSC recovery following sublethal irradiation. This is in contrast to the enhancing effect observed following various regimes of irradiation and bleeding.[7-10,28] In the latter case, the spleen plays an important role in the hemopoietic expansion, including that of the HSC compartment. However, there appears to be no significant splenic role in the early (i.e., first 2-week) stem cell response to low levels of gamma radiation alone.[29] The studies of Smith et al.[16,17] suggested an augmented recovery of HSC in hypertransfused sublethally irradiated animals, at least as far as the availability of HSC for differentiation is concerned. The results of the present study would suggest that if erythrocyte hypertransfusion enhances HSC radiation recovery, the available stem cells must be channeled into nonerythropoietic pathways of differentiation and are not utilized for HSC self-renewal per se. This preferential channeling of HSC into cell differentiation may reflect the already-large relative stem cell pool size.[30]

In marked contrast to the results with CFU-S, the BFU-E data indicate that plethora significantly reduces the rate of primitive erythropoietic progenitor cell recovery following

irradiation. The reduced BFU-E level cannot be attributed to the hypertransfusion-induced abrogation of erythropoietin levels, since in the unirradiated plethoric controls marrow BFU-E levels actually superseded normal controls (Figure 2). An alternative explanation for the reduced BFU-E regeneration might be found in the ability of these cells to "sense" the diminished demand for erythropoietic cell production. Likewise, late erythropoietic progenitors (CFU-E) also showed a diminished recovery in irradiated animals following hypertransfusion (Figure 3). However, CFU-E are known to be highly erythropoietin-dependent in vivo, consequently, much of the observed reduction might be attributed to the hypertransfusion-induced reduction in erythropoietin titers. This is only a partial explanation, however, since marrow CFU-E levels were consistently higher in *irradiated*, hypertransfused animals than in the plethoric controls.

Figure 4 summarizes the extent of correlation between the tibial levels of three progenitor cell types following exposure to 1.5 Gy or the combination of irradiation plus erythrocyte hypertransfusion. Although a slight positive correlation appears between the marrow levels of CFU-S and BFU-E in both experimental groups (Figure 4A), none is observed between CFU-E and CFU-S in either group (Figure 4B). In contrast to the above, these same marrow cell preparations gave a good correlation between the levels of CFU-E and their (distant) progenitors, 7-day BFU-E (Figure 4C). This correlation was observed in cell preparations containing both high (1.5 Gy) and low (1.5 Gy plus RBC) levels of BFU-E. These results appear to suggest a lack of correlation between the marrow frequencies of mature erythropoietic precursors (CFU-E) and their pluripotent progenitors (i.e., CFU-S) in an experimental situation where the demand for hemopoietic cell proliferation, in general, is high (1.5-Gy groups), but the demand for erythropoietic cell differentiation is low (irradiated plus hypertransfused groups).

ACKNOWLEDGMENTS

The research reported herein was supported by USPHS Grant AMDD-17735 from the NIH. F.C.M. is the recipient of a NIH Research Career Development Award (No. 1-K04-AM00200). The use of the ^{137}Cesium animal irradiation facility was made available by Dr. Jiri Palek of St. Elizabeth's Hospital, Tufts University School of Medicine, Medford, Mass. and is gratefully acknowledged.

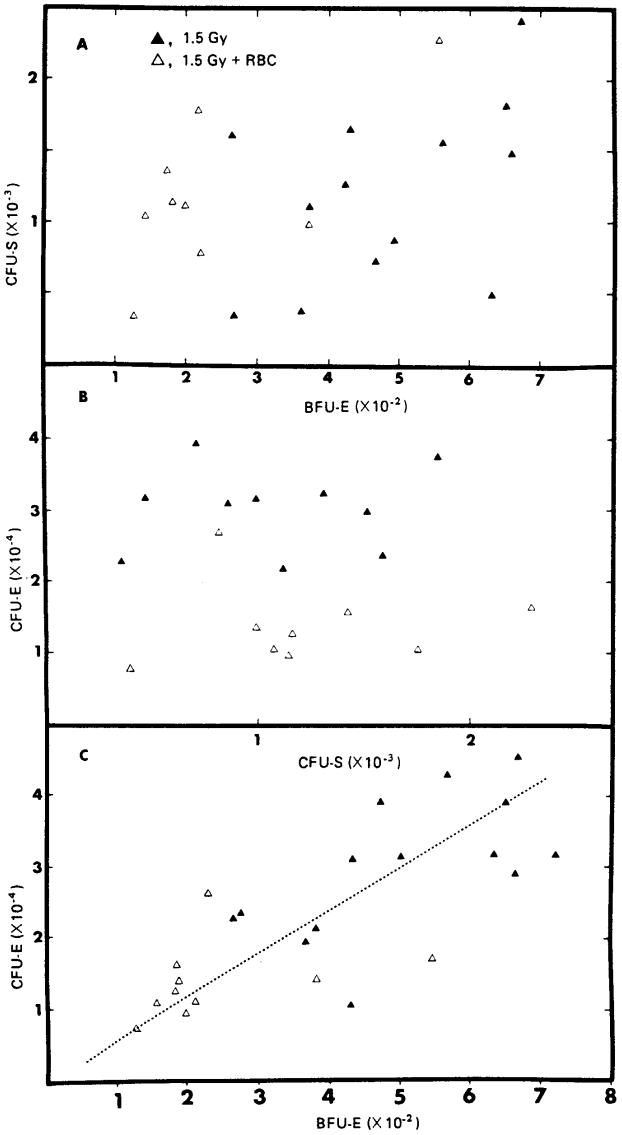

FIGURE 4. Correlation of tibial levels of cell progenitors following 1.5-Gy (150 R) total body irradiation (▲) or in combination with erythrocyte hypertransfusion (△). Data derived from Figures 1 to 3. (A) Compares CFU-S and BFU-E levels; (B) compares CFU-S and CFU-E levels; (C) compares CFU-E and BFU-E levels. The dashed line in C was drawn by eye.

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Chapter 8

COMBINATION OF IRRADIATION AND HYPERTRANSFUSION — A MODEL ANALYSIS*

Markus Loeffler and H.-Erich Wichmann

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I. ABSTRACT

The combination of irradiation and hypertransfusion with red cells leads to complex reactions of stem cells, erythropoietic and granulopietic progenitors, and precursors. The experimental findings have been analyzed by a mathematical model of stem cell regulation. The analysis suggests that the suppressed erythropoiesis stimulates the turnover rate of stem cells by intramedullary feedback. The resulting increased cell flux enlarges the granulopoietic cell compartments. This beneficial influence of hypertransfusion on granulopoiesis does not become effective immediately after irradiation but only after the stem cell number has recovered to a certain level.

II. INTRODUCTION

Experiments on a combination of irradiation and hypertransfusion have been performed mainly to find an effective mechanism of radioprotection. Indeed, it has been reported by several investigators that granulopoietic recovery after irradiation benefits from suppressed erythropoiesis.[1-4] This could either be achieved by hypertransfusion before[1,2] or after [3,4] the irradiation event or by a period of hypoxia preceding irradiation.[1,2] In any case, the number of granulopoietic progenitors and precursors in the bone marrow[1-4] and the number of blood granulocytes[4] recovered quicker and reached higher values. By some authors[3,4] these results have been considered as evidence for a stem cell competition concept with regulation of the determination process.

The experimental findings have been reviewed by Monette et al.[5] They can be summarized as follows, comparing the combination of irradiation and hypertransfusion with the effects of irradiation without hypertransfusion:

- The recovery of CFU-S from irradiation is hardly influenced by the additional hypertransfusion.
- In the combination experiment BFU-E recover slower than in the irradiated controls.
- CFU-E and erythropoietic precursors are suppressed by the additional hypertransfusion.
- Granulopoietic progenitors and precursors recover earlier and to a higher level if the irradiated mice are hypertransfused.

In the following we shall mathematically investigate the complex interaction of irradiation and hypertransfusion with a view to understanding the hemopoietic response. It should be noted that the experiment of Monette et al.[5] has been performed to improve the data base for such an analysis. The experiment has been designed together with a similar experiment on combination of irradiation and bleeding, and both protocols have been optimized using an earlier version of the stem cell model.[6,7] We are very obliged to Francis Monette and his group for this cooperation.

III. MATHEMATICAL METHODS

The combination of acute irradiation and red cell hypertransfusion is simulated using the mathematical model of stem cell regulation.[8-10] Since, in these experiments, the animals were hypertransfused 1 to 3 days after irradiation, the model simulation starts not on day 0 but on day 3. The measured values on day 3 are taken as initial values; missing values are chosen according to the rules developed for acute irradiation.[11]

Two experiments are simulated.

A. Experiment 1

In the experiment of Monette et al.,[5] mice have been irradiatd with 1.5 Gy on day 0 and hypertransfused on days 2 and 3. For S, BE, and CE the measurements of CFU-S, BFU-E, and CFU-E on day 3 are taken as initial values: S = 0.2, BE = 0.6, CE = 1.3 (irradiation) and S = 0.2, BE = 0.2, CE = 1.0 (irradiation + hypertransfusion).

For the other compartments, where no data are available, the initial values are chosen within the limits known for acute irradiation[11] as CG = G = 0.2 and E = 1 for both situations. Hypertransfusion is simulated by a theoretical curve of erythropoietin (EP) with reduced values for 10 days and recovery within 25 days (Figure 1), in the same way as described earlier.[12]

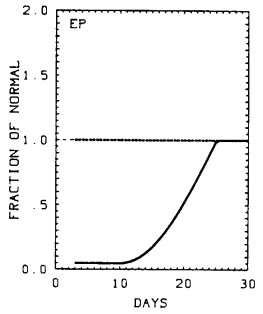


FIGURE 1. Combination of irradiation and hypertransfusion. Theoretical curves for erythropoietin (EP) under irradiation alone (---) and irradiation plus hypertransfusion (—). These curves are used as input for the simulations in Figures 2 to 4 and in Figures 5 to 9.

B. Experiment 2

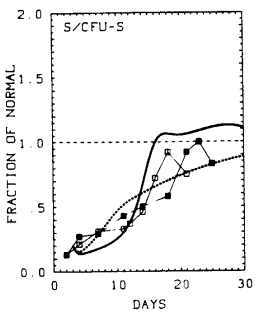
In the experiment of Smith et al.,[4] mice have been irradiated by 3.5 Gy on day 0 and hypertransfused 1 day later. Here the initial values are E = 0.2, CG = 0.1, G = 0.3 (irradiation) and E = 0.05, CG = 0.1, G = 0.3 (irradiation + hypertransfusion) according to the measurements of CFU-GM, erythropoietic, and granulopoietic precursors on day 3. For the other compartments, where no data are available, the initial values are chosen as S = BE = CE = 0.1 for both situations. Hypertransfusion is simulated by the theoretical EP curve in Figure 1, as for experiment 1.

IV. RESULTS

A. Model Calculations

Two sets of calculations are presented, simulating the combination of irradiation and hypertransfusion. They refer to two different radiation doses: (1) 1.5 Gy in Figures 2 to 4 and (2) 3.5 Gy in Figures 5 to 9. Despite the different degree of radiation damage, the model simulations of both experiments show the same characteristics.

Irradiation significantly reduces the number of early hemopoietic cells (dashed lines). During the first 3 days (for which the model curves are not shown), the differentiated cells recover quicker than the stem cells. This phase is characterized by enhanced differentiation, at the expense of stem cell renewal.[11] At day 3, the stem cells cycle fast (Figure 9) and the self-renewal probability "p" starts to increase (Figure 8). During the following 4 weeks, the compartments return to normal: the stem cells recover slowly (Figure 2), the early progenitors somewhat quicker (Figures 3 and 6), and the precursors rapidly (Figures 5 and 7).



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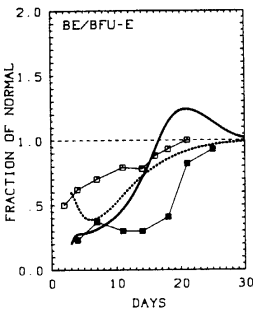

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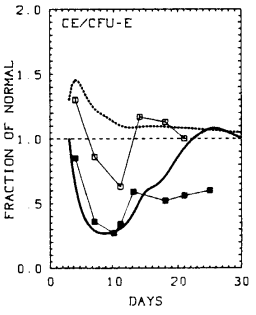
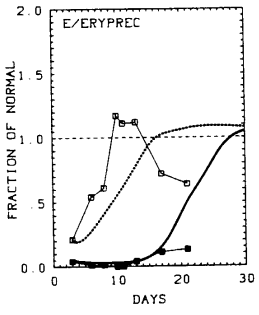


FIGURE 4

FIGURES 2 TO 4. Combination of irradiation and hypertransfusion. Comparison of model results and bone marrow data of mice from Monette et al.[5] Irradiation (---, □, 1.5 Gy); irradiation plus hypertransfusion on day 2 to 3 (—, ■, 1.5 Gy).



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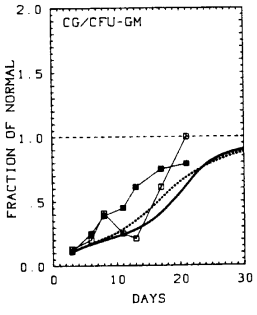

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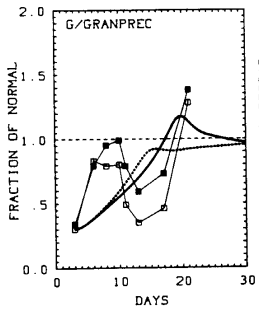

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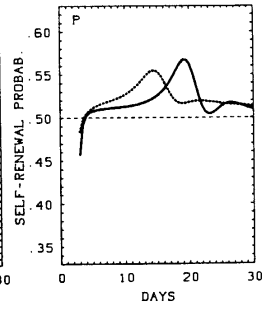

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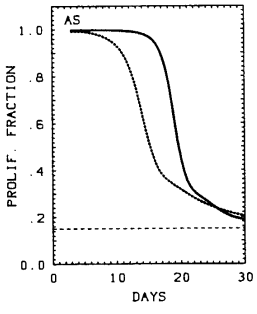

FIGURE 9

FIGURES 5 to 9. Combination of irradiation and hypertransfusion. Comparison of model results and bone marrow data of mice taken from Smith et al.[4] Irradiation (---, □, 3.5 Gy); irradiation plus hypertransfusion on day 1 (—, ■, 3.5 Gy).

The additional hypertransfusion (full lines) reduces the numbers of the EP-sensitive cells CE and E (Figures 4 and 5). This event activates the stem cell cycling (Figure 9) and reduces the self-renewal probability (Figure 8), compared with the irradiated controls. Therefore, one finds initially a slightly delayed recovery in S, BE, CG, and G after hypertransfusion (Figures 2, 3, 6, and 7). After 12 to 20 days, depending on the irradiation dosage, the pattern reverses: "p" now becomes higher for the additional hypertransfusion and the non-EP-sensitive cells recover quicker, compared with the irradiated controls (Figures 2, 3, 6, and 7). This effect may be called the beneficial effect of hypertransfusion for granulopoietic recovery. It is the indirect response of the intramedullary feedback due to the suppressed number of erythropoietic precursors E (Figure 5). The benefit only becomes effective if the stem cell number has reached 50% or more (Figure 2) and disappears as soon as EP returns to normal (Figure 1).

B. Comparison with Data

The model curves can be compared with data for CFU-S, BFU-E, and CFU-E from Monette et al.[5] (Figures 2 to 4) and with measurements for CFU-GM and precursor cells from Smith et al.[4] (Figures 5 to 7).

The following characteristics are reproduced by the model:

- CFU-S recovery does not differ significantly if hypertransfusion is added.
- CFU-E and erythropoietic precursors are suppressed by hypertransfusion.
- The granulopoietic precursors recover to higher values if hypertransfusion is applied to the irradiated animals.

The recovery pattern of BFU-E cannot be reproduced by the model curves. The measurement shows significantly reduced numbers if hypertransfusion is added (Figure 3), similar to the effect on CFU-E and erythroblasts (Figures 4 and 5). In the present model with EP-insensitive BE cells, this pattern cannot be explained.

For the precursors, a second difference between measurements and simulation is found. The experimental peaks on days 8 to 12 are missing in the calculations (Figures 5 and 7).

V. DISCUSSION

It has been demonstrated that the experimental observations can be reproduced by the mathematical model, although some discrepancies remain. Hypertransfusion reduces the number of erythropoietic bone marrow cells. In the interpretation suggested by the model this reduced cell number accelerates the turnover of the stem cells so that more stem cells enter differentiation.

Granulopoiesis profits from the increased influx of cells. This has already been found for hypertransfusion without irradiation.[12] The additional irradiation reduces the effect because stem cells of irradiated animals already cycle at their maximum rate and hypertransfusion cannot accelerate it further. Only after the stem cell number has recovered in part does hypertransfusion become effective. In the above figures this does not happen before day 10.

The model calculations have some practical consequences for the time scale of combined experiments of this type. Hypertransfusion should not start too early, because it has no influence during the first days after irradiation. Thus, the animal should be hypertransfused not before but some days after irradiation.

Furthermore, hypertransfusion should start later if a higher irradiation dose has been applied. A higher dosage reduces the stem cell number more severely and it takes longer to recover. Since the stem cells become responsive to hypertransfusion if they have recovered to about 50%, one has to adapt the time of hypertransfusion to the dosage of irradiation. Of course one can avoid this problem by repeated injections.

The beneficial effect of hypertransfusion has some clinical relevance. It has been demonstrated[13,14] that granulopoietic recovery was improved if children under chemotherapy for acute leukemias were hypertransfused. The number of CFU-GM recovered better[14] and the infection rate decreased.[13]

In summary, this chapter demonstrates how closely erythropoietic and granulopoietic feedback loops are linked together at the stem cell level and that the influence of red cell hypertransfusion on granulopoiesis can be interpreted as a stem cell effect.

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Chapter 9

IRON-55 EXPERIMENTS — EXPERIMENTAL RESULTS: EVIDENCE FOR INTRAMEDULLARY STEM CELL REGULATION

Ursula Reincke and Eugene P. Cronkite

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I. ABSTRACT

Iron-55, a radioactive isotope with extremely short range, caused cytocide of maturing erythroblasts when injected intravenously into mice. Within hours, the cytocide was followed by a corrective differentiation response, consisting of increased terminal differentiation of proerythroblasts, accompanied by a dramatic decrease of CFU-S. Since serum levels of erythropoietin remained normal, the findings were interpreted as evidence for an existing, intramedullary feedback control. As cytocide continued by way of reutilized isotope, the stem cell levels decreased further due partly to continued forced differentiation and partly to internal irradiation. The peripheral blood counts were stable for long periods, indicating regulation which accomplished a steady blood supply in spite of diminishing reserves.

II. INTRODUCTION

The experimental use of iron-55 as a cytocidal agent was the result of a search for a technique with which one could generate pressure for hemopoietic cell differentiation without causing direct damage to stem cells.

Iron-55 (^{55}Fe) is an Auger electron emitter whose particles have a range of 1 μm or less. It also emits X-rays (6 keV) with a 90% tissue range of about 1000 μm. These features offered an opportunity for a cytocidal experiment, i.e., for self-selected killing of erythroblasts by incorporated, ionic ^{55}Fe in bone marrow of isotope-injected mice. The cytocidal process was expected to perpetuate itself as fresh erythroblasts reutilized the isotope from disintegrated cells. The effects of a single dose were expected to be lasting because of the 2.7-year half-life of ^{55}Fe and also because iron is excreted exceedingly slowly.[1]

The experimental interest was focused on the physiological responses to the cytocidal effect. The forced demand for erythroblasts should stimulate differentiation and, concomitantly, the self-renewal activity of hemopoietic stem cells. With restrictions of physical damage to the hemoglobin-synthesizing cells, any changes in the stem cell kinetics should be attributable to physiological causes. The experiment was, therefore, expected to provide insight into the response of stem cells to losses not affecting their own, but a distant daughter population.

In reviewing early effects of an ^{55}Fe injection, this article describes the differentiation response that follows the initial cell kill of erythroblasts. Previously unpublished experiments are included which pertain to the levels of progenitor cell populations, serum erythropoietin, and to the response to ^{55}Fe in polycythemic mice.

The late effects of an ^{55}Fe injection are characterized by gradual loss of stem cells, followed by slow cellular depopulation of bone marrow. Peripheral blood counts over the remaining life span show that daily hemopoietic maintenance was kept stable at reduced levels for long periods and in spite of the gradual bone marrow decline.

III. MATERIALS AND METHODS

A. Isotope

Ionic ^{55}Fe of high specific activity was used so as to achieve sufficient isotope uptake in bone marrow without overload to other organs. We had established previously that up to 1 μg ionic iron could be given per mouse with good reproducibility and minimized shunting of nonutilized iron to the liver.[1] The efficiency of uptake and the reproducibility of the experiment was obviously dependent on the free iron-binding serum capacity which amounted to about 1 μg, and on the specific activity of the isotope.

Batches of "carrier-free", cyclotron-produced isotope were received from ICN (Irvine, Calif.) or from NEN (Boston) with specific activities ranging from 450 to 1300 mCi/mg iron. We stored and injected the isotope as $^{55}FeCl_3$ in 0.01 *N* HCl and used equivalent, stable iron chloride preparations to inject the control animals.

B. Experimental Animals

Random-bred Swiss albino mice or syngeneic C57Bl/J6 mice were used. They were bred and housed at Brookhaven National Laboratory (Upton, N.Y.). They were at least 9 weeks old and weighed at least 18 g when entered into the experiment. Tail vein injections with iron chloride were tolerated without iron poisoning when given very slowly at the rate of 1 μg iron per minute. Unless mentioned otherwise, all animals were previously untreated and received only one injection. They were either sacrificed within hours or days, or kept for long-term observation, in some studies for the rest of their lives.

Most of the experimental techniques were described in detail previously.[2] Some experiments are presented here originally, and their techniques were as follows.

1. Polycythemic Mice

In a first experiment, mice were kept at 0.4 atm in a hypobaric chamber 16 hr daily for 2 weeks, then at atmospheric pressure for 6 days before they were given ^{55}Fe. In a second experiment, mice received two intraperitoneal injections of 1 mℓ packed erythrocytes on day 6 and 7 before the ^{55}Fe or cold-iron injection.

2. Progenitor Cells

In vitro plasma clot bone marrow cultures[3] were set up with four to six petri dishes per point, using inoculates from 15,000 to 75,000 cells and erythropoietin additions from 1.5 to 10 units per milliliter. The most consistent results were found with 30,000 cells and with 2.5 to 5 units erythropoietin.

3. Erythropoietin

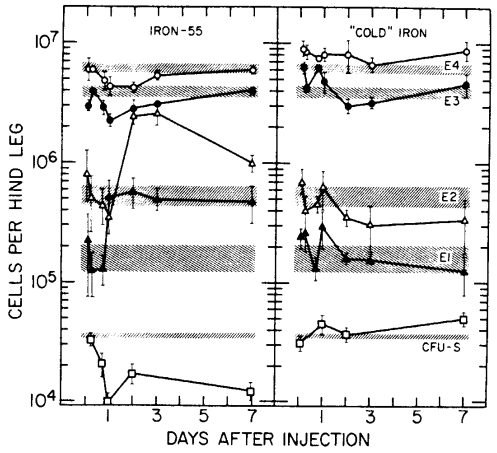
Erythropoietin was determined by radioimmunoassay, using a human erythropoietin standard preparation.[4] The measured blood loss was achieved by bleeding from the orbital sinus with thin, marked micropipettes under light ether anesthesia. Each mouse was bled twice. The first bleeding of 300 μℓ constituted the treatment, and the erythropoietin measured in the serum served as baseline value against which the post-treatment erythropoietin was measured at a second bleeding. All sera were individually coded, frozen, and stored until completion of the experiment, and then assayed at the Department of Biochemistry, University of Chicago by Professor E. Goldwasser.

4. Blood Counts

Blood counts were determined by Coulter counter from individual orbital blood samples of about 50 μℓ. Very light ether anesthesia and careful handling of the animals were important since the bone marrow-depleted mice were extremely sensitive to anesthetics. The blood sampling could be repeated monthly without causing further anemia.

5. Red Cell Survival Time

Ten donor mice per group were injected with 6 μCi ^{59}Fe. Their blood was collected after 6 days, the cells were washed, resuspended in saline, and injected intravenously into about 60 untreated, syngeneic recipients. The initial radioactivity in blood, measured 15 min later in five recipients, was taken to represent 100% of the labeled, transfused cells. Subsequently, three or four mice were killed at weekly intervals for determination of the residual blood radioactivity.[5]



A

FIGURE 1. Erythropoietic precursors and CFU-S in the hind leg (A) and fore leg (B) following ^{55}Fe injection of 440 μCi. Absolute differential counts were obtained by microscopic identification of at least 2000 cells per bone marrow sample, and multiplication of fractional counts with total nucleated cell count determined in the contralateral leg. Symbols denote means and standard errors in three to six mice. Proerythroblast (E1), basophilic normoblast (E2), polychromatic normoblast (E3). The CFU-S assays were performed using 12 syngeneic recipient mice and one donor mouse. Each symbol denotes one assay, mean, and s.e., corrected for an assumed seeding factor of 0.2. The shaded areas indicate one standard error above and below the mean of nine untreated mice for differential counts and of five untreated mice for CFU-S. (From Reincke, U., Brookoff, D., Burlington, H., Cronkite, E. P., and Gerard, E., *Experimental Hematology Today* 1979, Baum, S. J. and Ledney, G. D., Eds., Springer, New York, 1979, 27. With permission.)

IV. RESULTS

A. Early Effects of Iron-55

The first experiment revealed a significant reduction of hemoglobin-synthesizing cells as early as 6 hr after ^{55}Fe injection.[6] The finding was confirmed in subsequent studies of bone marrow differentials,[2] and typical cell population changes are shown in Figure 1. While even the ionic, elemental iron appeared to have some erythropoiesis-enhancing effect,[8] the changes in the isotope-treated mice were much more extensive, systemic, and sequential. The cytocide affected actively iron-incorporating precursors such as the basophilic, polychromatic, and orthochromatic erythroblasts (E2, 3, and 4), all of which were reduced dramatically when assayed 24 hr after the injection. At the same time a sharp increase was noted in the number of proerythroblasts (E1) which went from 1.3 ± 0.4 to $5.1 \pm 0.2 \times 10^6$ per hind leg, indicating the initiation of a corrective response. As equivalent increases followed sequentially in the basophilic, polychromatic, and orthochromatic cell population, the number of late normoblasts was soon normalized. The systemic nature of these effects was evident from their parallel occurrence in front and hind legs. The lack of comparable

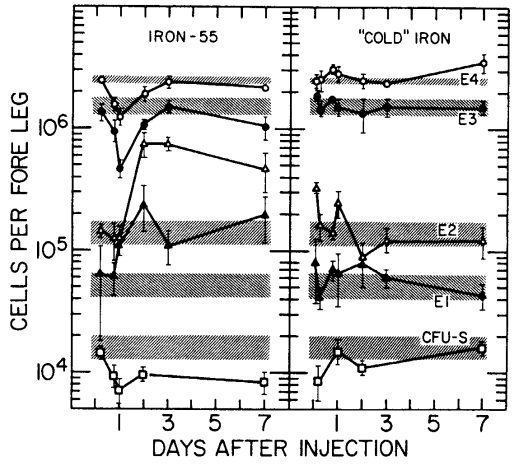


FIGURE 1B

changes among granulopoietic cells proved, on the other hand, the specificity of the response as well as the absence of detectable ^{55}Fe radiation damage in nonerythropoietic cells (Figure 2).

A threefold numerical increase persisted among early erythroblasts although the terminal E3 and E4 populations were restored after 3 days. Since no corresponding overshoot occurred, we concluded that only a third of the E2 precursors could have reached the E3 stage: this indicated a perpetuation of the cytocidal process, which was apparently compensated through continued overproduction of the specific precursor cells.

B. Changes among CFU-S

As shown in Figure 1 and confirming earlier results,[6] the CFU-S fell to a significantly lower level within 24 hr after the ^{55}Fe injection and remained decreased. Excluding direct radiation damage, we interpreted the acute loss of stem cells as a physiological component of the differentiation response to ^{55}Fe cytocide. The findings then could be explained by differentiation of CFU-S into progenitors no longer capable of forming macroscopic spleen colonies.

We were often asked how the CFU-S responded to ^{55}Fe if the isotope were given to polycythemic mice. Two such studies are presented in Table 1, the first in ex-hypoxic mice, the second in hypertransfused mice. Their CFU-S were always found within normal range when tested 24 hr after an ^{55}Fe injection. To identify what constituted normal range, we determined the 95% tolerance limits for blood and bone marrow counts as well as for CFU-S in nine untreated animals which were used in other experiments during the same time. In addition, we showed in untreated, in cold iron-treated and hypertransfused, as well as ^{55}Fe-injected control groups that the CFU-S behaved as predicted by previous experience. The results were consistent with the interpretation of stem cell loss as a physiological response: no cytocide, no pertubation. However, the possibility of ^{55}Fe radiation damage to CFU-S was not logically excluded by this particular experiment, because the almost nonexistent erythropoietic population may have discouraged ^{55}Fe uptake by the marrow in the first place. Absence, however, of early radiation damage to CFU-S can be deduced from the evident

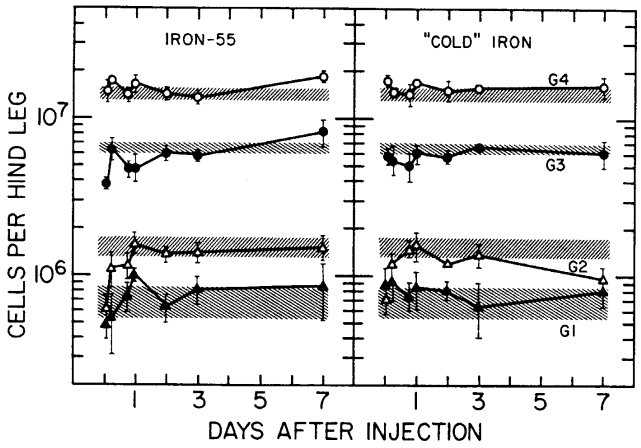


FIGURE 2. Granulopoietic precursors in the hind legs of the same animals whose data are presented in Figure 1. The symbols are the same, except for myeloblasts (G1), promyelocytes (G2), myelocytes (G3), and metamyelocytes, plus banded and segmented granulocytes (G4). (From Reincke, U., Brookoff, D., Burlington, H., Cronkite, E. P., and Gerard, E., *Experimental Hematology Today* 1979, Baum, S. J. and Ledney, G. D., Eds., Springer, New York, 1979, 27. With permission.)

lack of effect to granulopoietic cells (Figure 2 and Table 1) and is directly supported by experiments in which ^{55}Fe-treated marrow was frozen and stored for long time periods.[2,9]

C. Progenitor Cells

As a first step toward better understanding the corrective response we assayed various progenitor cell populations. We cultured bone marrow obtained 24 hr after donor injection, using the in vitro plasma clot technique.[3] The first experiment (Figure 3) showed that the granulopoietic progenitors (CFU-GM) were, on average, unimpaired and that the erythropoietic progenitors near terminal differentiation (CFU-E, 2- and 3-day colonies) were little affected. The more primitive progenitors (7- and 8-day erythropoietic bursts BFU-E) were substantially decreased. This result could not be reproduced in a repeating study. The assay technique was, at least in our hands, not sensitive to independent variables such as inoculate size and erythropoietin dose. When the results were expressed as percentages of comparable control values and then averaged over all dosage levels (Figure 3) no significant influence on early or late progenitors was discernable. It appears, therefore, that the differentiation response to ^{55}Fe was not correlated with significant changes in population size of specific progenitors.

D. Serum Erythropoietin

The findings suggested existence of a regulatory mechanism in which hemopoietic stem cells responded to cell death in the terminally differentiated population. Although this was observed in the erythropoietic line, erythropoietin seemed not to be involved. Erythropoietin levels were determined in serum of 12 ^{55}Fe-injected mice and found normal (Table 2). We performed a detailed study in collaboration with Professor E. Goldwasser (Department of Biochemistry, University of Chicago), measuring the erythropoietin response to acute bleeding (Figure 4) so as to see what might be expected under conditions where erythropoietin

Table 1
^{55}Fe TREATMENT OF POLYCYTHEMIC MICE

Treatment	Mice (#)	Blood (cells per milliliter) RBC (10^6)	WBC (10^3)	Plat. (10^6)	CFU-S	Total (10^6)	Bone marrow (cells per hind leg) ER (10^6)	GR (10^6)	LV	CFU-S	Spleen Total (10^6)	CFU-S
None	4	9.8 ± 0.1	6.5 ± 0.8	1.6 ± 0.1		51	13.9 ± 0.8	22.3 ± 0.0	13.5 ± 0.8	5,390 ± 490	—	—
700 μCi ^{55}Fe + 24 hr	4	9.6 ± 0.1	6.9 ± 1.0	1.1 ± 0.1		30[a]	5.5 ± 0.5	17.2 ± 0.0	7.0 ± 0.4	2,370[a] ± 300	77[a]	520 ± 90
1,400 μCi ^{55}Fe + 24 hr	4	9.8 ± 0.3	4.0 ± 0.7	1.1 ± 0.1		26[a]	1.9[a] ± 0.3	16.6 ± 1.1	7.0 ± 0.8	990[a] ± 180	73[a]	180 ± 80
Exhypoxic day 6: 1,400 μCi ^{55}Fe + 24 hr	4	12.4[a] ± 0.2	5.2 ± 0.5	1.4 ± 0.0		27[a]	0.3[a] ± 0.0	18.0 ± 0.2	7.9 ± 0.2	4,230 ± 690	67[a]	690 ± 80
Polycythemic day 6: cold Fe 1.2 μg + 24 hr	2	15.5[a]	7.8	—	41 ± 9	44	—	—	—	9,160 ± 910	109	3,850 ± 515
Polycythemic day 6: 500 μCi ^{55}Fe + 24 hr	2	15.3[a]	9.0	—	36 ± 6	51	—	—	—	8,125 ± 710	143	2,865 ± 39
Polycythemic day 6: 1,500 μCi ^{55}Fe + 24 hr	2	17.6[a]	8.5	—	46 ± 11	46	—	—	—	7,825 ± 600	118	4,150 ± 620
Untreated controls, 95% tolerance limits	9											
Upper		12.2	16.2	1.8	53	67	14.4	35.3	23.1	12,300	219	4,660
Lower		8.6	0.8	0.9	0	33	5.2	14.2	5.7	3,530	79	100

Note: The table presents two experiments: the first using ex-hypoxic, the second using hypertransfused mice. All were killed 24 hr after the ^{55}Fe or cold iron treatment. In order to provide a comparative measure of normalcy, we determined the 95% tolerance limits (with 90% confidence, Geigy Tables, 1962) for an additional untreated group of 9 mice.

[a] Outside the range of 95% tolerance limits for untreated mice corresponds to a significant difference at the 5% level.

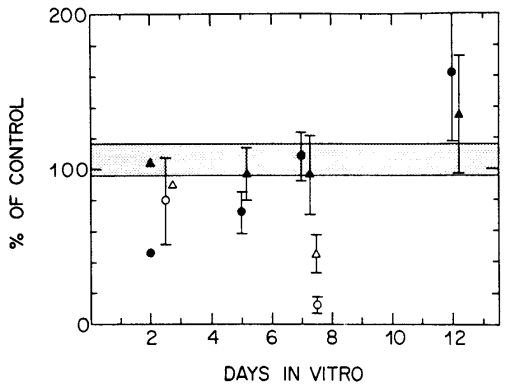


FIGURE 3. Erythropoietic colonies (○△) and bursts (●▲) in ^{55}Fe-treated mice are shown as percentages of control values in untreated mice. The open and closed symbols denote two different experiments. Each point indicates the average ± s.e. of three to six values obtained with various levels of erythropoietin or cell inoculate 24 hr after ^{55}Fe. The points without bar are based on two values. The shaded area represents the grand average ± s.e. of all granulopoietic colonies and bursts seen in the same experiments.

Table 2
SERUM ERYTHROPOIETIN CONCENTRATION (mU/mℓ) IN UNTREATED, BLED, AND ^{55}Fe-TREATED MICE

	N	$\bar{x}$ ± s.e.
Before bleeding	3	5.6 ± 1.4
Bled + 24 hr (300 μℓ)	3	16.8 ± 2.3
Cold iron (or untreated)	4	4.0 ± 1.5
^{55}Fe + 24 hr (500 μCi)	4	3.2 ± 0.7
^{55}Fe + 4 days (500 μCi)	1	2.7
^{55}Fe + 1—12 weeks (500 μCi)	7	6.3 ± 2.4

Note: Radioimmune assays were performed on coded sera, using a human erythropoietin standard.

was known to be the mediator of response. A detailed study of ^{55}Fe-injected mice was also planned but not performed because of difficulties in securing enough isotope.

Bleeding was followed by increased erythropoietin levels 2 1/2 hr after treatment and by further fluctuations and peaks over the 30-hr test period. Although we have, at present, no comparable time study in ^{55}Fe-injected mice, their absence of acute anemia (see Table 1) and lack of demonstrable changes in serum erythropoietin strongly suggest that the bone marrow response to cytocide was not mediated by circulating erythropoietin. We conclude it must be due to close-range feedback mechanisms of unknown origin and agency.

E. Late Effects of Iron-55

After dosages under 2000 μCi per mouse there was a symptom-free interval during which the animals gained weight and appeared active and healthy. Yet, serial sacrifices revealed

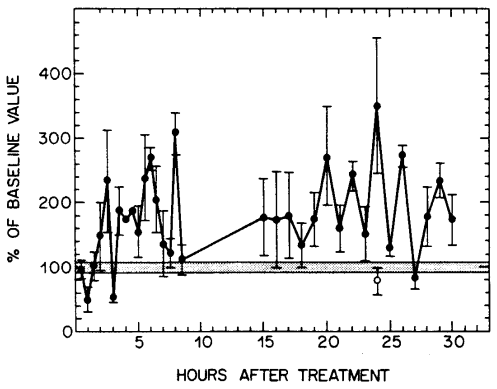


FIGURE 4. Erythropoietin response to bleeding. Mice were bled 300 μℓ from the orbital sinus and a second blood sample was taken at intervals ranging from 30 min to 30 hr later. The serum erythropoietin concentration is given as percentage of baseline levels, determined in each individual, and plotted as mean ± s.e. for three mice per point. The shaded area shows the variation in baseline levels of all 98 mice assayed in the bleeding experiment. Their erythropoietin level was 10.4 ± 0.7 mU/mℓ (average ± s.e.). The open circle refers to four ^{55}Fe-injected mice whose erythropoietin values are shown as percentage of values measured in four cold iron-injected mice.

that their stem cell levels were never restored but underwent further, gradual decrease. This occurred at different rates in different parts of the skeleton and was accompanied by slowly progressing hypocellularity. For instance, 20 weeks after treatment with 440 μCi, CFU-S were reduced to 10 and 30% in the hind and front legs, and the cellularity was 50 and 90% of normal at these sites. After 1300 μCi, the CFU-S were reduced to 2.5 and 4% with corresponding cellularities of 35 and 60%.[2] Histological examinations confirmed these findings and conveyed the general impression that — under this particular stress — the hind leg marrow was depleted first, followed by marrow in the front legs, while the spinal marrow appeared normal much longer.[10]

The cycling activity of stem cells was increased to about 40% and their functional competence remained high when judged by the effectiveness of the erythropoietic response to acute blood loss.[2] As a further measure of general hemopoietic maintenance peripheral blood counts were monitored in ^{55}Fe-injected mice kept for the duration of life. The design of this study and the pathological findings at autopsy have been published previously.[10,11] The blood counts are presented in Figures 5 to 8. Except when a very large isotope dosage was given, the counts assumed a stable but reduced level inversely proportional to ^{55}Fe dosage. The plateau lasted while the animals stayed clinically well and seemed to reflect the physiological base of the symptom-free latency interval. In other words, while CFU-S and bone marrow counts were gradually diminishing, the functional hemopoietic maintenance was held constant at lower settings.

Reduced values were demonstrated for white and red cell counts and for hemoglobin concentration. Since this could have been caused either by lowered production or early cell destruction, we determined the survival time in red cells, using ^{59}Fe-labeled red cell transfusions. The life span of erythrocytes from ^{55}Fe-treated donors was found to be normal (Table 3). This excluded hemolytic processes and argued for decreased production, although

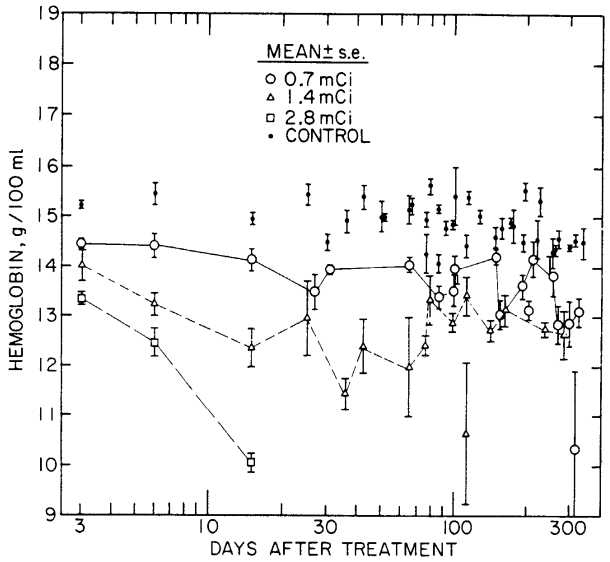


FIGURE 5. Hemoglobin (cyanmethemoglobin by Klett photometer) was determined in peripheral blood after ^{55}Fe or cold iron injection. Any severely depressed values indicate the onset of terminal bone marrow failure in the assayed group.

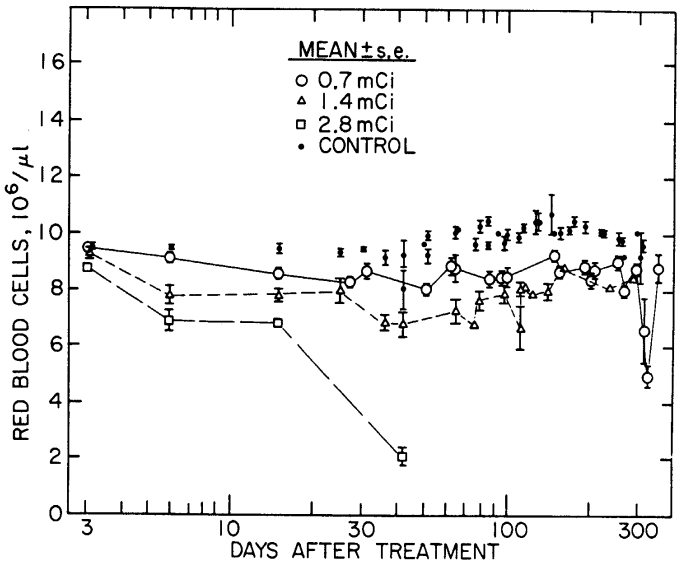


FIGURE 6. Red blood cell counts, determined in ^{55}Fe-injected mice and their controls.

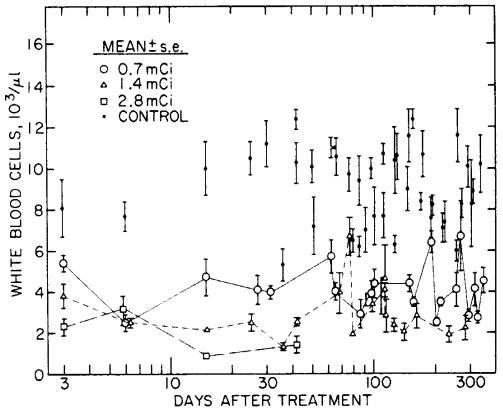


FIGURE 7. White blood cells, as in Figure 6. The lowered levels were due primarily to lack of lymphocytes although all white cell types were decreased.

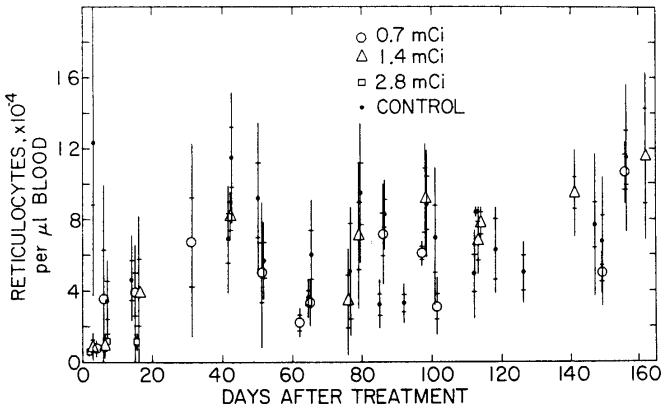


FIGURE 8. Reticulocyte counts (New Brillant Cresyl Blue Stain) were determined in the same animals whose red cell counts are shown in Figure 6.

we could not demonstrate decreased reticulocyte counts except very early after isotope injection (Figure 8). However, the error of the method was large, and a moderate reticulocyte decrease might have escaped our notice.

The final outcome of a single ^{55}Fe injection was a shortened life. The highest dosage given, 2800 μCi, caused bone marrow failure and death within 4 to 6 weeks. Doses below 2000 μCi led to hemopoietic adaptation for several months before bone marrow failure ensued. Doses below 1000 μCi were survived for a year and hemopoietic failure did not occur, for these animals succumbed to very high tumor incidence. The malignant tumors were ^{55}Fe induced. Most notable were osteosarcomas in pelvis and femur, not previously described in this particular mouse strain or with this radionuclide.[11]

Table 3
RED CELL LIFE SPAN

Red cell donor treatment	Survival time in transfused host (days, mean ± s.d.)
Cold iron + 3 months	47 ± 29
Cold iron + 3 months	53 ± 33
^{55}Fe, 500 μCi + 3 months	49 ± 37
^{55}Fe, 1,500 μCi + 3 months	56 ± 39

Note: Red cell life span was measured by the retention of ^{55}Fe in blood of mice which had received the labeled erythrocytes from Fe-treated or cold iron-injected donors. The values were read from eye-fitted data curves on linear probability paper.

V. DISCUSSION

Two findings from the ^{55}Fe experiments deserve further discussion. There was evidence for a corrective, intramedullary differentiation response apparently not mediated by erythropoietin, and there was evidence for stable but nonpermanent adjustment to continued stem cell loss.

The corrective differentiation response was prompt, specific, effective, and sustained. Its described components were a precipitous fall of CFU-S, no definitive changes among progenitors, and a significant increase of early erythropoietic precursors within 24 hr, all of which led to normalized late erythroblast counts after 3 days. An overall characteristic was stem cell depletion concomitant with cell increase in the terminally differentiated population. We have discussed the possible physiological base of this finding previously.[2] Briefly, the decrease of pluripotent stem cells can be explained, assuming that their initial rate of differentiation exceeded their initial replacement rate. This is reasonable if one bears in mind that it takes several hours for a cell from initiation of DNA synthesis until it actually produces the two daughter cells. Thus, if in a stem cell population the stimulus to differentiate and the stimulus to self-renew were simultaneously received, the rate of mitotic cell production would increase only several hours later and then serve to protect the population from further shrinking despite ongoing stress to differentiate. As the stimulus to self-renew was already active (judged by increased cycling of CFU-S) 1 hr after ^{55}Fe, and further depletion of CFU-S stopped after 24 hr,[2] less than 24 hr passed between initiation of DNA synthesis and the accomplished increase in self-renewal rate.

It is unknown which sensing and activating factors may have participated in starting and maintaining the regulatory chain. A detailed histological study[12] failed to reveal clues on the light microscopical level. The marrow of mice in whom the differentiation response had been demonstrated was inconspicuous. No differences from control marrow were discovered concerning numbers of pyknotic cell nuclei, megakaryocytes, hemosiderin deposition, or overall architecture.

The short-range feedback control whose existence, we believe, the present findings advertise, must play a part in everyday hemopoietic regulation and one wonders what that part may be. Since erythropoietin has little effect on CFU-S and early BFU-E but does accelerate proliferation of CFU-E and terminally differentiated erythroblasts, one could speculate that intramedullary factors are responsible for recruitment and differentiation of CFU-S into those progenitor populations which do respond to erythropoietin. Similar mechanisms would then have to be postulated for granulopoiesis, thrombopoiesis, etc. The ^{55}Fe may have generated a situation without biological precedent in that there was no appropriate stimulus for erythropoietin in spite of the demand for increased erythropoiesis. The erythropoietin-independent regulation could, perhaps, be discovered only under these particular circumstances.

The early differentiation response to cytocide was effective during a 1-week experimental period, but it did not prevent the later depopulation of bone marrow and spleen. The CFU-S diminished first, the marrow cellularity decreased less, and at a relative rate which paralleled the loss of CFU-S at the given site. Peripheral blood counts were only little or moderately affected and, more importantly, were held stable for up to a year until either hemopoietic failure or a malignant tumor terminated each individual's life. Since the chronic depletion of stem cells could have been partially caused by ^{55}Fe incorporated into CFU-S, we cannot ascribe it entirely to physiological mechanisms as we did the early stem cell decline. On the other hand, we believe that forced stem cell differentiation into erythropoietic precursors, subject to ongoing cytocide, was a contributory factor. In the end it was not irradiation (which decreased from 0.06 Gy/day to zero after 15 weeks post 750 μCi)[2] but the premature exhaustion of a limited regeneratory stem cell reserve[13-15] which led to bone marrow failure after a latency interval of dose-related length. Some regulation steered the cell production towards given but reduced peripheral settings. This pattern, which provided steady supply in spite of diminishing reserves, allowed for temporary well being of the animals and for their normal responses to acute blood loss. This may correspond to the in vitro phenomenon of transient recovery from radiation injury.[14,15] In both instances the limits to stem cell renewal were eventually exposed, and in both instances an inherent mechanism was operative which affected steady and near-normal output of functional cells for the longest possible time. The observation of this mechanism in culture suggests that its chief components are within the local hemopoietic microenvironment and that they are insensitive to transplantation.

The ^{55}Fe experiments have confirmed previous controversial findings[16,17] and provided alternative explanations for them. In addition, the experiments led to unexpected new findings which raised new possibilities to consider hemopoietic regulation. Although we were keen to study the mechanism of the corrective differentiation response, further ^{55}Fe experiments were temporarily halted because the high specific-activity isotope preparation[1] could not be obtained. Since commercially available ^{55}Fe can elicit the erythrocytocide,[18] one may resort to using the lower specific activity preparations in such studies which are not designed to avoid burdens of radioactivity outside the bone marrow.

ACKNOWLEDGMENTS

It is a pleasure to acknowledge the expert assistance of Naomi Pappas and Betty Heldman, and the generous involvement of our student helper Stefan Seibold.

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Chapter 10

IRON-55 — A MODEL ANALYSIS*

Peter Herkenrath, Markus Loeffler, and H.-Erich Wichmann

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I. ABSTRACT

After administration of the radioisotope, ^{55}Fe, three effects were found: (1) the maturing erythroblasts were reduced, (2) CFU-S decreased, and (3) the early erythroblasts increased. The maturing erythroblasts were probably reduced by severe radiation damage from the incorporated ^{55}Fe. However, as the mathematical analysis shows, the other findings cannot be explained solely by intramedullary feedback from the late erythroblasts. The model analysis suggests that ^{55}Fe may destroy CFU-S at a low rate and may induce additional mitoses in early erythropoietic cells.

II. INTRODUCTION

^{55}Fe is a radioactive and cytotoxic substance which is incorporated in erythropoietic precursor cells. As has been shown by Ganzoni,[1] iron incorporation in rats and mice takes place mainly in late precursors (60% accumulates in polychromatic and orthochromatic erythroblasts and 30% in reticulocytes) while the uptake by early precursors is only small (10% in proerythroblasts and basophilic erythroblasts). Therefore, one would expect that the direct cytotoxic effect of ^{55}Fe should be restricted to the late erythropoietic precursor cells. Reincke et al.,[2,3] indeed, have measured severe destruction of polychromatic and orthochromatic erythroblasts. After administration of 440 μCi ^{55}Fe the numbers of these cells decreased to about 50% of normal. On the other hand, the early precursors (proerythroblasts and basophilic erythroblasts) were not reduced but increased to three to five times normal. In addition, the number of CFU-S dropped to approximately 50%.

Two hypotheses are discussed by Reincke and Cronkite[3] to interpret their data. The first hypothesis is that ^{55}Fe only kills iron-incorporating late erythroblasts and has no direct influence on the other hemopoietic cells. In this case, the response action of CFU-S and the early erythroblasts would follow by intramedullary feedback control. The second theory postulates that ^{55}Fe damages CFU-S directly by internal irradiation and that the early erythroblasts increase due to erythropoietic stimulation of the developing anemia. In this case, intramedullary regulation would not be responsible for the experimental findings.

Reincke and Cronkite[3] argue that the absence of radiation damage to CFU-S could be shown in experiments in which ^{55}Fe-treated marrow was frozen and stored. Furthermore, there is no evidence for stimulation of the proerythroblasts and the basophilic erythroblasts by erythropoietin (Epo). Since the anemia after ^{55}Fe is not severe, the subsequent level of Epo is not sufficient to stimulate the demonstrated large increase of early precursors. In total, the authors come to the conclusion that intramedullary feedback, leading to a recruitment of CFU-S into the erythropoietic lineage, is the most likely explanation for their results.

In the following, the data will be analyzed mathematically. The investigation will be restricted to the early response of bone marrow cells after administration of 440 μCi ^{55}Fe.

III. MATHEMATICAL METHODS

The ^{55}Fe experiments are simulated using the mathematical model of stem cell regulation as described.[4-6] However, for the compartment of erythropoietic precursors a modification is necessary since iron incorporation in early and late precursors is different. Therefore, compartment E1-4 is divided into four subcompartments, E1 to E4. They correspond to the morphological classes used by Reincke and Cronkite.[2,3] The kinetic parameters of these cells have been taken from the published data on erythropoiesis in mice as reviewed by Wulff.[7] They are given in Table 1.

The differential equations for E1 to E4 have the form:

Table 1
SUBDIVISION OF THE COMPARTMENT E OF ERYTHROPOIETIC PRECURSOR CELLS

Morphological classification	Model compartment	Number of mitoses n	Transit time T (hr)
Proerythroblasts	E1	2	16
Basophilic erythroblasts	E2	2	16
Polychromatic erythroblasts	E3	2	16
Orthochromatic erythroblasts	E4	0	24
All erythropoietic precursors	E ≈ E1-4	6	72

$$\dot{E1}^* = \dot{E1}^{*in} - E1^*/T_{E1} \quad (1)$$

$$\dot{E2}^* = \dot{E2}^{*in} - E2^*/T_{E2} \quad (2)$$

$$\dot{E3}^* = \dot{E3}^{*in} - E3^*/T_{E3} \quad (3)$$

$$\dot{E4}^* = \dot{E4}^{*in} - E4^*/T_{E4} \quad (4)$$

As described earlier,[5] "*" indicates the absolute cell number in the corresponding compartment and the influx rate equals the efflux rate of the preceding compartment, multiplied with the factor of amplification. T_{E1}, T_{E2}, T_{E3} denote the transit times in the amplifying three compartments, while T_{E4} is the maturation time in E4.

Three possible influences of ^{55}Fe will be considered.

The E3 cells — These are reduced to 50% of normal, according to the data.[2,3] This can be simulated by the modified differential equation

$$\dot{E3}^* = \dot{E3}^{*in} - E3^*/T_{E3} - K_{E3} * E3^* \quad (5)$$

The loss coefficient K_{E3} equals 0.08/hr which corresponds to a selective irradiation of approximately 2.3 Gy/day on polychromatic erythroblasts (a loss coefficient of 0.03/hr corresponds approximately to 1 Gy/day[8]). Cytotoxicity of ^{55}Fe is restricted to E3 since the polychromatic erythroblasts are the proliferating cells which incorporate the largest amount of iron. The E4 cells incorporate even more ^{55}Fe, but since they are postmitotic, they will be less sensitive to internal irradiation.

The S cells — These are reduced to a plateau of 50%, according to the data.[2,3] This can be simulated by an initial value S = 0.5 and the differential equation

$$\dot{S} = (2p - 1) * S/T_S - K_S * S \quad (6)$$

with the loss coefficient K_S = 0.004/hr. As has been described in the chapter on chronic irradiation,[8] the latter corresponds approximately to a continuous dose of 0.12 Gy/day.

According to the rules previously described[8] this dose leads to a loss coefficient of 0.0027/hr in all differentiated compartments. Since such a small loss has no significant

influence on the model results it is neglected here. For the differentiated cells, only an initial reduction is considered using the initial values BE = CE = CG = 0.6, G = E1 = E2 = E3 = E4 = 0.8. However, even the choice of these initial values is of minor importance in the following.

The E1 and E2 cells — These increase initially to four times normal, according to the data.[2,3] This can be simulated by two additional mitoses assuming a severe erythropoietic stimulus during the first 2 days. Mathematically, the amplification Z_{CE} in compartment CE (late erythropoietic precursors, corresponding to CFU-E) is held at its maximum value $Z_{CE} = Z_{max}$ for 2 days.

IV. RESULTS

A. Model Calculations

The theoretical curves which have been generated to simulate the influence of ^{55}Fe are shown in Figures 1 to 8. They can be interpreted as follows.

1. Continuous Damage on E3 Cells

If only the E3 cells are destroyed by internal irradiation of approximately 2.3 Gy/day their number drops to 50% (Figure 4, dashed line). The decrease of E4 (Figure 5, dashed line) is then a consequence of the reduced influx from E3.

The low number of erythropoietic precursors stimulates the cycling of stem cells ("a_s") and reduces the self-renewal probability "p" (Figures 7 and 8, dashed lines). An intermediate dip of S and a slight elevation of E1, E2, and G is the consequence (Figures 2, 3, and 6, dashed lines).

2. Additional Destruction of S Cells

If 50% of the stem cells S are destroyed initially a continuous dose of approximately 0.12 Gy/day keeps their number at this plateau (Figure 1, full line).

3. Stimulation of Additional Mitoses

If two additional mitoses are induced in the late erythropoietic progenitors, CE, the number in E1 reaches a peak of four times normal after 3 days (Figure 2, full lines). One day later this peak occurs in E2 and E3 and after one further day it is found in E4 (Figures 3 to 5, full lines).

B. Comparison with the Data

The selective continuous damage of E3 cells obviously is not able to reproduce the experimental findings. Although the proposed changes in intramedullary regulation, induced by the depletion of late erythroblasts, produce effects of the right direction — temporary reduction of S and increase of E1 and E2 (dashed lines) — it cannot quantitatively explain the marked effects which have been measured.

Only if additional influences are considered can the experimental results be reproduced. This is shown by the full lines in Figures 1 to 8, where a mild direct damage of the stem cells and additional erythropoietic mitoses in E1 and E2 have been assumed to take place.

V. DISCUSSION

The destruction of E3 cells by ^{55}Fe can be predicted since it is known that iron is incorporated mainly in late erythroblasts.[1] The cytocidal process perpetuates itself because fresh erythroblasts reutilize the isotope from disintegrated cells.[3] In the model, only E3 cells are killed because they are proliferating and, therefore, radiosensitive. However, if one

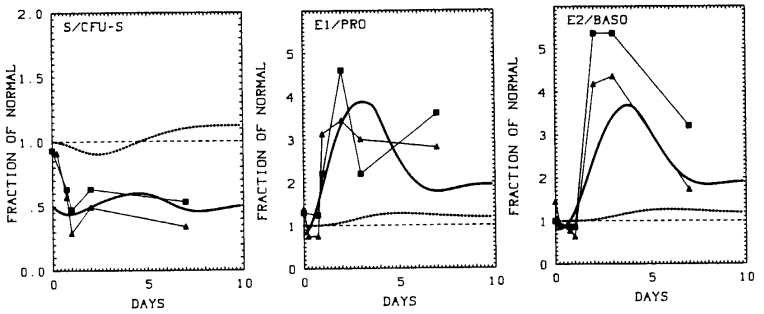

FIGURE 1

FIGURE 2

FIGURE 3

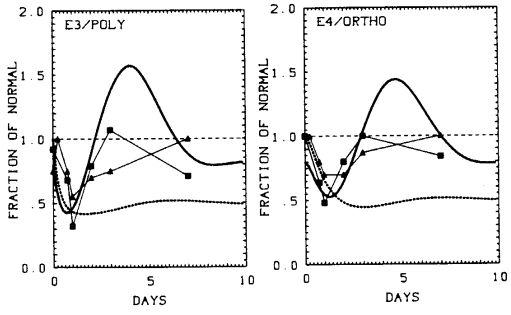

FIGURE 4

FIGURE 5

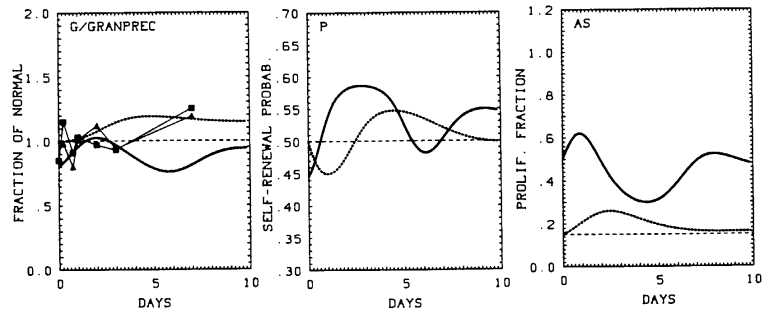

FIGURE 6

FIGURE 7

FIGURE 8

FIGURES 1 to 8. Iron-55 experiments — comparison of model results and data. Two model calculations have been performed: (– – –) ^{55}Fe damages only E3 cells; (—) in addition, S cells are damaged moderately and additional erythropoietic mitoses are induced. Data: cell numbers in the hind leg (▲) and fore leg (■) of mice following 440 μCi ^{55}Fe.[2,3] CFU-S, proerythroblasts, basophilic, polychromatic and orthochromatic erythroblasts, and granulopoietic precursors are compared with the model compartments S, E1-E4, and G.

assumes, in addition to E3 damage, a less pronounced destruction in E4, the results are similar.

The severe loss in E3 reproduces the experimentally found dip to 50% of normal. This loss corresponds to a chronic irradiation of approximately 2.3 Gy/day selectively directed to the polychromatic erythroblasts. Although this dosage can be estimated only very approximately from the loss coefficient of the model,[8] the order of magnitude is probably realistic.

As a clear result, it follows from the model calculations that the depletion of CFU-S and the increase of the early erythroblasts cannot be reproduced by the model-defined intramedullary feedback from the damaged late erythroblasts. In the model, intramedullary feedback is not strong enough for such large effects as have been measured.

Only additional assumptions about the influence of ^{55}Fe allow us to reproduce the data. However, these assumptions remain speculative because they are not based on direct measurements. Therefore, they cannot be interpreted as "model findings" but only as questions which might stimulate further investigations.

The first question is this: does a dosage of 440 μCi ^{55}Fe also kill other bone marrow cells? If the answer is yes, our calculations would propose an initial destruction of 50% of stem cells followed by a chronic destruction approximately corresponding to an irradiation of 0.12 Gy/day. It is of minor influence whether this internal irradiation may kill only stem cells or also other proliferative cells — the model curves are very similar in both cases.

The second question might be the following: does the administration of ^{55}Fe lead to stimulation of erythropoiesis? If the answer is yes, the model calculations would propose an initial stimulus which induces two additional mitoses of the early erythropoietic cells. The stimulus would explain the steep increase of E1 and E2 and the subsequent recovery of E3 and E4, as a consequence of the maturation of this cohort.

If both questions could be answered positively, a consistent interpretation of the data would be possible. But how realistic is this? Irradiation damage of the bone marrow cells by ^{55}Fe might be possible although no direct evidence exists.[3] However, the origin of the additional mitoses remains obscure. The simplest explanation, namely, a stimulation by erythropoietin (Epo), does not seem very probable. During the first 3 days of the experiment no anemia developed, and a remarkable increase of Epo was not found.[3]

The enlarged pool of iron may provide a better explanation of the additional mitoses: as has been shown by Ganzoni[1] the iron incorporation in erythropoietic cells increases for high concentrations of serum iron. The amount of injected ^{55}Fe would not be sufficient, as was demonstrated by Reincke and Cronkite[2,3] (in a control experiment they found no stimulatory influence of comparable quantities of "cold" iron on erythropoiesis). On the other hand, there might be an indirect influence of ^{55}Fe: after the destruction of late erythroblasts by internal irradiation the large iron content of these cells is released and might enlarge the pool of available iron significantly. Since serum iron was not measured[2,3] this argument remains speculative.

Other mechanisms might also be possible. Monette et al.[10] found that hemin selectively stimulates BFU-E by a factor ranging from 6 to 16. If a similar stimulus would also occur after ^{55}Fe, the cohort of cells seen in the early erythroblasts would originate from BFU-E and CFU-E a few days earlier. Unfortunately no reliable data on these cells are available.

Finally, stimulation of early erythropoiesis also has been measured after administration of other cytotoxic agents. Hydroxyurea leads to selective stimulation of erythropoietic progenitor cells within 3 to 4 days. The number of BFU-E increases twofold,[11] the CFU-E two- to fivefold,[11-13] and erythroblasts nearly fivefold.[13] Phenylhydrazine has similar effects which have been attributed to a higher sensitivity of erythropoietic cells to erythropoietin.[7,14,15] However, these phenomena are not understood and may have a similar explanation as the peaks of proerythroblasts and basophilic erythroblast cells after ^{55}Fe.

In summary, the fact that the additional assumptions reproduce the data does not mean that they are physiologically correct. The data might also be reproduced by different assumptions or by different concepts of stem cell regulation. Perhaps the idea of Reincke and Cronkite[3] which proposes an extra recruitment of CFU-S into the erythropoietic progenitor population provides a better explanation.

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Chapter 11

PHENYLHYDRAZINE-INDUCED HEMOLYTIC ANEMIA — EXPERIMENTAL RESULTS

G. S. Hodgson

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I. ABSTRACT

Injection of phenylhydrazine causes erythrocyte destruction and a rapidly developing anemia. In response to the anemia, plasma erythropoietin levels rise and there is a subsequent increase in erythropoiesis, both in marrow and, in rodents, even more markedly in spleen. The increase in splenic erythropoiesis is preceded by an increase in the blood content of BFU-E, CFU-S, and also CFU-GM. The CFU-S, BFU-E, and CFU-GM in spleen are increased severalfold, up to ten times normal. Marrow BFU-E, on the other hand, decreased markedly, CFU-GM somewhat less, and CFU-S slightly or not at all.

Experiments, in which repeated injections of endotoxin-free erythropoietin containing extracts were given, suggest that most of the effects observed in "phenylhydrazine anemia" are a consequence of the increased levels of erythropoietin (and possibly some other regulator[s] present in the extract of anemic human urine used). Blockage of the erythropoietic response to erythropoietin by actinomycin D, also prevents the increase in spleen CFU-S and CFU-GM (prevents cell migration).

II. INTRODUCTION

Following repeated injections of phenylhydrazine at 3- to 4-day intervals, a new "near steady state" of blood erythrocytes is established, with no or only slight anemia (so-called compensated hemolytic anemia). Under these conditions there is an increase in erythropoietin-responsive cells (erythropoietic progenitors), which have an increased responsiveness to erythropoietin.

The proliferative status of spleen CFU-S in phenylhydrazine anemia is variable. Some experiments show that spleen CFU-S are rapidly proliferating (sensitive to hydroxyurea and vinblastine) soon (3 to 5 days) after phenylhydrazine injection. Other experiments do not detect an increase of spleen CFU-S proliferation at any time after phenylhydrazine. Marrow CFU-S do not increase their proliferation rate until 6 to 7 days after phenylhydrazine injection.

Rodents with phenylhydrazine anemia respond to irradiation in a different manner to normal rodents. There is a prompt "abortive" rise of erythropoiesis after radiation (5.0 to 7.0 Gy), which is followed by a sustained recovery. Splenic erythropoiesis plays an important (about 50%) part in the erythropoiesis response. Multiple endogeneous colonies are seen in the spleen of anemic mice irradiated with 7.0 Gy. The CFU-S in the endogeneous colonies have a low "self-renewal" capacity as do the CFU-S circulating in the blood of phenylhydrazine-treated mice. The radiosensitivity of CFU-S in phenylhydrazine anemia is unchanged.

III. MATERIAL AND METHODS

Experiments were carried out in mice and rats and, in one case, in rabbits. The phenylhydrazine subcutaneous injection schedules used in mice were either one injection a day on days 0, 1, 2,[2,4] or 0, 1, 3.[3,7-9] In rats injections were given on days 0, 1, 2, 3, 4[1,2] or, in the case of chronic injections,[6] every 3 or 4 days for several weeks. Blood counts and differentials were done using standard techniques. CFU-S[2-5,8,9] were assayed using the Till and McCulloch method.[10] CFU-GM assays[4] used are an adaptation of the Bradley and Metcalf technique.[11] BFU-E and CFU-E[7] were assayed using the method of Iscove and Sieber.[12] The proliferative status of CFU-S was determined by measuring the kill produced by hydroxyurea[3,5,8] or vinblastine.[2] In one experimental series the effect of actinomycin D[9] and in two others[1,2] the effects of irradiation on phenylhydrazine-treated animals were examined. Erythropoietin levels were measured using the ^{59}Fe incorporation technique in fasted rats,[1] in transfused mice,[9] transfused rats,[6] or rabbit marrow culture.[6] The erythropoietin preparations used were extracts of anemic human urine.[1,6,9]

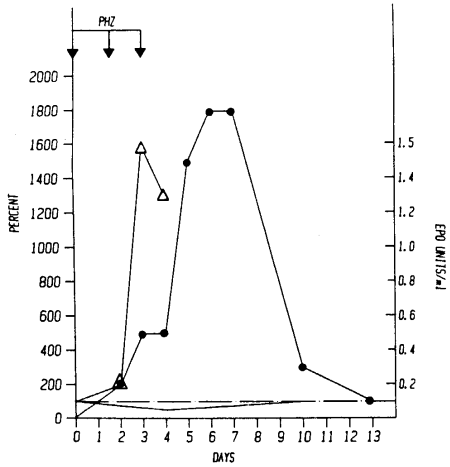

FIGURE 1. Changes in peripheral blood after triple injection of phenylhydrazine. Data presented for Epo (△) are reproduced from Quesenberry et al.,[9] for reticulocytes (●) from Rencricca et al.[3] The full line (—) indicates the behavior of the hematocrit as reported by several authors.[3,7]

IV. RESULTS

A. Erythropoietic Compartment

Injection of phenylhydrazine was followed in all experiments by a rapid decrease in erythrocytes to reach a value of about 50% of normal 4 days after initiation of treatment (Figure 1). Plasma erythropoietin (Epo) levels rose[1,9] to peak levels (1.5 to 2 $\mu m/m\ell^{9}$) 3 days after initiation of phenylhydrazine treatment (Figure 1). Reticulocytes increased sharply after 4 days and peaked at day 6 (Figure 1).[3,4,7] In the marrow erythropoietic precursors E (Figure 2) increased by 50% at day 2 and remained at this level until day 6.[3] In the spleen erythropoietic precursors started to rise sharply after day 3 and peaked at day 5.[3] At day 5 the spleen contained about 350×10^6 erythropoietic precursors, that is, about five times the estimated total marrow number (e.g., 20 × tibial content) of erythropoietic precursors. Marrow BFU-E[7] (Figure 2) decreased fourfold between days 2 and 4 and remained at this level for 10 days. Blood BFU-E showed a sharp, tenfold rise at day 2 and peaked at ten times normal at day 4, and then decreased at day 7. Marrow CFU-E (Figure 2) were increased by day 2, peaked at day 4 (ten times normal), decreased on day 7, but remained above normal. Spleen CFU-E increased to a peak level of about 200 times normal at day 4 and then declined. At the peak the spleen contained as many BFU-E and CFU-E as the total marrow.

B. CFU-S and CFU-GM

In some experiments[4,9] femur CFU-S remained unchanged after injection of phenylhydrazine; in others tibial CFU-S decreased to about 50% of normal.[3,8] Spleen CFU-S, on the other hand, increased markedly in all experiments peaking at about five to ten times the normal at day 5.

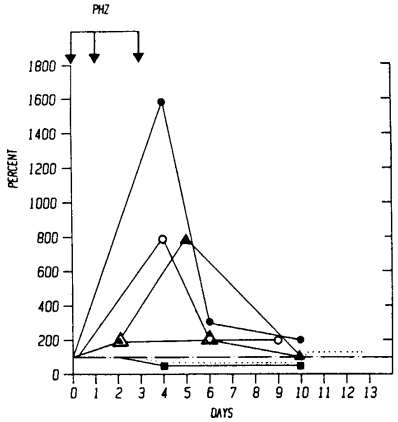

FIGURE 2. Changes in stem cells, erythropoietic progenitors, and precursors after triple injection of phenylhydrazine. Data for erythropoietic precursors (△) and CFU-E (○) in bone marrow are taken from Rencricca et al.[3] and Hara and Ogawa.[7] Values for the total numbers of BFU-E (■), CFU-E (●), and erythropoietic precursors (▲) are recalculated from marrow and spleen measurements reported by Hara and Ogawa[7] (■, ●) and Rencricca et al.[3] The dotted line (...) indicates the behavior of the total CFU-S numbers as can be calculated from data of various authors.[3,8]

The increase in spleen CFU-S was accompanied by a rise in blood CFU-S which increased to about 15 times normal.[4] The self-renewal capacity of blood CFU-S was low.[5] The proliferative status of spleen CFU-S was unchanged in some experiments[3] (insensitive to hydroxyurea), while in others spleen CFU-S were actively proliferating as judged from the effects of vinblastine[2] on day 3 or hydroxyurea[4,8] on days 4 and 5. Tibial marrow entered rapid proliferation after day 5, preceding the recovery from the depression.[3,8] Marrow CFU-GM decreased soon after phenylhydrazine injection and remained low.[4,9] Blood CFU-GM started to increase at day 2[4] and peaked at about ten times normal on day 4; spleen CFU-GM increased fivefold by day 5.[4,9]

C. Effect of Actinomycin D on Response to Phenylhydrazine

Injections of actinomycin D (75 μg/kg), once a day for 5 days which blocked erythropoietic differentiation, also prevented the increase in blood and spleen CFU-S and CFU-GM and the drop in marrow CFU-GM. Marrow CFU-S, which were not significantly depleted by phenylhydrazine, showed a significant rise after actinomycin D injection.[9]

D. Effects of Repeated Injections of Erythropoietin

Repeated injections of five units of endotoxin-free erythropoietin every 8 hr for 4 days, contained in extracts of anemic human urine, essentially mimicked the effects of phenylhydrazine: increasing blood and spleen CFU-S and CFU-GM and leaving marrow CFU-S unchanged. As in the phenylhydrazine response, actinomycin D blocked erythropoietic differentiation and the changes in blood and spleen CFU-S and CFU-GM induced by erythropoietin.[9]

E. Effect of Chronic Treatment with Phenylhydrazine

When injections of phenylhydrazine were given at 3- to 4-day intervals for several weeks, a steady-state erythrocyte level near normal values was established[6] (compensated hemolytic anemia). At this time, however, erythropoietin-sensitive cells were increased as was their sensitivity to erythropoietin.

F. Effects of Phenylhydrazine Anemia on the Response to Irradiation

Rodents pretreated with phenylhydrazine, before being exposed to irradiation, showed a prompt marked but abortive rise of erythropoiesis, followed by a sustained regeneration.[2,4] The spleen contributed about 50% to the erythropoietic response. Large numbers of endogeneous spleen colonies were seen on the spleens; the CFU-S in these colonies had a much lower "self-renewal" capacity than normal CFU-S.[4]

V. DISCUSSION

Following the induction of anemia by phenylhydrazine there was a marked expansion of the erythropoietic precursor compartment, from 5×10^7 to about 4×10^8; most of the expansion (3.5×10^8) took place in the spleen.

The total CFU-S pool, at most, was only slightly increased (30%) or slightly decreased in the experiments in which marrow CFU-S decreased in spite of the expansion of the spleen CFU-S.

Total BFU-E, on the other hand, decreased, as the pronounced marrow fall (from 8×10^4 to 2×10^4) was not made up by the increase in spleen BFU-E (2×10^4). The more mature erythropoietic progenitors, CFU-E, expanded from 6×10^5 to 1×10^7, half of the increase being due to the marked expansion of the spleen CFU-E. It is noteworthy that the ratios of erythroblasts to BFU-E were 4×10^3:1 in the marrow and 17×10^3:1 in spleen. The CFU-E to BFU-E ratios were about 300:1 and about 250:1, respectively, for marrow and spleen. It thus appears that either in the splenic environment many more erythroblasts are produced per CFU-E, or, alternatively, the mean residence time in marrow for erythroblasts is much shorter (some may migrate via bloodstream to the spleen). The total CFU-GM pool was depleted, for a marked expansion in spleen was insufficient to compensate for the marrow loss.

The changes that occur in acute phenylhydrazine hemolytic anemia are consistent with the response being due to increased levels of erythropoietin, which increased erythropoietic differentiation from BFU-E. As a consequence of the limited space available in marrow migration of CFU-S, BFU-E and CFU-GM occurred to the spleen via blood. Migration would account for the increase in spleen BFU-E and erythroblasts. In the case of spleen CFU-S, increased proliferation of these cells in the spleen may play a role in some mouse strains.

In the case of chronic hemolytic anemia, additional mechanisms, other than increased Epo levels, must come into play, since the excessive blood destruction was compensated by increased erythrocyte production, in the presence of near-normal levels of erythropoietin. This increased production was due to an expanded erythropoietic progenitor pool, with increased sensitivity to erythropoietin. The mechanism of the expansion and change of sensitivity is not known. One might speculate that production of local endogeneous factor[8] stimulating CFU-S proliferation begins after a 5-day delay. In the case of chronic phenylhydrazine treatment this control mechanism would maintain CFU-S in an active prolifer ation, producing increased quantities of erythropoietic progenitors with increased sensitivity to erythropoietin.

Any model describing the effects of phenylhydrazine hemolytic anemia must take into account the changes that occur in response to irradiation treatment, especially the marked

early abortive rise of erythropoiesis that occurs to which the spleen contributes importantly. Perhaps the higher level of erythropoietin attained under these circumstances has some effect on the differentiation of damaged stem cells with limited proliferative capacity.

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Chapter 12

PHENYLHYDRAZINE-INDUCED HEMOLYTIC ANEMIA — A MODEL ANALYSIS*

Markus Loeffler, H.-Erich Wichmann, and Alexander Jarczyk

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I. ABSTRACT

Phenylhydrazine (PHZ) is a potent hemolytic agent. Administered to mice, it produces severe anemia and shows additional effects: hemopoiesis is shifted from the bone marrow to the spleen and erythropoiesis is stimulated enormously. These changes lead to a significantly quicker hematocrit recovery than after comparable bleeding anemia. To understand these findings, two hypotheses have been formulated and tested by a mathematical model of stem cell regulation. In the first hypothesis, it is assumed that PHZ induces only hemolysis. This hypothesis leads only to a poor reproduction of the measurements. In the second hypothesis, two additional influences of PHZ are assumed: a loss of stem cells and progenitors (presumably occurring during a migration process from the bone marrow to the spleen) and an increased "sensitivity" of erythropoietic cells to erythropoietin. This hypothesis produces simulations which are in reasonable agreement with the data, although some discrepancies remain. Two reasons may be responsible for the phenomenon of increased erythropoietin sensitivity: an effect of the splenic microenvironment favoring erythropoiesis or a stimulatory effect of the iron being available for red cell formation after the preceding hemolysis. In total, the model analysis suggests that the experimental observations cannot be explained exclusively by the anemia provoked by the administration of PHZ.

II. INTRODUCTION

The behavior of hemopoietic stem cells and progenitor cells in mice after injection of phenylhydrazine (PHZ) has been studied by several authors.[1-6] Measurements of reticulocytes and hematocrit are generally available.[4-10] It is well established that PHZ induces severe (acute) hemolytic anemia.[7] In addition, it shifts hemopoiesis, particularly erythropoiesis, from the bone marrow to the spleen. As a third observation, erythropoiesis recovers earlier than after similar levels of anemia induced by bleeding and the hematocrit shows a temporary overshoot.

Changes of the early hemopoietic elements to triple PHZ injections (days 0, 1, 3) have been reviewed by Hodgson.[8] To summarize:

- In the spleen all hemopoietic cell stages (CFU-S, BFU-E, CFU-E, CFU-GM, erythroblasts, and myeloblasts) increase and reach their maximum at day 5. By day 20 the values have returned to normal.
- In the bone marrow, all cell stages except CFU-E and erythroblasts drop below normal in the first 5 days. Thereafter, CFU-S start a rapid recovery with a significant overshoot to at least 150% of normal on day 13. CFU-E and erythroblasts increase twofold and return to normal levels by day 10.
- The spleen's contribution to the total CFU-S population reaches 10 to 40% and for erythropoietic precursors it may be as high as 80%.[11]

In the following we investigate whether these findings can be understood within the frame of a mathematical model on stem cell regulation.

III. MATHEMATICAL METHODS

The effect of PHZ on hemopoiesis is simulated using a mathematical model of stem cell regulation.[12-14] Two hypotheses will be considered. The first one assumes that PHZ leads to acute hemolysis in the blood without further influences. In the second hypothesis, additional effects on early hemopoiesis are taken into account. In both calculations, anemia is indirectly simulated by theoretical EP curves (EP denotes the theoretical values of erythropoietin used in the model and "Epo" represents the experimental concentration of the hormone). Severe anemia is considered with a reduction of the hematocrit (Hct) to 0.55 of normal on day 15. This corresponds to the experimentally observed value after the second dose of PHZ.[3] A red cell reduction of this magnitude leads approximately to a 15-fold increase in experimental erythropoietin levels.[8,15,16]

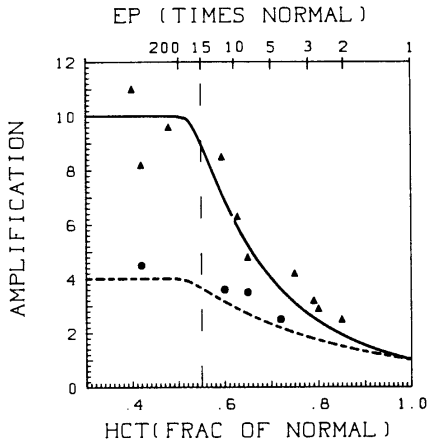


FIGURE 1. Dose-response of erythropoietic amplification (times normal). The curves show normal (---) and increased (—) sensitivity of erythropoietic amplification to erythropoietin.[16] The first dose-response curve is compared with data from bleeding anemia[20-24] (●), the second with data from PHZ anemia[3-6,9,10] (▲).

A. Hypothesis I: PHZ Induces Only Acute Hemolytic Anemia

In the model, an initial Hct of 0.55 of normal corresponds to EP of 15 times normal (Figure 1, vertical line). For the standard dose-response curve of the model which relates erythropoietic amplification and EP (Figure 1, dashed line), this stimulus corresponds to an amplification of 3.6 times normal. As a consequence, EP normalizes within 10 days (Figure 2, dashed line). This theoretical EP curve which has been derived from a mathematical model of mature murine erythropoiesis[16] is used as input for the stem cell model. Mathematically, the calculations are performed in the same way as described previously for bleeding anemia,[17] and the theoretical EP curve is consistent with the experimental findings on the recovery of the Hct after bleeding.[16]

B. Hypothesis II: PHZ Has Additional Influences on Immature Hemopoiesis

In this hypothesis, two additional influences of PHZ are considered:

1. Increased Sensitivity of Erythropoietic Progenitors to Erythropoietin

As has been discussed by Hodgson,[8] PHZ seems to increase the sensitivity of Epo-sensitive cells to Epo. Quantitatively, this can be seen from Figure 1. In PHZ-induced anemia (triangles) a significantly higher erythropoietic amplification is found than after bleeding (dots). For the purpose of our model analysis this finding can be expressed by an elevated dose-response curve (Figure 1, full curve), with a maximum amplification of ten times normal,

as has been suggested by Wulff.[16] Mathematically, the new dose-response curve is characterized by formula 15a in Reference 13 with the parameters being:

$$Z_{CE}^{max} = 320 \text{ (standard 128) and}$$

$$b = 1.0 \text{ (standard 0.7)}$$

For the Hct of 0.55 times normal, which will be simulated, again a theoretical EP value of 15 times normal follows (Figure 1, vertical line). But now, due to the enlarged erythropoietic production one has to consider a more rapid recovery behavior of the Hct and, consequently, of EP. Now EP decreases after 6 days and is suppressed for an additional 10 days (Figure 2, full curve). This theoretical EP curve has also been taken from Wulff[16] and it is consistent with experimental findings on the rapid recovery and overshoot of Hct[1,3,6,8,10] after PHZ (also, see References 16 and 17).

2. Cell Loss during Cell Migration to the Spleen

After administration of PHZ, the CFU-S counts in the bone marrow fall by approximately 50%. This has been attributed to a migration of stem cells from the marrow to the spleen.[2,3,8] However, already an approximate calculation[11] shows that a large fraction of migrating cells leaving the marrow never reaches the spleen (see Section V). We, therefore, make the assumption that stem cells migrate from the marrow to the spleen but that a certain fraction of them is lost. Similar arguments hold true for BFU-E[4] and CFU-GM.[1,6] If one considers the spleen/marrow ratio from these experiments,[11] one finds that about 0.6% of all stem cells and early progenitors are lost per hour over a 7-day period.

Mathematically, this is simulated in the following form (see Reference 13):

$$\dot{S} = (2p - 1) * S/T_S - K_L * S$$

$$\dot{BE}^* = \dot{BE}^{*in} - BE^*/T_{BE} - K_L * BE^*$$

$$\dot{CG}^* = \dot{CG}^{*in} - CG^*/T_{CG} - K_L * CG^*$$

with the loss coefficient, K_L, being 0.006 h^{-1} during the first 7 days and 0 thereafter.

In summary, hypothesis I simulates the "naive" assumption that the acute hemolysis induced by PHZ is substantially of the same type as bleeding anemia. In hypothesis II, additional experimental knowledge on increased erythropoietic amplification and on the loss of cells is incorporated.

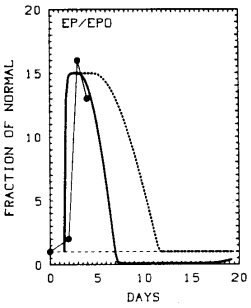
IV. RESULTS

A. Model Calculations

In the following, model curves for the simulation of hypothesis I (only acute hemolytic anemia after PHZ) and hypothesis II (additional influences of PHZ on early hemopoiesis) are compared (Figures 2 to 10). For both, an initial reduction of the Hct to 0.55 of normal is assumed.

1. Hypothesis I: PHZ Induces Only Acute Hemolytic Anemia (Dashed Lines)

Anemia leads to a 15-fold increase in EP (Figure 2) which stimulates CE and E (Figures 5 and 6). As has already been described in detail for bleeding anemia,[17] this leads to a drop of BE, CG, and G (Figures 4, 7, and 8) due to the suppressive effect of the elevated



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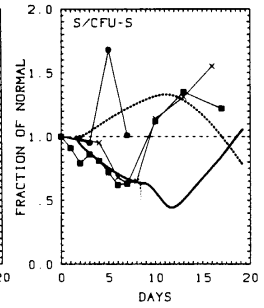

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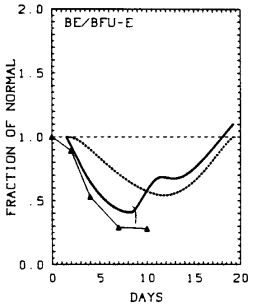

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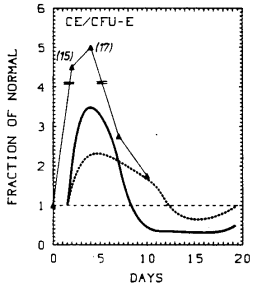

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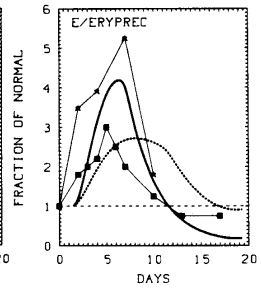

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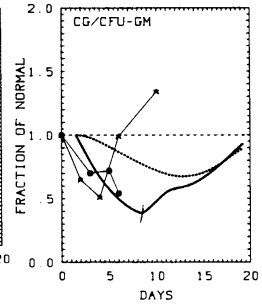

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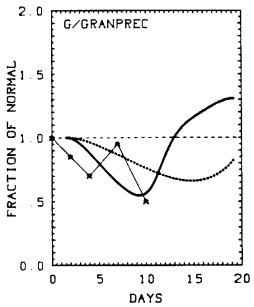

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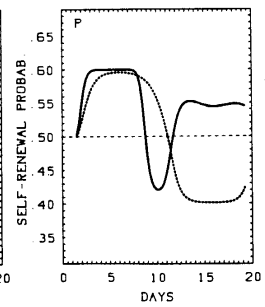

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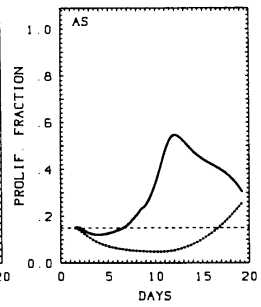

FIGURE 10

FIGURES 2 to 10. Administration of phenylhydrazine (PHZ) — comparison of model results and data. Two hypotheses are simulated by the model. Hypothesis I (---): PHZ induces only acute hemolytic anemia; hypothesis II (—): in addition to hemolytic anemia, PHZ leads to a loss of stem cells and progenitors (S, BE, CG) and increases the EP sensitivity of the erythropoietic cells. In both calculations, the same initial reduction of the Hct to 0.55 times normal is assumed. The theoretical EP curves in Figure 2 reflect the recovery of the hematocrit which is a consequence of the dose-response curves in Figure 1. The EP curves are used as input to the model. The model curves are compared with total cell counts of PHZ measurements (bone marrow plus spleen):[11] (BFU-E, CFU-E, ▲);[4] (CFU-S, CFU-GM, ●);[1] (CFU-S, erythroblasts, ■);[3] (CFU-GM, myeloblasts, erythroblasts, ★);[6] (CFU-S, X);[18] (Epo, ●).[15]

erythropoietic precursors on stem cell cycling (Figure 10). The number of stem cells, S (Figure 3), shows an increase above normal. After 10 days, EP returns to normal and the cell numbers start to normalize.

2. Hypothesis II: PHZ Has Additional Effects on Immature Hemopoiesis (Full Lines)

This simulation differs from that of hypothesis I in two features: (1) erythropoietic amplification is enlarged due to an increased EP sensitivity of erythropoietic progenitors (Figures 5 and 6). Consistent with the enlarged amplification, erythropoiesis recovers earlier and EP levels decline earlier (Figure 2); (2) during a 7-day period (of migration) a certain percentage of stem and progenitor cells is lost from hemopoiesis (Figures 3, 4, and 7).

In detail, two phases can be identified:

- Day 1.5 to 8: during this phase two effects are dominant. First, the increased EP sensitivity leads to pronounced peaks in CE and E, reaching maxima between 300 and 400% on day 5 (Figures 5 and 6). Second, the migratory loss of immature cells results in a drop of S, BE, and CG to about 50% of normal (Figures 3, 4, and 7). Although no migratory loss is assumed for G, it decreases to 70% because the cell flux from CG is reduced (Figure 8).
- Day 8 to 20: at day 8 the loss phase is terminated. The fate of stem cells is now exclusively governed by the feedback loop. At the same time EP drops below normal (Figure 2), thereby leading to a decline in CE and E to subnormal values (Figures 5 and 6). Although reduced in number, S does not start immediate recovery. This follows because the depressing effect of G on "p" is no longer compensated by high E values. Thus, "p" goes down and self-renewal of S is inhibited. In the meantime the cycling of stem cells ("a_S") is accelerated by the drop of CE and E. By day 12 "a_S" has reached its maximum. More cells enter differentiation leading to a recovery of BE, CG, and G. The normalization of G increases "p" and a rapid recovery of S follows.

In total, the numbers of the EP-sensitive cells (CE, E) are elevated while all other cell numbers are reduced, during the first 2 to 3 weeks after administration of PHZ. This result differs from that of hypothesis I in two aspects:

1. The behavior of S is reversed.
2. For all other cells the reactions are qualitatively the same, but more pronounced.

B. Comparison with the Data

The data shown in Figures 2 to 8 are derived from experiments in which three PHZ injections (on days 0, 1, 3) have been administered. Total cell numbers have been calculated by pooling bone marrow and spleen measurements in the way described elsewhere.[11]

These data can be partly understood if one assumes that PHZ induces only acute hemolysis (dashed lines): the increase of CFU-E and erythropoietic precursors, decrease of BFU-E and the granulopoietic cells are qualitatively reproduced. However, the quantitative agreement is poor. For the stem cells one finds a discrepancy: CFU-S decrease while S increases.

A clearly better agreement with the data is found, if one considers additional influences of PHZ on immature hemopoiesis (full lines): now the model curve for E gives a good reproduction of the erythropoietic precursors (Figure 6). The initial declines of S, BE, and CG are also reproduced much better than in the previous calculation. Qualitatively, the increase in stem cell cycling ("a_S") is in agreement with measurements of thymidine suicide in the bone marrow, which also indicates clear acceleration of stem cell proliferation.[3,18]

However, some discrepancies remain. The large increase of CFU-E to 17-fold normal values[2] cannot be reproduced. More severely, the increase of CFU-S,[3,18] measured after 8 days, appears many days later in the model. This discrepancy coincides with the findings on the rate of stem cell cycling. Thymidine suicide data[3,18,19] from the bone marrow suggest that stem cells proliferate at maximum rate on day 7, but "a_S" has its maximum on day 12 (Figure 10).

V. DISCUSSION

From a general point of view, the stimulus by erythropoietin is the dominating influence on hemopoiesis after PHZ-induced hemolytic anemia. This is reflected by the qualitative agreement of data and the dashed lines in Figures 2 to 8. However, the full lines show a significantly better quantitative agreement with the data. This tends to support the concept of additional influences of PHZ (migratory loss and high EP sensitivity of erythropoietic precursors).

Typically, a mouse spleen contains approximately 3000 CFU-S while the bone marrow includes about 20 times more (60,000).[11] Thus, the total CFU-S number in bone marrow and spleen equals approximately 63,000 CFU-S. If the bone marrow loses half of its CFU-S by migration,[2,3] one should expect an increase of splenic CFU-S to 33,000 (i.e., to 11 times normal), provided that all of the migrating CFU-S settled in the spleen. However, only an increase to 13,500 (i.e., 4.5 times normal) is observed in the spleen.[2,3] This finding suggests that about two thirds (19,500 of 30,000) of the migrating CFU-S are lost. It remains uncertain whether this loss reflects an in vivo seeding efficiency of $^1/_3$ or whether the cells are destroyed by the toxic effect of PHZ. Pooling spleen and marrow values shows that the total CFU-S number has dropped by 30% (43,500 of 63,000 remain). To account for this disappearance of stem cells and similarly for progenitor cells (BFU-E and CFU-GM), the assumption of a temporary (migratory) loss has been introduced. During a 7-day period a constant cell loss of 0.6%/hr has been chosen in the model calculations.

If PHZ-induced hemolytic anemia would be equivalent to bleeding anemia, erythropoietic recovery would be similar in both situations. However, after severe bleeding, anemia lasts at least 10 days before the Hct has returned to normal.[20-24] In contrast to this, after a comparable PHZ-induced anemia the Hct normalizes considerably quicker (approximately within 6 days) and shows an overshoot.[1,3,6,8,10] In addition, much higher reticulocyte numbers are found after PHZ than after bleeding.[3,6] These findings also support hypothesis II rather than hypothesis I. Both results can be explained by an increased sensitivity of the Epo-responsive cells after PHZ.

Biologically, this increased amplification after PHZ does not necessarily suggest that the Epo sensitivity of the individual Epo-sensitive cells is enlarged. It could as well be an environmental effect. It is known that the spleen has a favorable milieu for erythropoiesis. The spleen seems to support erythropoiesis with a higher amplification than in the marrow as is suggested by the enormous increase of CFU-E and erythroblasts in the spleen.[8] If more cells are shifted into this environment the whole system (marrow and spleen) seen as a whole seems to react with a greater amplification. In this context it is interesting that in rats (where the spleen plays a less important role than in mice) no pronounced differences are found between bleeding and PHZ-induced hemolytic anemia.[25-33]

A different "environmental" factor must also be taken into account: while after bleeding a large amount of iron is lost, in PHZ experiments the iron of the hemolyzed erythrocytes is left in the animal. The enlarged pool of serum iron could allow for a rapid incorporation into dividing erythroblasts which are already stimulated by erythropoietin. The effect would be an increased erythropoietic amplification. Such an effect has been measured in several experiments. Ganzoni[34] found a significant increase of iron incorporation after the substitution of iron. Reincke and Cronkite[35] measured an enlarged number of proerythroblasts and basophilic erythroblasts after the administration of ^{55}Fe. Here the free iron stems from the

late erythropoietic precursors which are selectively destroyed by the radioisotope.[36] Furthermore, in man erythropoietic amplification is two to three times higher in hemolytic anemia than after blood loss.[37,38]

Bearing these possible "environmental" explanations (effects of iron or splenic milieu) in mind, the notion of an "increased sensitivity" of the erythropoietic system to erythropoietin as a whole still seems justified as can be seen by the higher production rate of red cells.

In total, the above analysis suggests that PHZ has the following influences on hemopoiesis: it kills erythrocytes in the peripheral blood by hemolysis, it stimulates migration of early hemopoietic cells from the bone marrow to the spleen, some of which are lost, and it increases the amplification of erythropoietin-sensitive cells. It remains obscure whether the latter effect is a "real" increase of Epo sensitivity on a cellular level or a consequence of an easier availability of free iron or of the shift of erythropoiesis to the spleen where the same cells "physiologically" react to Epo more effectively than in the bone marrow.

Some discrepancies between the model calculations and the data remain (CFU-S recovery, cycling of stem cells). At least, in part, they may depend on the fact that local regulation in bone marrow and spleen might occur while the model considers only global regulation. Further investigation of these questions is necessary.

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Summary and Discussion

Chapter 13

SUMMARY OF THE RESULTS*

H.-Erich Wichmann and Markus Loeffler

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I. ABSTRACT

Twelve experimental situations have been analyzed with a mathematical model of stem cell regulation: acute, chronic, and post-chronic irradiation, bleeding anemia, administration of erythropoietin and hypoxia, hypertransfusion and ex-hypoxia, the combination of irradiation with bleeding and hypertransfusion, the administration of iron-55 and phenylhydrazine. This paper summarizes the results of the model analysis.

It is shown that the model may give a comprehensive interpretation of the experimental findings. Although not all details of every experiment can be reproduced (which is not the purpose of the presented model), the general features of the measurements can be identified in the theoretical curves.

II. INTRODUCTION

A mathematical model of stem cell regulation has been described in the context of its biological assumptions,[1] its mathematical properties,[2] and its system behavior.[3] The model

provides a new and comprehensive approach with which to interpret the response of the hemopoietic system to a broad spectrum of manipulations. The essential model assumptions are

- The cyclic activity (turnover) of stem cells (S) and their self-renewal probability are the most important variables.
- These activities are controlled by two feedback influences. One is the self-control by stem cells, the second is a negative feedback from the differentiated (granulopoietic and erythropoietic) precursor cells.
- Of the differentiated cells, erythropoietic precursors (E) rather than granulopoietic precursors (G) predominantly influence the cycling of stem cells. Granulopoietic precursors rather than E predominantly influence stem cell self-renewal.
- Peripheral demand influences (via erythropoietin) the erythropoietic amplification and changes the number of erythropoietic precursors. The stem cells react indirectly to these changed numbers.

The model has been applied to several experiments and the simulations compared with the available data. The essential features of this analysis will be summarized here, under the following general headings:

1. Technique of model simulation (Technique)
2. Model curves and their interpretation (Model Curves)
3. Comparison of the model curves with experimental data (Data)
4. Further properties and weak points of the model analysis (Comment)
5. Possible alternative explanations of the data (Alternatives)

III. RESULTS

A. Acute Irradiation

1. Technique

The effect of acute irradiation is simulated in the model by a reduction of the initial values of the cellular compartments.[4] These initial values are taken from the experimental minima between days 0 and 3.

2. Model Curves

Recovery after acute irradiation shows a triphasic pattern. In the first phase, exponential growth of stem cells is found. This phase is characterized by a maximum of both the self-renewal probability and the proliferation rate of the stem cells.

In the second phase, regulated (nonexponential) recovery takes place. Here the demand for differentiated cells achieves the higher priority, and differentiation may take place at the expense of stem cell recovery.

The third phase starts as soon as enough differentiated cells have been produced and is characterized by normalization of the stem cell number.

The presence or absence of these three phases as well as their duration depends on the initial depletion. For stem cell numbers below 6%, the first phase of exponential growth is obligatory. If stem cells and precursors are only moderately reduced (to values above 10%), the exponential phase may be missing and the stem cells may show an early plateau or even a dip.

The recovery of precursor cells often starts with an initial peak ("abortive rise"). This peak is pronounced if the differentiated cells are only moderately reduced and may be missing if they are severely reduced.

As a rule, the recovery of stem cells (S) is slow. The progenitors (BE, CG) recover quicker while the precursor cells (E, G) show an even quicker normalization. Only during the early phase may a different pattern be found.

3. Data

Comparison of the theoretical recovery curves with data[5] shows that the above findings can be identified in the measurements: exponential recovery of CFU-S after severe depletion, earlier normalization of the more mature cells, and sometimes an "abortive rise" for precursor cells.

4. Comment

It is unfortunate that dose-response relationships for all cell types are not available in a given mouse strain. Therefore, it was only possible to make qualitative statements about the radiosensitivity of the cell types. Comparing the initial reduction of stem cells with the initial reduction of differentiated cells obtained in the same experiments, one finds that the radiosensitivity of stem cells is 1 to 12 times as high as that of differentiated cells, with an enormous scattering of data between different experiments.

5. Alternatives

Different concepts have been proposed to explain the recovery curves after acute irradiation. Hellman et al.[6] assume that, during the first few days after irradiation, granulopoietic differentiation is preferred at the expense of erythropoiesis. Such a variable channeling of the efflux from the stem cell pool is not included (and not needed) in our model.

Chervenick and Boggs[7] propose a complete stop of differentiation if the stem cells are severely reduced. In the model, even during exponential growth 40% of the stem cells differentiate per cell cycle (compared to 50% under normal steady-state conditions).

The "abortive rise" of erythropoietic and granulopoietic precursors is explained by OKunewick[5] as a cohort of selectively activated cells traveling down the pathways, where the cells die with a delay. In the model, this "activated cohort" is the result of a regulatory process. Delayed death of some cells may occur but for us it is of minor importance.

B. Chronic Irradiation

1. Technique

Chronic irradiation is simulated in the model by constant cell loss proportional to the number of cells in each compartment.[8] Two loss coefficients are used, one for the stem cells and one for the differentiated cells.

For severe irradiation, a significant anemia and granulocytopenia develop. In the model the combined stimulatory influence of anemia and granulocytopenia is simulated as described below for bleeding anemia.

2. Model Curves

Biphasic model curves are found. The first phase is characterized by an exponential decrease of cell numbers. During this phase, cell death exceeds the formation of new cells. Thereafter a plateau is established.

The plateau values of the stem cells (expressed as fraction of normal) are slightly lower than those of the early progenitors. The precursors have clearly higher plateau values. If the daily destruction rate exceeds a critical value no plateau is found and the hemopoietic system dies out, because more stem cells are destroyed than can be formed.

The model calculations show that anemia and granulocytopenia which develop during chronic irradiation have severe influences on early hemopoiesis. They stimulate the amplification of precursor cells which can be maintained at much higher levels than without such peripheral stimulation. This maintenance of precursors leads to larger numbers of stem cells because the differentiation pressure is considerably reduced. In total, anemia and granulocytopenia have a protective effect on early hemopoiesis: the numbers of stem cells, progenitors, and precursors are significantly higher than they would be without peripheral stimulation.

3. Data

The data for CFU-S (as well as for the few available measurements of progenitors and precursors) demonstrate a biphasic pattern (initial decrease followed by a plateau). Furthermore, the more mature cells show higher plateau values than the earlier cells. In general, the data are more homogeneous than for acute irradiation, perhaps because most measurements are from one research group.[9]

4. Comment

The homogeneity of the data allows us to relate the loss coefficients of the model explicitly to the experimental doses. It is found that the radiosensitivity of stem cells is 50% higher than that of differentiated cells. The "limiting dosage" above which the hemopoietic system dies is 0.71 Gy/day in the model and 0.7 to 1 Gy/day in the experiments.

5. Alternatives

Other concepts on the influence of chronic destruction have been proposed. Mackey[10] has predicted that chronic irradiation might lead to an onset of sustained oscillations for a certain dose range. In the stem cell model presented here oscillations do not occur at any dose. This corresponds to the observations in normal mice and may be different for other species or for mouse strains with specific defects.

Blackett and Botnick[11] have proposed that differentiation from the stem cell pool may be induced by an *increased* number of precursors (rather than by a *reduced* number, as assumed in our model). Following their hypothesis, the anemia and granulocytopenia which develop during chronic irradiation would further deplete the stem cell pool and would make the situation worse. This is opposite to our model finding that the peripheral demand, to a certain extent, has a protective influence on stem cells.

A general comment on dose-response curves for chronic irradiation seems necessary. Many authors prefer to compare the cell survival with accumulated rather than daily doses. Accumulated doses may be useful to characterize residual damage, but they are misleading for intact hemopoiesis because the effect of a short exposure with a high daily dosage may be very different from the effect of long exposure with a low daily dosage. Therefore, we propose to use dose-response curves which relate the plateau values to the *daily* dosage rather than the *accumulated* dosage. After more than 20 days of chronic irradiation, the cell numbers are quite stable and steady-state dose-response curves can be drawn.

C. Post-Chronic Irradiation

1. Technique

The recovery of hemopoiesis after chronic irradiation is simulated by taking the measurements on the day of termination as initial values for the model.[12] Missing values are estimated from the theoretical plateau values of chronic irradiation.[8]

2. Model Curves

The theoretical recovery curves are similar to those found after single-dose irradiation: early and overshooting recovery of the late progenitors and precursors, exponential increase of stem cells for low initial values.

3. Data

Comparison of the theoretical curves with the measurements[13] shows a good agreement as long as the preceding phase of chronic irradiation does not exceed a dose of 0.5 Gy/day and a duration of 30 days. For higher doses or longer times of irradiation the measured cell numbers recover significantly slower than the theoretical curves and sometimes do not return to normal. This discrepancy between observed and predicted recovery may be interpreted by residual damage.

4. Comment

Here one important limitation of the model becomes apparent: it is not able to consider situations in which residual perturbations persist after the end of the experiment. The theoretical curves always return to normal after the stress has ended. For long-lasting severe irradiation the model in its present form is unable to realistically simulate on the data.

D. Bleeding Anemia

1.Technique

Since in the stem cell model mature erythrocytes are not considered, anemia is simulated indirectly by using a time course of erythropoietin changes as input for the model.[14] This theoretical curve is derived from a mathematical model of mature erythropoiesis.[15,16]

2. Model Curves

In the stem cell model, erythropoietin stimulates additional mitoses in the sensitive cells, leading to an increase of erythropoietic precursors (E). As a reaction to their change the cycling of stem cells (S) changes by intramedullary feedback. Since the feedback is negative the elevated number of cells in E tends to reduce the cycling rate in S so that the rate of cells entering differentiation is diminished. This reduced influx leads to reduced cell numbers in all differentiated compartments, except for erythropoietin-sensitive cells which are still stimulated by the high levels of erythropoietin. The impact of the erythropoietic precursors on the self-renewal of stem cells is small. Therefore, S shows no dramatic changes.

In total, one observes the following theoretical pattern in bleeding anemia: the cells sensitive to erythropoietin (CE, E) are elevated, the stem cells (S) fluctuate around their normal value, and the granulopoietic cells (CG, G) as well as the early erythropoietic progenitors (BE) are diminished.

3. Data

The measurements[17] show the same characteristics as the model, although there are some discrepancies in the magnitude of the reactions. CFU-S increase and decrease earlier and to a less pronounced degree than does the theoretical stem cell number. BFU-E sometimes show an initial peak before they decrease, which is not reproduced by the calculations. CFU-E and erythroblasts increase to a greater extent than predicted. Model predictions mirror the CFU-GM decrease.

4. Comment

Quantitatively, the model reaction is not yet satisfying. One reason may be that the spleen is not considered separately but only as part of a total hemopoietic organ with the same regulatory properties as in the bone marrow. This view may be too simple, especially in anemia, where the spleen plays an important role.[18]

E. Stimulation by Erythropoietin

1. Technique

In the model the administration of erythropoietin is simulated by an elevated theoretical curve for the hormone.[14]

2. Model Curves

The theoretical cell numbers behave similar to those noted after bleeding: the late erythropoietic progenitors (CE) and the precursors (E) increase significantly, the stem cell number remains close to normal, and the other differentiated cells decrease.

3. Data

These theoretical curves satisfactorily reproduce the experimental observations: a clear increase of CFU-E, only minor changes in CFU-S, and a decrease of BFU-E and CFU-GM. The quantitative agreement of theory and experiment is better than after bleeding.

F. Hypoxia

1. Technique

Like anemia, hypoxia is simulated by elevated erythropoietin values.[19] The theoretical curve for erythropoietin is high at the beginning of hypoxia and decreases exponentially to a supranormal plateau, consistent with experimental measurements.

2. Model Curves

During sustained hypoxia, the erythropoietin-sensitive cells CE and E are elevated, S is close to normal, and BE, CG, and G show subnormal plateau values.

3. Data

Comparison with the data[20] is somewhat sophisticated, because different strains of mice do not respond similarly to the hypoxic stimulus. In the responders, the increase of erythropoietin-sensitive cells (CFU-E, erythroblasts) is similar as in the model (CE, E). The intermediate increase found for the theoretical stem cell number (S) is found for CFU-S only in one experiment. The theoretical decrease of the progenitors and granulopoietic precursors corresponds to experimental findings for BFU-E, CFU-GM, and myeloblasts.

4. Comment

As after bleeding, some quantitative discrepancies remain. Again, the simplified consideration of splenic erythropoiesis may be responsible for this.

5. Alternatives

During hypoxia the increase of erythropoiesis is accompanied by a decrease in the numbers of nonerythropoietic cell lineages. This is similar to the situation during anemia, although the changes are more marked. The observations are consistent with the data as reduced granulopoietic progenitors and precursors as well as decreased granulocytes and thrombocytes in the peripheral blood have been noted. Rickard et al.[21] interpret these phenomena by competition of the cell lineages — in our model it is once more the consequence of a reduced cell flux from the stem cell compartment.

G. Hypertransfusion

1. Technique

Suppression of erythropoiesis is simulated indirectly by using a time course of erythropoietin as input for the model. In the time course, erythropoietin levels are close to zero.[22] This missing stimulus leads to the omission of amplifying divisions in the CE and E compartments.

2. Model Curves

The reduced number of erythropoietic precursors (E) activates the cycling of stem cells. The flux of cells into the differentiated lineages becomes enlarged and the number of early

progenitors (BE, CG) and of granulopoietic precursors (G) increases. Only the erythropoietin-sensitive cells remain at low levels because no erythropoietin is available to mediate amplification.

However, a second type of intramedullary feedback has to be considered here. This is the influence of the granulopoietic precursors (G) on the self-renewal probability of stem cells (S). The elevated number of G leads to an enlarged self-renewal of S. The consequence is an increase of the stem cell number after hypertransfusion.

In total, one finds reduced numbers of erythropoietin-sensitive cells, while all other compartments (stem cells, early erythropoietic progenitors, granulopoietic progenitors, and precursors) are enlarged. After normalization of the hematocrit the bone marrow cells also return to normal, after a delay.

3. Data

The distinct increase of CFU-S[23] (as found in most experiments) and granulopoietic precursors is reproduced by the model. The theoretical curves are also in reasonable agreement with the actual data for CFU-GM, CFU-E, and erythropoietic precursors.

4. Comment

Only two effects are not explained by the model: the experimental values of erythropoietin are only reduced to 50% of normal while the theoretical values are much lower. It is uncertain whether the difficulty in measuring subnormal erythropoietin concentrations can explain this discrepancy. Second, BFU-E show an initial decrease which is not found in the theoretical BE curves.

5. Alternatives

The experimental observations may also be explained by different hypotheses. As discussed by Monette,[23] one might assume that the step from BFU-E to CFU-E is blocked by the large number of red cells in the blood. This block would lead to the depletion of CFU-E and to an accumulation of cells in the BFU-E and earlier (CFU-S) compartments. These accumulated cells might then "pour" into other pathways giving rise to granulopoiesis.

A second popular approach assumes a competition between granulopoiesis and erythropoiesis for a limited cell supply.[23] If the number of red blood cells is enlarged, more progenitor cells are directed into the granulopoietic pathway.

Both hypotheses sound reasonable and simple for the explanation of hypertransfusion, but it remains questionable whether they lead to a consistent understanding of the other experiments analyzed in this volume.

H. Ex-Hypoxia

1. Technique

The normalization of hemopoiesis after termination of a hypoxic stimulation depends on the duration and the degree of the preceding hypoxia. The mathematical simulation uses the cell numbers at the end of the hypoxic phase as initial values for the model compartments.[24] The suppression of erythropoiesis during the first weeks posthypoxia is considered by assuming erythropoietin values close to zero.

2. Model Curves

The missing stimulus leads to a dramatic decrease of the initially elevated erythropoietin-sensitive cells. As described for hypertransfusion, this is followed by an increase of all cell numbers including differentiated compartments and stem cells.

3. Data

This theoretical behavior can be identified in the data.[25] CFU-E and erythroblasts decrease from elevated numbers to subnormal plateau values as seen in the theoretical curves. CFU-S, BFU-E, and CFU-GM recover from subnormal to supranormal values and a similar but less clear increase is found for granulopoietic precursors.

I. Irradiation and Bleeding

1. Technique

Irradiation is simulated by reduction of the initial values and the additional blood loss is described by elevated concentrations of erythropoietin.

2. Model Curves

In the model the recovery curves[26] are similar to those of irradiation: slow recovery of stem cells, slightly quicker recovery of (early) progenitors, early recovery of precursors. The additional anemia significantly influences only the erythropoietin-sensitive cells (CE and E) which now show a pronounced overshoot. All other cells (S, BE, CG, G) are not markedly affected.

3. Data

Comparison of the theoretical curves with the data of the one available experiment[27] shows a good agreement for CFU-S, BFU-E, and CFU-GM. Only for the CFU-E of the irradiated control is a discrepancy found.

J. Irradiation and Hypertransfusion

1. Technique

Irradiation is simulated by reduced initial values, and the influence of hypertransfusion is considered by reduced values of erythropoietin.

2. Model Curves

As already discussed, hypertransfusion decreases the erythropoietic cells but increases the number of stem cells and granulopoietic cells. When compared with irradiation alone, irradiation plus hypertransfusion results in earlier recovery of stem cells, progenitors, and granulopoietic cells.

3. Data

The few available experiments[29,30] do not clearly demonstrate the beneficial influence of hypertransfusion on granulopoiesis. Only the expected decrease of erythropoietic cells has been measured. In the experiments, hypertransfusion was added while stem cell numbers were still below 20%.

4. Comment

The model calculations predict a beneficial effect of hypertransfusion on the recovery of granulopoiesis after irradiation. The model analysis suggests that for this beneficial effect to be evident, the animals should be hypertransfused some days after irradiation rather than before or immediately after. The delay would depend on the radiation dose and should be longer for higher doses. This can be understood if it is realized that the beneficial effect of hypertransfusion on granulopoiesis follows by activation of stem cell cycling. In severely irradiated animals, cycling is already maximum and cannot be further increased. Therefore, hypertransfusion becomes most effective if applied after the stem cell numbers have already recovered to 50% of normal and stem cell cycling has declined from its plateau values.

K. Iron-55 Experiments

1. Technique

^{55}Fe is a cytotoxic radioisotope which is incorporated into erythropoietic precursor cells. In the model the effect of ^{55}Fe is simulated by destruction of polychromatic erythroblasts (E3).[31] For this purpose, the model compartment of the erythropoietic precursors is subdivided into four compartments which represent the proerythroblasts (E1), the basophilic (E2), polychromatic (E3), and orthochromatic (E4) erythroblasts. In the first step only cell destruction in E3 is simulated by a constant loss.

2. Model Curves

The theoretical curves show a decrease in E3 (by direct damage) and in E4 (by reduced influx from E3). These low numbers stimulate the cycling of stem cells and reduce their self-renewal probability. A slight decrease of the stem cell numbers (S) and a slight increase of the differentiated cells follows.

3. Data

Comparison of theoretical curves with the experimental data[32] (CFU-S, erythropoietic, and granulopoietic precursors) shows only poor agreement. Actual CFU-S numbers decrease to a plateau close to 50% of normal (after 440 μCi ^{55}Fe) and the proerythroblasts and basophilic erythroblasts (E1, E2) increase to three to five times normal. This cannot, in our model, be explained by intramedullary feedback from the reduced numbers in E3 and E4.

4. Comment

In a second step we have, therefore, looked for an alternative "reasonable" hypothesis of ^{55}Fe effects on hemopoiesis. An explanation consistent with the data can be given if three effects are assumed: (1) severe internal radiation damage of polychromatic erythroblasts (E3), (2) permanent destruction of stem cells (and possibly of other proliferative cells) at a low rate, and (3) additional mitoses of the early erythropoietic cells. However, at least the last two assumptions are speculative.

5. Alternative

A different explanation of the data is given by Reincke and Cronkite.[32] They assume that ^{55}Fe kills only iron-incorporating late erythroblasts and no stem cells. The decrease of CFU-S for them is a consequence of recruitment of CFU-S into the erythropoietic progenitor population.

L. Phenylhydrazine-Induced Hemolytic Anemia

1. Technique

Phenylhydrazine (PHZ) is a hemolytic agent. It leads to severe anemia, which is accompanied by a shift of hemopoiesis from the bone marrow to the spleen. In the mathematical analysis[33] the influence of PHZ is simulated as follows: (1) the severe anemia is considered indirectly by elevated erythropoietin values; (2) stem cells and progenitors migrate from the bone marrow to the spleen. On their way, a considerable proportion of cells is lost; (3) the sensitivity to erythropoietin is elevated.

2. Model Curves

With these assumptions, the theoretical curves show a decrease of S, BE, CG (migratory loss), and G (missing influx from CG). The erythropoietin-sensitive cells (CE, E) increase significantly.

3. Data

Comparison with the pooled data from bone marrow and spleen[34] shows a good reproduction by the theoretical curves of the behavior of BFU-E, CFU-GM, erythropoietic, and granulopoietic precursors. For CFU-S and CFU-E some quantitative discrepancies remain: CFU-S recover earlier and CFU-E increase higher than the model curves for S and CE.

4. Comment

It remains uncertain whether the discrepancies between observed and predicted behavior of CFU-S and S and CFU-E and E could be explained if the spleen was taken into account more appropriately. One might suspect that the elevated sensitivity of erythropoietic cells to erythropoietin is only a consequence of the general shift of hemopoiesis to the spleen. In the spleen, erythropoietic cells "physiologically" react more sensitive to erythropoietin than in the bone marrow. A second reason for the increased "sensitivity" to erythropoietin may be the stimulatory effect of the iron being available for red cell formation after the preceding hemolysis.

IV. DISCUSSION

A mathematical model of stem cell regulation has been applied to those perturbations of early hemopoiesis (in vivo, in mice) which have been most often studied. It was not our intention to fit model curves to individual measurements. Our intention was to start from an *a priori* concept for intramedullary feedback and to test whether the consequent application of this concept could reproduce the essentials of the measured patterns for many different situations.

We have tried to keep the model simple and the number of model parameters small. For the standard model 6 differential equations and 26 parameters have been used;[2] 20 of the parameters could be derived from measurements. Only six parameters are speculative and have been determined from the model simulations.

The proposed model should be understood as the consequent "thinking to the end" of one possible concept of regulation of early hemopoiesis rather than the solution of this complex problem. Several weak points remain:

- Only global regulatory control is considered and not local control in the bone marrow and spleen.
- Residual damage is not considered.
- The feedback dependencies are relatively well characterized for reduced cell numbers but are not sufficiently delineated for enlarged numbers.

A number of questions remain unanswered although they may be investigated by further refinement of the model. What is the influence of thrombopoiesis and mature granulopoiesis? How can the cyclic phenomena found in some animals (gray collie dogs, W/W^v mice, S1/S1^d mice) be understood? How strongly do cytotoxic drugs perturb regulatory control of early hemopoiesis? Can the model be applied to man?

Furthermore, the presented concept, which provides theoretical results which are consistently compatible with experimental data, may also indicate which experimental questions are of specific interest from the viewpoint of regulatory control:

- Can regulators of stem cell self-renewal be identified?
- How do the presumptive regulators of stem cell cycling experimentally found fit into the scheme of the model?

- How can the proposed asymmetry between feedback from granulopoiesis (dominant effect on self-renewal) and erythropoiesis (dominant effect on cycling) on stem cells be investigated more directly?
- Is the variable number of amplifying mitoses during differentiation an ingenious mechanism to protect the stem cells and to keep them in their normal (quiescent) situation as long as possible? Are there advantages of such a process against other possible mechanisms?
- Is it true that different hemopoietic lineages interact only indirectly via stem cells and their cell cycle status?
- Under which circumstances and how far can the regulatory processes themselves be altered?

The model in its present form may support the investigation of these questions in two ways. The first concerns the analysis of different concepts. This can be done by formulating the hypotheses mathematically and calculating their consequences.

The second use of the model for an experimentalist concerns the practical problem of the planning of new experiments and the analysis of the results. The model allows the experimental protocol (when to measure, what to measure) to be optimized and to give some information about the "theoretical" outcome of such a study.

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Chapter 14

DISCUSSION

Dr. C. D. R. Dunn, Dr. L. Kreja, Dr. M. Loeffler, Dr. F. C. Monette, Dr. M. J. Murphy, Dr. J. P. OKunewick, and Dr. H.-E. Wichmann

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I. INTRODUCTION

The discussion took place during the preparation of this volume (Baltimore, August 15, 1982). We (Wichmann, Loeffler) presented the basic ideas and the preliminary results of our model analysis to the authors of the experimental chapters at the 1982 meeting of the International Society of Experimental Hematology. Authors who could not participate had the chance to send written contributions to this discussion.

II. STEM CELL CONCEPTS

OKunewick — You are assuming only two lines of differentiation out of the stem cells, granulopoietic and erythropoietic differentiation, and I just wonder whether or not you should also make provisions for a lymphopoietic differentiation. I recognize that the concept of lymphopoietic cells developing out of the stem cells is not as well developed as for erythropoiesis and granulopoiesis, but I think that the data are such that one has to make allowances for that. Perhaps they don't follow exactly the same kinetic parameters and it may be that the pluripotent stem cells feed in at some constant fixed rate to the lymphopoietic compartment, which then has its own stem cells that develop out of that. I think you have to make some assumptions for it.

Wichmann — We have not incorporated the lymphopoietic cell lineage until now because we wanted to construct a model that is more or less sound and proven in all its details. Therefore, it was necessary to start at a very simple stage. In the first version we have only considered erythropoiesis as you know from our earlier papers. Now, in this more advanced version we have added the granulopoiesis to make the model more realistic. Of course it would be a further step on this way to incorporate the lymphopoietic cells. However, before we can do that, we need detailed information about lymphopoietic regulation which is not available at the present time.

Monette — I would like to argue that you have to keep it exactly the way you have it now because it makes a big difference on how one defines stem cells. It has been my belief that the CFU-S method does not measure lymphopoietic progenitors.

Dunn — I think that is the point. The stem cells in the model are CFU-S, not a more loosely defined stem cell, and while I think that there is some evidence that the lymphopoietic cells are coming from the CFU-S, it is not convincing.

OKunewick — I think that's fine then, if they are approaching it from that standpoint. The S of the model should then be very clearly defined as a common progenitor for the granulopoietic and the erythropoietic line and make an allowance for another arrow feeding in from a more primitive stem cell from which the lymphopoietic elements may derive.

Monette — In other words, you need to define exactly what you mean by "stem cell", what type of stem cell one is following and whether the model can, indeed, address any progenitors or the special stem cell that we are talking about. I do not know whether that is feasible.

OKunewick — There is evidence that T lymphocytes do carry the same chromosome markers as the transplanted CFU-S. Again, that does not mean that they are coming from the S compartment of the model. They may come from a more primitive compartment that the CFU-S assay does not measure.

Dunn — I think there was also some data that suggested that the repopulation of the lymphopoietic cells could appear to come from a CFU-S-type population at certain times posttransplantation and at other times it came from a non-CFU-S population.

Wichmann — I would agree with you that it is possible to subdivide our S compartment into an earlier one which might also be the progenitor for lymphopoietic cells and a later one which might be measured by the CFU-S. These later cells might be the stem cells of erythropoiesis and granulopoiesis only, as proposed by Dr. OKunewick. From the mathematical point of view, such a subdivision would present no serious problems. At the moment we have left it out because it seems to us a little speculative. However, if strong experimental arguments support this idea in the future, we will be able to consider it in the model. We think that it would not severely affect the model's present regulatory behavior.

Monette — The work by Rosendaal, Schofield, Hodgson, and others suggests that the important property of the self-renewal probability differs depending on where the cells are in the stem cell compartment. If the perturbations that we are inducing in the system are calling for different subpopulations, then that really affects the self-renewal probability in the entire population.

Hodgson (written contribution) — I really have only one comment to make on the model, which was already made in Baltimore. You assume that the stem cell population is a homogeneous one and that its behavior is adequately monitored by the CFU-S assay. I think that there is experimental evidence to indicate that the stem cell pool is heterogeneous and CFU-S assays are not a good measure of the stem cell pool. Whether including a more complex stem cell structure into the model will improve the fit between experimental data and model predictions remains to be tested.

Loeffler — You are talking about another substructure within the stem cell compartment reflecting a heterogeneity in self-renewal. We are aware of the discussion on this point, but, nevertheless, we use only one uniform stem cell compartment. We have the feeling that the particular perturbations considered in this book (irradiation, erythropoietic stimulation, and suppression) can satisfactorily be explained by this simple structure. Again, mathematically a subdivision would not be difficult but at present rather speculative. Therefore, our self-renewal probability has to be regarded as an average value and our stem cells as average stem cells.

OKunewick — I have one other thing to say with regard to the two compartments that we are looking at here (granulopoietic and erythropoietic precursors). All the evidence indicates that following irradiation damage or following transplantation these two compartments come back very rapidly. At the same time the evidence for the lymphopoietic compartment is that it comes back very very slowly. The immune response is not restored following transplantation for many months, and it may be that the perturbations that would be placed on the system by that would be very low. That might be one of the reasons why the evidence for the relationship is not too good because of the very long time that is involved into that restoration.

Murphy — It is probably indicative of the state of the model and its clarity that, as yet, we have not said a word about erythropoiesis. We have talked about almost everything else except that, so that this speaks very bold for what appears to be a very sound model of erythropoiesis. Does the model as it is allow you the latitude to take the next step? Clearly, the next step would be really designing good experiments for granulopoiesis, for monocyte-macrophage production, for megakaryopoiesis, as well as for lymphophoiesis. When we look at the scheme you have, there is no mention of CFU-Meg; and since, certainly, thrombopoietin regulates in vivo and Meg-CSF in vitro megakaryocytopoiesis, and since M-CSF and GM-CSF are becoming more highly purified, I think you may wish — although it's premature — to include some of these facts.

Wichmann — Our model is in an intermediate stage and we surely will include thrombopoiesis in the next step. In fact, we have already investigated thrombopoiesis separately and we hope it will be straightforward to incorporate this experience in a generalized model. We then will be able to investigate the influences of irradiation on thrombopoiesis or effects like thrombocytopenia in hypoxia — in other words, further interactions between the cell lines.

OKunewick — I think the model as we have it now defines very well what we know, and it is good from that standpoint. But I also agree with what Dr. Murphy is saying because what you are looking for in the long run is a model that is going to represent the entire system. The idea you propose of viewing it in terms of balance and demand probably is a very good means to initiate this; and if, indeed, it should work out that other elements of the hemopoietic system have lesser demands we would expect the perturbations in this area to affect the stem cell compartment less. The present existing model would, therefore, serve as a good first approximation to the whole system from which additional developments could be coming.

III. SELF-RENEWAL PROBABILITY

Dunn — What are the units for "p"?

Loeffler — It is a probability with values between 0 and 1.

Dunn — Is it percent, not percent per unit time?

Wichmann — You may express it as probability (between 0 and 1) or as percent (between 0 and 100%). In any case, it is not a rate (= percent per unit time).

Dunn — Then "p" is the proportion of cells that differentiates irrespective of time?

Loeffler — Let's assume the decision for stem cells to differentiate or to remain in the stem cell pool takes place at a certain step during the cell cycle. At any moment a number of cells enters this phase. Of these cells "1-p" is the proportion that differentiates and "p" is the proportion that remains in the stem cell pool; "p" is a simple probability (no rate), but since "p" is regulated, its value may, of course, change with time.

Monette — You haven't had to assume a low "p" value for granulopoietic and erythropoietic progenitors (CG and BE) at all?

Wichmann — No, we have not explicitly considered a self-renewal probability for non-stem cells. However, our concept of amplifying divisions in CG and BE can be transformed into the concept of "p" values for these cells which are lower than the "p" value of the stem cell in S.

Monette — I raise this point because you are probably aware of a paper published about 10 years ago by Udupa and Reissmann (*Blood*, 53, 1164, 1979) where they treated animals with Busulphan (Myleran) which wiped out all the stem cells. The remaining cells were BFU-E and CFU-E and their progeny, and those cells reconstituted erythropoiesis in the drug-treated animals if additional Epo was injected. Whether or not there is evidence for some self-renewal of these cell progenitors which are capable of extensive proliferation, I don't know.

Loeffler — We have made calculations (which are not incorporated in this book) on the Busulphan administration that you mention. That analysis suggests that the drug must have had a strong depressive influence on the self-renewal probability "p" of stem cells so that recovery of the stem cells is impaired. We believe that drugs like Busulphan disturb the regulation processes, at least for a limited time. On the other hand, the reactions of BFU-E and CFU-E are constistent with the model and can be explained on the basis of the Epo effects. The injected Epo seems to induce a maximum number of amplifying divisions so that erythropoiesis can be maintained by influx from the stem cells, although the stem cell counts are severely depleted.

Lord (written comment) — In fact, Schofield (*Blood Cells*, 4, 7, 1978) made the measurement experimentally and obtained a severe reduction in self-maintenance capacity of CFU-S after Busulphan.

Loeffler — It is interesting to note that according to our preliminary analysis, the situation is different for BCNU (Loeffler, dissertation, 1983). Here the self-renewal probability seems to be maximum for a while, although this is inadequate from a regulatory point of view. In conclusion, the drug-induced alterations of the regulatory process will need future attention.

Monette — Do you see an experimental way to determine "p" more directly? Should we be testing for changes in "p" in modulating the system which force "p" either up or down? By a self-renewal assay?

Wichmann — Well, we have a very sensitive assay for "p", and that is the CFU-S assay.

Monette — Just the numbers?

Wichmann — Just the numbers. At least the switching at $p = 0.5$ can be measured. If "p" is greater than 0.5, S will increase independent of whether the cell cycle is fast or slow. If the cycling is fast S increases steeper; if it is slow, it increases slowly but it will increase. Therefore, we have a switching point at $p = 0.5$ which is beautifully measurable by S. However, it is much more difficult to measure values of "p" different from 0.5, because then "p" and "a_S" have a combined influence on S. The number of S, e.g., will increase in the same way for $p = 0.55$ and $a_S = 1.0$ or for $p = 0.6$ and $a_S = 0.5$.

Monette — This really puts a different light into my thinking about the self-renewal probability "p". Here it is modulated completely by the system. It is just like every other parameter that can be changed on and off. I have tended to think of self-renewal probability as an endpoint, more or less of an intrinsic parameter of the stem cell itself. I just wonder whether one can really apply this experimentally, whether one can pursue some of these predictions.

Dunn — The limits of "p" are very close together, mathematically, but the biological effect might be very large if we are looking for differences between 0.4 and 0.6.

Monette — That's a huge, measurable difference of "p".

IV. IRRADIATION

Dunn — How do you simulate cell death by irradiation?

Loeffler — For an acute irradiation we simply reduce the initial values of the model compartments. We remove, e.g., 90% and let the system recover. This simulates an instantaneous loss of cells where the biological process of damage and subsequent cell death is not considered in detail. For chronic irradiation we permanently remove a certain fraction of cells.

Dunn — Did you notice that poster by John Millar (Millar, J. L., The effect of dose rate on the recovery of CFU-S after exposure to 60Co gamma-radiation, *Exp. Hematol.*, 10 [Suppl. 11], 160, 1982)? The data suggested that the acute effects of irradiation were not, but that the recovery effects were, dependent on the irradiation dose rate, which presumably you could not simulate in your model. The dose ranges that he used were quite wide (Eds: 0.04, 0.52, 3.7 Gy/min, up to a total dose of 8 Gy).

Loeffler — I don't think we can really explain this effect, but it gives me a chance to mention the problems with interpreting accumulated dose-response curves. Please look at the CFU-S behavior during continuous irradiation (Chapter 9, Volume 1). If you consider the curve for 0.1 Gy/day, then you have reached a plateau value of 65% of normal on day 100. This day corresponds to an accumulated dose of 10 Gy. The same accumulated dose with 0.5 Gy/day is already achieved at day 20. However, here the CFU-S are reduced to 20% of normal. Thus, for the same accumulated dose of 10 Gy you have totally different effects on the CFU-S. From our analysis we find, generally, that one cannot give reasonable dose-response relations for the stem cell number depending on the total dose. The dose rate is the important quantity, and in all situations where irradiation is not applied acutely (i.e., during a very short time interval), one should refer the stem cell effects to the dose rate rather than to the accumulated dose. This holds true for continuous irradiation as well as for fractionated irradiation.

Dunn — I appreciate that point. I don't think that it is quite the point that we were addressing. But I appreciate it.

Murphy — Dr. Loeffler, I would like to address a question just to your acute irradiation model. After whole body irradiation to the mouse many investigators showed a postirradiation dip in CFU-S. But erythropoietic progenitors in some cases showed an initial overshoot. What does your model predict?

Loeffler — In the model you sometimes get an initial rise in the CE (CFU-E) and sometimes not. That depends on the cell survival in the BE compartment (BFU-E). If many cells in BE survive after irradiation they are shifted to CE, developing the initial peak in this compartment. If you have only a small surviving fraction in BE you don't observe that peak in CE. That is just the difference between Dr. Monette's (Chapter 7) and Dr. Seidel's results (Chapter 5). The first measures an initial CFU-E peak but also a high initial BFU-E value, and the latter finds no initial CFU-E peak because — as we would explain — in his animals only a small number of BFU-E has survived irradiation. In addition, the post-irradiation dip of CFU-S (discussed in detail in Chapter 7, Volume I) can be explained on the basis of differentiation pressure exerted on stem cells by the demand for mature cells.

V. REGULATORY INFLUENCE OF ERYTHROPOIESIS AND GRANULOPOIESIS

Dunn — I am trying to phrase my question. What is the biological evidence for regarding different influences on the self-renewal probability "p" and on the fraction of actively cycling stem cells "a_s"? To ask more precisely, why do you assume in your model that the relative influence of erythropoietic (E) and granulopoietic (G) precursors is 1:4 on "p" but 3:1 on "a_s"?

Wichmann — The different influences of E and G become important when peripheral perturbations like anemia, hypoxia, or hypertransfusion are considered. We suggest that in those situations the active fraction of stem cells "a_s" is mainly influenced by E, while the self-renewal probability "p" is mainly influenced by G. Since, on the other hand, "p" is responsible for the behavior of S and "a_s" for the efflux from S, we can make the following somewhat oversimplified statement: If the cell numbers in the peripheral blood are changed, we have a direct stimulatory or suppressing influence on the progenitor and precursor cells in the bone marrow (E and/or G). This leads to indirect influences on stem cell regulation: the granulopoietic precursors are mainly responsible for the reaction of the stem cell number while the erythropoietic precursors are mainly responsible for the reaction of the stem cell cycling changing the cell flux from the stem cell pool into the differentiated cell lineages.

Loeffler — To say it briefly, the imbalance in these feedback influences reflects the experimental observations that CFU-S, CFU-E, and CFU-GM in hypertransfusion and in anemia do not behave as complete mirror images of each other as one would naively expect.

Dunn — Then I would question whether that is a biological assumption. That sounds to me as if that is a mathematical assumption to have the model fit the biological data.

Wichmann — You may put it that way. We have no direct information about these feedback influences, since they cannot be measured. The only thing we have found is that we need them to consistently reproduce the data found in hypertranfusion, hypoxia, posthypoxia, and anemia in one model. In this respect, they are a major result of our model analysis giving you quantitative information about the organization of the feedback loop.

VI. E-G INTERACTION

Monette — As I see it, your model has changed significantly in the last few years, largely because of a reciprocal relationship between G and E. When we talked 2 years ago about the hypertransfusion effect on stem cells, you had predicted no effect or only a slight decrease.

Wichmann — That is correct. We have learned a lot in the meantime, especially about hypertransfusion, and we now interpret the increase of CFU-S as a consequence of the increase of the granulopoietic precursors.

Monette — I realize the raw data scatter quite a bit and what you are drawing is the best reflection of that data. Not everyone necessarily sees large shifts in one when you manipulate the other. Is that really pointed out by the data?

Wichmann — I think the data show that there is an opposite behavior of erythropoietic and granulopoietic precursors. In anemia, e.g., you have an increase in erythropoietic cells and a decreased granulopoiesis and the opposite in hypertransfusion (Chapters 5 and 6.)

Monette — Have they been assayed side by side or is it from different laboratories?

Wichmann — Some have been assayed in parallel. You have measured BFU-E and CFU-E and Dr. Seidel and Dr. Kreja have measured BFU-E, CFU-E, and CFU-GM in one experiment. The rest of the data unfortunately could only be collected from different authors.

Monette — With the changes in anemia are they bringing down the hematocrit more or less to the same level? Have the data been filtered out so that it is more or less of the same magnitude and effect?

Wichmann — Yes, we consider only severe bleeding anemia where the red cell mass is reduced by 25 to 35% of its normal value.

Dunn — I would like to play the devil's advocate for a minute. In the hypertransfusion situation E goes down. That sends a message to S: "We need some more E." So "a_s" increases to increase BE which then tends to compensate for decreased E. But then, from an economic standpoint, I would have expected that the numbers of S and the numbers of G would not change. Furthermore, you are talking about influences on "a_s", but I cannot see any real reason why "p" should not change also to compensate for the change in "a_s". I guess that I am playing the devil's advocate because I am adapted to the approach that because mathematical equations fit the data it does not necessarily mean the mathematical equations are correct.

Wichmann — You say, and that is correct, that in hypertransfusion E are down and, therefore, "a_s" increases. That leads to an increased flux of cells into differentiation. This flux increases both erythropoietic and granulopoietic progenitors, since both are fed from the same source. If you keep the determination rates fixed (as we do in the model), every change of the cell flux out of S affects both erythropoiesis and granulopoiesis. The second effect is that the increase of the granulopoietic cells has an antagonistic influence, namely, to compensate for the modifications induced by erythropoiesis. The granulopoietic cells tell the stem cells: "We don't need any more cell supply, we (the granulopoietic compartment) have enough, keep your cells" and, therefore, S increases. In total, the regulatory system stabilizes at an intermediate position, where erythropoiesis is reduced and both granulopoiesis and stem cells are increased. If the counteraction of granulopoiesis would not exist, G would increase even higher.

Loeffler — Granulopoietic precursors limit and dampen the reactions which are induced by erythropoiesis and vice versa.

VII. FEEDBACK FROM EARLY PROGENITORS OR LATE PRECURSORS

Dunn — Why couldn't the influence on "p" be mediated by the progenitor cells BE and CG (corresponding to BFU-E and CFU-GM), the immediate successors of S instead of the later precursor cells?

Wichmann — In an earlier version of our model we had assumed exactly what you propose, namely, that the progenitor cells are responsible for the feedback on "p" and "a_s". This worked fine as long as the proliferative fraction of these cells was kept constant. Now we consider variable proliferative fractions "a_{BE}" and "a_{CG}" to make the model more

realistic. However, this variability destabilizes the whole system if feedback still comes from BE and CG. We only find an appropriate model behavior if the later precursor cells E1-4 and G1-4 are responsible for the regulation, and these quantitatively correspond to the total erythropoietic and granulopoietic cell numbers, E and G.

Dunn — The total cell numbers?

Wichmann — Yes, you see, if you add up all cells of one lineage, then, of course, the later cells dominate due to the amplification, so that the more mature cell stages are outnumbering the early stages.

Dunn — So, then, where is the influence of granulopoiesis on erythropoiesis in that system? From granulopoiesis to the BFU-E or from erythropoiesis on the BFU-E?

Loeffler — No, the important part of the interaction between granulopoiesis and erythropoiesis is mediated indirectly through the stem cells (via "a_S" and "p" which depend on E and G). To a less relevant extent cycling of CFU-GM is influenced by erythropoiesis and cycling of BFU-E by granulopoiesis ("a_{CG}" and "a_{BE}" depend on E and G, too).

Monette — You find the long-range loop from precursors to stem cells satisfying? To me, Dr. Dunn's short-range loop from BE on S, respectively, seems to be more efficient.

Loeffler — I think we disagree at this point. Our argument is based on some of your own measurements and Dr. Seidel's measurements about the behavior of BFU-E and CFU-GM after irradiation (Chapter 5 to 8). As irradiation data for BFU-E were not available before, we did not know that the BFU-E recover very slowly after irradiation, similar to the stem cells. We can understand this phenomenon if we assume that the BFU-E and CFU-GM have a variable fraction in G_0-phase just as the stem cells (for which experimental evidence exists). In the model we describe this by a variable proliferative fraction ("a_{BE}", "a_{CG}"). Consequently, the transit time for the BE cells (which correspond to BFU-E) is variable. If you would assume a constant transit time for BE, a cohort of cells leaving S would travel through BE, CE, and E with a fixed "velocity". You, consequently, would find repetitions of the same peak in each of these successive compartments, only shifted by the maturation delays. But you don't observe this. For example, after irradiation the CFU-E show an overshoot, but the BFU-E, which would be expected to have shown an overshoot some time before, recover very slowly. This is the argument for us to say that there is a regulation of the transit time mediated by a variable proliferative fraction. The consequence of this assumption is that the BE number depends not only on the influx rate but also on the variable transit time. A cell number which is under such complex influences is not a good reference compartment on which to build a feedback loop. It makes the system unstable. Therefore, feedback from later cells which have a constant proliferative fraction is more stable and efficient.

Wichmann — To complete this statement, we cannot say that it must be CE or E which are responsible for feedback. But it must be a population which has not the variability in the G_0-phase as Dr. Loeffler explained.

Loeffler — I want to comment on just one additional aspect and that is to what extent the CFU-S, BFU-E, and CFU-GM are similar cell populations. In the model all three stages are similar with respect to the proliferation rate. We have "real" stem cells (S) with a large variability of "a_S", we have a kind of intermediate cell stage (BE and CG) also with a (reduced) variability of their proliferative fraction, and we have more mature cells (CE, E, G) which have lost this property. For the self-renewal "p" this stepwise loss of stem cell property from one cell stage to the next is not explicitly assumed in the model. Only S has an effectively variable "p".

Dunn — Maybe this is then the next logical question: What evidence is there that the mature bone marrow cells influence CFU-S?

Wichmann — We have no direct biological support for this; we only suggest it from our model results.

Loeffler — I think this hits a very important point of model building, in general. What we know are cell cycle times, amplification rates, proliferation rates, but almost nothing is known about the biology of the regulatory mechanisms. Our reasoning is to make hypotheses about these regulation mechanisms, and we try to think about them as simply as possible. Our calculations give sound suggestions of how the real system could work. However, we cannot identify which molecular factors or microenvironmental processes are responsible for this kind of intramedullary feedback. We only can say that an influence of the mature bone marrow cells on self-renewal and active cycling of CFU-S, as assumed in the model, allows us to consistently understand the complex behavior of the measured cell numbers. We suggest a frame of thinking which can be filled by much more details.

Monette — Let me ask, what does it do if you change the minimum vs. maximum cycling rates for BE? It does not really have that much a big effect?

Wichmann — It does not have a big effect on the later cells which are the regulatory ones. The shift is only very short. That means the maturation wave entering into the later cell stages is transient and, therefore, it is of minor importance for regulation. However, the number in the BE and CG pool may be, of course, severely influenced.

Monette — I find this present model much more satisfying than your previous model (Loeffler, Wichmann, *Cell Tissue Kinet.*, 13, 543, 1980) mainly because it seems to be able to give you a more realistic picture. I think this has a lot to do with the way we are defining stem cells. We are defining it by the assay which seems to identify a progenitor for both CFU-GM and BFU-E. Would you predict that changes in CFU-Meg, which feed into megakaryopoiesis, have an important influence on your model? CFU-Meg appear to be the one major cell progenitor which is missing here.

Wichmann — I cannot make a clear statement concerning this point because we have not yet performed calculations considering CFU-Meg. However, I have the impression that the changes will only be minor.

Monette — That would be my feeling, also. I think how you are probably accounting for the major feedback to one particular class of stem cells, even though it does not consider all hemopoiesis. I think you have got a stem cell model here with the two major lines of differentiation that are probably responsible for feedback on it.

Wichmann — At the first step of modeling, when we did not consider granulopoiesis we thought that we would consider the most important influences of erythropoiesis, and I think we did. One of the points where we had problems was hypertransfusion. Hypertransfusion for us now seems to be determined by the granulopoietic influence which is quite indirect and a new understanding for us. Similarly, the introduction of thrombopoiesis might influence the interpretation of one or two specific experiments, but it surely will not make the analysis of the rest invalid.

VIII. STEM CELL COMPETITION

Monette — The concept of stem cell competition has been tossed around for a long time and it is still controversial. How have you considered it in your model?

Dunn — They do not assume stem cell competition.

Loeffler — That is correct. We do not have variable determination fractions into the different lineages. Nevertheless, we can reproduce phenomenologically everything that is attributed to stem cell competition like, e.g., anatogonistic behavior of E and G.

Monette — There are many ways you can look at this concept and the level that I was thinking of was very broad: when you manipulate the erythropoietic pathway, whatever the feedback loop is and whatever the response of the stem cells itself, the end result is a definite response by the opposite pathway.

Loeffler — Oh yes, in this broad sense we agree. The model exhibits this phenomenon although it was not explicitly designed in terms of regulated determination.

IX. TIME DELAY

OKunewick — Mentioning hypertransfusion, polycythemia. There is a question I wonder if you included that in the model. Are you allowing for, let's say, a lag time in adjustment? What I'm getting at is, when you, for instance, expose mice for a protracted period of time to hypoxic conditions you perturb the system and you get an increased number of erythropoietic cells coming out. The stem cell compartment adjusts to this. And I am sure your model contains that. However, when you take the mice out of the hypoxic conditions you find that there is an increase in the CFU-S compartment which takes about a week to come back down to the normal situation. That is what I am referring to about the lag time.

Wichmann — Yes, that is included in the model, and in the situation you refer to the model curves show the same time lag as the experimental data (Chapter 4).

Loeffler — The time lag that we have in the model stems from the maturation time that is needed for cells to process from the stem cells to the maturing stages which then determine the feedback. What we do not assume in the model is a lag-time for the feedback process itself. This process is assumed as being instantaneous. In other words, the assumption is that the time scales for the regulation process is much faster, this process must pass the microenvironment much quicker than the maturation process of the cells.

OKunewick — Maybe it is not instantaneous. But we all agree that it is rather rapid.

X. ERYTHROPOIETIC PRECURSORS

Hara (written contribution) — The morphologically identifiable erythropoietic cells in the bone marrow are usually equivalent with the erythroblasts. In the model you included these cells in one compartment with six mitoses. It is, however, generally accepted(with the exception of Dr. Weicker) that four generations with three or four mitoses are more appropriate. This is consistent with observations on morphology stathmokinetics, ^{3}HTdR autoradiography, and so on. On the basis of the biological observations you could, therefore, set up four compartments with a total of three to four mitoses and an additional marrow transit time after denucleation. This scheme would also correspond well to the model description for granulopoietic precursors.

Wichmann and Loeffler (written answer) — From our knowledge of the literature most investigators conclude that during normal steady state the erythroblasts perform approximately six mitoses in mice and six to seven mitoses in rats (see, e.g., Covelli et al., 1972, Mary et al., 1980, Tarbutt and Blackett, 1968, Monette, 1983; the full references are given in Chapter 4, Volume 1). We have chosen six mitoses in the model. A lower number of mitoses, however, would not severely affect the model results, since we consider relative cell numbers. Only during severe suppression of erythropoiesis the decrease of erythroblasts would be less pronounced: only three to four mitoses could be omitted if we follow your proposal, instead of six as assumed in the model. Concerning the number of compartments we agree that four precursor compartments can be introduced into the model. This has, in fact, been done in the chapter on iron-55 effects (this volume). However, in most situations it was sufficient to consider one comprehensive compartment for the erythroblasts and we have decided to choose this simpler form.

XI. INEFFECTIVE HEMOPOIESIS

Hara (written contribution) — I completely agree with the two-step feedback mechanism proposed. In the model committed progenitors such as CFU-GM and CFU-E are cells which have a kind of reserve function responding to specific stimulators. As a consequence I like to ask whether you made any consideration on cell loss of progenitors at the stage of differentiation.

Loeffler and Wichmann (written answer) — We do not take into account ineffective hemopoiesis and we do not need this assumption. The question is, what happens with CFU-E if, e.g., the stimulatory influence of Epo is missing? Their number drops although BFU-E remain largely normal. In the model the drop of CFU-E is neither interpreted as cell loss nor as presenescent death nor does it result from a block between BFU-E and CFU-E. In our interpretation we have a loss of amplification. All five mitoses in CFU-E may be omitted. The normal rate of cells enters from BFU-E but as five mitoses are omitted the erythropoietic production is only 1/32 of normal and the number of CFU-E and erythroblasts is drastically reduced.

XII. FINAL REMARKS

Murphy — Your modeling reminds me about the story of the three fellows that were shipwrecked on an island. One was a basis scientist, one was a physicist, and one was a theoretical mathematician. They were very hungry when they discovered a tin can of baked beans but they didn't have a can opener. The basic scientist said, "Don't worry about it. I will go off and I will fashion a can opener and I'll bring it back and we will open that tin can." The physicist said, "Give me a rock and I'll blast it open" and the mathematician said, "Let's assume that it's already been opened." Now we really still have this tin can which is quite closed in terms of hemopoiesis, and so you say, "Let's make the assumption that we do have it understood. Here is how it should respond."

Wichmann — We wanted to understand, first, what has been measured and believe we now have at least a feeling what might be going on. From this basis we want to come to the next important point, namely, to ask new questions. The model allows us to design new experiments and to test hypotheses about the site of action of physical, biochemical, or pharmacological perturbations on hemopoiesis. We believe that in this field a close "feedback loop" between experimentalists and model constructors will provide a new tool for understanding experimental hematology.

Murphy — I think you are doing an excellent job with your modeling. This is the first time that such an approach has been executed by a multiinstitutional consortium and it augurs well for the future of this vital field.

Appendixes

Appendix 1

A SUMMARY OF SOME OF THE IMPORTANT TECHNIQUES AVAILABLE TO ASSESS CELL PROLIFERATION IN EXPERIMENTAL HEMATOLOGY

C. D. R. Dunn

TABLE OF CONTENTS

I. ABSTRACT

The salient features are discussed of most of the important techniques available to experimental hematologists. It is with the numerous variations of these methods that the data were derived which are modeled in the other chapters of this volume. The methodological summaries are preceded by a short discussion of some of the limitations in data interpretation which impinge on the accuracy required in the mathematical simulations.

II. INTRODUCTION

Before summarizing the methodologies available to assess some of the more important parameters in experimental hematology, it is worth spending some time in emphasizing a few of the basic principles involved in biological measurement. An understanding of these principles is fundamental in assessing the validity of a set of experimental results. Only after the validity and accuracy of the results have been assessed is it reasonable for the biomathematicians to realistically attempt to simulate the data and use them as a basis for further predictive studies.

III. MEASUREMENT, OR ASSAY, OF A BIOLOGICAL SUBSTANCE

Condouris[1] has summarized the essential criteria for the measurement (i.e., assay) of any "material" which cannot be quantitated by a physical parameter such as weight or volume. Let "material" be equated in the first instance with a hormone such as erythropoietin (Epo). According to Condouris, the bioassay endpoint for this material should have some relevance to the anticipated in vivo role of the hormone. It should be assayed in a multidose protocol, against either "pure" hormone or an internationally recognized preparation as a standard, and a dose-response curve to the standard should be obtained in each assay. Ideally, a minimum of three doses of both the standard and unknown material should be used since one of the essential tests for validity of an assay is linearity of the dose-response relationship — it is always possible to draw a straight line through two points. Dose-response relationships of the original data or of suitably transformed results (e.g., log dose-response) should not only be linear, but also parallel to one another and demonstrate significant slopes by some conventional statistical criteria. To reduce experimental variability, a standard protocol should be employed and an estimate of the error of the measured potency (e.g., the 95% confidence limits) must be given. ONLY if these criteria are fulfilled is it justifiable to express the results in absolute terms — in this case units per milliliter.

Most Epo bioassays are based on the use of an endpoint, such as radioiron incorporation, which bears some relevance to what is presumed to be the in vivo role of the hormone. However, there are very few reports where the other criteria for statistically valid assays have been satisfied. Therefore, the quantitative significance of many of the studies on the role of Epo in the regulation of erythropoiesis has to be regarded as questionable. It could be argued that since Epo is not yet available for clinical use it may not be essential to know with great accuracy how many units per milliliter there are in a certain preparation, especially since many of the assays are time consuming and relatively insensitive. But in the absence of a dose-response relationship of demonstrated parallelism to a generally recognized standard, it is not possible to say with certainty that it is Epo which is being detected in the unknown sample. And it seems of limited value to argue the difference between, for example, sample A at 0.1 units/mℓ and sample B at 0.15 units/mℓ on the basis of a one-dose "test" when even multidose parallel line assays have difficulty detecting anything less than a twofold difference with any statistical certainty.[2,3] Nonparametric statistical evaluations could be used to show sample B was more potent than sample A on repeated measurement, but the magnitude of the difference would have to remain in doubt.

The foregoing discussion is, although pharmacologically realistic, perhaps a little harsh, given that the in vivo assays for Epo present some well-known logistical nightmares. However, there is little excuse for one-dose "tests" with sensitive in vitro systems. It is, for example, well recognized[4] that the various colony stimulating factors (CSF) have different characteristics (type of colony stimulated, maximum number of colonies, etc.). Given the relative simplicity and inexpense of an in vitro CSF bioassay it is really very hard to justify quantitation of CSF levels on the basis of one dose; nor is it justifiable to use an "optimal" or "saturating" dose of CSF and assume it is going to be either "optimal" or "saturating" under all conditions.

Dose-response curves are equally meaningful and essential when the "material" under study is the cells rather than the putative hormones used to stimulate them. To expand on the above example with CSF, let it be assumed that under condition X a concentration Z of CSF produces the maximum possible number of 100 colonies per 10,000 cells. After the bone marrow is subjected to some procedure and cultured under condition Y, concentration Z of CSF produces 50 colonies per 10,000 cells. It is an oversimplification to assume that this result reflects a 50% loss of colony-forming cells. Although such a loss is certainly a possibility, it could also equally well reflect a change in sensitivity of the cells; there may still be 100/10,000 cells (or even 200/10,000), but a concentration Z* of CSF is required for maximum stimulation. In the absence of dose-response relationships these two possibilities (with quite different implications) cannot be distinguished and the possibility that the two marrow populations are behaving differently to the stimulus (i.e., with different slopes in response to increasing CSF concentrations) will be missed. Likewise, the possibility of cell-to-cell interactions occurring in culture can only be realistically assessed by detailed dose-response relationships of the two, presumably interacting, populations.

For accurate quantitation, therefore, and in order to gain the maximum amount of information on cell-to-cell and cell-to-hormone interactions with the minimum potential for misinterpretation, there is no substitute for dose-response relationships. Furthermore, with the increasing use of in vitro methods in experimental hematology it is difficult to justify a one-dose "test" on the basis of cost, time, or convenience.

A brief word on that popular statistic, the standard error of the mean (SEM). In experimental hematology it is common practice to pool the marrow cells from a group of donor animals and, after appropriate cell counting, these cells are then "cultured" in a group of recipient animals or culture plates. At the termination of the experiment, colonies are counted and the SEM calculated. It should be clear that under these circumstances, the SEM reflects variability from culture plate-to-culture plate or from spleen-to-spleen (i.e., technical reproducibility). It indicates little or nothing about the variability from donor animal-to-donor animal, which is minimized by pooling the individual marrow suspensions, and in which most of us are more interested. The use of the SEM to indicate technical variability is valid, but to use this technical variability as a basis for statistical tests of difference between the groups of donor animals could produce potentially misleading conclusions.

Errors can also arise from the use of ratios of which the most commonly employed is probably the hematocrit — the ratio of red blood cell volume to total blood volume. Since the hematocrit is a ratio, its value will depend on changes in both numerator and/or denominator, so to deduce changes in one parameter (e.g., the red blood cell volume) from the ratio is potentially misleading. This is, perhaps, stating the obvious. However, somehow these concepts are all-too-often forgotten when ratios are used in other contexts such as in the analysis of experiments designed to investigate hemoglobin "switching", in interpreting tritiated thymidine and other "suicide" techniques (which essentially estimate the ratio of the number of cells in DNA synthesis [S phase] to the total number of cells in cycle), or when the results of multiple drug exposures are expressed as surviving fractions. To fully appreciate results presented in ratio form it is almost mandatory to provide the values of the numerator and/or denominator on which the ratios are based.

IV. WHAT TO MEASURE

In practice, the techniques in experimental hematology available to assess cell proliferation can be subdivided into the "functional" and the "morphological". If techniques such as the responsiveness of peripheral blood lymphocytes to mitogens are excluded (and these methods are really outside the scope of this book), then pragmatically "functional" can be equated to a very large extent with colony formation. In these procedures the assays depend on the functional ability of certain populations of cells to produce a clone of generally more mature progeny either in vivo or in vitro without any clear concept of the morphological identity of those colony-forming cells (CFC). Assays for these cells will be discussed first and the classification of those cells, which are morphologically recognizable by standard microscopic techniques, follows in a later section. It is implied in all the clonal assays that the colony-forming unit (CFU) must be a relatively immature cell with some potential for proliferation such that it can produce sufficient cells to yield a micro- or macroscopically visible colony under appropriate "culture" conditions.

A. Functional Assays for Hematopoietic Progenitors

1. The Exogenous Spleen Colony Assay in Vivo (CFU-S)

In 1957 Urso and Congdon[5] demonstrated that, in irradiated mice which had received a bone marrow graft, spleen size was directly related to the injected number of bone marrow cells. Subsequently, Till and McCulloch[6] showed that if the number of injected cells was reduced to near or below the minimum required for long-term survival of the host, the focal nature of the hemopoietic regeneration was demonstrated in the form of macroscopic colonies on the surface of the spleens. Because the clonal (i.e., one cell) origin of the colonies was a matter of uncertainty for some years the principle producing the colonies was designated a CFU — the term CFU-S (S for spleen) was introduced later to distinguish that entity from other CFU.

The assay is technically simple. Recipient mice are irradiated with a dose of irradiation (generally from a 60-Co or X-ray source) sufficient to ablate most, if not all, hemopoiesis. The dose required is in the region of 7.5 to 11.0 Gy.

Within 2 to 4 hr the mice are injected with a single cell suspension of bone marrow, spleen, blood, or other hemopoietic cells usually obtained from syngeneic mice. Usually about 10^5 marrow cells or 10^6 spleen cells in 0.25 mℓ are injected. The recipient mice are sacrificed and the spleens removed and placed into a suitable biological fixative such as Bouin's solution 7 to 12 days post-transplantation. Macroscopic "lumps" are visible as white spots on a dark reddish-brown spleen 2 to 4 hr later. An essential control is a group of mice not injected with cells where the absence of colonies is an indication of the efficiency of the irradiation and, indirectly, confirms the donor origin of the colonies. Colony numbers in the exogenous colony assay are linearly related to the number of cells transplanted over the approximate range 10,000 to 100,000 for mouse bone marrow and 100,000 to one million for mouse spleen cells, although for counting accuracy a maximum of 10 to 15 colonies represents a realistic limit. Overall survival of the recipients for the number of days necessary for colony development is good since the dose of radiation is not sufficient to induce "gut" death and the lethality due to hemopoietic aplasia only becomes a factor towards the 11th or 12th postirradiation day.

In contrast to the above-mentioned "exogenous" CFU-S assay, these cells can also be assessed by an "endogenous" or "autorepopulation" method. In this technique those CFU-S which survive whole-body sublethal irradiation (6.0 Gy)[7] or those which migrate from the shielded marrow of an otherwise lethally irradiated host,[8] directly seed the animal's own spleen. This method obviates the need for cell transplantation but introduces the additional variable of the rate of egress of the CFU-S from the shielded limb.

In addition to colonies in the recipient's spleen, foci of hemopoietic regeneration are also seen, in both "auto" and "exo" repopulation methods, in microscopic sections of the marrow cavities.

Histologically, most foci of regeneration in the spleen and bone marrow consist of one cell type (either erythropoietic, granulopoietic, or megakaryopoietic) during the first 9 days of colony development.[9] At later times the proportion of "mixed" colonies (generally of erythropoietic and granulopoietic precursors) increases. Erythropoietic colonies outnumber granulopoietic colonies in the spleen by 2:1 or 3:1, but the proportion of the two colony types in the bone marrow is reversed. The majority of both "pure" and "mixed" colonies is derived from one cell, i.e., they are of clonal origin.[10] Therefore, the increasing proportion of mixed colonies with time and the histological differences between spleen and bone marrow would appear to reflect some stochastic effect[11] or the migration of cells into microenvironmental niches with different differentiating influences.[12] The mixed nature of colonies derived from an individual CFU-S[13] confirms the multipotentiality of these cells. Retransplantation of colonies into secondary recipients yields additional colonies,[14] implying that the CFU-S are capable of self-maintenance. Thus, the CFU-S, by virtue of their multipotentiality and capacity for self-maintenance, appear to have the properties of multipotential stem cells. Tritiated thymidine suicide studies[15] and those involving other phase-specific cytotoxic agents, such as cytosine arabinoside,[16] methotrexate,[17] and hydroxyurea,[18] suggest the CFU-S are in a prolonged G_1 or distinct G_0 phase of the cell cycle, i.e., they are slowly proliferating.

The problems with the CFU-S assay are more interpretative than technical. The proportion (f) of CFU-S actually lodging in the spleen[19] (comparable to seeding efficiency in certain in vitro systems) is not known with any certainty nor is it fully appreciated how this might change as a consequence of manipulations of the donor and/or recipient. An f factor of 15% is often used but this cannot be considered as firmly established. Likewise, even if a potential CFC did lodge in the spleen, if it or its immediate progeny became fully committed towards differentiation during the early phases of colony development, then too few cells would be produced for a macroscopic colony to be visible on days 9 or 10 and, therefore, would not be scored. As many as 60% of potential CFU-S have been estimated to be lost by differentiation.[20] It is unclear how this is affected by donor and/or recipient manipulation and may well also change during the time course of colony development. One way to recognize these variables is to use the term CFC for all cells with the potential for spleen colony development and restrict the term CFU-S to those that actually do form colonies and, hence, are actually scored. The relationship of CFC to CFU-S is given by:

$$\text{number of CFC} = \text{number of CFU-S/f} \times (1 - \omega)$$

where f = seeding efficiency and ω = extinction coefficient (cells lost through differentiation), both expressed as fractions.[21]

Although the CFU-S assay in mice is most widely used, a comparable assay is available in rats,[22-24] and there has been at least one report of spleen colony development in hamsters.[25] In some circumstances rat CFU-S have been assessed by xenogeneic transplantation into irradiated mice.[26]

2. Granulocyte-Macrophage Colony Development in Vitro (CFU-GM)

In the mid 1960s, two groups[27,28] described in vitro colony formation from normal mouse bone marrow. The colonies were composed of granulocytes, macrophages, and mixtures of both of these cell lines. Colony numbers were linearly related to the number of cells plated down to limiting dilutions suggesting a clonal origin for each colony. However, in the absence of firm evidence to support the concept of clonogenicity and because of the ter-

minological precedent established by the CFU-S assay, the term CFU-A (A for agar) was introduced as a functional description for the principle producing the colony. (In some earlier reports the term CFC was used but this is, perhaps, best restricted to the absolute number of CFU-S — see above.) Although agar was used as a supporting medium in many of the early studies, it can be replaced by methyl cellulose such that CFU-C (C for culture) is a more utilitarian nomenclature for the in vitro CFU-GM.

The essential factors involved in the CFU-GM technique are hemopoietic cells, a suitable semisolid support medium (whose principal function is probably to maintain the proliferating cells in close geographical proximity, i.e., in the colony), and a diversity of specific stimulating factors now designated as CSF.[4] In theory the method is fairly straightforward. The hemopoietic cells from a variety of organs and, unlike the CFU-S assay, with no species limitation are suspended in an enriched liquid medium generally containing up to 30% of a serum supplement, usually fetal calf serum. The cell suspension is incorporated into the support medium (0.8% methyl cellulose of 0.3% agar) and aliquoted into petri dishes. The CSF, as a liquid, conditioned medium,[28] can be mixed into the cell suspension/support layer or directly provided by cells plated in a more viscous (0.5% agar) layer below that containing the target cells.[29,30] The cultures are incubated in a water-saturated atmosphere of 5 to 10% carbon dioxide for periods of times (7 to 21 days) which, in large part, appear to reflect the individuality of different investigators. Colonies can be counted under an inverted or dissecting microscope after flooding the dishes with a suitable stain if desired. There is no general agreement as to how many cells constitute a colony, but greater than 50 cells is widely used as a counting criterion. Aggregates of less than 50 cells are termed "clusters" and are frequently scored separately.

There are advantages in using methyl cellulose over agar,[31] and the complete replacement of serum with a variety of highly purified proteins[32] will allow a detailed evaluation of the relative importance, to granulopoiesis, of many of the putative regulatory molecules. The biological significance and properties of CSF have recently been reviewed.[4] It should be noted that CSF can be extracted from a variety of tissues but each source produces material with different properties with respect to the morphological type of colony stimulated and/or with different species specificity. If cell suspensions of "whole" hemopoietic tissue are cultured, particularly at high cell concentrations, CSF can be produced endogenously by non-CFU-GM in the cultures. To remove this potential complication, CSF-producing cells should be removed by adherence to glass or plastic prior to culture of the remaining cells.[33]

Considerable debate has taken place regarding the nature of the CFU-GM and its relationship to the CFU-S. There now appears to be a general acceptance that the CFU-GM represent a population of immature progenitor cells which are descendants of the CFU-S but which precede the myeloblasts (see, for example, Reference 34). The use of the term "committed stem cells" to describe the CFU-GM would appear appropriate as the cells appear to be "committed" to no more than two lines of differentiation (granulopoiesis and monocyte/macrophage production) and retain some ability for self-replication.[35] If, however, more emphasis is placed on the progeny character of CFU-GM (or granulo/macrophagopoiesis), then the term "determined granulopoietic progenitor cells" would also be appropriate. In a variety of physical parameters, such as size and density[36] as well as on the basis of proliferation rate,[18] the CFU-GM are distinct from the CFU-S. However, estimates of CFU-GM cell size and density indicate a considerable heterogeneity within this cell population.[37] It is possible that this reflects, in part, different levels of maturity (or of an age structure) within the CFU-GM such that it is not improbable that under suitable conditions some CFU-S might produce an in vitro colony and thus be scored as a CFU-GM.[38]

The CFU-GM technique can, of course, be used as an assay procedure for CSF. In such instances the variable is the material being assayed for colony-stimulating activity (CSA) while the number and type of hemopoietic target cells are held constant. One unit of CSF is defined as the quantity of material required to stimulate one CFU-GM of mouse bone marrow.[4]

3. Erythropoietic Colony Development in Vitro (BFU-E, CFU-E)

The growth of erythropoietic colonies in vitro (in this instance in the semisolid medium provided by a plasma clot) was first described by Stephenson et al. in 1971.[39] The small colonies (<64 cells) consisted of benzidine-positive (therefore, hemoglobin-synthesizing) aggregates which reached their maximum number and size, from mouse hemopoietic tissue, after 2 days in culture. Colony development was dependent on the presence of Epo. These erythropoietic CFU were designated CFU-E and were soon recognized to be a relatively mature progenitor cell, probably much more so than the CFU-GM, with an extremely limited proliferative capacity. Subsequent studies using either higher concentrations of Epo in methyl cellulose cultures[40] or by refeeding plasma clot cultures with Epo[41] both combined with longer incubation times, resulted in the appearance of large (in some instances macroscopic), multicentric colonies of benzidine-positive cells. The nature of these colonies resembled CFU-E that had "burst" so the CFU was designated a BFU-E, or burst forming unit-erythropoietic. Subsequent studies have demonstrated that while the primary stimulus for CFU-E development is Epo, the BFU-E requires a still poorly defined substance termed burst promoting factor (BPF) or burst promoting activity (BPA) which appears to be a product of T lymphocytes.[42] In BFU-E cultures, Epo is, however, still required for the hemoglobinization of the progeny. In addition, many sources of Epo (most notably the sheep plasma preparations) contain BPF. More recently a third population of CFU-E has been reported to occur in murine[43] and human[44] hemopoietic tissue. These cells appear to show a sensitivity to Epo and BPF intermediate to that of the BFU-E and CFU-E. They have not been designated with any unique terminology but are generally specified by the culture day on which they are scored, e.g., BFU-E-3d for those BFU-E in murine marrow cultures scored on culture day 3.

While culture conditions which permit the development of CFU-E in vitro have not in any way been standardized from laboratory to laboratory, certain general features appear evident. Thus, cell concentrations of 1 to 2 $\times$ 10^5 bone marrow or 1 to 2 $\times$ 10^6 spleen cells per milliliter are generally employed. Nonadherent cells may be removed prior to culture or "whole" marrow employed; 0.7 to 0.8% methyl cellulose or clotted bovine plasma provide the support matrix. A variety of liquid media are employed of which Alpha, NCTC-109, and Iscove's Modification of Dulbecco's Medium (IMDM) appear to be the most popular. Such media are supplemented with asparagine and 20 to 30% prescreened fetal calf serum, although the serum requirement can be reduced to less than 4% by the inclusion of iron-saturated transferrin, bovine serum albumin, and a variety of lipids (e.g., soybean "lecithin", oleic acid, dipalmitoyl lecithin, cholesterol). A sulphydryl compound, either thioglycerol or mercaptoethanol, is often included in the cultures at a concentration of approximately 0.1 m*M*. In addition, plasma clot cultures are supplemented with beef embryo extract. Epo is added to a final concentration of 0.1 to 0.3 U/mℓ for CFU-E and to concentrations approximately tenfold higher for BFU-E. BPF can be added as a "contaminant" of the Epo preparation or separately. In the latter instance, the conditioned medium from mixed leukocyte cultures or of mitogen-stimulated spleen cells is incorporated at 10 to 30% of the culture volume. Although smaller culture volumes are sometimes used (i.e., 0.1 mℓ aliquots cultured in microtiter plates), the incubation volume is usually 1.0 mℓ. These larger volumes are incubated in plastic petri dishes in a fully humidified atmosphere of 5 to 7.5% CO_2 in air. Unless stained the cultures can be scored sequentially (with the aid of microscope) for CFU-E, intermediate BFU-E, and immature BFU-E, between 2 and 14 days for murine cells, and between 7 and 21 days for human cells. Aggregates of 8 to 64 cells are generally considered to represent a colony derived from a CFU-E, while smaller

aggregates can be scored separately as erythopoietic clusters. Immature BFU-E produce colonies of greater than 200 cells. Depending on the particular type, erythropoietic colonies have one or more of the following characteristics which can be used to facilitate their identification and differentiation from CFU-GM-derived colonies: the cells are found in typical, closely packed aggregates (CFU-E) or as multicentric foci of these aggregates (BFU-E), they stain positively with benzidine, and the large "bursts" are frequently red in color due to the hemoglobin content. Staining of the cells can be accomplished *in situ* (by flooding the petri dish with stain) or by "plucking" individual colonies with a Pasteur pipette and smearing them onto a microscope slide for subsequent histochemical analysis. Typical colony numbers obtained under the above conditions range from 200 to 600 CFU-E to 60 to 100 BFU-E.

Detailed analyses using techniques to characterize cells on the basis of size, density, and proliferation rate have indicated that the erythropoietic CFC demonstrate a spectrum of properties.[43,44] Thus, the BFU-E appear to be small, slowly proliferating cells which are sensitive to BPF but show a questionable response to Epo. As cells mature through the progenitor cell compartments to the CFU-E, they increase in size and proliferation rate and show increased sensitivity to Epo and decreased sensitivity to BPF. The CFU-E appear to be closely related to the earliest morphologically recognizable erythropoietic cell, but a population of cells (erythroid cluster-forming cells) between the CFU-E and the pronormoblasts, has been recognized.[45] Thus, an apparent age structure within the erythroid progenitors is somewhat better defined than in the CFU-GM population. Population analyses[34] have demonstrated that the BFU-E are closely related to the multipotential stem cells (CFU-S) and are probably comparable to the CFU-GM of the granulopoietic series. The intermediate BFU-E may be the more important cell insofar as in vivo Epo sensitivity is concerned.[43]

Cultures of BFU-E have been used as an assay for BPF.[46] Cultures of either CFU-E[47] or of the cluster-forming cells[45] have been proposed as an appropriate assay for Epo, although in vivo techniques or suspension culture methods in vitro are currently more widely used.[48]

4. Megakaryopoietic Colonies in Vitro (CFU-Meg)

A few groups have described the in vitro growth of megakaryopoietic colonies from murine[49,50] and human[51] hemopoietic tissue. The characteristics of the CFU-Meg are not known with any certainty. At least in murine cell cultures two types of colonies appear to exist. One is composed of a few multinucleated megakaryocytes. A second type is composed of several mononuclear acetylcholine esterase-positive cells (and, therefore, identified as being in the megakaryopoietic lineage) which may represent the immediate precursors of the morphologically recognizable megakaryocytes. A specific growth factor does not yet appear to have been recognized, but "thrombopoietin" and the conditioned medium from the WEHI-3 cell line have been used. The number of megarkaryocytic colonies can be used as an endpoint to assay for "thrombopoietin"-like materials.[51]

The possibility cannot be excluded that the erythroid BFU-E are also capable of the production of megakaryocytes. Erythroid "bursts" have often been reported to contain cells resembling megakaryocytes, and more direct evidence for the bipotentiality of the BFU-E has recently been reported.[52]

5. Mixed Hematopoietic Colonies in Vitro (CFU-Mix)

At least two groups[53,54] have described the in vitro growth of hemopoietic colonies consisting of granulocytes, erythrocytes, megakaryocytes, and macrophage/monocytes. These colonies have been shown to be clonal and are considered to be derived from CFU-GEMM or CFU-Mix. The necessary culture conditions show only minor differences from those required for the development of BFU-E-derived colonies. The CFU-GEMM appear to have properties sufficiently similar to the in vivo murine CFU-S such that the possibility needs

to be seriously considered that multipotential stem cells are being assayed in an in vitro culture system. The adaptation of this system to human marrow[54,55] potentially opens the door for the study of human, multipotential stem cells.

6. The Measurement of Cell Cycle Characteristics

The most frequently used technique to obtain an estimate of the proliferation rate of morphologically unrecognizable cells is undoubtedly the so-called "suicide" method first described by Becker et al. in 1963.[15] With this method the cell suspension is divided into two parts; one aliquot is exposed for 20 to 30 min to high specific activity tritiated thymidine and the other to comparable concentrations of "cold" thymidine. The cells are washed by centrifugation and then both aliquots cultured under appropriate conditions or injected into lethally irradiated recipients for the CFU-S assay. Those cells which were either in DNA synthesis (S) at the time the thymidine was added or entered into S during the 20- to 30-min exposure will incorporate the isotope and will be sterilized by autoirradiation. The difference in the number of colonies obtained between the two aliquots gives an estimate of the proportion of cells in DNA synthesis. Exposure of the cells to the isotope can be done either in vivo or in vitro. Problems arise when cells exposed to tritiated thymidine are subsequently cultured in vitro. The dead cells release radioactive degradation products into the culture which are taken up by other proliferating cells leading to a "secondary" kill. This problem is not completely overcome by the incorporation of "cold" thymidine into the cultures in an effort to dilute out the radioactive material.[56] Variations on this method involve the use of other phase-specific cytotoxic agents of which cytosine arabinoside, methotrexate, and hydroxyurea[16-18] are most widely used. If the cells are repeatedly exposed to one of these agents over several hours, an absolute estimate of the cell cycle time can be obtained provided certain assumptions are made.[17]

7. In Vivo Assessment of Cell Proliferation

By far the most convenient method to assess red blood cell production is by the incorporation of ^{59}Fe into developing erythroblasts and the subsequent appearance of the label in the peripheral blood.[57] No comparable specific isotopic methods are available to study granulopoiesis or megakaryopoiesis, although $DF^{32}P$[58] has been used in the study of the former[58] and ^{35}S or ^{75}Se in the in vivo assessment of megakaryopoiesis.[59]

There are several important variations on the above methods which have been employed, although the characteristics of the responding cells have not been studied in any detail. Thus, the in vivo ^{59}Fe response to injected Epo[60] presumably measures a population of cells which can be realistically termed erythropoietin responsive cells (ERC), but there are many difficult-to-control variables involved with this method.[61]

The recovery of erythropoiesis[17] or of the peripheral blood granulocyte response to endotoxin[62] in otherwise lethally treated recipients of bone marrow grafts has been used to estimate the erythropoietic (or granulopoietic) repopulating ability (ERA, GRA). Presumably, these assays measure some type of "stem" cell but their relationship to one another, to the CFU-S, or to the BFU-E and CFU-GM has not been determined.

B. Morphological Methods

Most of the methods to be summarized in this section represent standard hematological techniques, details of which can be found in most reference texts (see, for example, Reference 63). A brief discussion is included here only for completeness.

1. Cell Counts

A variety of instruments are now available to accurately and rapidly perform cell counts on peripheral blood samples. These instruments have, to a large extent, replaced microscopes and hemocytometer chambers.

2. Cell Differentials

The later stages of the development of the mature blood cells can be followed in the hemopoietic tissues because the precursors have certain features in common with the "end" cells such that they are clearly recognizable as belonging to a certain cell lineage, e.g., erythropoiesis. However, the morphology of the various stages differs somewhat from species to species so only a general, overall description can be given. Most of the cell types can be differentiated on slides treated with such standard stains as Giemsa or Wright's, but a variety of special histochemical stains are available to further assist in the morphological recognition of hemopoietic cells.

The first, and, therefore, the most immature, cell which can be morphologically identified as belonging to the erythropoietic series is the pronormoblast, or proerythroblast,[64] in this book sometimes denoted as E1. This cell is of fairly large size with a nucleus occupying, perhaps, 80% of its volume. The nuclear chromatin is fine and dispersed in small clumps and one or two nucleoli are often visible. The next morphologically recognizable cell is the basophilic normoblast, (E2) so-called because the nucleus shows the characteristic dark violet basichromatin (heterochromatin) interspersed with pink-staining oxychromatin (euchromatin) in preparations stained with Wright's stain. The cytoplasm stains a deep blue and the cell is somewhat smaller than the pronormoblast. The next cell in the maturation process is the polychromatic normoblast (E3) which contains increasing quantities of hemoglobin within the cytoplasm and is named because of its increasingly polychromatophilic appearance in Wright's stained preparations. This cell is, in turn, smaller than the basophilic normoblast and has a proportionally smaller nucleus in which the heterochromatin is to be found in well-defined clumps. After a final mitotic division the intracellular hemoglobin concentration is at its peak, giving the cell a relatively uniform, or orthochromatic, appearance in stained preparations. This cell (E4) is smaller than the polychromatophilic normoblast and eventually extrudes its nucleus, and the resulting reticulocyte completes its maturation in the peripheral blood to become the nonnucleated, bi-concave, disc-shaped erythrocyte. This morphologically recognizable process involves approximately four cell divisions, a reduction in cell size, and an increase in intracellular hemoglobin concentration during maturation and is accomplished in 3 to 5 days in normal human bone marrow.

The division and maturation sequences within the morphologically recognizable granulopoietic precursors[65] follow a sequence not unlike that seen in the erythron. Thus, the myeloblast (in this book denoted as G1), the earliest cell definable as belonging to the granulopoietic series, is a large cell (15- to 20-μm diameter in human marrow) found only rarely in normal tissue. The myeloblast has a large, usually round nucleus with little cytoplasm. The nuclear chromatin is finely dispersed and myeloblasts can be distinguished from lymphoblasts (with which they are often confused) because the myeloblasts generally have two or more pale blue nucleoli and the cytoplasm is deeply basophilic with no clear zone about the nucleus. The promyelocyte (G2), which develops from the myeloblast, is smaller and is characterized by the presence of many coarse blue or violet granules in the cytoplasm. The myelocyte (G3) stage encompasses the most extensive morphological changes within the granulocytic series and is sometimes classified into several distinct categories. Myelocytes are of comparable size to the myeloblasts, but the nuclear chromatin is much more markedly clumped. The cytoplasm frequently shows a pinkish coloration and the granules are fairly homogeneous. It is within this stage that the cell-line-specific neutrophilic, basophilic, and eosinophilic granules are first recognized. The cytoplasmic characteristics of the mature granulocyte are first clearly evident in the meta-myelocyte stage — cells in which the cytoplasm is uniformly pink and the granules clearly delineate the different subtypes. Following maturation through a "stab" form in which the nucleus takes on the typical convoluted structure of the end cell, the cell assumes the appearance of the mature granulocyte (G4). This whole maturation scheme takes from 4 to 6 days in normal human bone marrow and is associated with three to seven divisions. Granulocyte kinetics are not as well understood as those associated with the developing erythroblasts due to the absence of specific radioisotopic labels and the fact that granulopoiesis has not been as amenable to suppression and stimulation. In addition, cell migration to and from the marrow and marginal pools has complicated the interpretation of cell kinetic data. An alternative classification scheme, listing the development stages as M1 (myeloblast) through M8 (senescent granulocyte) has been proposed.

The morphological sequences involved in the production of monocyte/macrophages are still not well understood, but a scheme similar to that described above for granulopoiesis has been reported.[66]

A morphological study of platelet production is really restricted to the megakaryocytes.[67] Precursors of these cells are recognized in some species, e.g., the mouse, by their characteristic reaction with acetylcholine esterase, but this is not a general phenomenon. Megakaryocytes are large, multinucleated cells in which development is associated with multiplication, if the nuclear replication is considered, or a purely maturational scheme in the nondividing cytoplasm.

3. Cell Kinetics

A most powerful approach to the study of cell proliferation within the morphologically recognizable cells is to combine the above classifications with studies which follow the incorporation, appearance, and cellular transfer of a radioisotope. The *a priori* examples of such techniques are those in which the appearance and disappearance of tritiated thymidine are followed in autoradiographic preparations of cells (see, for example, References 68 and 69). Exposure of cells, either in vivo or in vitro, to tracer (NOT suicidal) concentrations of tritiated thymidine results in incorporation of the isotope by cells in the S phase. The proportion of cells of a given morphological class which incorporate the isotope (estimated from the autoradiogram and otherwise known as the Labeling Index) will provide direct information about the cell cycle time if the entire population is dividing. Alternatively, the cell population(s) can be studied at various times after exposure to the thymidine and the "Percent of Labeled Mitoses" plotted as a function of time. From such graphs the durations of all phases of the cell cycle can be estimated with varying degrees of certainty and providing certain assumptions (mainly involving the reutilization of the isotope) are considered. The "dilution" of the label with time also allows an estimate to be made of the number of cell divisions in that cellular compartment.[70,71] Various refinements of the labeled mitoses method are available, of which the most popular are probably those utilizing a double labeling technique.

V. PHYSICAL METHODS FOR THE SEPARATION OF CELLS

Although not directly assessing cell proliferation, the characterization of cells by physical differences has frequently provided the basis for the identification of distinct populations. The methodologies for cell separation by unit gravity sedimentation (functioning primarily on the basis of cell size),[72] by either continuous[73] or discontinuous[30] density centrifugation (cell separation primarily by density), by electrophoresis,[74] or with the use of a fluorescent activated cell sorter (FACS)[75] can be found in the appropriate references.

VI. CONCLUSION

This paper was not intended to exhaustively discuss all the numerous methods available to the experimental hematologist. Rather, it was intended to summarize the types of techniques which have been employed to obtain the results which provide the background to

this volume. Quite obviously, the type of method employed is heavily dependent on whether it is possible to look through the microscope and recognize a particular cell type or whether the cell in question can only be studied by a functional test. There are, however, corollaries between the two situations — the thymidine suicide method applied to, for example, CFU-S differs essentially in only dose level from that used to construct a labeled mitoses curve for pronormoblasts. The use of such techniques has contributed greatly to our understanding of hemopoietic regulation, but it should be appreciated that many have pitfalls and limitations such that wherever possible, an assessment of the results by two independent tests is recommended.

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Appendix 2

THE ROLE OF THE SPLEEN IN HEMOPOIESIS*

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I. ABSTRACT

From measurements in the femur (tibia) and spleen simple formulas are derived to estimate the spleen's contribution to the total hemopoietic cell count. The formulas are based on the knowledge of the "bone marrow-to-spleen" ratio of normal cell counts, which differs for different cell types and mouse strains. With these formulas, a detailed reanalysis of published material has been performed in an attempt to understand under which circumstances and how the spleen takes over a considerable part of hemopoiesis in mice. The time courses of the spleen's contribution to hemopoiesis are constructed for many experimental situations. They show in which animals and during which phase of an experiment it is important to consider the role of the spleen.

II. INTRODUCTION

There is widespread uncertainty regarding the circumstances and extent to which the spleen quantitatively contributes to the total hemopoietic system. Authors often focus on enormous relative hemopoietic changes in the spleen but overlook that an even 100-fold increase from a very small base value may still be a small effect compared with the large bone marrow pool.

Since the importance of the spleen differs for different hemopoietic cells, formulas are needed which allow an easy calculation of the spleen's contribution to total hemopoiesis separately for each cell type. Using such formulas, one can explicitly show in which experiments and during which time interval the spleen has to be considered and where its role can be neglected.

This analysis is completely independent of the stem cell model discussed throughout the rest of this book. No kinetic modeling takes place. All information is derived from published experimental material by virtue of some simple formulas.

III. MATERIAL AND METHODS

A. Definitions

We define the variable "C" as standing for any of the following cell stages: CFU-S, BFU-E, CFU-E, CFU-GM, erythropoietic, and granulopoietic precursors. Reticulocytes, erythrocytes, and other cell stages will not be considered.

The *absolute cell number* of a particular cell stage will be denoted by C*. If the number has been determined in one femur, one tibia, or the spleen, it will be denoted by C^*_{fem}, C^*_{tib}, C^*_{spl}, respectively. The (calculated) absolute numbers in the whole bone marrow and the total body, consequently, will be written as C^*_{bm} and C^*_{tot}. The absolute cell numbers in normal steady state (= "controls") are denoted by the additional subscript "norm", e.g., $C^*_{fem,norm}$, $C^*_{tot,norm}$, etc.

The *relative cell number* in the respective organ will be denoted by C (without asterisks). It represents the ratio of absolute number and normal absolute number, e.g.,

$$C_{fem} = C^*_{fem}/C^*_{fem,norm},\ C_{spl} = C^*_{spl}/C^*_{spl,norm},\ \text{etc.}$$

The *fractional cell number* will be defined as ratio of the cell number in a particular organ to the total animal's cell number. It will be denoted by

$$C_{spl/tot} = C^*_{spl}/C^*_{tot},\ C_{bm/tot} = C^*_{bm}/C^*_{tot},\ \text{etc.}$$

If the fractional cell number is considered especially for normal (= control) values a special notation will be used:

$$K_{spl/tot} = C_{spl/tot,norm} = C^*_{spl,norm}/C^*_{tot,norm},$$

$$K_{bm/fem} = C_{bm/fem,norm} = C^*_{bm,norm}/C^*_{fem,norm},\ \text{etc.}$$

These ratios will be referred to as "spleen-to-total" ratio ($K_{spl/tot}$), "bone marrow-to-femur" ratio ($K_{bm/fem}$), etc.

B. Postulates

1. Postulate 1: Hemopoiesis Outside of Bone Marrow and Spleen is Negligible

The absolute number of hemopoietic progenitor and precursor cells in adult mice is constituted essentially by the cells in the bone marrow and the spleen:

$$C^*_{tot} = C^*_{bm} + C^*_{spl} \tag{1a}$$

The amount of progenitor and precursor cells in the liver or in circulation is negligible. This postulate leads to the consequence that the sum of the fractional cell numbers in both organs equals 1 (divide Formula 1a by C^*_{tot} and use the above definitions):

$$1 = C_{bm/tot} + C_{spl/tot} = K_{bm/tot} + K_{spl/tot} \tag{1b}$$

2. Postulate 2: All Parts of the Bone Marrow behave Similarly

In each part of the bone marrow (e.g., femur, tibia) cells of the various stages appear in the same concentrations. Everywhere the same relations (e.g., E/G ratio) hold. Each part is representative of the bone marrow for all situations. Therefore, the hemopoietic cells in one femur (or tibia) represent a constant fraction of the whole bone marrow. This constant is the same for all cell types. This postalate means that the ratio

$$K_{bm/fem} = C^*_{bm,norm}/C^*_{fem,norm} = C^*_{bm}/C^*_{fem} = \text{const} \tag{2a}$$

is identical for all cell stages C. We assume that this "bone marrow-to-femur" ratio does not change during one experiment as long as the experiment influences hemopoiesis in the whole body homogeneously (e.g., partial body irradiation is excluded). It is further assumed that the strain differences of the "marrow-to-femur" ratio are negligible.

Two consequences follow from Formula 2a: the absolute bone marrow count can be calculated, if the count in one femur is known, by

$$C^*_{bm} = C^*_{fem} * K_{bm/fem} \tag{2b}$$

and as the femur is representative for the bone marrow their relative counts are identical:

$$C_{bm} = C^*_{bm}/C^*_{bm,norm} = C^*_{fem}/C^*_{fem,norm} = C_{fem} \tag{2c}$$

(Everything that has been said for the femur holds, also, for the tibia. One simply has to exchange the subscript "fem" with "tib".)

3. Postulate 3: Bone Marrow and Spleen Hemopoiesis Differ

In contrast to the femur the spleen is not representative for the bone marrow cell counts. Thus, already in the normal steady state, the "spleen-to-total" and the "bone marrow-to-spleen" ratio may differ for different cell types:

$$K_{spl/tot} \text{ and } K_{bm/tot} \text{ differ for different C} \tag{3a}$$

Both ratios are connected by

$$K_{spl/tot} = \frac{1}{K_{bm/spl} + 1} \tag{3b}$$

This can be seen by inserting

$$K_{spl/tot} = C^*_{spl,norm}/C^*_{tot,norm} \text{ and } K_{bm/spl} = C^*_{bm,norm}/C^*_{spl,norm}$$

which reproduces Formula 1a. The connection of the "marrow-to-spleen" ratio to the "marrow-to-total" ratio is given by

$$K_{bm/tot} = 1 - K_{spl/tot} = \frac{K_{bm/spl}}{K_{bm/spl} + 1} \tag{3c}$$

This formula is obtained by inserting Formula 3b into Formula 1b. Finally, due to

$$K_{bm/spl} = K_{bm/fem} * K_{fem/spl} = K_{bm/fem} * \frac{C^*_{fem,norm}}{C^*_{spl,norm}} \tag{3d}$$

the "marrow-to-spleen" ratio ($K_{bm/spl}$) can be calculated from the "marrow-to-femur" ratio ($K_{bm/fem}$) and the normal values in femur and spleen ($C^*_{fem,norm}$, $C^*_{spl,norm}$).

C. Resulting Formulas

Using Formulas 1a and 2b it is easy to determine the absolute total cell numbers from absolute counts in the femur and spleen. However, to make experiments comparable from different laboratories with different animal strains it seems useful to consider relative cell numbers, which refer to the controls in the experiments.

1. Relative Cell Count in the Whole Body

The relative number C_{tot} can be written as

$$C_{tot} = C_{bm} * K_{bm/tot} + C_{spl} * K_{spl/tot} \tag{4a}$$

using Formula 1a and the above definitions. Making use of Formulas 3b and 3c one may replace the $K_{bm/tot}$ and $K_{spl/tot}$ by the "bone marrow-to-spleen" ratio $K_{bm/spl}$. One obtains

$$C_{tot} = \frac{C_{bm} * K_{bm/spl} + C_{spl}}{K_{bm/spl} + 1} \tag{4b}$$

This formula shows that the total cell number, C_{tot}, expressed relative to control, can be calculated from the relative numbers in the bone marrow and the spleen. Only the "bone marrow-to-spleen" ratio must be known. Since $C_{bm} = C_{fem} = C_{tib}$ (postulate 2), in fact, only the relative number in one femur or tibia must be available instead of C_{bm}. If the relative numbers in the marrow and the spleen were identical ($C_{bm} = C_{spl}$), Formula 4b would become trivial: $C_{tot} = C_{bm} = C_{spl}$. Therefore, this formula is always of practical relevance if marrow and spleen cell counts behave differently.

2. Fractional Cell Number in the Spleen

The fractional cell number $C_{spl/tot}$ can be written as

$$C_{spl/tot} = \frac{C^*_{spl}}{C^*_{tot}} = \frac{C_{spl}}{C_{tot}} * K_{spl/tot} \tag{5a}$$

using the definition $K_{spl/tot} = C^*_{spl,norm}/C^*_{tot,norm}$. Again one may replace $K_{spl/tot}$ and C_{tot} by Equations 3b and 4b and will find

$$C_{spl/tot} = \frac{C_{spl}}{C_{bm} * K_{bm/spl} + C_{spl}} \tag{5b}$$

Thus, the fractional contribution of the spleen to the total cell count can either be calculated from the absolute (Formula 5a) or relative (Formula 5b) counts in bone marrow (e.g., femur) and spleen if the "bone marrow-to-spleen" ratio ($K_{bm/spl}$) is known.

D. Applications

If the absolute cell counts C^*_{spl} from the spleen and C^*_{fem} from one femur are available, the absolute count C^*_{tot} of the whole animal can be calculated by Formulas 1a and 2b. One must only know the "bone marrow-to-femur" ratio which is quite constant for mice (see below). Then, the fractional cell number of the spleen can be determined from $C_{spl/tot} = C^*_{spl}/C^*_{tot}$ and the relative total number is found from $C_{tot} = C^*_{tot}/C^*_{tot,norm}$ using the normal (control) measurements. From relative cell counts in the spleen and one femur one may calculate C_{tot} and $C_{spl/tot}$ by Formulas 4b and 5b if the "bone marrow-to-spleen" ratio has been determined.

As an example we describe a hypothetical experiment with CFU-S measurements in femur and spleen (Table 1). To calculate the absolute total number, CFU-S^*_{tot}, by Formulas 1a and 2b the "bone marrow-to-femur" ratio $K_{bm/fem} = 17$ has been used. The relative total number, CFU-S_{tot}, and the fractional number of the spleen, CFU-$S_{spl/tot}$, then can be derived from the absolute cell counts. Alternatively, they can be calculated by Formulas 4b and 5b using the "bone marrow-to-spleen" ratio $K_{bm/spl} = 21.25$. Besides illustrating the calculations, Table 1 also shows how different combinations of femur and spleen numbers affect the total count and splenic fraction.

IV. RESULTS

A. Normal Bone Marrow Distribution in the Skeleton (Estimation of $K_{bm/fem}$, $K_{bm/tib}$)

In order to extrapolate from measurements in a part of the bone marrow (e.g., femur) to the total bone marrow cell count one has to know what proportion of the total system is represented by this part. This proportion is quantified by the "bone marrow-to-femur" ratio, $K_{bm/fem}$, or the "bone marrow-to-tibia" ratio, $K_{bm/tib}$. Table 2 gives the data for mice from two groups. The animals were injected with ^{59}Fe and the distribution of radioactive iron throughout the skeleton was assayed. According to these measurements one femur contains about 6% and one tibia about 3.5% of the total activity found in the skeleton. Provided that the composition of the bone marrow is identical in all parts of the skeleton for each hemopoietic stage, this corresponds to $K_{bm/fem} = 17$ and $K_{bm/tib} = 29$. In other words, the total bone marrow compartment for each cell stage is about 17 times larger (1/0.06) than the femur compartment and 29 times larger (1/0.035) than the tibia compartment.

B. "Bone Marrow-to-Spleen" Ratio (Estimation of $K_{bm/spl}$, $K_{fem/spl}$, and $K_{tib/spl}$)

The factors $K_{fem/spl}$ and $K_{tib/spl}$ are defined as the ratio of the normal cell count in the femur or tibia to the normal spleen count. Table 3 gives a survey of these ratios for CFU-S, BFU-E, CFU-E, CFU-GM, erythropoietic, and granulopoietic precursors (erythroblasts and my-

Table 1
HYPOTHETICAL EXPERIMENT

Time	Femur		Spleen		Total		Spleen's fraction
	$CFU\text{-}S'_{fem}$ absolute	$CFU\text{-}S_{fem}$ relative	$CFU\text{-}S'_{spl}$ absolute	$CFU\text{-}S_{spl}$ relative	$CFU\text{-}S'_{tot}$ absolute[a]	$CFU\text{-}S_{tot}$ relative	$CFU\text{-}S_{spl/tot}$ fractional[b]
Control	5,000	1.00	4,000	1.00	89,000	1.00	0.04
Day 2	5,000	1.00	8,000	2.00	93,000	1.04	0.09
Day 4	5,000	1.00	20,000	5.00	105,000	1.18	0.19
Day 6	10,000	2.00	20,000	5.00	190,000	2.13	0.11
Day 8	10,000	2.00	4,000	1.00	174,000	1.96	0.02

[a] $CFU\text{-}S'_{tot} = K_{bm/fem} * CFU\text{-}S'_{fem} + CFU\text{-}S'_{spl}$; $K_{bm/fem} = 17$ according to Equations 1 and 2b.

[b] $CFU\text{-}S_{spl/tot} = CFU\text{-}S_{spl}/CFU\text{-}S_{tot}$.

Table 2
CONTRIBUTION OF ONE FEMUR (TIBIA) TO HEMOPOIESIS OF THE WHOLE BONE MARROW

Organ	Fraction of total bone marrow	Values adopted in following calculations: Fraction of total bone marrow	Values adopted in following calculations: "Bone marrow to femur (tibia)" ratio	Ref.[a]
Femur	0.061	0.06	$K_{bm/fem} = 17$	a
	0.059			b
Tibia	0.034[b]	0.035	$K_{bm/tib} = 29$	a
	0.035			b

[a] a: C3H × C57B1 mice;[1]
b: C57B1 × DBA mice.[2]

[b] Tibia and fibula measured together.

eloblasts). Only those data have been analyzed where absolute normal cell counts in the spleen and femur or tibia were reported simultaneously. $K_{fem/spl}$ and $K_{tib/spl}$ are directly derived from these data. The "bone marrow-to-spleen" ratio $K_{bm/spl}$ follows by multiplying $K_{fem/spl}$ and $K_{tib/spl}$ with the appropriate values for $K_{bm/fem}$ (= 17) and $K_{bm/tib}$ (= 29) from Table 2. Although Table 3 does not claim completeness it gives an impression of the enormous heterogeneity of the data. It shows that only for CBA mice data for all cell stages are available, but even these were not measured in one laboratory. Table 4 summarizes the results giving the median "bone marrow-to-spleen" ratio and the "spleen-to-total" ratio. The latter is derived from Formula 3b and represents the normal fraction of hemopoietic cells in the spleen.

For the different cell stages one finds the following.

CFU-S — As can be seen from Table 3, in Swiss-Webster mice the spleen contributes 13% to the total CFU-S population ($K_{bm/spl} = 6.6$) and in CBA/Ca mice it contributes less than 1% ($K_{bm/spl}$ = 170-440). Leaving these extremes out of consideration, the number of splenic CFU-S still varies by more than a factor of 12 (1000 to 12,200 CFU-S) and by a factor of 7 in the femur (1500 to 11,800 CFU-S). The median "bone marrow-to-spleen" ratio of 22 suggests that about 5% of all CFU-S are normally found in the spleen. The percentage seems to be somewhat smaller in BDF_1 and CBA × C57Bl mice.

BFU-E — For different strains normal cell counts differ by a factor of 10 for the marrow (500 to 5300 BFU-E per femur) and 30 for the spleen (700 to 25,000 BFU-E per spleen). On average, about 8% of all BFU-E are normally found in the spleen. However, in C57Bl × DD mice this proportion is much higher (18%).

CFU-E — Swiss-Webster mice show an extraordinary large CFU-E content in the spleen (19%), while CBA/Ca mice show an extremely small splenic contribution (below 1%). Overall, about 5% of all CFU-E are found in the spleen, in the normal steady state.

Erythropoietic precursors — Only data for three strains are available, which differ by a factor of 2. With a median of more than 15%, the amount of erythroblasts in the spleen is higher than for any other cell stage. This emphasizes that the spleen has a favorable milieu for mature erythropoiesis.

CFU-GM — CBA/Ca mice seem to have virtually no splenic content of CFU-GM. In the remaining strains the spleen contains about 4% of all CFU-GM.

Granulopoietic precursors — Attention should be paid to the eightfold variability in spleen content. NMRI mice seem to favor granulopoiesis in the bone marrow, but the data

Table 3
NORMAL CELL COUNTS IN BONE MARROW AND SPLEEN AND CORRESPONDING "BONE MARROW-TO-SPLEEN" RATIO (ESTIMATION OF $K_{fem/spl}$, $K_{tib/spl}$, $K_{bm/spl}$)

Cell stage	Mouse strain	Absolute normal cell counts in: Femur or (tibia)	Absolute normal cell counts in: Spleen	$K_{fem/spl}$ ($K_{tib/spl}$)	$K_{bm/spl}$[a]	Figure no.[b]	Ref.
CFU-S	BALB/C	1.500	1.500	1.00	17.0	8	3
	BALB/C × DBA	7.200	6.500	1.11	18.9	—	4
	BALB/C × DBA	7.600	5.100	1.49	25.3	—	5
	$CAF_{1,}$	4.400	1.500	2.93	49.8	4	6
		2.900	1.000	2.90	49.3	4	
	CBA/ca	1.800—2600	100—180	10—26	170—440	1, 8	7
					50.0[c]		
	CF_1	(3.700)	4.800	(0.77)	22.3	8	8
	CF_1	3.400	3.200	1.06	18.0	4	9
	C3H/AKR	3.200	2.900	1.10	18.7	—	10
	C57Bl	1.900	1.800	1.06	18.0	8	11
	C57Bl	4.000	4.000	1.00	17.0	1	12
	C57Bl × CBA	10.000	3.500	2.85	48.5	1	13
	C57Bl × CBA	10.500	2.900	3.62	61.5	—	14
	C57Bl × DBA	4.700	2.900	1.62	27.5	8	15, 16
	C57Bl × DBA	11.800	12.200	0.97	16.5	4	17
	C57Bl × DBA	4.100	2.300	1.78	30.3	—	18
	C57Bl × DD	3.300	2.500	1.32	22.4	1	19
	NMRI	4.500	6.500	0.69	11.7	1	20
	Swiss-Webster	40.000	102.000	0.39	6.6	4	21
CFU-Mix	C57Bl × DBA	440	1.650	0.27	4.6	4	22
BFU-E (7d—9d)	BALB/C	4.000	1.500	2.67	4.5	9	23
	CBA/Ca	5.400—10.200	1.200—2.800	1.9—8.5	32—145	2, 9	7
					30.0[c]		
	CD_1	(3.200)	6.000	(0.53)	15.4	5, 9	24
	CD_1	(3.200)	8.100	(0.40)	11.6	—	25
	C3H	800	1.300	0.62	10.5	—	26
	C3H	500	700	0.71	12.1	9	27
	C57Bl × C3H f/f	3.450	4.220	0.73	12.4	—	28
	C57Bl × C3H t/t	4.070	4.720	0.86	14.6	—	28
	C57Bl × DD	5.200	19.000	0.27	4.6	5	29
		5.300	20.000	0.27	4.6	9	
		5.000	25.000	0.20	3.4	—	
CFU-E	BALB/C	35.000	30.000	1.17	19.9	10	23
	CBA/Ca	33.000—45.000	380—1.000	33—118	560—2.000[c]	2, 10	7
					150.0[c]		
	CD_1	(42.000)	115.000	(0.37)	10.7	6, 10	24
	CD_1	(15.100)	44.100	(0.34)	9.9	—	25
	C3H	1.600	1.500	1.07	18.2	—	26
	C3H	1.900	1.800	1.05	17.9	10	27
	C57Bl × C3H	18.400	46.400	0.40	6.8	—	28
	C57Bl × C3H	15.000	40.000	0.38	6.5	6	30
	C57Bl × C3H	20.000	20.000	1.00	17.0	—	31
	C57B1 × DD	25.000	15.000	1.67	28.4	10	29
		23.000	14.000	1.64	27.9	—	
		25.000	18.000	1.39	23.6	6	
	Swiss-Webster	97.000	388.000	0.25	4.3	6	21
Erythropoietic precursors	CBA	$1.8 * 10^6$	$9.64 * 10^6$	0.19	3.2	—	32
	CF_{1S}	$(3.4 * 10^6)$	$15 * 10^6$	(0.23)	6.7	11	33
		$(2.1 * 10^6)$	$13 * 10^6$	(0.16)	4.6	11	
	CF_1	$(2.5 * 10^6)$	$7 * 10^6$	(0.36)	10.4	11	8
	NMRI	$3.2 * 10^6$	$11 * 10^6$	0.29	4.9	—	20
	NMRI	$3.5 * 10^6$	$14.6 * 10^6$	0.24	4.1	—	32
CFU-GM	BALB/C	21.800	2.300	9.48	161.0	12	3
	CBA/Ca	6.600—12.000	100—180	37—120	630—2000	3, 12	7
	CD_1	(17.000)	19.000	(0.9)	150.0[c]	7, 12	24
					26.1		
	CD_1	(22.600)	40.200	(0.56)	16.2	—	25
	Swiss-Webster	10.000	48.000	0.21	3.6	7	21

Table 3 (continued)
NORMAL CELL COUNTS IN BONE MARROW AND SPLEEN AND CORRESPONDING "BONE MARROW-TO-SPLEEN" RATIO (ESTIMATION OF $K_{fem/spl}$, $K_{tib/spl}$, $K_{bm/spl}$)

Cell stage	Mouse strain	Absolute normal cell counts in Femur or (tibia)	Spleen	$K_{fem/spl}$ ($K_{tib/spl}$)	$K_{bm/spl}$[a]	Figure no.[b]	Ref.
Granulopoietic precursors	CBA	$12.32 * 10^6$	$25.8 * 10^6$	0.48	8.2	—	32
	CF_{1S}	$(8 * 10^6)$	$10 * 10^6$	(0.8)	23.2	13	33
		$(5.5 * 10^6)$	$10 * 10^6$	(0.55)	16.0	13	
	NMRI	$8.7 * 10^6$	$26 * 10^6$	0.33	5.6	—	34
	NMRI	$10 * 10^6$	$32 * 10^6$	0.31	5.3	—	20
	NMRI	$9.77 * 10^6$	$7.45 * 10^6$	1.31	22.3	—	32

[a] $K_{bm/spl} = K_{fem/spl} * 17 = K_{tib/spl} * 29$ with 17 and 29 taken from Table 1.
[b] The numbers indicate in which of the Figures 1 to 13 the values were used to calculate the time course of the splenic contributions.
[c] This value is taken although it represents on overestimation of the spleen's contribution. The values were chosen in order to generate nonzero data curves in Figures 1 to 13.

Table 4
BONE MARROW-TO-SPLEEN RATIO — MEDIAN VALUES

Cell stage	"Marrow-to-Spleen" ratio $K_{bm/spl}$ median (range)	Spleen's fraction of total hemopoiesis $1/(K_{bm/spl} + 1)$ (range)
CFU-S	22	0.043
	(6.6—61.5)	(0.016—0.13)
BFU-E	12	0.076
	(3.4—45.4)	(0.022—0.23)
CFU-E	18	0.053
	(4.3—150)	(0.007—0.23)
E1-4	5	0.17
	(3.2—10.4)	(0.088—0.24)
CFU-GM	24	0.037
	(3.6—161)	(0.006—0.14)
G1-4	12	0.076
	(5.3—23.2)	(0.04—0.16)

coming from the same laboratory[20,32,34] differ considerably. On average, 7% of the granulopoietic precursors can be found in the spleen of mice.

Comparison of the cell types — It is difficult to compare the spleen's contribution of the different cell types, due to the large variability of the data. Comparing the median values (Table 4) one finds that for CFU-S, CFU-E, CFU-GM, and granulopoietic precursors the spleen contributes about 5% of total. About 8% of the BFU-E and 15% of the erythroblasts are found in the spleen. In this comparison the spleen CFU-E content is similar to the CFU-GM and less than that for BFU-E. One would eventually have expected that the spleen's contribution to erythropoiesis increases the more mature the respective cell stages are (e.g., for CFU-E greater than for BFU-E). In fact, if one looks at parallel measurements of CFU-GM, BFU-E, and CFU-E in the same animals,[3,23-25] one finds the expected sequence with increasing contributions from CFU-GM, BFU-E, and CFU-E for BALB/C mice (ratio CFU-GM to BFU-E to CFU-E = 0.6:2:5%) and for CD_1 mice (6:8:10%). The unexpected result obtained above may have followed from the statistical pooling of all available data, which in this case may have lead to a bias.

C. Time Course of the Spleen's Contribution to Hemopoiesis

Figures 1 to 13 show how the spleen's contribution to the total hemopoietic system changes with time in different experimental situations. The values were obtained by applying Formula 5b for simultaneous measurements in marrow and spleen. For our purposes we separate the experiments into three classes.

1. Irradiation (Figures 1 to 3)

Following acute irradiation the splenic fraction of the total CFU-S count generally remains below 10% and never exceeds 20%. For BFU-E, CFU-E, and CFU-GM the role of the spleen is also of minor importance.

These results clearly demonstrate that the spleen does not significantly change its contribution to hemopoiesis while recovering from acute irradiation.

The data base for chronic or post chronic irradiation is small. The few measurements indicate that the spleen does not become a major organ under such circumstances.[35]

2. Erythropoietic Suppression (Figures 4 to 7)

Figures 4 to 7 show the spleen's contribution during red cell hypertransfusion (crosses) and ex-hypoxia (open and full symbols). Swiss-Webster mice behave differently than the

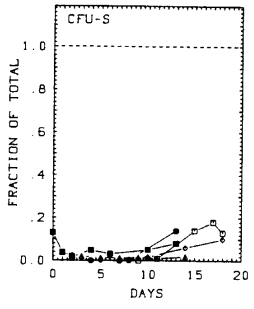


FIGURE 1

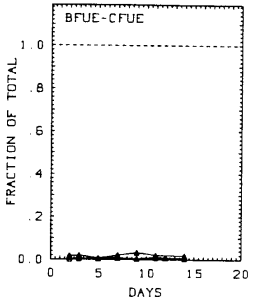


FIGURE 2

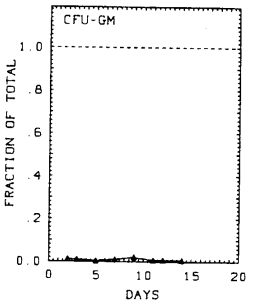


FIGURE 3

FIGURES 1 to 3. The role of the spleen after acute irradiation (expressed as fraction of the total hemopoiesis). Data are from : △ — Seidel and Kreja[7] (▲ for CFU-E); ◇ — Guzmann et al.;[12] □ — Vos;[13] ● — Hendry.[19] ■ — Beran.[20]

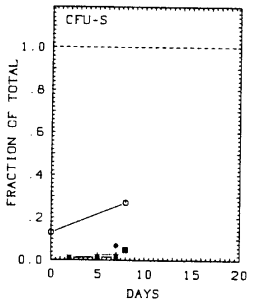


FIGURE 4

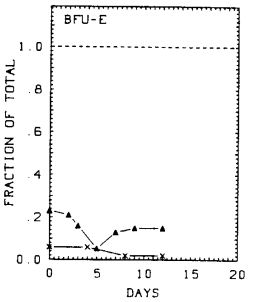
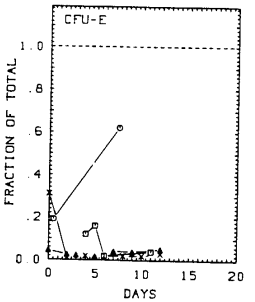


FIGURE 5



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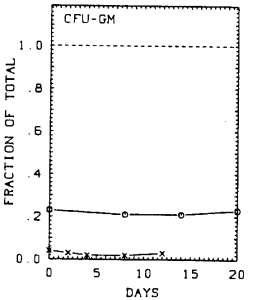

FIGURE 7

FIGURES 4 to 7. The role of the spleen in suppressed erythropoiesis. Data for hypertransfusion are from: □ — Gregory et al.;[30] ■ — McCarthy;[17] ○ — Erslev et al.;[21] ▲ — Hara and Ogawa;[29] ♦ — Shadduck et al.[6] Data for exhypoxia are from : *, + — Shadduck et al.;[9] × — Peschle et al.[24]

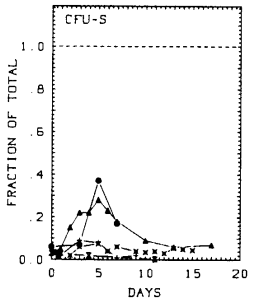

FIGURE 8

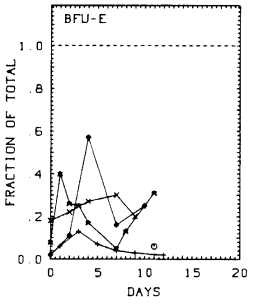

FIGURE 9

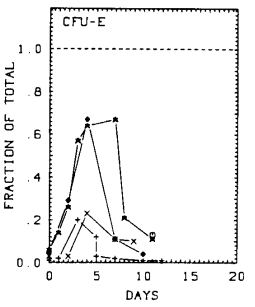

FIGURE 10

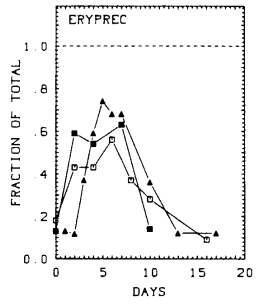

FIGURE 11

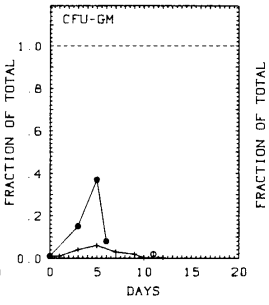

FIGURE 12

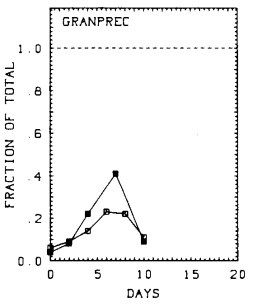

FIGURE 13

FIGURES 8 to 13. The role of the spleen in stimulated erythropoiesis. Data for bleeding anemia are shown as crosses: + — Seidel and Kreja;[7] × — Hara and Ogawa.[29] Data for hypoxia are shown as open symbols: ⊗ — Lord and Murphy;[15,16] ⧖ - Bruce and McCulloch;[11] □ — Rickard et al.;[33] ○ — Peschle et al.;[24] ☆ — Dunn et al.[27] Data for injection of phenylhydrazine as closed symbols: ■ — Rickard et al.;[33] ● — Hodgson et al.[3] ♦ — Hara and Ogawa;[23] ▲ — Rencricca et al.[8]

other strains. In most of the other animals the spleen's contribution decreases from the normal fraction to values below 5% in all cell stages and in no case did it exceed 20% of the total. Thus, again the spleen plays a marginal role compared with the marrow contribution, during an erythropoietic suppression.

3. Erythropoietic Stimulation (Figures 8 to 13)

Figures 8 to 13 show the spleen's behavior following bleeding, injection of phenylhydrazine, and during hypoxia.

Bleeding anemia (+, ×) — Following bleeding, all cell stages increase their number more intensively in the spleen than in the marrow. This is most pronounced for BFU-E and CFU-E for which the spleen's contribution exceeds 20% of total. This corresponds to an increase by more than a factor of 5.

Hypoxia (open symbols) — During hypoxia the splenic fraction of CFU-S does not change and its contribution remains below 10% of the total (Figure 8). For all other cell stages, however, the spleen plays an important role. Apparently, a considerable amount of erythropoiesis takes place in the spleen with an erythroblast contribution reaching up to 60%

of the total. Myeloblasts are also produced in the spleen in an increased amount reaching 25% of the total.

Phenylhydrazine (closed symbols) — Phenylhydrazine is a potent hemolytic agent. In addition to hemolysis it seems to induce a migration of marrow cells to the spleen and to increase the sensitivity to erythropoietin, so that the erythropoietic amplification is very large (see Reference 36). The data clearly show this dramatic effect and especially the predominant role of the spleen in the formation of CFU-E and erythroblasts (Figures 10 and 11). Up to 40% of all CFU-S are found in the spleen; for BFU-E, CFU-E, and erythropoietic precursors the percentages range between 60 and 80% of the total. Granulopoietic precursors increase up to 40% of the total.

Summarizing these results it is evident that the spleen plays an important role during stimulated erythropoiesis. The changes in the spleen's contribution to the total hemopoietic system are manyfold and generally considerably exceed the 20% level.

V. DISCUSSION

In the presented reanalysis an attempt is made to quantify the spleen's contribution to the whole animal's hemopoiesis. For this purpose it is first necessary to derive simple formulas for relative cell numbers and for the fractional cell number in the spleen. These formulas apply as long as two coefficients are available, namely, the "bone marrow-to-femur (tibia)" ratio and the "femur (tibia)-to-spleen" ratio. These coefficients can be found from the absolute cell numbers of the respective organs in normal steady state.

The derivation of the formulas is based on three assumptions (postulates 1 to 3). It is assumed that (1) hemopoiesis is restricted to bone marrow and spleen, (2) one femur (tibia) represents a constant fraction of the whole bone marrow for all hemopoietic cells, and (3) the fraction of total hemopoiesis found in the spleen differs for different cell types. The second assumption makes it possible to derive one "bone marrow-to-femur (tibia)" ratio for all cell stages from measurements of the iron-incorporating cells. This assumption is valid only if the bone marrow composition is homogeneous throughout the skeleton and if hemopoiesis behaves identically in all parts of the bone marrow even after perturbations of the steady state. Whether this postulate is valid can at present not be decided. But obviously there are experiments in which the latter condition of homogeneity is not fulfilled (e.g., total body irradiation with a shielded leg). Experiments of this type are excluded in this analysis.

Using the formulas one obtains a survey about the normal marrow-spleen ratio (Tables 2 and 3). It becomes apparent that considerable strain differences exist. In Swiss-Webster mice the spleen seems to play an important role, whereas it is negligible in CBA/Ca mice. The variability between strains and different laboratories (and, unfortunately, sometimes also within one laboratory) is enormous. However, it is a common feature that under normal circumstances erythropoietic cell stages are found in the spleen in greater amount than granulopoietic cells (Table 4).

More interesting than the analysis of the normal steady state is the investigation of changes of the spleen's role after perturbations. It has practical implications for the planning of experiments, because it may be possible to restrict the measurements to the bone marrow in some experiments while in others the contribution of the spleen should not be neglected. We consider the role of the spleen as relevant if its contribution exceeds 20% of the total for at least one cell stage. Figures 1 to 13 show that this criterion is only fulfilled for erythropoietic stimulation. (This does not hold true for Swiss-Webster mice, which even in the normal steady state have a significant contribution of the spleen to hemopoisis.) In these experiments it is necessary to measure simultaneously changes in the marrow and in the spleen if one is interested in the behavior of the total hemopoietic system.

In other experimental situations like acute irradiation or suppressed erythropoiesis the spleen generally plays a minor role and the marrow data represent a good approximation of the whole system.

The results obtained on the spleen's important role under erythropoietic stimulation are consistent with the observation that splenectomized mice exhibit a much slower recovery of hematocrit after bleeding than mice with an intact spleen.[37]

It should be mentioned that after hypoxia not all strains respond properly to the stimulus. According to Lord and Murphy,[16] C57Bl, CAF_1, and BALB/C mice fail to produce an adequate increase in hematocrit although they have a considerable splenic contribution. In contrast, C_3H mice[27] show an increase in hematocrit, but we are unable to detect a corresponding increase in the total number of erythropoietic progenitors and precursors. They do not show the expected amplification. It remains open how much of the hematocrit increases may be due to dehydration.[27]

In summary, the importance of the spleen has been shown in several different experiments. The analysis is based on the knowledge of the "bone marrow-to-spleen" ratio in the normal steady state, which differs for different cell types and different mouse strains.

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Appendix 3

DEFINITIONS

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I. INTRODUCTION

The terminology used in this volume is based on the proposals for concepts, definitions, and abbreviations in the field of hemopoietic stem cell regulation as summarized by Lajtha[1] and completed by Cronkite.[2] Some of these definitions have been slightly modified by us and further terms on cell kinetics have been added.

II. DEFINITIONS

Stem cell — Cell with extensive self-maintaining (self-renewal) capacity, extending throughout the whole (or most of the) life span of the organism. Differentiation potential is a property of some types of stem cells, but it is not an essential feature of "stem-ness". The pluripotent hemopoietic stem cell is a type of stem cell specialized for hemopoiesis, capable of giving rise to several sublines of progenitor cells. Example: CFU-S.

Progenitor cell — Member of a type of transit cell population, capable of numerous cell divisions but not self-maintenance, i.e., it has an age structure (due to a maturation process). Alternatively, these cells may be considered to have limited self-maintenance and no age structure. All immature, morphologically unrecognizable "committed" hemopoietic cells belong to this type. Examples: BFU-E, CFU-GM.

Precursor cell (morphologically recognizable precursor cell) — Member of a type of transit population which differs from the progenitor cells in that it is at a well-characterized "later" stage of differentiation. Such a cell is usually derived from a progenitor cell. It is a cell which already carries morphologically and cytochemically recognizable features of the nonproliferating end cell (e.g., erythrocyte or granulocyte), which is produced by its maturation. During this maturation, the limited proliferative capacity of the precursor cell produces amplification. Examples: normoblast and myelocyte.

Self-maintenance — Capability of extensive proliferation (not necessarily fast) without demonstrable change in the properties of the cells (extensive = > 50 to 100 cell cycles). Often used synonymously with self-renewal.

Differentiation — (1) Of stem cells: loss of self-maintenance; (2) of progenitor or precursor cells: development from one cell stage to another, always connected with maturation but not necessarily with amplification (mitoses).

Determination (= commitment) — Loss of pluripotentiality. Process of becoming unipotent. For stem cells it is useful to distinguish between the loss of the "stem-ness" (= self-maintenance) which will be called differentiation and the loss of pluripotentiallity which will be called determination. Although in hemopoiesis both processes go in parallel, they are, in principle, independent of each other.

Maturation — Quantitative change (as a rule) which results in a measurable age structure (e.g., more or less mature forms) in the cell population. Example: pronormoblast → erythrocyte, progenitor → precursor.

Amplification — Increase in cell numbers by cell division during differentiation. Property of some transit cell populations. Examples: BFU-E, CFU-E, CFU-GM, promyelocytes.

Self-renewal probability — The probability of a cell to remain, for the next cycle, of the same cell type. The classical abbreviation is "p". If the daughter cells keep the cellular properties of the mother cell, p = 1. If the daughter cells change to a different cell type, p = 0. For p = 0.5 on average one half of the daughter cells replace their mothers and the second half of the daughters change to a different cell type.

Proliferative fraction (= cyclic fraction) — Fraction of cells of a proliferative population which is not in a quiescent (G_0) state. The proliferative fraction also may be called fraction of actively cycling cells.

Cell cycle time — The time between two mitoses excluding the time where the cell is out of cycle (= cell cycle time of actively cycling cells).

Generation time — The average time between successive mitoses in a defined cell population (including resting and cycling cells). Cell cycle time and generation time are identical if all cells are actively cycling.

Doubling time — The time to double the population size. When the doubling time is greater than the generation time it indicates that there is either cellular death or elimination out of the population, e.g., by differentiation or migration.

Transit time (turnover time) — The average time it takes for the cells to pass through a cellular compartment. The variance of the transit time depends on the compartment kinetics. In the case of a homogeneous cell population with "first in and first out" pipeline kinetics, it is zero. In the case of random exit from the compartment the variance is large. The transit time is identical with the turnover time, which is the time it takes to replace the cells in a compartment.

Production rate (turnover rate) — The number of cells produced in a particular compartment per unit of time. In a steady-state maturation compartment the production rate is the quotient of the absolute cell number divided by the transit time. In an amplifying compartment the relation is more difficult.

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Appendix 4

ABBREVIATIONS

TABLE OF CONTENTS

I. EXPERIMENTS AND MODELS

Experiment		Model	
CFU-S	(colony-forming unit in the spleen)	S	(common stem cell for erythropoiesis, granulopoiesis, and thrombopoiesis)
CFU-Mix	(colony-forming unit with mixed colonies — in vitro)		
BFU-E	(burst forming unit-erythropoietic, obtained between day 7 and 9 in culture)	BE	(primitive, not erythropoietin-dependent erythropoietic progenitor cells)
CFU-E	(colony-forming unit-erythropoietic)	CE	(erythropoietin-dependent erythropoietic progenitor cells)
ERC	(erythropoietin responsive cell)		
PRO	(pronormoblasts)	E1	(first subopoulation of erythropoietic precursors, proliferating)
BASO	(basophilic normoblasts)	E2	(second subpopulation, proliferating)
POLY	(polychromatic normoblasts)	E3	(third subpopulation, proliferating)
ORTHO	(orthochromatic normoblasts)	E4	(fourth subpopulation, postmitotic)
ERYPREC	(all erythropoietic precursors)	E1-4	(all erythropoietic precursor cells)
		E	(sum of cells in BE, CE, and E1-4; practically identical with E1-4)
CFU-GM	(colony-forming unit-granulocyte/macrophage)	CG	(granulopoietic progenitors)
GRANPREC	(all granulopoietic precursors)	G1-4	(all granulopoietic precursor cells)
		G	(sum of cells in CG and G1-4; practically identical with G1-4)
TONUC	(total nucleated cells)	—	
Epo	(erythropoietin)	EP	(model erythropoietin)
BPA	(burst-promoting activity)	(Not explicitly included)	
CSA	(colony-stimulating activity, synonymous: CSF)	(Not explicitly included)	

II. MODEL SYMBOLS

Symbol	Explanation
p	Self-renewal probability of stem cells
a, a_S, a_{BE}, etc.	Proliferative fraction (= actively cycling fraction) of stem cells (S), progenitors (BE, CG), precursors (E1-4, G1-4)
Z_{CE}, Z_{E1-4}	Dose-response relations for EP effects on erythropoietic amplification

τ, τ_S, etc.	Cell cycle times
T^F, T_S^F, etc.	Generation times
T, T_S, T_{BE}, etc.	Compartment transit times
n, n_{BE}, n_{CE}, etc.	Number of mitoses
α_E, α_G	Fraction of stem cells undergoing determination into erythropoiesis or granulopoiesis
X	Weighted sum of all cell types, regulating a_S, a_{BE}, and a_{CG}
Y	Weighted sum of all cell types, regulating p
S*, BE*, etc.	Absolute cell number in the model compartment
S_{norm}, BE_{norm}, etc.	Normal cell numbers
S, BE, etc.	Relative cell numbers (e.g., BE = BE*/BE_{norm})

III. REMARKS ON THE NOMENCLATURE

In the theoretical chapters of this book the attentive reader will find several formulations and notations which are not totally correct. They have generally been used for our convenience:

- We do not differentiate between the name of a compartment and the symbol for the relative value, e.g., "S declines to 0.6" is a short term for "the cell content in compartment S declines to 60% of the normal value."
- We often talk of all erythropoietic and granulopoietic cells (using the symbols E and G) while really referring to the precursors alone (E1-4 and G1-4). This has been done if the number of progenitors is neglibibly small, compared with the precursors.
- We often talk synonymously about "bone marrow hemopoiesis" and "total hemopoiesis". In most cases this is quantitatively correct. Where misinterpretation is possible we tried to use the correct notation.
- We often say "stem cell regulation" meaning the regulation of immature hemopoiesis including stem and progenitor cells.
- We often talk about "the stem cell model" without mentioning that the parameters exclusively refer to mice.
- We relate the experimental cell numbers to their normal controls, without mentioning that only data from those authors have been included who have measured cell counts per organ. Concentrations of cells (e.g., CFU-S/10^5 bone marrow cells) are not considered (exception: chronic irradiation).

For this negligent use of terminology we have to apologize but we hope confusion will be limited.

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