

## Proliferation in murine epidermis after minor mechanical stimulation

### Part 2. Alterations in keratinocyte cell cycle fluxes

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**Abstract.** We have recently shown that a mild mechanical irritation (tape strip) of the epidermis on the back skin of adult mice induces a strong and long lasting increase in proliferative activity and cell production. This was revealed by following the fate of <sup>3</sup>HTdR-pulse labelled cells within the basal and suprabasal layers.

To obtain further insight into the dynamics of cell kinetic changes we also performed double labelling experiments with <sup>3</sup>HTdR and BrdUrd at various times after tape stripping. The technique for analysing the data had to account for a non stationary cell flux. A novel biometrical technique was developed which provides parameter estimates on the S-phase duration, the cell cycle duration and a parameter characterizing the degree of nonstationarity.

When applied to the mechanically irritated epidermis we observed that the cell flux through the S-phase in the basal layer was accelerated by a factor of 10 between 18 and 36 h post tape strip. This activation declined slightly in the subsequent days and remained 4–6 fold higher than in the normal steady state for over 7 days post tape strip. The duration of the S-phase was 3–5 h and showed little variation.

We conclude that mild mechanical irritation only affecting the stratum corneum has major stimulatory effects on the cell kinetics of proliferative keratinocytes in the basal layer of the epidermis indicating the existence of a powerful regulatory mechanism.

### INTRODUCTION

We have recently performed a series of experiments on the effect of a mild mechanical irritation of the back skin of the mouse (Potten *et al.*, 2000). It was our objective to obtain some further insight into the proliferation control mechanisms of the epidermis. For this purpose we wanted to apply a perturbation which does not by itself inflict any appreciable damage on proliferative or nonproliferative keratinocytes. Removal of parts of the stratum corneum by repeated tape stripping was deemed to fulfil these criteria. In a

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first set of experiments we observed that stimulation of the epidermis on the back of an adult mouse by tape stripping resulted in an increase of proliferative activity and cell production, that was dramatic and persisted for more than 7 days (Potten *et al.*, 2000). These studies involved a single pulse injection of [methyl-<sup>3</sup>H]-thymidine (<sup>3</sup>HTdR) at various times after tape stripping and the fate of the labelled cells within the basal layer and suprabasal layer was studied at various times after thymidine administration. The limitations of such experiments involving a single pulse labelling procedure are that flux parameters through the cell cycle cannot be determined on an absolute scale and hence, details of the cell kinetic changes in terms of cell cycle time and duration of S-phase cannot be estimated. In order to overcome this restriction, in a second set of experiments we performed a double labelling procedure consisting of a pulse injection of <sup>3</sup>HTdR as a primary marker at various times post tape stripping followed by a second dose of bromodeoxyuridine (BrdUrd), delivered 2 h after <sup>3</sup>HTdR.

Such double labelling procedures enable an estimation to be made of the rate of entry of cells into S and the rate of exit from S, and, using some additional assumptions, the cell cycle time ( $T_c$ ) and the duration of S-phase ( $T_s$ ) can be calculated. How this is done depends on appropriate assumptions on the state of the compartments of proliferating cells.

If the system is in a steady state the proliferating cells are expected to be uniformly distributed over the cell cycle. Then, according to Wimber & Quastler (1963) one can obtain estimates for cell cycle phase durations via the percentage of cells which are in a distinct phase of the cell cycle.

After double labelling three types of labelled cells can occur, i.e. labelled cells with marker 1 only, labelled cells with marker 2 only, and labelled cells with both markers. Although all three labelling patterns provide information they were not always commonly used. For example, Maurer *et al.* (1972) and Schultze *et al.* (1972) estimated the duration of the S-phase in cells in the jejunal crypts of mice only from the percentages of cells being labelled with marker M1 only and of cells labelled with marker M2 (with or without M1-label). In an analogous way Harriss & Hoelzer (1971) looked at leukaemic cells in rat and man. On the other hand, estimations of the duration of the S-phase based on the percentages of all three fractions of cells were done by Hilscher & Maurer (1962) for spermatogonia of rats and by Pilgrim & Maurer (1962) for a collection of cell types, including skin epithelial cells of mice and rats.

However, after tape stripping, the cells in the basal layer are not in a proliferative steady state. To cope with nonstationary growth and based on investigations of Steel and his coworkers (Steel & Bensted, 1965; Steel, Adams & Barrett, 1966; Steel, 1967; Steel & Hanes (1971) on exponentially growing tissues, Lennartz, Maurer & Eder (1968) derived a pair of equations which relate the percentages of cells in the three fractions to the duration of the S-phase, the cell cycle time, and the position of the S-phase within the cell cycle, i.e. the duration of G<sub>2</sub>- and M-phases together. An additional tacit assumption required was that after mitosis both daughter cells re-enter the cell cycle. As only two equations were available for three unknown parameters, the duration of G<sub>2</sub>- plus M-phase had to be estimated separately, either by the percentage of labelled mitoses method, as demonstrated for tumour growth, or by plausible assumptions. Knolle (1984) reformulated Lennartz's equations and showed that the duration of the S-phase and the cell cycle time can be approximately estimated without assumptions on the duration of the G<sub>2</sub>- plus M-phase. On the other hand it is rather unlikely that the skin post tape stripping can be treated like an exponentially growing tissue. Therefore, a novel strategy was adopted. Starting with a steady state approach,  $T_c$  and  $T_s$  were estimated for different

times post tape stripping. A check for consistency with the data revealed contradictions regarding influx to and efflux from the S-phase and resulted in a nonsteady state modification of the model. The technique is described in detail below. The results of these analyses show that the mechanical damage induced by tape stripping (which is not accompanied by any DNA damage or obvious cell killing) is followed by a dramatic long lasting increased cell flux through the S-phase, shortening of the cell cycle time,  $T_c$ , with  $T_s$  remaining relatively unchanged.

## MATERIALS AND METHODS

### Experimental procedures

Male DBA2 mice aged between 7 and 8 weeks were used for the experiments. Animals of this age have the hair follicles in the resting phase (telogen) of the hair production cycle. An area of skin on the back of the mice was clipped. The stubble of the hair was removed with a chemical depilatory cream (Immac, Anne French, London, UK). The skin was washed and dried, and then adhesive tape (Sellotape, Dunstable, UK) was applied and removed four times, each time in the opposing longitudinal direction. The protocols used were identical to those presented in the accompanying paper (Potten *et al.*, 2000). The different experimental procedures used here are described below.

The epidermis was stimulated by the mechanical action of multiple tape stripping at time 0. As a first marker [M1] 1.85MBq (50  $\mu$ Ci)  $^3$ HTdR/0.2 ml was injected at 14 time points of analysis chosen between 6 and 144 h after tape stripping. Groups of four mice were used.

As a second marker [M2], bromodeoxyuridine (BrdUrd) (Sigma-Aldrich, Poole, UK) was injected at a dose of 10 mg/0.2 ml. The interval between the two markers,  $\tau$ , was 2 h in all experiments. The animals were sacrificed 1 h after the BrdUrd injection. The timing of these experiments was similar to that described for single labelling (Potten *et al.*, 2000). Samples of skin were removed, placed flat on a piece of filter paper and fixed in Carnoy's fixative for 30 min prior to storage in 70% ethanol and subsequent tissue processing for histology. Sections 3–5  $\mu$ m thick were cut and immunohistochemistry was performed. The primary antibody used was rat anti-BrdUrd BU1/75 (ICR1) (Harlan-Sera Lab, Loughborough, UK), and the protocol used to detect those cells that had incorporated BrdUrd is described elsewhere (Potten *et al.*, 1992). The immunohistochemically stained slides were then processed for autoradiography as described for single labelled preparations (Ilford K5 diluted 1:1). Slides were exposed for 2 weeks, developed and lightly counter-stained with haematoxylin and eosin.

### Scoring procedures and counts

From the double labelled sections, the following parameters were measured by counting a minimum of 1000 basal cells per mouse: the number of cells that were unlabelled; labelled with  $^3$ HTdR alone [M1]; labelled with both  $^3$ HTdR and BrdUrd; and labelled with BrdUrd [M2] alone.

The following notations have been used in this paper:

$n_{00}$  number of cells not labelled with either marker (unlabelled),

$n_{10}$  number of cells labelled with M1 alone,

$n_{01}$  number of cells labelled with M2 alone,

$n_{11}$  number of cells labelled with both markers, i.e. double labelled cells,

where 'n' represents the sum of these counts, i.e. the total number of cells.

**Derived quantities**

From the counts that were made, the experimental labelling indices were estimated.

$$LI(M1 \overline{M2}) = \frac{n_{10}}{n} \quad \text{labelling with M1 alone, is related to the flux out of S (efflux)} \quad (1a)$$

$$LI(\overline{M1} M2) = \frac{n_{01}}{n} \quad \text{labelling with M2 alone, is related to the flux into S (influx)} \quad (1b)$$

$$LI(M1 M2) = \frac{n_{11}}{n} \quad \text{labelling index for cells double labelled with M1 and M2} \quad (1c)$$

The double labelled cohort of cells is an unusual biological parameter, but it is a useful parameter for the mathematical modelling.  $n_{10} + n_{11}/n$  or  $n_{01} + n_{11}/n$  are the more normal parameters used and are broadly equivalent to the labelling index as measured in the accompanying paper (Potten *et al.*, 2000). By introducing bars over symbols ( $\overline{M1}$ ,  $\overline{M2}$ ), we adopted the notation of probability theory. Let  $M1$  be an event, e.g. 'cell is labelled with M1', which occurs with probability  $P(M1)$ .  $\overline{M1}$  is the complementary event, i.e. 'cell is not labelled with M1', which occurs with probability  $P(\overline{M1}) = 1 - P(M1)$ .  $M1 \overline{M2}$  is the compound event 'labelled with M1 and not labelled with M2.' This event occurs with the probability  $P(M1 \overline{M2})$  which can be estimated by the labelling index  $LI(M1 \overline{M2})$ , i.e. the ratio of the number of M1-only labelled cells to the total number of cells.

**Estimation of the cell kinetic time parameters in steady state**

The cell kinetic parameters for a steady state model were derived for the basal cells. It was assumed that: (a) at least for the time span  $T_s + \tau$ , the cell kinetic flux is assumed to be in a stationary state; (b) all cells are proliferating, i.e. there are no resting cells; (c) the cell cycle time and duration of S-phase are identical throughout for all the cells being analysed; (d) the cells are equally distributed at random over the cell cycle with a duration of  $T_c$ ; (e) the labelling techniques are specific for S-phase cells.

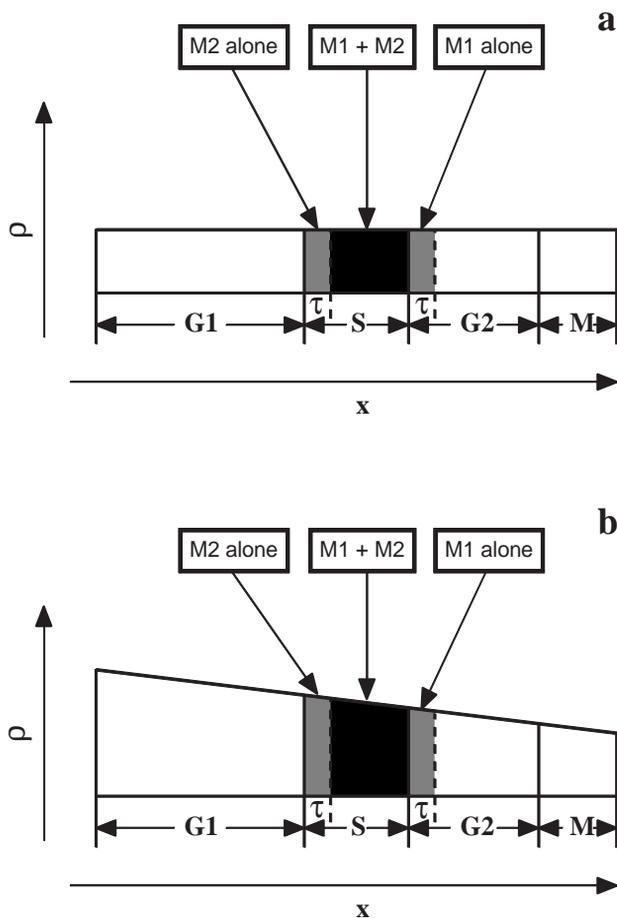
The relationship between the labelling indices and the flow of cells through the cell cycle is illustrated in Fig. 1(a) showing the state of the cells immediately after the second labelling. The abscissa represents the momentary state variable of the cell,  $x$ . The ordinate represents the density of cells,  $\rho$  (i.e. cells per state interval) which is constant in the steady state displayed. Therefore, the upper line in the diagram is parallel to the abscissa axis. Assuming that the velocity,  $v$ , with which the cells change their state is

$$v = 1 \left[ \frac{\text{state units}}{\text{time units}} \right]$$

then there is a constant flux of cells  $I = \rho \cdot v$  for all values of  $x$ . The areas of the hatched fields in Fig. 1 represent the fractions of single and double labelled cells. The probabilities of randomly chosen cells falling into one of the defined categories in this diagram are equal to the ratio of cell numbers in these categories and the total number of cells. Thus, model equations can be derived which relate these probabilities to the model parameters  $T_c$  and  $T_s$  and the time interval between first and second labelling  $\tau$ :

$$P(M1 \overline{M2}) = p_{10} = \frac{\tau}{T_c} \quad (2a)$$

$$P(\overline{M1} M2) = p_{01} = \frac{\tau}{T_c} \quad (2b)$$



**Figure 1.** (a) Schematic representation of the stationary state flux model of cells through the cell cycle. The light-shaded areas represent the influx to and efflux from S-phase. The dark-shaded area represents double labelled cells. The labelled fronts are drawn for the moment of labelling with M2. Within the time frame of the double labelling it is assumed that the duration of S-phase is constant. (b) Schematic representation of the accelerated flux model of cells through the cell cycle where the influx exceeds the efflux. The data suggest that model b (see Figure 4) is more appropriate, as will be shown. The number of cells labelled with either marker corresponds to the S-phase. M1: <sup>3</sup>HTdR; M2: BrdUrd.

$$P(M1 M2) = p_{11} = \frac{T_s - \tau}{T_c} \tag{2c}$$

$$P(\overline{M1} \overline{M2}) = p_{00} = \frac{T_c - T_s - \tau}{T_c} \tag{2d}$$

It follows that  $P(M1 \overline{M2})$  can be interpreted as the proportion of cells which leave the S-phase compartment during the time span  $\tau$ . In the same way  $P(\overline{M1} M2)$  can be seen to be the proportion of cells which enter the S-phase during  $\tau$ . In a steady state both are equal,

$$P(M1 \overline{M2}) = P(\overline{M1} M2) \tag{3}$$

The values of the probabilities depend on the values of the parameters  $T_c$  and  $T_s$ . The labelling indices for the two markers estimate the corresponding probabilities, i.e.  $LI(M1 \overline{M2})$

estimates  $P(M1 \overline{M2})$  providing the appropriate values of the parameters  $T_c$  and  $T_s$ . Thus, the system of model equations is overdetermined, and has no solution in the general case. Nevertheless, it is a useful tool to estimate  $T_c$  and  $T_s$ . One possibility is to reduce the system. For example, the equation

$$\frac{P(M1 M2) + P(\overline{M1} M2)}{P(M1 \overline{M2})} = \frac{T_s}{\tau} \quad (4)$$

results from summing equations (2b) and (2c) and dividing by equation (2a). This equation was used for the estimation of the duration of the S-phase by Maurer *et al.* (1972) and Schultze *et al.* (1972). Another way could be to combine equations (2a) and (2b) and to use the sum of the labelling indices for markers M1 only and M2 only as an estimate of  $2 \cdot \frac{\tau}{T_c}$ . In order to use all information in the data a fitting procedure was used which minimizes the weighted sum of squares of the differences between observed counts  $h_{ij}$  and expected counts  $n \cdot p_{ij}$  using the  $\chi^2$ -statistic

$$\chi^2 = \sum_{i=0}^1 \sum_{j=0}^1 \frac{[h_{ij} - n \cdot p_{ij}]^2}{n \cdot p_{ij}} \quad (5)$$

So for each time point observed a pair of point estimates ( $T_c, T_s$ ) was calculated. In order to obtain confidence limits for the parameter estimates a Monte Carlo approach was used. For each time point 100 independent simulation experiments were undertaken. Each experiment consisted of two steps: (1)  $n = 1000$  random numbers were assigned to the four categories 'nonlabelled', 'labelled with M1 only', 'labelled with M2 only', 'labelled with both M1 and M2' according to the model probabilities  $p_{ij}$  (equations (2a)–(2d)) using the point estimates for  $T_c$  and  $T_s$ ; (2) from the frequencies  $h_{ij}$  of hitting the above categories and the probabilities  $p_{ij}$  a pair of values ( $T_c, T_s$ ) was derived by minimizing  $\chi^2$  (eqn 5). Using 100 resulting such pairs ( $T_c, T_s$ ) and assuming a bivariate normal distribution, a 95% confidence ellipse was constructed and the projections onto the axes were used as (slightly) conservative confidence intervals. The numerical method has been adapted from Press *et al.* (1992).

### Estimation of the cell kinetic time parameters: adjusting for nonstationarity

As will be demonstrated by the results, the steady state assumption is not consistent with the data in our setting. Hence, we needed to consider that the flux of cells may be accelerated or decelerated during the time span,  $\tau$ . Within the framework of the models based on exponential growth (Steel & Bensted, 1965; Steel *et al.*, 1966; Steel, 1967; Steel & Hanes, 1971) this can happen if, on average, more than one daughter cell re-enters the compartment of proliferating cells after a cell division and the potential increase in cell number is not counterbalanced by cell loss within the cell cycle. Additionally, transient changes can result from changes of the velocity,  $v$ , or from cohorts of G0-cells which, after tape stripping, may rapidly enter the cell cycle (Gelfant, 1962, 1975; Pederson & Gelfant, 1970; Gelfant & Candelas, 1972; Boezeman *et al.*, 1987; Rijzewijk, Boezeman & Bauer, 1988a; Rijzewijk *et al.*, 1988b). Subsequently, the model assumption (a) can be altered to assume a uniformly accelerated or decelerated flux of cells during the time span  $T_s + \tau$  at any time point of evaluation. The appropriate schematic representation of this assumption can be seen in Fig. 1(b). The consequence of the adjustment is that the cell density,  $\rho$ , and the cell flux,  $I$ , which depend on time,  $t$ , and state,  $x$ , can be described in the form of a wave propagating to the right in the diagram:

$$I(t, x) = I_0 \cdot [1 + k \cdot (t - x + x_0)] \quad (6)$$

where  $x_0$  represents the middle of the S-phase. The acceleration parameter,  $k$ , describes the relative change of flux,  $I$ , per unit time through a state,  $x_0$  (e.g. if  $k = 0.10 \text{ h}^{-1}$ , then the flux of cells increases during 1 h by 10% of its previous value). Alternatively,  $k$  can be interpreted as the relative decline of the flux,  $I$ , per state unit at a given time,  $t$ . The acceleration parameter,  $k$ , is related to (but not identical with) the difference between influx and efflux. In the period of constant acceleration during the time span,  $\tau$ , a number of  $I_0 \cdot \tau \cdot (1 - k \cdot T_s/2)$  M1-only labelled cells leave the S-phase compartment and a number of  $I_0 \cdot \tau \cdot (1 + k \cdot T_s/2)$  cells enter the S-phase compartment and become M2-only labelled cells so that the net increase in cell number in the S-phase compartment equals  $I_0 \cdot \tau \cdot k \cdot T_s$ . A number of  $I_0 \cdot (T_s - \tau)$  cells move only within the S-phase compartment and become double labelled. If  $|k| \cdot T_c \ll 1$ , then there are approximately  $I_0 \cdot T_c$  cells in the system. Thus, the equations for the accelerated flow model which determine the probabilities for randomly chosen cells fall into one of the following defined categories:

$$P(M1 \overline{M2}) = p_{10} = \frac{\tau}{T_c} \cdot \left(1 - k \cdot \frac{T_s}{2}\right) \quad (7a)$$

$$P(\overline{M1} M2) = p_{01} = \frac{\tau}{T_c} \cdot \left(1 + k \cdot \frac{T_s}{2}\right) \quad (7b)$$

$$P(M1 M2) = p_{11} = \frac{T_s - \tau}{T_c} \quad (7c)$$

$$P(\overline{M1} \overline{M2}) = p_{00} = \frac{T_c - T_s - \tau}{T_c} \quad (7d)$$

The model can be seen as a linear approximation of a model of exponential growth. In contrast to the steady state model, the accelerated state model is not symmetrical with respect to the markers  $M1$  and  $M2$ . Thus, there is a system of three independent equations for the three unknown parameters  $T_c$ ,  $T_s$ , and  $k$ , which, after substitution of the probabilities by their estimates from the experimental data, can be solved by algebraic methods. However, the method to derive confidence intervals by simulation is restricted to stationarity and cannot therefore be applied to a nonstationary system.

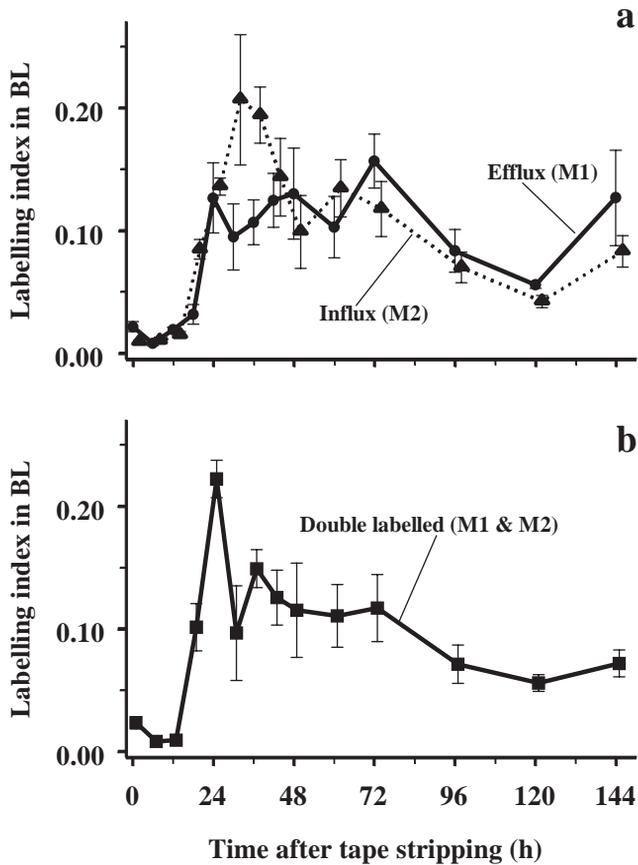
## RESULTS

### Labelling indices after mechanical irritation

The labelling indices for the individual animals were calculated and four animals were sampled from each treatment group. From these, the mean and the standard error of the mean were calculated. The results obtained for the various categories of labelling are shown in Fig. 2. Panel (a) shows the labelling index data for cells labelled with only one of the markers  $M1$  (i.e.  $^3\text{HTdR}$ ) or  $M2$  (i.e.  $\text{BrdUrd}$ ), according to the definitions given in equations (1a) and (1b) in the Materials and Methods. Obviously, at early times post tape strip, the influx into S-phase ( $M2$  alone) exceeds the efflux ( $M1$  alone), whilst at later times (e.g. 72 h) the efflux exceeds the influx. Panel (b) shows the labelling index data for cells labelled with both markers  $M1$  and  $M2$  simultaneously, according to the definition given in equation (1c).

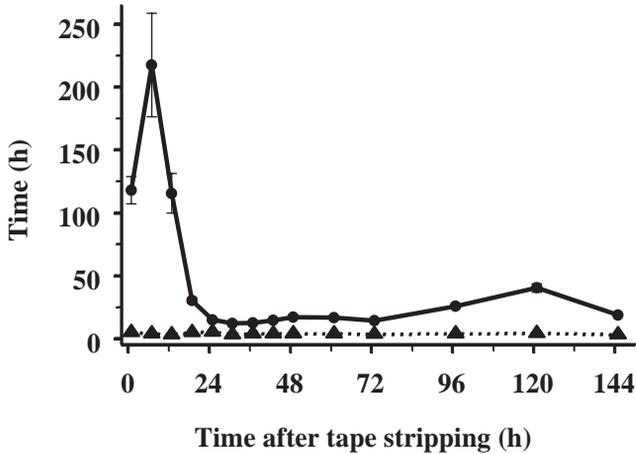
### Estimation of cell kinetic parameters using the steady state approach

Assuming that, during the relatively short time interval  $T_s + \tau$ , the flux through S-phase is in steady state, the values of  $T_c$  and  $T_s$  can be estimated as described in the Material and



**Figure 2.** Experimental data. Labelling indices obtained in a double labelling experiment at various times post tape stripping. (a) Proportions of cells which are only labelled with one marker but not with the other. M1:  $^3\text{HTdR}$ ; M2: BrdUrd. Data points over time of labelling with M1 (●, solid line) or M2 (▲, dotted line), respectively. (b) Proportion of cells which are double labelled with both markers (■, solid line). Data points shown are median time between labelling with M1 and M2. Error bars: means  $\pm$  SEM obtained from data (four mice per group).

Methods section. The parameters were estimated from the data independently for each time point after tape stripping. For each of the time points, 95% confidence intervals for  $T_c$  and  $T_s$  were calculated by a Monte Carlo simulation using the estimates as true values. The results are shown in Fig. 3. The undisturbed epidermis is characterized by a cell cycle time of  $118 \pm 11$  h and an S-phase duration of  $4.8 \pm 0.8$  h. At the earliest time point (7 h) post tape stripping, the cell cycle time estimate increased to  $218 \pm 41$  h. It then decreased dramatically to a value of  $15.0 \pm 0.8$  h at the end of the first day, and subsequently to a minimum value of  $12.1 \pm 0.4$  h at 31 h post tape stripping. In the later time period the cell cycle time estimates showed only a slight increase to  $40 \pm 3$  h while the duration of the S-phase ( $T_s$ ) remained relatively constant (range 3.1–5.4 h). Seven days post tape strip, the estimated cell cycle times in the basal layer were still remarkably shorter than seen in the initial time point. To check the model for consistency with the data, we compared labelling indices derived from the model with those observed experimentally. For this purpose we generated model confidence bands as shown in Fig. 4. When the labelling indices with both markers are



**Figure 3.** Time course for changes in the cell cycle time (●, solid line) and the duration of S-phase (▲, dotted line) after stimulation of the epidermis by tape stripping. Estimated using the steady state assumption. Data points shown are median time between labelling with M1 and M2. Error bars: 95% confidence intervals obtained by Monte Carlo simulation.

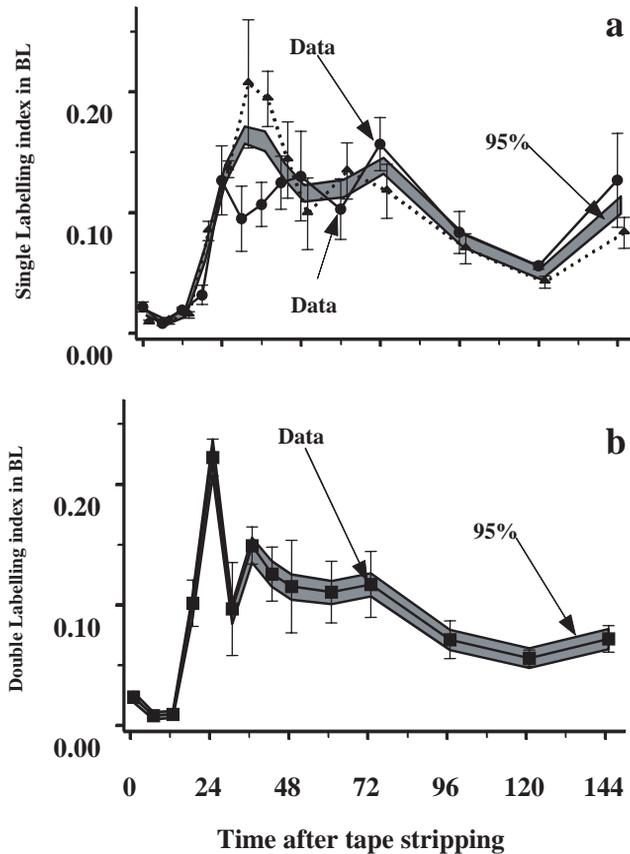
compared with the model predictions, the agreement is excellent (Fig. 4b). However, when the influx and efflux parameters from the data on  $^3\text{HTdR}$  alone and BrdUrd alone are compared with their model predictions (Fig. 4a), there are systematic deviations. These indicate that, for the first two days post stimulation, considerably more cells enter the S-phase compartment than leave it, and, after the second day this relationship reverses. Thus, the assumption of a stationary constant cell flux during this time-frame is invalid.

#### Estimation of cell kinetic parameters adjusting for non stationary fluxes

The method for non stationary fluxes which was described in the Material and methods section was used to obtain parameter estimates for  $T_c$ ,  $T_s$ , and the nonstationarity parameter,  $k$ . The results for the estimates for  $T_c$  and  $T_s$  deviate only slightly from those obtained using the steady state model (Fig. 3). The time course of the parameter  $k$  is shown in Fig. 5. The parameter  $k$  describing the percent change in cell flux per hour tends to be positive over the first two days and negative for the later time points. Positive values (acceleration) represent an influx greater than the efflux, i.e. more cells entering S-phase than leaving. Conversely, negative values (deceleration) represent more cells leaving S-phase than entering. Hence, we found a very strong proliferative activation which was switched on between 18 and 36 h post tape strip. Only a very slow switch off was observed after 72 h.

## DISCUSSION

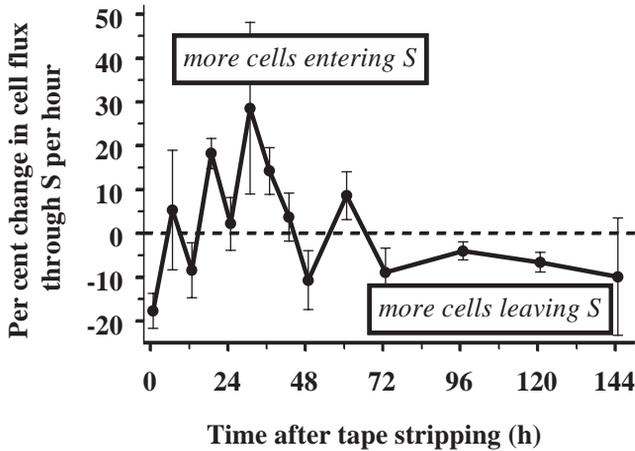
A technique has been developed to evaluate double labelling experiments in situations with stationary and nonstationary cell fluxes through the S-phase. In the case of stationary cell fluxes, the method is based on a parameter fitting procedure and is supplemented by a Monte Carlo procedure for construction of confidence limits, whereas in the case of nonstationary cell fluxes the parameters are calculated directly. The technique generates estimates for the average S-phase duration ( $T_s$ ), for the average cell cycle time ( $T_c$ ) and a parameter ( $k$ ) for the nonstationarity of this flux. By using simple assumptions, the reciprocal value of the cell cycle



**Figure 4.** Comparison of the experimental LIs (see Figure 2) with predictions made by the stationary flux model. Data points as shown in Figure 2. The shaded bands are the 95% confidence bands of the labelling indices for cells carrying only one single marker (a) or both markers (b). Note the excellent concordance for double labelling indices (b) and the adverse deviations of the single labelling indices (a), indicating that the stationary flux assumption is not appropriate.

time ( $1/T_c$ ) can be used as an estimate of the average cell flux in the middle of the S-phase. This methodology should be applicable generally for any proliferative tissue and is particularly appropriate in situations of transient changes of the fluxes (e.g. after perturbations to a stationary system).

The technique was applied to a set of double labelling data obtained from the basal layer of mechanically irritated murine epidermis. The analysis showed that, 19 h after tape stripping, a dramatic increase of the cell flux through the S-phase could be detected. At 25 h post irritation the flux rate had increased by a factor of 9.7. The cell cycle time had decreased from a normal 145 h to 14 h. For a period between, 19 and 37 h post tape stripping there were about 20% more cells entering S-phase per unit time than leaving it. At the same time the S-phase duration did not noticeably change. Hence, the data strongly suggest that the passage of cells through the cell cycle is dramatically accelerated in basal layer keratinocytes one day after tape strip. At 37 h post tape strip, the cell flux had reached its maximum and a slight deceleration was observed. However, by days 5–6 post tape strip, the cell fluxes were still increased four- to sevenfold above normal.



**Figure 5.** Time course of the acceleration parameter,  $k$  (see text), estimated using the accelerated flux model. The parameter describes changes in the flux rate of cells between labelling with M1 and M2. Values above 0 represent an increase in the flux of cells entering S (acceleration), whereas values less than 0 represent a situation where more cells are leaving S (deceleration or negative acceleration). Data points shown are median time between labelling with M1 and M2. Error bars: means  $\pm$  SEM obtained from data (four mice per group).

These observations are clearly consistent with a comprehensive set of experiments using single label protocols described by us separately (Potten *et al.*, 2000). In these experiments, label was injected at different times post tape strip and the passage through the basal and suprabasal layers was then followed from each of the time points onwards. The data clearly indicated a large increase of cell production and an accelerated cell migration through the suprabasal layers. Crude estimates for cell production and migration rates were obtained suggesting a 10–20 fold increase 1–2 days post tape strip. The above detailed analysis of double labelling data shown here confirms this impression and provides more precise quantification.

Taken together, the tape strip experiments described here and elsewhere (Potten *et al.*, 2000) strongly suggest the existence of a system of proliferation control which rapidly reacts to depletion of the stratum corneum. By the design of our experimental procedure, we attempted to avoid any direct damage to nucleated keratinocytes, such as occurs after ionizing radiation or ultraviolet light exposure. Thereby, we wanted to avoid confounding damage to, and reaction of, proliferating cells. Hence, our data strongly argue for an intraepithelial control process that is not merely related to cell densities but to some architectural and/or functional properties. By day 7 post tape strip, histological examinations showed that the suprabasal layers as well as the stratum spinosum and corneum have not yet returned to predamage architecture. We speculate that this is the reason why the proliferation has not yet switched off despite massive cellular overproduction and increased cellular density (Potten *et al.*, 2000).

There are several conceptual possibilities for a regulatory control process of epidermal cell proliferation. In order to gain a consistent view on the dynamics and interdependencies of these control processes, we are presently developing a simulation model to investigate which of these theories would be the most powerful and comprehensive. The model should provide a concept of proliferation control that can explain the cell kinetics, cell production, and cell migration in the normal steady state as well as after perturbations such as tape stripping,

ionizing irradiation and ultraviolet light exposure. The concept will have to incorporate the stem cell and cellular pedigree (lineage) concept suggested by us and colleagues based on separate sets of data (Potten & Lajtha, 1982; Potten *et al.*, 1982a,b, Potten, Saffhill & Maibach, 1987; Loeffler, Potten & Wichmann, 1987; Potten & Loeffler, 1987, 1990; Loeffler & Potten, 1997). It will also have to reflect cell count data obtained from basal layer sheet preparations as well as basal and suprabasal layer data obtained from sections.

With regard to the analysis of the double labelling data shown here, it is important to note that the analysis was done independently for each time point. However, biologically adjacent time points yield correlated results. Furthermore, one should remember that the technique relies only on observations on labelling which take place in the S-phase. In a nonstationary situation, the real cell cycle time for a cohort of cells (including its entire development) may, in fact, be very different from an estimate, which relies on an extrapolation from observations of a cell density distribution in the S-phase to the entire cell cycle. Finally, as indicated by the investigations of the groups of Gelfant (Gelfant, 1962; 1975; Pederson & Gelfant, 1970; Gelfant & Candelas, 1972) and Boezeman (Boezeman *et al.*, 1987; Rijzewijk *et al.*, 1988a,b), the model should allow for the inclusion of compartments of cells which do not proliferate in the normal skin (quiescent cells) but can re-enter the cell cycle after irritation.

The long lasting proliferative activation post tape stripping was an unexpected finding. Further experiments are under way to prolong the time frame and to also obtain further immunohistochemistry and microscopy data. Our working hypothesis is that proliferative down regulation cannot be fully restored before *restitutio ad integrum* has occurred in the stratum corneum.

In summary, a novel technique was used to analyse quantitatively nonstationary double labelling data obtained from mechanically irritated murine skin. The data clearly demonstrate a strong proliferative activation after tape stripping which is accompanied by a dramatic increase in cell production and cell migration.

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