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Epidermal cell proliferation

I. Changes with time in the proportion of isolated, paired and clustered labelled cells in sheets of murine epidermis

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Summary. A new technical approach to analysing labelled cells in sheets of epidermis is presented. The changes in the proportion of isolated single labelled cells, paired or clusters of 3, 4, or more than 4, labelled cells in sheets of epidermis from the back of the mouse have been analysed at various times up to 500 h after ^{3}HTdR administration at either 03.00 h or 15.00 h. The technique is not dependent on the relative number of labelled cells (i.e. the labelling index) but on the spatial distribution of labelled cells. The data cannot be adequately explained on the basis of a simple homogeneous stem cell population in the basal layer but can be better understood on the basis of an hierarchical stem cell-dividing transit proliferative model. The data are consistent with an average cell cycle time of about 100 h but there are suggestions of considerable cell kinetic heterogeneity. The data also suggest that the amount of lateral cell movement within the basal layer is small. The results may suggest that some stem cells either loose label in a manner similar to that suggested by Cairns (1975) i.e. through a process of selective segration of their DNA strands, or that they have an extremely short S phase duration as postulated earlier (Potten et al. 1982). The present data have been extensively mathematically modelled in an accompanying paper. The model which best fits all the data is an hierarchical scheme with three cell divisions in the transit population but some branches of the lineage may be prematurely terminated by the early production of post-mitotic cells. The average resisdence time for the post-mitotic cells can be estimated to be about 2 days for cells labelled at 15.00 h.

Key words: Epidermis – Clustered labelled cells – Mouse

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Introduction

It has recently been pointed out that an analysis of the spatial distribution of labelled cells in sheets of epidermis represents a relatively unexploited cell kinetic technique which has certain advantages (Loeffler et al. 1986). The technique is not dependent on the absolute number of labelled cells nor is it dependent on the ratio of labelled to unlabelled cells i.e. the labelling index. It is merely concerned with the spatial distribution, or clustering, of the labelled cell population. The changes in the proportion of isolated single labelled cells and those that are adjacent to another labelled cell, a labelled pair, over the first 24 h after tritiated thymidine labelling have been analysed elsewhere to provide information on the length of G_2 and the length of S (Loeffler et al. 1986).

The changes in the proportions of singles and pairs, as well as the changes in triplets, quadruplets, and larger clusters over longer periods should also be informative. Such data are analysed here for the first time for the back skin of mice when tritiated thymidine was administered at two different times of the day (03.00 h and 15.00 h) which represent the times of the peak and the trough in the LI circadian rhythms for our mice (Potten et al. 1977). The data can in principle provide information on the cell cycle time, on the proliferative organisation and on the degree of cell movement both within the basal layer and from the basal layer into the suprabasal stratum.

Materials and methods

Male BDF1 mice which were 7–8 weeks old were used thus ensuring that most hair follicles were in the resting, telogen, phase of the hair growth cycle. The mice received an intraperitoneal injection of 92.5 kBq (25 μCi) of tritiated thymidine (^{3}HTdR) (Amersham Int., PLC) in 0.2 ml saline at either

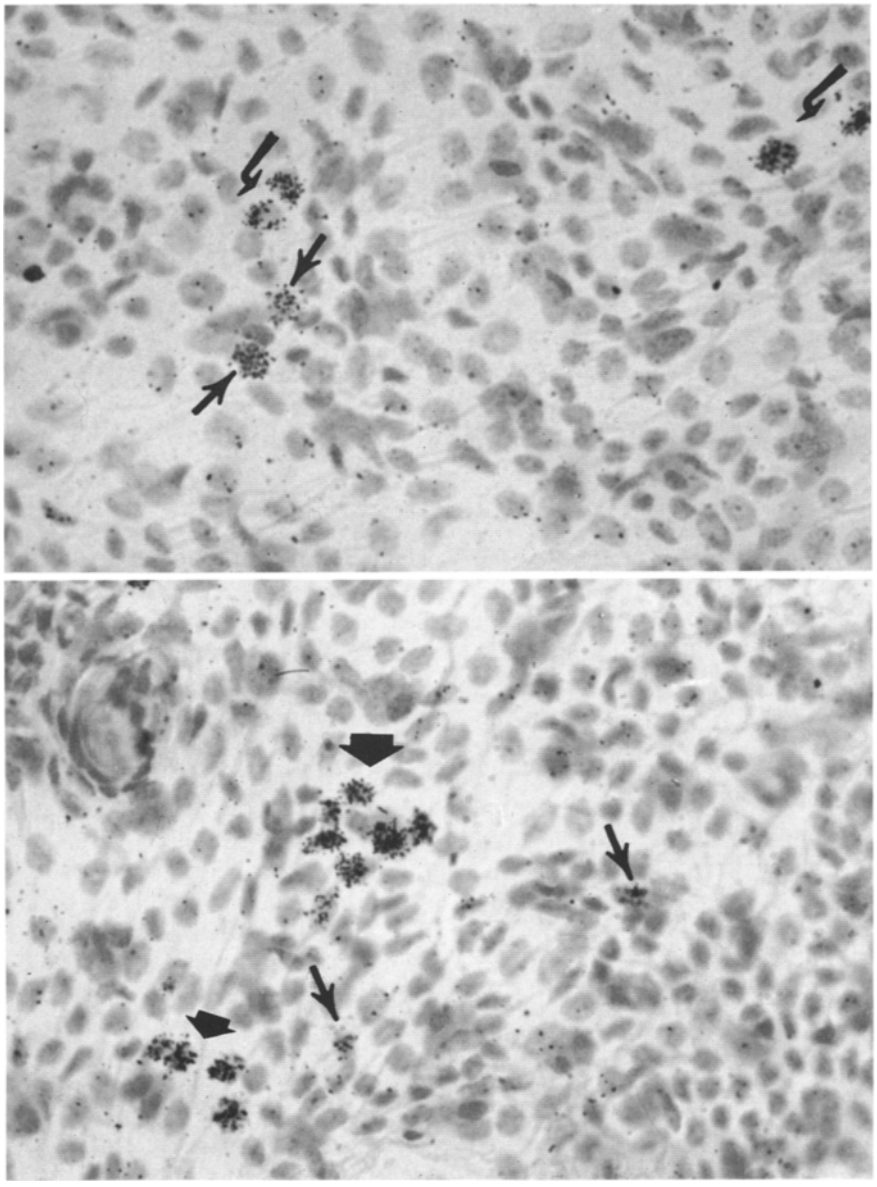

Fig. 1. Autoradiograph of an epidermal sheet prepared from a mouse that received 92.5 kBq of ^{3}HTdR 160 h before sacrifice and the fixing of a sample of skin. The autoradiograph shows many unlabelled basal nuclei, some single isolated labelled nuclei (*small arrows*), pairs (*curved arrows*), and larger clusters of 4 or 7 labelled nuclei (*heavy arrows*)

03.00 h or 15.00 h. The animals were housed in conventional plastic mouse boxes in humidity, temperature and light controlled (lights on 06.00–18.00 h) rooms and received water and food *ad libitum*. Groups of not less than four mice were killed at various times after the ^{3}HTdR administration starting at 40 min and ranging up to 500 h (20.8 days). Samples of skin were removed from the centre of the back, clipped free of hair and exposed to a chemical depilatory (Immac, Anne French Ltd.) for about 5 min before immersion of the skin in 0.5% acetic acid overnight at 4° C. Following this, the skin was fixed in Carnoy's fixative (20–30 min) and was then stored in 70% ethanol. The epidermis was then peeled from the skin using watch makers forceps and a dissecting microscope. The sheets of epidermis were then spread on a microscope slide in a drop of 70% ethanol with the cornified layers downwards. The sheets were dried onto the gelatinised slides and dipped in liquid emulsion (Ilford K 5 1:1 dilution with water). After exposure for 3–4 weeks the slides were developed and stained with haematoxylin. This exposure gives heavy labelling of S phase cells. Details of all these techniques have been presented elsewhere (Hamilton and Potten 1972; Potten 1974; Potten and Bullock 1983).

Using 4 grains or more to identify labelled cells, the labelling index and the clustering of labelled cells was determined. In some samples from the later time points the grain threshold for labelling was raised to 5 or 6 grains because the background increased as time passed. Using an eyepiece Whipple grid 10 grid areas were selected and the clustering of all the labelled cells was determined. Each labelled cell was analysed to determine if it was totally surrounded by neighbouring unlabelled cells in which case it was termed an isolated single labelled cell. If it was adjacent to one other labelled cell it was termed a pair. Three such adjacent cells were termed a triplet, four a quadraplet and any cluster with more than four labelled cells was termed a large cluster (see Fig. 1). The number of labelled cells in each category was determined, each pair being recorded as 2 labelled cells, a triplet as three etc. Clusters were scored

if more than half the labelled cells were within the grid. If equal numbers were within and outside the grid such clusters on two sides of the grid only were recorded. The total number of labelled cells analysed from the four mice in each group varied somewhat but was within the range of about 100–1500 cells per group in the 15.00 h experiment and 400–1300 in the 03.00 h experiment. The number being determined by the LI and the number of mice sampled and size and quality of the epidermal sheet. In such epidermal sheets labelled with a tritiated molecule once a cell migrates into the first suprabasal layer it is effectively lost from the analysis.

The results are presented as the proportion (%) of all labelled cells present as each category, singles, pairs, triplets etc. The data are presented graphically as the changes in the proportion of labelled singles, pairs, triplets etc with time after ^{3}HTdR administration. No curve fitting has been attempted since only general trends are to be discussed and an accompanying paper will present a mathematical modelling of the experiments where curve fitting has been attempted.

The data for the first 24 h have been analysed in detail elsewhere (Loeffler et al. 1986). The labelling index (LI) was determined by counting at least 1000 basal cells per mouse.

Results

Figure 2 illustrates the changes in the percentage of all labelled cells that appear as isolated single cells with time after ^{3}HTdR injection at either 03.00 h or 15.00 h. Both curves illustrate the same features. Initially the proportion of isolated single labelled cells is high (~67–85%). The proportion then falls rapidly to reach a minimum of about 5% between 10–40 h after ^{3}HTdR administration. The proportions then rise steadily with time over the next 400 h. The curve obtained after ^{3}HTdR administration at 03.00 h lags behind that for 15.00 h administration.

Figure 3 shows how the percentage of labelled pairs changes with time. As the singles fall so the pairs rise from initial low values to reach peak values of 50–65% (the 03.00 h values are lower). The proportion of labelled pairs then falls progressively with time reaching essentially zero at 500 h for the 15.00 h injection.

Figure 4 shows how the proportion of labelled triplets and quadruplets varies with time after ^{3}HTdR administration. Labelled triplets show an early rise within the first 12 h and then remain roughly constant for about the first 100 h and then rise to their highest levels at about 200 h after ^{3}HTdR administration. Labelled quadruplets, in contrast, rise rapidly over the first 20 h and reach peak levels of about 20% before falling steadily with time thereafter.

Figure 5 shows how the proportion of labelled clusters of more than 4 adjacent cells changes with time after ^{3}HTdR administration. These larger clusters included groups of up to 13 or 15 neighbouring labelled cells (03.00 h and 15.00 h respectively). The data for 03.00 h and 15.00 h appeared to differ somewhat with the 03.00 h sample reaching higher levels (~20%). The levels in both cases rose from essentially zero to reach a plateau level which persisted for about 200 h in contrast to the

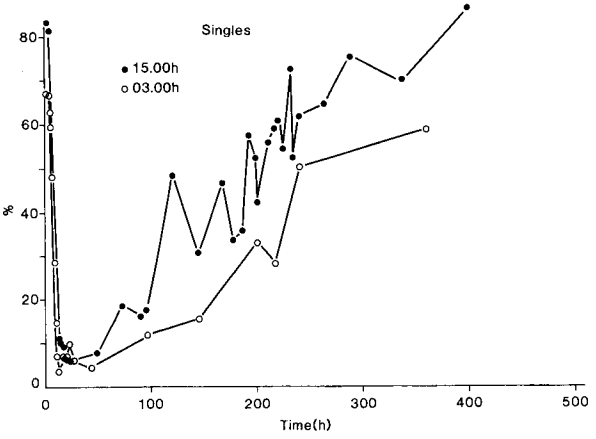

Fig. 2. The changes in the proportion (%) of all labelled cells that are observed as isolated single labelled cells with time after ^{3}HTdR labelling at either 03.00 h or 15.00 h

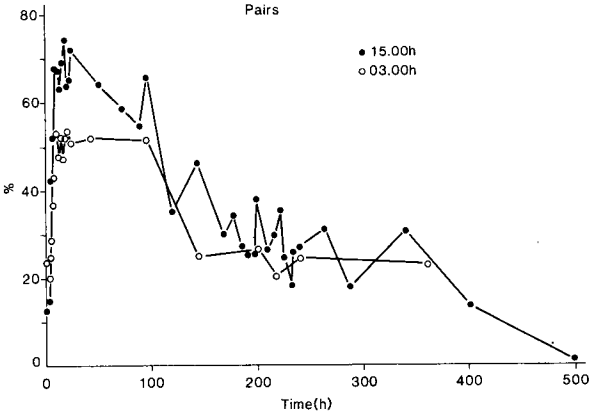

Fig. 3. The changes in the proportion (%) of all labelled cells that are observed as pairs with time after ^{3}HTdR labelling at either 03.00 h or 15.00 h with each pair being recorded as two labelled cells or one pair

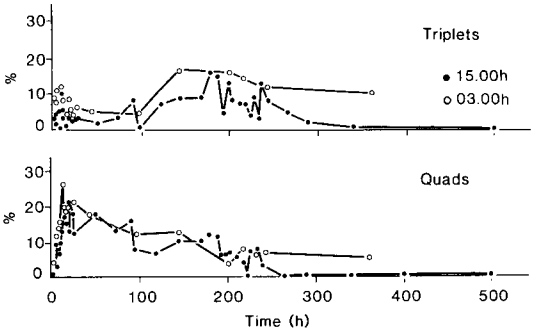

Fig. 4. The changes in the proportion (%) of all labelled cells that are observed as triplets (*upper panel*) and quadruplets (*lower panel*) with time after ^{3}HTdR labelling at either 03.00 h or 15.00 h

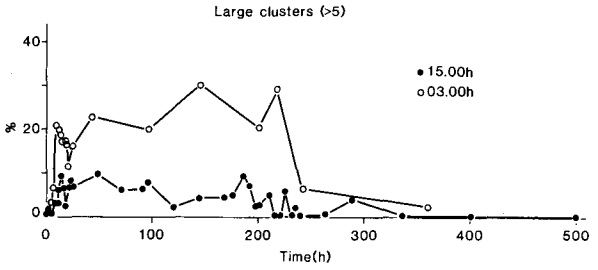

Fig. 5. The changes in the proportion (%) of all labelled cells that are observed as clusters of five or more labelled cells with time after ^{3}HTdR labelling at either 03.00 h or 15.00 h

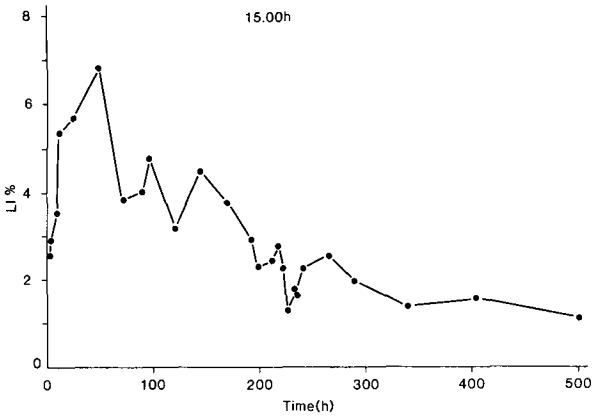

Fig. 6. The changes in the labelling index (LI, %) with time after ^{3}HTdR labelling at 15.00 h

data for quads which tended to decline steadily with time – once the initial peak had been reached. All clusters of a size greater than 2 essentially disappear at times beyond 200–300 h. Pairs persist for longer but disappear by 400–500 h. In contrast the numbers of singles rises steadily presumeably as the clusters become reduced to isolated labelled cells or become dispersed by unlabelled cells.

Figure 6 shows how the overall labelling index varied in the 15.00 h part of this experiment. Peak values in LI were obtained at the time of the trough in the proportion of isolated single labelled cells. A slight peak was observed at about 150 h i.e. about 100 h after the first peak and a third peak was observed at about 260 h i.e. about 100 h after the previous one and at a time slightly beyond the rise in the proportion of triplets. LI curves for 15.00 h and another curve for 03.00 h obtained from the same slides have been presented elsewhere (Potten et al. 1982, Potten and Bullock 1983).

Discussion

Although an analysis of clustering of labelled cells as a consequence of cell division activity could in principle be conducted using sectioned material it is really only on sheet preparations that the technique is fully effective. Initially labelled cells will tend to be scattered as isolated single cells. A small percentage of S phase cells would be expected to lie next to one another by chance and hence the frequency of labelled pairs at the beginning of the experiment will be determined by the initial LI.

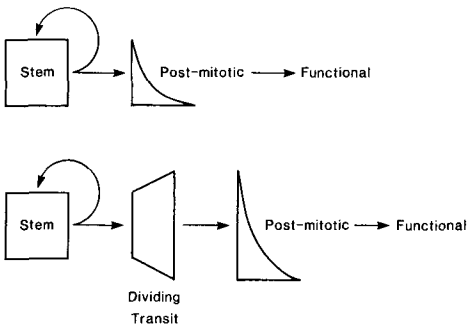

Fig. 7. Possible proliferative models for epidermis. Upper diagram: *Simple model* which assumes all proliferative cells in the basal layer are stem cells. The basal layer may also contain some post-mitotic cells. Lower diagram: *Hierarchical model* which assumes two types of proliferative cells in the basal layer – stem cells and dividing transit cells as well as post-mitotic cells

With time, the labelled cells will move through S and G_2 and will reach mitosis where each single should become a labelled pair. The length of time that the pair persists will depend on the amount of lateral movement in the basal layer, the length of the cell cycle (i.e. the time it would take for the pair to become a triplet or quadruplet) and finally on the time scale for the migration of one or both cells of a labelled pair into the next cell layer. A detailed analysis of the changes in singles and pairs over the first 24 h has been presented elsewhere (Loeffler et al. 1986). From such an analysis data can be obtained on the average length of the G_2 and S phases of the cell cycle. In principle an analysis of the changes over longer times may provide data on the cell cycle length. Such an analysis may also provide information on the proliferative organisation in epidermis and on the processes involved in cell movement and migration.

Two alternative models are possible for the proliferative organisation of murine epidermis (Fig. 7). A more extensive range of possibilities is presented in the accompanying paper. The simplest model assumes that all proliferative cells in the basal layer are identical and function as self-maintaining stem cells which produce differentiated cells that no longer divide (post-mitotic cells) and which migrate out of the basal layer according to

a rather poorly defined kinetic process. This model (simple model) is the one commonly assumed in the past but for which there is little, or no, direct evidence. An alternative model proposes that the stem cells produce a transit population within which some cell division may occur prior to entry into the post-mitotic compartment. The number of divisions in the transit population could in principle be anything, but in practice the most likely number is less than 4 (Potten and Hendry 1973; Potten 1974; Potten et al. 1982). Hence the basal layer can be considered to contain at least two types of proliferative cells as well as post mitotic cells i.e. an hierarchical model. The labelling index in the sheets of epidermis follows a pattern similar to that described previously (Potten and Bullock 1983). The slight peaks observed here (at about 50, 150 and 250 h) have been interpreted to be the consequence of successive rounds of cell division which are separated by times equivalent to the cell cycle time (about 100 h). One of the more striking features of these earlier LI data is that the LI declines progressively with time eventually reaching a plateau level of about 1% (Fig. 6 and Potten et al. 1982; Potten and Bullock 1983). This observation is virtually impossible to reconcile with the simple model in Fig. 7 which would predict a cyclic series of changes that oscillate from a constant level, from the initial LI i.e. about 2–4% for 15.00 h and 6–8% for 03.00 h (Potten et al. 1982).

The most likely overall average value for the cell cycle time in mouse epidermis is about 125 h with cycle times of about 100 h and 200 h for transit and stem cells if the more complex model is assumed: (Potten et al. 1982). The labelling seen at the end of the experiment (1%) represents a type of label retention (Bickenbach 1981) that is the consequence of a kinetic heterogeneity with some slow cycling cells. In fact the protocol here is similar to that used in some studies on label retaining cells (Morris et al. 1986).

The consequences of the two alternative models for the changing patterns of labelled cell clusters are as follows: For the simple model, the predictions are straight forward. The proportion of singles should fall as the pairs rise. However, of each new pair of labelled cells one daughter on average should disappear over a period equivalent to the cell cycle and so the singles and pairs should oscillate within the confines of a cell cycle time as shown in the top panels of Fig. 8. Clusters larger than pairs should not arise except from the occasional adjacent pairs of labelled cells. Any accummulation of large clusters could only occur by in-

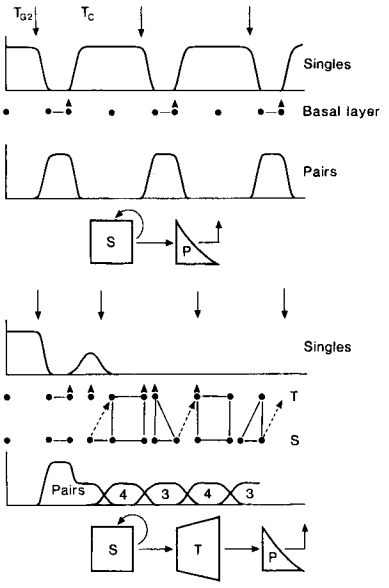

Fig. 8. Simple diagrammatic representation of how the clustering of labelled cells may change for the simple model (*upper diagrams*) and for the hierarchical model (*lower diagrams*). In the hierarchical model the overall proportions of clustered or single labelled cells will be determined by the sum of the patterns for each subpopulation and by the cell cycle times for the subpopulations. It has been suggested that the stem cells have a cycle time of about 200 h compared with a cycle time of about 100 h for cells in the later transit populations (Potten et al. 1982). Closed circles represent labelled cells and those closed circles joined by a line represent clusters. Arrowheads indicate loss of a labelled cell into the suprabasal layers. Arrows indicate cell divisions and dotted arrows differentiation from stem to transit cells. If more than one transit population is considered clusters of greater than 4 cells could develop

volving additional complex mechanisms such as a selective aggregation by migration of labelled cells – an unlikely process, or if the LI was very high. Triplets, quads and larger clusters clearly do occur and hence this argues against the simple model and for a more complex hierarchical model. Here, different patterns would be expected for the stem and dividing transit populations. For the last transit division the picture would be somewhat similar to that for the stem cells in the simple model (Fig. 8). The main difference would be that the singles might be expected to disappear quickly and then briefly reappear before disappearing permanently (Fig. 8). Although the data clearly show a dramatic and early fall in the proportion of singles (Fig. 2) there is also a steady slower but nonetheless dramatic rise in singles over the entire period of the experiment (400–500 h). The number of pairs would be expected to reflect the changes in the singles which on the whole was observed.

The length of time that the singles remain maximally depressed and the length of time that pairs

are at their maximum is a reflection of the kinetics of loss of new-born cells from the basal layer to the suprabasal layers i.e. the residence time for the post-mitotic cells in the basal layer. This would appear to be at least 2 days from the single and pair data for the 15.00 h injection. The data for pairs after 03.00 h suggest a much longer plateau of approximately one cell cycle (100 h). This could indicate the presence of different subpopulations in the more complex model which may be differently labelled at 15.00 h and 03.00 h.

When the hierarchical model is considered (Fig. 8) it can be seen that a labelled stem cell should generate labelled adjacent descendents i.e. larger clusters. In principle a labelled stem cell should generate a completely labelled EPU, while any EPU that had an unlabelled stem cell should eventually become completely unlabelled irrespective of whether or not it contained transit cells which were initially labelled. The size of the clusters would eventually be related to the number of transit cell generations, the size of the post-mitotic compartment and the total size of the EPU.

With one transit division only triplets and then quads would be expected. Although triplets, quads and large clusters are observed, which would be largely unexpected from the simple model, their changes with time do not follow the complete pattern expected from the more complex hierarchical model. The number of quads rises quickly and then declines gradually over a period of about 200 h (2 cell cycles) (Fig. 4). The number of triplets in contrast has perhaps an early peak and then rises steadily over the next 200 h. The proportion of larger clusters appears to rise and then remain roughly constant for about 200 h.

The data as a whole are obviously more consistent with the more complex two-tier, or hierarchical, model in Fig. 7. However, there are some inconsistencies (a) It is difficult to understand the steady increase in singles throughout most of the course of the experiment. (b) The increase in quads might be expected to follow the fall in pairs and be preceeded by a temporary rise in triplets which is not really what is observed. (c) A more progressive increase in the larger clusters would be expected.

The data suggest that mechanisms must be operating to break up clusters (or prevent the formation of large clusters) and to generate new singles. Four processes may be involved here: (1) some lateral cell movement may occur: – a jostling or repositioning of cells. This cannot be extensive otherwise no major clustering would be observed. (2) some labelled cells may move out of the basal layer into the suprabasal layer. This is clearly an important mechanism for reducing pairs to singles or reducing larger clusters by one labelled cell. (3) dilution of label due to successive cell divisions would reduce the label in some labelled cells to below the threshold for detection of label. Since the initial grain densities were high and longer exposures were used for some of the later times this process is not believed to result in the loss of many cells until times greater than two cell cycle times i.e. beyond 250 h. (4) other, largely unknown, mechanisms may exist whereby a labelled cell becomes unlabelled. One such mechanism which may apply to the stem cells is suggested by the hypothesis of non-random DNA segregation proposed by (Cairns 1975) for which there is some limited supportive evidence from other epithelial sites (Potten et al. 1978). This hypothesis would suggest that labelled stem cells would, on their second division, relinquish all remaining label to the daughter cell destined to become the dividing transit cell and so would generate an unlabelled stem cell where previously there had been a labelled one. This would tend to reduce the size of the larger clusters and would prevent any further increase in size that was dependent on labelled stem cell input. The presence of a somewhat higher proportion of stem cells would also mean that smaller clusters would be formed. This could be achieved by fewer transit divisions or by a form of asymmetric division in some transit cells (abbreviated lineages). Mathematical modelling of the data presented in this paper leads to the following conclusions; 1) lateral migration within the basal layer cannot be extensive; 2) the rise in singles with time in spite of the presence of large clusters can only be achieved by assuming some or all of the following: a) a low probability of labelling stem cells (ie a very short S phase); b) more than 10% of all cells are stem cells, 10% was assumed elsewhere (Potten et al. 1982; Potten and Hendry 1973; Potten 1974); c) an influence of grain dilution at later times; d) some selective segregation of DNA in some cells. None of these on their own are sufficient since on their own they would necessitate unrealistic levels of certain types of behaviour. For example, the data can be explained assuming only selective segregation but it would require this process in all of the early transit cell populations as well or the assumption of an unrealisticly high proportion of stem cells, both of which are unacceptable when other types of data are considered.

Reutilisation of tritium label soon after labelling has been suggested to explain some of the fine structure observed in labelling index and percent

labelled mitosis experiments (Wichmann and Fesser 1982). This however should not alter the clustering behaviour significantly. Reutilisation may also complicate the experiments, particularly at times beyond about 100 h from the initial labelling. However, this should tend to increase the level of pairs and other clusters as a result of subsequent cell divisions which was not observed in the data. There may also be some radiation cytoxicity but the complications introduced by this process are largely unknown. Our final conclusion is that the data can be best fitted by an hierarchical model with three transit generations but some abbreviation of the full lineage due to premature maturation (migration) of some cells. The best fit model also requires the assumption that the stem cells have either a very short S phase (as predicted elsewhere (Potten et al. 1982)) or they undergo selective DNA segration.

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