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Epidermal cell kinetics in pig skin

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Abstract

Investigations have been carried out on the cell population kinetics of the pig epidermis. Studies of the labelling index (LI) in the basal layer over a 24 hour period showed no significant diurnal variations. The mitotic index (MI), which was found to be low in the pig (0.51 ± 0.12‰), appeared to show small variations over a 24 h period, some of which did just reach accepted levels of significance. However, when compared with the observed animal to animal variation these slight differences in the diurnal MI were not considered to be of any major significance. Using a method of analysis based on the fraction of labelled cell clusters (FLC) after single pulse labelling with ^{3}H-TdR, values of the parameters T_S and $T_{G_2 + M}$ were calculated. The values obtained were comparable with those published using double labelling and fraction of labelled mitoses (FLM) methods.

Using the stathmokinetic technique the cell production rate (K), duration of mitosis (T_M) and the cell turnover time (T_T) were determined. For the basal layer of the epidermis these were 6.0 ± 0.2 cells/1000 cells/h, 40–54 min, and 167 ± 6 h, respectively.

Introduction

Rodent skin has been used extensively for cell kinetic studies on the epidermis. Recent investigations involving the mouse have suggested that the proliferative population of the epidermis is organised on an hierarchical basis, with a small sub-population of stem cells giving rise to a larger population of transit cells of limited proliferative potential (Potten *et al.* 1982).

Over the last decade pig skin has been used with increasing frequency in biomedical research with particular attention being given to radiobiological studies (Hopewell *et al.* 1978; Peel *et al.* 1984). It has been argued that this species provides a better model for human skin than that of other commonly used laboratory animals (Donovan, 1975; Hopewell & van den Aardweg, 1988). Despite this there have been surprisingly few reports on the cell kinetics of the epidermis in the pig. A basal cell density of 140–200 cells/mm has been reported (Osanov *et al.* 1976; Archambeau & Bennett, 1984, Morris & Hopewell, 1987) and a labelling index in the range 4–8% has been measured (Jurnovey *et al.* 1975; Archambeau & Bennett, 1984, Morris & Hopewell, 1985; 1987). Labelled cell nuclei have been observed throughout the viable epidermis of the pig, with the majority (~79%) being in the basal cell layer (L_1), 19.5% in the first suprabasal cell layer (L_2) and the remainder in the higher cell layers (Morris & Hopewell,

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1987). Labelled cells in L_2 have been shown to be actively proliferating, indicating that in the pig, as distinct from the mouse, the proliferative compartment of the epidermis is not restricted to the basal layer (Morris & Hopewell, 1987). These cell kinetic parameters derived for the pig have been shown to be comparable with analogous parameters for human skin (Morris & Hopewell, 1985; 1987). The transit time through the viable epidermis of the pig has been found to be 14 days and the turnover time of the whole epidermis, including the stratum corneum, 30 days (Weinstein, 1965).

The present paper is the third in a series of papers on the cell kinetics of normal pig epidermis. The objective of these papers has been to assess the suitability of this species as a model for human epidermis, particularly for radiobiological studies. In this paper diurnal variations in the mitotic and labelling indices of the epidermis of 16 week old pigs have been assessed. The fraction of labelled cell clusters (FLC) and the stathmokinetic technique have also been used to measure the parameters, T_S, T_{G2+M}, T_M and the cell production rate (K) for the pig epidermis so that they can be compared with the values of those parameters obtained using other techniques (Archambeau & Bennett, 1984; Morris & Hopewell, 1985; 1987).

Materials and methods A total of 12 Large White female pigs were used in these investigations. Animals were aged 16 weeks (25–30kg) at the commencement of all the experimental procedures. They were housed singly in indoor pens but were not subjected to a strictly controlled light/dark cycle.

The local *in-vivo* labelling of cells in DNA synthesis was accomplished by an intradermal injection of 0.1 ml of tritium labelled thymidine (^{3}H-TdR) into the skin of the upper rump and back. The tracer was diluted in normal saline containing disulphine blue (0.1ml/5ml saline) as a marker. Multiple injections of 370k Bq (10 μCi) ^{3}H-TdR were given to a group of four pigs, the injection sites being separated by ≧3cm. Biopsies of each injection site (two at each time point from each pig) were taken at 1, 2, 4, 6, 8, 10, 12, 15, 19, 22, 24, 27, and 30 h after injection.

An additional group of four pigs was used for the study related to the assessment of diurnal variations; biopsies, two per pig, were taken 30 min after each injection of ^{3}H-TdR at 10.00, 12.00, 15.00, 19.00, 24.00, 04.00, 07.00, and 10.00 h, throughout a 24 h cycle. Separate biopsies were taken at 10.00, 11.00, 13.00, 15.00, 19.00, 24.00, 04.00, 07.00 and 09.00, from the same group of pigs to examine changes in the mitotic index throughout a 24 h cycle.

Biopsy specimens were fixed in Bouin's fluid for 4 h and sectioned at 5 μm after embedding in Paraplast. There was a minimum gap of 35 μm between the sections scored to avoid duplication in subsequent cell counts. Slides, pre-stained with Mayer's haematoxylin, were dipped in Ilford K2 photographic emulsion (diluted 1:1 with distilled water) at 45°C. Autoradiographs were exposed for 3 weeks at 4°C, developed in Kodak D19 developer and fixed in 10% sodium thiosulphate. Either Euparal or Canada Balsam was used as the mounting media.

In autoradiographs a minimum of 300 labelled cells were counted for each biopsy. In routine histological sections between 75 and 150 mitoses (i.e. prophase, metaphase, anaphase or telophase) were scored per time point, per pig, to enable the assessment of diurnal changes in the mitotic index.

In the pigs receiving a single pulse label of ^{3}H-TdR the linear clustering of labelled cells in the basal and first suprabasal cell layers of the epidermis was also assessed for up to 30 h after labelling. Isolated labelled cells were termed 'singles', two adjacent labelled cells as a 'pair', three adjacent labelled cells as a 'triplet' and four or more adjacent labelled cells as 'multiples'. Triplets and multiples were seen infrequently and as a consequence were not considered in the subsequent evaluation. Changes in the proportions of single and paired labelled cells were assessed. Data were expressed as the fraction of all labelled cells (FLC) present as clusters. The values of the parameters T_S (duration of DNA synthesis) and T_{G_2+M} (durations of G_2 phase and mitotic phase) were calculated from this data using the method of Loeffler *et al.* (1986).

In the stathmokinetic study intravenous injections of 1.0 mg/kg (body weight) of vincristine sulphate (Oncovin, Lilly) were given, via an ear vein, to two pigs at 10.00 h. The vincristine was diluted in normal saline. Four biopsies were taken from the flank skin of each pig immediately before and at intervals of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 h after vincristine injection. Biopsy specimens were processed for histology in a manner identical to that given for autoradiography. Sections were stained with haematoxylin and eosin and mounted in Canada Balsam. A total of 100–150 metaphases were scored per time point per pig. In addition the number of prophases in a given section were counted so that changes in the mitotic index with time after vincristine administration could be determined.

Cell kinetic parameters were calculated according to the following standard methods:

1) Labelling Index (LI) = N_L/N_T, where N_L = number of labelled cells; N_T = total number of cells counted in the basal layer (L_1) or the first suprabasal layer (L_2).

2) Mitotic Index (MI) = N_M/N_T, where N_M = number of mitoses.

The line describing the accumulation of metaphases or mitoses with time after vincristine injection was fitted using linear regression analysis. The error on the fitted line, the slope, the standard error of the slope and the correlation coefficient were calculated using standard equations. Abercrombie's correction factor (f) as modified by Simnet (1968) was used to correct for differences in the diameters of mitotic and intermitotic nuclei. The factor 'f' was applied to metaphase counts, where $f = (D + T)/(D_{meta} + T)$; T = section thickness; D = interphase nuclear diameter; D_{meta} = metaphase nuclear diameter. D was measured as 6.0 ± 0.2 μm (L_1) and 6.2 ± 0.1 μm (L_2). D_{meta} values were 9.4 ± 0.2 μm (L_1) and 9.2 ± 0.6 μm (L_2).

The cell production rate (K) was assessed directly from the slope of the straight line fitted to the corrected metaphase accumulation data. Then:-

3) Cell Turnover Time (T_T) = $^1/_K$.

4) Duration of Mitosis (T_M) = MI_O/K, where MI_O = native mitotic index.

5) The duration of DNA synthesis (T_S) and T_{G_2+M}. These parameters were estimated using the fraction of labelled clusters (FLC) technique (Loeffler *et al.* 1986).

Results

Diurnal variation in the labelling and mitotic indices

There were minor diurnal variations in the values of the LI in layer L_1 over a 24 h period but none of the apparent differences were significant ($p>0.1$). LI values in layer L_2 were also found to be similar over a 24 h period ($p>0.1$) with the

exception of the LI at 24.00 h. This was significantly lower ($p<0.01$) than values at 12.00, 15.00 and 19.00 h (Fig. 1).

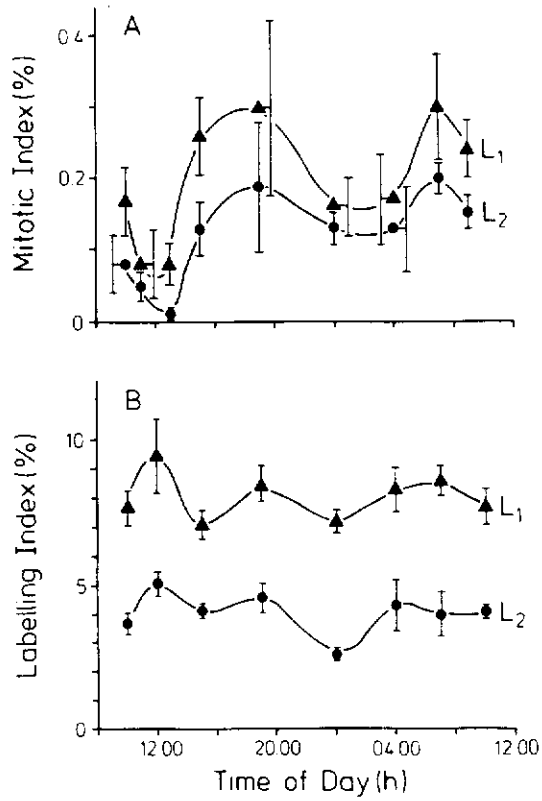

Fig. 1 Variation in the mitotic (A) and labelling (B) indices in the basal (L_1) and first suprabasal (L_2) layers of the epidermis over a 24 h period. (▲ basal layer; ● first suprabasal layer) Error bars ± SE.

In the four pigs used to examine changes in the MI of cells in layers L_1 and L_2 the index was very low, and never exceeded 0.3%. Although there was some suggestion of variations over a 24 h period, MI values were only significantly lower at 11.00 h and 13.00 h as compared with values at other time points and even then they only just reached accepted levels of significance ($p<0.05$).

The 24 h mean values of the LI calculated from the values given in Figure 1 were 8.1 ± 0.3% (L_1) and 4.1 ± 0.3% (L_2). Values of the 24 h mean MI were 0.20 ± 0.03% and 0.12 ± 0.03% for cell layers L_1 and L_2, respectively.

Singles and pairs of labelled cell nuclei in the epidermis

Labelled pairs of nuclei in cell layer L_1 were orientated either horizontally (both cells in L_1) or vertically (one cell in L_1 and the other immediately above it in L_2). The numbers of horizontally and vertically orientated labelled pairs in L_1 were similar ($p>0.1$) over a period of 30 h (Fig. 2). In cell layer L_2 the number of horizontally orientated pairs was greater than the number of vertically orientated pairs (one cell in L_2 and the other immediately above it in L_3) which remained relatively constant throughout a 30 h period (Fig. 3).

For the purposes of the subsequent analysis the numbers of horizontally and vertically orientated labelled pairs were combined (Figs. 2 and 3). In layer L_1 there

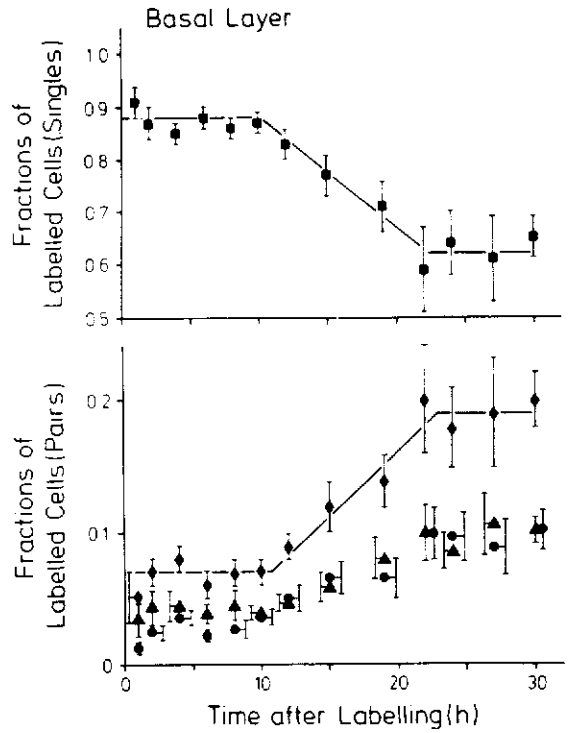

Fig. 2 Variation in the number of single (■) and pairs (♦) of labelled cells in the basal layer of the epidermis with time after single pulse labelling with ^{3}H-thymidine. Labelled nuclei 'pairs' were either vertically (●) or horizontally (▲) orientated. Error bars ± SE.

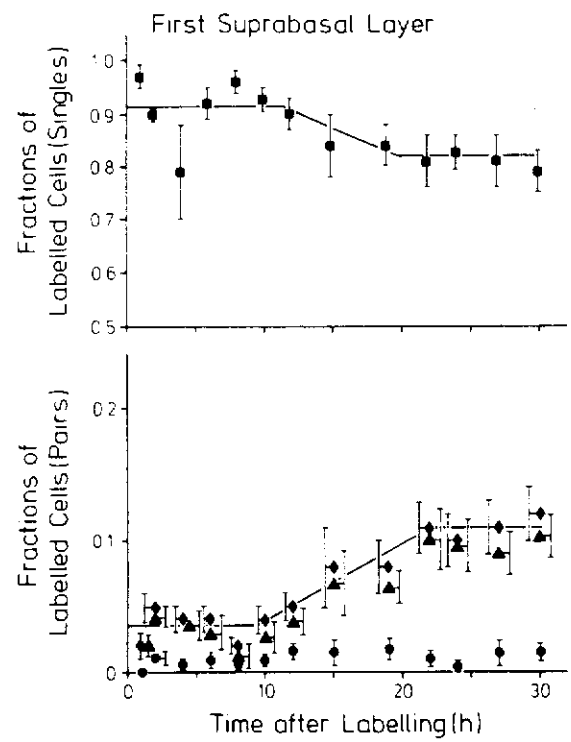

Fig. 3 Variation in the number of singles and pairs of labelled cells in the first suprabasal layer of the epidermis with time after a single pulsed labelling with ^{3}H-thymidine. For key to symbols refer to Fig. 2. Error bars ± SE.

was a progressive increase in the proportion of labelled pairs from 10 h to 22 h after labelling, whereafter values plateaued. In layer L_2 there was a less pronounced increase in the proportion of labelled pairs with time, and as a consequence the values for the parameters obtained were less accurate. Values of T_s and T_{G_2+M} calculated from this data are given in Table 1. The results obtained using single and pairs of labelled nuclei were similar.

Cell Layer	Singles or Pairs	T_S (h)	T_{G_2+M} (h)	$T_S + T_{G_2+M}$ (h)
L_1	Singles (best fit)	12 (0.1)	10 (0.1)	22
L_1	Pairs (best fit)	12 (0.1)	10 (0.1)	22
L_2	Singles (best fit)	9–13*	—	21–24
L_2	Pairs (best fit)	8–10	—	18–23

*A range of best fit values is given.
Variances could not be reliably estimated in cell layer L_2.

Table 1 Durations of DNA synthesis (T_S) and the G_2 plus M phases (T_{G_2+M}) of the cell cycle estimated from FLC data (variance of the phase duration is given in parenthesis).

Estimation of cell kinetic parameters using the stathmokinetic technique

A good correlation ($r \geqq 0.96$) was found between the accumulation of metaphases and the time after vincristine injection in cell layers L_1 and L_2 (Fig. 4). The correlation between the mitotic index (prophases plus metaphases) and time after vincristine administration was slightly less well defined, with correlation coefficients of 0.92 and 0.78 for cell layer L_1 and L_2, respectively (Fig. 5).

The cell production rate (K) calculated from the slope of the metaphase accumulation line (Fig. 4) was 6.0 ± 0.2 cells/1000 cells/h for L_1 and 4.2 ± 0.5 cells/1000 cells/h for L_2. The cell turnover times (T_T) derived from the reciprocal of K (i.e. $T_T = {}^1/_K$) were 167 ± 6 h (L_1) and 222 ± 23 h (L_2).

The native mitotic indices (MI_O) calculated from counts on biopsies taken between 10.00 and 12.00 h from all 12 pigs used in this study were 0.51 ± 0.12% (L_1) and 0.24 ± 0.04% (L_2). The values for the duration of mitosis (T_M) estimated from MI_O and K, were 40 ± 9 min (L_1) and 36 ± 6 min (L_2). Similar median values of T_M of 47 min (L_1) and 44 min (L_2) were obtained from the point of intersection of the mitosis accumulation line with the x-axis (Fig. 5). The value of T_M for L_2 included the addition of a delay period of about 30 min, which was evident from the shape of the metaphase accumulation curve for L_2 (Fig. 4). The range in the T_M values obtained from the mitosis accumulation line was estimated from the standard error of that line (Fig. 5). T_M varied from 41 min to 54 min (L_1) and 35 min to 56 min (L_2).

There was no histological evidence for metaphase degeneration during the course of the stathmokinetic study. However, it is apparent from Figure 4 that by 4 h after the injection of vincristine there was a falling off in the number of

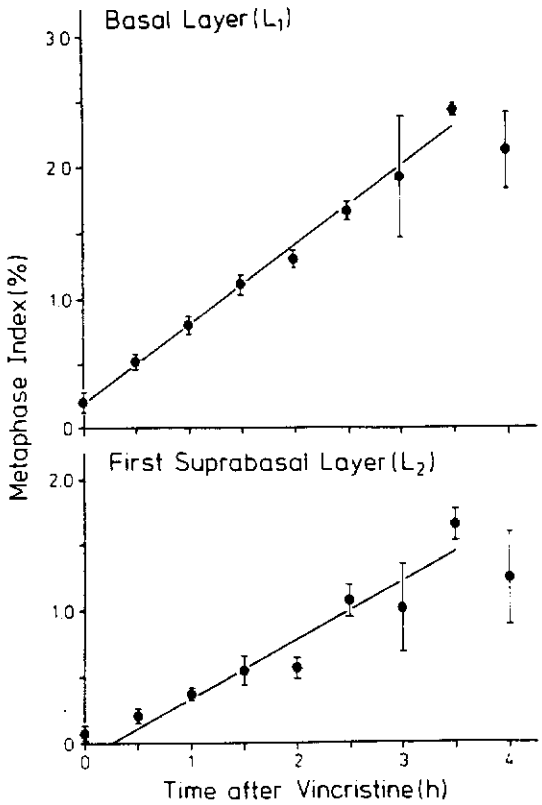

Fig. 4 Accumulation of metaphases in the cells of the basal (L_1) and first suprabasal (L_2) layers of the epidermis after the injection of vincristine. Error bars ± SE.

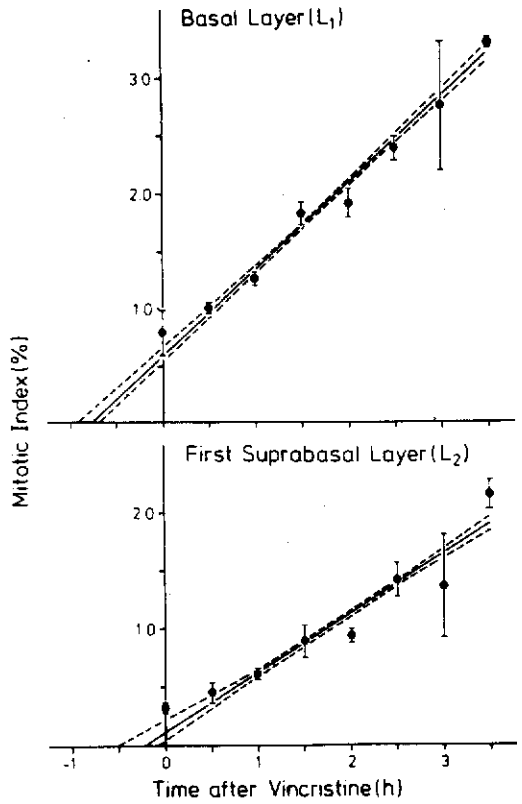

Fig. 5 Accumulation of mitotic figures in the cells of the basal (L_1) and first suprabasal (L_2) layers of the epidermis after the injection of vincristine. Error bars ± SE. Broken line indicates ± SE of the fitted linear regression line.

accumulated metaphases. This suggested that degeneration of metaphases had started by 4 h, that the effects of vincristine were reduced at this time, or that both these effects were operative.

Discussion

The epidermis of the pig is between 30 μm and 100 μm thick, which is similar to human epidermis, but considerably thicker than rodent epidermis from comparable sites (Potten et al., 1983; Morris, 1987). The viable epidermis of the pig is considerably undulated and in many areas rete ridges or papillae are seen. The stratum corneum is of varying thickness (10–15 layers) and contains elongated keratin squames (dead keratocytes). The granular layer is generally one cell thick and cells in this layer are flattened and have degenerating nuclei. The spinous layer is, on average, four cells thick in interpapillary regions. Cells in this region are large and have an ovoid profile. In the basal layer the cells are smaller than in the spinous layer and have a cuboidal shape (Morris, 1987).

In the thin epidermis of rodents, such as the mouse, observations indicate that proliferating cells are located almost exclusively in the basal layer (Smart, 1970; Potten *et al.* 1983). However, in the thicker epidermis of the pig it has been shown that approximately 20% of labelled cells are situated suprabasally and that these cells are actively proliferating (Morris & Hopewell, 1987). A number of reports have claimed that labelled cells are confined to the basal layer in human and pig epidermis (Brown & Oliver, 1968; Archambeau & Bennett, 1984) and although others have documented the presence of labelled suprabasal cells (Epstein & Maibach, 1965; Lachapelle, 1969) such cells have rarely been quantified. After a careful analysis of serial sections, Penneys *et al*, (1970) calculated that 32% of ^{3}H-TdR labelled cells were suprabasal in human epidermis. In guinea pig epidermis about 33% of labelled cells occurred suprabasally (Yamaguchi & Tabachnick, 1972). The precise kinetic relationship between the proliferating cells in the basal and suprabasal layers of the epidermis has not yet been established, and for this reason cell kinetic parameters for the basal (L_1) and first suprabasal layer (L_2) were evaluated separately in the present study.

In the basal layer of pig epidermis labelling studies over a 24 h period provided no evidence for any statistically significant diurnal variation in the LI. For the MI small variations were seen albeit in a group of pigs in which the mean MI was very low, i.e. 0.20 ± 0.03%. Closer examination of the MI data showed that interanimal variation was of greater significance than any possible diurnal changes. For example, the MI in cell layer L_1 calculated from pigs used in the evaluation of the fraction of labelled cell clusters (FLC) was 0.47 ± 0.03%, which compares with a MI of 0.13 ± 0.04% measured in the pigs used in the diurnal rhythm study or a value of 0.80 ± 0.16 from the pigs used in the stathmokinetic investigations. In all cases biopsies were taken between 10.00 h and 12.00 h during the winter months. This would seem to rule out the possibility that the interanimal difference in MI could be explained by seasonal variations which has already been proposed for human skin (Camplejohn *et al*, 1984).

A direct comparison of these results in the pig with other published data on diurnal variation in cell kinetic parameters is complicated by the lack of uniformity in the environmental conditions under which experimental animals were kept, and by strain and species differences. Rodent epidermis, and in particular mouse epidermis, has been demonstrated to have a pronounced diurnal variation in the mitotic and labelling indices. In many reports (Tvermyr, 1972;. Hegazy & Fowler,

1973; Clausen *et al.* 1979) the major peak in the LI was at 18.00–22.00 h, whereas the peak in the MI was at approximately 10.00 h (Pilgrim *et al.* 1963; Clausen *et al.* 1979). The situation with respect to diurnal variations in human epidermis is less clear, there being numerous conflicting reports in the literature. For example, Fisher (1968), Kahn *et al.* (1968) and Camplejohn *et al.* (1984) all reported significant variation in the MI over a 24 h period. However, a number of workers failed to detect any significant diurnal variation in mitotic activity (Milstein & Cornell 1973; Schell *et al.* 1980). Where studies have been carried out on a large number of human subjects, no significant diurnal variation in the LI has been detected (Pullmann *et al.* 1974; Gelfant *et al.* 1982; Camplejohn *et al.* 1984).

In the epidermis of the Yorkshire pig (Archambeau & Bennett, 1984) a broad peak in the LI was demonstrated between 14.00 h and 18.00 h and a nadir from 9.00 h to 11.00 h. However, the accuracy of this finding is questionable given that only one animal was used per data point, each point representing a different animal. Both male and female pigs were used without any indication of whether sex differences were important or not. These authors also demonstrated variations in the MI over a 24 h period, but no discernible diurnal rhythm was found.

A newly derived method of analysis of the kinetics of epithelial cells has been proposed by Loeffler *et al.* (1986). This involved an evaluation of the clustering behaviour of labelled cells in the epidermis after single pulse labelling with ^{3}H-TdR. Using this method T_S and T_{G_2+M} values can be estimated from the time-related changes in the number of single labelled cells or in the number of pairs of labelled cells. There was good agreement between the values of T_S and T_{G2+M} obtained using this technique and those previously obtained using the fraction of labelled mitoses (FLM) method (Table 2). This new analysis was carried out using the same autoradiographs that were used in the FLM study of Morris and Hopewell (1987), to enable a direct comparison of the results obtained using the two methods. The number of labelled cell pairs was also quantified by Archambeau and Bennett (1984) in pig epidermis. The number of pairs had peaked by 24 h after labelling indicating a value for $T_S + T_{G2+M}$ similar to that obtained in the present investigation.

In the present stathmokinetic studies vincristine was injected intravenously in the pig since preliminary studies (Morris, 1987) had indicated that intradermal injections of the drugs failed to yield any significant accumulation of arrested metaphases. A similar phenomenon has been reported in normal human epidermis after the intradermal injection of a range of doses of vincristine or colcemid (Camplejohn *et al.* 1981). An intravenous dose of vincristine was highly effective as a stathmokinetic agent in the pig. The cell production rates (K) calculated from the metaphase accumulation lines were 6.0 ± 0.2 cells/1000 cells/h (L_1) and 4.5 ± 0.5 cells/1000 cells/h (L_2). These values are comparable with K values of 7.4 ± 0.7 cells/1000 cells/h (L_1) and 4.2 ± 1.2 cells/1000 cells/h (L_2) obtained after double pulse labelling studies in the epidermis of the pig (Morris & Hopewell, 1987). The calculated T_T values were also similar (Table 2).

Although the stathmokinetic method has been used extensively, data obtained using this technique are limited for the normal epidermis. After the intravenous injection of colchicine, Archambeau and Bennett (1984) obtained an estimate of the epidermal cell production rate of 4–5 cells/1000 cells/h for the Yorkshire pig. This would appear to be an underestimate because biopsies were not taken until 6 h after the injection, at which time numerous metaphases had degenerated.

KINETIC PARAMETERS	INVESTIGATOR			
	Present Study	Morris and Hopewell (1987)	Archambeau and Bennett (1984)	Osanov *et al.* (1976) A Jurnovoy *et al.* (1975) B
BCD (cells/mm)	—	142.0–144.8	198.3–207.9	151.7 A
MI (%)	0.20–0.51	—	1.70–3.20	—
LI (%)	—	7.6–8.2	3.8–5.0	4.7 B
T_M (min)	Stathmokinesis 40–47	—	—	—
T_S (h)	—	Double labelling 8.7–10.2	Graint count halving 7.9–12.3	—
T_S (h)	FLC 11.9–12.1	FLM 9.8	FLM 10.2–15.2	—
$T_{G_2+½M}$	—	FLM 7.2	FLM 4.2–5.2	—
T_{G_2+M}	FLC 9.9–10.1	—	—	—
K (cells/1000 cells/h)	Stathmokinesis 5.8–6.2	Double labelling 6.7–8.1	—	—
T_T (h)	Stathmokinesis 161–173	T_S/LI 118–140	Continuous labelling 136	—
T_T (h)	—	—	T_S/LI 263–389	—

Table 2 Comparison of cell kinetic parameters for the basal layer of pig epidermis (BCD = basal cell density; MI = mitotic index; LI = labelling index; T_M = duration of mitosis; T_S = duration of DNA synthesis; T_{G2} = duration of G_2 phase; K = cell production rate; T_T = turnover time).

Using a topical application of colchicine, Fisher (1968) estimated a cell production rate of 1.3 cells/1000 cells/h for normal human epidermis. This is considerably below the value of 5.6 cells/1000 cells/h estimated from MI and LI data (Potten, 1975), suggesting that colchicine, applied topically, was ineffective as a stathmokinetic agent. Indeed, as mentioned above, Camplejohn *et al* (1981) failed to observe any significant accumulation of metaphases in human epidermis after the intradermal injection of colcemid. In the dorsal skin of the mouse stathmokinetic studies have provided values of K in the range 5.0–6.0 cells/1000 cells/h (e.g. Iversen & Evensen, 1962; Evensen & Heldaas, 1964).

Surprisingly few estimates of the value of T_M have been published based on this method. A value of 1.5 h for T_M was calculated for human epidermis (Fisher, 1968) but the accuracy of this estimate is questionable, because the stathmokinetic agent (colchicine) was applied topically. In hairless mice a value for T_M of about 1.0 h was obtained (Evensen & Haldaas, 1964). This value is similar to those estimated for the epidermis of the pig.

In conclusion, there would appear to be no appreciable diurnal variation in the LI and the MI for pig epidermis. Values for the MI were relatively low and considerable interanimal variation was found. The calculated values of kinetic parameters in pig epidermis, obtained using the fraction of labelled cell clusters (FLC) and the stathmokinetic technique, were comparable with values obtained using the double pulse labelling and fraction of labelled mitoses (FLM) methods (Morris, 1987; Morris & Hopewell, 1987). There was good agreement between the

values of the parameters T_S, T_{G_2} and T_M for cell layers L_1 and L_2 in pig epidermis, but values for the parameters K and T_T differed between the two cell layers. The differences in K and T_T between cell layers L_1 and L_2 probably reflect the fact that there is a higher proportion of non-proliferative, differentiated cells in L_2, and consequently a lower growth fraction.

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