THE IN VITRO CELL CYCLE OF ADULT MAMMALIAN EPIDERMIS: A PRELIMINARY STUDY*

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ABSTRACT

Cultured epidermal cells of albino guinea pigs were studied autoradiographically to determine the lengths of the $G_1$ (gap preceding DNA synthesis), S (DNA synthesis), $G_2$ (gap following DNA synthesis and immediately preceding cell division), and M (mitosis) periods of the cell cycle.

The epidermal cells were grown in vitro using a chamber culture technique. Skin slices containing dermis and epidermis were surgically removed from the ear and then incubated for ten minutes in a trypsin solution to remove the underlying dermis. The cells were teased apart and cultured in Fischer’s Medium supplemented with 15% fetal calf serum and a penicillin-streptomycin mixture. $^3$H-thymidine was added at $t = 0$ to make a final concentration of 0.21 μC/ml (s.a. 3.0 c/m mole). Progress in the cycle was monitored hourly by stopping growth in a portion of the chambers. Coverglasses with cells attached were then prepared for autoradiography. Kodak AR-10 Stripping Film was used. Development was performed seven days after exposure began.

One thousand cells on each slide were scored to determine the percentage of cells labeled, labeled cells in mitosis, and the percentage of labeled and unlabeled cells in metaphase. From this data, the total length of the cell cycle was found to be approximately 15 hours; $G_2$ was minimally 3 hours; S lasted approximately 5.4 hours; the duration of the M phase was .4 hours; and $G_1$ was 6.2 hours long. A marked increase in DNA synthetic activity was noted at 7 to 8 hours. Partial synchrony of the cell population was thought to be achieved upon removal of the ear tissue from the guinea pig. This apparent synchrony supports the proposal that cells in $G_1$ prior to injury are directed to synthesis when removal occurs and suggests that the real control of the cell cycle may lie in the $G_1$ period.

Since the original work of Howard and Pelc in 1953 (1), an enormous amount of research has been done on the “cell mitotic cycle”, or the interval between two successive cell divisions. It is now generally accepted that cells which are multiplying by mitotic division do indeed pass through a series of physiological and biochemical events that comprise this cycle (2). The cycle, recently reviewed extensively by Baserga (3, 4) is characterized by four distinct stages: 1) $G_1$, the period after the completion of mitosis and before the onset of DNA synthesis; 2) S, a stage during which DNA is actively synthesized, and which is necessary for, and generally indicative of impending mitosis; 3) $G_2$, the period between completion of DNA synthesis and mitosis; 4) M, the period in which mitosis occurs. Baserga has further hypothesized that there is a $G_0$ stage in which the cells are quiescent but able to be stimulated to move into the synthesis phase if necessary. The entire hypothesized cell cycle is pictured in Figure 1. It should be noted, however, that the $G_0$ or quiescent stage is disputed by many investigators (2, 5) who credit the $G_1$ phase with all regulatory mechanisms. In this preliminary investigation we wish to describe how the technique of autoradiography was employed to determine the length of the in vitro cell cycle in guinea pig epidermis as well as to establish the duration of each of the four distinct phases.

MATERIALS AND METHODS

Preparation of cell cultures. Epidermal cells from the ears of young albino guinea pigs weighing from 250–350 gms were used for this investigation. After thorough cleansing, skin slices containing dermis and epidermis were surgically removed from the ear. Immediately after removal, the skin slices were incubated for 10 minutes at $37^\circ$ C in 2 ml of 15% Bacto-trypsin, previously constituted by dissolving the contents of one vial in Fischer’s Medium for Leukemic Cells of Mice. After incubation, the fragments of skin were easily separated into sheets of epidermis and dermis by teasing apart with needles. The epidermal sheets were washed twice in 2 ml aliquots of Fischer’s medium to remove excess trypsin. The culture medium was prepared by combining 100 ml of Fischer’s Medium for Leukemic Cells...
of Mice with 15 ml of fetal calf serum, and 1 ml of a penicillin-streptomycin mixture (10,000 units penicillin per ml plus 10,000 units streptomycin per ml). The sheets of epidermal cells were then teased apart with needles and agitated to prepare single cells and small clumps of cells. The separated cells were diluted in enough medium to prepare 12 to 15 glass culture chambers, each with a capacity of 2.5 ml. By preparing this number of chambers from the tissue obtained from both ears of the pig, it was found that a relatively constant final concentration of 100,000 cells per ml was obtained. Before the chambers were sealed, a specific labeled precursor of DNA, 3H-thymidine (3HTDN), was added to make a final concentration of 0.21 μC/ml (s.a. 3.0c/m mole). After filling, the coverslip culture chambers were sealed with paraffin and incubated continuously with the isolate at 37° C for periods of time ranging from 2 to 24 hours.

Preparation of slides for autoradiography. During each hour of the experiment, a portion of the chambers containing cells and isotopes were removed from the incubator and the medium containing all unattached cells was removed. Coverslips with cells attached were removed from each chamber and put into successive rinses of ethanol (50%, 60%, 70%, 80%, 90%, 100%) to facilitate drying of the cells without rupturing cell membranes. Each coverslip was then mounted on a slide, attached cell side up, and allowed to dry in a dust-free atmosphere.

Autoradiographic techniques. The actual autoradiographic procedure (stripping film technique) was patterned after methods proposed by Joffes (6), Kopriva and Leblond (7), and Pelc (8). The concentration of 3HTDN that was used was found to provide adequate labeling of the genetic material in those cells actively producing DNA. Kodak AR-10 Stripping Film was used for most of the slides. It was found that, although the technique used is slightly more time consuming than the popular emulsion technique, the grains produced using stripping film are larger and therefore easier to score. A small portion of the slides was prepared using Ilford L-4 Emulsion. The resultant slides using this technique had a higher background count (roughly 3 grains per cell) than the 1 grain per cell background achieved with the stripping film, even though both techniques were carried out in total darkness, therefore, where possible, slides prepared with emulsion were disregarded. However, when used, the data from emulsion slides was corrected for the difference in the sensitivity of the stripping film and the emulsion (9). Each prepared slide was stored in a light tight box at 4° C and the time of exposure was 7 days.

Staining techniques. After developing the radioautographic slides, they were stained with Azure B (Bromide). Azure B is a metachromatic, basic, thiazine dye that is a derivative of Azure A and is prepared by chloroform extraction (10). The dye used in this experiment was obtained in a powdered form from Allied Chemical Co., and had a total dye content of 77%. A 1.33% solution was prepared and stored at 4° C between uses. Stain was applied at room temperature for 20 minutes, and was followed immediately by a 10 minute gentle tap rinse. Excellent metachromatic resolution was usually obtained. As outlined by Flax and Himes (10), the concentration of the dye, pH of the solution, temperature of the solution, and length of the staining period had a marked effect on the apparent color of the various cellular organelles and the general effectiveness of the stain.

Counting methods. On each slide, 1000 cells were scored to determine the percentage of labeled cells in each hour of the cycle. For each hour at least 3 separate slides representing separate experiments were scored to insure validity and reproducibility of results. Additionally, the number of mitotic figures, both labeled and unlabeled, and the number of labeled and unlabeled metaphases were scored to determine the respective percentages at each hour. These data are graphically presented in Figures 2-5. Each point represents an average of 3 or more separate determinations. Any cell showing a group of clearly distinguishable chromosomes with attendant absence of the nuclear membrane was considered to be a cell in mitosis. When the chromosomes were occupying a more or less central position in the cell and were in a regular order (as if organized around a metaphase plate), the cell was additionally said to be in metaphase. Grains over metaphase figures were scanned but not counted. Finally, any cell with 5 or more silver grains over the nuclear region was considered to be labeled.

RESULTS

Analysis of the results was done using methods proposed by Stammers and Till (11). Their mathematical formulation of the problem of cell cycle length determination in L-strain mouse cells was easily applied to this experiment.

Fig. 2 Demonstrates the total number of cells per 1000 scored that were labeled with 3H-thymidine. Each point represents at least 3 separate determinations.
Partial synchrony of the cell culture population was thought to have taken place, although no chemical agents were applied to achieve this result. Apparently, the technique used to remove the cells from the guinea pig and/or culturing them in vitro caused sufficient shock to the cell population to impose this partial synchrony. It is also possible that the deviation from the asynchronous expectations (a fixed percentage of cells passing through each point of the cycle at any given time, logarithmic growth, and a relatively constant mitotic index) as can be seen in Figure 2, may represent accumulation of labeled cells. Additionally, in Figures 3 and 4 it is possible that the synchrony is shown to have appeared when the M phase of the cell cycle was examined, since the mitotic index has rather large variation in the twelfth hour.

However, even though some synchrony may have taken place, the results (especially those used in the determination of the lengths of the S and G₂ phases of the cell cycle) will be treated as if they were from a totally asynchronous experiment. The justification for this assumption lies in the fact that the mitotic index was relatively constant during the first 8 hours of the experiment (Figure 3). The mitotic index is a sensitive indicator of the degree of synchrony present in any given cell culture (11). In the calculation of the length of the S phase, only data for the hours less than 8 were used. Deviation from asynchrony after 8 hours does not affect the value of S found using the results from the initial asynchronous portion.

**Determination of the generation time (total length of the mitotic cycle).** The total length of the mitotic cycle was found by analyzing the percentages of labeled cells found at each hour. There was a marked increase in the number of labeled cells during the eight and twenty-third hours (Figure 2). Comparative values for hours 8 and 23 for three separate determinations are as follows: 8 hours—101, 166, and 120; 23 hours—191, 192, and 120. These hours were accepted as representing the end of the S periods for two consecutive generations of the synchronized cells. Therefore, the length of time required to move through the entire mitotic cycle was found to be 15 hours. The data for hour 16 as graphically presented, are also significant. However, other experimental data from our laboratory using pulse labeling and liquid scintillation techniques led us to disregard hour 16 and consider only hour 23 in our final calculations (12).

**Determination of the length of the G₂ period.** The time required for labeled DNA to show up in metaphase chromosomes gave a good measure of the length of the G₂ period (4). If label was picked up by the cells synthesizing DNA at the time of removal from the ear and addition of H-thymidine (t = 0), then the post-synthetic gap would be the period from t = 0 to the first appearance of labeled metaphase figures. This period can be graphically determined as seen in Figure 5. In this figure, the percent of all metaphase figures that were labeled is plotted against time. Note that no labeled metaphases were found before the fourth hour. Therefore, the length of the G₂ period was taken to be 3 to 4 hours. This must, however, be viewed as a minimum length, since if all cells had the same G₂ length the percentage of labeled metaphases should have risen to 100% at 3 to 4 hours rather than rising erratically as it does in Figure 5 (5).

**Determination of the length of the M period.** The mitotic phase of the cycle is very much shorter than any other period. The length of time

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**Fig. 3** Demonstrates the variation in the mitotic index with time.

**Fig. 4** Indicates the percentage of cells in metaphase. This is a close approximation of the mitotic index.

**Fig. 5** Demonstrates the percent of all metaphase figures that were labeled with ³H-thymidine.
any particular cell spends in mitosis can be calculated using the following equation (11).

\[ T_m = \frac{MT}{\ln 2} \]

Where \( T_m \) is the time in mitosis, \( M \times 100 \) is the mitotic index, and \( T \) is the generation time or total length of the cell cycle. Using the mean value of the mitotic index, 0.018, the equation yields a value of 0.39 hours for \( T_m \).

**Determination of the length of the S period.** Stanners and Till (11) again provided the method used to analyze the results and calculate the experimental value for the length of the S period in the in vitro cell cycle of the epidermis of the guinea pig. The experimental percentage of labeled cells had to be corrected before being used to calculate the desired value. First, an adjustment had to be made for the ever increasing number of cells in the culture chamber (see Table). The following equation was used for that purpose.

\[ L(t) = \frac{1}{N_o} \left[ L(t)_\text{exp} - \int_0^T P(t - t_m)\,dN(t)\,dt \right] \]

\( L(t) \) represents the percentage of labeled cells divided by 100, \( N_o \) is the initial concentration of cells in the culture chamber, \( N(t) \) is the cell concentration as a function of time, \( a \) is equal to \( \ln 2/T \) where \( T \) is the generation time of the cells, and \( P(t - t_m) \) is the fraction of labeled cells entering division. The first term in the brackets represents the total number of labeled cells seen, and is therefore a correction for division of unlabeled cells. The second term represents the number of labeled cells due to division of already labeled cells. In this experiment, the percentage of cells labeled was low and the second term of the above equation was found to be insignificant. It should be noted that hours much larger than \( G_1 \) were not used to determine S, because doing so would require a large correction for cells that divided after addition of label, but which had not been in the S period in the presence of label. A final correction was made to account for the fact that roughly 85% of the cell never took up the label.

The actual determination of the length of the S period was done with the following equation, again from Stanners and Till (11).

\[ S = \frac{1}{a} \ln \left( \frac{L(t)_\text{corr}}{t < G_1} - (G_2 + t) \right) \]

In this equation \( S \) is the length of the period, \( G_2 \) is the length of the \( G_2 \) period, \( t \) is the hour, and \( L(t) \times 100 \) is the corrected percentage of cells determined by the previous equation. The data is represented in the Table. Discarding the questionable values found for hour 6, a mean value of 5.4 hours was found for the length of the S period. We have discarded the values for hour 6 in this experiment because we feel that if the data were valid, we should have noted a straight line rise in the mitotic index from hours 2 through 8 rather than a fall at hour 6.

**Determination of the length of the G1 period.** The post-mitotic and pre-synthetic gap, \( G_1 \), can be found by subtracting the length of the other three periods from the total length of the cell cycle, yielding a \( G_1 \) period of 6.2 hours.

\[ G_1 = T - (G_2 + S + M) \]

**DISCUSSION**

The mitotic cycle of the ear epidermis of the albino guinea pig is roughly comparable to the cycle of other mammalian cells as reported by other investigators. The cell cycle is characterized by a long pre-synthetic and post-mitotic gap involving slightly less than one-half of the generation time, followed by an S period of roughly one-third the length of the cell cycle, which in turn followed by a post-synthetic gap of approximately one-fifth of the generation time. The division process is very rapid and requires less than one-half hour for completion.

Perhaps one of the more interesting observations in this investigation was the appearance of the temporary partial synchrony. Many workers have experienced this phenomenon, and some have proposed possible control theories to explain the synchrony (13, 14). Block and his associates (15) have proposed that perhaps an inhibitor substance exists in tissues to control cell division. The mechanism, it is assumed, involves a type of feedback or negative inhibition affecting the \( G_0 \) or quiescent cells. If an inhibitor is present in great enough concentration, cells are held in \( G_0 \) and when the concentration of the inhibitor is lowered either by wounding, or in this case by removal of tissue from the ear, the cells held in \( G_0 \) are allowed to proceed immediately to the S phase of the cell cycle. Bullough has done a great deal of work on such inhibitor substances and several laboratories are now attempting to identify the "chalones" that he described (16).
The results of this investigation may lend plausibility to the existence of such control substances and mechanisms. The large increase in the number of labeled cells in the eighth hour is consistent with these views if it is assumed that a lag period of approximately 2 hours exists during which the cells become adjusted to the new environment of the chamber culture (Figure 2). (Again we wish to acknowledge that partial synchrony may not exist and we are merely observing an accumulation of labeled cells.) The concentration of the inhibiting substance at the end of 2 hours is then too low in the culture chamber to effectively act as an inhibitor and cells begin to synthesize DNA as soon as they are able. The synchrony is experienced because all cells in G1, whether 1 or 5 hours into the period are stimulated to proceed to DNA synthesis at once. Some of the cells of the epidermis may not have been affected by the culturing technique and did, therefore, not experience a lag in the beginning of DNA synthesis. Since the synthesis period is roughly 5.4 hours long, this may explain the increase in the number of labeled cells in the fifth hour.

The mitotic index supports the proposed cycle very well (Figs. 3 and 4). As can be seen, there is an increase in mitosis at hour 12. If synthesis ends at about hour 8, as is proposed, and G2 is minimally 3 hours, then mitosis in hour 12 of the experiment is indeed expected. The small increase noted in the fifth hour probably represents cells that were in the synthesis phase at the time of removal from the ear, that is near the end of the S phase. With the 2 hour lag and a 3 hour G2 period, this increase is in an expected position.

Finally, due to the low amount of cell labeling in this experiment, the value of S, though probably a close approximation of the true length of the period, has not been conclusively established. Further work is being done to substantiate this value. These experiments involve the use of scintillation counting and colcemid synchrony of the cell population. In the future, similar investigations, using a labeled amino acid to locate times of protein synthesis and labeled uridine to trace the production of RNA, will fully determine the timing of other events in the cell cycle of guinea pig epidermis (12).

REFERENCES


EPIDERMAL CELL CYCLE In Vitro