Cell proliferation and displacement in the lens epithelium of young rats injected with tritiated thymidine

Adrian G. Mikulicich and Richard W. Young

Cell proliferation and displacement were studied autoradiographically in the lens epithelium of young rats injected with H-3-thymidine at 6 days of age and sacrificed at intervals between 1 hour and 3 weeks thereafter. A "proliferative zone" characterized by a high incidence of DNA synthesis (H-3-thymidine incorporation) and mitosis was detected slightly anterior to the equator. Cell generation time was shortest in this zone, and progressively longer toward the anterior pole. Differences in generation time were mainly due to variations in the length of the G1 period of the proliferative cycle, although minor variations in the lengths of the G2 and mitotic periods were also documented. The duration of DNA synthesis was relatively constant (about 10¼ hours). Cells produced by mitosis in the proliferative zone were displaced toward the equator, where cell division ceased and further cell specialization was initiated. Differences in the functional activities of the common epithelial cell type in different regions of the lens were attributed to regionally varying cellular microenvironments.

The crystalline lens, unlike any other organ in the body, is entirely derived from a single cell type. As growth proceeds, older cells are retained within the lens, so that all stages in the life history of the common cell are simultaneously in evidence. Such cell systems are amenable to study by autoradiography following injection of H-3-thymidine. The labeled nucleoside, which is specifically incorporated by cells undergoing DNA synthesis, is otherwise rapidly degraded and excreted. The fate of the labeled cells may then be followed by serial analysis at progressively longer intervals after injection.

With regard to the lens, prior autoradiographic studies have generally used H-3-thymidine labeling as an indicator of impending mitosis, or as a means for documenting the conversion of lens epithelium into lens fibers. In the present investigation of the lens in young rats, this technique has been used (1) to define the regions in which DNA is synthesized by lens epithelial cells, (2) to record the passage of these labeled cells through the several stages of the cell proliferative cycle, and (3) to trace the varied fates of their descendants.

Methods

Thirty-seven 6-day-old Long-Evans rats were injected intraperitoneally with 1 μc per gram

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This work was supported by United States Public Health Service grants AM-04243-03 and B-3807.
body weight of H-3-thymidine* diluted with isotonic saline in 0.1 ml. Animals were sacrificed at intervals ranging from 1 hour to 3 weeks after injection. One newborn and one 35-day-old rat also received 1 mc per gram H-3-thymidine, and were sacrificed 1 hour later.

Eyes were fixed in Bouin-Hollande solution, double embedded in nitrocellulose-paraffin, and sectioned sagittally at 5 μ. Only sections at or near the midsagittal plane were used. Some sections were stained with hematoxylin and eosin, or with fast green-van Gieson. The remainder were prepared for autoradiography with Kodak NTB2 liquid emulsion. These were stained with periodic acid-Schiff (PAS) before development and hematoxylin alone after development. Autoradiograms were exposed at 4° C. under low humidity for 6 weeks, and were developed in Dektol (Eastman Kodak) for 2 minutes at 17° C.

Eight or more sections of one lens from each animal were analyzed autoradiographically. At the equator, the first lens epithelial cell presenting a distinguishable inner cytoplasmic membrane (Figs. 1 and 2) was considered to be cell No. 1. From this landmark, moving anteriorly, it was recorded for each successive epithelial cell whether that cell was (1) labeled, (2) unlabeled, (3) labeled and undergoing mitosis (all recognizable stages), or (4) unlabeled and undergoing mitosis. The last cell counted was comparable to cell No. 1 on the opposite side of the lens. The first 20 immature lens fibers immediately posterior to cell No. 1 were also analyzed for frequency of labeling at each interval. These analyses were carried out by the senior author (A. G. M.).

All counts from sections of each lens were combined, according to cell position. The observations from one half of each lens were combined with those of the opposite half (the last cell with cell No. 1, the next to last with cell No. 2, etc.). These data were then grouped by lens, i.e., cells 1 to 10, 11 to 20, etc., up to cell 150. All cells beyond 150 (corresponding to the anterior pole of the lens) were combined in a single group. The immature lens fibers were placed in two categories (-1 to -10, and -11 to -20), according to their distance from cell No. 1. The percentage of dividing cells and the percentage of labeled cells were then computed for each of these several groups in each animal.

Results

1. Frequency of mitosis. Lens fibers did not divide. Within the epithelium, how-
Fig. 2. Enlarged view of the equatorial region of the lens in a 6-day-old rat. Arrow indicates the first cell with an intact inner cytoplasmic membrane (cell No. 1). The first 10 cells posterior (below) to cell No. 1 were designated as lens fibers -1 to -10. Cell No. 1 and the succeeding 9 cells anterior to it were designated as epithelial cells 1 to 10. Note that polarization and elongation of the epithelial cells is initiated approximately 10 cells anterior to cell No. 1. (Hematoxylin and eosin. x320.)

However, regional differences in mitotic frequency were apparent (Table I). In cell positions 1 to 10 the incidence of mitosis, 1.0 per cent at birth, did not exceed 0.2 per cent at any age studied thereafter. In the immediately adjacent portion of the epithelium (cells 11 to 20), the frequency of cell division was generally slightly increased. At all ages, practically without exception, the occurrence of dividing cells was greatest in cell positions 21 to 70. Anterior to this region (hereafter called the proliferative zone), the frequency of mitosis decreased gradually, and seldom exceeded 1.5 per cent beyond position 90.

The incidence of mitosis decreased with age (Table I). In the newborn animal the average percentage of dividing cells was 4.7; at 6 days it was reduced to 1.7 per cent. Thereafter the gradual decline continued, reaching 0.8 per cent by 35 days. The zone of concentrated proliferative activity maintained a constant spatial relationship to cell No. 1 throughout the period of study.

2. Incorporation of H-3-thymidine. Labeled nuclei (Fig. 3) were observed in the lens epithelium of the newborn, 6-day-, and 35-day-old rats sacrificed 1 hour after injection. The percentage of cells which were labeled decreased with age (Table II). At birth it was 12.1 per cent and at 6 days, 9.5 per cent; by 35 days it had dropped to 4.5 per cent. Reactive nuclei were seen throughout the epithelium, but no labeled lens fibers were observed. At all three ages, labeling was more frequent in the proliferative zone (cells 21 to 70). The highest incidence of H-3-thymidine incorporation, therefore, coincided with the region of greatest mitotic activity. The frequency of labeling, however, always exceeded the incidence of mitosis.

3. Mitosis of labeled cells. In the series of animals injected at 6 days of age and sacrificed at varying intervals thereafter, the percentage of labeled mitotic figures was computed separately at each interval for (1) the proliferative zone, and (2) all other regions of the epithelium (Figs. 4 and 5, Table III). Labeled mitotic cells were observed in the proliferative zone as early as 2 hours after injection, but were not evident elsewhere until 2½ hours. Thereafter, labeled mitoses rapidly increased in frequency. During this ascending phase, the percentage of labeled mitoses was (with one exception) consistently higher in the proliferative zone. By 7 hours, effectively 100 per cent of dividing cells were labeled in the proliferative zone. The point at which this maximum was attained in the remainder of the epithelium was apparently slightly later, between 7 and 8 hours. After
the plateau at about 100 per cent labeled dividing cells, the curves separated again as the frequency of labeled mitoses progressively declined. During the descending portion of the curve, the decrease was generally in advance in the proliferative zone, compared to the remainder of the epithelium. The interval between the initial attainment of 50 per cent labeled mitoses and subsequent drop to 50 per cent was similar in both (about 10½ hours).

At all intervals between 20 and 122 hours the percentage of radioactive mitotic figures was higher in the proliferative zone (Table III). There was no evidence of a second wave of increased labeled mitoses.

4. Temporal variations in cellular labeling. The location and frequency of nuclear labeling at various intervals after an initial injection at 6 days of age are given in Table IV. During the first 3 or 4 hours, the

<table>
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<th>Age (days)</th>
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<th>11-20</th>
<th>21-30</th>
<th>31-40</th>
<th>41-50</th>
<th>51-60</th>
<th>61-70</th>
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<th>91-100</th>
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Table I. Percentage of cells undergoing mitosis according to cell position in the lens epithelium from birth to 35 days
number and spatial distribution of labeled cells was relatively stable. Immature lens fibers were unreactive, and the contiguous epithelial cells (positions 1 to 10) were seldom labeled. Labeling was more frequent in cells 11 to 20, and was further augmented in the proliferative zone, in which the highest incidence of reactive nuclei occurred in cells 31 to 50 (roughly 20 per cent). Beyond position 70, labeling decreased considerably, seldom exceeding 7 per cent after position 90.

Within a few hours, the incidence of labeling began to increase in all regions.

Table III. Percentage of labeled mitoses in the proliferative zone and other regions of the lens epithelium at intervals between 24 hours and 3 weeks after injection of H-3-thymidine

<table>
<thead>
<tr>
<th>Hour after injection</th>
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<tr>
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<tr>
<td>24</td>
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<td>10</td>
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<tr>
<td>3 weeks</td>
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In the proliferative zone, percentages began to rise at about 4 to 5½ hours, reaching a peak between about 20 and 30 hours. Nearer the anterior pole (cells 91 and above), the increase in labeling was not clearly evident until 8 to 10 hours after injection, and leveled off at about 20 hours. At its maximum, labeling was roughly double that observed initially.

Prominent changes in labeling occurred in cell positions near the equator. Shortly after 4 hours, the number of labeled cells in positions 11 to 20 began to increase. By 16 hours, labeling in this region was more than three times as frequent as it had been prior to 4 hours (25 per cent compared to 8 per cent). By 24 hours, a plateau averaging about 40 per cent was attained. This was comparable to the incidence of maximum labeling observed in the proliferative zone. In 11 of the 12 animals sacrificed between 32 and 122 hours, moreover, the percentage of labeled cells in positions 11 to 20 exceeded that in all parts of the proliferative zone.

Prior to 4½ hours, labeling in cells 1 to 10 averaged less than 1 per cent. The percentages began to increase some 9 or 10 hours after injection. By 24 hours, nearly 1 out of 4 nuclei was labeled in this zone. The increase continued, reaching a peak somewhere between 36 and 45 hours. Between 45 and 122 hours, labeling was comparable to that in the adjoining region, cells 11 to 20.

The first labeled nuclei appeared in immature lens fibers at 32 hours, and were
consistently observed thereafter (Fig. 6). A labeling incidence comparable to that of the proliferative zone was attained by 58 hours in fibers -1 to -10, and by 72 hours in fibers -11 to -20.

As early as 2 days after injection, a drop in labeling frequency was perceptible in the proliferative zone. The average percentage of labeled cells between 20 and 36 hours, for example, exceeded that recorded in the same region between 50 and 96 hours by a factor of roughly one half. After 122 hours, the incidence of labeled nuclei progressively diminished in all zones. In the older animals (3 weeks, 35 days) the formerly cuboidal epithelium had become squamous anterior to the proliferative zone. Consequently, the enlarged surface of the lens was covered by fewer cells than it was at earlier ages.

Discussion

Between birth and 35 days, in the lens epithelium of these young rats, mitosis occurred throughout the epithelium, but was more concentrated in a narrow band, roughly 50 cells in width, disposed circumferentially near the equator. The peripheral edge of this “proliferative zone” was about 10 cells anterior to the site of initial cellular polarization and elongation, and about 20 cells from the last, intact, inner cytoplasmic membrane. DNA synthesis (H-3-thymidine incorporation) also occurred throughout the lens epithelium, and was similarly concentrated in the band of increased mitotic activity. The proximity of the highly vascular, metabolically active ciliary processes (Figs. 1 to 4) is presumably an important microenvironmental factor which influences cellular activity within the proliferative zone.

The concentration of DNA synthesis and mitosis in the same region indicates that in the lens epithelium, cells synthesize DNA and divide without significant intervening migration (as contrasted with the immature retina). The labeled cells did not immediately divide, however. None were observed in mitosis at 1 or 1½ hours after injection. Labeled mitoses were first observed in the proliferative zone at 2 hours, and in the remainder of the epithelium at 2½ hours, which indicated that the minimum G2 period (between DNA synthesis and mitosis) was slightly shorter.

![Figure 5](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933143/)
in the former (1½ to 2 hours) than in the latter (2 to 2½ hours).

The passage through $G_2$ and mitosis ($M$) of cells labeled in the preceding DNA synthetic period ($S$) was reflected in the progressive rise in frequency of reactive dividing cells. This process was slightly in advance in the proliferative zone. Effectively, all the unlabeled cells initially present in $G_s$ and $M$ had completed these phases between 5½ and 7 hours after injection in this region, which indicated that the maximum duration of $G_s + M$ was 5½ to 7 hours. In the remainder of the epithelium, the maximum $G_s + M$ was apparently slightly longer, probably between 7 and 8 hours (Fig. 5). The average duration of DNA synthesis may be estimated from the interval between the initial attainment of 50 per cent labeled mitoses and the subsequent decline to that level.

This proved to be about 10½ hours in both cases. With the assumption that cells were randomly distributed in the various phases of the proliferative cycle, the proportion of cells initially labeled provides an estimate of the segment of the total cycle spent in DNA syn-
thesis. In the center of the proliferative zone, about 20 per cent of cells were in this S phase of 10½ hours average duration (at 1 hour, Table IV). Thus, about 80 per cent of the cycle was spent in other phases, lasting approximately 42 hours. The total proliferative cycle (one cell generation) must, therefore, have been about 52½ hours. The greater part of the cycle (roughly 36 hours) is evidently spent in the $G_1$ period of interphase, which follows mitosis and precedes DNA synthesis.

The decrease in cells engaged in DNA synthesis at increasing distances from the center of the proliferative zone implies a progressively lengthened proliferative cycle (e.g., almost 300 hours beyond position 150). Since, however, the duration of $S$ and $G_1 + M$ was practically the same in all regions, these changes in cell generation time must be mainly due to variations in the duration of $G_1$. The decrease in cells engaged in DNA synthesis at increasing distances from the center of the proliferative zone implies a progressively lengthened proliferative cycle (e.g., almost 300 hours beyond position 150). Since, however, the duration of $S$ and $G_1 + M$ was practically the same in all regions, these changes in cell generation time must be mainly due to variations in the duration of $G_1$. The decrease in cells engaged in DNA synthesis at increasing distances from the center of the proliferative zone implies a progressively lengthened proliferative cycle (e.g., almost 300 hours beyond position 150). Since, however, the duration of $S$ and $G_1 + M$ was practically the same in all regions, these changes in cell generation time must be mainly due to variations in the duration of $G_1$. The decrease in cells engaged in DNA synthesis at increasing distances from the center of the proliferative zone implies a progressively lengthened proliferative cycle (e.g., almost 300 hours beyond position 150). Since, however, the duration of $S$ and $G_1 + M$ was practically the same in all regions, these changes in cell generation time must be mainly due to variations in the duration of $G_1$. The decrease in cells engaged in DNA synthesis at increasing distances from the center of the proliferative zone implies a progressively lengthened proliferative cycle (e.g., almost 300 hours beyond position 150). Since, however, the duration of $S$ and $G_1 + M$ was practically the same in all regions, these changes in cell generation time must be mainly due to variations in the duration of $G_1$. The decrease in cells engaged in DNA synthesis at increasing distances from the center of the proliferative zone implies a progressively lengthened proliferative cycle (e.g., almost 300 hours beyond position 150). Since, however, the duration of $S$ and $G_1 + M$ was practically the same in all regions, these changes in cell generation time must be mainly due to variations in the duration of $G_1$.

The finding that $G_1 + M$ may be slightly increased in conjunction with extended generation time has also been observed in the adrenal cortex and bones of these animals. In these tissues, the average $S$ period duration was about 8 hours. In the lens epithelium, however, the $S$ period averaged 10½ hours. These differences in the average duration of DNA synthesis do not appear to be related to cell generation time. They suggest, however, that the duration of $S$ may vary slightly in different cell types, or might indicate that unique environmental conditions may be operative in the case of the lens. An extended duration of $S$ in ear epithelium of the mouse has been attributed to lower temperatures at this site. Since it appears that temperatures on the anterior surface of the lens are slightly below core temperature, this may conceivably account for the lengthened duration of $S$ in the lens epithelium.

This cell system is characterized by a progressively diminishing rate of cell proliferation. Predictions regarding cell generation time in any particular region of the epithelium are thus only approximations of the general rate of cell proliferation at the age studied. Because of individual cell variability, labeled cells, "synchronous" in the $S$ period at the time of injection, soon become randomly distributed within the proliferative cycle, yielding a continued, low frequency of labeled mitoses. The excess of labeled mitoses in the proliferative zone between 20 hours and 1 week probably reflects the fact that initially there were more labeled cells in this region.

As a result of the shortened $G_1 + M$ period in the proliferative zone, the increase in labeling incidence following the first division of reactive cells occurred slightly earlier in that region. Practically all labeled cells had divided by 20 hours. Labeling throughout the epithelium was

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*Note, in this regard, that the frequency of cells in DNA synthesis exceeds the incidence of cells in mitosis (Tables 1 and 27).

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Fig. 6. Equatorial region of the lens in a 10-day-old rat injected with H-3-thymidine 88 hours previously. Note the presence of labeled and unlabeled nuclei in immature lens fibers. (Autoradiogram, PAS-hematoxylin. ×640.)
then roughly twice that initially observed (Fig. 7). During the following day, the frequency of labeled nuclei in the proliferative zone began to decrease, as a result of division of unlabeled cells. Within about 3 days after injection, the incidence of labeling in this region had returned to its initial level, a condition not achieved in the more slowly proliferating anterior regions for 2 or 3 weeks. Ultimately, continued, frequent mitosis in the proliferative zone diluted the radioactivity of some labeled cells beyond the limits detectable at the exposures used, so that after 1 week the incidence of labeling in this region dropped below its initial level.

Superimposed on these proliferative phenomena was a continuing displacement of cells toward the equator, reflected in the rapid rise in labeling frequency in that region (Fig. 7). Labeling equilibrium with the proliferative zone was attained by cells 11 to 20 within 1 day, by cells 1 to 10 in less than 2 days, by the first 10 lens fibers in slightly more than 2 days (58 hours), and by fibers 10 to 20 within 3 days. This suggests that almost 15 cells per day were displaced out of the proliferative zone into the equatorial region of lens fiber formation. If all cells within the proliferative zone participate in supplying cells to the equatorial zone, the former must be replaced in slightly more than 3 days at this age in these rats, a figure reasonably consistent with the predicted average generation time in this region (10½ hours ÷ 17 per cent initial labeling × 100 = 62 hours).

There is no indication in this material that cells are displaced from the proliferative zone toward the anterior pole. Occasional cell division in this latter region, in association with a progressive tendency for the cells to become squamous, is appa-
ently adequate to compensate for the gradually increasing surface area of the lens. In the absence of apparent cell death, the decrease in the total number of cells which coat this surface in older animals suggests that, with age, the rate of lens fiber formation may exceed the rate of cell proliferation, in the process of which some cells originally anterior to the proliferative zone may be displaced equatorially into that zone.

The cells of the lens epithelium, while of a common type, exist within several uncommon microenvironments. Near the anterior pole, conditions for mitosis are marginal; cells are "stalled" in the G, period of interphase, and only rarely replicate their DNA and divide. Cells progressively nearer the equator encounter altered conditions which tend to shorten the duration of G, and thus the interval between successive mitoses. The shortest cell generation times occur in the center of the proliferative zone (which is probably under the influence of the nearby ciliary processes). Continued rapid proliferation in this environmental "field" displaces cells toward the equator, and into a region which will not support mitosis. Instead, the equatorial microenvironment predisposes the lens epithelial cells to activate another of the functions in their limited repertory, the transformation of their cellular structure into lens fibers. This process, which involves an increase in RNA and protein synthesis (unpublished material), appears to be irreversible. Consequently, it represents a true cellular differentiation.<ref>1</ref> 4

The authors gratefully acknowledge the invaluable technical assistance of Mrs. Mirdza Berzins. Miss Anna Lou Woodbridge and Mr. Philip Marcus were of considerable help in tabulating the findings.

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