Studies on Lymphocytes
VI. Evidence Showing Different Generation Times for Cytologically Different Lymphoid Cell Lines in the Thoracic Duct of the Calf

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LYMPHOCYTES circulating in the body represent a mixed population in respect to lifespans and size. The relationships between morphology, staining characteristics, function and proliferative patterns of the precursor cells are obscure. Different estimates for DNA synthesis and generation times have been reported for lymphoid cells. So far, no evidence has been published showing different DNA synthesis and generation times for different populations of lymphoid cells within the same animal. Previous studies on proliferation of lymphoid cells were complicated by a considerable degree of reutilization of labeled DNA breakdown products and the difficulty of studying a proliferating cell line in which there is no detectable cytoplasmic differentiation. Lastly, in small laboratory animals one cannot obtain sufficient samples of tissues from the same animal to describe the proliferative pattern adequately.

In the present experiment the proliferative pattern of lymphoid cells is correlated with cytologic features. In earlier studies it was shown that calf thoracic duct lymph is particularly desirable for the study of the kinetics of lymphoid cells following the administration of tritiated thymidine to label the cells in DNA synthesis because (1) general anesthesia is not needed, (2) large numbers of cells are collected in short time intervals (about 10^6 per minute) with many in mitosis, and (3) a great number of samples can be obtained from the same animal without detectably disturbing the steady state.

In earlier studies it was shown that an analysis combining the flow of labeled cells through mitosis (mitotic labeling index, MLI) and the mean grain count per labeled mitotic figure (MGC/LMF) as a function of time after administration of tritiated thymidine necessitated fewer assumptions in evaluating the time parameters of the cell generative cycle. In the present par-
per the cytologic characteristics of mitotic figures were expressed by grading the size, the cytoplasmic-nuclear ratio and the cytoplasmic basophilia.

**Materials and Methods**

A 4-month-old female Holstein calf and a 5-month-old male Holstein calf, weighing 200 and 325 lbs., respectively, were used in this study. Both calves received 48 hours of extracorporeal irradiation of the circulating blood (ECIB), during which their circulating red cells accumulated a total dose of $66 \times 10^3$ to $110 \times 10^3$ rads.\(^{14,15}\) Shortly after termination of the ECIB the thoracic duct of each animal was cannulated and a single intravenous injection of 0.2 μc./Gm. body weight of $^3$H-thymidine (Schwartz Bioresearch, specific activity 1.9 c./μM, concentration 100 μc./ml.) was given to both calves at 4:43 p.m. and 5:00 p.m., respectively. After injection of tritiated thymidine, lymph samples were taken at 10-minute intervals during the first 3 hours, at 30-minute intervals up to 10 hours, and at 1-hour intervals up to 24 hours. The lymph was centrifuged (1000 r.p.m., 10 minutes, 21 C.) and the supernatant reinfused. Smears prepared from the sedimented cells were dried briefly, fixed in absolute methyl alcohol, and processed for autoradiography using NTB-2 liquid emulsion (Eastman Kodak). Autoradiographic exposure was for 14 days at 4 C. in dehumidified air. After development, the preparations were stained with Giemsa solution 1:20 at pH 6.0, dried and covered.

One-hundred or more mitotic figures were found on the slides of most lymph samples taken. Questionable early prophases and latest telophases were not registered. The mitotic figures were classified according to the mitotic phase and graded in respect to size (grades 1 to 6, smallest to largest), the cytoplasmic-nuclear ratio (grades 1 to 6, lowest to highest), and the degree of the cytoplasmic basophilia (grades 1 to 6, lowest to highest), as reported previously.\(^{1} \) Various mitotic figures with different cytologic characteristics are shown in Figure 1. The grain number over the nuclear material of each mitotic figure was registered and background corrections made as indicated previously.\(^{12} \)

ECIB has been shown not to influence the generation time of at least a major portion of the lymphoid cells in the thoracic duct of the calf, and since generation and DNA synthesis times were found not to vary significantly from one animal to another,\(^{12} \) the data obtained from the two calves used in this study were pooled. The study was facilitated by using ECIB since this depletes the animals of a large proportion of the small nondividing lymphocytes, thus making the search for mitotic cells much easier and faster.

**Results**

As found earlier\(^{13} \) mitotic figures, with rare exceptions, were all placed in size classes 3 to 6. Lymphoid cells of the size classes 1 and 2 are clearly non-dividing as long as they maintain the morphological characteristics classically associated with the small lymphocyte. The changes in the MLI and/or MGC/LMF as a function of time after injection of tritiated thymidine was the same if one considers size classes 3 to 6 separately or broken into two categories of medium or large cells. In addition, the proliferative behavior of cells with different cytoplasmic nuclear ratio was similar. However, when the more basophilic cells were compared to the less basophilic cells, differences in the proliferative pattern were observed. This is illustrated in Figure 2, where the MLI curves for mitotic figures with cytoplasmic basophilia grades 3 to 6 (more basophilic) are compared to mitotic figures with basophilia grades 1 and 2 (less basophilic). In Figure 2, MLI curves are plotted using 2, 4, 6, or 8 grains, respectively, as a threshold for labeling. The solid lines are the background-corrected MLI curves. The MLI curves for the more basophilic mitotic figures rise steeply at the maximal rate of approximately 4.2 per cent.
Fig. 1.—Examples of classification of mitotic figures in smear preparations of centrifuged calf thoracic duct lymph with three values according to their size (1 to 6, smallest to largest), to their cytoplasmic-nuclear ratio (1 to 6, lowest to highest), and to the degree of their cytoplasmic basophilia (1 to 6, lowest to highest). Autoradiographs of calf thoracic duct cells removed 15 hours after a single i.v. injection of "H-thymidine (Giemsa; yellow filter; enlargement x 2000).

a. Prophase (6-2-3).
b. Prophase (6-2-3).
c. Metaphase (4-3-1).
d. Metaphase (3-1-3).
e. Anaphase (5-3-2).
f. Metaphase (4-3-3),
g. Anaphase (4-3-3).
h. Telophase (4-3-6).

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Fig. 2—Variation of the mitotic labeling index of calf thoracic duct lymphoid cells with more (grades 3 to 6: upper half of figure) or less (grades 1 and 2: lower half of figure) basophilic cytoplasm, as a function of time after a single i.v. injection of \(^{3}H\)-thymidine. The different curves represent the variation of the mitotic labeling index if all mitotic figures with more than 2, 4, 6 or 8 grains, respectively, were considered to be labeled. Pooled data from two calves which had received 48 hours of extracorporeal irradiation of the blood immediately prior to the \(^{3}H\)-thymidine injection.

Per minute. The curve reaches 100 or close to 100 per cent and remains there for about 4 hours. Actually, 9 out of 15 observations between 1 and 4 hours were at 100 per cent. The ML1 drops and rises to a second peak at about 8 hours and thereafter periodically oscillates. When the threshold for labeling of mitotic figures is raised arbitrarily, the first valley and the second peak become more evident.

The ML1 curves for the less basophilic mitotic figures (basophilia grades 1 and 2, lower half of Figure 2) rise less steeply with a maximal slope of about 2.8 per cent per minute. Between 1 and 4 hours, 10 out of 15 observations were less than 100 per cent for the background-corrected ML1 curve. After 4 hours there is considerable oscillation, but a clearly defined drop is not seen until 9½ hours. This is not followed by a well-defined second peak.

Individual grain counts of labeled mitotic figures and the MGC/LMF curves for the more and less basophilic cells are compared in Figure 3. The MGC/LMF curves for the more basophilic mitotic figures in the upper half of Figure 3 shows two definite peaks separated by a time interval of 6 hours. The mean grain count at the second peak is approximately half the mean grain count at the first peak. From 12 hours on, a definite periodicity is not maintained. Two successive waves of high individual grain counts (>50)
![Graphs showing grain count per labeled mitotic figure and variation of the background-corrected mean grain count per labeled mitotic figure of calf thoracic duct lymphoid cells with more (grades 3 to 6) or less (grades 1 and 2) basophilic cytoplasm, as a function of time after a single i.v. injection of $^3$H-thymidine. Pooled data from two calves which had received 48 hours of extracorporeal irradiation of the blood immediately prior to the $^3$H-thymidine injection.](image)

Fig. 3.—Grains per labeled mitotic figure and variation of the background-corrected mean grain count per labeled mitotic figure of calf thoracic duct lymphoid cells with more (grades 3 to 6: upper half of figure) or less (grades 1 and 2: lower half of figure) basophilic cytoplasm, as a function of time after a single i.v. injection of $^3$H-thymidine. Pooled data from two calves which had received 48 hours of extracorporeal irradiation of the blood immediately prior to the $^3$H-thymidine injection.

were observed during the first 10 hours after injection of $^3$H-thymidine. Thereafter, cells with such grain counts were not observed.

The MGC/LMF curve for less basophilic mitotic figures (basophilia grades 1 and 2, lower half of Fig. 3) oscillates more than that for basophilic cells graded 3 to 6 (upper half of Fig. 3) and does not show a clearly recognizable periodicity. High individual grain counts (>50) are distributed almost throughout the first 10 hours after injection of $^3$H-thymidine, and some appear even after 10 hours.

The corresponding periodicity of the MLI and MGC/LMF curves is shown in the upper half of Figure 4 for mitotic figures with cytoplasmic basophilia grades 3 to 6. The waves are in step for a period of at least 12 hours following the injection of $^3$H-thymidine. A similar periodicity of successive peaks is not observed in the case of the less basophilic cells. When the MLI and MGC/LMF of dividing cells with pale-staining cytoplasm (basophilia grades 1 and 2) are plotted against time after injection of tritiated thymidine (lower half of Fig. 4), the shape of the two curves is clearly different.

**DISCUSSION**

Three cytologic criteria were used to describe types of cells in mitosis: size, cytoplasmic-nuclear ratio, and degree of cytoplasmic basophilia. Only the latter was useful in detection of populations of lymphoid cells with different
proliferative patterns. Size classes 1 and 2 are nondividing cells. Size classes
3 to 6, when broken into a medium and large cell line, have similar DNA
synthesis and generation times. This is comparable to observations on the
erthroblastic series in the dog. However, more data are needed since
very few mitotic figures were observed in size classes 5 and 6.

The more basophilic (pyroninophilic) cells in the thoracic duct of the calf
have very little variance in the time parameters of their generative cycle.
From the steep rise of the MLI commencing 30 minutes after injection of
\(^{3}\text{H-thymidine}, it can be concluded that the minimum \(G_2\) period is about 30
to 40 minutes followed by a mitotic time of 17 minutes. The rapid drop of
the MLI at 4 hours after administration of thymidine indicates a minimum
DNA synthesis time of 34 hours. The mean DNA synthesis time is probably
of the order of 4 hours. Since the MLI did not fall to zero, it is not possible
to estimate the maximum DNA synthesis time for these data. As discussed
previously, reutilization of labeled nuclear material probably influences the
shape of the MLI curves and thus makes it difficult, if not impossible, to
determine precisely the generation time of lymphoid cells based on MLI
data alone. The curve of the MGC/LMF in the upper half of Figure 3 shows
two significant facts: (1) The time interval between the first two successive
peaks of 5\% to 6 hours is the same as was found in MLI curve. (2) The
second peak grain count is one-half of the first peak. These findings provide
strong evidence in favor of a generation time of approximately 5\% to 6 hours
for the majority of the proliferating cells with more basophilic cytoplasm.
The second peak of the MGC/LMF curve most probably represents a wave
of cells going through the second mitosis after the injection of \(^{3}\text{H-thymidine}
because the grain count of the labeled mitotic figures is about one-half that
observed in the first peak.

The pattern of the MLI and MGC/LMF of lymphoid cells with less baso-
philic cytoplasm (grades 1 and 2) after injection of \(^{3}\text{H-thymidine differs
considerably from that seen in the case of the more basophilic cells. The \(G_2\)
period for the less basophilic cells is also in the order of 30 to 40 minutes.
The subsequent time parameters of these curves deviate from those obtained
for the basophilic elements as follows:

1. The ascending part of the MLI curve between 30 and 120 minutes has a maximum
slope of 2.8 per cent per minute as compared to 4.2 per cent per minute for the more
basophilic cells.
2. Between 1 hour and 4 hours after injection of \(^{3}\text{H-thymidine}, more points are below
the 100 per cent in the case of the less basophilic cells than the more basophilic cells, and
the intermittent depressions below 100 per cent are greater in the case of the less basophilic
cells.
3. In the case of the more basophilic cells, the MLI curve drops precipitously after 4
hours, whereas there is very little depression in the case of the less basophilic cells. Actually,
the MLI oscillates around 90 per cent until 10 hours after labeling and then falls slowly
to lower levels.
4. The MGC/LMF curve does not have the two separate peaks at 2 and 8 hours as is
evident in the cells with the more basophilic cytoplasm. In contrast to the steady drop from
2 to 6 hours in the more basophilic cells, there is an oscillation around 20 to 30 grains per
labeled mitotic figure with the peaks becoming progressively slightly lower.
Fig. 4.—Comparison of the variation of the mitotic labeling index (only mitotic figures with more than 4 grains were considered as labeled) and of the background-corrected mean grain count per labeled mitotic figure for thoracic duct lymphoid cells with more (grades 3 to 6: upper half of figure) or less (grades 1 and 2: lower half of figure) basophilic cytoplasm, all as a function of time after a single i.v. injection of $^3$H-thymidine. Pooled data from two calves which had received 48 hours of extracorporeal irradiation of the blood immediately prior to the $^3$H-thymidine injection.

5. Although the mean grain count at 8 hours is about one-half of the 2-hour value, the intervening wide oscillation makes the interpretation impossible.

6. The successive waves of high grain count mitoses as observed in the upper half of Figure 2 is not present for the less basophilic cells in the bottom half of Figure 2. Furthermore, there is a lack of corresponding periodicity in the MLI and MGC/LMF curves, as illustrated in Figure 4. The failure to observe a corresponding periodicity in the MLI and MGC/LMF curves in the case of the less basophilic cells probably indicates a heterogeneous population in regard to the duration of the various phases of the generative cycle. It is conceivable that these cells develop through successive mitoses with unequal speed and on the average more slowly than basophilic cells. One might speculate that several cell lines with unequal time parameters of their proliferative pattern could be detected if it were possible to distinguish between cell lines within the less basophilic cell group. Alternatively, pronounced straggling of cells of the same line might contribute to the different MLI and MGC/LMF curves observed for lymphoid cells with pale staining cytoplasm.

For several reasons it is conceivable that the more basophilic mitotic figures in the thoracic duct represent immunologically active sensitized lymphoid cells:

1. Since the lymph flow in thoracic duct is rather rapid, it is highly probable that most of the dividing cells within the duct originated within intestinal and mesenteric lymphoid tissue. The latter is known to be exposed to a multitude of antigenic stimuli arising in the intestinal contents. One might therefore assume that immunologically committed cells commence the cell cycle within the intestinal and mesenteric lymphoid tissue, become detached, and continue to proliferate within the lymph.
2. The experimental data presented within this paper indicated the generation time of the more basophilic elements to be of the order of 5½ to 6 hours. When one calculated the generation time for cells in the germinal centers of the calf from the observed mitotic index of 5 per cent and mitotic time of proliferating lymph cells in the calf thoracic duct of 17 minutes, one obtained a generation time of about 6 hours.

3. Cells with the morphologic criteria characteristic of plasma cell precursors have been found in thoracic duct by electron microscopy.

4. Thoracic duct lymphoid cells have been found to develop into plasma cells under appropriate culture conditions.

It is more difficult to assign a possible function and characteristics to the cell line represented by the less basophilic mitotic figures in the thoracic duct of the calf. Are these mitotic figures from proliferating immunologically noncommitted cells and/or macrophage precursors? It has been found that lymphoid cells in the thymus of newborn mice have a generation time of approximately 10 hours. Immunologically noncommitted lymphoid cells may have longer generation time than the committed cells. It is also possible that at least some of the less basophilic mitotic figures in the thoracic duct of the calf represent proliferating macrophage precursors. This last possibility is suggested by the experimental fact that under some conditions lymphoid cells from the thoracic duct have been found to develop into macrophages.

**SUMMARY**

Two calves received 48 hours of extracorporeal irradiation of the circulating blood (ECIB) immediately prior to cannulation of the thoracic duct and administration of a single intravenous injection of $^3$H-thymidine. Lymph samples were then removed at short time intervals during 24 hours and smears from sedimented cells were processed for autoradiography. For different groups of mitotic figures classified according to size, cytoplasmic-nuclear ratio and degree of cytoplasmic basophilia, the variation of the mitotic labeling index (MLI) and of the mean grain count per labeled mitotic figure (MGC/LMF), as a function of time after a single injection of $^3$H-thymidine, was analyzed. Based on the pooled data obtained from the two animals, it was found that tinctorial properties of the cytoplasm permit differentiation into at least two populations of lymphoid cells in the thoracic duct of the calf: one of more basophilic cells with a mean DNA synthesis time of 4 hours and a mean generation time of 5½ to 6 hours and one or more populations of less basophilic cells with longer DNA synthesis and generation times.
diferentiation de al minus duo populationes de cellulas lymphoide in le ducto thoracic del vitello. Le un de istos ha cellulas plus basophilic con un synthese medie de acido deoxyribonucleic de 4 horas e un tempore medie de generation de 5li a 6 horas. Le altere (o le alteres) ha cellulas minus basophilic con plus longe tempores de synthese de acido deoxyribonucleic e de generation.

REFERENCES


18. Unpublished data.


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