Identity and Proliferation of Small Lymphocyte Precursors in Cultures of Lymphocyte-rich Fractions of Guinea Pig Bone Marrow

By Y. Yoshida and D. G. Osmond

Radioautography with $^{3}H$-thymidine was used to examine the proliferative activity of bone marrow lymphoid cells and to identify the precursor cells of small lymphocytes in short-term cultures of lymphocyte-rich marrow fractions. High concentrations of small lymphocytes (nuclear diameters less than 8.0 \( \mu \) in smears) together with large lymphoid ("transitional") cells (nuclear diameters greater than 8.0 \( \mu \)) were separated from suspensions of guinea pig bone marrow by centrifugation in linear sucrose-serum density gradients. When such lymphocyte-rich marrow fractions were cultured in vitro the labeling and mitotic indices following either continuous or terminal exposure to $^{3}H$-thymidine indicated that the large lymphoid cells were confined mainly to the pre-DNA-synthetic (G1) and early DNA-synthetic (S) phases at first, but proceeded subsequently through S phase and mitosis. From these data tentative values were derived for the in vitro duration of G1 (12 hours) and S (13.7 hours). Further cultures were followed radioautographically after a 1-hour pulse of $^{3}H$-thymidine at 6–7 hours of culture. The absolute numbers of labeled large lymphoid cells declined during the subsequent 21 hours but, simultaneously, labeled small lymphocytes appeared and increased progressively in absolute numbers to 44.4 ± 8.1 per cent of the initial numbers of labeled large lymphoid cells. The mean grain count of labeled small lymphocytes was half that of the initially labeled large lymphoid cells. Very few labeled undifferentiated cells other than large lymphoid cells were observed. The results demonstrate that lymphocyte-rich marrow fractions are capable of sustaining the production of small lymphocytes in short-term cultures and that the immediate precursors of marrow small lymphocytes are contained within a population of large lymphoid cells.

Guinea pig bone marrow contains large numbers of small lymphocytes which are renewed continuously by the proliferation of precursor cells within the marrow. Similar findings in the rat confirm the marrow to be a major site of small lymphocyte production, but the identity and proliferative kinetics of the lymphocyte progenitors have not been resolved.

In addition to typical small lymphocytes the marrow contains a population of dividing cells, named "transitional cells" by Yoffey, which are generally larger than small lymphocytes but exhibit some lymphocyte-like morphological features. Considerable controversy surrounds the functional role(s) of these
It has been proposed that they are hemopoietic stem cells, capable of giving rise to erythroid and granulocytic cells\(^9,11,12\) and that they are themselves derived from the enlargement of small lymphocytes.\(^3,9\) On the other hand, it has also been suggested that they are the precursors of marrow small lymphocytes.\(^3,5,13\) For descriptive convenience throughout the present work both small lymphocytes and transitional cells have been grouped together morphologically as marrow lymphoid cells. However, the use of this term was not intended to presuppose any functional relationship between them.

As a necessary prerequisite for further studies of marrow lymphocytopoiesis, the present experiments were designed to identify unequivocally the marrow small lymphocyte precursors. This has not been possible in previous work because of the cellular complexity of whole marrow, but has now been facilitated by the separation of lymphocyte-rich fractions of simplified cellular composition from suspensions of guinea pig marrow.\(^14,15\)

The lymphoid cells in both whole marrow and lymphocyte-rich marrow fractions have been analyzed, using \(^3\)H-thymidine radioautography and cell measurements, and classified into two groups: nondividing small lymphocytes and a group of larger cells, many of which were proliferating, tentatively designated large lymphoid cells. In cultures of lymphocyte-rich marrow fractions, exposed either continuously or terminally to \(^3\)H-thymidine, the progress of large lymphoid cells through the mitotic cycle was determined. From the wave of proliferation which was observed in these cultures, preliminary values for the in vitro cell cycle times of large lymphoid cells have been derived. Finally, using these data to derive appropriate culture conditions, the appearance of newly-formed small lymphocytes has been studied quantitatively following a brief exposure of lymphocyte-rich marrow fractions to \(^3\)H-thymidine in vitro, and the origin of small lymphocytes from cells in the large lymphoid group has been established.

**Materials and Methods**

All experiments were performed on male Hartley guinea pigs weighing approximately 400 Gm. \(^3\)H-thymidine (New England Nuclear Corp.) was used throughout at a specific activity of 6.7 Ci./mM.

**In Vivo Incorporation of \(^3\)H-Thymidine by Marrow Lymphoid Cells**

Animals were killed 1 hour after a single intracardiac injection of \(^3\)H-thymidine (1 μCi./Gm. body weight). Marrow samples from each femur, tibia and humerus were suspended in homologous serum by gentle shaking, pooled, washed in Eagle's minimum essential medium (MEM), smeared, and stained with MacNeal's tetrachrome stain. Radioautographs of methanol-fixed marrow smears were prepared by dipping in a melted NTB3 emulsion (Eastman Kodak), exposed for 14 days, processed and stained through the fixed emulsion with a modified MacNeal's stain. The nuclear diameters of 500 lymphoid cells were measured to 0.1 μ in regions of comparable cell density in each radioautograph, using an ocular micrometer. Cells with more than three grains over the nucleus were recorded as being labeled.

**Separation of Lymphocyte-rich Fractions from Marrow Cell Suspensions**

Suspensions of marrow cells were fractionated by centrifugation in linear sucrose-serum density gradients as described elsewhere.\(^14,15\) Briefly, washed marrow cell suspensions were
layered onto 14 ml. linear gradients of 5–15 per cent sucrose (5% sucrose in half-strength Hank's balanced salt solution; 15% sucrose in water) plus 20 per cent fresh homologous serum. After centrifugation for 6–8 minutes at 100 × g. and room temperature, approximately one-quarter of the marrow lymphoid cells were recovered from a low-density fraction in which they sedimented slowly together with a well-defined band of erythrocytes. In some experiments, this lymphocyte-rich marrow fraction was further fractionated by centrifugation in a second similar density gradient for 5–6 minutes at 100 × g. Nucleated cells in fractions and in subsequent cultures were enumerated with a Coulter particle counter, model B (Coulter Electronics).

**Cellular Proliferation in Short-term Cultures of Lymphocyte-rich Marrow Fractions**

One hour after the intracardiac injection of $^{3}$H-thymidine (1.0 μCi./Gm. body weight) the labeling indices of lymphoid cells in marrow and marrow fractions were compared. Samples of freshly prepared unlabeled lymphocyte-rich fractions, containing 3–4 × 10⁶ nucleated cells were then cultured in Eagle's MEM (2 ml.) supplemented with fresh guinea pig serum (1 ml.), penicillin (150 units) and streptomycin (150 μg.) in sterile plastic culture tubes (Falcon Plastics). The cells were incubated at 37°C with either continuous exposure to $^{3}$H-thymidine (0.1 μCi./ml.) for periods ranging from 2 to 24 hours or the addition of $^{3}$H-thymidine (1.0 μCi./ml.) for the terminal hour of culture at 1–27 hours. After washing the cells three times with Eagle's MEM radioautographs of smears were prepared as described above and exposed for 7 days.

In each case, 2000–4000 lymphoid cells were examined throughout the entire length of smears to determine the percentage of labeling together with the incidence and labeling of mitotic figures.

![Fig. 1.—Percentage size distribution of marrow lymphoid cells in normal guinea pigs, based on measurements on radioautographic smears 1 hour after an intracardiac injection of $^{3}$H-thymidine (1 μCi./Gm.).](image-url)
In Vitro Production of Marrow Small Lymphocytes and Identification of Precursor Cells

The DNA synthesizing cells of lymphocyte-rich fractions were labeled by exposure to $^3$H-thymidine (1.0 µCi/ml) for 1 hour after 6 hours prior incubation. Cells were washed three times with Eagle's MEM, resuspended in fresh Eagle's MEM serum medium and incubated in aliquots for further periods of 1, 6, 16 and 21 hours. After enumeration of nucleated cells, smears were made, processed for radioautography and exposed for only 48 hours to facilitate grain counting.

The absolute numbers and mean grain counts of labeled small lymphocytes and large lymphoid cells were derived from nucleated cell counts and radioautographic examination of 2000-4000 lymphoid cells in each culture.

RESULTS

Classification of Marrow Lymphoid Cells

Figure 1 shows the size distribution of lymphoid cells in radioautographs of marrow smears prepared 1 hour after $^3$H-thymidine injection. With few borderline exceptions (1.8%), lymphoid cells with nuclear diameters of less than 8.0 µ showed no direct incorporation of $^3$H-thymidine. These cells possessed the morphological characteristics of small lymphocytes, namely a dense pachychromatic nucleus and scanty cytoplasm often concentrated at one pole of the cell (Fig. 5A). On the other hand, while also having scanty cytoplasm, many cells with nuclear diameters greater than 8.0 µ exhibited a relatively leptochromatic nucleus and varying degrees of cytoplasmic basophilia (Fig. 5A). Unlike small lymphocytes many of these cells showed active DNA synthesis (Figs. 1 and 5B). In separate counts from 6 experiments of approximately 3000 lymphoid cells of this size range the overall labeling index was 35.2 ± 1.4 per cent.

Because it was necessary in the following studies to distinguish objectively between nonproliferative and proliferative cells, the marrow lymphoid cells were classified into two groups on the basis of nuclear diameters in smears, (1) small lymphocytes, with nuclear diameters less than 8.0 µ; (2) large lymphoid cells, with nuclear diameters of 8.0 µ or more. The latter group consisted predominantly of cells previously named transitional cells. The proportion of large lymphoid cells in DNA synthesis increased progressively with respect to cell size (Fig. 1). Thus, the labeling indices for cells with nuclear diameters of 8.0-8.9 µ, 9.0-10.4 µ and greater than 10.5 µ were 19-30 per cent, 42-67 per cent and 81-86 per cent, respectively. The lower proportion of DNA-synthesizing cells among smaller members of the large lymphoid group might be explained by an overlap in size between proliferating cells and non-proliferating small lymphocytes and/or by a relative predominance of cells in the pre-DNA-synthetic ($G_1$) phase of the cell cycle.

Cell Populations in Lymphocyte-rich Marrow Fractions

Lymphocyte-rich fractions derived from a single density gradient centrifugation usually contained $30-50 \times 10^6$ nucleated cells, approximately 80 per cent of which were lymphoid cells (Table 1, Fig. 5A). Most contaminants were late erythroblasts and granulocytes. Few unclassifiable blasts were seen (<
Table 1.—Composition of Lymphocyte-rich Fractions Separated From Guinea Pig Bone Marrow

<table>
<thead>
<tr>
<th></th>
<th>Lymphocyte-rich Fraction Recovered from</th>
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<tbody>
<tr>
<td></td>
<td>First Gradient *</td>
</tr>
<tr>
<td></td>
<td>Second Gradient †</td>
</tr>
<tr>
<td>Nucleated cell count (× 10^6)</td>
<td>9.1±2.8</td>
</tr>
<tr>
<td></td>
<td>43.1±4.1</td>
</tr>
<tr>
<td>Differential count (%)</td>
<td></td>
</tr>
<tr>
<td>Lymphoid</td>
<td></td>
</tr>
<tr>
<td>Small lymphocytes</td>
<td>65.8±5.3</td>
</tr>
<tr>
<td>Large lymphoid cells</td>
<td>16.4±2.1</td>
</tr>
<tr>
<td>Total</td>
<td>82.2±3.8</td>
</tr>
<tr>
<td>Granulocytic</td>
<td></td>
</tr>
<tr>
<td>Band and segmented</td>
<td>4.8±1.5</td>
</tr>
<tr>
<td>Metamyelocytes</td>
<td>0.8±0.2</td>
</tr>
<tr>
<td>Myelocytes</td>
<td>0.2±0.1</td>
</tr>
<tr>
<td>Total</td>
<td>5.8±3.8</td>
</tr>
<tr>
<td>Erythroid</td>
<td></td>
</tr>
<tr>
<td>Orthochromatic</td>
<td>2.0±0.4</td>
</tr>
<tr>
<td>Polychromatic</td>
<td>6.9±1.8</td>
</tr>
<tr>
<td>Basophilic</td>
<td>0.3±0.1</td>
</tr>
<tr>
<td>Total</td>
<td>9.2±5.2</td>
</tr>
<tr>
<td>Blast cells</td>
<td>2.7±1.1</td>
</tr>
<tr>
<td>Others</td>
<td>0.1</td>
</tr>
</tbody>
</table>

* Mean ± standard error (5 experiments).
† Mean ± standard error (3 experiments).

3 %). The morphological clarity of the preparations was comparable with that of unfractonated marrow smears. Small lymphocytes comprised approximately 80 per cent of all lymphoid cells in the lymphocyte-rich fractions. Successively deeper fractions of the gradients showed a sharp rise in the numbers of granulocytic cells, and a progressive increase in erythroblasts, large blast-like cells and other cell types.15

One hour after the in vivo administration of 3H-thymidine, marrow cell suspensions were centrifuged in sucrose-serum gradients and the large lymphoid cells were examined in radioautographs of consecutive fractions throughout the gradients. The results are illustrated in Figs. 2 and 5B. In six experiments the proportion of large lymphoid cells which had incorporated 3H-thymidine prior to fractionation was consistently lower in the slowly sedimenting lymphocyte-rich fractions (14.1 ± 1.5%) than in the original marrow (35.2 ± 1.4%). Large lymphoid cells in deeper fractions showed progressively increasing labeling indices which exceeded those in the original marrow.

Centrifugation of the lymphocyte-rich fractions in a second similar density gradient resulted in the recovery of a fraction in which approximately 95 per cent of the nucleated cells were lymphoid cells (Table 1), while the proportion of large lymphoid cells in DNA synthesis was further reduced to 5.4 ± 1.4 per cent (Fig. 2). Small lymphocytes remained unlabeled, thus demonstrating that no cell shrinkage had occurred in the course of separation.

These results indicate that the large lymphoid cells in the lymphocyte-rich fractions were not a random sample of the marrow population but were selected with respect to their position in cell cycle. Further experiments were therefore undertaken to test the ability of separated large lymphoid cells...
Fig. 2.—Percentage of labeled large lymphoid cells in density gradients. Marrow cell suspensions were fractionated in one or two successive gradients 1 hour after an intracardiac injection of ³H-thymidine (1 μCi./Gm.). The lymphocyte-rich fractions are fractions number 1. Hatched area represents the labeling index (mean ± standard deviation) within the original bone marrow.

to progress into the DNA synthetic (S) phase and to undergo mitosis during culture.

Cell Cycle Characteristics of Large Lymphoid Cells in Cultures of Lymphocyte-rich Marrow Fractions

Figure 3 summarizes the results in approximately 100 cultures of lymphocyte-rich marrow fractions, which had been exposed to ³H-thymidine either continuously or in the terminal hour. In each case, the percentage of labeled large lymphoid cells showed a linear and identical increase, reaching approximately 40 per cent at 9 hours (Fig. 5C). The labeling index then showed a brief plateau and a decline in the pulse-labeled cultures but continued to rise in the continuously labeled cultures. However, the maximum labeling indices in continuously labeled cultures did not exceed 50 per cent although, even after 24 hours, the culture medium still contained available ³H-labeled DNA precursor(s) as shown by its ability to label the usual percentage of large lymphoid cells when incubated for 1 hour with previously unlabeled marrow fractions.

The percentage of large lymphoid cells in lymphocyte-rich fractions labeled in vitro soon after recovery from the density gradients approximated closely to that seen after in vivo labeling of the original marrow (Figs. 2 and 3), thus
suggesting that cells which were synthesizing DNA in vivo continued to do so in vitro and that the separation technique did not depress DNA synthesis.

Mitotic figures, rarely seen (0-0.2%) at the outset of cultures, increased in incidence between 9 and 27 hours (Fig. 4). During continuous exposure to \(^{3}\)H-thymidine no labeled mitoses were seen until 6 hours, but virtually all mitoses were labeled after 8 hours.

These results demonstrate that the large lymphoid cells in the lymphocyte-rich fractions were confined mainly to the \(G_1\) and early \(S\) phases of the cell cycle at first and proceeded into DNA synthesis and mitosis during culture. Thus, very few cells were in mitosis initially, and the time lag before the onset of the mitotic wave and in the appearance of labeled mitoses in continuously labeled cultures suggests that few cells were in the post-DNA-synthetic (\(G_2\)) or late \(S\) phases at the beginning of culture, assuming the \(G_2\) phase to be relatively short (2-3 hours) as for other marrow cells.

The identical increase in labeling index during the first 9 hours of culture with either continuous or pulse labeling indicates that, throughout this period, cells entered \(S\) phase continuously but did not leave \(S\) phase in substantial numbers. Consequently, the cells initially synthesizing DNA were predominantly in early \(S\) phase. On the other hand, cells were spread randomly throughout the \(G_1\) phase at first, since the rate of flow of cells from \(G_1\) to \(S\)
Fig. 4.—Percentage of large lymphoid cells in mitosis in cultures of lymphocyte-rich marrow fractions. The bars represent the standard error.

Phase remained constant for the first 12 hours of culture, as shown by the linearity of the increase in labeling index in continuously labeled cultures.

Approximately half of the large lymphoid cells were in cell cycle during the culture period, as indicated by their maximum labeling index with continuous exposure to $^3$H-thymidine. Many of the remaining unlabeled cells were probably the largest members of the nonproliferating lymphocyte population, which, as already noted, may exceed 8.0 μ nuclear diameter to some extent. In addition, because a small number of cells with nuclear diameters greater than 9.0 μ remained unlabeled, some normally proliferative cells may have failed to proceed through cell cycle under the prevailing experimental conditions.

Small Lymphocyte Production in Cultures of Lymphocyte-rich Marrow Fractions

Cultures of lymphocyte-rich fractions were examined radioautographically at intervals after 1 hour exposure to $^3$H-thymidine at 6-7 hours. The time of the pulse labeling was selected from the experiments described above so as to label many large lymphoid cells in DNA synthesis just prior to the onset of the mitotic wave. Absolute numbers per culture and mean grain counts of labeled large lymphoid cells and small lymphocytes were determined. The results of three experiments are shown in Table 2 in which the absolute numbers of labeled cells and their mean grain counts are expressed as percentages of the respective values for the labeled large lymphoid cells 1 hour after
**Table 2.** Absolute Number and Mean Grain Count of Labeled Lymphoid Cells in Cultures of Lymphocyte-rich Marrow Fractions

<table>
<thead>
<tr>
<th>Labeled Cells</th>
<th>Hours after (^3)H-thymidine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Large lymphoid cells</td>
<td>100</td>
</tr>
<tr>
<td>Small lymphocytes</td>
<td>3.8±0.5</td>
</tr>
<tr>
<td>Mean grain count *</td>
<td></td>
</tr>
<tr>
<td>Large lymphoid cells</td>
<td>100</td>
</tr>
<tr>
<td>Small lymphocytes</td>
<td>67.1±3.8</td>
</tr>
</tbody>
</table>

* Expressed as a percentage of the values for labeled large lymphoid cells, 1 hour after \(^3\)H-thymidine administration.

\(^3\)H-thymidine (absolute number, 100–200 × 10^2; mean grain count, 30–38). The absolute number of labeled large lymphoid cells tended to fall by approximately 20 per cent of the initial value during the next 21 hours, some of them being observed in mitosis (Fig. 5D). On the other hand, labeled small lymphocytes progressively increased in absolute number from 6 hours onwards (Figs. 5E and 5F). By 21 hours after the pulse of \(^3\)H-thymidine they totaled 44.4±8.1 per cent of the initial number of labeled large lymphoid cells.

Because, with few borderline exceptions, small lymphocytes as defined in this study were incapable of DNA synthesis, the labeled small lymphocytes must have arisen from the division of labeled precursor cells within the cultures. Moreover, assuming the labeled small lymphocytes to be all first generation daughter cells, their immediate precursors must have been so numerous, that they could only have been contained within the population of labeled large lymphoid cells. The observed labeling intensities were fully in agreement with this interpretation because the mean grain count of the labeled small lymphocytes from 6 to 21 hours after the \(^3\)H-thymidine pulse was half that of the initially labeled large lymphoid cells (Table 2). A few lymphocytes with nuclear diameters slightly less than 8.0 \(\mu\) were labeled 1 hour after \(^3\)H-thymidine. They showed a higher mean grain count than that of labeled small lymphocytes appearing subsequently (Table 2) and represent the smallest extreme of the proliferating lymphoid cell size range.

**DISCUSSION**

The present study demonstrates that lymphocyte-rich fractions separated from guinea pig bone marrow by centrifugation in sucrose-serum density gradients are capable of sustaining the production of small lymphocytes in short-term cultures and that the immediate precursors of the nondividing marrow small lymphocytes are contained within a population of large lymphoid cells, referred to previously as transitional cells.

Large lymphoid cells with nuclear diameters of 8 \(\mu\) or more in marrow smears show evidence of rapid proliferation, namely a high proportion of cells in DNA synthesis in vivo (Fig. 1) and a rapid rate of entry into DNA synthesis and mitosis in vitro (Figs. 3 and 4). However, in accordance with previous observations in vivo, fewer DNA-synthesizing cells are seen among the smaller cells
Fig. 5.—Photomicrographs of cells in smears of lymphocyte-rich marrow fractions. × 1125. (A) Lymphocyte-rich fraction prior to culture, showing small lymphocytes and large lymphoid cells. (B) Labeled large lymphoid cell in a radioautograph of a lymphocyte-rich fraction separated from bone marrow 1 hour after an intracardiac injection of ³H-thymidine. (C) Labeled large lymphoid cells in a radioautograph of a lymphocyte-rich fraction which had been cultured for 9 hours with the addition of ³H-thymidine for the terminal hour. (D) Labeled mitotic figures in a radioautograph of a 23-hour culture of a lymphocyte-rich fraction to which ³H-thymidine was added for 1 hour at 6–7 hours. (E) and (F) Labeled small lymphocytes in a radioautograph of a 28-hour culture of a lymphocyte-rich fraction to which ³H-thymidine was added for 1 hour at 6–7 hours.

of this group (8.0–8.9 μ nuclear diameter) than among the larger cells (greater than 9.0 μ nuclear diameter). Thus, although the marrow lymphoid cell population as a whole shows an unbroken range of cell sizes and morphology, the ³H-thymidine incorporation data suggest that there are two basic subpopulations of cells, nonproliferating small lymphocytes and proliferating large lymphoid cells, which overlap in size and morphology, mainly between 8.0 and 8.9 μ nuclear diameter. In the absence of specific morphological criteria by which to recognize the degree of maturity of marrow lymphocytes, the cell size provides the most objective and reliable basis for their classification. The present classi-
Small lymphocyte precursors in bone marrow

fication of small lymphocytes as being cells of less than 8.0 μ nuclear diameter in smears limits this cell group almost entirely to nondividing cells, but, at the same time, excludes some nondividing cells, functionally small lymphocytes, which have nuclear diameters somewhat greater than 8.0 μ. Such cells form a relatively greater proportion of the lymphoid population in lymphocyte-rich fractions than in the original marrow because of the selective displacement of the larger cells from the lymphocyte-rich fractions by the separation technique. As already noted, the overlap in size between nondividing and dividing cells may largely account for the observation that continuous exposure of lymphocyte-rich marrow fractions to 3H-thymidine in vitro labels only approximately half of the large lymphoid cells. Thus, the cell classification used in the present study results in a minimum estimate of the production of small lymphocytes. When 6-hour cultures of lymphocyte-rich fractions were given a 1 hour pulse of 3H-thymidine, one third of large lymphoid cells were labeled (Fig. 3). Labeled small lymphocytes, which were rarely seen initially, appeared in increasing numbers during the subsequent 21-hour period and finally totalled two-fifths of the initial number of labeled large lymphoid cells (Table 2). However, not all the newly-formed labeled small lymphocytes were necessarily included in these figures because some of them may have been slightly larger than 8.0 μ nuclear diameter and thus would have been classified as large lymphoid cells.

The demonstration that some large lymphoid cells divide in vitro to produce small lymphocytes does not necessarily rule out the reverse possibility that, under appropriate circumstances, some small lymphocytes may enlarge and so exhibit large lymphoid morphology. A variety of stimuli in vitro have been shown to cause the enlargement of marrow small lymphocytes into dividing blast-like cells. As yet, there is no evidence that this normally occurs within the marrow in vivo. Nor, do the present experiments reveal the production of cells other than lymphocytes from the large lymphoid cells. In the present experiments the mean grain count of labeled large lymphoid cells declined after a pulse exposure to 3H-thymidine in vitro (Table 2). Because this decline was evident in some labeled cells larger than 9.0 μ nuclear diameter it cannot be explained solely by the production of labeled small lymphocytes which overlapped in size with the large lymphoid cells, but indicates that some labeled large lymphoid mitoses gave rise to more large lymphoid cells in the cultures. Further data are required to determine whether some of the large lymphoid cells divide repeatedly in a self-sustaining manner and to estimate the number of precursor cell generations leading to the production of small lymphocytes.

Tenative figures for the duration of cell cycle of the large lymphoid cells in vitro may be derived from the flow of partially synchronized cells through S phase and mitosis. Initially, as described above, the large lymphoid cells in lymphocyte-rich marrow fractions were distributed predominantly throughout G1, and the early part of S phase. The DNA-synthesizing cells were concentrated in deeper fractions of the gradients (Fig. 2) in accordance with observations of others that the sedimentation rate of cells in sucrose density gradients
may be dependent upon their position in the cell cycle. Continuous exposure to \(^3\)H-thymidine showed that large lymphoid cells continued to enter DNA synthesis for the first 12 hours of culture, suggesting that this represents the maximum duration of \(G_1\) (Fig. 3). Assuming that this procedure labeled all the large lymphoid cells in cell cycle, the slope of the labeling index curve indicates that from zero to 100 per cent labeling of the cycling cells would take 16.7 hours, i.e., the portion of the cell cycle occupied by the synchronized cells in the marrow fractions was approximately 16.7 hours long. This figure is compatible with the observed duration of the subsequent mitotic wave (Fig. 4). Because \(G_1\) accounted for approximately 12 hours, the DNA-synthesizing cells occupied initially the first 4.7 hours of \(S\) phase. This value may be obtained by extrapolating the labeling index curve back to the baseline. The remainder of \(S\) phase was approximately 9 hours, after which cells began to leave \(S\) phase in significant numbers as indicated by the onset of a plateau in the \(^3\)H-thymidine pulse labeling index (Fig. 3). Again, this figure is generally compatible with the rise of the mitotic index curve (Fig. 4) and with the appearance of labeled mitoses after continuous \(^3\)H-thymidine labeling, except for a relatively small proportion of cells which were apparently in advance of the main wave. With these exceptions, therefore, the total duration of \(S\) phase was in the order of 13.7 hours. On the basis of a \(G_1\) phase of 12 hours and an \(S\) phase of 13.7 hours a total cell cycle time of approximately 28–29 hours may be postulated. Under the in vitro experimental conditions it is probable that these cell cycle times, especially the duration of \(S\) phase, are significantly longer than the corresponding values in vivo. Large marrow lymphoid cells (nuclear diameter greater than 9.0 \(\mu\)) have been found to show a mean grain count halving time of 18 hours following a single administration of \(^3\)H-thymidine to guinea pigs. Further studies are necessary for the more detailed characterization of the in vivo cell cycle times of marrow lymphocyte precursors.

One of the practical difficulties in elucidating the proliferative characteristics of large lymphoid cells has been that of identifying them in mitosis in normal marrow, although large numbers of such mitoses occur in guinea pig marrow during recovery from sublethal whole-body irradiation. Lymphocyte-rich fractions provide a suitable opportunity for detecting these mitoses in normal marrow because of the paucity of dividing cells other than large lymphoid cells, together with the partial synchrony of the separated large lymphoid cells in culture. In the present experiments, all stages of mitosis were observed from 9 to 27 hours of culture, metaphases being most common (Fig. 5D). They exhibited thick clumps of chromosomes, and varying degrees of cytoplasmic basophilia and were characterized by a relatively small volume of cytoplasm throughout mitosis.

The viability of marrow cells recovered from sucrose-serum density gradients, previously demonstrated by exclusion of trypan blue, radioautographic studies of RNA and protein synthesis, and response to blastogenic stimuli, is further attested to by the present studies. The preparation of suspensions of

\[x - \frac{y}{t}\text{ per cent per hour where, } x = \text{maximum labeling index } (\%), y = \text{initial labeling index } (\%), t = \text{time between } x \text{ and } y (\text{hours}).]
SMALL LYMPHOCYTE PRECURSORS IN BONE MARROW

viable marrow cells which may be either enriched or depleted of lymphocytes provides a suitable technique for use in a variety of experimental systems designed to further elucidate the production and functional potentialities of marrow lymphoid cells.

ACKNOWLEDGMENTS

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REFERENCES


5. ——: Lymphocyte production in the bone marrow: radioautographic studies in polycythaemic guinea pigs. In J. M. Yoffey (Ed.): The Lymphocyte in Immunology and Haemopoiesis. London, Edward Arnold, 1967, p. 120.


21. Morris, N. R., Cramer, J. W., and...

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