Morphologic and Autoradiographic Observations of H\(^3\)-Thymidine-Labeled Thoracic Duct Lymphocytes Cultured in Vivo

By John C. Schooley and Irwin Berman

The in vivo method of tissue culture using cellulose ester filter diffusion chambers developed by Algire and co-workers\(^1\) combined with the autoradiographic method of observing cells labeled with the specific DNA precursor, tritiated thymidine, has been used in the present experiments to study lymphocytopoiesis in an isolated population of lymphoid cells. In addition, data are presented concerning the transforming abilities of thoracic duct cells when cultured in the diffusion chambers, either separately or combined with bone marrow cells.

Methods

The modification of Algire's diffusion chambers described by Berman and Kaplan\(^2\) was used in these experiments. The chambers were constructed of Lucite rings (0.75 inch outside diameter, 0.5 inch inside diameter, 1/16 inch thick) which served as supports for Millipore filter membranes (obtained from Millipore Filter Corp., Watertown, Mass.). The membranes (Type HA, 150 \(\mu\) thick, average pore diameter 0.45 \(\mu\)) were sealed to the Lucite rings with a solution of 1 per cent Lucite in acetone. The chambers were sterilized with ultraviolet light before use.

Sprague-Dawley rats were used as donors and hosts. In separate experiments C57Bl mice were used. Lymph was collected from the thoracic duct at its entrance into the jugulo-subclavian veins as previously described.\(^3\) Approximately \(3 \times 10^6\) thoracic duct lymphocytes were introduced into each chamber, and the filter membranes were sealed to both sides of the Lucite rings. The chambers were placed in the peritoneal cavity of animals under ether anesthesia through a small incision on the midventral surface. Usually only one chamber was placed in each recipient, although in one series several chambers were introduced into the peritoneal cavity of one rat to compare the behavior of cultured H\(^3\)-thymidine-labeled and normal unlabeled lymphocytes. In one rat series, lymph collected from an intestinal lymphatic was placed in the chamber, and the chamber was introduced into the peritoneal cavity of the same animal after leakage from the lymphatic used for lymph collection had ceased.

Labeled thoracic duct lymphocytes were obtained from donor rats and mice about 6 to 8 hours after the intravenous injection of H\(^3\)-thymidine (1 \(\mu\)c per gram body weight, 360 mc. per millimole, Schwarz Lab.). Nonlabeled bone marrow was obtained from rats or mice by gently suspending the femoral marrow in modified Locke's solution. The marrow suspension was then mixed with labeled thoracic duct lymphocytes and placed in the chambers. This inoculum contained about \(6 \times 10^6\) cells, lymphocytes comprising about one-half of the inoculated cells.

At various times after implantation the recipient animals were killed, the chambers removed and their surfaces cleared of adherent host cells. In some instances the host animals were injected intraperitoneally with a 1 per cent trypan blue-saline solution.

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several hours before the chambers were removed. The Millipore membranes were then cut from the rings and fixed in Bouin's fluid or buffered formalin. Serum smears were made of the fluid within the culture chambers and were fixed in methyl alcohol. Cover-slip cultures of the cells found in this fluid were also examined. Autoradiographs were made of the smears and of many of the membranes using Kodak Ltd. AR-10 stripping film. The technic of autoradiograph preparation was similar to that of Pelc. The films were exposed in the cold for 30 days, developed, fixed and stained with dilute Giemsa at pH 6.8. The membranes were treated like tissue sections and stained with hematoxylin and eosin.

Observations

Behavior of Cultured Thoracic Duct Lymphocytes

The combined in vitro and in vivo conditions present in the diffusion chambers within the peritoneal cavity allow excellent growth of thoracic duct lymphocytes for at least 4 or 5 days, if not longer. Before discussing the behavior and morphologic appearance of cultured cells, some remarks regarding the cellular composition of the initial inoculum of thoracic duct lymph are pertinent. In our experience almost all of these cells in both rats and mice are mononuclear. Observations of living cells in cover-slip cultures and in stained smears indicate that practically all of these mononuclear cells are typical lymphocytes. Macrophages and monocytes are, however, occasionally found and their presence in the initial inoculum seems certain. Typical mature plasma cells and reticulum cells are not observed in normal animals. The differential size distribution of thoracic duct lymphocytes, based on measurements to the nearest micron of the nuclear diameters of cells found in smears made with serum or in smears made when thoracic duct lymph is mixed with leukocyte-free whole blood, is shown in figure 1. The nuclear diameters range as a continuum from 4 to 12 μ or greater.

![Graph](null)

**Fig. 1.—**Differential size distribution of thoracic duct lymphocytes based on measurements to the nearest micron of the nuclear diameters. Lymph + serum based on measurements of about 10,000 cells and lymph + blood on 5000 cells. (Data of J. C. Schooley and L. S. Kelly)
presence of red cells produces a shift in the size distribution, and more larger cells are found. No modal distribution of cell sizes occurs in either type of preparation; and any separation into different size categories is necessarily arbitrary. Serum smears have been used in these experiments so that more reliable comparisons can be made with data obtained previously from serum smears of cellular suspensions of lymph nodes. Morphologically little difference is observed in those cells having a nuclear diameter of 9 $\mu$ or less, although there is some tendency for the smaller cells to have pachychromatic nuclei, whereas the larger cells have more leptochromatic nuclei. If the morphologic descriptions given by Sundberg and Sundberg and Downey are utilized, many of the remaining lymphocytes having a nuclear diameter equal to or greater than 10 $\mu$ are considered to be reticular lymphocytes. Some of the cells within this size range, especially those having a nuclear diameter of 10 or 11 $\mu$ are typical large lymphocytes. Some of the largest cells within this group with intensely basophilic cytoplasm may be plasma cell precursors. The morphologic characteristics which permit a separation into these different cell types are not striking, and often their similarities are greater than their differences; therefore, we have for convenience termed all of these larger cells large lymphocytes.

A considerable number of cultured thoracic duct lymphocytes can be observed in mitosis during the first 4 days of cultivation (fig. 2a, c). Most of these dividing cells are of the larger variety; however, what appears to be a dividing small lymphocyte has occasionally been observed (fig. 2b). The differential distribution of cell sizes found in smears of cells living within the diffusion chambers does not change much during the first four days. There is, however, a slight but steady increase in the percentage of pyknotic nuclei observed. It is our impression, although no adequate method of quantitation is available, that during this time the number of cells in the fluid within the chambers does not appear to change significantly. Commencing with day 4, an increasing number of pyknotic nuclei, cells undergoing karyorrhexis, and increasing amounts of nuclear debris are observed (fig. 2c–h). The superficial resemblance between those cells undergoing karyorrhexis and early neutrophils suggests that careful evaluation of experiments which propose a transformation of lymphocytes into neutrophils is necessary. In some instances, mitotic figures have been observed in cultures over two weeks old; however, large numbers of dying or dead cells were also found. In addition, a network of fibroblastic cells has been observed in some of these older cultures (fig. 2l). It is conceivable that these fibroblasts were the result of a lymphocyte transformation; however, it is equally possible that they are simply the result of rapid proliferation of some other cell present in the initial inoculum.

Isotransplanted mouse thoracic duct lymphocytes did not appear to live longer than homotransplanted rat thoracic duct lymphocytes; however, cultures of mouse lymphocytes were more often contaminated with bacteria, probably because of the technical difficulties involved in the collection of an initial sterile inoculum of mouse lymph. Autotransplanted rat lymphocytes did survive for several days longer than either the rat homotransplants or
Fig. 2.—See legend, facing page.
H^-THYMIDINE-LABELED THORACIC DUCT LYMPHOCYTES

mouse isotransplants. Preliminary observations indicate that homotransplants of suspensions of lymph node cells survive even longer, possible because of the presence of reticulum cells in the initial inoculum. The increasingly poor nutrient conditions within the diffusion chambers brought about by the growth of host cells on the exterior of the Millipore filter undoubtedly adversely affect the growth of the cultured cells. Gregoire7 has observed that the lymphoid elements of thymus cultivated in vivo rapidly die and the cultures become composed of epithelial cells. Williams et al.8 have observed that extensive death of the mature cells of transplants of thymus within the anterior chambers of the eyes of mice occurs progressively for 2 to 3 days after transplantation. The remaining immature types of cell proliferate, and by 20 days the transplants may consist of mature lymphoid cells. The mature lymphocytes of thoracic duct lymph appear to survive no longer in the in vivo conditions of the Millipore cultures than in in vitro conditions.9 Perhaps some substance which cannot pass through the Millipore filters, the presence of other cell types, or a particular arrangement of the cells comprising the tissue, is necessary for the continual development of thoracic duct lymphocytes.

Autoradiographic Observations of Cultured H^-Thymidine-Labeled Thoracic Duct Lymphocytes

The basic premises involved in the use of labeled thymidine are: (a) thymidine is a specific precursor for DNA, and is available for only a short time after injection; (b) thymidine is incorporated into DNA only at the time of its synthesis in preparation for cell division, and the activity is distributed to the daughter cells at mitosis; (c) in a nondividing cell, formed from a labeled cell, the activity is stable for the life of the cell; and (d) radiation damage produced by the intranuclear irradiation is negligible at the dosage used. These and other problems relevant to the use of cellular labels have been discussed at a recent conference.10 Johnson and Cronkite11 report that an intraperitoneal dose of 1 μc. per gram of tritiated thymidine in mice produces an effect on spermatogonia comparable to that produced by 5 r of acute whole-body gamma irradiation. The behavior of cultured thoracic duct lymphocytes labeled with H^-thymidine was similar to that described for unlabeled cells. In some labeled cultures a few aberrant mitotic figures

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Fig. 2.—Photomicrographs of cells on the surface of the Millipore filter. Hematoxylin and Eosin, magnification 1000x.

a. Normal appearance of cultured thoracic duct lymphocytes; 1 day culture; mouse. b, Division of small lymphocyte; 1 day culture; mouse. c. Mitotic figure, metaphase; 3 day culture; rat. d, Aberrant mitotic figure, anaphase; 4 day culture of labeled thoracic duct lymphocytes; rat. e, f, g, h, Abnormal cells undergoing karvorrhexis (f and g, 4 day culture, mouse; h, 4 day culture, rat; e, 7 day culture, rat). i, Aberrant mitotic figure, metaphase; many monocytoid cells; 3 day culture of labeled thoracic duct cells; rat. j, Reticulum cell?; 1 day culture; mouse. k, Monocytoid cell, prophase in large reticular lymphocyte?; 3 day culture; rat. l, Fibroblasts; 9 day culture; mouse.
were found (fig. 2d, i), whereas none was seen in cultures of unlabeled cells. However, too few mitotic figures were examined to decide whether the rare abnormal ones were the result of radiation damage. It is assumed that these aberrant mitoses occur too rarely to affect significantly the following studies of lymphocytopoiesis.

The presence and temporal pattern of appearance of tritiated thymidine labeled mononuclear cells in the blood, marrow and lymph has been reported for humans and mice, dogs, guinea pigs and rats. In previous experiments an attempt was made to determine the generation times and mean-life of the various classes of lymphocytes by analyzing the temporal pattern of labeling of these cells following a single injection of labeled thymidine in mice. In the in vivo experiments a rapid decrease in the percentage of labeled large and medium lymphocytes occurred in lymph nodes, lymph, blood and thymus during the first week. It was assumed that this decrease was primarily the result of cellular division and subsequent passage of the larger cells into the smaller size categories. It was not possible to assess to what extent cell migration into or out of the observed population or reticulum cell lymphocytogenesis influenced these observed changes in the labeling pattern. These factors are absent in cultures of thoracic duct lymphocytes, and any changes observed in the temporal pattern of labeling are the result of homoplastic lymphopoiesis or cell death.

The temporal pattern of labeling in cultured lymphocytes during the first four days is summarized in figure 3. The cells were separated into categories on the basis of nuclear diameters; small, medium and large lymphocytes having, respectively, nuclear diameters of 7 μ or less, 8 or 9 μ and 10 μ or greater. Cells having less than 3 silver grains above representative background areas were arbitrarily considered to be unlabeled. At least 1000 small, 200 medium and 100 large lymphocytes were counted at each time interval.

![Graph](image)

Fig. 3.—Temporal pattern of labeling of lymphocytes present in cultures of rat thoracic duct lymph.
The decrease in the percentage of labeled large and medium lymphocytes is similar to that found in vivo in the mouse and rat except that detectible labeled cells disappeared from the cultured population more rapidly. Presumably this is because there are no labeled stem cells present whose divisions would continually add labeled large lymphocytes to the population. It must be re-emphasized that these larger cells are only a very small part of the total lymphocyte population. The relative importance of the labeled small, medium and large lymphocytes is shown more clearly in figure 4, in which the relative number of labeled cells in each size category per thousand lymphocytes counted is given for the initial inoculum and after one, two and three days of cultivation. Increasing numbers of labeled small lymphocytes are observed during growth of the culture, whereas the number of labeled medium and large cells decreases. This increase in labeled small lymphocytes could be the result of either selective death of older unlabeled small lymphocytes or of formation of labeled small cells by division of labeled large and medium cells. A large number of unlabeled small lymphocytes must die in order to account for the doubling in the relative number of labeled cells present in the two-day culture when compared to the initial inoculum. Since no really great difference apparently occurred in the total number of cells in the chambers during this time, it is concluded that small lymphocytes do arise from division of medium and large lymphocytes.

Despite the many unknown variables of autoradiography, it seems reasonable to conclude that the number of silver grains observed above a labeled cell is proportional to the amount of activity in that cell. The decrease in the mean grain count of a population of labeled cells with time should be a measure of the generation time of those cells, assuming that no further activity can enter the observed population.

The mean grain count of the labeled cells found in the different size categories of lymphocytes is given in table 1. The half-times of the decrease in mean grain counts indicates that the maximum generation times of large and medium lymphocytes are about 15 and 24 hours, respectively. Similar calculations for lymph nodes of rats in vivo (unpublished data of J. C. Schooley and L. S. Kelly) give values of about 12 hours for large and medium lymphocytes; however, in this case further activity may enter the population by division of labeled stem cells, and migration into and out of the population can occur. The mean grain count of the small lymphocyte increases

<table>
<thead>
<tr>
<th>Time</th>
<th>Large</th>
<th>Medium</th>
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<tr>
<td>6 hour</td>
<td>64.2</td>
<td>41.7</td>
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<td>1 day</td>
<td>27.5</td>
<td>24.9</td>
<td>9.5</td>
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<td>2 days</td>
<td>10.8</td>
<td>15.1</td>
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<td>3 days</td>
<td>0</td>
<td>0</td>
<td>13.3</td>
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<tr>
<td>4 days</td>
<td>0</td>
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Large lymphocytes, >10μ nuclear diam.
Medium lymphocytes, 8-9μ nuclear diam.
Small lymphocytes, 7μ or < nuclear diam.

Fig. 4.—Relative number of labeled cells observed per thousand lymphocytes counted in the initial inoculum of rat thoracic duct lymphocytes and after 1, 2 and 3 days of cultivation.

during the life of the culture. This fact also argues in favor of the production of small lymphocytes from larger lymphocytes.

The Question of Transformation

Numerous investigators have suggested that the small lymphocyte is a primitive cell capable of transforming into a wide variety of other cell types such as monocytes, macrophages, fibroblasts, plasma cells, myeloid and erythroid cells. Trowell has recently reviewed this controversial subject. Bloom initially described the transformation of thoracic duct lymphocytes into monocytes and macrophages, but other workers have not been able to repeat these observations. Medawar carefully repeated Bloom's experiments and concluded that such transformations occurred only when the initial inoculum contained a large number of monocytes. Trowell has observed in cultured rat lymph nodes that after 4 days many of the small lymphocytes resemble monocytes. Interestingly, he observed this change only among these
cells in the reticular tissue network. Reticulum fibers were not observed in the present thoracic duct lymph cultures even though there was some suggestion that a few of the larger reticular lymphocytes had become even less mature (fig. 2f). Increased numbers of monocytoid cells were observed on the surface of the Millipore filters of lymph cultures after 2 days (fig. 2i, k). Examination of living cells in cover-slip cultures and stained smears of the cells taken from the fluid within the culture chambers of animals which had been injected with trypan blue several hours before sampling demonstrated the presence of only a few cells having the ability to segregate this colloidal dye. Cells having this ability were more frequently observed on the surface of the Millipore filter. Possibly the surface of the filter substitutes for a reticulum network and permits a transformation of lymphocytes to monocytes. Comparisons of the grain count of the few monocytoid cells found in smears of the chamber fluid would indicate that many of these monocytoid cells had been derived from large or medium lymphocytes rather than from small lymphocytes.

Lymphocytes must, undoubtedly, be exposed to the proper milieu before transformations to any other cell type can occur, assuming that such transformations do occur. In some experiments labeled thoracic duct lymphocytes were mixed with unlabeled bone marrow and were cultured. It was hoped that the conditions in these cultures would simulate those occurring normally in marrow and would promote the metamorphosis of labeled lymphocytes into other marrow cells, a possibility which has often been suggested. The appearance of labeling in marrow cells can be interpreted as evidence for transformation only if reutilization of labeled DNA fragments, released from dying lymphocytes, does not occur. Although this possibility, which has been suggested by some workers, cannot definitely be excluded, various observations argue against such a process in these cultures. During the life of these cultures no evidence of phagocytosis of labeled or unlabeled fragments of DNA by any cell was observed. Labeling of the youngest cells declined rapidly, and no later increase in the labeling of these cells was observed. Reutilization of the labeled nucleotides released from degradation of DNA might occur, but this activity would be available to all DNA synthesizing cells of the culture, and therefore its detection autoradiographically would be minimal.

Over 40 labeled plasma-like cells were found in these mixed cultures after 12 hours of cultivation. All the other labeled cells present in these cultures were morphologically indistinguishable from the cells of the initial inoculum (fig. 5m). The temporal pattern of labeling of the nonplasma cells was essentially similar to the pattern observed when labeled thoracic duct cells were cultured alone. The plasma cells were large with eccentric nuclei and a considerable amount of flaky, quite basophilic cytoplasm (fig. 5n, o). A perinuclear clear area and vacuoles in the cytoplasm were often observed. The nucleus of these cells was identical to that of typical small lymphocytes. The large number of silver grains over these cells often obscured the nuclear detail. In some cases coordinates of the labeled cells were taken, the auto-
Fig. 5.—Photomicrographs of autoradiographs of labeled cells found in initial inoculum of thoracic duct lymph (m) and of a typical plasma cell found in smears from cultures of labeled thoracic duct lymphocytes and unlabeled bone marrow after 36 hours. n is focused on the silver grains of the emulsion, and o is focused on the cell. m, 500×; n and o, 1100×.

radiographic film removed, and comparisons made with plasma cells present in antigenically stimulated lymph nodes. The labeled plasma cell such as shown in figure 5o was identical to the plasma cells of this size found in the stimulated nodes. The more mature labeled plasma cells observed in 60 hour cultures were generally smaller, and the cartwheel nucleus was not as pronounced as in the stimulated node. Comparisons of the grain counts of labeled plasma cells with labeled cells of lymph would favor a direct origin from the larger cells of lymph rather than from the small lymphocyte. The identity of these larger cells of lymph has already been discussed, and it was noted that many of these cells might be plasma cell precursors. Reinhardt and Yoffey20 have observed such cells in guinea pig thoracic duct lymph and have come to a similar conclusion. Apparently conditions exist within these mixed cultures which supply the appropriate stimulus for the transformation or development of these plasma cell precursors of lymph into mature plasma cells. It is not known whether this stimulus is the result of the presence of marrow cells or the antigenic nature of these cultures. Plasma cells were not observed in cultures of thoracic duct lymph to which bone marrow had not been added. Future work using histocompatible donor animals is necessary to determine more specifically the nature of this stimulus and the precise cell types involved in this transformation.

Summary

1. The behavior of mouse and rat thoracic duct lymphocytes cultivated in diffusion chambers implanted into the peritoneal cavity of recipient mice and rats has been described.

2. The temporal pattern of labeling of cultured thoracic duct lymphocytes labeled with H²-thymidine has been described. From an analysis of this pattern and the changes in the mean grain count of the different classes of lymphocytes a maximum generation time for large and medium lymphocytes
of 15 and 24 hours has been calculated. The results of these experiments favor an origin of small lymphocytes from the division of large and medium lymphocytes.

3. Some evidence for the transformation of thoracic duct lymph cells into monocytoid cells was found. In homologous cultures of labeled thoracic duct lymph cells and unlabeled bone marrow apparent evidence for transformation of labeled cells into plasma cells was found. The data suggest that neither the monocytoid cells nor the plasma cells arose necessarily from small lymphocytes. It was concluded that some unidentified cells, presumably the largest cells which are normally present in thoracic duct lymph, can be transformed into these other cell types when appropriately stimulated.

SUMMARIO IN INTERLINGUA

1. Es describite le comportamento de lymphocytos prendite ab le ducto thoracic de muses e rattos e cultivate in cameras de diffusion que esseva implanitate in le cavitate peritonee de recipiente muses e rattos.

2. Es describite le progressive incorporation de thymidina $^3$H in tal culturas in vivo. Per medio de un analyse del modo de progresso de ille incorporation e del alterationes in le numeration medie del granos in le varie classes de lymphocytos, maximos de 15 e 24 horas esseva calculate pro le tempore de generation de lymphocytos grande e intermediari, respectivemente. Le resultatos de iste experimentos supporta le theoria del origine de micre lymphocytos ab le division de lymphocytos grande e intermediari.

3. Esseva trovate indicios de untransformation de lymphocytos ab le ducto thoracic a in cellulas monocytoide. In culturas homologe de marcate lymphocytos ab le ducto thoracic con non-marcate medulla ossee, indicios esseva trovate de un transformation de marcate cellulas a in plasmocytos. Le datos pare indicar que ni le cellulas monocytoide ni le plasmocytos habeva lor origine necessarimente in le micre lymphocytos. Esseva concluidite que certe non-identificate cellulas, probablemente le plus grande cellulas que es normalmente presente in le lympha del ducto thoracic, pote—in le presentia de appropriate stimulos—transformar se a in le altere typos de cellulas mentionate.

REFERENCES

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**IN VITRO INCORPORATION OF **$^{32}P$** INTO NUCLEIC ACIDS OF LYMPHATIC CELLS.**


$^{32}P$-orthophosphate is incorporated into both types of nucleic acids in vitro, and even isolated nuclei from rabbit appendix retain some activity in incorporating it. In the present studies, intact free lymphatic cells and Ehrlich ascites carcinoma cells were used to determine the nature of inhibition of DNA synthesis by noxious agents such as arsenate, X-rays and hypertonic sucrose solution. The inhibition for DNA was sometimes much more severe than that for RNA. In lymphatic cells, X-irradiation or hypertonic sucrose produced a moderate inhibition of incorporation into both nucleic acids. In Ehrlich carcinoma cells the effect of these two agents was not remarkable and probably not specific to nucleic acids.—O. P. J.
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