STUDIES ON ANTIBODY-PRODUCING CELLS

III. Identification of Young Plaque-Forming Cells by Thymidine-^{3}H Labeling*

By FRED G. GUDAT,‡ M.D., T. N. HARRIS,§ M.D., SUSANNA HARRIS, PH.D. and KLAUS HUMMELER, M.D.

(*From The Children's Hospital of Philadelphia, Philadelphia, Pennsylvania 19146, and the Department of Pediatrics, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104*)

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Earlier studies of the ultrastructure of antibody-producing cells, identified by their production of hemolytic antibody plaques (1, 2) or rosettes (2) with the antigenic sheep red blood cells (SRBC),[1] showed morphologic forms of both the lymphocytic and plasmacytic categories among both plaque-forming cells (PFC) and rosette-forming cells (RFC). The presence of cells of both categories among these has also been reported in other studies (3–7). In the second paper referred to (2), in which considerably greater numbers of cells were examined, some of the large lymphocytes contained a few short, narrow channels of endoplasmic reticulum (ER), which showed some degree of parallel orientation. The width of these channels was fairly constant and barely enough to detect some precipitated material within them. These cells were classified as transitional lymphocytes and were found among both RFC and PFC. Avrameas and Leduc (8) have also recently described apparently intermediate forms between the lymphocytic and the plasmacytic categories among antibody-producing cells.

The finding of cells which are actively synthesizing antibody in two morphologically designated categories poses the question of whether there are two cell lines, each with the faculty of synthesizing antibody, or whether all of these cells are of one line, the morphologic differences reflecting differences in development or differentiation, or variations in storage of antibody in addition to synthesis. The present paper describes an approach to this question: to identify the youngest antibody-producing cells among those which could be isolated, by labeling

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‡ Present address: Pathologisches Institut, University of Basel, Basel, Switzerland.

§ Reprint requests should be sent to Dr. T. N. Harris at The Children's Hospital of Philadelphia, 1740 Bainbridge St., Philadelphia, Pa. 19146.

[1] *Abbreviations used in this paper:* ER, endoplasmic reticulum; PFC, plaque-forming cells; RFC, rosette-forming cells; SRBC, sheep red blood cells.

with thymidine-^{3}H in vivo, and to see whether the distribution of these young cells between the lymphocytic and plasmacytic categories differed from the distribution which we have described in the entire population of antibody-producing cells identified by the same method.

Materials and Methods

Animals, Immunization, and Labeling with Thymidine-^{3}H.—Female BALB/c mice (The Jackson Laboratory, Bar Harbor, Maine) were injected with 0.1 ml of a 20% SRBC suspension subcutaneously into both forelegs. On the 4th day, three injections, each of 10 μCi of thymidine-^{3}H, (New England Nuclear Corp., Boston, Mass., Lot 444-134, specific activity 16.1 Ci/mM) were given by the same route at intervals of 2 hr. The animals were sacrificed 2, 24, or 48 hr after the last injection. Single cell suspensions of the brachial lymph nodes of three mice per group were tested for hemolytic plaque formation. For the in vitro labeling 0.5 ml of 50% SRBC were injected into both hind footpads of rabbits. Politeal lymph nodes were excised on the 5th day, and single cell suspensions in concentration of 40×10^6 cells/ml were incubated for 60 min at 37°C in 1 ml of Eagle's minimal essential medium containing 20 μCi of thymidine-^{3}H. After two washings the cells were ready for the hemolytic plaque test.

Plaque Formation and Electron Microscopy.—The hemolytic antibody plaque technic (9, 10) was modified, on the basis of a recent adaptation for thin plating (11), to yield a sufficiently thin layer of SRBC in agarose for collecting individual plaque-forming cells with braking pipettes. After preliminary tests by the usual plating procedure to determine the number of PFC/10^6 cells, the lymph node cells were suspended at a concentration which would allow for the loss of PFC in the thin-plating procedure. Of this suspension 0.6 ml was mixed with 1 ml of 0.9% agarose (L'Industrie Biologique Francaise S.A., Gennevilliers, France) containing 12.5% SRBC. This was spread on the hardened agar layer in the plate and the excess then poured out, with an additional flip of the wrist, leaving only the adherent layer of agar. After incubation with complement, single cells at the center of plaques were picked with a braking pipet, under 100 magnification using a dissecting microscope, and transferred to the embedding capsules. Sections were processed for radioautography as described in detail elsewhere (12).

RESULTS

In Vivo Labeling Experiments.—

PFC labeled on day of sacrifice: Lymph node cells were obtained from mice on the same day as the injections of thymidine-^{3}H, 4 days after the injection of SRBC. Of the PFC isolated for electron microscopy, 26 were found to be labeled. Of these, 17 were large lymphocytes and nine were plasmablasts (Table I). No mature plasma cells were seen. The 17 lymphocytes included five of the classical, least-differentiated type, shown in Fig. 1, and 12 transitional lymphocytes, which we have observed among RFC and PFC (2), as described in the introduction. A cell of the latter type is shown in Fig. 2. The remaining nine cells were plasmablasts with low levels of differentiation. The most highly differentiated of these is shown in Fig. 3.

PFC labeled 1 day before sacrifice: In other experiments, mice were given thymidine-^{3}H on the same schedule of injections, relative to the time of antigen in-

TABLE I

Electron Microscopic Classification of Thymidine-^{3}H-Labeled Plaque-Forming Cells of Mouse Lymph Node, Labeled In Vivo

Interval after last injection of thymidine-^{3}H	Large lymphocytes	Transitional lymphocytes	Plasmablasts	Plasma cells	Total
hr					
2	5	12	9	—	26
24	3	7	9	5	24
48	—	6	6	—	12

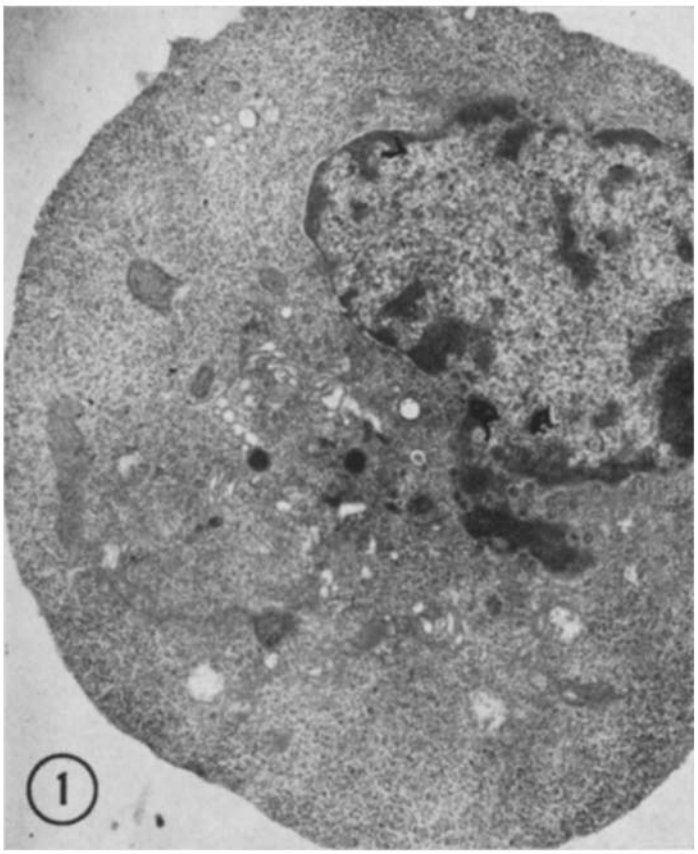

FIG. 1. Labeled PFC, mouse lymph node, 4 days after injection of antigen, obtained day on of injection of thymdine-^{3}H. Lymphocyte with cytoplasm rich in ribosomes and essentially no ER. × 16,800.

jection, but were sacrificed 1 day after the injections of thymidine-^{3}H. Among the PFC obtained from these lymph nodes, 24 labeled cells were examined by electron microscopy. Of these, 10 were lymphocytes and 14 of the plasmacytic category. The lymphocytes included three classical large lymphocytes and seven

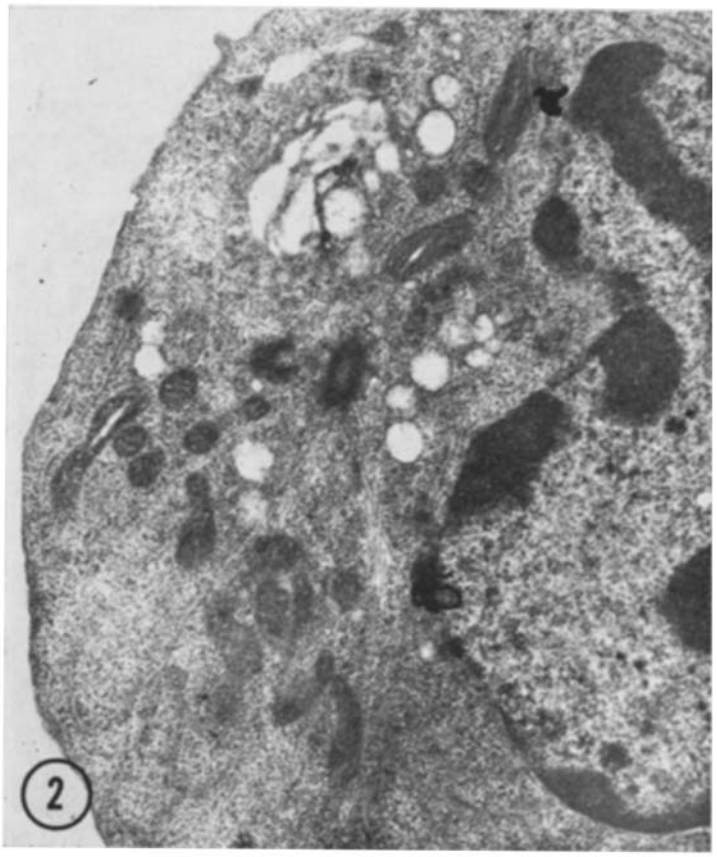

FIG. 2. Same as Fig. 1. Lymphocyte with very few channels of ER. These are relatively short, of constant width, and so narrow as to show either very little precipitated protein or none. × 20,400.

transitional lymphocytes, such as that shown in Fig. 4. Of the plasmacytic cells, 11 were plasmablasts and three were mature plasmacytes (Table I). The plasmablasts found showed relatively advanced levels of differentiation (Fig. 5), markedly greater than the degrees of differentiation found among the labeled cells so classified which had been isolated on the day of thymidine-^{3}H injection (the most advanced of which was shown in Fig. 3). The mature plasmacytes were far

advanced in differentiation, including cells with vesicular ER and with some diffusion of nuclear chromatin (Fig. 6).

A smaller number of experiments was done with mice injected with thymidine-^{3}H on the same schedule but sacrificed 2 days later. Of six labeled PFC isolated, three were transitional lymphocytes and three were plasmablasts.

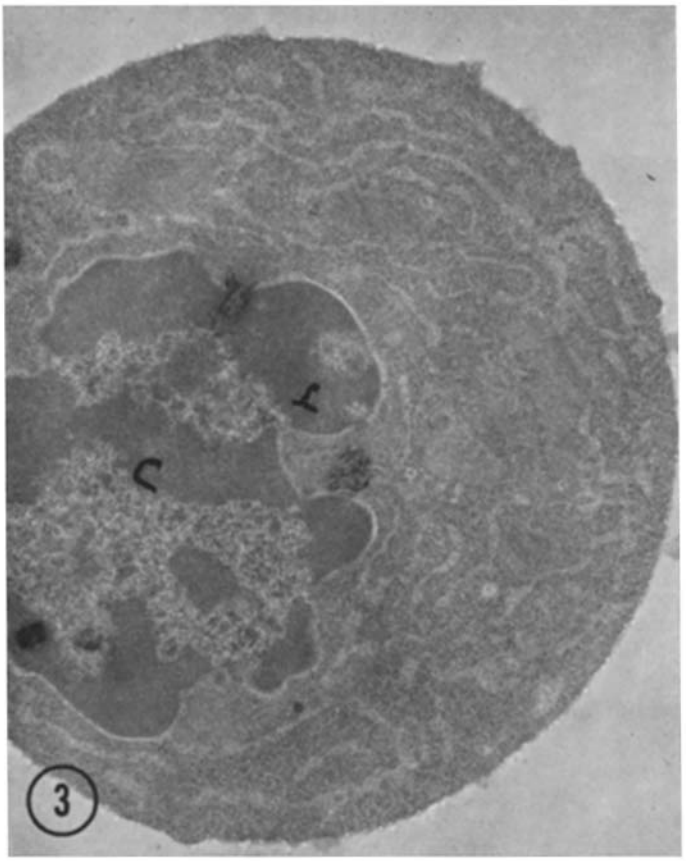

FIG. 3. Same as Fig. 1. Plasmablast. Substantial development of ER. Channels wider, of variable width, containing precipitated material. This is the most highly differentiated of the plasmablasts found on the day of thymidine-^{3}H injection. × 15,800.

In Vitro PFC Labeled.—In one series of experiments, PFC obtained from rabbit lymph nodes were labeled with thymidine-^{3}H in vitro as described above and examined by electron microscopy. A representative experiment of this kind is summarized in Table II. Of 32 PFC, 17 were labeled with thymidine-^{3}H. Four cells were classified as lymphocytes, one a classical large lymphocyte and three

transitional lymphocytes (Fig. 7). The majority of labeled cells were typical plasmablasts, like that shown in Fig. 8. An unexpected finding was that of mature plasma cells, apparently fully differentiated, among these labeled cells. Some of these cells had a narrow cytoplasm with prominent ER, and in some

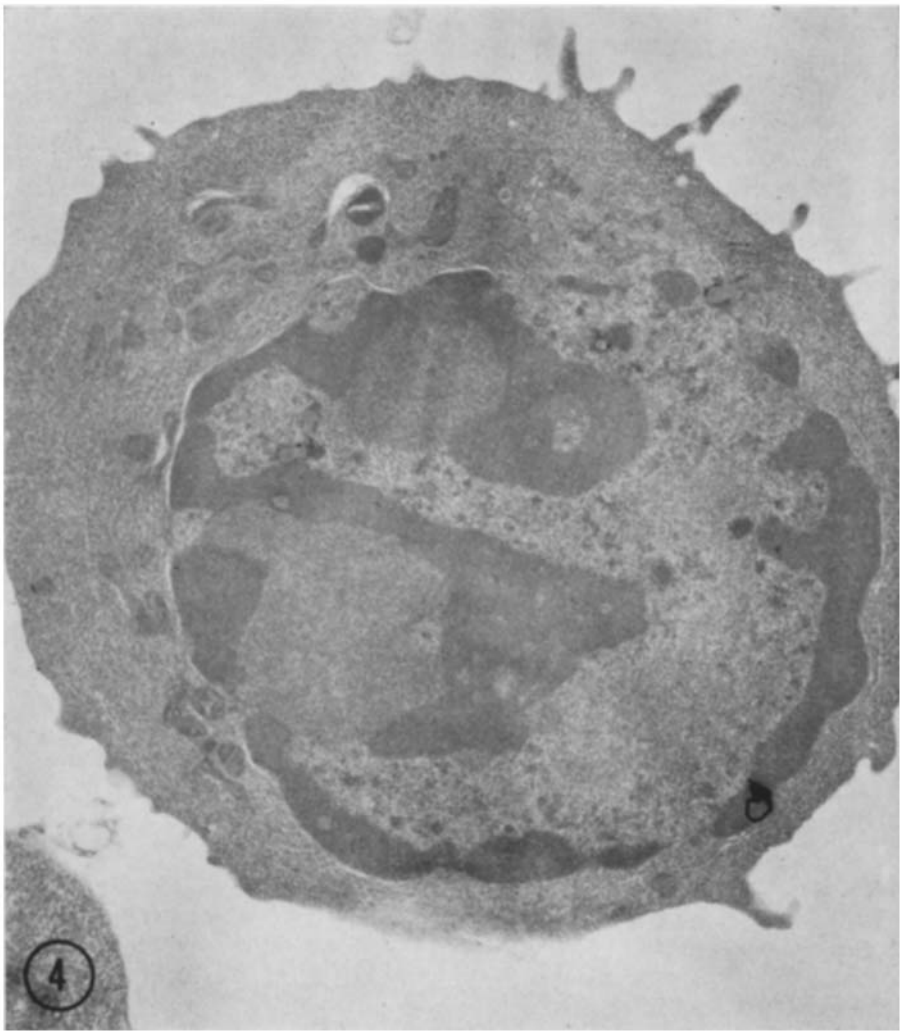

FIG. 4. Labeled PFC obtained on day after injection of thymidine-^{3}H. Lymphocyte with few short channels of ER, of constant width, and in most cases too narrow for detection of precipitated protein. × 14,700.

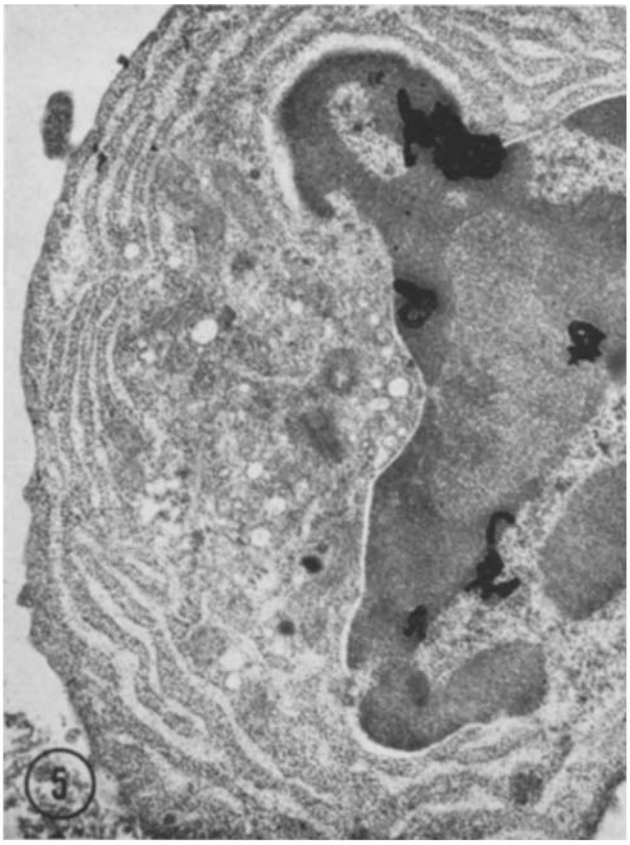

FIG. 5. Same as Fig. 4. Plasmablast in relatively advanced state of differentiation. Channels of ER of substantial and variable width and clear tendency toward parallel orientation. × 18,9000.

cases with loss of definition of nuclear chromatin (Fig. 9). Unlabeled cells from these experiments showed a similar distribution.

DISCUSSION

The Relation of Plasma Cells to Lymphocytes.—The recent observations, in several laboratories, that antibodies can be synthesized and secreted by cells which have been classified morphologically in two categories posed the problem of whether there are one or two cell lines with the apparatus for synthesizing antibody. The present approach to this problem was to label an active population of antibody-producing cells with thymidine-^{3}H and seek labeled cells among

these. The rationale for labeling the youngest cells in a replicating population of antibody-producing cells was that if there are two cell lines, the label should appear in cells of both categories with a frequency generally similar to the frequency of occurrence of the two categories of cells in the entire antibody-produc-

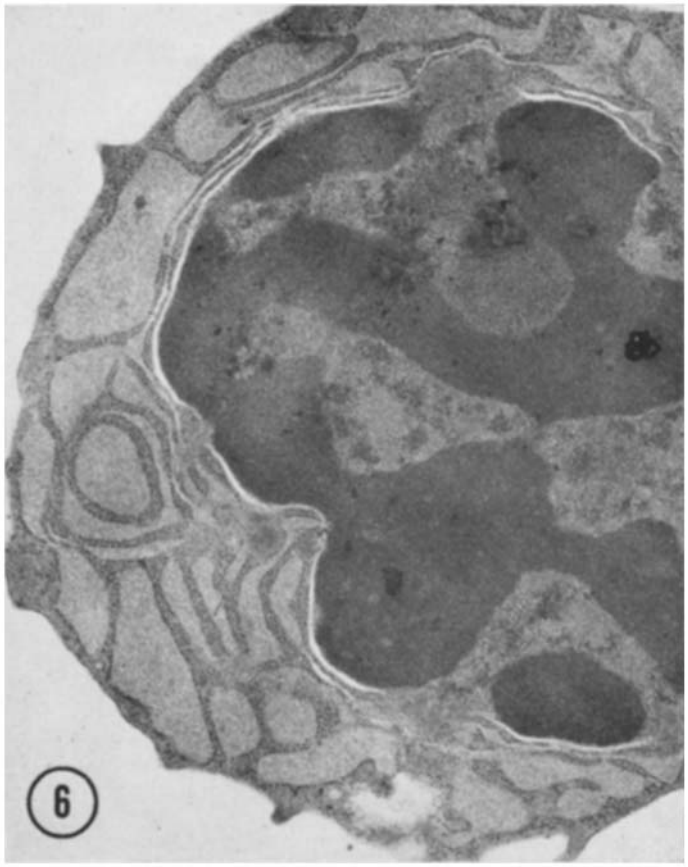

FIG. 6. Same as Fig. 4. Mature plasma cell with vesicular ER. × 17,800.

TABLE II

Electron Microscopic Classification of Plaque-Forming Cells of Rabbit Lymph Node after In Vitro Labeling with Thymidine-^{3}H

	Large lymphocytes	Transitional lymphocytes	Plasmablasts	Mature plasma cells	Total
Unlabeled	3	0	6	6	15
Labeled	1	3	7	4	15

ing population. On the other hand, if there is only one line of antibody producing cells, then one morphologic category should predominate among the labeled antibody-producing cells.

For such experiments two methods of isolating antibody-producing cells were

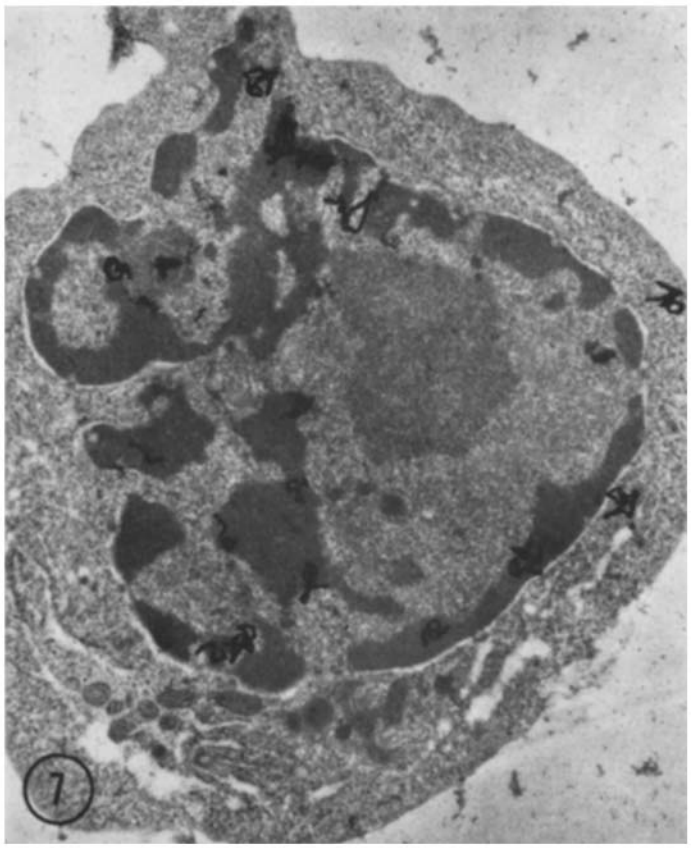

FIG. 7. Rabbit lymph node 4 days after injection of antigen. PFC labeled in vitro with thymidine-^{3}H. Lymphocyte with few short, narrow channels of ER. × 12,900.

available: the formation of rosettes or plaques. In an earlier study of lymph nodes and spleens, we had found the great majority of the RFC to be of the lymphocytic category (2, 12), and the great majority of the PFC to be plasmacytic (2). If there is a direct relation between the cells designated respectively as lymphocytes and plasmacytes, in that one type is directly derived from the other by further differentiation, then the original or basic form of this single cell

line would almost certainly be the less-differentiated lymphocyte. Since it was among the PFC of the lymph node that the lymphocytes had been found to be in the minority, it was necessary to use plaque formation for the isolation of antibody-forming cells in the present study, so that an increase in frequency would be detectable.

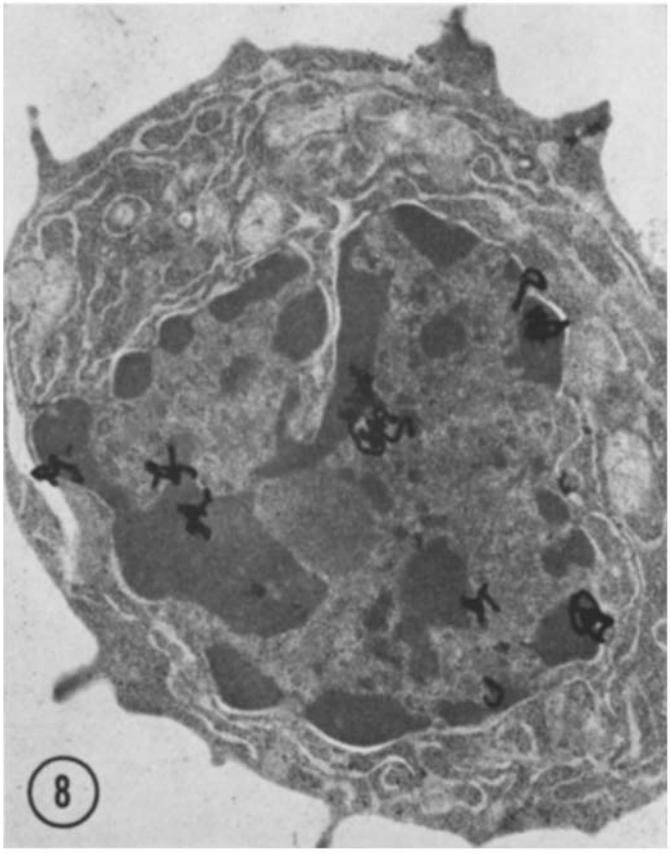

FIG. 8. Same as Fig. 7. Plasmablast with irregular distribution of ER of variable width. × 15,800.

In this study, 65% the labeled PFC isolated from mice on the day of injection of thymidine-^{3}H were of the lymphocytic category, in comparison with the 13% which we found in this category in the entire population of PFC in the lymph node or spleen (2). These data favor the hypothesis of a single line of antibody-producing cells, one form, the original one, being the cell recognized as the lymphocyte, and another more highly or specially differentiated form, the plasma

cell. This suggestion receives further support from the striking shift of these labeled cells in distribution between the categories, and in degree of differentiation within the plasmacytic series, from the day of thymidine-^{3}H injection to 1 day later. Even the degree of differentiation found among the labeled lympho-

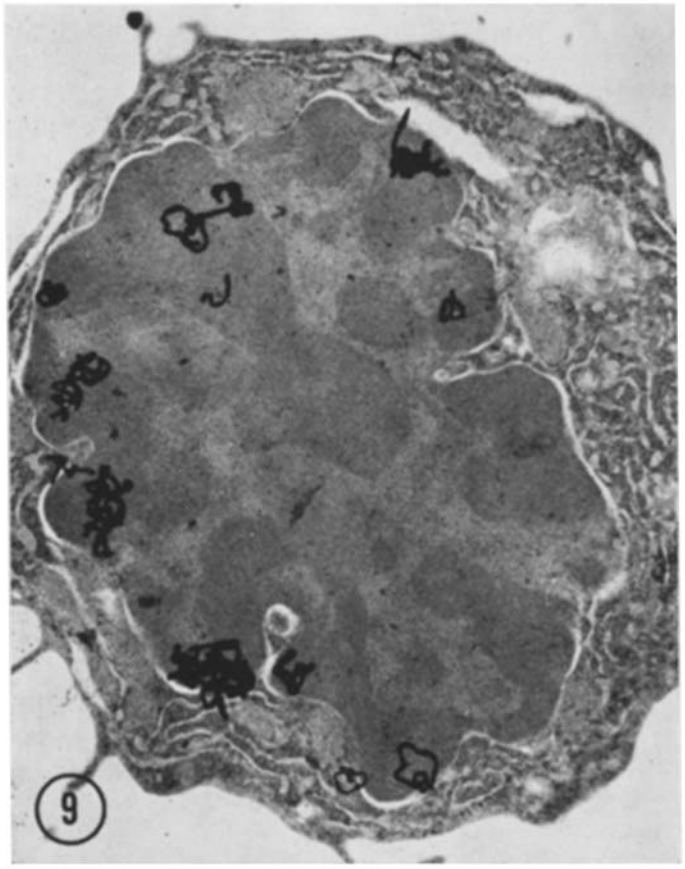

FIG. 9. Same as Fig. 7. Plasma cell, fully differentiated, with ER filling most of the cytoplasm and beginning loss of definition of nuclear chromatin. × 17,800.

cytes on the day of thymidine injection may reflect the beginnings of a differentiation of some antibody-producing lymphocytes toward a plasmacytic form, since the 6 hr between the first injection of thymidine-^{3}H and sacrifice made available the label to some cells during a major part of the entire S phase of a mitotic cycle (13, 14). (The spreading of the thymidine-^{3}H injections over this period was to avoid a "burning out" of antibody-producing cells, a possibility

indicated by the work of Dutton [15]. This would be especially undesirable here since a burning out of the cells which would be taking up the label would cause not only a decrease in the number of PFC but, more important, a skewing of the distribution of such cells, with a disproportionate degree of elimination of precisely the cells of interest in this study.)

If the plasma cell does in fact arise by direct differentiation from the antibody-producing lymphocyte, then the finding of some plasmablasts among the labeled PFC even on the day of injection of thymidine-^{3}H gives evidence of preparation for another mitosis by a cell of the relatively advanced state of differentiation within this system. This presents an interesting parallel to other situations in which a replicating population of cells gives rise to a more highly differentiated end cell, with the cells in the final stage of differentiation still giving evidence of a small number of mitotic divisions possible at that stage. In the reticulocyte-erythrocyte relation, Holtzer (16) has presented evidence of a limited number of mitoses among cells which already contain hemoglobin, and among cardiac myoblasts it has similarly been shown that cells which are already synthesizing myosin may yield a small progeny (17).

The relation between the lymphocyte and plasma cell has been a subject of considerable discussion in the literature, because of the elements of morphologic similarity between the two categories of cells, and of the evidence for the association of each of these cell types with immune processes. On the basis of morphologic forms as found in the entire cell populations of lymph nodes and spleens, there have been generally two schools of thought. One of these regarded each of the categories of cells as arising perhaps from a common ancestor, the reticulum cell, but by separate lines of differentiation, each through its own blast forms (18). The other school, which has developed especially with the use of electron microscopy, has seen some evidence of transition from the lymphocyte to the plasma cell, as in the work of Bessis (19) and Movat and Fernando (20). The present study gives evidence in favor of a relationship of direct differentiation from the antibody-producing lymphocyte to the plasma cell by examination of a functionally selected group of these cells, those involved in the synthesis of antibody. Consistent with this suggestion is the finding, also among antibody-producing cells, identified by specific binding of antigen, of cells of intermediate morphology between lymphocyte and plasma cell, by Avrameas and Leduc (8), and by the reports of lymphocytes with fine gradations of ER from rudimentary to abundant both in number and width of channels. The latter finding has been recorded in both RFC and PFC of lymph nodes and spleens of the mouse and rabbit by Harris, Harris, and Gudat (21) and in cells of efferent lymph emerging from active lymph nodes of sheep by Hall et al. (22). Although the study by Hall et al. was not of cells individually identified as antibody producing, the cells of lymph obtained from the efferent lymphatic vessel of an active lymph node have been shown in earlier work from this laboratory to be particularly rich in antibody production (23).

The Rate of Differentiation of Antibody-Producing Cells.—The distribution and appearance of the plaque-forming cells of mice obtained 1 day after the injection of thymidine-^{3}H are of interest in a different connection. Accepting the label in the cells as an indication that these represent the same population as the labeled cells obtained on the day of thymidine-^{3}H injection (day 0), comparison of the labeled cells obtained on day 1 with those of day 0 (Table I) showed a substantial degree of increased differentiation, which must have occurred within 1 day. The numbers of the cells in the various categories also showed a shift in the direction of more differentiated forms, with fewer lymphocytes, more plasmablasts, and even some mature plasmacytes, of which none had been seen labeled on the day of thymidine-^{3}H injection. In fact, the mature plasma cells found in this group included a cell which was as advanced in differentiation as any antibody-synthesizing cell we have thus far examined (Fig. 6).

The few cells that we were able to isolate and examine of those obtained two days after the injection of thymidine-^{3}H indicated no further degree of differentiation, suggesting that if there is indeed a progressive differentiation from lymphocyte to plasma cell, this can be essentially complete within 1 day.

The In Vitro Labeled PFC.—In the experiment involving in vitro uptake of tritiated thymidine by cells obtained from rabbit lymph nodes engaged in antibody production, the distribution of labeled PFC was different from that of the in vivo labeled cells. Here, of 15 labeled cells found, only four were of the lymphocytic category. Of the plasmacytic cells, the seven plasmablasts were generally of a higher level of differentiation than those plasmablasts which had been found among labeled PFC in the in vivo labeling experiments on day 0, and four of these cells were actually mature plasma cells. In assessing the significance of the difference between this distribution and that of the cells labeled in vivo on day 0, it is pertinent to consider recent reports which have demonstrated a reentry into S phase, with resultant uptake of thymidine in mature cells, as a response to explantation in vitro. This has been shown in chondrocytes by Abbott and Holtzer (24) and by Chako et al. (25). The possibility of such a response to explanation on the part of mature, fully differentiated cells would affect the interpretation of the results of an in vitro study of uptake of thymidine-^{3}H, by placing more mature cells among those labeled. Thus, where any difference is found between the data of such experiments carried out in vivo and in vitro, the in vivo experiment would be considered to be more representative of the normal activity of the cells.

SUMMARY

Mice injected with sheep RBC and then, 4 days later with thymidine-^{3}H, were sacrificed on the day of thymidine-^{3}H injection or 1 or 2 days later. Hemolytic antibody plaque preparations were made of cells from the draining lymph nodes by a thin-plating procedure permitting collection of isolated PFC for electron microscopic examination and radioautography.

Of cells obtained on the day of thymidine-^{3}H injections, 65% of the labeled PFC were in the lymphocytic category, in comparison with 13% found previously in the entire population of such cells. The remaining 35% were plasmablasts in early stages of differentiation. Cells obtained 1 day after the thymidine-^{3}H injections showed a shift to a majority of labeled cells in the plasmacytic category. Also, the plasmablasts were substantially more differentiated than those of the previous day, and some mature plasma cells were now seen. The labeled PFC obtained on day 2 gave no indication of further differentiation.

Cells of rabbit lymph nodes labeled in vitro with thymidine-^{3}H showed a range of labeled PFC. The majority were in the plasmacytic category, including some mature plasma cells.

The data from the experiments with in vivo labeling suggest a direct differentiation from antibody-synthesizing lymphocytes to plasma cells. Further, the in vivo experiments indicated that differentiation from nascent lymphocyte to plasma cell could be essentially completed within 1 day.

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