CYTOLOGY OF IMMUNOLOGIC MEMORY

A Morphologic Study of Lymphoid Cells During the Anamnestic Response*

By CESARE BOSMAN,‡ M.D., and JOSEPH D. FELDMAN, M.D.

(From the Department of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, California 92037)

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Recent studies on immunologic memory have implicated either the small lymphocyte (1, 2) or the blast cell (3) as the cellular element imbued with recall capacity. The best evidence implicates the small lymphocyte. In this paper we present light and electron microscopic evidence that some small proportion of apparently inactive lymphocytes are committed to the anamnestic synthesis of specific antibody, and that the process is characterized by transformation of these cells to immature forms which are mitotically active and which differentiate into plasma cells.

Materials and Methods

Pairs of male Lewis rats, each about 200 g in body weight, were immunized by two intravenous injections of 0.5 mg of keyhole limpet (*Megathura crenulata*) hemocyanin (KLH)[1] given 1 wk apart. At the time of the first injection of antigen and for the following 14 days, the rats were administered thymidine-methyl-^{3}H(^{3}H-Td)[2] intraperitoneally, 3 × daily in a dose of 0.5 μc/g of body weight, in order to label the DNA of lymphoid cells committed during the primary response. Pairs of rats were killed 5 hr and 5, 10, 25, 30, and 50 days after the last injection of isotope. The 5-hr pair was used as controls to determine the extent of labeling and the numbers of tagged cell types in lymphoid tissues. 3 days before autopsy one of each pair of rats (except the 5-hr pair) was given 0.5 mg of KLH intravenously (specific challenge) and the other rat 0.5 mg of bovine gamma globulin (BGG)[3] (control). The rats injected with nonspecific antigen (BGG) provided reference levels of labeling for comparison with those animals administered specific antigen (KLH).

Another set of rat pairs were immunized with KLH, as described above, but were injected intraperitoneally with 5-iodo-2′-deoxyuridine-^{125}I (^{125}I-DU)[4] 3 × daily for 10 days in doses

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[1] Purified and generously supplied to us by Miss P. McConahey and Dr. F. J. Dixon.

[2] Schwarz Bio-Research Laboratories, Orangeburg, N. Y. Specific activity 6.0 c/mM, concentration 1 mc/ml.

[3] Fraction II from bovine plasma. Armour Pharmaceutical Co., Kankakee, Ill.

[4] Schwarz Bio-Research Laboratories, Orangeburg, N. Y. Specific activity 41.4 c/mM, concentration 0.5 mc/ml in CH_3CH OH, 50%.

of 0.75 μc/g of body weight. 5 hr after the last dose of ^{125}I-DU, one pair was killed and a second pair 10 days later, 3 days after the injection of specific (KLH) and nonspecific (BGG) antigens. ^{125}I-DU was used as DNA precursor (4–7) which was not reutilized (8). This experiment was designed to assess whether reutilization of ^{3}H-Td might have influenced the observations.

At autopsy, the lymphoid organs (spleen, thymus, cervical, and mesenteric lymph nodes), liver, kidney, and small intestine were fixed in formalin and processed for both light microscopic examination of hematoxylin and eosin stains and for radioautography. In addition, aliquots of lymphoid organs were fixed in either 1% osmic acid or 2% glutaraldehyde followed by 1% osmic acid. The tissues were processed for electron radioautography (9, 10).

A second experiment was devised to test the specificity of the responses of labeled cells. The design of the experiment is illustrated in Fig. 1. Its purpose was to determine the effect, if any, of an indifferent anamnestic response (BGG–anti-BGG) on the secondary response elicited by KLH. Lymphoid tissues were prepared at autopsy for light microscopic study of radioauto-

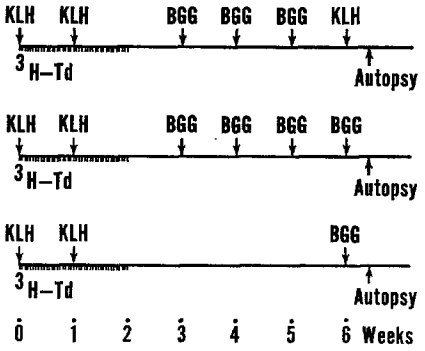

FIG. 1. Diagram of design of Experiment 2. Three groups of rats were labeled with ^{3}H-Td during immunization with KLH. Top: specific challenge with KLH following immunization with BGG. Middle: irrelevant challenge with BGG following immunization with BGG. Bottom: control, primary injection of BGG 3 days before autopsy.

graphs and hematoxylin and eosin sections. No ultrastructural observations were made on this material.

Sera of all animals from both experiments were examined for antibodies to KLH or BGG 5 days after the second injection of either of these antigens and also at the time of autopsy, 3 days after administration of antigen. Antibodies to KLH or to BGG were detected by double diffusion techniques in agar (Ouchterlony plates) or by radioimmunoelectrophoresis.

To record the behavior of labeled committed cells during the secondary response, a biometric analysis was performed at two levels. In the light microscope, radioautographs of lymphoid tissues were studied with an ocular reticule of 100 equal squares. The number of cells in 10 random squares of 100 was counted and the total within the reticule calculated by multiplying by 10. The number of labeled cells (5 grains or more) in all 100 squares was counted and the percentage calculated. Originally 25 fields enclosed by the reticule were counted but later 10 sufficed since it was found that the results were statistically not significantly different when 10 or 25 random fields were enumerated.

Originally we prepared radioautographs of smears of cell suspensions derived from labeled tissues. A comparison of the percentage of tagged cells in smears with that in tissues revealed that the former numbers were higher, probably because of an incomplete representation of the tissue cells in smears, i.e., only a selected population was being removed from the minced

organs. In addition, background grains over smears tended to be high. For these reasons, radioautographs of smears for biometric purposes were discarded.

In the electron microscope, radioautographs of the different lymphoid organs were perused and a differential of cell types recorded. Cells were classified as monoribosomal, polyribosomal, or mixed lymphocytes (11), blast, immature, and mature plasma cells; over 1700 were tabulated.

Radioautographic preparations of both light and electron microscopic sections were used only if background grains were less than 1 per 25 μ^{-2} in the former and less than 1 per 50 μ^{-2} in the latter.

Since the percentages of tagged cells in histologic preparations 10 or more days after cessation of labeling was low and since differences were small, the precision of the radioautographic results was determined by counting 10 random fields of the same slide five different times; three such preparations were used. The standard error of the method was 2.7% (4.05 ± 0.11).

RESULTS

The light microscopic data of the first experiment are presented in Table I A. At selected time intervals in the four different lymphatic organs, from 5 to 50 days after termination of labeling, the percentage of tagged cells was significantly lower in the tissues of rats injected with specific antigen (KLH) 3 days before, than that in the tissues of animals injected with nonspecific antigen (BGG). There were two exceptions in the thymus (see Table I A). Also the number of labeled cells in thymus was significantly smaller than that of the other lymphoid organs.

A plot of the percentage of labeled cells over the 50-day period produced a complex curve (Fig. 2). When the percentages were plotted on semilog paper, there were at least two components: a relatively rapid one occurring within the first 10 days with a half-life calculated at about 6 days, and a slow decay from 10 to 50 days with an estimated half-life of 45 days. This type of curve indicated the presence of at least two mononuclear cell populations with different life spans, an observation already noted by several other investigators (12–15).

50 days after cessation of ^{3}H-Td injections, a small number of labeled lymphoid cells was detected in the lymphatic sheaths of the spleen and in the cortical follicles of lymph nodes. Despite the small percentages of labeled elements, the differences in results between specific and nonspecific stimulation by antigen were significant.

The problem of isotope reutilization (8, 16–20), at least with regard to the results presented in this paper, was examined by using ^{125}I-DU. In Table I B, the data obtained with this reagent were parallel to but lower than those obtained with ^{3}H-Td.

The anamnestic response, then, was characterized by a diminution of labeled cells. It was desirable to identify the labeled elements in the electron microscope and to determine the frequency of the different lymphoid types, i.e., to perform a differential count of tagged cells in lymphoid tissues.

In Table II, the differential counts of labeled cells are shown. In the lymphoid

organs of the control animals (5-hr and BGG-injected), approximately 70% of labeled elements were lymphocytes. If this category was subdivided into monoribosomal (mature and resting), mixed, and polyribosomal (immature), the monoribosomal subclass (Fig. 3), at any time interval examined, ranged from 40 to 60% in the BGG rats. By contrast, in KLH rats the percentage of mono-

TABLE I

Average Per Cent of Labeled Cells in Lymphoid Tissues after Stimulation by Specific (KLH) and Nonspecific (BGG) Antigens, Light Microscopic Radioautography

Label	Time after	No. of rats	Antigen	Mesenteric lymph node	Cervical lymph node	Spleen	Thymus
A. ^{3}H-Td	5 hr	2	—	20.3 ± 1.4*	13.5 ± 0.95	18.8 ± 1.0	3.7 ± 0.2
	5 days	1	KLH	7.9 ± 0.4	5.4 ± 0.4	4.0 ± 0.3	1.5 ± 0.1
		1	BGG	13.4 ± 0.85	8.0 ± 0.5	6.3 ± 0.3	2.4 ± 0.2
	10 "	1	KLH	5.6 ± 0.3	4.7 ± 0.2‡	3.7 ± 0.3	*0.9 ± 0.15*
		1	BGG	7.5 ± 0.4	6.5 ± 0.5‡	6.0 ± 0.3	*1.4 ± 0.25*
	25 "	7	KLH	1.6 ± 0.07	1.3 ± 0.05	1.6 ± 0.06	0.3 ± 0.03
		3	BGG	4.3 ± 0.1	4.5 ± 0.2	2.9 ± 0.1	1.4 ± 0.1
	30 "	2	KLH	1.8 ± 0.1	1.6 ± 0.04	1.7 ± 0.07	*0.25 ± 0.04*
		4	BGG	3.6 ± 0.1	3.2 ± 0.15	3.0 ± 0.08	*0.3 ± 0.04*
	50 "	3	KLH	2.1 ± 0.1	1.6 ± 0.06	2.0 ± 0.1	0.4 ± 0.04
		3	BGG	3.0 ± 0.1	2.6 ± 0.1	2.8 ± 0.2	0.8 ± 0.07
B. ^{125}I-DU	5 hr	2	—	—	8.3 ± 0.4	7.3 ± 0.5	1.2 ± 0.1
	10 days	1	KLH	2.1 ± 0.2	1.2 ± 0.07	1.0 ± 0.10	0.2 ± 0.03
		1	BGG	4.0 ± 0.3	3.0 ± 0.10	2.7 ± 0.14	0.5 ± 0.05

* Standard error of mean. The differences between means are statistically significant at less than 0.1%, except for the two pairs in italics

‡ Significant differences between 0.5 and 0.1%.

ribosomal lymphocytes at all time intervals, for all lymphoid organs, was 20–30% or less. In the opposite direction, the BGG control lymphoid organs revealed roughly 10% polyribosomal lymphocytes (Fig. 4), and 3% mature plasma cells (Fig. 8), while the lymphoid organs of rats injected with KLH yielded over 20% polyribosomal lymphocytes and about 10% plasma cells. A total of nine labeled mitoses (Fig. 6) were observed, three in control and six in KLH-treated organs. This number was too small to be classified separately and analyzed statistically. In brief, the stimulation by specific antigen in the second-

ary response was accompanied by a decrease of mature cells and an increase of immature cells.

The injection of KLH after primary immunization did not substantially change the number of labeled blast cells (Fig. 5) or of labeled immature plasma cells (Fig. 7) compared with those counted in the lymphoid tissues of rats injected with BGG for the first time. We have assumed that these categories of cells were rapidly dividing and differentiating so that their numbers were low at all times, both in animals undergoing a secondary and a primary response.

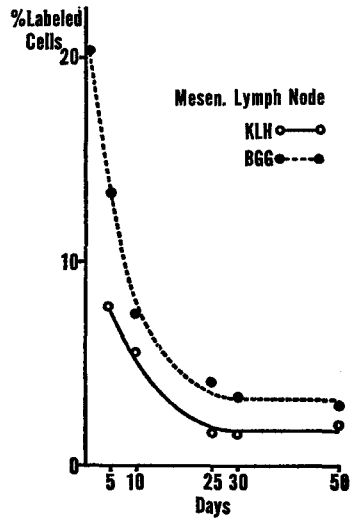

FIG. 2. Linear plot of percentages of labeled mesenteric lymph node cells against time. Differences between KLH challenged and BGG injected animals are significant at all time intervals. Curve reflects the existence of at least two populations of lymphoid cells with short and long life spans. Almost identical curves were plotted for labeled cells in mesenteric lymph nodes, spleen, and thymus.

At least we have other evidence, with cells halted in metaphase by colchicine, that supported this view since the number of labeled blast cells increased markedly when mitoses were arrested.[5]

To be certain that a secondary response, per se, of an irrelevant system did not influence the results obtained using specific antigen, the control experiment represented in Table III was carried out. The data were very similar to those shown in Table I A. The specific antigen caused a diminution of labeled cells in all lymphoid organs compared with the percentage of labeled cells in animals stimulated with BGG. In brief, a secondary reaction in an irrelevant system did not significantly alter the number of tagged cells present 30 days after labeling,

[5] Bosman, C., and J. D. Feldman. Unpublished observation.

TABLE II

Differential of Labeled Cells, Electron Radioautography

Label	Time after	Antigen	Lymphocytes						Blast cells		Immature plasma cells		Mature plasma cells		Miscellaneous*		Total	
			Monoribosomal		Mixed		Polyribosomal											
			Per cent	Absolute	Per cent	Absolute	Per cent	Absolute	Per cent	Abs	Per cent	Abs	Per cent	Abs	Per cent	Abs	Per cent	Abs
A. ^{3}H-Td	5 hr	—	39.6	44	10.8	12	21.6	24	7.2	8	2.7	3	6.3	7	11.7	13	99.9	111
	10 days	KLH	33.3	21	9.5	6	27.0	17	0.0	0	6.3	4	6.3	4	17.4	11	99.8	63
		BGG	42.9	21	8.2	4	8.2	4	2.0	1	2.0	1	4.1	2	32.6	16	100.0	49
	25–30 days	KLH	28.3	99	12.8	45	26.0	91	3.4	12	4.0	14	10.6	37	14.8	52	99.9	350
		BGG	47.7	113	13.5	32	8.4	20	5.1	12	5.1	12	5.1	12	15.2	36	100.1	237
	50 days	KLH	27.4	73	15.4	41	39.1	104	6.4	17	0.75	2	1.1	3	9.8	26	99.5	266
		BGG	60.2	103	10.5	18	12.8	22	2.3	4	2.9	5	0.0	0	11.1	19	99.8	171
	Total (10–50 days)	KLH	28.4	193	13.5	92	31.2	212	4.3	29	2.9	20	6.5	44	13.1	89	99.9	679
		BGG	51.9	237	11.8	54	10.1	46	3.7	17	3.9	18	3.1	14	15.5	71	100.0	457
B. ^{125}I-DU	5 hr	—	54.5	134	6.1	15	17.5	43	2.4	6	2.4	6	8.5	21	8.5	21	99.9	246
	10 days	KLH	15.2	9	11.9	7	39.0	23	5.1	3	3.4	2	15.3	9	10.2	6	100.1	59
		BGG	53.8	100	14.0	26	19.3	36	3.2	6	2.1	4	3.2	6	4.3	8	99.9	186

* Includes monocytes, macrophages, reticular, endothelial cells, et al.

while a secondary reaction induced by specific antigen caused a drop in the number of labeled cells.

The serologic data showed that all labeled rats given two intravenous injections of KLH 1 wk apart produced antibody to KLH and those immunized with BGG yielded detectable circulating antibody to BGG.

DISCUSSION

The goal of these experiments was to identify, if possible, the cell type or types with immunologic memory, and to record the integrated cytologic events that were associated with the anamnestic response. The observations disclosed

TABLE III*

Per Cent of Labeled Cells in Lymphoid Tissues after Stimulation by Specific (KLH) and Nonspecific (BGG) Antigens,‡ Light Microscopic Radioautography

Group	No. of animals	Antigen	Mesenteric lymph node	Cervical lymph node	Spleen	Thymus
A. Secondary response to KLH	3	KLH	2.3 ± 0.06§	2.3 ± 0.09	2.1 ± 0.07	0.2 ± 0.03‖
B. Secondary response to BGG	3	BGG	4.1 ± 0.07	3.6 ± 0.09	3.7 ± 0.14	0.4 ± 0.04
C. Primary response to BGG	2	BGG	4.0 ± 0.13	4.0 ± 0.14	3.2 ± 0.13	0.4 ± 0.06

* See Fig. 3.

‡ 30 days after cessation of labeling with ^{3}H-Td.

§ Standard error of mean. Results of group A are significantly different statistically from those in groups B and C at less than 0.1% (except ‖, which is statistically different between 0.5 and 0.1%).

several changes that occurred simultaneously and were apparently related: a sudden decrease of tagged cells within the total labeled pool, a marked diminution of mature lymphocytes; an increase of immature cells, and an expansion of the plasma cell class. This discussion will attempt to interpret these phenomena as transformation of mature lymphocytes, the memory cells, into immature elements that divide and eventually differentiate into plasma cells.

It was assumed that not all cells committed to antibody synthesis were labeled; nor that all labeled cells were immunized by KLH; nor, finally, that all cells which were immunized during the period of KLH injections were committed to production of anti-KLH antibody. Presumably, however, a good proportion of tagged elements were sensitized to KLH and the difference between the per cent of labeled cells in KLH rats and the per cent in BGG rats represented a large portion of these particular committed elements. At any time interval after the phenomena of primary immunization subsided, the per

cent of labeled lymphoid cells invariably dropped significantly after re-exposure to specific antigen.

Such a sudden reduction of tagged cells might have been the result either of active proliferation with loss of marker or of cell destruction by cytotoxic complexes of antigen, antibody, and complement. There is considerable evidence to support the former, since re-exposure to antigen has been shown repeatedly to trigger mitotic activity in vitro and in vivo (21–26, see also references 1 and 27). Furthermore, in experiments similar to those presented in this paper, numerous mitoses accumulated when colchicine was injected several hours after antigenic stimulation and before autopsy.[5] On the other hand, histologic and ultrastructural examination of many sections disclosed no evidence of cell necrosis or augmented phagocytosis of cell debris in the tissues of the KLH group. In addition, antigen-antibody interactions per se, as revealed in the BGG–anti-BGG experiment, did not affect the pool of cells labeled during immunization with KLH. This latter experiment also demonstrated the specificity of the cytologic phenomena.

The percentage of labeled cells, in both KLH and control groups of rats, between 10 and 50 days after ^{3}H-Td administration, slowly diminished. The gradual decline might be attributed either to natural death of long-lived cells or to a transformation and division induced by numerous antigens other than KLH. The 45-day half-life calculated for long-lived cells in these experiments was based on disapperance of label, not of cells, and was similar to the life span calculated by other methods (28, 29).

The ultrastructural classification of labeled lymphoid elements revealed significant differences in the lymphoid organs of KLH and control animals. These differences, although static profiles of dynamic changes, were constant at all time intervals studied and most likely reflected the processes of transformation, cell division, and differentiation in vivo. A reduction of approximately 50% of labeled mature lymphocytes in the tissues stimulated by specific antigen must be interpreted as a change of morphologic character, since mature lymphocytes have not been shown to be capable of division (1) and since there was no evidence of cell destruction. The doubling of labeled immature lymphocytes in the KLH rats was most likely effected by the enlargement (transformation) of tagged mature lymphocytes. An increase of polyribosomal lymphocytes could be derived either from labeled monoribosomal lymphocytes or from labeled blast cells that were differentiating in the opposite direction. If the latter pathway prevailed, the number of tagged mature lymphocytes should have increased rather than decreased. Since this was not the case, the flow of events must have been from mature to immature. The expansion of the plasma cell class was likewise derived either from labeled mature lymphocytes by modulation, from labeled immature precursors capable of dividing and differentiating, or from long-lived plasma cells capable of division and surviving from the original im-

munization. There is no morphologic evidence that mature lymphocytes can modulate directly into plasma cells. Further, several studies have indicated that plasma cells, at least the majority of them, are end cells and have a relatively short life span of several days (24, 30, 31). One the other hand, numerous observations have demonstrated a burst of mitoses preceding the appearance of plasma cells and a morphologic sequence from immature blast cells to mature plasma cells. In our investigation we have no data that disclosed the derivation of tagged plasma cells, either directly from polyribosomal lymphocytes and blast cells without division, or by differentiation following one or more mitoses.

In two categories of cells, the blast and immature plasma cells, the percentage of labeled elements was low at all time intervals studied and of the same magnitude in stimulated and control tissues. Such young forms containing detectable isotope 50 days after labeling could have issued from either long-lived immature cells that did not divide, from transforming labeled mature lymphocytes, or from dividing cells reutilizing isotope. Kinetic studies of cell division and maturation have shown that blast and immature plasma cells divide repeatedly and/or differentiate (32). They do not retain visible nuclear marker for long periods as immature elements. We felt, therefore, that these categories of labeled young cells were derived from labeled transforming mature lymphocytes. The fact that the percentage of these cell types was small was probably due to rapid proliferation and/or maturation, i.e., label was lost or the immature cell passed over into another category. The failure to detect significant percentile differences between stimulated and control tissues might be attributed to our choice of time to examine tissues after antigenic challenge. The peak of mitotic activity probably occurred prior to 3 days after KLH stimulation. In similar current work with colchicine, the number of mitoses reached a maximum at 36–48 hr after KLH challenge.[5]

The problem of reutilization of ^{3}H-Td has been discussed by a number of investigators and in our experiments, if it occurred in a visible form, it should not have affected the results. Only cells with 5 grains or more were considered labeled, even with very low background. Under the conditions of labeling, cells with this number of grains would not be found as a result of reutilization (18). In addition, the data obtained when ^{125}I-DU was used, a reagent that is not reutilized, paralleled those observed with ^{3}H-Td, although at a lower level of incorporation. Finally, if reutilization had occurred and was detectable by radioautography, it should have occurred in experimental and control rats to a similar degree.

The percentage of labeled cells in the thymus, at all time intervals including the 5-hr period, was low and indicated rapid turnover of cells by division (14, 33), or emigration from the gland (33, 34). In addition, at 10 and 30 days after isotope, the percentile differences between labeled cells in the thymuses of KLH and control animals were not significant. The data implied that the thy-

mus was not primarily involved with immunologic memory. However, our experiments were not designed to test whether this was true or whether the thymus discharged its mature labeled lymphocyte to settle elsewhere.

SUMMARY

Pairs of rats were immunized with keyhole limpet hemocyanin (KLH) and simultaneously labeled with thymidine-methyl-^{3}H or 5-iodo-2′-deoxyuridine-^{125}I. From 10–50 days later, their lymphoid organs were examined 3 days after anamnestic stimulation with KLH or after primary injection of BGG. Light and electron microscopic study of the labeled cells revealed that immunologic memory resided in the mature resting monoribosomal lymphocyte which, upon stimulation, transformed to an immature polyribosomal lymphocyte and mitotically active blast cell. These latter elements differentiated into plasma cells directly or after mitosis.

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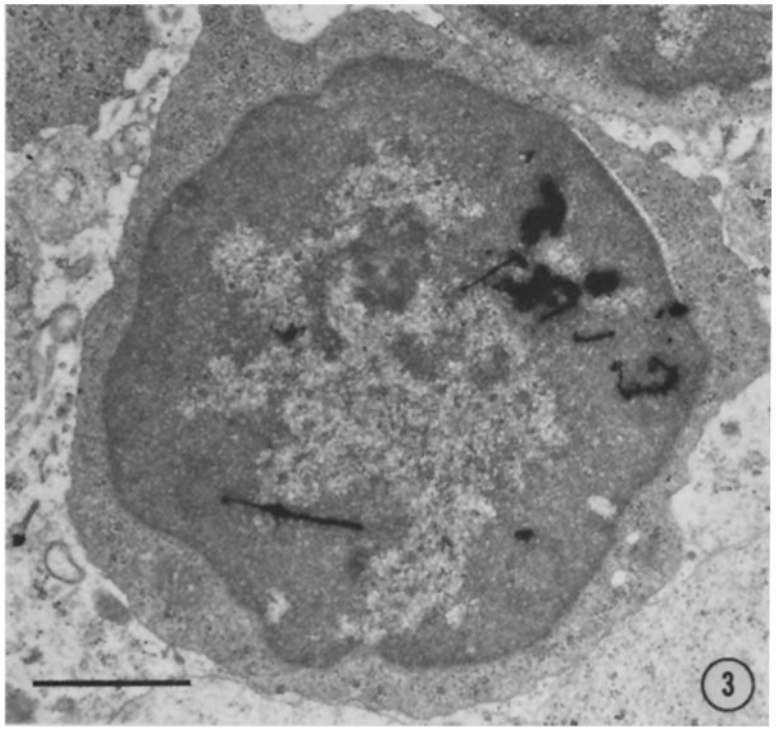
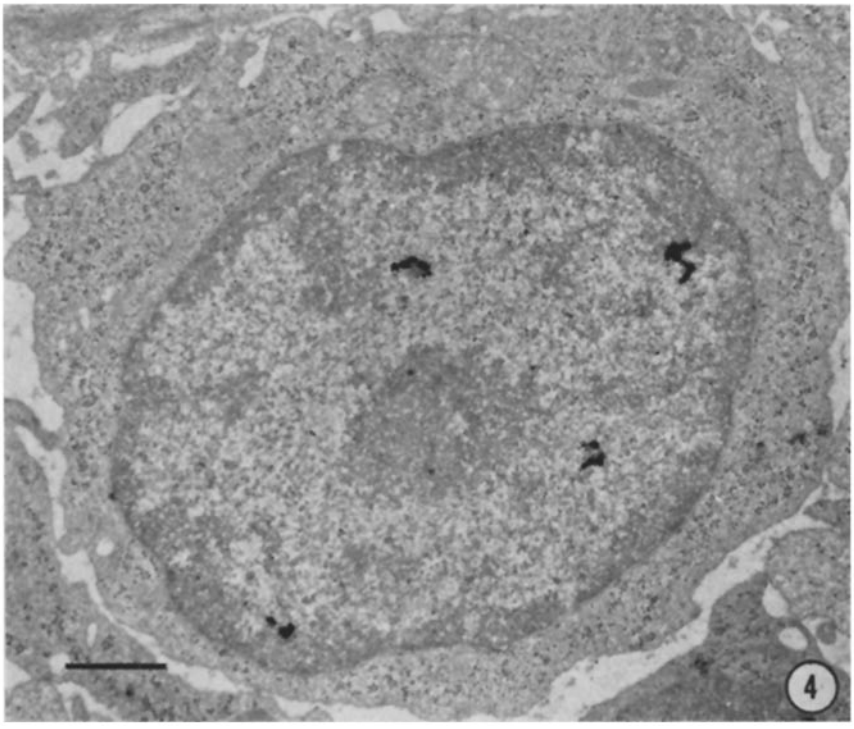
FIGS. 3–8. Electron microscopic radioautographs of lymphoid cells labeled with ^{3}H-Td. All tissues were fixed in 2% glutaraldehyde and 1% osmic acid, embedded in Vestopal W, coated with L 4 Ilford, and stained with uranyl acetate and lead citrate. The line in each picture represents 1 μ.

FIG. 3. Monoribosomal lymphocyte from spleen, characterized by scanty cytoplasm containing scattered free single ribosomes and nucleus with dense chromatin. 50 days after ^{3}H-Td. × 21,000.

FIG. 4. Polyribosomal lymphocyte from thymus, characterized by numerous polyribosomes in more abundant cytoplasm and large, less compact nucleus. 50 days after ^{3}H-Td. × 13,500.


3


4

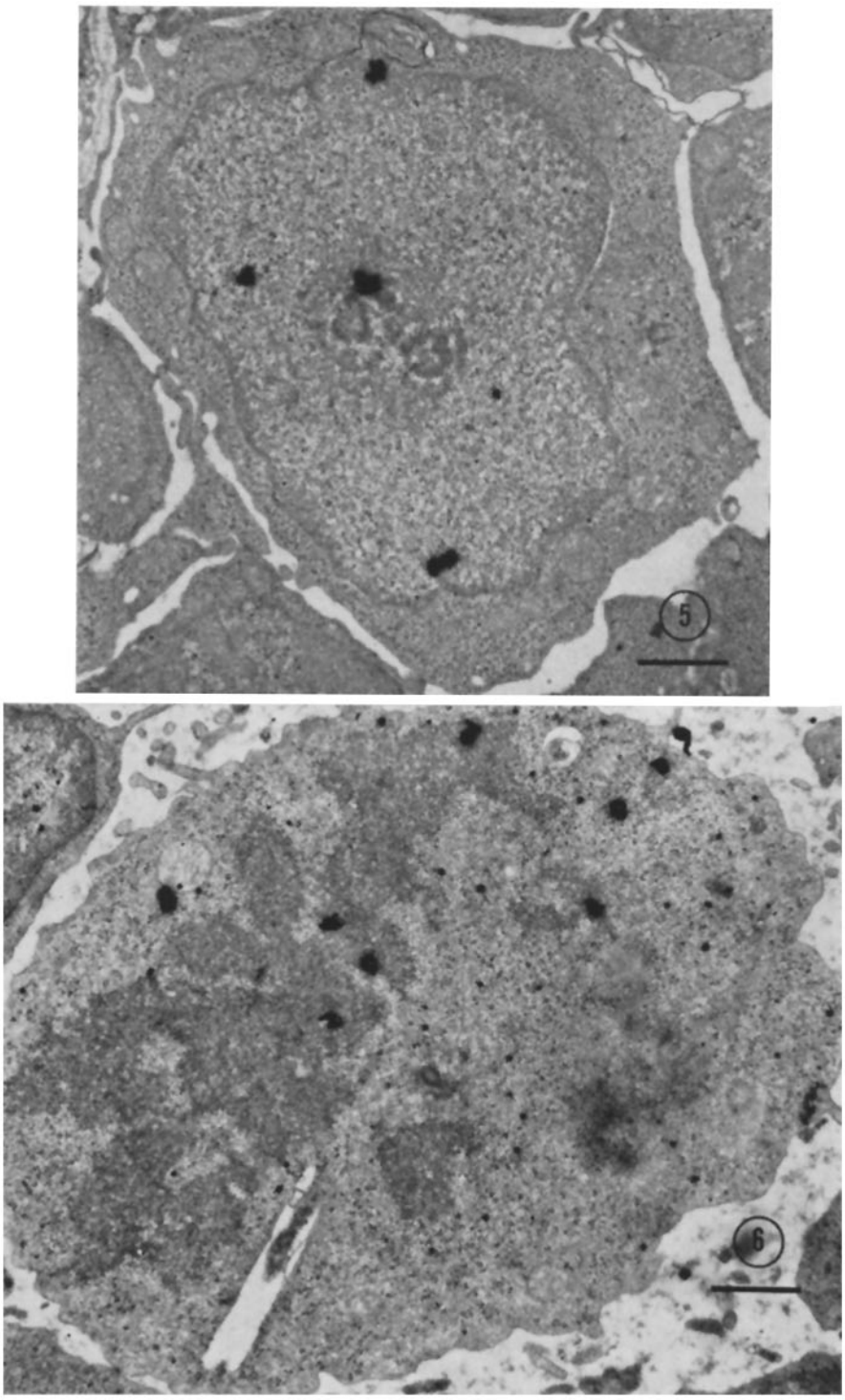
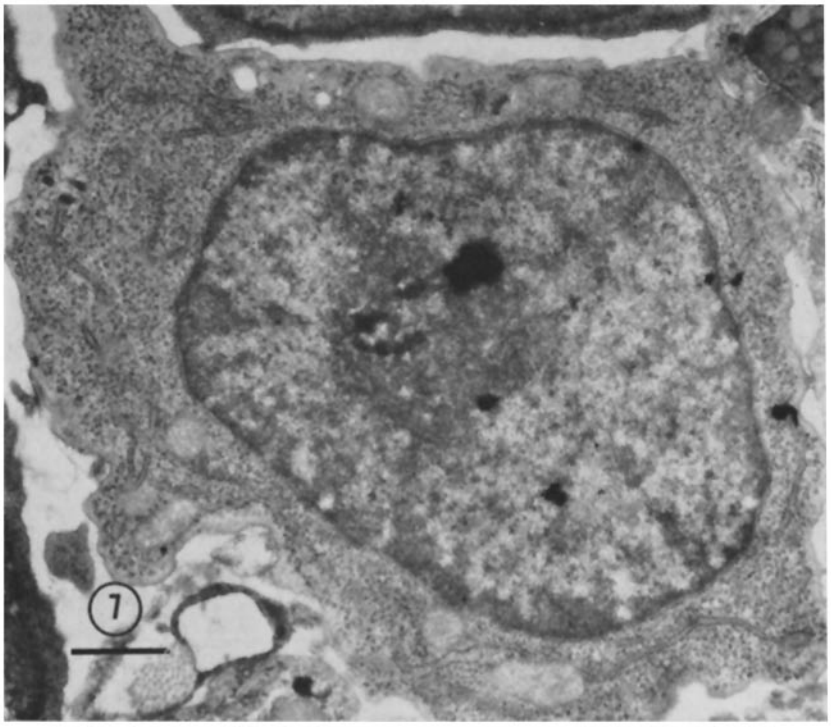


FIG. 5. Blast cell from cervical lymph node, characterized by numerous polyribosomes in abundant cytoplasm and large pale nucleus. 30 days after ^{3}H-Td. × 12,000.

FIG. 6. Blast cell from mesenteric lymph node, in mitosis, with numerous polyribosomes in cytoplasm. 50 days after ^{3}H-Td. × 12,000.



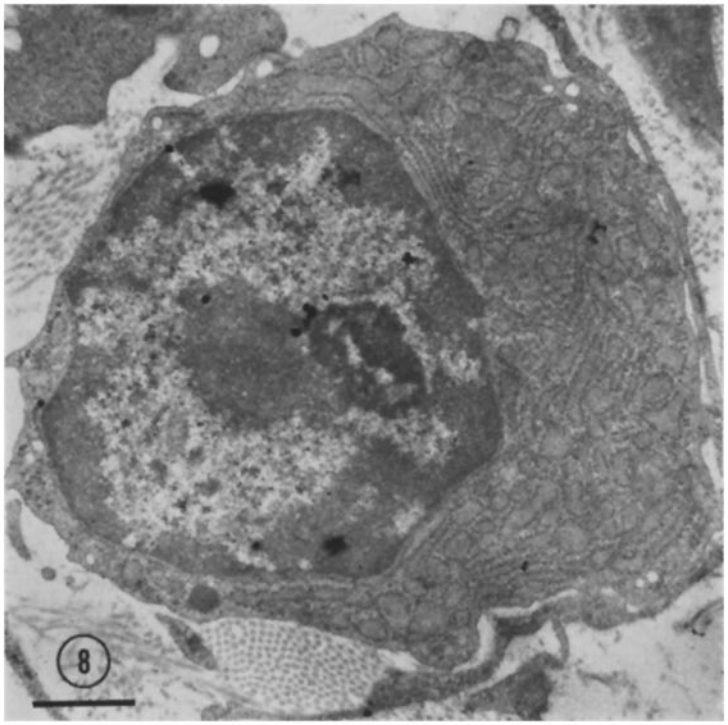


FIG. 7. Immature plasma cell from mesenteric lymph node, characterized by numerous polyribosomes and a rudimentary rough-surfaced endoplasmic reticulum in abundant cytoplasm. 50 days after ^{3}H-Td. × 12,500.

FIG. 8. Mature plasma cells from cervical lymph node with extensive rough-surfaced endoplasmic reticulum containing electron-dense material. 50 days after ^{3}H-Td. × 13,000.