

# MOUSE THYMUS-INDEPENDENT AND THYMUS-DERIVED LYMPHOID CELLS

## II. ULTRASTRUCTURAL STUDIES\*

By A. MATTER, B. LISOWSKA-BERNSTEIN, J. E. RYSER, J.-P. LAMELIN,  
AND P. VASSALLI

(From the Department of Pathology and Institute of Histology, University of Geneva,  
Geneva, Switzerland)

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A companion paper (1) showed that it is possible to identify by immunofluorescence mouse thymus-independent (B)<sup>1</sup> and thymus-derived (T) lymphoid cells, using rabbit antibodies directed against differentiation antigens present on the cell membrane, mouse-specific lymphocyte antigens (MSLA) or brain-associated  $\theta$  antigen (BA $\theta$ ) for T cells, and mouse-specific bone marrow-derived lymphocyte antigens (MBLA) for B cells. In the present work, the ultrastructural features of B-, T-, and surface immunoglobulin (sIg)-bearing lymphoid cells have been studied using rabbit antisera against MSLA, BA $\theta$ , MBLA, and mouse Ig. The fixation on cells of these antibodies has been detected by peroxidase-labeled anti-rabbit Ig antibodies (2, 3), or by a "bridge technique" using southern bean mosaic virus (SBMV) (4) or bacteriophage T4, as markers. Pathways of differentiation were observed among the B and T cell lines; lymphoid cells bearing sIg had the ultrastructural features of the B cells.

### *Materials and Methods*

*Animals.*—CBA/J mice (obtained from C.S.E.A.L.-Centre National de La Recherche Scientifique, Orléans-la-Source, France) were immunized either with *Bordetella pertussis* (vaccine pertussis Berna, from the Institut sérothérapique et vaccinal, Bern, Switzerland),  $2.5 \times 10^8$  bacteria per footpad and/or  $10^9$  bacteria intraperitoneally, or with cells of DBA/2 mastocytoma P-185 ( $2 \times 10^7$  per footpad), and killed at various times after priming or boosting.

*Antigens.*—Bacteriophage T4 were grown and purified on sucrose gradients according to Dickson et al. (5). SBMV (the original inoculum being a kind gift of Dr. U. Hämmerling)

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<sup>1</sup> *Abbreviations used in this paper:* aMSLA, aBA $\theta$ , and aMBLA, rabbit antisera anti-MSLA, etc.; BA $\theta$ , brain-associated theta antigen; B cells, thymus-independent lymphocytes; ER, rough endoplasmic reticulum; HBSS, Hanks' balanced salt solution; MBLA, mouse-specific bone marrow-derived lymphocyte antigens; MSLA, mouse-specific lymphocyte antigens; PBS, phosphate-buffered saline; Pox, horseradish peroxidase; RaMIg, rabbit anti-mouse Ig; SaRIg, sheep anti-rabbit Ig; SBMV, southern bean mosaic virus; sIg, surface immunoglobulin; T cells, thymus-derived lymphocytes; TL, thymus leukemia.

were grown, prepared, and purified according to Steere (6), the final purification being obtained by ultracentrifugation on a 10–40% linear sucrose gradient. T4 and SBMV preparations were examined by negative staining, using 0.5% neutralized phosphotungstic acid as staining solution, and found to be free of contaminating particles and almost devoid of empty capsids.

*Antisera.*—Ig fractions of rabbit anti-mouse Ig (RaMIg) and of sheep anti-rabbit Ig (SaRIg), rabbit antisera anti-MSLA (aMSLA), anti-brain-associated  $\theta$  (aBA $\theta$ ), and anti-MBLA (or the Ig fraction of this last antiserum) (aMBLA) were the same as used in the companion paper (1). SaRIg antibodies were purified by immunoabsorption on Sepharose 4 B coupled to rabbit Ig according to Axen et al. (7), followed by elution in 0.2 M propionic acid, neutralization, and concentration by pressure dialysis. To obtain antibodies against T4 and SBMV, rabbits received three intramuscular injections of 2 mg of either antigen in complete Freund's adjuvant, followed by weekly boosts with 0.5 mg of antigen intramuscularly or 0.2 mg intravenously during several months. Sera of several rabbits were pooled. Purified antibodies were prepared by immune precipitation of the antisera with their respective antigens, repeated washings of the precipitates in phosphate-buffered saline (PBS), followed by dissociation of the immune complexes in 1.0 M propionic acid for 30 min at 37°C and sedimentation of the viral particles by centrifugation (30 min at 60,000 g for T4; 30 min at 105,000 g for SBMV). The supernatants, containing the specific antibodies, were neutralized and concentrated by pressure dialysis against PBS.

*Coupling of Antibodies to Peroxidase.*—SaRIg antibodies, purified as described above, were coupled to horseradish peroxidase (Pox) (Sigma Chemical Co., St. Louis, Mo., and Behringwerke, Marburg-Lahn, West Germany) by the one-step (8) or two-step (9) procedure described by Avrameas. Antibodies coupled with the one-step procedure were purified again by immunoabsorption on Sepharose rabbit Ig to get rid of the unconjugated Pox.

*Preparation of Cells.*—Spleens, lymph nodes, and thymuses were minced with scissors in Hanks' balanced salt solution (HBSS), squeezed through wire mesh No. 100, filtered through eight sheets of nylon stocking, and sedimented for 5 min to remove cell clumps. The cell suspension was incubated in 0.83% ammonium chloride to lyse contaminating erythrocytes and further washed three times in HBSS at 4°C; the cells were counted and dead cells determined by the trypan blue dye exclusion test.

*Cells Labeling with Antibodies.*— $30 \times 10^6$  cells were incubated for 30–60 min at 0°C in 1 ml of HBSS containing 5% bovine serum albumin (Difco Labs., Inc., Detroit, Mich., 30% stock solution) and aMSLA, aBA $\theta$ , or aMBLA, at  $\frac{1}{4}$  final concentration, RaMIg serum ( $\frac{1}{10}$  final concentration), or RaMIg Ig fraction (1–2 mg/ml final concentration). In some cases, an Ig fraction of aMBLA (10) was used at a final concentration of 1–2 mg/ml. After washing, the detection of rabbit Ig bound on cell surfaces was carried out by one or two methods: (a) Incubation with SaRIg coupled to Pox (SaRIg-Pox, see above), 0.1–1 mg/ml in HBSS, for 30 min at 0°C, followed by washing. (b) A bridge technique (11) using purified SaRIg (0.1 mg/ml), followed by purified rabbit antibodies against T4 or SBMV (usually 0.1 mg/ml) and finally T4 or SBMV (0.1–1 mg/ml).

*Electron Microscopic Techniques.*—After final incubation the washed cells were transferred into small cellulose tubes, pelleted, and fixed by layering 3 ml of Karnovsky's fixative (12) diluted 1:1 with distilled water, on top of the pellet. After 2 hr of fixation, the fixative was replaced by PBS containing 5% sucrose. This limited time of fixation was found to be essential to preserve the activity of exogenous Pox. After overnight washing in sucrose-PBS, the pellet was removed with forceps and suspended in Graham and Karnovsky's medium for detection of Pox (13). This was performed in two steps: 1 hr incubation with diaminobenzidine (Fluka AG, Buchs, Switzerland) 0.05% in tris(hydroxymethyl)aminomethane buffer, 0.05 M, pH 7.6, followed by 1 hr in the same medium with added H<sub>2</sub>O<sub>2</sub> 0.01%. Postosmication for 1 hr in phosphate-buffered 2% OsO<sub>4</sub> was followed by dehydration in alcohol and embedding in

Epon (14). Since the histochemical detection of Pox was done on small blocks the depth of penetration of the substrates was limited, even in prolonged incubations. Therefore semithin sections were controlled for endogenous Pox or catalase activity by phase-contrast microscopy and selected areas were trimmed for further fine sectioning. Ultrathin sections were cut with glass knives on a LKB ultratome III (LKB Produkter, Stockholm, Sweden), stained first with a 5% aqueous solution of uranyl acetate and then with lead citrate (15).

Radioautography was performed by means of the loop method of Caro and van Tubergen (16), using Ilford L4 (Ilford Ltd., Ilford, Essex, England), diluted 1:1 with distilled water as emulsion. Development was carried out 4–8 wk after storage at 4°C, using Microdol (Eastman Kodak Co., Rochester, N. Y.) for 5 min. After rinsing in distilled water the grids were fixed during 5 min in Kodak acid fixer, washed for 30 min, dried, and stained as indicated above.

Observations were carried out on a Philips EM 300 microscope (Philips Electronic Instruments, Mount Vernon, N. Y.), with original enlargements ranging between 3000 and 40,000.

#### RESULTS

*Technical Considerations.*—The two types of methods used for the ultrastructural detection of antibodies fixed on cell surfaces presented each different advantages and pitfalls which require some comments. The use of Pox-labeled antibodies usually allowed clear detection at low magnification of the dark product of the enzymatic reaction, thus facilitating rapid screening of a large number of cells. The main pitfalls appeared to be either excessively strong or excessively weak enzymatic reactions. With strong reactions, the reaction product had a tendency to “flow” at some distance from the enzyme (and therefore from the antigen), which not only made localization on the cell surface imprecise but also produced false labeling of the membranes of cells in close contact with a labeled cell. Excessive weakness or absence of the enzymatic reaction, leading to a failure to detect the antigen and therefore to properly identify the cells, appeared to result from two main causes: (a) inhibition of Pox activity by prolonged fixation (over 2–4 hr) with glutaraldehyde (which, however, did not inhibit endogenous Pox); (b) insufficient histochemical detection resulting from an inappropriate penetration of the substrate in the glutaraldehyde-fixed cell pellet: semithin section must always be checked by phase-contrast microscopy for the presence of at least endogenous Pox or catalase activity (for instance in granulocytes or erythrocytes), and only areas of the blocks showing such activity must be selected for further thin sectioning. With recognition of these pitfalls, the method proved quite reliable. The specificity of the staining was checked by either replacing the rabbit Ig antibody by normal rabbit Ig, or replacing the SaRIg-Pox by normal sheep Ig coupled to Pox, or simply omitting the intermediate step. Some Pox-labeled antibodies were slightly “sticky” and gave occasionally a very faint labeling of some cells, which usually could not be mistaken for specific labeling. An example of such unspecific labeling is given on Fig. 15.

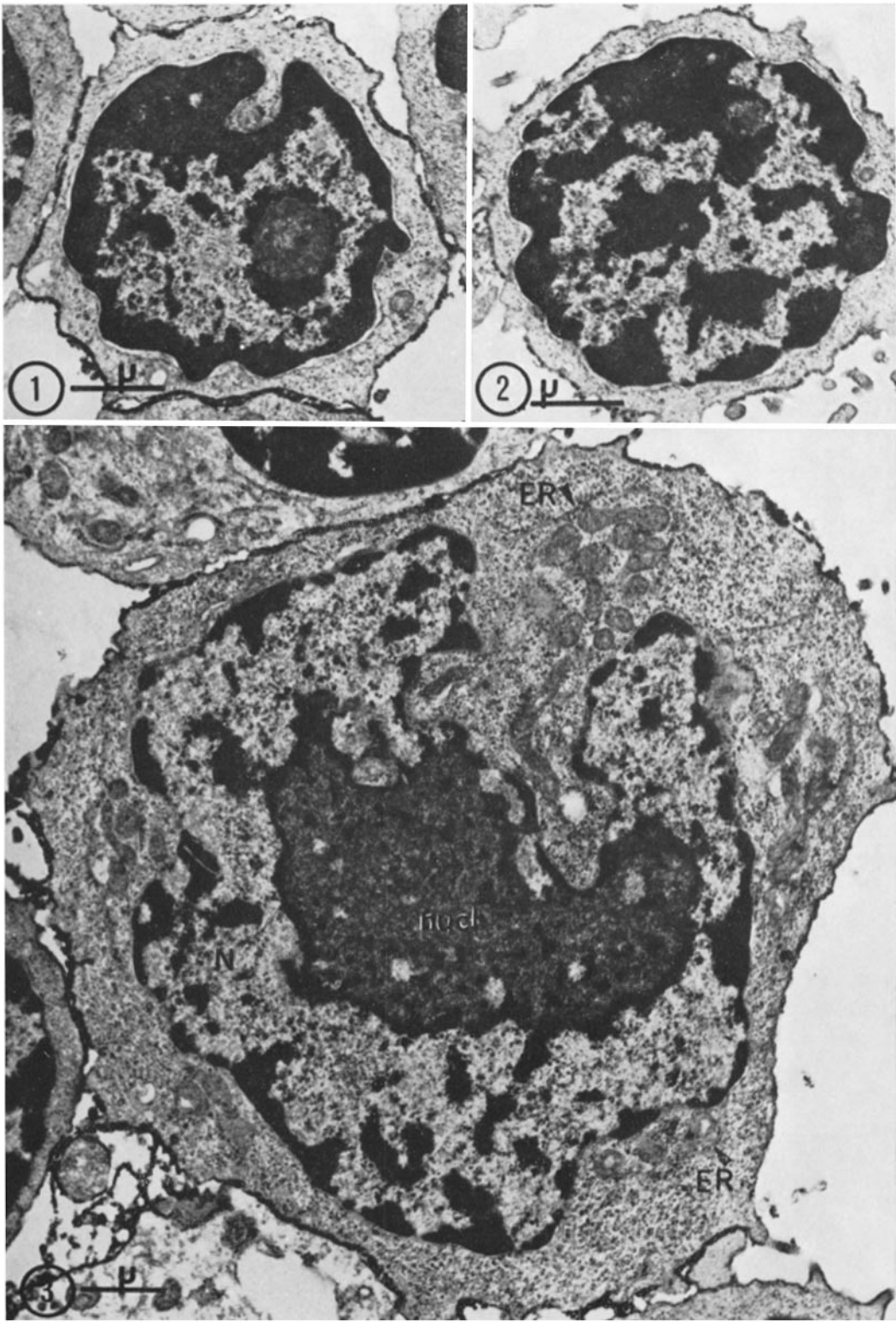
The bridge technique, in spite of the succession of incubations and washings that it requires, was surprisingly specific and sensitive (provided, of course, that purified antibodies to the first antibody and to the viral particles were

used). Labeling with T4, which is easier to see at low magnifications, tended to give a less precise localization than SBMV, the phage particles being not very densely packed and attached by the tail as well as by the head, so that depending upon the orientation of the phage, the head could be barely visible or absent from the section. Controls, performed as for Pox-labeled antibodies, indicated excellent specificity, since only occasional viral particles were observed on rare cells, and could not be mistaken for specific labeling, especially in the case of SBMV where the regular lining of virus along segments of the cell membrane is characteristic of specific localization.

Finally, it must be realized that the quantity of surface antigen available for detection poses a much more serious problem for electron microscopy than for immunofluorescence, since an ultrathin section passing at the cell equator exposes only about 1% of the cell surface. It was noticed that some cells had decreased amounts of surface differentiation antigens, notably "senescent" cells (such as fully mature plasma cells), blast cells, and especially cells in mitosis; the absence of label on ultrathin sections on these cells must be interpreted with caution, since it could lead to erroneous interpretation as to the B or T nature of the cell.

*B Cells (MBLA+ Cells).*—Only lymphoid cells labeled by the aMBLA serum will be described, and not macrophages, which were also MBLA+ (1). MBLA+ lymphoid cells can be divided into three categories: lymphocytes (Figs. 1 and 2), blasts (Figs. 3 and 4), and plasma cells (Figs. 5 and 6) and their immediate precursors. The most frequently observed B cells were small to medium-sized lymphocytes (Figs. 1 and 2), with a relatively clear cytoplasm and a central nucleus showing marginated and patchy heterochromatin and sometimes one or two nucleoli. The amount of cytoplasm and of intracellular organelles was quite variable with usually a few mitochondria, a small Golgi complex, a mixture of mono- and polyribosomes, occasionally a few profiles of rough endoplasmic reticulum (ER), dense granules, pinocytosis vesicles, and multivesicular bodies. The marker on the cell membrane had a patchy localization but was usually present on rather large areas. When the cells were left for a few minutes at 37°C after final incubation with SaRIg-Pox, pinocytosis of the marker was evident (Fig. 4, inset). The B blasts were huge cells, often with giant nucleoli, with abundant mono- and polyribosomes, but without conspicuous amounts of microfilament system (a characteristic of certain T cells, see below) (Fig. 3). With decrease in cell size, increasing amounts of ER (Fig. 4), these cells appeared to transform progressively into fully mature plasma cells. Blasts had often lesser amounts of marker on their membranes than lymphocytes, and this appeared also to be true of the most mature plasma cells. Cells labeled by aMBLA were very rare among thymic cells, had the same ultrastructural features as the cells described above, and included plasma cells.

*Cells Bearing Surface Ig (sIg).*—The cells had the same characteristics as the B lymphocytes described above, with a patchy localization of the marker



(Fig. 7). When the cells were incubated for a few minutes at 37°C after fixation of SaRIg-Pox, striking pinocytosis of the marker by lymphocytes was observed (Fig. 7, inset). sIg could also be detected on blasts (Fig. 8), but usually in much smaller amounts than on lymphocytes. sIg was very rarely seen on plasma cells.

*T Cells (MSLA+ or BAθ+ Cells).*—The lymphoid cells labeled by the aMSLA or aBAθ serum could be classified into three categories which will be called, for convenience, T<sub>1</sub>, T<sub>2</sub>, and T<sub>3</sub>. T<sub>1</sub> lymphocytes (Fig. 12) were the least characteristic, being small to medium-sized lymphocytes with a relatively clear cytoplasm resembling B lymphocytes. Figs. 9 and 10 allow the comparison of B and T<sub>1</sub> lymphocytes, the cells in Fig. 9 being labeled with aMBLA and in Fig. 10 with aMSLA. The most distinctive features of T<sub>1</sub> lymphocytes (Figs. 9–11) were their paucity in all organelles other than ribosomes (Figs. 9 and 10), and their apparent lack of polyribosomes (Fig. 11).

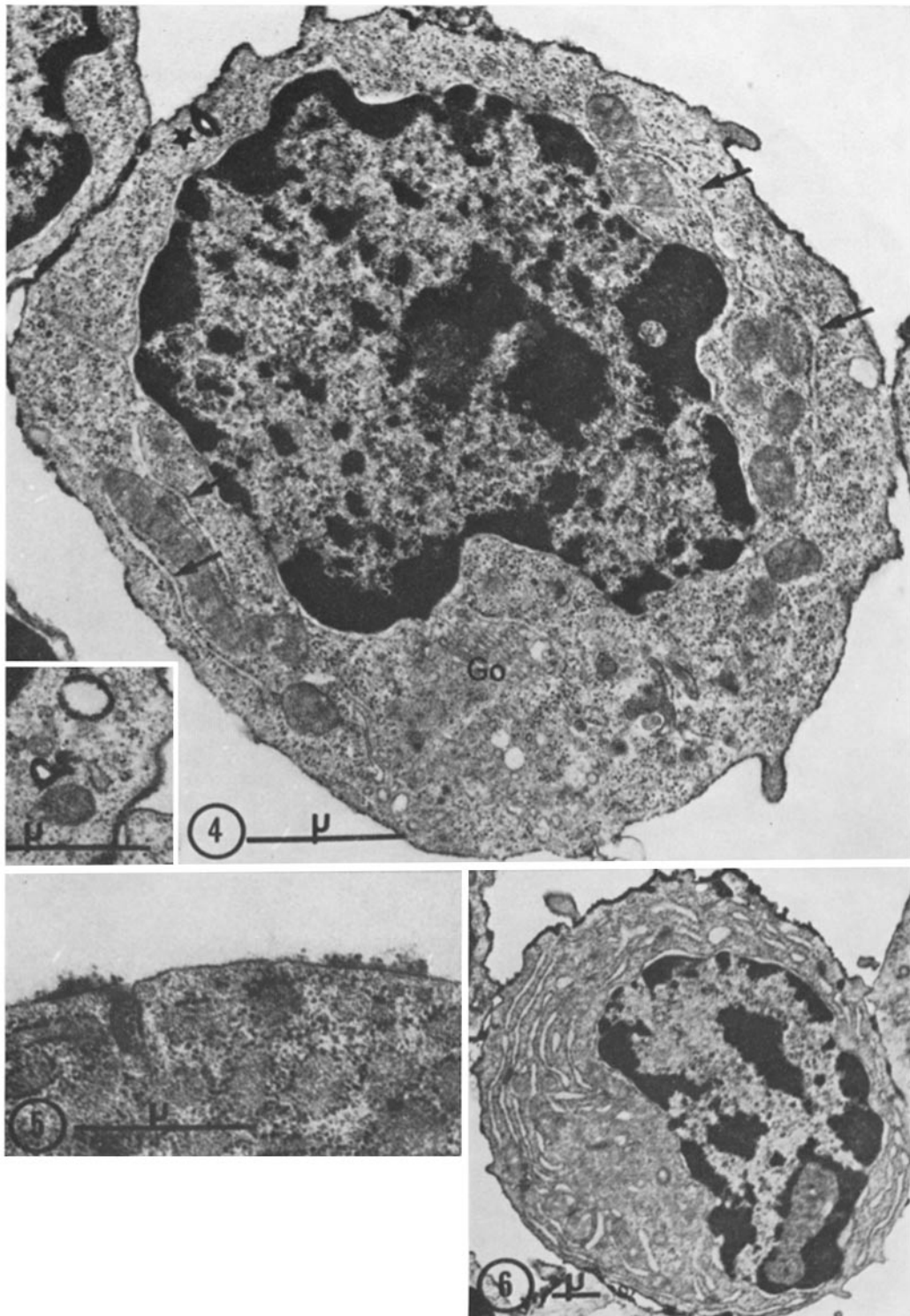
The T<sub>2</sub> lymphocytes (Figs. 13 and 15) were large, often blast-like cells showing considerable amounts of polyribosomes (Fig. 13) and occasionally some profiles of rough ER (Fig. 14). The most striking features of the cells classified as T<sub>2</sub> were: (a) the accumulation of polyribosomes (Figs. 13 and 14), often assuming a “rosette” configuration (Fig. 14) and (b) the existence of a grayish, apparently amorphous material, present as a continuous peripheral layer beneath the cell membrane (Figs. 14 and 15), and, also not uncommonly, between the polyribosomes (Fig. 15); at high magnification, this material consisted of densely packed microfilaments (see below). A number of these cells showed, in addition, membrane-bound granules (Figs. 15 and 17), of a diameter of 0.2–0.4 μ containing a homogeneous electron-opaque matrix.

Cells classified as T<sub>3</sub> were small and medium-sized lymphocytes exhibiting the most characteristic features within this cell class (Figs. 18–23); they could be recognized at low magnification even in the absence of a specific marker because of their relatively dark appearance (Fig. 7). Two cytoplasmic features seemed to contribute to this darkness: (a) clusters of densely packed mono-ribosomes, frequently in large amounts (Figs. 19, 20, 23); (b) large, irregular grayish areas, usually devoid of cell organelles (Figs. 18 and 19), which were found to consist at high magnification of a dense network of microfilaments (Fig. 22). A number of these cells also showed a few, long profiles of ER and numerous elongated or round vesicles, devoid of any detectable content (Figs. 18, 19, 21, 23), by places clearly belonging to the Golgi complex (Figs. 21 and

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FIGS. 1 and 2. Lymph node cells labeled with aMBLA followed by SaRIg-Pox. Enzymatic activity of Pox leads to a black reaction product which is visible on the cell membrane. The two labeled cells, with their clear cytoplasm containing few organelles, are typical examples of small B lymphocytes. × 14,000.

FIG. 3. Lymph node blast (4 days after priming with *B. pertussis*) labeled with aMBLA and SaRIg-Pox. The cytoplasm contains large amounts of mono- and polyribosomes and very little ER profiles (ER). The large mainly euchromatic nucleus (N) shows a huge nucleolus (nucl). × 12,000.



23). The  $T_3$  cells may have an irregular outline with finger-like projections (Figs. 18–21) and in some instances showed a polarization of the cell cytoplasm which formed a large “uropod” (Fig. 23); the uropod, bearing the marker, might show numerous infoldings of the cell membrane surmounting an area of intricate vesicles and vacuoles, and, closer to the nucleus, a Golgi complex.

The  $T_2$  lymphocytes were rare in the spleen and lymph nodes of nonimmunized animals; their number increased sharply in the days after immunization with *B. pertussis* or DBA/2 mastocytoma, and their proportions might reach about 20–30% of the T cells in the lymph nodes. During the same period, the other T cells were about equally divided between  $T_1$  and  $T_3$  lymphocytes. It is worth mentioning that a suspension of spleen cells from a nude mouse, examined by electron microscopy, was found to contain very rare but unmistakable  $T_3$  cells, which is in agreement with the finding of a very small percentage of MSLA+ cells by immunofluorescence (1).

When thymic cells were incubated with aMSLA or aBA $\theta$ , practically all the cells were strongly labeled. The ultrastructure of thymic cells will not be described here, except to mention that  $T_3$  lymphocytes were observed only exceptionally (see Discussion).

*Ultrastructure of the Short-Lived Lymphocytes.*—Pooled spleen and lymph nodes of nonimmunized mice injected every 4 hr for 36 hr with thymidine- $^3\text{H}$  (TdR- $^3\text{H}$ , 1  $\mu\text{Ci/g}$ ) were incubated with aMBLA or aMSLA (1) and processed for radioautography. The most frequently labeled T cells were  $T_3$  cells; the rare  $T_2$  cells were usually labeled (Fig. 24), but only very few labeled  $T_1$  cells were observed. Among B cells, the labeled cells were mostly medium-sized B lymphocytes, the blasts, not numerous, being also labeled.

#### DISCUSSION

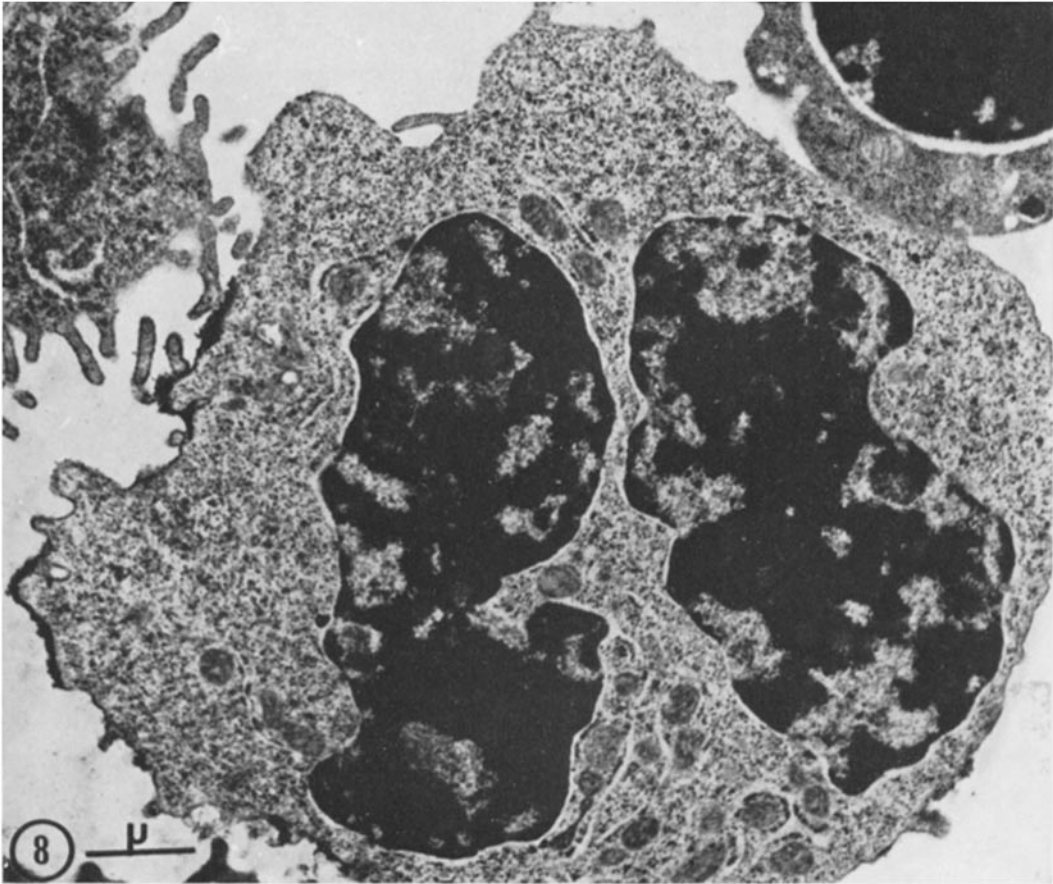
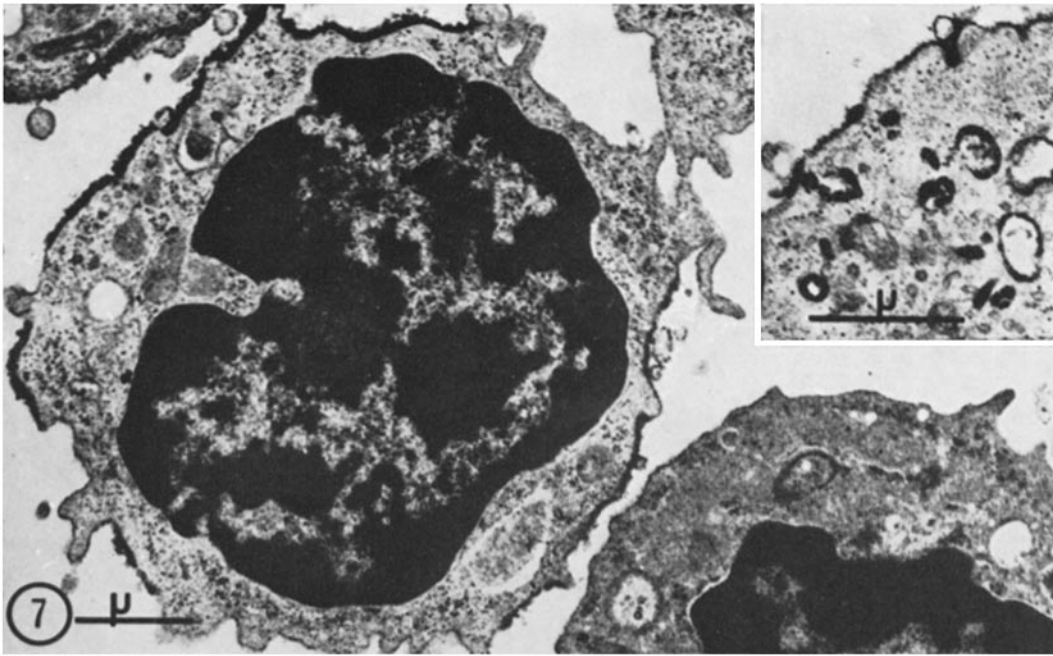
Different methods of ultrastructural labeling of the cell surface have been used in the present study. When some of the pitfalls mentioned in the Results section are taken into consideration, all these methods proved valid. The results obtained with the bridge technique showed that the ultrastructural localization

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FIG. 4. Lymph node cells (hyperimmune animal) labeled with aMBLA and SaRIg-Pox. This large cell contains many ribosomes and several elongated ER profiles (arrows). Cells of this type appear to be intermediates between the B blasts and the plasma cells. MBLA was usually less abundant, as judged from the labeling pattern, on blasts and large cells than on small and medium-sized lymphocytes. On the upper left part of the cell membrane (★), there is beginning pinocytosis of the label. *Go*, Golgi complex. When the B lymphocytes were briefly incubated at 37°C after exposure to SaRIg-Pox, there was evident pinocytosis of the label (inset).  $\times 23,000$ ; inset,  $\times 21,000$ .

FIGS. 5 and 6. Plasma cells labeled by incubations with aMBLA, followed by the “bridge” technique with SBMV (Fig. 5) or by SaRIg-Pox (Fig. 6). Plasma cells, especially when fully mature, showed also a reduced amount of MBLA compared with B lymphocytes. Fig. 5,  $\times 30,000$ ; Fig. 6,  $\times 7500$ .





of antigenic determinants on the cell membrane can be studied not only with hybrid antibodies (17) or with antibodies coupled to tracer molecules, such as ferritin or peroxidase, but also with methods requiring no manipulation of the antibody molecules other than purification. In spite of the four successive steps of incubation and washings which are involved, the bridge technique, especially with the SBMV particles, appears to be of excellent accuracy and allows, as in all indirect techniques, easy control of labeling specificity, by omission of the first incubation or its replacement by exposure to Ig devoid of specific antibody activity. When screening of large number of cells is concerned, however, Pox-labeled antibodies present the advantage of faster cell identification, because of the easy detection of the dark reaction product.

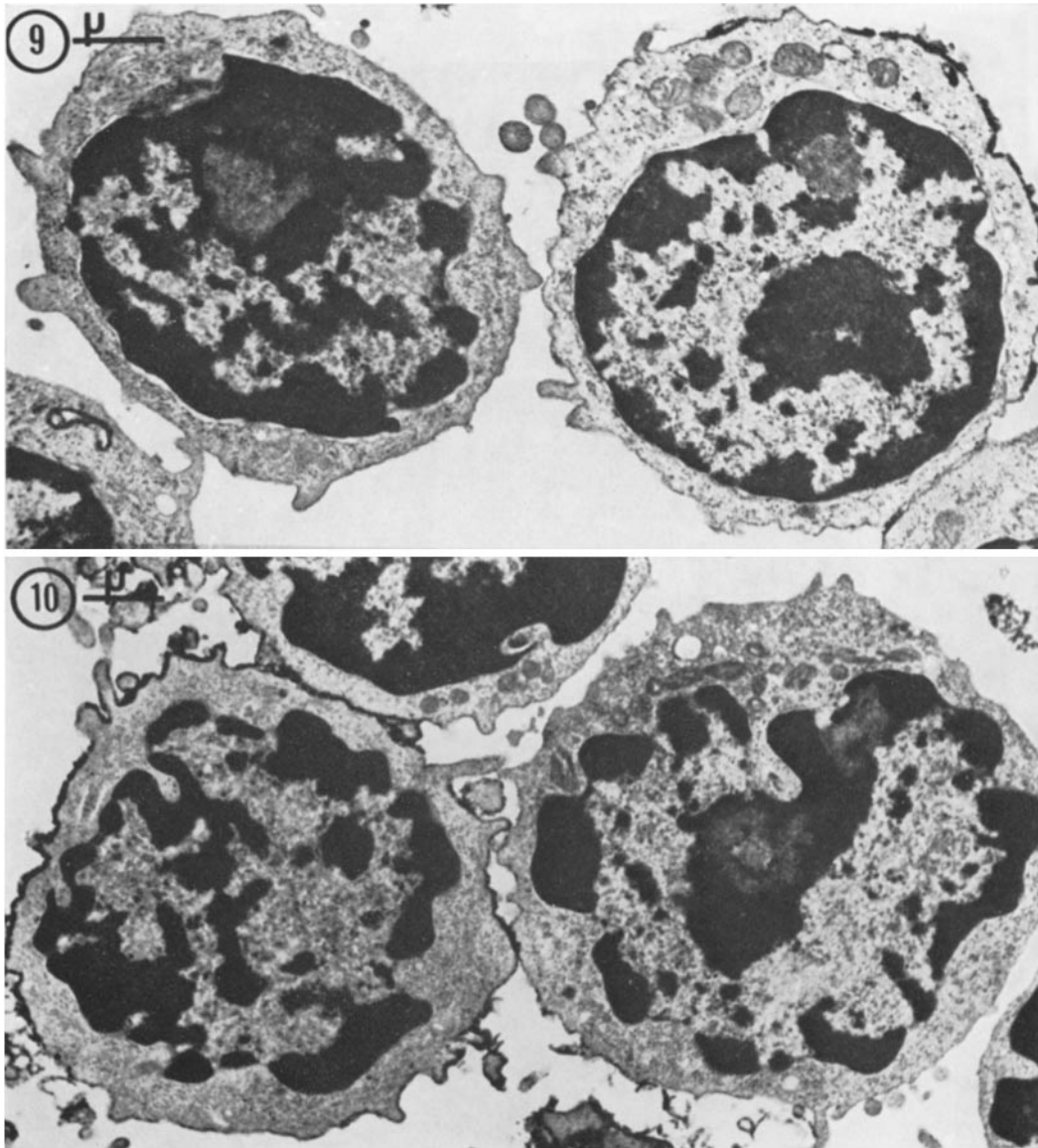
MBLA+ lymphoid cells showed a whole range of ultrastructural patterns: small and medium-sized lymphocytes, blasts and large cells with various amounts of ER, plasma cells at various stages of maturation. If these patterns are consistent with a differentiation pathway leading from small lymphocytes to mature plasma cells, there was no morphological evidence for a second differentiation pathway ending into cells clearly different from plasma cells and with distinct ultrastructural features, which could represent the B "memory cells" (18). Occasional MBLA+ cells classed in the plasma cells category had a somewhat darker cytoplasm and less regular ER than other plasma cells, and thus might have corresponded to the "lymphoplasmocytes" described by Avrameas and Leduc, a cell type tentatively identified as short-term memory cells (19). It was not felt, however, that the ultrastructural patterns of various plasma cells were different enough to subdivide them into various categories; it must be pointed out that the fixation procedure used in the present study differed from that described to favor the identification of lymphoplasmocytes (19).

sIg was found to be focally distributed on the membranes of cells with ultrastructural features identical with small and medium-sized B lymphocytes, and was never detected on the dark, characteristic T<sub>3</sub> cells; it was less easily detectable on large blasts and very rarely on plasma cells. This fits well with the results obtained by immunofluorescence in the companion paper (1), and contrasts with the results of Hämmerling and Rajewsky (20) who found, using hybrid antibodies and SBMV as the tracer, 95% of the mouse lymph node

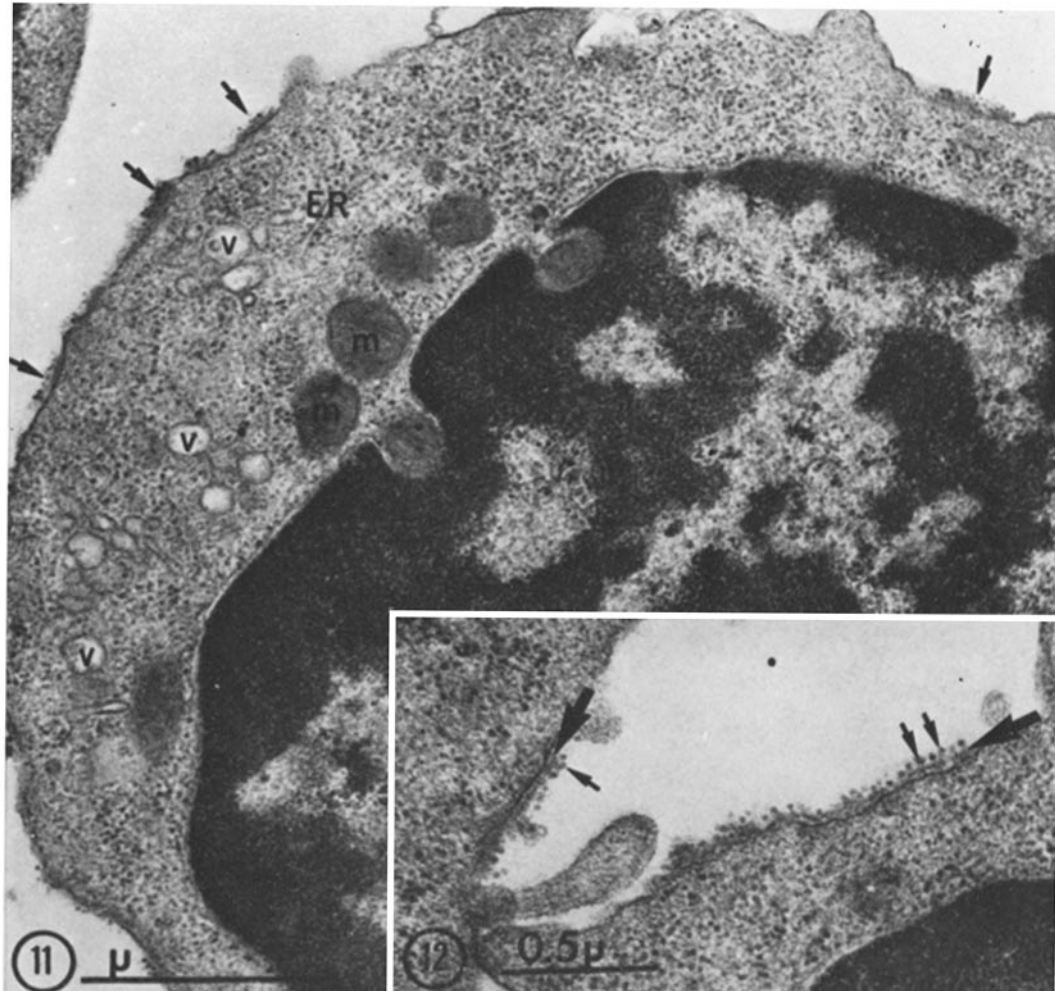
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FIG. 7. Spleen lymphocytes (hyperimmune animal) labeled with RaMIg followed by SaRIg-Pox, thus detecting the sIg. The cell on the left shows sIg in a patchy distribution and has the appearance of a medium-sized B lymphocyte. The cell on the right does not show sIg, and its dark cytoplasm is typical of T<sub>3</sub> lymphocytes (see Figs. 18–23). When cells labeled for detection of sIg were incubated at 37°C after fixation of SaRIg-Pox, there was marked pinocytosis of the label (inset).  $\times 15,000$ ; inset,  $\times 20,000$ .

FIG. 8. Spleen cells (secondary response to *B. pertussis*) labeled for sIg with RaMIg and SaRIg-Pox. Large cell showing numerous ribosomes and some ER profiles, resembling the B cell shown in Fig. 4. Cell in the upper right corner is an erythroblast.  $\times 14,000$ .



FIGS. 9 and 10. Spleen cells allowing a comparison between  $T_1$  and B lymphocytes. Labeling in Fig. 9 is done with aMBLA and in Fig. 10 with aMSLA, followed by SaRig-Pox. Thus in Fig. 9 there is a labeled B lymphocyte on the right and an unlabeled, presumably  $T_1$ , lymphocyte on the left; on Fig. 10 there is a labeled  $T_1$  lymphocyte on the left and an unlabeled, presumably B, lymphocyte on the right. Note the resemblance between both cell types (as opposed to the contrast between the two lymphocytes shown in Fig. 7) and the scarcity of organelles in  $T_1$  cells.  $\times 11,500$ .



FIGS. 11 and 12. Spleen cells labeled with aMSLA detected by the bridge technique with SBMV. The distribution of SBMV particles (arrows) on the cell membranes is patchy. At high magnification (Fig. 12) a thin opaque layer (large arrow), probably representing the antibody complexes of the "bridge," is seen between the cell membrane and the viral particles (small arrows). The cytoplasm is clear and contains mainly monoribosomes. There are also some mitochondria (*m*), vesicles (*v*), and short ER (*ER*) profiles. Fig. 11,  $\times 30,000$ ; Fig. 12,  $\times 40,000$ .

lymphocytes bearing  $\mu$ - and  $\kappa$ -chains, and thus concluded that both T and B lymphocytes have sIg in comparable amounts. This discrepancy cannot be ascribed to a difference in the sensitivity of the methods of detection used, which were of similar nature. Using the still more sensitive technique of radio-

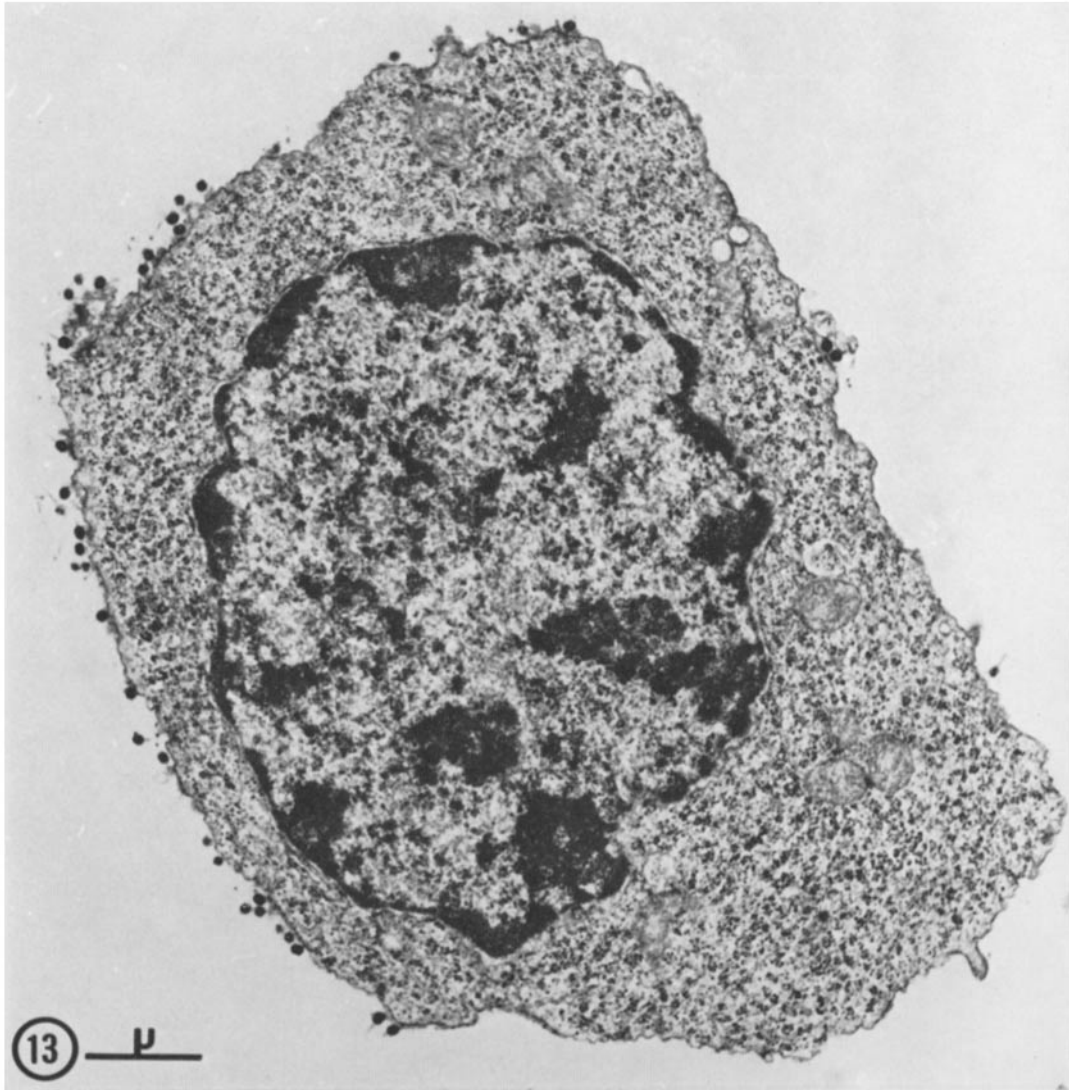


FIG. 13. Spleen cell (10 days after alloantigenic immunization with DBA/2 mastocytoma) labeled with aMSLA detected by the bridge technique with phage T<sub>4</sub>. Some of the phage heads are sectioned tangentially and therefore barely visible. This large blast-like cell, classified as T<sub>2</sub> lymphocyte, is mostly characterized by its very large content in polyribosomes.  $\times 15,000$ .

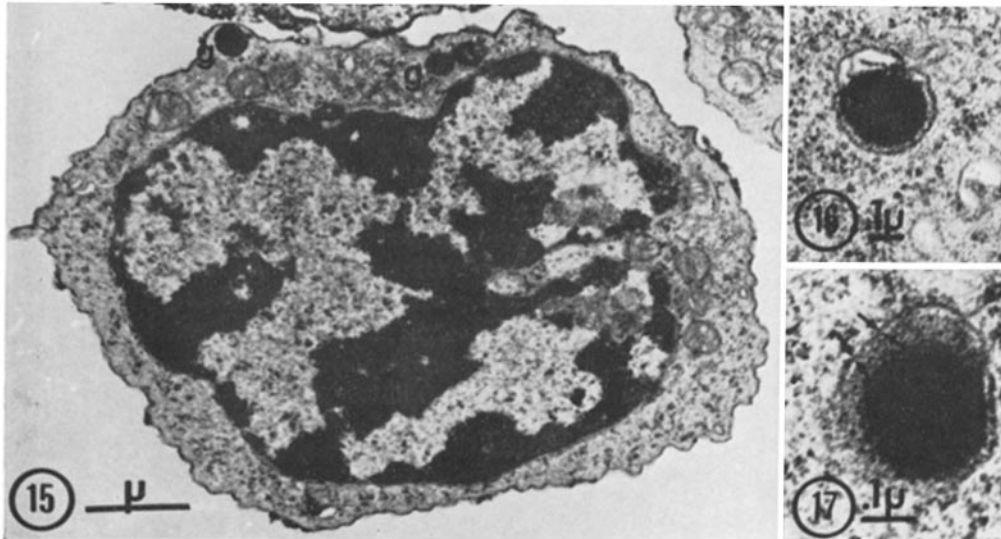
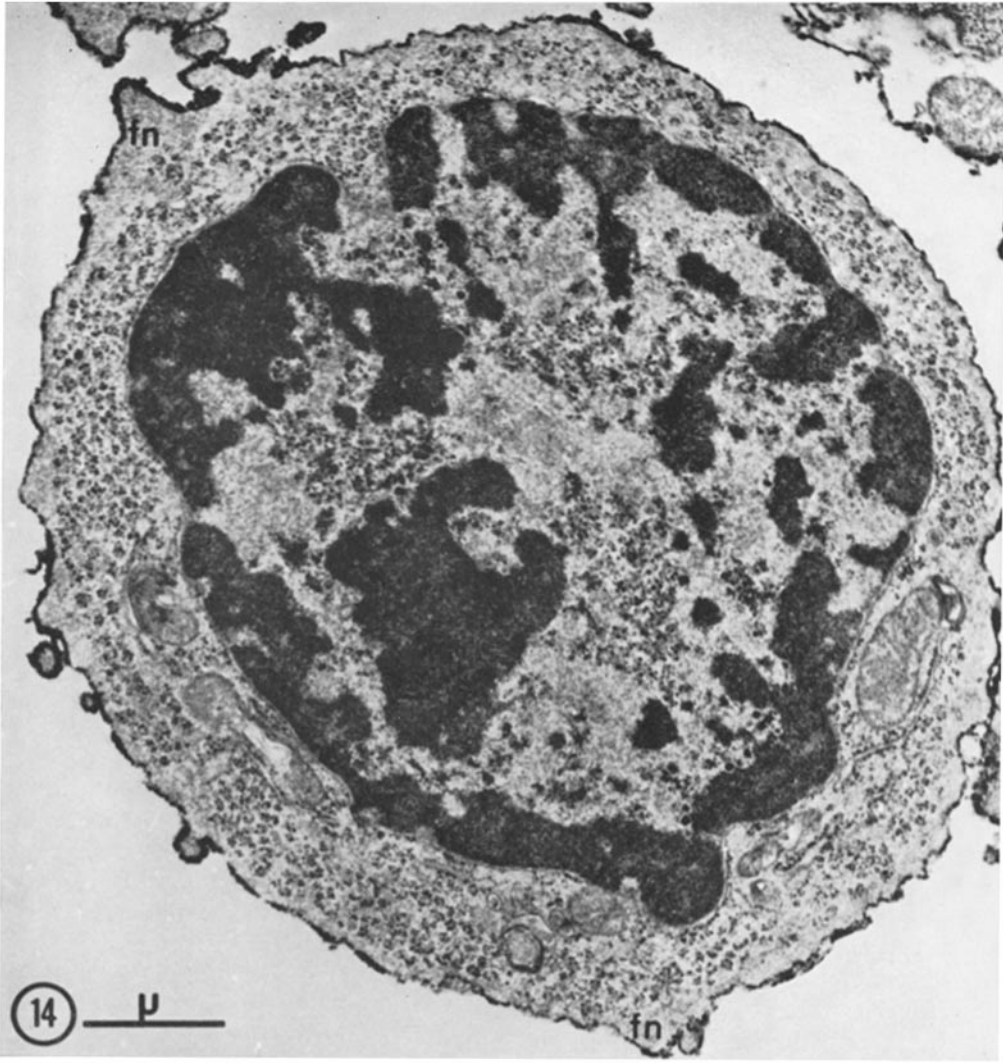
autography with <sup>125</sup>I-labeled antibodies, Perkins et al. found on electron micrographs a percentage of sIg-bearing lymphocytes in the range of 40% (21), which is in reasonable agreement with our own immunofluorescent (1) and ultrastructural observations.

The ultrastructural aspects of MSLA+ or BA $\theta$ + cells allowed their classification into three categories. The blasts and large cells, called T<sub>2</sub> lymphocytes, were especially conspicuous during the early immune response to *B. pertussis* and DBA/2 mastocytoma. The existence of two other, very distinct cell types suggest a process of differentiation leading, through a series of cell divisions, from one of the cell types to the other (Fig. 25). Since the T<sub>3</sub> cells were characterized by their larger number of ribosomes and a striking development of a filamentous network, while the T<sub>1</sub> cells lacked any peculiar feature suggestive of a state of differentiation, it would appear that T<sub>1</sub> lymphocytes, under antigenic stimulation, transform into T<sub>2</sub> and finally T<sub>3</sub> cells. Strong support for this concept has been obtained by the electron microscopic study of hydrocortisone-resistant and TL- thymocytes<sup>2</sup> (see below). The cells persisting in the thymus after hydrocortisone treatment are much more immunocompetent than the average thymus population, as judged by their capacity to elicit graft-vs.-host reactions in semiallogenic hosts (22, 23); the same is true, in the mouse strains whose thymocytes carry the "thymus leukemia" or TL antigens (24), of the cells persisting after treatment of thymocytes with anti-TL antisera in the presence of complement (25). It appears that these hydrocortisone-resistant TL- cells are mature thymocytes present in the medulla and ready to leave the thymus to become peripheral T lymphocytes. The ultrastructural aspect of these cells is similar to that of T<sub>1</sub> lymphocytes.<sup>2</sup> Furthermore, when such cells are injected into lethally irradiated allogenic recipients according to the protocol of Cerottini et al. (26), they proliferate massively in the host spleen, from which they can be obtained after 5 days and shown to be strongly cytotoxic in vitro for cells of the host strain (26). The electron microscope appearance of these cells is that of T<sub>2</sub> cells, with an abundance of polyribosomes, filamentous network at different degrees of development, and frequent dark granules.<sup>2</sup> It seems therefore likely that T<sub>1</sub> cells represent "virgin," T<sub>2</sub> "stimulated," and T<sub>3</sub> "differentiated" T cells.

The dark appearance of the differentiated T<sub>3</sub> cells, made them, under the conditions of cell preparation used in the present experiments, so characteristic that they could easily be recognized without surface marker. This darkness corresponded to an over-all increase in cytoplasmic density, especially conspicuous in some cells where there were large zones of cytoplasm devoid of cell organelles. At high resolution the increased cytoplasmic density was found to result from an accumulation of microfilaments, generally forming a dense network. This ultrastructural pattern was identical to the "microfilamentous network" found in other cells to be sensitive to the action of cytochalasin B (27, 28), and considered for this reason to be one of the contractile elements of

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<sup>2</sup> A. Matter, B. Lisowska-Bernstein, and P. Vassalli, unpublished observations. We are grateful to Dr. L. Old and to Miss E. Stockert for a generous gift of mouse anti-TL antiserum.



the cells, since this drug inhibits a wide variety of cellular movements. This microfilamentous network is present in every cell (28), but its very striking development and dense accumulation appears to be one of the characteristics of activation and differentiation of T cells. This would suggest that the activated T<sub>2</sub> cells and, still more, the differentiated T<sub>3</sub> cells may be cells presenting a high degree of motility. T<sub>3</sub> cells indeed presented different shapes, uropods, and vesicles consistent with strong pinocytic activity; however, they had been incubated at 0°C before fixation, a condition which certainly does not favor cell movement, so that this problem remains to be explored by other ways. Whether this postulated high motility might be related to the role of activated and differentiated T lymphocytes in immune surveillance and their function as killer cells is a matter of speculation,<sup>3</sup> it is however, interesting to mention in this respect that the *in vitro* killing of allogenic cells by T cells of sensitized mice is totally suppressed by cytochalasin B (29). As for the dark granules found in stimulated T cells, one can also speculate about their possible relationship to the cytotoxic activity of these cells, or to the release of lymphokines (30).

The radioautographic study of the "short-lived" spleen cells of normal mice showed that most of the labeled T lymphocytes were not T<sub>1</sub>, but T<sub>3</sub> cells, the rare T<sub>2</sub> cells being usually also labeled. It is not known whether this reflected labeling resulting from "spontaneous" immunization or whether T<sub>3</sub> cells are able to periodically divide even in the absence of antigenic stimulation.

Finally, a word should be said concerning the usually focal nature of all the antigens, B, T, or sIg, detected on the cell membranes. By immunofluorescence, monovalent antibodies to sIg gave a smooth ring, and not a patchy membrane staining (1), and they did not "modulate" sIg at 37°C except when they were themselves exposed to divalent antibodies in the indirect fluorescent technique

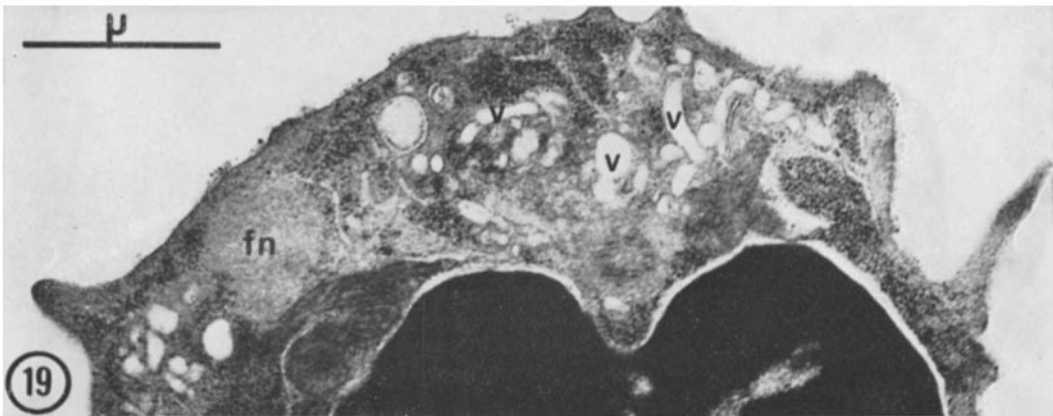
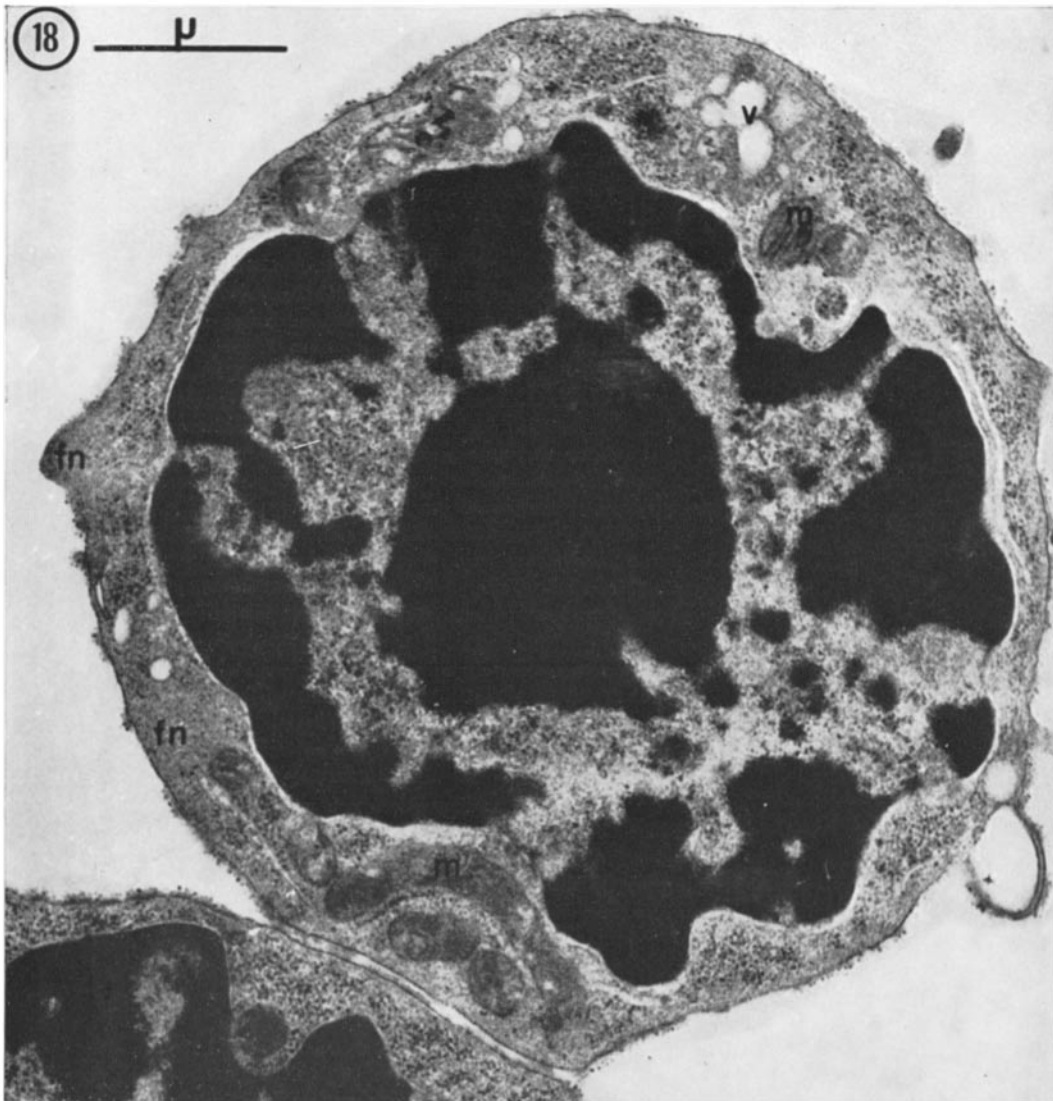
<sup>3</sup> Differentiated T<sub>3</sub> lymphocytes do not appear to recirculate on a large scale, since they are very rarely found in the thoracic duct lymph of normal mice. A. Matter, D. Guy-Grand, C. Griscelli, and P. Vassalli.

FIG. 14. Lymph node cells (4 days after immunization with DBA/2 mastocytoma) labeled with aBAθ followed by SaRIg-Pox. This cell classified as T<sub>2</sub> lymphocyte is characterized by its large content in polyribosomes, often forming "rosettes," and by an enveloping filamentous network (*fn*), appearing at this magnification as a peripheral grayish area. × 18,500.

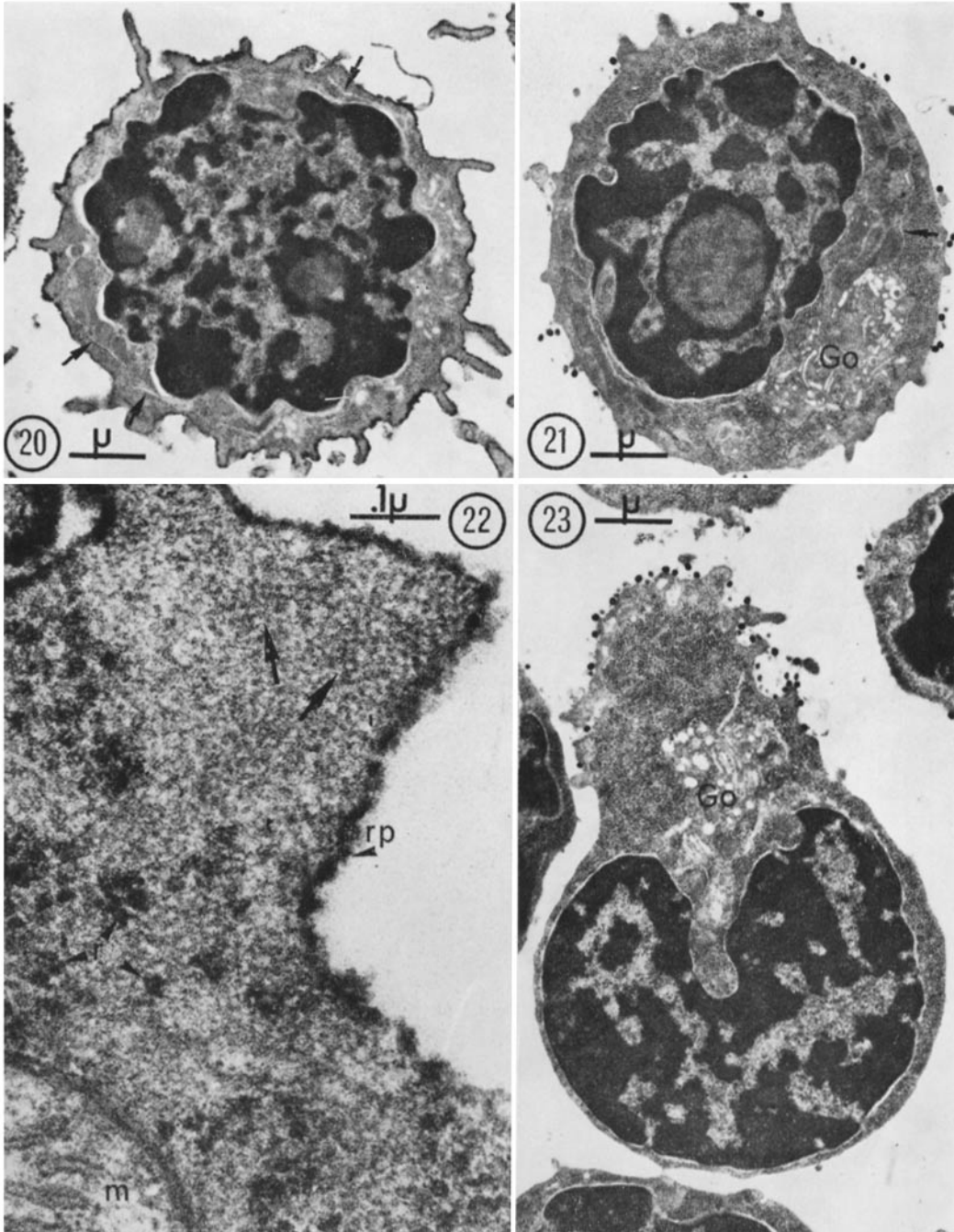
FIG. 15. Lymph node cell (4 days after immunization with DBA/2 mastocytoma) labeled with aMBLA and SaRIg-Pox. These discrete deposits of reaction product slightly blackening the cell membrane give an example of the degree of unspecific staining which can be observed on occasional cells with some samples of SaRIg-Pox. This large cell is characteristic of T<sub>2</sub> lymphocytes, in spite of the less numerous polyribosomes, because of the marked development of the filamentous network, giving a grayish appearance. It shows two dark granules (*g*) which are not rare in this cell type. × 13,500.

FIGS. 16 and 17. Higher magnification of granules observed in T<sub>2</sub> lymphocytes. The granules are membrane bound, the membrane being about 75 Å, as seen between arrows in Fig. 17. Fig. 16, × 50,000; Fig. 17, × 68,000.





For legends Figs. 18-23, see page 1026.



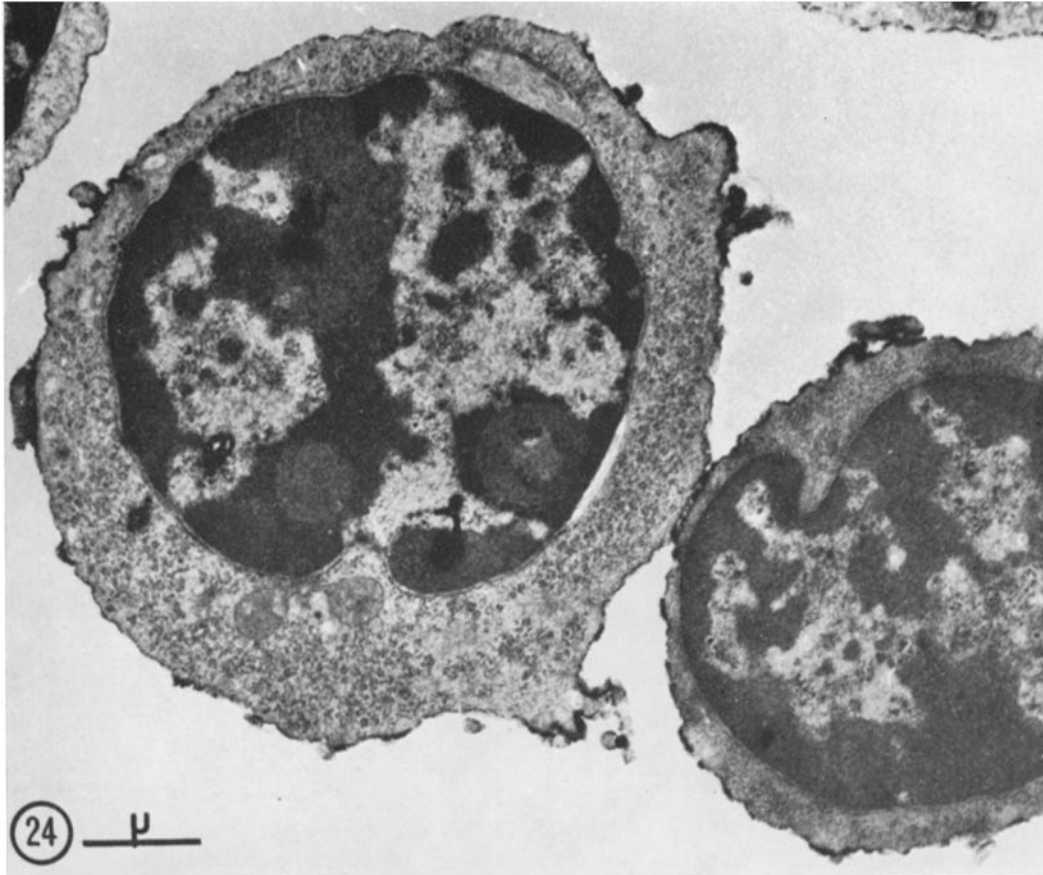


FIG. 24. Radioautography after *in vivo* labeling with thymidine- $^3\text{H}$  of spleen cells incubated with aMSLA, followed by SaRIg-Pox. The large  $\text{T}_2$  cell on the left, but not the dark, smaller  $\text{T}_3$  lymphocyte on the right, shows nuclear grains.  $\times 15,000$ .

FIGS. 18-23. Examples of  $\text{T}_3$  (or differentiated T) cells from spleen or lymph nodes, labeled with aMSLA followed by the bridge technique with SBMV on Figs. 18 and 19, with phage  $\text{T}_4$  on Figs. 21 and 23, and by SaRIg-Pox on Figs. 20 and 22. All the cells are dark, small to medium-sized lymphocytes, with dense clusters of monoribosomes (Figs. 19, 21, 23) and a grayish filamentous network (*fn*), which appears by places as grayish zones devoid of cell organelles (Figs. 18 and 19), and at high magnification corresponds to a microfilamentous network (Fig. 22: arrow; microfilamentous network; *r*, ribosomes; *m*, mitochondrion; *rp*, reaction product of peroxidase). Well-developed Golgi (*Go*) complexes are seen in Figs. 21 and 23, elaborated systems of vesicles (*v*) on Figs. 18 and 19, and a few, long ER profiles (arrows) on Figs. 20 and 21. Differences in cell shapes are marked, with microvilli well developed on Fig. 20 and a characteristic uropod on Fig. 23. Figs. 18 and 19,  $\times 24,000$ ; Figs. 20, 21, and 23,  $\times 11,000$ ; Fig. 22,  $\times 125,000$ .

(1, 31). The focal distribution of B and T antigens as detected in the present study, which used not only divalent antibodies but also indirect technique with "piggy back" effects, might thus not represent the actual antigen distribution in the living state (32).

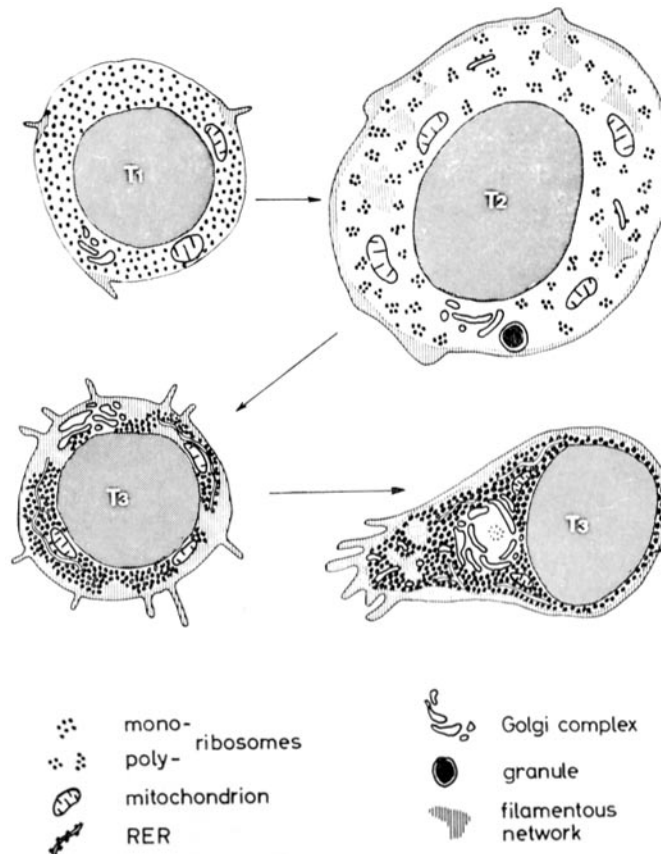


FIG. 25. A possible pathway for T cell differentiation. For explanation see text.

#### SUMMARY

The ultrastructural features of B-, T-, and surface Ig(sIg)-bearing cells have been studied on cell suspensions from lymphoid organs of mice at different stages of immunization. The cells were identified by exposure to rabbit antibodies against mouse-specific lymphocyte antigens (MSLA) or brain-associated  $\theta$  antigen (BA $\theta$ ) for T cells, mouse-specific bone marrow-derived lymphocyte antigens (MBLA) for B cells, and mouse Ig for sIg-bearing cells. The rabbit antibodies fixed on the cell surfaces were detected by peroxidase-labeled sheep

anti-rabbit Ig antibodies or by a "bridge" technique using southern bean mosaic virus or bacteriophage T4 as the final markers. In some experiments, short-lived lymphoid cells were labeled in vivo with repeated tritiated thymidine and the ultrastructural detection of their surface antigens was combined with radioautography.

MBLA+ lymphoid cells showed a whole range of ultrastructural patterns. Most were small and medium-sized lymphocytes with a clear cytoplasm containing mono- and polyribosomes, but they comprised also blasts and large cells with various amounts of endoplasmic reticulum, as well as plasma cells at different stages of maturation. sIg-bearing cells appeared to be identical with MBLA+ cells, except that sIg was less easily detectable on large blasts, and only very rarely observed on plasma cells. MSLA+ and BA $\theta$ + cells fell into three categories. One of them (T<sub>1</sub> cells) consisted of small to medium-sized lymphocytes with a clear cytoplasm and few organelles, mostly monoribosomes. A second consisted of large cells (T<sub>2</sub> cells) characterized by numerous polyribosomes often in a "rosette"-like pattern, occasional dark, membrane-bound granules, and a developing "filamentous network." The third, very characteristic type, (T<sub>3</sub> cells) was represented by dark small to medium-sized lymphocytes, usually containing large amounts of closely packed ribosomes and showing a striking accumulation of filamentous network, often condensed in large areas devoid of cell organelles. This filamentous network appeared to correspond to the cytochalasin B-sensitive system of microfilaments found in other cells and considered to be one of the contractile elements of the cell. The T<sub>3</sub> lymphocytes showed frequently vesicles suggestive of a strong pinocytotic activity, and assumed a variety of shapes, including uropods. Evidence is presented that T<sub>1</sub> lymphocytes represent "virgin" T cells, T<sub>2</sub> "activated," and T<sub>3</sub> "differentiated" lymphocytes.

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