DETECTION OF SIMULTANEOUS ANTIBODY SYNTHESIS IN PLASMA CELLS AND SPECIALIZED LYMPHOCYTES IN RABBIT LYMPH NODES*

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The problem of determining the cellular basis of humoral antibody production is compounded by the heterogeneity of cell types in lymphoid tissues, the synthesis of several types of antibodies, and the variations in response produced by different antigens. The role of plasma cells in antibody synthesis is well established (14, 21) but other cells, especially the lymphocyte, also have been implicated (6, 8, 12, 13, 25). The lymphocyte, furthermore, has been the chief candidate for the function of carrier of immunological memory (5, 9).

The recent introduction of techniques for localizing specific antibodies within cells by electron microscopy (1, 14, 15) promises to make possible the identification of the cells responsible for all aspects of antibody synthesis. The present investigation, utilizing the procedure for visualizing intracellular antibody to horseradish peroxidase (14), was initiated to review the sequence of events in the popliteal lymph node of the rabbit during the primary and secondary responses.

Under the conditions employed in this study, namely after the injection of a soluble, enzyme antigen plus Freund's adjuvant, antibody was found in three types of cells: (*a*) large numbers of typical plasma cells, (*b*) a few large lymphocytes or lymphoblasts, and (*c*) very abundant modified or specialized small lymphocytes which bear some characteristics of plasma cells. We tentatively call the specialized lymphocyte a "lymphoplasmacyte" and we present evidence that it may be the immunologic memory cell.

Materials and Methods

Animals.—1 yr old albino rabbits weighing approximately 2.5–3 kg were used.

Antigen.—Horseradish peroxidase RZ3 possessing a special activity of 295 purpurogallin units/mg was obtained from Sigma Chemical Co., St. Louis, Mo.

Antisera.—Sheep serum anti-rabbit γ-globulins and horse serum anti-whole rabbit serum were purchased fron L'Institut Pasteur, Paris, France. Goat serum anti-rabbit immunoglobulins was prepared by repeated injections of rabbit antibodies incorporated in complete Freund's adjuvant. The rabbit antibodies were isolated from whole antisera by use of immunoadsorbents (2).

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Injection of Rabbits.—5 mg of peroxidase dissolved in 0.5 ml of saline was dispersed in 0.5 ml of complete Freund's adjuvant (Difco Laboratories, Inc., Detroit, Mich.). This emulsion was injected into both hind foot pads of each rabbit. Animals were bled and killed 4, 6, 8, 10, 12–14, 17, 21, 28, and 34 days after injection (2 rabbits each on days 8, 10, 12). A second group of rabbits was killed 1 through 8 days after a second injection of 2.5 mg of peroxidase in saline, given 14 days after the first injection, and a third group 1–4 and 6 days after a booster injection given 34 days after the first antigen administration. Thus, a total of 27 rabbits was used for revealing antibody to peroxidase, 14 for a single antigen injection and 13 for a second antigen administration. In addition, eight rabbits hyperimmunized with other enzymes including glucose oxidase, tyrosinase, and lactic dehydrogenase, were donors of control tissues.

Antibody Analysis.—In the sera of the rabbits the level of antibody to peroxidase was measured by passive hemagglutination following a procedure described in detail elsewhere (3). According to this method, peroxidase was covalently attached to the red cell by the use of glutaraldehyde. To distinguish between 19S and 7S antibody, passive hemagglutination was carried out either with or without treatment of the antisera with 2-mercaptoethanol. To do this, the sera were diluted 1:4 with buffered saline and reacted at 37°C for 90 min with 2-mercaptoethanol at a final concentration of 0.1 M. The sera were then dialyzed overnight at 4°C against buffered saline. Control sera were treated in the same way but buffered saline was added in the place of mercaptoethanol.

Isolation of the Anti-Peroxidase Antibody.—Rabbit anti-peroxidase antibodies were specifically isolated by passage of the corresponding whole antisera on an insoluble peroxidase immunoadsorbent. This immunoadsorbent was prepared by copolymerization at pH 5 of five parts of bovine serum albumin and one part of peroxidase using glutaraldehyde as the cross-linking agent. The isolation of antibodies was carried out following the batching procedure and by using glycine-CLH buffer 0.1 M, pH 2.5–2.8 as the eluting medium of the adsorbed antibodies (3). Isolated antibody was concentrated on an Amicon ultrafiltration cell equipped with a Diaflo UM.10 membrane and then dialyzed against saline at 4°C.

Characterization of Anti-Peroxidase Antibody by Enzyme Immunoelectrophoresis.—To characterize the antibodies, sera from normal and immunized rabbits were subjected to immunoelectrophoresis and bands developed with either sheep serum anti-rabbit γ-globulins or goat anti-rabbit immunoglobulins or horse anti-whole rabbit serum. The immunoelectrophoretic slides were washed for 3 days in buffered saline and then immersed overnight at room temperature in buffered saline containing 0.1 mg of peroxidase per ml. Following this the slides were washed again for 5 days in saline and dried under filter paper. The enzyme, which was thus fixed on its homologous precipitated antibody, was then revealed by incubating the slides in the same substrate employed to detect intracellular peroxidase by light microscopy.

Light Microscopy.—Rabbits were sacrificed at various times after immunization. Popliteal lymph nodes were removed, stripped of fat, and placed in Hanks's medium at 4°C. Nodes were teased gently with forceps and the suspension was passed through a 70 μ porosity stainless steel sieve and washed three times with the Hanks's medium by low speed centrifugation. The final washed cell pellet was suspended in an equal volume of Hanks's medium and the cells were smeared on slides. The slides containing the air-dried cells were fixed for 30 min in 60:40 alcohol:ether, washed in saline and incubated for 30 min in buffered saline containing 0.1 mg/ml of peroxidase. The slides were then washed again with buffered saline and the sites of bound peroxidase were revealed with the substrate used for electron microscopy but omitting postincubation of the stained preparations in osmium tetroxide. For each cellular preparation about 5000 nucleated cells were randomly counted and the positive cells were noted.

Electron Microscopy.—Samples were taken from both popliteal lymph nodes of each rabbit and fixed in a 1% formaldehyde solution, freshly prepared from paraformaldehyde, in 0.2 M cacodylate buffer, pH 7.3, containing 0.25 M sucrose (15). The tissue was cut into blocks of

approximately 1 mm^3 in a drop of fixative on a card, then transferred to a vial containing a large volume of fixative which was changed at least twice. This entire procedure was carried out at room temperature, 18–22°C, and lasted 15–20 min. The vials containing the tissue and fixative were then placed on an agitator at 3°C for 45 min. Subsequently, the fixative was removed by 3 changes, 30–60 min each, of buffer-sucrose (0.2 M Na-cacodylate–0.25 M sucrose) and the tissue stored in this solution at 3°C for at least a day up to 2 wk.

To carry out the reaction for revealing the sites of antibody, the blocks of tissue were cut into thinner slices with the Smith-Farquhar tissue chopper (Sorvall TC-2, Sorvall Laboratory Equipment, Norwalk, Conn.) set at 20–40 μ. Several of the 1 mm^3 blocks of tissue were piled on the stage of the chopper at one time; this resulted in slices of varying thickness but it was preferable to the use of a single, larger, sausage-shaped block of tissue as recommended for use with the tissue chopper because fixation was better in the smaller blocks. The resulting sections were subsequently handled in conical centrifuge tubes and the sections were sedimented by low speed centrifugation for each solution change.

The steps for carrying out the immunocytochemical reaction were the same as previously reported (14, 15) and include: (*a*) rapid wash, 2 x 5 min. in cacodylate buffer to remove the sucrose; (*b*) exposure to a solution of the antigen, horseradish peroxidase, 1 mg/ml of buffer, for 1 hr at room temperature, during which time the antigen combines with the antibody in the tissue; (*c*) wash, 2 x 10 min in buffer to remove unbound peroxidase; (*d*) postfixation in 2.5% glutaraldehyde in the cacodylate buffer without sucrose for 15 min at room temperature to assure firm binding of the antigen-antibody complex to the tissue; (*e*) wash, 3 x 10 min in buffer to remove unbound fixative; (*f*) incubation for 30 min at room temperature in the substrate medium to reveal peroxidase activity (10), 5 ml of tris(hydroxymethyl)aminomethane (Tris)–HCl pH 7.5 containing 2.5 mg diaminobenzidine (DAB) and H_2O_2; (*g*) rinse in distilled water; (*h*) postfixation in 2% OsO_4 in cacodylate buffer pH 7.3 for 1 hr at room temperature; (*i*) dehydration in alcohol or acetone and embed in Epon; (*j*) examination of sections without staining.

The incubation medium was prepared just before use. Its pH was checked after addition of DAB to the buffer and before addition of H_2O_2. Some samples of DAB caused a drop in pH only to 7.0–7.2 and these were employed successfully without adjustment of the pH; other samples reduced the pH to 4.5–4.8 and these were discarded or the pH adjusted to neutrality. The quantity of stock H_2O_2 solution to be added to produce the largest amount of precipitation product was determined at about 2 wk intervals by adding varying quantities of the stock solution to a series of vials containing the Tris buffer, DAB, and peroxidase, because an excess of H_2O_2 inhibits the peroxidase reaction. After incubation and before postfixation with OsO_4, during rinsing in distilled water, the tissue slices were examined with a dissecting microscope and those that were sufficiently thin for complete penetration of the reagents were selected for study.

The ultrathin sections were examined with a Siemens Elmiskop I at 80 kv with 50 μ objective apertures.

RESULTS

Identification of Circulating Antibodies.—The antibody titers in the sera of the immunized animals at different times after the injection of the antigen are shown in Fig. 1. The titers of the b-mercaptoethanol–treated antisera were almost always lower than those of untreated antisera whether the antisera were from rabbits injected once or twice with the antigen. In general, the decrease in antibody titers was more pronounced in antisera having high titers than in antisera of low titers. However, the decrease was always a small one and never

exceeded three dilutions for high and one dilution for low titer antisera. When antibodies from low titer antisera were isolated and then treated with b-mercaptoethanol, a similar but more pronounced difference between the titers of the treated and untreated preparation was observed.

Enzyme immunoelectrophoresis revealed in all antisera the presence of IgG antiperoxidase antibody. In high titer antisera and in preparations of antibodies isolated from low titer antisera, the presence of a very small amount of IgM antiperoxidase antibody was also detectable (Fig. 2).

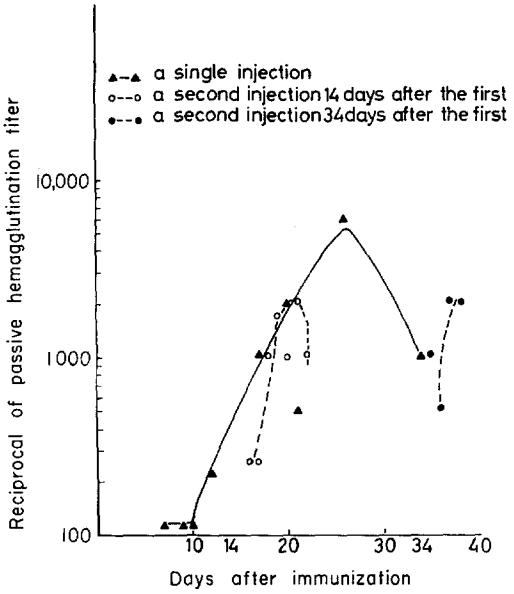


FIG. 1. Antibody titers in sera of rabbits after a single or a second injection of peroxidase.

Light Microscopy—Two cell types containing antibody were seen in smears of the stimulated nodes: cells which corresponded to the larger plasma cell series, and small round cells having morphologic characteristics of small lymphocytes (Fig. 3). The two cell types were present at all times of observation. Heavily, moderately, and lightly positive cells were observed in the plasma cell series while the small round cells usually were heavily stained.

In Fig. 4 are seen the number of antibody-containing cells per thousand of nucleated cells found in the popliteal lymph nodes of immunized rabbits, examined at different times after the injection of peroxidase. Comparison of Figs. 1 and 4 reveals that the number of antibody-containing cells is roughly proportional to the concentration of circulating antibody.

Electron Microscopy.—The use of nonfrozen, 20–40 μ thick sections prepared by the TC-2 tissue chopper, instead of thicker blocks of tissue as used in an earlier study (14), permitted the observation of a larger number of reactive cells with ease. Numerous positive cells often were found in a single field (Fig.

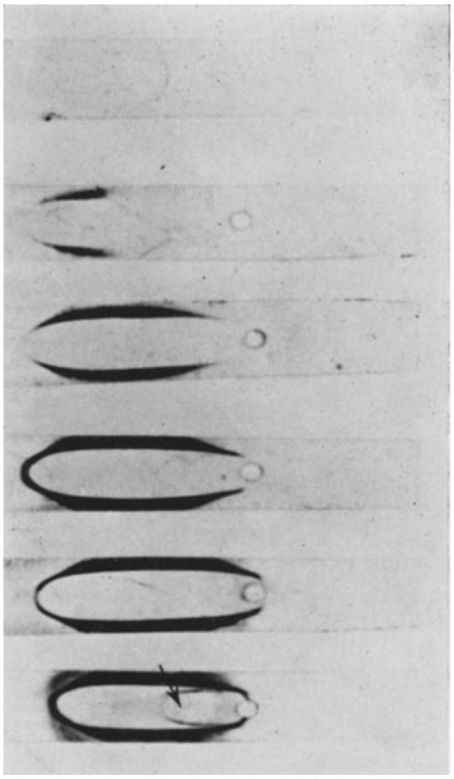


FIG. 2. Characterization of rabbit antiperoxidase antibody by enzyme immunoelectrophoresis. Rabbit sera were developed with goat anti-rabbit immunoglobulin antibody. From top to bottom: normal rabbit serum, 11, 12, 13, 14, and 18 days after a single injection of peroxidase. Note that IgM is seen in the 18 day preparation (arrow). In the original slide, faint positive reaction on the line of IgM is also noted in days 13 and 14.

5) and even when the number of antibody-containing cells was low, whenever they were detectable by light microscopy they also were found in preparations made for electron microscopy.

Prefixation in a dilute formaldehyde solution tended to provide somewhat less sharply defined images than when gluteraldehyde prefixation (14) was used and some types of cells, namely, reticular cells and some large lymphocytes,

appeared to be more fragile and often were damaged by subsequent cytochemical procedures for ultrastructural localization of antibody (Fig. 5). However, the formaldehyde fixation not only revealed a positive reaction on the ribosomes within a few large lymphocytes (15) but also, in this study, has permitted the identification of two different families of immunocytes which synthesize and secrete antibody (Fig. 6).

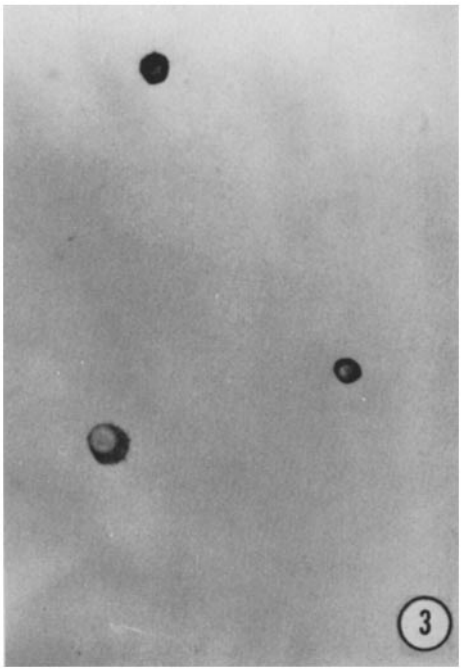


FIG. 3. Light microscopic detection of antiperoxidase antibody in a cell suspension from a stimulated lymph node: three positive cells—a typical plasma cell at lower left, and two small round cells resembling small lymphocytes. Abundant negative cells in the field have too little contrast to be discernible. × 400.

One type of antibody-synthesizing cell is the typical plasma cell (Figs. 6, 7, 9, 10). It is relatively large and its prominent interchromatin regions persist in the nucleus throughout its development from the antibody-producing blast stage to the final mature plasma cell. Its cytoplasm is always clear, and some cisternae of its endoplasmic reticulum become progressively enlarged with the accumulation of antibody until, eventually, localized segments become antibody-positive Russell bodies. The second type of antibody-synthesizing cell (Figs. 6, 8, and 11–18), distinguishable from the above in tissue prefixed with formaldehyde but not glutaraldehyde, is smaller than the one described above;

its nucleus is completely hyperchromatic, like that of small lymphocytes, with little or no interchromatinic regions during all of its development except the initial blast stage; its cytoplasm is very dense, also like that of small lymphocytes. However, unlike other small lymphocytes but like the plasma cells, it develops an elaborate, antibody-containing endoplasmic reticulum. This rough-surfaced endoplasmic reticulum (RER), however, becomes only slightly distended, even when the cell reaches its peak content of antibody. For the purposes of the present paper we have called the latter cell the *lymphoplasmacyte*.

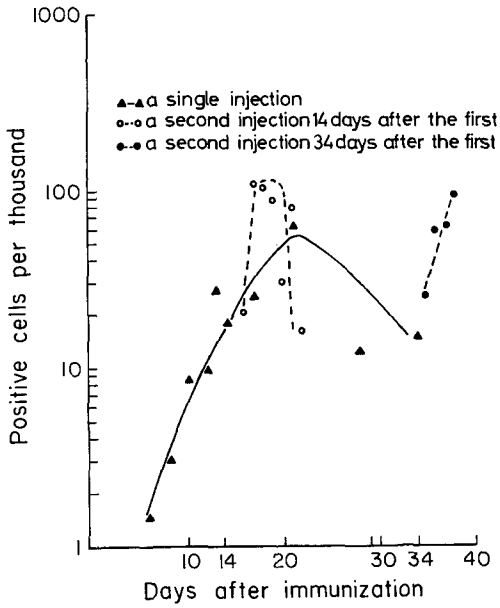
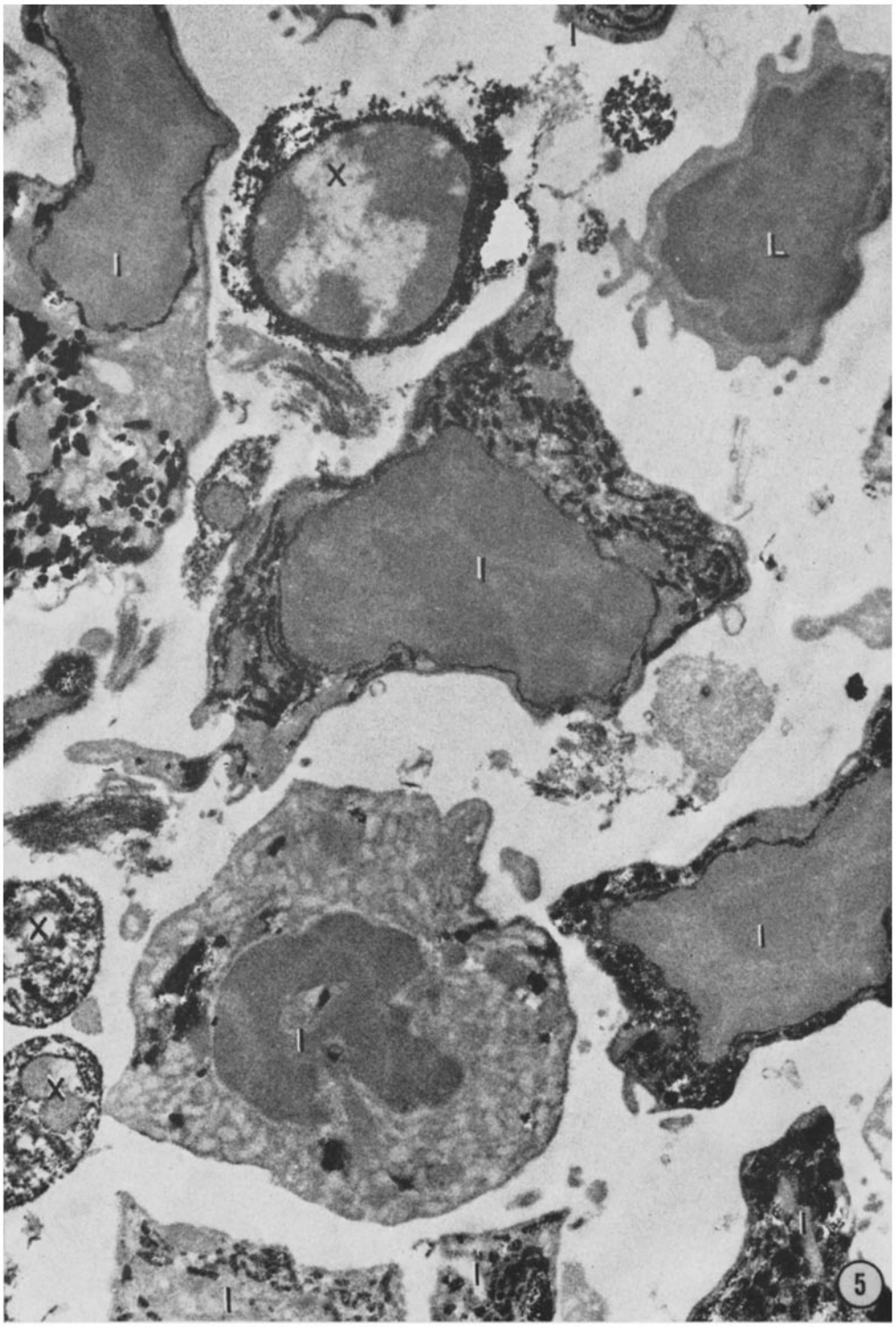


FIG. 4. Antibody-containing cells in stimulated lymph nodes of rabbits after a single or a second injection of peroxidase.

The terminology used in this paper to describe the stages of plasma cell differentiation is the same as in a previous publication (14). The continuous sequence is divided into four stages: *hemocytoblast*, characterized by the presence of a large nucleus, a large and intricate nucleolus, relatively little rough-surfaced endoplasmic reticulum (RER) and many free ribosomes; *plasmablast* in which the nuclear diameter is reduced and the RER is more extensive; *immature plasma cell* in which the Golgi apparatus, when included in the section, occupies a large area beside a nucleus of smaller size and the rest of the cytoplasm is compactly filled with a well developed RER which still has flat cisternae; and *mature plasma cell* in which the nucleus reaches its smallest diameter,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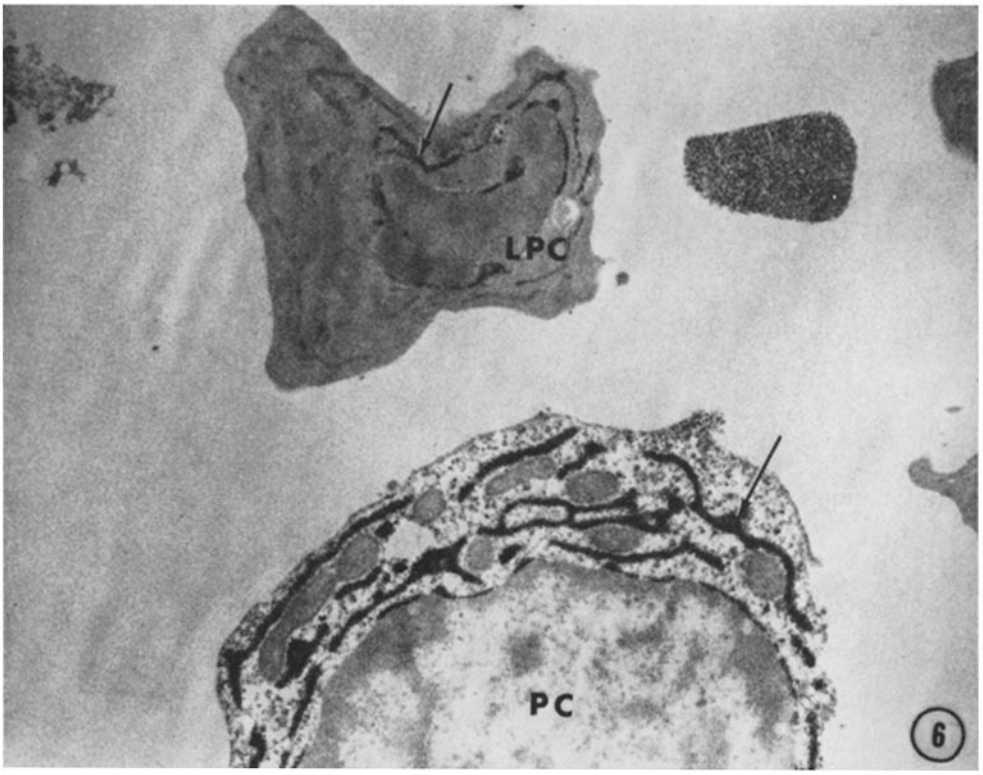
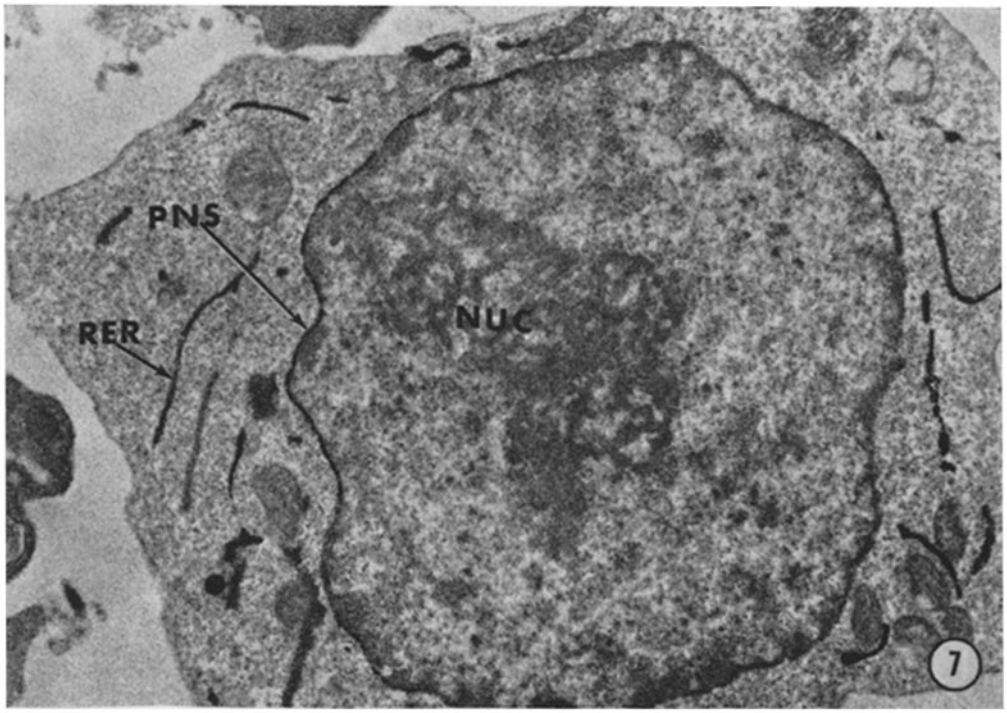


FIG. 6. Electron microscopic comparison of antibody producing, large, clear, plasma cell (*PC*) and small, dense, lymphoplasmacyte (*LPC*) day 3 after second antigen injection. The antibody reaction appears as an intense, electron-opaque product in the cisternae of the RER (arrows). × 9000.

the RER cisternae reach their maximum distention, and morphological evidence of secretion is visible. A similar sequence of differentiation can be followed in the lymphoplasmacyte.

We previously reported (14) that specific antibody antiperoxidase first arises in the perinuclear space (PNS) of hemocytoblasts. In this study, we have confirmed this and found it to occur simultaneously in both types of immunocytes.

FIG. 5. 5th day after second antigen injection. A low magnification electron micrograph illustrating numerous antibody-containing cells. The eight immunocytes (*I*) are all lymphoplasmacytes and have varying amounts and distributions of antibody. Also present is a small lymphocyte (*L*) without antibody and portions of three cells, possibly medium to large lymphocytes or lymphoblasts (*X*), in which a positive reaction occurs on cytoplasmic ribosomes; the one near the top of the page exhibits two typical features: a nucleus with a clearly defined interchromatin region and a fragile cytoplasm easily damaged by the procedures employed. × 10,500.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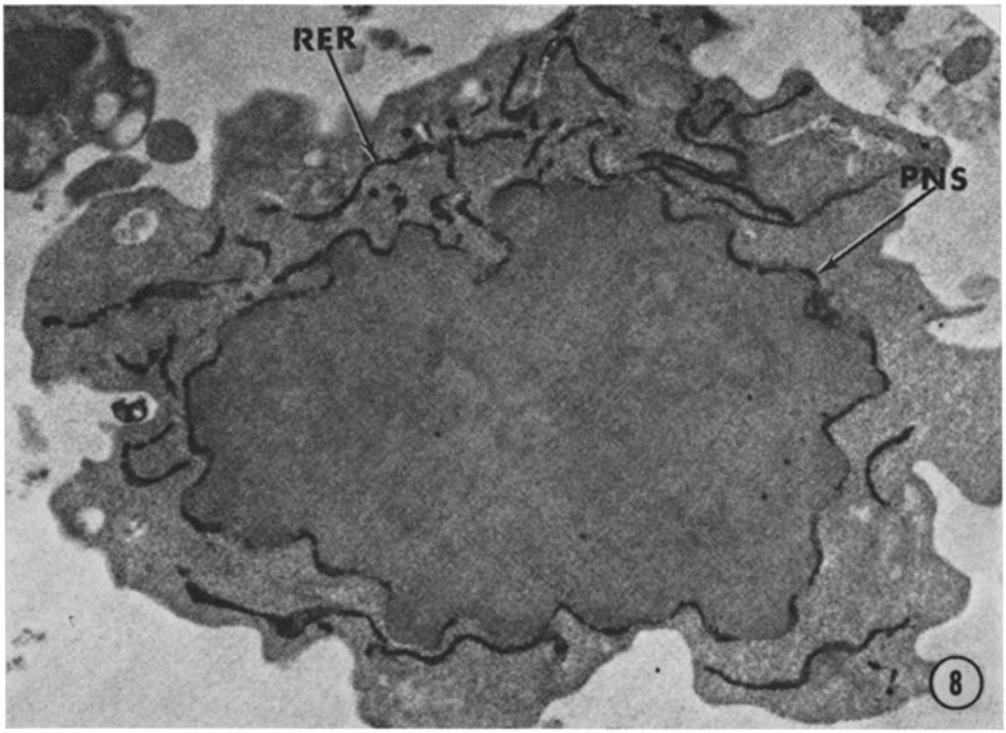
RER
PNS
8

Furthermore, in both cell types the antibody then becomes distributed throughout the cisternae of the developing rough-surfaced endoplasmic reticulum in the plasmablast and immature plasma cell (Fig. 9) on the one hand, and the lymphoplasmablast (Fig. 8) and immature lymphoplasmacyte (Fig. 11) on the other hand. Subsequent localization of antibody differs in the two cell types, except for the fact that in both the PNS becomes antibody-free.

In the mature stage of the large clear plasma cell, as previously reported (14), specific antibody disappears not only from the PNS but also from certain segments of the RER randomly distributed in the cytoplasm, and it accumulates in a few greatly distended RER cisternae which eventually correspond to the "Russell bodies" first described by light microscopy (Fig. 10). The mode of release of antibody from the Russell body cell is not certain, but Fig. 8 suggests that it may be by apocrine secretion. No other type of mature large clear plasma cells was seen and we have no observations on the ultimate fate of these cells. As long as they are recognizable by the methods employed here, they always have their characteristic clear cytoplasm and interchromatin regions in the nucleus and they exhibit no sign of cytoplasmic degeneration and cell death.

In the mature stage of the lymphoplasmacyte, Russell bodies are not formed. During maximal antibody production the RER cisternae are only slightly distended (Fig. 12). There follows a peripheral displacement of antibody, in that it disappear first from the PNS (Fig. 12), then from the RER near the nucleus (Fig. 13), eventually to be found only in a few cisternae at the cell periphery (Fig.14). Secretion of antibody appears very likely to be apocrine, since there are numerous deep infoldings of the plasma membrane into the cytoplasm (Fig.15) or long protrusions of cytoplasm (Fig. 16) that suggest that fragments of peripheral cytoplasm, including antibody-containing RER cisternae, are being pinched off and released. Occasional apparently free fragments are seen around the cells (Figs. 14, 17, and 18). When the antibody-containing cisternae of the RER are limited to the periphery of the cell, the rest of the cytoplasm is filled with flat, antibody-negative RER cisternae (Fig. 14). We have interpreted this stage as the end of the primary response in the lymphoplasmacyte.

In all lymph nodes examined 17 or more days after a single injection of peroxidase and adjuvant, and in nodes examined on the first 6 days after a

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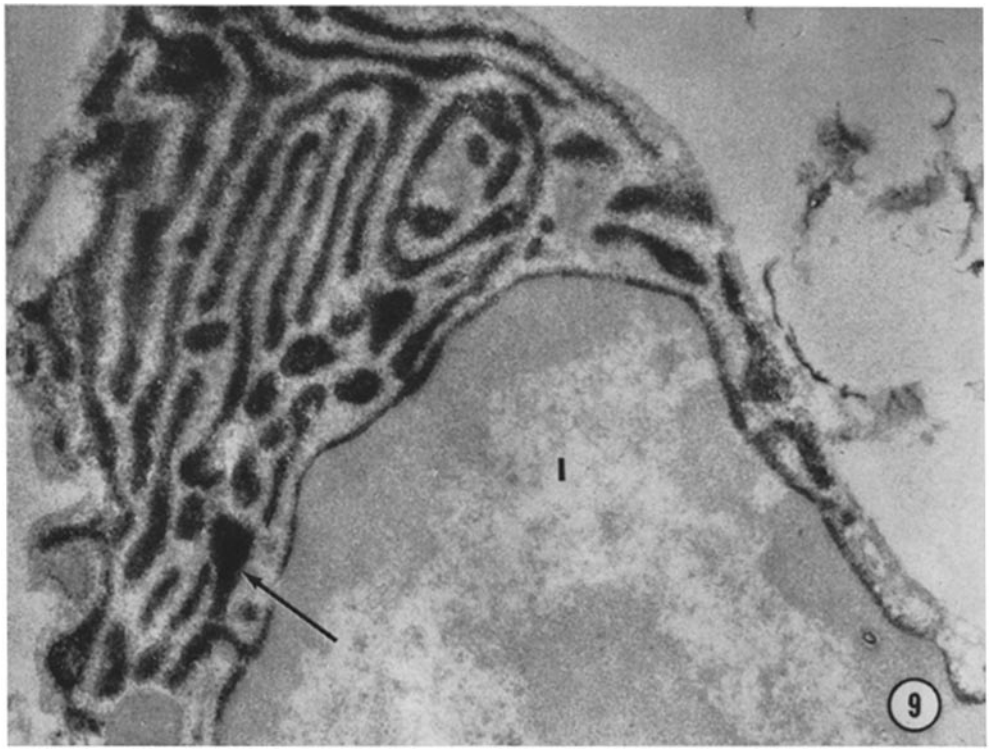
FIGS. 7 and 8. 6th day after primary injection of antigen, the 1st day that antibody-containing cells were detectable.

FIG. 7. Large, clear hemocytoblast with well developed nucleolus (*NUC*) and only a few flattened cisternae of rough endoplasmic reticulum (*RER*). Antibody is present in the perinuclear space (*PNS*) and some, but not all, of the RER cisternae. × 14,000.

FIG. 8. A somewhat smaller, dense lymphoplasmablast in which the nucleolus is barely discernible (but see Fig. 18). Antibody is present in the *PNS* and *RER*. × 14,000.

second injection given either 14 or 34 days after the first, another distribution of antibody was found in many of the lymphoplasmacytes. Antibody often occurred in peripheral cisternae with none in the RER nearer the nucleus, as described above and interpreted to signify the end of the primary response, but



FIGS. 9 and 10. Further differentiation of the large, clear plasma cells, sequences which follow the hemocytoblast stage in Fig. 7. Note that in both illustrations the interchromatin region of the nucleus is clearly discernible (*IC*) in spite of the absence of any stain in these sections except the reaction for antibody.

FIG. 9. 6th day after second antigen injection. Immature plasma cell stage in which antibody is still present in the perinuclear space and extends throughout the well developed endoplasmic reticulum. A few cisternae of the RER are slightly distended (arrow) but most are still flattened. Interchromatinic region (*I*) is clear. × 25,000.

there also was specific antibody around the nucleus, in the PNS, as found in the blast stage during the early phase of antibody production and cell differentiation (Figs. 17 and 18). Furthermore, at this stage a transient appearance of interchromatin regions in the nucleus is sometimes evident (Fig. 18); it is usually found only in the blast stage of the lymphoplasmacyte. We have interpreted

this as the beginning of a second cycle of antibody production, initiated before the first cycle was completed. Large numbers of lymphoplasmacytes in this phase of secretion were found. We have not observed an identical reappearance of specific antibody in the PNS of well differentiated, large clear plasma cells,

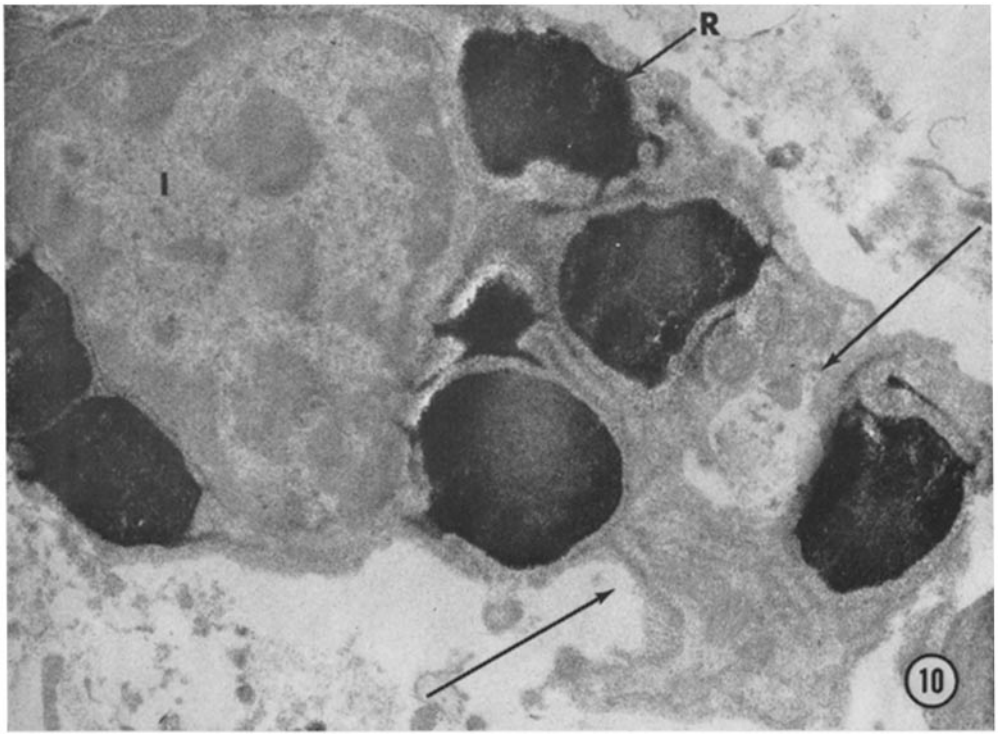
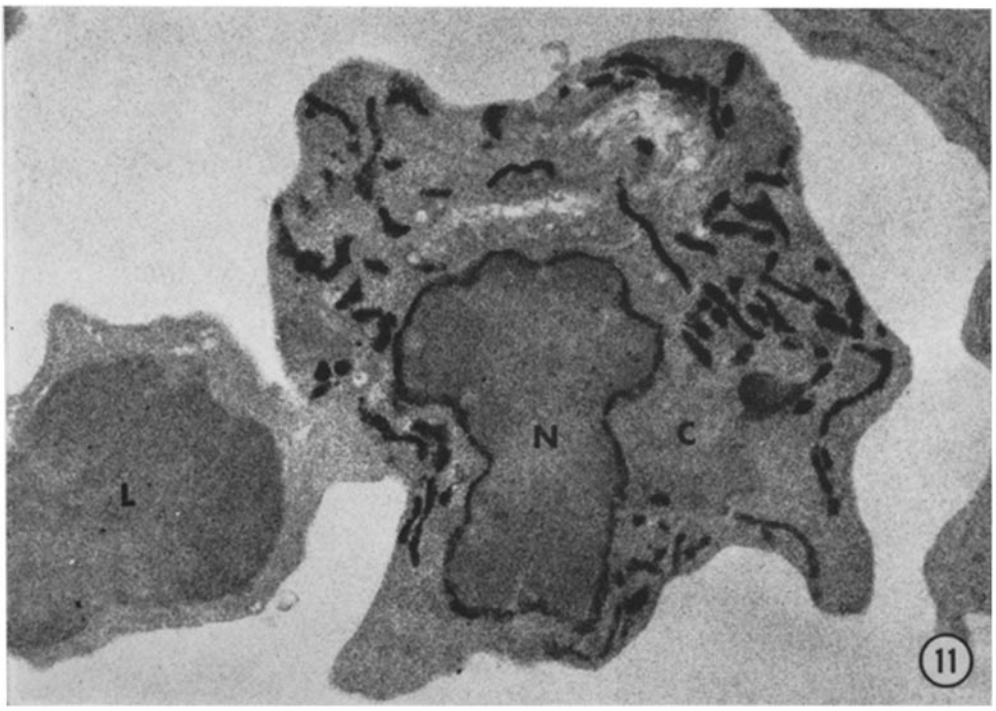


FIG. 10. 3rd day after second antigen injection. Final stage of differentiation of large clear plasma cells in which the antibody has accumulated in a few highly distended cisternae of the RER to form Russell bodies (*R*). The perinuclear space and non-distended RER cisternae are now almost completely antibody-negative. Arrows indicate apposing deep infoldings of the cell surface, a phenomenon suggesting a possible apocrine secretion of the Russell bodies along with a portion of adjacent cytoplasm. Interchromatin region (*I*). × 15,000.

with the possible exception of one mature plasma cell in which the PNS was strongly reactive and the cytoplasm was filled with moderately distended RER cisternae of which about one-third contained antibody.

The types of immunocytes present and the stages of their development at various intervals after a primary or secondary antigenic stimulation varied. In the *primary reaction* on *day 4* after administration of antigen, before any anti-

body was detectable by either light or electron microscopy, cells were present in the lymph node that could be identified morphologically as the hemocytoblast stage of both the large clear plasma cell and the small dense lymphoplasmacyte. No older or more differentiated stages of either type of cell were found, but an



FIGS. 11 and 12. Further differentiation of the lymphoplasmacytes, sequences which follow the hemocytoblast stage in Fig. 8. Note that both its nucleus (*N*) and cytoplasm (*C*) have the same density as those of the small lymphocyte (*L*) at the left in Fig. 11.

FIG. 11. 2nd day after second antigen injection. A small lymphocyte (*L*) without antibody and a lymphoplasmacyte with antibody in the PNS and RER cisternae. × 15,000.

extensive search was not made. On *day 6*, the first day that intracellular antibody was detected but before circulating antibody was found, antibody appeared simultaneously in both the plasma cells (Fig. 7) and the lymphoplasmacytes (Fig. 8), primarily in the hemocytoblast stage of both cell types but also in the large clear plasmablast and the smaller, dense lymphoplasmablasts.

On subsequent days there was a difference in the stages of differentiation of the two types of cells that contained specific antibody. The *plasma cell* on *day 8*

was represented by hemocytoblasts, plasmablasts, and immature plasma cells with antibody in both the PNS and RER; on *day 10*, it was represented by these three types plus the mature stage with antibody in most of the RER but no longer in the PNS; on *day 12*, by the preceding four stages plus Russell

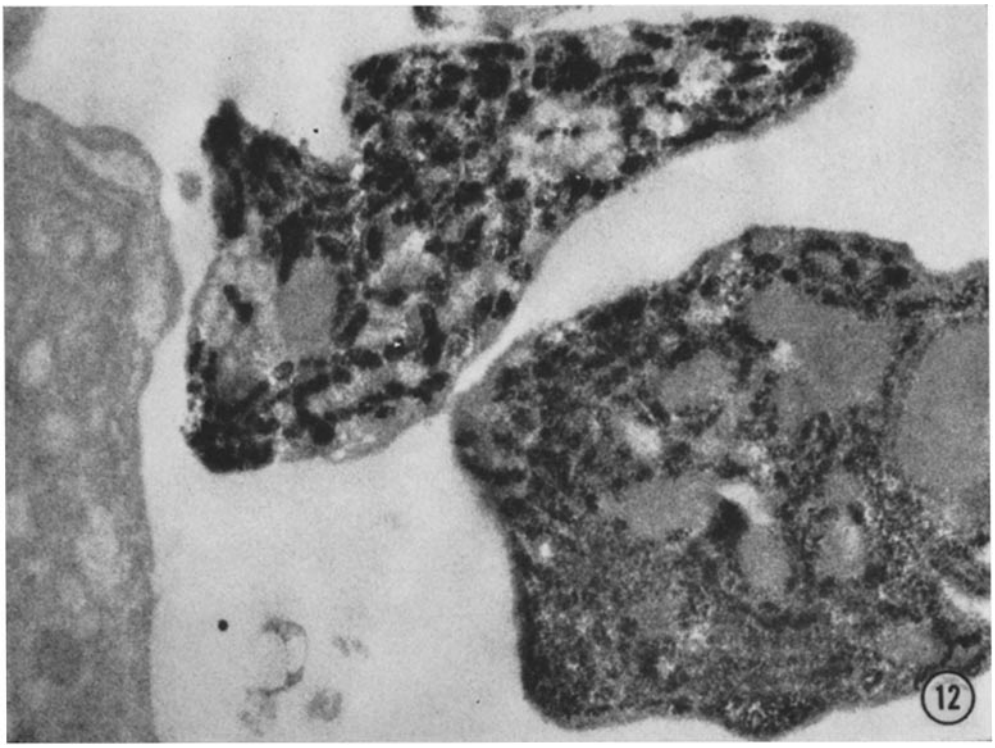


FIG. 12. 5th day after second antigen injection. The lymphoplasmacyte at the far left is already devoid of antibody; the cisternae of the other two are as distended with antibody as they ever become in this type of immunocyte. Although all cisternae are positive in the cell at the right, the perinuclear space no longer contains antibody. × 15,000.

body-containing cells. Subsequently, on *days 13, 14, 17, 21, and 28*, all types were present, but the hemocytoblasts, plasmablasts, and Russell body cells were always relatively few in number in comparison to the mature stage. The mature plasma cells were particularly abundant on days 10–21. On days 13–34 they contained mixtures of antibody-positive and negative RER cisternae, as previously described (14). In summary, in the primary response there was a gradual maturation of the large clear plasma cell from the time of its first appearance

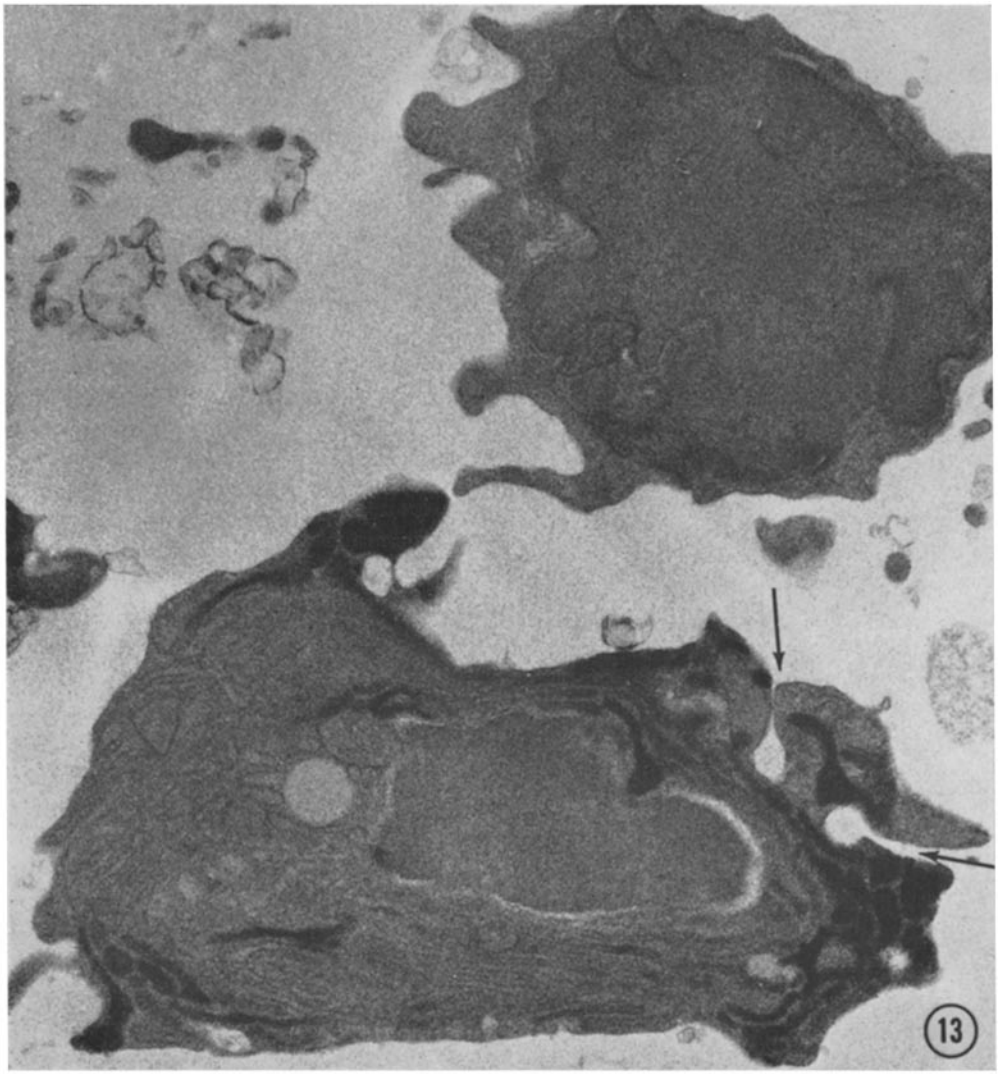


FIG. 13. 22nd day after primary injection of antigen. The cell at the top is a small lymphocyte. Below is a lymphoplasmacyte of similar density in which antibody is limited to cisternae at the periphery of the cell. Deep infoldings of the cell membrane (arrows) around segments of antibody-containing cisternae suggest that the antibody is released by apocrine secretion. × 18,000

as a hemocytoblast on day 6 to the appearance of Russell body cells on day 12, and all stages continued to be present through day 28, although the mature stage predominated, and only a few mature cells persisted on day 34 (Table I).

In the *lymphoplasmacyte line* on *day 8* there were dense hemocytoblasts,

lymphoplasmablasts, and immature lymphoplasmacytes with antibody in both the PNS and RER, and a few mature ones with antibody throughout the well developed RER but no longer in the PNS. On *days 10 and 12* there were only immature and mature lymphoplasmacytes like those above. No lymphoplasma-

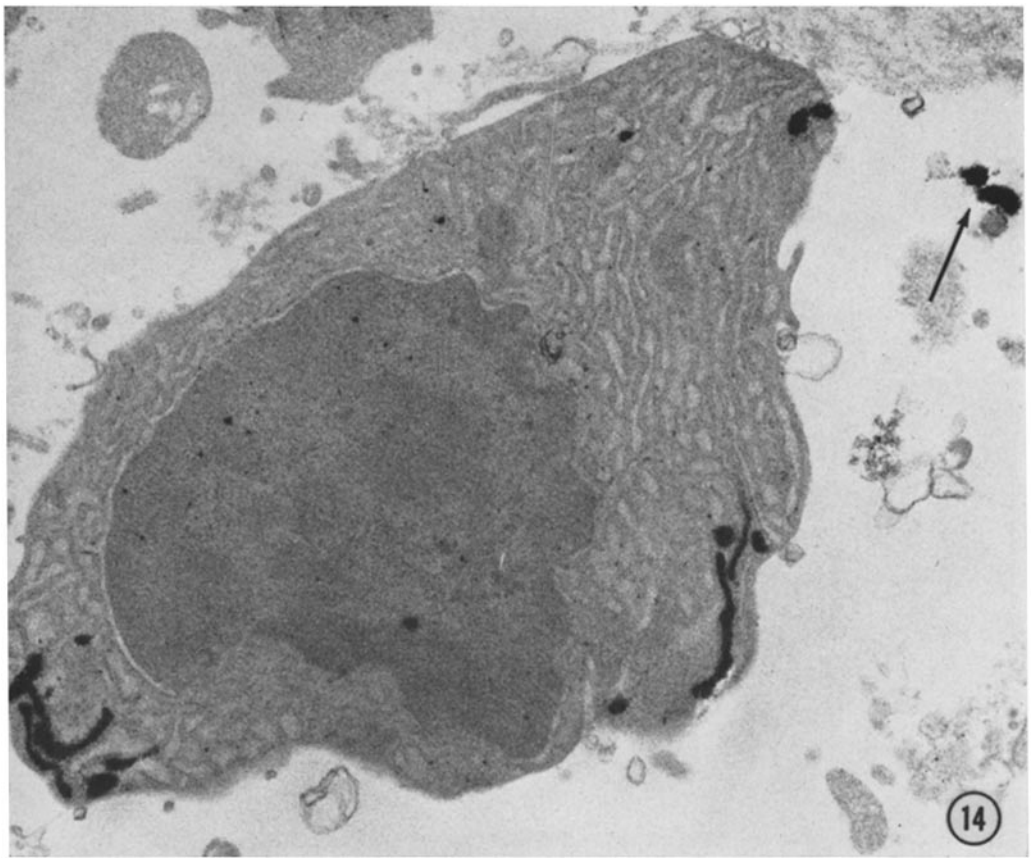


FIG. 14. 18th day after primary injection of antigen. A lymphoplasmacyte in what is interpreted as the final stage in which antibody can still be found in a few peripheral cisternae. Although the antibody-synthesizing cycle of this cell seems to be at an end, an abundant endoplasmic reticulum persists. At arrow there appears to be some extracellular antibody. × 15,000.

cytes were found on days 13 and 15, probably because all blocks were in the follicular part of the lymph node (see below, page 1159). On days 17, 21, 28, and 34 there were only mature lymphoplasmacytes, but these exhibited four types of antibody distribution, as follows: (*a*) those with antibody throughout the RER, as above; (*b*) those with antibody persisting only in peripheral RER cisternae

FIG. 15. Higher magnification of the border of a small dense plasma cell to illustrate infolding of the cell membrane that may pinch off segments of the cytoplasm. × 35,000.

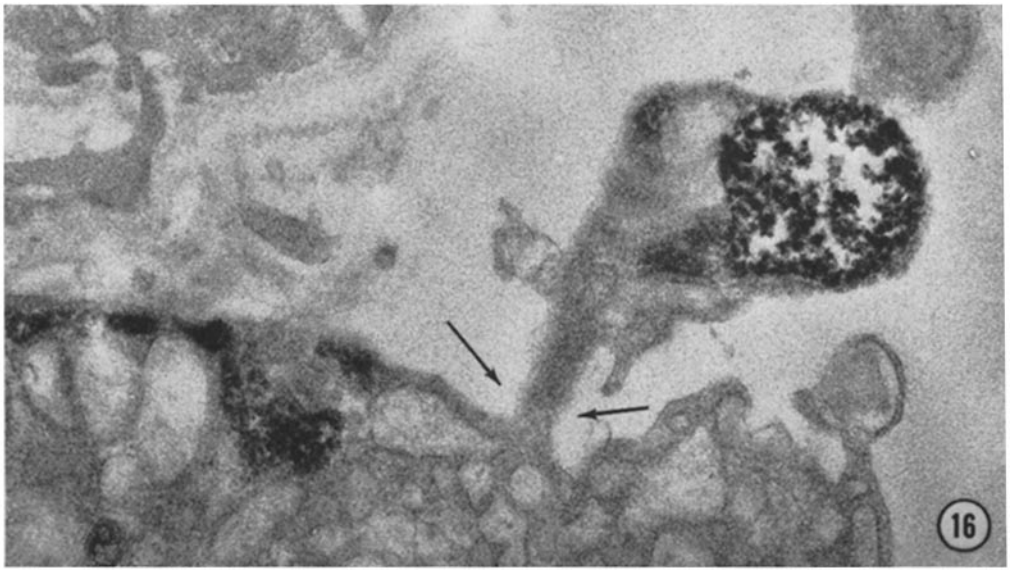


FIG. 16. 35th day after primary injection of antigen. Edge of a lymphoplasmacyte with a portion of its cytoplasm nearly pinched off at arrows, an apparent apocrine secretion of antibody. Highly distended, antibody-negative cisternae, like those in this cell, occurred rarely. × 35,000.

near the plasma membrane; (*c*) those with antibody in the peripheral cisternae and also in the PNS; (*d*) those containing a highly differentiated but antibody-free RER, but with antibody in the PNS. In summary, there was a shift in the stage of differentiation with time after antigen injection. Antibody-positive

TABLE 1

*Developmental Stages of Large Clear Plasma Cells (Top) and Small Dense Lymphoplasmacytes (Bottom)**

Day	Clear hemocytoblast	Plasmablast	Immature plasma cells PNS + RER	Mature plasma cells RER only	Mature plasma cells Russell body
6	+	+			
8	+	+	+		
10	+	+	+	+	
12	+	+	+	+	+
13		+		+	+
14		+		+	+
17	+	+		+	
21	+	+		+	+
28	+				
34				+	
1	+	+			
2		+			
3	+	+			
4	+		+		
5	+	+	+	+	
6	+	+	+	+	
7	+	+	+	+	+
8				+	
6	+	+			
8	+	+	+	+	

Day	Dense hemocytoblast	Dense LPC blast	Dense LPC PNS + RER	LPC RER only	LPC peripheral RER	LPC peripheral RER + PNS
6	+	+				
8	+	+	+	+		
10			+	+		
12			+	+		
13						
14						
17				+	+	+
21				+	+	+
28					+	+
34				+		+
1		+	+	+	+	+
2	+		+	+		+
3			+	+	+	
4			+	+		
5			+			+
6		+	+	+	+	+
7						
8				+		

* Cells were present 6–34 days after a single administration of antigen plus Freund's adjuvant and 1–8 days after a 2nd injection of antigen.

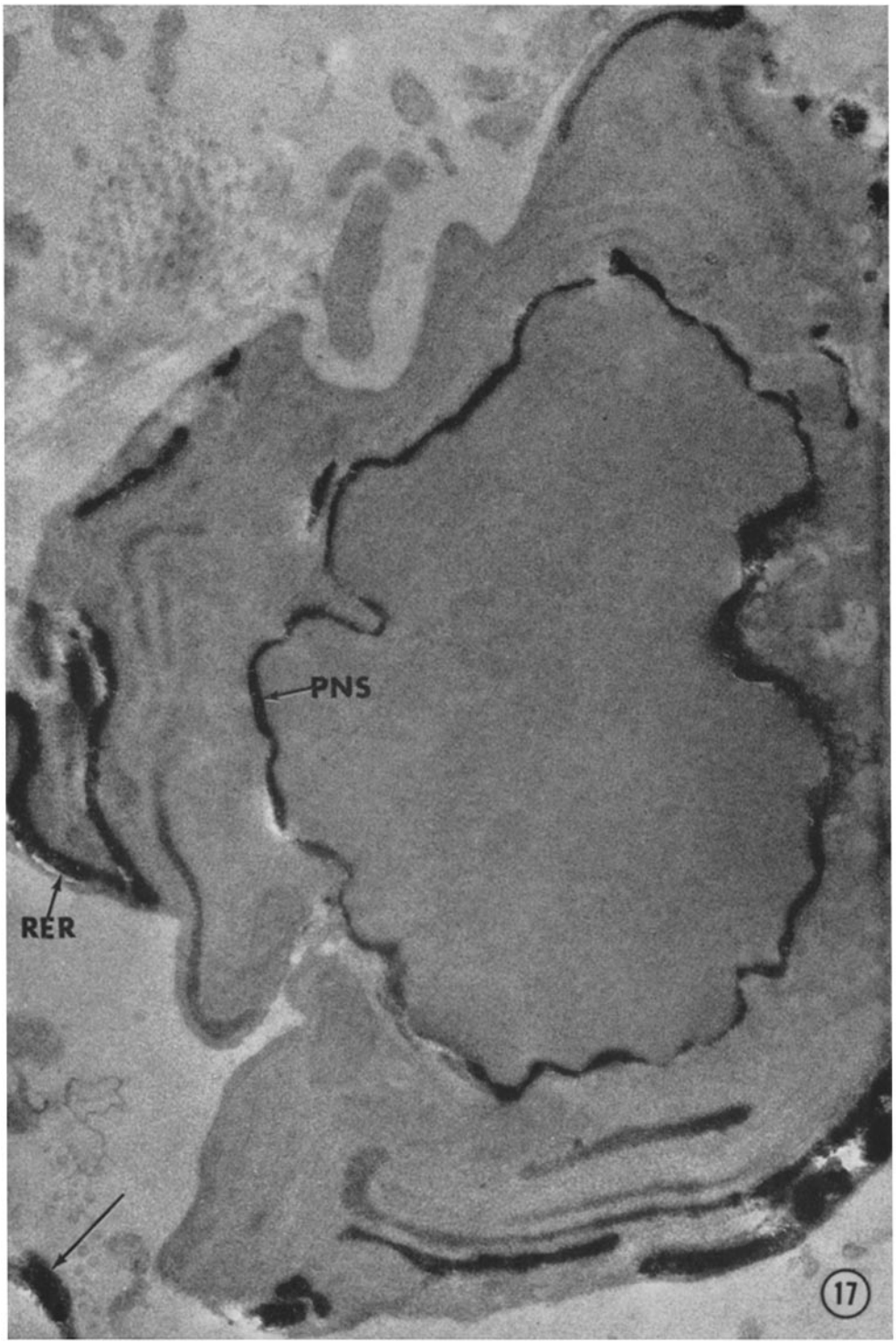


FIG. 17. 6th day after a second injection of antigen. A lymphoplasmacyte which, on the one hand, has reached the end of one cycle of antibody synthesis as indicated by the localization of antibody in the RER cisternae at the periphery of the cell but not in those near the nucleus, and, on the other hand, has begun another cycle of synthesis as revealed by the appearance of antibody in the perinuclear space (*PNS*). Note possible extracellular antibody at arrow. × 20,000.

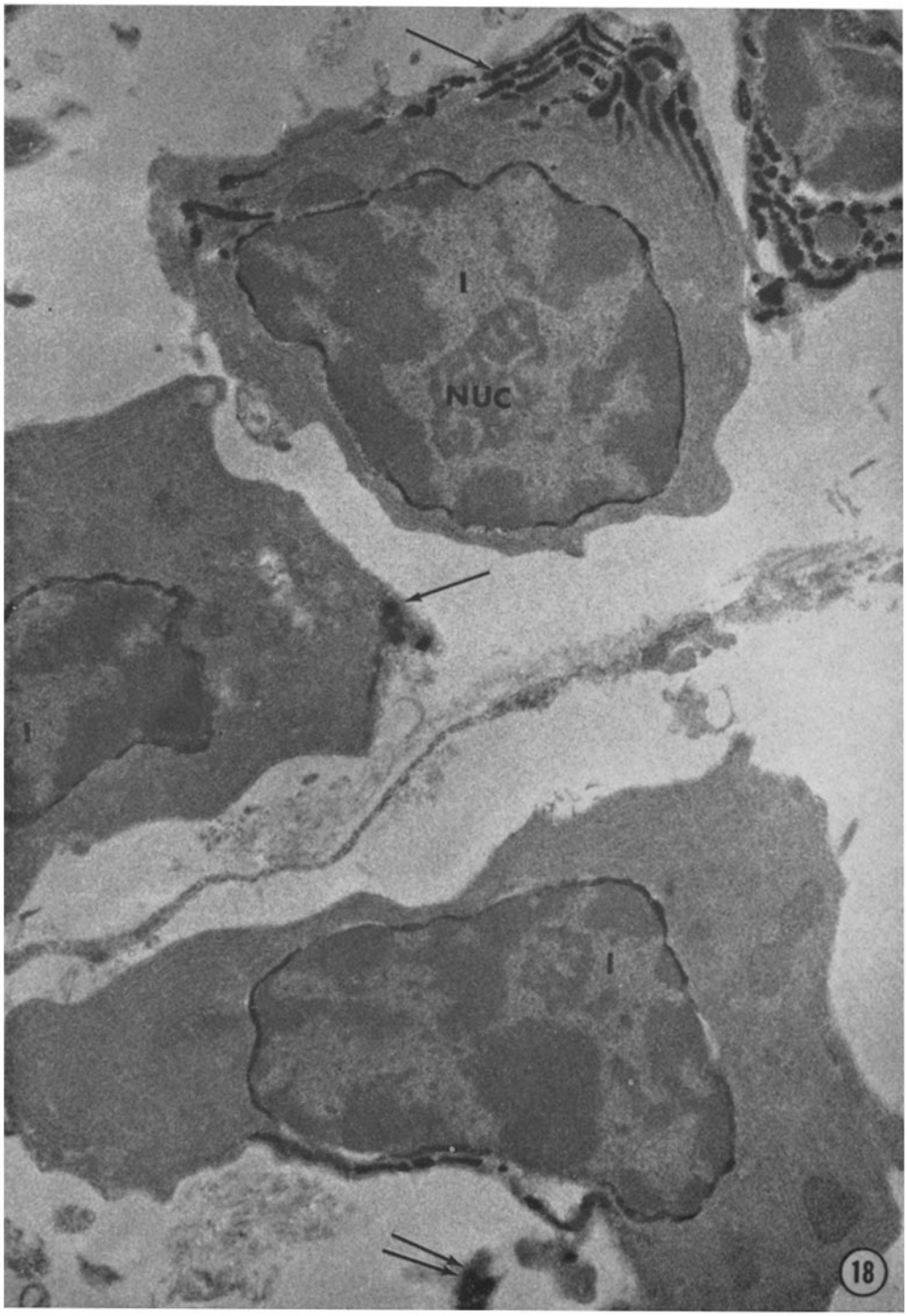
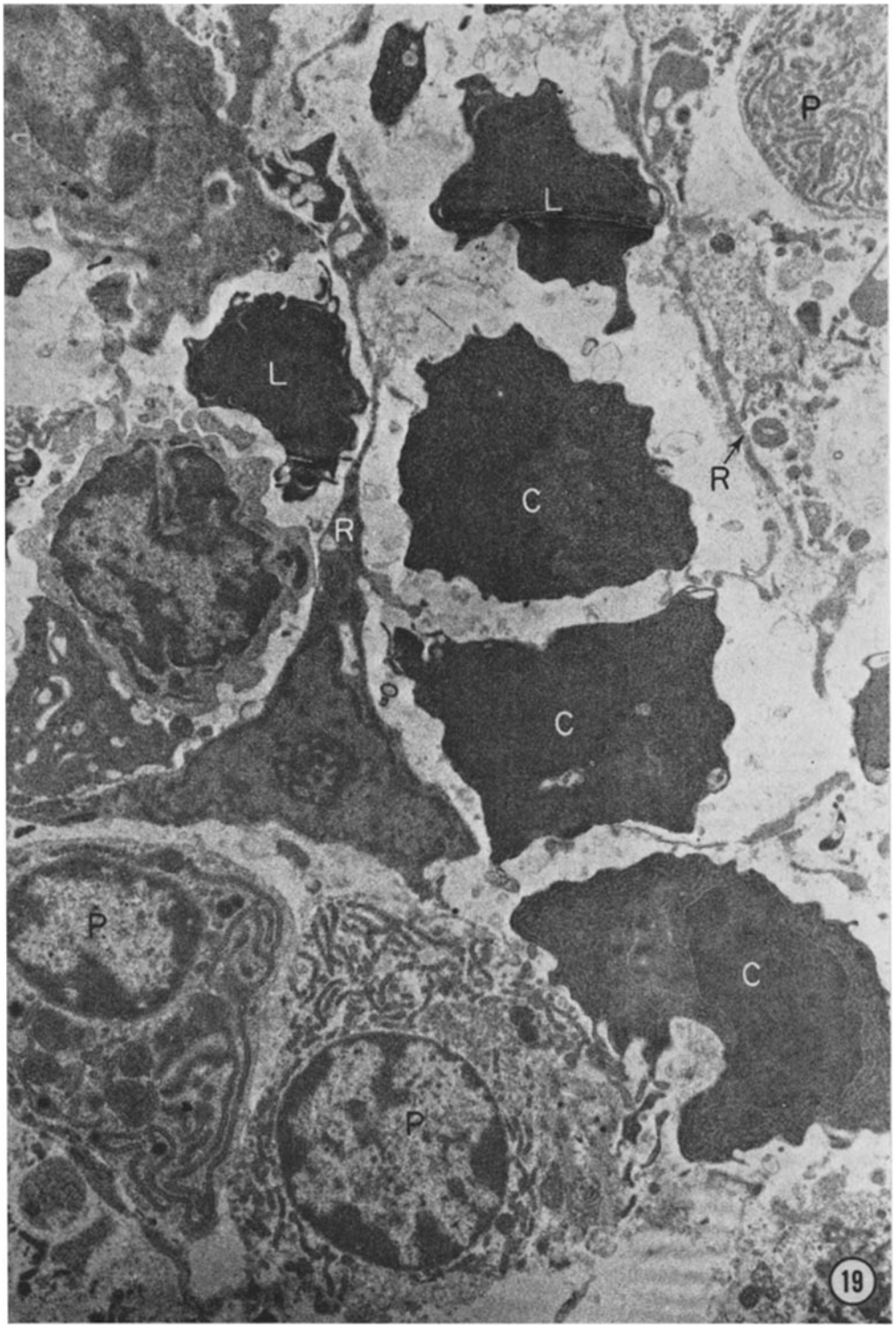


FIG. 18. 8th day after a second antigen injection. Three lymphoplasmacytes in which remnants of antibody from one cycle of synthesis persist at the edge of the cell (arrows), and a second round of synthesis is beginning as indicated by a positive reaction in the perinuclear space. The rest of the RER is negative for antibody and can barely be discerned in this dense cytoplasm without metallic "stains." It is when antibody first appears in the PNS that interchromatin regions (*I*) and nucleoli (*NUC*) can be discerned in this type of immunocyte. At the bottom of the micrograph (double arrow) there is a small segment of cytoplasm containing antibody which may be either continuous with the cell at another level outside the plane of section, or pinched off completely from the cell. × 14,000.


P
L
L
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R
R
C
C
P
P
P
19

dense hemocytoblasts and lymphoplasmablasts were found only on days 6 and 8; immature lymphoplasmacytes, only on days 8, 10, and 12; mature ones with all RER reactive, on days 8 through 34; mature cells with most or all of the RER antibody free but with a positive antibody reaction in either the peripheral RER cisternae, the PNS, or both, on days 17–34 (Table I).

Secondary response: There was no difference between lymph nodes of rabbits that received a second injection of antigen 14 or 34 days after the first injection. The antibody-containing large clear *plasma cell* line was represented by a few hemocytoblasts and plasmablasts and numerous immature plasma cells on *days 1, 2, and 3*. These stages continued to be present through *days 4, 5, 6 and 7*, but the mature stages were also present and constituted the majority of the clear type of cell. The antibody-containing small dense *lymphoplasmacyte* line was represented chiefly by mature stages of differentiation on *days 1–7* after the second injection of antigen. Both cell types were present on *day 8* but very few contained antibody (Table I).

No attempt was made to determine relative numbers of cells. After the single administration of antigen plus adjuvant, except for days 13 and 15 (see above), the two families of cells that contained antibody appeared to be equally represented, that is, present in moderate numbers on days 6 and 8; very numerous on days 10, 12, 18, 22 and 29; and rare on day 35. After the second antigen injection, on the other hand, although both types were very abundant on days 1, 2, and 3, the lymphoplasmacytes constituted a clear majority at later stages. It is uncertain whether this is a significant evaluation. For an accurate count of positive cell types, precautions would be necessary to ensure that only one section from each of many samples of tissue would be used, and that both cortical and medullary areas of each lymph node be included.

Examination of sections from many samples of the day 6 lymph nodes, the first day of antibody production, revealed the presence of many plasma cells and lymphoplasmacytes which contained no detectable specific antibody against peroxidase and which had differentiated to the stages of immature plasma cells and mature lymphoplasmacytes (Figs. 19 and 20). It is possible that they were reacting to some other antigen than the peroxidase, perhaps some component of the adjuvant. Although both cell types occurred at all sites within the lymph node, the plasma cells were predominant in the perifollicular nodules (Fig. 20) and medullary cords (Fig. 19) and the lymphoplasmacytes were predominant

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FIG. 19. 6th day after a single antigen administration. Plasma cells (*P*) are in medullary cords, lined by processes of reticulocytes (*R*). Lymphoplasmacytes (*C*) are in sinusoid. The lymphoplasmacytes are as dense as the nearby small lymphocytes (*L*) and the elaborate RER in their cytoplasm can barely be discerned without a counterstain. Although plasma cells and lymphoplasmacytes in other fields of the same section contained antibody, none of these contain detectable antibody. × 6000.

in the sinusoids (Fig. 19). This distribution of the two types of cells, including those containing specific antibody anti-peroxidase, seemed to persist throughout the primary and secondary responses, but we made no effort to find the perinodular accumulations of plasma cells in each lymph node and it was not always possible to distinguish clearly the precise distribution of cells in the medulla (Fig. 5). Occasionally, the ultrathin sections of an incubated, 40 μ

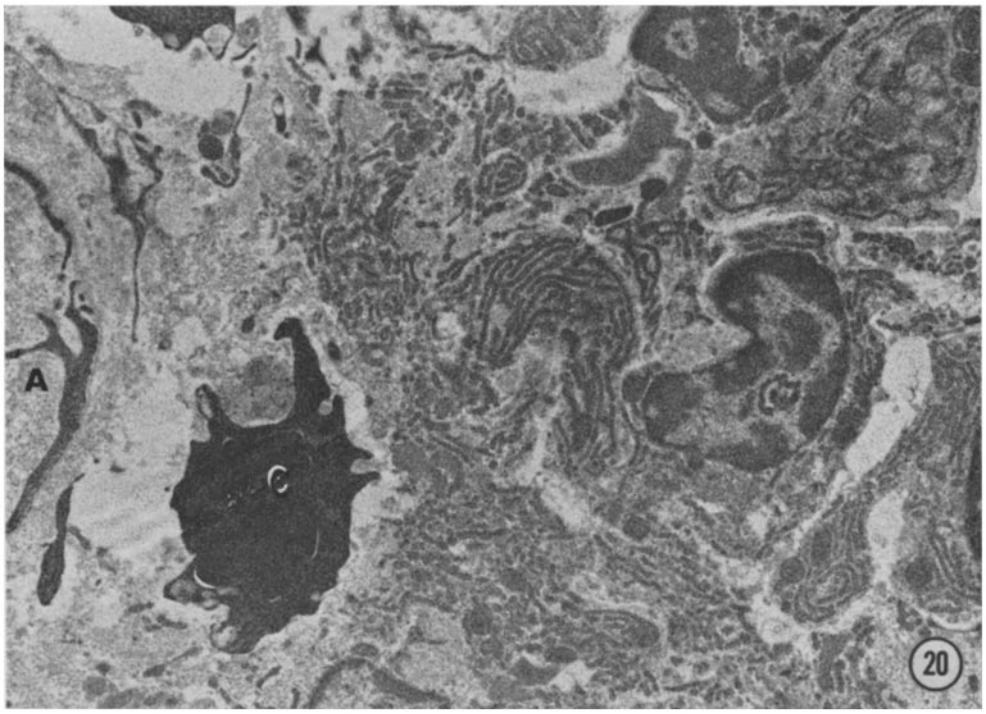

FIG. 20. 6th day after a single antigen administration. One lymphoplasmacyte (*C*) lies near an arteriole (*A*) at the left; all the rest of the cells, to the right in this perifollicular nodule, are plasma cells. × 6000.

thick slice of tissue contained no positive cell whatsoever, whereas sections of another slice of tissue from the same lymph node was filled with positive cells. Hence, this may represent the development of antibody-containing cells in groups or clones.

Mention must be made of the other cell type that exhibits a positive reaction for antibody anti-peroxidase in tissue prefixed in a formaldehyde solution. In a previous publication (15) we called it a lymphoblast, but this now appears erroneous since we do not know the fate of the cell, so we shall tentatively refer

to it as a *large lymphocyte*. Unlike either plasma cells or lymphoplasmacytes, the antibody reaction does not appear within the endoplasmic reticulum; neither the PNS nor the occasional short segments of RER are positive. The reaction occurs only on the ribosomes of this monoribosomal cell (Fig. 21). The cell

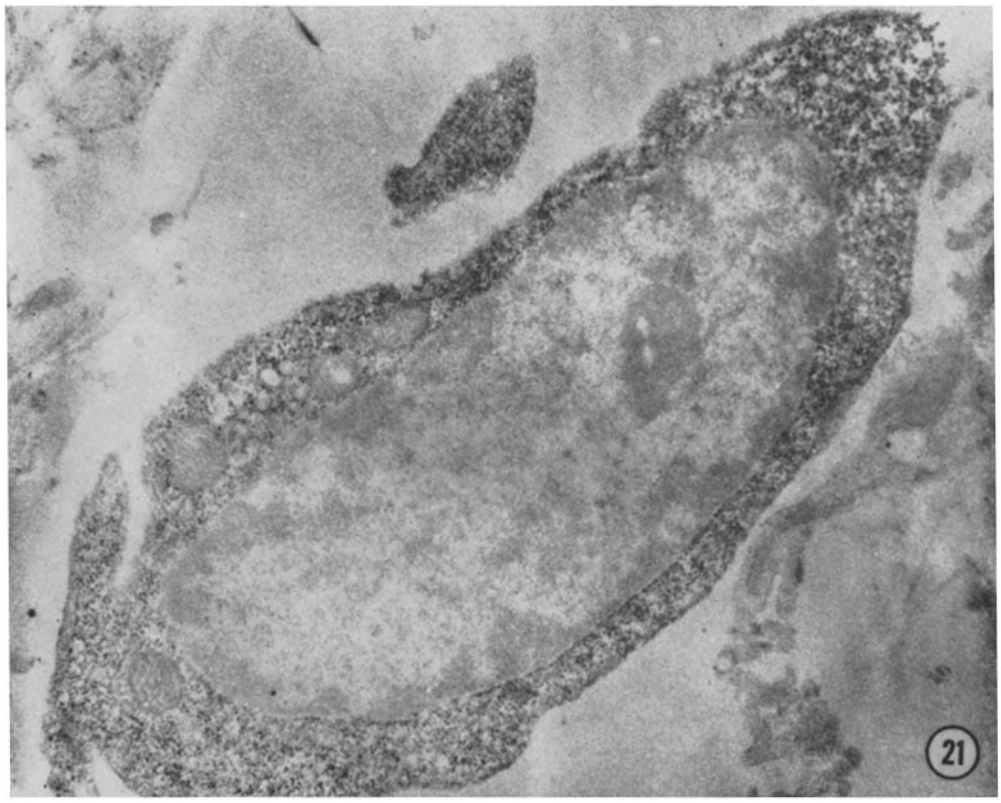

FIG. 21. 6th day after a second antigen administration. A medium to large lymphocyte with clear interchromatin regions exhibits a positive reaction for antibody on its ribosomes throughout the cytoplasm. × 16,000.

resembles the plasma cell line with respect to the presence of clear interchromatin regions in the nucleus and a clear cytoplasm. It appears to vary in size and contains several mitochondria; it usually occurs in the sinusoids and sometimes it is fragmented, as if fragile and easily damaged by our preparative techniques. This cell type was rare in these experiments. It was found on days 18 and 29 after the primary antigen injection and on days 4 and 7 after a second antigen injection. Since there were so few present even in these lymph nodes, their presence at other stages could easily have been missed.

Control sections were examined of all the lymph nodes containing antibody to peroxidase. These were exposed to the cytochemical substrate for peroxidase activity without preliminary exposure to peroxidase. Other controls consisted of lymph nodes of rabbits immunized with other enzymes and then exposed both to peroxidase and its substrate. The only reaction found in any of the control sections was endogenous activity typically found in the specific granules of eosinophils and in some phagosomes of macrophages.

DISCUSSION

This investigation has revealed by direct, electron microscopic, cytochemical localization of antibody to horseradish peroxidase, that the synthesis and secretion of humoral antibodies is the function of at least two distinct families of cells, one group of typical plasma cells and another group of specialized lymphocytes. Because the latter exhibit certain morphological characteristics of both small lymphocytes and plasma cells, we have tentatively called them "lymphoplasmacytes." The lymphoplasmacyte, like the plasma cell, can be induced by a primary antigenic stimulation to differentiate from a lymphoid precursor that has only a rudimentary endoplasmic reticulum, consisting chiefly of the nuclear envelope, to a cell containing an elaborate, ribosome-studded endoplasmic reticulum (RER) that is characteristic of cells that synthesize proteins for export. Unlike the plasma cell, however, the lymphoplasmacyte retains the condensed, hyperchromatic nucleus of the small lymphocyte; its cytoplasm has the same electron density as small lymphocytes after prefixation with formaldehyde and, although the cell does enlarge somewhat as its cytoplasm becomes organized into RER, it is generally somewhat smaller than the plasma cell.

We believe that in the past, in our laboratory and in others, the lymphoplasmacytes have been identified either as lymphocytes or as plasma cells, depending on the methods used to examine them. At the light microscope level, in this study, the antibody-containing cells can clearly be separated into two classes, one corresponding to the larger cells of the plasma cell series and the other having the size, shape and nuclear characteristic of small lymphocytes. Other investigators (4, 21, 25, 27) also have reported the presence in stimulated lymphoid organs of cells with morphological characteristics of the small lymphocyte which contained antibodies and were able to divide. At the electron microscope level there have been several reports of small lymphocytes with unusually well developed endoplasmic reticulum (6, 12, 13, 20). Feldman and Nordquist (6) found a greater number of these cells in immunized rats than in nonimmunized ones. Harris et al. (12) showed by the hemolytic-antibody plaque technique that antibody-synthesizing cells from rabbit lymph nodes included lymphocytes containing varying amounts of RER, as well as plasma cells. In a previous study of the secondary response in the spleen (14), we failed

to recognize the lymphoplasmacytes because prefixation was carried out with glutaraldehyde instead of formaldehyde. Unusual cells were described (14, Fig. 11) which had well developed RER but with antibody only in the PNS, but since they had the same density of cytoplasm and nucleus as plasma cells, they were mistaken for plasma cells. Vazquez (25) describes antibody-forming cells "similar to small and medium-sized lymphocytes" which he has called "lymphokinecytes" because they incorporate H^3-thymidine and divide. The lymphokinecytes may be the same as the lymphoplasmacytes.

In a comparison of the lymph node cells (12) with extranodal plaque-forming cells, Hummeler et al. (13) found that the latter were lymphocytes: those from the efferent lymph vessels and thoracic duct varying from relative scarcity to abundant RER, and those from peripheral blood containing cytoplasm wholly organized into RER. Hummeler et al.'s observations agree with this study, namely, that the majority of the plasma cells appear to be fixed cells in the medullary cords and perifollicular nodules whereas the lymphoplasmacytes predominate in the sinusoids and, therefore, appear to be part of a circulating population of cells.

The plasma cell and the lymphoplasmacyte apparently arise from different immediate precursors or stem cells, that is, one does not differentiate directly into or from the other, because the hemocytoblast stages of both cell types appeared simultaneously 6 days after an initial antigenic stimulation and no intermediate forms were subsequently found. It seems possible that the large clear plasma cell is derived from a tissue-bound cell resembling a large lymphocyte or blast cell while the dense lymphoplasmacyte arises from a circulating small lymphocyte. It is even possible that the lymphoplasmacytes receive their antigenic stimulus at the site of injection and the plasma cell reacts to antigen that has been carried to the lymph node. Therefore, one can assume that these cells represent different clones and may have different functions. It is tempting to hypothesize that the lymphoplasmacyte line may be thymus dependent and the plasma cell line, thymus independent (16).

In this study, complete Freund's adjuvant was administered with the antigen to increase the number of responding cells in order to facilitate adequate sampling for electron microscopy. This method of injection results in the slow release of small quantities of antigen over a long period of time and, hence, the antibody synthesis in response to this one injection is not just a primary one but also a secondary one (7) and this must be taken into account in this discussion. The plasma cells and the lymphoplasmacytes appear to have reacted differently to this situation. In the plasma cell line, the early, antibody-containing hemocytoblast and plasmablast stages were detected in every rabbit except two (on days 13 and 15) from days 6–28. In the lymphoplasmacyte line, on the other hand, the dense hemocytoblasts and lymphoplasmablasts were present only on days 6 and 8; on subsequent days after antigen injection, there

was a complete shift to more highly differentiated stages. On this basis alone, one could conclude that the plasma cell precursors were undergoing constant stimulation and new cells were being continuously fed into the plasma cell population, whereas the lymphoplasmacytes all differentiated from those stem cells that were stimulated only at the beginning of the response. Since both families of cells appeared to be equally abundant, it would seem to be necessary that the lymphoplasmacyte line would have a higher mitotic activity and longer span of active antibody synthesis than the plasma cell line in order to produce an equivalent population of antibody-forming cells from fewer stem cells. This will remain hypothetical until one can identify lymphoplasmablasts under conditions in which mitotic indices can be measured.

A difference in response of plasma cells and lymphoplasmacytes was also evident after a second injection of antigen. Again, the plasma cell hemocytoblasts and plasmablasts were present continuously days 1–7, and the more mature stages were present on days 5–8. On the other hand, the corresponding blast stages of lymphoplasmacytes were rare after a second injection of peroxidase, but the mature stages were present throughout the observation period. This would suggest that blast stages were no longer necessary, that is, that large numbers of lymphoplasmacytes were already differentiated (instructed?) and ready to respond rapidly to a new antigenic stimulus.

The most striking finding in this study was the observation that a lymphoplasmacyte that is just completing one cycle of antibody synthesis may begin a new, secondary one. As we reported previously (1, 14), antibody synthesis begins immediately around the nucleus and the first intracisternal antibody that is detectable accumulates in the perinuclear space (PNS) between the two membranes of the nuclear envelope, and subsequently fills the developing RER cisternae. In the lymphoplasmacyte, antibody synthesis apparently stops at some time after the RER cisternae wholly occupy the cytoplasm and are filled with the specific γ-globulin. The cell then secretes the antibody by clasmatosis or apocrine secretion, that is, by pinching off fragments of peripheral cytoplasm containing the antibody-rich cisternae. This process has been seen in microcinematographs of plasma cells (24). As this process occurs, the cell appears to continue to produce new RER but not antibody, so that eventually the lymphoplasmacyte contains RER throughout its cytoplasm, but specific antibody, if it persists at all, has been displaced to the peripheral RER cisternae near the plasma membrane. It is in such cells that a new cycle of antibody to peroxidase was found beginning again in the PNS from 17–34 days after the single injection of antigen with Freund's adjuvant, i.e. the secondary reaction after one antigen injection due to the presence of the adjuvant, and from day 1–6 after a second antigen injection. These cells, therefore, not only "remembered" the antigen but they also had the complete subcellular organelle, the RER, presumably with all the necessary enzymic components, ready for an immediate

reaction to a new stimulus. This, plus the fact that large numbers of these cells were formed during the primary reaction, would account for the characteristic rapid appearance of a high titer of antibody in the secondary response. Therefore, we propose that under the conditions used in these experiments the lymphoplasmacyte is the memory cell and that the memory cell can be a cell that has already produced the antibody at an earlier time.

Several studies have shown the existence of cells endowed with immunological memory (9, 17, 22, 23), and it was thought that the small lymphocyte played this role (5, 9). In these and other investigations, a net parallelism between "antibody-producing cells" and "memory cells" is apparent. For example, the kinetics of antibody synthesis and memory were similar (21, 26) and suppression of the antibody response by excess antigen (21, 22) or by blockade of the reticuloendothelial system (18) also suppressed the development of immunological memory.

The indication of two cycles of antibody synthesis in an individual lymphoplasmacyte suggests that antibody synthesis may be periodic or intermittent and, therefore, may be regulated by the antigen. Under other conditions, in which the level of antigen may differ both at the site of injection and in the draining lymph node, at least two other situations might exist. First, the one mature plasma cell which we found that contained antibody in the perinuclear space as well as in a few distended RER cisternae suggests that the plasma cell as well as the lymphoplasmacyte might have the potential of cyclic antibody synthesis and memory. Since the plasma cells appear to be relatively fixed in the lymph nodes, in these experiments they may have received less antigenic stimulation than the lymphoplasmacytes which appear to be migrating and, therefore, could recirculate through the site of injection where antigen persisted. Second, if the original level of antigen is low, it is possible that certain stem cells might not receive a sufficient antigenic stimulation to start antibody synthesis but the stimulus might be sufficient to allow the cell to respond rapidly to a second antigenic stimulation. Such a cell would also have immunologic memory. In other words, antibody synthesis and memory may be two different expressions or activities of the same cell.

If we accept that certain small lymphocytes may be stimulated to become antibody-secreting lymphoplasmacytes and then memory cells, what is their morphology when they are no longer synthesizing antibody? At best, the "memory" depicted in this study is a very short term one. Do these cells retain the elaborate RER for extensive periods of time, or can there be a reverse differentiation from lymphoplasmacyte to small lymphocyte by continued shedding of the cytoplasm? Long term experiments are in progress to attempt to answer this question. Experimental evidence that antibody-synthesizing cells may become small lymphocytes has been reported (11, 27).

By using b-mercaptoethanol and immunoelectrophoresis it was found that

almost all of the antisera in the experimental animals in this study contained primarily IgG as well as small amounts of IgM antibody to peroxidase. This means that under our experimental conditions the synthesis of 19S antibody did not precede that of 7S and it is possible that the synthesis of IgG and IgM occur quite independently. Schoenberg et al. (19) who, as in this study, used a simultaneous injection of antigen and complete Freund's adjuvant, presented evidence that IgM is produced primarily by large mononuclear cells, and IgG by plasma cells. Furthermore, in some primitive sharks that produce only IgM, large lymphocytes are present but there are no plasma cells.[1] Because the level of 19S antibody was always low in our antisera, it may be that it was produced by the small numbers of large lymphocytes in which we found a positive reaction associated with the ribosomes, instead of the RER cisternae, cells that would correspond to the large mononuclear cells of Schoenberg et al. (19). If so, the 7S antibodies are associated with the plasma cells and lymphoplasmacytes. It remains to be determined if both produce IgG.

SUMMARY

Antibody to horseradish peroxidase was localized by electron microscopic immunocytochemistry in cells of the popliteal lymph nodes of the rabbit after a single injection of antigen with complete Freund's adjuvant and after a second antigen administration. Synthesis of antibody, chiefly of 7S type, occurred simultaneously in two types of cells: large, clear, fixed, typical plasma cells, and small, dense, circulating cells which exhibit morphological characteristics of both small lymphocytes and plasma cells. We call the latter "lymphoplasmacytes" and propose that they arise from small lymphocytes. They secrete antibody by clasmatosis and continue to develop an elaborate endoplasmic reticulum after specific antibody synthesis ceases. In the presence of an additional antigenic stimulation, a second cycle of antibody synthesis may begin around the nucleus in the same cell, with antibody accumulating in the perinuclear space sometimes even before the previously synthesized antibody has been entirely secreted at the cell periphery. On this basis, we propose that the lymphoplasmacyte is a memory cell and that memory and antibody synthesis are two different activities of the same cell. The appearance of a small amount of 19S antibody may be correlated with the presence of a small number of antibody-containing, large lymphocytes.

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Note added to proof: Since this manuscript was submitted for publication, we have also identified lymphoplasmacytes in mice, rats, and guinea pigs.

[1] Marchalonis, J. J. Personal communication.

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