

ELECTRON MICROSCOPIC OBSERVATIONS ON ANTIBODY-
PRODUCING LYMPH NODE CELLS*

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PLATES 27 TO 32

(Received for publication 20 August 1965)

The direct demonstration by McMaster and Hudack (1) of the production of antibody in lymph nodes led to a long search for the cell in the lymph node which synthesized antibody. Early evidence by Harris, Grimm, Mertens, and Ehrlich (2), Dougherty, Chase, and White (3), and Harris and Harris (4) that the lymphocyte was the cell of synthesis was followed by the studies of Björneboe and Gormsen (5), and Fagraeus (6) indicating the plasma cell as the cell which produced antibody. The following years produced studies of the cellular source of antibody by at least six experimental approaches, each of which led some authors to conclude that the plasmacytic series of cells was involved, and others, the lymphocytic series (7).

The culmination, in 1955, of this decade and a half of intensive research was the actual finding of antibody within plasma cells, by Coons and his colleagues (8-10), using the indirect fluorescent antibody technique. These cells were found in antibody-producing lymph nodes; antibody-containing plasma cells were soon also found in other situations, i.e., in sites of deposition of transferred lymph node cells, by Dixon and his colleagues (11-13), and in chambers containing antigen-stimulated lymph node cells, by several groups of workers (14-16). Also supporting this association was the observation of Nossal (17), in single cell preparations from active lymph nodes, that virtually all the cells which were identified as antibody-producing were plasma cells.

More recently, however, a number of reported observations have again given direct evidence of a role of the lymphocyte in antibody formation. In single cell droplet studies which involved a more sensitive antibody assay than that of Nossal (17), Attardi, Cohn, Horibata, and Lennox (18) found lymphocytes among the antibody-forming cells, the frequency of antibody-producing cells among the lymphocytes found being roughly one-third that found among plasma cells. Balfour, Cooper, and Alpen (19) found, after a secondary injection of diphtheria toxoid, that of the cells in the regional lymph node identified by

* This study was supported by Research Grants HE-04598(AI), AI-04911, and AI-02405 of the United States Public Health Service.

† Career Development Award K3-HD-22708.

specific fluorescence as containing antibody, a number clearly resembled lymphocytes. Significant recent evidence for the role of the lymphocyte in the production of antibody has come from the application by McGregor and Gowans (20) of Gowans' (21) ingenious method of prolonged cannulation of the thoracic duct. In rats severely depleted specifically of lymphocytes there was marked depression of antibody formation, and this was restored on infusion of thoracic duct cells from other rats of the same inbred strain.

Since it is difficult to ascertain the amount or concentration of antibody necessary for its detection in a cell by the indirect fluorescent antibody technique, the small number of cells thus identified in active lymph nodes following primary stimulation (9) raised the question of whether only the most active antibody-synthesizing cells were selected by the immunofluorescent technique. The recent studies referred to above have made this question the more pertinent. In one, which involved only lymphocytes (20), evidence of an inductive role by these cells was found. In the others, which yielded evidence of antibody produced by, or contained in, lymphocytes (18, 19), antibody-producing plasma cells were also found, and with substantially greater frequency. It was, therefore, desirable to examine antibody-producing cells at some stages between the induction of antibody formation by immunocompetent cells and the presence of completed antibody in such cells in an amount necessary for detection by immunofluorescence. An opportunity for such a study was offered by the recent descriptions by Jerne and Nordin (22), and by Ingraham and Bussard (23) of a method for detecting single cells which have produced antibody *in vitro*.

The availability of such a method for the identification of antibody-producing cells, and the far greater resolution in cytologic observations by electron microscopy than by light microscopy, led to the present study of such cells by electron microscopy.

Materials and Methods

The Hemolytic-Antibody Plaque Procedure.—Popliteal lymph nodes of rabbits injected with sheep erythrocytes were excised and teased to release cells, which were washed twice and suspended at 1:6 in Earle's (24) solution. Plastic Petri plates 5 cm in diameter were used, in which a 3 ml layer of 1.1% agar in Earle's solution had been allowed to harden. A suspension of lymph node cells, 0.6 ml of an appropriate dilution, was mixed with 1 ml of 1.1% agar (Ionagar, No. 2, "Oxoid," Consolidated Laboratories, Inc., Chicago Heights, Illinois) containing 2% sheep erythrocytes. (The dilution of the lymph node cell suspension was made as high as possible in order to increase the frequency of plaques which would contain only a single nucleated cell.) Of this mixture, 1 ml was placed on the agar layer in the plate and agitated until it covered the area of the plate. A 0.5 mm layer of agar containing lymph node cells and erythrocytes was thus produced. When the agar had hardened, the plate was placed in the incubator for 1 hr. Normal guinea pig serum (1 ml of 1:3 in Earle's solution) was then added to supply complement, and incubation was continued for an additional $\frac{1}{2}$ hr. At the end of this time the cells were fixed by adding 2 ml of 0.5% phosphate-buffered osmic acid (25) to each plate without removing it from the incubator. After 15 min

of fixation at 37°C, the osmic acid was largely removed by repeated washings with 70% ethanol.

Preparation and Embedding of Plaques for Electron Microscopy.—The agar layers containing the cells were further dehydrated by addition of increasing concentrations of ethanol (80 and 95%), each used three times for 5 min. After removal of the 95% ethanol, the plates were examined with a dissecting microscope on an illuminator, and cylindrical cuts 2 mm in diameter were made through the agar around the clearest plaques by a J-tipped Pasteur pipette. The partial dehydration facilitated the manipulation, leaving clearly punched discs without any material remaining in the lumen of the pipette. After a sufficient number of plaques were cut out of the agar layer, 100% ethanol was added, and the agar discs were gently floated into the alcohol with a jewelers' forceps. They were collected with a wide-tipped pipette and further dehydrated in a separate container in two additional changes of 100% ethanol.

RESULTS

Immunologic Data.—In the eight experiments which yielded cells for electron microscopic study in this experiment, 2 or 3 rabbits were given a single injection of sheep erythrocytes (0.2 ml of a 50% suspension) in each hind foot-pad. After 4 days the popliteal lymph nodes were excised and teased as described above, and the pooled cell suspension was used for plating. In such pooled suspensions, the yield of cells per lymph node was in the range of 285 to 480 million. Such cell suspensions were examined for the number of plaque-producing cells by plating 0.6 ml volumes, as described above, at five successive two-fold dilutions, between 200 and 3200. Plaque-producing cells were found in these experiments in the range of 1 per 4400 cells to 1 per 12,600 cells.

In experiments in which a 3 day interval was allowed between injection of sheep erythrocytes and excision of the lymph node, the frequency of plaque-producing cells was considerably less, approximately one-tenth that found in the 4 day interval. These were not examined in the present study.

Electron Microscopic Preparations.—The problem of finding and examining single plaque-producing cells was solved in the following way. After the dehydration of the plaque-containing agar discs, described above, these were exposed to an epoxy embedding resin (26) for 16 to 18 hr. Each agar disc was then embedded separately by transferring it to a capsule containing one drop of the resin and orienting the disc under a dissecting microscope to face the tip of the capsule. Subsequently the capsules were filled with the embedding medium and transferred to 60°C for polymerization. The blocks were then trimmed with a diamond knife to obtain a smooth surface. This made it possible to see the plaque in detail by light microscopy, with the resin blocks in a vertical position. Plaques for further processing were selected on the following criteria.

1. Blocks were used only if no more than one nucleated cell was seen in the center of the plaque. This eliminated a number of preparations in which the plaques contained more than one cell, a not infrequent occurrence.

2. Plaques were preferred which contained little or no erythrocyte background, i.e., where the cell had produced enough antibody to lyse all erythro-

cytes through the whole layer of agar. This type of plaque was found infrequently, and therefore some cells were also processed which were near the bottom of the agar layer, with some unlysed erythrocytes above them. In such cases, the erythrocytes, and the accompanying nonantibody-producing lymph node cells, could be eliminated by trimming the block, leaving a clear plaque without background. The final screening of the resin-embedded preparations was done at a magnification of 400. A plaque that met the above criteria is shown in Fig. 1.

The blocks selected were further processed as follows: Under a dissecting microscope excess resin was removed with a razor blade to a level close to the embedded material, and the block face was made smaller. The blocks were sectioned again with a diamond knife. The distance of the cell from the block surface was estimated with a light microscope having a calibrated fine adjustment. This distance was noted for each block, and these were further trimmed on the ultramicrotome to within $1\ \mu$ of the cell. After a further incubation, at 60°C for 1 to 2 days, the preparations were ready for serial sectioning.

The Fine Structure of Antibody-Producing Cells.—Two classes of cells, with distinct morphological features, were found to produce hemolysis after a single injection of sheep erythrocytes. The cells were in the category either of lymphocytes or of plasma cells. Both kinds of cells were found in about equal numbers, although the relatively small number of cells studied so far would not yield meaningful data on the relative frequency of occurrence of the two kinds of cells in such preparations.

The lymphocytes ranged in size from 5 to $7\ \mu$ in diameter. Representatives of the two extremes can be seen in Figs. 2 and 3. These lymphocytes contained some morphological features both of the small lymphocyte and of the lymphoblast, according to the current terminology. The eccentric nucleus showed deep indentations, with the chromatin in part condensed. Most of the cells had a relatively large and honeycombed nucleolus, which is illustrated in Fig. 4. The cytoplasm was fine and granular, with most organelles confined to the larger pole of the cell. The number of small mitochondria varied greatly, the larger lymphocytes containing a greater number of mitochondria than the smaller ones, even in relation to their size. A Golgi apparatus was usually present but it was small, with few smooth vesicles.

The most interesting feature in these antibody-producing lymphocytes was the rough endoplasmic reticulum. In the smaller lymphocytes a small amount of endoplasmic reticulum was found, as has been described in the recent literature (27, 28). This was sparse, and was consistently widened. The resulting vacuoles, ringed with ribosomes, were partially filled with a grayish, granular material, as demonstrated in Fig. 5. In the larger lymphocytes, however, endoplasmic reticulum of a different form was also found, in relative abundance. The

channels were narrow. Short pieces were observed, cut longitudinally, with apparently random orientation. No organization into lamellae was apparent. These features can be seen in Fig. 3, and in more detail in Fig. 6.

While the lymphocytes exhibited a certain morphological unity, differing primarily in size and in degree of cytoplasmic organization, the plasma cells showed considerable pleomorphism. Some of the small plasma cells were very fragile, so that it was impossible to maintain their structural integrity with the same methods of handling which were apparently not injurious to the lymphocytes. It is likely, therefore, that this reflected their natural state. The plasma cells were found in several forms, which may represent various stages of development, especially in view of the appearance of the endoplasmic reticulum in these cells. All of the cells in this group were characterized by a well developed and flattened endoplasmic reticulum, a distinct Golgi apparatus, and a round nucleus with evenly dispersed chromatin. Their size varied from 6 to 9 μ .

A cell typical of those with the least developed endoplasmic reticulum in this group is shown in Fig. 7. The sacs were parallel, with wide interspaces. The cytoplasm was still relatively small, compared to the size of the nucleus, and the Golgi apparatus was well developed with numerous smooth vesicles. These features can be seen at a higher magnification in Fig. 8.

The cell shown in Fig. 9 resembled a mature plasma cell. The whole of the cytoplasm was taken up by endoplasmic reticulum, which can be seen in detail in Fig. 10. Transversely cut sacs contained a granular material.

The third type of cell in this group is demonstrated in Fig. 11. It can be seen that most of the endoplasmic reticulum was widened and filled with material, and that the surrounding cytoplasm, the plasmalemma, and a mitochondrion had disintegrated.

In sections outside the clear plaques typical small cells of the lymphocytic series were observed frequently, as well as an occasional eosinophile. Unlysed erythrocytes were always seen in the proximity of these cells, whereas those near the antibody-producing cells, within the plaque, were effectively lysed, and presented themselves as ghosts, as can be seen in Fig. 12.

DISCUSSION

The observations reported here indicate that cells which are clearly shown to have produced antibody include both plasma cells and lymphocytes. That cells of the morphologic classification of plasma cells can synthesize antibody is well accepted in the current literature. However, in the case of the lymphocyte, also, there has been recorded evidence that these cells can produce antibody. Evidence that a function of the lymphocyte is the synthesis of antibody was originally presented in the 1940's, in cells of lymph node and spleen, in the mouse and rabbit, and with cellular, bacterial, and viral antigens (2-4). Subsequently, however, in view of the evidence for the plasma cell as a source of anti-

body, culminating in the finding of antibody within the plasma cell (8-10), the emerging descriptions of these two cell types by electron microscopy gave rise to a generally accepted view that the synthesis of antibody could be expected in the plasma cell, with its well developed endoplasmic reticulum and associated organelles, but not in the lymphocyte, with its paucity of cytoplasmic differentiation.

In the lymphocytes found as single cells in the center of plaques in this study, however, there were definitely more of the structural units generally associated with synthetic functions than have been described thus far for the small lymphocyte (27, 28); Golgi bodies, nucleoli, and the short channels of endoplasmic reticulum. The significance of these structures for antibody synthesis in these cells is borne out by the fact that they were not found in the lymphocytes examined which were at the edges of plaques, and which were, therefore, not producing the antibody.

These differences in fine structure between antibody-synthesizing and presumably inactive lymphocytes, respectively, is of considerable importance for the interpretation of the earlier electron microscopic descriptions of lymphocytes (27, 28) and of the differences between those descriptions and the features of the lymphocytes described here. As indicated above, the frequency of occurrence of plaque-producing cells in these suspensions was about one in 8000 cells. In the study of Ingraham and Bussard referred to, plaque-producing cells were found with a frequency of approximately one per 9000 and one per 10,000 in cells obtained from lymph nodes and spleens of rabbits injected with sheep erythrocytes (23). In recent studies with this antigen in the mouse and the rat, the frequency of plaque-producing cells in splenic suspensions has been reported in the range of one cell per 200 to one per 5000 (29-32). In the case of bacterial lipopolysaccharides, for which plaque tests have been developed by bacteriolysis, for *Escherichia coli* (33), or by hemolysis of normal erythrocytes to which salmonella lipopolysaccharide had been adsorbed (34, 35), the frequency of plaque-producing cells has been found to be in the range of one per 400 to one per 2000 (34, 36). Therefore, although cells of lower degrees of response to the injected antigen, and cells producing antibody to unknown antigens, may well be present in a lymphatic organ, the chance of encountering a demonstrably active cell among the lymphocytes of a lymph node or spleen studied by electron microscopy is extremely small, and it is highly probable that the lymphocytes described in the literature thus far (27, 28, 37) have been inactive with respect to antibody formation.

The observations on the antibody-synthesizing lymphocytes are also of interest in relation to the protein-synthetic mechanism of the mammalian cell. Although many of the ribosomes of the lymphocytes were found lining the scattered channels of apparently developing endoplasmic reticulum, the substantial majority appeared to be free in the cytoplasm. Thus it may be that

this represents an instance in mammalian cells of a secreted protein synthesized by ribosomes not bordering an organized endoplasmic reticulum. The effectiveness of these free ribosomes in the synthesis of secreted protein is indicated by the fact that thus far no association has been observed between the size of the plaque and the development of endoplasmic reticulum in the cell. In this study plaques were not selected for size, but were chosen for maximum clarity and distinctness, and thus represent a selection from among the most actively secreting cells. The fact that single cell plaques selected on a basis of maximum clarity contained a lymphocyte or a plasma cell with roughly equal frequency suggests that the cells of the two cytologic classes do not differ widely in their rate of secretion of antibody to the external medium. Rather, the plasma cell appears to represent a cell type or a stage of development in which the cell synthesizes antibody at a higher rate but stores some of it within its endoplasmic reticulum until disintegration of the cell releases this additional antibody.

The evidence for secretion of antibody by active lymphocytes, with morphologic evidence of activity of the cell at the end of a period of such secretion, confirms the results of the ingenious double labeling experiments by Helmreich, Kern, and Eisen (38) which led to the conclusion that in active lymph node cells antibody is produced by a continuous secretory process and that the cell may still be active after a period of secretion of antibody.

Among those plaque-producing cells classified as plasma cells, three types were recognized, illustrated in Figs. 7, 9, and 11. These present interesting comparisons. The cells in Figs. 7 and 9 might well represent different stages of development of a given cell type, the endoplasmic reticulum being relatively sparse in the former and filling the cytoplasm in characteristic parallel bundles in the latter. The cell of Fig. 11 appears to be in the final stage of dissolution, with swollen endoplasmic reticulum.

In a recent study by light microscopy, Bussard and Hannoun (39) have also reported pleomorphism of plaque-producing cells, without regard to their conventional classification in the lymphocytic or plasmacytic grouping.

The finding of these diverse cell types, lymphocytes larger and smaller, with endoplasmic reticulum of apparently different kinds or of different degrees of development, and plasma cells with different amounts of endoplasmic reticulum, raises the question of whether there are, in fact, two different cell lines which produce antibody or whether all of these forms may represent different stages of development of a single cell line.

Various attempts have been made in the past to find a role for the two cell types in antibody formation, the lymphocyte perhaps being involved in the primary response and the plasma cell in the secondary. However, these efforts have not succeeded in assigning such roles to these cells. For example, Ward, Johnson, and Abell, who had found in rabbits given a primary injection of bovine gamma globulin that plasma cells were still increasing in the spleen after

the level of antibody in the serum had reached its peak and begun to decline (40), repeated the study with secondary injections of the antigen, but made the same cytologic observations (41). On the other hand, there have been several observations which suggested the possibility of transformation of lymphocytes into plasma cells, e.g., at sites of deposition of transferred lymph node cells (11-13) or in diffusion chambers (14). Other recent studies of the potentialities of the small lymphocyte for change into presumably more active forms, which cannot be discussed here, have arisen from the work of Gowans with thoracic duct cells, referred to above, and from the discovery of blastogenesis in peripheral blood lymphocytes caused by phytohemagglutinins (42, 43) and by known antigens (44, 45). In the present study a group of cells selected by their activity in the synthesis of this 19S antibody have shown pleomorphism of both kinds of cell, and a progression of development of endoplasmic reticulum through a series of cell forms which includes both lymphocytes and plasma cells. These observations suggest that such developmental change of the small lymphocyte can, in fact, occur, as this cell, in its remarkable response to a stimulus for rapid synthesis of a special protein, develops a synthetic apparatus before producing the protein itself. Such a reactive development of the lymphocyte thus stimulated, which had been suggested elsewhere (46), could account for the various cell-types which have been classed as being of both the lymphocytic and plasmacytic series.

SUMMARY

Lymph node cells of rabbits injected with sheep erythrocytes, identified as antibody-producing by their ability to produce plaques of hemolysis in erythrocyte-containing agar layers, have been examined by electron microscopy, by the use of a procedure devised for subjecting single cells to such examination.

The antibody-producing cells thus examined were found to fall into two classes, according to the current terminology: some were in the category of lymphocytes, and others, in the category of plasma cells. Within each class, cells were found to vary in certain characteristics, especially in the degree of development of such organelles as the nucleolus, Golgi apparatus, and the endoplasmic reticulum. In the case of the endoplasmic reticulum especially, it could be seen that a series of these plaque-producing cells, ranked in order of increasing size and development of the endoplasmic reticulum, would extend over a considerable range from those lymphocytes with the least developed organelles to the mature plasma cells with the greatest development of these structures.

The authors are indebted to Mr. Natale Tomassini for his invaluable technical assistance.

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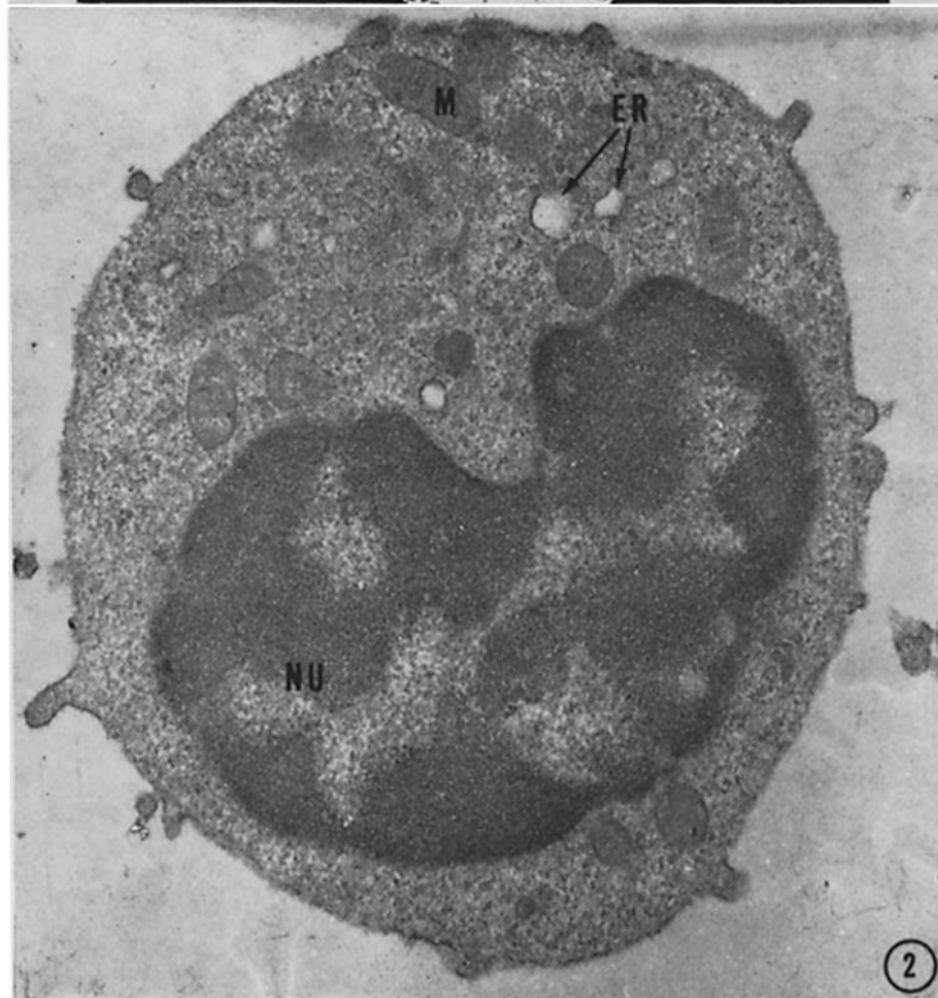
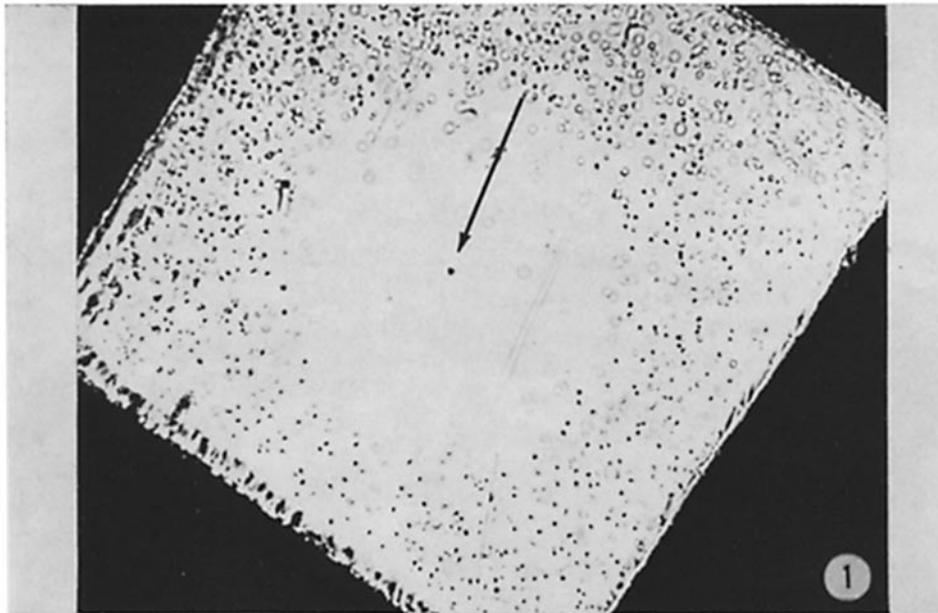
EXPLANATION OF PLATES

NU, nucleus*M*, mitochondrion*NOS*, nucleolus*ER*, endoplasmic reticulum*G*, Golgi apparatus

PLATE 27

FIG. 1. The appearance of a plaque embedded in epoxy resin with the antibody-producing cell in the center. $\times 100$.

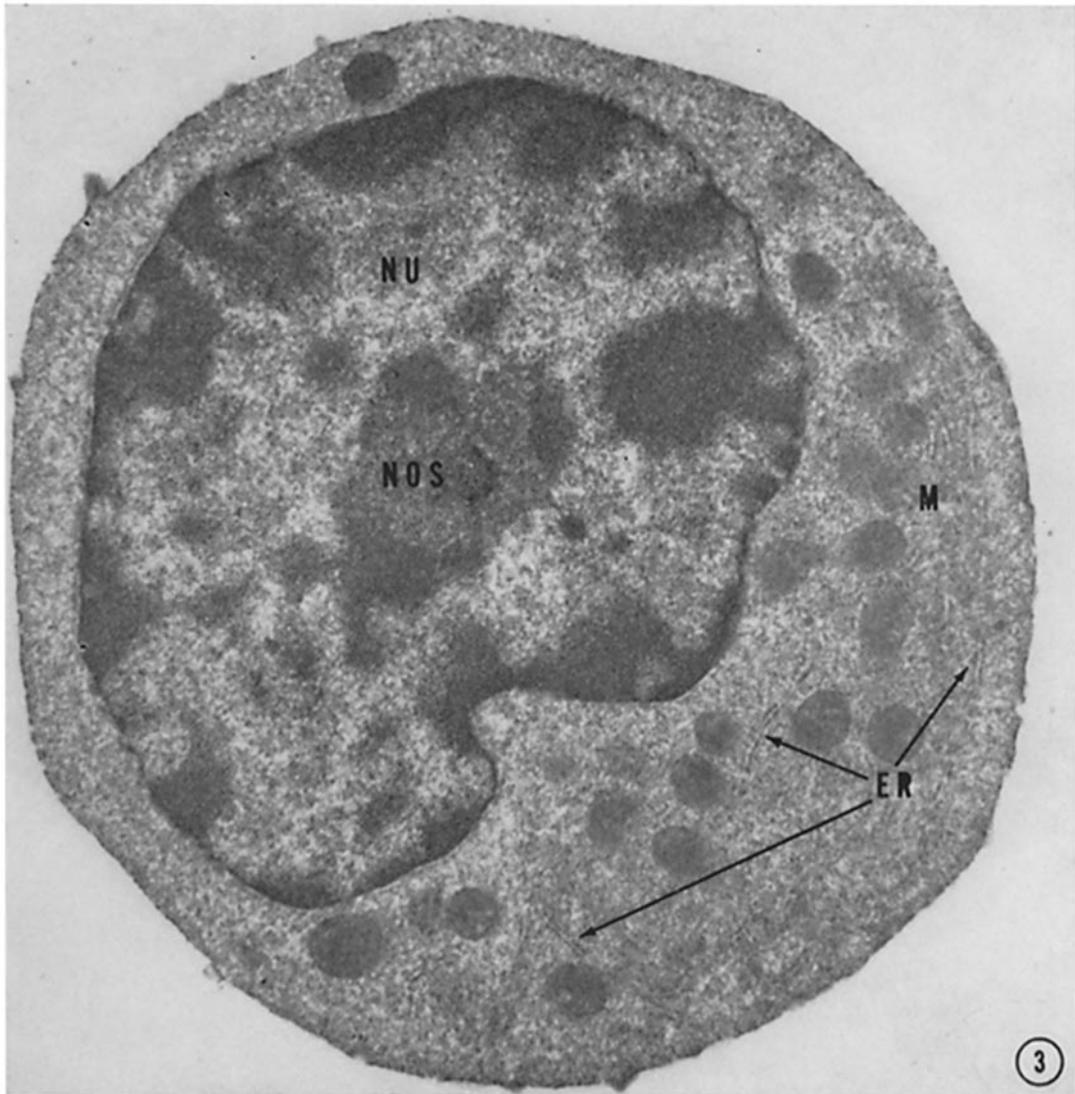
FIG. 2. Small antibody-producing lymphocyte. Eccentric and indented nucleus. Organelles concentrated in one cell pole. Endoplasmic reticulum sparse but widened. $\times 24,000$.



(Harris et al.: Antibody-producing lymph node cells)

PLATE 28

FIG. 3. Large antibody-producing lymphocyte. Eccentric nucleus with large nucleolus. Numerous mitochondria in one cell pole. Many narrow channels of endoplasmic reticulum, which are not organized. $\times 21,600$.



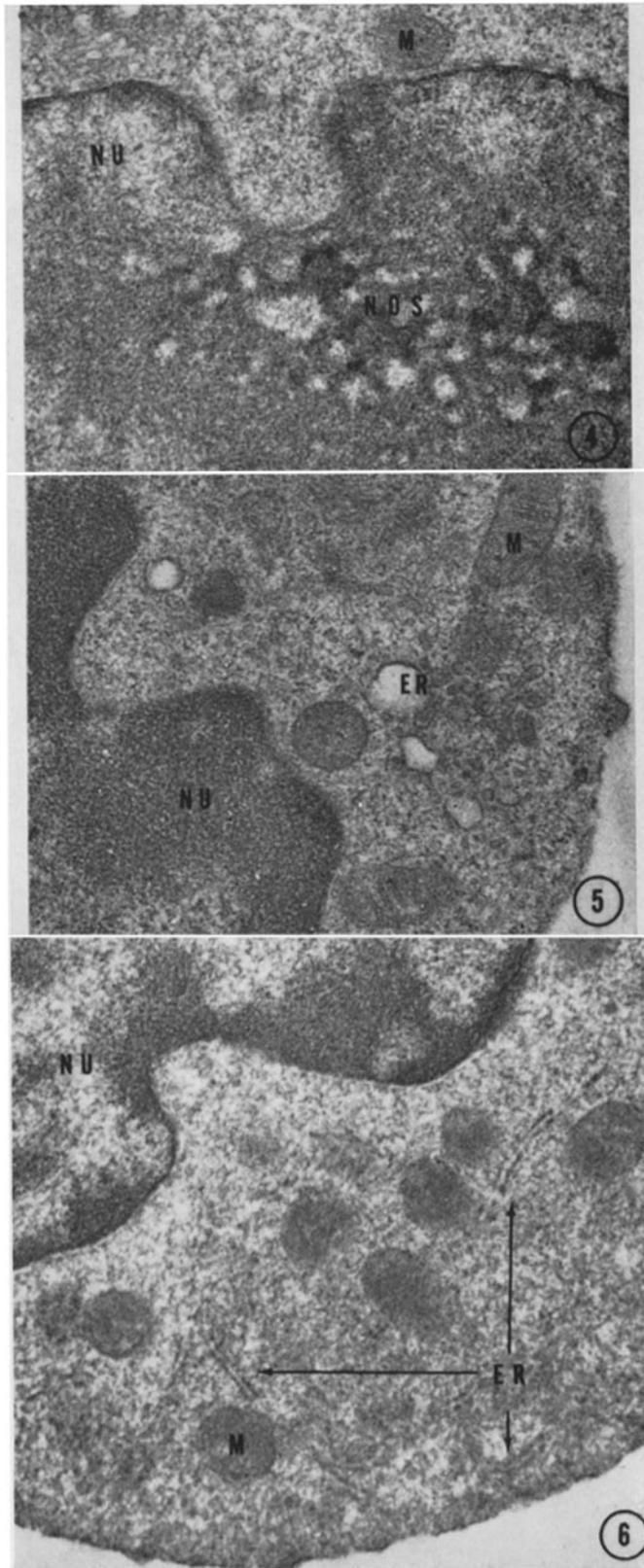
(Harris et al.: Antibody-producing lymph node cells)

PLATE 29

FIG. 4. Details of nucleolus in a large antibody-producing lymphocyte. Nucleolus of cell shown in Fig. 3. It is large and has a honeycomb appearance. $\times 23,000$.

FIG. 5. Details of widened endoplasmic reticulum in small antibody-producing lymphocyte shown in Fig. 2. The ribosome ringed vacuoles contain granular material. $\times 40,000$.

FIG. 6. Details of unorganized channels of endoplasmic reticulum in large antibody-producing lymphocyte shown in Fig. 3. The consistently narrow channels are apparent throughout the cytoplasm, sectioned at various planes. $\times 29,000$.

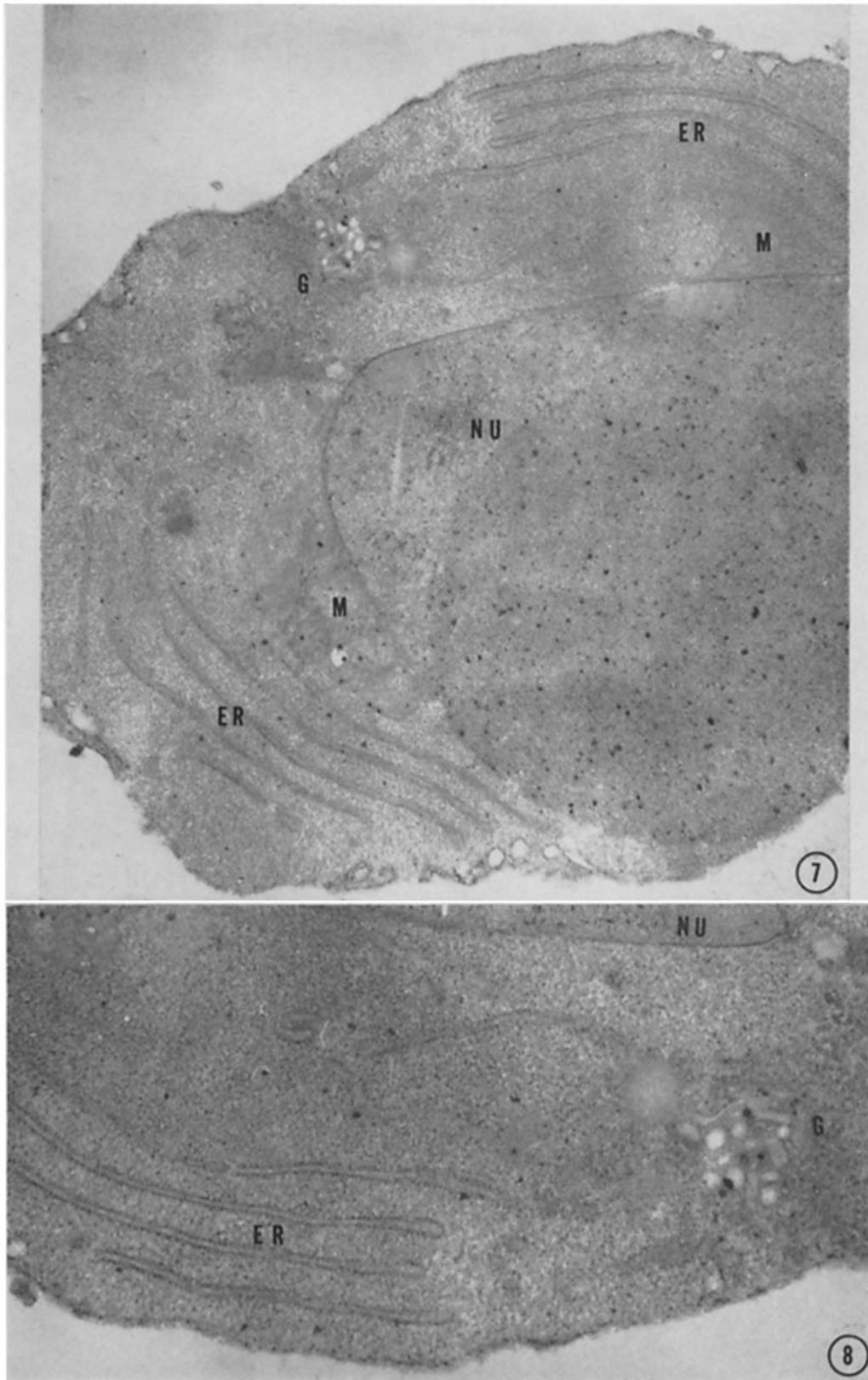


(Harris et al.: Antibody-producing lymph node cells)

PLATE 30

FIG. 7. Antibody-producing cell resembling young plasmacyte with sparse, widely spaced but organized endoplasmic reticulum, and well developed Golgi body. $\times 19,000$.

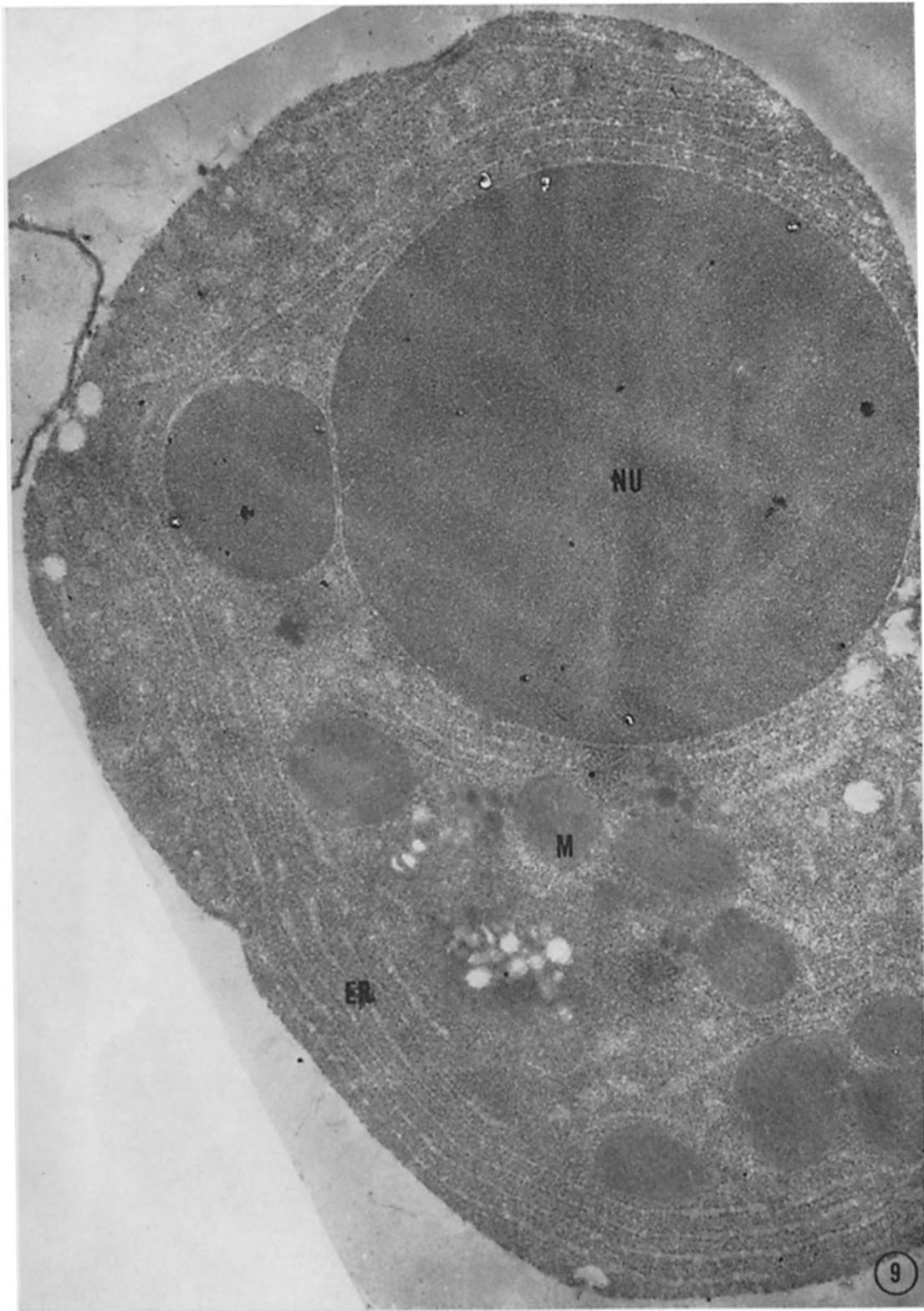
FIG. 8. Details of endoplasmic reticulum and Golgi body of the cell in Fig. 7. The channels of endoplasmic reticulum are still sparse but well organized. Ribosomes can be seen in the cytoplasmic matrix. $\times 32,000$.



(Harris et al.: Antibody-producing lymph node cells)

PLATE 31

FIG. 9. Antibody-producing plasma cell. Well developed endoplasmic reticulum organized into lamellae. The cell appears fragile. No details apparent in the nucleus or mitochondria. $\times 22,500$.



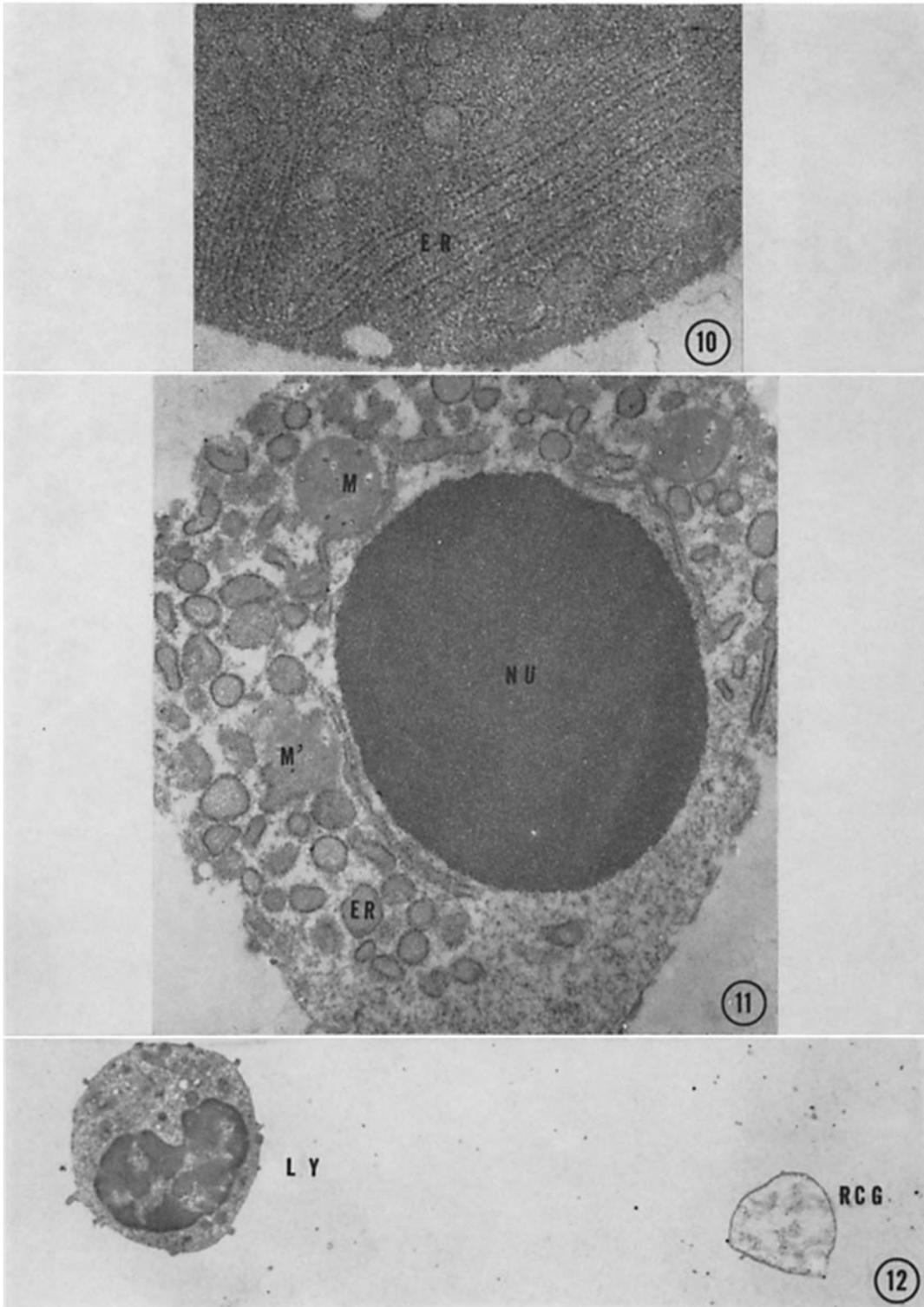
(Harris et al.: Antibody-producing lymph node cells)

PLATE 32

FIG. 10. Details of endoplasmic reticulum in cell similar to the one shown in Fig. 9. Distinct organization of the endoplasmic reticulum into lamellae which are lined with ribosomes. $\times 29,000$.

FIG. 11. Antibody-producing plasma cell. Most endoplasmic reticulum channels are extremely widened and contain a granular substance. Cytoplasmic matrix, plasma-lemma and a mitochondrion (M') disintegrating. $\times 17,000$.

FIG. 12. Red cell ghost (RCG) in proximity to the antibody-producing small lymphocyte (Ly) in the center of a plaque. This is the same cell as that shown in Fig. 2. $\times 4,300$.



(Harris et al.: Antibody-producing lymph node cells)