ANTIGENS IN IMMUNITY

XIV. Electron Microscopic Radioautographic Studies of Antigen Capture in the Lymph Node Medulla*

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Plates 37–42

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It is by now well established that antigens entering lymph nodes become localized in two chief areas (1–7). All antigens are taken up, though to varying extents, by macrophage cells present in large numbers in the medulla and in much smaller numbers elsewhere in the node. In the presence of specific natural or immune antibody, antigen will also be specifically trapped in the cortical lymphoid follicles, where it does not enter typical macrophages. The bulk of follicular antigen is retained on the surface of reticular cell processes forming a dendritic web (4, 6, 8, 9). We do not know the relevance of either of the above two antigen-capturing mechanisms to the inductive events of immunity. The present study is an attempt to enlarge our understanding of both mechanisms through the use of the electron microscope. Microgram quantities of purified *Salmonella adelaide* flagella were labeled with carrier-free ^{125}I and injected into one hind foot-pad of adult rats. Localization of antigen in the popliteal lymph nodes at various subsequent time points was studied by the technique of electron-microscopic radioautography. The sequence of events in the lymph node medulla forms the basis of the present paper. The entirely different phenomena occurring simultaneously in the cortex are described in the accompanying paper.

From a number of recent publications (10–13) we now have a fairly comprehensive picture of the way in which phagocytic cells handle small particles. Material enters cells by the formation of small vacuoles lined by portions of the plasma membrane (pinocytosis, athrocytosis, phagocytosis, and endocytosis). Then such vacuoles fuse both with each other and with a variety of cell inclusions which contain hydrolytic enzymes. There is good evidence that these hydrolases are synthesized, as other proteins, on polyribosomes; transported via the endoplasmic reticulum to the Golgi region; sequestered into tiny

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Golgi vesicles (protolysomes); and preserved for eventual reutilization in larger intracellular granules (lysosomes, telolysosomes) (11, 12).

Our study of antigen capture in the lymph node medulla shares many features with the work of Cohn and Hirsch (10, 11) but differs from it in four important respects: (*a*) it is an in vivo rather than an in vitro study; (*b*) the particle we used was highly immunogenic and, because of the high specific activity of labeling achieved, quite minute amounts could be used; (*c*) attention could be given not only to the details of phagocytosis but also to the coincident cellular events occurring in the lymph node; and (*d*) no attempt has been made to study the enzyme content of the various cell inclusions encountered, and thus any reference to lysosomes or phagloysosomes rests on morphological inference.

Materials and Methods

Animals.—In all but one small experiment, randomly-bred Wistar rats of both sexes were used. In one experiment, inbred Lewis rats, bred from a breeding pair obtained from Dr. W. K. Silvers, University of Pennsylvania, were used and as the results showed no obvious differences they will be included. Rats were aged 12 wk at first immunization.

Antigen and Iodination Procedures.—Intact flagella from *Salmonella adelaide* were used. The method of shearing the flagella off the bacteria and purifying them by differential centrifugation has been previously described (14). After having been labeled with ^{125}I, 20 μg of antigen was injected into the right foot-pad on a single occasion in saline without adjuvants. Some rats had received a primary stimulus of uniodinated flagella 6 wk previously.

The chloramine T method (15) of iodination was used. Carrier-free ^{125}I was obtained from the Radiochemical Centre, Amersham, England. Substitution rates of 0.6–1.2 g atoms of iodine/38,000 g of antigen (specific activities 30–60 μ Ci/μg) were achieved.

Experimental Design.—Rats were killed at 3 min–21 days after antigen injection by a sharp blow on the head while under light ether anesthesia. A total of 24 rats was studied over a period of 2 yr. A prime objective was to select for electron-microscopic examination areas the location of which in the lymph node was exactly known. This was accomplished by following strictly the routine described below.

Electron Microscopy.—After excision, the draining popliteal lymph node was cut in half and each piece immediately immersed in one of the two chosen fixatives; i.e., osmium tetroxide in veronal-acetate buffer at pH 7.3 (16), or 5% glutaraldehyde in 0.1 M phosphate buffer (17). After a short interval in the cold fixative the tissue was diced into pieces of about 2 mm and fixed for 2 hr or overnight at 4°C. Glutaraldehyde-fixed material was then washed for 3 hr in six changes of 0.1 M phosphate buffer followed by 2 hr osmium postfixation. Tissues then received a common treatment, following a distilled water wash, of dehydration through a graded methanol series and finally acetone before infiltration and embedding in the epoxy resin, araldite.

After polymerization the radioactivity of individual blocks was determined to establish a priority of cutting sequence. A thick (2 μ) section, embracing the complete cross-section of tissue was first cut and mounted on a gelatin-coated glass slide for light microscope radioautography. Prior to the application of Kodak NTB2 emulsion, the Araldite was removed, using sodium methoxide (18), to permit subsequent staining with methyl green–pyronin.

Radioautographic exposure of up to 1 day was sufficient to demonstrate the labeling pattern of medullary and cortical regions on light microscope examination. The related block face was then further trimmed by razor blade to contain the desired area of cortex or medulla within the more restricted area chosen for electron microscope study.

A Cambridge "Huxley" ultramicrotome, using glass knives, was employed to cut silver-colored ultrathin sections in a ribbon which terminated with a 1 μ section. This section was mounted for light microscopy as described and was used for correlative studies as well as providing a guide to the required exposure period for the ultrathin sections. These latter were mounted for electron microscope radioautography on collodion-coated glass slides according to the method of Salpeter and Bachmann (19), using Kodak NTE emulsion, with the modifications previously described (8) but including the added refinement of using 1% hydrofluoric acid[1] when a section was reluctant to strip from the glass. Sections were stained, floating freely, in 5% aqueous uranyl acetate for 2–12 hr prior to mounting on the slide.

After exposure and development the sections were mounted for electron microscope examination on either NEW 100 Athene grids (Smethurst High-Light Ltd., Bolton, Lancashire, England) or annular copper discs of 0.5 or 1 mm internal diameter (20). The latter type of support proved invaluable in light microscope correlative studies as continuous, uninterrupted viewing of the complete section area was possible in the electron microscope and allowed the compilation of low power photomontages of clearly established follicular areas to permit the study of the spatial relationships of cells at the fine structural level.

RESULTS

Structure of the Medulla before Antigenic Stimulation.—The electron microscopic appearance of the lymph node medulla has been fully described by a number of authors (21–26). The medulla consists essentially of cords and sinuses. The cords are continuous with the lymph node cortex and, in our rats, were relatively nonprominent in unimmunized rats. They contained lymphocytes and mature plasma cells, occasional macrophages, and usually a blood vessel. They were separated from the sinuses by a layer of elongated sinus-lining macrophages but this clearly did not represent a complete barrier, there being no basement membrane and many cells being noted in apparent transit between sinuses and cords. The medullary sinuses were in free communication with the cortical circular sinus via trabecular sinuses which were frequently quite short. The predominant class of cell found in the sinuses was the macrophage. These were large cells with ovoid nuclei having a light chromatin pattern and only occasionally showing the deep indentation characteristic of blood monocytes. The cytoplasm was usually extensive, and frequently broad blunt processes could be seen. The Golgi apparatus was always well developed, and vesicles similar in appearance to Golgi vesicles were liberally scattered right throughout the cytoplasm. Mitochondria were moderately frequent; they were usually rounded, 0.5–1 μ in diameter, and possessed well-defined cristae. The elongated variety was rarely seen. Occasional profiles of rough-surfaced endoplasmic reticulum were present, and free clusters of polyribosomes were frequent. Macrophages contained a great variety of dense granules and vacuoles which will be described in detail below. Those portions of the plasma membrane of macrophages which were in contact with the lymph were covered with a layer of an electron-opaque material of 20–100 mμ thickness. Frequently this

[1] Merrillees, N. C. R. Personal Communication.

layer had a somewhat beaded appearance, which was more apparent in the glutaraldehyde-fixed material. Often, contiguous sheets of macrophages could be seen straddling the sinus. Some lay apparently free; others were closely related to bundles of collagen fibrils. In the latter case, the collagen was frequently surrounded by macrophage cytoplasm on all sides, as if it had become engulfed by extensive invaginations of the macrophage plasma membrane. Where the immurant membranes lay in apposition, specialized intermediate junctions or zonula adhaerens (27) were often observed. Many of these features are shown in Figs. 1–16 (Plates 37–41).

Mast cells and lymphocytes were also present in moderate numbers in the sinuses, and occasional polymorphonuclear leukocytes and erythrocytes could be seen.

In the descriptions of antigen trapping which follow, reference will be made to the labeling pattern of radioautographs at various time points after antigen injection, but it is not intended to make direct quantitative comparisons of grain frequency. Exposure times of radioautographs varied from 1 day to 3 months and prints have been chosen for publication where the silver grains do not obscure underlying detail. Previous study (4) with the light microscope has shown that after foot-pad injection of ^{125}I-flagella, grain counts over medullary macrophages in the popliteal node reach a maximum 4 hr later. After this the grain count declines slowly over a period of weeks, the half-life varying between 1 and 3 wk (4, 8).

Entry of Antigen into Medullary Macrophages.—Uptake of antigen by medullary macrophages was very rapid. 3 min after foot-pad injection significant radioactivity was already present in the popliteal node, and the amount trapped rose rapidly and progressively over the first 4 hr. Two basic mechanisms of entry seemed to be at work. Some of the antigen entered the cell by pinocytosis (Figs. 2–5). First, grains were seen over the electron-opaque layer outside the plasma membrane, though no evidence for a prolonged adsorption phase was obtained. Then, invagination caused the formation of caveolae, and soon budding off of the inverted plasma membrane resulted in the formation of pinocytic vacuoles. Even at the earliest time points, these varied widely in size. The range of diameters encountered was from 40 mμ (micropinocytic vacuole) to 500 mμ (phagocytic vacuole) though the majority were 100–200 mμ across. Moreover, the vacuoles varied in electron opacity. Some showed a moderate homogeneous electron opacity from the very beginning (Fig. 3), perhaps due to filling by the material which had coated the outside of the cell. Others (Figs. 1, 2, 4, and 9) were electron translucent except for their inner lining, which was electron opaque and often somewhat beaded. Such vesicles have been termed "coated vesicles" (28, 13). Many early vesicles showed inhomogeneous electron opacity, creating a speckled appearance (Figs. 1, 4, 7, and 9). The second mechanism of entry appeared to be a direct penetration of the plasma membrane

without the formation of a resolvable vacuole. Many single grains and clumps were found lying apparently free in the cytoplasm at time points up to 30 min, and occasionally thereafter. It is possible that in some cases the grain itself might have obscured a small, underlying vesicle but this is unlikely to be the explanation in all cases (Figs. 1–6). As in the case of our light microscopic studies (4), no differences in the entry pattern could be noted in the primary versus the secondary response.

Processing of Antigen by Macrophages.—Shortly after the pinocytic vacuoles were formed, they began to fuse with other vacuoles. A frequent appearance (Figs. 7–8) between 30 min and 4 hr after antigen injection was a cluster of small vesicles around an antigen-laden vacuole, sometimes forming a complete ring. The small vesicles frequently contained an electron-opaque material and may well have represented the hydrolase-containing protolysosomes of other authors (11, 12). In fact, the resemblance between many of our pictures 30 min after antigen injection and the published pictures of Cohn and Hirsch (11) is noteworthy. The result of such fusion was frequently the formation of a more electron-opaque membrane-bound antigen containing granule (Figs. 6–9). Though we have performed no enzyme studies, we feel that these dense bodies might well have corresponded to the telolysosomes of Gordon, Miller, and Bensch (12).

Within less than 4 hr the free cytoplasmic antigen had also been sequestered into membrane-bounded inclusions. A clue as to how this may have happened is given in Figs. 4 and 6. These show areas of label embedded in electron-opaque material which is, however, not yet bounded by a membrane. Clustered around this are numerous small, dense vesicles. The appearances suggest that a dense granule (lysosome?) is being rapidly constructed around free cytoplasmic antigen. A less frequent finding at these time points was a vacuole with an incomplete wall surrounding silver grains (Fig. 6). Such an appearance could have been due to a fixation artifact or could have represented another sequestration mechanism. In any event, antigen free in the cytoplasm was seen extremely rarely after 4 hr.

Beyond the 4 hr time point, the key happening was fusion of antigen-containing inclusions with each other to form larger and more complex inclusions. A bewildering variety of types of inclusions resulted, which we are provisionally terming phagolysosomes. Numerous examples are shown in Figs. 10–16. In the primary response these events can be followed without the complicating feature of preexisting phagolysosomes resulting from a previous antigenic stimulation. At 1 day (Fig. 13), many of the vacuoles were relatively electron translucent but showed a lining of electron-opaque material, possibly the material that had been lining the outside of the macrophage. Also, many showed label predominantly at their periphery. Often, though not always, such label was associated with discrete, electron-opaque blobs that may have represented antigen-con-

taining telolysosomes that had fused with the larger vacuole (Fig. 15). By 3 days (Figs. 12 and 14) most inclusions were more electron opaque and their median size was larger. In many cases, the rounded dense granules remained visible; label was associated with these and was also scattered generally throughout the inclusion. Our overall impression was that as time went on, less label was associated with the round dense intravacuolar bodies and more was scattered randomly throughout the phagolysosome (cf. Figs. 15 and 17). The inclusions became progressively larger and more complex in ultrastructure. Fig. 16 represents a typical late inclusion. It shows variegated, inhomogeneous electron-opaque material and scattered electron translucent areas as well as occasional round dense granules. The electron translucent areas were the least heavily labeled. Rather rarely, a labeled inclusion had the appearance of a myelinic figure (Fig. 17).

Even at 3 wk, though large complex inclusions dominated the picture, some label remained associated with smaller, simpler vacuoles and granules basically similar to those seen at 4 hr.

We did not follow the progress of labeled material for longer than 3 wk. However, from examination of sections taken at early time points after *secondary* injection, we can report that phagolysosomes (in this case unlabeled because the first injection had consisted of unlabeled flagella) persist for at least 6 wk. A macrophage that had taken up antigen 6 wk previously can still ingest and process new material, and this it appears to do in a way no different from the way it handled the first pulse. Thus, the one cell can contain old phagolysosomes and new, labeled small inclusions (Fig. 10). These must rapidly fuse with the larger ones and with each other because from the 1-day time point on, we noted no differences between the primary and the secondary response in macrophages.

At no time was significant labeling noticed in the nuclei of macrophages.

Cellular Changes Associated with Antigenic Stimulation.—The basic sequence of cellular changes in the lymph node medulla which follow antigenic stimulation have been described by many authors (reviewed in reference 23). In essence these consist of transient polymorphonuclear leukocyte invasion of the sinuses and progressive plasmacytopoiesis in the cords. From 4 to 24 hr we noted a massive increase in the number of granulocytes in the sinuses. At 4 hr this was so intense that the sinuses were almost filled. Most of these cells were neutrophils but eosinophils were not uncommon. Granulocytes were usually unlabeled and occasionally showed light labeling in small vesicles (Fig. 11); their relative absence of labeling was in striking contrast to the intense labeling of macrophages at this time point, and strongly suggested that the granulocytes were attracted to the region after the bulk of antigen had already been trapped by macrophages.

The intense plasmacytopoiesis, noted at 3 days, reached its height at 5 days.

Though extensive in the primary response, it was even more intense in the secondary. The cords increased considerably in thickness and confluent sheets of blasts, immature and mature plasma cells could frequently be seen. Labeled macrophages and their broad cell processes were scattered through the cords, but we could discover no evidence of a special anatomical relationship between them and the plasma cells or their precursors. No cytoplasmic bridges were found, nor was any rosette pattern of plasma cells around macrophages noted. In fact, the total absence of labeled macrophages in many areas crowded with plasma cells was a salient feature. Occasionally, degenerating plasma cells were seen and these appeared soon to be engulfed by macrophages. However, cell death was not nearly as prominent a feature as in germinal centers, and no cells similar to the tingible body macrophages of germinal centers were seen in the medulla.

Recently, Roberts (29) reported greatly increased numbers of mast cells in mouse lymph nodes after antigenic stimulation. Our unstimulated rats always had considerable numbers of mast cells in the medulla and no clear-cut increase followed antigenic stimulation. In some sections, a small percentage of the mast cells showed a marked heterogeneity of staining reaction to uranyl acetate amongst individual granules. This may have represented an early stage of degeneration, as occasionally ruptured mast cells were encountered. Mast cells were not labeled in our experiments.

Question of Antigen Entry into Plasma Cells.—Occasional primitive lymphoid cells and plasma cells in the medulla showed single silver grains over the nucleus or cytoplasm (Fig. 14). However, we are reluctant to ascribe significance to this observation. These scattered grains always appeared in areas close to heavily-labeled macrophages and we know that the more energetic emissions of ^{125}I can travel considerable distances. Moreover, it is always possible that the knife edge might have dragged labeled molecules some distance from their true in vivo location. Plasma cell areas remote from sinuses and free of heavily labeled macrophages showed no labeling. In all, no evidence for antigen entry into plasma cells in the medulla was obtained.

DISCUSSION

In recent years, the function of macrophages has been intensively investigated. Broadly speaking, there have been two major areas of study. On the one hand, immunologists have attempted to uncover the role of macrophages in immune responses with special reference to the production of RNA fractions effective in the induction of antibody formation (30–32). On the other hand, cell biologists have addressed themselves to the details of the phagocytic event itself, from both ultrastructural (10–13) and biochemical (11, 33) viewpoints. The current paper is an attempt to bring these two universes somewhat closer together. Electron microscopic radioautography has been used

to study the capture and processing of a small colloidal particle by macrophages situated in an antibody-forming organ. The particle used was ^{125}I-labeled *Salmonella adelaide* flagella, highly immunogenic in the dose of 20 μg. Moreover, the specific activities of labeling achieved were such as to ensure heavy silver grain development over macrophages with relatively short exposure periods. The experimental design allowed us to make parallel observations on antigen distribution and antigen-induced cellular and proliferative changes in the node.

In order to set limits on the usefulness of both positive and negative observations, we should consider the sensitivity of the present technique. Let us regard one unit of monomeric flagellin (mol wt 38,000) effectively as one molecule of antigen, and let us take a ^{125}I substitution rate of one atom per molecule as typical for the present experiments. Moreover, let us assume for the moment that deiodination of antigen does not occur inside the node. We have estimated (31) that one disintegration of ^{125}I may produce about 0.04 grains in an electron microscopic radioautograph using NTE emulsion and recent work with ^{3}H (34) suggests that this is a reasonable first approximation. Bearing in mind that some time elapses between the injection of the isotope and the dipping of the ultrathin section in radioautographic emulsion, we can see that with an exposure period of somewhat over one half-life, a single silver grain denotes the presence of about 50 molecules of antigen in the underlying section. If a cell is a sphere of 10 μ diameter, a section of 800 A thickness taken though its center will have a volume about $\frac{1}{80}$ the volume of the whole cell. Thus, if one silver grain is noted over a cell section and if antigen had been uniformly spread throughout the whole cell, its total antigen content would have been about 4000 molecules. Naturally, most of the above assumptions involve gross over-simplifications, but is is important to point out that this technique is more than two orders of magnitude less sensitive than when light microscopic radioautographs of smears of whole cells were studied (5, 35, 36). Another factor limiting the sensitivity of the present method is the fact that little significance can be attached to single silver grains over cells close to heavily-labeled macrophages. Such grains could have arisen through the emergence from the section of more energetic electrons taking an oblique track or through displacement of labeled molecules by the knife edge. Thus, less meaning attaches to the *absence* of labeling in certain cell types in this study than in our previous work (5, 36). Nonetheless, we would like to stress the fact that following the injection of only 20 μg of labeled antigen into the foot-pad, radioautographs showing many hundreds of grains over cells from the draining lymph node could be prepared. Thus the technique cannot be regarded as totally lacking in sensitivity. Moreover, though it compares unfavorably with light microscopic radioautography, particularly of cell smears, in sensitivity, the present technique has the great advantage of much better resolution.

Despite the absence of histochemical data on our preparations, we believe this study supports the almost identical concepts of lysosome formation recently proposed by Gordon, Miller, and Bensch (12), and by Hirsch and Cohn (11). In their work, the typical dense granules, diameter about 500 mμ and rich in acid hydrolases, are believed to result from the fusion of certain Golgi vesicles with pinocytic vacu-

oles. The Golgi vesicles themselves contain hydrolytic enzymes and may represent the original "protolysosomes." In our work, fusion of tiny vesicles with antigen-containing vacuoles was an early and prominent feature. Further, the vacuoles fused extensively with each other, forming "phagolysosomes." The preservation, inside such phagolysosomes, of dense granules resembling in ultrastructure the "telolysosomes" of Gordon et al. (12) may have represented an attempt by the macrophage to conserve hydrolases for subsequent use. In fact, the flagellar antigen appears to be relatively resistant to digestion, as evidenced by the prolonged retention of label in phagolysosomes and the unchanged appearance of these bodies between 2 and 6 wk after antigen injection. The following further differences between our studies and the previous ones are worth mentioning: (*a*) the pace of events seemed to be quicker in our in vivo work, with extensive vacuolar fusion present already 30 min after antigen injection; (*b*) migration of inclusions to the Golgi region was not noted; in fact, frequently, labeled inclusions were scattered right throughout the cytoplasm of the cell, and late phagolysosomes were often so large that a single inclusion virtually filled the cell; and (*c*) autophagic vacuoles containing mitochondria or other organelles were very infrequent in medullary macrophages.

While much of our antigen entered macrophages by pinocytosis, a substantial fraction appears to have entered by direct penetration of the plasma membrane, and to have enjoyed a brief period of lying free in the cytoplasm. A similar phenomenon has been noted by Moore, Mumaw, and Schoenberg (37), and by Florey (13) for other colloidal particles. However, such freely-scattered antigen was incorporated into membrane-bounded inclusions, probably within minutes. Possibly, Golgi vesicles were attracted to the sites of deposition and coalesced to form an antigen-containing lysosome. This aspect seems a promising area for further study using a strong, low molecular weight antigen such as soluble flagellin.

While a morphologic study can clearly give only limited insights into biochemical problems, we believe our results create some difficulties for proponents of the view that macrophages synthesize RNA molecules with inductive properties (30, 31). If we have correctly interpreted the sequence of events occurring in macrophages, then antigens are rapidly sequestered from the rest of the cytoplasm and subjected to digestive enzymes. In many studies of the role of macrophage RNA in antibody synthesis particles larger than our flagella, e.g. phages or red cells, have been used as antigens. Thus, most likely the period of residence of such antigens free in the cytoplasm would have been very brief or absent.

We have obtained no evidence for the exit of labeled antigen fragments from lysosomes, or for the entry of antigen into either the macrophage nucleus or into surrounding lymphocytes or plasma cells. Admittedly, such processes could have been occurring to an extent undetectable by present techniques. The following questions are thus raised: Is it likely that antigen-induced messenger RNA or RNA-antigen complexes are formed in lysosomes? If not, where are they formed? If they are formed, how do they leave the lysosome and reach

the antigen-sensitive precursor of antibody-forming cells? We have discovered no special or favored anatomical relationship between macrophages and primitive lymphoid cells, nor any cytoplasmic bridges between them. In fact, many sections between 3 and 5 days after antigenic stimulation showed confluent sheets of unlabeled primitive lymphoid and plasma cells with a striking absence of labeled macrophages. These remarks are not intended to disparage the role of the macrophage in induction, but rather to point out some of the important problems which our study has raised.

The results have not shed much new light on the role of eosinophils, neutrophils, or mast cells in the immune response. Recent studies using hapteneprotein conjugates as antigens have found extensive antigen localization in eosinophils (7, 29). We have occasionally seen some label over tiny vesicles in eosinophils, but these and also the invading neutrophil polymorphonuclear leukocytes were always much less heavily labeled than macrophages. The characteristic eosinophil granule and the mast cell granules were never labeled in our study.

Since we first drew attention to the fact that antibody-forming cells need not contain detectable antigen (38), this finding has been confirmed several times (5–7, 39). Again, in the present ultrastructural study no evidence for passage of labeled antigen into plasma cells was obtained but this finding is not a very telling one as it would have taken some thousands of molecules of antigen per cell to have caused convincing plasma cell labeling.

There exist in antibody-forming organs at least two other types of cells involved in antigen trapping. Their structure and function is discussed in the accompanying paper (40).

SUMMARY

Details of antigen trapping and processing in the rat lymph node have been investigated by the technique of high resolution radioautography. A series of 24 adult rats was injected with 20 μg of ^{125}I-labeled *Salmonella adelaide* flagella, given as either a primary or a secondary stimulus into one hind foot-pad. At intervals ranging from 3 min to 3 wk, rats were killed and the popliteal nodes were processed for electron microscopic radioautography using Kodak NTE emulsion. The present paper deals with events in the lymph node medulla, and an accompanying report describes the radically different behavior of antigen in the cortical follicles.

In the medulla, lightly labeled granulocytes were transiently encountered, but by far the greatest bulk of antigen was in macrophages. Antigen entered these cells in two ways: by direct penetration of the plasma membrane; and by pinocytosis. In either case, the antigen rapidly became surrounded by tiny vesicles which may have represented Golgi-derived "protolysosomes." Vacuolar fusion ensued and a series of progressively larger and more complex antigen-

containing "phagolysosomes" was formed. Substantial amounts of antigen could be detected in such bodies for at least 3 wk.

The antigen injection, as expected, caused extensive plasma-cytopoiesis. No evidence of label in plasma cells was obtained. No special anatomic relationship between plasma cells and antigen depot sites was discovered. These results are briefly discussed in relation to current theories of immune induction.

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EXPLANATION OF PLATES

Fixation is by osmium tetroxide unless otherwise stated.

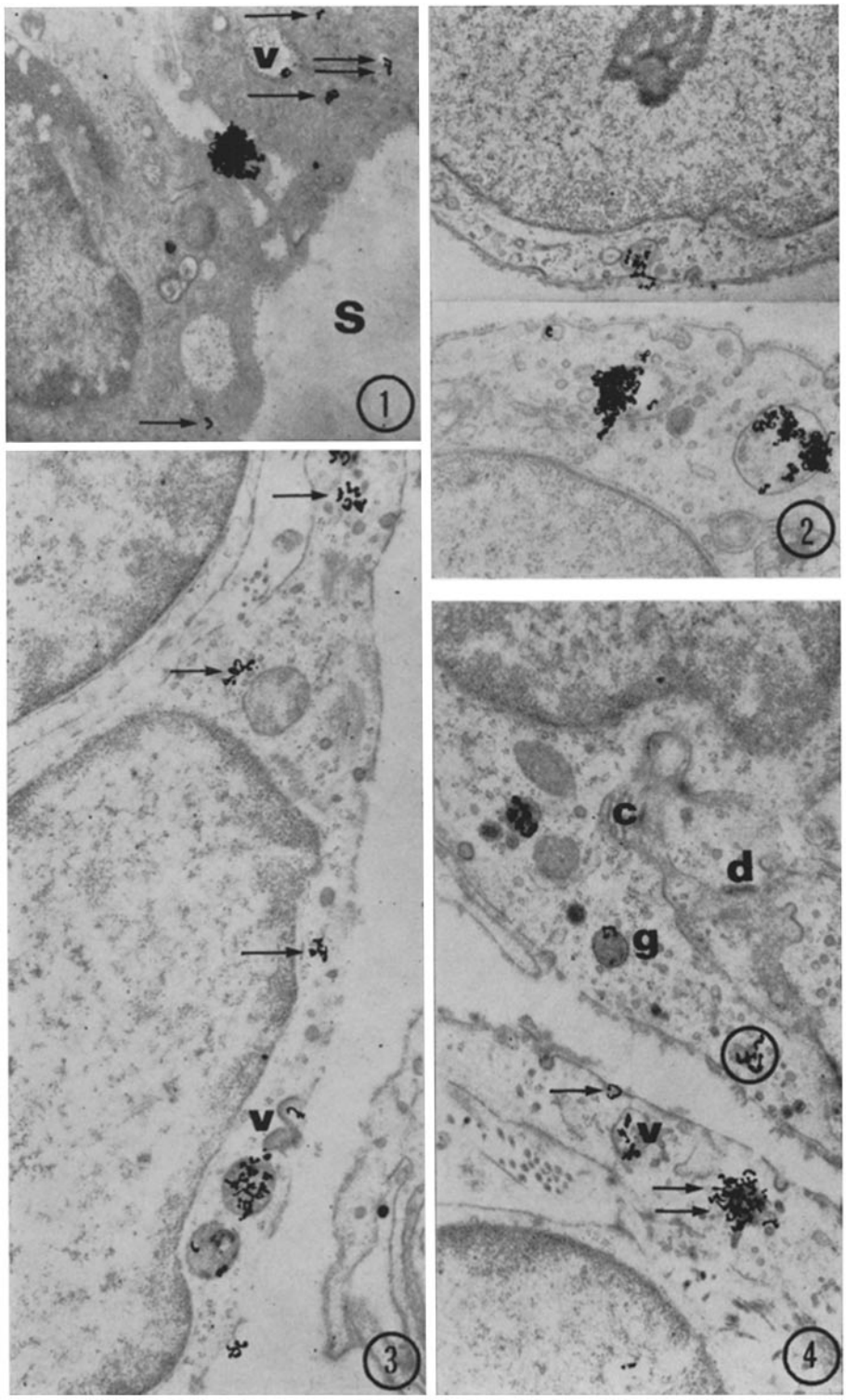
PLATE 37

FIG. 1. 10 min after antigen injection; primary response. Portions of two medullary macrophages; note the scattered free silver grains (single arrows), the vesicle with the somewhat beaded lining (*v*), and the micropinocytic vesicle (double arrow). The beaded nature of the electron-opaque coating on the outside of the plasma membrane is well shown. A large clump of label is also present, obscuring underlying detail. *S* indicates sinus. Glutaraldehyde fixation; × 12,350.

FIG. 2. 30 min after antigen; primary response. Portions of two littoral macrophages in the medullary sinus; the electron-opaque material is again evident on the luminal aspect of the cell membranes and that coating on the border of the upper cell has associated label in close proximity to the point where a labeled pinocytic vacuole has apparently just separated from the surface. This cell displays typical nuclear chromatin pattern and the well defined nucleolus is a not uncommon feature. In the cytoplasm of the lower cell numerous pinocytic vesicles are shown in progressive stages of formation; one of these containing a single grain has recently detached from the cell surface. The larger labeled vesicles have both marginal and diffuse moderate electron-opacity. Well developed Golgi membranes can be seen in the paranuclear region at lower left. × 15,600.

FIG. 3. 30 min after antigen injection; primary response. Electron-opaque pinocytic vesicles are forming (*v*); many clumps of grains are apparently free in the cytoplasm (arrows). Some of these may represent antigen in minute vacuoles but it is not likely that all do. Note the broad cell process extending to upper right. × 16,250.

FIG. 4. 30 min after antigen; secondary response. Some of the antigen is still free in the cytoplasm (circle); some is in a pinocytic vacuole (*v*); some is in electron-opaque membrane-bound inclusions which we will term dense granules (*g*). The single arrow points to an area where antigen may be passing directly through the plasma membrane. The double arrow points to an area where small vesicles and electron-opaque material appear to be surrounding what may originally have been free antigen. The macrophage cytoplasm surrounds collagen fibrils (*c*) and a number of zonulae adherens (*d*) are evident. × 16,250.



(Nossal et al.: Antigen capture by macrophages)

PLATE 38

FIG. 5. 30 min after antigen; secondary response. A heavily labeled area showing many similar features to Fig. 4. This is included chiefly to show how heavy the labeling in the medulla can be following the injection of only 20 μg of labeled antigen. Encloistered collagen fibres (*c*) are shown at mid-right and the adhesion plates (*d*) at the conterminal surfaces of the immurant membranes are well demonstrated. × 19,500.

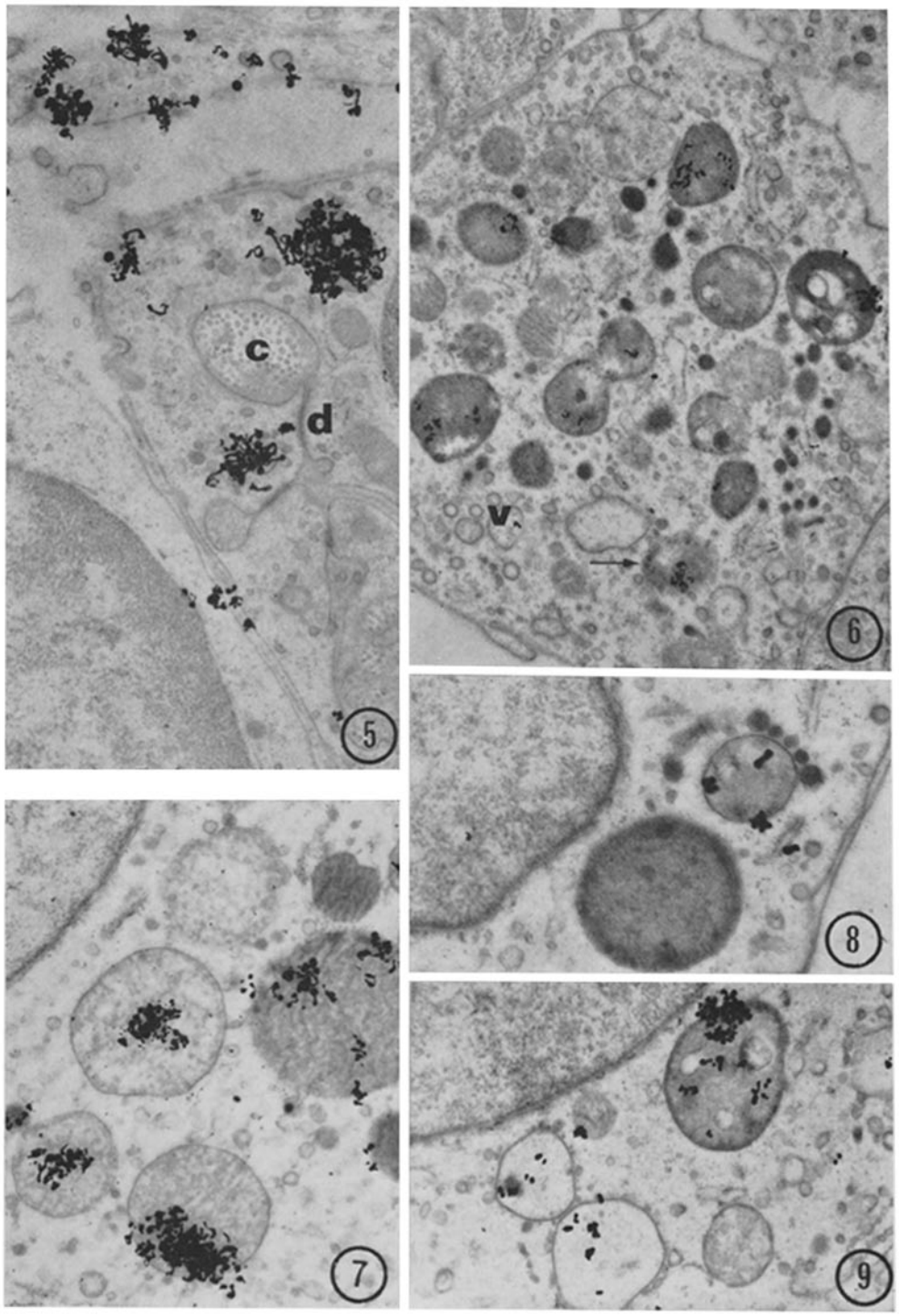
FIG. 6. 30 min after antigen injection; secondary response. This portion of macrophage cytoplasm shows labeled dense granules rather larger than those seen in Figs. 4 and 5. There is some apparent fusion. The arrow points to a particularly interesting area where the confluence of tiny vesicles appears to be leading to the actual formation of a dense granule around what may have been free antigen. The membrane of the vesicle (*v*) appears incomplete. Is this a fixation artifact or is membrane actually being built around the antigen to sequester it? × 13,650.

FIGS. 7–9. Examples of fusion of tiny vesicles and small dense bodies with antigen-containing vacuoles to form a labeled inclusion of increasing electron opacity.

FIG. 7. 30 min after antigen. Labeled granules of varied electron opacity in the paranuclear region. There are numerous small vesicles in the locality. × 16,250.

FIG. 8. 30 min after antigen. Note the spatial relationship of the labeled inclusion and the impendent array of small electron-opaque granules. × 24,375.

FIG. 9. 4 hr after antigen. Numerous small vesicles are seen in peripheral location with relation to labeled electron-lucent vacuoles. × 16,250.



(Nossal et al.: Antigen capture by macrophages)

PLATE 39

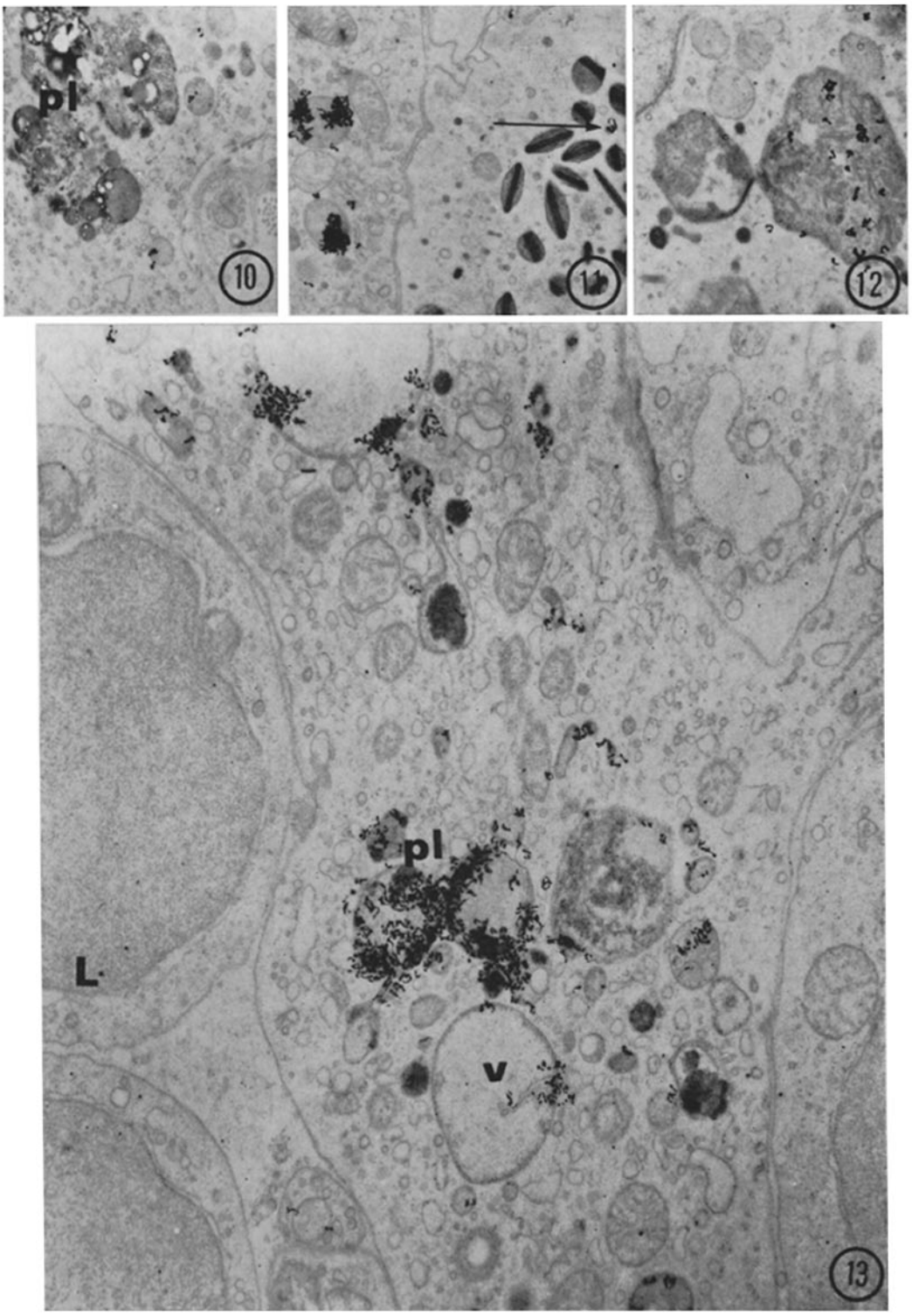
FIG. 10. Portion of a macrophage 30 min after the injection of labeled antigen into a rat that had been immunized 6 wk previously with unlabeled flagella. Note two complex inclusions that are unlabeled and which probably represent phagolysosomes (*pl*) formed after the first antigen pulse; and two small labeled inclusions formed after the recent antigen injection. × 10,400.

FIG. 11. Lymph node medulla, 4 hr after antigen injection; primary response. Labeled dense granules are present in the macrophage at left. Portion of an eosinophil is shown on the right and one small vesicle (arrow) is labeled. The characteristic eosinophilic granules are not labeled. Neither eosinophil nor neutrophil polymorphonuclear leukocytes ever showed intense labeling in this study. × 11,375.

FIG. 12–13. Examples of coalescence of antigen-containing inclusions resulting in the formation of complex structures of variegated appearance, tentatively termed phagolysosomes.

FIG. 12. 3 days after antigen; primary response. Paranuclear region. × 13,000.

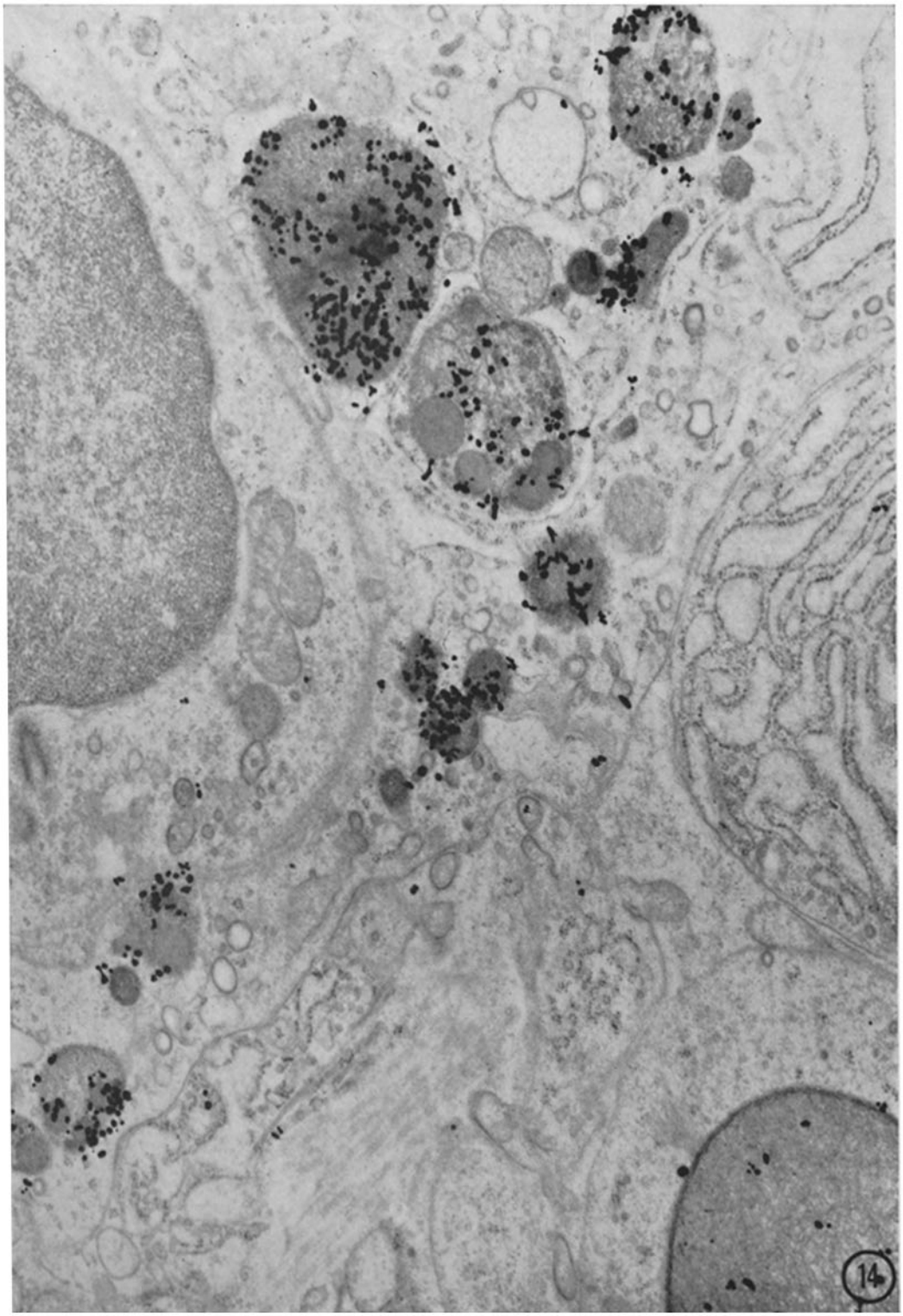
FIG. 13. A broad macrophage process; 1 day primary response. The vacuoles (*v*) contain an electron-opaque layer just inside their limiting membrane; most of the antigen is attached to or close to the wall. In the region marked *pl*, we appear to be watching the imminent fusion of at least eight inclusions, including two large ones, into a phagolysosome. Note that discrete, rounded electron-opaque entities are still visible within the larger vacuoles. The cytoplasm also includes numerous smaller labeled inclusions of varying sizes and degrees of electron opacity. The appearance is most characteristic of the 1-day time point. *L*, lymphocytes. × 16,250.



(Nossal et al.: Antigen capture by macrophages)

PLATE 40

FIGS. 14. 3 days after antigen; secondary response. The broad process of a heavily labeled macrophage protrudes between a plasma cell at right and a lymphocyte with distinct centriole at left, and continues on to bottom left. At bottom right is portion of another macrophage with a somewhat unusual inclusion. Note the varied size, shape and ultrastructural features of the labeled inclusions. A few single grains are also present, including one in the plasma cells; the significance of these is discussed in the text. × 21,125.

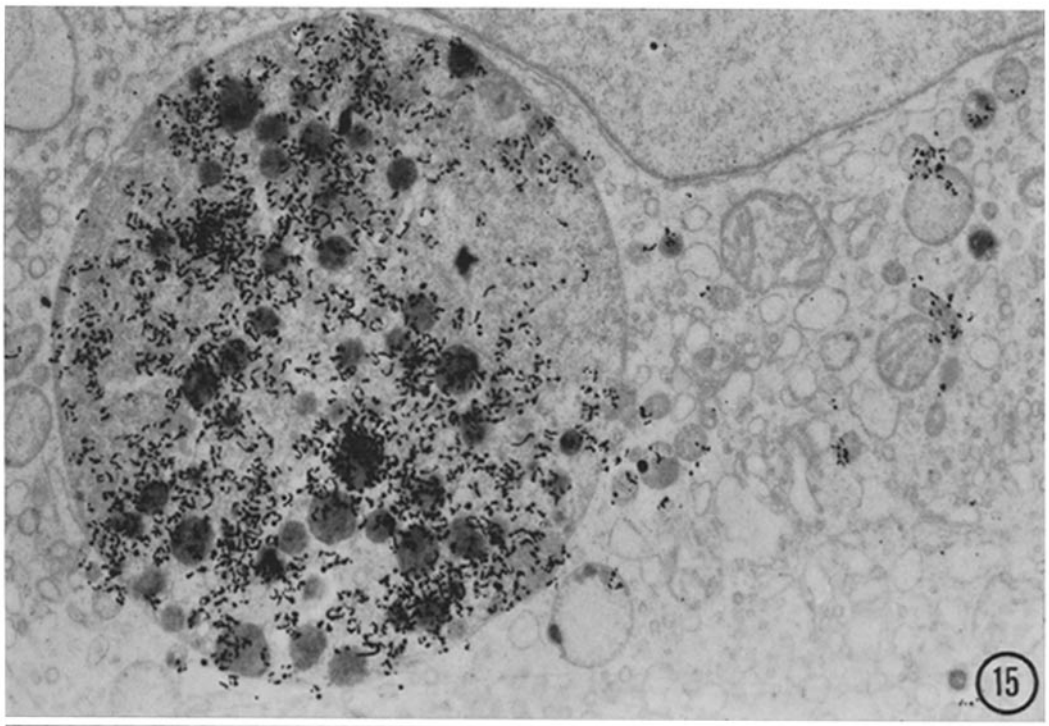


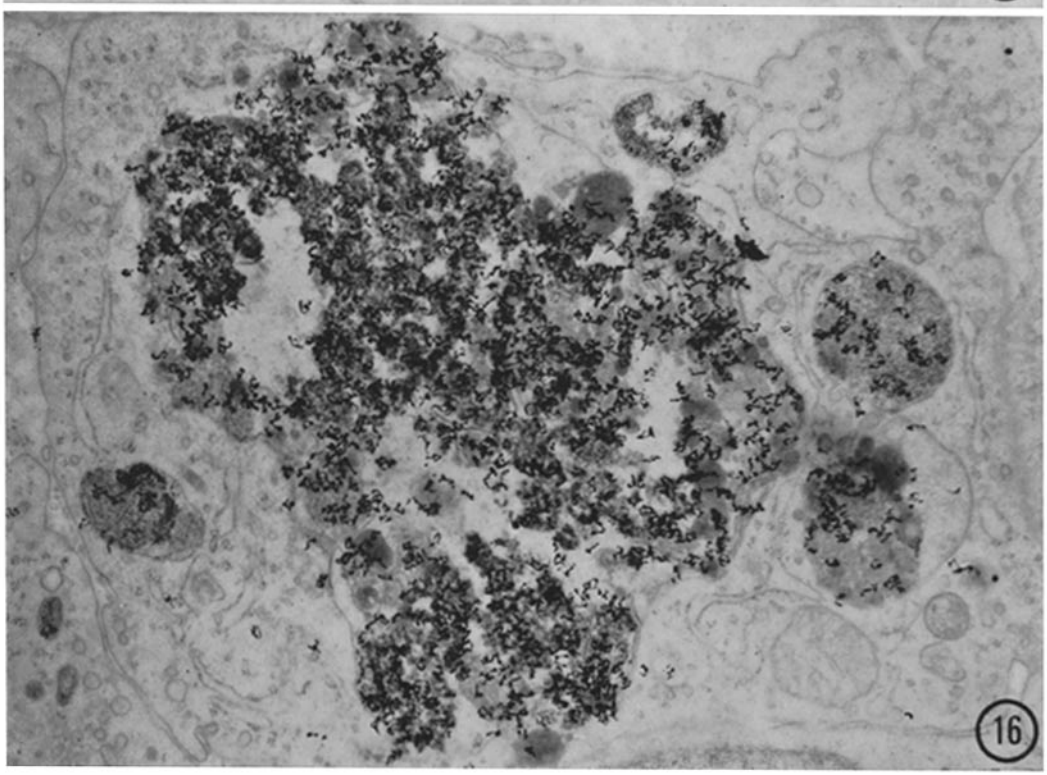
(Nossal et al.: Antigen capture by macrophages)

PLATE 41

FIG. 15. A large phagolysosome, 1 day after antigen; primary response. Though the vacuole is atypically large for this time point, several features differentiate it from the "late" phagolysosome (Fig. 16). These include the fact that dense granules within the big inclusion are so well preserved and that they contain a large proportion of the label and also the relatively homogeneous appearance of the rest of the vacuole. Note that further small labeled inclusions are still present. They may be about to fuse with the big vacuole. × 19,500.

FIG. 16. Typical appearance of macrophage 2 wk after antigen injection; primary response. A large and very complex inclusion, almost equal in size to the nucleus of the cell, is present. Inside the inclusion, some discrete dense granules are still present together with much inhomogeneous electron-opaque material the general appearance of which is a characteristic hallmark of these late phagolysosomes. × 17,225.



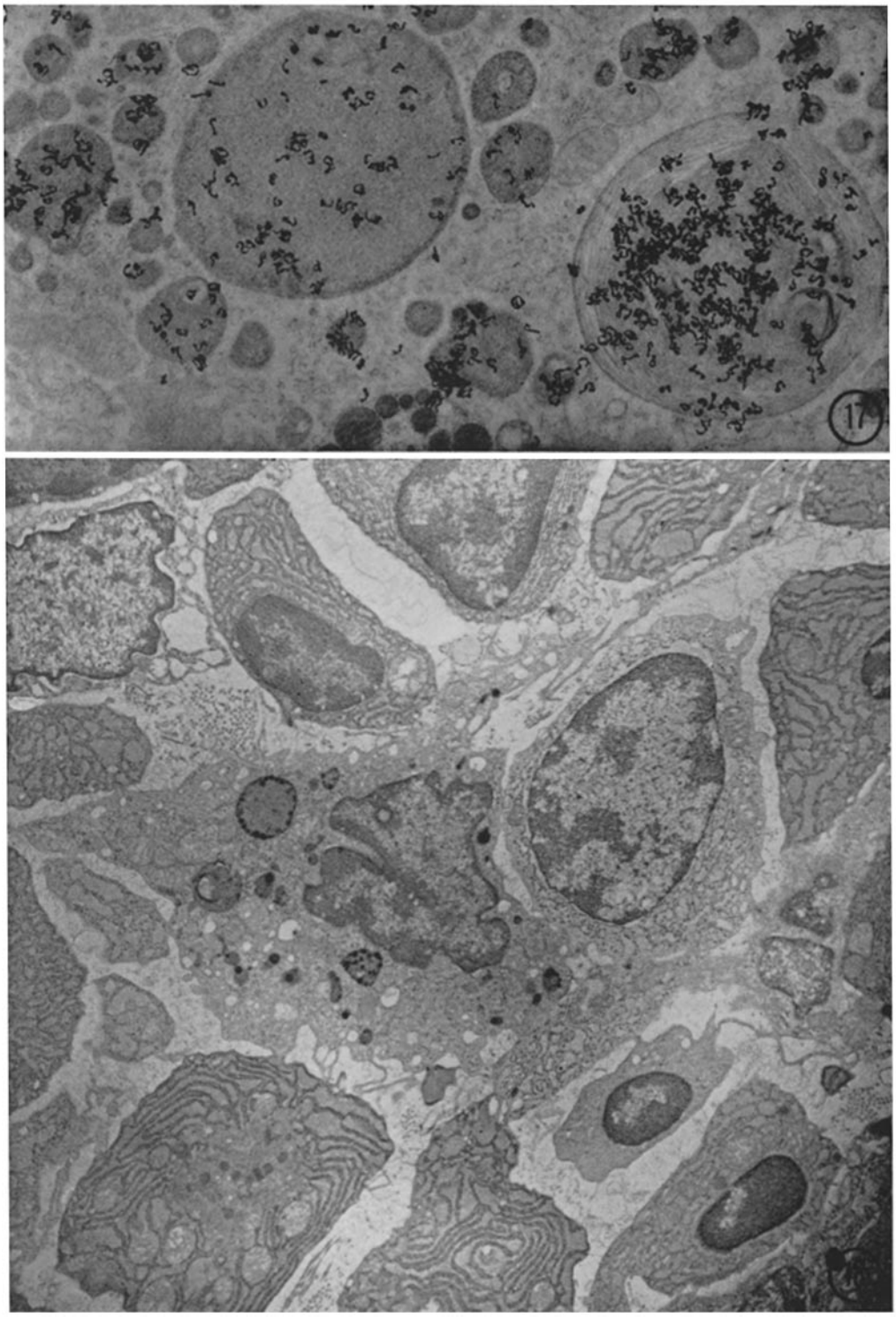


(Nossal et al.: Antigen capture by macrophages)

PLATE 42

FIG. 17. 1 day after antigen; secondary response. At least five of the labeled inclusions on the left show myelinic configurations. This type of granule is infrequently seen. × 19,500.

FIG. 18. 1 day after antigen; secondary response. A macrophage with a few lightly labeled inclusions is seen centrally located among many unlabeled plasma cells in the lymph node medulla. These plasma cells look much too mature to be related to the recent antigen injection. More likely, they were evoked by the primary dose of unlabeled antigen. Many sheets of plasma cells could be seen in both primary and secondary responses without macrophages in their immediate vicinity. This picture seen in isolation might support the concept of rosettes of plasma cells developing around antigen-containing macrophages. Review of all the material, however, does not favor this interpretation. × 7150.



(Nossal et al.: Antigen capture by macrophages)