EXPERIMENTAL INOCULATION OF HUMAN LEPROSY IN LABORATORY ANIMALS[1, 2]

II. ELECTRON MICROSCOPE STUDY

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In the preceding article of this series ([5]) we reported transmission to the golden hamster of a mycobacterium obtained from borderline leprosy patients, transfer of the lesion produced in that animal through several passages, and failure to obtain growth of that mycobacterium in any culture medium suitable for mycobacteria generally. Undoubtedly, this bacillus is one of the uncultivable pathogenic mycobacteria, such as *M. leprae* or *M. leprae murium*, and it was suggested that the infection in the hamster might be caused by the former of those microorganisms. The present investigation comprises studies with the electron microscope of the hamster lesion which shows, both in the bacilli and in its environment, structures characteristic of *M. leprae*.

In recent years, electron microscope studies applied in the field of leprosy research have elucidated submicroscopic features of both *M. leprae* and various tissue reactions in the polar types of leprosy ([1, 2, 8, 9, 10, 17, 18, 21]). These studies have revealed especially the bacterial environments characteristic of leprosy lesions. Therefore, to determine the type of the bacillus transmitted to the hamster from leprosy patients, electron microscopic examination of animal lesions caused by that microorganism is of great interest.

The fine structures of both the bacillus and its environment in the passaged animal lesions, as compared with human and murine leprosy lesions, are reported in this paper.

MATERIAL AND METHOD

The materials used were the same as those described in our previous report. They were: the first passage lesion after 8 months, those of subsequent passages after 3-4 months, and year-old lesions of the second passage in hamsters, as well as a 3-months lesion developed in a white mouse with material of the third passage in the hamster. The fifth passage in the hamster was the last one examined in this study.

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Biopsy specimens were taken from the ear lesions without local anesthesia. The tissue chips were fixed with one per cent osmium tetroxide buffered with 2,4,6,-collidine (0.2 M) at pH 7.3 for 5 hours ([7]), then dehydrated with acetone and embedded in a mixture of methyl and butyl methacrylate. Ultra-thin sections were made with a Fernández-Morán-type Leitz ultramicrotome equipped with a diamond knife, and they were examined with the Siemens Elmiskop I and Hitachi HS-6 electron microscopes. The sections were stained with uranyl-acetate solution before electron microscopy ([19]).

RESULTS

First passage in the hamster (8 months).—This lesion is mainly composed of many cells containing bacilli. Their cytoplasm contains various organelles which show no noteworthy alterations. Rough-surfaced endoplasmic reticulum is abundantly distributed, together with small vesicles. Many bacilli are observed among the cytoplasmic organelles (Figs. 1-4). The majority of the bacilli are accumulated as groups, each enclosed by a membrane. A small group of the bacilli is sometimes surrounded by moderately-dense substance, that being separated from the bacilli by a narrow transparent zone (Fig. 3). Infrequently, a foamy structure is visible around the bacilli (Fig. 4). When the bacilli form a large agglomeration, however, neither moderately-dense substance nor foamy structure is visible. Rupture of the cell membranes of host cells is occasionally observed in such a case; thus, the large agglomeration of bacilli is exposed to the extracellular environment (Fig. 2).

Most of the bacilli, especially those forming agglomerations, appear to be electron-transparent but contain electron-dense fragments, corresponding to the debris of bacterial cytoplasmic components. This feature may represent bacterial degeneration. However, the bacilli in the small groups are laden with the cytoplasm and possess the complex membranous configuration described later. Since the details of the bacilli are the same as seen in subsequent passage materials, they will be described in a later paragraph.

Membrane-limited vacuoles with fine granular matrix are visible in the host-cell cytoplasm, and contain an intensely dense substance varying in size and in shape (Figs. 1, 3 and 4). These vacuoles may represent the disintegration of phatocytosed melanin. Sometimes bacilli surrounded by moderately-dense droplets are found in these vacuoles.

In the cutaneous nerves of this material, no pathologic changes of Schwann cells, axons or myelin sheath are observed. The bacillus is not contained in either the Schwann cells or the axons.

Second passage in the hamster (3 months).—The material examined 3 months after transmission from the first-passage animal shows the lesion to consist of bacillated cells similar to those of the first passage. Frequently multinuclear cells containing the bacilli are also observed in this lesion (Fig. 8).

The bacilli are dispersed abundantly in the cytoplasm as single in-

dividuals or as small groups, always enclosed by a membrane. However, they are not so closely aggregated as those seen in the first passage, but are separated from each other in the same enclosing membrane. Very small amounts of a moderately-dense droplet cling to the bacilli.

The majority of the bacilli are laden with the cytoplasmic components. A moderately-dense cell wall approximately 10 mμ in thickness covers the bacillary body. Outside the cell wall, a moderately dense diffuse layer separated from the former by a low density space is discernible (Fig. 5). As observed on the surface of *M. leprae* ([10]), the low-density space may represent the coating substance covering the cell wall. The outermost diffuse layer is probably a substance absorbed on the coating substance.

A dense layer 30 Å thick is closely adjacent to the inner surface of the cell wall. Separated from this dense layer by a less dense space 30 Å in thickness, a dense layer 30 Å thick borders on the bacillary cytoplasm. These two dense layers may represent the plasma membrane of the bacillus. In other words, the plasma membrane consists of a double dense layer separated by the low density space.

In some regions, the plasma membrane enters the bacterial cytoplasm and is transformed into a complex beehive-like configuration (Fig. 5). This configuration corresponds to the "intracytoplasmic membrane system" observed in some mycobacteria ([10, 15]).

A homogeneously dense substance not delimited by any membrane, believed to be a polyphosphate body, is seen in the bacterial cytoplasm (Fig. 5). A dense, irregularly-shaped substance, representing the nuclear apparatus, is also observed, and is frequently revealed in the transparent area. It is surmised that this appearance of the nuclear substance may be caused by shrinkage during methacrylate polymerization.

Two bacilli flattened end to end are seen in Fig. 10. The cytoplasm of each is delimited by a membranous layer. In the study of cultivable mycobacteria, such a picture appears in the last stage of cell division. In consequence, this feature is believed to represent bacterial cell division occurring in a host cell.

In the cytoplasm of the host cells, large electron-transparent vacuoles occasionally contain dense fragments surrounded by a dense membrane (Figs. 6 and 7). Sometimes this dense membrane appears to be discontinuous. Since the dimension of this dense membrane conforms to that of bacterial cell wall, and also since membrane-enclosed fragments display an appearance similar to that of the debris of cytoplas mic components of the bacilli, it is reasonable to believe that these features may represent the disintegrating process of degenerated bacilli.

Second passage in the hamster (1 year).—There is no morphologic difference between the host cells of the 1-year material and those of the 3 months lesion. However, the bacilli within the cells are more abundant

than in the earlier stages of this passage. Furthermore, the bacilli showing fragmented cytoplasm (i.e., degenerating bacilli), are numerous (Fig. 9). Usually the bacilli are entirely wrapped in moderately-dense droplets, which are comparable to the "opaque droplets" observed in lepra cells of the human leprosy lesion ([8, 9, 18, 21]). Of course, the enclosed space containing the bacilli with small amounts of the moderately-dense droplet (Figs. 9, 11 and 12) is also found in this material. On occasion, the droplet containing denser droplets, believed to be of lipid nature, is attached to the outer surface of the enclosing membrane (Fig. 9).

It should be noted that a low-density space is always discernible between the moderately-dense droplet and the cell wall (Fig. 12). This means that the droplet cannot be attached directly to the cell wall, because of the presence of the coating substance of low density.

Several nerve twigs, composed of both myelinated and nonmyelinated fibers, are found among the bacillate cells (Fig. 14). Perineurial cells poor in cytoplasmic components enclose these nerve fibers and usually form a single lamellar layer. Infrequently, the Schwann cell cytoplasm contains many transparent vacuoles, but cytoplasmic organelles are very scarce. No bacilli are evident in these nerve elements, although the nerves are surrounded by the cells containing many bacilli.

Third, fourth and fifth passages in hamsters (3-4 months).—The lesions of these passages show an appearance similar to that of the earlier passages. No difference in the submicroscopic structures of either the bacilli or the host cells is observed.

Mouse lesion (3 months).—This lesion was provoked by the bacilli from the third passage in the hamster (Fig. 13). The distribution of the bacilli in the host cells is not different from that of the hamster lesion.

The bacilli are accompanied by various amounts of moderately-dense droplets, and each is separated from the cytoplasm of the host cell by a membrane. This finding is very important in considering the identification of the bacillus inoculated in the mouse, especially in distinguishing *M. leprae* from *M. leprae murium*, as described later.

DISCUSSION

The bacillus.—The cell surface of the passaged bacillus is composed of the outermost diffuse layer, the low-density coating substance, and the less-dense cell wall. The bacterial plasma membrane consists of a double dense layer, and its outer layer adheres closely to the inner surface of the cell wall. This double plasma membrane enters the bacterial cytoplasm and forms a complex configuration, which is believed to be a bacterial organelle because bacterial enzymes are generally located in the plasma membrane ([16]).

The submicroscopic structures of *M. leprae*, reported by several au-

thors ([2, 21]) and recently described most clearly by Imaeda and Convit ([10]), such as the bacterial surface, the plasma membrane and the complex membranous configuration (the latter called the "intracytoplasmic membrane system"), are quite similar to those of that bacillus as found in animal lesions. However, the fine structure of *M. leprae murium* ([3]), which is also one of the noncultivable mycobacteria, shows a close similarity to the bacillus transmitted to animals. This being so, one cannot discuss the identification of the transmitted bacillus from its morphologic appearance.

It should be mentioned that the majority of the bacilli forming agglomerations in the first passage contain fragmented cytoplasm. In subsequent passages, the bacilli laden with their cytoplasm are scattered as small groups in the host cells and do not accumulate as agglomerations. In the study of leprosy, it is substantiated that *M. leprae* grows very rapidly and is clustered as "globi" in the lepromatous and some borderline and reactional tuberculoid cases ([1, 8, 9, 18, 21]). In addition, clumped bacilli generally show the so-called "cytoplasmic condensation" (plasmolysis in the bacteriologic sense), which is a sign of bacterial degeneration. Thus, there is an intimate relationship between globus-formation and bacterial degeneration; the bacillus may degenerate soon after the globus is formed as a result of rapid growth. The fact that in most borderline cases, and also in peripheral nerve elements of various leprosy cases, the majority of the bacilli are laden with cytoplasmic components suggests that these bacilli may actively subsist and not quickly degenerate ([10, 12]). These morphological differences of *M. leprae* in various lesions can best be explained as different phases of the multiplication of the bacillus. Of course, the bacterial growth depends closely on the type of leprosy.

This opinion regarding the multiplication of *M. leprae* can be adduced to explain various features shown by the bacilli inoculated in animals. The multiplication phase of the transmitted bacillus may be dissimilar in the first and the subsequent passages. The bacillus may multiply rapidly in the first passage, forming agglomerations, and then rapidly degenerate, as seen in lepromatous leprosy lesions. In subsequent passages, the evidence that the nodule formation in animals occurs within three months is indicative of the fact that the bacillus may positively multiply in animal tissues. However, they are never clumped as agglomerations, nor do they degenerate quickly. Since the tissue response of the kind of animal used for the present study may not be different in different individuals, the individual animal tissue may not influence bacterial growth. Therefore, the change of growth rates in subsequent passages is probably due to adaptation or mutation of the bacillus transmitted in animals.

Bacterial environment.—In animal lesions, a single bacillus or a small group of bacilli are usually enclosed by a membrane, supposedly

a remnant of the cell membrane taken into the cytoplasm of the host cell during phagocytosis, and they are thus separated from the host-cell cytoplasm. Frequently a small amount of the moderately-dense droplet material is closely attached to the bacillus in the enclosed space, even in the mouse lesion. These bacterial distributions are all like those seen in a borderline leprosy lesion by Imaeda and Convit ([10]). Murine leprosy lesions also show similar appearances ([3, 22]), but they do not contain any moderately-dense droplets. From this feature, the possibility of *M. leprae murium* infection in the present study can be excluded.

Moderately-dense droplets entirely enclosing the bacillus in the 1-year lesion resemble closely the "opaque droplets" observed in lepra cells of human leprosy lesions. Concerning the occurrence of the opaque droplet in the lepra cell of lepromatous leprosy, Imaeda ([8]) postulated that pinocytotic lipoprotein droplets accumulate around the bacilli as a result of the disturbance of the lipid metabolism in that form of the disease. In the transmitted animal lesions, moderately-dense droplets, presumably of lipoprotein origin, cling to the enclosing membrane as seen in Fig. 9, showing that the pinocytotic droplet attaches to the enclosing membrane, passing through it and accumulating around the bacilli. In addition, the fact that the quantity of these droplets increases in older lesions implies that the accumulation of droplets occurs very gradually, as in the human leprosy lesion. This correlation between the moderately-dense droplet and the bacillus in animal lesions suggests that the transmitted bacillus may correspond to *M. leprae;* but this phenomenon is not shown exclusively by *M. leprae,* since *Salmonella enteritidis* is found to be surrounded by a similar droplet, as reported by Yamamoto and Nakano ([20]).

Besides the droplets, a foamy structure identical with that of lepra cells appears around the bacilli. This foamy structure, however, is evident only in the first passage and not in any of the later passages, even in the 1-year lesion. It is presumed that the foamy structure in the first passage might have been contained in the inoculum taken from the patient, and simply phagocytosed by histiocytic cells in animals. As already discussed, bacterial multiplication in later passages may be different from that of the first passage, resulting from mutation during animal transmission. According to Clifton ([4]), "frequently the mutation gives rise to loss of some characteristic possessed by the parent cells." Therefore, it may also be interpreted that the foamy structure is not formed in subsequent passages because of changes of the bacterial activity caused by the mutation.

Cutaneous nerves.—The cutaneous nerves of hamsters' lesions do not contain any bacilli, in spite of the fact that the nerves are completely surrounded by granulomatous tissue composed of bacillated cells. Furthermore, pathologic changes of nerves, such as degenerative

or regenerative features and cytoplasmic changes of Schwann cells, are not observed, although the cytoplasm of some of the Schwann cells is vacuolated.

In lepromatous and borderline skin lesions, both axons and Schwann cells are frequently invaded by *M. leprae,* and the cytoplasmic organelles of the Schwann cells show a tendency to increase in numbers as a sign of pathologic changes of the nerves ([11, 13]) In tuberculoid skin lesions, alteration of both the Schwann cell and the axon, and epithelioid change of peri- and endo-neurial cells, are observed ([11, 13]). These features of peripheral nerve lesions of human leprosy imply an intimate relationship between *M. leprae* and nerves, as postulated by several authors ([6, 14, 18]).

As compared with human leprosy lesions, the absence of nerve lesions in the hamster does not strongly support the possibility of *M. leprae* infection. However, it is reasonable to believe that the bacillus may lose the characteristic of invading preferentially the nerves and, as a result of bacterial mutation, no longer affects them, even if the transmitted bacillus corresponds to *M. leprae.*

CONCLUSION

Examining the animal lesions caused by the bacillus obtained from human leprosy patients, one can find some features similar to those of the human leprosy lesions; i.e., the bacterial fine structure, the opaque droplet around the bacillus, and the intracellular distribution of the bacillus. Since these features are characteristic of *M. leprae* infection in human tissues, it seems very likely that the animal lesions studied were caused by *M. leprae,* although none of these features can identify the bacillus conclusively. In studies of animal transmission of human leprosy by other workers, the materials employed were obtained from lepromatous lesions in which *M. leprae* are abundant. As described in our first report, the majority of the bacilli contained in these lepromas show degeneration, and, furthermore, may be particularly adapted to human tissue. On the other hand, the inoculum used in the present study was obtained from a borderline leprosy lesion, which contains relatively few *M. leprae* which, however, show intact features ([10, 11, 12]). From this point of view, it is possible to say that *M. leprae* from borderline lesions may adapt and multiply in animal tissues differently than in its proper human tissue host.

However, various problems of the animal lesion, such as the absence of nerve lesions and no formation of either globi or foamy structures in the subsequent passages, are still unsolved. In order to clarify these problems, further studies will be made, including immunologic experiments.

SUMMARY

1. The nodules produced in the ears of golden hamsters primarily, and in white mice after transfer, by acid-fast bacilli obtained from a patient with borderline leprosy, were examined by the electron microscope.

2. In the material of the first passage, which required 8 months to develop, a majority of the bacilli showing plasmolysis are accumulated as agglomerations in the host-cell cytoplasm. Both moderately-dense droplets and foamy structures are seen around the bacilli.

3. Most of the bacilli in later passages are laden with bacterial cytoplasm, even in the oldest lesion, and they are enclosed by moderately-dense droplets, but foamy structures are not evident in the later passages.

4. The mouse lesion exhibits structures entirely similar to those observed in the hamsters, differing from the lesion provoked by *M. leprae murium* infection.

5. Cutaneous nerves of the hamster lesion do not display any lesions caused by bacterial invasion.

6. The submicroscopic structure of hamsters' lesions, compared with both human and murine leprosy lesions, shows greater similarity to human leprosy.

RESUMEN

1. Los nódulos producidos en las orejas de *Criceti aurati* primariamente, y en ratones blancos después del pase a ellos, por bacilos ácidorresistentes obtenidos de un enfermo con lepra limítrofe, fueron examinados con el microscopio electrónico.

2. En el material del primer pase, que requirió 8 meses para desarrollarse, la mayoría de los bacilos que revelan plasmólisis se acumula en aglomeraciones en el citoplasma de la célula huésped. Alrededoe de los bacilos se observan tanto gotillas moderadamente espesas como estructuras espumosas.

3. La mayor parte de los bacilos de los pases subsiguientes está cargada de citoplasma bacteriano, aun en la lesión más antigua, y está rodeada de gotillas moderamente espesas, mas no se divisan estructuras espumosas en los pases más recientes.

4. La lesión murina manifiesta estructuras absolutamente semejantes a las observadas en los *Criceti*, discrepando de la lesión provocada por la infección con *M. leprae murium*.

5. Los nervios cutáneos de la lesión del *Cricetus* no despliegan ninguna lesión ocasionada por la invasión bacteriana.

6. Comparada con las lesiones de la lepra tanto humana como murina, la estructura submicroscópica de las lesiones del *Cricetus* revela mayor semejanza con la lepra humana.

RESUMÉ

1. On a examiné par le microscope électronique des nodules produits, soit d'emblée dans les oreilles de hamsters dorés, soit secondairement après transfert chez la souris blanche, par des bacilles acido-résistants obtenus d'un malade atteint de lèpre border-line.

2. Lors du premier passage, qui requit 8 mois pour se développer, la majorité des bacilles montrant de la plasmolyse se sont accumulés en agglomérats dans le cytoplasme des cellules hôtes. On a constaté à la fois des gouttelettes modérément opaques et des structures spumeuses autour des bacilles.

3. Lors des passages ultérieurs, la plupart des bacilles sont chargés de cytoplasme bacillaire, même dans les lésions les plus anciennes, et ils sont enfermés dans des gouttelettes modérément denses. Les structures spumeuses ne sont pas évidentes lors de ces passages plus tardifs.

4. Chez les souris, les lésions développées témoignent de structures entièrement similaires à celles observées chez les hamsters, différant du type de lésions provoquées par l'infection par *M. leprae murium*.

5. Dans la lésion du hamster, les nerfs cutanés ne révèlent pas d'atteinte causée par l'invasion bacillaire.

6. Par comparaison avec les lésions de la lèpre humaine et de la lèpre murine, la structure submicroscopique des lésions des hamsters présente une plus grande ressemblance avec la lèpre humaine.

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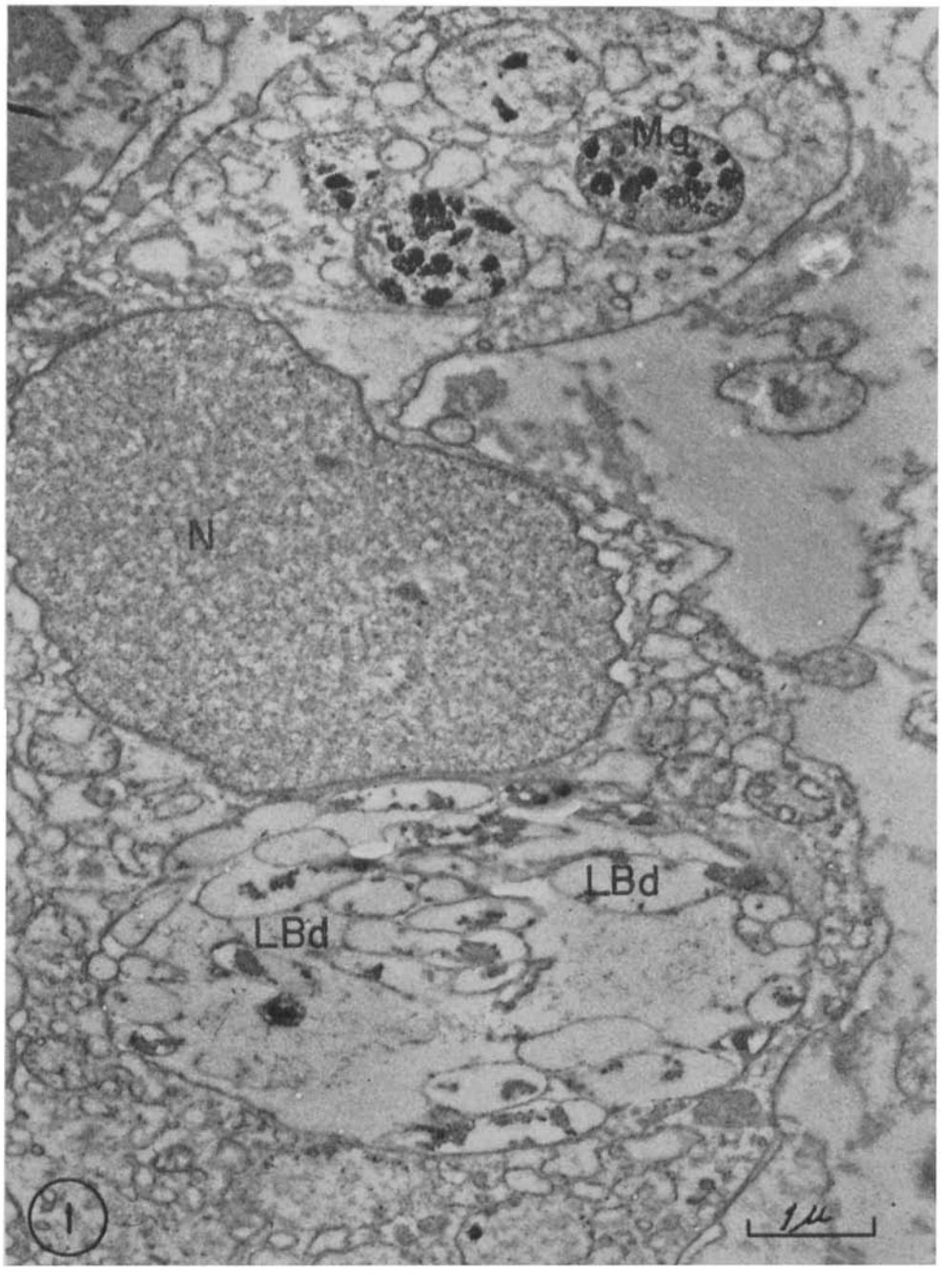


FIG. 1. The first-passage lesion in the hamster. Clumped degenerated bacilli (LBd) containing fragmented cytoplasm are enclosed by a membrane in the host cell cytoplasm. Phagocytosed melanin granules (Mg) are also seen in the upper part. N: Nucleus of host cell. Magnification, 19,000×.

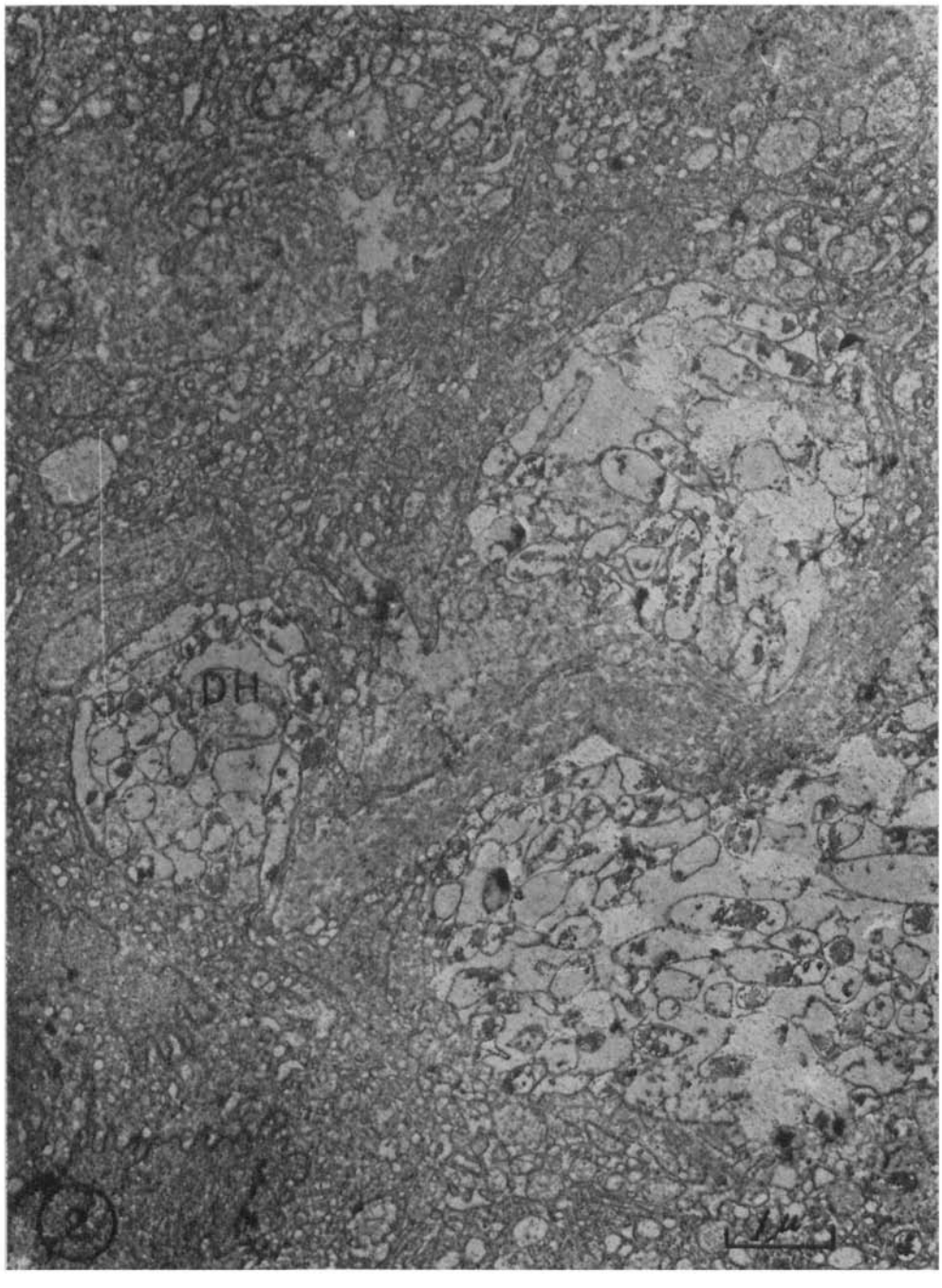
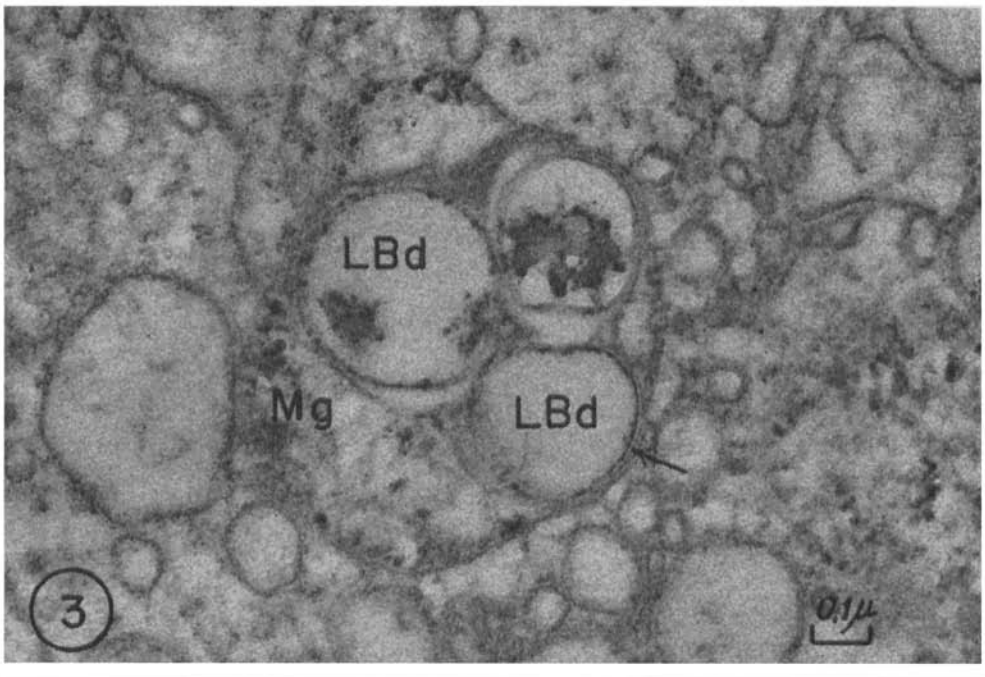


FIG. 2. Three agglomerations of the bacilli showing plasmolysis. The upper right one is exposed to the extracellular environment by rupture of the cell membrane, together with the cytoplasmic organelles of the host cell. Debris of the host cell cytoplasm (DH) is found in the agglomeration. Magnification, 16,500×.



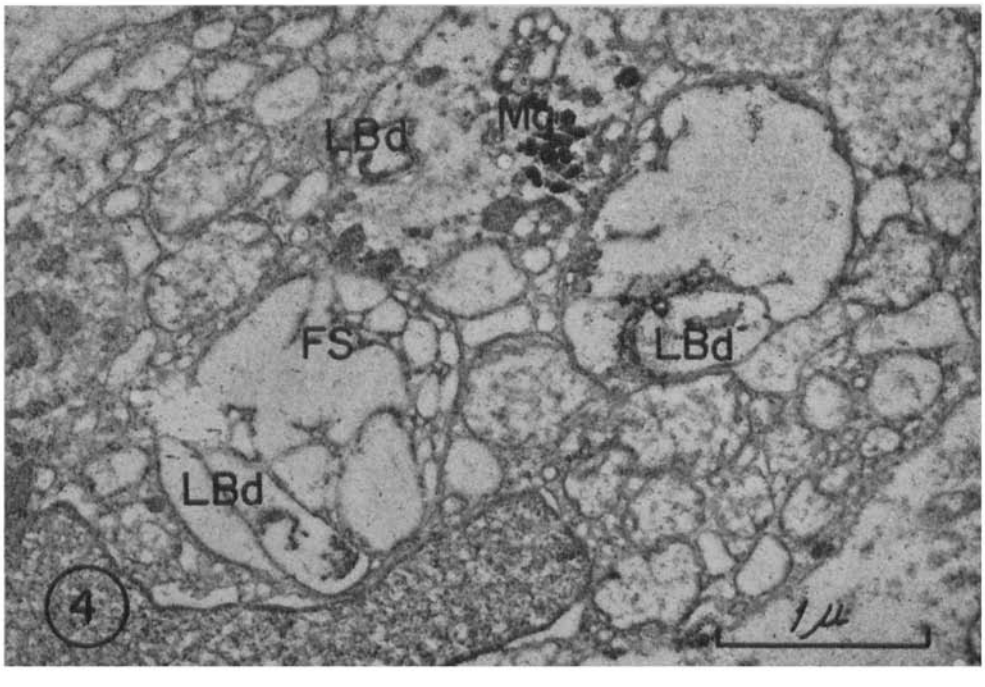


FIG. 3. Around the degenerated bacilli (LBd) is a moderately dense droplet. Melanin granules (Mg) are also evident in the droplet. Note a low-density space (arrow) between the bacterial surface and the droplet. Magnification, 80,000×.

FIG. 4. A foamy structure (FS) around bacilli in the first-passage lesion. Melanin granules are contained, together with the bacilli, in the same vacuoles. Magnification, 30,000×.

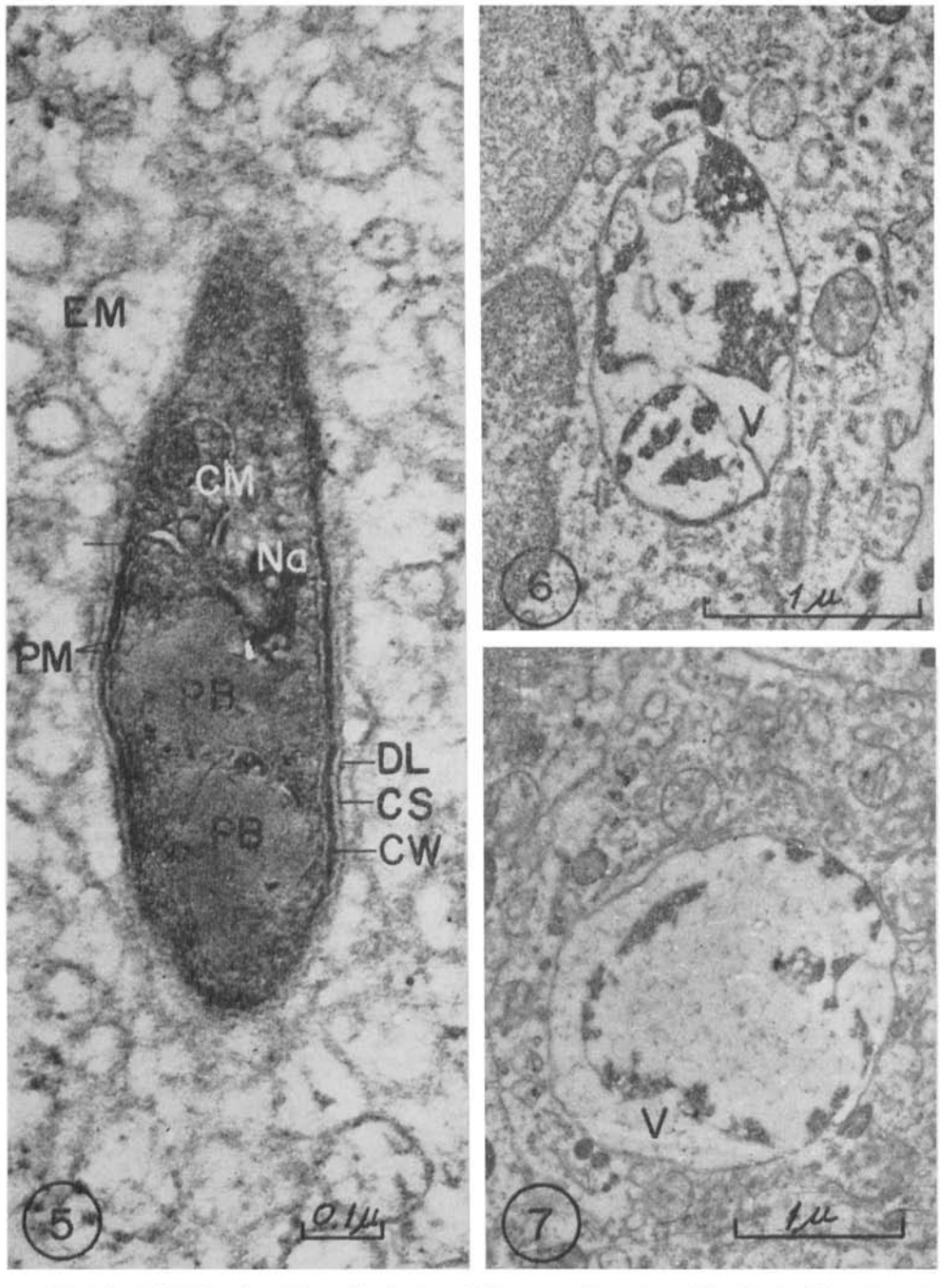


FIG. 5. A bacillus in a 4-months lesion of the second passage. The bacterial surface is composed of the diffuse layer (DL), the coating substance of low density (CS), and the cell wall (CW). The plasma membrane (PM), consisting of a double dense layer separated by a less dense space, is seen between the cell wall and the bacterial cytoplasm. Its outer layer closely adheres to the cell wall. Both layers of the plasma membrane enter the cytoplasm (arrow), to be transformed into the intracytoplasmic membrane system (CM). PB: Polyphosphate bodies. Na: Bacterial nuclear apparatus. EM: Enclosing membrane. Magnification, 120,000×.

FIGS. 6 & 7. Three-months lesion of the second passage. Dense fragments surrounded by a membrane are observed in the vacuoles (V). These features may represent the distintegrating process of degenerated bacilli. Magnification, Fig. 6, 32,000×; Fig. 7, 25,000×.

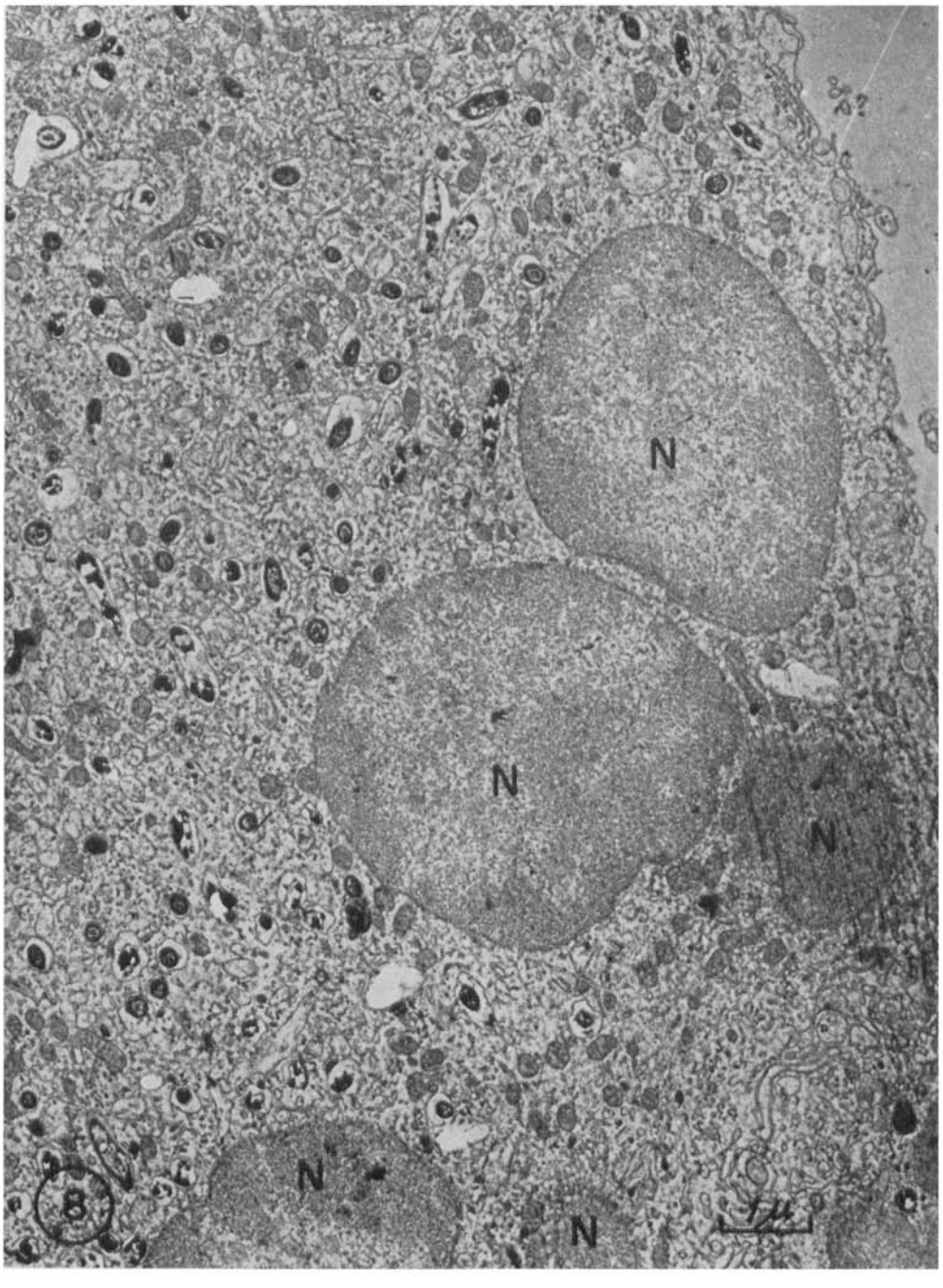


FIG. 8. A multinuclate cell containing many bacilli in the 4-month second passage lesion. The majority of the bacilli are enclosed by membranes in the host cell cytoplasm, and are not accumulated as agglomerations. Magnification, 13,500×.

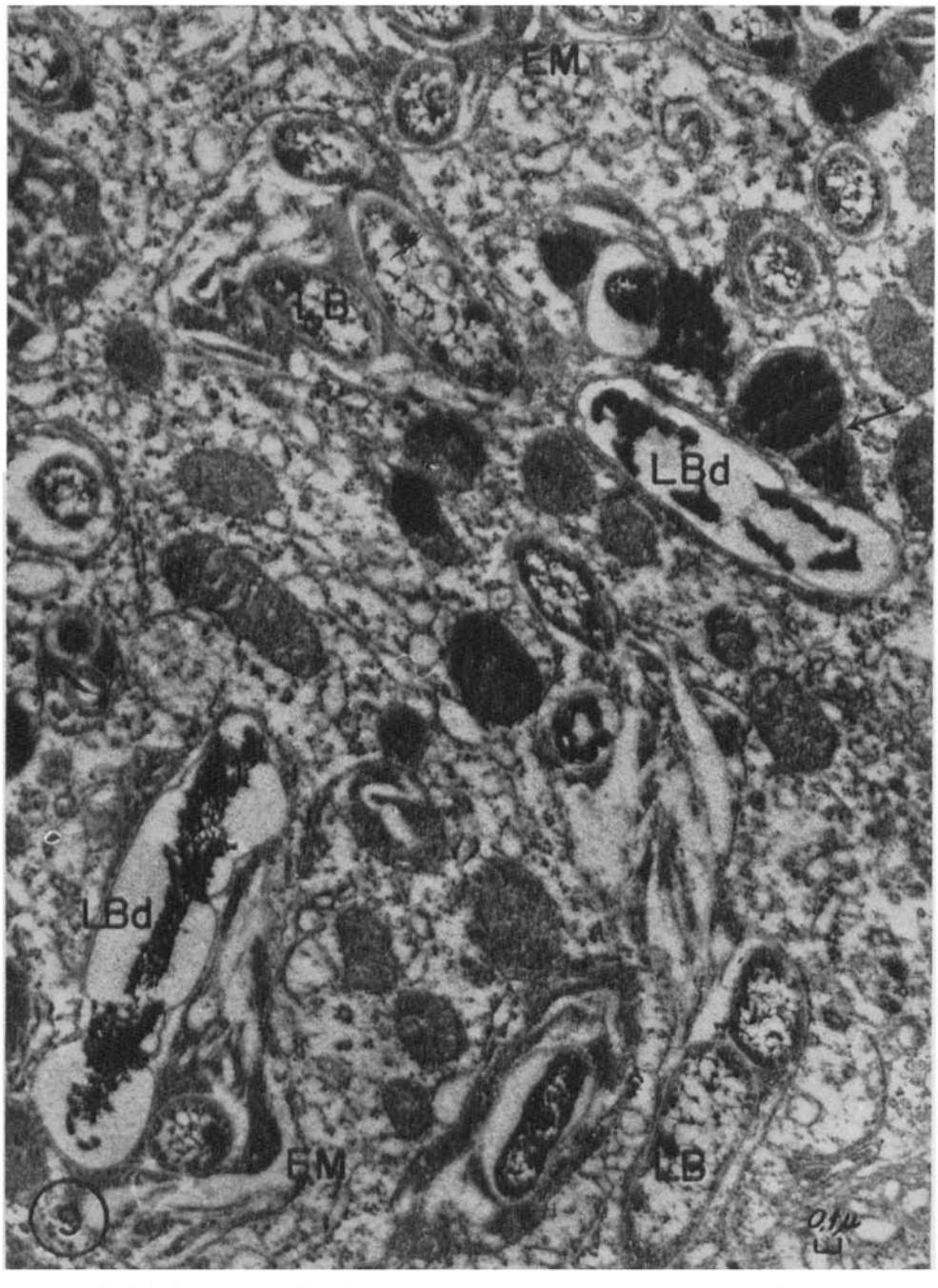


FIG. 9. One-year lesion of the second passage. The bacilli (LB) form small groups, each enclosed by a membrane. Many vesicles are to be seen in the bacterial cytoplasm. Degenerating bacilli (LBd) are also seen in this host cell. Moderately-dense droplets are attached to the bacilli, but the narrow space of low density is always visible between the bacillus and the droplets. The droplet containing denser droplets clings to the outer surface of the enclosing membrane (arrow). Magnification, 35,000×.

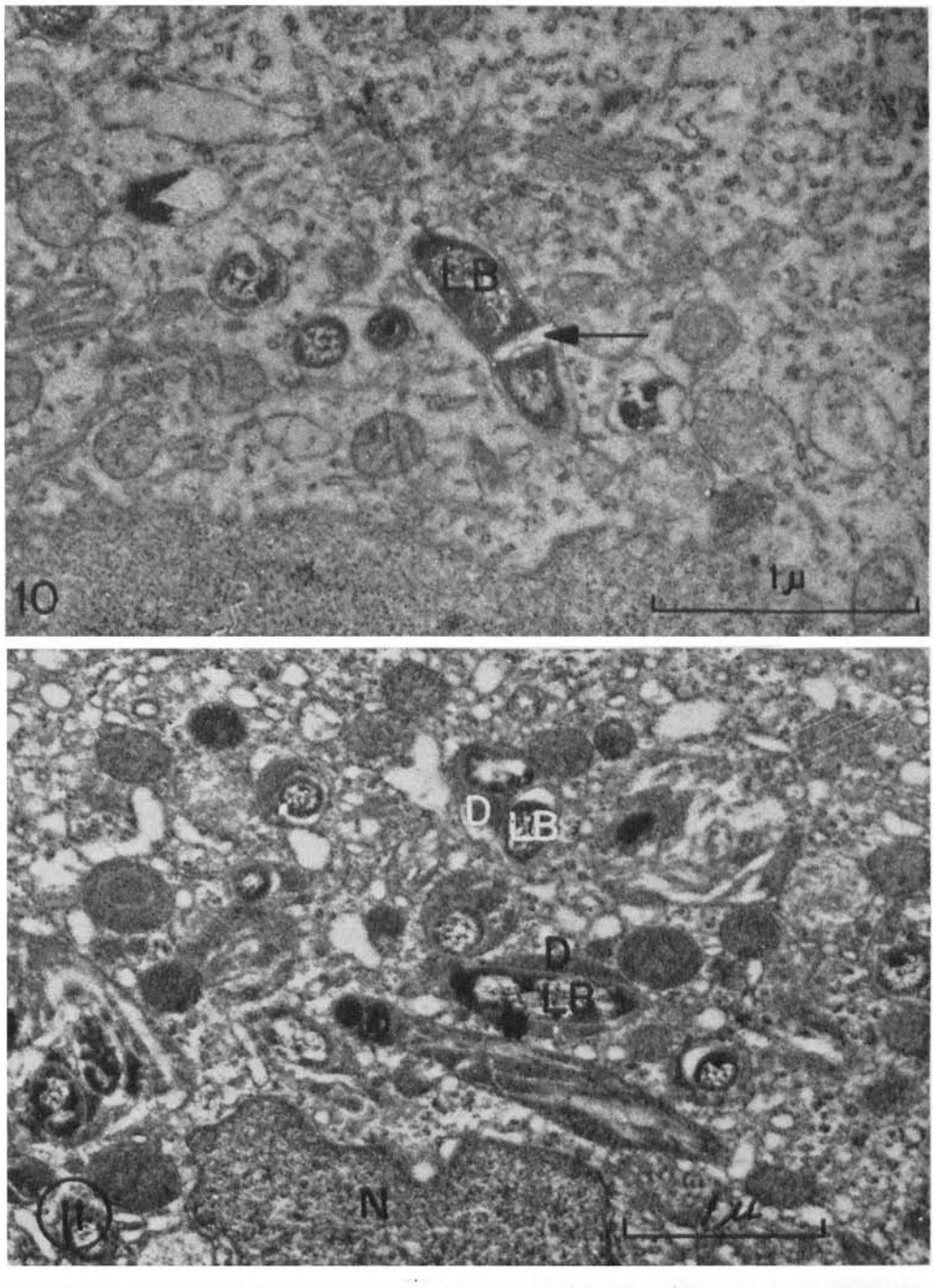


FIG. 10. Three-months lesion of the second passage. Membranous layers are visible between two bacilli (arrow). This feature may represent the process of bacterial cell division. Magnification, 40,000×.

FIG. 11. Moderately-dense droplet (D) surrounding the bacillus in the second passage after one year. This appearance is identical with the "opaque droplet" in lepra cells of the human leprosy lesion. Magnification, 30,000×.

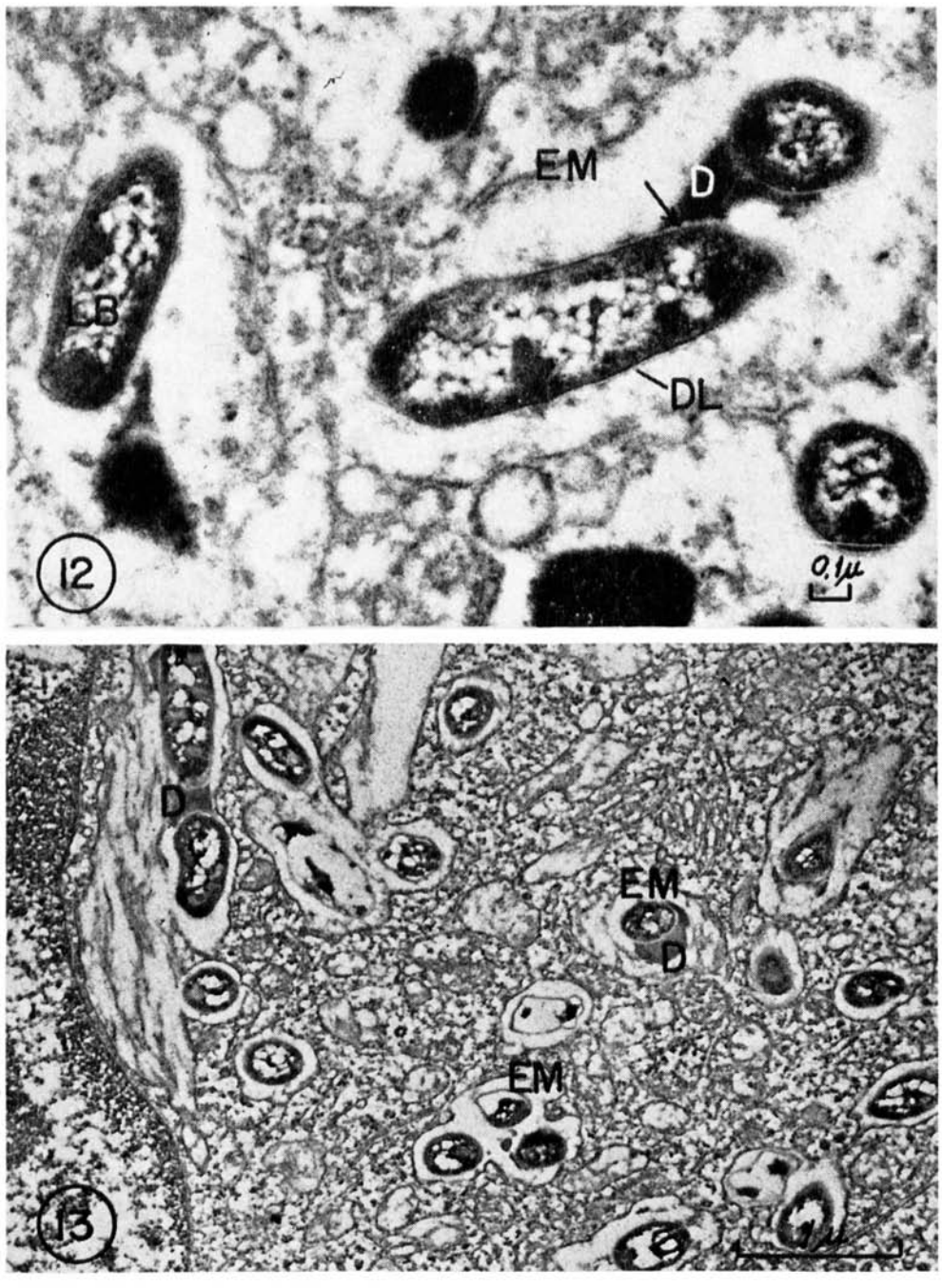


FIG. 12. This picture shows the diffuse layer (DL) of the bacillus observed in one-year material of the second passage. Note the low-density space (arrow) between the bacterial surface and the moderately-dense droplet. Magnification, 56,000×.

FIG. 13. Four-months lesion in a white mouse. Moderately-dense droplets are noted around the bacilli. Magnification, 24,000×.

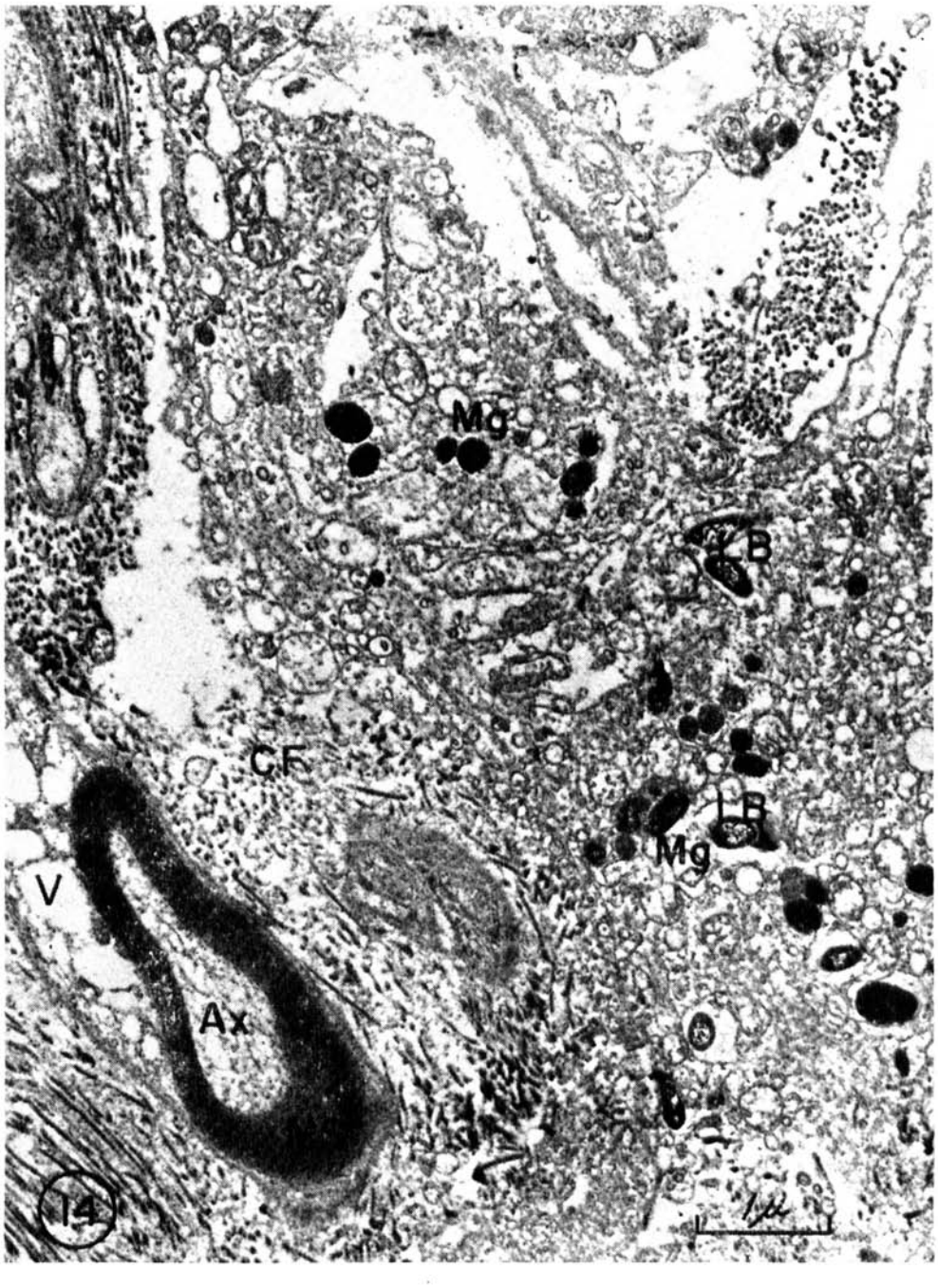


FIG. 14. Cutaneous nerves surrounded by bacillated cells in the one-year lesion of the second passage. The cytoplasm of the Schwann cell (lower left) contains many vacuoles (V), but the cytoplasmic organelles are not increased in number. No bacilli are found in the nerve elements. In the upper left is demonstrated a nonmyelinated fiber surrounded by collagen fibers. CF: Collagen fibers. MS: Myelin sheath. Ax: Axon. Magnification, 20,000×.