ELECTRON MICROSCOPIC ANALYSIS OF THE COMPONENTS OF LEPRA CELLS

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Histochemical studies of lepra cells have demonstrated the presence of lipids, and have shown that the chemical composition of the lipids varies during the development of the lepra cells. In recent years electron microscope studies have elucidated the submicroscopic structures of lepra cells, and have shown that an "opaque droplet" appears around the leprosy bacilli in the initial stage of the cells and finally develops into a "foamy structure" ([5, 15, 25]). It has been suggested, also, that these submicroscopic structures correspond to sudanophilic lipids observed by light microscopy.

In the present study, the chemical composition of various figures which appear in lepra cells has been analyzed physicochemically by electron staining. For the purpose of comparative study of electron stains, various purified lipids have been examined with the electron microscope.

MATERIALS AND METHODS

Lepromatous nodules from 10 cases, and lepromatous great auricular nerves from 3 cases, were used in this study. The biopsy specimens were fixed approximately 4 hours at 4°C in 1 per cent osmium tetroxide buffered with phosphate (1/50 mol) containing 7.5 per cent sucrose, dehydrated for 2 hours in graded ethyl alcohols, imbedded in a 6:4 mixture of butyl and methyl methacrylate, and polymerized with benzoyl peroxide at 55°C. Blocks were sectioned with glass knives in a Nippon Ultramicrotome. Two electron microscopes, an Akashi Tronscope 50 and a Hitachi HU 10, were used in examining the sections.

Some sections were floated on a solution containing hydrogen peroxide and hydrochloric acid. By this treatment, initiated by Merriam ([13]), the osmium which is combined with the tissues is almost entirely removed from the sections.

In order to examine the electron stains of various lipids, they were dissolved in ethyl ether (sphingomyelin was dissolved in a 9:1 petroleum ether-methanol mixture) and thin films were then made on a water surface. These were mounted on copper grids covered with formvar, and then exposed to a vapor of osmium tetroxide for 2 hours. The lipids used in the present study were an unsaturated fatty acid (oleic acid), two saturated fatty acids (stearic acid and palmitic acid), neutral fat extracted by ethyl alcohol from human subcutaneous fat tissue, two phosphatides (ovolecithin and lung sphingomyelin), and cholesterol.

OBSERVATIONS AND DISCUSSION

Observed with the electron microscope, lepra cells are characterized by the presence of opaque droplets and foamy structures. Opaque droplets, so designated by our group ([25]), are a moderately dense sub-

stance surrounding leprosy bacilli. Foamy structures, named by Brieger and Glauert ([5]), we have found to be the disintegrated substance of opaque droplets ([25]). These two submicroscopic structures are thought to be lipids stainable by histochemical procedures.

With various purified lipids, electron staining gives the following results. Neither the saturated fatty acids used nor cholesterol exhibit an osmiophilic property, and consequently they cause hardly any scattering of the electron beam. Unsaturated fatty acid and its ester (neutral fat) show intense electron staining. Phosphatides, however, which also possess unsaturated fatty acids, show moderate electron density. These results are in accord with experiments carried out by Bahr ([2, 3]).

In this article are discussed the origin, or mechanism of formation, and the composition of the opaque droplet, and also these features of the foamy structure, comparing them with the results of electron stains for various lipids.

FORMATION AND COMPOSITION OF THE OPAQUE DROPLET

Three opinions have been advanced regarding the origin of the lipid substances observed in lepra cells with the light microscope: 1. They originate by the accumulation of pinocytotic fat drops supplied from the surrounding fat tissue ([16]).[1] 2. They arise from the reactive products of the cytoplasm caused by the invasion of the leprosy bacilli ([8, 14, 24]). 3. They are the break-down products of the degeneration of bacillary bodies ([23]).

In electron micrographs of lepra cells there are seen scattered in the cytoplasm very small, moderately dense droplets measuring less than 100 mμ in diameter, thought to be pinocytotic droplets. They usually consist of a moderately dense, finely granular substance surrounded by a single membrane, as shown in Figs. 1, 2, 5 and 7.

Concerning the pinocytotic droplets in lepra cells, Ogata ([16]) concluded from studies with the light microscope that they consist mainly of neutral fat supplied from fat cells. However, it has been substantiated in the present study, what was found in a previous study of fat cells ([10]), that neutral fat drops have a homogeneous electron density. Therefore, I cannot agree that the pinocytotic droplets of lepra cells consist of neutral fat.

It may be supposed, from the finely granular appearance of pinocytotic droplets, that they may be a lipoprotein derived from the blood stream. According to Brandt ([4]), the limiting membrane may be interpreted as a plasma membrane which is taken into the cytoplasm by

[1] To avoid any uncertainty on the part of noncytologists about its meaning, the following two definitions of "pinocytosis" (from the Greek *pinein*, to drink) are quoted. From the New Gould: Drinking by cells, as opposed to phagocytosis, eating by cells. Used to indicate microscopically visible "drinking" or engulfing of globules of fluid by pseudopodia. The fluid later becomes part of the cytoplasm. From Dorland: The absorption of liquids by cells; especially the phenomenon in which minute incuppings or invaginations are formed in the surface of leucocytes, which close to form fluid-filled vacuoles.—EDITOR.

pinocytosis, but it may be digested in the course of time.

Although nucleoprotein particles (Palade's particles) may conglomerate in small masses and exhibit an appearance similar to pinocytotic droplets, they are easily distinguished from the latter because they lack the limiting membrane and are larger in size.

Pinocytotic droplets coalesce with each other to produce round or oval, moderately dense "coalescent droplets" measuring 300-500 mμ in diameter, as is seen in Figs. 1, 2, 3 and 7. Palade [19] observed similar round bodies of various sizes in seminal epithelia, and he also assumed that these bodies represented a terminal appearance of phagocytized vacuoles. Since these coalescent lipoprotein droplets are not abundantly found in normal cells, the fact that they are numerous in the cytoplasm of lepra cells suggests a deviation from normal lipid metabolism on the part of those cells. Furthermore, as noted by Azulay and de Andrade [1], the histiocytes of the leproma contain lipids after the leprosy bacilli have disappeared. It is surmised, therefore, that a disorder of lipid metabolism may be characteristic of the lesions of lepromatous cases.

Infrequently, a texture resembling mitochondrial cristae is resolved in the coalescent droplets, as in Fig. 3. These figures are regarded variously as the visualization of a changing functional process of mitochondria [10, 12, 20], as mitochondrial degeneration [6, 22], and as steps in the production of different cytoplasmic granules [21]. Under the complicated conditions of lepra cells, however, it is difficult to confirm that these figures belong to any of these three categories.

Coalescent droplets accumulate around leprosy bacilli and develop into opaque droplets (Fig. 3). The opaque droplet is resolved as an agglomerate of fine granules, delimited from the cytoplasm by a single dense membrane measuring about 50Å in thickness (Figs. 1, 5, 6 and 7). In addition to this evidence, sections treated by hydrogen peroxide, as in Fig. 4, demonstrate that the opaque droplet retains its density after osmium is removed. In this picture, the protein components of cytoplasmic organelles and nuclei show electron scattering due to their high molecular property. This evidence signifies that the opaque droplet is not made of either single lipids or a complicated mixture of lipids, but that it may be composed of a lipoprotein colloid. The histochemical study of Harada [8] has shown that lepra cells in their initial stage contain lipoprotein. This lipoprotein in the initial stage may correspond to that of the opaque droplets in electron micrographs.

From the occurrence of opaque droplets, and the constitution of that body, it is inferred that lipoprotein is present in the form of a hydrophilic colloid. Since this colloid droplet is located in the cytoplasm, which also consists of hydrophilic colloids, lipoprotein molecules arrange themselves on the surface of the droplet to decrease the free energy of the surface. This surface arrangement may be resolved as the limiting membrane around the opaque droplets.

In some circumstances, however, there may be a different explanation of the limiting membrane. When groups of bacilli accompanied by remnants of opaque droplets are discharged into the extracellular environment by the breakdown of lepra cells, they are again phagocytized by histiocytic cells. Some of the limiting membranes, therefore, might be a remnant of the plasma membrane resulting from phagocytosis.

CHEMICAL COMPOSITION OF THE FOAMY STRUCTURE

With the proliferation of leprosy bacilli in opaque droplets, minute vesicles occur in association with them—frequently around them, but sometimes apparently independent of them (Fig. 5). In my study of xanthoma cells ([11]), which is one of the cells with lipid deposition caused by abnormal lipid metabolism, it was found that the vesiculation occurs in dense bodies composed of lipoprotein and develops into large lucent vacuoles. Since the tissue of the leproma deviates from the normal lipid metabolism, the disintegration of lipoprotein in the lepra cell may be attributed not only to bacillary enzymes but also to abnormal enzymatic activities of the cell proper.

As regards the composition of plasma lipoprotein, Oncley *et al.* ([18]) proved that: "Approximately 75% of plasma lipid is bound in β-lipoprotein, which is mainly composed of polypeptide, phosphatides, esterified and free cholesterol." Since the lipoprotein of the opaque droplet originates mostly in pinocytotic plasma lipid, disintegration products may also comprise protein, phosphatides and cholesterol. Furthermore, minute vesicles consist of a limiting membrane of 50Å in thickness and contain moderately dense particles 50Å in diameter. These measurements are in accord with a double length of the lecithin molecule.

Histochemical studies carried out by Ueda ([24]) and by Sugai ([23]) proved that lecithin is present in lepra cells. Considering the physicochemical analysis of electron micrographs and the results reported by other workers, I offer the speculative hypothesis that the minute vesicles may consist mainly of stable micelles of phosphatides. Their wall may represent a double leaflet the molecules of which are arranged to face their hydrophilic surface to the outside aqueous phase, and the moderately dense particles of the interior may be spherical micelles of phosphatide radially oriented so as to present a hydrophilic surface. It is thought, therefore, that the protein moiety is disintegrated earlier than the phosphatide.

With the further development of vesicles, they coalesce and finally form the electron-transparent foamy structures (Fig. 5). The results of electron stains indicate that the lucent interior of the foamy structure is not composed of unsaturated fatty acids, their esters and phosphatides. Fite ([7]) reported from a histochemical study of lepra cells that the lipids of those cells are free of cholesterol. In the present investigation, observation with the polarizing microscope has demon-

strated no refractile substance in the lepromas used for the electron staining. Furthermore, I have observed (9) that in the lepra cells the activities of various phosphatases are increased, which means that phosphate compounds are disintegrated by enzymes. Based on these facts, it is thought that the lucent area of the foamy structure consists of a saturated fatty acid, or its ester, or lower molecules, which do not scatter the electron beam.

Sugai (23) has also found fatty acids in his histochemical study of lepra cells. This presence of fatty acids may correspond to the stage of the foamy structure.

At the boundary between the foamy structure and the opaque droplet, there is present a limiting membrane measuring 50Å in thickness which is interpreted as a double layer of phosphatide, as suggested in connection with the formation of the minute vesicles.

Besides foamy structures, Nishiura (15) has observed an electron-transparent zone around leprosy bacilli which he believes is ascribable to the metabolites of the bacilli, related to bacillary growth. These transparent zones are delimited from the opaque droplet by a dense membrane measuring about 50Å in thickness (Figs. 1, 2 and 7). In analogy to the formation of the membrane between the opaque droplet and the cytoplasm, the presence of this limiting membrane implies that the physicochemical state of the transparent zone is under the same conditions as that of the surrounding cytoplasm.

At the site of a narrow opaque droplet, this membrane and the outer enclosing membrane are arranged side by side separated by a less dense interspace (Figs. 6 and 7). With the rupture of these limiting membranes, cytoplasmic elements are blended with the components of the transparent zone (Fig. 6). On the evidence of these observations, it is suggested that the transparent zone around leprosy bacilli is not an oil phase but a water phase, in which various metabolites of the bacilli are dissolved.

Infrequently, an irregular arrangement of dense granules is seen in the transparent area (Fig. 7). This picture may be factitious, arising from the diffusion of the osmium molecules during the preparation for electron microscopic examination, as pointed out by Ogura *et al.* (17).

Summarizing succinctly the results presented, it is concluded that pinocytotic lipoprotein droplets accumulate around the leprosy bacilli in the lepra cell and form the opaque droplet, that these opaque droplets undergo degenerate change to vesicles composed of phosphatide micelles, and that finally the opaque-droplet complex degenerates to form the foamy structures containing saturated fatty acids, their esters, or lower molecular substances. The electron-transparent zone surrounding the leprosy bacilli is thought to be an aqueous phase in which various metabolites of the bacilli are dissolved.

SUMMARY

1. The submicroscopic structures of lepra cells have been analyzed physicochemically, in comparison with the results of electron stains of various purified lipids.

2. The opaque droplet originates from the accumulation of pinocytotic droplets, and it may consist of lipoprotein derived from the blood.

3. The minute vesicles arise from the disintegration of the opaque droplet. From the physicochemical analysis it is suggested that the minute vesicles may be composed of phosphatide micelles.

4. The foamy structure is a terminal appearance of the opaque droplet. The present observation introduces the possibility that the foamy structure contains saturated fatty acids, their esters, or lower molecular substances.

5. The electron-transparent zone around leprosy bacilli does not consist of a lipid phase, but of a water phase in which are dissolved the metabolites of the bacilli.

6. From various points of view, it is concluded that there is a disorder of lipid metabolism in the essential lesion cells of the leproma.

RESUMEN

1. Se analizaron físicoquímicamente las estructuras submicroscópicas de las células leprosas, comparándolas con los resultados de las coloraciones electrónicas de varios lípidos purificados.

2. La gotilla opaca procede de la acumulación de gotillas pinocitóticas, pudiendo constar de lipoproteína derivada de la sangre.

3. Las vesículas minúsculas proceden de la desintegración de la gotilla opaca. A juzgar por el análisis físicoquímico, se sugiere que dichas vesículas quizás estén compuestas de tagmas de fosfátidos.

4. La estructura espumosa representa el aspecto terminal de la gotilla opaca. La observación actual introduce la posibilidad de que dicha estructura contenga ácidos grasos saturados, ésteres de éstos o substancias moleculares más inferiores.

5. La zona electrono-transparente alrededor de los bacilos leprosos no consta de una fase lípida, sino de una fase acuosa en la cual están disueltos los metabolitos de los bacilos.

6. Desde varios puntos de vista, se deduce que existe un trastorno del metabolismo lípido en las células de la lesión esencial del leproma.

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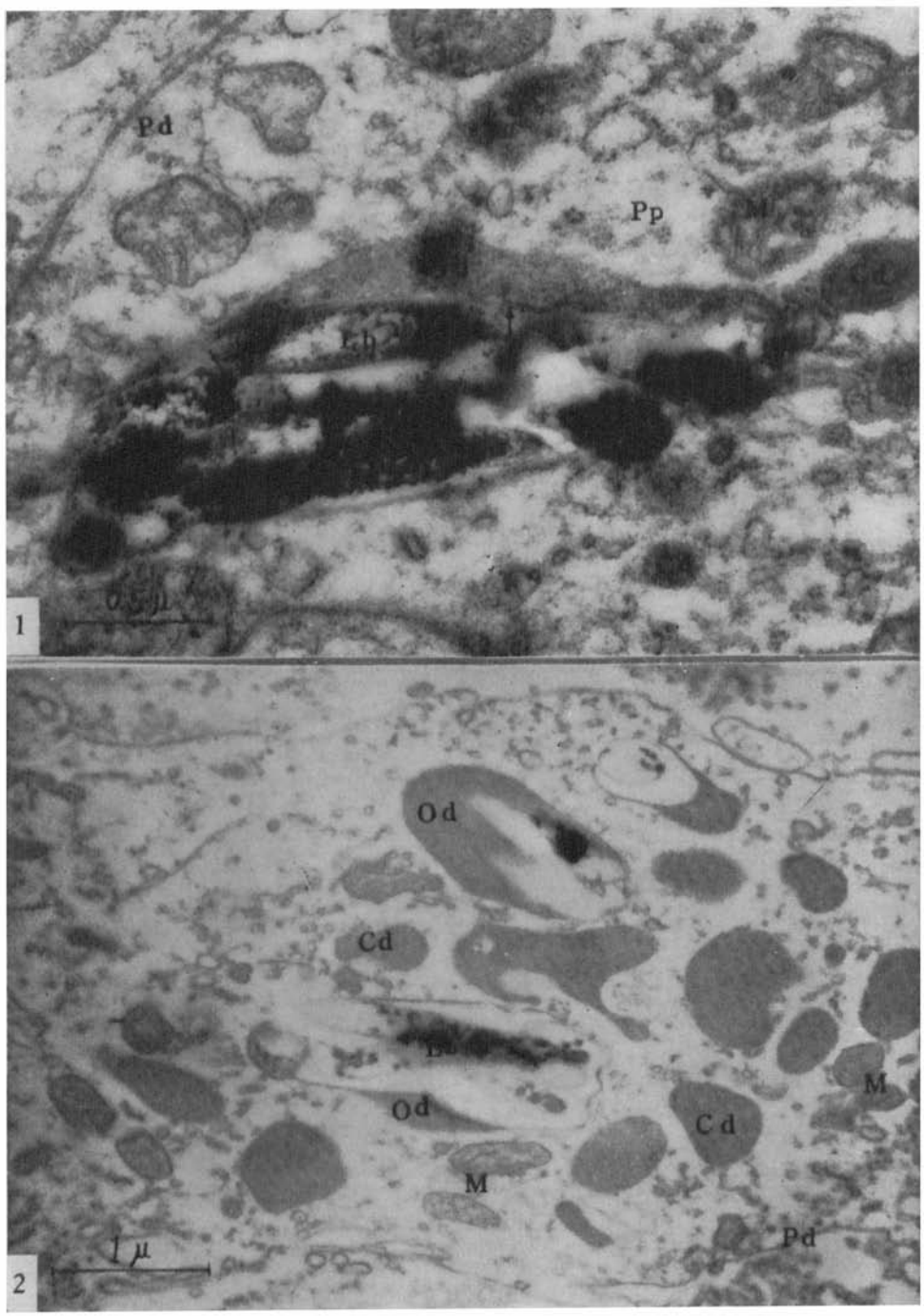
[Illustrations Follow]

DESCRIPTION OF FIGURES

FIG. 1. An ultra-thin section of a lepra cell in a skin nodule of a 34-year-old patient. Leprosy bacilli (Lb) are enveloped in an opaque droplet (Od), which is limited by a dense single membrane. Pinocytotic droplets, small bodies less than 100 mμ in diameter (Pd); the coalescent droplets, 300-500 mμ in diameter, formed by fusion of pinocytotic droplets (Cd); and numerous mitochondria (M) are distributed in the cytoplasm. Neither the pinocytotic droplets nor opaque droplets show a homogeneous appearance; actually, both kinds consist of aggregations of fine granules. Between the opaque droplet and the bacilli there is a limiting membrane (arrow). Palade's particles (Pp) are scattered in the cytoplasm, and develop a tendency to conglomerate into small masses.

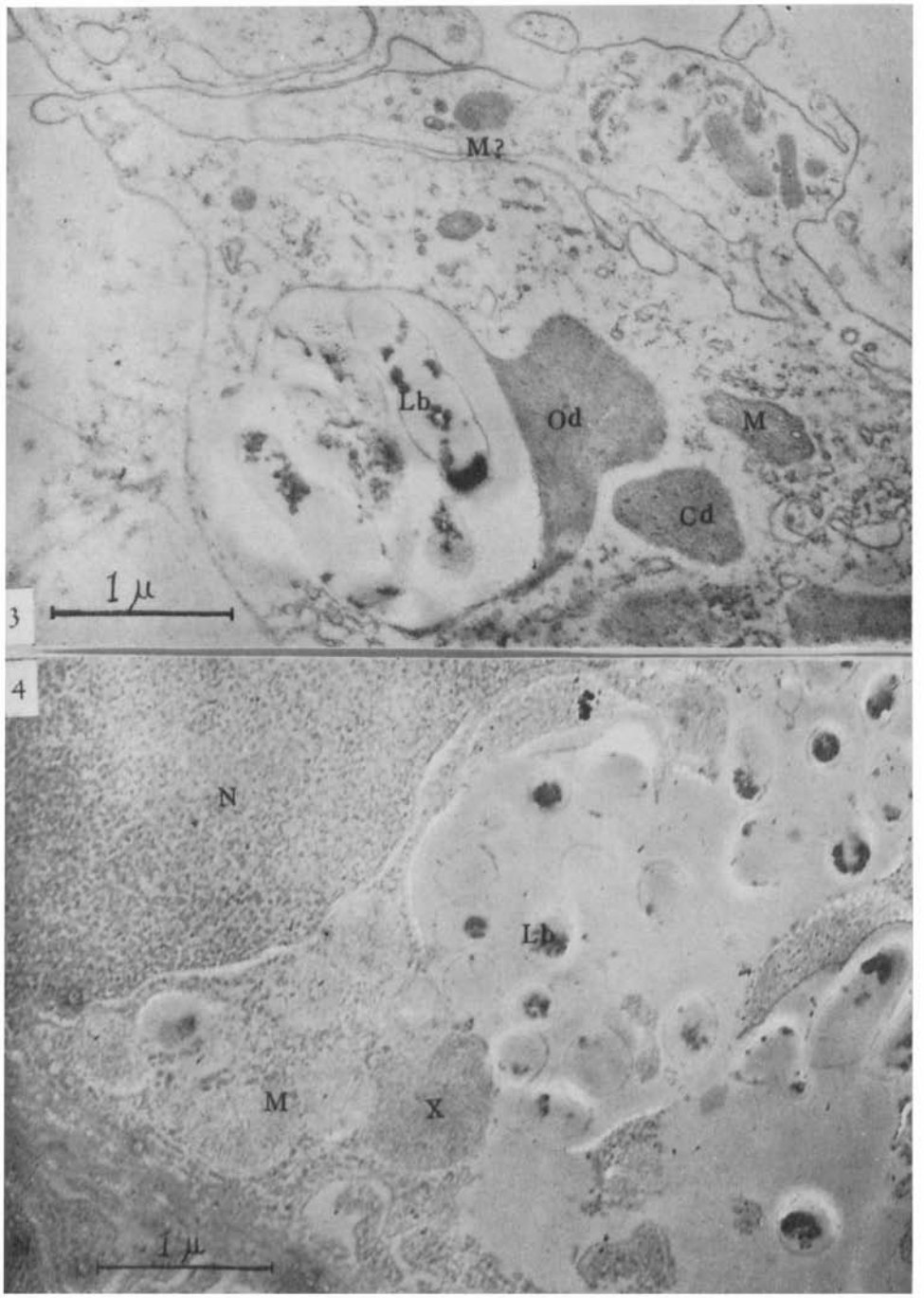
FIG. 2. One of the lepra cells in a skin nodule of a 56-year-old patient. Numbers of pinocytotic droplets (Pd) and their coalescent droplets (Cd) are observed in the cytoplasm. Some of the latter present an appearance as if developing into opaque droplets (Od). Normal mitochondria (M) are also demonstrated, and leprosy bacilli (Lb) are present.


Pd
Pp
M
Eb
0.5μ
1
Od
Cd
Eb
Od
M
Cd
M
Pd
1μ
2

DESCRIPTION OF FIGURES

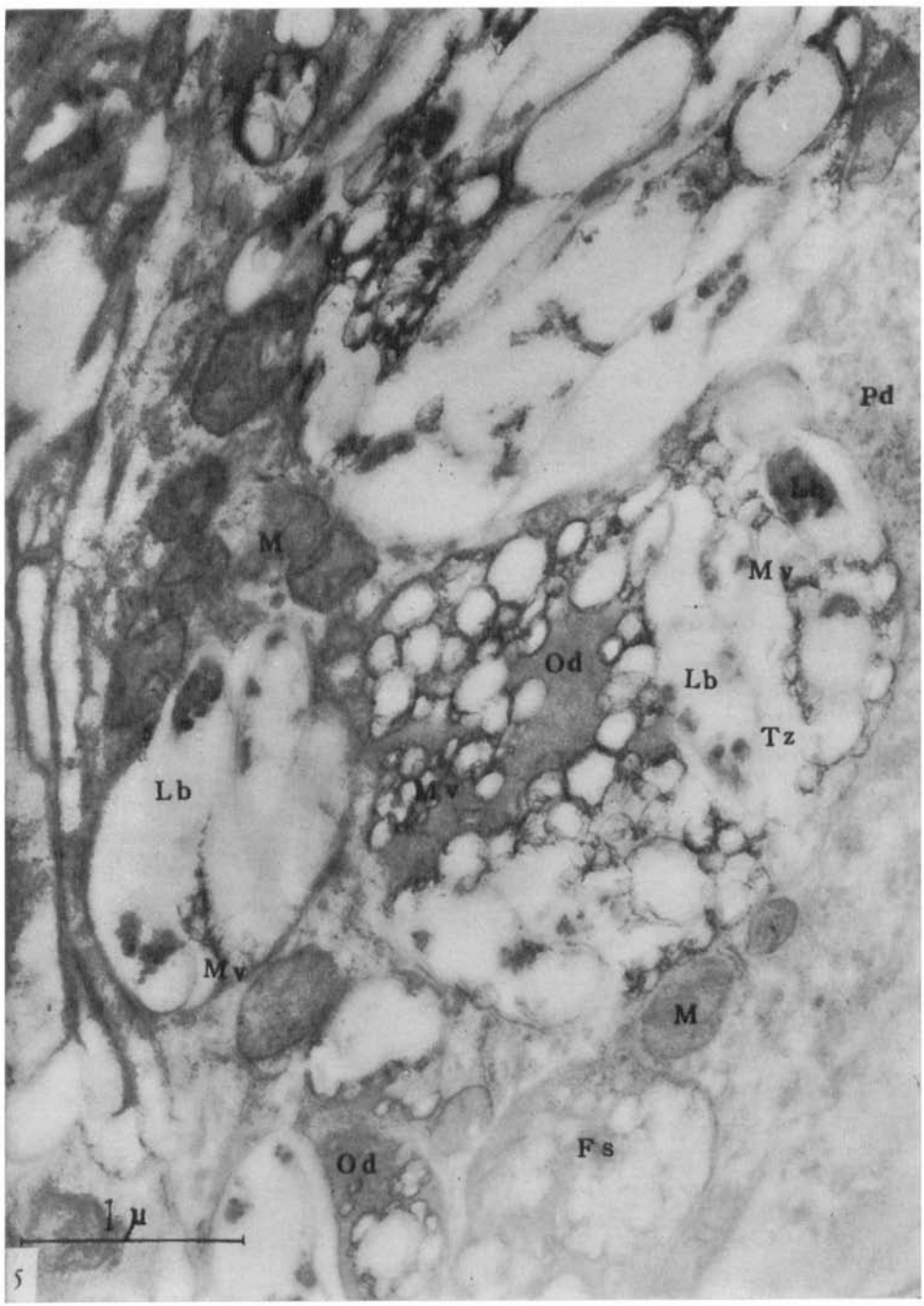
FIG. 3. One of the lepra cells in a skin nodule of a 48-year-old patient. This picture is indicative of the fact that the opaque droplet (Od) results from the accumulation of pinocytotic droplets, or of their coalescent droplets (Cd), around leprosy bacilli (Lb). In the upper part, a cristae-like texture is perceptible in the granular matrix (M?). Note the difference between this figure and typical texture of the mitochondria (M).

FIG. 4. This picture is of a section treated with hydrogen peroxide. Although the osmium is almost entirely removed from the tissue, the nucleoprotein and lipoprotein particles retain their electron-scattering property. At the same time, the area (X) with an opaque droplet or a coalescent droplet also shows electron scattering. Leprosy bacilli, Lb. Mitochondria, M. Nucleus of lepra cell, N.


M?
Lb
Od
M
Cd
1 μ
3
4
N
Lb
M
X
1 μ

DESCRIPTION OF FIGURE

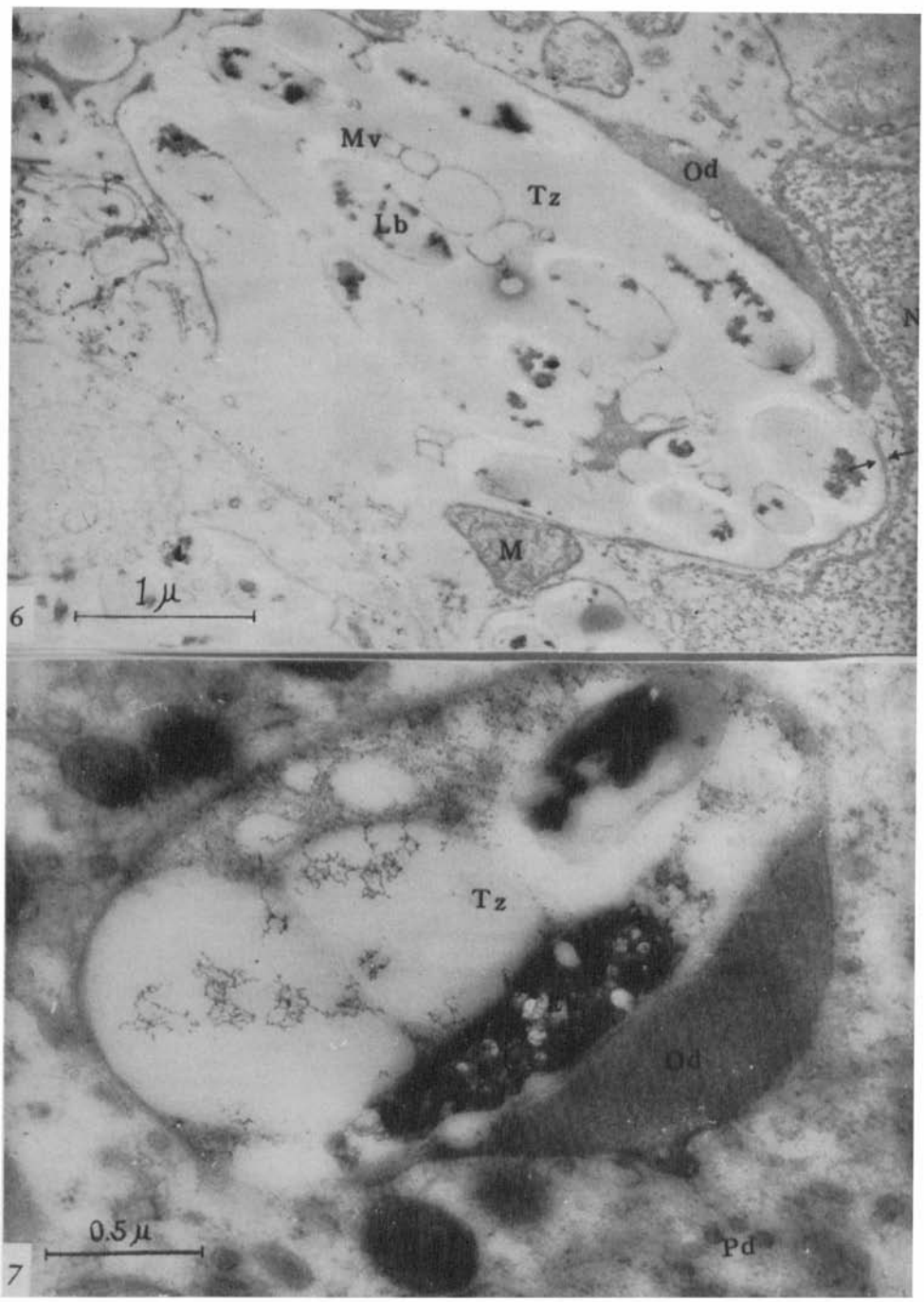
FIG. 5. One of the well-developed lepra cells in a skin nodule of a 48-year-old patient. Here is a striking demonstration of minute vesicles (Mv), which appear mostly around leprosy bacilli (Lb), although some vesicles occur in opaque droplets (Od) independently of bacilli. Note the presence of minute vesicles in the electron-transparent zone (Tz) around the bacilli. These minute vesicles consist of fine, less-dense granules surrounded by a single dense layer. In the lower part of the picture are seen typical foamy structures (Fs), and they include the remnants of the fine granules of minute vesicles. Mitochondria, M. Pinocytotic droplets, Pd.


Pd
Lb
M
Mv
Od
Lb
Tz
Lb
Mv
Mv
M
Od
Fs
1 μ
5

DESCRIPTION OF FIGURES

FIG. 6. One of the lepra cells in the same leproma as Fig. 3. Enclosed by an opaque droplet of which only a narrow zone is apparent on one side (Od), leprosy bacilli (Lb) are situated in the electron-transparent zone (Tz). The inner limiting membrane of the opaque droplet is adjacent to the other membrane, and therefore they look like a double membrane (arrow). At a break in these enclosing membranes, the content of the transparent zone diffuses into the cytoplasm. Structural changes of the cytoplasmic organelles are not observed. Mitochondria, M. Nucleus of lepra cell, N. Minute vesicles, Mv.

FIG. 7. One of the lepra cells of a nerve lesion of the same patient as Fig. 1. An opaque droplet (Od) is delimited by a dense membrane from the cytoplasm, and also from the electron-transparent zone (Tz). An irregular arrangement of dense granules, thought to be osmium molecules, is diffused into the transparent zone. Pinocytotic droplets, Pd. Leprosy bacilli, Lb. Coalescent droplets, Cd.


Mv
Od
Tz
Lb
N
M
1 μ
6
Tz
Od
0.5 μ
Pd
7