

Ultrastructural Changes in Cells of the Mouse Footpad Infected with *Mycobacterium leprae*

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Ultrastructural changes in cells of the mouse footpad are described which occurred during the log phase of multiplication, the plateau, and the stationary phase of growth of *Mycobacterium leprae*. BALB/c mice were inoculated in the right hind footpad with 5×10^3 organisms and sacrificed in pairs at 86 to 173 days after inoculation. Tissue samples were prepared for electron microscopy by standard techniques. During the early growth phase of *M. leprae* in the mouse footpad, few organisms can be detected. Those present are in macrophages and are bound by a single membrane. The cytoplasm of the macrophage is less dense around the organism. There are few lysosomes, and the bacteria do not appear to be degenerating. At the peak of the growth phase, the organisms within a macrophage are bound by either a single or double membrane. There is an increased number of vacuoles, which are also bound by a double membrane, and lysosomes. During the stationary phase, most of the macrophages have taken on a vacuolar appearance and contain lysosomes. The vacuoles are bound by a double membrane, as are most of the organisms within the macrophage. Many of these organisms appear to be degenerating. Occasionally, organisms are encountered in the sarcoplasm of striated muscle. They are usually bound by a single membrane and do not appear to be degenerating.

The relationship between *Mycobacterium leprae* and the tissues they infect is of considerable interest. In tissue samples from human leprosy patients and from mice and rats infected with *M. leprae*, bacteria are found in macrophages of the loose connective tissues and in the cells making up nerve and muscle tissue (1, 8). Within tissue macrophages, the bacilli usually are surrounded by an electron-transparent area bounded by a membrane, and many appear to be in various stages of degeneration. In nerve and muscle cells, not as many bacilli appear to be degenerating (5, 6).

The mouse footpad infected with *M. leprae* is a suitable system in which to study the host-parasite relationship in leprosy because it permits serial sampling of the tissue as the infection progresses. After the inoculation of a small amount of viable *M. leprae* into the footpad of an immunologically competent mouse, there is a lag phase lasting 30 to 60 days, during which period there is no detectable increase in the number of organisms (9). Then, *M. leprae* enters a logarithmic phase of multiplication, during which time the number of organisms increases, with an

average doubling time of 12.5 days. Toward the end of the logarithmic phase, numbers of acid-fast bacilli (AFB) may be readily seen within cells lying in the loose connective tissue just beneath the plantar skin and surrounding the nerve-and-vessel bundles. Despite the presence of the organisms, there is a minimum of inflammatory response in the footpad tissues. Shortly after the number of organisms passes 10^6 per footpad (the "plateau," 130 to 150 days after inoculation), multiplication ceases; at about this time, the footpad tissue displays evidence of a low-grade inflammatory process characterized by the presence of a round-cell infiltrate. At no time is there gross disease of the footpad. After cessation of multiplication, death of *M. leprae* begins. Although the number of AFB does not change very much during this stationary phase, death of organisms can be demonstrated by mouse passage (4, 10).

The purpose of the study reported below was to describe ultrastructural changes in cells of the mouse footpad as the leprosy infection progressed through the logarithmic phase of multiplication, the plateau, and the beginning of the stationary phase.

MATERIALS AND METHODS

Shepard's mouse footpad technique for the cultivation of *M. leprae* was used in these experiments (9, 12). Inoculation of the mice and the harvesting of *M. leprae* were performed in San Francisco. The mice studied were taken from groups of locally bred

BALB/c mice inoculated with *M. leprae* in other experiments. The *M. leprae* were all from the same strain of organisms originally isolated from a patient by Shepard and carried since then in mouse passage both in Shepard's laboratory and in San Francisco. From each group of mice from which individuals were ta-

TABLE 1. Summary of experiments from which mice were taken for electron microscopy (EM)

| Expt no. | Time of harvest ^a (days) | Yield of <i>M. leprae</i> ^b ($\times 10^6$) | Mean time to plateau ^{a, c} (days) | Time of sacrifice for EM ^a (days) | Time before (+) or after (-) plateau (days) | Designation of EM prepn |
|-----------|--|--|---|--|---|----------------------------|
| m 6-8-70 | 126 | 7.6 | 145 | 86 | +59 | A |
| | | 4.1 | | | | |
| | 5.4 | | | | | |
| | 13.0 | | | | | |
| 147 | 12.6 | | | | | |
| | 5.9 | | | | | |
| 148 | 5.4 | | | | | |
| m 9-3-69 | 117 | 0.4 | 176 | 120 | +56 | B |
| | | 0.4 | | | | |
| | | 0.2 | | | | |
| | | 1.4 | | | | |
| | 166 | 5.9 | | | | |
| | 167 | 3.2 | | | | |
| | 168 | 11.3 | | | | |
| 175 | 11.2 | | | | | |
| m 10-8-69 | 117 | 2.2 | 138 | 90 | +48 | C |
| | | 4.2 | | | | |
| | | 19.9 | | | | |
| | | 6.5 | | | | |
| | | 11.1 | | | | |
| | | 19.0 | | | | |
| | | 14.4 | | | | |
| | | 19.4 | | | | |
| | | 30.4 | | | | |
| | | 30.1 | | | | |
| | | 21.3 | | | | |
| | | 208 | | | | |
| m 5-19-70 | 139 | 11.5 | 145 | 106 | +39 | D |
| m 5-13-70 | 139 | 21.0 | 125 | 112 | +13 | E |
| | 154 | 11.5 | | | | |
| m 8-5-69 | 162 | 18.4 | 151 | 150 | +1 | F |
| m 4-9-70 | 160 | 37.5 | 135 | 142 | -7 | G |
| m 4-30-70 | 139 | 24.5 | 117 | 125 | -8 | H |
| | 152 | 21.0 | | | | |
| m 3-17-70 | 146 | 11.9 | 143 | 168 | -25 | I |
| m 3-11-70 | 152 | 25.9 | 134 | 173 | -39 | J |

^a Days since inoculation.

^b Number of organisms per footpad, based on a harvest from the pooled tissue of four footpads.

^c The time to plateau is calculated assuming a constant doubling time of 13 days for this strain of *M. leprae* in these BALB/c mice.

ken for histologic study, at least one harvest of *M. leprae* was performed. Harvests were carried out on the pooled footpad tissue of at least four mice. The time to plateau was calculated from the number of organisms first inoculated, the number harvested, and the time from inoculation to harvest, assuming a constant doubling time during logarithmic multiplication.

Because killing of *M. leprae* in the mice begins shortly after the plateau of multiplication is achieved, the proportion of viable organisms will vary among harvests and among the inocula prepared from the harvests. Therefore, the time course of multiplication of *M. leprae* will vary from experiment to experiment. To be able to superimpose growth curves from more than one experiment, it is necessary to use a time scale related to the time to plateau rather than to the time of inoculation. This method was used here.

Electron microscopy studies were carried out in Menlo Park, Calif. The footpad was dissected free and fixed in 2% gluteraldehyde buffered with cacodylate to pH 7.2 for up to 24 hr. It was then sliced, washed in buffer, postfixed in 1% OsO₄ containing 7.5% sucrose buffered with Veronal acetate to pH 7.2, dehydrated in alcohol, and embedded flat in Beem capsules with Araldite. Samples for light microscopy were cut with a glass knife at 1.0 μm and stained with Toluidine Blue. Samples for electron microscopy were cut with a diamond knife on a Porter Blum MT2 ultramicrotome, placed on nickel grids, stained with uranyl acetate and lead citrate, and viewed with a Philips 200 electron microscope.

RESULTS

The data describing the experiments from which mice were taken for electron microscopic study of the infected footpad tissue are presented in Table 1. The results of harvests for each experiment are shown, together with the calculated time to plateau, the time mice were taken for electron microscopic study, and the time of study relative to the time of plateau. Figure 1, in which the log number of *M. leprae* recovered in each of the harvests listed in Table 1 has been plotted as a function of the time before or after plateau, demonstrates that the timing of the electron microscopic studies in the manner used in this experiment is valid. The results of the harvests may be seen to fall along two straight lines, the regression equations for which are:

$$\log \text{ no. of } M. \text{ leprae} = 5.83 + 0.022(7.74 - \text{no. of days before plateau}) \quad (1)$$

$$\log \text{ no. of } M. \text{ leprae} = 6.32 + 0.0006(\text{no. of days after plateau} - 43.33) \quad (2)$$

Line 1, which describes the logarithmic phase of multiplication, has a correlation coefficient of 0.93; the logarithmic increase in bacterial numbers of 0.022 per day represents a doubling time of 13.7 days. The logarithmic phase of multiplication may be seen to extend from 59 days before until 15 days after the plateau value of 10⁶ *M. leprae* per footpad has been achieved.

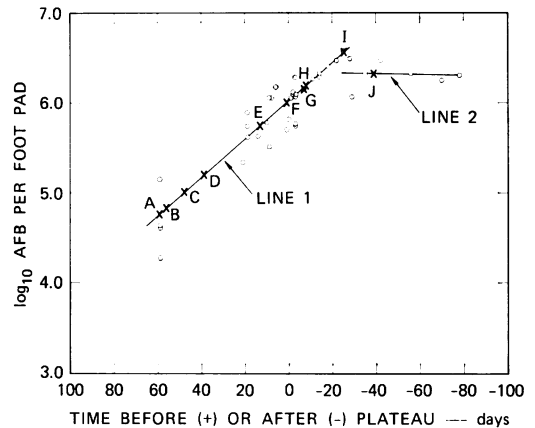


FIG. 1. Composite growth curve of *Mycobacterium leprae* in the 10 groups of mice from which individuals were taken for histologic study. The log number of *M. leprae* recovered per footpad in each harvest is plotted as a function of the time before or after the plateau of 10⁶ organisms/footpad was achieved in each group of mice. Curve may be resolved into two components: line 1, logarithmic phase; and line 2, stationary phase of multiplication. The letters A through J indicate the time with respect to plateau when mice were sacrificed for electron microscopy.

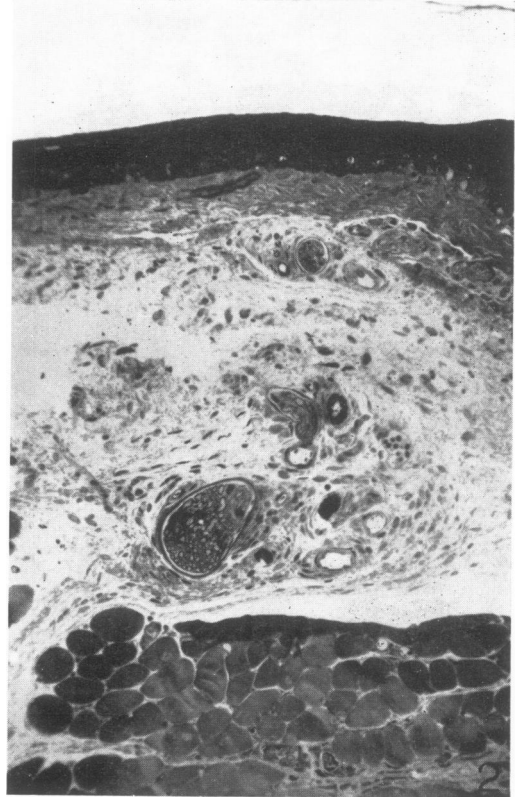


FIG. 2. Low-power view of a mouse footpad. Sectioned at 1 μm and stained with Toluidine Blue. × 125.

Line 2 describes a stationary phase of bacterial multiplication. Although bacterial numbers are not decreasing at this time, the proportion of viable organisms is shown to decrease after the peak of multiplication has been reached (4, 10).

Preparations A through D represent early

logarithmic multiplication, whereas E was made late in the logarithmic phase; F, G, and H represent the plateau, and preparations I and J were made during the end of logarithmic multiplication and the early portion of the stationary phase.

An example of the area of mouse footpad tis-

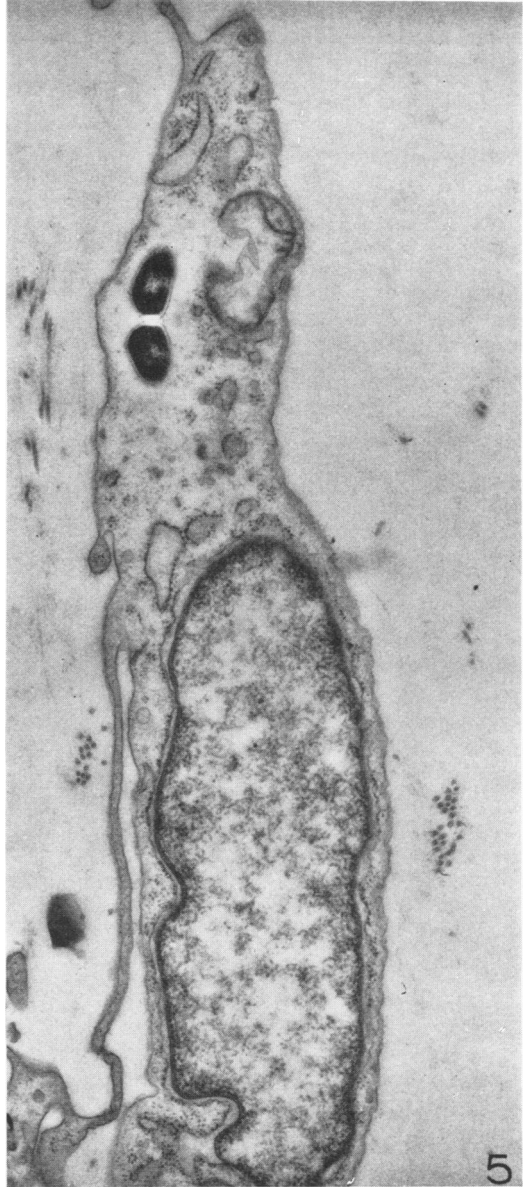
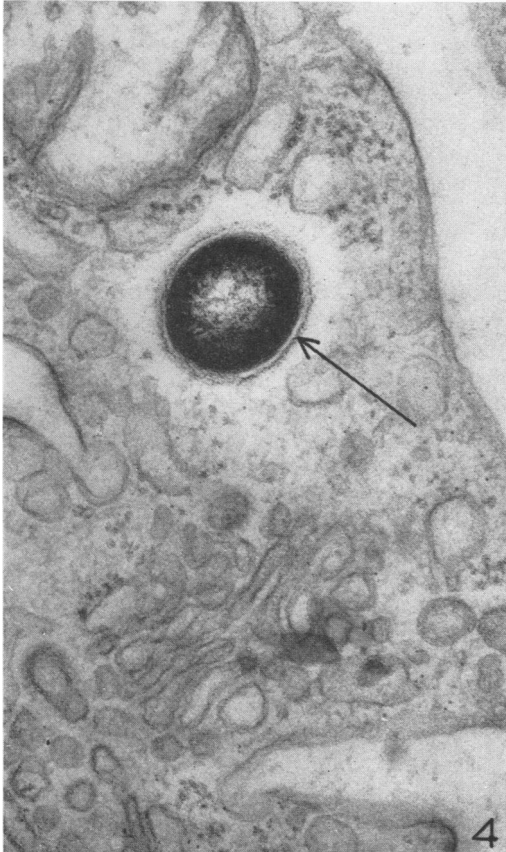
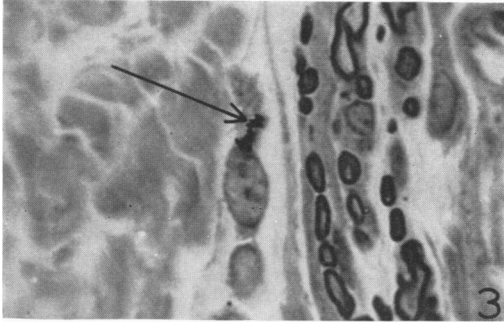


FIG. 3. Light micrograph of organisms (arrow) in a macrophage of the loose connective tissue, 56 days before plateau was reached. $\times 1,250$.

FIG. 4. Organisms in macrophages 13 days before plateau. They are surrounded by a single membrane. Note lack of lysosomes, vacuoles, and residual bodies. $\times 17,500$.

FIG. 5. An organism in a macrophage 13 days before plateau. Note the cytological detail in the organism, the single membrane surrounding it (arrow), and the less dense area of cytoplasm that surrounds it (halo). $\times 67,250$.

sue that was studied is illustrated in Fig. 2. Cross sections of the footpad like this allowed us to study the epidermis, hypodermis, muscles, nerves, tendons, and the loose connective tissue around these structures. These sections were scanned with the light microscope, and an area containing organisms was selected. This area was then trimmed and sectioned for electron microscopy.

The footpads from 10 different mice were used to study the logarithmic phase of *M. leprae*. Eight animals were sacrificed at times from 59 to 39 days before plateau was reached (preparations A through D). In these tissue sections, very few organisms were observed. Two mice were sacrificed 13 days before plateau was reached (preparation E). They were similar to the other ani-

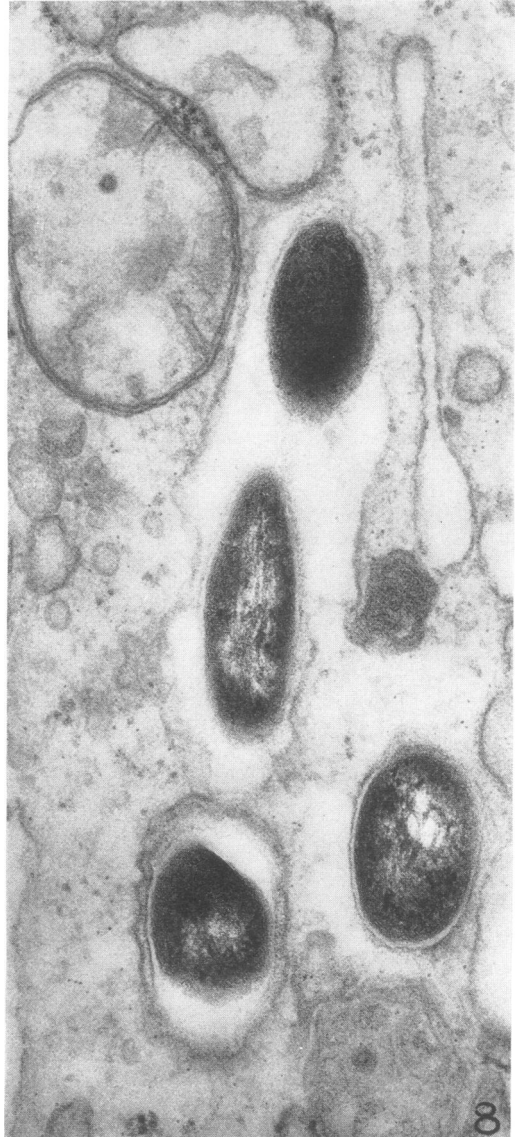
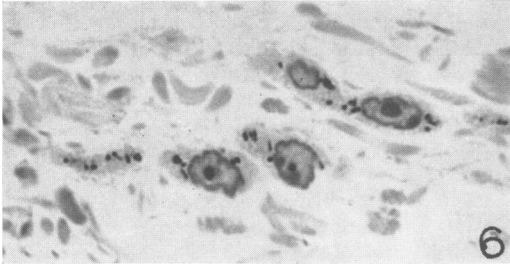


FIG. 6. Light micrograph of organisms in macrophages 1 day before plateau. $\times 1,250$.

FIG. 7. Organisms in macrophages 8 days after plateau. Notice the membrane that has formed around the periphery of the halo and encloses the organisms (arrow); also membrane-bound residual bodies (R). $\times 17,500$.

FIG. 8. Examples of organisms in the cytoplasm of a macrophage 8 days after plateau: (i) bound by a single membrane and surrounded by a halo; (ii) bound by a single membrane, surrounded by a halo, with another membrane at the periphery of the halo; (iii) surrounded by a double membrane. $\times 67,250$.

mals except that there were more organisms. The organisms were usually within fibroblast-like cells of the loose connective tissue (Fig. 3). Because these cells contain organisms, we have referred to them as macrophages; however, they contain very few lysosomes, vacuoles, or residual bodies (Fig. 4). The organisms in the macrophages usually have good cytological detail and are surrounded by a single membrane. Outside this membrane, the cytoplasm of the cell is usually less

dense, forming a halo around the organisms (Fig. 5). There are no other membranes around the organisms at this time. The macrophages containing organisms tend to be in groups in the loose connective tissue. No organisms are seen in the extracellular space or in striated muscle fibers.

The footpads from six mice were used to study the plateau of *M. leprae* infection (preparations F through H). The animals were sacrificed at in-

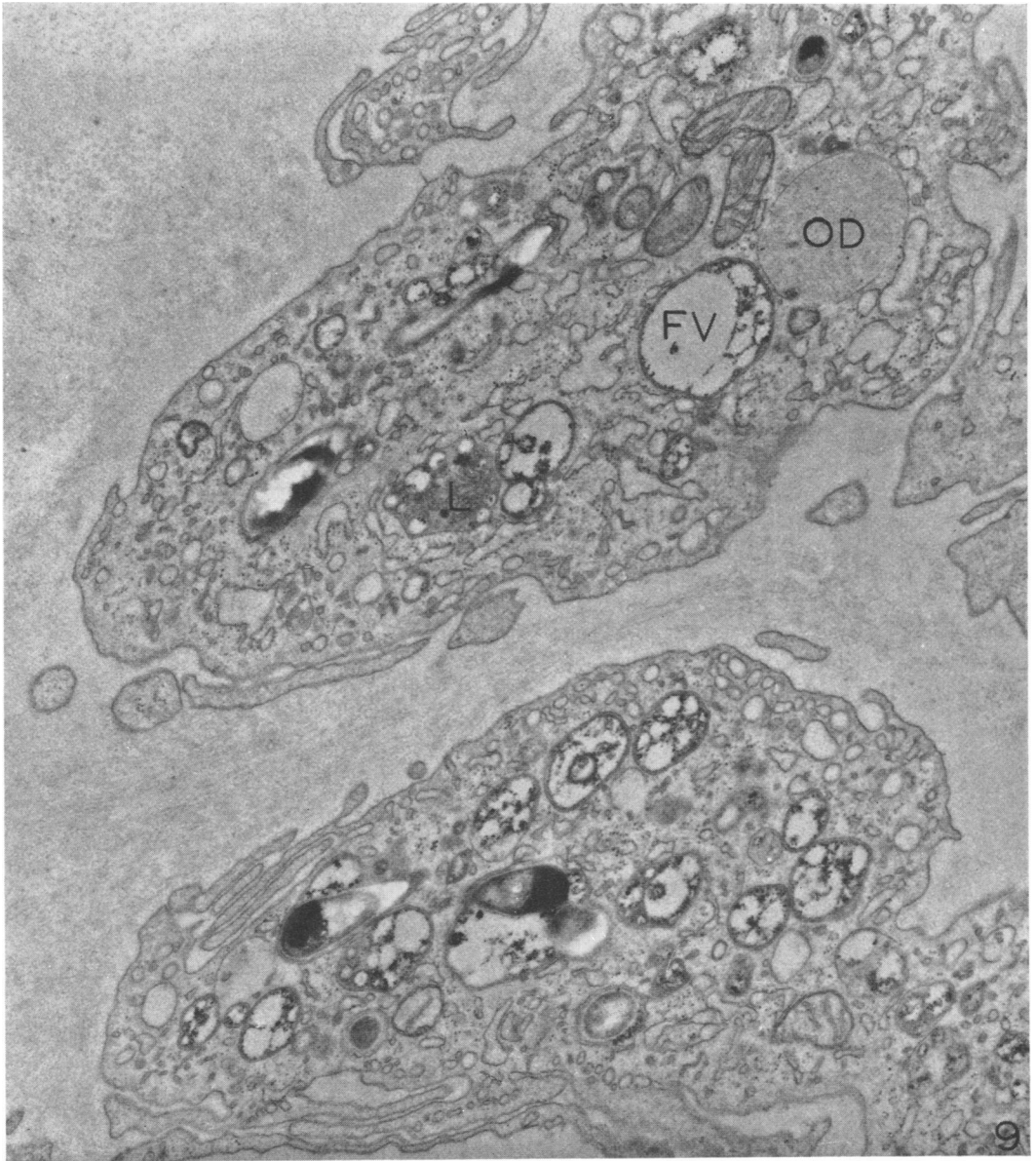


FIG. 9. Organisms in foamy macrophages 1 day before plateau. In contrast to other macrophages at this time and earlier times (Fig. 4), there are opaque droplets (OD), foamy vacuoles (FV), and lysosomes (L). $\times 22,750$.

tervals from 1 day before to 8 days after plateau had been reached. Many more organisms were found at this time (Fig. 6). Almost all are located in macrophages of the hypodermis and the loose connective tissue around muscle fibers. Upon closer analysis, it was found that the organisms were in three different conditions and that there was a strong tendency for all of them to be in the

same condition in any given cell. In the first condition, the organisms are surrounded by a single membrane, and few lysosomes or residual bodies are present in the macrophage. These cells are similar to macrophages reported in the early growth phase (Fig. 4 and 5). Secondly, the organisms are surrounded by two membranes (Fig. 7 and 8). In these cells, there may or may not be a

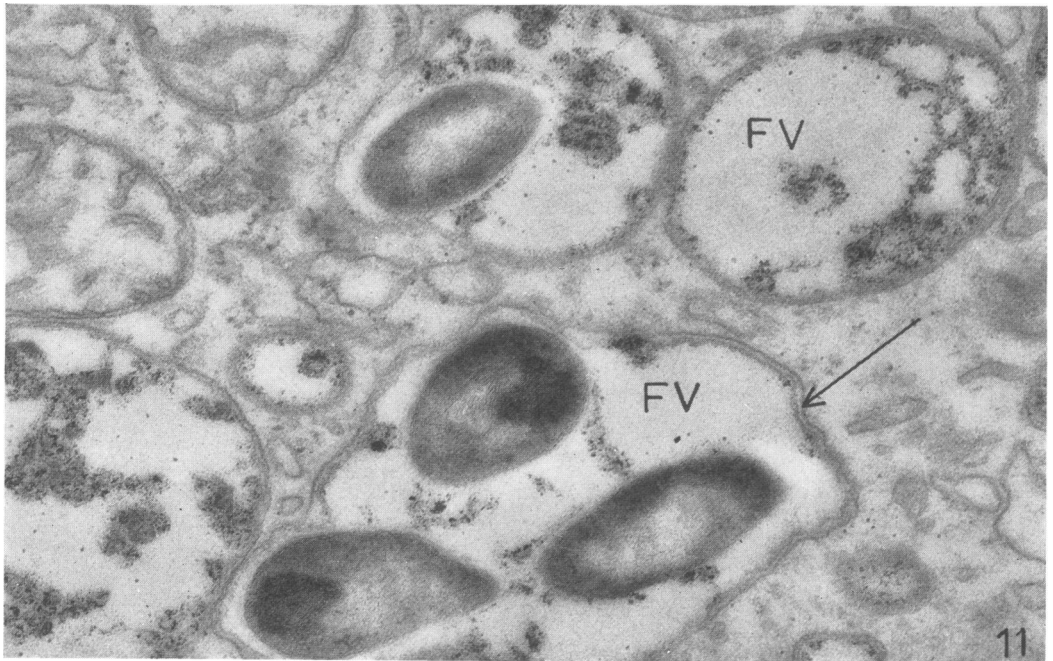


FIG. 10 and 11. Organisms in a macrophage 1 day before plateau, showing the relationship of opaque droplets (OD), foamy vacuoles (FV), and a lysosome-like combination (C) of these to the organisms. Note the double membranes around these structures (arrows). $\times 39,000$.

significant number of lysosomes and residual bodies. Figure 8 shows an organism surrounded by a double membrane. Notice that the second membrane forms at the periphery of the halo around the organism. The two membranes appear to come together so that the organisms are surrounded tightly. Finally, organisms are observed in "foamy" macrophages and appear to have de-

generated (Fig. 9, 10, 11). There are numerous vacuolated structures, some containing organisms, together with occasional lysosomes and opaque droplets (Fig. 9). In foamy macrophages, bacteria may be found inside vacuoles that contain (i) opaque droplets, (ii) dense granules similar to those in the foamy structures, or (iii) a combination of the two (Fig. 10 and 11). Macrophages in

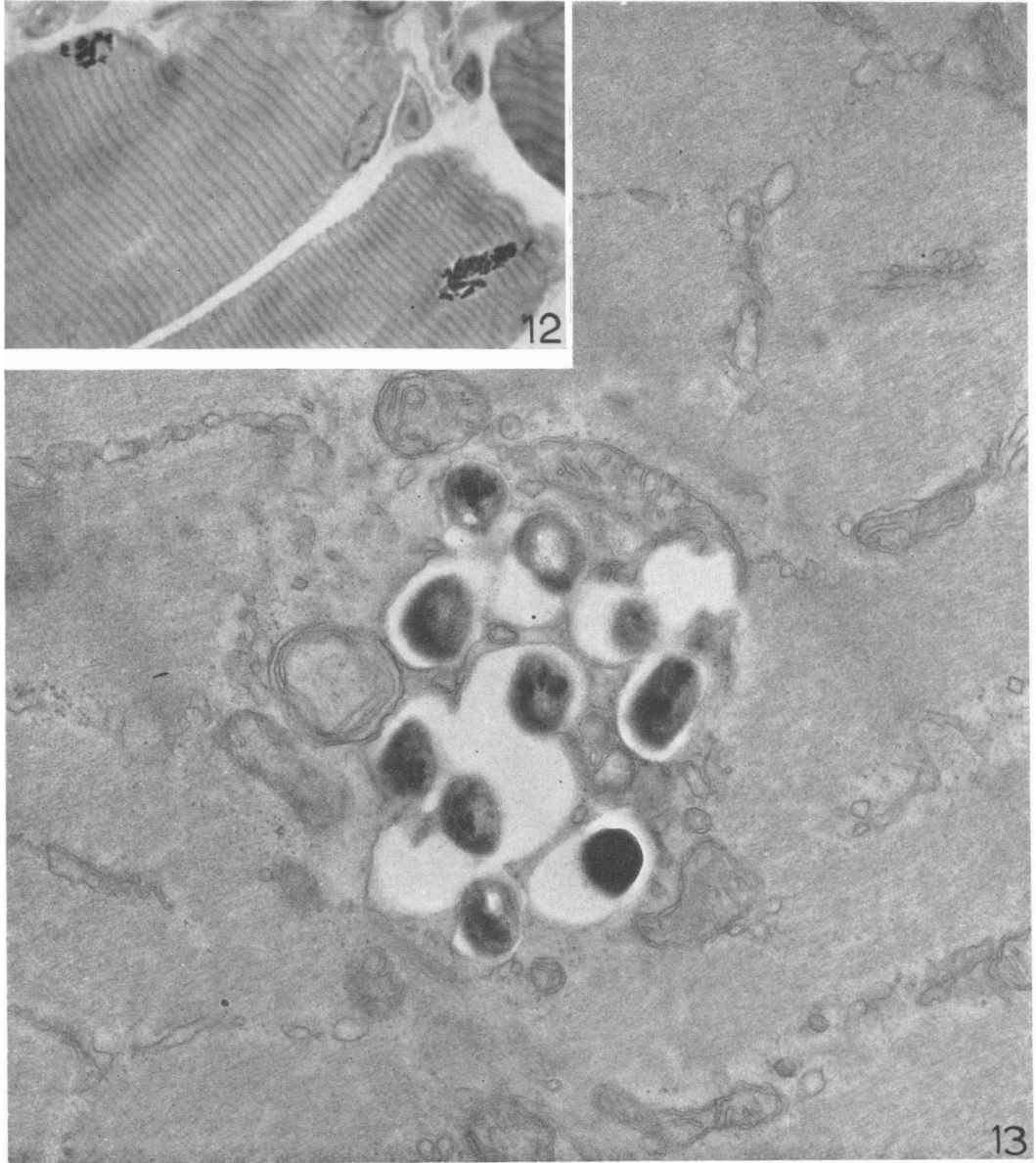


FIG. 12. Light micrograph of organisms in a striated muscle fiber 25 days after plateau. $\times 1,250$.

FIG. 13. Electron micrograph of same tissue as in Fig. 11. The organisms lie in the sarcoplasm and are not associated with double membranes as are organisms in the macrophages. $\times 39,000$.

the second and third condition are characteristic of immunologically activated cells (3, 15, 16).

The footpads from four mice were used to study the stationary phase (25 to 39 days after plateau) of *M. leprae* infection (preparations I and J). There is an increase in the number of macrophages, forming a diffuse granuloma in the loose connective tissue. Most of the macrophages that contain organisms are vacuolated and look like those in Fig. 9. However, an occasional macrophage was observed that contained bacteria surrounded by a single or double membrane similar to those in Fig. 4 and 7. At this time, organisms were also found in the striated muscle fibers of two animals (Fig. 12). The organisms are in the sarcoplasm and are not surrounded by a double membrane; they did not appear to be degenerating (Fig. 13).

DISCUSSION

These cellular changes are consistent with tissue changes reported during the development of delayed hypersensitivity and cellular immunity during the pathogenesis of tuberculosis (3). We did not directly measure delayed hypersensitivity; however, we detected ultrastructural changes in macrophages that are associated with the onset of cellular immunity. Development of cellular immunity requires activation of macrophages to increase their digestive and microbicidal capabilities, and proliferation to increase their number (2, 3). During the logarithmic growth phase, there were very few ultrastructural changes in tissue macrophages that would signify their activation. However, when the number of organisms reached about 10^6 , a point after which immunological factors have been shown to check the growth of *M. leprae* in the footpads of mice (8, 11), there were changes in macrophages that are associated with the onset of cellular immunity (1, 13, 14). These macrophages contained lysosomes, residual bodies, and organisms in vacuoles, and there was an increase in the number of macrophages. These observations suggest that the macrophage population had been immunologically activated.

Activation of the macrophages was also associated with the appearance of a second membrane around the organism. During the logarithmic growth phase, when the macrophages were not activated, most organisms were surrounded by a single membrane. However, as the plateau was reached and the macrophages were activated, most organisms were now surrounded by two membranes. The second membrane may be associated with lysosome-bacillus interaction since bacteria

bounded by double membranes are seen with lysosomes and vacuoles, whereas organisms bound by single membranes are not. We have no direct evidence from which to determine whether the second membrane is of cytoplasmic or bacterial origin. However, it appears first at some distance from the organism, at the outer limit of the electron-transparent area, suggesting that it is produced by the cytoplasm.

M. leprae has been reported in striated muscle fibers, and it was suggested that this may be an important site for their multiplication (5, 6, 7). In the present study, the majority of the organisms we observed were in macrophages. They were not found in muscle cells until after plateau was reached. Because most multiplication of organisms takes place during the growth phase before plateau is reached, it suggests that the organisms multiply in macrophages (1, 2). The main argument against the macrophage as a site for multiplication of *M. leprae* is the unfavorable environment produced by an activated macrophage (6). We have shown that during the logarithmic phase the macrophages do not appear to be activated. If this is the case, then the macrophages would not inhibit multiplication of the organisms. However, after plateau, when the macrophages are activated, they may not be as favorable a site for bacterial multiplication. At this time multiplication in muscle cells might be the preferred site.

ACKNOWLEDGMENT

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