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# In Vitro Response of Rabbit Alveolar Macrophages to Infection with *Nocardia asteroides*

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The interaction of *Nocardia asteroides* with cultured "normal" nonimmune rabbit alveolar macrophages was studied by light and electron microscopy. It was shown that the alveolar macrophage response to the more virulent strain (*N. asteroides* 14759) was quite different from the response to the less virulent organism (*N. asteroides* 10905). *N. asteroides* 14759 elicited a dramatic in vitro response of the macrophages toward the nocardial infection. Within a few hours postinfection, there was a migration of macrophages toward other cells actively infected with viable nocardia, so that at 6 h considerable macrophage aggregation on the cover slips had occurred. Many of the macrophages within these aggregates exhibited tight cell-to-cell contact, whereas others were observed to fuse, forming multinucleate giant cells, with many containing more than 10 nuclei. Upon continued incubation, these giant cells appeared to destroy the intracellular nocardia, so that, at 24 h postinfection, gram-positive, ultrastructurally intact bacteria could not be observed. At the same time, some of the macrophage aggregates that did not fuse appeared to be unable to stop the intracellular growth of nocardia. At 12 to 24 h large numbers of gram-positive, acid-fast filaments were observed growing out from within these macrophage aggregates. The macrophage response seemed dependent upon the strain of *Nocardia* infecting them, since *N. asteroides* 10905 did not induce a similar response within the macrophage population.

*Nocardia asteroides* ATCC 14759 induces a granulomatous response during experimental infections in rabbits (9). When saline suspensions of this organism were injected either intraperitoneally or intravenously into Swiss Webster mice, progressive metastatic lesions were formed that ultimately led to animal death. These lesions were either abscesses, containing predominately polymorphonuclear neutrophils and macrophages, or occasionally granulomas, consisting almost entirely of macrophages and lymphocytes (Beaman, unpublished data). Furthermore, there were always lesions in the lungs irrespective of the route of inoculation. Because of these observations, Beaman and Smathers studied the interaction of this strain of *Nocardia* in cultured rabbit alveolar macrophages to determine whether virulent strains of *N. asteroides* grew as intracellular parasites within macrophage populations (3). It was observed that the macrophage response toward infection with this strain was significantly different from that of the response towards the less virulent *N. asteroides* 10905.

When $10^6$ colony-forming units (CFU) of stationary-phase *N. asteroides* 14759 was added to $10^6$ rabbit alveolar macrophages, approximately 99% of the bacterial cells were phagocytized by 2 to 3 h (3). Within 6 h postinfection, the number of CFU isolated from the macrophages had increased significantly (3). At the same time it was shown that little or no growth occurred extracellularly within the culture medium (3). During this 6 h postinfection, a dramatic response of the macrophages toward *N. asteroides* 14759 was observed. In contrast, when $10^6$ CFU of stationary-phase *N. asteroides* 10905 was added to the same number of macrophages, there was a rapid uptake of bacterial cells, with a subsequent decrease in organisms that could be isolated from the macrophage population. No significant macrophage response toward infection with *N. asteroides* 10905 was evident (3). This report presents a more detailed analysis of the in vitro alveolar macrophage response to infection with *N. asteroides* as observed by light and electron microscopy.

## MATERIALS AND METHODS

**Microorganisms.** Clones of *N. asteroides* 14759 and 10905 were maintained on brain heart infusion agar as previously described (1, 3).

**Animals.** New Zealand white rabbits, weighing

approximately 2.3 to 2.7 kg (5 to 6 lb.), were obtained from the B and H Rabbitry (Rockville, MD.) and from Nitabell Rabbitry (Hayward, Calif.). They were used as the source of the alveolar macrophages.

**Collection and maintenance of alveolar macrophages.** Alveolar macrophages were obtained and cultured as described by Beaman and Smathers (3).

**Infection of macrophages.** The same methods previously employed by Beaman and Smathers (3) were used in the experiments described below.

TABLE 1. *Multinucleated giant cells at 6 h postinfection*

| Organism | Avg no./1,000 macrophages | |
|---|---|---|
| | 4 to 5 nuclei/cell | >5 nuclei/cell |
| *Nocardia asteroides* 14759 | 40.5 ± 2[a] | 16.0 ± 3 |
| *N. asteroides* 10905 | 3.0 ± 1 | 0.5 ± 0.2 |
| Control (uninfected) | 1.5 ± 0.5 | 0 |

[a] Standard deviation of the mean.

**Light microscopy.** Essentially the methods described by Beaman and Smathers (3) and Bourgeois and Beaman (4) were used. All samples were done in duplicate on at least four different rabbits for each nocardial strain. The data shown in Table 1 represent blind counts of 1,000 macrophages per individually coded cover slip, and the standard deviation of the mean values was tabulated as shown (Table 1). All light micrographs were taken of Gram-stained preparations by a Ziess research microscope and Kodak Plus X film. A standard green filter (Asahi Optical Co.) was used to enhance contrast.

**Transmission electron microscopy.** Control and infected macrophages were removed from cover slips, fixed in 3.0% glutaraldehyde, postfixed in 1.0% osmium tetroxide, and embedded in Maraglas (Ladd) as described by Bourgeois and Beaman (4). Blocks were sectioned with a diamond knife (DuPont) on an MT-2 Porter Blum ultramicrotome, Sections were stained with lead citrate for 60s and photographed through a Philips EM300 electron microscope operated at 60 kV (1). Each experiment was repeated three times.

**Scanning electron microscopy.** Cover slips were

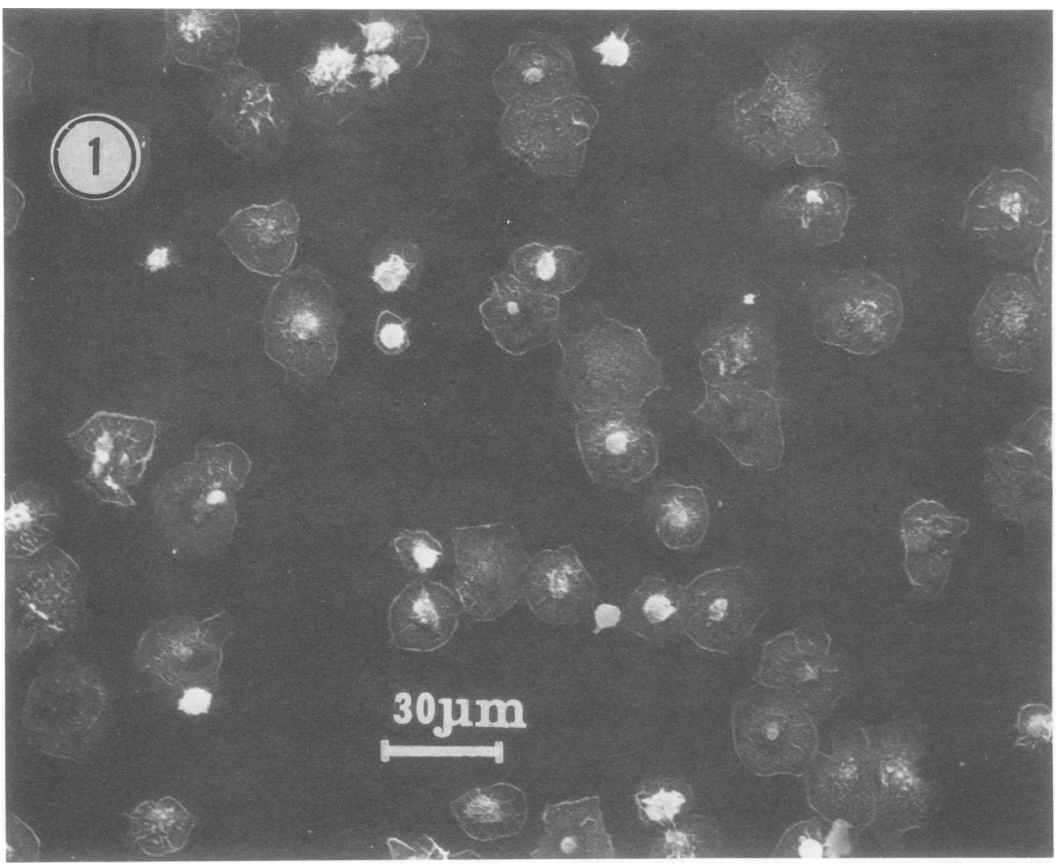


FIG. 1. *Scanning electron micrography of uninfected "normal" rabbit alveolar macrophages maintained on cover slips for 24 h. These macrophages served as uninfected controls. Low magnification shows random distribution of the cells and demonstrates that most of the adherent cells have spread onto the cover slip.*

fixed in 3.0% glutaraldehyde in Kellenberger buffer (pH 6.5) for approximately 1 h. The cover slips were then washed with buffer, dehydrated through a series of ethanols (50, 75, 95, and 100%), and critically point dried in a Sorvall critical-point dryer with liquid $CO_2$. Dried specimens were coated with gold-palladium in a Hummer (Technics, Inc.). The samples were photographed by using Polaroid film 55P/N and an Etec Autoscan scanning electron microscope operated at 20 kV. All experiments were repeated three times.

## RESULTS

Scanning electron microscopy revealed that after 24 h of incubation, unstimulated rabbit alveolar macrophages had spread onto the surface of glass cover slips so that the peripheral cytoplasm was flat and smooth-surfaced (Fig. 1). The central or nuclear portion of the cells was raised, and it frequently had a prominent ruffled surface (Fig. 1). In the presence of *Nocardia*, the macrophages became rounded, phagocytic processes came into contact with the bacterial cells, and surrounded them, and the organisms became phagocytosed (Fig. 2). By 3 h postinfection, few bacteria could be observed at the surface of the cell; however, transmission electron microscopy demonstrated bacteria intracellularly (see Fig. 8 and 10). The macrophages that had phagocytosed nocardia remained rounded, and it was observed that they no longer spread out onto the glass surface as did the uninfected control cells. The macrophage response and surface topography upon continued incubation depended upon the strain of *Nocardia* used for infection.

Two cellular events were observed in macrophages infected with the virulent *N. asteroides* 14759. Initially, a migration of the macrophages toward cells infected with nocardia occurred so that at 6 h postinfection there was extensive macrophage aggregation (Fig. 3B) as compared with the uninfected control (Fig. 3A). Many of the macrophages within these aggregates exhibited tight cell-to-cell contact (Fig. 4 and 5), whereas others were observed to fuse, forming multinucleate giant cells (Fig. 6). The giant-cell formation appeared to be a specific response of the macrophages to the virulent strain of *N. asteroides*, since very few giant cells were observed in macrophages infected with the less virulent *N. asteroides* 10905 or in the uninfected macrophage controls (Table 1). The number of multinucleated giant cells found on cover slips 12 and 24 h postinfection per 1,000 macrophages did not increase beyond the levels shown in Table 1; in fact, there appeared to be a slight decrease. However, a marked change in the morphology and nuclear arrangement of the giant cells was observed at 24 h postinfection (Fig. 6).

Upon continued incubation, the giant cells seemed to destroy the intracellular nocardia (Fig. 7, arrow). Therefore, at 12 to 24 h, gram-

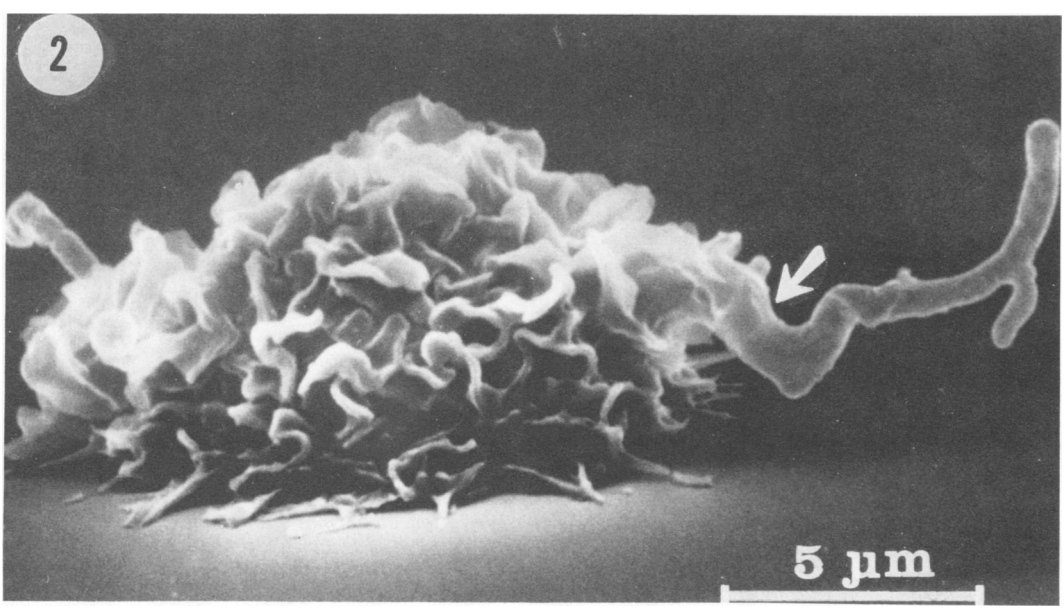


FIG. 2. *Scanning electron micrographs of N. asteroides being phagocytosed by rabbit alveolar macrophages (as in Fig. 1). Side view of a macrophage 1 h postinfection. The arrow notes area where nocardial cell is being engulfed by the macrophage. Note that the macrophage is raised, and a ruffled surface is prominent.*

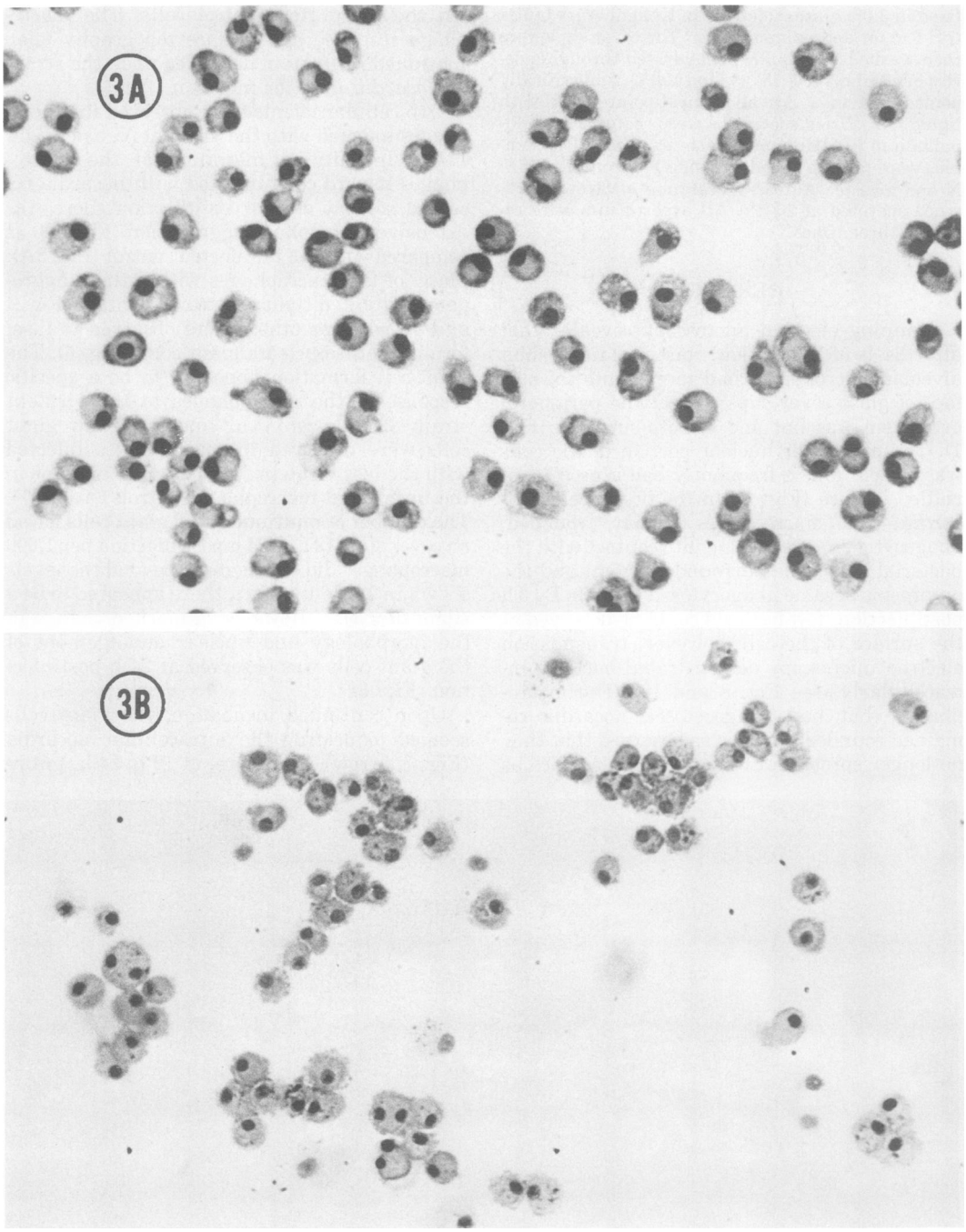


FIG. 3. *(A) Light micrograph of a Gram-stained preparation of normal rabbit alveolar macrophages cultured in vitro for 30 h. Note the random distribution of the cells (uninfected controls of 3B). (B) Light micrograph of a Gram-stained preparation of normal rabbit alveolar macrophages 6 h postinfection with N. asteroides 14759. Note that most of the infected macrophages have formed aggregates.*

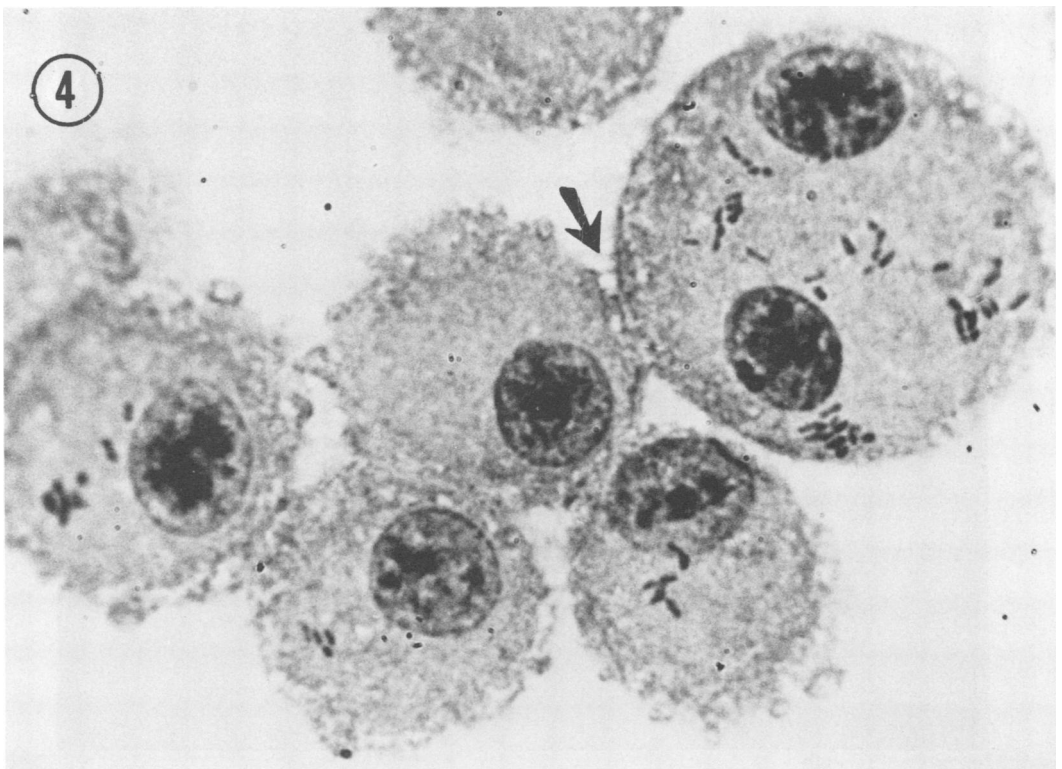


FIG. 4. *Light micrograph of rabbit alveolar macrophages 6 h postinfection with N. asteroides 14759. Note the macrophage aggregation and close cell-to-cell contact. Arrow points to area where two macrophages may be in the process of cell fusion.*

positive organisms could not be observed within them (Fig. 6). Furthermore, many of the giant cells had undergone nuclear rearrangement, so that at 24 h of incubation many appeared as typical "Langhan's giant cells," with the nuclei arranged in a peripheral circle (Fig. 6). This type of giant cell was never observed in the uninfected control macrophages or in macrophages infected with the avirulent *N. asteroides* 10905.

The second and most frequent cellular response of the macrophages toward infection with *N. asteroides* 14759 was the failure of the macrophage aggregates to fuse (Fig. 8). The nocardial cells were able to grow freely within these macrophages (Table 2). Figure 8A and Table 2 show that at 3 h postinfection, the macrophages infected with the virulent *N. asteroides* 14759 possessed approximately three gram-positive cells per macrophage. Light microscopy revealed that these bacterial cells were short rods or cocci. Long, gram-positive filaments were not observed in Gram-stained preparations. Furthermore, these cells were not acid-fast. At 6 h, there was both an increase in numbers of gram-positive cells per macrophage and in bacterial cell length (Fig. 8B and Table 2). A few of these organisms were also acid-fast. At 24 h postinfection, large numbers of strongly acid-fast, gram-positive branching filaments were observed growing out of individual macrophages as well as macrophage aggregates (Fig. 8C and Table 2). Acid-fastness appeared to be strictly associated with growth within the macrophage, since filaments growing within the tissue culture medium without macrophages were not acid-fast. In addition, it was observed that macrophages that contained large numbers of growing nocardia became very smooth-surfaced spheres, with the nocardial filaments protruding through the cytoplasm (Fig. 9A and B). Transmission electron microscopy revealed, however, that these filaments were still surrounded by a thin layer of macrophage cytoplasmic membrane (Fig. 10). Macrophage aggregation and nocardial cell outgrowth were not observed in macrophages infected with *N. asteroides* 10905 (Table 2).

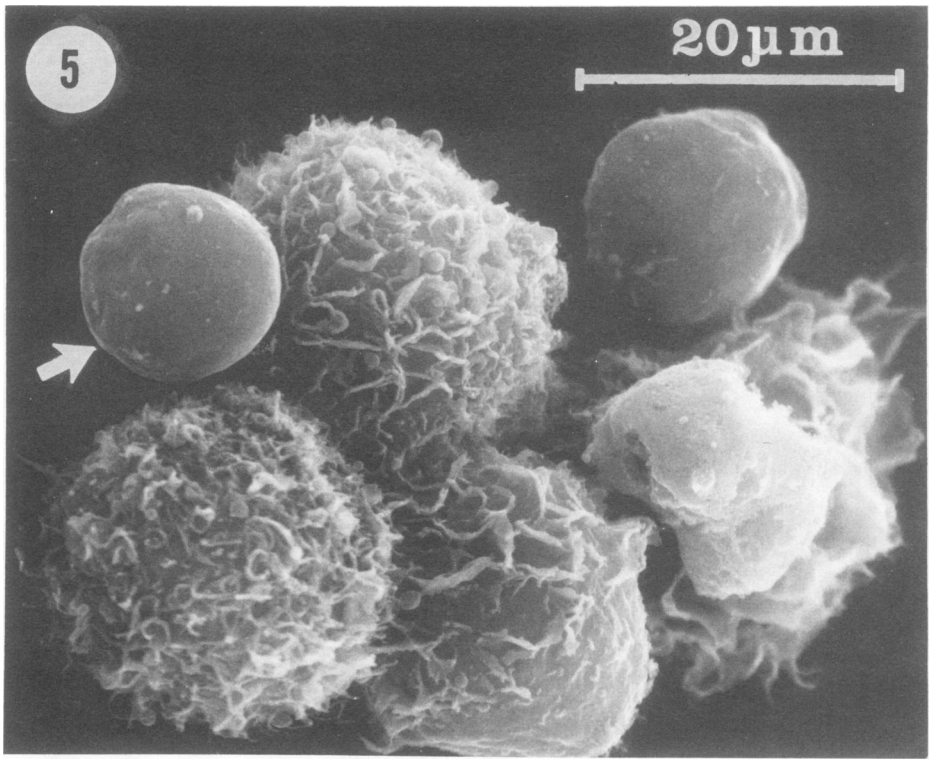


FIG. 5. *Scanning electron micrograph of alveolar macrophage aggregation 6 h postinfection with N. asteroides 14759. Compare with uninfected controls in Fig. 1. Note that the infected macrophages are rounded into spherically shaped cells with a highly ruffled surface. Also note the presence of smaller, smooth-surfaced cells (arrow); these may be lymphocytes.*

## DISCUSSION

There are few reports in the literature concerning the interaction between cultured macrophages and *Nocardia* (3, 4, 6). There are a few studies of macrophage interactions with specific nocardial antigens (13–16). These studies showed that these antigens could inhibit macrophage migration within appropriately sensitized populations, thus demonstrating a cell-mediated immune response in vitro (13–16). Bourgois and Beaman (4) and Beaman and Smathers (3) showed that *N. asteroides* can function as facultative intracellular parasites, which is quite similar to observations regarding mycobacteria (19). However, from the work of Beaman and Smathers it became clear that the response of the nocardia to macrophages as well as the specific macrophage response towards *Nocardia* depended upon the strain of *Nocardia* being studied (3).

An ultrastructural study of the specific *Nocardia*-alveolar macrophage interaction has not been previously reported. However, the macrophage response toward mycobacteria and many other foreign substances have been studied in detail, and the literature is so voluminous that it is not possible to discuss completely the numerous observations here (19). For example, it is well established that macrophages become spread out and the cytoplasm somewhat flattened, when they attach to a glass surface (19). Furthermore, the surface of the phagocytic cell is ruffled (Fig. 1). Under appropriate conditions the macrophage will migrate to areas that contain foreign substances, especially in response to certain lymphocyte products. This represents a basic mechanism in cell-mediated immunity (19). Additionally, when a macrophage comes in contact with most particulate substances, it will engulf them (18). Figure 2 clearly shows this phagocytic process toward nocardial cells. Once the bacteria are within the macrophage, several events occur (2, 19). As a final result, (i) the bacterial cell may be killed and digested, or (ii) it may remain viable and either persist or replicate (2). Since both processes are occurring within the macrophage population, one is tempted to speculate that there are at least two different "macro-

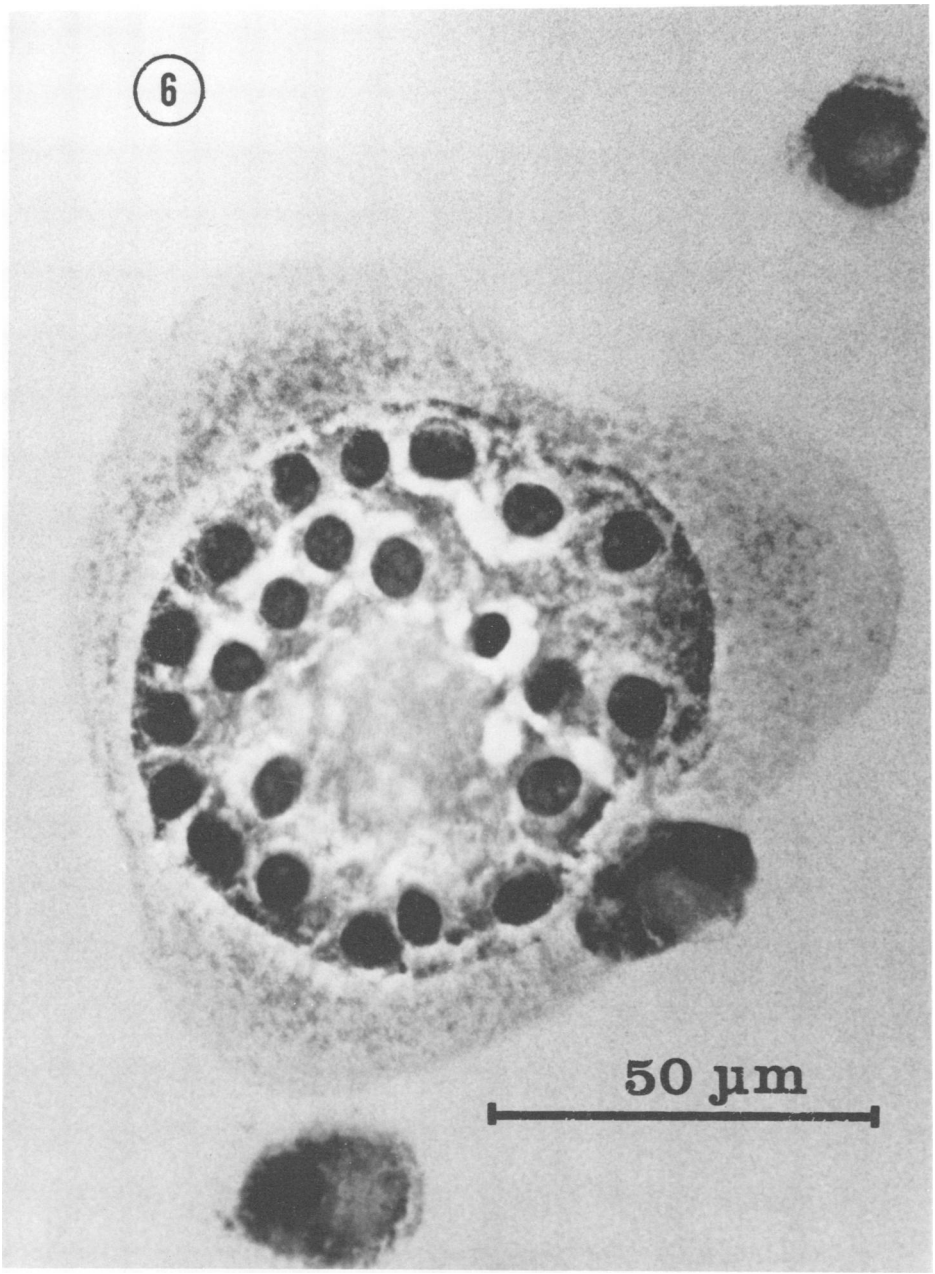


FIG. 6. *A "Langhan's"-like giant cell found in alveolar macrophage populations 24 h postinfection with N. asteroides 14759. This type of cell was not observed in either $10^4$ control macrophages or in $10^4$ macrophages infected with N. asteroides 10905. Note the characteristic peripheral arrangement of the nuclei within the giant cell (containing more than 20 nuclei). Also note the absence of intact (gram-positive) bacterial cells within the giant cell. (Compare with Fig. 8C.)*

phage types" present on the cover slips. Figure 8 demonstrates that in some of the macrophages the cells of the virulent *N. asteroides* 14759 are not destroyed, but instead they are able to grow until they extend out of the macrophage (Fig. 9 and 10). In contrast, some of the *N. asteroides* 14759 cells elicit a migratory response of some of the alveolar macrophages, so that several macrophages become closely associated with the infected cells (Fig. 3B and 4).

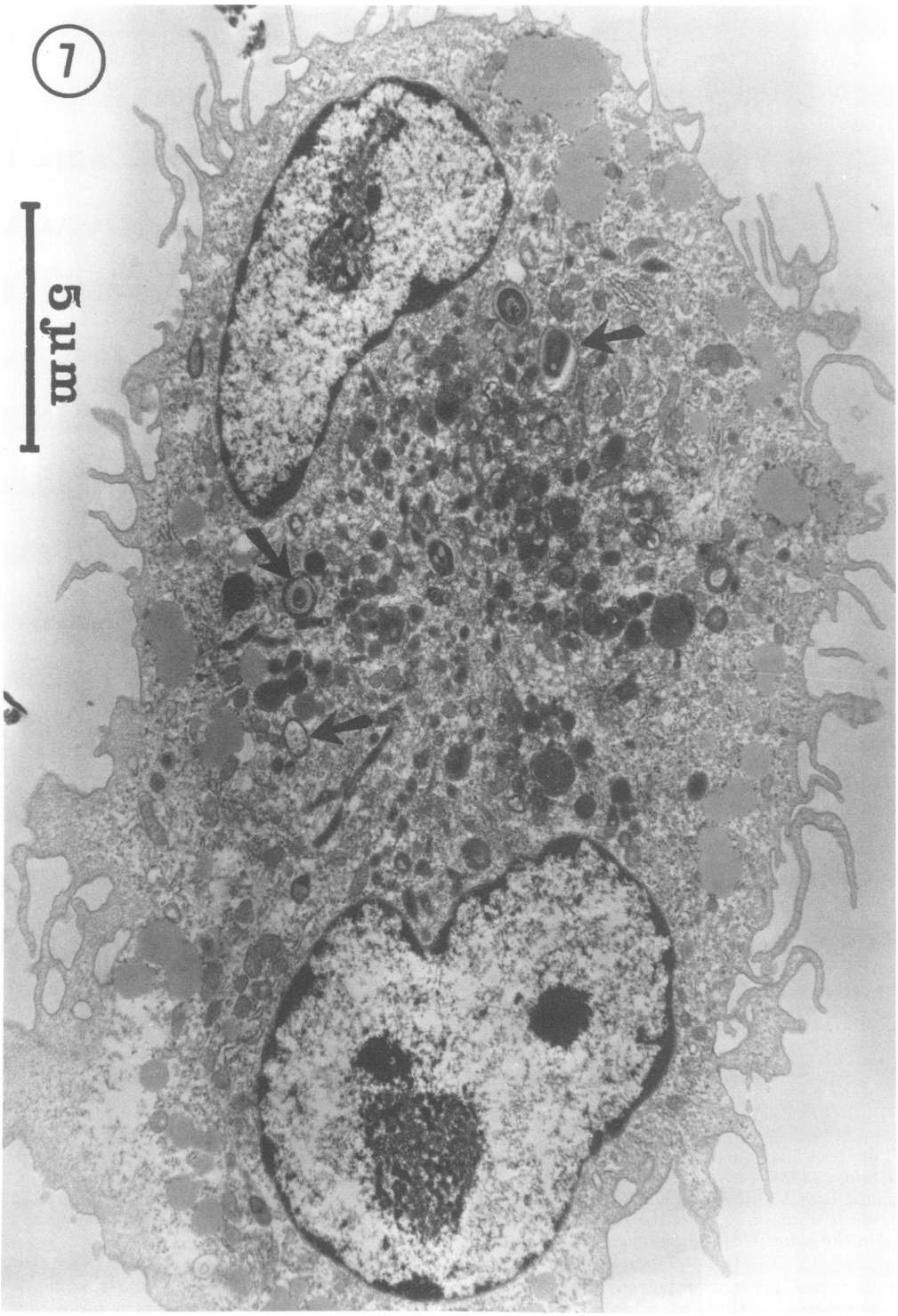


FIG. 7. *An electron micrograph of a thin section of a multinucleate cell 6 h postinfection. Note the presence of large numbers of pleomorphic, osmiophilic bodies (presumably lysosomal bodies) characteristic of "activated" macrophages (arrows). Note the presence of nocardial cells in various stages of destruction.*

TABLE 2. *Relative growth of Nocardia asteroides in cultured rabbit alveolar macrophages*[a]

| Organism | Time postinfection (h) | No. of CFU/macrophage | No. of gram-positive organisms/ infected macrophage |
|---|---|---|---|
| *N. asteroides* 10905 | 3 | 1.4 | 5.8 |
| | 6 | 0.8 | 4.9 |
| | 24 | 0.4 | 4.1 |
| | 48 | 0.1 | 2.1 |
| *N. asteroides* 14759 | 3 | 0.9 | 3.4 |
| | 6 | 3.5 | 6.1 |
| | 24 | 6.3 | 8.1 |
| | 36 | (complete overgrowth) | |

[a] The values represent an average of three determinations.

Many of these macrophages fuse as the result of the nocardial infection, thus forming multinucleate giant cells (Fig. 6).

In vitro macrophage fusion to form multinucleate giant cells similar to those found in granulomatous reactions in vivo is not a new observation. Giant cells were described in vitro as early as 1912 by Lambert (10). It is now known that multinucleate cells can be formed either as the result of nuclear division without cytoplasmic division (8) or by the fusion of the cytoplasms of several mononucleated cells (8). The data presented above show that the latter event occurred as a result of macrophage contact with *N. asteroides* 14759 but not with *N. asteroides* 10905. The fusion of macrophages in vitro to form giant cells may occur as a nonspecific event (5, 7, 11, 17, 18) or as an antigen-mediated event within macrophages from specifically sensitized animals (8). Thus, there are immunologically induced giant cells and foreign body-induced giant cells similar to what is observed in vivo (5, 7, 8, 11, 17, 18).

Draper indicated that giant-cell formation was induced in vitro after overnight incubation of nonsensitized macrophage cultures with suspensions of *Mycobacterium leprae* (7). In contrast, Galindo observed mycobacterial-induced macrophage fusion occurring in cells taken from rabbits that had been immunized with heat-killed *Mycobacterium tuberculosis* and then exposed in vitro to killed *M. tuberculosis*. These macrophages did not form multinucleate giant cells when no mycobacteria were added or when *Escherichia coli, Bacillus subtilis,* latex particles, etc., were added (8). He found that the addition of immune sera against *M. tuberculosis* potentiated giant-cell formation. Therefore, he concluded that fusion of rabbit alveolar macrophages was mediated by an immunological mechanism (8). He noted that heat-killed cells of *Nocardia brasiliensis* induced some giant cell formation in the macrophages obtained from *Mycobacterium*-sensitized animals, and he suggested that this was the result of antigenic cross-reactivity (8). Galindo did not indicate whether or not heat-killed cells of *N. brasiliensis* induced cell fusion in the nonsensitized cell population (8).

Presumably, the cells obtained from the "normal" rabbits during the current series of experiments described above have not been presensitized for either *Mycobacterium* or *Nocardia*, especially since macrophages from rabbits procurred at Georgetown University (see Materials and Methods) and those obtained at the University of California (Davis) gave the same response toward *N. asteroides* 14759 (see Table 1). Furthermore, if the macrophage response towards *N. asteroides* 14759 was mediated strictly by an immunological mechanism, then one would expect to see fusion of macrophages exposed to *N. asteroides* 10905 as well. This did not occur. Therefore, it is suggested that there is some response of the macrophages toward specific components present in or on *N. asteroides* 14759 (but not present in *N. asteroides* 10905) that results in membrane fusion. It is further suggested from the data (Fig. 5) that perhaps lymphocytes are present during the cell aggregation and fusion process; their specific function is unknown at the present time. It is well established that certain viruses can interact with mammalian cell membranes, causing cells to fuse (12). It is tempting to speculate that a similar kind of mechanism may be involved in nocardia-induced fusion of alveolar macrophages. It should be pointed out, however, that the data presented above do not completely rule out the possibility of a specific cell-mediated immune response. It is possible that all of the rabbits used had a low level of sensitization towards either nocardia or mycobacteria. Since the avirulent strain was killed while the virulent strain *N. asteroides* 14759 grew within the macrophages, the results observed might be due to differences in antigen concentration. Furthermore, it is possible that the replicating virulent organism could be producing substances that would have a mitogenic effect on

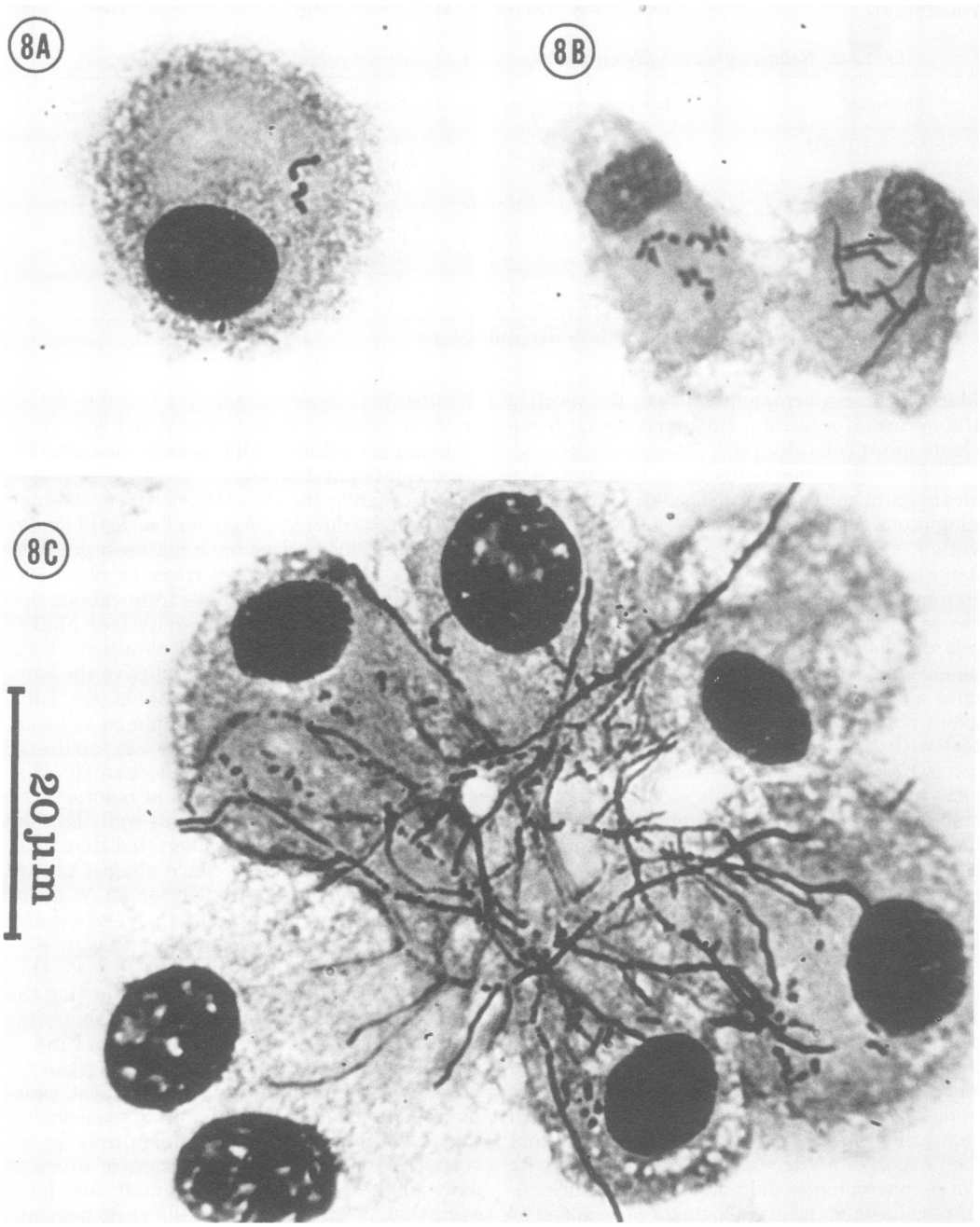


FIG. 8. *(A) Light micrograph of a Gram stain of an alveolar macrophage 3 h postinfection with N. asteroides 14759. Note that there are three gram-positive intracellular bacteria. The macrophages were incubated with an approximate 1:1 ratio of CFU to macrophages. (B) Gram stain of the above aveolar macrophage preparation 6 h postinfection. Note the interaction occurring between two infected macrophages (from the same cover slip preparation as Fig. 4A). At this time, the CFU/macrophage had increased significantly over the 3-h sample. Note the elongation of several nocardial cells within the macrophages. (C) Gram stain of the above alveolar macrophage preparation 24 h postinfection. Note the close aggregation of seven macrophages, some of which appear to have fused (from the same cover slip preparation as the cell in Fig. 6). Note the extensive outgrowth of nocardial filaments from within the alveolar macrophages. (All micrographs in Fig. 8 are magnified approximately the same.)*

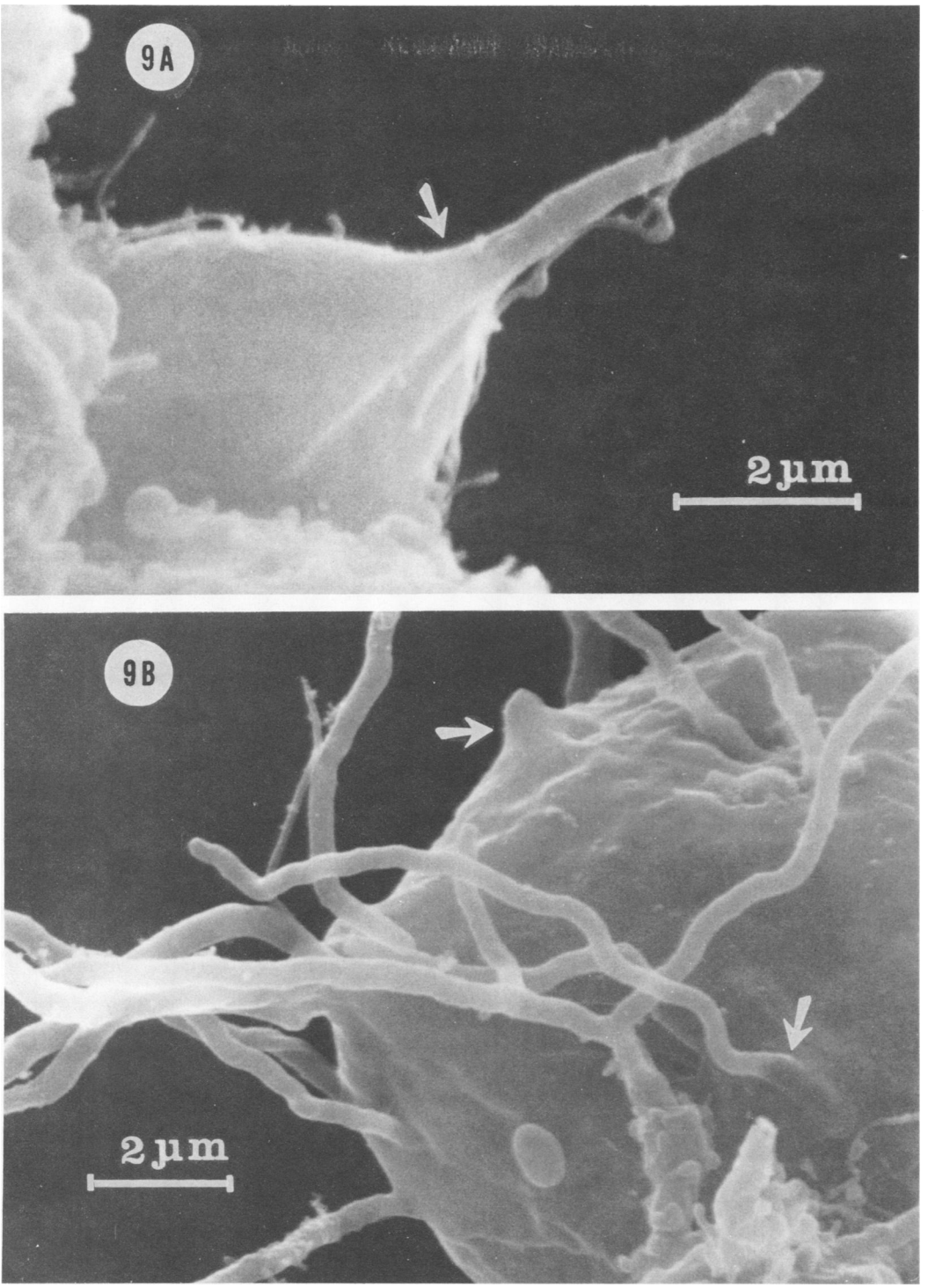


FIG. 9. *(A) Scanning electron micrograph of the surface of a rabbit alveolar macrophage 24 h postinfection with N. asteroides 14759. Note the outgrowth of a single nocardial filament and the smooth, stretched surface of the macrophage. The arrow notes that the outer surface of the macrophage membrane is continuous with the nocardial filament. Contrast this process with that of phagocytosis shown in Fig. 2. (B) The same as in 9(A) except numerous nocardial filaments have emerged from inside of the macrophage. The arrows indicate filaments in the process of beginning to push though the macrophage cytoplasmic membrane. It is important to note that these macrophages remained attached to glass; they were not lysed by this nocardial outgrowth; and they still excluded trypan blue. Therefore, we conclude that they are viable cells.*

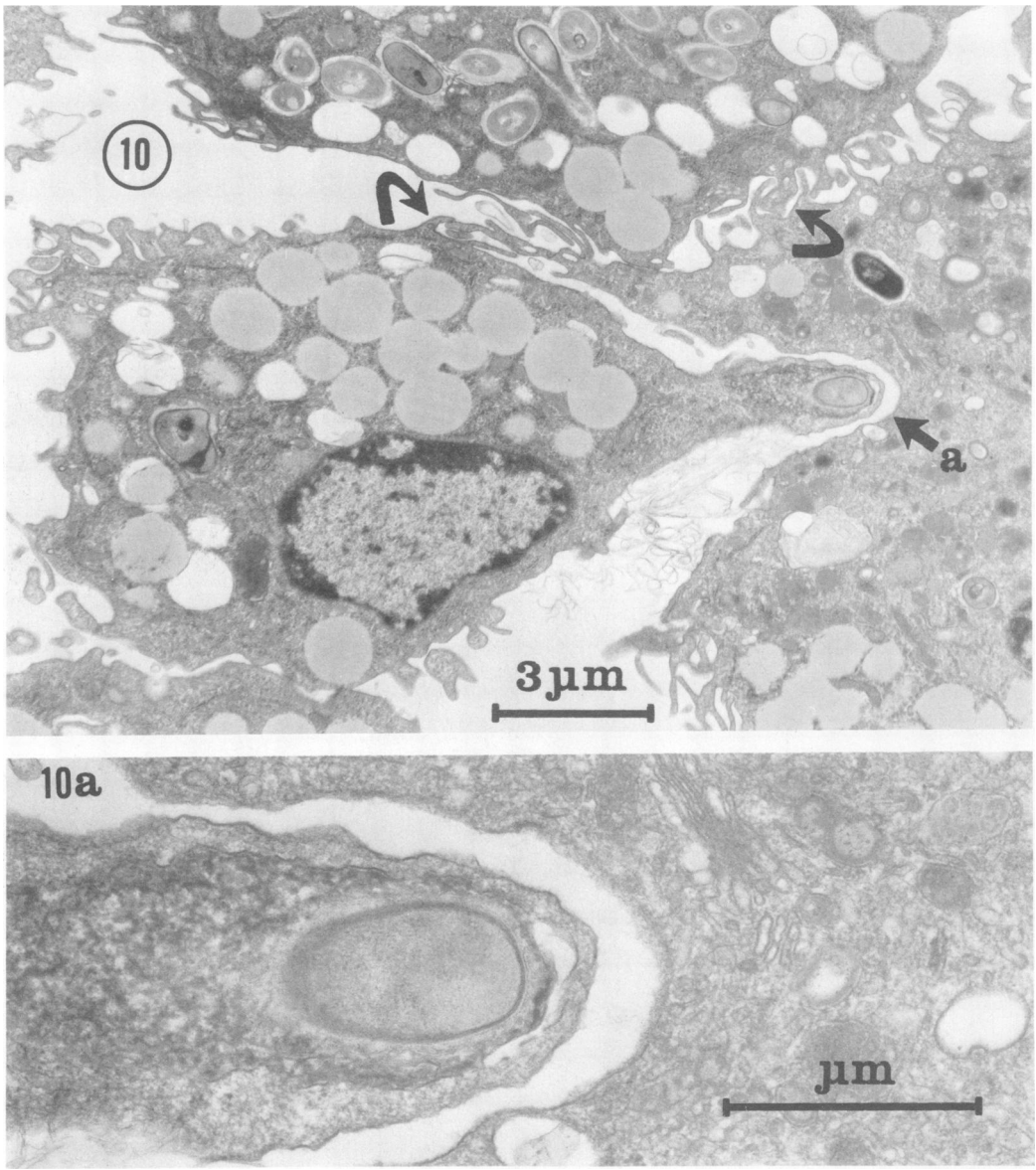


FIG. 10. *Thin section of alveolar macrophages 24 h postinfection with N. asteroides 14759. Note the large accumulation of lipid inclusions within the macrophages. Furthermore, there is a very close association (cell-to-cell contact) among the macrophages (bent arrows). The arrow at point (a) shows a nocardial cell that is in the process of active outgrowth (based on serial sections showing that this cell is a long filament arising from deep within the macrophage). (Insert a) High-magnification view of the nocardial filament growing out of the macrophage. Compare Fig. 10 with 9A. It is important to note that the nocardial cell is entirely within the macrophage and apparently within a phagosome. It is clear that even though the nocardial cells appear to be extending from the cytoplasm during outgrowth (Fig. 9), they remain surrounded by macrophage membrane. Only when the filaments penetrate through the cytoplasmic membrane of the macrophage do the macrophages appear to detach from the glass, many becoming lysed, and show an uptake of trypan blue. Therefore, it seems that extensive outgrowth with subsequent rupture of the macrophage cytoplasmic membrane is responsible for macrophage death.*

the lymphocytes, which in turn would mediate specific responses within the macrophage population. Macrophage fusion does not work to the advantage of the nocardial cell, since the multinucleate giant cells appear to be more destructive to the intracellular bacteria (Fig. 6 and 7). Similarly, Galindo noted that mycobacteria were more effectively destroyed by multinucleated giant cells than by individual macrophages, and he suggested that the phenomenon of giant-cell formation represents a process of cell cooperation resulting in greater antibacterial capacity. (8).

We are presently studying in more detail the specific mechanisms of nocardia-induced macrophage fusion. Some preliminary data suggest that specific components in the complex cell envelope may be responsible for this host-parasite response. This will require additional experimental data.

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