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An Ultrastructural Analysis of *Nocardia* During Experimental Infections in Mice

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Several strains of *Nocardia* that varied from virulent to avirulent were injected intraperitoneally into young mice. Histological and ultrastructural analysis of the resultant infections revealed that the bacteria and the lesions they induced were different depending upon the strain of organism used. Further, the morphological and tinctorial characteristics of the bacteria grown in vitro changes during growth in vivo. These observations strongly suggested that chemical and physical alterations occurred in the cell envelope of the *Nocardia* when grown in mice. Electron microscopy confirmed that significant structural modification occurred, especially in the cell envelope, when the nocardial cells established themselves within the host tissue. It was shown that the least virulent strain exhibited the most dramatic changes whereas the most virulent organism appeared to be affected the least.

There have been few studies reported on the mechanisms of nocardial pathogenesis. Uesaka et al. (31) reported on the pathogenicity of 76 strains of *Nocardia* for mice. Their work dealt with the gross histological characteristics of induced infections and the effects of mucin in enhancing virulence. They confirmed and expanded the observations made earlier by Destombes et al. (5), Mohapatra and Pine (20), Macotela-Ruiz and Mariat (17), and Runyon (28) that adjuvants such as oil or mucin increased nocardial virulence for experimental animals. However, none of these studied the host-parasite interactions at the ultrastructural level, nor did they examine the differences between in vitro- and in vivo-grown *Nocardia*. A review of the literature has revealed a single electron microscopy investigation of *Nocardia braziliensis* granules found within purulent exudate obtained from a man suffering from nocardial mycetoma (16).

Virulent, lowly virulent, and avirulent strains of *Nocardia* were studied in vitro and in vivo to ascertain how these organisms differed from one another and to establish which differences were relevant to nocardial pathogenesis. The results obtained revealed that the lesions induced in mice by the lowly virulent organisms differed histologically from those caused by the more virulent strains. Further, it was observed that there were corresponding changes in the morphological and tinctorial properties of the nocardial cells when grown in vivo. This communication deals primarily with the ultrastructure of these organisms grown both in vivo and in vitro.

MATERIALS AND METHODS

Organisms. *Nocardia farcinica* type C, originally isolated from a bovine infection in Africa, and *N. asteroides* 10905 were kindly supplied by Jacob Rozanis (University of Western Ontario, London, Canada). *N. asteroides* Mahvi, obtained from a fatal human infection, was kindly supplied by T. A. Mahvi (South Carolina Medical College, Charleston, S.C.). *N. rubra*-721-A was isolated from the air (4).

Culture conditions. All nocardial strains were maintained on brain heart infusion (BHI) agar. Prior to use each organism was streaked onto fresh BHI agar slants and incubated for 1 week at 34 C. The resultant growth was resuspended in sterile saline (0.85% NaCl) and transferred to BHI broth. The broth cultures were incubated in a water bath incubator at 34 C with gentle agitation for either 3 or 18 days.

Mouse inoculation and nocardial virulence. All cells were collected for use after 3 or 18 days of incubation to determine whether or not culture age affected the relative virulence of *Nocardia*. They were washed three times with 0.85% saline and pelleted at 10,000 $\times$ *g* for 30 min, and the excess liquid was removed. The pellets were recorded as packed wet weight of cells. Dead cell controls were prepared by suspending the organisms in 3.0% glutaraldehyde in Kellenberger buffer (pH 6.5) for 24 h at 4 C (13). These cells were then washed five times in sterile

saline to remove the glutaraldehyde and pelleted as described above. Each cell preparation was suspended in either sterile physiological saline or emulsified with Freund incomplete adjuvant (oil) to give a final concentration of 50 mg packed wet weight per ml. A 1-ml portion of each sample was plated to determine purity and viability.

Each of five mice were injected intraperitoneally (i.p.) with 0.2 ml of a preparation (representing 10 mg wet weight of cells), placed in isolation facilities, and monitored daily. Control mice were injected i.p. with 0.2 ml of either sterile saline or saline emulsified in incomplete Freund adjuvant without organisms added. Mice from each group were sacrificed at 3 days and weekly thereafter up to a total of 10 weeks. These procedures were repeated, although the mice were kept for 10 weeks to determine animal death. Relative virulence was based on the ability of the organism to kill animals in less than 10 weeks when suspended in either saline or incomplete Freund adjuvant (see Table 1). Death of the mice in less than 1 week indicated acute infections whereas death between 1 and 4 weeks meant that the infections were chronic and progressive.

Animals. Both male and female white Dub:(ICR) mice were used during this investigation. The animals were all approximately 3 weeks old and weighed an average of 18 g at the time of inoculation. The animals were obtained from Flow Laboratories (Dublin, Va.) and maintained on Purina Laboratory Chow. The animals were kept in individual cages (five animals per cage) within an animal isolator (Germfree Lab) supplied with filtered air.

Light microscopy. The mice were sacrificed by cervical dislocation, and the peritoneal and thoracic cavities were opened. Portions of the viscera and body fluid were streaked onto Lowenstein-Jensen medium and BHI agar to test for recovery of viable organisms as well as to determine contamination. The internal organs of the mice were then flooded with 3.0% glutaraldehyde fixative prepared in Kellenberger buffer (13) with the final pH adjusted to 6.5. Any infected regions as well as the lungs, heart, liver, spleen, and kidneys were removed from the animal and partially perfused with additional fixative. This tissue was then placed in 3 to 5 ml of fixative and stored at 4 C for at least 24 h. The fixed samples were washed with several changes of Kellenberger buffer (pH 6.5), dehydrated through a series of ethanol, cleared, and then embedded in paraffin as described by Luna (14). Thin sections were cut with a Spencer microtome (AO Company), affixed to glass slides, and stained by the Brown and Brenn modification of the Gram stain (14), by the Kinyoun acid-fast stain using 1% HCl in 70% ethanol as the decolorizing agent (14), and with the Giemsa stain as modified by Luna and Parker (15).

Electron microscopy. Selected portions of the animal were removed after fixation as described earlier and cut into small fragments about 1 mm^3. These fragments were resuspended in fresh 3% glutaraldehyde fixative (pH 6.5) as described above. After 18 h at 4 C, the tissue was washed twice with fresh Kellenberger buffer (pH 6.5), resuspended in 1% osmium tetroxide in Kellenberger buffer (pH 6.5), and stored at 4 C for 18 h. The tissue was washed twice with fresh buffer (pH 6.5) and resuspended in 0.5% uranyl acetate in Kellenberger buffer (pH 6.5) for 3 h at 4 C. The tissue was dehydrated through a series of ethanol, propylene oxide, and finally Maraglass. The tissues were embedded in Maraglass and polymerized at 60 C for 48 h, trimmed, and sectioned. Thick sections were cut first to permit specific orientation within the block, and then thin sections were cut by use of a DuPont diamond knife on an MT-2 Porter Blum ultramicrotome. Sections with interference colors of silver-gold were collected on either nickel or copper athene grids, and stained for 60 s with lead citrate as described by Reynolds (13). The sections were photographed through a Philips EM 300 electron microscope with a 20-μm gold objective aperature and operated at 60 kV.

RESULTS

Mouse infections and nocardial virulence. *N. farcinica* C, *N. asteroides* Mahvi, and *N. asteroides* 10905 produced either a chronic, progressive disease or an acute fatal infection in mice when these organisms were emulsified in incomplete Freund adjuvant (Table 1). *N. asteroides* Mahvi in saline established both acute and chronic infections in mice (Table 1). Some of the mice died within 5 days after inoculation, and the remainder developed firm, nodular masses within 4 weeks (Fig. 1). *N. farcinica* C, suspended in saline, established a progressive infection in all of the mice injected. However, only 20% of these died within 10 weeks (Table 1). In contrast, *N. asteriodes* 10905 was emulsified in oil, and all of the mice developed progressive infections resulting in a 70% mortality rate (Table 1). Viable nocardial cells could always be isolated from the animals, even after 10 weeks. *N. rubra* 721-A in incomplete Freund adjuvant induced the formation of soft tissue masses; however, no mice died (Table 1). These lesions were self limiting and appeared to be the same as those produced by the dead nocardial cell controls. *N. rubra* 721-A suspended in saline did not produce infections in any of the mice. Based on these results it appeared that *N. asteriodes* Mahvi and *N. farcinica* C were most virulent for mice as defined by Uesaka et al. (31). *N. asteriodes* 10905 was less virulent, whereas *N. rubra* 721-A was not virulent (Table 1).

Light microscopy of in vitro- and in vivo-grown nocardia. Both *N. farcinica* and *N. asteroides* Mahvi induced granulomatous nodules of varying size in the spleen, liver, kidneys, and peritoneal lymph nodes (Fig. 1) when actively growing cultures suspended in saline were injected i.p. into young mice. Unique to *N.*

TABLE 1. *Relative virulence of selected strains of nocardia: mouse death after i.p. injection*

Organism (72 h BHI)[a]	Inoculum (10 mg wet wt/mouse)	No.[b] of mice injected	No. of mice dead at 1 week	No. of mice dead at 4 weeks	Cumulative mortality (%)
N. asteroides Mahvi	Saline	10	1	3	40
N. asteroides Mahvi	Oil	5	5		100
N. asteroides Mahvi (dead cell control)	Saline	10	0	0	0
N. asteroides Mahvi (dead cell control)	Oil	5	0	0	0
N. farcinica C	Saline	10	0	2	20
N. farcinica C	Oil	10	0	6	60
N. farcinica C (dead cell control)	Saline	10	0	0	0
N. farcinica C (dead cell control)	Oil	10	0	1[c]	10
N. asteroides 10905	Saline	20	0	0	0
N. asteroides 10905	Oil	10	2	5	70
N. asteroides 10905 (dead cell control)	Saline	10	0	0	0
N. asteroides 10905 (dead cell control)	Oil	20	0	1[c]	5
N. rubra 721-A	Saline	10	0	0	0
N. rubra 721-A	Oil	10	0	0	0
N. rubra 721-A (dead cell control)	Saline	10	0	0	0
N. rubra 721-A (dead cell control)	Oil	10	0	0	0
No organisms	Saline	20	0	1[c]	5
No organisms	Oil	20	0	0	0

[a] Similar results were obtained using organisms grown on BHI for 18 days.
[b] The values represent a total of two or more experiments.
[c] The three dead mice came from the same lot composed of 30 mice; none of the remaining mice of this group died (the deaths appeared to be unrelated to the experimental procedure).

asteroides Mahvi was the formation of aggregates of acid-fast bacilli within mononuclear cells, producing what appeared to be "globi" (Fig. 2). Raich, Casey, and Hall (26) reported that microscopy examination of exudate from an individual with cutaneous nocardiosis revealed numerous intracellular acid-fast organisms arranged in globi similar to what we observed. Frequently the central region of the larger nodules contained scattered foci of colonies of bacteria accompanied by tissue necrosis (Fig. 3). In addition there was a characteristic infiltration of the granulomatous tissue by individual filaments of *Nocardia* (Fig. 5). The lesions induced by these organisms suspended in oil were indistinguishable from those found in mice receiving organisms in saline. *N. asteroides* 10905 (Fig. 4) emulsified in incomplete Freund adjuvant induced the formation of firm nodules that contained colonies of bacteria localized within the central region of a granulomatous mass similar to that designated as the "Braziliensis" type of lesion by Uesaka et al (31). This strain of *N. asteroides*, suspended in saline, did not induce progressive lesions in mice. Dead cell controls as well as *N. rubra* 721-A, when emulsified in incomplete Freund adjuvant, induced the formation of soft, nodular masses accompanied by tissue proliferation which reached a peak after 2 weeks and then subsided. Sections revealed large vacuolar areas surrounded by mononuclear cells. The bacteria could be found within the vacuoles; however, we were unable to locate the organisms within the tissue (Fig. 6).

None of the strains of *Nocardia* used during this investigation was acid fast when grown in BHI, but all of them were strongly and uniformly gram positive (Table 2). However, these strains became strongly acid fast when grown in mice. Further, in vivo *N. asteriodes* 10905 underwent dramatic morphological changes (Table 2) with the formation of bulbous and club-shaped cells (arrows, Fig. 4) similar to that observed when *N. rubra* 721-A (smooth) was grown for 12 h on BHI agar (4). These cells lacked the ultrastructural complexity of *N. rubra* 721 reported by Beaman and Shankel (4). *N. asteriodes* 10905 appeared to be gram variable in tissue, with most of the cells being gram negative (Table 2). In contrast, *N. farcinica* C was strongly gram positive. *N. asteriodes* Mahvi was intermediate in the Gram reaction. Most of the cells of this latter strain appeared as gram-positive, beaded filaments or short rods (Table 2).

Electron microscopy of nocardial infections in mice. The cells of *N. asteriodes* 10905 grown for 18 days in BHI were predominantly

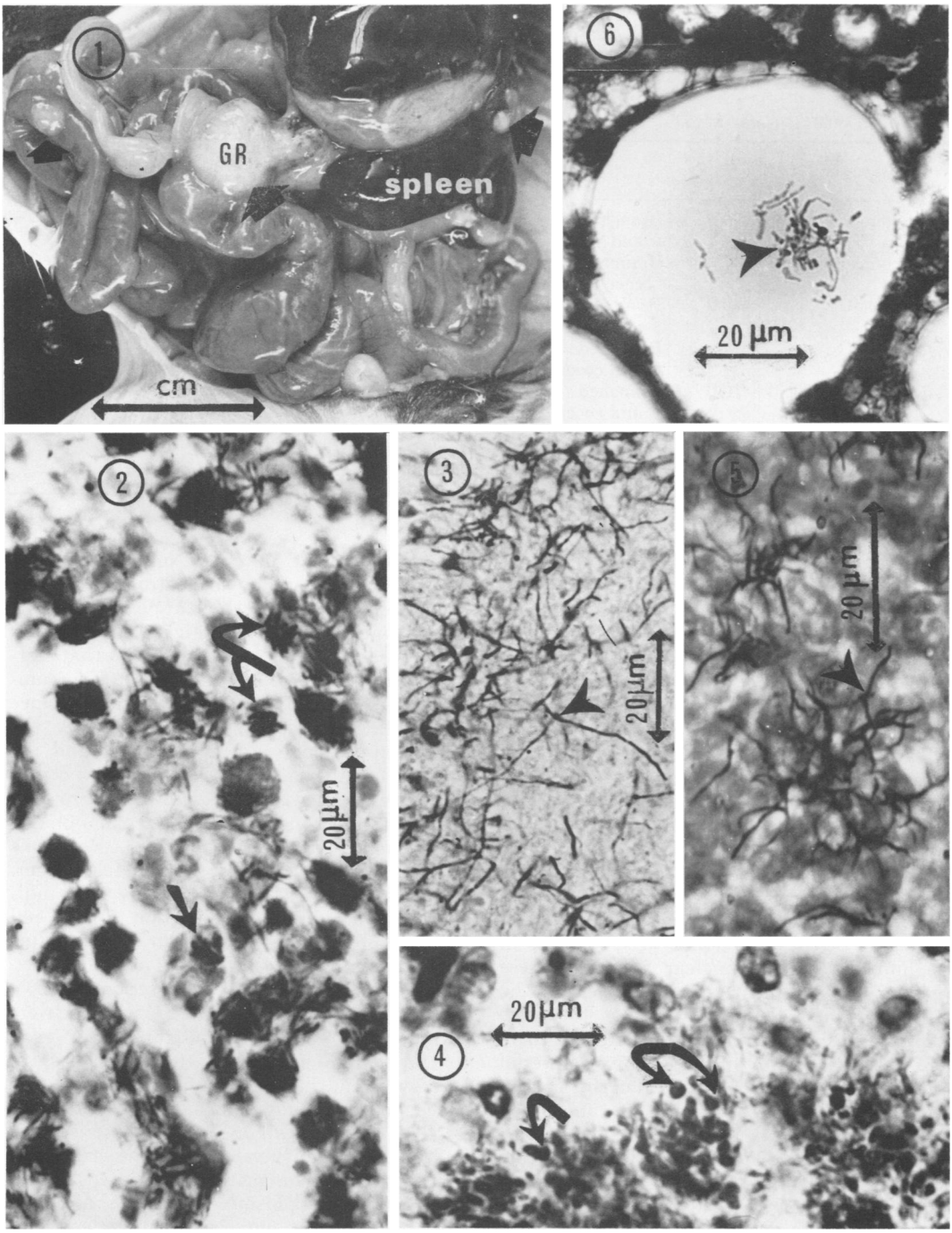

FIG. 1. *Peritoneal cavity of a mouse 4 weeks after injection with N. asteroides Mahvi in saline. Arrows indicate firm, granulomatous nodules or granules (GR).*

FIG. 2. *An acid-fast stain of a paraffin section of a pericardial nodule formed 3 days after i.p. injection with N. asteroides Mahvi in saline. The arrows indicate clusters of bacilli (globi) within large mononuclear cells.*

FIG. 3. *An acid-fast stain of the central, necrotic region of a nodule formed in the peritoneal cavity 1 week after injection of N. asteroides Mahvi in saline. The pointer notes the formation of long, branched filaments.*

FIG. 4. *A modified Giemsa stain of the periphery of a colony of bacteria located within the central region of a nodule formed within the lung 4 weeks after i.p. injection with N. asteroides 10905 suspended in incomplete Freund adjuvant. The arrows indicate enlarged, bulbous, and club-shaped organisms. (N. asteroides GUH-1 appeared to be similar.)*

FIG. 5. *A Gram stain of N. farcinica C within the tissues of a nodule formed in the kidney 4 weeks after i.p. injection with organisms in saline. The pointer indicates the presence of branched, filamentous bacteria.*

FIG. 6. *A Gram stain of N. rubra 721A 2 weeks after i.p. inoculation with organisms in incomplete Freund adjuvant. The pointer notes the presence of pleomorphic cells, some of which appear to be degraded. (Similar lesions were observed in mice after injection of dead organisms in incomplete adjuvant.)*

TABLE 2. *Comparison of in vitro- and in vivo-grown nocardial cells by light microscopy*

Organism	Source	Morphology	Gram stain	Acid fast	Beaded filaments	Lesion[a] type
N. asteroides Mahvi	BHI	Branched filaments, rods, and cocci	+	−	−	
N. asteroides Mahvi	Mouse	Branched filaments, rods, cocci, spirals, and globi	+ (Some −)	+	+	"Asteroides"
N. farcinica C	BHI	Branched filaments, rods, and cocci	+	−	−	
N. farcinica C	Mouse	Branched filaments, rods, and cocci	+	+	±	"Asteroides"
N. asteroides 10905	BHI	Branched filaments, rods, and cocci	+	−	−	
N. asteroides 10905	Mouse	Pleomorphic, large spheres, clubs and bulbs, rods, cocci, and bizarre forms	− (Few +)	+	±	"Braziliensis"

[a] As described by Uesaka et al. (31).

rod shaped; however, some filaments and cocci were present (Table 2). The cell wall of these organisms, grown under these conditions, was a relatively thick, single layer (Fig. 7a insert). The outer layers that were usually observed in the cell walls of the other nocardial strains studied were either absent or greatly reduced (compare the cell wall profiles of the organisms in Fig. 7a, 11, and 13). The outer layer of the cytoplasmic membrane, adjacent to the cell wall, was significantly thickened and more electron dense than the inner layer (Fig. 7a). This was not observed in the in vivo-grown bacteria (compare the cell envelope of the organisms in Fig. 7a and 8a and see Table 3).

The bacterial cells localized within the central region of the granuloma were variable in size and shape. The nuclear region of these organisms was frequently more diffuse than the in vitro-grown cells used to infect the mice (compare Fig. 7, 8, and 9B). In addition there were large amounts of amorphous material associated with the outer region of the in vivo-grown bacteria (arrow, Fig. 8), and many of these cells possessed a layered cell envelope (pointer, Fig. 8). The club-shaped filaments near the periphery of the bacterial mass within the lesion were surrounded by a wide layer of amorphous material (pointer, Fig. 9B). This substance appeared to have a single membrane boundary that was distinctly separable from the phagosomal membrane of the host (arrows, Fig. 9B), and it seemed to be of bacterial origin. This layer was always present in varying amounts in mice infected with *N. asteroides* 10905, but it was absent in the animals infected with the other *Nocardia*. Killed cells of *N. asteroides* 10905 emulsified in incomplete Freund adjuvant did not possess this outer amorphous layer. Only one mouse out of 10 that were inoculated with *N. farcinica* C in oil had small numbers of bacteria enclosed within an electron-dense layer of amorphous material which appeared to lack a limiting membrane (arrow, Fig. 9A). This material appeared to be different from that surrounding the cells of *N. asteroides* 10905 (compare Fig. 9A and B). The amorphous substance surrounding the cells shown in Fig. 9B, the altered appearance of the cell wall, and the changed cellular morphology was observed with this same strain of *Nocardia* grown in vitro after passage through mouse peritoneal macrophages from which L-forms were isolated (L. Bourgeois and B. L. Beaman, Abstr. Annu. Meet. Amer. Soc. Microbiol., 1973, p. 66). These data indicate that considerable alteration of the cell envelope of this nocardial strain occurred during in vivo growth (see Tables 2 and 3).

In marked contrast to *N. asteriodes* 10905, most of the cells of *N. farcinica* C in tissue were surrounded by an electron-transparent zone that appeared empty (Fig. 10). The cell envelope was multilayered, and the periplasmic space was uniformly thickened (Fig. 10A and B). This strain grown on BHI characteristically had a layered cell envelope, a thin periplasmic space, a thickened, more electron-dense outer region of the cytoplasmic membrane, an indistinct nuclear area, and fewer polysomes (compare the cells in Fig. 10A and B with 11A and B; Table 3). In addition, the size of the bacteria did not vary significantly between the in vivo- and in vitro-grown cells (contrast the cells shown in Fig. 10 and 11 with Fig. 7, 8, 9). Further, we were unable to demonstrate any differences in the ultrastructural appearance of *N. farcinica* C in mouse lesions after i.p. injection of these organisms suspended in either saline or oil.

N. farcinica C grown on BHI formed hard,

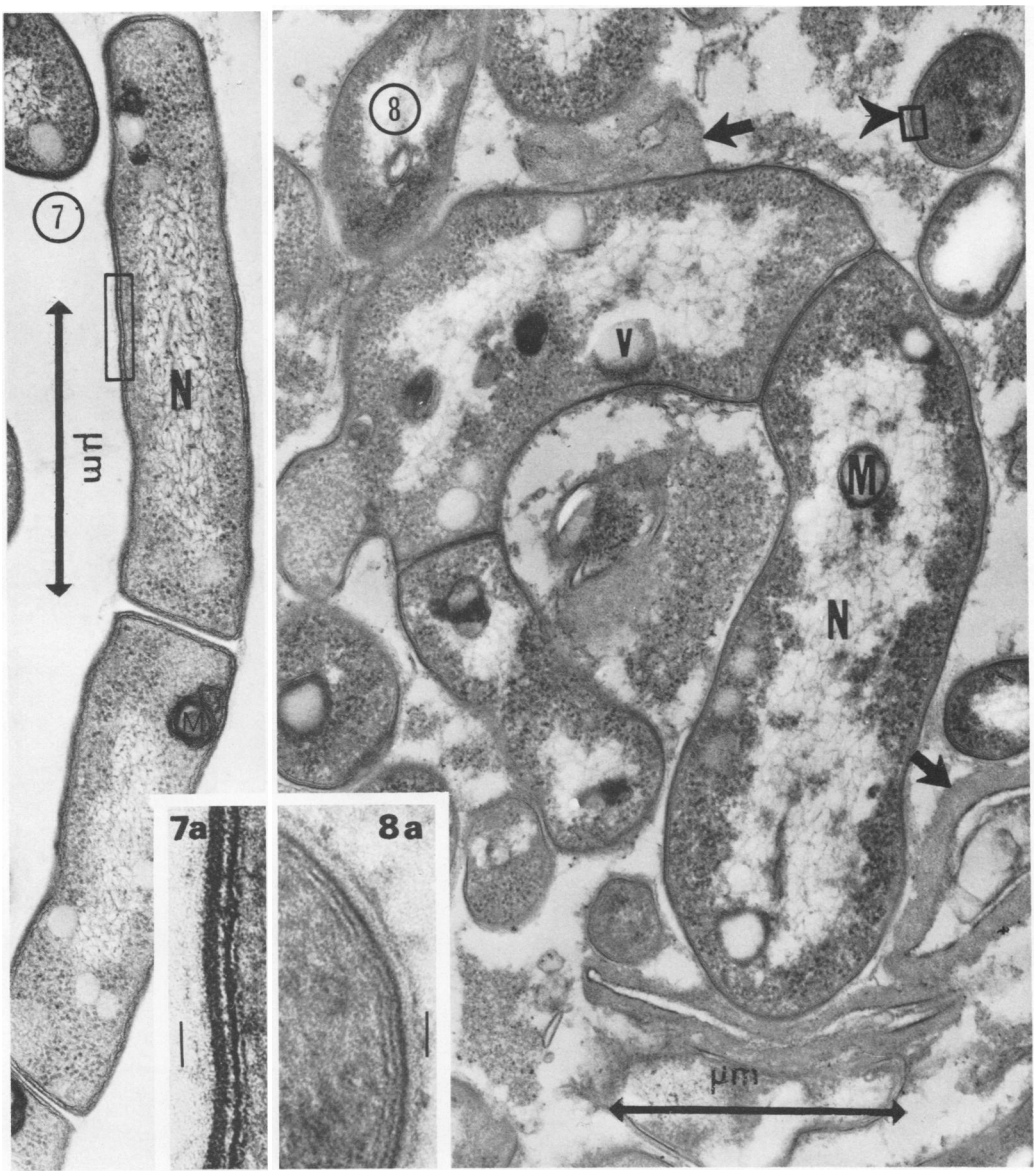

FIG. 7. *An electron micrograph of N. asteroides 10905 grown on BHI-A for 18 days. Cells from this preparation were used as the inocula that induced the lesions shown in Fig. 4, 8, and 9B. Abbreviations: N, nuclear region, M, mesosome. Insert a: high magnification of a portion of the cell wall. The bar is approximately 30 nm.*

FIG. 8. *A thin section of N. asteroides 10905 located within the central region of a granule formed within the capsule of the spleen. The mouse was sacrificed 4 weeks after i.p. injection of organisms in incomplete Freund adjuvant. The arrows indicate amorphous material associated with the outer layer surrounding a thicker, more electron-dense region forming a triple-layered cell wall. Abbreviation: V, vacuole (probably lipid inclusion). Insert a: high magnification of a portion of the cell wall. The bar is approximately 30 nm.*

tenacious colonies that were difficult to suspend in saline. Figure 11B (arrow) showed that the outermost layer of the cell wall was usually continuous from one cell to the next. This could account for the tenacious characteristic of many of the nocardial colonies, and it might possibly enhance the cells' resistance to phagocytosis.

Both *N. farcinica* and *N. asteriodes* Mahvi were frequently found deep within mononuclear cells which contained numerous vacuoles and

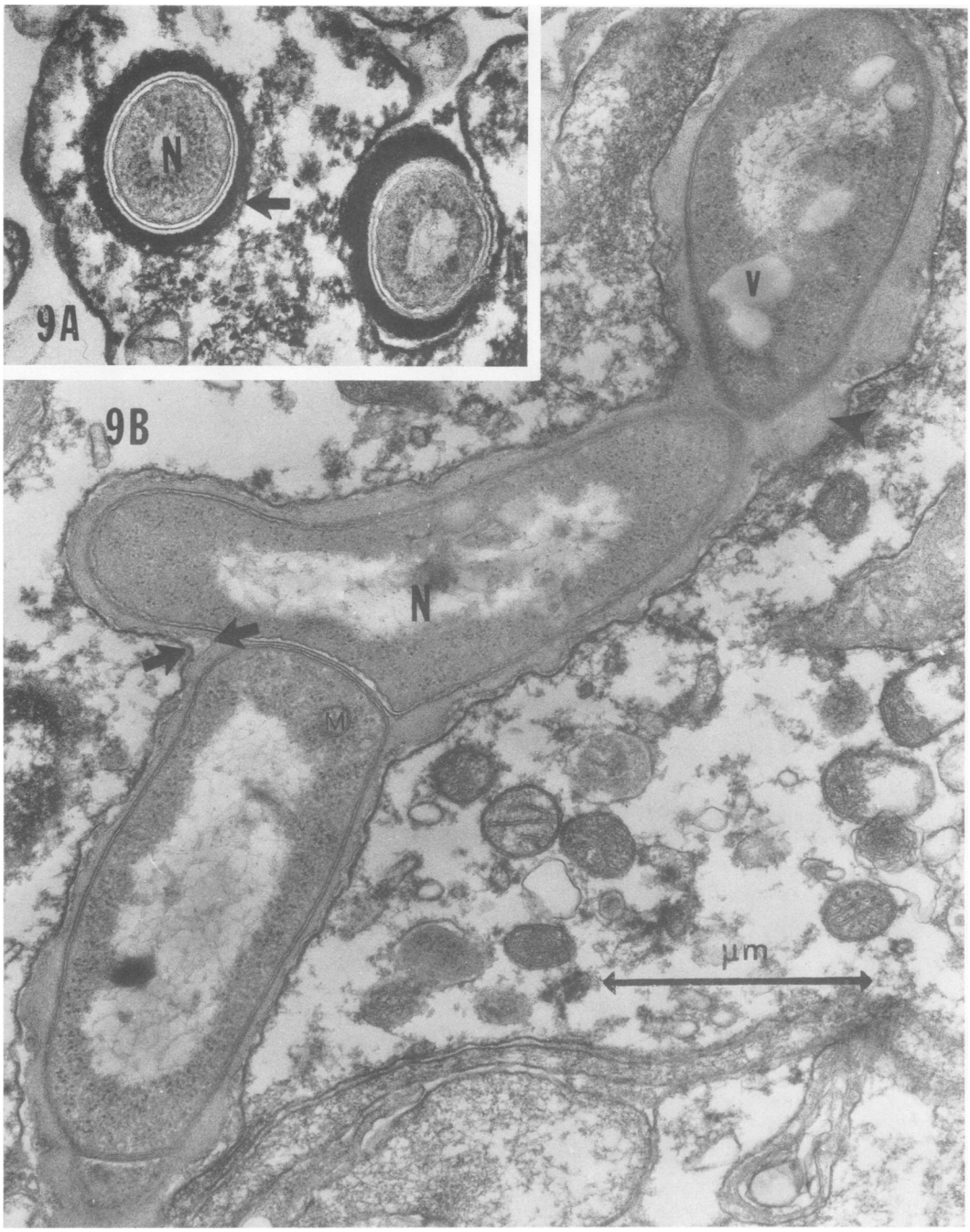

FIG. 9. *A, N. farcinica C in a nodule 8 weeks after i.p. injection of organisms in incomplete Freund adjuvant. The arrow indicates a region of strongly osmophilic, amorphous material surrounding the bacteria. (This was observed only in one mouse out of more than a dozen receiving N. farcinica C suspended in either saline or oil.) B, N. asteroides 10905 located at the periphery of the bacterial mass within a nodule. Four weeks prior to sacrifice the mouse had received an i.p. injection of organisms in incomplete Freund adjuvant. The pointer notes the presence of a layer of amorphous material associated with the surface of the bacterial cell. (This material was consistently present with N. asteroides 10905 in vivo but not with the other strains of Nocardia studied during this investigation.) The arrows indicate a membrane surrounding the material that is clearly separable from the phagosomal membrane of the animal cell.*

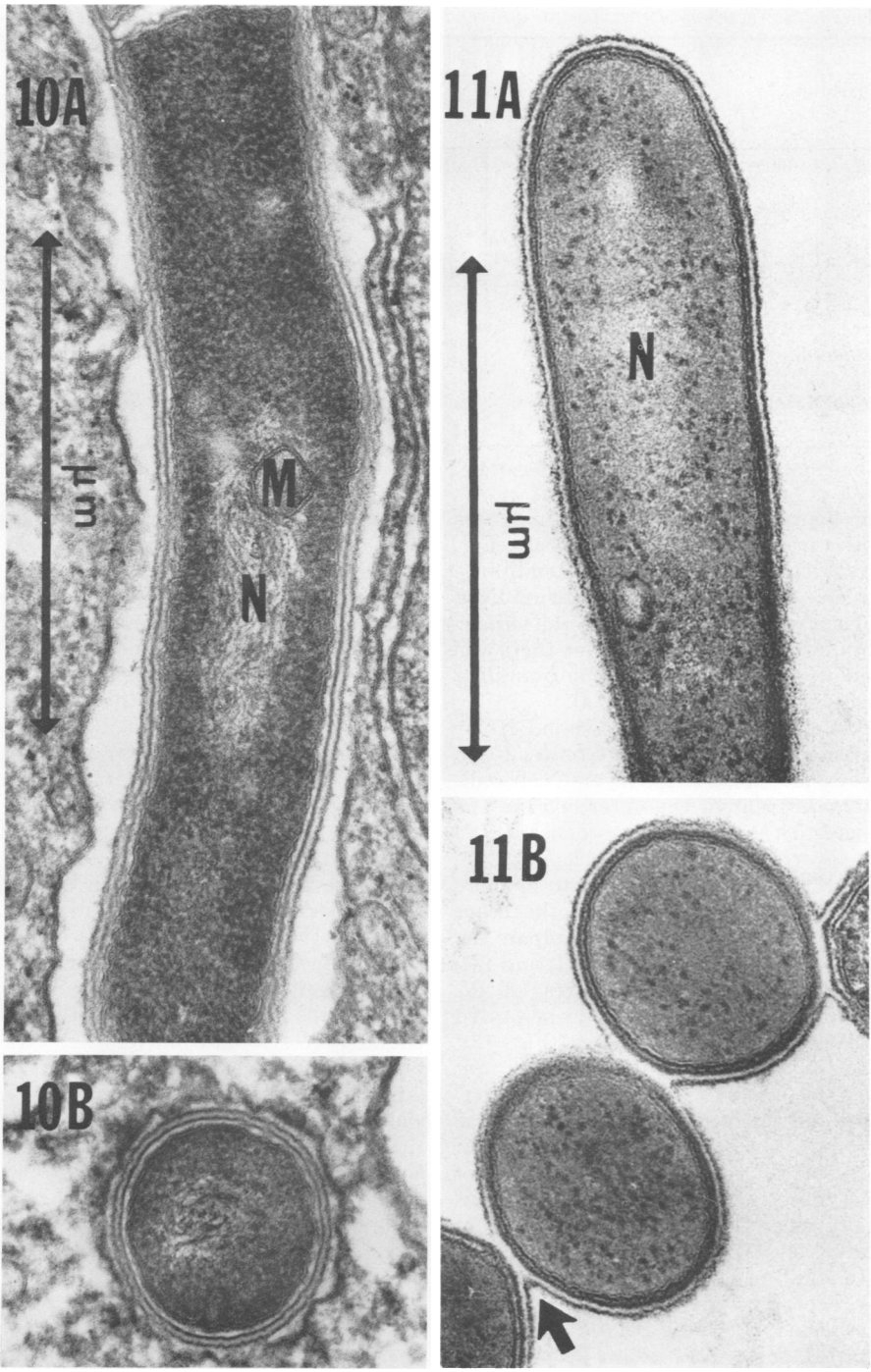

FIG. 10. *A, Longitudinal section of N. farcinica C found within a macrophage in a granuloma formed in the spleen. The animal had received an injection of the organisms in incomplete Freund adjuvant 4 weeks prior to sacrifice. B, A cross-section of N. farcinica C found within the tissues of a granuloma (from the same animal as Fig. 10A).*

FIG. 11. *A, N. farcinica C grown on BHI for 18 days. Cells from this preparation were used to induce the lesions shown in Fig. 10 and 12. B, N. farcinica C. grown on BHI for 18 days (cross-section). Arrow notes that the thick outer layer of the cell wall is continuous from one cell to the next.*

TABLE 3. *Summary of ultrastructural differences between in vivo- and in vitro-grown nocardia*

Organism	Fig.	Source	Cell wall[a] (inner layer) (nm)	Cell wall[a] (nm)	Cell wall (profile)	Periplasmic space[a] (nm)	Cytoplasmic membrane
N. asteroides 10905	7	BHI	15	15	Single layer	6	Outer dense layer
N. asteroides 10905	8	Mouse	5	20	Triple layer	6	All layers uniform
N. farcinica C	10	Mouse	6	30	Triple layer	15	All layers uniform
N. farcinica C	11	BHI	8	30	Triple layer	5	Outer dense layer
N. asteroides Mahvi	13	BHI	6	25	Triple layer	5	All layers uniform
N. asteroides Mahvi	14B	Mouse	15	60	Triple layer	5	All layers Uniform

[a] These are approximate values that represent an average of five random measurements.

membrane-bound structures of varying sizes and shapes (arrows, Fig. 12). The insert (Fig. 12a) shows a bacterial cell within a membrane-bound vesicle. Tubular structures arising from the cytoplasm were continuous with the vesicular membrane (arrow, Fig. 12a). Often there was an accumulation of dense material surrounding the "phagosome" (pointer, Fig. 12a).

Cells of *N. asteroides* Mahvi grown in BHI for 72 h differed from those of *N. asteroides* 10905 in that they possessed a triple-layered cell wall. In *N. asteroides* Mahvi, the outer layer of the wall was as thick and as electron dense as the innermost layer. In contrast, each layer of the cell wall of *N. farcinica* C appeared to be more osmophilic than those observed in the other organisms used during this study (compare the cell walls of the organisms in Fig. 7, 11, and 13). In actively growing BHI cultures of *N. asteroides* Mahvi many of the cells possessed clusters of electron-lucent granules (Fig. 13). Barksdale (2) noted that *Corynebacterium diphtheriae* $C7_s(-)$ tox^- grown maximally contained large clusters of granules similar to those shown in Fig. 13. Barksdale suggested that these represented lipoidal material in regions of intense biosynthetic activity (2). These observations were consistent with our demonstration of polar staining of many of the cells with Sudan black B. In addition to these diffuse regions the cells also contained well-defined "lipid vacuoles," especially in those bacteria grown in vivo (Fig. 14). Figure 14 shows a nocardial filament within a membrane-bound vesicle of a mononuclear cell. The triple-layered nature of the bacterial cell wall is evident (arrows, Fig. 14a); however, the outer layer is less distinct and thickened (contrast the cell walls of the organisms in Fig. 13 with Fig. 14). Large numbers of vacuoles, presumably lipid in nature, occurred giving the filaments a beaded appearance when stained for light microscopy. The granular regions shown in Fig. 13 were not observed in vivo. What appeared to be a cross-section of a nocardial cell being phagocytized is shown in Fig. 14B. Note the thickened inner layer of the cell wall and the less distinct outer layer (Table 3). In addition there is a large amount of membranous material in close association with the outer region of the wall (arrow, Fig. 14B). Compare this cell with the cell shown in Fig. 13.

Light microscopy revealed that *N. asteroides* Mahvi often occurred intracellularly as clusters of bacilli (Fig. 2). Figure 14C is a thin section through one of these mononuclear cells. Present were numerous intact bacteria (arrows) as well as cell walls (pointers). Apparently some of the bacilli were lysed within the host cell, leaving behind the undigested cell wall. This permitted a better visualization of the many layers of the wall and how these layers were altered within the host (compare Fig. 13 with Fig. 14C). It also suggested that the bacterial cytoplasm and membrane, not the cell wall, was degraded first, and that in fact the cell walls were less sensitive to the lytic enzymes released by the host cell.

DISCUSSION

During the past several years there have been several hundred published case histories of nocardial infections in both man and animals (5, 6, 9, 10, 16–18, 21, 25–27, 30). In addition there have been numerous reports concerning nocardial pathogenicity in mice, rabbits, and

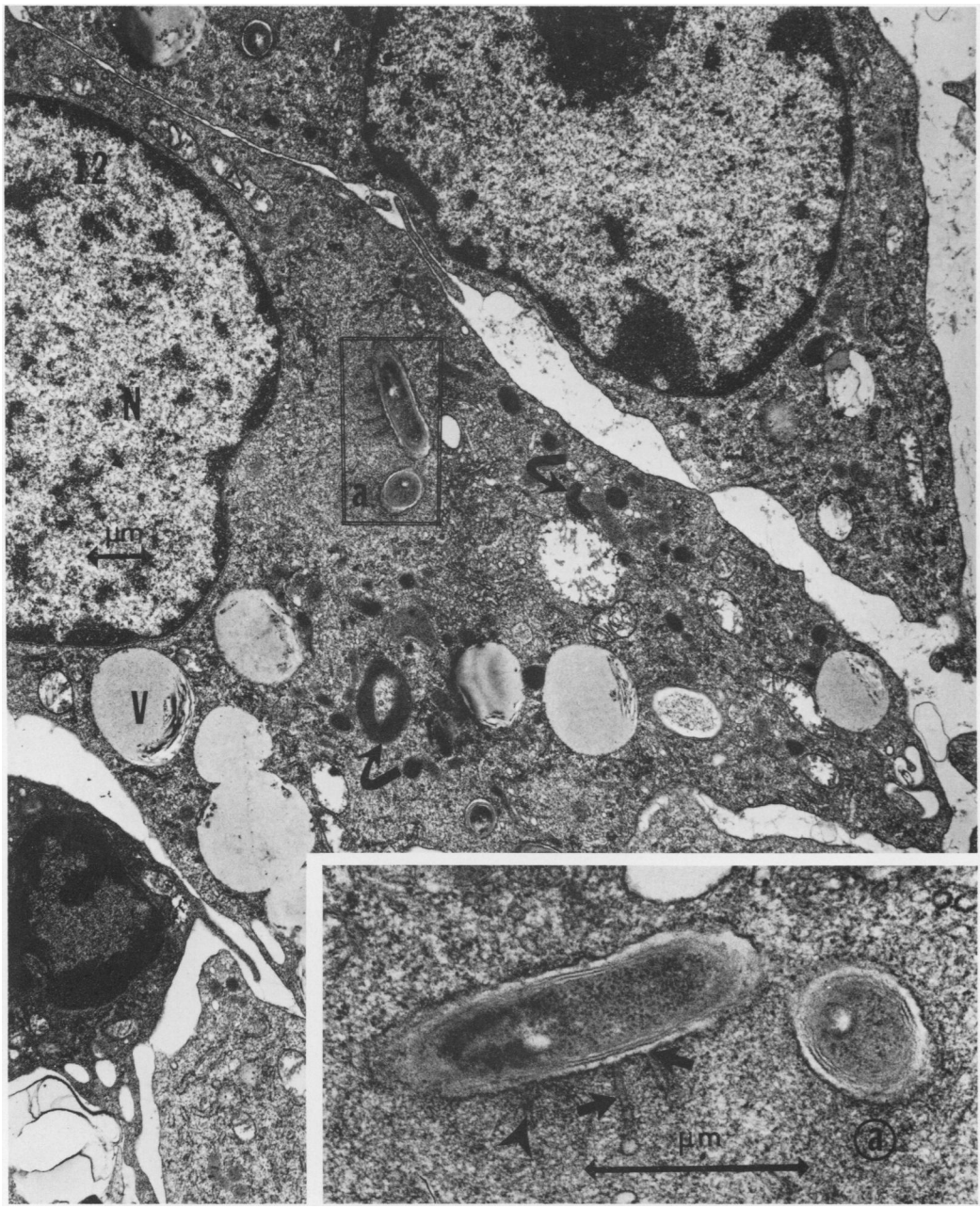

FIG. 12. *N. farcinica C within a macrophage in a granuloma induced by i.p. injection of the organism 4 weeks earlier. Insert a more clearly demonstrates the bacteria within a phagosomal vesicle. Arrows indicate tubular structures continuous with the membrane of the vesicle. The pointer notes the presence of dense granular material surrounding the phagosome.*

guinea pigs (6, 17–20, 28, 31). Most of these described the histology and pathology of the resultant infections. From these reports we concluded that nocardial infections were varied and reliable symptomatology was not always present. Histological and pathological descriptions varied from acute, purulent abscesses resembling those caused by the pyogenic cocci

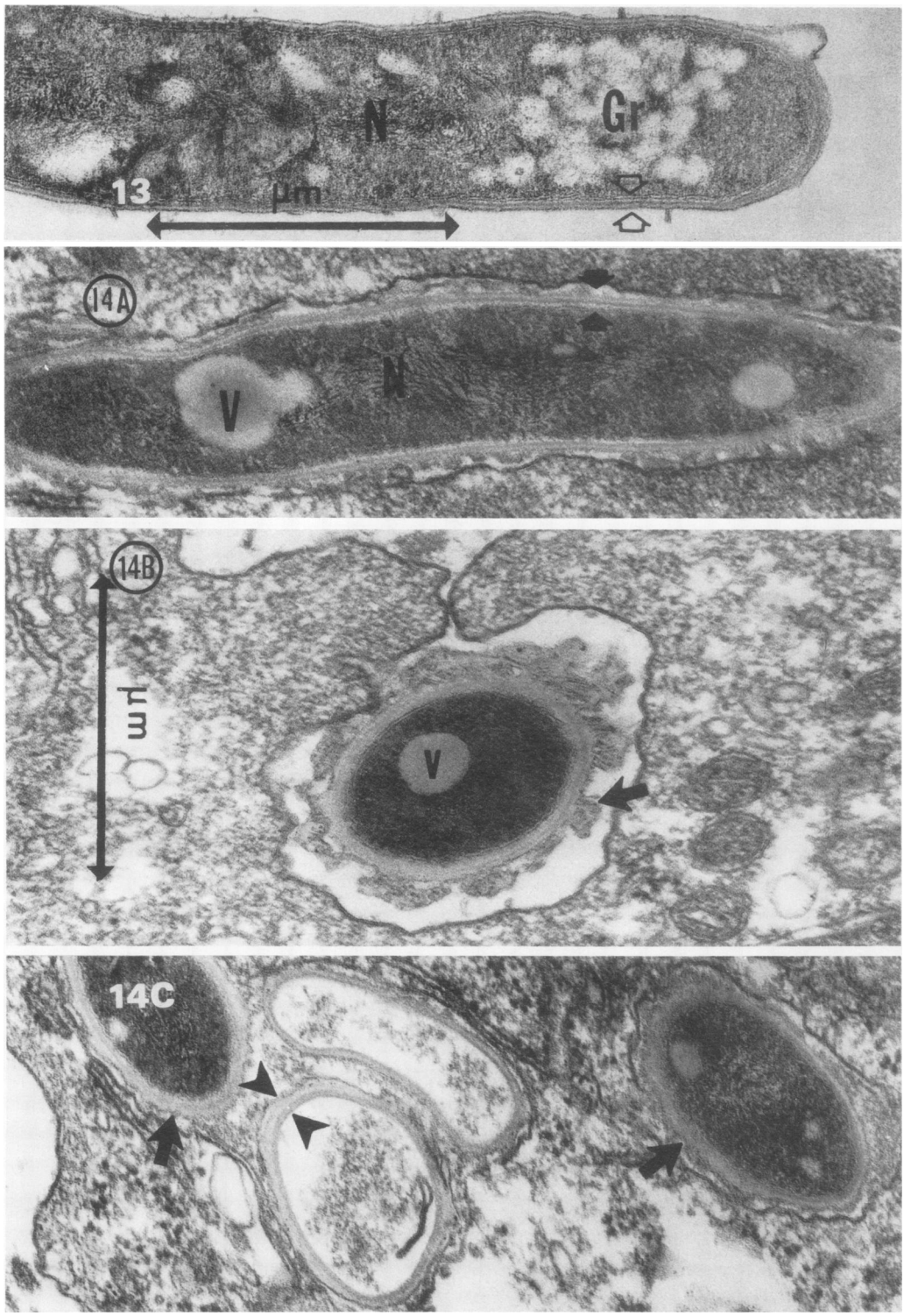

FIG. 13. *N. asteroides Mahvi grown on BHI for 72 h. Cells from this preparation were used to infect the mice studied in Fig. 14. Abbreviation: GR, diffuse electron-lucent granules that may represent lipid material (based on Sudan black B staining). The arrows indicate the nature of the layered cell wall.*

FIG. 14. *A, N. asteroides Mahvi within a macrophage in a nodule induced by i.p. injection of a saline suspension of organisms 72 h earlier. Arrows point out the nature of the cell wall. B, N. asteroides Mahvi in the process of being phagocytized (from the same animal as in Fig. 14A). The arrow notes that the cell wall has thickened and appears to have additional membranous or fibrillar material associated with it. C, Clusters of cells of N. asteroides Mahvi within a macrophage (see Fig. 2). Arrows note an outer membranous material associated with the cell wall. The pointers indicate the thickness of the cell wall after digestion of the cytoplasm. Note how the wall has changed from that seen in Fig. 13.*

to progressive granulomas developing over a period of several years similar to those caused by mycobacteria (10, 21, 25–27, 30).

Macotela-Ruiz and Gonzalez-Angulo (16) reported that granules of *N. braziliensis* isolated from man contained a central region of tangles of fine filaments with the periphery composed of club-shaped elements similar to those observed within sulfur granules produced by *Actinomyces* in mice (23, 24). Destombes et al. (5) described small-grain mycetomas in man and experimental animals similar to those described later by Uesaka et al. (31) as the "braziliensis type" of nodule formation. A second type of lesion most frequently induced by strains of *N. asteroides* was characterized by Uesaka et al. (31) as a granuloma encapsulated by epithelioid cells. They noted that these lesions were similar to those produced by mycobacteria and designated them as the "Asteroides type" (31). Numerous other investigators have studied nocardial infections in mice, guinea pigs, and rabbits, and they also observed that some strains formed granules similar to *Actinomyces* whereas others more closely resembled mycobacteria (6, 17–20, 23, 24, 28). Most of these experimental studies stressed that nocardial virulence could be enhanced by simultaneoulsy injecting adjuvants such as mucin or oil with little change in the type of lesion produced.

In this investigation we were attempting to determine what structural changes occurred in the bacteria as they adapted to in vivo growth. By comparing organisms emulsified in oil with those suspended in saline we were able to find nocardial strains that varied from nonvirulent to fully virulent forms. The basic questions we asked was how these strains differed structurally and biochemically.

Electron microscopy revealed that the virulent strains, *N. asteroides* Mahvi and *N. farcinica* C, underwent the least structural modification during growth in vivo. *N. asteroides* 10905 demonstrated altered morphology. All of the organisms, except *N. rubra*, showed significant changes in the structure of the cytoplasmic membrane, with *N. farcinica* C possessing the most pronounced periplasmic region. Cells of *N. asteroides* 10905 located at the periphery of the granule were often bulbous and surrounded by fine granular material limited by a single membrane. Macotela-Ruiz and Gonzalez-Angulo (16) noted the presence of a similar substance surrounding cells of *N. braziliensis* and suggested that this material was responsible for the bulbous nature of the cells when observed by light microscopy. In addition, they felt that this material represented deposits of calcium salts. Amorphous material surrounding the cells of *Actinomyces* have also been reported (23, 24). We found similar material associated with cells of *N. asteroides* 10905 when isolated from in vitro-grown macrophages (L. Bourgeois B. L. Beaman, Abstr. Annu. Meet. Amer. Soc. Microbiol. 1973, p. 66). The other strains of *Nocardia* we studied were contained within a phagocytic vacuole with an electron-transparent zone between the host cell membrane and the bacterial cell wall similar to that observed with intracellular mycobacteria (11, 29). We found that *N. farcinica* C localized deep within mononuclear cells (presumably macrophages) were often accompanied by an accumulation of dense granular material closely associated with the phagosome. Glick, Getnick, and Cole (8) reported similar dense material surrounding recently formed phagocytic vacuoles in human monocytes that ingested streptococci. In contrast to our observations, these authors noted that this material disappeared as the phagosome approached the nucleus of the monocyte. They suggested that this material represented a mechanism of transporting phagocytic vacuoles into the central region of the cell (8). Others have postulated that this material represented condensed cytoplasmic filaments associated with the phagocytic mechanism (7, 22). Since we observed this material deep within the cytoplasm of host cells and the nocardia were probably existing as intracellular parasites in well-established lesions (up to 10 weeks after inoculation), we believed that this material was not associated with the phagocytic process per se, but instead it was associated with the presence of the organism. However, the true nature and significance of this dense material remains unknown. In addition, tubular structures were occasionally observed to be continuous with the phagosomal membrane. These structures did not appear to be typical lysosomes, and their exact identity remained undefined.

Since we observed changes in the Gram reaction, acid fastness, and other staining properties of in vivo-grown nocardia, it was reasonable to assume that some chemical and structural modifications were taking place as the organisms adapted to in vivo growth. The present ultrastructural analysis confirmed that structural changes were occurring. As a consequence, it is tempting to speculate on the nature and role of the various layers observed in the nocardial cell envelope. The chemical structure of the cell walls of nocardiae have not been fully

elucidated; however, many components have been isolated from whole cells of nocardia that have also been isolated from cell walls of mycobacteria. For example, "cord factor" (trehalose-6,6-dimycolate) is found in the cell walls of virulent mycobacteria (1). Recently, cord factor was isolated from *N. asteroides* (12). Its location within the nocardial cell is probably the cell wall. There are other lipids, glycolipids, and peptidolipids present in both nocardia and mycobacteria in which the specified location within the cell is not known (1). Many or all of these components could be part of the cell envelope. Beaman et al. (3) have shown that the outer layers of the nocardial cell wall were removable by alkaline-ethanol. This material appeared to be composed of several amino acids either as peptide or protein closely associated with lipoidal material. We are currently studying these cell wall components in more detail to determine their role in the host-parasite interaction.

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