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Ultrastructural Analysis of Growth of *Nocardia asteroides* during Invasion of the Murine Brain

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BALB/c mice were infected with 10^6 CFU of log-phase cells of *Nocardia asteroides* GUH-2 by tail vein injection (at this lethal inoculum dose, approximately 800 to 1,000 CFU becomes deposited in the brain). At 24 h after infection, the ultrastructural interactions of the nocardiae during growth within the murine brain were investigated. The nocardiae grew perivascularly in the pons, substantia nigra, hypothalamus, and thalamus portions of the brain, where they were either within or associated with most brain cell types. There appeared to be a propensity for growth within the soma of neurons and their axonal extensions. The nocardial cells were surrounded by 1 to 30 layers of membrane, and the innermost membrane was usually tightly adherent to the cell wall. This compartmentalization of nocardiae within brain cells could contribute to their failure to induce an inflammatory response or a cytopathic effect. Nevertheless, the bacteria were able to obtain adequate nutrients from the host to grow within the brain. The nocardiae were not completely inert, since some of the brain cells showed signs of degeneration. The myelin sheaths of axons were the most strongly affected, and there was evidence of demyelinization and axonal degeneration. Nocardiae growing within brain cells were phagocytized by compact, dense cells that were probably microglia. There was no ultrastructural evidence that the nocardiae were damaged by these phagocytes 24 h after infection; nevertheless, these cells may be important for the elimination of nocardiae from the brain during a nonlethal infection.

Nocardia asteroides causes central nervous system infections in humans and other animals, and it can be a primary brain pathogen (2). Several studies have focused on the mechanisms of pathogenesis and host interactions with *Nocardia* spp. by using *N. asteroides* GUH-2 as a model (1, 3–5, 8). It was shown that GUH-2 has a specific predilection for the brain following intravenous (i.v.) injection into mice (2, 16, 17). Kohbata and Beaman (16) demonstrated that a nonlethal dose of log-phase GUH-2 cells injected i.v. into mice induced a variety of permanent neurologic signs. Furthermore, in about 10 to 15% of these animals, a levodopa (L-dopa) movement disorder that shared many features with Parkinson's disease in humans was produced (16).

By using this murine model, it was shown that a nonlethal i.v. injection of GUH-2 resulted in a silent, self-limited invasion of the brain (16–18). Light microscopy of coronal sections of the infected brain suggested that the bacteria adhered to capillaries and that, by some mechanism, the organisms grew into the brain tissue without destroying the integrity of the blood-brain barrier (16). In addition, there was no evidence for an inflammatory response at the site of invasion (16). To better understand this process of nocardial invasion of the brain, an ultrastructural analysis was initiated (6) since no electron-microscopic studies on nocardial interactions in the brain have been reported. Attachment to capillary endothelial cells in the regions of the pons and midbrain was visualized following a 15-min intra-arterial perfusion with a suspension of log-phase cells of *N. asteroides* GUH-2 (6). It was shown that the attachment occurred mostly at the growing apex of the nocardial filament. There was adherence between the outer region of the nocardial cell wall and the cytoplasmic membrane of the endothelial cell, and there appeared to be specificity for this binding (6). This attachment process was followed by a rapid penetration of the endothelium, wherein the bacteria were internalized within the host cell. The bacteria remained within vesicles in the endothelial cells and appeared to pass through these cells to the basement membrane. The membrane surrounding the basement membrane appeared to form numerous caveolae, which appeared to interact with the vesicle containing the nocardiae (6). By a mechanism that is not clearly defined, the nocardial cell crossed to the parenchymal side of the basement membrane (16).

Light microscopy results suggested that the nocardiae invaded cells within the brain; however, the exact process, the types of cells involved, the types of damage, the host response, and specific features of nocardial growth within brain tissue are not known. As a consequence, an electron-microscopic analysis of the growth of *N. asteroides* in the murine brain was undertaken, and the results are described below.

MATERIALS AND METHODS

Microorganism. *N. asteroides* GUH-2 was isolated from an individual with a fatal infection at Georgetown University Hospital, Washington, D.C. It was grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) as described previously (17).

Inoculum. Suspensions of single cells (<0.1% cell aggregates based on phase-contrast microscopy) of GUH-2 during log phase were prepared from brain heart infusion broth cultures by differential centrifugation as described in detail elsewhere (17).

Animals. Female BALB/c mice weighing 18 to 20 g were obtained from Simonsens, Gilroy, Calif. The animals were maintained by the Animal Resource Services, University of California, Davis, as previously described (17). Infected mice were placed in a special self-contained animal room supplied with filtered air under negative pressure (17).

Infection of the brain. A uniform suspension of log-phase

cells of *N. asteroides* GUH-2 was prepared from brain heart infusion broth and adjusted to a concentration of approximately 10^7 CFU/ml. To facilitate visualization of the localized growth of the nocardiae in the brain, a lethal dose of 10^6 CFU per mouse (0.1 ml of the bacterial suspension) was injected into the tail vein. It was shown that, at this dose, approximately 800 to 1,000 CFU became localized within the brain 3 h after injection (16–18).

Preparation of the infected brain for electron microscopy. At 24 h after infection, the mice were anesthetized with 0.5 ml of sodium nembutal (5 mg/ml) injected intraperitoneally, and the chest cavity was opened. The mice were perfused intra-arterially with sterile phosphate-buffered saline to remove the blood and then perfused with 4% (wt/vol) paraformaldehyde in 0.1 M phosphate buffer (pH 7.3) at a flow rate of 1 ml/min for 15 min by using a peristaltic pump. The perfused, fixed brains were removed, and 100-μm-thick coronal sections were cut by using a Vibratome. The sections that included the substantia nigra region of the brain were postfixed for 1 h in 0.5% (wt/vol) OsO_4 in phosphate buffer (pH 7.3). The coronal sections were then cut into small segments that specifically included the substantia nigra, red nucleus area, and the pons (the cerebellum was embedded separately). These portions of the brain were placed in 0.5% (wt/vol) uranyl acetate for 30 min and then dehydrated through a series of ethanol solutions and embedded in med-cast epoxy resin (Ted Pella, Inc., Redding, Calif.). After polymerization, thick sections (approximately 1 μm thick) were cut with a glass knife and stained with aqueous carbol-fuchsin (0.2%, wt/vol) to visualize bacteria under the light microscope. When bacteria were located, the blocks were trimmed and sectioned with a diamond knife for electron microscopy. Gold to silver sections were placed on copper grids (200 mesh), stained for 15 min with 0.5% (wt/vol) uranyl acetate in 50% (vol/vol) methanol–filtered distilled water, rinsed, and stained for 5 min with 0.1% lead citrate. The sections were washed in deionized water and dried; they were visualized and photographed by using a Philips model 400 electron microscope operated at 80 kV.

RESULTS

Perivascular growth. Within 24 h after i.v. injection into the tail vein, *N. asteroides* GUH-2 cells had invaded the brain (Fig. 1). There was extensive growth throughout localized regions such as in the substantia nigra (Fig. 1A, pointers). The nocardiae appeared to be associated with the basic types of brain cells (i.e., oligodendrocytes, astrocytes, neurons, and microglia) during this perivascular growth (Fig. 1). Furthermore, there was no ultrastructural evidence of a host response induced by these bacterial cells (Fig. 1). Instead, the nocardiae were bound by membrane that was adherent to the outer layer of the bacterial cell wall (Fig. 1B and 2A).

Interaction with glial cells. Figure 2A shows a nocardial cell within a glial cell (probably an oligodendrocyte) just beneath the basement membrane. The nocardial cell was tightly bound by a unit membrane (Fig. 2A, curved arrow) that appeared to be of host origin. There appeared to be evidence for a modicum of nocardia-induced damage to the glial cell, since the cytoplasm appeared to be less dense than it should have been. This abnormality was accompanied by some loss of structural integrity with a contraction of the cytoplasmic membrane away from neighboring cells (Fig. 2A, pointer). However, both intercellular tight junctions forming the blood-brain barrier and the structure of the surrounding cells appeared to be normal (Fig. 2A). Even though some brain cells appeared to show evidence of nocardia-induced damage, most did not (Fig. 2). Often the nocardiae grew among both myelinated and nonmyelinated axons. As shown in Fig. 2B, such nocardial interactions did not result in any recognizable cytopathic effect since the membranes, microtubules, microfilaments, and mitochondria appeared undamaged even when they were close to the nocardial cell (Fig. 2B).

Growth in neurons. Figure 3 shows a nocardial cell within the cytoplasm of the soma of a neuron. The structural integrity of the cell was not altered, and both the nocardial cell and the neuron appeared to be ultrastructurally intact as evidenced by a lack of damage to the Golgi apparatus and mitochondria (Fig. 3A). Interestingly, the nocardiae growing within the cells of the brain were often surrounded by layers of membrane such as shown in Fig. 3. Usually, there was a membranous layer closely adherent to the cell wall of the bacterium (Fig. 3B, arrow), which was then surrounded by more loosely associated layers (Fig. 3B). The number of layers of membrane surrounding the nocardiae varied from as few as 1 to as many as 30 (approximately 10 layers surrounded the cell shown in Fig. 3B). The source and nature of these membranes are not clear, but in some instances the membranes appeared to be associated with, or derived from, axonal myelin sheaths (Fig. 4).

Growth in axons. The nocardiae frequently extended into both myelinated and nonmyelinated axons (Fig. 4). The nocardiae did not usually cause structural alterations in the nonmyelinated axons (Fig. 4A). Indeed, axonal microtubules were intact even when they were closely associated with the bacterial cell (Fig. 4A, arrows). On the other hand, the interactions of nocardiae with myelinated axons resulted in a disruptive response to the myelin sheaths (Fig. 4B and C). Frequently, there was a demyelination and the outer layers of the myelin became tightly adherent to the nocardial cell wall (Fig. 4B, arrow). Occasionally, the nocardiae grew between the layers of the myelin, resulting in a displacement of the axonal body as well as disruption of the surrounding sheath (Fig. 4C).

Nocardia-induced axonal degeneration. Figure 5 depicts an area of the substantia nigra in which numerous nocardiae are growing. Even though there was extensive nocardial growth, it should be noted that there was little evidence of damage to brain cells and that there was no infiltration by inflammatory cells (Fig. 5A). Nevertheless, there was induction of a neurodegenerative response involving the axons (Fig. 5A, arrows) as shown by axonal degeneration and myelin disruption associated with the nocardial cells (Fig. 5B).

Interaction of nocardiae with probable microglia. Compact, more electron-dense cells (probably microglia) were occasionally observed in areas where the nocardiae had invaded the brain tissue (Fig. 6). These cells were phagocytic, and they engulfed small regions of the brain that contained nocardial cells (Fig. 6A). Thus, the nocardiae became internalized within these probable microglia (Fig. 6B). It is not known whether microglia kill *Nocardia* cells, but these host cells might represent a means by which the nocardiae were eliminated from the brain during a sublethal infection. However, in this study of a lethal infection, there was no ultrastructural evidence that the nocardiae were damaged by these phagocytic cells (Fig. 6).

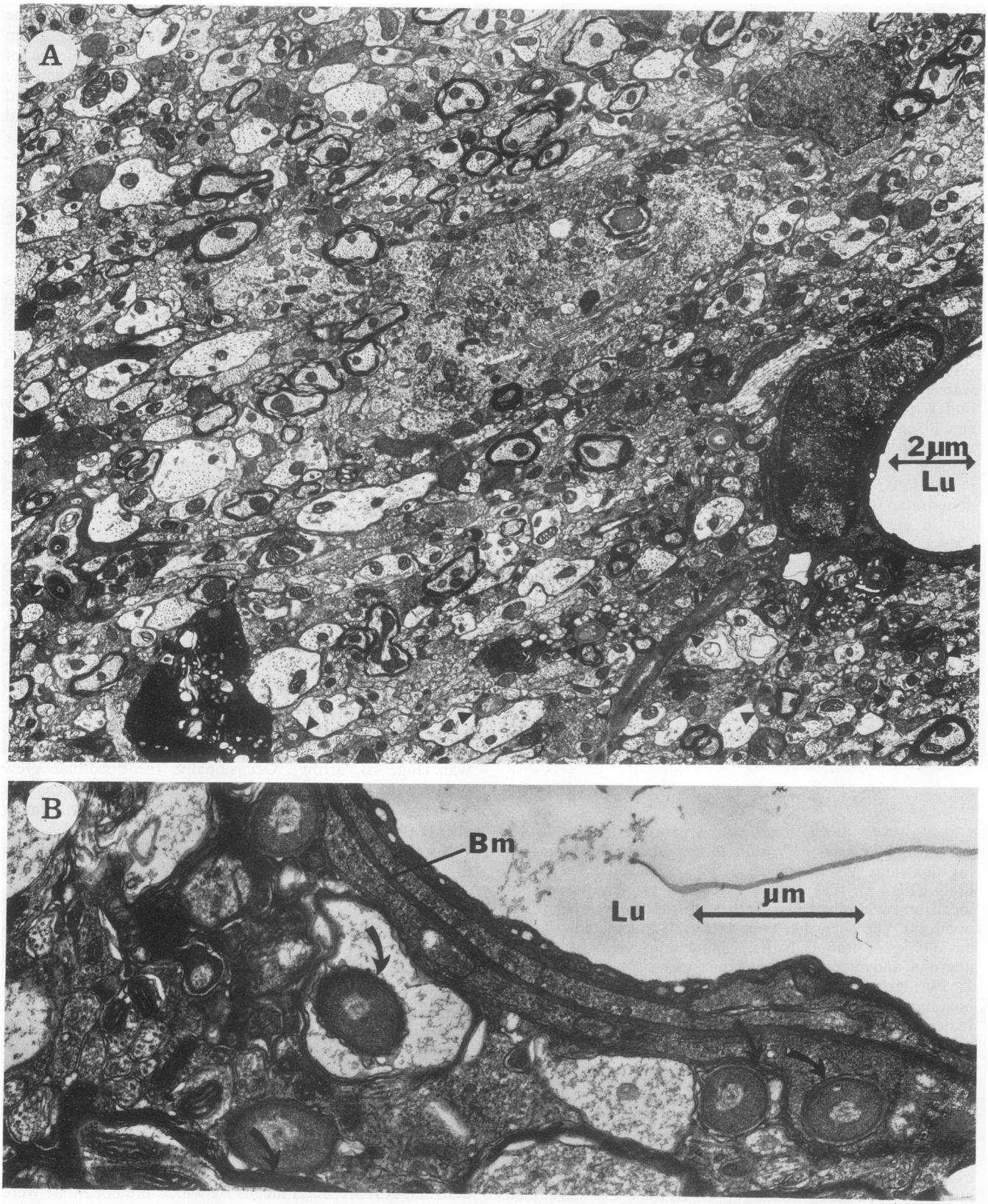

FIG. 1. Perivascular growth of *N. asteroides* GUH-2 in the region of the substantia nigra 24 h after infection. (A) Low-magnification view of an area showing the nocardiae growing extensively throughout the brain tissue (note the absence of inflammation). Lu, lumen of the capillary. Each triangular pointer indicates a nocardial cell (at least 14 bacteria are present in this section). Bar, 2.0 μm. (B) Enlargement of a region near a capillary similar to that in panel A. Numerous bacteria are growing in pericytes and other brain cells. Arrows indicate a close association between the nocardial cell wall and host membrane. Bar, 1.0 μm. Lu, lumen of the capillary; Bm, basement membrane beneath capillary endothelial cells.

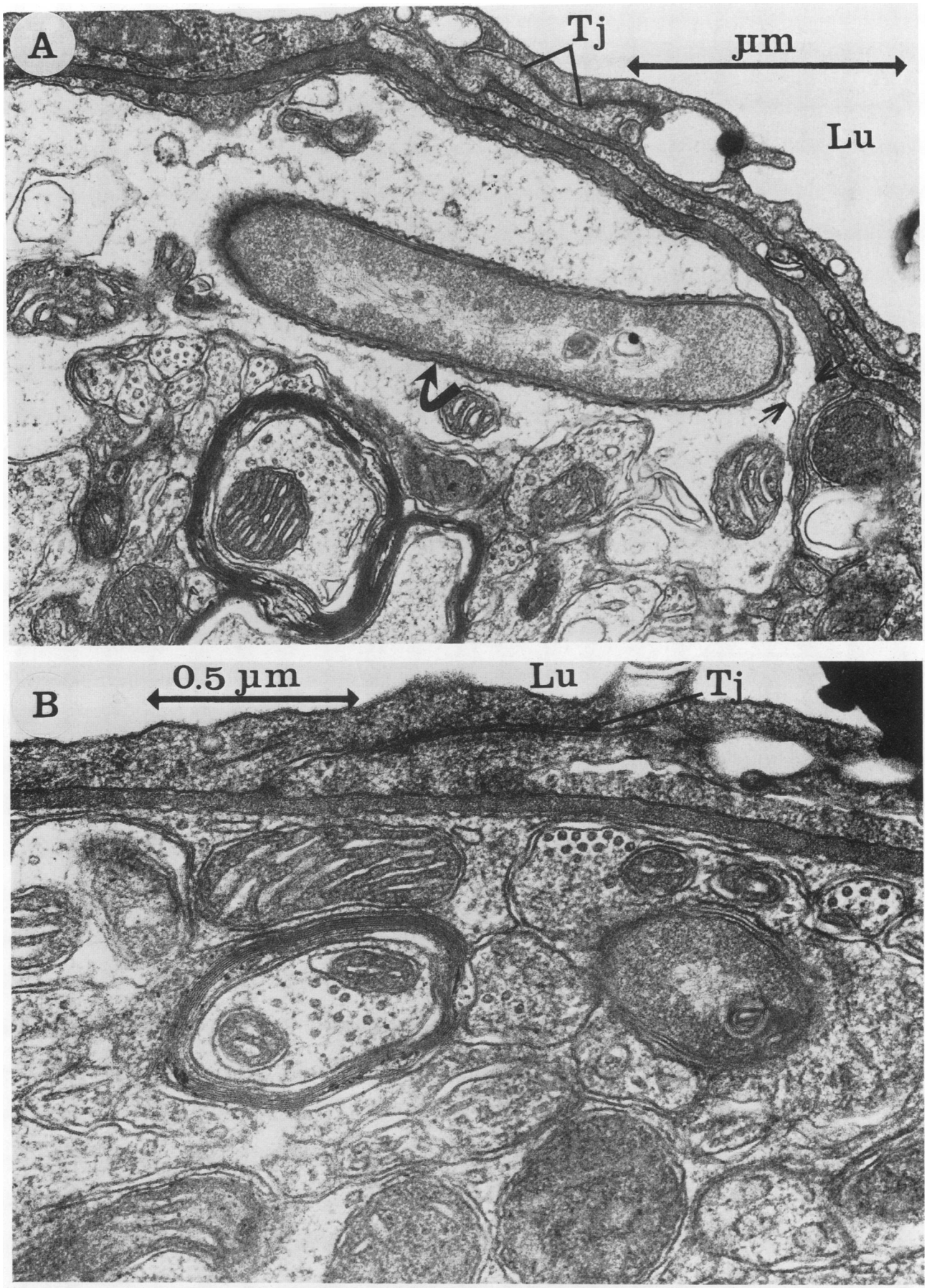

FIG. 2. Growth of *N. asteroides* GUH-2 within cells beneath the basement membrane of the capillary 24 h after infection. (A) Nocardial cell in the cytoplasm of a glia cell (a probable oligodendrocyte). Tj, tight intercellular junctions forming the blood-brain barrier; Lu, lumen of the capillary. The pointer notes the pulling away of the cytoplasmic membrane from adjacent cells; the arrow indicates host membrane tightly bound to the surface of the nocardial cell wall. Bar, 1 µm. (B) Nocardial cell in (or among) nonmyelinated neuronal extensions along the basement membrane surrounding a capillary. Tj, tight junction; Lu, lumen of the capillary. Bar, 0.5 µm.

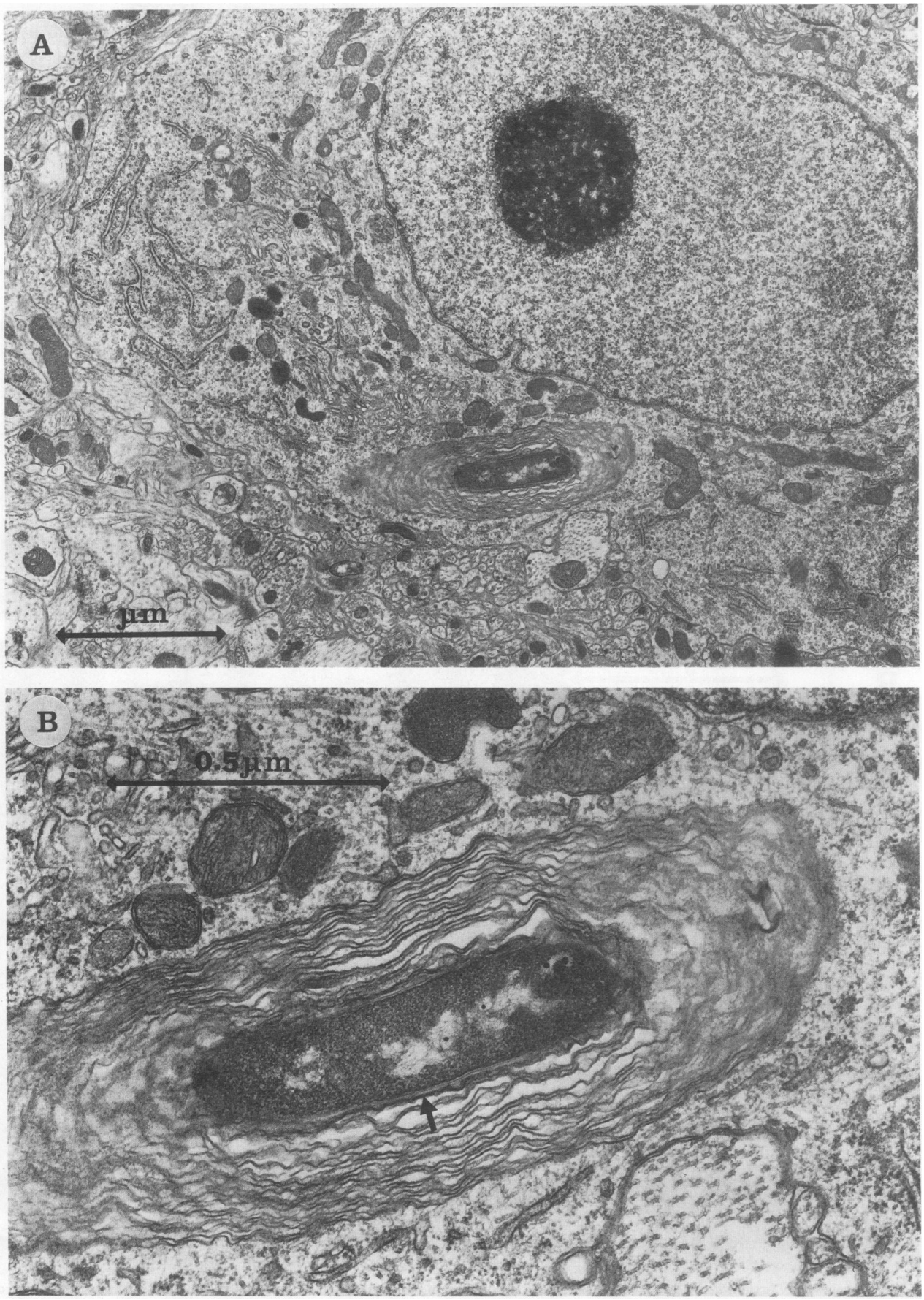

FIG. 3. Growth of *N. asteroides* GUH-2 in a neuron 24 h after infection. (A) Low-magnification view showing that there is no apparent damage to the neuronal cell (no cytopathic effect). Bar, 1 μm. (B) High-magnification insert of the bacterial cell from panel A showing that the ultrastructural integrity of the organism is unaltered even though the bacterium is surrounded by numerous layers of membrane. The arrow points to membrane adherent to the bacterial cell wall. Bar, 0.5 μm.

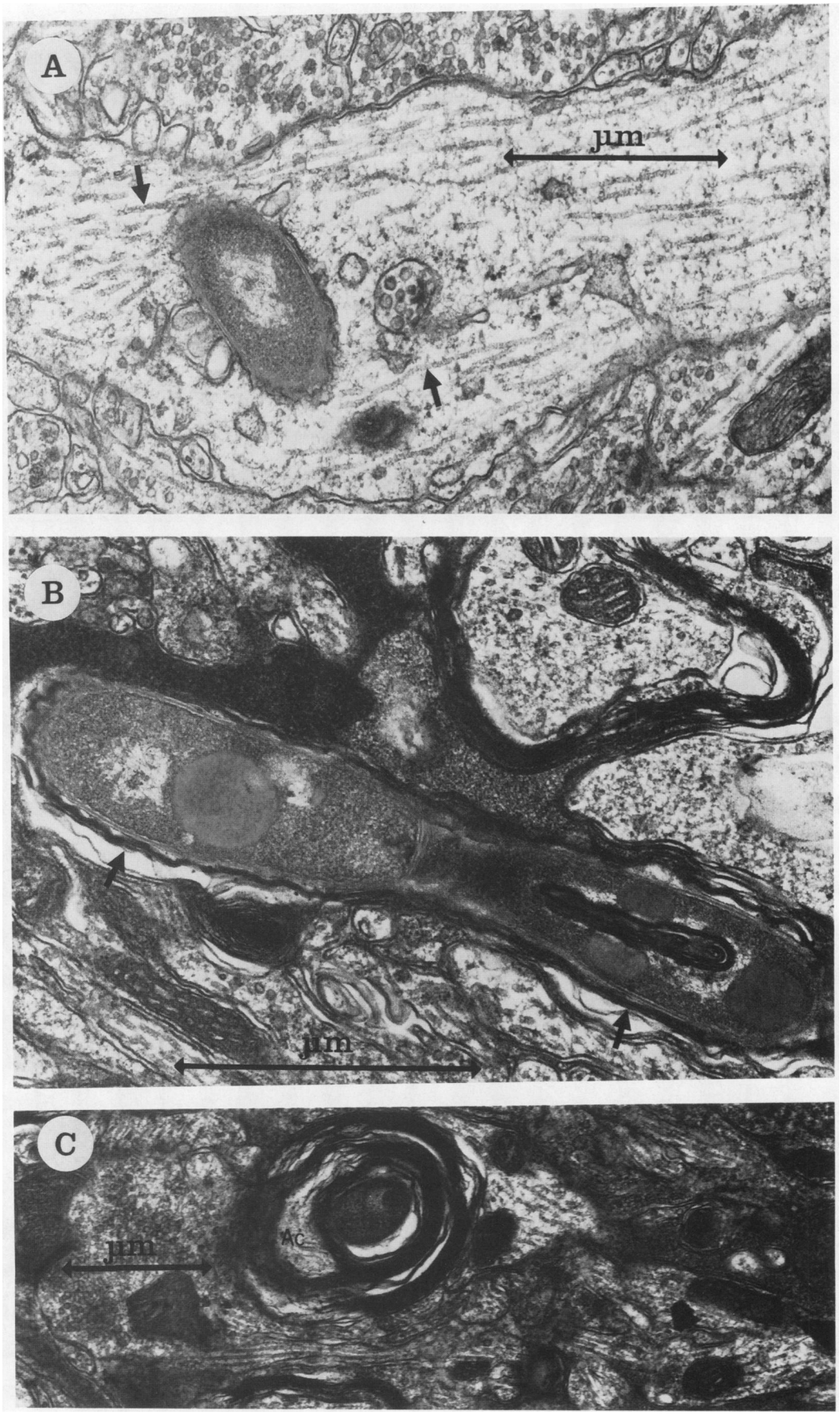

FIG. 4. Interactions of *N. asteroides* GUH-2 with axons 24 h after infection. (A) Extension (growth) into a nonmyelinated axon. Arrows point to axonal microtubules. Bar, 1.0 μm. (B) Growth of nocardiae between myelin membranes along a myelinated axon. Arrows point to layers of membrane tightly adherent to the nocardial cell wall. Bar, 1.0 μm. (C) Growth of the nocardial cell along a myelinated axon between layers of the myelin sheath, resulting in separation of the myelin and compression of the axonal core (Ac). Bar, 1.0 μm.

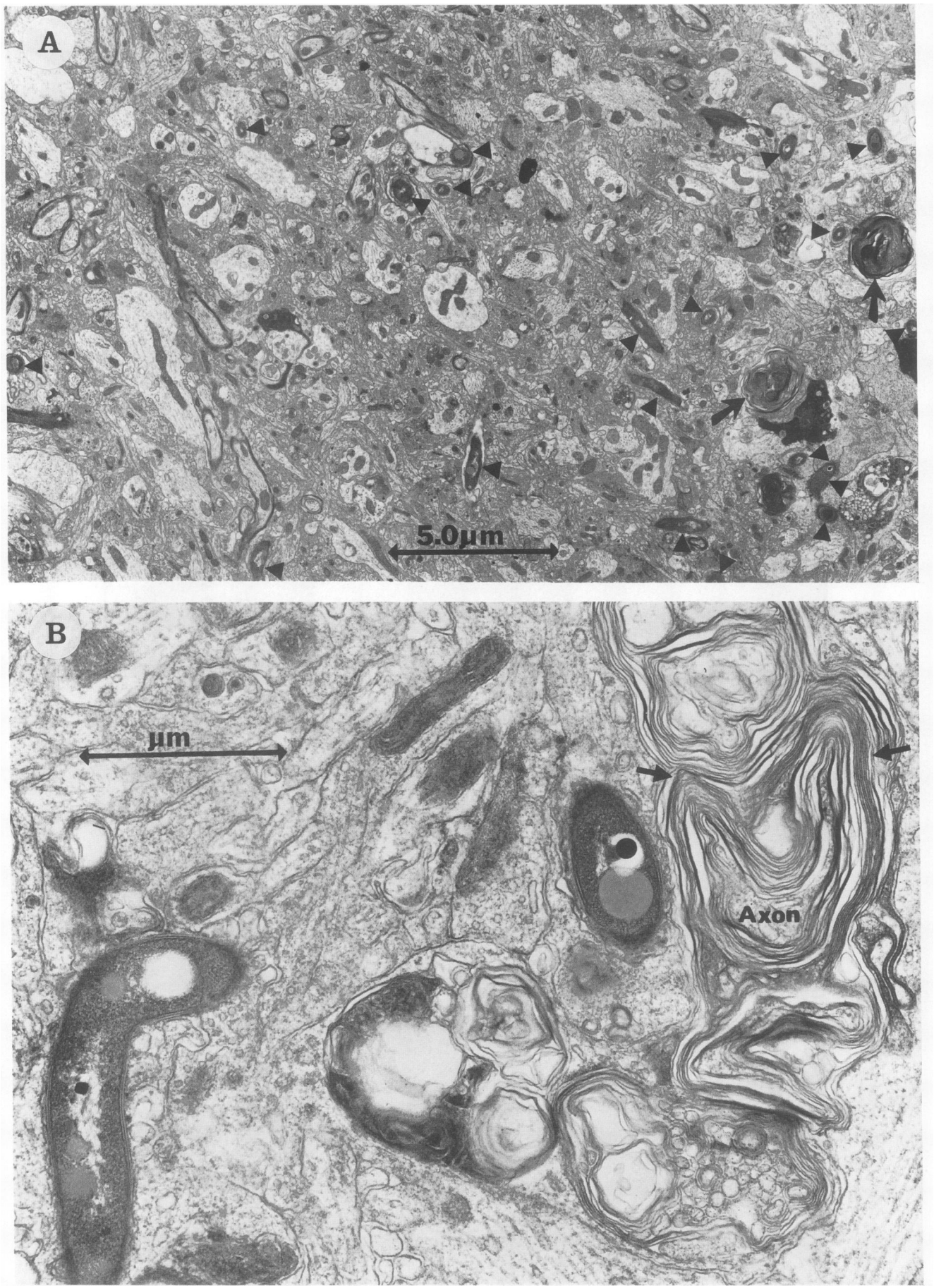

FIG. 5. Nocardia-induced axonal degeneration and myelin disruption 24 h after infection. (A) Low-magnification view showing nocardial cells growing extensively throughout this region of the brain tissue (note the absence of inflammation). The arrows indicate evidence of induction of neurodegeneration involving the myelinated axons. Each triangular pointer indicates a nocardial cell (at least 19 bacteria are present in this section). Bar, 5.0 µm. (B) Enlargement of an area adjacent to panel A showing axonal degeneration (Axon) and myelin disruption (arrows). There are two nocardial cells in proximity to these degenerating myelinated axons. Bar, 1.0 µm.

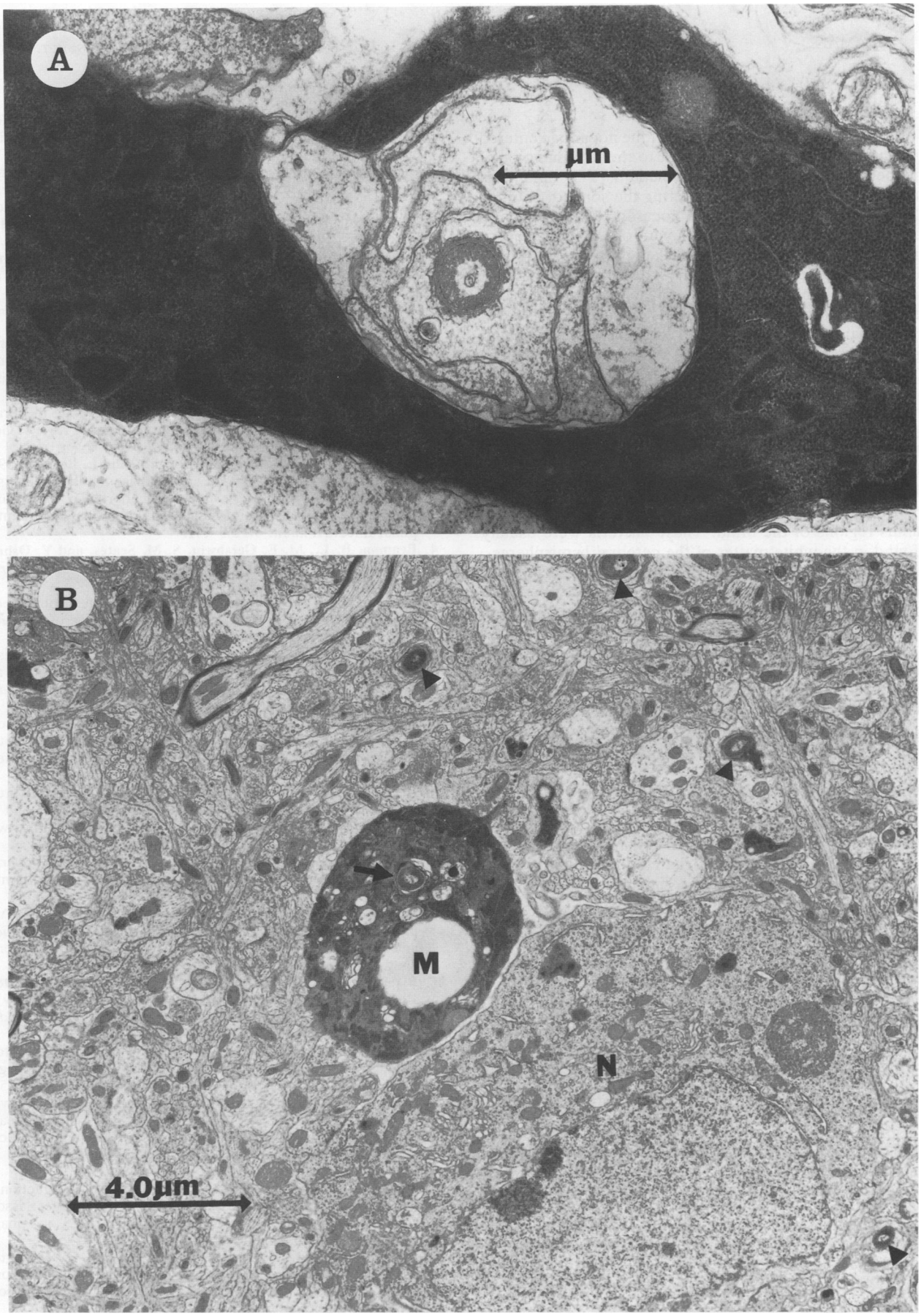

FIG. 6. Interaction of phagocytic cells (probable microglia) with *N. asteroides* GUH-2 in the brain 24 h after infection. (A) Dense, phagocytic cell in the process of phagocytizing a small region of the brain containing a nocardial cell. Bar, 1.0 μm. (B) Low-magnification view of a section of the brain showing a neuron (N), several nocardial cells (pointers), and a more dense microglia (M) that had phagocytized a nocardial cell (arrow). Bar, 4.0 μm.

DISCUSSION

Ultrastructural analysis showed that the nocardiae entered perivascular glial cells lying adjacent to the basement membrane beneath the capillary endothelium. The organisms then grew through these cells, entering the soma of neurons and their axonal extensions. The nocardial cells frequently became surrounded by multiple layers of additional membrane. This process appeared to mask the organism and may have prevented recognition of the nocardiae by the host since there was generally no recognizable host response at the sites of nocardial infiltration. This extensive invasion of the brain parenchyma resulted in little damage to cellular integrity; thus, there was minimal cytopathic effect and a general absence of inflammation. Nevertheless, the nocardiae did damage some of the cells, especially myelinated axons, and this resulted in demyelinization and axonal degeneration.

The nocardiae were observed within different types of cells within the cerebrum, but they were most frequently associated with neurons in the pons region of the brain stem and the substantia nigra, red nucleus, thalamus, and hypothalamus regions of the brain. These bacteria were usually not found in the white matter of the cerebral hemispheres.

Small numbers of compact, phagocytic cells were scattered throughout the brain. These were probably microglia, which are macrophage-equivalent cells within the brain (9). The microglia engulfed areas of tissue that contained the invading nocardial cells, and nocardiae were observed within these more distinctive cells. However, at 24 h after infection with a lethal dose of nocardiae, there was no ultrastructural evidence of damage to the bacteria by these phagocytes. Indeed, the nocardiae appeared to be growing in some of the microglia (data not shown). Nevertheless, it seems likely that microglia were critical to preventing continued growth of nocardiae in the brain during a sublethal infection (9, 16). Furthermore, microglia probably represented the most important means by which the nocardiae were eliminated from the brain 10 to 14 days after infection, because it was shown that microglia cultured in vitro for 14 days were able to prevent nocardial growth whereas in vitro-grown astroglia were not (9).

The mechanisms whereby *N. asteroides* GUH-2 invades and grows within the murine brain are not known. It has been shown that *N. asteroides* is a facultative intracellular pathogen that grows within macrophages and polymorphonuclear neutrophils from mice, rabbits and humans (1, 3, 8, 11, 15). The mechanisms that permit virulent *Nocardia* strains to grow within these phagocytes have been studied. It was demonstrated that *N. asteroides* GUH-2 inhibits phagosome-lysosome fusion (14), blocks phagosomal acidification (12), alters lysosomal enzyme content (11, 13), and resists oxidative killing mechanisms by secreting a unique superoxide dismutase (3, 7). Furthermore, it was observed that GUH-2 cells can utilize both glutamate and acid phosphatase as the sole carbon source and that when they are grown in glutamate, acid phosphatase synergistically stimulates their growth (10). Also, *N. asteroides* GUH-2 metabolizes tyrosine to produce an iron-binding compound (19). Since glutamate, acid phosphatase, and tyrosine are important components in specific regions of the brain (20, 21), the ability of GUH-2 to preferentially metabolize these compounds may be significant for its survival and preferential growth within neurons in regions such as the pons, substantia nigra, red nucleus, thalamus, and hypothalamus (18).

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