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Probable L-Forms of *Nocardia asteroides* Induced in Cultured Mouse Peritoneal Macrophages

L. BOURGEOIS AND B. L. BEAMAN

Department of Microbiology, Georgetown University, Schools of Medicine and Dentistry, Washington, D.C. 20007

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Mouse peritoneal macrophages were infected with varying numbers of *Nocardia asteroides* 10905, and the fate of the ingested organisms was determined by viable plate count (VPC), light microscopy (LM), immunofluorescent microscopy (IM), and electron microscopy (EM). The results obtained with these methods differed. VPC indicated that intracellular *Nocardia* decreased in numbers during the first 12 days, followed by significant increases after 16 days. LM suggested that *N. asteroides* 10905 was slowly degraded by macrophages with no subsequent increases observed. In contrast, IM demonstrated large numbers of intracellular *Nocardia* throughout the experiment. EM studies of infected macrophages failed to demonstrate intact bacteria after 8 days; however, wall-less and spheroplast-like organisms were seen. These results suggested that *N. asteroides* 10905 was present within the macrophages in an altered form. By using hypertonic culture medium, we were able to isolate, from infected macrophages, organisms which exhibited many of the properties of bacterial L-forms. IM demonstrated these variants to be of nocardial origin. These altered forms also reverted to typical nocardial cells either spontaneously or upon transfer into broth. These findings indicate that *N. asteroides* 10905 is capable of existing within macrophages in an altered state. Further investigation is in progress to determine whether these altered forms represent L-forms or transitional-phase variants.

Nocardia asteroides causes both localized and disseminated infections in man (12, 22). Primary infections of the lungs, skin, and central nervous system, and secondary infections in such debilitating illnesses as Hodgkin's disease, leukemia, and emphysema have been reported (12, 17, 26, 32). Diagnosis of nocardial infection is often difficult since, in many cases, the organisms cannot be isolated from infected individuals and, therefore, diagnosis is not made until autopsy (17, 22, 26). In addition, nocardial infections may be mistaken for tuberculosis, bacterial pneumonia, or actinomycosis (17, 22, 26).

The mechanisms controlling primary and secondary nocardial infections are not known. In vivo studies of nocardial pathogenesis have dealt with pathology of infection and the relative virulence of various *Nocardia* species (2, 7, 28). Preliminary studies done in this laboratory with experimentally infected mice have shown that *Nocardia* undergo ultrastructural changes within the host which might be associated with virulence (2). In vitro studies of the mechanisms of nocardial pathogenesis have not been reported. Friedhiem and Baroni (8), in 1929, attempted to ascertain the fate of *N. asteroides* in cultures of rat fibroblast and mouse embryonic tissue. However, they were unable to maintain cultures long enough to obtain meaningful results (8). A study of nocardial pathogenesis in an in vitro system is important in determining the host-parasite relationship at the cellular level. The interactions that occur between *Nocardia* and specific host cells may direct the course of infection.

The chronic, progressive nature and pathology of nocardial infections suggest that *Nocardia*, like *Mycobacterium tuberculosis*, are facultative intracellular parasites (1, 11, 24). To investigate the intracellular capabilities of *Nocardia*, we studied the fate of *N. asteroides* 10905 in cultured mouse peritoneal macrophages. Ingested *N. asteroides* 10905 were analyzed by viable plate counts, light microscopy, immunofluorescent microscopy, and electron microscopy. The results obtained using these techniques indicated that this strain of *N.*

asteroides existed intracellularly in an altered state that exhibited many of the properties of bacterial L-forms.

MATERIALS AND METHODS

Microorganisms. *N. asteroides* 10905 was supplied by J. Rozanis, University of Western Ontario, London, Canada. Stock cultures were maintained on brain heart infusion agar (BHI-A) slants at 4 C. For the infection of macrophage cultures, *N. asteroides* 10905 was grown on BHI-A plates for 5 days at 34 C. The organisms were washed from the plates with saline, and the bacterial cell suspension was centrifuged at a low speed to remove clumps. The number of *Nocardia* in the suspension was standardized by absorbance at a wavelength of 450 nm with a Spectronic 20 spectrophotometer (Beckman). In addition, samples were subcultured to determine the viability and cell purity of the preparation. The nocardial suspension was diluted to give a multiplicity of infection of 5 *Nocardia* per macrophage. These dilutions were made in Eagle minimum essential medium containing Earle salts (MEM) supplemented with 20% heat-inactivated fetal calf serum and *l*-glutamine (1 μg/ml).

Tissue culture. Macrophages were obtained from the unstimulated peritoneal cavity of 3- to 4-week-old female Swiss-Webster mice. The cells were harvested and cultured by a modification of the method of Chang (5). The macrophages were harvested in cold MEM containing heparin (1:20,000), and were maintained in MEM (pH 7.2) containing 20% heat-inactivated fetal calf serum and *l*-glutamine. The macrophages (10^6 cells) were added to petri dishes (Falcon Plastics; 35 by 10 mm), containing cover slips, and incubated at 37 C under 5% CO_2 in air.

After 24 h of incubation, the cell cultures were washed once with MEM, followed by the addition of a suspension of *N. asteroides* 10905. After 2 h at 37 C, the cultures were washed twice to remove extracellular bacteria, and fresh maintenance medium containing penicillin (40 μg/ml) and fungizone (1 μg/ml) was added. Uninfected macrophage cultures served as controls. The culture medium was changed every 3 days. Both infected and control macrophages remained viable for more than 20 days.

Mouse infections. Experimental infections were induced in 3- to 4-week-old female Swiss-Webster mice by intraperitoneal injections of *N. asteroides* 10905 as described previously (2). Granules from these mice were processed for electron microscopy (2), and thin sections were prepared.

Viable plate counts. At specific times postinfection, the cover slips containing infected macrophages were washed twice with MEM, and the cells were removed from the glass with a rubber policeman. Dilutions of the intact cells were spread on culture media to determine the number of viable intracellular *Nocardia*. The following media were used to recover the *Nocardia* from macrophages: BHI-A; BHI-A containing 0.2 M sucrose; brain heart infusion broth supplemented with 20% heat-inactivated fetal calf serum, 0.7% agar, and 5% sodium chloride; and Barile, Yaguchi, and Evelyn (BYE) broth containing 15% heat-inactivated fetal calf serum and 0.7% agar. Duplicate inoculated plates were incubated either at 34 C aerobically or 37 C under 5% CO_2 in air. After 17 to 21 days of incubation, the mean number of nocardial colonies per 10^4 macrophages was determined.

Uninfected control macrophages and portions of the MEM and serum used in the culture medium were also cultured to determine whether they were contaminated with other bacteria or *Mycoplasma*.

Light microscopy. Various staining techniques were used to ascertain the number of intracellular *Nocardia* at specific times postinfection. The Ziehl-Nielsen acid-fast stain (19) and the Brown and Brenn modification of the Gram stain (19) were inconclusive. The standard Gram stain gave the most consistent results.

Cover slips were taken from control and infected cultures at 2 h, and 1, 3, 5, 7, 10, 18, and 20 days and fixed in methanol (undiluted) for 5 min. The cover slips were Gram stained by using acetone-alcohol (1:1) as the decolorizing agent. After being counterstained with safranin, they were washed, dried, and mounted on slides with Permount.

Stained control and infected macrophages were counted, and the number of gram-positive particles per 10^2 macrophages were determined. The mean number of gram-positive particles observed per 10^4 control macrophages was calculated throughout the experiment. The confidence interval of the control mean was determined at the 5.0% level by the Poisson distribution (10). Mean values computed for infected cultures at specific intervals postinfection were compared to the control mean. Values within the control distribution were considered to be free from *Nocardia*. The counts were done on cover slips that had previously been color-coded and scored independently by other individuals. This was done to eliminate possible counting bias. Further tabulation of the results was not done until the experiment was completed.

Antiserum. Five-day cultures of *N. asteroides* 10905 grown in brain heart infusion broth were fixed in 4% Formalin solution overnight and washed three times with deionized water. A uniform suspension was prepared by homogenization with a Virtis 45 blender. Thirty milliliters of the bacterial suspension was combined with 40 g of 0.1-mm glass beads (Glasperlen) and broken in a carbon dioxide-cooled Braun disintegrator for 4 min. The extent of breakage was determined by phase-contrast microscopy. The broken-cell suspension was separated from the glass beads and centrifuged (Beckman J-21) at approximately 20,000 × *g* for 1 h. The resulting supernatant served as a cytoplasmic extract. The protein content of this extract was determined by the method of Lowry et al. (18). Rabbits were injected with 6 mg of protein intravenously and 4 mg of protein, emulsified in incomplete Freund adjuvant, at various sites (footpads, shanks, and nuchal region) subcutaneously. The immunization schedule was as follows. Rabbits received intravenous injection of 1 mg of protein suspended in saline every 3 days for 2 weeks, and 2 mg of protein in incomplete Freund adjuvant on days 1 and 14. The sera obtained from animals bled 7 days

after the last injection were pooled and used for indirect immunofluorescent staining.

Indirect immunofluorescence. Indirect immunofluorescent stains (9) were done on cover slips at various intervals postinfection. Before staining, the cover slips were rinsed in phosphate-buffered saline and fixed in cold methanol for 5 to 10 min. Commercially prepared fluorescien-conjugated goat, anti-rabbit immunoglobulin G (Pentex, Research Products Divisions, Miles Laboratories, Inc., Kankakee, Ill.) was used in the indirect method. A 0.01% solution of Evan blue was used as a counterstain (9). Photographs were made with a Nikon fluorescent microscope. High-speed Ektachrome film (Kodak) was exposed for 20 min, and the resultant slides were copied by using Kodak Plus-X film.

Electron microscopy. Control and infected macrophages were removed from their cover slips with a rubber policeman. Cell suspensions were fixed in 3.0% glutaraldehyde fixative prepared in Kellenberger buffer (pH 6.5) (16). After 24 h at 4 C, the cells were gently pelleted and washed twice with this buffer (pH 6.5). The pellet was suspended in Kellenberger buffer (pH 6.5) containing 1.0% osmium tetroxide and stored at 4 C for 18 h. The pellet was washed twice with Kellenberger buffer (pH 6.5) and suspended in Kellenberger buffer (pH 6.5) containing 0.5% uranyl acetate for 3 h at 4 C. The pellet was dehydrated through a series of ethanol, propylene oxide, and Maraglass. The pellet was finally embedded in Maraglass and polymerized at 60 C for 48 h. Blocks were sectioned with a DuPont diamond knife on an MT-2 Porter-Blum ultramicrotome. Sections were stained with lead citrate for 60 s (16) and photographed through a Philips EM300 electron microscope operated at 60 kV.

Normal nocardial colonies and colonies of altered forms were fixed and processed for electron microscopy by the same procedure. Negative stains of L-forms were prepared by suspending the colonies in 2% ammonium molybdate (16) made up in 20% sucrose solution. Stained preparations were mounted on Formvar- or parlodion-coated grids.

Scanning microscopy. Normal nocardial colonies and colonies of altered forms on agar were mounted on cover slips and fixed in osmium tetroxide vapors. The specimens were then coated with gold-palladium under vacuum. After coating, the specimens were examined in a Joel JSM-U3 scanning electron microscope, and photomicrographs were taken on Polaroid film type 55 P/N.

RESULTS

Fate of ingested organisms. The viable count of intracellular *Nocardia* decreased during the first 12 days, followed by significant increases in the number of intracellular *Nocardia* after 16 days (Fig. 1). Viable counts were performed by plating dilutions of infected macrophages on brain heart infusion agar, and the number of viable *Nocardia* per 10^4 macrophages was determined. In addition, the number of extracellular organisms in the medium was determined at specific intervals after infection. This number never exceeded 400 *Nocardia* per ml.

Gram stains of infected macrophages gave results that differed from the viable plate count. Cover slips of control and infected cells were gram stained to determine the mean number of gram-positive intracellular organisms per 100 macrophages. Gram-positive organisms were not observed in macrophages after 7 days of culture, and gram-positive intracellular *Nocardia* were not demonstrable after 15 days of incubation even though the number of viable *Nocardia* recovered had increased significantly (Fig. 1). In contrast, indirect immunofluorescent staining of infected macrophages with

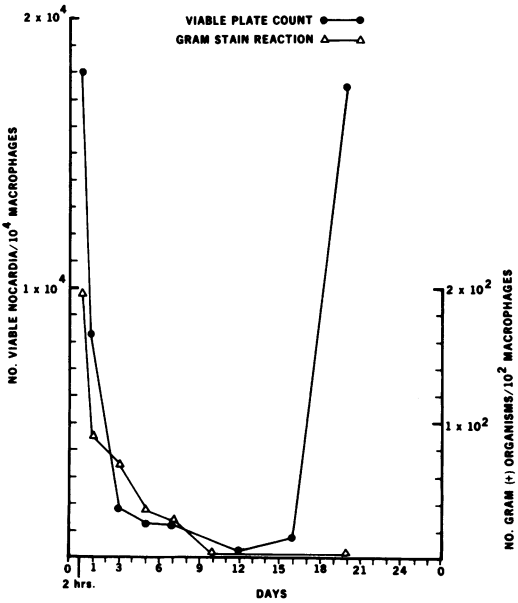


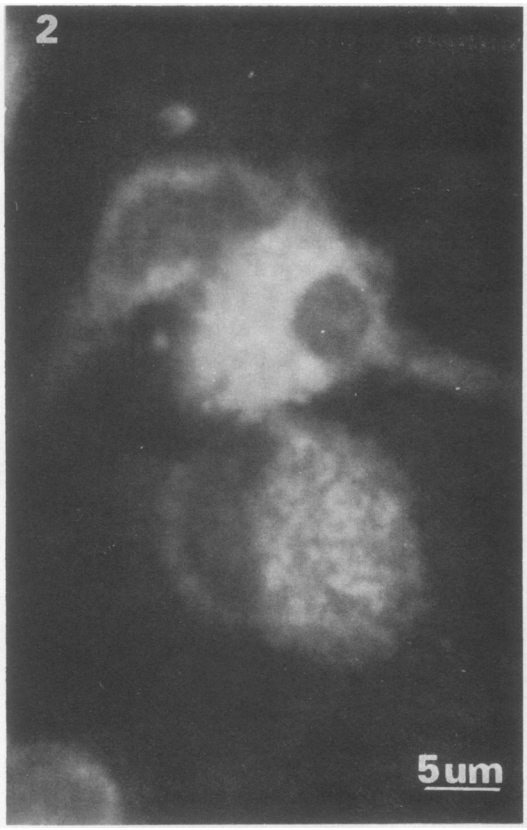
FIG. 1. *Curves contrasting the number of intracellular Nocardia determined by viable plate counts with the number of gram-positive, intracellular organisms as determined by the Gram stain reaction. Each point represents an average of three determinations.*

FIG. 2. *Indirect immunofluorescent stain of peritoneal macrophages with intracellular N. asteroides 10905 (2 h postinfection).*

FIG. 3. *Indirect immunofluorescent stain of peritoneal macrophages with intracellular N. asteroides 10905 (15 days postinfection).*

FIG. 4. *Indirect immunofluorescent stain of infected macrophages (2 h postinfection). Normal rabbit serum was substituted for anti-nocardial serum.*

FIG. 5. *Indirect immunofluorescent stain of infected peritoneal macrophages (15 days postinfection). Anti-staphyloccal antibody was substituted for anti-nocardial serum.*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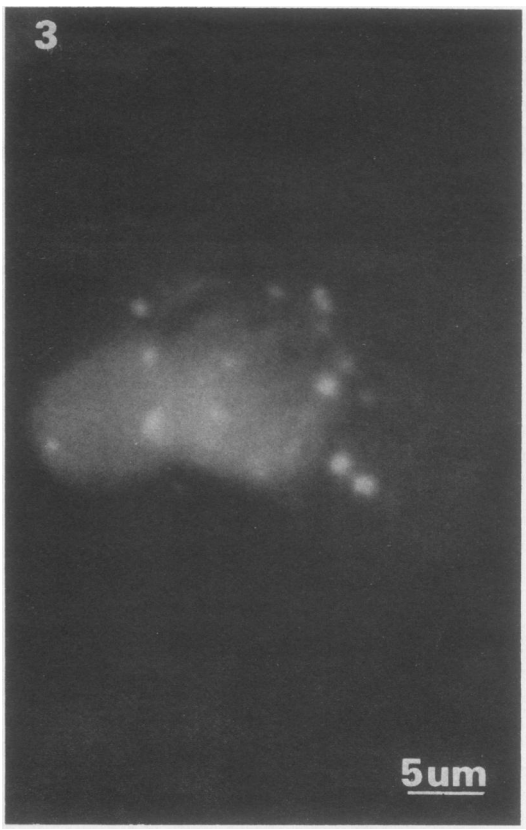
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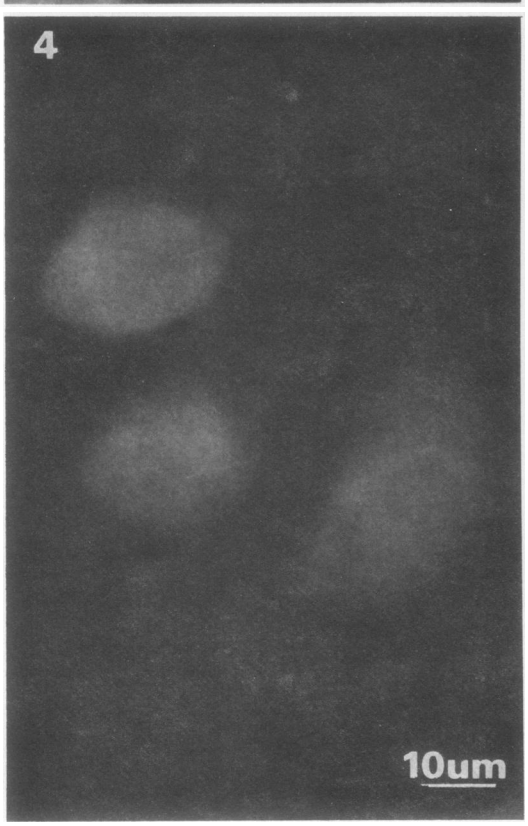
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specific anti-nocardial serum showed that nocardial antigens were present in macrophages throughout the experiment. At 2 h, large numbers of *Nocardia* were visible in infected cells (Fig. 2), and after 15 days fluorescent antibody staining still demonstrated large numbers of intracellular organisms (Fig. 3). Treatment of control and infected macrophages with specific anti-staphylococcal antibody, as well as with normal rabbit serum, gave no fluorescence (Fig. 4 and 5).

Ultrastructural studies of *N. asteroides* 10905 in macrophages showed the cell wall to be dramatically altered during the course of infection. At 2 h, intracellular organisms had the trilayered cell wall characteristic of in vitro-grown *N. asteroides* 10905 (compare cell wall profiles of organisms in Fig. 6, 6a, 7, and 7a). However, after 8 days, typical *Nocardia* were not demonstrated in thin sections of infected macrophages. Instead, organisms that resembled spheroplasts were observed. These wall-less nocardial cells were found to have either single- or double-unit membranes (Fig. 8). In addition, spheroplast-like organisms that retained portions of their cell wall were seen in infected cells at later stages of infection (Fig. 9). Similar structures were not observed in thin sections of control macrophages. The data obtained from these experiments indicated that *N. asteriodes* 10905 underwent considerable structural alteration within the macrophages. Finding wall-less and spheroplast-like organisms suggested that this strain of *Nocardia* existed intracellularly as L-forms. Subsequent experiments were designed to facilitate recovery of altered forms from infected macrophages.

Recovery of probable nocardial L-forms

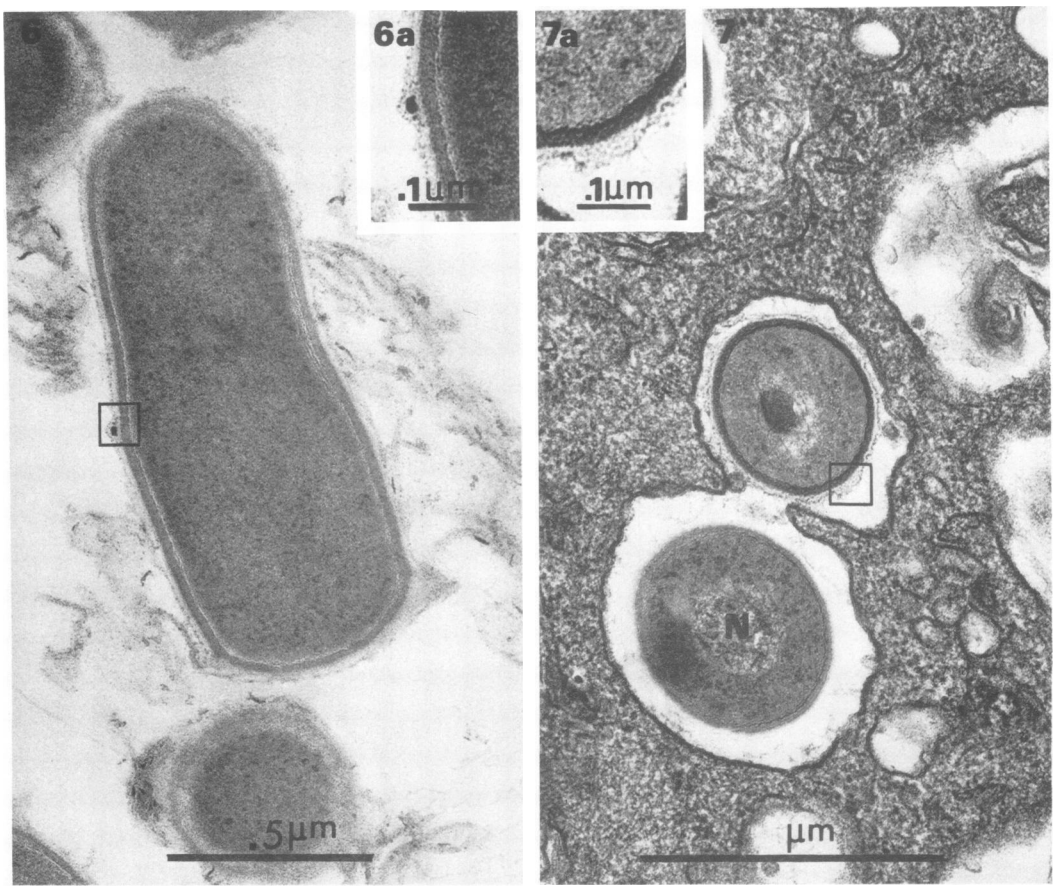


FIG. 6. *Thin section of N. asteroides 10905 cells grown on brain heart infusion agar for 5 days at 34 C. Insert (6a), High magnification of the cell wall of N. asteroides 10905, showing its trilayered structure.*

FIG. 7. *Thin section of N. asteroides 10905 in peritoneal macrophages (2 h postinfection). N, Nuclear region. Insert (7a), High magnification of the cell wall of intracellular N. asteroides 10905 (2 h postinfection). The trilayered structure is still clearly evident.*

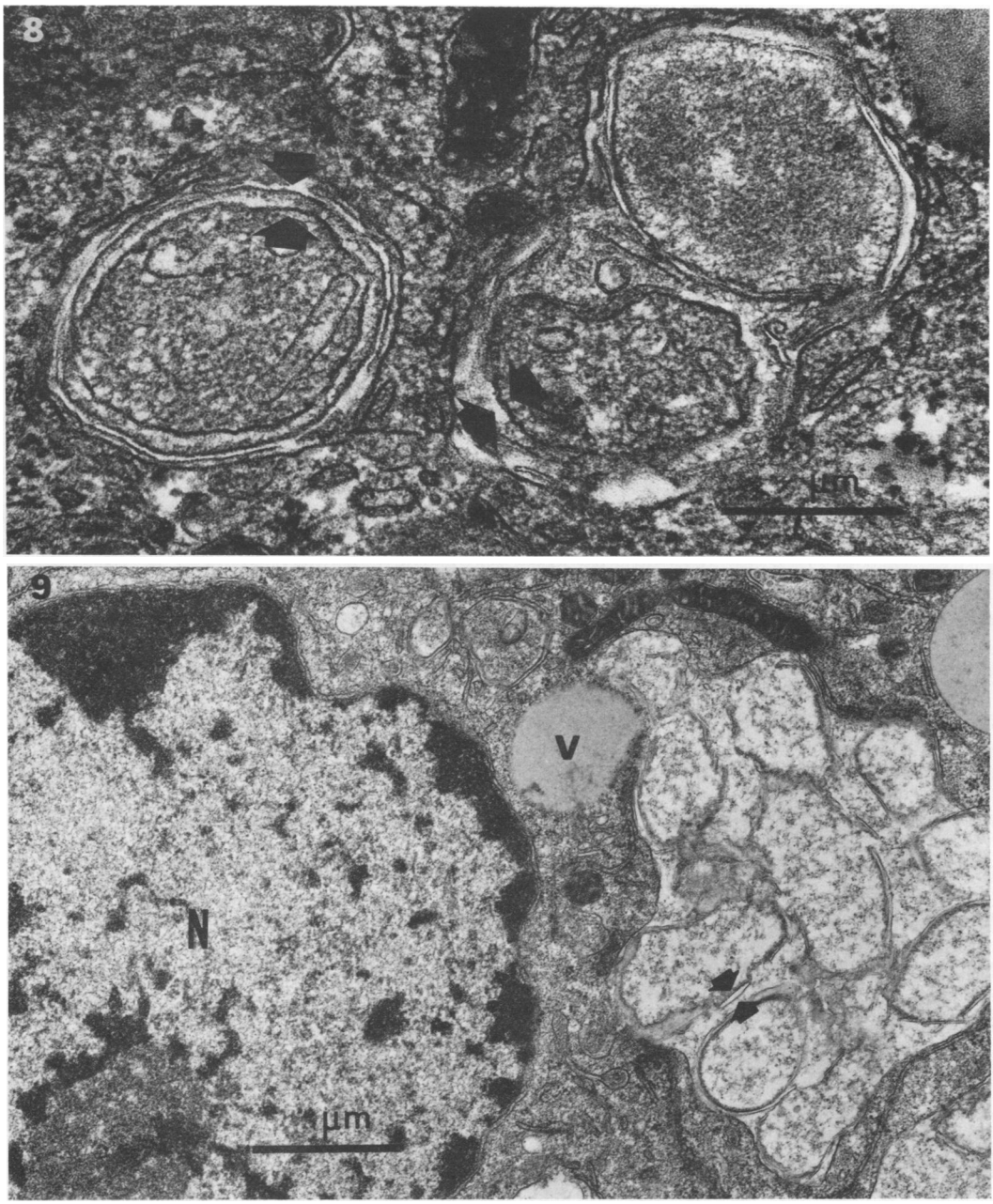


FIG. 8. *N. asteroides 10905 in peritoneal macrophages (16 days postinfection). The trilayered cell wall seen on in vitro-grown N. asteroides 10905 and on the intracellular organisms at 2 h postinfection is absent. The arrows indicate the single- and double-unit membranes surrounding these wall-less forms.*

FIG. 9. *Electron micrograph of spheroplast-like nocardial cells in peritoneal macrophages (27 days postinfection). Note that spheroplast-like organisms retained a portion of their cell wall (arrows). N, Nucleus; V, vacuole.*

from cultured macrophages. Organisms resembling bacterial L-forms were isolated directly from intact macrophages by plating dilutions of infected cells on hypertonic media. Dilutions of infected macrophages were made in Eagle MEM containing 20% heat-inactivated fetal calf serum and 0.34 M sucrose as an osmotic stabilizer. The altered forms of

Nocardia were also isolated from disrupted macrophages by a modification of the techniques described by Hatten and Sulkin for recovery of *Brucella* L-forms (13). Infected macrophages were maintained in Eagle MEM for 12 days as described above. These cells were then disrupted by incubation for 7 days in an antibiotic-free medium containing 0.34 M sucrose at pH 7.8. The cell debris was then pelleted by low-speed centrifugation, and these pellets were inoculated into various types of hypertonic culture media or fixed for electron microscopy. Control cells and samples of serum and MEM were also plated on hypertonic medium to rule out contamination by *Mycoplasma*.

The different media used to recover aberrant forms of *Nocardia* from infected macrophages are given in Table 1. Optimal recovery occurred on plates containing low concentrations of agar and osmotically active substances, such as NaCl, incorporated into the media. Recovery was also improved when plates were incubated with increased CO_2. Brain heart infusion broth containing 20% heat-inactivated fetal calf serum, 5% NaCl, and 0.7% agar was selective for growth of altered forms of *N. asteroides* 10905. Stock cultures of this strain inoculated directly onto this media did not grow. This indicated that macrophage passage was necessary before growth of *Nocardia* occurred on this medium.

Thin sections of disrupted macrophage pellets showed that membrane-bound organisms were present in cell debris (Fig. 10, 11, and 11a). These organisms varied considerably in their size and shape, with spherical bodies and large pleomorphic forms predominating (Fig. 10 and 11).

Characteristics of altered forms of Nocardia. Altered forms of *Nocardia*, isolated from infected macrophages, exhibited many of the properties of bacterial L-forms. For the purpose of clarity, we considered L-forms to be wall-defective microbial variants which exhibit the following characteristics: (i) pleomorphism due to the absence of cell wall or the lack of a rigid cell wall (6, 21, 29); (ii) reproduction and growth on artificial media in their altered form (6, 21, 29); and (iii) formation of colonies composed of granules and large bodies which exhibit "fried-egg" morphology (6, 21, 29).

TABLE 1. *Recovery of altered and bacterial forms of N. asteriodes 10905 from cultured macrophages*[a]

Medium	Altered forms	Bacterial forms
BHI + 1.5% agar	ND	+
BHI + 0.2 M sucrose + 1.5% agar	ND	+
BHI + 20% Δ FCS + 5% NaCl + 5% NaCl + 0.7% agar	+	ND
BYE + 15% Δ FCS + 0.7% agar	+	+
BYE + 15% Δ FCS + 1.5% agar	ND	+

[a] Δ FCS, Heat-inactivated fetal calf serum; BHI, brain heart infusion broth; BYE, Barile, Yarguchi, and Eveland broth; ND, not detected.

Altered forms of *Nocardia* grown on artificial media differed markedly from their parent organism. Phase-contrast microscopy showed that these forms were composed of small, dense granules and large refractile bodies (Fig. 12 and 13). Colonies were small and embedded in the surface of the agar. Many colonies formed thin films on the agar which gave them a classic fried-egg appearance (Fig. 14). Cover slip impressions of colony material stained gram negative or not at all. Their Gram reaction suggested that these altered forms may be similar to the cell wall-defective variants of *N. rubra* strain VAC-321 isolated by Prasad and Bradley (25).

Analysis of the surface fine structure of *N. asteroides* 10905 and its altered form, by scanning electron microscopy, demonstrated dramatic differences between the two cell types. The surface morphology of *N. asteroides* 10905 grown for 2 weeks under increased CO_2 and on BYE media containing 15% heat-inactivated fetal calf serum and 0.7% agar was characteristic of the genus *Nocardia* (Fig. 15). A previous study of several genera of *Actinomycetes* showed *N. rubra* to exhibit similar fine structure (31). The surface morphology of altered forms of *N. asteroides* 10905 isolated from infected macrophages and grown under identical conditions (media, time, and CO_2) appeared granular in nature (Fig. 16). Individual granules were flattened and irregular in shape. Branching filaments were not seen. The granular surface of altered forms of 10905 was similar to that seen on L-forms of *Streptococcus* strain ADA (4). L-form colonies of this streptoccal strain became granular after transfer to an agar surface. The granules were thought to arise from large bodies which condensed and collapsed during growth on agar (4).

Altered forms of *N. asteroides* 10905 were unstable, as indicated by their frequent reversion to typical *Nocardia* (Fig. 14). This reversion occurred either spontaneously or upon subsequent transfer into broth. In addition, the altered forms were osmotically fragile since the number of *Nocardia* recovered from infected macrophages was greatly reduced or eliminated

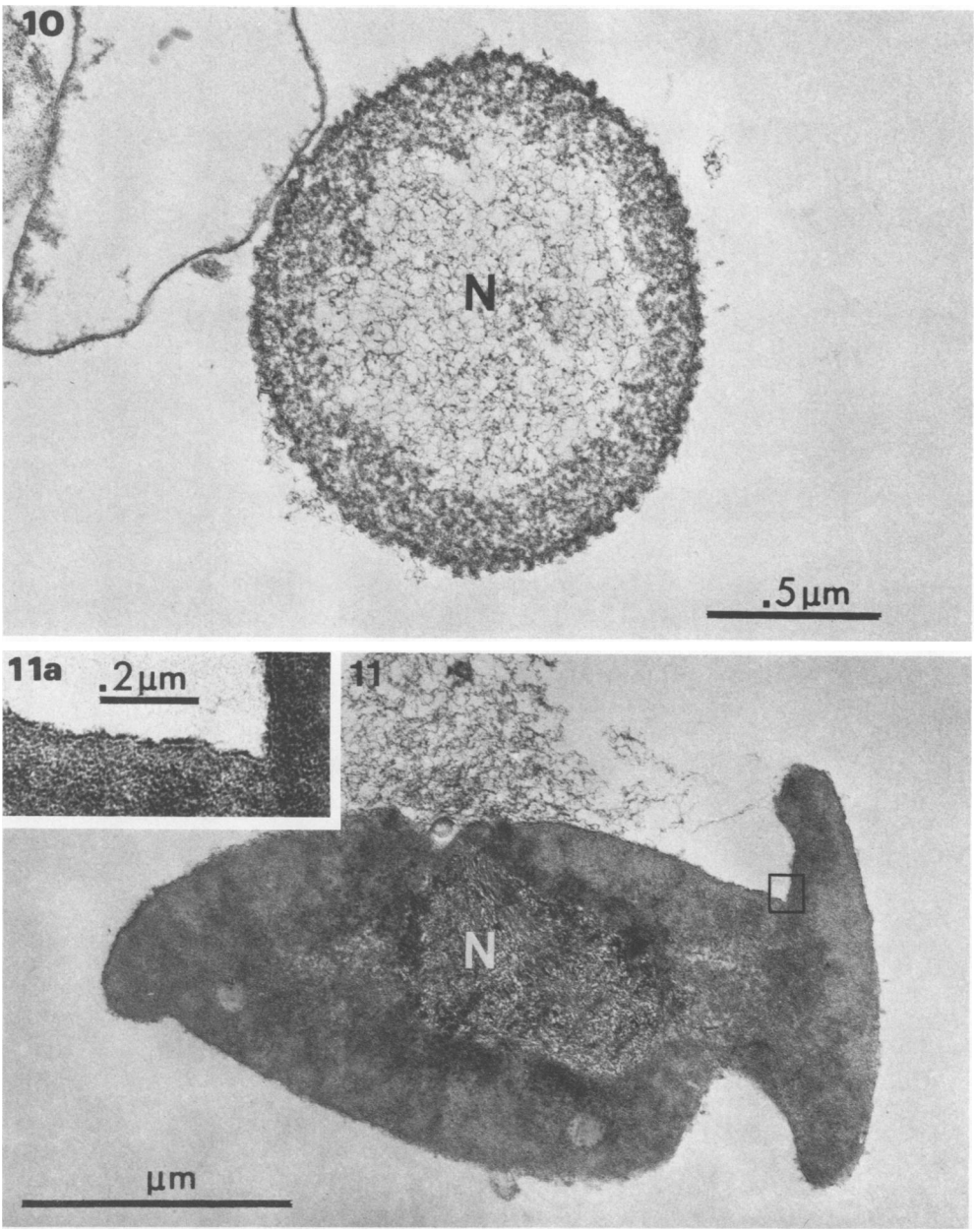
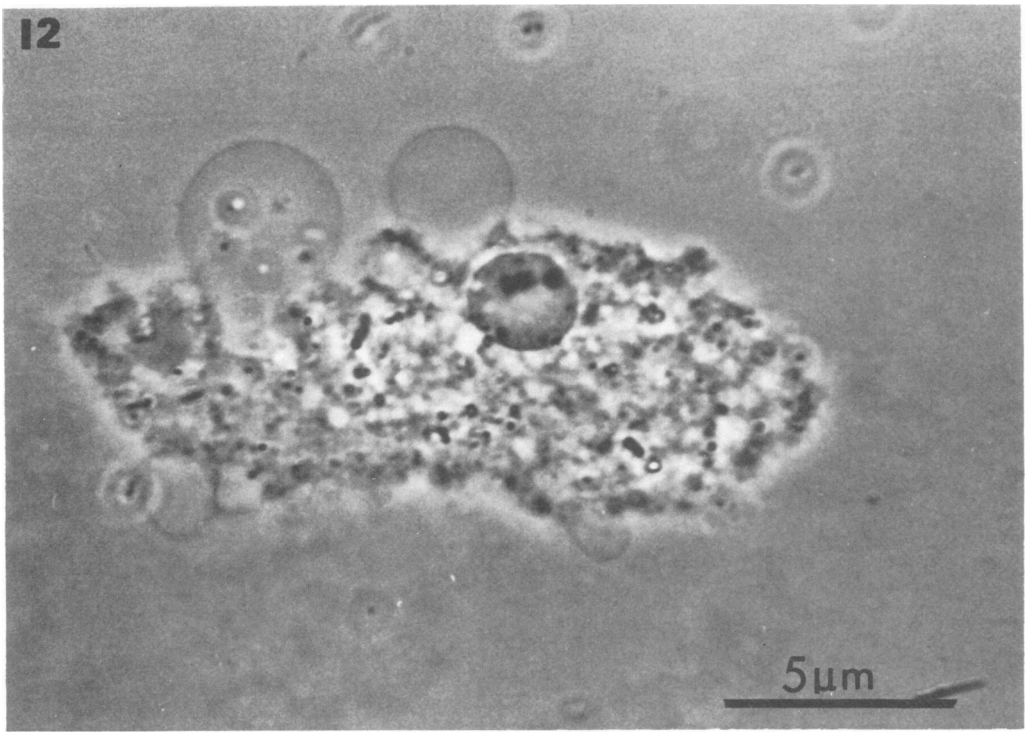


FIG. 10. *Thin section of a membrane-bound nocardial cell found in the disrupted macrophage pellet (17 days postinfection). N, Nuclear region.*

FIG. 11. *Thin section of a membrane-bound nocardial cell found in the disrupted macrophage pellet (17 days postinfection). Note the pleomorphic morphology of this form. N, Nuclear region. Insert (11a), High magnification of the nocardial cell periphery. This wall-less nocardial cell is bound by a single-unit membrane.*

when the macrophages were disrupted by osmotic shock.

Electron microscopy showed that the altered colonies were composed of two distinct morphological types. Membrane-bound organisms that appeared to be free from cell wall material and large pleomorphic forms, which retained remnants of their outer cell wall, were present. Wall-less organisms were bound at their periphery by a unit membrane, and they resembled



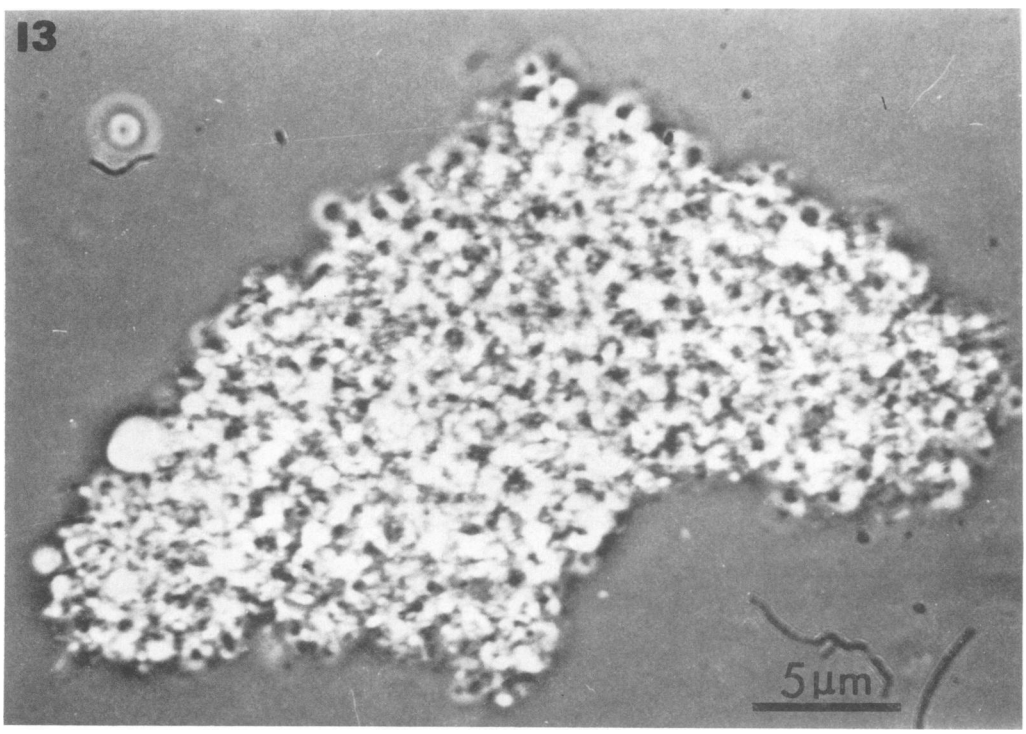


FIG. 12 and 13. *Phase-contrast micrograph of altered forms of N. asteroides 10905 grown for 3 weeks on brain heart infusion broth containing 20% heat-inactivated fetal calf serum, 5% NaCl, and 0.7% agar (incubation was at 37 C in a CO_2 incubator). Altered forms appeared to be composed of granules and large membranous bodies.*

FIG. 14. *Phase-contrast micrograph of a colony of altered forms of N. asteroides 10905 grown on brain heart infusion agar as described in the legend to Fig. 12. These colonies formed a thin film on the agar which gave them a fried-egg morphology. Spontaneous revertants were occasionally observed (R).*

FIG. 15. *Scanning electron micrograph showing the surface structure of N. asteroides 10905 grown for 2 weeks on BYE media containing 15% heat-inactivated fetal calf serum and 0.7% agar and incubated at 37 C in CO_2. The branching filaments are characteristic of Nocardia.*

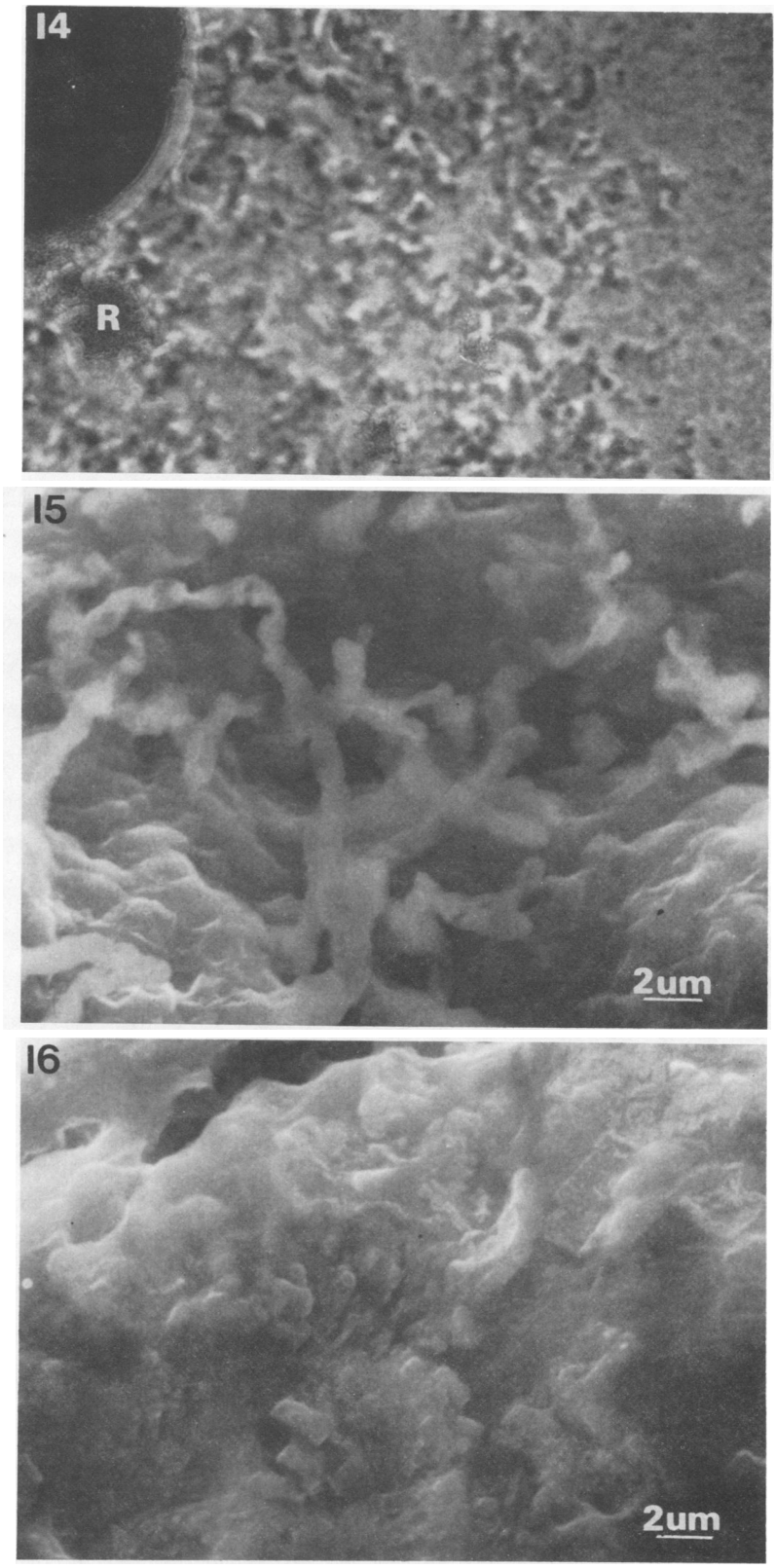


FIG. 16. *Scanning electron micrograph showing the surface structure of altered forms of N. asteroides 10905 isolated from peritoneal macrophages and grown as described in the legend to Fig. 15. The altered forms appear flattened and irregular, and branching filaments are not present.*

the membrane-bound organisms previously seen in infected macrophages and in disrupted macrophage pellets (compare Fig. 8, 10, 11, and 17). Negatively stained preparations showed the pleomorphic and membranous nature of these forms (Fig. 19). Organisms that retained the outer layer of the cell wall appeared to be surrounded by a double membrane. The inner layer of the cell envelope appeared greatly reduced and was difficult to distinguish from the plasma membrane (Fig. 18). Higher magnifications of the cell envelope revealed that the inner layer was very granular and irregular (Fig. 18a). In contrast, the parent organisms had a thicker, more uniform cell wall (compare Fig. 6a, 7a, and 18a). These forms were also surrounded by a wide layer of amorphous material (Fig. 18). This layer was present in much smaller amounts in both in vitro-grown *Nocardia* and in organisms present in macrophages at 2-h postinfection (compare Fig. 6, 7, 18). This amorphous material was previously shown surrounding this strain of *Nocardia* in lesions of experimentally infected mice (Fig. 21), and it

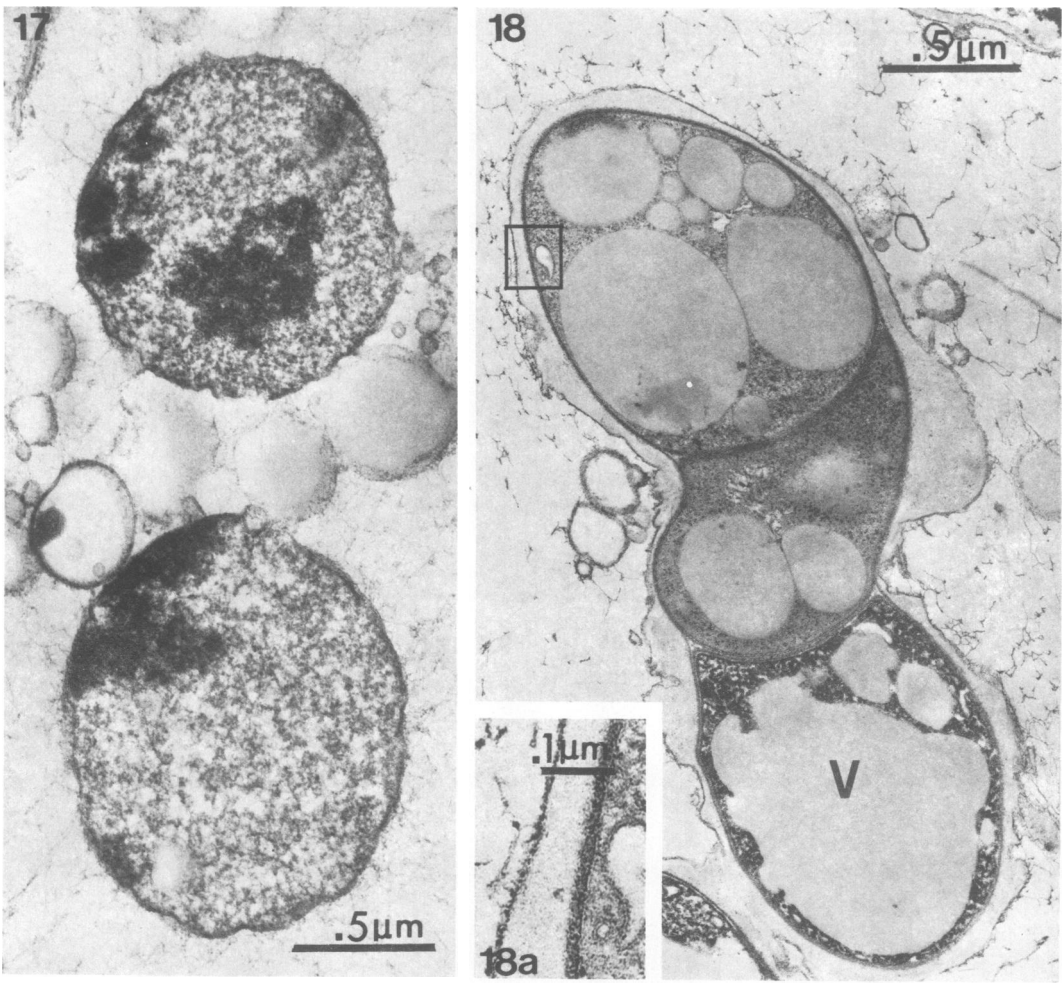


FIG. 17. *Thin section of an altered form of N. asteroides 10905 isolated from peritoneal macrophages and grown for 3 weeks on brain heart infusion broth containing 20% heat-inactivated fetal calf serum, 5% NaCl, and 0.7% agar (incubated at 37 C in a CO_2 incubator). These forms resemble type A L-forms in that they were bound only by a unit membrane.*

FIG. 18. *Thin section of an altered form of N. asteroides 10905 isolated from peritoneal macrophages and grown for 3 weeks on brain heart infusion broth as described in Fig. 17. This form resembles type B L-forms in that it retained the outer layers of its cell wall. V, Lipid vacuole. Insert (18a), High magnification of the cell wall of an altered form of N. asteroides 10905 resembling the type B L-form. The peptidoglycan layer appears greatly reduced and irregular.*

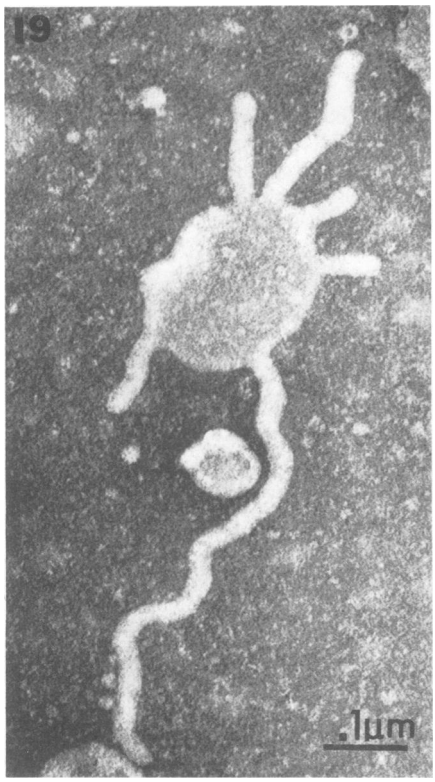
was thought to be of bacterial origin since it was absent in mice injected with killed cells of *N. asteroides* 10905 (2). These results indicated that considerable alteration of the cell wall of *N. asteroides* 10905 had occurred after passage through macrophages. The failure to observe similar morphological changes in this strain of *Nocardia* grown under identical conditions but not passed through macrophages suggested that these alterations were macrophage induced. The altered colonial and cellular morphology of these organisms and their ability to propagate in their altered form suggested that they were bacterial L-forms.

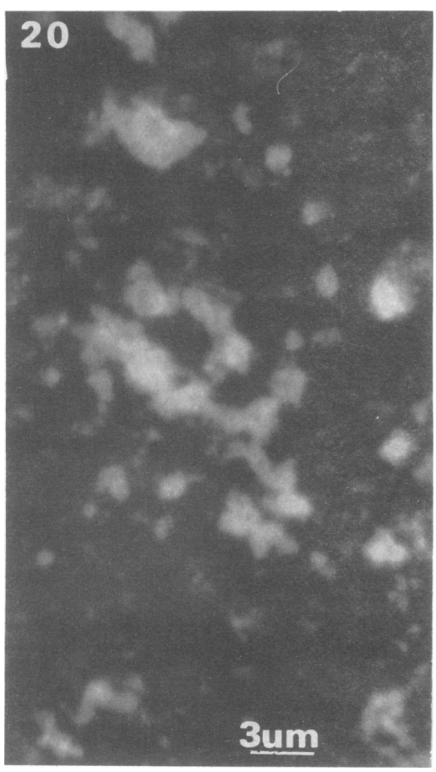
The relationship between the recovered altered forms and the infecting strain of *N. asteroides* was determined by treating colonies with specific anti-nocardial antibody. Indirect immunofluorescence demonstrated that the altered forms were derived from *N. asteroides* 10905 (Fig. 20). Control colonies treated with specific anti-staphylococcal as well as with normal rabbit serum gave no fluorescence.

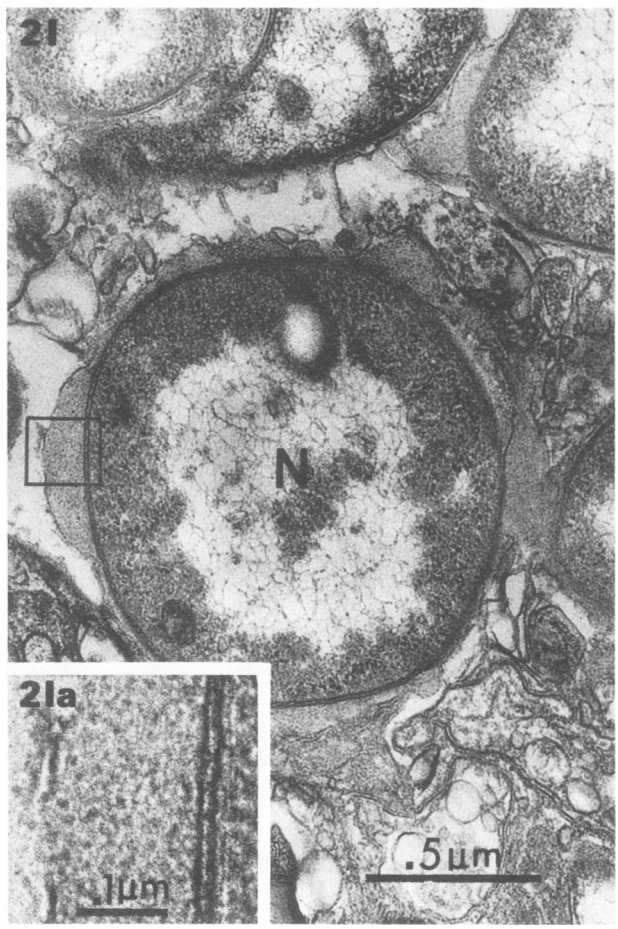
DISCUSSION

L-forms are defined as wall-defective microbial variants which grow as non-rigid cells due to the absence of cell wall or lack of a rigid cell wall (6, 20, 29). They can grow indefinitely in their altered form, and on solid media they produce distinctive colonies which may have a fried-egg appearance (6, 20, 29). In addition, L-forms are gram negative, and individual organisms may appear as large, refractile bodies or small, dense granules (20). Altered forms of *N. asteroides* 10905 isolated from infected macrophages exhibited these properties. The cellular and colonial morphology, the Gram reaction, and the osmotic sensitivity of these forms suggested that they may be L-forms. Ultrastructural studies of these altered forms of *N. asteriodes* 10905 showed that they occurred as two distinct morphological types which resemble the type A and type B L-forms observed in *Proteus*, *Salmonella*, and *Escherichia* (6). The altered forms resembling type A L-forms had no apparent cell wall and were bound only by a unit membrane. Altered forms resembling type B L-forms, however, retained the outer most layers of their cell wall. These cells appeared greatly enlarged, with a pleomorphic morphology that suggested that the cell wall had lost its rigidity. Although the chemical structure of nocardial cell wall has not been fully elucidated, evidence indicates that the outer layer is composed of several amino acids, either as peptides or proteins associated with lipoidal material (3). Amino acid analysis of the remaining basal layer suggests that this layer represents the peptidoglycan (3). The cell wall component responsible for nocardial morphology has not been determined. Previous studies with other organisms have demonstrated that the peptidoglycan has a role in cellular morphology. Inhibition of peptidoglycan synthesis or its degradation by lysozyme leads to the formation of protoplasts or spheroplasts (29). It has also been shown that proteins in the cell wall may function in determining cellular morphology (14). Martin and Wiedul have shown that a covalently interlinked complex of peptidoglycan and protein, termed the "rigid layer," determines shape in the enterobacterial cell wall (21, 30). As previously cited, the nocardial cell wall contains amino acids in the form of peptides or proteins in its outer layer (3). These peptides or proteins, in addition to the peptidoglycan, may contribute to cellular morphology as rigid-layer proteins do in *Proteus mirabilis* (15, 21). In altered forms of *Nocardia* resembling type B L-forms, the cell wall appears to have lost its shape-determining ability. In thin sections of these altered forms, the peptidoglycan appeared greatly reduced and irregular when compared with the parent organism (compare cell wall profiles in Fig. 6, 7, 18). Unfortunately, thin sections of altered forms of *Nocardia* are not useful for localizing the specific site of chemical damage in the cell wall which gives rise to these altered forms.

Although our results suggested that altered forms of *N. asteriodes* 10905 were L-forms, we were unable to determine whether these altered forms were stable. The altered forms of *Nocardia* frequently reverted back to typical *Nocardia*, and the colonies, although atypical, did not always exhibit fried-egg morphology. The instability of these altered forms suggested that they were unstable L-forms (20) or transitional-phase variants (20). It is interesting to note, however, that the instability of these forms varied with the media used for isolation. Brain heart infusion broth containing 20% heat-inactivated fetal calf serum, 5% NaCl, and 0.7% agar was selective for growth of the altered forms of *N. asteroides* 10905. This media appeared to stabilize these forms and reversion rarely occurred. The differentiation of stable L-forms from unstable L-forms and transitional-phase variants depends on the ability of these altered forms to propagate serially as distinctive L-form colonies on agar (20). Unfortunately, we have been unable to develop a satisfactory method for passage of these altered forms.


19
.1µm


20
3um


21
N
21a
.1µm
.5µm

After primary isolation, subculturing of altered forms on media selective for altered forms of *Nocardia* consistently resulted in no growth. Spontaneous revertants subcultured on this media also did not grow. Additional work must be done before we can state that these altered forms of *Nocardia* are L-forms or transitional-phase variants.

Wall-less and spheroplast-like organisms were observed in infected macrophages, disrupted macrophage pellets, and colonies of altered forms isolated from infected macrophages. These results, plus the failure to observe similar morphological changes when *N. asteroides* 10905 was grown under identical conditions but not passed through macrophages, indicated that these altered forms were macrophage induced. However, the possibility exists that antibiotics or serum factors may play some role in the induction of these altered forms. Complement plus serum lysozyme has been shown to convert gram-negative bacteria to spheroplasts (23), and antibiotics are well-known inducers of protoplasts, spheroplasts, and L-forms (29). Evidence indicates, however, that these factors do not function alone in the induction of altered forms of *Nocardia*. During this investigation, heat-inactivated serum was used exclusively, and growth of *N. asteriodes* 10905 in MEM containing 20% heat-inactivated fetal calf serum, penicillin (40 μg/ml), fungizone (1 μg/ml), and *l*-glutamine (1 μg/ml) or in brain heart infusion broth containing 20% heat-inactivated fetal calf serum and penicillin (1,000 μ/ml) had no apparent effect on cellular morphology.

The isolation of altered forms of *N. asteroides* 10905 from cultured macrophages raises the question of what role, if any, these forms play in nocardial infection. No attempt has been made to isolate wall-defective forms from individuals with nocardial infections. The difficulty frequently encountered in isolating *Nocardia* from infected individuals (17, 22, 26) suggests that the organisms may be present in an altered form. Histological and ultrastructural studies done on mice infected with *N. asteriodes* 10905 have shown that these organisms become altered in vivo (2). Like altered forms of *Nocardia* isolated from infected macrophages, those found in situ in mice retained their outer cell wall, but the innermost layer of the cell envelope was greatly reduced (compare Fig. 18, 18a, 21, and 21a). In addition, the altered forms of *Nocardia* obtained from macrophages and present within infected tissue were surrounded by a wide layer of amorphous material (compare Fig. 18 and 21). The persistance of these altered forms of *Nocardia* in cultured macrophages suggests that they may contribute to the chronic nature of nocardial infection. However, this conclusion is purely speculative. Further experiments are needed to evaluate the true clinical importance of altered forms of *Nocardia* in disease.

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FIG. 19. *Negative-stained preparation of an altered form of N. asteroides 10905 grown on brain heart infusion broth as described in the legend to Fig. 17 (stained with ammonium molybdate). Note the pleomorphism of this cell.*

FIG. 20. *An indirect immunofluorescent stain of colonies of altered forms of N. asteroides 10905 grown on brain heart infusion broth as described in the legend to Fig. 17. This clearly demonstrates the relationship between the recovered altered forms and the infecting strain of Nocardia asteroides.*

FIG. 21. *Thin section of N. asteroides 10905 in mouse lung tissue. Note the similarity between in vivo-grown Nocardia and isolated altered forms of Nocardia resembling type B L-forms shown in Fig. 18. N, Nuclear region. Insert (21a), Higher magnification of the cell wall of the in vivo-grown N. asteroides 10905. The peptidoglycan layer appears greatly reduced and is difficult to distinguish from the plasma membrane.*

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