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Induction of L-Phase Variants of *Nocardia caviae* Within Intact Murine Lungs

BLAINE L. BEAMAN

Department of Medical Microbiology, School of Medicine, University of California, Davis, California 95616

The data presented show that cells of *Nocardia caviae* 112 are converted to cell wall-deficient microbial variants within the intact murine lung after intranasal administration. At the time that these L-phase variants were recovered in large numbers from the lung, there was a correspondingly enhanced inflammation leading to alveolar consolidation and animal death. During the peak of this response (at 1 week after infection), normal nocardial cells were neither isolated from nor seen within the lung. It is suggested that the conversion of these normal nocardial cells to their L-phase variant leads to this extensive pulmonary damage. Furthermore, the L-phase organisms appear to play an active role in this pathological effect since introduction of similar amounts of killed nocardial cells into the lungs of the mice failed to produce a similar response.

Nocardia caviae may cause either acute or chronic pulmonary disease as well as mycetomas in humans (1). Furthermore, it is a frequent cause of mastitis in cows (unpublished data), and it is pathogenic for a variety of animals. The specific nature of the host-parasite relationship and the progression of the disease within the host have not been studied adequately.

We analyzed the pulmonary response of mice after intranasal administration of several strains of *Nocardia* (2, 4). It was found that *N. caviae* 112 was significantly more virulent for mice when administered by this route than was *N. asteroides* GUH-2. Thus, the 50% lethal dose of *N. caviae* 112 in mice after intranasal infection was about 6×10^5 colony-forming units per lung (4). *N. asteroides* GUH-2 differed significantly from *N. caviae* 112 in that the organisms could be recovered from lung homogenates of dying mice, and at the peak of animal death increasing numbers of cells of *N. asteroides* could be isolated. Gram-stained preparations of paraffin sections of these infected lungs demonstrated large numbers of gram-positive branched filaments extending throughout the lung (2). In sharp contrast, *N. caviae* frequently could not be isolated from the homogenates of infected lungs obtained from dying mice, yet macroscopic examination of these lungs indicated extensive consolidation and abscess formation. The inability to isolate nocardia from these lungs suggested that a toxic response to some component present in the organisms was responsible for the lesions within the lungs which resulted in animal death. However, when similar amounts of dead cells (killed either by Formalin or heat) were introduced intranasally, similar results were not observed; that is, no mice died and the lungs maintained a normal macroscopic appearance. These findings strongly suggested that a living bacterial process was necessary for the induction of pulmonary consolidation leading to animal death, even though few or no bacteria were recovered from the lungs.

It had been shown previously that cells of *N. asteroides* 10905 disappeared within in vitro-cultured peritoneal macrophages obtained from mice (7). After several days, these macrophages were destroyed, even though typical nocardial cells were not recovered. It was shown that, as the nocardial cells were phagocytized, some of them survived as cell wall-defective microbial variants (L-phase variants) which ultimately increased in numbers within the macrophages. This increase in L-phase variants resulted in death of the macrophages. The same results were obtained when *N. asteroides* 10905 were phagocytized by rabbit alveolar macrophages (5). In addition, it was shown that several strains of *Nocardia* could be induced to grow as L-phase variants in vitro by growing them in an osmotically stabilized environment containing large amounts of glycine and lysozyme (6). One of the most readily induced of these L-phase variants was obtained from *N. caviae* 112. Thus, the observations concerning the induction of cell wall-defective variants of *N. asteroides* 10905 in alveolar macrophages in combination with the ease with which L-phase variants of *N. caviae* 112 could be induced in vitro suggested that this process might be responsible for the damage to the lungs and animal death after intranasal administration of cells of *N. caviae* 112. The data presented herein establish the role of L-phase

variants in pulmonary infections in mice after intranasal administration of early stationary-phase cells of *N. caviae* 112.

MATERIALS AND METHODS

Microorganism. *N. caviae* 112 was isolated from a human infection and obtained from W. Causey (University of Chicago Hospitals and Clinics, Chicago, Ill.). The organism was grown and maintained as previously described (3).

Animals. Female Swiss Webster mice (18 to 20 g) were obtained from Simonson's (Gilroy, Calif.). All infected mice were maintained in maximum isolation in a germfree isolator (Germfree Laboratories) equipped with positive-pressure filtered air. This unit is located in a special animal room supplied with filtered air. They were fed Purina Laboratory Chow ad lib.

Intranasal infection. Cells of *N. caviae* were grown to early stationary phase of growth (48 h) in brain heart infusion (BHI) broth as described previously (3). Single-cell suspensions of the cocco-bacillary forms were obtained by differential centrifugation (3) and suspended in sterile saline (0.85%, wt/vol). Intranasal infection was accomplished by lightly anesthetizing 100 mice with vapors of a mixture of 95% ethanol, chloroform, and diethyl ether (1:2:3, vol/vol/vol) and then introducing 0.05 ml of a saline suspension (approximately 1.0×10^7 colony-forming units per ml) of nocardia into the nares. At 30 min, and 6, 24, 48, 96, and 168 h after infection, five mice from each group were sacrificed.

Analysis of the lungs. The left lobe of the lungs of each mouse was aseptically removed and placed in 3.0 ml of sterile sucrose (20%, wt/vol) and homogenized for 30 s with a Tekmar Tissuemizer. Serial dilutions of the homogenate were prepared in sterile sucrose solutions and plated on either brain heart infusion agar or Barile, Yarguchi Eveland agar (BYE-L agar) as previously described for the isolation of nocardial L-forms (6, 7). The plates were incubated at 37°C in a CO_2 (5%) incubator, and the numbers of L-phase variant and normal nocardial colonies were quantitated (6, 7). The histological relationships of nocardia and phagocytes were determined in the right lung by perfusing it with approximately 1.0 ml of 3% glutaraldehyde in Kellenberger buffer (pH 6.5) containing 20% sucrose (wt/vol). Samples were then prepared for electron microscopy as described previously (6, 7). Histological examination was carried out on paraffin-embedded sections that were stained either by hematoxylin and eosin or the Brown and Brenn tissue Gram stain (2).

Indirect immunofluorescence. The nocardial L-phase variants and their revertants were shown to be derived from *N. caviae* 112 by indirect immunofluorescent staining and by physiological and biochemical characteristics of the revertants as compared with the parental form. Coverslip impressions of the L-phase colonies and smears of the L-phase organisms isolated from the lungs of infected mice were fixed with cold methanol and processed as previously described (6). The antiserum used was prepared by immunizing guinea pigs with cell-free cytoplasmic extracts of *N. caviae*, and it was shown to be specific for *N. caviae* by the methods previously described (6). Thus, to determine the specificity of the antiserum, 3 strains of *N. caviae*, 10 strains of *N. asteroides*, 3 strains of *Rhodococcus*, and 2 strains of *N. brasiliensis* were studied. The smears were treated with specific antiserum for 30 min at 37°C and washed thoroughly with phosphate-buffered saline (pH 7.5). The smear was stained with fluorescein-labeled goat anti-guinea pig immunoglobulin G (6) for 30 min at 37°C and rinsed thoroughly with buffered saline. It was shown that only the three strains of *N. caviae* gave strong, specific immunofluorescence. Photographs of the immunofluorescent slides were made with a Zeiss Mercury Vapor Ultraviolet Epi-fluorescent Illuminator on a Zeiss research microscope equipped with a 100× planachromatic objective.

Electron microscopy. Normal cells and colonies of L-phase variants isolated from the lungs of infected mice were fixed for electron microscopy as previously described (6, 7).

Animal susceptibility to intranasal infection by *N. caviae* 112. Groups of Swiss Webster mice were infected intranasally as described above. These mice were maintained for several weeks to determine lethality of a given dose of organisms.

Observation of developing L-phase colonies. To observe the growth and development of L-phase and normal nocardial colonies, sterile cover slips were placed over the inoculated regions of the BYE-L plates, and specific regions were marked and observed daily for 3 to 4 weeks with a 40× phase-contrast objective on a Zeiss research microscope equipped with a long-working distant phase substage condenser. These developing L-phase colonies and their reversion to normal colonies were photographed with Kodak Panatomic X-film through a green filter.

RESULTS

After intranasal instillation of early stationary-phase cells of *N. caviae*, it was found that clearance of these bacteria from the lung began rapidly, so that after 24 h approximately 90% of the organisms were lost when these lungs were cultured on BHI agar (Fig. 1). During the first 24 h after infection, no L-phase variants were isolated from the lung homogenates plated on BYE-L agar. In sharp contrast, at 48 h there was a continued decrease in the ability to isolate bacteria on BHI agar; however, more than 10^5 L-phase organisms per left lobe were recovered from the lungs when the homogenates were plated on BYE-L agar (Fig. 1). The numbers of L-phase variants within the lungs appeared to remain relatively constant for the next 5 days, whereas the ability to isolate normal nocardial cells on BHI agar decreased to near zero at 1 week. At 48 h after infection, the mice appeared relatively healthy, but, at 72 h they appeared acutely ill (100%). At 96 h after infection, some of the mice died, and the deaths continued for the next 72 h. The peak of animal death was

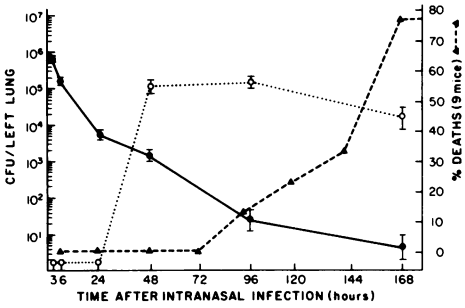


FIG. 1. *Curves contrasting the numbers of normal colonies of N. caviae 112 isolated on BHI agar from homogenates of the left lung at specific times after intranasal infection (●) with the numbers of L-phase variants when the same samples are placed on BYE-L agar for L-forms (○). Nine mice were placed in a separate cage after intranasal inoculation and monitored for animal death. The bars show the standard error of the mean based on five animals at each point.*

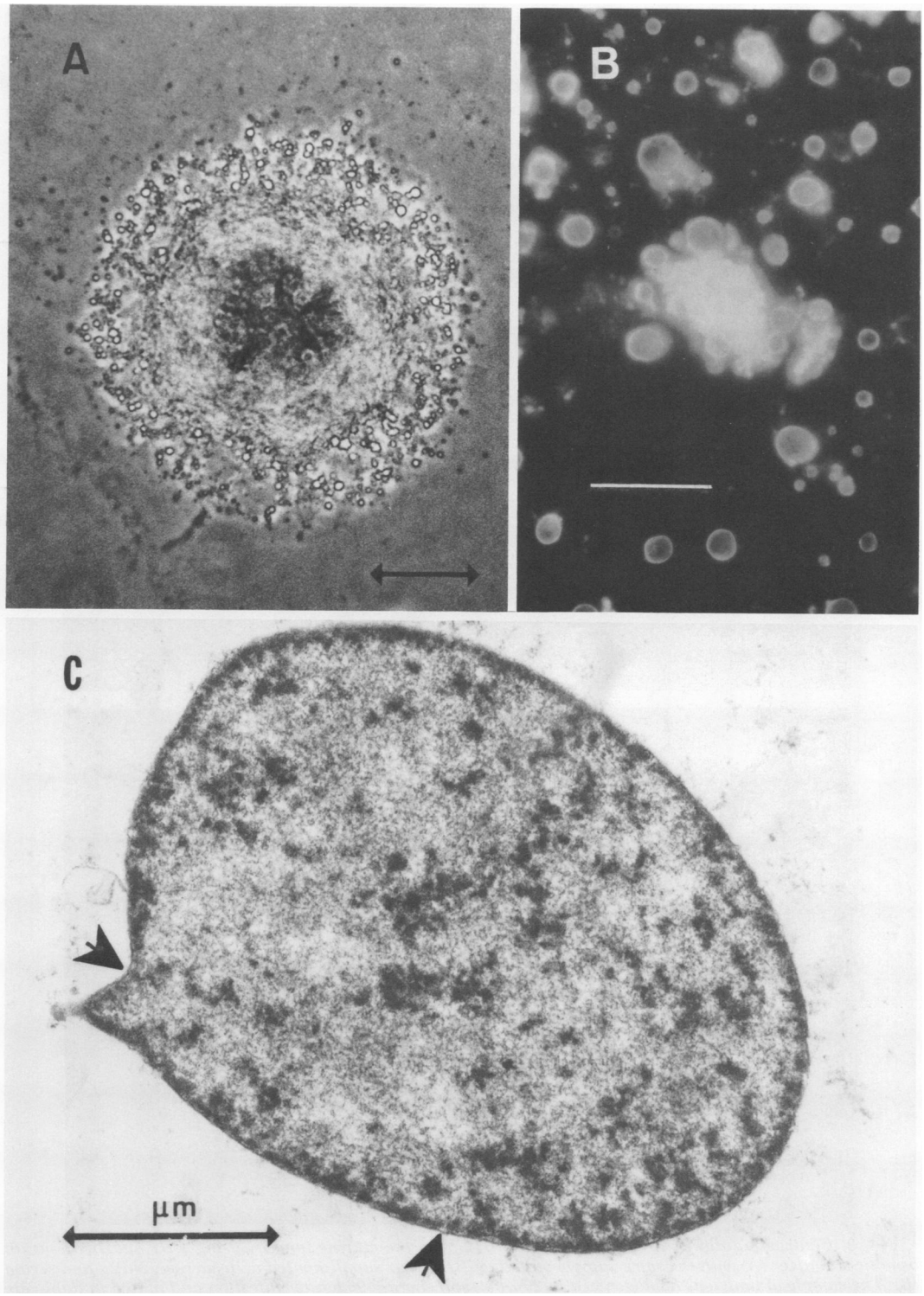
between 144 and 168 h (1 week) after infection (Fig. 1). At this time, no normal organisms could be isolated on BHI agar, while approximately 10^4 L-phase colonies per left lung were recovered on BYE-L agar (Fig. 1).

The organisms isolated from the lung homogenates when plated on BYE-L agar were L-phase variants and not stable L-forms, because upon continued incubation (3 to 4 weeks) or upon transfer to fresh BYE-L agar they always reverted to typical colonies of *N. caviae*. The initial L-phase colonies were composed entirely of refractile spheres, granules, and large membrane sacks surrounding a dense core of granules and spheres, as observed by phase-contrast microscopy (Fig. 2A). When viewed with transmitted light, these colonies had the typical fried egg appearance characteristic of L-forms. Some of the L-phase variant colonies isolated from the lungs were transferred to cover slips by impression smears which were then air dried and fixed in methanol. Immunofluorescent staining specific against *N. caviae* cytoplasm and membrane was carried out on these smears. As shown in Fig. 2B, there was strong immunofluorescent staining of L-phase cells, indicating that they were derived from *N. caviae*. When impression smears of colonies not reacted with antibody were examined, there was a very slight autofluorescence, but it was negligible when compared with the sample reacted with specific antibody. The L-phase colonies that were reacted with antiserum against the cytoplasm of *N. asteroides* had no specific fluorescence. Electron microscopy demonstrated the presence of spheroplast and protoplast-like cells and no normal organisms were observed until after the colonies initiated reversion (Fig. 2C). The L-phase organisms required an osmotically stabilized medium supplemented with serum to grow as shown in Fig. 3. Figure 3A shows the colonies of a 10^{-1} dilution of a lung homogenate plated on BHI agar and incubated for 4 weeks. Only 13 colonies were present in this lowest dilution. In contrast, Fig. 3B shows the colonies from the same dilution blank, as in Fig. 3A, only plated on BYE-L agar instead of BHI. This plate also was incubated for 4 weeks. Note that there are about 17 large colonies which represent the growth of normal nocardial cells. However, there are more than 1,000 colonies that initially grew as L-phase variants (Fig. 2) but over the 4-week period of incubation cells within these colonies reverted, giving rise to the much smaller colonies composed of a mixture of spheres, granules, and typical nocardial filaments (Fig. 3B). Upon transfer to fresh BYE-L or BHI agar, these revertant colonies grew as typical cells of *N. caviae* 112, and they could no longer be distinguished.

Histological analysis of the lungs obtained from acutely ill mice 96 h after infection demonstrated considerable consolidation within the alveoli (Fig. 4A). Mononuclear cells predominated within the lesions, and lightly stained spheres of varying size were seen within the tissue and in the alveolar spaces when stained by hematoxylin and eosin. A Gram stain (Fig. 4B) of a section of the same lung did not reveal filamentous forms of the nocardia in the tissue sections. Instead, large numbers of gram-variable (mostly gram-negative) spheres of varying size were prominent within the inflammatory infiltrate (Fig. 4B).

Electron microscopy of the lungs obtained from mice 96 h after infection demonstrated large numbers of protoplast-like bodies within macrophages (compare Fig. 4C with 2C). Rarely, an intact nocardial cell was observed showing the typical ultrastructural profile of the intact cell wall. Figure 4C presents a normal nocardial cell lying next to an unmistakable protoplast within the same macrophage (Fig. 4C).

FIG. 2. *(A) Phase-contrast micrograph of L-phase variant colony of N. caviae 112 isolated from the lungs of a mouse 96 h after intranasal administration. Bar represents 100 μm. (B) Immunofluorescent stain of cover slip impression smear of L-phase colony as shown in A above. The bright green fluorescence is specific against antigens of N. caviae 112. Bar represents 10 μm. (C) Electron micrograph of L-phase organism from an L-phase colony as shown in A. The arrow points to single unit membrane surrounding the protoplast-like cell. No cell wall material is evident (compare with Fig. 4C).*


A
B
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DISCUSSION

The pathogenic potential of L-phase variants of bacteria for humans and laboratory animals has been the subject of numerous investigations and reviews (10, 11, 14, 15). The results of many of these reports have been inconclusive or contradictory, and the role, if any, of microbial cell wall-defective variants in disease is clouded with controversy. There is, however, a considerable amount of experimental evidence supporting the concept that cell wall-defective microorganisms

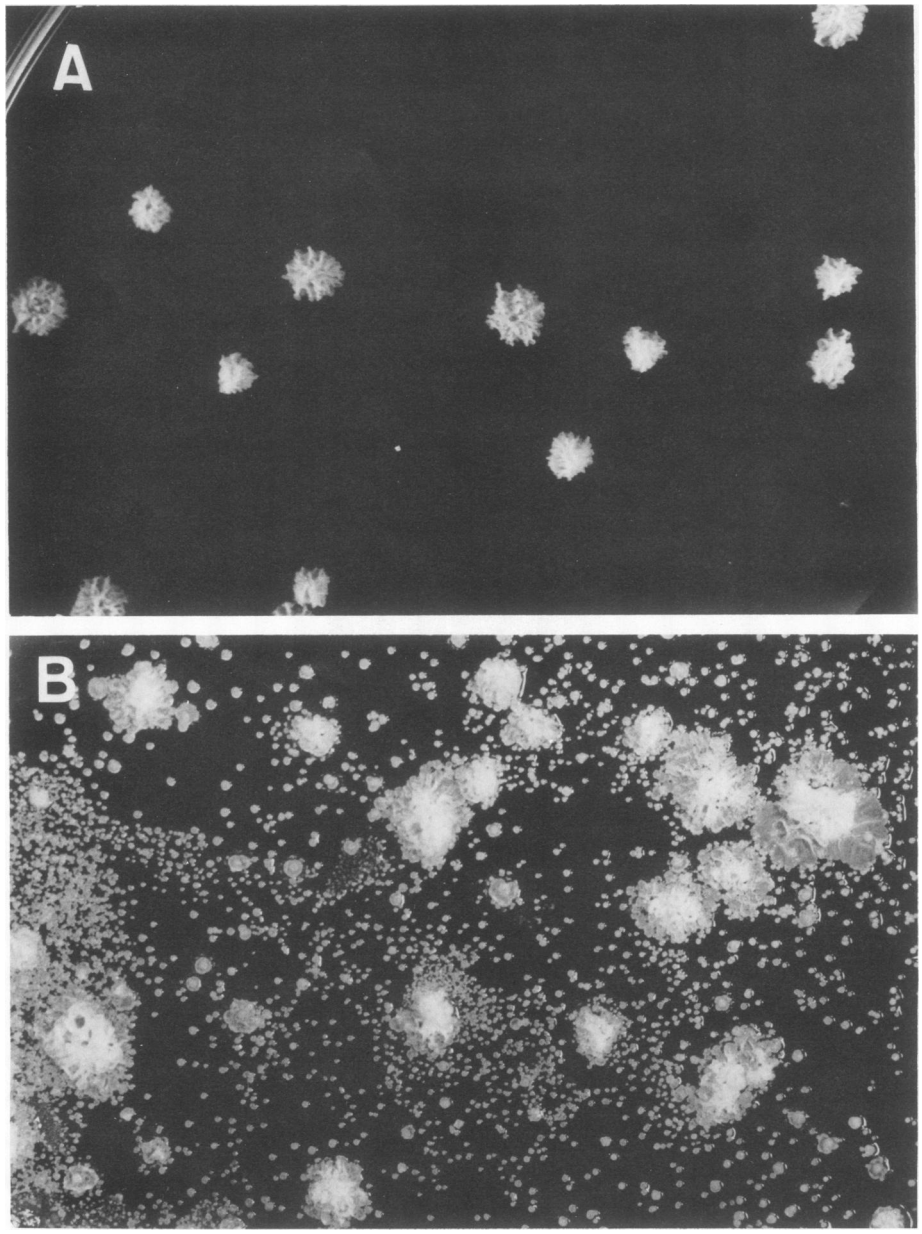

FIG. 3. *Contrasting ability to isolate N. caviae 112 from the murine lung by using BHI agar without an osmotic stabilizer (A) with the same sample plated on BHI agar supplemented with sucrose, NaCl, and serum (B). The sample of lung was homogenized in 20% (wt/vol) sucrose 96 h after infection and plated in duplicate on BHI agar and BYE-L agar as shown. The numerous small colonies in B are L-phase revertants of N. caviae 112. The large colonies in A and B appear to represent the normal growth of cells of N. caviae that were not converted to the cell wall-deficient state.*

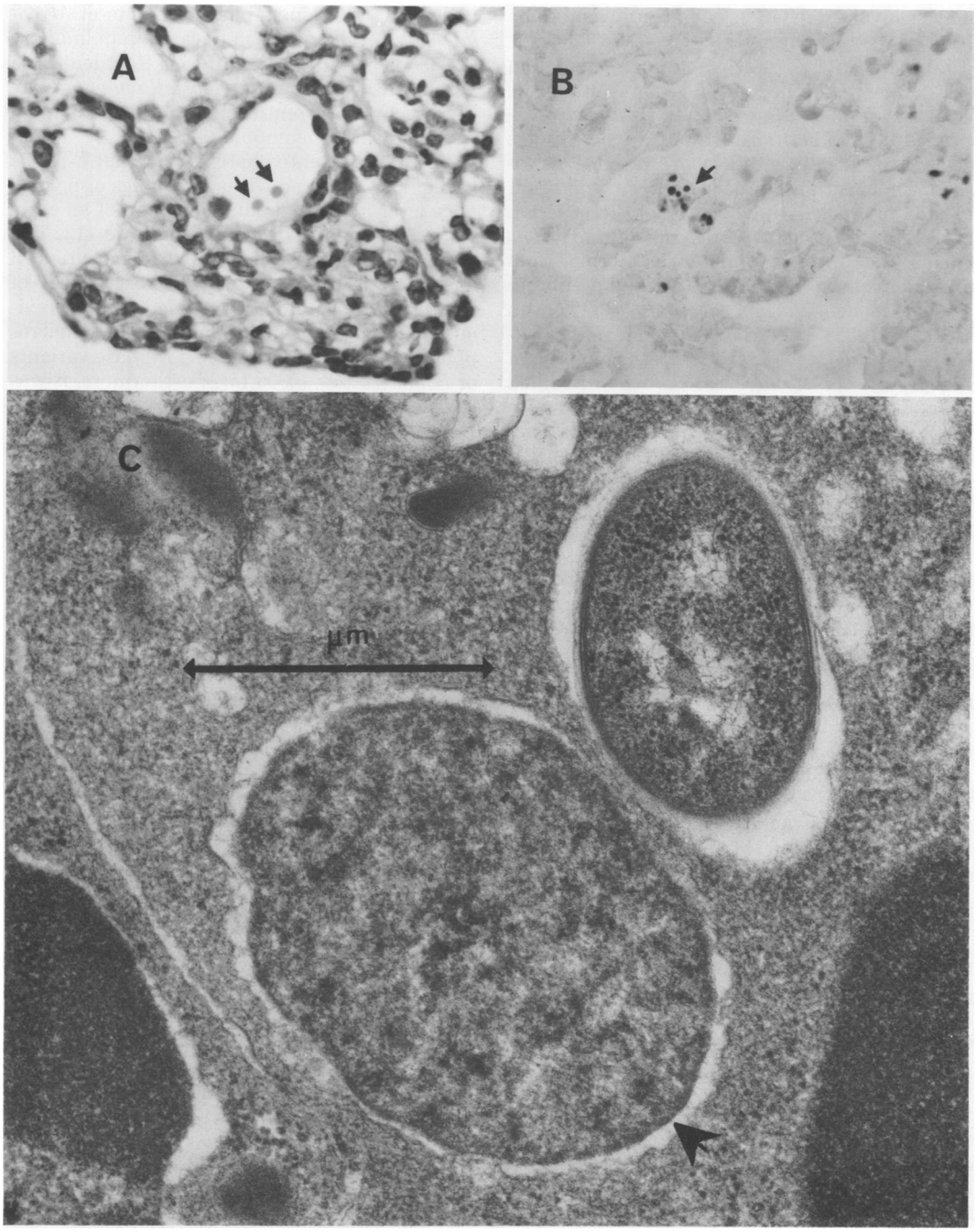

FIG. 4. *In vivo induction of spheroplast-like cells of N. caviae within the intact murine lung. (A) Hematoxylin-eosin stain of the Formalin-perfused lung of a mouse 96 h after intranasal administration of early stationary-phase cells of N. caviae 112. Arrows indicate large, lightly staining spheroidal bodies within the alveolus. Similar spheres of varying size are also detectable within the inflammatory infiltration. (B) Brown and Brenn Gram stain of the section as in A. Note the large numbers of gram-variable spheres of varying size within the pulmonary infiltrate. (C) Electron micrograph of a thin section of the lung (as in A) perfused with glutaraldehyde in buffered sucrose. Note that within the cytoplasm of a macrophage there is a normal nocardial cell that possesses an intact cell wall. Lying next to this apparently intact cell is an unmistakable cell wall-deficient variant (compare with Fig. 2). The arrow points to the cytoplasmic membrane surrounding the cell and the apparent absence of cell wall material.*

may be involved in latency of infection and some L-forms may be pathogenic (10, 11, 14, 15).

Little information is available on the induction of L-forms within the lung and the resultant role of these variants in induction of pulmonary disease (15). Furthermore, there are no studies dealing specifically with L-phase variants of *Nocardia* in infections of the lung. Brem and Reidt (8) and others (21) have reported the isolation of cell wall-deficient forms of *Streptococcus pneumoniae* from individuals suffering from pneumonia, and L-phase variants and spheroplasts of *Mycobacterium tuberculosis* have been identified (15, 17, 22). Ratnam and Chandrasekhar attempted to study the pathogenicity of spheroplasts of *M. tuberculosis* when instilled intratracheally into guinea pigs (22). These investigators injected approximately 4×10^3 colony-forming units of a spheroplast suspension into each guinea pig. They found that this low number of spheroplasts failed to induce pulmonary infection, and the guinea pigs remained negative to tuberculin. However, after several weeks some of the animals were found to have tuberculous lesions in the lung, and the guinea pigs reacted positively to tuberculin. Ratnam and Chandrasekhar found that acid-fast parental tubercle bacilli were always present in animals that developed disease and tuberculin hypersensitivity (22), and felt that the spheroplasts of *M. tuberculosis* were not capable of causing disease per se but, instead, had to revert to the parental form before disease could occur. Their results clearly establish that spheroplasts can remain viable within the host lung for a period of time, thus suggesting a role for L-phase organisms in latency of disease which is very characteristic of tuberculosis (22).

The L-form of *Streptobacillus moniliformis* has been shown to induce fatal pulmonary disease in mice, and it is known that this organism is converted to the L-phase of growth with great frequency (15). Although the observations regarding the pathogenic role of L-forms of *S. moniliformis* were initially made in 1937 by Dienes and Edsall (13), there still exists some controversy regarding the pathogenic potential of these cell wall-deficient forms. Wittler studied the pathogenicity of L-forms of *Haemophilus pertussis* administered intranasally to mice. This investigator presented some evidence that the L-forms could increase in numbers within the intact murine lung, and it was suggested that the L-form of this organism might be pathogenic when administered intranasally (23). McKay et al. (16) found that protoplasts of *Haemophilus parainfluenzae* induced a pneumonic process when inoculated intratracheally into 1-week-old piglets. In addition, L-phase variants of *Mycobacterium avium* were implicated in pulmonary disease in an individual suffering from leukemia (15). L-phase variants of *Klebsiella pneumoniae* were recovered from the lungs of an individual who died with pneumonia (9).

The data presented herein show that L-phase variants are induced within the lungs of normal mice after intranasal administration of an early stationary-phase culture of *N. caviae* 112. The alveolar macrophage is the first phagocytic cell encountered when entering the host by way of the respiratory route. It has been shown previously that in vitro-maintained alveolar macrophages are capable of inducing L-phase variants of *N. asteroides* 10905 (5). Furthermore, it was shown that glycine and lysozyme will induce the L-phase of *N. caviae* 112 when grown in vitro (6). These observations suggest a possible mechanism for the induction of L-phase variants within the murine lung, since it has been shown that alveolar macrophages contain a high level of lysozyme activity and tend to be more bacteriostatic for intracellular parasites and less bactericidal for these cells when compared with peritoneal macrophages (12, 18–20).

The L-phase variants of *N. caviae* induced within the lungs appear to play a major role in the infectious process because they are present in large numbers at a time when normal bacteria cannot be recovered. At the same time, there is considerable cellular infiltration within the alveoli leading to an acute pneumonic process and death. Therefore, one must consider the cell wall-defective variant of *N. caviae* as an alternate pathogenic form. This should be considered when diagnosing and treating individuals with pulmonary disease of unknown etiology or in individuals with pulmonary nocardiosis.

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