Role of L-Forms of *Nocardia caviae* in the Development of Chronic Mycetomas in Normal and Immunodeficient Murine Models

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Single-cell suspensions of *Nocardia caviae* 112 were injected into normal, athymic, and asplenic mice by several different routes. The 50% lethal dose values, kill curve characteristics, histological and electron microscopic properties, organ clearance patterns, and induction of L-forms during the acute and chronic phase of disease were determined in groups of mice for up to 2 years after infection. From these data we concluded the following. (i) Athymic and asplenic animals were significantly more susceptible to *N. caviae* than their littermate controls regardless of inoculation route. (ii) All mice were most susceptible to lethal infection after intranasal administration and least affected when the organisms were injected into the peritoneal cavity. (iii) Chronic, progressive disease leading to the formation of mycetomas occurred only in mice injected intravenously. (iv) T-cell-deficient animals were impaired in the development of typical mycetomas. (v) L-forms of *N. caviae* were induced within immunocompetent hosts, whereas the cell wall-less state of the bacteria was not observed in the immunodeficient animals. (vi) Two colony types of the cell wall-deficient state were isolated from infected animals. (vii) These cell wall-deficient organisms were intimately involved in the pathogenesis of disease and bacterial persistence within the host. Finally (viii), with this strain of *Nocardia*, cell wall-deficient organisms played a major role in the development of the characteristic bacterial granule formed within the mycetomatous lesions 6 months to 1 year after intravenous inoculation.

Mycetomas have been recognized in humans from antiquity. They are chronic granulomatous diseases caused by various fungi and by members of the *Actinomycetales*. It is generally believed that the infection begins as a painless nodular mass at the site of an injury such as the introduction of a thorn or splinter into a foot or hand (2). After a period of time which may vary from 1 month to 4 years (23), the lesion enlarges and discharges a purulent exudate. The entire region affected becomes swollen, and multiple secondary nodules develop followed by the formation of multiple sinus tracts that exude pus, serous, and sero-sanguinous fluid. Within the discharge, granules composed of colonies of the organism are usually found. These granules are both microscopically and macroscopically characteristic of the etiology of the mycetoma. The lesions become indurated, and the abscesses are surrounded by granulomatous inflammation. The infection usually remains localized, but the lesions progressively enlarge over a period of several months to years with direct extension into the tissue. The muscle and bone are frequently involved; however, mycetomas may develop in any region of the body (2, 18, 23).

Mycetomas occur worldwide; however, they are most frequently recognized in Central and South America, Central and Northern Africa, and India (18, 32, 35-37). Mycetomas are occasionally diagnosed in the United States (4, 22, 23). The most frequent etiologies of this disease are the *Actinomycetales* (18, 35). *Nocardia brasiliensis* causes most actinomycetomas; however, *Nocardia asteroides* and *Nocardia caviae* also are important etiological agents (1, 18, 32, 35-37).

N. caviae was first identified as a pathogen in guinea pigs by Snijders (34) in 1924; however, it was not recognized as causing disease in humans until 1965 (17, 24). More recently, there have been several reports of infections caused by *N. caviae* in humans as well as a variety of other animals. The spectrum of disease varies from pulmonary and systemic nocardiosis to mycetomas, and from acute, fulminating infection to chronic, progressive tissue destruction. Nevertheless, 51.7% of all reported infections caused

by *N. caviae* in humans are mycetomas (1, 4, 10, 11, 21, 31, 37).

There have been a few reports describing experimental infections of mice, rabbits, and guinea pigs caused by *N. caviae* (19, 20, 26, 33); however, there have been no studies concerning the mechanisms of host resistance to these organisms. Even though mycetomas have been induced in laboratory animals by *N. brasiliensis*, there have been no investigations published on the mechanisms of development of experimentally induced mycetomas (28). This is due in part to the lack of a good experimental model. In the present study, normal and genetically immunodeficient mice were infected with *N. caviae* by several different routes, and the course of infection and the development of nocardial mycetoma were studied over the ensuing 2 years to determine the mechanisms of induction of mycetomas. Further, this report documents the induction and role of L-phase, cell wall-deficient forms of *N. caviae* in the pathogenesis of chronic actinomycetomas.

MATERIALS AND METHODS

Microorganism. *N. caviae* 112 was isolated from a fatal human infection (11) and obtained from W. Causey (University of Chicago Hospitals and Clinics, Chicago, Ill.). The organism was grown and maintained as previously described (7). Early stationary-phase cells (48-h cultures) were prepared by differential centrifugation to remove clumps, and individual cells were suspended in 0.85% sterile saline as described (7).

Animals. Congenitally athymic mice of a BALB/c background, hereditarily asplenic mice of a B6·CBA background, and Swiss Webster mice were maintained at the Animal Resources Service, University of California, Davis, as previously described (5, 6). All infected mice were maintained in a special animal room supplied with filtered air and fed Purina Laboratory Chow ad libidum. The nude mice were maintained in sterile isolators with filtered air and provided with acid water (pH 2.5 to 2.8) (5).

Infection schedules. Mice were infected by the following routes: intravenously (i.v.), subcutaneously (s.c.) in the footpad, intraperitoneally (i.p.), and by intranasal (i.n.) administration. They were monitored daily. At the end of 3 months, 50% lethal dose (LD_{50}) values were determined (12) and surviving mice were necropsied and evaluated for macroscopic and microscopic lesions and cultured for nocardial cells and L-forms. In addition, groups of more than 150 mice were injected i.v. with approximately 6×10^6 colony-forming units (CFU) of *N. caviae*, and specific organs were cultured for normal as well as cell wall-deficient organisms at daily, weekly, and monthly intervals (at least 5 mice per time period). In addition, tissues of duplicate mice were prepared for histology and electron microscopy (3, 6, 27), and tissue impression smears were prepared for immunofluorescent microscopy (3). These experiments were carried out for 1 year. Fur-

ther, surviving mice (more than 50 mice) were monitored for 2 years after i.v. challenge with sublethal doses of *N. caviae*. All experiments were repeated at least once with similar results, and more than 1,000 mice were evaluated during this investigation.

Quantitation of organisms. The numbers of bacteria and cell wall-deficient organisms from the organs of five mice from each group were counted. The mice were killed by an overdose of ether, and the blood was removed and plated directly on duplicate plates of brain heart infusion agar (BHI) and Barile, Yarguchi, Eveland agar (BYE-L agar). The bone marrow, brain, eyes, kidneys, liver, lungs, spinal cord, and spleen were aseptically removed and placed in 3.0 ml of sterile 20% (wt/vol) sucrose and homogenized for 30 s with a Tissumizer high-speed blender as described (3, 7). Serial dilutions of the homogenized tissues were prepared in sterile 20% sucrose and plated in duplicate on both BHI agar and BYE-L agar. The plates were incubated at 37°C for 1 to 4 weeks, and the colonies were counted (L-form and L-form variant colonies were quantitated using 30× magnification on a Zeiss Microscope).

Observation of developing L-form colonies. Samples of homogenized tissues were plated on BYE-L agar, and sterile cover slips were placed over the inoculated regions of the plates that demonstrated possible growth of organisms. 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-distance-phase substage condensor. These developing L-form colonies and their reversion to normal colonies were compared to plates inoculated with in vitro-induced, cell wall-deficient organisms of *N. caviae* 112 (3, 9) which served as controls. The colonies were photographed with Kodak Panatomic-X film through a green filter.

Indirect immunofluorescence. The L-forms, L-form 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 as previously described (3, 8).

Light microscopy. The mice were killed as described above, and the internal organs as well as mycetomatous regions were flooded with 10% buffered Formalin. These tissues were removed from the animals and partially perfused with additional fixative. The fixed samples were washed with buffer, dehydrated through a series of ethanol, cleared, and then embedded in paraffin as described earlier (3, 6, 7). Thin sections were cut with a Spencer microtome (AO Co.), affixed to glass slides, and stained by the Brown and Brenn modification of the Gram stain (27), by the Kinyoun acid-fast stain using 1% HCl in 70% ethanol as the decolorizing agent (27), and by hematoxylin and eosin (27). The stained sections were observed with a Zeiss research microscope.

Electron microscopy. Normal cells and colonies of L-forms isolated from the tissues and samples of infected tissue were fixed as previously described (3), embedded in Maraglass, and sectioned. The sections were stained for 30 min with uranyl acetate and for 60 s with lead citrate and were photographed using a Philips-400 electron microscope.

Definition of terms. The terms cell wall-deficient forms, wall-defective variants, protoplasts, spheroplasts, and L-forms were previously defined by Madoff and Pachas (29) and by Mattman (30). These terms are defined as follows. Wall-defective variant is a general term indicating that an organism has undergone change as the result of alterations in its cell wall. Cell wall-deficient form generally indicates that the organism has an altered morphological state as the result of deficiencies in cell wall structure or synthesis, or both. They are generally osmotically fragile and Gram negative. Protoplasts are osmotically fragile, spherical-shaped cells that have lost all cell wall constituents; they are not able to reproduce serially. Spheroplasts are osmotically fragile, spherical-shaped cells that still possess some cell wall constituents; however, they are not able to reproduce serially. L-forms are cell wall-deficient forms that produce distinctive "fried-egg" colonial morphology on agar; they can be serially passaged without reversion to the classical bacterial stage. L-forms may be nonreverting or they may readily revert to the parental bacterium once the inducing agent is removed (unstable L-form). Some cell wall-deficient organisms grow on agar to form colonies that do not have the classical fried-egg appearance. These are referred to as L-form variants (29, 30).

RESULTS

Host susceptibility to *N. caviae*. Survival data and LD_{50} values were calculated for normal and immunodeficient mice infected by different routes with single-cell suspensions of *N. caviae* (Table 1). The data showed that host resistance depended upon the route of infection as well as

TABLE 1. LD_{50} *values for N. caviae 112 (48 h) inoculated by different routes into different murine hosts*[a]

Mouse strain	No. of mice	Route	LD_{50} (CFU/ mouse)[b]
Swiss Webster	45	i.v.	1.7×10^7
BALB/c (Nu/+)	30	i.v.	4.1×10^6
Nude (BALB/c Nu/Nu)	30	i.v.	8.9×10^5
B·6 CBA (control)	29	i.v.	1.2×10^7
B·6 CBA (asplenic)	32	i.v.	1.7×10^6
Swiss Webster	30	i.p.	2.5×10^8
BALB/c (Nu/+)	21	i.p.	3.8×10^8
Nude (BALB/c Nu/Nu)	22	i.p.	1.9×10^6
B·6 CBA (control)	30	i.p.	5.6×10^7
B·6 CBA (asplenic)	27	i.p.	2.8×10^7
Swiss Webster	40	i.n.	5.5×10^5
BALB/c (Nu/+)	30	i.n.	3.8×10^5
Nude (BALB/c Nu/Nu)	30	i.n.	1.7×10^5
B·6 CBA (control)	25	i.n.	3.5×10^6
B·6 CBA (asplenic)	23	i.n.	4.5×10^4
Swiss Webster	30	Footpad	1.1×10^7
B·6 CBA (control)	18	Footpad	1.0×10^7
B·6 CBA (asplenic)	18	Footpad	3.2×10^6

[a] LD_{50} values determined 3 months after inoculation.

[b] Calculated by the Reed-Muench method (12).

the immunological capabilities of the host. All animals except the athymic mice demonstrated an acute onset of illness, which resulted in some deaths, followed by apparent recovery and the development of chronic, progressive disease by surviving mice when infected by the i.v. route. In contrast, nude mice were initially more resistant to infection, and athymic mice did not die of the acute form of the infection. However, both athymic and asplenic mice infected i.v. by an LD_{50} dose succumbed significantly earlier in the chronic stage of the disease than did either the CBA, heterozygous nude, or Swiss Webster controls.

Nude mice were more susceptible to lethal infection than their heterozygous littermates regardless of inoculation route; however, the greatest difference was noted after i.p. infection by which the heterozygous mice were 200 times more resistant than the athymic animals (Table 1). In sharp contrast to nude mice, the asplenic animals demonstrated only a twofold difference from the CBA controls when *N. caviae* was injected i.p. (Table 1). Whereas CBA mice were 6 times more resistant to i.n. infection than the Swiss Webster animals, they were 78 times more resistant than their asplenic littermates. Finally, asplenic mice were 10 times more susceptible to i.v. challenge than their CBA controls (Table 1).

Induction of mycetomas. After i.v. injection with approximately 6×10^6 CFU, Swiss Webster, heterozygous BALB/c (Nu/+), and CBA mice were acutely ill at 48 h. Few mice died during the first 2 weeks; however, all mice developed either a spinning syndrome or uncontrolled bobbing of the head. The animals initially lost weight, but at 1 month after infection the mice appeared healthy except for the involuntary bobbing of the head. Many mice raced around the cage in a tight circle (chasing their tails), and some mice were partially paralyzed in the posterior part of the body. These conditions persisted for 6 months or more. Between 6 and 10 months all control mice developed multinodular lesions that progressively enlarged for several additional months before killing the animals. The lesions usually developed at the base of the skull or near the posterior, terminal end of the spine and extended through the surrounding tissues (Fig. 1A and B). In some mice, these lesions developed multiple draining sinus tracts and bacterial granules were present in the exudate. When excised from the animal, some lesions were found to weigh as much as 50 g (Fig. 1B). The spleens at this time were greatly enlarged, with a mean weight of 2.5 (±0.5) g (Fig. 1B). Histological analysis revealed these lesions to be similar to the chronic actinomycetomas described in humans with granules of organisms

surrounded by inflammatory cells (Fig. 1C). Mycetomas were observed to develop only after i.v. injection.

Three months after i.v. injection of approximately 10^6 organisms, some of the surviving athymic mice developed large abscesses at the base of the skull and at the posterior portion of the spine (compare Fig. 1A with Fig. 2A). More than 10^8 normal bacteria could be isolated on BHI agar from these lesions (Fig. 2B). In contrast to the control mice, however, the bacterial granule was not prominent within the lesions. Instead, light and electron microscopy revealed that normal-appearing organisms grew as dispersed filaments and rods within macrophages and polymorphonuclear neutrophils (Fig. 2C). There were few lymphocytes infiltrating the lesions, and the abscesses were not encapsulated by granulomatous tissue. The progressive lesions shown in Fig. 1 and 2 were not observed in asplenic mice and they were not found in animals injected either i.p., i.n., or in the footpad even 2 years after infection.

Distribution and growth of *N. caviae* after i.v. injection. To study the initial host response during the acute phase of infection, the bone marrow, brain, kidneys, liver, spinal cord, and spleen were removed at 3, 24, 48, 72, 96, 168, and 336 h after infection, and the number of organisms per organ was quantitated. Most of the organisms were deposited in the liver at 3 h, and few bacteria were in the blood. At 48 h after infection, the blood was sterile and remained so throughout the experiment. On the other hand, the liver gradually cleared the organisms and no lesions were observed within the liver. In contrast, at 72 h after infection, the numbers of *N. caviae* increased in the brain, kidneys, spleen, and spinal cord; however, at 2 weeks the numbers of bacteria in the spleen were decreased whereas in the other organs they were increased. The numbers of organisms in the bone marrow persisted at low levels during the 2-week period. At 4 weeks after infection, the numbers of organisms remained constant in the kidneys but decreased in the brain and spinal cord. At this time the liver, spleen, and blood were sterile and

only the kidneys had macroscopic evidence of infection, with small abscesses and discolorations (7).

To determine the chronic persistence of *N. caviae* within the host, the organisms were quantitated at 1, 2, 3, 4, 7, and 12 months after infection (Fig. 3). During this 12-month period normal organisms could never be isolated from the liver, lungs, spleen, or blood (not one colony of *N. caviae* was recovered on BHI agar from these organs obtained from 35 mice, and the limit of sensitivity is 5 CFU per organ). The eye is particularly noteworthy since at 6 months to 1 year after infection a significant number of mice (15/35) developed opacification of the eye and became blind (see Fig. 6B), but normal organisms were not usually isolated. Uninfected control mice never developed this blindness (0/10). The kidneys and brains of these mice had variable numbers of bacteria (Fig. 3). Large numbers of bacteria persisted for 1 year within the kidneys of some of the infected mice; however, many of the kidneys appeared to be sterile since normal bacteria could not be isolated on BHI agar (Fig. 3). At 3 months, none of the mice studied had bacteria that could be recovered from the brain (Fig. 3); however, at 7 months after infection bacteria could be recovered from 60% of the brains of the mice (Fig. 3). In addition, normal bacteria could not be recovered from any of the organs of 5 of 35 mice studied, yet 100% (52/52) of the mice maintained for 2 years developed mycetomatous lesions as shown in Fig. 1.

L-forms and L-form variants of *N. caviae*. With the exception of asplenic and nude mice, all animals that recovered from the acute phase of infection after i.v. challenge with more than 6×10^6 CFU of *N. caviae* always developed progressive mycetomatous lesions. However, attempts to recover normal cells of *N. caviae* on BHI agar from these infected animals before the emergence of the mycetomas frequently failed. It was demonstrated previously that *N. caviae* can be induced to grow in the cell wall-deficient form on BYE-L agar but not on BHI agar (9). In addition, it was shown that L-forms were in-

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FIG. 1. *Formation of mycetomatous lesions in Swiss Webster mice. (A) A normal mouse 1 year after i.v. injection of 6.8 × 10⁶ CFU of N. caviae 112. Arrows indicate the formation of chronic, progressive lesions originating from the base of the skull and spine (compare with Fig. 2). (Note: At 9 months after inoculation this mouse had no visible evidence of infection; however, lesions were discernable at 10 months.) (B) The mouse as in (A) showing the multinodular development of the mycetomatous lesions (M) extending from the spine (pointer) through the muscle and involving the leg. Note the greatly enlarged spleen (S) which weighed approximately 2.0 g. (C) A light micrograph of a hematoxylin-and-eosin-stained paraffin section from (B). The lesions in (B) were composed of large numbers of granules (G) surrounded by polymorphonuclear neutrophils, lymphocytes, and macrophages and encapsulated by fibrotic tissue. The granules combined with the pathology of this lesion are characteristic of nocardial mycetomas.*
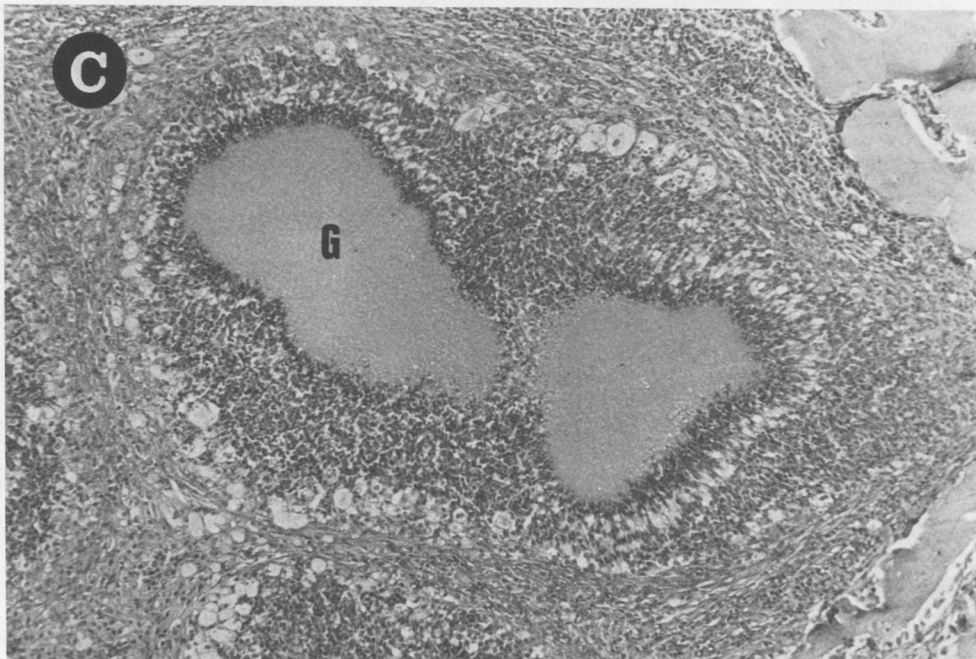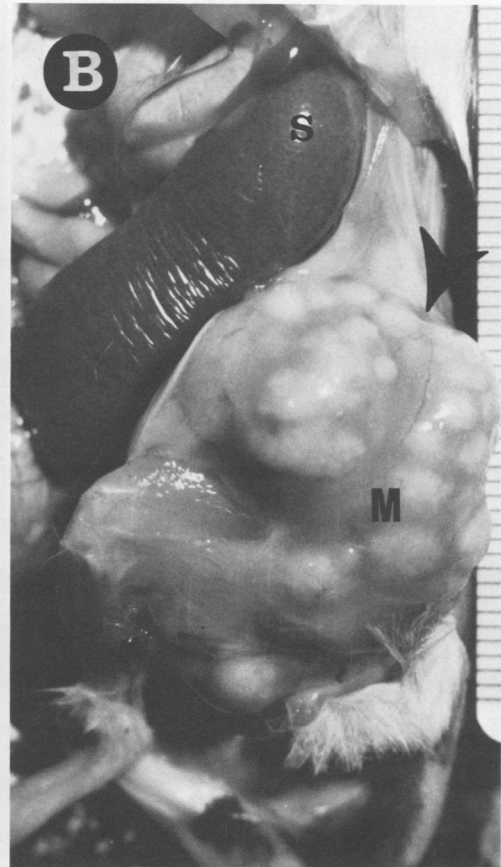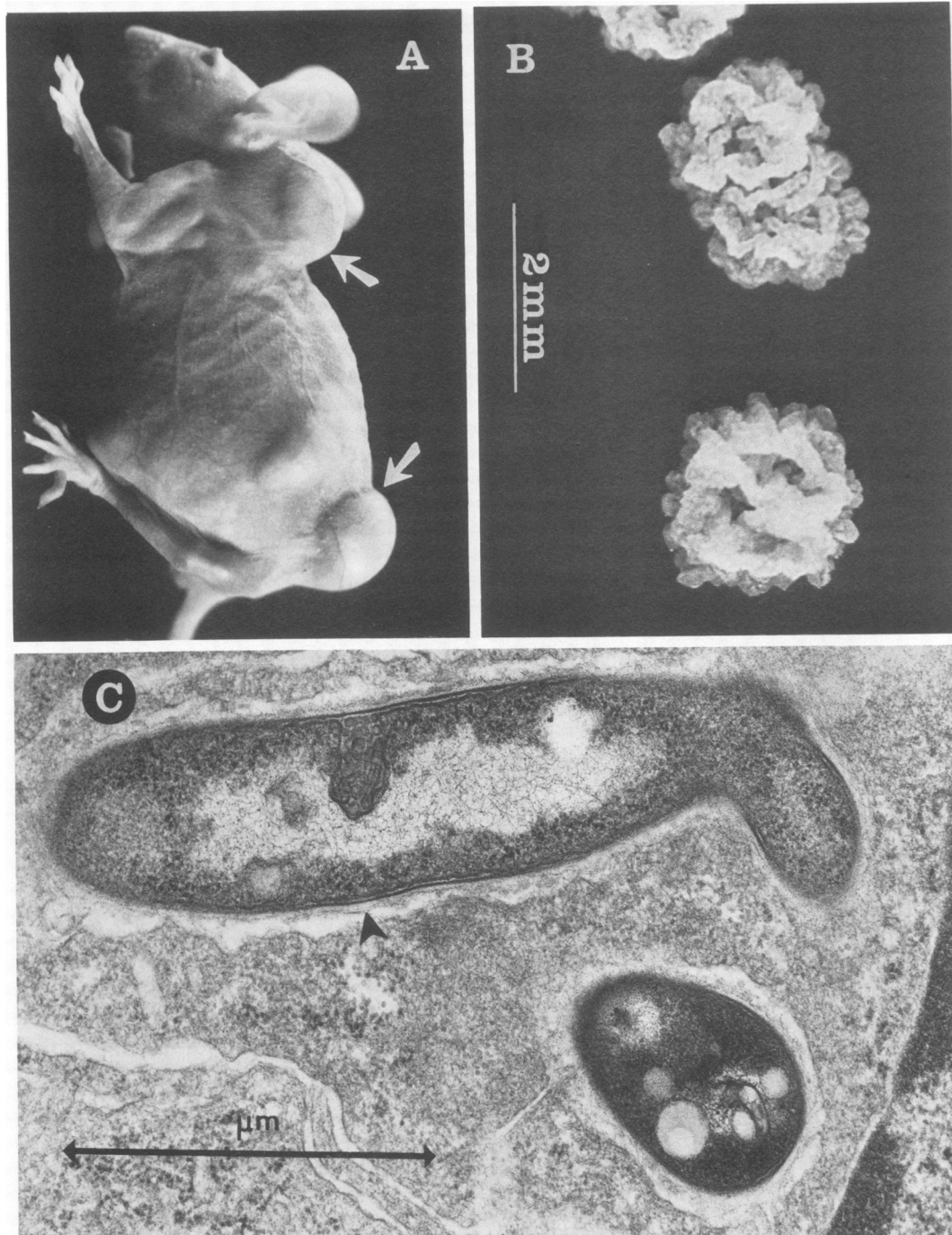
FIG. 2. *Interaction of N. caviae 112 within the athymic mouse. (A) Nude mouse 12 weeks after i.v. injection of 1.3 × 10⁶ CFU. Arrows indicate the formation of chronic, progressive lesions orginating from the base of the skull and spine (compare with Fig. 1). (B) Normal-appearing colonies of N. caviae isolated from the lesions of the nude mouse inoculated onto BYE-L agar and incubated for 1 week at 37°C. (Note: L-forms and L-form variant colonies were not observed on any of the BYE-L plates inoculated with samples obtained from nude or asplenic mice [compare Fig. 2B with Fig. 4 and 5].) (C) Electron micrograph of lesion shown in (A). Note the presence of normal nocardial cells within the phagosome of a macrophage. Pointer indicates the typical, intact, trilayered cell wall characteristic of actively growing cells of Nocardia.*
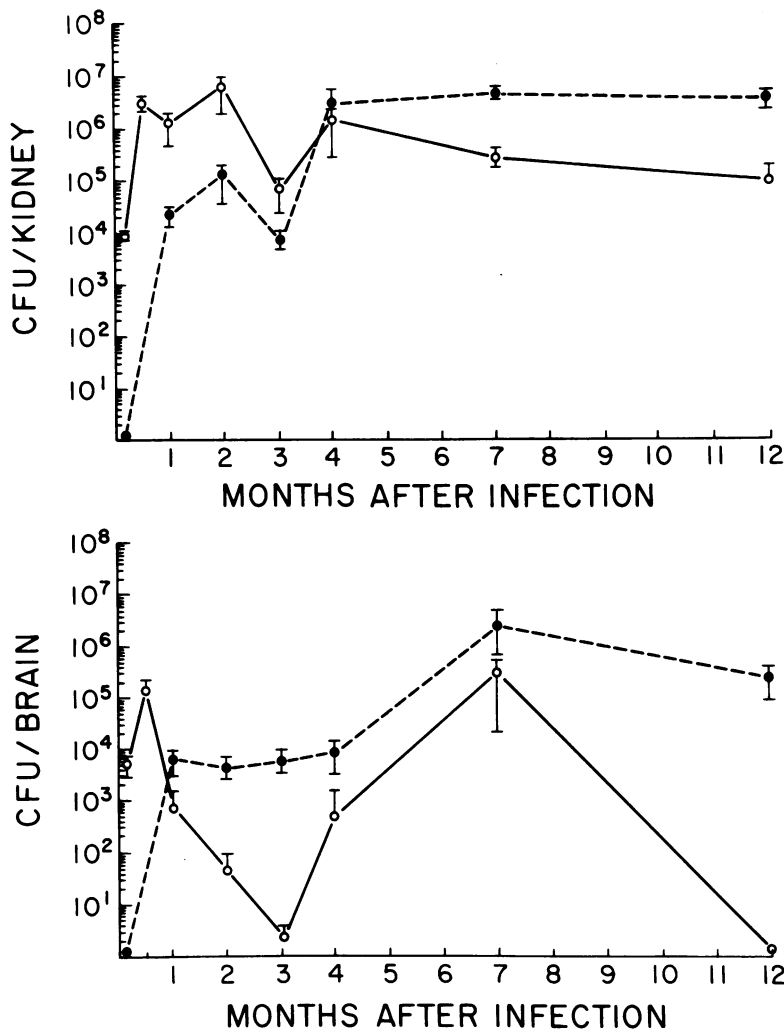
FIG. 3. *Curves contrasting the numbers (in the kidneys and brains) of normal colonies of N. caviae isolated on BHI agar* (O) *with the numbers of L-forms when the same samples were plated on BYE-L agar* (●). *Each point represents the mean of five mice; the bars represent the standard error.*

duced in the lungs of normal mice when cells of *N. caviae* were administered i.n. (3). The observations described herein suggested that a cell wall-deficient form of *N. caviae* persisted within the host during the latent period between the acute phase of the illness and the development of the mycetomas. Thus, for 1 year after i.v. infection, more than 150 mice were assessed for the presence of L-forms in the blood, bone marrow, brain, eyes, kidneys, lungs, liver, and spleen as well as within the mycetomatous lesions that developed. These studies demonstrated the presence of two distinct colony types of wall-deficient cells (Fig. 4 and 5).

In one series of experiments, 28 mice were studied for 1 year after infection to determine the presence of normal nocardiae, L-forms (Fig. 4), and L-form variants (Fig. 5) in the blood, bone marrow, brain, eyes, kidneys, liver, lungs, spinal cord, and spleen. Normal cells of *N. caviae* were isolated from the kidneys of 67.9% (19/28) of the mice, whereas 21.4% of the animals had normal organisms in the brain, 25% had normal organisms in the spinal cord, 10.7% had normal organisms in the eyes, and 7.1% had normal organisms in the bone marrow. As shown previously, none of the mice studied had organisms in the blood, liver, lungs, or spleen. Approximately 18% of the mice had no normal organisms in any organ cultured. In sharp con-
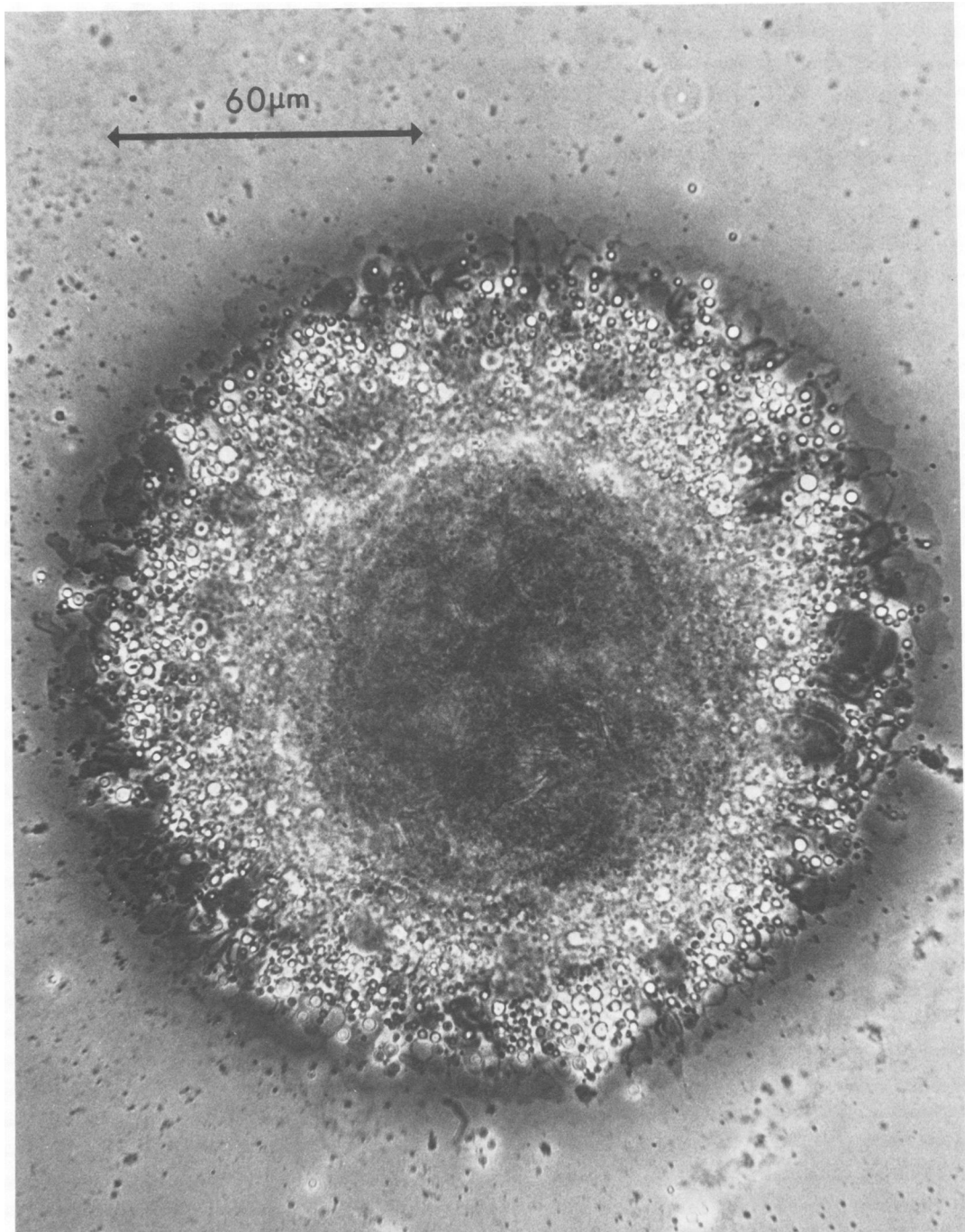
FIG. 4. *Phase-contrast light micrograph of an L-form colony grown for 2 weeks on BYE-L agar. This colony was isolated from the brain of a mouse 3 months after i.v. injection of 6.8 × 10⁶ CFU (see Fig. 3). At this time period no normal nocardial colonies were isolated from this mouse. Note the characteristic fried-egg morphology with dense core surrounded by a periphery of granules, spheres, and membranous structures. This colony represents the most frequent form of wall-defective cells of N. caviae recovered from the mice.*

trast, either L-forms or L-form variants could be isolated from one or more organ in 100% (28/28) of the mice.

Cell wall-defective organisms were readily isolated from the brains and kidneys of infected mice. The colonies were typical of bacterial L-
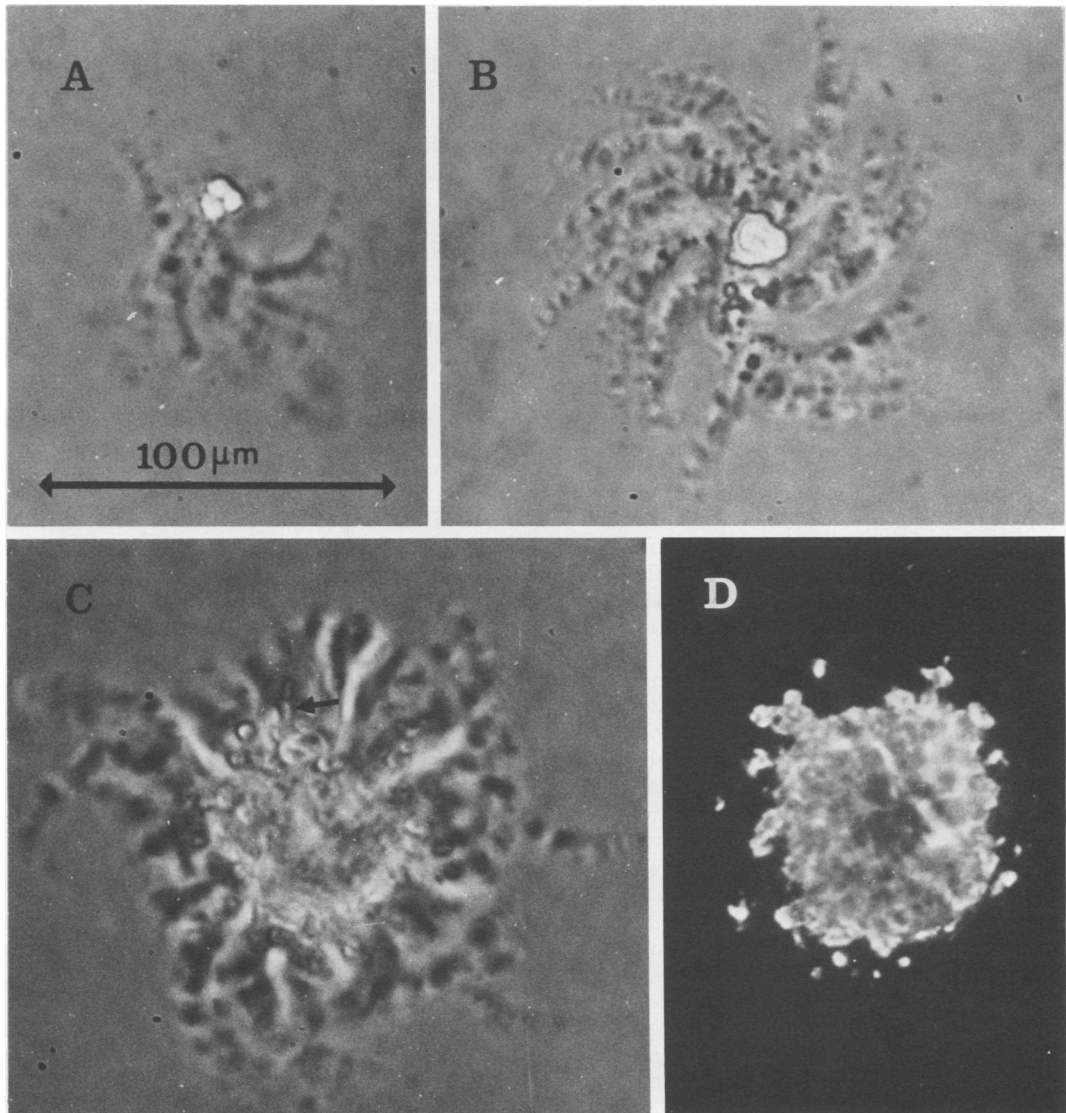
FIG. 5. *Development and microscopic characterization of L-form variants of N. caviae 112 incubated on BYE-L agar at 37°C. (A) Phase-contrast micrograph of developing L-form variant colony 72 h after inoculation of BYE-L agar with spinal cord homogenates of a mouse infected for 6 months. Note the large refractile spheres and the radiating spiral arms. (B) Phase-contrast micrograph of the same colony shown in (A) but incubated for 7 days at 37°C. (Note: The refractile spheres have fused to form one large, irregular, refractile central body.) There are numerous spheres and granules forming within the radial growth. (C) Same colony as in (A) and (B) but incubated for 14 days at 37°C. The central large body has been replaced by a compact aggregation of spheres and granules of varying size and density. The radiating spiral arms are less distinct. What may be a reverting cell is evident (arrow). This type of colony does not readily revert to the parental form. (D) Indirect immunofluorescent stain of L-form variant colony as above using guinea pig antiserum specific for N. caviae 112 (3).*

forms in that they consisted of a compact core of cells growing into the agar surrounded by a periphery of spheres, granules, and membranes (Fig. 4). The L-forms shown in Fig. 4 are the predominant organisms isolated from mycetomas as well as from the brains and kidneys of infected mice, and their colonial morphology is the same as described for other wall-defective variants of bacteria (30). In contrast, an unusual colony was frequently isolated from the spinal cord, eyes, and bone marrow of infected mice (Fig. 5). This second type of colony is defined as

an L-form variant. (Compare with the parental colony morphology [Fig. 2B] and with the typical L-form colony [Fig. 4].) The distribution of normal cells, L-forms, and L-form variants of *N. caviae* in mice 1 year after i.v. inoculation is shown in Table 2. The spinal cord had three colony types present whereas only L-form variants were recovered from the bone marrow and eyes (Table 2 and Fig. 6). Because the L-form variant colonies were not typical of L-forms but instead they superficially resembled "pseudocolonies," rigorous criteria were used to prove the true identity of these colonies. Thus, these L-form variants were shown to be viable cells derived from *N. caviae* and not related to pseudocolonies by the following properties. (i) They could be serially transferred on fresh medium. (ii) They were gram negative and osmotically fragile. (iii) They followed serial dilutions of the infected tissues and were never recovered from uninfected controls. (iv) They were partially acid fast using auramine-rhodamine fluorescent staining as described for L-forms of *Mycobacterium* (30). (v) They stained green for deoxyribonucleic acid and orange for ribonucleic acid with acridine orange fluorescent microscopy described for L-forms (30). (vi) They gave positive immunofluorescence when treated with antiserum against *N. caviae* but not when treated with either normal serum or antiserum against *N. asteroides* (Fig. 5D). (vii) Electron microscopy demonstrated that these colonies were composed of membrane-bound cells and vesicles surrounded by diffuse granular material and membranes similar to those seen for other L-forms of bacteria (Fig. 6). And (viii), most importantly, they could be induced to revert to yield the parental form of *N. caviae* (as the colony shown in Fig. 2B). The electron microscopic appearance of the cells within these colonies as well as reversion to the parental bacterial type are nec-

essary for conclusive identification of these colonies.

In addition, chemical analysis of the L-forms and L-form variants of *N. caviae*, like those shown in Fig. 4 and 5, revealed the presence of 10-methyl stearic acid (tuberculostearic acid) which is found only in the cytoplasmic membranes and cell walls of *Mycobacterium* and *Nocardia*. In addition to this unique marker, only small amounts of arabinose and galactose (cell wall sugars) were present whereas larger amounts of glucose were detected. *Meso*-diaminopimelic acid (found only in the cell walls) was not detected in any of the cell wall-deficient forms of *N. caviae* even though it was demonstrated readily in whole-cell hydrolysates of the L-form revertants.

Mycetomas and cell wall-deficient organisms. Light microscopy of sections of mycetomatous lesions produced in mice several months to 1 year after i.v. inoculation of single cells of *N. caviae* demonstrated that most of the granules were gram negative and composed of spheres and pleomorphic, "involuted" bacterial cells (Fig. 7A). The data suggested that these granules were initially cell wall-deficient cells that replicated within the host to form small aggregates. As the aggregates enlarged, a host response ensued that surrounded the aggregates of L-forms. As the aggregates increased further, some of the L-forms reverted to a walled state and grew as gram-positive to gram-variable filaments at the periphery of the aggregate (Fig. 7B). An almost identical process was shown to occur in vitro when artificially induced L-forms of *N. caviae* 112 were plated on BYE-L agar without inducing agents and incubated for 2 to 4 weeks at 37°C. Electron microscopy of the developing granules clearly demonstrated that spheroplasts (or protoplasts) were present within the lesion (Fig. 7C). Some bacteria at the

TABLE 2. *L-form variants and L-forms of N. caviae 112 isolated from mice 1 year after infection*[a]

Organ	No. of normal cells isolated on BHI agar	No. of L-forms isolated on BYE-L agar	No. of L-form variants isolated on BYE-L agar
Blood (0.1 ml)	0[b]	0	0
Bone marrow	0	0	1.9×10^5 ($\pm 1.9 \times 10^5$)[c]
Brain	0	2.1×10^5 ($\pm 1.3 \times 10^5$)	0
Eyes	0	0	5.8×10^5 ($\pm 3.5 \times 10^5$)
Kidneys	1.4×10^5 ($\pm 1.3 \times 10^5$)	4.1×10^6 ($\pm 1.5 \times 10^6$)	0
Liver	0	0	0
Lungs	0	0	0
Mycetoma	8.8×10^4 ($\pm 2.8 \times 10^4$)[d]	2.8×10^7 ($\pm 2.3 \times 10^7$)[d]	0
Spinal cord	3.3×10^2 ($\pm 1.9 \times 10^2$)	2.1×10^6 ($\pm 1.5 \times 10^6$)	4.2×10^6 ($\pm 4.2 \times 10^6$)
Spleen	0	0	0

[a] Infection consisted of 6.3×10^6 CFU injected i.v. into Swiss Webster mice.

[b] 0, None detected in any of five mice (the practical limit of detection represents about 5 CFU per organ).

[c] Numbers in parentheses indicate ± standard error.

[d] Measured in colony-forming units per gram.

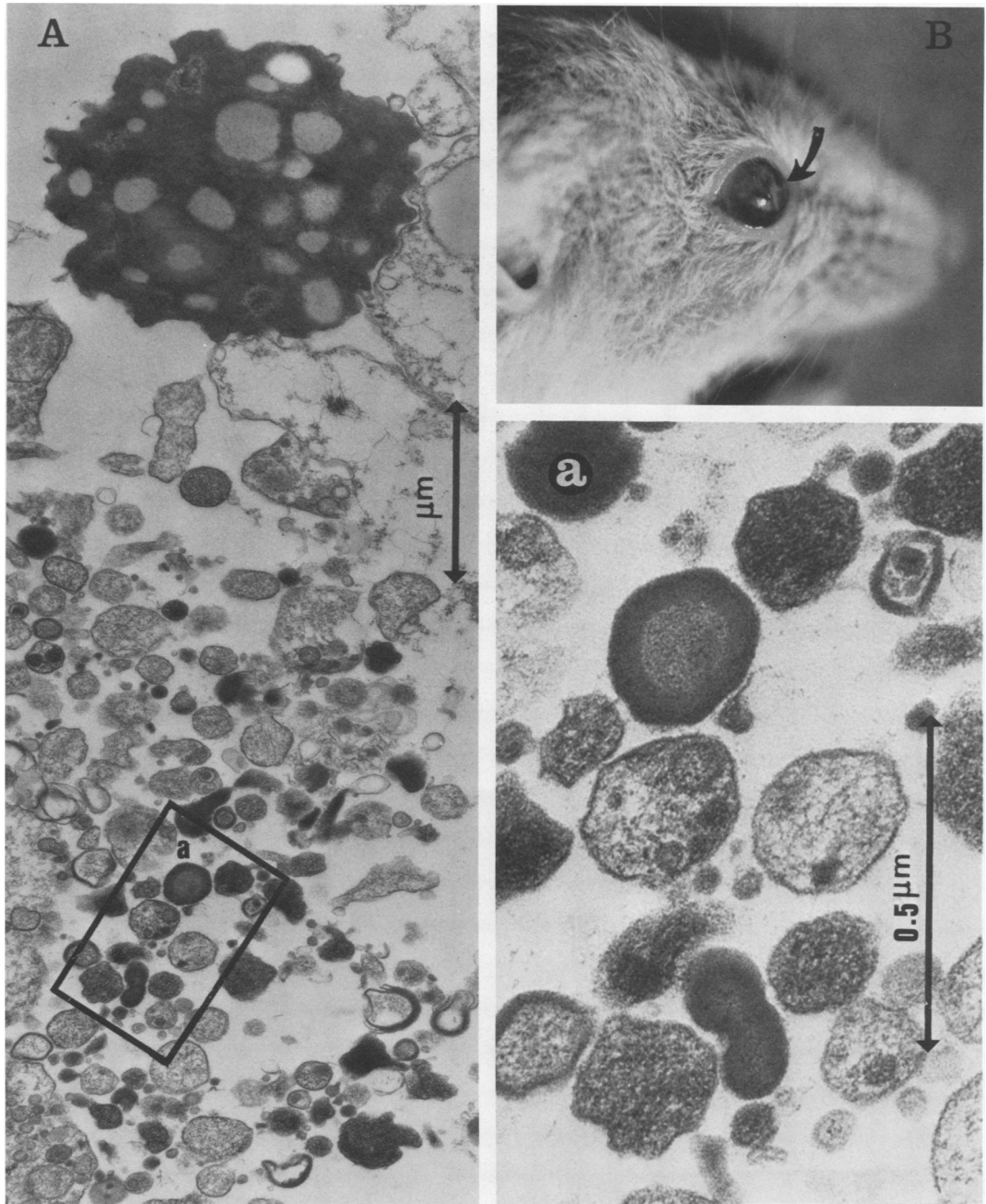FIG. 6. *L-form variants from eyes of mouse infected with N. caviae 112. (A) Electron micrograph of the L-form variant (Fig. 5) demonstrating the presence of large, irregular spheroplasts surrounded by masses of smaller membranous and spherical cells. (Insert a) High magnification of a small portion of the L-form variant colony showing the small irregular cells surrounded by a single cytoplasmic membrane. There is no evidence of cell wall material. Many of the small bodies have a more dense cytoplasm and nuclear region whereas others are much more diffuse. (B) The eye of a mouse 1 year after i.v. injection with 6.8×10^6 CFU of N. caviae 112. Only L-form variants as shown in Fig. 5 and 6A above were isolated from this eye. Arrow notes the presence of white opacifications within the eye. L-form variants were isolated only from eyes showing this type of opacification and were never isolated from the eyes of control mice.*

periphery of the granules possessed cell walls; however, the cells in the central portion of the granule were wall-less or had a modified cell wall possessing only the outer layers. The peptidogly-
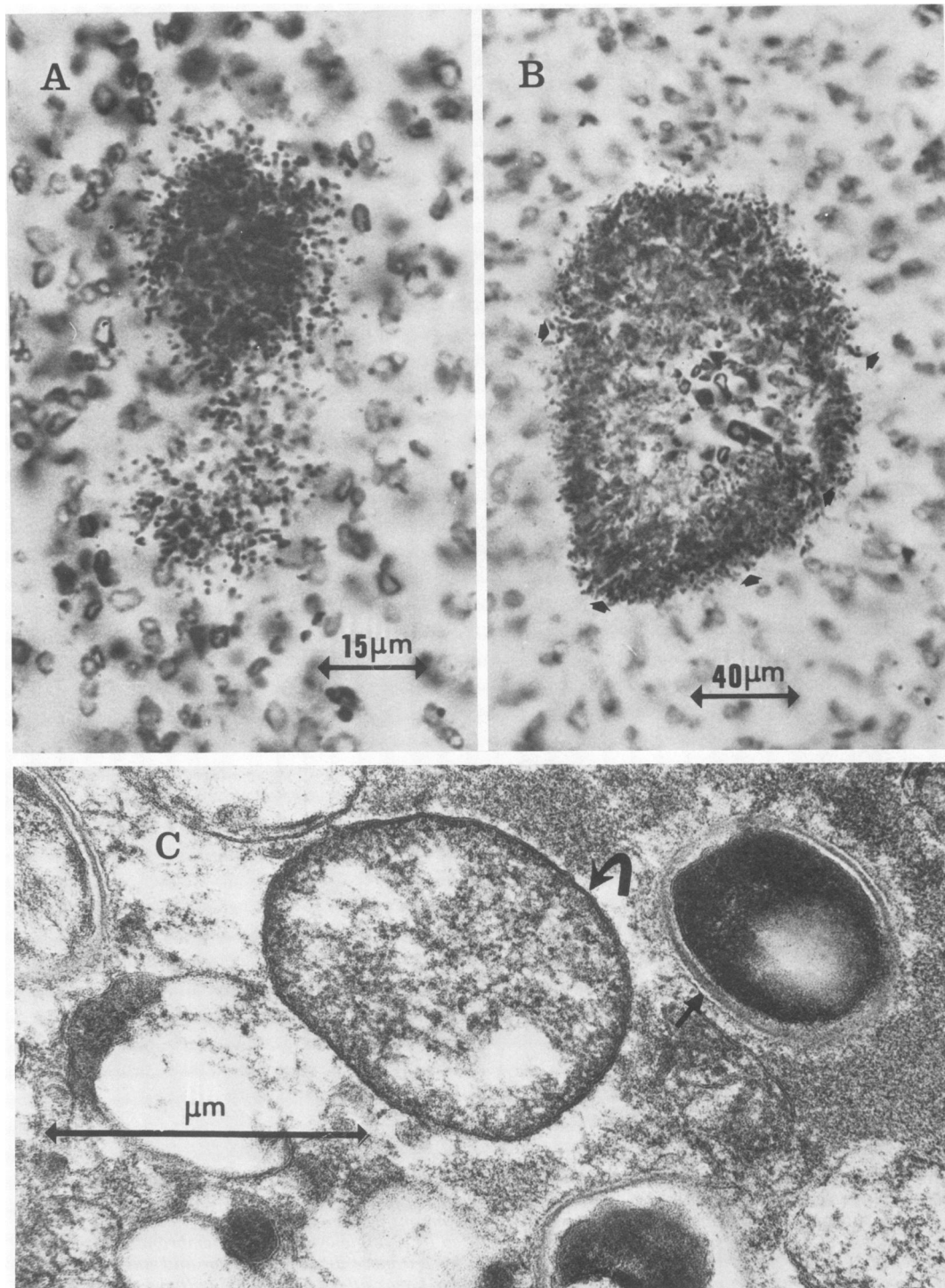
FIG. 7. Microscopic and ultrastructural characterization of the granules formed within the chronic lesions 1 year after infection with N. caviae 112 as shown in Fig. 1. (A) Gram stain of a small, probably developing granule (based on serial sections) showing an aggregation of gram-negative spheres of various sizes. No gram-positive organisms are present. The cells were enhanced photographically by using a green filter. (B) A larger, more advanced granule noting the presence of pleomorphic cells and the presence of gram-positive

can portion of the wall was missing or significantly decreased (Fig. 7C). Culture data demonstrated that the cell wall-deficient organisms which could be recovered from the mycetomatous lesions were about 300 times more numerous than the normal organisms (Table 2). These numbers are relative, however, since homogenization of the lesions has been shown to kill some of the L-forms and incompletely disperse the bacterial granules. It is important to note that in three mice, the mycetomatous lesions failed to yield any normal organisms on BHI agar and only L-forms were isolated on BYE agar from these three animals. Thus, L-forms of *N. caviae* 112 are present within the granules and appear to contribute to the induction and formation of the mycetomatous lesions.

DISCUSSION

The data presented herein show the following. (i) T-cell-deficient animals (25) are more susceptible to *N. caviae* than their littermate controls regardless of route of exposure. (ii) Asplenic mice which have a major age-dependent deficiency of T-cell function as well as a quantitative reduction in serum immunoglobulins (14) are most susceptible to acute, systemic infection. (iii) Asplenic mice always died before progressive mycetomas could be established or the mice appeared to remain uninfected, whereas in control mice, the progressive localized mycetomas always appeared 6 to 10 months after i.v. inoculation. Athymic mice usually succumbed to infection within 12 weeks; however, those nude mice surviving longer than 3 months usually developed leisons. In contrast, normal mice never developed mycetomatous lesions in less than 6 months after infection. (iv) Cell wall-defective cells of *N. caviae* are intimately involved in the persistence of the organisms in the tissues of normal mice for prolonged periods of time. (v) L-forms appear to play a major role in the formation of the bacterial granule and induction of the characteristic mycetomatous lesion. (vi) L-forms are induced and maintained within immunologically competent animals but they are not recovered from T-cell-deficient mice. (vii) *N. caviae* 112, when inoculated i.v. into mice, provides an excellent model for studying the development of chronic mycetomas. Further, the animal model established with *N. caviae* 112 parallels the development of disease this

organism caused in the human host and suggests that L-forms are an integral part of nocardial pathogenesis (11).

The data suggested that as *N. caviae* grew within the host during the early stages of infection there was a cell-mediated response that involved T-lymphocytes, macrophages, and polymorphonuclear leukocytes that could effectively inhibit the growth of the organism. The organisms that were thus contained were gradually stripped of their cell wall, probably by the interaction of highly activated macrophages (8, 15, 16). The resulting protoplasts or spheroplasts appeared to be refractile to the milieu of degradative enzymes that were released, and the cells survived within certain tissues. During the prolonged incubation within the host cells, these wall-deficient forms appeared to replicate, and some of them probably reverted to the parental state; however, in the immunocompetent host these revertants were readily destroyed or had their wall stripped away again. Outside the lung, this equilibrium within the competent host continued for an extended period of time and there was frequently no pathological evidence of infection; only L-forms could be recovered. As the L-forms replicated within the host, small clusters of spheres and granules accumulated to form bacterial granules. Wall-deficient cells within this developing granule reverted to the walled state and these revertant cells continued to grow at the periphery of the granule. This process induced the host response with an influx of polymorphonuclear leukocytes followed by lymphocytes and macrophages. The bacterial granule continued to enlarge, and the host cells were ineffective in eliminating the nocardial cells. As a consequence, the lesions developed the characteristic mycetomatous appearance (Fig. 1).

The induction and pathogenic roles of L-form variants were more uncertain. There was no doubt that these microbial forms were solely responsible for the infections of the eyes of the mice that resulted in blindness. However, examination of the pathology of the infected eyes demonstrated an absence of abscess or granuloma formation. Instead there was a polymorphonuclear leukocyte infiltrate with the presence of extracellular debris composed of spheres and a diffuse precipitate in the vitreous humor. In the brain, spinal cord, and bone marrow where these L-form variants persisted in high

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bacteria especially at the periphery of the granule (arrows). Some of the large granules were surrounded by large numbers of gram-positive filaments whereas the central portion of the granule was composed of gram-negative spheres and pleomorphic cells. (C) Electron micrograph of the periphery of a granule such as shown in (B). Note the presence of a protoplast-like cell that possesses no evidence of cell wall (bent arrow). In contrast, an adjacent cell that has a typical cell wall (arrow) is shown.

numbers, it was not possible to document, with certainty, a pathological response induced solely by them. Either typical L-forms or normal cells of *N. caviae* were always recovered from lesions visible within these organs. Thus, within these tissues the L-from variant appeared to play a greater role in the persistence of the bacterial genome rather than the induction of overt disease.

The unusual colonial morphology of the L-form variants was described previously by Fernandes and Panos (13). They isolated colonies that appeared to be identical to those shown in Fig. 5 from a renal biopsy of an individual with acute, proliferative, diffuse glomerulonephritis. No normal bacteria were recovered from the biopsy, and these L-form colonies did not revert to a bacterial type. However, both *Mycoplasma* and pseudocolonies were ruled out. It is well established that *Nocardia* can cause kidney infections, and we have isolated nocardial L-forms from the kidneys of mice. Because of the unique properties of our L-form variant colonies, it is possible that the L-forms with similar colonial morphology isolated from the human renal biopsy by Fernandes and Panos might be related (13). This suggestion was supported further by the isolation of L-form variants of *N. asteroides* GUH-5 from in vitro-induced spheroplast perparations (B. L. Beaman, unpublished data).

Nocardiae are quite variable in their colonial morphology, and several distinct colony types are regularly isolated from a single culture. Some of these colony types represent stable mutations and can be maintained whereas others remain variable. Thus, it is not surprising that at least two colony morphologies are found in cell wall-less variants of *Nocardia*. Further, because one of the colonial variants resembles pseudocolonies, it is possible that these L-form variants are recovered frequently from clinical material but discarded without further evaluation. Since the data presented herein show the potential pathogenicity of these L-form variants, it is important to emphasize that pseudocolonies isolated from clinical material should be examined to distinguish between true pseudocolonies and those of L-forms.

ACKNOWLEDGMENTS

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