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Interaction of *Nocardia asteroides* with Cultured Rabbit Alveolar Macrophages

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The interaction between virulent and less virulent strains of *Nocardia asteroides* and cultured rabbit alveolar macrophages was studied. It was shown that cells of the less virulent strain (*N. asteroides* 10905) were rapidly phagocytized and destroyed. However, some cells were able to avoid being killed, and they persisted within the macrophage in an altered, gram-negative form. These variants apparently increased in numbers after several days within the macrophage population, so that at 9 days postinfection more colony-forming units per macrophage were recovered than at 3 h. Little or no extracellular growth was observed in the tissue culture medium. During the increase at 9 days, both transitional-phase variants and L-forms of *N. asteroides* were isolated from the macrophages but not from the medium. Gram-positive bacterial cells were never observed in 9-day infected macrophages. In contrast, cells of the more virulent strain (*N. asteroides* 14759) were not destroyed after being ingested. After 6 h postinfection, it was observed that the number of colony-forming units per macrophage had increased significantly. There was no corresponding increase in extracellular organisms observed in the culture medium. Therefore, cells of *N. asteroides* 14759 were able to grow rapidly within cultured rabbit alveolar macrophages. Upon continued incubation of the infected cells (24 h postinfection), it was shown that this strain of *Nocardia* grew out of the macrophages as acid-fast branching filaments. From these data, it is clear that the initial interaction between *N. asteroides* and unstimulated, nonimmune alveolar macrophages depends upon the relative virulence of the nocardial strain.

In the United States, most nocardial infections are caused by *Nocardia asteroides*. Further, these infections usually involve the lung. It is well established that one of the major lines of lung defense against infectious agents is the alveolar macrophage (7). Therefore, the initial stages of lung infection should involve a nocardial-alveolar macrophage interaction. There are no published reports concerning the fate of *Nocardia* in alveolar macrophages.

We designed experiments to study the interaction of two strains of *N. asteroides* within in vitro cultured rabbit alveolar macrophages. One strain, *N. asteroides* 10905, was previously shown to be of low virulence (1), and it was found to persist in an altered form within cultured mouse peritoneal macrophages (3). The second strain, *N. asteroides* 14759, was shown by us to be virulent for mice and was reported to produce granulomas in rabbits (6). Both strains were initially isolated from human infections (6; J. Rozanis, personal communication.)

MATERIALS AND METHODS

Microorganisms. *N. asteroides* 10905 was supplied by J. Rozanis, University of Western Ontario, London, Canada. The beige clones were maintained on brain heart infusion (BHI) agar slants and used during this study. *N. asteroides* 14759 was obtained from the American Type Culture Collection (Rockville, Md.). Both gray and white clones (6) were maintained on BHI agar slants, and the white clone was used for this study.

Animals. New Zealand white rabbits weighing 5 to 6 lb (ca. 2.3 to 2.7 kg) were obtained from the B and H Rabbitry (Rockville, Md.) and used as the source of alveolar macrophages for all experiments.

Collection and maintenance of alveolar macrophages. Alveolar macrophages were obtained and cultured by modifying the procedures of Myrvik et al. (9). Rabbits were killed by injection of sodium pentobarbital into the marginal ear vein. The trachea was exposed and clamped shut, and the lungs and heart were carefully removed. A tube equipped with a two-way valve was inserted into the lower portion of the trachea, and the lungs were washed with 6 volumes (30 ml each) of saline containing the following: 0.9% (wt/vol) NaCl, 0.5 U of heparin per

ml, and 4 μg of garamycin per ml. The lavage saline was prewarmed to 37 C. The macrophage-rich fluid was pooled and centrifuged at 500 × g for 15 min at 5 C. The macrophage pellet was resuspended in cold (5 C) attachment medium, and cells were counted on a hemocytometer, using trypan blue exclusion as an indicator of cell viability. The cells were diluted to 2 × 10^6 per ml in the attachment medium, which was composed of medium 199 supplemented with 100 U of penicillin G per ml, 1 μg of fungizone per ml, 4 μg of garamycin per ml, 100 mg of L-glutamine per liter, and 0.01% (wt/vol) bovine serum albumin. One milliliter of the macrophage suspension was added to cover slips in small petri dishes (33 by 10 mm) and allowed to attach for 2 h at 37 C in 5% CO_2 in air. After attachment, the macrophages were washed, and fresh maintenance medium containing medium 199 supplemented with penicillin, fungizone, and L-glutamine, as described above, plus 20% heat-inactivated rabbit serum was added. All macrophages were maintained for 24 h before being infected with *Nocardia*. In addition, samples were cultured on BHI agar, blood agar, and Barile, Yarguchi, and Eveland agar (3) to monitor for possible contamination.

Infection of macrophages. The nocardiae were incubated in BHI broth for 4 days so that most of the cells were coccoid (stationary phase of growth). Samples of the bacterial suspension were centrifuged at approximately 100 × g for 5 min to remove clumps of bacteria. The supernatant was collected and centrifuged at 500 × g for 15 min. The pellet was resuspended in 10 ml of BHI broth, and the optical density was determined at 580 nm with a Spectronic 20 spectrophotometer (Beckman). The optical density was compared to standard curves determining colony-forming units versus optical density. A sample of the bacterial suspension was then diluted into an appropriate volume of the macrophage maintenance medium to give approximately 10^6 CFU/ml. Dilutions of this suspension were prepared, and the actual CFU per milliliter was determined. Two milliliters of the bacterial suspension in maintenance medium was added to the macrophage culture and incubated. After 3 h the bacterial suspension was removed from the macrophages, and the number of bacterial remaining in the medium was determined. The control and infected macrophages were washed twice with Hanks balanced salt solution (pH 6.5). Fresh maintenance medium was then added to all plates, and they were incubated in 5% CO_2 in air at 37 C. The medium was changed on control and infected macrophages at each sampling time period.

Viable plate counts. At specific times postinfection, triplicate cover slips containing infected macrophages were washed twice with Hanks balanced salt solution, and the macrophages were scraped from the cover slips with a rubber policeman. The number of macrophages was determined in a hemocytometer, and the CFU per macrophage was determined as previously described (3). In addition, dilutions of the maintenance medium were cultured on BHI agar to determine the number of extracellular organisms.

Light microscopy. At each time period, cover slips of uninfected control and infected macrophages were briefly rinsed with 0.5% saline, air dried, heat fixed, and either Gram stained or stained for acid fastness as previously described (1, 3). The brief wash with 0.5% saline was essential to cause the macrophages to swell, revealing the intracellular bacteria. Unwashed infected macrophages were rounded, and the bacteria could not be easily visualized. The number of bacteria within the macrophages was determined as described by Bourgeois and Beaman (3). The relative number of infected and uninfected macrophages adhering to the cover slips at each time period was determined at ×400 magnification as described by Wilder and Edberg (13). In addition, all samples were prepared for transmission and scanning electron microscopy, which will be discussed in a subsequent manuscript.

RESULTS

During the first 3 h there was a rapid uptake and killing of *N. asteroides* 10905 by cultured rabbit alveolar macrophages (Fig. 1–3).

After 24 h of incubation, 4.8 × 10^3 CFU of nocardia were recovered from 10^5 macrophages, whereas only 67 CFU were found extracellularly per ml of medium (Fig. 1). During the first 24 h after infection there was a decrease in glass-adherent cells, which indicated that the infectious process resulted in a loss of macrophage viability (Fig. 2). At this time period, about 64% of the macrophages contained gram-positive organisms, with an average of 4.3 bacteria per infected macrophage (Fig. 3).

At 3 days postinfection, very few CFU were recovered from the macrophages and no extracellular organisms were recovered from the medium (Fig. 1). The number of macrophages attached to the cover slips did not decrease significantly during this period of time (Fig. 2). Light microscopy showed that about 22% of the macrophages had gram-positive organisms, with an average of only 2.4 per infected macrophage (Fig. 3).

The data in Fig. 1 and 3 suggested that the macrophages killed 100% of the bacteria within 6 days. However, because we found that *N. asteroides* 10905 persisted within mouse peritoneal macrophages in an altered form for several days before a dramatic increase in recovery of CFU (3), we decided to continue attempts to isolate organisms from the alevolar macrophages. At 9 days postinfection, we recovered large numbers of cells of *N. asteroides* 10905 from the macrophage cultures. In addition, we successfully isolated cell wall-defective variants from these macrophages. This dramatic increase in CFU was not a result of extracellular growth, since we were able to detect only 13 organisms in the medium, whereas 1.8 × 10^4 CFU were recovered from 10^5 macrophages (Fig. 1). Acid-fast and Gram-stained prepara-

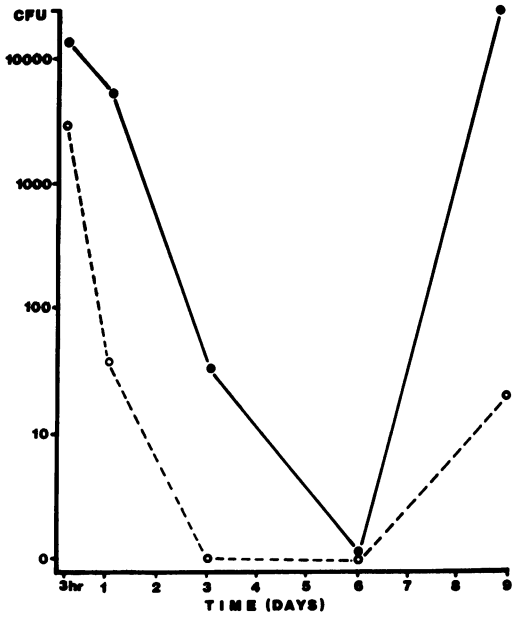

FIG. 1. *Determination of viable N. asteroides 10905 in cultured rabbit alveolar macrophages. Symbols: (●) number of CFU obtained from 10^5 macrophages; (○) number of CFU isolated from 1 ml of tissue culture medium.*

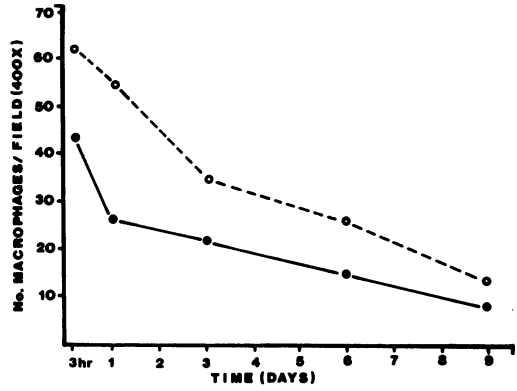

FIG. 2. *Macrophage destruction after infection with N. asteroides 10905. Symbols: (●) infected macrophages; (○) control macrophages.*

tions failed to reveal any acid-fast or gram-positive organisms (Fig. 3). Therefore, results obtained in cultured rabbit alveolar macrophages were similar to those previously reported with *N. asteroides* 10905 in cultured mouse peritoneal macrophages (3).

Since *N. asteroides* 10905 was shown to be of low virulence for laboratory animals, we wished to determine the fate of more virulent strains within macrophages. We found that *N. asteroides* 14759 was at least 100 times more invasive in mice than *N. asteroides* 10905.

Approximately 1.7×10^6 CFU of *N. asteroides* 14759 were incubated with 10^6 alveolar macrophages. After 3 h, the cells were thoroughly washed, and the macrophages containing nocardia were enumerated as described under Materials and Methods. At this time, 6.2×10^4 CFU per 10^5 macrophages were recovered (Fig. 4). The data presented in Fig. 4–6 indicated that there was uptake of *N. asteroides* 14759 cells accompanied by some killing; however, the killing was significantly less than that observed with *N. asteroides* 10905.

After 6 h of incubation, there was a 10-fold increase in organisms recovered from the macrophages (Fig. 4). This increase could not have resulted from continued phagocytosis of organisms in the medium (Fig. 4) because it was found that *N. asteroides* 14759 in the absence of macrophages did not increase significantly in numbers during a 12-h incubation period. Further, light microscopy revealed an increase in number of intracellular gram-positive organisms, without a significant increase in percentage of macrophages infected (Fig. 6).

We observed that at 6 h postinfection, many of the macrophages had migrated together to form large aggregates. Further, many of them fused to form multinucleate giant cells with as many as 10 nuclei. This macrophage aggregation and giant cell formation was not observed either in the control macrophage cultures or in the macrophages infected with *N. asteroides* 10905. These observations as well as the ultrastructural analysis of the nocardial-macrophage interaction will be the topic of a subsequent mnuscript and therefore will not be discussed here.

At 12 h postinfection, the CFU per 10^5 macrophages had decreased (Fig. 4). Thus, it seemed that the macrophages were now killing the bac-

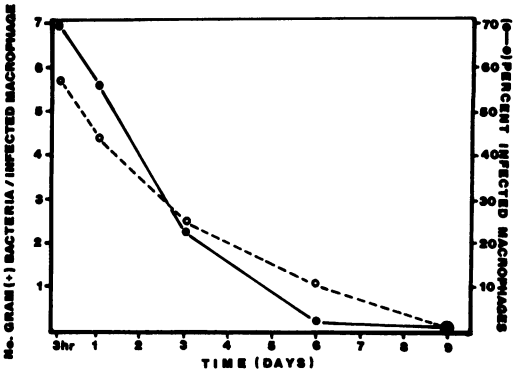

FIG. 3. *Microscope determination of gram-positive cells of N. asteroides 10905 within alveolar macrophages. Symbols: (○) number of gram-positive cells per infected macrophage; (●) percentage of macrophages containing gram-positive organisms.*

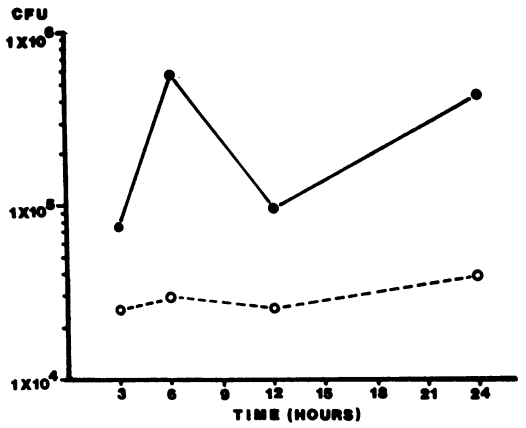

FIG. 4. *Determination of viable N. asteroides 14759 in cultured rabbit alveolar macrophages. Symbols: (●) number of CFU obtained from 10^5 macrophages; (○) number of CFU per 1 ml of tissue culture medium.*

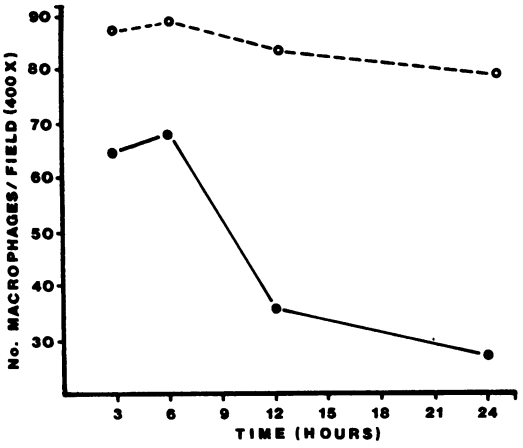

FIG. 5. *Macrophage destruction after infection with N. asteroides 14759. Symbols: (●) infected macrophages; (○) control macrophages.*

teria. However, light microscopy revealed that many of the macrophage-nocardial aggregates had floated off the cover slip, and in the medium one could find many aggregates with masses of nocardial filaments growing from them. The number of extracellular nocardia recovered from the medium did not reflect these floating masses, because each clump would only form one colony. Therefore, the number of CFU in the medium remained constant at about 3×10^4 per ml, even though one could find nocardial-macrophage masses floating in the medium. To support this view, counts of macrophages adherent to the cover slip decreased dramatically at this time period (Fig. 5). At the same time, the relative percentage of infected macrophages increased to 81%, whereas the average number of gram-positive bacteria per infected macrophage decreased to 4.4 (Fig. 6). There were still clumps of macrophages adhering to the cover slips with nocardial filaments growing out in spidery masses. These macrophage-nocardial clumps were not included in the counts shown in Fig. 6 because it was impossible to estimate the number of cells per macrophage. We believe the data at 12 h reflected a destruction of many of the macrophages by outgrowth of the intracellular nocardia, followed by rephagocytosis of some of the organisms now released into the medium. These bacteria once again grew within many of the macrophages, repeating a cycle, so that at 24 h, 4.7×10^5 CFU per 10^5 macrophages were recovered (Fig. 4 and 6). Although the experiments were carried out an additional 12 h, the data were not included here because of massive overgrowth of the macrophages by the nocardia with resultant excessive growth extracellularly.

We believe these data show that cells of *N. asteroides* 14759 grew as facultative intracellular parasites within cultured alveolar macrophages. Further, these macrophages were unable to destroy the 14759 cells as readily as they did the 10905 cells. *N. asteroides* 10905 persisted within the macrophages in an altered form, and transitional-phase variants and L-forms could be isolated after several days of incubation within the macrophage population. In contrast, transitional-phase variants or L-forms of *N. asteroides* 14759 were not recovered.

DISCUSSION

It is well established that many pathogenic organisms grow in unstimulated, nonimmune

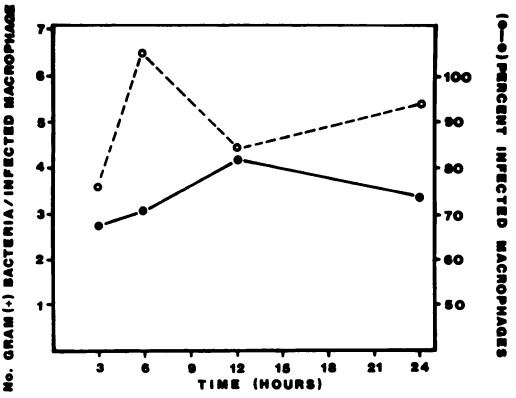

FIG. 6. *Microscopy determination of gram-positive cells of N. asteroides 14759 within alveolar macrophages. Symbols: (○) number of gram-positive cells per infected macrophage; (●) percentage of macrophages containing gram-positive organisms.*

macrophages (4, 5, 8, 11–13). It was shown that virulent strains of *Mycobacterium* and *Brucella* (5, 8, 11, 12) grew better than avirulent strains. Similar observations were reported with virulent and avirulent strains of *Listeria monocytogenes* (13). In fact, the literature is so voluminous with these kinds of studies that it is impossible to discuss them here. Many of these studies present problems in determining whether or not the organism is an intracellular parasite, or simply growing extracellularly and being constantly phagocytized from the medium. Several investigators restricted extracellular growth by adding antibiotics that inhibited the growth of the organism being studied, whereas other researchers removed the medium containing the extracellular organisms faster than the organisms grew (5, 8, 11–13). In the present study, we added penicillin and fungizone to control contamination that might occur during the lung lavage and preparation of macrophages. It was found that these compounds did not inhibit the growth of *N. asteroides* 10905 and 14749 in the culture medium. Therefore, we changed the medium every few hours to eliminate or restrict extracellular growth.

Our data indicated that cells of *N. asteroides* 10905 were rapidly phagocytized and inactivated by rabbit alveolar macrophages. It is difficult to explain how no organisms could be recovered from 10^5 macrophages or from the medium on day 6 postinfection, but at day 9 approximately 1.8×10^4 CFU per 10^5 macrophages were isolated. These were shown to be macrophage-associated, since only 13 CFU were found in the medium. Gram stains and acid-fast stains failed to reveal the presence of gram-positive or acid-fast organisms. Although the numbers were slightly different from one experimental rabbit to the next, the results were always the same.

The only explanation that might account for all of the observations is that the nocardial cells evade total destruction within the phagocytes and are capable of persisting within the macrophages in altered, gram-negative form, since gram-negative, spherical bodies were observed in many of the macrophages. Apparently, there is a short period required for a stabilization of the cell wall-defective form so that it can express itself on appropriate culture media. During this "stabilization period" neither normal nor altered cell types could be cultivated on artificial medium. At some later interval the ability to recover nocardial variants dramatically increased. The viable CFU isolated on BHI agar appeared to be "normal" nocardial cells; however, we believe these represented revertants of transitional-phase variants present within the macrophages. At the same time, L-forms and transitional forms were detected on Barile, Yarguchi, and Eveland agar medium used to grow bacterial L-forms (3).

This interpretation of our data is supported further by the work of Bourgeois and Beaman (3). They demonstrated that cells of *N. asteroides* 10905 persisted within cultured mouse peritoneal macrophages as both transitional-phase variants and L-forms (3). Further, the induction of L-forms by rabbit alveolar macrophages has been found to occur with other bacteria such as *L. monocytogenes* (10).

In contrast, cells of the more virulent strain (*N. asteroides* 14759) were phagocytized by cultured rabbit alveolar macrophages, but, as determined by light and electron microscopy and viable plate counts, most of these organisms survived the initial contact with the phagocyte. Upon continued incubation, the nocardial cells grew within the macrophage until the host cell was destroyed. Further, the cell increase within the phagocyte appeared to be more rapid than previously observed in BHI broth or in the tissue culture medium (2). No transitional-phase variants or L-forms were isolated from macrophages infected with *N. asteroides* 14759. However, there was evidence that the bacterial cells were altered within the macrophages. These observations will be presented and discussed in more detail in subsequent manuscripts.

The data presented here established that the more virulent strain of *N. asteroides* grew as a facultative intracellular parasite in cultured rabbit alveolar macrophages. Most of the less virulent *N. asteroides* 10905 cells were destroyed or greatly altered within these phagocytic cells, and some were able to persist for long periods of time in a cell wall-defective form.

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LITERATURE CITED

1. **Beaman, B. L.** 1973. An ultrastructural analysis of *Nocardia* during experimental infections in mice. Infect. Immun. 8:828–840.
2. **Beaman, B. L.** 1975. Structural and biochemical alterations of *Nocardia asteroides* cell walls during its growth cycle. J. Bacteriol. 123:1235–1253.
3. **Bourgeois, L., and B. L. Beaman.** 1974. Probable L-forms of *Nocardia asteroides* induced in cultured

mouse peritoneal macrophages. Infect. Immun. 9:576-590.
4. **Bulmer, G. S., and J. R. Tacker.** 1975. Phagocytosis of *Cryptococcus neoformans* by alveolar macrophages. Infect. Immun. 11:73-79.
5. **Freeman, B. A., D. J. Kross and R. Circo.** 1961. Host-parasite relationships in brucellosis. II. Destruction of macrophage cultures by *Brucella* of different virulence. J. Infect. Dis. 108:333-338.
6. **Gorrill, R. H., and R. H. Heptinstall.** 1954. The animal pathogenicity of *Nocardia sebivorans* nov. spec. J. Pathol. Bacteriol. 68:387-393.
7. **Green, G. M.** 1973. Lung defense mechanisms. Med. Clin. North Am. 57:547-562.
8. **Maxwell, K. W., and S. Marcus.** 1968. Phagocytosis and intracellular fate of *Mycobacterium tuberculosis:* in vitro studies with guinea pig peritoneal and alveolar mononuclear phagocytes. J. Immunol. 101:176-182.
9. **Myrvik, Q. N., E. S. Leake, and B. Farris.** 1961. Studies on pulmonary alveolar macrophages from the normal rabbit: a technique to procure them in a high state of purity. J. Immunol. 86:128-132.
10. **O'Beirne, A. J., and W. C. Eveland.** 1974. Induction of the L-phase of *Listeria monocytogenes* by rabbit alveolar macrophages in vitro. Can. J. Microbiol. 20:963-966.
11. **Patterson, R. J., and G. P. Youmans.** 1970. Multiplication of *Mycobacterium tuberculosis* within normal and "immune" mouse macrophages cultivated with and without streptomycin. Infect. Immun. 1:30-40.
12. **Solotorovsky, M., and L. S. F. Soderberg.** 1972. Host-parasite interactions with macrophages in culture, p. 77-123. *In* A. Laskin and H. Lechevalier (ed.), Macrophages and cellular immunity. CRC Press, Cleveland, Ohio.
13. **Wilder, M. S., and J. C. Edberg.** 1973. Interaction of virulent and avirulent *Listeria monocytogenes* with cultured mouse peritoneal macrophages. Infect. Immun. 7:409-415.