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Interaction of *Nocardia asteroides* with Rabbit Alveolar Macrophages: Effect of Growth Phase and Viability on Phagosome-Lysosome Fusion

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Rabbit alveolar macrophages were infected in vitro with cells of *Nocardia asteroides* GUH-2 in either logarithmic or early or late stationary phases of growth. Previous studies have established that during the growth cycle dramatic changes occur both in cell wall composition and structure and in the virulence of this organism. This study establishes the correlation between the relative virulence of the phase of growth of the infecting organisms and the degree of inhibition of macrophage phagosome-lysosome fusion. The occurrence of phagosome-lysosome fusion in infected macrophages was determined by both fluorescent and electron microscopy. It was found that relatively few phagosomes containing the highly virulent log-phase organisms had any evidence of lysosomal fusion; more of the phagosomes containing early stationary-phase cells had evidence of fusion. The greatest amount of phagosome-lysosome fusion was observed with the least virulent late stationary-phase cells. Electron microscopic evaluation of infected macrophages indicated that this increase in fusion was not associated with an increase in cell damage. Comparison of macrophages infected with either viable or nonviable organisms indicated that loss of viability did not decrease inhibition of fusion by early or late stationary-phase cells. In contrast, loss of viability did decrease inhibition of fusion by log-phase cells.

Typically, morphological changes occur during the growth cycle of the nocardiae. Filamentous forms which are characteristic of logarithmic-phase cells fragment during stationary phase, and the resultant rods and cocci repeat the cycle by growing into filamentous forms when they enter log phase. Previous ultrastructural analysis of cells of *Nocardia asteroides* during lag, log, and stationary phase indicated that significant structural modification occurred, especially in the cell envelope. In addition, chemical analysis indicated corresponding changes in the relative amounts of peptidoglycan, lipid, and fatty acid components present in the cell wall, thus establishing that the structure and composition of the cell wall of *N. asteroides* change dramatically during the growth cycle (3).

Subsequent studies established that the structural and chemical changes which occur in the cell wall during the growth cycle greatly affect the virulence of *N. asteroides* for mice (6) and rabbit alveolar macrophages infected in vitro (4). In mice, greater than 1,000 times more cells at stationary phase were necessary to achieve the same mortality induced by log-phase cells. Organisms in either the lag phase or early stationary phase of growth were intermediate in the number of cells required (6).

Several possible reasons for this enhanced virulence of log-phase cells were suggested by the study of macrophages infected in vitro. This study showed that log-phase cells were more resistant to phagocytosis than stationary-phase cells, and that even after ingestion, the log-phase cells were more toxic and grew more rapidly. An additional possibility was suggested by our observations on nocardial inhibition of macrophage phagosome-lysosome fusion (9). This recent study established the association of the relative virulence of three strains of *N. asteroides* with their resistance to killing by macrophages, maintenance of ultrastructural integrity, and inhibition of phagosome-lysosome fusion.

In the current study, we have further analyzed the association of virulence, viability, and inhibition of macrophage phagosome-lysosome fusion by assessing the interaction of rabbit alveolar macrophages with cells of *N. asteroides* that differ in their phase of growth.

MATERIALS AND METHODS

Bacteria. *N. asteroides* GUH-2 was isolated from a fatal human infection and maintained as previously described (5). Its growth cycle and pathogenicity for mice have been described (5, 6). Single-cell suspensions of the organisms were prepared from growing cultures in brain heart infusion broth (Difco Labora-

tories, Detroit, Mich.) at logarithmic phase (16 h), at early stationary phase (3 days), and at late stationary phase (1 week) as previously described (3, 6). In experiments using nonviable organisms, the cells were killed by exposure to 4% formaldehyde for 1 h and were subsequently washed thoroughly before incubation with macrophages.

Collection, maintenance, and infection of alveolar macrophages. Unelicited alveolar macrophages were lavaged from normal female New Zealand White rabbits (Herbert's Rabbitry, Plymouth, Calif.) by a modification of the technique of Myrvik et al. (18) and maintained as previously described (7). After a 3-h attachment to sterile cover slips, the macrophages were washed and subsequently infected as previously described (4, 7).

For these studies comparable numbers of organisms at each phase of growth were used to infect the rabbit alveolar macrophages for 3 h. In vitro infection of alveolar macrophages from a single rabbit per experiment permitted us to utilize several methods concurrently to assess the relative interaction of the nocardial cells at different phases of growth.

Dark-field fluorescent microscopy. After attachment to sterile cover slips, the macrophages were rinsed and incubated for 10 min with the lysosomotropic fluorochrome acridine orange at a final concentration of 5 μg/ml in Hanks balanced salt solution at 37°C (15). The macrophages were then rinsed thoroughly, infected for 3 h, and processed as previously described (9). Macrophage phagosomes with no fusion of lysosomes were not labeled and thus appeared dark, but those in which fusion of lysosomes had occurred were labeled a brilliant orange and thus were easily identifiable. Random blind fields were selected, and both the total number of infected macrophages and the number of macrophages with acridine orange-labeled phagosomes were counted and tabulated.

Electron microscopy. (i) Acid phosphatase. After 3 h of infection, the macrophages were rinsed, and the lysosomes were labeled with lead phosphate by an adaptation of the technique of Gomori et al. (14) and processed as previously described (8).

(ii) Horseradish peroxidase. After attachment, the macrophages were rinsed and incubated with 5 mg of horseradish peroxidase (type II; Sigma Chemical Co., St. Louis, Mo.) per ml for 2.5 h at 37°C in medium 199 (GIBCO, Grand Island, N.Y.) containing 40% fetal calf serum (19). They were then rinsed thoroughly and incubated in medium without peroxidase for 0.5 h. Subsequently the labeled macrophages were infected for 3 h, rinsed, and processed by the technique of Graham and Karnovsky (12) with the exception that a pH 6.0 buffer was used instead of pH 7.6 (2). The macrophages were then postfixed overnight in 1.0% osmium tetroxide and processed as previously described (8).

(iii) Assessment of damage. The organisms were considered to be damaged if there was any appearance of abnormality such as breaks in cell wall or membrane, disorganization of cytoplasm, or disorganization of the nuclear region.

(iv) Assessment of fusion. Both of these electron microscopic techniques labeled the macrophage lysosomes with an electron-dense substance; thus lysosomal fusion was identified by the presence of the dark electron-dense label within the phago-lysosome.

(v) Quantitation of fusion. To statistically assess phagosome-lysosome fusion and morphological damage to the organisms, we examined 50 infected macrophage profiles for each of the phases of growth in each experiment. The structural integrity of the ingested nocardiae was evaluated, and the occurrence or absence of fusion was recorded. The total number of profiles of phagocytized nocardiae evaluated was between 900 and 1,200 for each phase of growth. In all experiments care was taken to avoid serial sections.

RESULTS

Fluorescent microscopic observation of acridine orange-labeled macrophages indicated that after a 3-h infection with the highly virulent log-phase cells, only 12% of the infected macrophages had any evidence of lysosomal fusion, whereas 23% of the macrophages infected with the early stationary-phase cells had evidence of fusion, and 42% of those infected with the least virulent late stationary-phase cells had evidence of fusion. Electron microscopic observation further substantiated the association of phase of growth with inhibition of macrophage phagosome-lysosome fusion. Analysis of infected macrophages labeled for lysosomal acid phosphatase activity indicated that 21% of the phagosomes containing the log-phase organisms had evidence of fusion, whereas 34% of the phagosomes containing early stationary-phase cells showed signs of fusion. In contrast, 50% of the phagosomes containing late stationary-phase cells had evidence of fusion (Table 1).

Results from the electron microscopic evaluation of cell damage of phagocytized nocardiae at log, early stationary, or late stationary phase are presented in Fig. 1A. For phagocytized log- and early stationary-phase cells there appeared to be a general trend of increasing cell damage with increasing cell age. However, these differences were within the range of experimental error. The total percentage of phagocytized late stationary-phase cells with evidence of ultrastructural damage was higher than that of the log- or early stationary-phase cells, with the most notable difference occurring in the percentage of damaged cells showing no evidence of

TABLE 1. *Incidence of phagosome-lysosome fusion*

Phase of growth	% of infected macrophages with fusion[a]	% of phagocytized nocardiae with fusion[a]
Log	12.3 ± 3	21.3 ± 2
Early stationary	23.3 ± 3	33.7 ± 3
Late stationary	41.5 ± 9	49.5 ± 8

[a] ± Standard deviation.

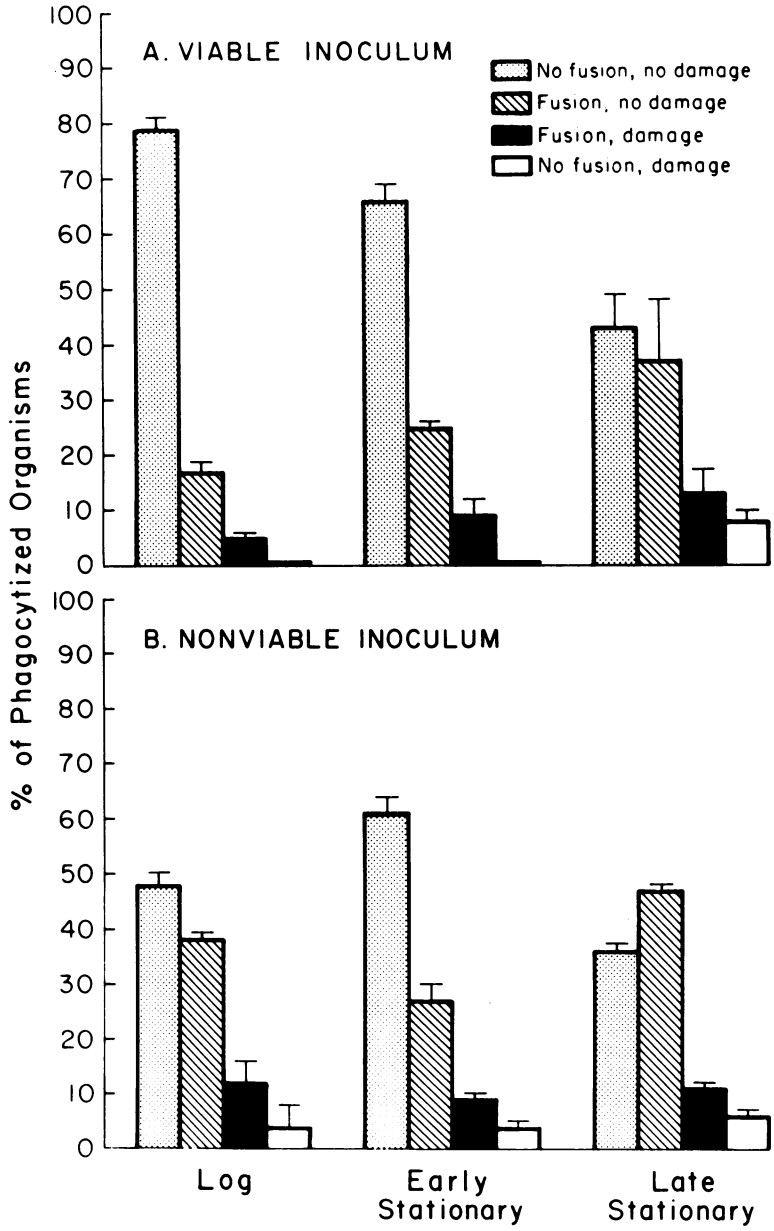

FIG. 1. *Incidence of phagosome-lysosome fusion and nocardial cell damage with viable and nonviable inocula at log, early stationary and late stationary phase. Error bars indicate the standard deviation. The total number of profiles of phagocytized nocardiae evaluated was between 900 and 1,200 for each phase of growth.*

fusion. This lack of fusion in obviously damaged and presumably nonviable cells suggested that perhaps viability was not necessary for inhibition of phagosome-lysosome fusion.

The data from fluorescent microscopic observation and quantitation of acridine orange-labeled macrophages infected with viable or formaldehyde-killed organisms at various stages of growth are presented in Fig. 2. The loss of viability of late and early stationary-phase cells had essentially no effect on the ability of these organisms to inhibit macrophage phagosome-lysosome fusion. In contrast, loss of ability to inhibit fusion was noted when the log-phase cells

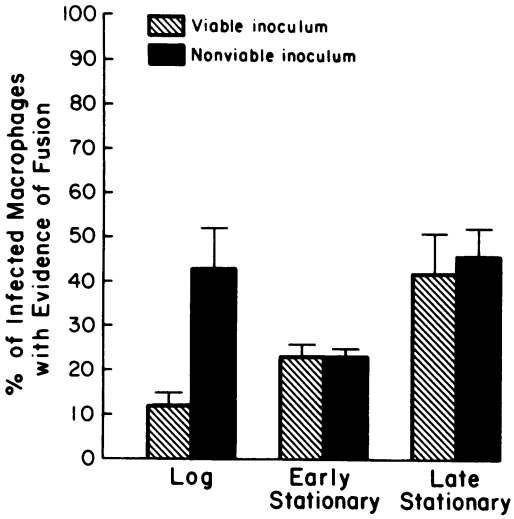

FIG. 2. *Effect of inoculum viability on incidence of phagosome-lysosome fusion at log, early stationary, and late stationary phase. Error bars indicate the standard deviation. An average of 800 infected macrophages was evaluated for each phase of growth.*

were killed by treatment with formaldehyde (Fig. 2).

The data from electron microscopic observation and quantitation correlated well with the fluorescent microscopic data. There was only a slight increase in the total percentage of phagocytized nocardiae with evidence of fusion in the macrophages exposed to the nonviable inoculum of late stationary-phase cells, and no apparent change in macrophages inoculated with the early stationary-phase organisms. However, there was a large increase in the total percentage of nocardia-containing phagosomes, with evidence of fusion in macrophages that had ingested the nonviable log-phase organisms (compare Fig. 1A and 1B).

Ultrastructural observation of macrophages infected with nonviable cells at either early stationary or late stationary phase indicated that there was no change in the incidence of damage after 3 h of infection. However, there was a slight increase in the incidence of damaged log-phase cells (compare Fig. 1A and B).

DISCUSSION

The association of the relative virulence of three strains of *N. asteroides* with their resistance to killing by macrophages, maintenance of ultrastructural integrity, and inhibition of macrophage phagosome-lysosome fusion has been previously established (9). Of the three strains evaluated, *N. asteroides* GUH-2 was the most virulent for mice and was the most resistant to killing by macrophages. In addition, this virulent strain had the least amount of cell damage after 3 h of incubation and induced the greatest amount of inhibition of phagosome-lysosome fusion. Previous reports on *N. asteroides* GUH-2 have established that the structural and chemical changes that occur in the cell wall during the growth cycle greatly affect the virulence of this organism. Results of these previous studies indicated that the most virulent phase of growth of *N. asteroides* GUH-2 is the logarithmic phase (6).

Whereas our previous study indicated a correlation between the degree of inhibition of macrophage phagosome-lysosome fusion and the relative virulence of the infecting strain (9), the results presented in this report show a further correlation between the degree of inhibition of fusion and the relative virulence associated with the phase of growth of the infecting organism. Thus, the greatest inhibition of fusion occurred when macrophages were infected with the more virulent log-phase cells of *N. asteroides* GUH-2. This inhibition of fusion was apparent both by fluorescent microscopic observation of acridine orange-labeled infected macrophages and by electron microscopic observation and quantitation of phagocytized nocardiae. Previous reports on inhibition of macrophage phagosome-lysosome fusion suggest that this ability may be important for intracellular survival of some pathogens (1, 9–11, 16, 17, 20). The results of this current study provide additional evidence that inhibition of macrophage phagosome-lysosome fusion may be one of the mechanisms of nocardial pathogenesis.

Although our previous study with three strains of *N. asteroides* of different virulence indicated a correlation between the degree of inhibition of fusion and resistance to damage after ingestion by macrophages, this correlation was not evident in this study involving the phase of growth. The results presented in Fig. 1A indicate only a slight suggestion of increasing cell damage with increasing cell age, whereas the correlation of decreasing inhibition of fusion with increasing cell age is very evident. The degree of lack of damage to the log-phase cells was especially surprising, since previous work has shown that 15% of cells at this phase of growth are killed after a 3-h infection of macrophages (4). This observation serves to emphasize that ultrastructural evaluation alone is not sufficient to evaluate the viability of these organisms, since seemingly intact organisms may be nonviable.

A small, but notable, increase in the percent-

age of damaged cells with no indication of fusion was evident in macrophages that had phagocytized late stationary-phase cells. It is suggested that this increase in obviously damaged and presumably nonviable cells may be due to the natural loss of viability associated with autolysis in maturing cell populations. This increase is probably not due to macrophage activity, since previous work has shown that after a 3-h incubation with macrophages only 3% of late stationary-phase cells were not recovered (4). Thus these damaged cells may represent nonviable cells present in the initial inoculum.

The results presented in Fig. 2 indicate that formaldehyde-killed cells of *N. asteroides* GUH-2 at early or late stationary phase are just as effective in inhibition of fusion as are viable ones. However, a previous report by Armstrong and Hart indicated that whereas viable *Mycobacterium tuberculosis* organisms inhibit macrophage phagosome-lysosome fusion, nonviable organisms do not (1). It is possible that this apparent difference may be reconciled by the observation that Armstrong and Hart made their evaluations of infected macrophages 4 days after infection, whereas the rapid growth of *N. asteroides* made it necessary that our evaluations be made 3 h after infection.

The data presented in Fig. 1 indicate that except for a small increase observed with nonviable log-phase cells, loss of viability did not affect the incidence of damaged cells observed by electron microscopy. These results are entirely consistent with the data presented on lack of lysosomal fusion with phagosomes containing nonviable cells, since, aside from autolysis, exposure to the hydrolytic lysosomal contents is most likely necessary to increase the incidence of damaged intraphagosomal cells.

In contrast to the results obtained with stationary-phase cells, evaluation of macrophages infected with the log-phase cells indicated that loss of inoculum viability greatly decreased inhibition of phagosome-lysosome fusion. This loss of inhibition was apparent both in the percentage of macrophages with evidence of fusion as measured by fluorescent microscopy (Fig. 2) and in the percentage of phagosomes with evidence of fusion as measured by electron microscopy (compare Fig. 1A and B). These results may not indicate that actual viability is important in inhibition of fusion by cells at this phase of growth, since complete loss of viability did not abrogate inhibition of fusion but instead reduced it. A more likely alternative, based on previous studies on structural biochemistry of the nocardial cell wall (3), is that the nocardial component responsible for inhibition of fusion is more exposed or more labile during the log phase of growth and thus is more susceptible to inactivation during the killing process. The role of cell surface components in inhibition of macrophage phagosome-lysosome fusion has been suggested by previous studies with *M. tuberculosis*. Goren et al. demonstrated that anionic sulfolipids isolated from the cell walls of virulent *M. tuberculosis* inhibit fusion in mouse peritoneal macrophages (11). It may be that the cell walls of virulent *N. asteroides* possess similar compounds. Studies are now in progress to further analyze the nocardial cell wall and to assess its role in pathogenicity.

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