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Intracellular Acid Phosphatase Content and Ability of Different Macrophage Populations to Kill *Nocardia asteroides*

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It has been reported that the activity of lysosomal acid phosphatase decreases inversely with numbers of ingested virulent *Nocardia* spp. in normal murine peritoneal and alveolar macrophages. These studies suggested that this relationship correlated with the effectiveness of these macrophage populations in killing *Nocardia asteroides*. Experiments were designed to determine if acid phosphatase activity is affected by infection with *N. asteroides* in four different macrophage populations isolated from normal and nocardia-immunized mice. Macrophages were also tested simultaneously for their ability to kill *N. asteroides*. Peritoneal, alveolar, and splenic macrophages and Kupffer cells were infected in vitro with strains of *N. asteroides* of differing virulence. Uptake and killing assays were performed. Acid phosphatase levels and numbers of intracellular nocardiae were quantitated in the same macrophages, using a computer-assisted cytophotometry system. Acid phosphatase activity decreased inversely with numbers of intracellular nocardiae in macrophages that could not kill or inhibit this pathogen. Acid phosphatase activity was not significantly changed in macrophages that inhibited growth of, but did not kill, *N. asteroides*, whereas activity was increased or enhanced in macrophages that killed most of the ingested nocardiae. The order of nocardicidal effectiveness (and resistance to enzyme activity reduction with infection) for normal macrophages was splenic > peritoneal > alveolar > Kupffer. In contrast, the order of these two parameters for macrophages isolated from immunized mice was Kupffer > peritoneal > alveolar > splenic. These results demonstrate that lysosomal acid phosphatase activity is an effective marker of the ability of macrophages to inhibit growth of and kill *N. asteroides* and that macrophages isolated from different anatomical sites differ functionally from each other with respect to nocardicidal and acid phosphatase activities.

Nocardia asteroides is now recognized as an important pathogen of normal and immunocompromised individuals (2, 22, 26). Nocardial infections result from ineffective elimination of this intracellular pathogen by phagocytes such as macrophages (5, 8). The interactions of macrophages with nocardiae vary with both the virulence of the strain of *N. asteroides* and the anatomical source and immune status of the macrophage population (1, 9, 12, 13). For example, the virulent strain *N. asteroides* GUH-2 inhibits phagosome-lysosome fusion and is resistant to intracellular killing by normal macrophages, whereas the less virulent strain *N. asteroides* 10905 does not inhibit fusion and is killed within these same macrophages (1, 9, 12, 13).

Few reports exist that include comparisons of macrophage populations isolated simultaneously from several different anatomical regions. Since macrophages resident in different sites differ in both phagocytic and microbicidal capabilities (25), and since nocardiosis can be a systemic disease, the importance of studying the interactions between nocardiae and different macrophage populations becomes evident.

We have previously demonstrated a relationship between the virulence of strains of *N. asteroides* and the extent of reductions in lysosomal acid phosphatase activity of alveolar and peritoneal macrophages obtained from normal mice (9). Although acid phosphatase is not known to be directly involved in bactericidal activity, it was used as a marker of the interaction between ingested bacteria and macrophage lysosomal enzyme systems. The present study was designed to determine if the demonstrated relationship between enzyme activity reductions and virulence correlates directly with the effectiveness of nocardicidal activity of macrophages isolated from different anatomical sites in normal and immunized mice. Thus, the acid phosphatase activity in alveolar, peritoneal, splenic, and hepatic macrophages was determined simultaneously with the ability of these macrophages to kill *N. asteroides*. Whereas the nocardicidal capability of the macrophages was determined by standard killing assays, both acid phosphatase activity and intracellular nocardiae were quantitated within individual macrophages by using a computer-assisted cytospectrophotometer. These methods permitted further assessment of nocardiae-macrophage interactions by direct comparison of acid phosphatase activity with degree of infection and nocardicidal effectiveness in the same macrophages.

MATERIALS AND METHODS

Microorganisms. *N. asteroides* strain GUH-2 was a clinical isolate from a patient with disseminated nocardiosis at Georgetown University Hospital, Washington, D.C. *N. asteroides* strain 10905 was furnished originally by J. Rozanis (University of Western Ontario, London, Ont., Canada). The virulence of these strains for mice has been characterized: strain GUH-2 is highly virulent and strain 10905 is of low virulence (6). Both strains were grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) at 37°C on a rotary shaker as previously described (7, 10).

Cultures were harvested at the early stationary phase of growth during which the bacterial cells were typically in the coccobacillary form. Single-cell suspensions were made by glass-wool column filtration and centrifugation as previously described (9). Concentrations of bacteria were determined by direct cell count, using a Neubauer hemacytometer, and

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then diluted to the inoculum dose in medium 199 plus 12% fetal calf serum (M199 + 12% FCS) (GIBCO Laboratories, Grand Island, N.Y.). The actual numbers of viable nocardiae in the inocula were determined by plating dilutions onto agar.

Killed cultures were prepared in 4% Formalin at 4°C and then treated similarly to the live cultures as described above and previously (9).

Immunization of mice. Female BALB/c mice (Bantin and Kingman, Fremont, Calif.) were immunized with 4% Formalin-killed early stationary-phase cells of *N. asteroides* GUH-2. Each mouse received 18 mg of cells (0.2 ml) in incomplete Freund adjuvant subcutaneously and then at 2-week intervals received two footpad boosts of 5 mg of cells (0.05 ml) and one intraperitoneal boost of 10 mg of cells (0.1 ml) in sterile saline. Immunization of mice was verified by solid-phase radioimmunoassay measurement of an antibody titer at least fourfold greater than that observed for unimmunized mice.

Isolation, maintenance, and infection of macrophages. Macrophages were obtained from the peritoneal cavities, lungs, spleens, and livers of normal and immunized female BALB/c mice. Peritoneal and alveolar macrophages were collected by lavaging the unstimulated peritoneum and the lungs of the same mice, using previously described methods (9). Splenic macrophages were obtained by forcing the removed spleens of these mice through a stainless-steel wire grid with a sterile syringe barrel and 3 ml of M199 + 12% FCS. The resulting suspension was filtered through a loosely packed glass-wool column and then diluted 10-fold with 0.16 M ammonium chloride in Tris buffer (pH 7.2) and incubated for 5 min at 37°C to lyse erythrocytes. The cell suspension was then centrifuged at 200 $\times$ *g* for 5 min, washed three times in Hanks balanced salt solution (HBSS) (GIBCO Laboratories) and suspended to 10^7 cells per ml in HBSS with 5% FCS. Lymphocytes were removed from the cell population by a panning technique (19, 31). This procedure uses monoclonal antibodies directed against immunoglobulins to remove B-lymphocytes in the first step and then an indirect (sandwich) technique in the second step, using an antibody directed against a T-lymphocyte surface antigen to remove T-lymphocytes. The remaining macrophage-rich cell population was then suspended in M199 + 12% FCS.

Kupffer cells were isolated from the liver by a modification of the procedure of Garvey and Caperna (15). Livers were removed from the mice, minced with sterile scissors, and incubated in 0.05% collagenase type IV (Sigma Chemical Co., St. Louis, Mo.) at 37°C for 30 min with shaking. The suspension was centrifuged at 300 $\times$ *g* for 5 min and suspended in HBSS. These cells were then centrifuged at 10 $\times$ *g* for 3 min. The supernatant was centrifuged at 300 $\times$ *g* for 5 min and suspended in 5 ml of Gey balanced salt solution (GIBCO Laboratories). This suspension was added to 7 ml of filter-sterilized 30% metrizamide (wt/vol) (Sigma Chemical Co.) in Gey balanced salt solution, mixed, and overlaid with 0.5 ml of Gey balanced salt solution. This gradient was centrifuged for 15 min at 1,400 $\times$ *g* and 20°C. Cells were collected from the gradient interface, washed three times with HBSS, and suspended in M199 + 12% FCS.

For each type of cell preparation described, the number of macrophages present was determined by a nonspecific esterase stain. Macrophages were maintained at 37°C in a 5% CO_2-humidified atmosphere as previously described (8).

Macrophage suspensions were allowed to adhere for 1 h in culture chamber wells on glass slides as previously described (9). All macrophage suspensions were used at the same approximate concentration. The expected differences in efficiency of adherence between macrophage populations resulted in only minor variations in cell density on the slides which did not interfere with subsequent functional measurements on the cells. After adherence, macrophages were washed and infected at a multiplicity of 5 nocardiae per macrophage by incubating them for 1 h with suspensions of live *N. asteroides* strain GUH-2, 4% Formalin-killed strain GUH-2, or live *N. asteroides* strain 10905. Thus, in each well, 5×10^4 macrophages were infected with 2.5×10^5 nocardiae. Uninfected control macrophages were incubated in M199 + 12% FCS alone for the 1-h period. After 1 h, the macrophages were washed to remove extracellular nocardiae and then incubated for an additional 2 h in M199 + 12% FCS to achieve a total infection time of 3 h. Under these conditions, the unopsonized nocardiae were quickly phagocytized by macrophages. It has been verified by electron microscopy that all of the nocardiae present in this system after the 3-h infection period are intracellular (10, 12, 13).

Nocardicidal assays. Macrophages were allowed to adhere to microtiter wells at concentrations of 2×10^5 per well in a 100-μl volume for 1 h. Each well was washed two times with 0.1 ml of HBSS. Single-cell suspensions of either *N. asteroides* strain GUH-2 or *N. asteroides* strain 10905 were added to each well at a multiplicity of 5 nocardiae per macrophage (10^6 nocardiae per well) and incubated for 1 h. The supernatants were then collected and the macrophages were washed two times with HBSS. The washings were added to the supernatant from three of the wells (triplicate wells per treatment group), diluted, and plated on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.). The macrophages in these same three wells were lysed by treatment with 0.1 ml of sterile distilled water at 37°C and then diluted and plated on Trypticase soy agar. Thus, at 1 h after infection, viable counts were obtained for both the extracellular nocardiae present in the well supernatants and ingested nocardiae present within the macrophages. The remaining wells were incubated in M199 + 12% FCS, and the viable nocardiae were measured at 3 and 7 h postinfection in a similar manner. After determination of the numbers of nocardiae in the inocula by plate counts, the percent recovery of the inoculum at 1 h was determined by dividing the sum of the supernatant and macrophage-associated organisms by the total number of nocardiae added to the well. The percent phagocytosis was calculated by dividing the macrophage-associated organisms by the sum of the supernatant and macrophage-associated organisms. Killing was defined as the difference between the number of organisms recovered from the wells infected for 1 h and the number of organisms recovered from subsequently sampled wells.

Histochemical determination of acid phosphatase activity. Activity of acid phosphatase within macrophages was detected by a substrate-staining procedure as described before (9, 20). This procedure used hexazotized pararosanilin as the coupling agent for the hydrolyzed substrate to form an insoluble red dye complex at the site of enzyme activity. After fixation for 30 min in 4% paraformaldehyde–1% $CaCl_2$ at 4°C, the macrophages were incubated in the enzyme-staining solution for 1.5 h at room temperature. The slides were then washed, air dried, and mounted under cover slips, using a water-based mounting medium (Aquamount; Lerner Laboratories, Stamford, Conn.). Acid phosphatase activity and cell area were measured quantitatively in 100 to 200 randomly chosen macrophages for each of the 32 different treatment groups (four infection groups $\times$ four macrophage populations $\times$ normal and immunized murine sources).

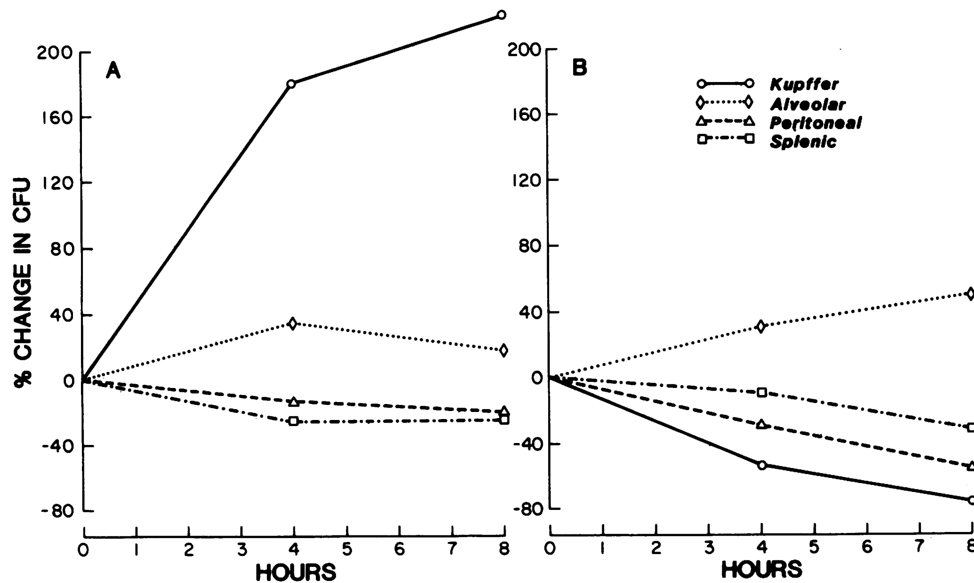

FIG. 1. Effect of four different macrophage populations on viability of virulent *N. asteroides* GUH-2 after phagocytosis. Killing is represented as reductions of the initial macrophage-associated count (expressed as zero on the y axis); intracellular proliferation is represented as positive values. (A) Macrophages isolated from normal mice. (B) Macrophages isolated from mice immunized against *N. asteroides* GUH-2.

Enzyme quantitation within individual macrophages was performed by using a computer-assisted cytospectrophotometry system consisting of a microscope equipped with a photometer and scanning stage connected online with a computer as described previously (9, 16). By means of the HIDACSYS programs for interactive scanning cytophotometry, the background-corrected optical densities of stained individual cells were determined (28, 29). The resultant optical density of the macrophage is proportional to the amount of chromophore present and is thus a measure of cellular enzyme activity.

Quantitation of intracellular nocardiae. After intracellular acid phosphatase activity was quantitated, cover slips were removed from the slides by soaking in phosphate-buffered saline. The macrophages were then stained by the Brown and Brenn method of the Gram stain to visualize intracellular nocardiae (21). Individual macrophages previously measured for enzyme activity were then relocated under computer control. With this method, 93.0 to 99.2% of each macrophage population scanned for enzyme levels was successfully relocated by the computer. For each relocated macrophage, the number of intracellular nocardiae was recorded and compared with the integrated enzyme optical density value of that cell. This comparison enabled the direct determination of the relationship between the degree of infection of the macrophage to the amount of acid phosphatase activity present. The results presented are based on a total evaluation of approximately 3,500 macrophages.

The results were categorized into four groups based upon increasing infectivities. These groups consisted of (i) macrophages exposed to but uninfected by nocardiae, (ii) macrophages containing one or two nocardiae, (iii) macrophages containing three or four nocardiae, and (iv) macrophages containing five or more nocardiae. Statistical analyses of the results were performed by using the Student's *t* test and analysis of variance.

Incubation of macrophages with nocardial culture supernatants. In an attempt to characterize any differences in acid phosphatase activity between unexposed control macrophages and macrophages exposed to but uninfected by nocardiae, a separate experiment was performed. Cultures of *N. asteroides* strain GUH-2 were grown as described above. Upon reaching the early stationary phase of growth, the cultures were centrifuged at 100 × *g* for 5 min, and the supernatants were filter sterilized with 0.45-μm membrane filters (Millipore Corp., Bedford, Mass.). The sterile culture supernatants were diluted in M199 + 12% FCS, using dilutions corresponding to the appropriate inoculum dose based on direct hemacytometer counts before centrifugation of the cultures. Peritoneal macrophages collected, adhered, and maintained as described above were incubated with the culture supernatant suspensions for 3 h. Control macrophages were incubated with dilutions of brain heart infusion broth in M199 + 12% FCS, with M199 + 12% FCS alone, or with M199 + 12% FCS-diluted suspensions of the nocardiae prepared as described above at multiplicities of 10 nocardiae per macrophage. In addition, one group of control macrophages was incubated with the filter-sterilized supernatant collected from a sample of macrophages preinfected for 3 h with live *N. asteroides* strain GUH-2.

After 3 h, macrophages were washed and fixed as described above. Acid phosphatase activity and numbers of intracellular nocardiae (where applicable) were quantitated and the results were analyzed as described previously.

RESULTS

Although the macrophage populations were exposed to approximately equal numbers of nocardiae at the same multiplicities of infection, larger numbers of macrophages ingested the less virulent nocardiae (killed strain GUH-2 and live strain 10905) than macrophages that ingested the more virulent live strain GUH-2. In all macrophage populations, the number of ingested nocardiae per macrophage was lowest for macrophages containing live cells of the virulent strain GUH-2. These observations are in agreement with our previous report that live cells of the virulent strain GUH-2 are more resistant to phagocytosis by murine macrophages

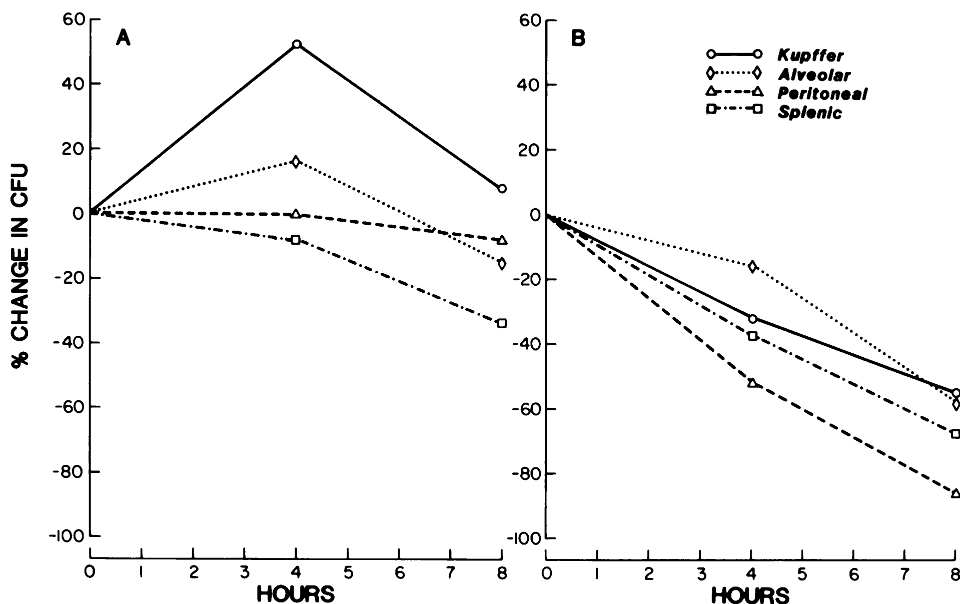

FIG. 2. Effect of four different macrophage populations on viability of *N. asteroides* 10905 after phagocytosis. Killing is represented as reductions of the initial macrophage-associated count (expressed as zero on the y axis); intracellular proliferation is represented as positive values. (A) Macrophages isolated from normal mice. (B) Macrophages isolated from mice immunized against *N. asteroides* GUH-2.

than are either killed cells of this strain or live cells of the less virulent strain 10905 (9).

Phagocytic capacity as measured by uptake of nocardiae from the inoculating suspension was greater for macrophages isolated from immunized mice than for normal macrophages in all populations. The order of phagocytic capacity of the macrophage populations studied was peritoneal > alveolar > Kupffer > splenic.

The results of the nocardicial assays were plotted as shown in Fig. 1 and 2 as percent change in CFU per time in hours. None of the four different macrophage populations isolated from normal mice and incubated with virulent strain GUH-2 was able to kill strain GUH-2 effectively (Fig. 1A). The greatest reduction in counts was only 27% less than the initial macrophage-associated counts observed for the normal splenic macrophages after 8 h of incubation. The least effective of the normal macrophage populations against virulent strain GUH-2 were the Kupffer cells: CFU increased 225% over the initial level in 8 h. Cells of strain GUH-2 also proliferated within the normal alveolar macrophages. The viable counts of strain GUH-2 within the normal peritoneal macrophages remained approximately the same during 8 h; thus, this population was unable to kill strain GUH-2 but appeared to successfully retard an increase in CFU.

The macrophage populations isolated from immunized mice were more effective than the normal macrophages in inhibiting strain GUH-2 (Fig. 1B). The most dramatic difference in behavior occurred with the Kupffer cells. Thus, Kupffer cells from normal mice were unable to retard the growth of strain GUH-2, whereas Kupffer cells from immunized mice effectively reduced the viable counts by >80% after 8 h. The peritoneal macrophages from immunized mice achieved a >50% reduction in counts from initial level, whereas the splenic and alveolar macrophages from immunized mice proved less effective against strain GUH-2. The

TABLE 1. Acid phosphatase activities (expressed as total optical density) in murine macrophage populations containing ingested cells of *N. asteroides* strains of differing virulence

Infecting strain	Enzyme activity, mean ± SE (no. of macrophages)[a]							
	Normal mice				Immunized mice			
	Alveolar	Peritoneal	Splenic	Kupffer	Alveolar[b]	Peritoneal[b]	Splenic	Kupffer
GUH-2								
Live	11,840 ± 586 (55)[c]	4,329 ± 205 (57)[c]	3,457 ± 185 (94)[c]	5,392 ± 496 (91)[c]	3,776 ± 176 (78)[c]	3,719 ± 243 (99)[c]	3,681 ± 220 (124)[c]	5,277 ± 353 (98)[c]
Killed	14,360 ± 803 (52)	7,880 ± 556 (55)	4,224 ± 319 (54)	8,036 ± 814 (72)	7,115 ± 381 (78)	ND[d]	4,715 ± 340 (71)	8,394 ± 766 (66)
10905 (live)	13,610 ± 830 (52)	8,183 ± 458 (42)	4,031 ± 331 (41)	8,663 ± 859 (72)	5,942 ± 274 (68)	4,562 ± 417 (74)	5,059 ± 525 (78)	10,550 ± 863 (81)
None (control)	18,840 ± 561 (99)	11,000 ± 333 (99)	6,495 ± 419 (99)	6,596 ± 760 (99)	6,107 ± 254 (99)	5,470 ± 395 (99)	4,452 ± 272 (99)	9,989 ± 656 (99)

[a] Units were calculated as the sum of optical density measurements at 0.5-μm intervals. Numbers of macrophages shown vary because they do not include the portion of the population which did not ingest nocardiae.
[b] Enzyme staining performed under slightly different conditions. Comparisons internal only.
[c] $P < 0.05$ as compared with control and other infecting strains.
[d] ND, Not determined.

splenic macrophages inhibited its growth, whereas cells of strain GUH-2 proliferated within the alveolar macrophages.

The less virulent strain 10905 was killed more efficiently by macrophages than was strain GUH-2 (Fig. 2). Although the CFU initially increased with time in normal Kupffer cells, a significant reduction in viable counts occurred during the 4- to 8-h time period (Fig. 2A). The normal alveolar, peritoneal, and splenic macrophage populations successfully inhibited the growth of strain 10905, but failed to reduce viable counts by more than 30% below initial levels. In no case, however, did cells of strain 10905 notably proliferate within macrophages after 8 h, as was observed with strain GUH-2. All four macrophage populations isolated from immunized mice were highly effective in killing strain 10905 (Fig. 2B).

Levels of acid phosphatase activity in the differently treated populations of macrophages are compared in Table 1. The enzyme activities shown represent mean population

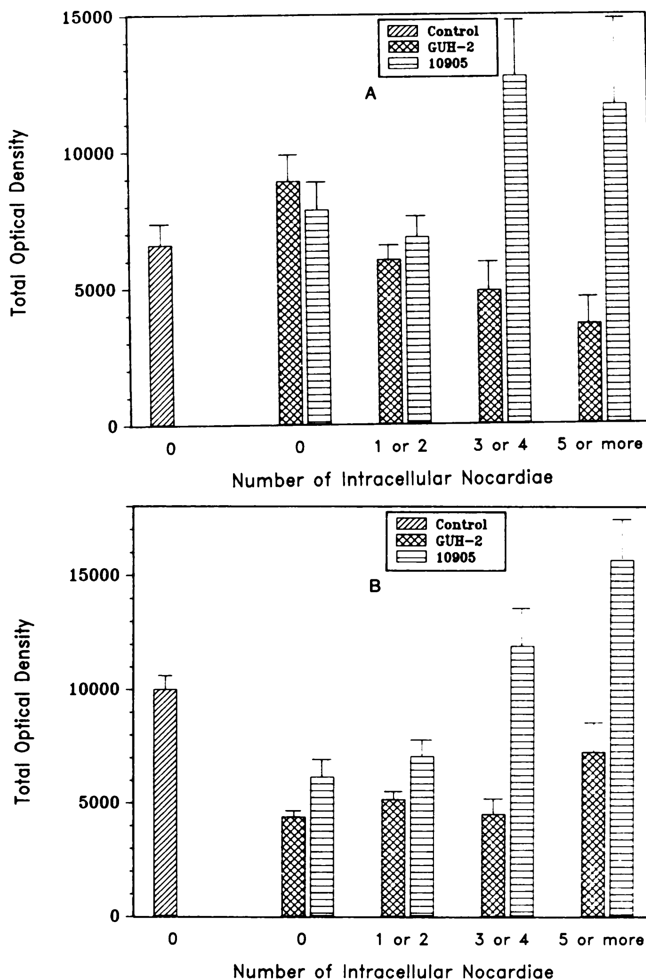

FIG. 3. Effect of degree of infection of murine Kupffer cells with two strains of *N. asteroides* of different virulence on acid phosphatase activity, expressed as total optical density. Summaries of the simultaneously measured nocardicidal effectiveness of these macrophage populations (from Fig. 1 and 2) are as follows: (A) Macrophages isolated from normal mice: no killing of strain GUH-2, significant killing of strain 10905. (B) Macrophages isolated from mice immunized against *N. asteroides* GUH-2: significant killing of both strains. Error bars indicate the standard error.

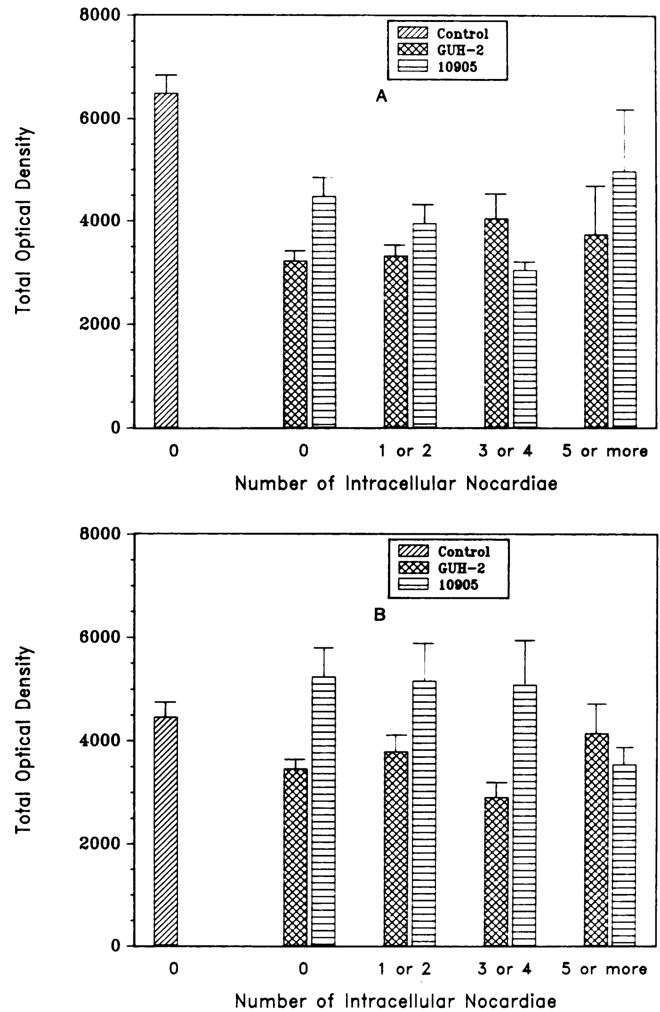

FIG. 4. Effect of degree of infection of murine splenic macrophages with two strains of *N. asteroides* of different virulence on acid phosphatase activity, expressed as total optical density. Summaries of the simultaneously measured nocardicidal effectiveness of these macrophage populations (from Fig. 1 and 2) are as follows. (A) Macrophages isolated from normal mice: inhibited growth of both strains. (B) Macrophages isolated from mice immunized against *N. asteroides* GUH-2: inhibited growth of both strains. Error bars indicate the standard error.

values and include macrophages ranging in multiplicity of infection from 1 to 5 or more nocardiae. For all macrophage populations, acid phosphatase activities were significantly lower ($P < 0.05$) in macrophages containing live virulent strain GUH-2 than in uninfected control macrophages. In contrast, we were not able to demonstrate a significant difference between control values and acid phosphatase levels in the macrophage populations containing killed nocardiae or live strain 10905.

Macrophage acid phosphatase levels in response to increasing degrees of nocardial infection are plotted in Fig. 3 to 6. To aid the direct comparison of the nocardicidal activity of the macrophage populations (shown in Fig. 1 and 2) with intracellular acid phosphatase levels, the killing assay results were summarized in one of three statements: (i) no killing (which also indicates no inhibition of growth); (ii) inhibited growth; or (iii) >50% killed.

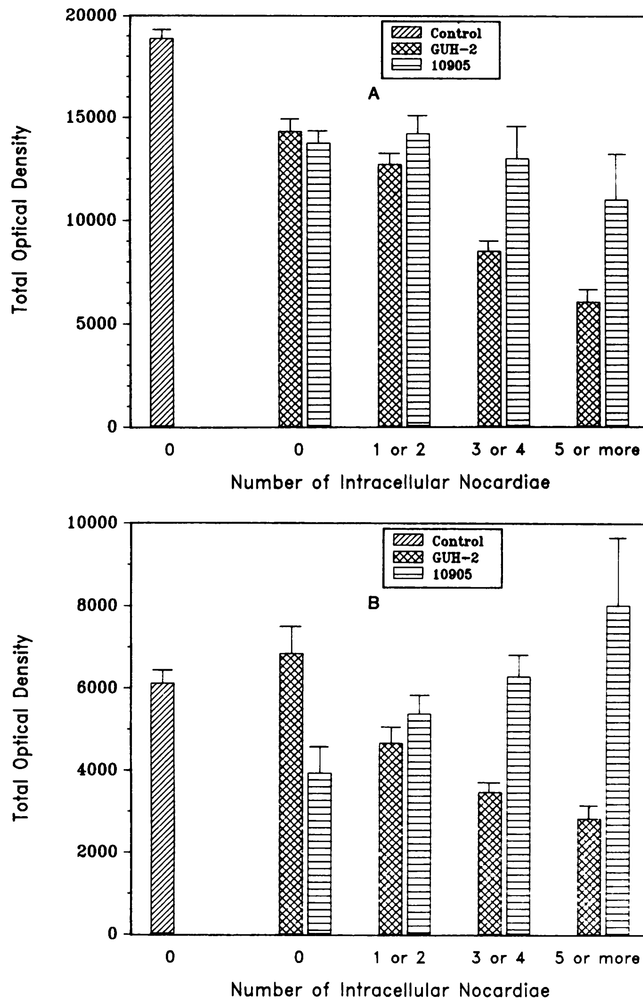

FIG. 5. Effect of degree of infection of murine alveolar macrophages with two strains of *N. asteroides* of different virulence on acid phosphatase activity, expressed as total optical density. Summaries of the nocardicidal effectiveness of these macrophage populations (from Fig. 1 and 2) are as follows. (A) Macrophages isolated from normal mice: no killing of strain GUH-2, inhibited growth of strain 10905. (B) Macrophages isolated from mice immunized against *N. asteroides* GUH-2: no killing of strain GUH-2, significant killing of strain 10905. Error bars indicate the standard error.

Figure 3A illustrates that in the Kupffer cells collected from normal mice a significant reduction in acid phosphatase activity occurred with increasing numbers of ingested cells of strain GUH-2 ($P < 0.025$ for macrophages infected with one to four nocardiae; $P < 0.001$ for macrophages infected with five or more nocardiae). In the nocardicidal assay, cells of strain GUH-2 proliferated 225% above inoculum levels within this normal Kupffer cell population (Fig. 1A). In contrast, strain 10905 was successfully killed by the normal Kupffer cells, and acid phosphatase activity for Kupffer cells infected with strain 10905 was enhanced with increasing infection ($P < 0.05$).

The Kupffer cells collected from immunized mice killed more than 50% of both strains of *N. asteroides*, and acid phosphatase activity was enhanced with increasing degree of infection with both strains ($P < 0.05$ for macrophages infected with GUH-2, and $P < 0.01$ for macrophages infected with strain 10905) (Fig. 3B). Enhancement for enzyme activity was greater, however, with the less virulent strain.

Splenic macrophages isolated from both normal and immunized mice behaved similarly when infected with the two strains of *N. asteroides* (Fig. 4A and B): bacterial growth was inhibited, but counts were reduced <50%, and acid phosphatase levels remained at nearly the same values regardless of increasing numbers of ingested nocardiae. The small differences in enzyme activity shown in Fig. 4 are not statistically significant.

Figures 5 and 6 illustrate that similar relationships between nocardicidal effectiveness and acid phosphatase activity with increasing nocardial infection existed for the alveolar and peritoneal macrophage populations. Neither of the alveolar macrophage populations isolated from normal and immunized mice was able to reduce the bacterial counts or inhibit the growth of strain GUH-2; thus these two populations showed significant decreases in acid phosphatase activity with increasing numbers of intracellular nocardiae of strain GUH-2 ($P < 0.01$) (Fig. 5A and B). Normal

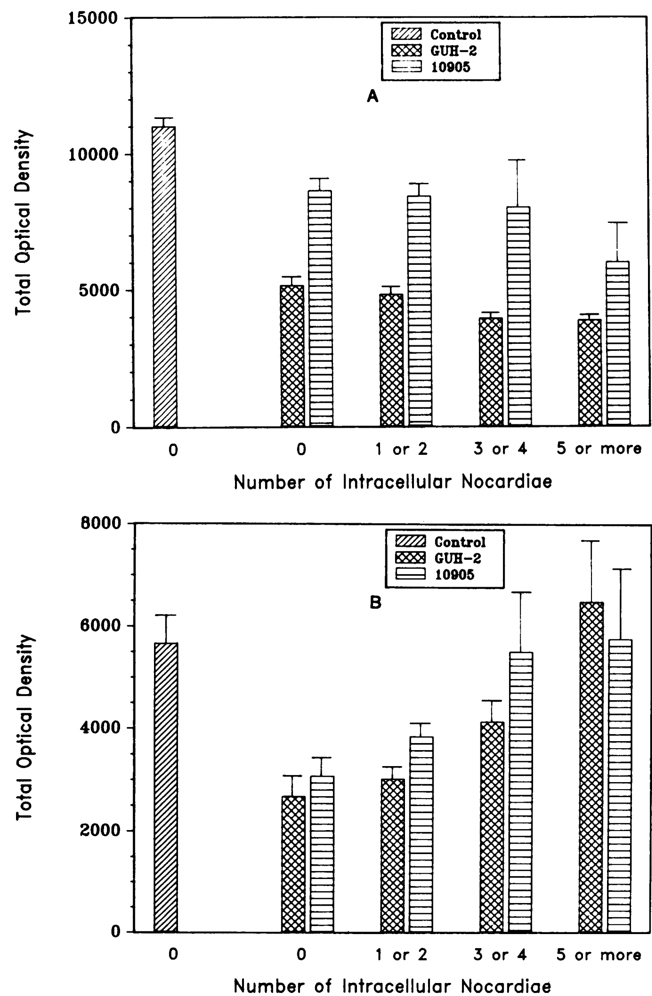

FIG. 6. Effect of degree of infection of murine peritoneal macrophages with two strains of *N. asteroides* of different virulence on acid phosphatase activity, expressed as total optical density. Summaries of the nocardicidal effectiveness of these macrophage populations (from Fig. 1 and 2) are as follows. (A) Macrophages isolated from normal mice: inhibited growth of both strains. (B) Macrophages isolated from mice immunized against *N. asteroides* GUH-2: significant killing of both strains. Error bars indicate standard error.

TABLE 2. Acid phosphatase activities (expressed as optical density) in murine peritoneal macrophages exposed to sterile culture supernatants or live cells of virulent *N. asteroides* GUH-2

Cells	Enzyme activity, mean ± SE (no. of macrophages), in given treatment group[a]				
	GUH-2 live cells	GUH-2 supernatant	BHI control[b]	3-h macrophage supernatant[c]	Untreated control
All cells in sample	5,074 ± 555 (48)[d]				
Uninfected cells only	4,264 ± 529 (24)[d]	6,802 ± 707 (50)	7,659 ± 1,222 (50)	8,126 ± 1,097 (51)	7,815 ± 603 (100)

[a] Units were calculated as the sum of optical density measurements at 0.5-μm intervals.
[b] Brain heart infusion broth (BHI) diluted in tissue culture medium.
[c] Supernatant collected from a separate sample of macrophages preinfected for 3 h with live *N. asteroides* strain GUH-2 and filter sterilized.
[d] $P < 0.05$ as compared with the unexposed control.

peritoneal macrophages which failed to effectively reduce bacterial counts of strain GUH-2 but successfully retarded an increase in numbers showed no significant change in acid phosphatase activity with increasing infectivity of intracellular nocardiae (Fig. 6A). Finally, peritoneal macrophages from immunized mice reduced bacterial counts of strain GUH-2 by 56% and showed enhancement of acid phosphatase levels with increasing degree of infection ($P < 0.05$) (Fig. 6B). The response of the alveolar and peritoneal macrophage populations to infection with strain 10905 was similar: when bacterial counts were reduced >50%, enzyme activity was enhanced with increasing numbers of intracellular nocardiae ($P < 0.05$ for peritoneal macrophages from immunized mice [Fig. 6B], and $P < 0.01$ for alveolar macrophages from immunized mice [Fig. 5B]). When strain 10905 was not effectively killed but its growth was inhibited, levels of enzyme activity did not significantly change with increasing degree of infection (Fig. 5A and 6A).

The data corresponding to the response of macrophage populations to ingestion of killed cells of strain GUH-2 were similar to those shown for live cells of strain 10905 and are not included.

Experiments in which macrophages were incubated with cell-free supernatants of nocardial cultures were performed to elucidate the differences observed in enzyme activity between unexposed control macrophages and exposed but uninfected macrophages (Fig. 3B and 6B; Table 2). Peritoneal macrophage acid phosphatase activities in response to exposure to sterile culture supernatants of virulent strain GUH-2 are shown in Table 2. The enzyme activities shown in the first row represent mean population values. The live cell infection control (column 2) includes macrophages infected with zero to five or more nocardiae. The values in the second row include only macrophages containing no ingested nocardiae. As expected, acid phosphatase activities were significantly lower ($P < 0.05$) in macrophages infected with live cells of strain GUH-2 than in untreated control macrophages. In addition, acid phosphatase activity was significantly lower than the control value in macrophages exposed to, but not infected by, strain GUH-2 ($P < 0.05$). Macrophages exposed to cell-free supernatants of strain GUH-2 showed no significant difference in enzyme activity from untreated control values. Control macrophages exposed to dilutions of brain heart infusion broth or to sterile supernatants from strain GUH-2-infected macrophages showed no significant difference from untreated control values in acid phosphatase activity.

DISCUSSION

The importance of the initial interaction of intracellular pathogens with macrophages in establishing infection is well documented (14, 17, 27). Previous studies have demonstrated correlations between the relative virulence of bacterial strains for laboratory animals and (i) their resistance to bactericidal attack by macrophages (12, 14, 23, 30), (ii) their resistance to phagocytosis (9, 12), and (iii) reduction in intracellular levels of lysosomal enzyme (9). In addition, reductions in lysosomal acid phosphatase coincide with impairment of the bactericidal activity of alveolar macrophages (16). The results presented herein corroborate these reports, in that they demonstrate the use of lysosomal enzyme activity levels as a marker of bactericidal effectiveness against nocardial strains of different virulence.

Previous studies comparing macrophage populations isolated from different anatomical sites indicate that hierarchies based on antimicrobial functions vary according to the agent used. Simpson et al. demonstrated that murine alveolar macrophages killed the yeast *Saccharomyces* more efficiently than either peritoneal or splenic macrophages, which killed the yeast cells similarly (25). Furthermore, peritoneal macrophages outperformed the alveolar population in two different phagocytic assays, but in these same assays the performance of the alveolar macrophages exceeded that of the splenic population (25). At least two additional reports demonstrated that alveolar macrophages killed *Candida* and *Aspergillus* cells more effectively than did peritoneal populations (18, 24). In contrast, studies performed with *Pasteurella* and *Nocardia* species revealed that peritoneal macrophages killed these respiratory bacterial pathogens more competently than did alveolar macrophages (9, 11). The results presented in this report further define functional hierarchies by examining four different populations simultaneously and by studying the effect of immunization of the mice on these functional orders. The order of nocardicidal effectiveness (and resistance to the loss of enzyme activity) of the macrophage populations isolated from normal mice was splenic > peritoneal > alveolar > Kupffer. In contrast, the ranking for macrophage populations collected from immunized mice was Kupffer > peritoneal > alveolar > splenic. Thus, the populations of macrophages which killed more effectively exhibited smaller decreases in acid phosphatase activity in response to increasing infection.

It was the Kupffer cell population that was most affected by immunization of the mice and the splenic macrophage population which was affected least. Indeed, for all parameters measured using strain GUH-2, the behavior of the splenic macrophages isolated from normal and immunized mice was virtually indistinguishable. If it can be presumed that immunization serves to activate portions of macrophage populations, then these results suggest that the differences observed among macrophages isolated from various anatomical sites may be due to unequal states of macrophage activation. Alternatively, these differences may be due to the presence of functionally different subpopulations of

macrophages within these anatomical sites. If macrophages within the spleens of normal mice were already in a state of activation, then this would account for the lack of the effect of immunization on the splenic macrophages. It follows, then, that the Kupffer cell population within the liver of the normal mouse is not in an activated state; thus, Kupffer cells from normal versus immunized mice would behave quite differently in functional assays, as was observed in the present study. Finally, alveolar and peritoneal macrophage populations would theoretically be in intermediate states of activation within the lungs and peritoneum of the normal mouse, since smaller differences were observed in the functional assays between the populations isolated from normal versus immunized mice. To study these possibilities further, the state of activation of the different macrophage populations must be determined with different parameters, such as tumoricidal activity.

The conclusion from the in vitro results that increased nocardicidal activity occurs in alveolar macrophages and Kupffer cells but not in splenic macrophages after immunization agrees with previous data from in vivo experiments. Normal mice intranasally challenged with *N. asteroides* GUH-2 did not begin to clear the bacteria from their lungs until after 24 h, whereas the preimmunized mice began to clear strain GUH-2 from their lungs immediately after challenge (3). Since in the present study normal alveolar macrophages were less effective against strain GUH-2 than were alveolar macrophages from immunized mice, the delay in clearance of the normal mouse lung reflects a requirement for activation of macrophages before clearance can occur (4, 5). Experiments on rates of liver clearance after intravenous inoculation produced similar results, in that the normal mouse livers did not begin to clear the nocardiae until several days after the challenge, whereas the livers of the immunized mice began to clear the bacteria immediately. These results correspond to the presently reported finding that Kupffer cells from immunized mice were dramatically more effective against strain GUH-2 than were the normal Kupffer cells. Clearance of nocardiae from the spleen was not similar to that observed for the lungs and livers of mice. Thus, the clearance rates and patterns within the spleen were the same for both normal and immunized mice. Prior immunization of the animals failed to improve the efficiency of bacterial clearance from the spleen. These observations are in accord with the findings of the present study that no differences existed in nocardicidal effectiveness of splenic macrophages from normal versus immunized mice.

The difference in acid phosphatase activity observed in some experiments for the nocardia-exposed but uninfected macrophages as compared to the unexposed control macrophages (Fig. 3B and 6B; Table 2) suggested that the nocardiae may induce an effect upon the macrophages which occurs independently of phagocytic uptake. To test this possibility, separate experiments were performed in which peritoneal macrophages were incubated with filtered supernatants of nocardial cultures. Results of these experiments in which the macrophages exposed to the strain GUH-2 supernatant showed no difference in enzyme activity from the unexposed control indicate that it is probably not a soluble nocardial product that is responsible for the observed difference. The lack of effect on enzyme levels of macrophages incubated with the diluted brain heart infusion broth and the infected macrophage supernatants indicates that the observed difference is also not likely to be due to a toxic component of the growth media or a macrophage product in response to infection with strain GUH-2. These results suggest that a subpopulation of macrophages may exist within the assayed macrophage populations which is defined by the following two characteristics: (i) inability to phagocytize nocardiae, and (ii) significantly lower levels of activity of acid phosphatase than that of the total untreated macrophage population. Even when the macrophages were exposed to 10 bacteria per macrophage, a significant number of these macrophages remained free of microorganisms, indicating an inability to phagocytize bacteria. To determine whether this macrophage subpopulation may be a consequence of differences in cellular maturation, separate experiments measuring myeloperoxidase activity within these macrophages were performed with similar techniques. Results of this study revealed that the described macrophage subpopulation did not contain myeloperoxidase; that is, enzyme levels were not different from those observed in untreated controls (data not shown). The described macrophage subpopulation thus does not consist of immature macrophages (or monocytes) as defined by the presence of intracellular myeloperoxidase. Investigations which would verify and further characterize this apparent macrophage subpopulation remain to be performed.

The mechanism by which *N. asteroides* infection reduces the acid phosphatase activity of macrophages has yet to be explained. The question as to whether this may represent a mechanism for pathogenicity by virtue of increased intracellular survival is a much more difficult one to assess. We are currently assaying the effect of nocardial infection on other lysosomal enzymes to determine if a similar relationship exists. This would seem to indicate whether losses in enzyme activity are due to degranulation of the macrophage. In addition, the possibility that reductions in enzyme activity may be due to denaturation or some means of selective activity loss is currently under investigation.

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