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Effects of Activated Macrophages on *Nocardia asteroides*

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The mechanism(s) of host resistance against *Nocardia asteroides* has not been well defined. Since disease due to *N. asteroides* frequently occurs in patients with impaired cell-mediated immunity, we studied the interaction of *N. asteroides* with activated and control mouse peritoneal macrophages. Activated macrophages were from mice infected with *Toxoplasma gondii* or injected with *Corynebacterium parvum*. *N. asteroides* in the early stationary phase (>99% in the coccobacillary form) was used for challenge of macrophage monolayers. Growth of two strains of *N. asteroides* was markedly inhibited in activated macrophages, whereas *N. asteroides* grew well in control macrophages. Quantitation of macrophage-associated *N. asteroides* indicated that activated macrophages killed 40 to 50% of *N. asteroides* within 6 h ($P < 0.002$). In control macrophage preparations, it appeared as if *Nocardia* filaments extended from within macrophages to the outside, and many of these filaments appeared to have extended to and then grown through neighboring macrophages. In activated macrophage preparations, *Nocardia* remained in the coccobacillary form in most macrophages. Control macrophage monolayers were almost completely overgrown with and destroyed by *Nocardia* 20 h after challenge, whereas activated macrophage monolayers remained intact. *Nocardia* that grew in control macrophages were not acid-alcohol fast or only weakly so, whereas the few *Nocardia* that grew in activated macrophages were strongly acid-alcohol fast. Our results indicate that activated macrophages may be important in host defense against *N. asteroides*.

Nocardia asteroides causes severe, often fatal infection in immunocompromised patients (19, 23) and *Nocardia* infection has been increasingly recognized in recent years (5). At Stanford University Hospital, *Nocardia* infection had occurred in 21 of 165 patients who had received heart transplants as of December 1978 (G. L. Simpson, E. B. Stinson, and J. S. Remington, manuscript in preparation). In persons without demonstrable evidence of a predisposing condition, *Nocardia* infection occurs less commonly but still with serious consequences (23).

The mechanism(s) of resistance to *Nocardia* infection is not known. Prior studies in a mouse model by Krick and Remington suggest that activated macrophages are important in resistance (18). To elucidate the role of activated macrophages in resistance to *Nocardia* infection, we examined the interactions of *N. asteroides* with activated and control mouse peritoneal macrophages in vitro.

(Portions of these results were presented at and appeared in the abstracts of the 11th International Congress of Chemotherapy and the 19th Interscience Conference on Antimicrobial Agents and Chemotherapy, 1 to 5 October, 1979, Boston, Mass.)

MATERIALS AND METHODS

N. asteroides. Both strains of *N. asteroides* we used had been isolated originally from patients with disseminated nocardiosis. Strain GUH-2 is highly virulent for mice; strain 14759 (American Type Culture Collection [13]) is less virulent for mice. Intravenous doses of early stationary-phase organisms which kill 50% of mice are 8.7×10^5 and 8.5×10^6 for strain GUH-2 and strain 14759, respectively (7). These strains have been used extensively in experiments in mice (6, 7, 13), rabbits (13), and guinea pigs (13). The effect of rabbit alveolar macrophages on these strains has also been reported (3, 4, 9).

Before use, the strains were maintained in broth and were passaged through mice. Infected kidneys from the mice were transferred into brain heart infusion broth, as previously described (7). To obtain preparations in which the majority of *Nocardia* were in the coccobacillary form, the organisms were harvested during the early stationary phase of growth (8). After the brain heart infusion broth cultures were centrifuged at $60 \times g$ for 5 min to remove clumps and filaments, the supernatants containing coccobacillary forms were centrifuged at $900 \times g$ for 15 min and

suspended in phosphate-buffered saline, pH 7.2. The concentrations of organisms in the suspensions were determined by optical density at 580 nm. The organisms were brought to the desired concentration in medium 199 (M199) plus 10% fetal calf serum (FCS). (M199 and FCS were obtained from GIBCO Laboratories, Grand Island, N.Y.).

Sources of macrophages. Macrophages were obtained from the peritoneal cavities of Swiss Webster female mice (Simonsen Laboratories, Gilroy, Calif.). Activated macrophages (defined by their ability to inhibit the growth of *Toxoplasma gondii* [1, 21]) were obtained from 13-week-old mice chronically infected with *T. gondii* (14) or from 6-week-old mice injected 7 days earlier with *Corynebacterium parvum* (7 mg, dry weight, per ml; lot CA 592A; kindly provided by John Whisnant, Burroughs Wellcome Co., Research Triangle Park, N.C.), as previously described (29). Control macrophages were from age-matched littermates, except in one experiment in which activated macrophages from mice that were injected with *C. parvum* were compared with peritoneal macrophages from mice that had chronic renal infection with *N. asteroides* GUH-2. Peritoneal macrophages from the *Nocardia*-infected mice were not activated to inhibit the growth of *T. gondii*.

Macrophage culture. Peritoneal exudate cells were harvested in Hanks balanced salt solution, as previously described (22), and suspended in M199 + 10% fetal calf serum at a concentration of 4 to 5.7 cells/ml. From 0.5 to 0.7 ml of the cell suspensions was seeded onto glass cover slips (22 by 11 mm or 22 mm^2) in plastic petri dishes (35 by 10 mm) or into each chamber of four-chamber Lab-Tek slides (Lab-Tek Products, Naperville, Ill.). For all experiments, concentrations and inoculum sizes were such that 8.3×10^5 peritoneal exudate cells were seeded onto each square centimeter of the cover slips or the floors of the chambers. After approximately 3 h of incubation at 37°C in 5% CO_2, nonadherent cells were washed off with saline, and the remaining cells were challenged immediately thereafter. Approximately 50% of peritoneal exudate cells obtained in this way are adherent, and approximately 95% of the adherent cells have the morphology of macrophages and phagocytose heat-killed *Candida albicans* (29).

Challenge of macrophage monolayers with *N. asteroides*. Monolayers were challenged with *Nocardia* in a ratio of 2 to 8 organisms per macrophage and incubated for 1 to 3 h. The choice of organism-to-macrophage ratio for these experiments was based on the earlier observation that a similar ratio caused infection of approximately 50% of normal macrophages with from one to five organisms in each infected macrophage (B. L. Beaman, unpublished data). The monolayers were then washed twice with 1 ml of saline each time, fresh medium was added, and the preparations were reincubated. At various intervals, the numbers of viable *Nocardia* associated with macrophages and in supernatant mediums were determined as described below. Lab-Tek slides or cover slips run in parallel were stained with either Gram stain or Kinyoun modified acid-fast stain.

For enumeration of viable non-macrophage-associated *Nocardia*, supernatants were removed from the cover slip cultures, and the cover slips were washed three times with 1 ml of saline each time. The washes were added to the supernatants, and the resultant suspensions were dispersed by vigorous mixing in a Vortex blender and then serially diluted. Portions of the dilutions were plated on brain heart infusion agar. In preliminary experiments to determine the efficiency of this washing procedure, *Nocardia* were added to cell-free cover slips in petri dishes, the supernatants were removed after 30 min, and the cover slips were washed in the manner described. When these methods were used, approximately 99% of *Nocardia* was recovered.

For enumeration of viable macrophage-associated *Nocardia*, macrophages on the washed cover slips were lysed by incubating the cover slips in 2 ml of distilled water for 15 min. The cover slips were then scraped with a rubber policeman and washed twice with 1 ml of distilled water each time. The washes were combined with the lysates, and the resultant suspensions were dispersed by vigorous mixing in a Vortex blender and then serially diluted. Portions of the dilutions were plated on brain heart infusion agar. Suspension of these *Nocardia* strains in distilled water for 15 min does not affect their viability (unpublished data).

Challenge of macrophage monolayers with *T. gondii*. In parallel with *Nocardia* challenge, macrophage monolayers were challenged with *T. gondii*, as previously described (1). Macrophages were considered activated if they inhibited multiplication of *T. gondii* (1, 21).

Analysis of the effects of macrophages on macrophage-associated *Nocardia*. To analyze the effects of macrophages on the *Nocardia* that were macrophage associated at the end of the 1- to 3-h challenge periods, the numbers of macrophage-associated *Nocardia* at the end of the challenge periods were compared with the numbers of *Nocardia* in the whole preparations (sum of *Nocardia* associated with macrophages and *Nocardia* in the supernatant medium) at later times. Immediately after the challenge suspensions were removed and the monolayers were washed, essentially all *Nocardia* in the preparations were macrophage associated. Therefore, *Nocardia* that were isolated from the whole preparations at later time points originated from the populations of macrophage-associated *Nocardia* present immediately after challenge.

Statistics. Analyses of the differences between means were performed by using the Student's *t* test.

RESULTS

The results of interactions of *N. asteroides* GUH-2 with activated macrophages from mice chronically infected with *Toxoplasma* and with macrophages from age-matched controls are shown in Fig. 1. One hour after challenge, many *Nocardia* in control macrophages were in the form of short filaments (Fig. 1A), whereas almost all *Nocardia* in activated macrophages were in

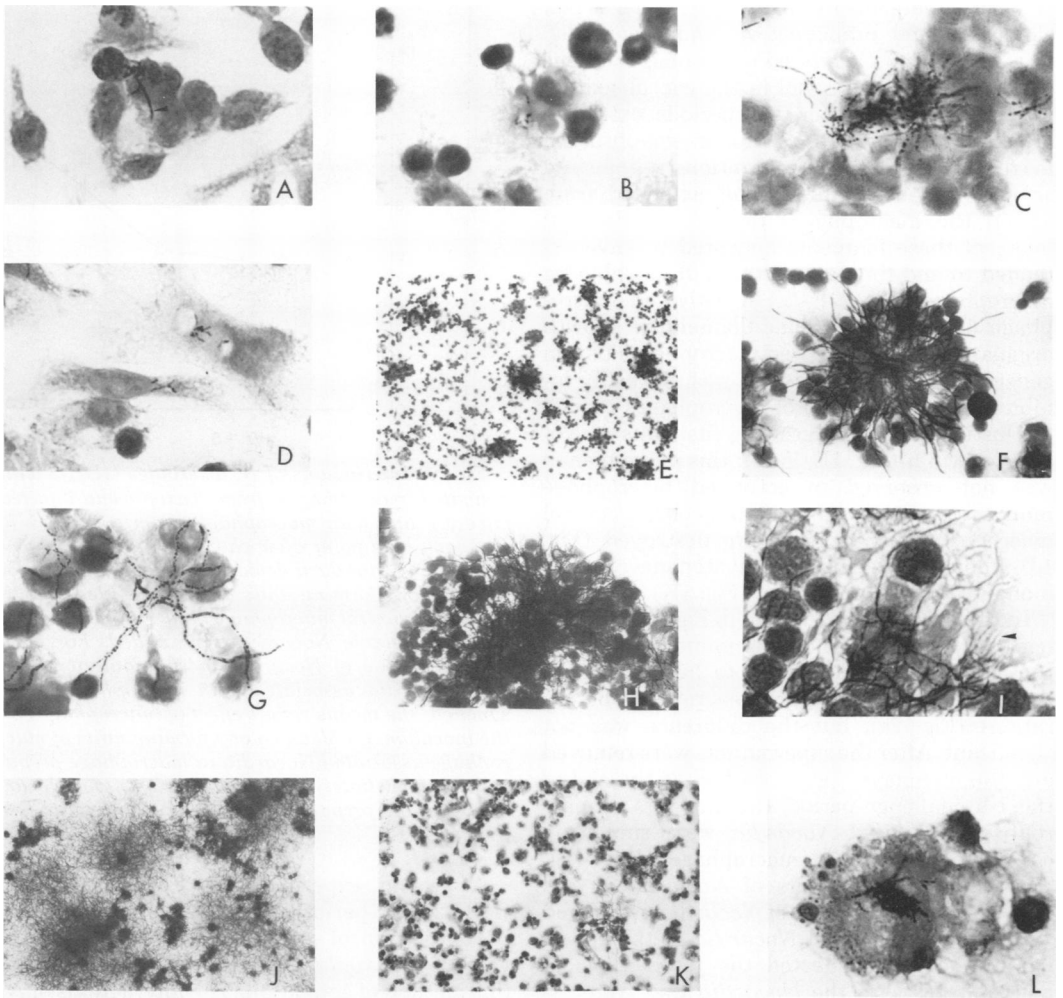

FIG. 1. *Interactions of N. asteroides GUH-2 with activated macrophages from Toxoplasma-infected mice and with macrophages from controls (Gram-stained preparations). (A) At 1 h after initiation of challenge, filament formation has begun in Nocardia (arrow) within control macrophages (×200). (B) At 1 h after initiation of challenge, Nocardia within activated macrophages are in coccobacillary form (×200). (C) At 3 h, Nocardia in many control macrophages have grown extensively (×200). (D) At 3 h, Nocardia in most activated macrophages remain in the coccobacillary form (×200). (E) At 6 h, macrophages in control preparations appear to have migrated toward cells containing Nocardia and form multicellular plaques (×35). (F and G) Higher magnifications (×100 and ×200, respectively) of preparation shown in E reveal growth of Nocardia which apparently stimulated plaque formation. (H) At 12 h, growth of Nocardia in control preparations is more extensive, and the plaques surrounding centers of growth have enlarged (×80). (I) On higher magnification of preparation shown in H, Nocardia filaments intertwine extensively through and around macrophages; some filaments clearly appear to be in vacuoles (arrow) (×200). (J) At 20 h, control macrophage monolayers are overgrown and destroyed by Nocardia (×20). (K) At 20 h, although Nocardia have grown in some areas, the activated macrophage monolayers are still intact (×20). (L) On higher magnification of preparation shown in K, growth of Nocardia is still markedly inhibited in many activated macrophages (×200).*

the coccobacillary form (Fig. 1B) and appeared as they did in the original inoculum. Most *Nocardia* appeared to be intracellular, although some may have been adherent to macrophage surfaces. At 1-, 3-, and 6-h time points in this and the two experiments described below, the densities of activated and control macrophage monolayers were similar. By 3 h after challenge,

Nocardia filaments in control macrophages had elongated, and branching was apparent (Fig. 1C). In almost all activated macrophages at this time, *Nocardia* were still in the coccobacillary form (Fig. 1D). At later time periods, extensive arborization of *Nocardia* filaments was observed in control macrophage preparations; it appeared as if *Nocardia* filaments had extended from within the macrophages to the outside, and many of these filaments appeared to have extended to and then grown through neighboring macrophages (Fig. 1F–I). In activated macrophage monolayers, similar filamentous growth originated in a few isolated macrophages but, in general, growth of *Nocardia* was markedly inhibited (Fig. 1L). Control macrophages tended to cluster around the growing filaments of *Nocardia* by 6 h (Fig. 1E, F, H); this phenomenon was not observed in activated macrophage monolayers. By 20 h, almost all cells in control macrophage monolayers were destroyed (Fig. 1J), whereas most cells in activated macrophage monolayers were still intact (Fig. 1K).

In the experiment depicted in Fig. 1, the number of *Nocardia* recoverable immediately after the 1-h challenge appeared to be fewer from activated than from control macrophage preparations (Fig. 2a), but the difference was not significant. After the supernatants were removed and the monolayers were washed at the end of the 1-h challenge period, the numbers of macrophage-associated *Nocardia* were similar in control and activated macrophages (Fig. 2b). Thereafter, the numbers of *Nocardia* in the whole preparations (sum of *Nocardia* associated with macrophages and *Nocardia* in the supernatant medium) reflected the interactions of macrophages with the *Nocardia* that were associated with macrophages at the end of the 1-h challenge period (see Materials and Methods). Three hours after challenge, the numbers of *Nocardia* in activated and control macrophage preparations had not changed from those observed at the end of the challenge period. By 6 h, the numbers of *Nocardia* in activated macrophage preparations were significantly reduced when compared with the numbers in similar preparations at 3 h ($P < 0.0001$) or with the numbers in control macrophage preparations at 6 h ($P < 0.002$) (Fig. 2b). By 12 h, the numbers of *Nocardia* increased in both types of macrophage preparations, but the numbers in activated macrophage preparations were still significantly less than in control macrophage preparations ($P < 0.001$). After 12 h, extracellular organisms grew profusely in a pellicle on the surface of the medium in both types of macrophage preparations, precluding meaningful interpretation of colony counts.

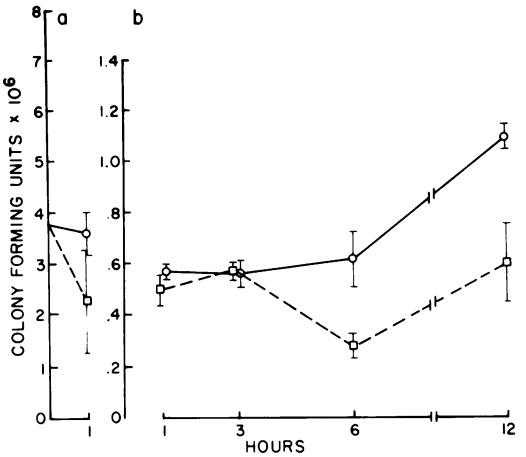

FIG. 2. *Interactions of N. asteroides GUH-2 with activated macrophages from Toxoplasma-infected mice* (□) *and with macrophages from controls* (○). *For each time point after challenge, data represent the mean* (± *standard deviation) of four samples. (a) Mean colony-forming units of N. asteroides in activated and control macrophage preparations 1 h after* 3.8×10^6 *viable Nocardia were added. For each sample, counts of Nocardia in supernatant media and Nocardia associated with macrophages were summed; the means reflect effect of macrophages on the inoculum. (b) Mean colony-forming units of macrophage-associated Nocardia in macrophage preparations immediately after washing (1 h) and Nocardia in whole preparations at later time points (see text).*

A second experiment with *N. asteroides* GUH-2 was performed to determine whether the inhibition of growth of macrophages activated by *Toxoplasma* infection was unique to this method of activation. The interactions of *N. asteroides* GUH-2 with macrophages from *C. parvum*-injected mice (macrophages activated to kill *Toxoplasma*) were compared with the interactions of *N. asteroides* GUH-2 with macrophages from *Nocardia*-infected mice (macrophages which were not activated to kill *Toxoplasma* and served as controls). Examination of stained cover slips and results of cultures in this experiment revealed that growth of *Nocardia* in macrophages from *Nocardia*-infected mice was similar to that in control macrophages in other experiments. Growth of *Nocardia* in *C. parvum*-activated macrophage preparations was markedly inhibited when compared with growth in macrophage preparations from *Nocardia*-infected mice. The inhibition observed with *C. parvum*-activated macrophages in this experiment was similar to that observed with *Toxoplasma*-activated macrophages in the experiment described above.

To determine whether differences between in-

teractions of control and activated macrophages with *Nocardia* were unique to the GUH-2 strain, interactions of the less virulent 14759 strain of *Nocardia* with macrophages from *C. parvum*-injected mice and age-matched controls were studied. Examination of stained preparations indicated that proliferation of *Nocardia* occurred in control macrophages but not in activated macrophages, as in the previously described experiments. Colony counts indicated that the numbers of *Nocardia* decreased in the first hour in both control and activated macrophage preparations (Fig. 3a). However, immediately after the monolayers were washed at 1 h, there were significantly more *Nocardia* associated with activated than with control macrophages ($P < 0.005$) (Fig. 3b). The numbers of *Nocardia* in control macrophage preparations remained the same at 1, 3, and 6 h and then increased by 12 h. In contrast, there were significantly fewer *Nocardia* in activated macrophage preparations at 3 h than at 1 h ($P < 0.002$). Although counts rose in activated and control macrophage preparations by 12 h, there were significantly fewer *Nocardia* in activated than in control macrophage preparations ($P < 0.001$) at this time.

At 6 h and at later time points, the few *Nocardia* that had elongated in activated macrophages were strongly acid-alcohol fast. In contrast, *Nocardia* that had elongated in control macrophages were not acid-alcohol fast or only weakly so. The *Nocardia* in the challenge suspensions and those that had remained in the coccobacillary form in activated macrophages were not acid-alcohol fast.

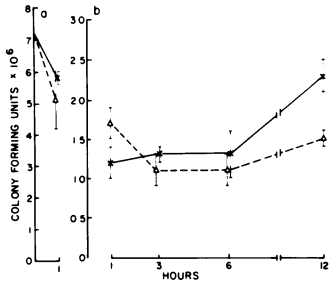

FIG. 3. *Interactions of N. asteroides 14759 with activated macrophages from C. parvum-treated mice (△) and with macrophages from controls (×). For each time point after challenge, data represent the mean (± standard deviation) of four samples. (a) Mean colony-forming units of N. asteroides in activated and control macrophage preparations 1 h after 7.2 × 10⁶ viable Nocardia were added. Means are derived as described in the legend to Fig. 2a. (b) Mean colony-forming units of macrophage-associated Nocardia in macrophage preparations immediately after washing (1 h) and Nocardia in whole preparations at later time points. Means are derived as in Fig. 2b.*

DISCUSSION

These data provide evidence that activated macrophages may be important in defense against *Nocardia* infection. The ability of activated peritoneal macrophages to inhibit the growth of *N. asteroides* as compared with that of control peritoneal macrophages was apparent both by microscopy and by quantitation of viable *Nocardia* in macrophage preparations at various intervals after challenge. The results of the quantitative analyses revealed that activated macrophages killed a significant portion of the *Nocardia* in the challenge inocula. The effects of activated macrophages were not limited to one method of activation or to one strain of *Nocardia*; this lends further support for the importance of this line of defense. Additional evidence that the effects of activated macrophages upon *Nocardia* differed from those of control macrophages was the observation that the few *Nocardia* that grew in activated macrophage preparations were strongly acid-alcohol fast, whereas those that grew in control macrophage preparations were not. The degree of resistance of *Nocardia* to decolorization with acid-alcohol depends upon the conditions of growth. Strains of *Nocardia* growing in tissues of animals or humans are often acid-alcohol fast, whereas the same strains grown in cell-free media are often not. Enhanced resistance of *Nocardia* to decolorization with acid-alcohol reflects altered lipid composition of the cell envelope and may be related to enhanced ability to evade destruction by host defenses. Further study of such changes in the cell envelope of *Nocardia* may lead to a better understanding of the pathogenesis of *Nocardia* infection. Recent studies in rabbits suggest that alveolar macrophages may also be activated to inhibit *Nocardia* (4).

In control macrophage preparations, it appeared as if macrophages migrated toward infected cells, and large plaques were formed within hours. Similar migration has been observed in studies of interactions of *N. asteroides* 14759 with alveolar macrophages from normal rabbits (3) and in studies of interactions of *Listeria monocytogenes* with peritoneal macrophages from mice that had previously been immunized with *L. monocytogenes* (20).

In previous studies, a number of strains of *N. asteroides* were found to grow intracellularly to various degrees in vitro when incubated with mouse peritoneal macrophages (10), guinea pig peritoneal and alveolar macrophages (24, 25), and rabbit alveolar macrophages (3, 9). Bour-

geois and Beaman (10) found that a strain of low virulence for mice was able to survive within normal mouse peritoneal macrophages. The *Nocardia* could not be detected by Gram stain, and only low numbers were detectable by culture on brain heart infusion agar. However, by 8 days of incubation, L-form-like variants were observed within macrophages by electron microscopy and could be isolated on hypertonic media. It is possible that reductions in numbers of viable *Nocardia* recoverable from activated macrophages in the present study may have reflected transformation of *Nocardia* into L-form-like variants. If this did occur, it still does not refute the observation that growth of typical forms of *Nocardia* was markedly inhibited by activated macrophages.

Results of earlier in vivo experiments with mice (18) suggested that activated macrophages are important in host defense against *Nocardia*. Immunization with *N. asteroides* resulted in protection against subsequent challenge with *N. asteroides* injected intraperitoneally in hog gastric mucin. Marked protection was also observed in mice known to have activated peritoneal macrophages as a result of infection with other organisms. Peritoneal macrophages from mice immunized with *N. asteroides* 3 weeks earlier inhibited uptake of tritiated thymidine by tumor target cells, which in the model used correlates with activation of macrophages (17). Transfer of serum did not protect against subsequent challenge with *Nocardia* in this model. To our knowledge, transfer of resistance by serum has only been reported by Nelson and Henrici. In an abstract by E. Nelson and A. T. Henrici, (Proc. Soc. Exp. Biol. Med. **19**:351, 1922), they stated that transfer of serum from vaccinated rabbits partially protected guinea pigs against challenge with *Actinomyces gypsoides* (renamed *N. asteroides* [11]). Although the studies of Krick and Remington (18) provided evidence that peritoneal macrophages from *Nocardia*-infected mice are activated whereas peritoneal macrophages from *Nocardia*-infected mice in the present study were not activated, the data in these two studies are not necessarily inconsistent. Different strains, doses, and routes of inoculation of *N. asteroides* were used, and macrophages were studied with different assays of activation and at different intervals after infection.

Previous investigations (6, 12) have revealed that athymic mice were more susceptible than their heterozygous littermates or normal mice to challenge with *Nocardia*, suggesting a role for thymus-derived (T) lymphocytes in protection against *Nocardia* infection. In the Krick and Remington model, attempts to transfer resistance to *Nocardia* infection by transferring spleen cells from immune to normal mice were unsuccessful when they used doses that had been sufficient for transfer of resistance to other infections (18). Sundararaj and Agarwal (26–28) concluded from their studies in which a guinea pig model was used that resistance to *Nocardia* infection could be transferred by spleen cells, but the small numbers of animals employed in their studies and the assays of resistance performed make the findings difficult to interpret. Since transfer of spleen cells was not between isogeneic animals, genetic differences between donor and recipient cells may have prevented optimal immunological function. The available data suggest that resistance to experimental *Nocardia* infection is mediated at least in part by T-lymphocytes.

Nocardiosis in humans occurs most frequently in immunocompromised patients, most of whom have had underlying conditions or therapy known to result in marked defects in cell-mediated immunity (5, 19, 23, 30; G. L. Simpson, E. B. Stinson, and J. S. Remington, manuscript in preparation). Data in animals now suggest that T-lymphocytes and macrophages play important roles. Nocardiosis has also been reported in patients with chronic granulomatous disease (16) in which there is a defect in oxidative killing mechanisms in polymorphonuclear leukocytes, monocytes, and alveolar macrophages (2, 15). Which component(s) of the immune system in humans is important in defense against nocardiosis remains to be determined.

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