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Tumor Necrosis Factor and Macrophage Activation Are Important in Clearance of *Nocardia brasiliensis* from the Livers and Spleens of Mice

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The roles of tumor necrosis factor (TNF) and macrophage activation in clearance of *Nocardia brasiliensis* from BALB/c mouse livers and spleens were evaluated. TNF activity was detectable in sera from animals at all stages of infection. Treatment of infected mice with an antiserum against TNF significantly enhanced the experimental infection as judged by enumeration of CFU in the spleens and livers of infected mice. In another set of experiments, a population of activated macrophages from the peritoneal cavities of *N. brasiliensis*-infected mice was studied by using a cytostatic assay. The observed cytotoxic activity of these activated macrophages against L929 cells was mediated by TNF, since this activity was inhibited by anti-TNF antiserum treatment. The level of TNF activity generated in vitro in the presence of lipopolysaccharide (LPS) by peritoneal macrophages from infected mice was higher than that of adherent peritoneal cells obtained from normal mice after challenge with LPS. When the nocardiacidal activity of peritoneal cells from *N. brasiliensis*-infected mice was estimated in vitro, a significant decrease in the number of CFU recovered was observed. Moreover, nocardiacidal activity of peritoneal cells obtained from *N. brasiliensis*-infected mice previously treated with anti-TNF antiserum was significantly reduced compared with the activity of cells obtained from infected mice previously treated with normal rabbit serum and that of cells from uninfected mice. These data suggest a role for TNF in resistance to *N. brasiliensis* infection.

Nocardia brasiliensis is an important agent of actinomycotic mycetoma in tropical and subtropical areas of the Americas and, to a lesser extent, in other parts of the world (9). Mycetoma arises from soil contamination of skin wounds, usually on the feet and hands of laborers. Its development is slow and insidious, being characterized by localized subcutaneous abscesses that spread by direct extension after breaking through the skin surface to form chronic, draining, ulcerated, and crusted lesions (14).

A model for *N. brasiliensis* infection in mice in which a granuloma is produced with large numbers of foam-laden macrophages has been established (8). Electron microscopic studies demonstrated that these macrophages contain organisms in various stages of degeneration within their cytoplasm.

The mechanism(s) of resistance to *N. brasiliensis* infection is not known. Previous studies with a mouse model have suggested that activated macrophages are important in resistance to *Nocardia* spp. (13). Monocytes/macrophages play an important role in host defense both by their direct microbicidal capacity and by their synthesis of regulatory proteins termed cytokines (4). Release of cytokines such as tumor necrosis factor (TNF) by activated macrophages could explain these features of the disease. TNF, released by mononuclear phagocytes in response to microbial components or other stimuli simulating host invasion (11), elicits a complex repertoire of metabolic reactions during inflammation (4). Activated macrophages are the likely source of TNF in tuberculosis (23), leprosy (25), and paracoccidioidomycosis (24) patients. In the present communication, we report that TNF produced by activated macrophages from *N. brasiliensis*-infected mice is an essential factor in host defense against *N. brasiliensis* infection.

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MATERIALS AND METHODS

Bacterial strain. *N. brasiliensis* 519 was the generous gift of Carlos S. Lacaz, School of Medicine, University of São Paulo (the strain number refers to Lacaz' collection). This strain was originally isolated from an actinomycotic mycetoma patient and identified according to the procedure described by Moore and Jaciow (15).

Preparation of an *N. brasiliensis* suspension. A suspension of *N. brasiliensis* predominantly in the coccobacillary form and without grossly visible particles was prepared. Briefly, 100 ml of Trypticase soy broth in a 250-ml flask was inoculated with several colonies of *N. brasiliensis* from a Trypticase soy agar plate and incubated at 37°C in a shaking water bath for 6 days. Organisms were collected by centrifugation at 12,000 × *g* for 20 min, washed twice in saline, and resuspended in 10 ml of saline with vigorous shaking. The suspension was allowed to settle for 30 min, by which time a layer of particles on the surface and a heavy sediment appeared. The cloudy suspension between the surface layer and sediment was removed and allowed to settle for another 2 h. A small amount of sediment again formed, but the suspension above this contained only a few visible particles. A standardized curve of optical density at 650 nm versus concentration of viable organisms was prepared, and the concentration of organisms in subsequent suspensions was estimated from this curve.

Quantitation of *N. brasiliensis* in mouse organs. At various intervals after infection, four mice chosen at random were killed by ether inhalation, and different organs were removed. The entire organ was homogenized in a Teflon-pestle

tissue grinder containing 5 ml of 0.85% NaCl. This procedure did not alter viability of *N. brasiliensis* cells as determined by the culture of a suspension before and after homogenization. Serial dilutions of the homogenate were cultured on Trypticase soy agar, the plates were incubated for 7 days, and the results were expressed as CFU per organ. In another set of experiments, mice were injected intravenously with 1 mg of whole rabbit serum containing anti-recombinant murine TNF antiserum (approximately 2×10^5 neutralizing TNF units/mg of protein [donated by J. M. Belizario from the Butantan Institute, São Paulo, Brazil]), or with 1 mg of normal rabbit serum as a control, 2 h before infection with 10^6 *N. brasiliensis* cells and with four doses of the antiserum at 5-day intervals during infection. The splenic bacterial load was assessed as described above.

Peritoneal macrophages. Peritoneal cells from infected or normal mice injected intraperitoneally with 3 ml of thioglycolate medium (3%) (Difco) 4 days before harvest were aseptically collected after intraperitoneal injection of 5 ml of RPMI 1640 medium supplemented with 8 U of heparin, 100 U of penicillin, and 100 μg of streptomycin per ml. Cells from several mice in each group were pooled, centrifuged at $1,000 \times g$ for 5 min at room temperature, and washed twice with heparin-free medium. The concentration was adjusted to 10^7 cells per ml after the cells were counted in a Neubauer chamber. Cell viability, revealed by the trypan blue dye exclusion test, was usually about 98%. Cells were incubated for 3 h on a plastic petri dish (30 mm in diameter) at 37°C in 5% CO_2 and then washed three times with RPMI 1640 medium to remove nonadherent cells.

In vitro test of mouse peritoneal macrophage activation. A cytostatic assay shown previously to differentiate between activated and normal macrophages was employed (12). Briefly, monolayers of peritoneal macrophages from mice infected with *N. brasiliensis* and from control mice (5×10^4 cells per well in 96-well Titertec plates) were prepared and challenged with tumor target cells (10^4 L929 cells per well). The cytostatic effect of macrophages on the target cells was judged by measuring the ability of the target cells to incorporate [^{3}H]thymidine ([^{3}H]TdR). Eighteen hours after the addition of target cells to macrophage monolayers, triplicate cultures were pulsed for 6 h with 2 μCi of [^{3}H]TdR (specific activity of 6.7 Ci/mmol) (New England Nuclear). At the end of the pulse period, cells were harvested and the amount of [^{3}H]TdR incorporated was determined with a liquid scintillation counter.

Measurement of TNF levels. L929 mouse tumor cells were used to measure TNF levels in plasma and in the macrophage supernatants as previously described (22). Briefly, L929 cells in RPMI 1640 medium containing 5% fetal calf serum were seeded at 3×10^4 cells per well in 96-well Linbro microdilution plates (Flow Laboratories, Inc., McLean, Va.) and incubated overnight at 37°C in an atmosphere of 5% CO_2 in air. Serial 1:2 dilutions of plasma or macrophage supernatants were made with the medium described above but containing 1.0 μg of actinomycin D (Sigma Chemical Co., St. Louis, Mo.) per ml, and 100-μl volumes of each dilution were added to the wells. On the next day, cell survival was assessed by fixing and staining the cells with crystal violet (0.2% in 20% methanol), and 0.1 ml of 1% sodium dodecyl sulfate was added to each well to solubilize the stained cells. The A_{490} of each well was read with a model BT-100 Microelisa Autoreader (Bio-Tek). Percent cytotoxicity was calculated as $[1 - (A_{490}$ of sample$/A_{490}$ of control$)] \times 100$. Titers were expressed as the reciprocal of the dilution of the sample in which 50% of the cells in the monolayers were lysed. For characterization of the cytotoxic activity in serum or in the macrophage supernatants, the samples were incubated with an excess of whole rabbit serum containing anti-recombinant murine TNF antiserum or with control rabbit serum. After 2 h at 37°C, residual cytotoxicity was determined by adding the test samples to L929 cells.

Challenge of macrophage monolayers with *N. brasiliensis*. Peritoneal cells were from mice which had been injected intravenously with normal rabbit serum or with rabbit anti-TNF antiserum 2 h before being injected intravenously with 10^6 *N. brasiliensis* cells or saline only; they were given thioglycolate medium (3%) intraperitoneally 4 days later. Peritoneal cells were collected on day 8 of the first antiserum treatment, and adherent cells were obtained as described above. Monolayers were challenged with *N. brasiliensis* at a ratio of 5 to 10 organisms per macrophage (2×10^7 viable *N. brasiliensis* cells) and incubated for 3 h. The monolayers were then washed twice with 1 ml of saline each time to remove nonphagocytized cells, fresh medium was added, and the preparations were reincubated for 6 h at 37°C in a CO_2 incubator. The numbers of viable *N. brasiliensis* cells associated with macrophages were determined as described previously (7).

Statistical evaluation of the data. Data were expressed as means ± standard deviation, and Student's *t* test was used to determine the significance of the differences between the control and experimental groups.

RESULTS

Fate of *N. brasiliensis* in mouse organs. After intravenous injection of normal mice with 10^6 CFU of *N. brasiliensis*, the number of organisms in the livers and spleens decreased by more than 2 log units within 3 weeks (Fig. 1A). Many mice became sick, showing loss of body weight, slow movement, and ataxia. None of them died for up to 5 weeks of the experiment. Intraperitoneal injection of 10^6 *N. brasiliensis* organisms in saline produced clinical illness similar to that observed at the beginning of the intravenous infection. Moreover, at 4 weeks after injection, *N. brasiliensis* cells were not recoverable from the livers or spleens of infected mice (Fig. 1B).

TNF production in *N. brasiliensis*-infected mice in vivo. After mice were infected intravenously with 10^6 CFU of *N. brasiliensis*, TNF activity of the pooled sera from four mice was monitored at various times. The results shown in Fig. 2 demonstrated that TNF was produced endogenously by the infected mice. To evaluate whether the observed cytotoxic activity against L929 cells was mediated by TNF, the experimental sera were pretreated with anti-TNF antiserum before testing. As is also shown by Fig. 2, cytotoxicity was significantly inhibited by the TNF-specific antiserum. In contrast, a low level of TNF activity was detectable in all of the samples from control mice examined.

Administration of an antiserum against TNF decreases resistance against *N. brasiliensis*. Mice were injected intravenously with rabbit anti-mouse TNF antiserum or with normal rabbit serum as a control 2 h before infection and were given four doses at 5-day intervals during infection, and the hepatic bacterial load was assessed. As suggested by Fig. 3, elimination of endogenous TNF led to a significant increase in the number of CFU recovered from livers of infected mice after 2 weeks of infection ($P < 0.001$).

Activated peritoneal macrophages in mice infected with *N. brasiliensis*. In order to determine whether infection with *N. brasiliensis* results in a population of activated macro-

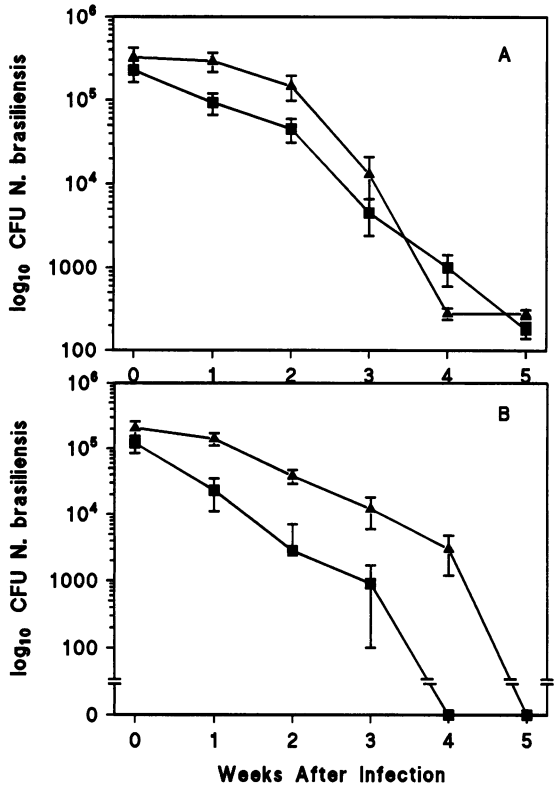

FIG. 1. Growth of *N. brasiliensis* in spleens (▲) and livers (■) of mice treated intravenously (A) or intraperitoneally (B) with 10^6 CFU of *N. brasiliensis*. Vertical bars represent the standard deviations for four mice. Representative results of one experiment repeated three times with similar results are given.

phages, peritoneal macrophages from infected mice (2 weeks after intraperitoneal infection) and normal mice (given thioglycolate medium intraperitoneally 4 days before harvest) were tested for their ability to inhibit [^{3}H]TdR uptake by

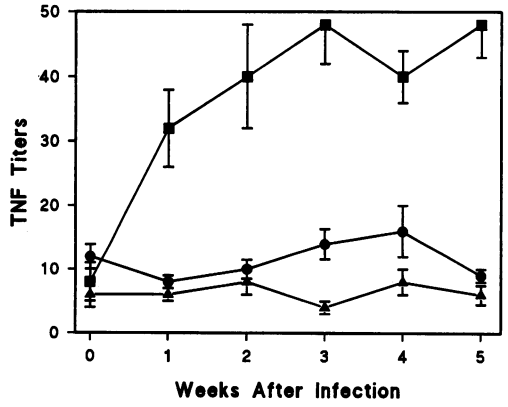

FIG. 2. Production of TNF in the bloodstream of mice following *N. brasiliensis* infection. Mice were injected intravenously with 10^6 CFU of *N. brasiliensis* cells (■), and blood was taken at different times postinjection. Mice injected with saline were used as controls (▲). TNF activities in the experimental sera previously treated with anti-TNF antiserum were also determined (●). Vertical bars represent the standard deviations for four mice. Representative results of one experiment repeated twice with similar results are given.

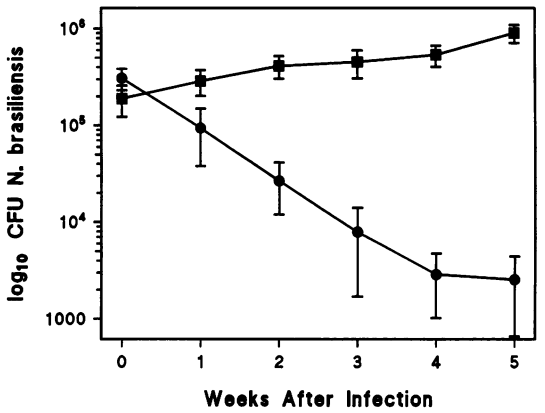

FIG. 3. Growth of 10^6 CFU of *N. brasiliensis* in livers of mice treated with anti-TNF antiserum (■) or normal rabbit serum (●) 2 h before intravenous infection and given four doses at 5-day intervals during infection. Vertical bars represent the standard deviations for four mice. Representative results of one experiment repeated twice with similar results are given.

tumor target cells in a cytostatic assay. The results, shown in Fig. 4, revealed that macrophages from infected mice inhibited [^{3}H]TdR uptake by the target cells more than did normal macrophages ($P < 0.001$). To evaluate whether the observed cytotoxic activity against L929 cells was mediated by TNF, the activated peritoneal macrophages were pretreated with anti-TNF antiserum before testing. As shown in Fig. 4, the cytotoxic activity against L929 cells can be fully neutralized by the TNF-specific antiserum but not by control serum.

TNF released in vitro by peritoneal macrophages from *N. brasiliensis*-infected mice. Peritoneal macrophages were recovered from normal mice and from mice infected intraperitoneally 2 weeks before with 10^6 *N. brasiliensis* cells and incubated in the presence or absence of lipopolysaccharide (LPS) (10 μg/ml). Figure 5 shows that the TNF activity generated by LPS-treated peritoneal macrophages from infected mice was higher than that observed with LPS-treated peritoneal cells which were obtained from normal mice. Moreover, spontaneous TNF liberation into the culture

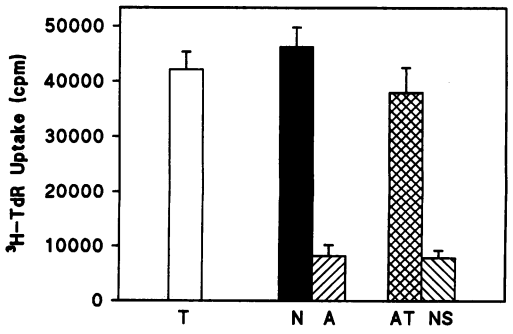

FIG. 4. [^{3}H]TdR uptake by L929 target cells alone (T) or cultured with normal (N) or activated (A) macrophages. Activated macrophages were obtained 2 weeks after intraperitoneal inoculation of mice with 10^6 *N. brasiliensis* cells. Cytotoxicity against L929 cells by activated macrophages was also determined in the presence of anti-TNF antiserum (AT) or in the presence of normal rabbit serum (NS) as a control. Vertical bars represent the standard deviations for triplicate cultures. Representative results of one experiment repeated twice with similar results are given.

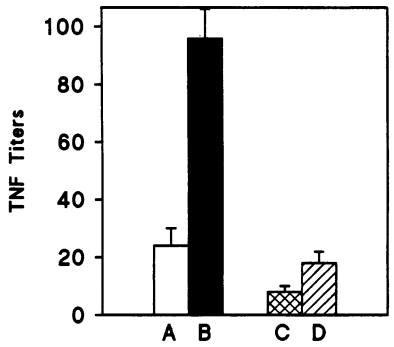

FIG. 5. Comparison of TNF activity in supernatants of peritoneal cells from infected mice (inoculated 2 weeks before with 10^6 *N. brasiliensis* organisms) or normal mice. Peritoneal cells from infected mice were incubated in the absence (A) or presence (B) of LPS (10 μg/ml) as a stimulant. Peritoneal cells from normal mice were also incubated in the absence (C) or presence (D) of LPS. Vertical bars represent the standard deviations for triplicate cultures. Representative results of one experiment repeated twice with similar results are given.

supernatant (in the absence of LPS) by peritoneal macrophages from infected mice was also observed, the amount of TNF in this case being less than that observed when the cells were incubated in the presence of LPS.

Effect of administration of anti-TNF antibody on nocardiacidal activity of peritoneal macrophages obtained from *N. brasiliensis*-infected mice. We investigated whether administration of anti-TNF antiserum to mice would interfere with the induction of activated macrophages during *N. brasiliensis* infection. Mice were injected intravenously with normal rabbit serum or rabbit anti-TNF antiserum 2 h before being injected intravenously with 10^6 CFU of *N. brasiliensis* cells or saline only; they were given thioglycolate medium intraperitoneally 4 days later. Peritoneal cells were harvested on day 8 of the antiserum treatment, and their nocardiacidal activities were estimated in vitro (Table 1). The nocardiacidal activity of peritoneal cells obtained from *N. brasiliensis*-infected mice treated with normal rabbit serum was significantly higher than that of the uninfected controls ($P < 0.02$). However, the increase in the activity induced by the infection was significantly suppressed in mice injected with anti-TNF antiserum ($P < 0.001$).

TABLE 1. Effect of in vivo administration of anti-TNF antiserum on in vitro nocardiacidal activity of peritoneal macrophages

Pretreatment[a]	No. (10^5) of viable intracellular *N. brasiliensis* cells at[b]:	
	0 h	6 h
N. brasiliensis alone	5.30 ± 0.56	0.28 ± 0.11[c]
Rabbit serum	6.80 ± 0.42	7.20 ± 0.57[d]
Rabbit serum + *N. brasiliensis*	4.90 ± 0.29	0.46 ± 0.12[c]
Anti-TNF + *N. brasiliensis*	5.70 ± 0.47	6.40 ± 0.33[d]
Anti-TNF	4.30 ± 0.38	4.70 ± 0.39[d]

[a] Mice were pretreated and the number of viable *N. brasiliensis* cells was determined as described in the text.

[b] Each result represents the mean CFU ± standard deviation for triplicate macrophage cultures.

[c] $P < 0.001$ compared with value for corresponding group at 0 h.

[d] $P < 0.001$ compared with value for group at 6 h without antiserum pretreatment.

DISCUSSION

Although the precise pathogenesis of experimental *N. brasiliensis* infection in mice is unknown, we used the mouse model to determine whether host resistance to *N. brasiliensis* exists and, if so, by what mechanism it operates. In every instance of both intraperitoneal and intravenous inoculation of *N. brasiliensis*, there was a decrease in the number of viable organisms in the spleens and livers of infected mice. These results clearly demonstrate that mechanisms of host resistance to *N. brasiliensis* infection may be operating.

Peritoneal macrophages from *N. brasiliensis*-infected mice inhibited the uptake of a radiolabeled DNA precursor by L929 cells to a significantly greater extent than did macrophages from uninfected control mice; this difference indicated that infection of mice with *N. brasiliensis* produces a population of activated peritoneal macrophages. Murine infection with certain intracellular parasites, such as *Toxoplasma gondii* and *Listeria monocytogenes*, also produced a population of activated macrophages, which are considered to be of fundamental importance in protecting the animal from challenge with homologous and heterologous intracellular organisms (19). The mechanisms by which activated macrophages selectively recognize and destroy *N. brasiliensis* and their regulatory role in the generation of the inflammatory reaction induced by this microorganism are unknown, but they may involve the release of mediators. Thus, a number of lines of evidence suggest that activated macrophages or their products are important in resistance to infection. TNF, a protein mainly produced by macrophages, has diverse effects on a wide variety of cells throughout the body, including leukocytes, tumor cells, and fibroblasts, and has profound and important biological effects in the intact host (3, 16). On the basis of these observations, the studies presented here demonstrated that TNF was produced endogenously, probably by activated macrophages, and played an essential role in host defense against *N. brasiliensis* infection. Administration to the infected mice of rabbit antibody against murine recombinant TNF inhibited the generation of activated macrophages and abolished the clearance of bacteria from the spleens and livers of infected mice.

The ability of activated peritoneal macrophages recovered from *N. brasiliensis*-infected mice to inhibit the growth of *N. brasiliensis* in vitro compared with that of control peritoneal macrophages was evident by quantitation of viable organisms in monolayers of macrophage preparations 6 h after challenge. The results of the quantitative analysis revealed that activated macrophages killed a significant portion of *N. brasiliensis* in the challenge inocula.

Nocardiosis has been associated with many factors, some of them concerning the host response, as in the case of immunocompromised hosts (1), and others related to bacterial competence, such as bacterial strain, virulence, and cell constituents (5, 6, 10). Several constituents of *N. brasiliensis* have been characterized and have been correlated with virulence and pathogenicity (5, 6). Lipids and polysaccharides have been shown to be the most important of these components. In a recent study, we have shown that these fractions, mainly the glycolipid trehalose dimycolate fraction, are able to induce an inflammatory response in mice, a fact suggesting that they play a role in pathogenicity (21). It has also been demonstrated that this glycolipid is one of the immunostimulants and toxic constituents of the cell walls of several strains of bacteria, including mycobacteria (17), nocardiae (26), and corynebacteria (27). The capacity of mycobacteria to enhance the nonspecific resistance of mice

to tumors (2, 20) and bacterial infections (18) is related to the presence of the glycolipid trehalose dimycolate. The immunostimulant properties of this glycolipid are well known, and activation occurs at the level of the mononuclear phagocytic system (21), with TNF being the main mediator released during the activation process (22). Taken together, these findings may contribute to the elucidation of the roles of TNF and macrophage activation in the natural mechanism of mouse resistance to *N. brasiliensis* infection.

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