Immune Response to *Nocardi a brasiliensis* Antigens in an Experimental Model of Actinomycetoma in BALB/c Mice

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Nine- to twelve-week-old BALB/c mice were injected in footpads with $10^7$ CFU of a *Nocardi a brasiliensis* cell suspension. Typical actinomycetoma lesions, characterized by severe local inflammation with abscesses and fistula formation, were fully established by day 28 after infection. These changes presented for 90 days, and then tissue repair with scar formation slowly appeared, with complete healing after 150 days of infection. Some animals developed bone destruction in the affected area. Histopathology showed an intense inflammatory response, with polymorphonuclear cells and hyaloid material around the colonies of the bacteria, some of which were discharged from draining abscesses. Sera from experimental animals were analyzed by Western blotting, and immunodominant antigens P61 and P24 were found as major targets for antibody response. Anti-P24 immunoglobulin M (IgM) isotype antibodies were present as early as 7 days, IgG peaking 45 days after infection. Lymphocyte proliferation with spleen and popliteal lymph node cells demonstrated thymidine incorporation at 7 days, the stimulation index decreasing by day 60. Levels of interleukin-1 (IL-1), IL-2, IL-4, IL-6, tumor necrosis factor alpha, and gamma interferon (IFN-γ) were determined by enzyme-linked immunosorbent assay in the sera of infected animals. The circulating levels of IFN-γ increased more than 10 times the basal levels; levels of IL-4, IL-6 and IL-10 also increased during the first 4 days of infection.

Intracellular facultative pathogens such as *Mycobacterium tuberculosis*, *Nocardi a brasiliensis*, and others acquire effective, evasive mechanisms that prevent their destruction and adapt to multiply in host cells. Among these cells, the phagocytes are the principal targets, ingesting the bacteria opsonized with antibody or complement components, which in the case of extracellular pathogens leads to their destruction but in the case of intracellular bacteria may help to spread the infection (11). Some cytokines such as gamma interferon (IFN-γ) may induce or activate bactericidal mechanisms of the infected macrophages and help to clear the infection (12). Bacterial clearance or disease progression is related to pathogenic or virulence factors of the offending microbe on the one hand and the immune response killing ability of the host on the other. Nonspecific killing ability of the macrophages is dramatically increased by specific T lymphocytes during the course of an infection (10).

*N. brasiliensis* is a bacterium that lives as a saprophyte in the soil and enters the skin by traumatic inoculation. Even though many persons are accidentally inoculated, few develop the actinomycetoma lesion; host mechanisms that control and heal the lesion are unknown. Anti-*N. brasiliensis* antibodies have been demonstrated both in human patients and in experimental animals (15, 16). The role of these antibodies in host protection is not clear (2, 17); in humans, the presence of anti-*N. brasiliensis* antibodies has been helpful in serodiagnosis and has recently been introduced for use in routine clinical laboratories (18). Animal models have been used to study the nocardial infections that induce mycetoma both in mice and in rats (4–6, 8, 9, 21). More recently, Zlotnik and Buckley described the experimental production in BALB/c mice of actinomycetoma resembling the typical chronic mycetoma lesion (22). However, the immune response to *N. brasiliensis* antigens has been studied to only a limited extent (14). In the present work we describe the clinical and histopathologic changes in an experimental model of actinomycetoma in mice. The anti-*N. brasiliensis* antibody response and lymphocyte proliferation were also studied. Th1 and Th2 cytokines were determined during the evolution of mycetoma lesion. Potential utility of this mycetoma model to dissect the complex host-parasite relationship can, perhaps, be extended to other intracellular pathogens.

**MATERIALS AND METHODS**

**Animals.** We used 9- to 12-week-old male and female BALB/c mice. These animals were derived from the colony kindly donated by Carl Hansen (Small Animal Section, Veterinary Resources Branch, National Institutes of Health, Bethesda, Md.) and kept under ordinary conditions with Purina rodent food and water available ad libitum.

**Bacterial strain.** *N. brasiliensis* HUJEG-1 was isolated from a patient with human actinomycetoma who was attending the Dr. José E. González University Hospital, Monterrey, Mexico. Jane Brown (Actinomycete Laboratory, Centers for Disease Control and Prevention, Atlanta, Ga.) kindly reconfirmed the identification. This strain is maintained in Sauvouraud agar culture and is registered as ATCC 700358.

**Experimental mycetoma induction.** *N. brasiliensis* was cultured in brain heart infusion medium to prepare a unicellular suspension containing $10^7$ CFU per ml in the log phase of growth; 100-μl aliquots of the suspension were injected in saline solution without adjuvant in the footpad. Animals were observed daily to evaluate inflammation, formation of abscesses and fistulae, and presence of secretion. A group of five animals was sacrificed by cervical dislocation every week after the infection up to 300 days postinfection. Serum samples were obtained for anti-*N. brasiliensis* antibody determination by enzyme-linked immunosorbent assay (ELISA), Western blot analysis, and cytokine quantification. The affected feet were removed for histopathology study; the spleen and draining popliteal lymph nodes from each animal were aseptically removed for culturing and flow cytometric study.
N. brasiliensis antigen preparation. Soluble protein antigen was prepared for Western blotting and as starting material for immunodominant antigen purification for the ELISA and the lymphocyte proliferation assay. The technique for preparing cell extracts has been published elsewhere (18). Briefly, N. brasiliensis was cultured in 1-liter Erlenmeyer flasks with 170 ml of brain heart infusion medium (Difco Laboratories, Detroit, Mich.) for 7 days at 37°C. Bacterial mass was extensively washed with distilled water and defatted with ethanol-ethyl ether; protein antigens were extracted with 0.01 M Tris-HCl containing 0.01 M magnesium acetate by stirring. The supernatant was obtained by ultracentrifugation and dialyzed. This crude antigen will hereafter be called the crude cellular extract (CCE).

Purification of immunodominant antigens. N. brasiliensis CCE was precipitated with 50% ammonium sulfate solution; the supernatant was extensively dialyzed and lyophilized. After being reconstituted with 1 ml of phosphate-buffered saline (pH 7.2), it was incubated for 2 h at 37°C with 150 μg of DNase I (Sigma Chemical Co., St. Louis, Mo.) and then applied to a Sephadex G-100 column as previously described (16, 18). Fractions containing the immunodominant antigen P24 were collected and used as purified antigen for ELISA and for lymphocyte stimulation in culture.

Anti-N. brasiliensis antibody quantification by ELISA. Ninety-six-well polystyrene plates (Costar product no. 9017) were incubated with purified antigen at a concentration of 0.5 μg per well as previously described (18). Plates were then washed and blocked with 5% skimmed milk in phosphate-buffered saline; sera from five immunized mice bled at days 0, 7, 14, 28, 45, 60, 80, 90, and 160 were diluted 1:50 before the assay; after incubation at 37°C for 60 min, the plates were washed again and 100 μl of goat anti-mouse antibody conjugated to peroxidase was added. We used a chromogen substrate solution containing hydrogen peroxide and o-phenylenediamine. The A₄₉₂ was read with a semiautomatic ELISA plate reader. Antibody isotyping was done by an ELISA technique using rabbit anti-mouse immunoglobulin G (IgG) and anti-μ-chain-specific sera (Sigma) as secondary antibodies.

Immunodominant antigen identification by Western blotting. The CCE was first resolved by polyacrylamide gel electrophoresis (PAGE) with sodium dodecyl sulfate (SDS). We used the Laemmli discontinuous buffering system (7) with an 8 to 18% gradient resolving gel and a 5% stacking gel. After electrophoresis, protein bands were transferred to nitrocellulose membranes by using a Trans-blot cell (Bio-Rad) as specified by Towbin et al. (19) and incubated with the serum samples from immunized mice; individual nitrocellulose strips were then washed. An anti-mouse IgG-peroxidase conjugate was added, and 0.2% hydrogen peroxide with diaminobenzidine (Sigma) was used as the chromogen substrate solution.

Lymphocyte proliferation assay. The animals were killed by cervical dislocation; draining popliteal lymph nodes and spleen were aseptically removed and teased. Mononuclear cells were suspended at a concentration of 10⁶ per ml of RPMI 1640 culture medium supplemented with 2 mM glutamine, 25 mM HEPES, 100 U of penicillin per ml, and 10% heat-inactivated fetal calf serum. Cultures containing 2 × 10⁵ cells in 0.2 μl were stimulated with either UV-killed N. brasiliensis, CCE, or purified immunodominant antigen and incubated in 96-well flat-bottom sterile plates (Falcon 3020; Becton Dickinson, Oxnard, Calif.) at 37°C for 5 days. Tritiated thymidine (37 kBq/well; Amersham) was added 16 h before harvesting with a semiautomatic microharvester (MASH II); thymidine incorporation was quantitated with a liquid scintillation counter (Beckman).

The results are expressed as mean counts per minute of triplicate cultures, and the stimulation index was calculated by dividing the mean values for stimulated cultures by mean values for of unstimulated cultures.

Cytokine quantification. Sera from animals at different times of infection were obtained and diluted 1:5 as instructed by the manufacturer (Endogen, Woburn, Massachusetts). The cytokine level was determined by ELISA using commercial kits (Endogen) according to the manufacturer’s instructions. The results are presented as mean cytokine levels ± SEM for triplicate cultures.

FIG. 1. Clinical evolution of mycetoma in BALB/c mice. (A) Normal hind footpad before infection; (B) severe edema greater than 7 mm at 15 days of infection; (C) edema abscess and sinus 28 days after infection.

FIG. 2. Typical full-blown mycetoma lesion, with 19-mm edema open abscess, fistula formation, and healing scars, 90 days after infection.
Quantitative ELISAs in microplates were used for interleukin-1β (IL-1β), IL-2, IL-4, IL-6, IL-10, tumor necrosis factor alpha (TNF-α), and IFN-γ.

RESULTS

Clinical evolution of the mycetoma. Inflammation was present as early as 72 h after injection of *N. brasiliensis* cells; average diameter of the hind footpad before injection was 3 to 4 mm, reaching 7 mm after 3 days. Increasing edema continued during daily observations. Hyperemia and small abscesses were also present until day 14; a clear decrease in edema was detected over the subsequent 14 days. Figure 1 shows morphologic changes in control and infected animals. We recorded signs of inflammation from day 1 to 200 days after infection, using the following clinical scale for evaluation: +, slight edema; ++, 7-mm edema plus abscess and ulceration; ++++, edema, abscess, and granule discharge; +++++, full-blown mycetoma. Eighty percent of infected mice developed a typical mycetoma lesion as presented in Fig. 2, where all of these signs are apparent; bone destruction was also present in the chronic disease, as evidenced in an X-ray film not shown. About 60% of infected BALB/c mice recovered from infection spontaneously after 150 days. In other experiments (data not shown), we demonstrated that the production of mycetoma was easier and reproducible when *N. brasiliensis* in filaments characteristic of the log phase rather than cells from the stationary phase were used. The number of injected bacteria also determines the production of mycetoma lesions; for example, neither 10⁶ nor 10⁷ CFU/ml induced mycetoma, inflammation of the popliteal

FIG. 3. Histopathological evolution of mycetoma. Paraffin-embedded sections stained with hematoxylin and cosin demonstrate multiple microabscesses (A; magnification, ×40), foamy macrophages limiting the abscess by day 10 (B), polymorphonuclear and mononuclear cells, with giant vacuolar macrophages also visible (C; magnification, ×40), and *N. brasiliensis* microcolony 90 days after infection (D).

FIG. 4. Western blot identification of immunodominant antigens. The nitrocellulose strips contain soluble crude cellular extract from *N. brasiliensis* incubated with sera from infected mice at different times after infection. Strip A, negative control before infection; strips B and C, sera from infected BALB/c mice 30 and 90 days; strip D, serum from BALB/c mouse 160 days after infection.
lymph node in these animals being so slight that recovery of cells for proliferation assay was not possible.

**Histopathology of experimental mycetoma.** Freshly obtained purulent material from mycetoma lesions was stained on a glass slide by using Kinyoun carbol fuchsin and methylene blue. The lesion was characterized by the presence of mononuclear and polymorphonuclear cells containing abundant acid-fast bacilli in aggregates and filamentous formation. Hema-toxyl-in-and-cosin-stained sections from paraffin-embedded tissue demonstrated an intense inflammatory response characterized by highly packed granulocytes in multiple microabscesses with poorly defined limits during the first 7 days after infection. By day 10, we observed an increasing number of macrophages at the periphery of the lesion as well as histiocytes with the classical foamy appearance as seen in lepromatous leprosy infection. By day 30 after infection, well-defined multiple abscesses were clearly limited by these foamy macrophages, with central *N. brasiliensis* microcolonies; a typical *N. brasiliensis* lesion is shown in Fig. 3.

**SDS-PAGE analysis of *N. brasiliensis* antigens.** The CCE obtained as described above and resolved by SDS-PAGE on an 8 to 18% gradient polyacrylamide gel shows a complex mixture of bands with molecular mobility from 10 to 66 kDa as previously published (16); CCE and purified fraction were used for Western blot identification of immunodominant antigens. The purified fraction was used to stimulate in vitro lymphocyte proliferation.

**Immunodominant antigen identification.** Sera from BALB/c mice injected with live bacteria were collected at different times after infection. It was clear that 30 days after bacterial inoculation, the IgG anti-*N. brasiliensis* antibodies reacted with bands with molecular mobilities identical to those recognized by sera from human actinomycetoma patients. The P61 and P24 bands are immunodominant antigens in infected mice, as shown in Fig. 4.

**Anti-*N. brasiliensis* antibody quantification by ELISA.** A purified antigen preparation containing immunodominant antigen P24 was used in an ELISA to quantitate the IgG and IgM in sera from mice at different times after infection. As shown in Fig. 5, IgM antibodies were present as early as 7 days (standard deviation [SD], ±0.070), and peaked at day 14 (SD, ±0.140), while the IgG isotype increased by day 28 (SD, ±0.647) and continued to increase up to 163 days after infection (SD, ±0.053).

**Lymphocyte proliferation assay.** Popliteal lymph node lymphocytes from infected BALB/c mice exhibited high stimulation index when stimulated in vitro with UV-killed *N. brasiliensis* cells compared with CCE and purified P24. Spleen cells showed the opposite effect, its highest proliferation being induced with the soluble CCE. Thymidine incorporation by proliferating lymphocytes was present at 7 days for cells from the draining popliteal lymph nodes and at 14 days for those from spleen lymphocytes. Figure 6 summarizes the results of the proliferation assay.

**Cytokine quantification in sera from infected animals.** Circulating levels of IL-1β, IL-2, IL-4, IL-6, IL-10, TNF-α, and IFN-γ were determined in sera from infected mice at weekly intervals. In the first group of experiments, IL-10 and IFN-γ increased during the first week after bacterial infection. In other experiments, we collected blood daily and, as shown in Table 1, found that levels of IL-4, IL-6, IL-10, and IFN-γ dramatically increased as early as 24 h after infection. Cytokine basal levels rose 4- to 10-fold during the first week of infection. On the other hand, IL-β, IL-2, and TNF-α levels showed no variation.

### Table 1. Cytokine quantification after infection with *N. brasiliensis*

<table>
<thead>
<tr>
<th>Day after infection</th>
<th>Conc. (pg/ml; mean ± SD)*</th>
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<tbody>
<tr>
<td></td>
<td>IL-4</td>
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<tr>
<td>0</td>
<td>39 ± 11</td>
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<tr>
<td>1</td>
<td>192 ± 14</td>
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<tr>
<td>2</td>
<td>250 ± 66</td>
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<td>3</td>
<td>233 ± 52</td>
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<td>250 ± 45</td>
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<td>7</td>
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<tr>
<td>28</td>
<td>82 ± 10</td>
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<td>300</td>
<td>83 ± 31</td>
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* Mean value for five BALB/c mice from day 0 (before infection) to 300 days (after infection). ND, not determined.
DISCUSSION

The experimental model of actinomycetoma described in this report is reproducible and resembles the histopathological changes described for human infections. There is no need to inject incomplete Freund’s adjuvant, thus avoiding the severe inflammation and high mortality rate associated with its use. The experimental model of actinomycetoma in mice described by Zlotnik and colleagues also appeared 28 days after infection with $1.2 \times 10^7$ CFU per ml, but with the disadvantage of using incomplete Freund’s adjuvant (14, 22). More recently, Welsh-Lozano and coworkers have developed a mycetoma model in rats which may be of help in identifying mechanisms of resistance to nocardial infection (21). The anti-\(N.\) brasiliensis antibody response in BALB/c mice in our experimental model is identical to that of the mycetoma patients detected by Western blotting. Moreover, its titer in the quantitative ELISA is high during the infectious process as it is in human patients. In both cases, IgG antibodies react with the same immunodominant antigens. The role of these anti-\(N.\) brasiliensis antibodies in host protection is not clear; some authors have suggested that their presence is not important (2, 3, 13). However, adaptive immunity by passively transferring sera from sensitized mice protected BALB/c mice from developing mycetoma (17). More experiments are needed to understand this observation, and the experimental model used in this work may be helpful in this regard. Serum levels of both Th1 and Th2 cytokines such as IFN-\(\gamma\), IL-4, IL-6, and IL-10 were dramatically increased from days 1 to 4 of infection. The role of Th1 cytokines such as IFN-\(\gamma\) in protection against some intracellular pathogens is clear, but data on nocardial infections are lacking. In the experiments described here, we found that IFN-\(\gamma\), IL-4, IL-6, and IL-10 concentrations increased during the first week of infection, when inflammation is very important too. Surprisingly, there was no increase in IL-1 and TNF-\(\alpha\) levels, for which we have no explanation. However, the role of these mediators in host protection was not studied and may not be as simple as recently proposed (1). The experimental model of intracellular pathogen infection described in this work and results with other intracellular pathogens used by other investigators may clarify the role of cell-mediated effectors in killing intracellular parasites (11). In addition, the mycetoma model described here will be of great help in dissecting the complex and dynamic host-parasite relationship during the acute, chronic, and healing phases of the infection: pathogenic mechanisms and virulence factors of \(N.\) brasiliensis may be examined as well. Furthermore, the availability of congenic BALB/c strains, mice with knockouts of several genes, and anti-CD monoclonal antibodies may be valuable for understanding the host protective response that leads to resolution of the infectious process, knowledge necessary for design of a vaccine. Finally, the findings obtained with this model may be extended to studies using \(M.\) tuberculosis, another facultative intracellular microbe that shares with \(N.\) brasiliensis chemical composition and antigens and induces similar histopathological changes in affected areas, with many advantages regarding both reproducibility and safety in handling a human pathogen.

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