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Monoclonal Antibodies to P24 and P61 Immunodominant Antigens from *Nocardia brasiliensis*

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We prepared a *Nocardia brasiliensis* cell extract and purified two immunodominant antigens with molecular weights of 61,000 and 24,000. The isolated proteins were shown to be reasonably pure when analyzed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (8 to 18% polyacrylamide gradient) and stained with Coomassie blue and silver nitrate. By using an immunoelectrotransfer blot method (Western blotting), we demonstrated that these two purified proteins reacted strongly with serum from *N. brasiliensis*-infected mycetoma patients. To obtain anti-P61 and anti-P24 monoclonal antibodies (MAbs), we used an *N. brasiliensis* cell extract as the antigen for the first immunization; 2 weeks later female mice were reimmunized with a semipurified antigen containing the P24 or P61 fraction. A booster injection was given 3 days before the fusion was carried out. Two hybrids that reacted strongly with P24 were cloned by limiting dilution, the generated MAbs were analyzed for isotyping, and their specificity was tested in a Western blot assay with cell extracts from *Nocardia asteroides* and *Mycobacterium tuberculosis* cultures. Anti-P24 MAbs were shown to be specific for *N. brasiliensis* HUJEG-1 and did not cross-react with either the *N. asteroides* or *M. tuberculosis* strains used. However, additional studies with several *N. asteroides* and *N. brasiliensis* strains are needed to investigate whether there are cross-reactions between strains or species when these MAbs are used. The anti-P61 and anti-P24 MAbs were used to locate the antigen in *N. brasiliensis* cells by immunofluorescence. The lack of reaction with intact cells suggests that the P24 and P61 antigens are not exposed in the complete bacterial cell surface or that the recognized epitopes are different. Only one anti-P61 MAb that reacted specifically with the *N. brasiliensis* cell extract was obtained.

Nocardia brasiliensis is a gram-positive bacterium, related to mycolic acid-containing actinomycetes like the genera *Mycobacterium*, *Corynebacterium*, *Gordona*, etc. (5, 10, 17), that lives in the soil as a saprophyte. *N. brasiliensis* infects humans by traumatic inoculation through the skin; some of these infected persons may develop actinomycetoma (21). Mycetoma is a chronic infectious disease that involves skin and subcutaneous tissues, and it can be produced by fungi or by bacteria such as *N. brasiliensis*. Although mycetoma is usually a local lesion, in some cases it can extend to the bones and destroy adjacent organs (19, 20). On the other hand, *Nocardia asteroides* produces the systemic disease nocardiosis and may infect the brain and lungs, producing an abscess (1). *N. asteroides* is sometimes isolated from mycetoma patients. However, this determination may not always be correct because traditional methods of identifying Nocardia species are inaccurate, and more complete species identification methodology has recently been published (19). The role of the immune response in resistance to *N. brasiliensis* infection is not clear. In both murine models (4, 13) and human cases, it has been suggested that cell-mediated immunity plays an important role in resistance (22). However, the chemical nature of the involved epitopes has never been identified because purified antigens have not been available. In a previous work we demonstrated that a crude extract from *N. brasiliensis* reacted strongly with sera from infected patients in a Western blot assay, and we concluded that P61, P26, and P24 were immunodominant antigens (14). We isolated and purified two of these immunodominant antigens with molecular weights of 61,000 and 24,000 by using simple physicochemical methods (18). The serodiagnosis of nocardial infection has been difficult in the past due in part to the low sensitivity of the techniques used and to cross-reactions between the sera from the infected patients and the sera from mycobacterium-infected individuals (2, 6, 23). In 1986, El-Zaatari et al. obtained two monoclonal antibodies (MAbs) against *N. asteroides* proteins that lacked cross-reactivity with mycobacterial antigens (3). More recently, Jiménez et al. (7) produced six stable cell lines that secrete antinocardia MAbs. However, these MAbs cross-react with *N. asteroides* and with a culture filtrate from several *Mycobacterium* strains. Using the P24 and P26 protein fractions, we developed a conventional solid-phase enzyme-linked immunosorbent assay (ELISA) with great specificity and sensitivity. This assay was very helpful in the serological diagnosis of *N. brasiliensis* infection and in assessing response to medical treatment. Furthermore, we found a close correlation between anti-P24 and anti-P26 antibody concentration and the clinical condition of mycetoma patients (15). Purified antigens are needed to define their chemical composition and to study their role in host protection.

In this work we describe the generation of MAbs that reacted with the P61 and P24 immunodominant antigens from *N. brasiliensis*. We were unable to locate the P24 and P61 antigens in various cell preparations using an immunofluorescence technique. The possible use of these antibodies in purifying antigen for serodiagnosis, exploring species specificity, analyzing cross-reactions, and screening an *N. brasiliensis* gene library is now at hand.

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MATERIALS AND METHODS

***Nocardia* strain.** In this study we used *N. brasiliensis* HUJEG-1, isolated from a mycetoma patient at the José E. González University Hospital; its identification

was made by microbiological methods and kindly confirmed by L. Ajello at the Centers for Disease Control and Prevention (Atlanta, Ga.). Batch cultures for antigen purification were grown in 1-liter flasks, each containing 170 ml of brain heart infusion medium (Difco Laboratories, Detroit, Mich.); after 7 days of incubation at 37°C, cells were harvested and washed extensively with distilled water.

***N. brasiliensis* crude extract.** The bacterial mass was defatted with ethanol-ethylic ether (1:1) and desiccated under a vacuum at room temperature. To obtain a crude extract from the *N. brasiliensis* cell cultures, we used the technique described in detail elsewhere (15) with modifications. Dried cells were ground with glass powder, suspended in 0.01 M Tris-HCl (pH 7.4)–0.01 M magnesium acetate, and magnetically stirred overnight at 4°C. Unbroken cells and debris were removed by centrifugation at 1,200 × *g* for 15 min. The soluble fraction was obtained by centrifugation at 144,000 × *g* for 3 h at 4°C in an L8-70M ultracentrifuge (Beckman, Palo Alto, Calif.). The yellowish clear supernatant was dialyzed for 24 h against distilled water, lyophilized, and stored at −20°C until use. Protein concentration was determined by the method of Lowry et al. (11) with bovine serum albumin used as a standard. We also prepared similar extracts from *N. asteroides* and from *M. tuberculosis* H37-Ra.

***N. brasiliensis* antigen purification.** We precipitated 50 mg of bacterial crude extract with a 50% saturated ammonium sulfate solution. P61 was present in the precipitate and P24 was present in the supernatant. To further purify P61, we used a previously published method (18) with modifications. An aliquot of the precipitate containing P61 was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), with an 8 to 18% polyacrylamide gradient as the resolving gel and a 4% stacking gel, by using the Laemmli (9) discontinuous buffer system. After electrophoresis was completed, we cut out the gel slice containing P61 and electroeluted it (Electroeluter; Bio-Rad Laboratories, Richmond, Calif.) at 120 V for 2 h. To purify P24 from the supernatant obtained in the salting-out procedure described above, we used the same published method, briefly summarized as follows. After extensive dialysis for 72 h against saline solution, the sample was lyophilized and reconstituted with 1 ml of 0.01 M phosphate-buffered saline (PBS), pH 7.2, and incubated with DNase I (Sigma Chemical Co., St. Louis, Mo.) for 2 h at 37°C. The sample was applied to a Sephadex G-100 column, equilibrated with 0.01 M PBS, and eluted with the same buffer; 2-ml fractions were then collected, analyzed by SDS-PAGE, and stained with Coomassie blue R-250.

Immunization protocol. Six female, 12-week-old BALB/c mice were injected twice subcutaneously with the *N. brasiliensis* extract emulsified with incomplete Freund's adjuvant (vol/vol). Injections with the antigens were given every 2 weeks in the footpad, and in the case of P24, mice were given a third immunization with the purified antigen. Three days before the fusion was carried out, an additional 5 μg of the purified antigen with either P61 or P24 was injected intraperitoneally.

Fusion protocol. An immunized mouse was sacrificed by cervical dislocation, the spleen was aseptically removed, and a cell suspension in RPMI 1640 was mixed with NS-1 myeloma cells by the hybridoma technique described by Köhler and Milstein (8) with modifications. Spleen and myeloma cells in a ratio of 3:1 were centrifuged; after the supernatant was discarded, the pellet was suspended in 1 ml of RPMI 1640 culture medium and mixed with 1 ml of 50% polyethylene glycol 1300-1600 sterile solution. After 90 s we slowly added 20 ml of culture medium and centrifuged to remove the supernatant; finally, cells were suspended in RPMI 1640 containing 100 μM hypoxanthine, 2×10^{-5} M aminopterin, 16 μM thymidine, and 10% fetal bovine serum (HAT) and cultured in 96-well tissue culture plates (Falcon, Oxnard, Calif.) at a concentration of 2×10^5 cells in 200 μl per well. The cells were cultured at 37°C with 5% CO_2 and fed 4 days later with 100 μl of RPMI 1640 containing 100 μM hypoxanthine, 16 μM thymidine, and 10% fetal bovine serum (HT). At 14 to 21 days after fusion, we collected the supernatant from the wells having microscopic evidence of hybridoma formation and screened for anti-*N. brasiliensis* antibodies using a solid-phase ELISA. The cells from antibody-positive wells were transferred to larger culture plates to expand. Selected hybrids were cloned twice by limiting dilution and tested again for antibody formation by using an immunotransference assay (Western blotting).

ELISA screening for anti-*N. brasiliensis* antibodies. We used a microfiltration unit (Bio-Rad) with nitrocellulose membranes (0.45-μm pore size) humidified with Tris-buffered saline (20 mM Tris-HCl, 500 mM NaCl), pH 7.5. The *N. brasiliensis* cell extract (50 μg) was added to each well and incubated for 30 min at room temperature, and 1% bovine serum albumin in Tris-buffered saline was used as a blocking agent. Undiluted supernatants from positive hybrids were added and incubated for 1 h, and, after 3 washings, goat anti-mouse immunoglobulin G (IgG) conjugated to peroxidase (Sigma) was added; the chromogen substrate used contained 0.2% hydrogen peroxide and 3,3′-diaminobenzidine (Sigma) in PBS. Positive controls included serum from a rabbit and from BALB/c mice immunized with the *N. brasiliensis* antigen.

MAb isotyping. We used an ELISA to investigate the isotypes of the generated MAbs. Polystyrene 96-well plates (Costar, Cambridge, Mass.) were used in these assays. The *N. brasiliensis* crude extract (50 μg per well) was dissolved in 200 μl of acetate buffer, pH 5, and incubated overnight at 4°C; wells were washed three times with 0.01 M PBS, pH 7.4, containing 1:1,000 Tween 20. Skim milk dissolved in PBS-Tween was used as a blocking agent for 2 h at 37°C, and wells were washed five times with the washing solution; then 200 μl of tested supernatants were added to different wells, which were incubated and washed again. Rabbit anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgA, and IgM (Mouse Typer Subisotyping Kit; Bio-Rad) were added and incubated for 1 h at 37°C. After washing, 200 μl of an anti-rabbit immunoglobulin conjugated to peroxidase (Sigma) was used at a 1:1,000 dilution, hydrogen peroxide and *o*-phenylenediamine were added as chromogen substrates, and the reaction was stopped with 1 N sulfuric acid. The A_{492} was read with a microassay reader (Diamedix, Miami, Fla.).

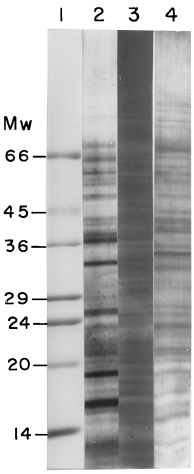

FIG. 1. Bacterial cell extracts analyzed by gradient SDS-PAGE and stained with silver nitrate. Lanes: 1, molecular weight markers (numbers on the left are in thousands); 2, *N. brasiliensis* cell extract; 3, *N. asteroides* cell extract; 4, *M. tuberculosis* extract.

MAb specificity analyzed by Western blotting. We used SDS-PAGE under denaturating conditions with an 8.75 to 18% polyacrylamide gradient and a 5% stacking gel. Samples and molecular mass markers were boiled for 2 min in a solution containing 10% SDS, 10% mercaptoethanol (vol/vol), 50% glycerol, 125 mM Tris-HCl (pH 6.8), and 0.2% bromophenol blue. Electrophoresis was carried out at 150 V with the Laemmli buffering system (9) and stopped when the blue color reached the bottom of the gel. A gel slice was stained with silver nitrate, and the rest was transferred to a nitrocellulose membrane according to Towbin et al. (16). We used the Mini Trans Blot II (Bio-Rad) and a solution containing 192 mM glycine, 20% methanol, and 25 mM Tris buffer (pH 8.3). After 1 h of transferring at 100 V, the membrane was blocked with fish gelatin (Highpure liquid gelatin; Norland Products, Inc., New Brunswick, N.J.) at a 3% concentration in PBS for 1 h at 37°C. The membrane was washed with PBS-Tween 20 and cut, and the individual strips were incubated with the cloned supernatants and washed with the above-described solution. A peroxidase-conjugated anti-mouse immunoglobulin was added, and the reaction developed color after we added the chromogen substrate solution composed of 3,3′-diaminobenzidine and hydrogen peroxide as described above. We also tested the crude extract from an *N. asteroides* culture and from *Mycobacterium* strain H37-Ra by both SDS-PAGE and Western blotting.

Indirect immunofluorescence to locate P24 and P61. An immunocytochemistry technique was used to locate the P24 and P61 antigens in the intact and delipidized *N. brasiliensis* cells. Bacteria were centrifuged in Eppendorf tubes at a concentration of 6×10^5 CFU, and the cell pellet was suspended in 200 μl of the cloned supernatants and incubated at 37°C for 1 h. After five washes, a goat anti-mouse IgG conjugated to fluorescein was added, the cells were washed again, and a smear was prepared by fixing with methanol for 30 min. Finally, the smear was examined in a fluorescence microscope (Zeiss, Oberkochen, Germany).

RESULTS

SDS-PAGE analysis of *N. brasiliensis*, *N. asteroides*, and *M. tuberculosis* antigens. The crude cell extracts from *N. brasiliensis*, *N. asteroides*, and *M. tuberculosis* exhibited a complex protein composition, as shown in Fig. 1. Interestingly, the P24 band from *N. brasiliensis* does not develop color with silver nitrate, but it does strongly stain with Coomassie blue R-250. A complete crude extract from *N. brasiliensis*, as shown in Fig. 1, was used for mouse initial immunization. The molecular

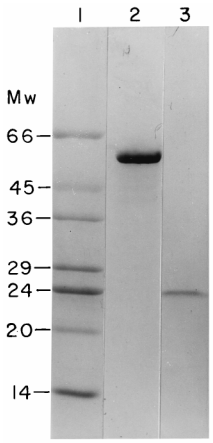

FIG. 2. SDS-PAGE analysis of *N. brasiliensis* purified proteins in an 8.75 to 18% gradient gel stained with Coomassie blue R-250. Lanes: 1, molecular weight markers (numbers on the left are in thousands); 2, purified P61; 3, purified P24.

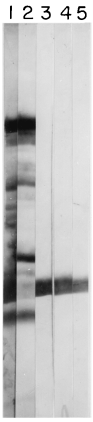

FIG. 4. Anti-P24 Western blot analysis of the *N. brasiliensis* cell extract after limiting dilution. Lanes: 1, serum from a mycetoma patient; 2, serum from the hyperimmune mouse; 3, A2 supernatant clone; 4, MAb from clone A4; 5, MAb from clone H11.

weight bands varied from 12 to 100 and were used in the Western blot assays to study the specificity of isolated MAbs. Figure 2 shows the SDS-PAGE gradient gel, which demonstrates that the major component of this fraction is P24, which had been injected as booster antigen 3 days before the fusion.

Identification of the MAbs. The supernatants of the hybrids selected in the HAT culture medium were tested in a Western blot assay using an *N. brasiliensis* cell extract. As is clear in Fig. 3, more than one hybrid product was present in the samples before cloning. After limiting dilution, we selected and expanded three clones, each producing a single MAb, as shown in Fig. 4. The anti-P61 MAb, on the other hand, was shown in the Western blot assay to be highly reactive.

MAb isotyping. The results of the ELISA to identify the isotypes of the anti-P24 MAbs are shown in Table 1; it is clear that the isotype that dominates is IgG2a, as expected according to the immunization protocol of the donor mice for a T-dependent antigen. Anti-P24 circulating polyclonal antibodies in the BALB/c mice also belong to the IgG2a isotype. Neither IgM nor IgA isotypes were found in the isolated MAbs. On the contrary, the anti-P61 MAb belongs to the IgG2b isotype.

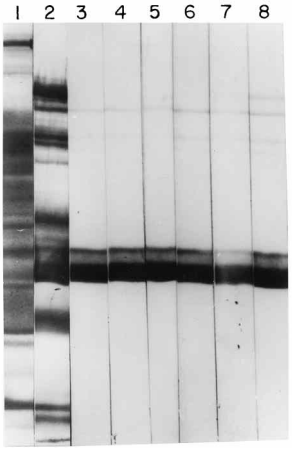

FIG. 3. Western blot analysis of the *N. brasiliensis* cell extract. Lanes: 1, serum from a mycetoma patient; 2, serum from the hyperimmune donor mouse; 3 to 8, reactions of culture supernatants from six positive hybrids with P24 and other bands.

Anti-P24 and anti-P61 MAb specificity analyzed by Western blotting. The cellular extracts from *N. brasiliensis*, *N. asteroides*, and *M. tuberculosis* were tested in a Western blot assay with the MAbs produced by clones A2, A4, and H11; the results presented in Fig. 5 demonstrate that these MAbs react only with the P24 band present in the extract from *N. brasiliensis* and not with the *N. asteroides* or *M. tuberculosis* antigens. The anti-P61 MAb was also shown to be specific, since it does not react with *M. tuberculosis* or *N. asteroides* (Fig. 6).

Immunocytochemistry studies. Indirect immunofluorescence with defatted or intact *N. brasiliensis* cells shows a lack of reactivity with the MAbs isolated in this study. The surfaces of intact or delipidized cells show no fluorescence. In other experiments with whole bacterial cells, we used peroxidase-conjugated anti-mouse IgG instead of the fluorescein-labeled antibody, but no reaction was observed. BALB/c normal and hyperimmune sera were also included in these experiments, with the same negative results.

DISCUSSION

We obtained three different clones (A2, A4, and H11) that produce MAbs which react with the *N. brasiliensis* P24 protein. It should be noted that immunized mice recognize the P24 protein as an immunodominant antigen when they are injected with the crude cellular extract, just as actinomycetoma patients did. We previously have shown as well that sera from *N. brasiliensis*-infected mycetoma patients do react with P24 in a Western blot assay, suggesting that this protein is one of the three major targets of the antibody response (14). In addition, anti-P24 antibody response in mycetoma patients is also an IgG response and is directly correlated with mycetoma pro-

TABLE 1. Anti-P24 MAb isotyping determined by ELISA

Sample	A_{492} obtained with anti-mouse antibodies			
	IgG1	IgG2a	IgG2b	IgG3
Serum from spleen donor mouse	1.402	1.218	1.300	0.153
Clone A2	0.170	0.436	0.153	0.122
Clone A4	0.189	0.550	0.148	0.106
Clone H11	0.151	0.542	0.149	0.124
Clone 4E2 (anti-P61)	0.154	0.158	1.202	0.076

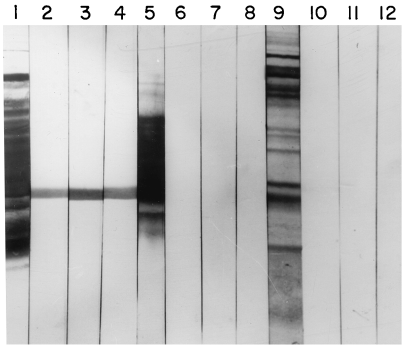

FIG. 5. Specificity of anti-P24 MAbs A2 (lanes 2, 6, and 10), A4 (lanes 3, 7, and 11), and H11 (lanes 4, 8, and 12) by Western blot analysis. Cell extracts from *N. brasiliensis* (lanes 1 to 4), *N. asteroides* (lanes 5 to 8), and *M. tuberculosis* (lanes 9 to 12) were also reacted with serum from an *N. brasiliensis*-infected mycetoma patient (lane 1), serum from an *N. asteroides*-infected patient (lane 5), or serum from a lung tuberculosis patient (lane 9).

gression as determined by ELISA (15). The anti-P24 MAbs isolated in this study belong to the IgG2a isotype; this is in contrast to the findings by Jiménez and coworkers, who reported that four of six MAbs were of the IgM isotype (7). This can be explained in part because those authors immunized only once, before the fusion booster, and we immunized three times to obtain a secondary humoral response. Moreover, it is possible that the IgM-isotype MAbs reported by Jiménez et al. were directed to T-independent antigens located on bacterial surfaces, which would explain their cross-reactivity with *N. asteroides* (7). In contrast, none of the three IgG anti-P24 MAbs that we generated cross-reacted with *M. tuberculosis* or with *N. asteroides* extracts in a Western blot assay. These three anti-P24 MAbs failed to react with *N. brasiliensis* cells in the immunocytochemistry studies; this may be explained either because the target antigen (P24) is not located on the surface or because the antigen epitope that strongly reacts in the Western blot assay is not exposed to or is modified by the experimental conditions of the cytochemistry technique. In similar studies with *M. tuberculosis*, Rambukkana and coworkers (12) also reported that 80% of the MAbs they tested reacted with the antigen in a blot assay but not in their immunofluorescence studies. In other experiments, not reported here, we found that the A4 anti-P24 MAb does not react with the *N. brasiliensis* antigens isolated in nondenaturing electrophoresis, supporting the notion that the epitope recognized by the MAb is not accessible in intact cells.

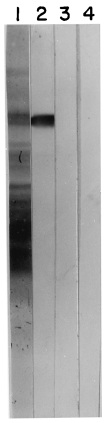

FIG. 6. Specificity of the anti-P61 MAb. Lane 1, *N. brasiliensis* cell extract reacted with serum from an *N. brasiliensis*-infected mycetoma patient, lanes 2 through 4, anti-P61 MAb with *N. brasiliensis* extract, *N. asteroides* cell extract, and *M. tuberculosis* cell extract, respectively.

The anti-P24 MAbs reported in this study may be of help in purifying the P24 immunodominant protein by affinity chromatography under denaturating conditions. This antigen's utility has been demonstrated in serodiagnosis of mycetoma patients (15). These MAbs can also be used to further investigate the role of passive immunity in host-parasite interaction in experimental mycetoma in BALB/c mice. The P61 protein from *N. brasiliensis* was recognized by the anti-P61 MAb (4E2). This antibody can also be used to purify the P61 protein and to isolate the encoding DNA from a gene library. In addition, the specificity of the generated anti-P24 and anti-P61 MAbs for different nocardial strains can be investigated, since in this study we tested only one *N. brasiliensis* strain and one *N. asteroides* strain; however, a more complete species identification methodology for the genus *Nocardia* has recently been published (19).

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