Monoclonal Antibodies to Nocardia asteroides and Nocardia brasiliensis Antigens

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Nocardia asteroides and Nocardia brasiliensis whole-cell extracts were used as antigens to generate monoclonal antibodies (MAbs). Six stable hybrid cell lines secreting anti-Nocardia spp. MAbs were obtained. These were characterized by enzyme-linked immunosorbent assay, Western blot (immunoblot), and immunofluorescence assay. Although all the MAbs exhibited different degrees of cross-reactivity with N. asteroides and N. brasiliensis antigens as well as with culture-filtrate antigens from Mycobacteria spp., they have the potential for use as reagents in the purification of Nocardia antigens.

Nocardia asteroides, Nocardia brasiliensis, and Nocardia otitidiscaviarum can act as primary pathogens in cases of actinomycetoma or appear as causative agents of secondary opportunistic infections (i.e., nocardiosis) in patients suffering from diseases or conditions that suppress their normal immune responses (6). In either case, the diagnosis of Nocardia infections relies on obtaining an appropriate clinical sample, which may require the use of invasive procedures, especially in cases of nocardiosis, and the subsequent isolation of the agent in culture followed by its biochemical identification. Growth in primary culture and identification often requires several days to several weeks, as nocardiae are slow growers. Therefore, serological tests for the diagnosis of Nocardia infections have been investigated as alternatives to current microbiological laboratory procedures. However, most of the serological tests that have been developed have failed to yield the desired sensitivity or specificity (or both). Cross-reactions of Nocardia culture-filtrate or whole-cell-extract antigens with sera from cases of tuberculosis or leprosy (or both) have been reported by several investigators (3, 13), as has a lack of reactivity in sera from documented cases of Nocardia infections (3). These findings have led to the search of specific Nocardia antigens of diagnostic value.

In 1985, Sugar et al. succeeded in identifying two immunodominant N. asteroides antigens by the use of the Western blotting (immunoblot) technique (14). One of them, a 55-kilodalton protein, has been found to be present also in N. brasiliensis and N. otitidiscaviarum and has been tested in serological assays yielding promising results (1, 2). However, the nature or localization (or both) of those antigens remains unknown.

In 1986, El-Zaatari and co-workers separated Nocardia antigens by electrofocusig and used these antigens to generate several monoclonal antibodies (MAbs) (5). Only two of these were found to lack cross-reactivity with mycobacterial antigens. Therefore, the investigators envisioned that they could be used as probes for N. asteroides antigens of clinical significance.

In the present work, we report the generation of a series of MAbs to antigens of Nocardia spp. These were characte

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peritoneally with 50 μg of *N. brasiensis* 27-78, and two mice were injected intraperitoneally with 50 μg of *N. asteroides* 71 antigens in complete Freund adjuvant. At 28 days after the first injection, the mice received a second dose of 50 μg of antigen in saline. Three days after this booster, mice were bled from the retroorbital plexus and sacrificed by cervical dislocation.

**Production of hybridomas and isotyping of clones.** The technique of Kohler and Milstein was used (8). Briefly, spleens were removed aseptically from the immunized mice and minced. The minings were washed with RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.), and spleen cells were centrifuged at 1,200 rpm for 8 min. The supernatant fluid was decanted, and cells were suspended in RPMI 1640 and underlaid with Ficoll-Hypaque solution. After centrifugation at 1,200 rpm for 20 min, the lymphocytes and mononuclear cells were removed from the gradient interface. These were washed twice with serum-free RPMI 1640 and were adjusted to a concentration of 2.5 × 10⁶ cells per ml. Murine myeloma cells (SP2/0-Ag 14) were mixed in a ratio of 3:10 with the splenic lymphocytes. The mixed cells were centrifuged at 1,400 rpm for 5 min, the supernatant was decanted, and 1 ml of a 50% solution of sterile polyethylene glycol 1540 (Ph. 7.8) (Hana Biologics, Inc., Berkeley, Calif.) was added. The pellet was gently shaken and left undisturbed for 2 min, and 1 ml of RPMI 1640 was added. Fifteen milliliters more of RPMI 1640 was then added at a rate of 3 ml/min. Cells were spun down at 1,200 rpm for 4 min, suspended in complete Dulbecco modified Eagle medium (containing 10⁻⁴ M hypoxanthine, 4 × 10⁻⁷ M aminopterin, 1.6 × 10⁻³ M thymidine [HAT medium], and 10% fetal calf serum), and plated into 96-well plates (Costar, Cambridge, Mass.). The cells were fed HT medium (50% volume) on day 5 after the fusion. When the growth of hybrids was detected microscopically (usually 10 to 14 days after the fusion), the supernatant culture fluid was removed and assayed with an ELISA (see below). Cells from antibody-positive wells were transferred to 24-well plates (Costar) in HT medium and then were cloned twice by limiting dilution. Positive subclones were expanded to large cultures or injected into mice to produce ascites and were frozen in liquid nitrogen. Isotyping of the MAbs was done with the Ouchterlony double diffusion test as previously described (12). Monoclonal immunoglobulins from either hybridoma supernatant fluid or ascites were placed in the outside wells of an Ouchterlony plate. The center wells contained goat anti-mouse antibody to either immunoglobulin G (IgG) or IgM (Sigma Chemical Co., St. Louis, Mo.).

**ELISA for detection of Nocardia-specific MAbs.** Indirect ELISAs were performed with disposable polystyrene 96-well microtiter plates (Costar) for the solid phase. The *Nocardia* antigens (and later mycobacterial antigens) were appropriately diluted in phosphate-buffered saline (PBS), pH 7.4, to a concentration of 30 μg/ml. Two hundred microliters of antigen (6 μg) was added per well, and the plates were stored at 4°C overnight. When the plates were used, the wells were emptied by shaking and each well was washed three times with PBS-0.05% Tween 20 buffer.

Sera, culture fluids, or ascites to be tested were appropriately diluted in PBS, and the solution (100 μl per well) was added to the plates. Culture fluids from the original fused hybrid cells were screened undiluted for antibody activity; ascites were diluted 1:4. Plates were incubated at room temperature for 1 h; then they were shaken dry and washed four times with the PBS-Tween 20 buffer. Goat anti-mouse immunoglobulins conjugated with peroxidase (Sigma) were added (100 μl per well; 1:1,000 dilution in PBS with 1% bovine serum albumin), and the plates were incubated for 45 min at room temperature. The conjugate was discarded, plates were washed five times with PBS-Tween 20, and freshly prepared *ortho-*phenylenediamine (40 mg in 100 ml of citric acid-phosphate buffer [24.3 ml of 0.1 M citric acid, 25.7 ml of 0.2 M phosphate, and 50 ml of distilled water] containing 40 μl of 30% hydrogen peroxide) was added (100 μl per well). After 20 min of incubation at room temperature, the reaction was stopped by the addition of 25 μl of 8 N sulfuric acid and the wells were read with an ELISA Minireader II (Dynatech Laboratories, Inc., Alexandria, Va.). Positive, negative, and conjugate controls were included. Readings of more than 0.2 were considered positive.

**Immunoblotting procedure.** Sodium dodecyl sulfate (10%) gels were prepared by the method of Laemmli (10). Antigen samples containing 60 μg of protein were solubilized in sample buffer and heated for 2 min at 100°C. Electrophoresis was carried out until the dye front reached the bottom of the gel. Molecular weight standards were included in each run. Immunoblotting was performed by a method similar to that of Towbin et al. (16). Briefly, the proteins were electrophoretically transferred to nitrocellulose paper at 100 mA for 19 hr. One portion of the membrane containing the molecular weight standards and one lane of each of the antigens was stained with amido black. The other portion of the membrane was incubated with 5% nonfat dry milk in Tris-buffered saline (0.05 M Tris, pH 7.4, containing 0.5 M NaCl; TBS) for 30 min. The sheets were subsequently incubated with antiserum, undiluted hybridoma supernatant fluids, or a 1:100 dilution of ascites and then were washed four times with TBS-0.5% Tween 20 buffer for 30 min. After this, the membranes were incubated for 1 h with peroxidase-conjugated goat anti-mouse IgG (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) that had been diluted 1:300 in TBS-0.5% gelatin. This was followed by two washes in TBS-Tween 20 and two washes in TBS. Finally, the antigenic bands were developed by the addition of 4-chloro-1-naphthol and hydrogen peroxide.

**Indirect immunofluorescence.** Drops (100 μl) of 5-day-old *Nocardia* cultures grown in Sabouraud glucose broth with shaking were placed on clean slides and mixed with 50 μl of 0.01% bovine serum albumin. Smears were carefully prepared, allowed to air dry, and fixed with ethanol, chloroform, and Formalin (60:30:10) for 3 min. The smears were then washed with 95% ethanol for 4 min and twice in distilled water and were allowed to air dry. Fixed smears of *M. tuberculosis* (gift of the Center for Control of Tuberculosis from the San Juan Department of Health, San Juan, P.R.) and *Corynebacterium xerosis* were also treated in the same manner.

Undiluted hybridoma supernatant fluids, a 1:20 dilution of ascites, or a 1:4,000 dilution of mouse polyclonal antiserum in PBS was added to the smears. The samples were incubated for 30 min at 37°C in a humidified chamber. Samples were then washed three times in PBS and later in distilled water. A 200-μl sample of fluorescein isothiocyanate-conjugated goat anti-mouse IgG or IgM (Boehringer Mannheim) diluted 1:40 in PBS and mixed 1 to 1 with a 1:1,000 Evans blue solution (final dilution, 1:80) was then applied to each smear and incubated for an additional 30 min in a humidified chamber. Finally, the slides were washed three times in PBS for 5 min, once with distilled water, and allowed to air dry. Coverslips were mounted with 1 drop of 90% glycerol in PBS. The preparations were examined with a fluorescence microscope (Olympus Corp. of America, New Hyde Park,
N. asteroides, N. brasiliensis, M. intracellularle, M. scrofulaceum, M. kansasi, and M. fortuitum were used in an ELISA to determine the possible cross-reactivity of the anti-Nocardia spp. MAbs with antigens from Mycobacteria spp. We determined that all MAbs exhibited different degrees of cross-reactivity with the mycobacterial antigens (Table 3). MAbs 54.7.2 and 54.7.3, which reacted strongly with Nocardia spp., were the ones that showed the highest degree of cross-reactivity with Mycobacteria spp.

### RESULTS

Isolation and characterization of MAbs. Six hybrid cell lines, designated 3.13.10, 3.14.12, 36.6.5, 47.2.4, 54.7.2, and 54.7.3, were recovered from fusions involving the SP2/0 myeloma cells and spleen cells from BALB/c mice immunized as previously described. Hybrid cell lines 36.6.5, 47.2.4, 54.7.2, and 54.7.3 were produced from mice immunized against *N. brasiliensis* whole-cell-extract antigens. The other two cell lines, 3.13.10 and 3.14.12, were produced against *N. asteroides* antigens. The isotypes of the six MAbs secreted by the parental cell lines were determined by double immunodiffusion. MAbs 3.13.10, 3.14.12, 54.7.2, and 54.7.3 were of the IgM isotype, whereas the other two were of the IgG class (Table 1). All of the MAbs reacted with both *N. asteroides* and *N. brasiliensis* antigens when tested by ELISA (Table 1).

Identification of Nocardia antigens recognized by the MAbs. The Western blotting technique was used to identify *Nocardia* cellular components which had specific antigenic reactivity for the MAbs. Samples of *N. asteroides* and *N. brasiliensis* whole-cell extracts containing 60 μg of protein were run in 10% sodium dodecyl sulfate-polyacrylamide gels (Fig. 1). The separated polypeptides were then transferred to nitrocellulose sheets for immunological analysis. Table 2 shows the results obtained. MAbs 3.13.10, 3.14.12, 54.7.2, and 54.7.3 recognized more than one polypeptide band in both *N. asteroides* and *N. brasiliensis* antigens (Table 2). In contrast, MAb 47.2.4 only recognized one single polypeptide band in the *N. brasiliensis* preparation. MAb 36.6.5 did not react in the blots.

Indirect immunofluorescence assays. In indirect immunofluorescence assays, the six MAbs tested reacted with both *N. asteroides* and *N. brasiliensis* filaments (Fig. 2). Moreover, the MAbs recognized the bacterial surface (Fig. 2b). When tested with *M. tuberculosis* cells, a high degree of nonspecific fluorescence was observed, making it difficult to assess the degree of reactivity of the MAbs. However, with *C. xerosis*, no fluorescence was observed (Table 2).

Cross-reactivity of anti-Nocardia spp. MAbs with mycobacterial antigens. Culture-filterate antigens from *M. tuberculosis*, *M. intracellularle*, *M. scrofulaceum*, *M. kansasi*, and *M. fortuitum* were taken with protein precipitated in the ELISAs against *asteroides* and *N.* designated. The results obtained were compared with the antigens used in the ELISAs in a concentration of 4 μg per well.

### DISCUSSION

The primary purpose of this study was to develop and characterize a panel of MAbs against *N. asteroides* and *N. brasiliensis* that could be used for the analysis of Nocardia antigens and, ultimately, for the development of diagnostic tests and the study of the immune response to *Nocardia* spp.

A total of three fusions were performed between SP2/0-Ag14 myeloma cells and lymphocytes from BALB/c mice immunized with *N. asteroides* or *N. brasiliensis* whole-cell extracts. We obtained six stable hybrid cell lines secreting MAbs which were further characterized by ELISA, Western blot, and the indirect immunofluorescence assay. In ELISA, all the MAbs were found to react with homologous and heterologous Nocardia antigens. In addition, they reacted with antigens from various Mycobacterium spp. in the
ELISA. This was not totally unexpected because both genera are antigenically related (4, 9, 17) and because the antigen was given in Freund complete adjuvant. Since the latter includes killed Mycobacterium butyricum cells as part of its formulation, it is possible that shared antigens between Nocardia and Mycobacterium spp. acted as immunodominant epitopes in the antigen mixture. Although this cross-reactivity may be viewed as undesirable, the fact that the MAbs recognize surface antigenic components of the bacteria, as demonstrated by immunofluorescence, could be of significance in the purification of Nocardia spp.-specific antigens, such as the ones described by Sugar et al. (14).

The Western blot experiments with the anti-Nocardia spp. MAbs revealed some interesting findings. Four of the six MAbs (3.13.10, 3.14.12, 54.7.2, 54.7.3) detected multiple bands on the blots containing N. asteroides and N. brasiliensis antigens separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Since those antibodies also reacted with the mycobacterial antigens in ELISA, it is likely that they recognize shared lipid, carbohydrate, or peptide epitopes (7, 17). Moreover, one could infer that the epitopes were located in the surface of the Nocardia cells, since the cell walls bound the MAbs as observed in the indirect immunofluorescence experiments.

Of the other two MAbs tested on the blots, one (47.2.4) detected a single protein band in the N. brasiliensis-separated antigens, while the other (36.6.5) did not exhibit any reactivity. This discordant behavior of MAb 36.6.5, which gave positive ELISA and indirect immunofluorescence assay reactions, is possibly due to its recognition of conformational epitopes that are lost when the antigens are subjected to the sodium dodecyl sulfate-polyacrylamide gel electrophoresis procedure (15).

While the absolute specificity of MAbs may be an obvious

<table>
<thead>
<tr>
<th>Reagent</th>
<th>A_{490} in ELISA for:</th>
<th>No. of bands in Western blot for:</th>
<th>Presence (+) or absence (−) of fluorescence in indirect immunofluorescence assay for:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N. asteroides 71</td>
<td>N. brasiliensis 71</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N. asteroides 27</td>
<td>N. brasiliensis 27</td>
<td></td>
</tr>
<tr>
<td>3.13.10</td>
<td>1.26</td>
<td>1.20</td>
<td>+</td>
</tr>
<tr>
<td>3.14.12</td>
<td>1.06</td>
<td>1.10</td>
<td>3</td>
</tr>
<tr>
<td>36.6.5</td>
<td>1.32</td>
<td>1.60</td>
<td>None</td>
</tr>
<tr>
<td>47.2.4</td>
<td>0.49</td>
<td>0.66</td>
<td>None</td>
</tr>
<tr>
<td>54.7.2</td>
<td>1.15</td>
<td>1.45</td>
<td>10</td>
</tr>
<tr>
<td>54.7.3</td>
<td>1.61</td>
<td>1.52</td>
<td>1</td>
</tr>
<tr>
<td>Anti-N. brasiliensis 27 mouse serum</td>
<td>1.00</td>
<td>1.33</td>
<td>3</td>
</tr>
<tr>
<td>N. asteroides 63 mouse serum</td>
<td>1.15</td>
<td>0.68</td>
<td>1</td>
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<tr>
<td>PBS</td>
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<td>ND</td>
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<tr>
<td>cDMEM ³</td>
<td>0.00</td>
<td>0.00</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Fluorescence difficult to assess because of background.
* ND, Not done.
³ Complete Dulbecco modified Eagle medium.

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FIG. 2. Indirect immunofluorescence photographs of N. brasiliensis 27-78 incubated with heterologous monoclonal antibody 3.14.12 (a) and N. asteroides 71 incubated with heterologous monoclonal antibody 3.13.10 (b) (magnification, ×1,200).
TABLE 3. Reactivity of MAbs against mycobacterial antigens as determined by ELISA

<table>
<thead>
<tr>
<th>Mycobacterial antigen</th>
<th>$A_{max}$ for indicated MAb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3.13.10$^a$ 3.14.12$^b$ 36.6.5$^c$ 47.2.4$^d$ 54.7.2$^e$ 54.7.3$^e$</td>
</tr>
<tr>
<td>$M. tuberculosis$</td>
<td>0.37 0.28 0.26 0.25 1.29 1.58</td>
</tr>
<tr>
<td>$M. intracellulare$</td>
<td>0.33 0.28 0.23 0.22 1.2 1.26</td>
</tr>
<tr>
<td>$M. scrofulaceum$</td>
<td>0.38 0.38 0.31 0.29 0.69 0.77</td>
</tr>
<tr>
<td>$M. kansasii$</td>
<td>0.46 0.57 0.40 0.25 0.95 1.07</td>
</tr>
<tr>
<td>$M. fortuitum$</td>
<td>0.40 0.52 0.31 0.22 0.50 0.60</td>
</tr>
</tbody>
</table>

$^a$ Analyzed with ascites diluted 1:4.
$^b$ Analyzed with undiluted culture supernatant fluid.

requirement for a diagnostic test, cross-reactive antibodies such as the ones described here have the potential for application as reagents that recognize shared mycobacterial and nocardial antigens. The use of such cross-reactive reagents could result in the purification of Nocardia spp.-specific antigens by affinity chromatography which, in turn, may prove to be of value in the development of a non-cross-reactive, highly specific diagnostic reagent or in the definition of the epitopes responsible for stimulating host cellular reactions during invasive infection.

ACKNOWLEDGMENTS

This work was supported in part by Public Health Service grants RR 08224-04 and SO7 RR 05419-26 from the National Institutes of Health.

We thank J. A. Lavergne for the use of his tissue culture facilities and Ana Estrada for excellent secretarial assistance.

LITERATURE CITED