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Identification of a Common Immunodominant Protein in Culture Filtrates of Three *Nocardia* Species and Use in Etiologic Diagnosis of Mycetoma

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Diagnosis of systemic nocardiosis has been hampered by difficulty in the rapid isolation and identification of the organism and by the frequent need for invasive biopsies. We show that a 55,000-M_r protein previously found in *Nocardia asteroides* is also present in *N. brasiliensis* and *N. caviae* culture filtrates. We also used the antigen in a dot blot assay to identify patients infected with *N. brasiliensis*. Sera from patients with mycetoma secondary to *N. brasiliensis* (*n* = 6) or *Actinomadura madurae* (*n* = 2) and 10 hospitalized controls were tested. Six (100%) of the sera from the patients with nocardial mycetoma had positive dot blots to purified antigen, whereas all 10 of the control sera were negative. Serum from one of two patients infected with *A. madurae* also reacted with the antigen. Thus, the 55-kilodalton protein appears to be produced by three medically important species of *Nocardia* and is useful when used in an immunoblot assay for identifying patients infected with these organisms.

Two different forms of nocardiosis have been recognized as distinct clinical entities (7). The first, presumably secondary to the inhalation of organisms into the lungs, is caused primarily by *Nocardia asteroides*. The second, chronic subcutaneous disease such as mycetoma, is often caused by direct inoculation of *N. brasiliensis* or *N. caviae* into the affected area. While both syndromes have been caused by each of the three species, the above generalization is a useful concept in that *N. asteroides* is most often associated with systemic disease and that the other two *Nocardia* spp. usually cause local infection. Furthermore, despite the ubiquitous nature of all the *Nocardia* spp. (7), systemic nocardiosis due to *N. asteroides* is the most common form of the disease found in North America. In contrast, mycetoma caused by the other two *Nocardia* spp. is the most common form of the disease in South America and India (8). Thus, the clinical suspicion of nocardiosis tends to implicate one of the organisms based on presentation of the illness and the geographic location of the patient. The problems with making a diagnosis of nocardiosis have been reviewed in the past (2, 5, 8–10) and need not be reviewed here.

The role of the humoral immune response to infection with *Nocardia* spp. is not well understood. A major contributing factor hindering investigation into the role of antibody in the pathogenesis of different forms of nocardiosis has been the lack of a suitable antigen with which to measure such a response. We have previously documented the presence of a 55-kilodalton (kDa) protein in culture filtrates of *N. asteroides* (10). This protein has been shown to be a sensitive marker for identifying patients infected with *N. asteroides*. In addition, it is a highly specific molecule, demonstrating no cross reactions with sera obtained from patients infected with *Mycobacteria* spp. (1; unpublished observations). Here we report that the 55-kDa protein is also found in culture filtrates obtained from *N. brasiliensis* and *N. caviae*. Using dot blots to detect antibody, we further demonstrate that patients with mycetoma infected with either of these strains produce immunoglobulin G (IgG) directed against this protein.

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MATERIALS AND METHODS

Antigen. The antigen used in this assay was prepared as described previously (10). Briefly, *N. asteroides* 47N (obtained from Allan Pier) and GUH-2 (obtained from Blaine Beaman), *N. brasiliensis*, and *N. caviae* were grown for 2 weeks in 2-liter Erlenmeyer flasks containing 200 ml of Long medium at 37°C on a rotary shaker. The organisms were removed by centrifugation, and the supernatant fluid was precipitated with 65% (wt/vol) ammonium sulfate. We used the supernatant resulting from this precipitation step, which was the same fraction used in our previous study (1).

Sera. Sera were kindly provided by Jose Santos (Hospital Infantil de Mexico, Mexico City, Mexico). Six patients had mycetoma and positive cultures for *N. brasiliensis*. Two additional patients with mycetoma were infected with *Actinomadura madurae*, an organism with a close taxonomic relationship to *Nocardia* spp. (4). Organisms were identified by standard techniques (4).

For negative controls, sera were obtained from randomly selected hospitalized patients at The University Hospital, Boston University Medical Center. None of these patients was known to be infected with *Nocardia* or *Mycobacterium* spp.

Polyclonal antisera were obtained from a rabbit that was immunized with supernatant 3b, which was the fraction that resulted from the ammonium sulfate precipitation step used in the previous study (1). The initial 1-ml intramuscular injection contained the antigen (25 μg of protein) emulsified in incomplete Freund adjuvant. At weekly intervals for 2 weeks, 1 ml of 25 μg of the antigen was administered intramuscularly in saline. One month later the antigen (12 μg) was administered intravenously in 0.5 ml of saline. The rabbit was bled from the marginal ear vein 1 week after the last booster. In the enzyme-linked immunosorbent assay

with supernatant 3b, the titer of the rabbit sera used in this study was 1:32,768.

Electrophoresis. Reducing gel electrophoresis (sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE]) modified from the procedure described by Laemmli (6) was performed. Gels were stained with silver as directed by the manufacturer (Bio-Rad Laboratories, Richmond, Calif.). High-molecular-weight standards were obtained from Sigma Chemical Co., St. Louis, Mo., and included phosphorylase *b* (molecular weight, 97,400), bovine albumin (molecular weight, 66,000); egg albumin (molecular weight, 45,000); and carbonic anhydrase (molecular weight, 29,000). To obtain pure 55-kDa antigen for the dot blot studies, the protein with an apparent molecular weight of 55,000 (R_f = 0.35) was cut out of the gel and then eluted in 2 ml of 0.01 M Tris-buffered saline (TBS; pH 7.4) by rotating the gel at 4°C for 2 h and then allowing it to stand overnight at 4°C. The gel strip was removed, and the protein in solution (the concentration was 1 μg/ml, as determined by the protein assay described by Bradford [3] by using bovine albumin as the standard) was directly applied in 20-μl fractions to nitrocellulose squares (2 by 2 cm; total volume, 80 μl).

Immunoblotting (dot blot). The nitrocellulose squares containing the 55-kDa protein were placed into sterile petri dishes (10 by 35 mm), and then the incubation solutions were added sequentially. The first antibody, diluted in TBS–1% gelatin (polyclonal rabbit sera to semipurified *N. asteroides*-derived culture filtrate [1:20 dilution] or human sera [1:40 dilution]), was incubated with the antigen for 2 h at 37°C. The nitrocellulose squares were then washed twice with TBS-Tween 20 (0.05%) for 10 min at 25°C. The second incubation was with anti-human (lot 224650; Cappel Laboratories, Cochranville, Pa.) or anti-rabbit horseradish peroxidase-conjugated IgG (lot E428; 61-202; Miles Laboratories, Inc., Elkhart, Ind.) diluted 1:500 in TBS–1% gelatin for 1 h at 37°C. The squares were again washed, developed immediately with 4-chloro-1-naphthol (60 μg; Bio-Rad), and dissolved in 20 ml of 100% methanol–TBS with 0.015% hydrogen peroxide. The development was stopped after 5 to 10 min by replacing the development solution with distilled water. A positive reaction was one in which color appeared in the location of the antigen and was graded on a scale of 0 to 4+. No color development was noted if the primary antibody was not included in the assay.

RESULTS

The presence of a 55-kDa band in each of the three species of *Nocardia* was demonstrated by SDS-PAGE (Fig. 1). The band located at an M_r of 55,000 was cut out of the gel, eluted, lyophilized, and again subjected to SDS-PAGE. This resulted in a single band that was identifiable at a molecular weight of 55,000 when stained with silver reagent. We then investigated whether sera obtained from patients with mycetoma contained antibody to this *Nocardia*-derived antigen. When sera from patients infected with *N. brasiliensis* were incubated with the 55-kDa protein (derived from either *N. asteroides* or *N. brasiliensis*) adsorbed onto nitrocellulose, detection of IgG directed to the antigen was demonstrated. Dot blot analysis of human sera revealed that for 10 control sera, 6 sera containing *N. brasiliensis*, and 2 sera containing *A. madurae*, results were positive for 0, 6, and 1 serum samples, respectively, and were negative for 10, 0, and 1 serum samples, respectively. Six of six *N. brasiliensis*-infected patients were positive. Two patients infected with *A. madurae* were tested and one was positive. Sera from

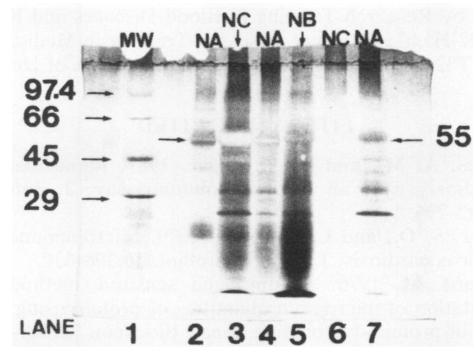

FIG. 1. Silver-stained 10% SDS-PAGE of culture filtrates derived from *N. asteroides* (NA), *N. brasiliensis* (NB), and *N. caviae* (NC). Some negatively stained bands were due to overloading of the wells. Lane 1, molecular weight (MW) markers; lane 2, *N. asteroides* 47N; lane 3, *N. caviae*; lane 4, *N. asteroides* GUH-2; lane 5, *N. brasiliensis*; lane 6, *N. caviae*; a different batch of antigen from that in lane 3 was used; lane 7, *N. asteroides* GUH-2; different batch of antigen from that shown in lane 4 was used.

randomly selected hospitalized patients were always negative (no color development).

DISCUSSION

We have previously demonstrated that a 55-kDa protein isolated from culture filtrates of *N. asteroides* was immunogenic in humans and rabbits and could be incorporated into a diagnostic enzyme immunoassay (1, 10). In this study, we extended our previous observations to show that this 55-kDa protein is present in culture filtrates obtained from two other medically important *Nocardia* spp. (*N. brasiliensis* and *N. caviae*). The isolation of an apparent immunodominant protein that can be used as a diagnostic reagent will permit a more rapid diagnosis of nocardial infections and timely initiation of appropriate antimicrobial therapy and may obviate the need for invasive biopsies. Of course, further studies are needed to assess the utility of the dot blot or other related assays in making an etiologic diagnosis in patients with mycetoma. One potential problem might be the persistence of antibody produced during a past infection with *Nocardia* spp. This would make interpretation of the test problematic. A possible solution to this problem could be to measure antibody titer, or to determine the presence or absence of IgM to the 55-kDa protein.

The demonstration of the presence of this 55,000-M_r protein among these three *Nocardia* spp. has not been previously noted and represents confirmation that, despite the different clinical presentations of nocardiosis and infection with any one of three different nocardial species, production of IgG directed against the 55-kDa protein can be found. The availability of a suitable antigen may further our understanding of the pathogenesis of nocardiosis and provide us with a probe to study the humoral immunologic response to nocardiae. The presence or absence of this 55-kDa protein in other actinomycetes also needs to be evaluated so that potential cross-reactive organisms can be identified. Moreover, such a survey may also have relevance to taxonomic placement of related actinomycetes.

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