JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 1990, p. 328–331
0095-1137/90/020328-04$02.00/0

Vol. 28, No. 2

Use of Partially Purified 54-Kilodalton Antigen for Diagnosis of Nocardiosis by Western Blot (Immunoblot) Assay

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A Western blot (immunoblot) assay is presented for the diagnosis of nocardiosis with a specific immunodominant 54-kilodalton (kDa) antigen purified from a culture filtrate of *Nocardia asteroides* by immunoaffinity chromatography. The chromatography column was prepared with immunoglobulin G obtained from sera from patients with lepromatous leprosy. Unbound solutes consisted of specific, partially purified *N. asteroides* antigens, primarily a 54-kDa band, accompanied by two others of 31 and 62 kDa. The Western blot technique was applied to detecting the immunologic response to nocardiae. Immunodetection was performed according to the biotin-avidin system, which greatly improved the detection of antibodies even in immunosuppressed hosts. Each of 16 serum samples from immunosuppressed or immunocompetent patients infected with *N. asteroides* reacted with the 54-kDa band, and two reacted with the 31- and 62-kDa bands. Each of the serum specimens obtained from patients with mycetoma caused by *Nocardia brasiliensis* or *Rhodococcus rhodochrous* reacted with the 54-kDa band. There was no reaction to either the 54- or the 31-kDa antigen with all serum samples obtained from patients with tuberculosis, except one, with all serum samples obtained from patients with leprosy, or with all sera obtained from healthy controls. The 54-kDa protein is a candidate to be used as a probe to study the humoral immunologic response to nocardiae.

Nocardiae are gram-positive, partially acid-fast, branching, filamentous bacteria. *Nocardia asteroides*, responsible for most *Nocardia* infections, is generally associated with localized pulmonary and disseminated involvement. Nocardiosis is an opportunistic infection in patients with an impaired host defense mechanism (1, 14, 24), but infection in normal hosts has also been reported (5).

Nocardiosis is difficult to recognize on the basis of clinical, radiological, or histological findings. A definitive diagnosis depends on the isolation and identification of a *Nocardia* sp., a procedure which can take 2 to 3 weeks (19). However, early recognition of nocardiosis, even by aggressive, invasive diagnostic efforts, is often necessary, since specific antibiotic therapy improves the prognosis. The need for a reliable serological test to detect specific antibodies in the sera of nocardiosis patients and to minimize the need for invasive diagnostic procedures is therefore obvious.

Sugar et al. have previously documented the presence of a 55-kilodalton (kDa) protein in culture filtrates of *N. asteroides* (25). This protein has been shown to be a highly specific marker for identifying patients infected with *N. asteroides*, *Nocardia brasiliensis*, or *Nocardia otitidiscaviarum* (2). Previous studies (6, 15, 23) have indicated a remarkable degree of cross-reactivity of nocardiae with related genera. We take advantage of the high degree of cross-reactivity between nocardiae and mycobacteria to purify nocardia-specific antigens by immunoaffinity chromatography. Here we report the partial purification of this protein from culture filtrates obtained with *N. asteroides* and the investigation of the usefulness of Western blot (immunoblot assay) in the specific diagnosis of *Nocardia* infection.

MATERIALS AND METHODS

Sera. Sera from 21 patients with documented culture-proven nocardia infection caused by *N. asteroides* (15 cases) and by *N. brasiliensis* (6 cases of mycetoma) were obtained from the serum bank of the Mycology Unit, National Reference Center on Mycoses and Antifungal Agents, Pasteur Institute. Of the 15 patients infected with *N. asteroides*, 5 (one with cervical abscess and four with pulmonary abscesses) were without other discernible disease; the remainder were under immunosuppressive therapy (one had a cardiac transplant, four had renal transplants, and five were on high-dose steroids) and had documented pulmonary or disseminated infection (B. Dupont and P. Boiron, Annual Activity Report of National Reference Center on Mycoses and Antifungal Agents, 1988, unpublished data). In all instances, the serum samples were obtained at the time of bacteriological diagnosis. In addition, serum specimens from five patients with mycetoma caused by *Actinomadura madurae* (three cases) or *Rhodococcus rhodochrous* (two cases) (kindly provided by L. C. Severo) (22), eight patients with lepromatous leprosy (kindly provided by J. Millan), five patients with *Mycobacterium tuberculosis* infection (kindly provided by H. David), and seven uninfected normal individuals (obtained from randomly selected consulting patients at the Pasteur Hospital) were examined.

Antigen preparation. *N. asteroides* 4265, from the Culture Collection of the Pasteur Institute, was originally isolated from a human cerebral abscess. It was identified by the method of Gordon (13). This strain was used because of its high animal pathogenicity and its regular reaction with tested sera. The medium used for the antigenic preparation was prepared according to a modification of the double-dialysis method of Edwards (11). A dialyzable culture medium was prepared from Bennett liquid medium. It consisted of pancreatic digest of casein, 2 g; yeast extract, 1 g; beef extract, 1 g; glucose, 10 g; distilled water, 1,000 ml; final pH 7.3. Dialysis was performed by ultrafiltration (Pellicon; Millipore Corp., Bedford, Mass.; nominal molecular weight limit of membrane, 10,000). The organisms were grown for 14 days at 32°C in filtrate without shaking. The bacteria were then filtered (Whatman no. 1 filter; Whatman, Inc., Clifton, N.J.) to remove cell debris and concentrated by ultrafiltration (the

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same membrane as for the first dialysis was used), and the concentrate was dialyzed against distilled water before lyophilization.

Antigen purification. (i) Immunoglobulin purification. Immunoglobulin G (IgG) was obtained from serum specimens from three patients with lepromatous leprosy. Pooled serum specimens were centrifuged at 12,000 × *g* for 5 min to clarify the fluid. A column of CM-Affi-Gel Blue (1 by 10 cm) (Bio-Rad Laboratories, Richmond, Calif.) was equilibrated with 0.01 M potassium phosphate buffer, pH 7.25, containing 0.15 M NaCl and 0.02% NaN_3, and the clarified pooled serum specimens were applied. The column was washed with the same buffer, and the effluent, which contained the serum proteins minus plasminogen and albumin, was collected. Saturated ammonium sulfate was added to the effluent to a final concentration of 45% (wt/vol). The precipitate was collected by centrifugation at 18,000 × *g* for 20 min at 4°C and was dissolved in sterile distilled water. The solution was then dialyzed extensively against 0.1 M sodium bicarbonate, pH 8.0 (coupling buffer). After dialysis, the solution was centrifuged at 18,000 × *g* for 20 min to remove any particulate matter. Protein determination was performed by the method of Bradford (Bio-Rad protein assay), with bovine serum albumin as a standard (10).

(ii) Preparation of an immunoaffinity chromatography column. A 3-ml portion of the IgG fraction (4.8 mg/ml) was combined with 5 ml of activated Affi-Gel 10 resin (Bio-Rad). The mixture was placed on a rocker table at 4°C for 16 h to complete the coupling. N-linked IgG was removed and assayed for protein to determine the efficiency of coupling. Excess activated groups were blocked by 0.2 M glycine, pH 8.0, for 2 h at room temperature. The reacted resin was washed with coupling buffer until the gel was free of reactants, as detected by optical density at 280 nm. Prior to use, the column was stored at 4°C in the presence of 0.2% sodium azide.

(iii) Purification of specific antigen. All antigen purification procedures were done at 4°C. Lyophilized, double-dialyzed *N. asteroides* antigen was reconstituted and extensively dialyzed against coupling buffer and clarified by centrifugation at 18,000 × *g* for 20 min. The supernatant solution (100 μl) was transferred to an IgG Affi-Gel resin-containing column. Then, the column was stirred slowly at 4°C for 2 h, and unbound solutes were decanted to obtain specific purified *N. asteroides* antigens.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Purified antigens were denatured in a boiling solution containing 2-mercaptoethanol and 1% sodium dodecyl sulfate and subjected to electrophoresis. The discontinuous buffer system of Laemmli (16) was used with a 12% polyacrylamide resolving gel and a 5% stacking gel (7 by 9 by 0.15 cm). Electrophoresis was performed for 2 h at 50 mA. The gel was stained by silver staining with a modification of the method of Merril et al. (18). The molecular weight standards consisted of phosphorylase *b* (M_r, 92,500), bovine serum albumin (M_r, 66,200), ovalbumin (M_r, 45,000), carbonic anhydrase (M_r, 31,000), soybean trypsin inhibitor (M_r, 21,500), and lysozyme (M_r, 14,000) (Bio-Rad).

Electrophoretic transfer. The transfer buffer was 50 mM sodium phosphate, pH 6.5. Proteins were transferred from the gel to nitrocellulose paper at 75 V for 2 h immediately after completion of polyacrylamide gel electrophoresis. The nitrocellulose paper was dried, and strips were cut from each run. Strips were stained with 0.2% Ponceau S and developed immunologically as described below.

Nitrocellulose strip development. Immunodetection of antigens on nitrocellulose was performed with Vectastain ABC-Immunoperoxidase reagents (Vector Laboratories, Burlingame, Calif.) by following the procedure of the manufacturer. Briefly, the first step of this procedure is to incubate the blot with the serum sample (primary antibody). Next, a biotin-labeled anti-human IgG is added. This introduces biotin into the blot at the location of the primary antibody. The preformed avidin-biotinylated horseradish peroxidase complex is then added and binds to the biotinylated secondary antibody. Bound human antibodies were detected by incubation in a colorigenic peroxidase substrate solution which was 0.05% diaminobenzidine in 0.1 M Tris hydrochloride, pH 7.6, plus 3% H_2O_2.

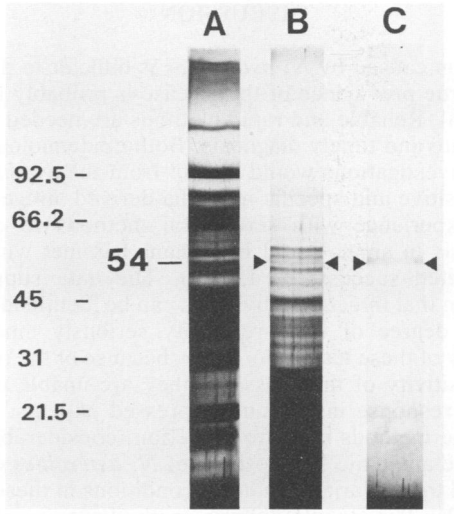

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of silver-stained nocardia antigens. Lane A, Molecular weight markers; lane B, unpurified double-dialyzed *N. asteroides* antigens; lane C, antigens purified by immunoaffinity chromatography. Molecular weights, in thousands, are shown on the left.

RESULTS

Approximately 35 bands were present in lyophilized double-dialyzed *N. asteroides* antigen when stained with silver staining on 12% polyacrylamide gels. Five bands at molecular masses of 23, 28, 45, 52, and 58 kDa were consistently more heavily stained than the other bands (Fig. 1). On a polyacrylamide gel of purified *N. asteroides* antigens, three bands were stained, at molecular masses of 31, 54, and 62 kDa.

The reactions of 46 different serum specimens from patients with nocardiosis; mycetoma caused by *N. brasiliensis*, *A. madurae*, or *R. rhodochrous*; lepromatous leprosy; and tuberculosis and from uninfected normal individuals were evaluated with Western blots of purified antigens. Each of the serum samples from patients, either immunosuppressed or immunocompetent, infected with *N. asteroides* reacted with the 54-kDa band, and two reacted with the 31- and 62-kDa bands. Each of the serum specimens obtained from patients with mycetoma caused by *N. brasiliensis* or *R. rhodochrous* reacted with the 54-kDa band.

Three cross-reactions were observed. One sample from a patient with tuberculosis reacted with the 54-kDa antigen, and two samples, one serum specimen from a patient with leprosy and one from a healthy control, reacted with the 62-kDa antigen.

DISCUSSION

Infection caused by *N. asteroides* is difficult to diagnose, and the true prevalence of the disease is probably underestimated (4). Reliable and rapid methods are needed to make an accurate and timely diagnosis. Both epidemiological and clinical investigations would benefit from the availability of more sensitive and specific nocardia-derived antigens.

Past experience with serological methods to diagnose nocardiosis in animals and in humans has met with somewhat limited success (6, 15, 23). The data support the suggestion that infected individuals can be identified but that the high degree of cross-reactivity seriously hinders the specificity of these tests. Moreover, because of the relatively poor sensitivity of these assays, they are unable to detect antibody response in immunosuppressed patients. In addition to the methods used for detection, considerable variation of the antigenic composition of *N. asteroides* could be attributed to the variable cultural conditions in these studies (6, 15, 23). The double-dialysis method was used in this study to eliminate interference from extraneous proteins in the analysis of *N. asteroides* antigens.

It is well-known that members of the genera *Nocardia* and *Mycobacterium* have many properties in common, as attested by similarities of morphological, cultural, chemotaxonomic, and particularly serological characteristics (20). Because of the high degree of cross-reactivity between these organisms, nocardia-specific antigens should be in the minority. Therefore, immunoaffinity chromatography should be an efficient technique with which to purify such a specific antigen.

The presence of a 55-kDa protein in culture filtrates of *N. asteroides*, *N. brasiliensis*, and *N. otitidiscaviarum* was previously documented (2, 25). This protein has been shown to be a sensitive marker for identifying patients infected with *N. asteroides*. It is a highly specific molecule demonstrating no cross-reactions with sera obtained from patients infected with *Mycobacterium* spp. (3). We partially purified this protein—54 kDa in our hands—by immunoaffinity chromatography that was prepared with IgG isolated from patients suffering from lepromatous leprosy (Fig. 1). The other bands of 31 and 62 kDa were also decanted with unbound solutes. The previous study of Sugar et al. (25) also reported the presence of bands of ≈24 and 65 kDa in culture filtrates which were consistently more heavily stained than were the other bands. The Western blot technique has been applied to detect the immunologic response to nocardiae. We have demonstrated a particular pattern of immunological reactivity in patients with infection due to *N. asteroides* (reactivity to the 54-kDa subunit with or without reactivity to the 31- and the 62-kDa subunits) (Table 1).

We decided to measure the antibody response to the 54-kDa protein by the technique of Western blotting because the indirect enzyme immunoassay was not usable, probably because minute quantities of linked IgG from leprosy patients were detached from immunoaffinity resins during chromatography and were mixed with purified *N. asteroides* antigens.

Nocardiosis is encountered with increasing frequency in high-risk immunocompromised hosts, who often produce little antibody during systemic infections. Current serological methods, including enzyme immunoassay, failed to detect a serological response to nocardia antigens in such patients (7, 8). The high sensitivity of the biotin-avidin system, because of the amplification due to multiple-site binding, greatly improves the detection of antibodies, and a positive serological response became evident in these immunosuppressed hosts.

TABLE 1. Analysis of sera from 46 patients with nocardiosis, mycetoma, leprosy, or tuberculosis and from seven healthy individuals by Western blot technique

Disease	No. of patients	No. of patients positive with proteins of:		
		31 kDa	54 kDa	62 kDa
Nocardiosis				
Nonimmunocompromised	5	2	5	2
Immunosuppressed	10	0	10	0
Mycetoma				
N. brasiliensis	6	0	6	0
A. madurae	3	0	0	0
R. rhodochrous	2	0	2	0
Leprosy	8	0	0	1
Tuberculosis	5	0	1	0
None	7	0	0	1

The 54-kDa protein, in an unpurified form, was shown to be produced by the three medically important species of *Nocardia* (2). As expected, our assay, in which a partially purified protein was used, gave positive results with sera from patients with mycetoma caused by *N. brasiliensis*.

A previous study on sera from patients with mycetoma secondary to *A. madurae*, an organism with a close taxonomic relationship to *Nocardia* spp. (13), revealed that one of two sera reacted with the 54-kDa antigen by dot blot analysis (2). Our assay failed to detect any positive-reaction sera containing *A. madurae*, but more sera from confirmed cases are needed for significant evaluation.

Close serological relationships between strains of nocardiae and rhodococci were previously demonstrated (9). In particular, the presence of common antigens (e.g., precipitinogen β), which may be responsible for intergenus cross-reactions, was found in about half of the *Nocardia* and *Rhodococcus* strains (17). False-positive reactions with the 54-kDa antigen were observed in sera from patients with mycetoma due to *R. rhodochrous*. However, this assay is not plagued by broad cross-reactivity to sera from patients infected with *M. tuberculosis* and *M. leprae*, which are also closely related to *Nocardia* spp. (20, 21).

Antigenic factors displaying a specificity for *N. asteroides* were characterized by El-Zaatari et al. (12) by the enzyme-linked immunoelectrotransfer blot technique adapted to isofocused polyacrylamide gels. The relationship, if any, between those proteins and the proteins we studied is unknown.

The role of the humoral immune response to infection with *Nocardia* spp. is not well understood. The availability of a suitable antigen may further our understanding of the pathogenesis of nocardiosis. The 54-kDa protein is a candidate to be used as a probe to study the humoral immunologic response to nocardiae.

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