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Immunoblot Analysis of Antibody Responses to *Sporothrix schenckii*

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The serologic response to *Sporothrix schenckii* was investigated in patients with sporotrichosis by solid-phase enzyme-linked immunosorbent assays (ELISAs) and Western immunoblot techniques. A soluble antigen preparation derived from an *S. schenckii* isolate contained 15 protein staining components ranging in molecular size from 22 to 70 kilodaltons (kDa) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Sera from 40 patients with sporotrichosis demonstrated *Sporothrix* immunoglobulin G antibody by ELISA with titers between 128 and 65,200. No sera from 300 healthy individuals or 100 patients with various systemic mycoses other than sporotrichosis had ELISA titers greater than 64. By Western immunoblotting of the antigens separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, sera from 10 patients with cutaneous sporotrichosis reacted with 8 to 10 antigen components (range, 40 to 70 kDa), while sera from 15 patients with extracutaneous sporotrichosis reacted with a greater number of antigen components (15 to 20 bands) over a wider range of molecular sizes (22 to 70 kDa). Antibody to 40- and 70-kDa antigen components was detected by immunoblots in all sera tested from patients with sporotrichosis. Antibody to 22- to 36-kDa antigen components was present in sera from 13 of 15 patients with extracutaneous sporotrichosis, but these lower-molecular-weight components were not detected by sera from patients with cutaneous sporotrichosis. Antibody to these components was not detected by Western blotting in sera from 19 of 20 patients with other fungal diseases or from 30 healthy individuals. Purification of these specific antigen fractions could provide the basis of a sensitive and specific serodiagnostic test to indicate the presence and activity of extracutaneous sporotrichosis.

Immunoblot techniques provide a means of analyzing serologic responses to complex antigen systems. In recent years, these techniques have been applied to fungal antigens, especially those of *Candida* and *Aspergillus* species (2, 6, 9–11, 16). We utilized Western immunoblotting techniques to analyze human antibody responses to the antigens of *Sporothrix schenckii* to determine whether there are antigen-specific profile differences between the serologic responses of patients with extracutaneous sporotrichosis compared with the immune responses in patients with cutaneous sporotrichosis. In general, the antigen preparations used in serologic assays for *Sporothrix* antibody have been undefined, and it is not known what relation, if any, the various antigens may have to the differences in the pathogenesis of cutaneous and invasive forms of the disease. Differentiation among those *Sporothrix* antigens which are active in the production of self-limited infection or disseminated disease would allow development of an antibody test which might be of value in forecasting the prognosis and in the selection of an optimal therapeutic regimen.

(Portions of this study were presented previously in abstract form [E. N. Scott, Program Abstr. 27th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 1342, 1987].)

MATERIALS AND METHODS

Organism. The *S. schenckii* isolate (OK/SS7) utilized in this study was obtained in 1981 from an Oklahoma patient with lymphocutaneous sporotrichosis. The initial culture of this isolate was identified by typical colony morphology and microscopic appearance of growth on culture medium at 25 and 37°C. A wrinkled, black colony was obtained on Sabouraud dextrose agar (Difco Laboratories, Detroit, Mich.) after 5 days of incubation at 25°C.

Microscopically, the mycelial growth consisted of branched, septate hyaline hyphae (1 to 2 μm in diameter) with conidiophores at right angles to the hyphae bearing hyaline pyriform conidia (1 to 2 μm) arranged in "daisy" clusters. At 35°C on brain heart infusion (BHI) agar containing 5% sheep blood, this isolate was readily converted to the yeast phase containing spherical to cigar-shaped blastoconidia (average, 2 by 8 μm). Since initial isolation, this isolate has been maintained in the yeast phase by weekly transfer to BHI agar slants supplemented with 2% glucose and 0.1% cysteine and grown at 35°C. In the present study, BHI agar or broth refers to this supplemented formulation of BHI. This OK/SS7 isolate was selected for this study because it is easily maintained in the yeast phase (>80% yeast forms with few hyphae or conidia), it has the yeast morphology typical of most clinical isolates of *S. schenckii*, and it has been utilized in our studies on *S. schenckii* since 1981 (14, 15).

Soluble antigen preparation. For yeast-phase soluble antigen preparation (4, 17), yeast cells were grown in yeast nitrogen-Casamino Acids-glucose (YCG) medium consisting of (grams per liter of distilled water): yeast nitrogen base, 6.7; Casamino Acids (Difco), 2.5; glucose, 50; and 1 ml of a vitamin mixture (containing [per 100 ml] thiamine hydrochloride, 50 mg; riboflavin, 50 mg; calcium pantothenate, 50 mg; nicotinic acid, 50 mg; pyridoxine hydrochloride, 10 mg; *p*-aminobenzoic acid, 10 mg; inositol, 10 mg; folic acid, 1 mg; biotin, 0.4 mg) (17). Cells from 48-h-old cultures on BHI agar were suspended in BHI broth and inoculated into tubes containing 20 ml of BHI broth followed by incubation on a culture roller (3.5 rpm) at 35°C for 48 h in air, without added carbon dioxide. The BHI broth cultures were centrifuged (10 min at 1,000 × *g*), the yeast cells were suspended in saline, and this yeast suspension was inoculated into 200 ml of YCG in 1-liter Erlenmeyer flasks. These starter cultures were incubated for 3 days with shaking (100 rpm, orbital shaker;

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Queue Systems, Inc., Parkersburg, W.Va.) at 35°C in air. Following centrifugation as before, the pelleted yeast cells from the starter cultures were transferred to 3 liters of YCG medium (in 4-liter Erlenmeyer flasks) and incubated at 35°C with shaking (as before) for 7 days. Cultures were killed by heat (62°C for 2.5 h) and allowed to stand at room temperature for 48 h. The yeast cells were removed by filtration (Whatman no. 41 filter paper), and thimerosal (1:5,000, final concentration) was added to the filtrates as a preservative (4). Further removal of the yeast cells was accomplished by centrifugation (5,000 × *g* for 60 min at 4°C) of the culture filtrates. The final supernatant preparations (containing soluble antigens) were dialyzed against distilled water (three changes over 48 h at 4°C), lyophilized, divided into aliquots, and stored at −70°C. Analytical assays for total protein content were performed on this antigen preparation by using a Coomassie blue protein-binding assay (Bio-Rad Laboratories, Richmond, Calif.).

ELISA. An indirect solid-phase enzyme-linked immunosorbent assay (ELISA) was performed on serum samples by standard methods as previously described (14, 15). An optimal concentration of the soluble *Sporothrix* antigen (prepared above) was added to microplate wells (in 0.2-ml volumes). This concentration of *Sporothrix* protein was determined by checkerboard titration of twofold dilutions of antigen and high-titered human serum. The plates were incubated overnight at 4°C and washed in phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T), and nonspecific binding sites were blocked with PBS containing 0.1% gelatin. Serial twofold dilutions of serum were added to the antigen-coated wells and incubated for 2 h at 25°C. Control wells were coated with either uninoculated YCG medium or negative human serum. The plates were washed in PBS-T, an optimal dilution of alkaline phosphatase-conjugated anti-human immunoglobulin G (heavy- and light-chain specific; Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.) was added to the wells, and the plates were incubated for 2 h at 25°C followed by washing in PBS-T. Enzyme substrate (*p*-nitrophenyl phosphate; Sigma Chemical Co., St. Louis, Mo.) was added to all wells, followed by further incubation for 30 min. The reaction was stopped with NaOH, and the A_{405} was determined in a spectrophotometer (MicroELISA Reader 580; Dynatech Laboratories, Inc., Alexandria, Va.). Endpoint titers were determined as the reciprocals of the dilutions of the test serum with an optical density exceeding the absorbance (mean + 2 standard deviations) of the negative serum controls. An ELISA titer of 8 or greater was considered to be positive.

SDS-PAGE. Antigen samples (containing 20 μg of protein) for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were solubilized under reducing conditions in electrophoresis sample buffer (62.5 mM Tris, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.01% phenol red, pH 6.8), boiled for 2 min, cooled, and centrifuged. Following solubilization, the antigen samples were electrophoresed on 10% polyacrylamide gels in the presence of SDS as described by Laemmli (7). The soluble antigen fractions were applied to a vertical slab gel apparatus (Hoefer Scientific Instruments, San Francisco, Calif.) and electrophoresed at 30 mA per gel for 5 h (with cooling). Standard molecular size markers included a mixture of proteins from 20 to 66 kilodaltons (kDa) (Sigma). Gels were stained for protein (Coomassie blue) or processed for Western immunoblotting.

Western immunoblot assays. Proteins from SDS-PAGE were electrotransferred onto nitrocellulose membranes (0.45-μm pore size) in a transblotting chamber (Hoefer Scientific) with a buffer consisting of Tris-glycine-methanol (pH 8.3) (5). Adjacent wells containing molecular size standards were also transblotted. The transblotting was done for 18 h at 80 mA. The efficiency of transfer was established by staining the acrylamide gel with Coomassie blue, and samples of the nitrocellulose strips containing *Sporothrix* antigen or molecular size standards were stained for protein (amido black). For immunoblot assays, the nitrocellulose membranes were incubated for 1 h at 37°C in PBS containing 3% bovine serum albumin to block any remaining protein-reactive sites. The nitrocellulose was then cut lengthwise into 0.5-cm strips, and each strip was placed in a separate tray and incubated with 1:100 dilutions (in PBS–3% bovine serum albumin) of human serum for 2 h at 25°C. The strips were washed three times in PBS-T and then incubated for 2 h at 25°C with a 1:500 dilution of alkaline phosphatase-conjugated anti-human IgG (Sigma). After additional PBS-T washes, the strips were incubated in a substrate which consisted of equal volumes of naphthol AS-MX phosphate (0.4 mg/ml of distilled water) and fast red TR salt (6 mg/ml in 0.2 M Tris, pH 8.2) (11, 12). After development of maximum color with minimum background, the strips were washed with distilled water and dried. Antigen-antibody-containing bands were made visible by the deposition of red stain.

Human serum specimens. Serum specimens for use in these studies were obtained after informed consent from 40 patients with sporotrichosis, from 100 patients with fungal diseases other than sporotrichosis (including 12 with histoplasmosis, 20 with cryptococcosis, 18 with coccidioidomycosis, 30 with candidiasis, and 20 with aspergillosis), and from 300 normal human volunteer subjects. The term cutaneous sporotrichosis in this study refers to patients who showed ascending lesions (ulcers and nodules) interconnected by palpable local lymphatic channels; none of our patients had fixed cutaneous lesions. The patients in this study with extracutaneous sporotrichosis are those with lesions in deeper tissues, including lung, joint (articular), or central nervous system. Sera from the various patient groups have been collected at this medical center over several years, and aliquots have been stored at −70°C. The serum specimens from patients with sporotrichosis used in this study were obtained before antifungal therapy was initiated, or shortly thereafter.

RESULTS

Solid-phase ELISA titers of antibody to *S. schenckii* were determined for all sera, including those from healthy volunteers and patient groups. Serum samples were obtained from 300 volunteer subjects constituting a random sampling of Oklahoma residents. Of these normal sera, 18% had detectable antibody by ELISA. The positive titers in these sera showed a normal curve distribution by density function analysis with a median titer of 8 and a range of 8 to 32. In serum samples from 100 patients with various systemic fungal diseases other than sporotrichosis (including patients with active histoplasmosis, aspergillosis, candidiasis, or cryptococcosis), 20% of the serum samples had detectable antibody with a median titer of 16 (range from 8 to 64), similar to the normal population (Table 1).

Solid-phase ELISA antibody titers were also determined for serum samples from 26 patients with extracutaneous sporotrichosis and 14 patients with cutaneous sporotrichosis. The patients with extracutaneous sporotrichosis included 12 with articular disease, 10 with pulmonary disease,

TABLE 1. ELISA titer of IgG antibody to *S. schenckii*

Group	No. positive/ no. tested (%)	Titer range (median) of positive sera
Patients		
Sporotrichosis		
Extracutaneous	26/26 (100)	256–65,200 (4,096)
Cutaneous	14/14 (100)	128–4,096 (512)
Other mycoses	20/100 (20)	8–64 (16)
Normal subjects	54/300 (18)	8–32 (8)

and 4 with central nervous system disease. Sera from all patients with sporotrichosis had detectable *Sporothrix* antibody, and these titers ranged from 128 to 65,200. However, the sera from patients with extracutaneous sporotrichosis contained much higher antibody titers (ELISA median titer of 4,096) than did the sera from patients with cutaneous disease (median titer of 512).

Fifteen protein-containing bands ranging from 22 to 70 kDa were identified in the soluble antigen preparation by protein staining of the SDS-PAGE strip from this isolate (Fig. 1). The heterogeneity of the antibody response to *S. schenckii* was investigated by immunoblotting with serum samples from 25 of the 40 patients with sporotrichosis (15 with extracutaneous and 10 with cutaneous disease). Protein bands visualized by Western blotting were assigned molecular weights by comparing their relative mobilities with those of the proteins included in the mixture of the molecular weight protein standard that was electrophoresed in an adjacent gel lane, electroblotted to nitrocellulose, and stained with amido black (nonspecific protein stain). Antibody to 15 to 20 antigenic components ranging from 22 to 70 kDa was present in the sera from the 15 patients with extracutaneous sporotrichosis. Only 8 to 10 bands, ranging from 40 to 70 kDa, were detected by serum from the 10 cutaneous sporotrichosis patients. Representative immunoblots from five patients of each group are shown in Fig. 2. The ELISA titers for sera from these same representative patients shown in Fig. 2 are as follows: patients with extracutaneous disease, lane 1, 4,096; lane 2, 512; lane 3, 2,048; lane 4, 16,000; lane 5, 32,000; and patients with cutaneous disease, lane 6, 256; lane 7, 512; lane 8, 128; lane 9, 128; lane 10, 1,024. Utilizing our soluble antigen preparation, sera from all 25 patients with sporotrichosis tested by Western blotting had antibody to major proteins at 40 and 70 kDa, while unique bands with relative molecular sizes between 22 and 36 kDa were present only in the sera from patients with extracutaneous sporotrichosis. Antibody to a single molecular species (32 kDa) was dominant in sera from 13 of 15 patients with extracutaneous disease (sera from 5 patients are shown in Fig. 2, lanes 1 to 5).

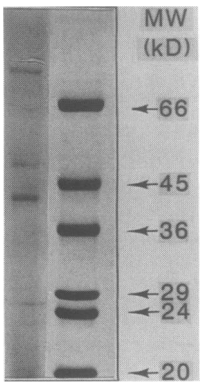

FIG. 1. Left lane, SDS-PAGE of *Sporothrix* antigen preparation; right lane, molecular size standards, 20 to 66 kDa. The gels were stained with Coomassie blue.

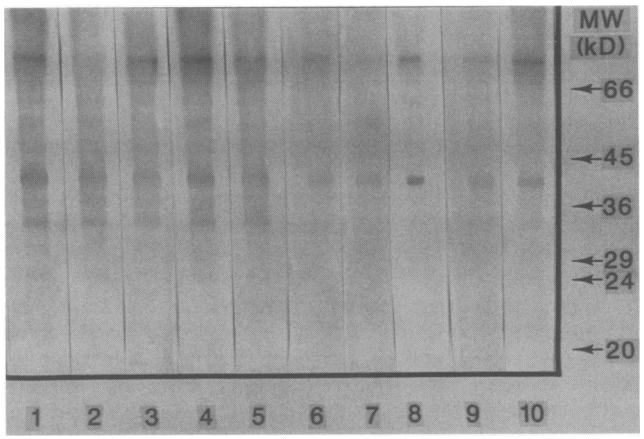

FIG. 2. Western immunoblots of the IgG response in patients with sporotrichosis (extracutaneous, lanes 1 to 5; cutaneous, lanes 6 to 10). The serum samples from patients with extracutaneous disease include two with pulmonary disease (lanes 1 and 2), two with articular disease (lanes 3 and 4), and one with meningeal disease (lane 5).

Western blot bands obtained with serum samples from patients with extracutaneous sporotrichosis were generally darker than bands obtained with serum samples from patients with cutaneous sporotrichosis, even when the sera had equivalent ELISA titers of antibody. For example, in Fig. 2, the sera used in lanes 2 and 7 had ELISA titers of 512, but lane 2 contained serum from a patient with pulmonary sporotrichosis, while lane 7 contained serum from a patient with cutaneous disease. Representative immunoblots of sera from individuals without sporotrichosis are shown in Fig. 3 (other mycoses, lanes 1 to 5; normal individuals, lanes 6 to 10). Western blots detected no antibody in serum samples

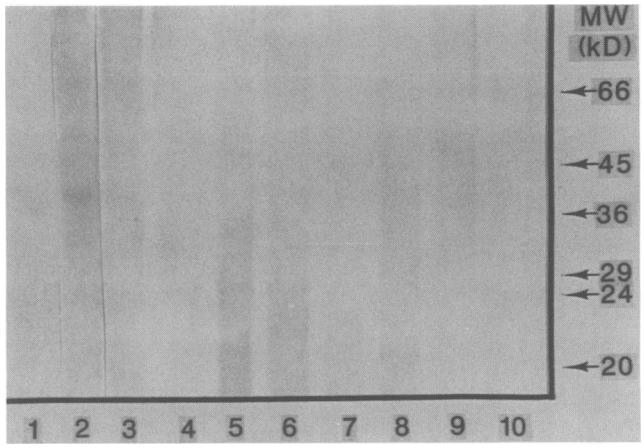

FIG. 3. Western immunoblots of sera from patients with other mycoses (lanes 1 to 5) and from normal individuals (lanes 6 to 10). Other mycoses include cryptococcosis (lane 1), histoplasmosis (lane 2), aspergillosis (lane 3), candidiasis (lane 4), and coccidioidomycosis (lane 5).

from 30 normal individuals or from 20 patients with other fungal infections, except for serum from one histoplasmosis patient, which demonstrated a faint band to the 40-kDa antigen (Fig. 3, lane 2). *Sporothrix* immunoblotting was not carried out on the other remaining serum samples from 80 patients with other mycoses or 270 healthy individuals.

DISCUSSION

There was little evidence of cross-reactivity between our *Sporothrix* antigen and the antibodies in the sera from patients with other systemic mycoses as determined by both solid-phase ELISA and Western immunoblots. From the ELISA results presented, no overlap occurred in titers between the normal sera or sera from patients with other fungal diseases and sera from our patient population, i.e., no patient with sporotrichosis had initial serum titers of less than 128, whereas no sera from normal individuals or patients with other fungal diseases had titers greater than 64 (Table 1). Serum from one patient with disseminated histoplasmosis (Fig. 3, lane 2) showed a faint Western blot antibody band to the 40-kDa antigen. This serum showed an ELISA titer of 64 against the *Sporothrix* antigen. We do not know whether this *Sporothrix* antibody to the 40-kDa antigen represents cross-reactivity or dual infection. Sera from two other patients with histoplasmosis had *Sporothrix* titers of 16 by ELISA, but did not show any detectable *Sporothrix* reactivity by Western blotting (data not shown). Sera from several of the healthy volunteer subjects also contained low ELISA titers against the *Sporothrix* antigen but no reactivity by Western blotting. In our survey of random sera from normal Oklahoma residents, we showed that 18% of these individuals have low titers of *Sporothrix* ELISA antibody, i.e., titers of 8 to 32, which may represent past subclinical infection.

Although these preliminary studies utilized a relatively crude soluble antigen preparation, it is apparent that the antibody responses against *S. schenckii* are heterogeneous. A single strain of *S. schenckii* was used throughout this study so that antibody responses of the patients could be compared against a standard antigen profile. By SDS-PAGE, 15 protein bands were identified in the soluble antigen preparation obtained from one cutaneous isolate (Fig. 1). In 1967, Bièvre (3) found 22 to 24 protein-staining bands by electrophoresis on 15% acrylamide gel of an extract obtained from broken *Sporothrix* yeast cells (cytoplasmic contents), but no comment was made as to the relative molecular weight mobilities of these proteins.

All the 25 sporotrichosis serum samples tested showed antibody reacting with antigens of 40 and 70 kDa. The 40-kDa bands were prominent in all sera, while the 70-kDa bands showed more variability in densities. We cannot explain the individual differences in the intensity of band staining between the sera from patients with cutaneous and extracutaneous sporotrichosis. The differences in band intensities (particularly with the 40- and 70-kDa proteins) in sera with the same ELISA titers (Fig. 2, lanes 2 and 7, sera from patients with pulmonary and cutaneous disease, respectively) could be accounted for by individual differences in the amount and type of antibody produced to specific antigen components. Differences in the affinity and avidity of the antigen to bind to nitrocellulose membranes versus polystyrene wells could also lead to these staining variations. Additionally, the differences in the conditions under which the assays were run could account for the differences in binding of antibody. For the ELISA, whole soluble antigen was attached to the polystyrene wells in the native state, whereas the immunoblots were run on reduced antigen preparations. This latter treatment may alter the structure of the antigen such that both the affinity and avidity of the antibody to bind to the antigen is affected. Additionally, ELISA measures total IgG binding to whole antigen, while immunoblots detect binding of specific IgG molecules to antigens separated by molecular weight.

Other than the one histoplasmosis serum mentioned above (Fig. 3), 40- and 70-kDa bands were not present in the 50 nonsporotrichosis serum samples tested by immunoblotting. This suggests that a serologic test utilizing the 40-kDa antigen (or both the 40- and 70-kDa antigens) would be useful for early identification of sporotrichosis. The lower-molecular-size (22- to 36-kDa) proteins in our soluble antigen preparation appear to correspond to proteinases of similar molecular size (18 to 37 kDa) isolated from *S. schenckii* by Tsuboi et al. (19, 20). With our soluble antigen preparation, sera from patients with extracutaneous sporotrichosis reacted with more antigenic determinants, and over a wider range of molecular sizes, than did sera from patients with cutaneous sporotrichosis. It appears that patients with extracutaneous disease produce detectable antibody to several more antigens contained in the soluble antigen preparation than do the patients with extracutaneous sporotrichosis. An immunodominant 32-kDa band occurred in sera from patients with extracutaneous disease, but this band was not seen in sera from patients with cutaneous disease (Fig. 2). This suggests that antibody characteristic of extracutaneous disease is produced against this 32-kDa antigen.

Studies are in progress to purify the immunodominant antigens (32, 40, and 70 kDa) from our crude soluble antigen preparation. Once purified, these antigens can be assessed for homogeneity by gel filtration, SDS-PAGE, and crossed immunoelectrophoresis; and they can be analyzed for various activities, including proteinase, and their possible relationship to pathogenesis. Additionally, experiments are needed to determine whether antigenically related proteins having the lower molecular weight are modifications of a common protein or are fragments of higher-molecular-weight proteins which were split under the denaturing conditions used in this study.

It is not known whether specific proteins or enzymes produced by *S. schenckii* contribute to the pathogenic potential of this fungal organism. However, in studies of human candidal infections, *Candida albicans* polypeptide profiles by immunoblot analysis have shown that antibody to certain polypeptides predominates in patients with systemic candidiasis, while antibody to mannans appears to predominate in sera of patients with superficial infections (2). In addition, other investigators have postulated that extracellular proteinases produced by various fungal organisms (*Microsporum* spp., *Trichophyton* spp., *Candida* spp.) may be linked to their virulence and pathogenesis (1, 8, 13, 18). Recently, Tsuboi and co-workers (19, 20) have reported the production of extracellular proteinases by *S. schenckii*, but these enzymes have not yet been linked to the pathogenesis of this disease. Identification of the significant antigenic determinants of *S. schenckii* and purification of these specific proteins should lead to the development of specific and sensitive ELISA and immunoblot diagnostic tests for sporotrichosis which might be useful to indicate the presence of unsuspected extracutaneous disease or to identify the cause of deep tissue disease when the organism is difficult (or impossible) to culture, e.g., sporotrichosis meningitis (14).

These tests would also provide a seroepidemiologic tool for identifying patients with self-limited or past infection.

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