JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 1987, p. 358–363
0095-1137/87/020358-06$02.00/0

Vol. 25, No. 2

Electrophoretic and Immunoblot Analyses of *Rhizopus arrhizus* Antigens

DEBORAH R. WYSONG[1] AND ALAYN R. WALDORF[1,2]*

*Evans Memorial Department of Clinical Research and the Department of Medicine, University Hospital, Boston University Medical Center, Boston, Massachusetts 02118,[1] and Department of Biomedical Health Sciences, School of Public Health, University of California, Berkeley, California 94720[2]**

Received 16 June 1986/Accepted 4 November 1986

Four antigen preparations from *Rhizopus arrhizus* were made and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and column chromatography. Electrophoretic analyses of these antigens indicated that there are 18 to 28 component bands with a molecular mass range of approximately 10,500 to 83,000 daltons. Seven of these bands appear to be components common to three antigen preparations. Several of the bands identified by SDS-PAGE were composed of glycoproteins or carbohydrates as determined by their affinity for concanavalin A. Western blots, using sera from five patients with mucormycosis, consistently identified five different determinants in the *R. arrhizus* antigens separated by SDS-PAGE. This suggests that several of the *Rhizopus* antigens are present during mucormycosis. Four of the antigenic determinants recognized by patient sera reacted with the concanavalin A-peroxidase stain, indicating that they are composed of glycoproteins or carbohydrate. Enzyme-linked immunosorbent assays of sera from five patients with mucormycosis and with rabbit antisera resulted in antibody titers ranging from 1:64 to 1:32,000 for the *R. arrhizus* antigens.

Rhizopus arrhizus (*R. oryzae*) is the most common agent of mucormycosis, an opportunistic fungal infection in which the mechanisms of host defense are not completely understood (16). There is evidence of acquired immunity in experimental mucormycosis; mice previously exposed to sublethal doses of spores are resistant to subsequent intravenous challenge (2). However, spore agglutinins do not restrict spore germination in vitro or in vivo (20), and vaccination with nonviable spores does not induce significant protection (2). Thus, if humoral immunity plays a role in host defense, antibodies directed against the hyphal forms are more effective than those directed against spores. Passive transfer of specific antisera produced against mycelial homogenates increases the resistance to morbidity of mice with mucormycosis, although spore germination and infection occur (17). The mechanism(s) by which the antisera increased the resistance to fatal mucormycosis is unknown, and the fungal antigens with which the antibodies react are also unknown. Characterization of the mycelial antigens and the reactivities of these antigens with sera from patients with mucormycosis are prerequisites for further studies of antibody-mediated host defenses.

To identify specific *Rhizopus* constituents which occur during mucormycotic infection and which may be important in host defense, young mycelial homogenates, water-soluble fractions, and culture filtrate antigens from *R. arrhizus* were prepared. Electrophoretic fractionation in sodium dodecyl sulfate (SDS)-containing polyacrylamide gels was used to characterize the major components of four *R. arrhizus* antigens, allowing reproducible, high-resolution analytical fractionation of antigenic components (6). In addition, sensitive methods of carbohydrate staining by concanavalin A (ConA) binding allowed further characterization of these mycelial and culture filtrate antigens.

Separation of *R. arrhizus* antigens by SDS-polyacrylamide gel electrophoresis (PAGE) followed by electrophoretic transfer of the proteins to nitrocellulose membranes produced a replica or Western blot of the original gel (8, 14). The antigens present in *R. arrhizus* mycelial homogenates and filtrates were detected with specific antisera from immunized rabbits and from five patients with mucormycosis. The use of an enzyme-linked immunosorbent assay (ELISA) for the determination of antibody titers is also described. Antigens derived from *R. arrhizus* elicited a strong response in the Western blot assay when reacted with sera obtained from patients with mucormycosis. The results demonstrate that several components in hyphal homogenate antigens of *R. arrhizus* react with antibodies in sera from patients with mucormycosis.

MATERIALS AND METHODS

Fungi and preparation of antigens. An isolate of *R. arrhizus* (ATCC 11886, *R. oryzae*) was maintained on potato dextrose agar, and sporangiospores were harvested as previously described (19, 21). *R. arrhizus* spores (10^6) were inoculated into 1 liter of asparagine broth (12) and incubated at 37°C on a gyratory shaker (150 rpm; Braun Autoshake) for 3 days. The mycelial growth was then harvested by filtration over Whatman no. 1 filter paper and washed in either phosphate-buffered saline (PBS) or trichloroacetic acid before being mechanically homogenized at 4°C with a tissue grinder (Tri-R Instruments, Rockville Centre, N.Y.). Hyphae were disrupted into fragments of approximately 10 μm (determined by microscopic observation). The PBS-washed homogenate was centrifuged at 16,500 × *g* for 60 min, and the supernatant was dialyzed against distilled water for 24 h at 4°C. The dialyzed material was lyophilized and designated the homogenate antigen (17).

A second mycelial antigen, referred to as the trichloroacetic acid antigen, was prepared by precipitating the trichloroacetic acid-washed homogenate supernatant with an equal volume of acetone (at 4°C), collecting the precipitate by centrifugation at 16,500 × *g* for 60 min, and drying this precipitate overnight in an evacuated desiccator (11).

* Corresponding author.

A third mycelial antigen was obtained by stirring the PBS-washed homogenate with Triton X-100 (0.5% [vol/vol]) in 0.05 M NH_4HCO_3 at pH 8.0 and 30°C for two 2-h periods to solubilize wall-associated constituents (7). The detergent-soluble extracts were dialyzed and concentrated approximately 30-fold against 0.015 M NaCl containing 10% (wt/vol) polyethylene glycol 6000. The soluble supernatants were stored at 4°C overnight and designated for column fractionation, as described below.

A metabolic antigen was prepared by inoculating 1 liter of asparagine broth with 10^6 *R. arrhizus* spores and incubating the static culture at 37°C for 5 weeks. After incubation, the supernatant was collected and precipitated with an equal volume of 4°C acetone. The precipitate was dried overnight in an evacuated desiccator (11).

At least three lots of each antigen were prepared and analyzed for protein and carbohydrate content and by SDS-PAGE (see below). All antigens were stored at −20°C until used.

Affinity chromatography. The mycelial antigen processed with Triton to solubilize cell wall constituents was subjected to affinity chromatography on ConA (Sigma Chemical Co., St. Louis, Mo.)-bound agarose (7). A column (0.9 by 10 cm) of ConA-Sepharose 4B (Sigma) was equilibrated with 0.01 M Tris hydrochloride buffer (pH 7.2) containing 1 mM CaCl, 1 mM $MnCl_2$, and 0.025 M NaCl. The antigen (30 mg of protein), equilibrated by dialysis in the column buffer, was applied to the column, and effluent was monitored for protein content at A_{280}. After the elution of the Triton, 0.2 M methyl α-D-mannopyranoside (MMP, grade II; Sigma) was added to the buffer, and the elution was continued. The eluate fraction was designated as the column-purified antigen.

Protein and carbohydrate assays. The total protein was estimated in the four *R. arrhizus* antigens by a dye binding assay using bovine serum albumin as the protein standard (1). Carbohydrate was determined colormetrically with phenol-sulfuric acid, using either mannose or fucose as a reference standard (5). Assays were performed in triplicate and the mean ± standard deviation of protein and carbohydrate content, from lots of like antigens, were compared using the Student *t* test for independent means (two-tailed).

Rabbit sera. Preimmune rabbit sera, for use as a control, was obtained from New Zealand White rabbits before injection of the *R. arrhizus* antigens. Two groups of three rabbits each were immunized by intramuscular injections of either the homogenate antigen or the metabolic precipitate antigen. Each rabbit was injected with a 1-ml emulsion of the antigen (containing 0.5 mg of carbohydrate) dissolved in sterile saline and Freund incomplete adjuvant (13, 18). For intravenous injections, each antigen contained 0.5 mg of carbohydrate per ml of saline. All sera were divided into aliquots and stored at −70°C until needed.

Human sera. Human sera were a generous gift from Leo Kaufman at the Centers for Disease Control in Atlanta, Ga. The sera were from five patients diagnosed as having mucormycosis caused by either *R. arrhizus* or *R. oryzae*. Additional information regarding these patients, including predisposing conditions or underlying diseases, was not available. Control sera were obtained from healthy human volunteers. All sera were divided into aliquots and stored at −70°C.

PAGE. *R. arrhizus* antigens were prepared for discontinuous SDS-PAGE by boiling for 3 min in sample buffer (0.0625 M Tris hydrochloride [pH 6.8], 2% SDS, 10% glycerol, 0.5% 2-mercaptoethanol, 0.001% bromphenol blue) (6). *R. arrhizus* antigens were electrophoresed in 12, 15, or 5 to 20% gradient polyacrylamide slab gels (140 by 160 by 1.5 mm) (6, 10, 23). Samples of *R. arrhizus* antigen (20 to 30 μl) contained 100 to 200 μg of protein. Electrophoresis was performed at 30 mA constant current for 4 to 5 h in electrode buffer (0.025 M Tris, 0.192 M glycine, 0.1% SDS [pH 8.3]). After electrophoresis, the gels were stained for visualization with either silver stain (Bio-Rad Laboratories, Richmond, Calif.) or ConA-peroxidase, or they were electrophoretically transferred to nitrocellulose (see below).

Detection of ConA-binding constituents. Carbohydrates in the *R. arrhizus* antigens separated by SDS-PAGE were identified by a ConA-peroxidase labeling technique, because it is more sensitive for mannose-containing glycoproteins than the periodic acid-Schiff method (22). Briefly, after electrophoresis, the slab gels were removed and sliced into vertical segments. One segment was silver stained for protein, and SDS was removed from the remaining two segments (22). One of the washed gel slices was immersed in a solution containing ConA (0.5 mg/ml) in PBS (pH 7.2), and the control gel was immersed in the ConA solution containing, in addition, 0.2 M MMP, for 45 min. The gels were then washed three times (30 min each) with PBS and immersed in horseradish peroxidase (0.1 mg/ml; Sigma) in PBS for 45 min. After an additional series of washes in PBS, the gels were incubated in PBS containing 50 mg of 3,3′-diaminobenzidine (Sigma) and 15 μl of hydrogen peroxide (30%).

Immunochemical detection of antigens on nitrocellulose paper. The SDS-PAGE preparations were equilibrated with transfer buffer (0.025 M Tris, 0.192 M glycine, 20% methanol [pH 8.3]) for 30 min, and *R. arrhizus* antigens were transferred to a nitrocellulose membrane (0.45-μm pore size; Schleicher & Schuell, Keene, N.H.) for 30 to 45 min at 0.6 to 1.2 A using a Hoefer Transphor unit (Hoefer Scientific Instruments, San Francisco, Calif.) (14). Nonfat dry milk was used in the incubation medium for blocking nonspecific reactions and in the wash buffer (8). Nitrocellulose strips of the transferred components were incubated in milk buffer (5% [wt/vol] nonfat dry milk, 0.01% Antifoam A [Sigma], and 0.0001% merthiolate in PBS) for 60 min at 37°C to block any unbound sites on the membrane. The strips were then incubated overnight at 4°C with patient, rabbit, or normal control sera which were diluted in milk buffer. Strips were then washed (three times for 20 min) in milk buffer. Indicator antibodies, horseradish peroxidase-conjugated, affinity-purified goat anti-human or anti-rabbit immunoglobulin G (Cooper Biomedical, Inc., Malvern, Pa.), were diluted (1:200) in the milk buffer and incubated for 1.5 to 2 h at 37°C. After the strips were washed, the chromogenic substrate, 4-chloro-1-naphthol (Bio-Rad), was added (8). Development was stopped by washing the strips in distilled water.

Enzyme immunoassay. Antibody titers in sera were determined by an indirect ELISA (15). *R. arrhizus* antigens (50 μg of protein per ml of buffer) were solubilized in carbonate-bicarbonate coating buffer (0.05 M [pH 9.6]), and 150 μl per well was adsorbed overnight at 4°C to 96-well microtiter plates (Immulon I; Dynatech Laboratories, Inc., Alexandria, Va.). The plates were then washed three times with PBS-Tween (0.05% Tween 20). Serial twofold dilutions (1:2 to 1:32,000) of sera from patients, rabbits, or controls were added (0.1 ml per well), and the plates were incubated for 2 h at 37°C. After washing, 200 μl of horseradish peroxidase-conjugated anti-human (1:1,000) or anti-rabbit (1:5,000) IgG was added. Incubation was continued for 1 h, the plates were washed, and the chromogenic substrate, *o*-phenylene-

TABLE 1. Protein and carbohydrate content of *R. arrhizus* antigens[a]

Antigen prepn	Protein (μg/mg)	Carbohydrate (μg/mg)
Homogenate	52.7 (±4.26)	65.3 (±3.2)
Trichloroacetic acid	101.1 (±15.2)	81.0 (±9.1)
Metabolic	5.1 (±0.2)	15.7 (±4.0)
ConA column purified	27.4 (±4.8)	ND

[a] Contents are given in micrograms per milligram of lyophilized or vacuum-dried material. Values are means (± standard deviation) for at least three antigen preparations. ND, Not determined.

diamine (400 μg/ml; Sigma), and hydrogen peroxide (40 μl of 30%) were added. The plates were incubated for 30 min before the reaction was stopped with H_2SO_4 (25 μl of 4 M). The plates were read photometrically in an ELISA reader (Dynatech) at 488 nm. Normal human or preimmune rabbit sera were used as negative controls on each plate. Titers were determined as the net absorbance readings after subtraction of the controls. A positive result was recorded as the highest dilution with a net A_{488} greater than 0.1 above that of a known negative sample.

RESULTS

Chemical analysis of antigens. The protein and carbohydrate concentrations in each of the *R. arrhizus* antigenic preparations varied significantly between the four antigens ($P < 0.001$) but were consistent between lots of like antigens (not significant; Table 1). Considerable amounts of water were retained in the lyophilized and vacuum-dried antigens, as evidenced by the low protein and carbohydrate yields.

SDS-PAGE. Antigenic components of the *R. arrhizus* preparations were separated by SDS-PAGE and silver stained (Fig. 1). Because of the anomalous behavior of glycoproteins in single-concentration gels (6), the molecular masses of the *R. arrhizus* antigens were determined on gradient gels. An SDS-PAGE profile of the homogenate antigen is shown in Fig. 1, lane 1; approximately 18 bands ranging in molecular mass from 14.4 to 66 kilodaltons (kDa) could be routinely detected. The major antigens had apparent molecular masses of 14.4, 14.6, 15.3, 15.7, 20.7, 23, 25, 28, 35.5, 42, 54, 58, 60, and 66 kDa. The seven major bands and two minor bands which were consistently detected in the trichloroacetic acid antigen are shown in Fig. 1, lane 2, with apparent molecular masses of 14.4, 15.3, 15.7, 21, 29, 60, and 83 kDa and 16 and 54 kDa, respectively. Dark, nonspecific staining of the trichloroacetic acid antigen by silver stain suggested the presence of large amounts of carbohydrate. The metabolic antigen was less complex, with four major and two minor bands with approximate molecular masses of 12.5, 15.3, 16.5, and 42 kDa and 10.5 and 68 kDa, respectively (Fig. 1, lane 3).

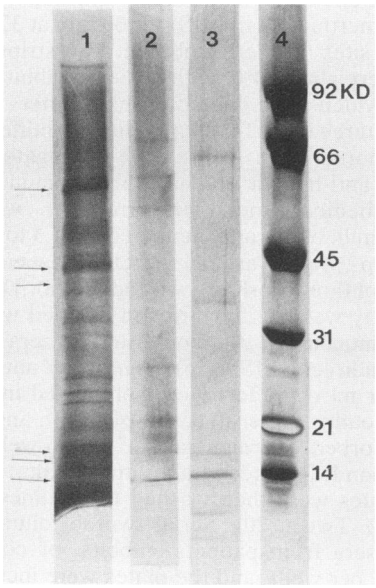

FIG. 1. Silver stain of 5 to 20% gradient SDS-PAGE of *R. arrhizus* antigens. Lanes: 1, homogenate antigen; 2, trichloroacetic acid antigen; 3, metabolic antigen; 4, molecular mass markers. Arrows on the left indicate the positions of the common antigens.

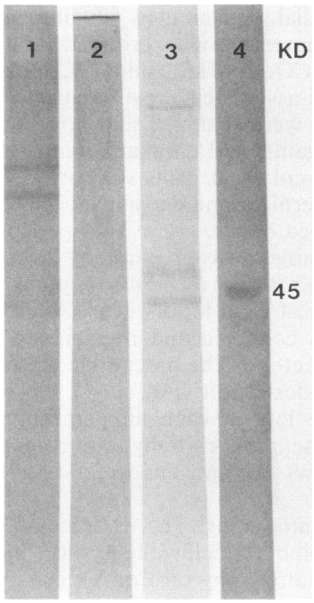

FIG. 2. SDS-PAGE of *R. arrhizus* antigens on 5 to 20% gradient gels stained with ConA-peroxidase. Lanes: 1, homogenate antigen; 2, trichloroacetic acid antigen; 3, metabolic antigen; 4, molecular mass standard.

The composition of the *R. arrhizus* antigens was also studied. Some of the gels were stained specifically by ConA-peroxidase to locate carbohydrate constituents. Two bands in the homogenate antigen were stained by this technique (Fig. 2, lane 1). One of the bands, with an approximate molecular mass of 58 kDa, was a major protein by silver staining. The second band was only a minor band by silver staining and had an approximate molecular mass of 54 kDa. The trichloroacetic acid antigen did not stain by the ConA method (Fig. 2, lane 2). The metabolic antigen had three components which stained by ConA-peroxidase, only one of which was detected by silver staining, at 68 kDa (Fig. 2, lane 3). The remaining metabolic antigen components that were stained by ConA had molecular masses of 43 and 45 kDa. The specific binding of ConA was confirmed in the gels by the complete abolition of the reaction with MMP (not shown).

The column-purified antigen, obtained after affinity chromatography with ConA, was also fractionated by SDS-

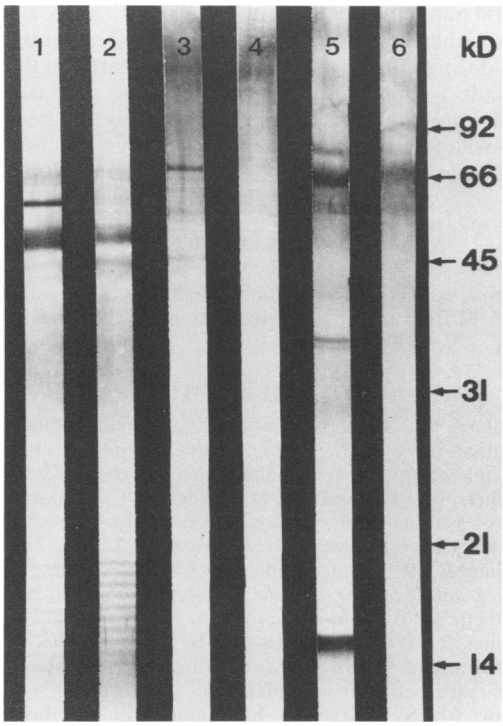

FIG. 3. Western blot analysis of electrophoretically resolved antigens from *R. arrhizus* transferred to nitrocellulose paper and reacted with either immune rabbit sera (lanes 1, 3, and 5) or a serum from a patient with mucormycosis (lanes 2, 4, and 6). Lanes: 1 and 2, homogenate antigen; 3 and 4, trichloroacetic acid antigen; 5 and 6, metabolic antigen. The arrows on the right indicate the positions of molecular mass markers.

PAGE and compared with the precolumn material and with the effluent fraction. The precolumn material contained at least 15 components ranging in molecular mass from 15 to 77.6 kDa. The bound and unbound fractions were identical, with the exception of a 23-kDa component which was eluted from the ConA after treatment with MMP. The remaining components had approximate molecular masses of 15, 29.5, 31, 32.4, 38.9, 46, and 56.2 kDa (not shown).

Reaction of sera with *R. arrhizus* antigens by immunoblotting. The results for the immunoblots of the *R. arrhizus* antigens from SDS-PAGE with antisera from immunized rabbits or from patients with mucormycosis are shown in Fig. 3. Comparison of the SDS-PAGE profiles and the immunoblots shows that the rabbit antisera reacted with several of the antigenic bands in the *R. arrhizus* homogenate antigen (Fig. 3, lane 1). The most intense reactions occurred with components of approximate molecular masses of 35.5, 42, 54, 60, and 66 kDa. Five sera from patients with mucormycosis were reacted with the *R. arrhizus* homogenate antigen. Reactions consistently occurred at the same sites as those where rabbit antiserum reactions were observed, with the exception of the 60-kDa component, to which the human sera showed little or no reaction, and a 42-kDa component, in which an increased reaction was observed (representative reaction is shown in Fig. 3, lane 2). Rabbit antisera to the homogenate antigen recognized three components of the trichloroacetic acid antigen at approximate molecular masses of 42, 61, and 83 kDa (Fig. 3, lane 3). The sera from patients with mucormycosis did not react with the trichloroacetic acid antigen when Western blotted (Fig. 3, lane 4). Rabbit antisera produced against the metabolic antigen recognized components with approximate molecular masses of 15.3, 38, 60, 68, and 83 kDa (Fig. 3, lane 5). The patient sera reacted with components of the metabolic antigen with approximate molecular masses of 60, 68, and 83 kDa (Fig. 3, lane 6). Rabbit antisera produced against the metabolic antigen or the homogenate antigen and patient sera did not react with the column-purified antigen (not shown). Normal control human sera and preimmune rabbit sera did not react with any of the antigen preparations blotted onto nitrocellulose (not shown).

ELISA. Peak antibody titers of sera from patients with mucormycosis, from rabbits immunized with *R. arrhizus* antigens, and from normal human donors or preimmunized rabbits are shown in Table 2. The sera from the five patients with mucormycosis had antibody titers ranging from 1:32,768 to 1:1,024 for three *R. arrhizus* antigens. Normal human control and preimmunized rabbit sera had titers of <1:256 and <1:64, respectively.

DISCUSSION

Antigenic preparations from *R. arrhizus* mycelium and culture filtrates were analyzed by SDS-PAGE and contained complex antigenic mixtures composed of proteins, glycoproteins, and, possibly, polysaccharides. Approximately 18 to 28 components were found in the homogenate, trichloroacetic acid, and metabolic antigen by SDS-PAGE. Eight additional components were found in the ConA-column-purified antigen. The antigenic components with molecular masses of 14.4, 15.3, 15.7, 42, 54, 60, and 83 kDa appear to be common antigenic components. Of these common bands, only the 14.4-, 15.3-, and 60-kDa bands appear to be of protein alone, because the others also stained by the ConA technique. Numerous other components appear to be unique to the method of antigen preparation, suggesting that variations in preparation method can significantly alter antigenic components.

Because the hyphal walls of fungi in the order *Mucorales* consist of fucose, mannose, glucuronic acids, chitosan, chitin, and polyphosphate (3, 4), an attempt to stain carbohydrate and glycoprotein components in the *R. arrhizus* antigens was made using a ConA gel staining method. The plant lectin, ConA, possesses a specific capacity to bind primarily to alpha-D-glucose or mannose residues on macromolecules and also, via free binding sites, to horseradish peroxidase. Several components in the homogenate and metabolic antigen from *R. arrhizus* were stained by the

TABLE 2. ELISA endpoint titers

Sample	Antigen titer[a]		
	Homogenate	Precipitate	Trichloroacetic acid
Patient[b] serum			
1	16,384	32,768	32,768
2	16,384	32,768	16,384
3	16,384	16,384	16,384
4	1,024	16,384	32,768
5	4,096	32,768	32,768
Rabbit antiserum	>32,768	32,768	32,768
Normal human serum	<128	<256	<128
Normal rabbit serum	<64	<64	<64

[a] Endpoint titer. The last dilution of antiserum which yielded an absorbance value of at least 0.1 above that of the corresponding control (negative) serum.
[b] Patients diagnosed as having mucormycosis caused by either *R. arrhizus* or *R. oryzae*.

ConA-peroxidase. These components ranged in molecular mass from 43 to 83 kDa. The 58- and 68-kDa bands appear to be glycoproteins, because they stained by both silver and ConA methods. Fractionation by affinity chromatography indicated the presence of a ConA binding component with a molecular mass of 23 kDa.

By using Western immunoblotting (14), several immunodominant determinants in the complex antigenic mixtures could be identified. Antigenic components of the homogenate and metabolic antigen were recognized by five mucormycotic patient sera. The strongest of these reactions occurred with components of approximate molecular masses of 42, 54, 60, 68, and 83 kDa. The 42-, 54-, and 68-kDa components appear to be the same as those stained by the ConA-peroxidase, suggesting that carbohydrate(s) or glycoproteins and, possibly, hyphal wall constituents are recognized by the host during infection and, perhaps, are the site of humoral defense against these organisms.

Significant ELISA titers were obtained from sera of patients with mucormycosis, suggesting that the ELISA is sensitive in detecting anti-*Rhizopus* antibodies. Control sera from normal human donors or from normal rabbits had titers against each *R. arrhizus* antigen, presumably as the result of environmental exposure to this ubiquitous organism. Because case histories from the five patients were not available, it was not possible to determine what caused the underlying immunosuppression and allowed mucormycosis to occur in these five individuals. Thus, sera from patients with similar underlying diseases but who did not have mucormycosis were not evaluated. Further work is necessary to determine the specificity of the reagents and their possible role as immunologic probes.

Because mucormycosis is often a rapidly fatal infection, an accurate and timely diagnosis is important. Unfortunately, there are no standardized, rapid serologic methods (9). In addition, epidemiological and clinical investigations would benefit from the availability of more sensitive and specific antigens. The results presented here represent a preliminary step in the identification of antigenic fractions that could be used for antibody detection. These antigens could also be used to generate specific antibodies or to detect antigen in the tissue or blood of patients with mucormycosis. Furthermore, such antigens may be useful for research in host defense mechanisms. Importantly, all five of the patient sera responded to the *R. arrhizus* antigens and consistently recognized only a few of the 20 to 30 bands as identified by SDS-PAGE. The antigenicity of these antigens was preserved under the reducing conditions of electrophoresis. High ELISA titers in patient sera, with respect to the trichloroacetic acid antigen, and yet a lack of reactivity to this antigen by immunoblot underscore the sensitivity of the ELISA for detecting antibody and suggest that reducing conditions of SDS-PAGE destroyed important immunodeterminants in the trichloroacetic acid-extracted antigen.

Although widely distributed in nature, *R. arrhizus* is a fungus that rarely causes life-threatening infection in normal individuals. However, some patients, immunocompromised by either drugs or disease, are particularly susceptible to widespread mucormycotic infection. Specific antisera, produced against hyphal forms, are effective in reducing pathology in mice with mucormycosis (17), although the mechanism of protection is unknown. Analyses of *R. arrhizus* antigens and antibody responses to different antigens of *R. arrhizus* will help determine the role humoral immunity plays in host defense against *R. arrhizus* infection. The recognition of several antigenic components of *R. arrhizus* by sera from patients with mucormycosis indicates that these antigens are present during infection. The antigens prepared in this study were made from hyphal organisms on the premise that if antibodies are important in host defense they will be directed against wall-associated antigens. The physical location of these antigens on *R. arrhizus* remains to be determined.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants AI21474 and AI23494 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. **Bradford, M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–260.
2. **Corbel, M. J., and S. M. Eades.** 1976. Experimental phycomycosis in mice; examination of the role of acquired immunity in resistance to *Absidia ramosa*. J. Hyg. **77**:221–233.
3. **Datema, R., H. Van den Ende, and J. G. H. Wessels.** 1977. The hyphal wall of *Mucor mucedo*. 1. Polyanionic polymers. Eur. J. Biochem. **80**:611–619.
4. **Datema, R., J. G. H. Wessels, and H. Van den Ende.** 1977. The hyphal wall of *Mucor mucedo*. 2. Hexosamine-containing polymers. Eur. J. Biochem. **80**:621–626.
5. **Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28**:350–356.
6. **Hames, B. D.** 1984. An introduction to polyacrylamide gel electrophoresis, p. 1–86. *In* B. D. Hames and D. Rickwood (ed.), Gel electrophoresis of proteins—a practical approach. IRL Press, Washington, D.C.
7. **Hearn, V. M., and D. W. R. Mackenzie.** 1980. The preparation and partial purification of fractions from mycelial fungi with antigenic activity. Mol. Immunol. **17**:1097–1103.
8. **Johnson, D. A., J. W. Gautsch, J. R. Sportsman, and J. H. Elder.** 1984. Improved technique utilizing nonfat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. Genet. Anal. Technol. **1**:3–8.
9. **Jones, K. W., and L. Kaufman.** 1978. Development and evaluation of an immunodiffusion test for diagnosis of systemic zygomycosis (mucormycosis): preliminary report. J. Clin. Microbiol. **7**:97–103.
10. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
11. **Palmer, D. F., L. Kaufman, W. Kaplan, and J. Casallarn.** 1977. Immunodiffusion test for aspergillosis, p. 111–122. *In* D. F. Palmer (ed.), Serodiagnosis of mycotic diseases. Charles C Thomas, Springfield, Ill.
12. **Rippon, J. W.** 1982. Medical mycology. The pathogenic fungi. The pathogenic actinomycetes, p. 781. The W. B. Saunders Co., Philadelphia.
13. **Standard, P. G., and L. Kaufman.** 1976. Specific immunological test for the rapid identification of members of the genus *Histoplasma*. J. Clin. Microbiol. **3**:191–199.
14. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
15. **Voller, A., D. E. Bidwell, and A. Bartlet.** 1979. Practical aspects, p. 19–43. *In* A. Voller and D. E. Bidwell (ed.), Enzyme linked immunosorbent assay (ELISA). Dynatech Laboratories, Inc., Alexandria, Va.
16. **Waldorf, A. R.** 1986. Host-parasite relationship in opportunistic mycoses. Crit. Rev. Microbiol. **13**:133–172.
17. **Waldorf, A. R., C. Halde, and N. A. Vedros.** 1983. Passive immunization in murine mucormycosis. Mycopathologia **83**:149–155.

18. **Waldorf, A. R., C. Halde, and N. A. Vedros.** 1983. Imm unodiffusion and complement fixation assays with sera from mucormycotic-infected mice. Mycopathologia **83**:157–160.
19. **Waldorf, A. R., S. M. Levitz, and R. D. Diamond.** 1984. In vivo bronchoalveolar macrophage defense against *Rhizopus oryzae* and *Aspergillus fumigatus*. J. Infect. Dis. **150**:752–760.
20. **Waldorf, A. R., L. Peter, and A. Polak.** 1984. Mucormycotic infection in mice following prolonged incubation of spores in vivo and the role of spore agglutinating antibodies on spore germination. Sabouraudia **22**:101–108.
21. **Waldorf, A. R., N. Ruderman, and R. D. Diamond.** 1984. Specific susceptibility to mucormycosis and bronchoalveolar macrophage defense against *Rhizopus*. J. Clin. Invest. **74**:150–160.
22. **Wood, J. G., and F. O. Sarinana.** 1975. The staining of sciatic nerve glycoproteins on polyacrylamide gels with concanavalin A-peroxidase. Anal. Biochem. **69**:320–322.
23. **Wu, F. S., and M. Y. Wang.** 1984. Extraction of proteins for sodium dodecyl sulfate-polyacrylamide gel electrophoresis from protease-rich plant tissues. Anal. Biochem. **139**:100–103.