INFECTION AND IMMUNITY, Feb. 1984, p. 715–721
0019-9567/84/020715-07$02.00/0

Vol. 43, No. 2

Identification and Molecular Weight Characterization of Antigens from *Candida albicans* That Are Recognized by Human Sera

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Received 17 August 1983/Accepted 31 October 1983

Antigenic components in the cytoplasmic extract of *Candida albicans* were examined after fractionation by concanavalin A-Sepharose and DEAE-Sephacel ion-exchange chromatography. Fractions from the DEAE column were tested by fused rocket immunoelectrophoresis for their reactivity with antibodies in the sera of 20 patients with disseminated candidiasis. Three groups of fractions (regions A, B, and C) from the DEAE column were defined by their reactivity with these sera. Immunoblot analysis with 20 human sera identified 18 antigenic components in regions A, B, and C. Region A contained nine antigens, region B contained four antigens, and region C contained five antigens. Region A contained an antigen with an apparent molecular weight of 48,000 that was recognized by 7 of 10 sera from patients with disseminated candidiasis. Immunoprecipitation experiments with labeled proteins from region A and 51 human sera also demonstrated the presence of a major antigen whose apparent molecular weight is 48,000 to 52,000. The 48- to 52-kilodalton protein is an abundant protein in region A and is the most frequently recognized protein by antibodies in the sera of patients with disseminated candidiasis. Patients with disseminated candidiasis had significantly higher levels of antibody (immunoglobulin G) ($P < 0.001$) directed against the 48- to 52-kilodalton protein than did patients with noninvasive forms of candidiasis, patients with other fungal infections, or normal, healthy persons.

Over the past 30 years, the incidence of disseminated candidiasis has increased markedly (4, 5, 15, 22, 28, 29, 32, 33). As a major cause of morbidity and mortality among immunocompromised patients, severe *Candida* spp. infections have received a great deal of clinical and research investigation. Despite these efforts, the diagnosis of disseminated candidiasis is still problematic. Numerous serological tests have been described to facilitate the diagnosis of *Candida* infections. One approach to the serodiagnosis of disseminated *Candida* spp. infections has been the detection of antibodies directed against the cell wall or cytoplasmic components of *Candida albicans*. Techniques employed for *Candida* spp. antibody detection have been reviewed by Odds (30). Recently, quantitative techniques for the detection of *Candida albicans*-specific antibodies have been reported (7, 17, 25, 26). Another approach to the serodiagnosis of disseminated disease has been the detection of antigens or metabolites in patient sera or tissue (1, 3, 11, 12, 16, 18, 24, 27, 31). These methods are popular, as the test results are not dependent upon the ability of the patient to mount a humoral immune response. With so many serodiagnostic techniques in use, there is considerable disagreement regarding the sensitivity, specificity, and predictive value positive of many of the serological tests. Explanations for the conflicting results emphasize differences between antigen preparations, testing methods, and patient populations.

The identification and characterization of serologically important antigens of *C. albicans* would greatly facilitate the development of an accurate serodiagnostic test. Several investigators have studied the antigenic structure of the cell wall and cytoplasmic extracts from the yeast and mycelial phase cells of *C. albicans* (2, 6, 8, 10, 13, 23, 36, 37). Little research has been reported that identifies and characterizes antigens in the cytoplasmic extract that are recognized during disseminated candidiasis. One report by Greenfield and Jones (13) describes the purification and characterization of a major cytoplasmic antigen of *C. albicans*. The purpose of this research has been to fractionate, identify, and characterize by apparent molecular weight the antigenic components in the cytoplasmic extract of *C. albicans* that are recognized by patients with disseminated candidiasis.

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MATERIALS AND METHODS

Culture methods. *C. albicans*, serotype A (ATCC 32354), was grown on Sabouraud slants for 18 to 24 h at 25°C. The growth from one slant was inoculated into 50 ml of liquid synthetic medium (21) and incubated on a gyratory shaker at 150 rpm at 25°C. After 18 h, 1-ml samples of this growth were added into 100 ml of liquid synthetic medium and rotated at 150 rpm for 24 h at 37°C. The cells were harvested by centrifugation and suspended with 0.05 M Tris-hydrochloride (pH 7.4) with 0.02% (wt/vol) NaN_3. The cell slurry was stored at −80°C until used for preparation of extracts. Cells grown under these conditions were 90% mycelium and 10% yeast cells.

Antigen preparation. (i) Preparation of cytoplasmic extract. The mycelial cytoplasmic extract was prepared by mechanical disruption essentially as described by Syverson et al. (37). Samples were examined microscopically for cell breakage. The reactivity of the mycelial cytoplasmic extract prepared from different batches of cells was tested against sera from patients with systemic candidiasis with crossed immunoaffinoelectrophoresis in the presence of concanavalin A (ConA) and was found to be reproducible.

(ii) ConA affinity chromatography. The mycelial cytoplasmic extract was passed through ConA-Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) affinity columns to remove the cell wall mannan, glucan, and mannan-protein and glucan-protein complexes. The loading and running buffer was 0.05 M Tris-hydrochloride (pH 7.4) with 0.02% (wt/vol) NaN_3, 0.001 M $MnCl_2$, 0.001 M $MgCl_2$, and 0.001 M $CaCl_2$. The mannan-depleted material (nonbound fraction) was pooled, dialyzed against 0.05 M $(NH_4)_2HCO_3$ (pH 8.0),

lyophilized, and suspended in 0.05 M Tris-hydrochloride (pH 7.8) with 0.02% (wt/vol) NaN_3 for ion-exchange chromatography. The removal of mannan-containing components was monitored by crossed immunoaffinoelectrophoresis with ConA.

(iii) Ion-exchange chromatography. The mannan-depleted mycelial cytoplasmic extract was fractionated by ion-exchange chromatography through a DEAE-Sephacel (Pharmacia) column. Fractions were eluted with a linear salt gradient, 0 to 0.4 M NaCl in 0.05 M Tris-hydrochloride (pH 7.8) with 0.02% (wt/vol) NaN_3. Conductivity was measured by using a conductivity bridge (model RC 16B2; Industrial Instruments, Cedar Grove, N.J.); the gradient was found to be linear from 0 to 0.25 M NaCl (data not shown). Tightly bound material was eliminated from the column with a 1.0 M NaCl wash.

Immunoelectrophoretic techniques. (i) Fused rocket immunoelectrophoresis. Fused rocket immunoelectrophoresis was performed as previously described (35). Fractions (35 μl) eluted from the DEAE-Sephacel column were placed in each well and electrophoresed against sera from patients with disseminated candidiasis for 16 to 20 h at 2 to 3 V/cm at 10°C.

(ii) Immunoblot analysis. Fractions from the DEAE column representing regions A, B, and C (Fig. 1) were prepared for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (20) and separated on 12% acrylamide gels. When electrophoresis was completed, the gels were removed, and the separated proteins were electrophoretically transferred to nitrocellulose paper (NCP) (Schleicher & Schuell, Inc., Keene, N.H.; BA 85, 0.45 μm) essentially as described by Towbin et al. (39). The NCP gel was electrophoresed overnight under constant current at 150 mA at 10°C in a Transblot cell (Bio-Rad Laboratories, Richmond, Calif.). After electrophoresis, the NCP was either stained immunochemically or with amido black (0.1% [wt/vol] in 45% [vol/vol] methanol, 10% [vol/vol] acetic acid, and 45% [vol/vol] distilled water. Immunochemical staining was performed by first blocking the NCP with 2.5% (wt/vol) casein in Tris-buffered saline (pH 7 to 8) for 4 h at 45°C. All subsequent manipulations of the NCP were done at room temperature. The NCP was incubated overnight with human sera diluted 1:50 or 1:100 in PBST (0.05 M phosphate buffer [pH 7.4] containing 0.8% [wt/vol] NaCl and 0.1% [wt/vol] Tween 20) followed by extensive washing (four times for 30 min) in PBST and incubation in 4.5 μg of biotinylated anti-human immunoglobulin G (IgG) (H + L) (Vector Laboratories, Burlingame, Calif.) per ml overnight. The NCP was washed as described above and then incubated in 10 μg of avidin D (Vector Laboratories) per ml in 0.015 M carbonate buffer (pH 9.6) for 30 min. The avidin D was washed away as described above, and the NCP was incubated in 2.5 μg of biotinylated horseradish peroxidase (Vector Laboratories) per ml in PBST for 30 min. The NCP was washed again as described above and developed with 0.005% (wt/vol) 3,3'-diaminobenzidine tetrahydrochloride (Litton Bionetics, Kensington, Md.) and 0.03% (vol/vol) H_2O_2 in PBST for 30 min.

Immunochemical techniques. (i) Iodination procedures. Iodination of the region A proteins from the mannan-depleted mycelial cytoplasmic extract was performed by the diphenylglycouril technique (9). The labeled proteins were separated from the unreacted iodide by gel filtration through a G-25 Sephadex (Pharmacia) column (10 by 200 mm). Gelatin phosphate buffer (0.1 M phosphate buffer, pH 7.4, containing 0.2% [wt/vol] gelatin and 0.02% [wt/vol] NaN_3) was used as the equilibration and running buffer. The excluded peak (labeled proteins) was pooled and stored frozen as working samples at −80°C.

(ii) Immunoprecipitation. Immunoprecipitation was performed essentially as described by Kessler (19). The washing buffer employed throughout these experiments was RIPA (0.01 M Tris-hydrochloride [pH 7.4], 0.5 M NaCl, 0.001 M EDTA, 1.0% [wt/vol] Triton X-100, 1.0% [wt/vol] sodium desoxycholate, 0.1% [wt/vol] SDS, and 0.02% [wt/vol] NaN_3). The labeled antigen extract from region A in gelatin phosphate buffer was precleared by adding it to the pellet from 8.0 ml of a 10% (wt/vol) suspension of heat-killed, formalinized *Staphylococcus aureus* Cowan I cells (Pansorbin; Calbiochem-Behring, La Jolla, Calif.). The antigen and the *S. aureus* were incubated on ice for 1 h at 4°C with periodic vortexing, and the bacteria were removed by centrifugation. Labeled antigen (precleared, 10^6 cpm of trichloroacetic acid-precipitable material) was then added to 5 μl of human serum and incubated for 18 h at 4°C. Excess *S. aureus* suspension (500 μl) was added and incubated for an additional 90 min at 4°C. The bacteria were collected by centrifugation and washed three times with RIPA buffer. The pellet was then resuspended in 2× sample buffer (20) and heated for 5 min in a boiling-water bath. The bacteria were removed by centrifugation, and the supernatant was applied to SDS–12% polyacrylamide gels. After electrophoresis of

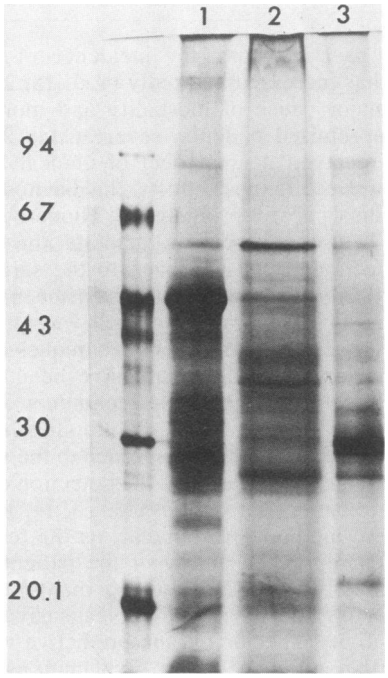

FIG. 1. SDS-PAGE of the proteins from regions A, B, and C of the DEAE-Sephacel column. Lanes 1, 2, and 3 contain pooled fractions from regions A, B, and C, respectively. The gel was stained by the silver technique. The molecular weight standards (Pharmacia) represent the following: phosphorylase *b*, 94,000; bovine serum albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; and soybean trypsin inhibitor, 20,100. Previous gels run with different lots of molecular weight standards have shown a single band for ovalbumin that migrates, with respect to the other standard proteins, with the lower form of this protein. Reported molecular weights for ovalbumin range from 43,000 to 46,000. The degree of glycosylation may contribute to the different molecular weight forms of this protein. Based on linear regression analysis of the standard curve, the lower band of ovalbumin was chosen as the 43-kd standard.

TABLE 1. Reactivity of 20 human sera with the *C. albicans* proteins in the pooled fractions from regions A, B, and C of the DEAE-Sephacel column[a]

Mol wt (in thousands)	Disseminated candidiasis in serum sample (n = 10):										Locally invasive candidiasis (n = 1)
	1	2	3	4	5	6	7	8	9	10	
Region A											
122 (119 to 135)	+	+	+		+	+			+		+
123 (116 to 129)	+		+			+			+		+
89 (85 to 91)	+	+	+		+	+			+		
65 (62 to 67)						+		+			
59 (58 to 60)		+	+			+	+	+			
48 (44 to 52)	+	+	+	+	+				+	+	+
42 (42 to 43)	+				+	+					
36 (33 to 39)	+										
29 (29 to 30)	+										
Region B											
91 (90 to 92)						+					
81 (81 to 82)			+			+				+	
58 (56 to 60)	+	+	+			+				+	
55 (52 to 58)	+					+					
Region C											
96 (94 to 97)	+	+	+						+		
64 (62 to 67)									+		
34 (33 to 35)	+			+							
31 (29 to 33)	+			+	+				+		
20 (20 to 21)	+										

[a] +, Presence of a band. The columns each represent a blot stained with an individual human serum. Of the sera from five patients with superficial noninvasive candidiasis tested, two sera reacted with two proteins in region C (96 and 31). Serum from four, normal, healthy men and women did not react with any of the fractions tested. There was no reaction when blots were treated with either biotinylated second antibody, avidin, biotinylated horseradish peroxidase and substrate (no human antiserum), or substrate alone and tested for endogenous peroxidase activity.

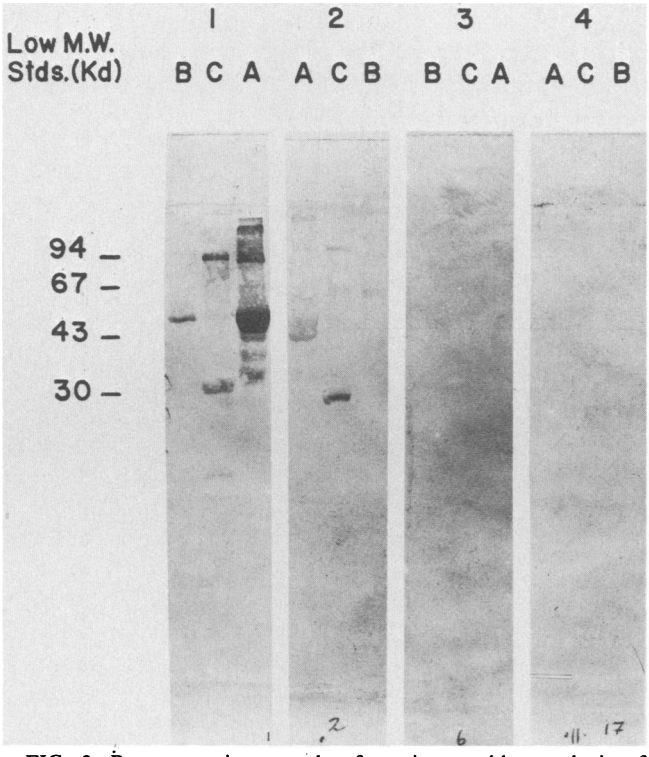

FIG. 2. Representative samples from immunoblot analysis of pooled fractions from regions A, B, and C of the DEAE-Sephacel column. The letters above each blot refer to the regions (A, B, or C) analyzed in that lane. Blots 1 and 2 were treated with sera from patients with disseminated candidiasis. Blot 3 was treated with serum from a patient with noninvasive, superficial candidiasis. Blot 4 was treated with serum from a normal, healthy person. The control blots for binding of the second antibody and for endogenous peroxidase activity were identical to blots 3 and 4 (data not shown).

the immunoprecipitates, the gels were fixed and stained with Coomassie blue G250 (0.3% [wt/vol] in 10% [vol/vol] CH_3COOH, 10% [vol/vol] CH_3OH, and 80% [vol/vol] distilled water), destained, and dried on filter paper. The gels were exposed to Kodak AR-5 film with Du Pont Cronex Hi-Plus intensifying screens. The amount of each antigen immunoprecipitated by the sera was determined by cutting out the band from the gel and counting it in a Gamma Trac 1191 gamma counter (Tracor Analytic, Elk Grove Village, Ill.). Statistical analysis was performed by using one-way analysis of variance with fixed effects (34).

Classification of human sera. Human sera were provided by the Mycology Serology Laboratory, Harvard School of Public Health, Boston, Mass.; by N. A. Hyslop, Infectious Disease Unit, Massachusetts General Hospital, Boston; and by the Immuno-serology Laboratory, Temple University Hospital, Philadelphia. Sera were divided into four groups as follows.

(i) Deep tissue or disseminated candidiasis. The first category includes sera from patients diagnosed as having deep tissue or disseminated candidiasis. The criteria for this diagnosis include (i) repeated isolation (more than one separated by 24 h) of *Candida* spp. from blood; (ii) isolation of *Candida* spp. from normally sterile body fluids (excluding urine) or aseptically obtained deep tissue material; (iii) histological evidence of deep tissue invasion not in communication with a nonsterile surface; or (iv) endophthalmitis with typical candidal ophthalmoscopic findings and a clinical condition consistent with *Candida* spp. sepsis. Included in this group were two sera from patients with locally invasive candidiasis and two sera from a patient with endocarditis due to *Candida parapsilosis*.

(ii) Noninvasive candidiasis. The second group includes sera from patients at risk of acquiring, but apparently without, disseminated candidiasis when the sera were

drawn. Patients in this group had surgery, burns, wounds, or cancer as the predisposing underlying condition. *Candida* spp. were isolated from the mucosal surfaces and the urine of many of these patients.

(iii) Other fungal infections. The third group includes sera from patients with fungal infections due to *Histoplasma capsulatum*, *Cryptococcus neoformans*, *Coccidioides immitis*, and *Aspergillus* spp.

(iv) Normal, healthy persons. The fourth group includes sera from healthy, nonhospitalized men and women.

RESULTS

Distribution of antigens in the fractionated cytoplasmic extract. The fractions from the DEAE column were screened by fused rocket immunoelectrophoresis for their reactivity with antibodies in the sera from 20 patients with disseminated candidiasis (DC sera). From this screening, it was found that the 20 DC sera tested reacted almost exclusively with fractions that eluted between 0.028 and 0.066 M NaCl, 0.079 and 0.090 M NaCl, and 0.010 and 0.014 M NaCl. These three groups of fractions were designated as regions A, B, and C, respectively. The percentages of DC sera that reacted with each region were as follows: region A, 80%; region B, 35%; and region C, 40% (data not shown). Figure 1 shows fractions from regions A, B, and C as analyzed by SDS-polyacrylamide gel electrophoresis.

Immunoblot analysis of regions A, B, and C. Regions A, B, and C from the DEAE column were studied by immunoblot analysis to identify and characterize by apparent molecular weight the antigenic components in each region. The presence of antibodies in 20 human sera against the *Candida* spp. antigens was detected immunochemically with the sensitive bridged avidin-biotin technique (14). The blots were scored qualitatively, and the data are summarized by region and apparent molecular weight in Table 1. Representative blots are shown in Fig. 2. These data demonstrated that 18 antigenic components from regions A, B, and C were recognized by antibodies in DC sera. Region A contained nine antigens, region B contained four antigens, and region C contained five antigens. Region A not only contained the largest number of antigenic components, but also contained an immunodominant antigen of apparent molecular weight of 48,000 (44,000 to 52,000) that reacted with 7 of the 10 DC sera. The reactivity of regions A, B, and C as detected by immunoblot analysis was similar to the results obtained by fused rocket immunoelectrophoresis. The percentages of DC sera that reacted with each region were as follows: region A, 100%; region B, 50%; and region C, 60%. The higher percentage of reactivity detected by immunoblot analysis as compared to that detected by fused rocket immunoelectrophoresis may reflect the detection of non-precipitating as well as precipitating antibodies.

Immunoprecipitation of *C. albicans* antigens from region A. The incidence of antibodies directed against antigens from region A was studied in 51 human sera by *S. aureus* protein A immunoprecipitation. The antigen used for the immunoprecipitation was prepared by iodination of the material eluting in region A from the DEAE-Sephacel column (9). From preliminary titration experiments (data not shown) an amount of labeled antigen in excess of the binding capacity of the serum and an amount of *S. aureus* cells in excess of the amount of antigen-antibody complexes were chosen for these experiments. Figure 3 shows representative autoradiographs of the 51 human sera studied by immunoprecipita-

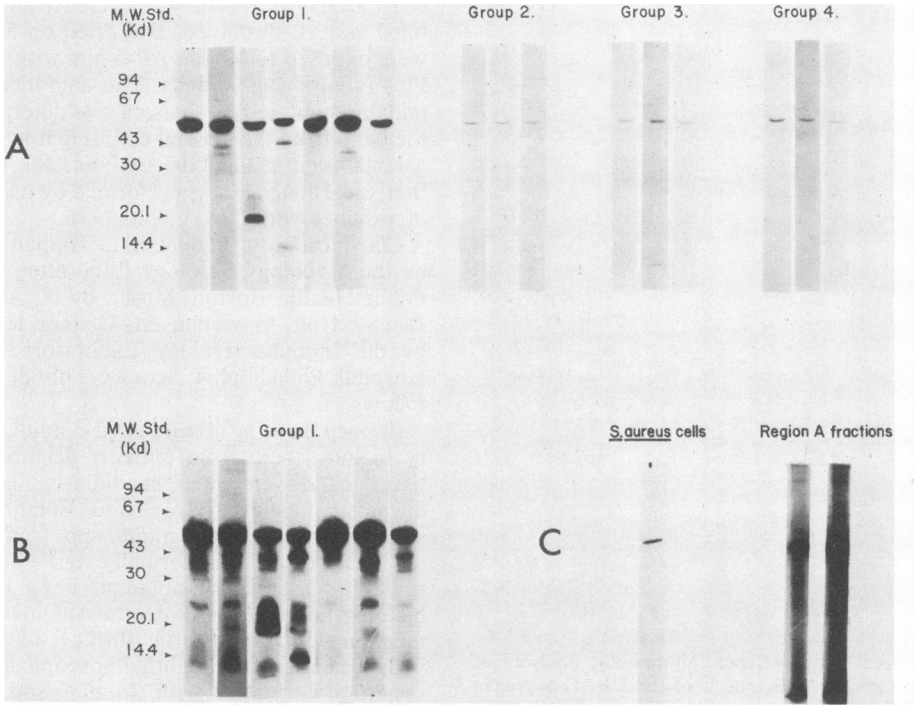

FIG. 3. *S. aureus* protein A immunoprecipitation of *C. albicans* proteins from region A of the DEAE column by antibodies in human sera. Region A proteins were labeled with ^{125}I by the diphenylglycouril technique. Representative autoradiographs from sera in each group are shown. Group 1 contains sera from patients with disseminated candidiasis; group 2 contains sera from patients with noninvasive, superficial candidiasis; group 3 contains sera from patients with other fungal infections; and group 4 contains sera from healthy, nonhospitalized men and women. Panel A represents autoradiographs exposed for 18.5 to 23 h. Panel B represents a 67-h exposure of the sera from group 1 in panel A. Panel C is a 21-h exposure of the antigen precipitated by *S. aureus* cells alone and the ^{125}I-labeled proteins from region A.

tion. To quantitate the amount of each antigen from region A that was immunoprecipitated by the human sera, bands were cut out of the SDS gel and counted in a gamma counter. The amount of the immunodominant 48- to 52-kilodaltons (kd) antigen from region A that was immunoprecipitated by the human serum is shown in Fig. 4.

Of the proteins from region A that were immunoprecipitated by the human sera, the 48 to 52-kd protein showed the smallest degree of variance within and between test groups. For this reason, an analysis of variance was performed to determine whether the antibody response among the four groups of sera to this antigen was statistically significant. A one-way analysis of variance with fixed effects of the data presented in Fig. 4 showed a statistically significant difference between groups with $P < 0.001$. Within the three control groups, there was no statistically significant difference in their response to the 48- to 52-kd protein at the 95% confidence interval in a difference of means test (34).

DISCUSSION

Syverson et al. (38) used the crossed immunoaffinoelectrophoretic technique with ConA to screen sera from patients suspected of having disseminated candidiasis for antibody production to *C. albicans*. It was observed that removal from the cytoplasmic extract of glyco components (mannans, glucans, mannan-proteins, glucan-proteins, etc.) by adsorption to ConA increased the predictive value positive of the precipitin test for the diagnosis of systemic candidiasis (38). In the present study, experiments were designed to identify and characterize by apparent molecular weight the antigenic components in the cytoplasmic extract that do not bind to ConA. To facilitate the identification and characterization of the serodiagnostically important antigens, the unbound material from the ConA-Sepharose affinity column was fractionated by DEAE ion-exchange chromatography. The fractions from the DEAE column were screened for their reactivity with antibodies in the sera of patients with disseminated candidiasis. Three groups of fractions (regions A, B, and C) were defined by their reactivity with these sera.

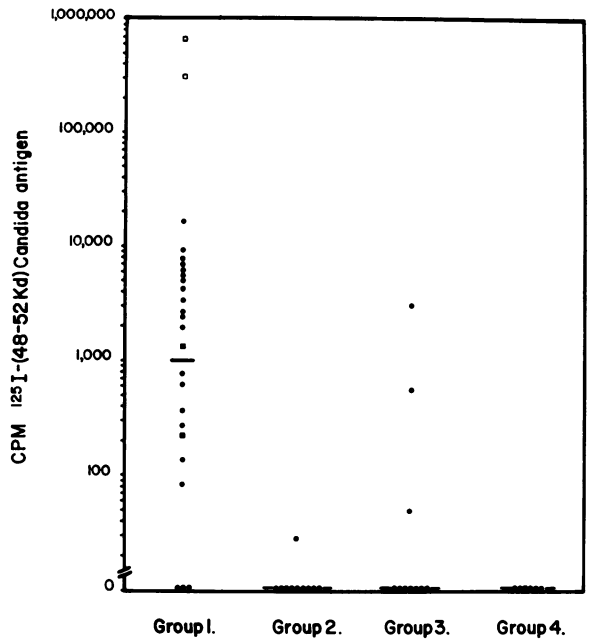

FIG. 4. *S. aureus* immunoprecipitation of the 48- to 52-kd antigen of *C. albicans* by antibodies in human sera. The antigen was labeled with ^{125}I by the diphenylglycouril technique. The data were obtained by cutting out the 48- to 52-kd antigen from an SDS-polyacrylamide gel and counting it in a gamma counter. Each point represents the specific counts precipitated of the 48- to 52-kd antigen, i.e., sample counts per minute minus *S. aureus* cells alone counts per minute. Group 1 contains 25 sera from patients with disseminated or invasive candidiasis. Symbols: ■, sera from the two patients with locally invasive candidiasis; □, serum from a patient with endocarditis due to *C. parapsilosis*. Group 2 contains 10 sera from patients at risk of candidiasis. These patients had noninvasive, superficial candidiasis. Group 3 contains 10 sera from patients with other fungal infections. Group 4 contains six sera from healthy, nonhospitalized men and women. The geometric means and standard errors of the mean are as follows: group 1, 1,000.24 (212.90 to 4,699.45) cpm; group 2, 1.97 (1.07 to 3.63) cpm; group 3, 6.22 (1.96 to 19.73) cpm; group 4, 0 (0) cpm. The F value, obtained by using a one-way analysis of variance with fixed effects (34), is 20.27, which is highly significant at $P < 0.001$.

Immunoblot analysis of 20 human sera and fractions from regions A, B, and C identified 18 antigenic components. Region A contained nine of these. Region A also contained the most frequently recognized antigens. One antigen of apparent molecular weight of 48,000 (44,000 to 52,000) was recognized by antibodies in the sera from 7 of 10 patients with disseminated candidiasis, one patient with a local infection due to *C. albicans*, and none of the control patients.

Since immunoblot analysis was performed after the antigens had been denatured (boiling 5 min in 2-mercaptoethanol and SDS) and separated by molecular weight on an SDS-polyacrylamide gel, the *S. aureus* protein A immunoprecipitation technique was employed to study the incidence of antibodies in the sera of patients with disseminated candidiasis to antigens in their native state. Material from region A was chosen as the antigen for exogenous labeling with ^{125}I due to its large number of antigenic components. The results (Fig. 3) of the staphylococcal protein A immunoprecipitation with labeled region A proteins and 51 human sera demonstrated a major antigen of apparent molecular mass of 48,000 to 52,000. The 48- to 52-kd protein is an abundant protein in region A (Fig. 1 and 3) and is the most frequently recognized protein by antibodies in the sera of patients with disseminated candidiasis (Fig. 3 and 4). Patients with disseminated candidiasis had significantly higher levels of antibodies (IgG) ($P < 0.001$) against the 48- to 52-kd protein than did patients with noninvasive forms of candidiasis, patients with other fungal infections, or normal, healthy persons (Fig. 4).

The 48- to 52-kd protein identified by immunoprecipitation is likely to be the 48-kd (44- to 52-kd) protein identified by immunoblot analysis, since several sera used in both immunoprecipitation and immunoblot analysis identified a major protein with an apparent molecular weight of 48,000. Several other antigenic proteins, including a 120- to 135-kd and a 35- to 38-kd protein, were identified by immunoprecipitation. Proteins of similar apparent molecular weights were also identified by immunoblot analysis. Direct comparison by limited proteolytic digestion of the antigens detected by immunoblot analysis and *S. aureus* protein A immunoprecipitation has not been done.

The relationship of the major 48- to 52-kd antigen from region A to the major 54.3-kd (48.9- to 59.7-kd) antigen reported by Greenfield and Jones (13) is not clear. In addition to the similarity in apparent molecular weight, both proteins were purified by similar separation techniques, and both proteins were eluted from a DEAE column with buffers of similar ionic strength. Both proteins are highly antigenic

and relatively abundant in the cytoplasmic extract. A minor difference between the two proteins is the source of material from which they were purified. The 48- to 52-kd antigen from region A was purified from mycelial-phase cells, whereas the 54.3-kd antigen was purified from yeast-phase cells. Further experiments are needed to determine the relationship of these two antigens.

ACKNOWLEDGMENTS

We sincerely thank Gerald Pearlman for his patience and excellent photography and Gregory Harvey for his editorial assistance in the preparation of this manuscript. We also thank Cheryl Hayden, for her help with the statistical analysis. We are grateful to Newton Hyslop, Massachusetts General Hospital, Boston, Mass., for supplying the sera used in this study.

This work was supported by Public Health Service grant AI 14695 and by Public Health Service training grant for Medical Mycology AI 07141, both from the National Institutes of Health.

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