Extracellular Proteins of *Cryptococcus neoformans* and Host Antibody Response

LIN-CHI CHEN,¹ LIISE-ANNE PIROFSKI,^{1,2} AND ARTURO CASADEVALL^{1,2*}

Department of Microbiology and Immunology¹ and Division of Infectious Diseases of the Department of Medicine,² Albert Einstein College of Medicine, Bronx, New York 10461

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Proteins secreted by the fungal pathogen *Cryptococcus neoformans* may be involved in invasion and could be useful in vaccine design. Despite the medical importance of this fungus, little is known about its extracellular proteins or the immune response to these antigens. To study *C. neoformans* extracellular proteins, 12 strains were metabolically radiolabeled and protein supernatants were analyzed. Both strain- and growth condition-dependent differences were observed. Enzymatic assays of filtered culture supernatants revealed butyrate esterase and caprylate esterase lipase activity for 11 of 12 strains, as well as acid phosphatase, naphthol-AS-BI-phosphohydrolase, and β -glucosidase activities in some strains. Serum from infected rodents immunoprecipitated several secreted proteins, consistent with in vivo expression and development of an antibody response. For strain 24067, two immunodominant species, of approximately 75 and 30 kDa, were recognized. The relative intensity of the autoradiographic bands depended on the route of infection for both rats and mice. In summary, our results indicate that (i) there are multiple proteins in *C. neoformans* culture supernatants, (ii) there are strain differences in supernatant protein profiles, (iii) there are differences in supernatant protein profile depending on the growth conditions, (iv) there are several new extracellular and/or cell-associated enzymatic activities, and (v) antibodies to several supernatant proteins are made in the course of infection.

Cryptococcus neoformans is an opportunistic fungal pathogen which causes an incurable, life-threatening meningoencephalitis in 6 to 8% of patients with AIDS. In New York City alone, there are over 1,000 cases of cryptococcosis per year (15). The immune response to and biological effects of the *C. neoformans* polysaccharide antigen have been extensively studied (13, 23, 36, 43, 45). The polysaccharide antigen glucuronoxylomannan (GXM) is the major component of the capsule and has an apparent molecular mass of ~1,000 kDa (6). Antigenic differences in GXM provide the basis for separating *C. neoformans* strains into four serotypes (13). *C. neoformans* strains lacking the polysaccharide capsule are avirulent (10, 20, 31). Antibodies to GXM have been shown to protect mice against cryptococcal infection (19, 41, 42). In contrast, little is known about the proteins secreted or released by *C. neoformans*, despite evidence that they elicit important immune responses. Immunization with a concentrated filtrate of cell culture supernatant known as CneF produced a delayed-type hypersensitivity response (44), although the active protein antigens were not identified. To date, systematic studies of *C. neoformans* supernatant proteins have not been undertaken, but several proteins have been described (1, 11, 22, 25, 26, 50, 54, 55).

Identification and characterization of *C. neoformans* extracellular proteins are important because proteinases, esterases, and lipases have been associated with virulence in other pathogens (14, 30, 34, 37, 52, 53) and cryptococcal protein antigens in culture supernatants elicit strong immune responses (23, 27, 46). Furthermore, characterization of cryptococcal protein antigens could identify potential protein carriers for the synthesis of polysaccharide-protein conjugate vaccines to *C. neoformans*.

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MATERIALS AND METHODS

Strains. *C. neoformans* 24067, 52817, 24065, and 32608 were obtained from the American Type Tissue Collection (Rockville, Md.); J9, J22, J11 (16), and 69 are clinical isolates from the Bronx, N.Y. NIH 34 was obtained from Robert Cherniak NIH 371 was obtained from J. E. Bennett, and SB4A and SB6A were obtained from E. Spitzer. Serotype A is represented by strains NIH 371, J11, SB4A, and SB6A; serotype B is represented by strain 24065; serotype C is represented by strains NIH 34 and ATCC 32608; and serotype D is represented by strains 24067, J9, and J22 (4). Strain 52817 is an acapsular strain.

Media and culture conditions. Strains were maintained in Sabouraud's dextrose agar (Difco Laboratories, Detroit, Mich.) at 4°C. Growth media were as indicated, either Sabouraud's dextrose broth (Difco Laboratories), a chemically defined medium (carbon source as indicated in the text, 10 mM MgSO₄, 29.4 mM KH₂PO₄, 13 mM glycine, 3 μ M thiamine [Sigma Chemical Co., St. Louis, Mo.] [pH 4.5]) (11, 57), or an acetate protein medium (15 mM sodium acetate, 1% peptone, 10 mM MgSO₄, 29.4 mM KH₂PO₄, 3 μ M thiamine).

Growth analysis. Approximately 10⁵ cells of strain 24067 were incubated in 100 ml of chemically defined media at either 30 or 37°C. At various times, aliquots were removed for hemacytometer counts and plating on Sabouraud's dextrose agar plates for CFU determination.

Preparation of radiolabeled culture supernatants. Cells were incubated for 96 to 312 h at 30 or 37°C in chemically defined medium containing [³⁵S]methionine (Amersham Life Science, Arlington Heights, Ill.) (10³ cells/ml, 25 μ Ci/ml) for the times indicated and then sedimented (1,200 $\times$ g for 20 min at 22°C). The supernatants were filtered (0.8- μ m syringe filters; Nalge Co., Rochester, N.Y.) and stored at -80°C with 0.02% sodium azide. Some supernatant samples were concentrated (up to 80-fold) in Centrifu-10 units (Amicon Inc., Beverly, Mass.) at 4°C. The protein concentration in supernatants was determined by a protein assay (Bio-Rad Laboratories, Hercules, Calif.) which is based on the Bradford method (7). For analysis, a 4:1 ratio of sample (concentrated or unconcentrated without proteinase inhibitors) was mixed with sample buffer, immediately boiled for 3 min, and centrifuged, and 35 μ l was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 4°C, and visualized by Coomassie blue treatment, silver staining, or autoradiography as indicated. A control

* Corresponding author. Mailing address: Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461. Phone: (718) 430-4259. Fax: (718) 430-8968. E-mail: casadeva@acem.yu.edu.

experiment with washed cells was resuspended in fresh media with or without 2% sodium azide, incubated (48 to 72 h at 30°C and 200 rpm), and screened for autoradiographic bands.

Enzymatic analysis. Suspensions of *C. neoformans* cells grown in chemically defined media with 0.3% glucose or the cell-free supernatants were tested for enzymatic activity with the Api-Zym system (Bio-Mérieux/Vitek, Hazelwood, Mo.) as specified by the manufacturer. Briefly, filtered supernatants or cultures were pipetted into individual cupules containing buffer and enzyme substrate, and the assay mixtures were incubated at 35 to 37°C overnight. The presence of enzymatic activity was determined by the addition of color reagents (Fast Blue BB, lauryl sulfate, methoxyethanol, and Tris) and compared to a chart for the quantity of hydrolyzed substrate. Uninoculated media, media concentrated 10-fold, autoclaved cultures, and chymotrypsin (Sigma Chemical Co.) were used as controls. A reading was considered positive if the color change was significantly different than that observed with media alone. The substrates and enzymes tested were (i) 2-naphthyl phosphate for alkaline phosphatase; (ii) 2-naphthyl butyrate for butyrate esterase; (iii) 2-naphthyl caprylate for caprylate esterase; (iv) 2-naphthyl myristate for myristate lipase; (v) L-leucyl-2-naphthylamide for leucine arylamidase; (vi) L-valyl-2-naphthylamide for valine arylamidase; (vii) L-cystyl-naphthylamide for cystine arylamidase; (viii) N-benzoyl-DL-arginine-2-naphthylamide for trypsin; (ix) 2-naphthyl phosphate for acid phosphatase; (x) naphthol-AS-BI-phosphate for naphthol-AS-BI-phosphohydrolase; (xi) 6-Br-2-naphthyl- α -D-galactopyranoside for α -galactosidase; (xii) 2-naphthyl- β -D-galactopyranoside for β -galactosidase; (xiii) naphthol-AS-BI- β -D-glucuronide for β -glucuronidase; (xiv) 2-naphthyl- α -D-glucopyranoside for α -glucosidase; (xv) 6-Br-2-naphthyl- β -D-glucopyranoside for β -glucosidase; (xvi) 1-naphthyl-N-acetyl- β -D-glucosaminide for N-acetyl- β -glucosaminidase; (xvii) 6-Br-2-naphthyl- α -D-mannopyranoside for α -mannosidase; (xviii) N-glutaryl-phenylalanine-2-naphthylamide for chymotrypsin; and (xix) 2-naphthyl- α -L-fucopyranoside for α -fucosidase.

Sera. (i) **Murine sera.** Female A/JCr mice (National Cancer Institute, Bethesda, Md.) were infected with 10^4 cells of *C. neoformans* 24067 in ~0.5 ml of sterile phosphate-buffered saline (PBS; pH 7.4) either intraperitoneally (i.p.) or intratracheally (i.t.) (19). Blood samples were obtained before infection and at 14 and 28 days postinfection.

(ii) **Rat sera.** Fischer rats (National Cancer Institute) were anesthetized and infected with 10^7 cells of *C. neoformans* 24067 i.p. or i.t. as described previously (21). Blood was obtained before infection and from chronically infected rats after 9 and 16 months. Blood samples were centrifuged in a microcentrifuge (5 to 10 min at 4°C) and stored at -20°C.

(iii) **Human sera.** Human sera were obtained from two human immunodeficiency virus (HIV)-positive cryptococcosis patients and from normal volunteers by using a protocol approved by the Institutional Review Board of our institution.

Immunoprecipitation. Samples (100 μ l) of concentrated [35 S]methionine-labeled culture supernatants were incubated with 50 μ l of serum (4°C for 8 to 14 h) or with antibodies to human and mycobacterial heat shock proteins (StressGen Biotechnologies Corp., Victoria, British Columbia, Canada). Immune complexes were precipitated with 20 μ l of insoluble protein A (100 mg/ml in PBS) (Sigma Chemical Co.) for 4 h at room temperature or 12 h at 4°C. The samples were then centrifuged in a microcentrifuge (8,000 $\times$ g for 30 min at 4°C), and the supernatants were removed. The pellets were washed twice with PBS before being resuspended in sample buffer and subjected to gel electrophoresis, and autoradiography.

RESULTS

Proteins in supernatants of *C. neoformans* cultures. *C. neoformans* cultures were radiolabeled (Fig. 1), and the supernatant proteins were examined by autoradiography. Under the conditions used, no proteins were apparent before 84 h of culture when the cell density was 1×10^8 to 2.6×10^8 cells/ml. At later times, proteins secreted or released from cells accumulated in the medium. After 10 days of growth, the total protein concentration in the medium was <1 μ g/ml (~40 μ g/ml in 80-fold-concentrated samples). For all time studies, the colony and hemacytometer counts were essentially identical, indicating that the cultures were healthy (Fig. 1A).

The number and size of supernatant proteins are dependent on the growth medium and the *C. neoformans* strain. Twelve *C. neoformans* strains were grown and radiolabeled in three different media, and the supernatant proteins were compared (Fig. 2). There was significant variation among the strains in the number and intensity of the protein bands detected by autoradiography. The protein levels in filtered defined media prior to concentration were as follows for each strain: SB6A, 0.10 μ g/ml; NIH 371, 0.19 μ g/ml; 52817, 0.22 μ g/ml; 24067,

FIG. 1. Proteins secreted by *C. neoformans* 24067. (A) Typical growth curve for *C. neoformans*. Cells were grown as described in Materials and Methods, and the total number of cells per milliliter and CFU per milliliter are shown for various times. Points correspond to an average of three or four measurements from one culture, and error bars denote one standard deviation. (For most points, the standard deviation is smaller than the symbol used to denote the mean in the graph.) Similar curves were observed in five separate experiments. (B) Autoradiograph of secreted proteins. Labeling and gel analysis of unconcentrated samples are described in Materials and Methods. The molecular mass is given in kilodaltons. Similar results were obtained in four independent experiments. A 4-month exposure is shown. Panels A and B are from separate experiments.

0.29 μ g/ml; J22, 0.33 μ g/ml; J11, 0.40 μ g/ml; 69, 0.53 μ g/ml; NIH 34, 0.57 μ g/ml; SB4A, 0.58 μ g/ml; J9, 0.58 μ g/ml; 24065, 1.57 μ g/ml; and 32608, 1.60 μ g/ml. When individual strains were grown in different media, the supernatant protein profiles were also markedly distinct (Fig. 2B).

Effect of glucose and temperature on supernatant protein profiles. Most studies of *C. neoformans* have been done in media with 2% glucose and grown at 30°C (5, 8, 23, 40, 43, 45, 50). Some studies have been conducted at 37°C (24–26). No studies have investigated the similarities or differences in supernatant protein profiles resulting from changes in the temperature or carbon source. We addressed the effect of either the carbon source or the temperature on the supernatant protein profile. Proteins secreted by strain 24067 grown in the presence of various supplements were examined (Fig. 3). For larger polypeptides, the profile was similar under all conditions compared with chemically defined media, but additional bands were observed in Sabouraud's dextrose broth (lanes 8) or in the presence of 10% fetal calf serum (FCS) (lanes 1). A prom-

FIG. 2. Secreted proteins of 12 *C. neoformans* strains. (A) Cultures grown in defined chemical medium were radiolabeled as in Materials and Methods. Each lane contains a solution of ~30-fold-concentrated filtered medium of 168-h cultures and sample buffer as described in Materials and Methods. Strain designations are shown above the lanes. A 1-week exposure is shown. (B) Camara lucida depiction of the secreted protein profile of 12 strains grown in (i) defined chemical medium, (ii) acetate-peptone medium, and (iii) Sabouraud's dextrose broth. Data from three sets of gels (168-h cultures, ~30-fold-concentrated media) were combined to construct this figure. Thick squares denote broad bands. Shades of gray denote intensity in the autoradiogram and should be interpreted relative to other bands in the same lane. The molecular mass is given in kilodaltons.

FIG. 3. Autoradiograph of the SDS-PAGE gel for strain 24067 grown under eight medium conditions. (A) Exposure (5 days) of an SDS-PAGE gel (7.5% polyacrylamide). Lanes: 1, 10% FCS in PBS; 2, 0.3% glucose; 3, 0.9% raffinose; 4, 1% glucose; 5, 1% raffinose; 6, 2% glucose; 7, 2% raffinose; 8, Sabouraud's dextrose broth. Cultures containing glucose or raffinose also included additional salts and nutrients as described for the chemically defined medium solution in Materials and Methods. The samples are unconcentrated. Lane 1 bands are distorted because the gel was overloaded with proteins from the FCS medium. (B) Exposure (14 days) of an SDS-PAGE gel (15% polyacrylamide). The lanes are as in panel A. The molecular mass is given in kilodaltons. Protein standards of 29 and 24 kDa migrate aberrantly for their stated molecular masses, but the observed profile corresponds to that described by the manufacturer.

inent band of higher mobility (~35 kDa) was present only in the absence of glucose (lanes 1, 3, 5, 7, and 8); others seemed specific to growth in FCS or Sabouraud's dextrose broth (lanes 1 and 8).

Laboratory studies have commonly been conducted at 30°C because the fungus grows well at this temperature. However, this temperature does not reflect the conditions during infection. Hence, the protein profiles of culture supernatants were analyzed at 30 and 37°C (Fig. 4). Strain 24067 was selected because it had been used in prior studies (11, 19, 41, 56, 57) and because it has been studied extensively in other laboratories (17, 28, 35, 47, 58). Growth at 37°C was slower than at 30°C, with doubling times of 7.7 and 4.3 h, respectively; therefore, the samples were analyzed at comparable cell densities (>7 days, ~10⁸ cells/ml), when the cultures were still fully viable (as assayed by CFU determination [Fig. 4A]). Protein profiles from cultures grown at 30 and 37°C were similar, but some proteins were found in supernatants from cells grown at 30°C and not at 37°C (Fig. 4B). Specifically, a band of approx-

B

FIG. 4. Comparison of cultures of strain 24067 grown at 30 and 37°C in defined chemical media. (A) Growth curves, as in Fig. 1. Points correspond to an average of three or four measurements from one culture, and error bars denote one standard deviation. (B) Exposure (14 days) of an SDS-PAGE gel (15% polyacrylamide) showing the time course of labeling at 30°C (top) or 37°C (bottom). Hours of labeling are shown above lanes. The lanes contain preparations of unconcentrated supernatants as described in Materials and Methods. The molecular mass is given in kilodaltons.

TABLE 1. Strains and extracellular enzyme activity

Strain	Activity ^a							
	C4E	C8EL	AcPh	β-Glc	N-Ph	α-Glc	β-Glc	AlkPh
J9	+	+	—	—	+	—	+	—
J11	+	+	—	—	—	—	—	—
NIH 371 ^b	+	+	—	—	+	—	—	—
SB4A	+	+	+	—	—	—	—	—
SB6A	+	+	+	—	—	—	—	—
24065	+	+	—	—	—	—	—	—
32608	+	+	+	—	—	—	—	—
NIH 34	+	—	—	—	—	—	—	—
24067	+	+	—	—	—	—	—	—
J22	+	+	+	—	—	—	+	—
69	+	—	—	—	—	—	—	—
52817	+	+	—	—	—	ND ^c	ND	—

^a +, activity detected; —, no activity detected. Enzyme abbreviations: C4E, butyrate esterase; C8EL, caprylate esterase lipase; AcPh, acid phosphatase; β-Glc, β-glucuronidase; N-Ph, naphthol-AS-BI-phosphohydrolase; α-Glc, α-glucosidase; β-Glc, β-glucosidase; AlkPh, alkaline phosphatase.

^b Cell culture supernatants for all strains except 371 were concentrated 9- to 10-fold before the enzyme assay.

^c ND, not done.

imately 16 kDa was present in the 30°C culture that was not present, or not detectable, in the 37°C culture.

Reproducibility of supernatant protein profiles. The supernatant protein profiles were highly reproducible when measurements were made with cultures grown on different days. Strain 24067 was studied in defined medium plus 0.3% glucose a minimum of six times, and for each experiment the autoradiograms were similar. The reproducibility of protein profiles was also observed with strain 24067 in acetate-peptone medium and Sabouraud's dextrose broth in experiments done four and three times, respectively. For strain 52817, protein profiles were reproducible in the three experiments done in defined medium and the two experiments done in acetate-peptone medium. All other strains were studied twice in defined medium, and the protein profiles were reproducible.

Enzymatic activities of culture supernatants. Analysis of defined medium supernatants demonstrated butyrate esterase and caprylate esterase lipase activities in supernatants from 11 of the 12 strains (Table 1). By concentrating the supernatants, we detected additional activities in some strains: acid phosphatase, naphthol-AS-BI-phosphohydrolase, and β-glucosidase (Table 1). Protease activity assays with *N*-glutaryl-phenylalanine-2-naphthylamide or *N*-benzoyl-DL-arginine-2-naphthylamide as the substrate were negative, indicating that the *C. neoformans* proteolytic activity is not chymotrypsin- or trypsin-like. Negative controls of media, 10-fold-concentrated media, and autoclaved supernatants all had little to no background activity. Whole-cell assays revealed many additional enzymatic activities, including alkaline phosphatase, myristate lipase, three arylamidases (leucine, valine, and cystine), β-galactosidase, *N*-acetyl-β-glucosaminidase, and α-mannosidase (Table 2). No enzyme activities were detectable for the heat-killed organisms.

Antibody response to supernatant proteins. Microbial proteins often elicit host antibody responses during infection. To investigate whether this is true for the proteins observed in culture supernatants, we immunoprecipitated radiolabeled proteins with sera from humans, mice, and rats with *C. neoformans* infection (Fig. 5A). Serum antibody from mice and rats infected with strain 24067 i.t. precipitated proteins of ~125, 75, and 30 kDa. Serum antibody from mice infected i.p. with strain 24067 precipitated radiolabeled polypeptides of

TABLE 2. Cell-associated enzyme activity

Strain	Activity ^a																
	AlkPh	C4E	C8EL	C14L	LArg	VArg	CArg	Tryp	AcPh	N-Ph	α-Gal	β-Gal	β-Glc	α-Gluc	β-Gluc	N-βg	α-Man
J9	+	+	+	+	+	+	+	+	+	+	—	—	+	+	+	+	+
J11	+	+	+	+	+	+	+	—	+	+	—	—	+	+	+	+	+
NIH 371	+	+	+	+	+	+	—	—	+	—	—	—	+	+	+	+	+
SB4A	+	+	+	+	+	+	+	—	+	—	—	—	+	+	+	+	+
SB6A	+	+	+	+	+	+	+	—	+	—	—	—	+	+	+	+	—
24065	+	+	+	+	+	+	+	—	+	+	—	—	+	+	+	+	—
32608	+	+	+	+	+	+	+	—	+	+	—	—	+	+	+	+	—
NIH34	+	+	+	+	+	+	+	—	+	+	—	—	+	+	+	—	—
24067	+	+	+	+	+	+	—	—	+	—	—	—	+	+	+	+	—
J22	+	+	+	+	+	+	+	—	+	—	—	+	+	+	+	+	+
69	+	+	+	+	+	+	+	—	+	+	—	—	+	+	+	+	—
52817	+	+	+	+	+	+	—	—	+	—	—	—	—	+	+	—	—

^a +, activity detected; —, no activity detected. Enzyme abbreviations: AlkPh, alkaline phosphatase; C4E, butyrate esterase; C8EL, caprylate esterase lipase; C14L, myristate lipase; LArg, leucine arylamidase; VArg, valine arylamidase; CArg, cystine arylamidase; Tryp, trypsin; AcPh, acid phosphatase; N-Ph, naphthol-AS-BI-phosphohydrolase; α-Gal, α-galactosidase; β-Gal, β-galactosidase; β-Glc, β-glucuronidase; α-Gluc, α-glucosidase; β-Gluc, β-glucosidase; N-βg, *N*-acetyl-β-glucosaminidase; α-Man, α-mannosidase. Chymotrypsin and α-fucosidase results are not shown since all the strains were negative for these activities.

similar size but with different relative intensities. Sera from rodents without *C. neoformans* infection did not precipitate *C. neoformans* proteins (Fig. 5B). Antibodies that precipitate *C. neoformans* proteins did not appear in murine sera until after 4 weeks of infection (Fig. 5B). No precipitating antibodies were detected in the sera of HIV-positive patients by this method and/or at this exposure (Fig. 5A, lanes 6 to 10).

The nonreactivity of human sera to strain 24067 could potentially be due to antigenic differences between strains. For one patient, the infecting strain of *C. neoformans* was available. We analyzed the patient's serum antibody response to proteins released from that strain as well as strain 24067 but detected no protein antigens (data not shown). Sera from rats infected with strain 24067 did recognize a protein secreted by this patient's isolate, however, but with a different M_r than that of the proteins from strain 24067. No proteins were precipitated with antibodies to mycobacterial heat shock proteins which cross-react with cryptococcal heat shock proteins (47a).

DISCUSSION

The known extracellular antigens of *C. neoformans* are the polysaccharide capsule and an associated mannoprotein (12, 13, 49, 54). We have now identified many additional proteins in culture supernatants by using [³⁵S]methionine labeling, electrophoresis, and autoradiography. Protein bands were detected by autoradiography beginning in the early stationary phase. This suggests one of the following explanations: (i) extracellular proteins are not produced during the lag and early logarithmic growth phases; (ii) proteins are made in small amounts during growth, but detectable amounts do not accumulate until the late logarithmic phase; and/or (iii) the presence of secreted proteins is growth and/or nutrient regulated and occurs in the early stationary phase when nutrient depletion occurs and waste products accumulate in the culture medium.

The bands observed in autoradiograms are probably secreted or shed proteins, not proteins released due to cell lysis. The reasons for this conclusion are as follows. (i) The extracellular protein profile shows a few well-defined protein bands rather than the smear of bands that occur upon cell lysis. (ii) Enzymatic activities are found when whole cells are tested with the API-Zym kit that are not present in the culture filtrates. (iii) Autoradiograms of supernatants of azide-killed cells incubated for 3 days show either faint or no bands in comparison to

those observed for live cultures (data not shown). (iv) the same strain can give very different supernatant protein profiles when grown under different conditions. (v) The cultures are healthy, as shown by the correlation between cell counts and CFU. Taken together, these observations are consistent with the notion that the supernatant proteins are secreted or released with the caveat that transport and secretion from the cell has not been established for any of them.

The number and intensity of radiolabeled supernatant proteins varied depending on the growth media. The smallest number of protein bands was consistently observed in Sabouraud's dextrose broth (rich medium with 2% glucose), and the largest number was seen in chemically defined medium (defined medium with 0.3% glucose), suggesting that protein secretion or release is regulated in response to the growth conditions. The presence of proteins found in nutritionally poorer media could reflect an upregulation of genes, protein synthesis, and protein release and secretion in an attempt by cells to modify the environment and increase the available nutrients.

Immunization with culture filtrate concentrates of one strain has been shown to elicit delayed-type hypersensitivity in sensitized mice (27). The observation of large strain-to-strain variations suggests the possibility that immunization with concentrates from one strain does not protect against other strains. Previous studies have demonstrated *C. neoformans* strain differences in serotype, capsular polysaccharide structure, and virulence (3, 10, 13, 20, 32), and in this study we report strain-to-strain variation in the number and intensity of protein bands detected by autoradiography even among strains assigned to a given serotype. This variation may result in differences in virulence and immune response to infection. Hence, the *C. neoformans* strain and the growth conditions are important in the design and preparation of culture filtrate derived vaccines.

The proteins found in *C. neoformans* spanned a range of <20 to >200 kDa. Previously, we identified proteinase activity associated with proteins of approximately 200, 100, and 50 kDa (11). The butyrate esterase and caprylate esterase lipase activities in *C. neoformans* supernatants are interesting because lipase and esterase activity has been associated with *Candida albicans* virulence and these enzymes could also contribute to virulence in *C. neoformans* (29, 39, 53). Acid phosphatase activity was also detected in concentrated culture supernatants, confirming earlier reports of this activity in *C. neoformans* (9,

FIG. 5. Immunoprecipitation of strain 24067 culture supernatants with sera from rodents and humans. Immunoprecipitation done as described in Materials and Methods. Protein A-precipitated immune complex pellets were suspended in 30 μ l of sample buffer, boiled, and centrifuged, and the supernatant was analyzed. (A) A 1-week exposure of an SDS-PAGE gel (7.5% polyacrylamide). Lanes: 1, no serum; 2, preinfection sera from mice; 3, serum 28 days after i.t. infection in mice; 4, serum 28 days after i.p. infection in mice; 5, serum 9 months after i.t. infection in rats; 6, serum from a patient infected with strain 69 on day 22 of the hospital stay; 7, serum from a patient infected with strain 69 on day 2 of the hospital stay; 8, serum from infected patient no. 5; 9, serum from a control individual who is a laboratory worker and is exposed to *C. neoformans*; 10, serum from a normal, unexposed control. Immunoprecipitation was carried out as described in Materials and Methods. The experiments in lanes 1 to 5 were repeated four times with similar results. Immunoprecipitation in lanes 6 to 10 was done twice with similar results. (B) A 1-week exposure of an SDS-PAGE gel (10% polyacrylamide) showing the time course of immunoreactivity in mice infected i.p. or rats infected i.t. Lanes: 1, PBS; 2, preimmune sera; 3, 2 weeks postinfection; 4, 4 weeks postinfection; 5, rat day 0; 6, rat 9 months. The molecular mass is given in kilodaltons.

18, 33, 38). Acid phosphatase has also been implicated in the virulence of bacteria and parasites (2, 51), where it is believed to enhance microbial survival by suppressing the neutrophil respiratory burst (48) and superoxide anion production.

Some radiolabeled supernatant proteins were precipitated by sera from infected rodents, suggesting that these antigens elicited antibody responses during infection. In rodents, proteins precipitated by immune sera differed depending on the route of infection. A 75-kDa protein was the dominant band in immunoprecipitates obtained with sera from rodents infected i.t., while a 30-kDa protein was the dominant band in animals infected i.p. This difference, which was apparent at 4 weeks of infection in mice, became less prominent at later times and may be a result of differences in the antigens and/or antigen

presentation depending on the route of infection. The appearance of antibody responses after 2 weeks of infection suggests a T-cell-dependent immunoglobulin G response to protein antigens, consistent with immunoprecipitation occurring with protein A. Sera from patients with *C. neoformans* infection did not react with the secreted proteins. This is not surprising, because such patients are generally severely immunocompromised, e.g., with HIV. Control serum from an individual with no known exposure to *C. neoformans* showed no immunoreactivity. Studies are in progress to determine the host antibody response to *C. neoformans* proteins by Western blot analysis.

We have systematically studied the secreted or released proteins of *C. neoformans*. We observed that culture supernatants contained multiple proteins whose expression varied with the culture conditions and the *C. neoformans* strain used. For a given growth condition, supernatant protein profiles were reproducible from experiment to experiment. Several new extracellular and/or cell-associated enzymatic activities were identified, including esterolytic and lipolytic activities that may facilitate fungal infection and invasion of mammalian host cells. The supernatant-associated proteins which are made in vivo are candidates for future studies of virulence factors and extracellular antigens that elicit immune responses.

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