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Cryptococcal Culture Filtrate Antigen for Detection of Delayed-Type Hypersensitivity in Cryptococcosis

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Previous studies on a cryptococcal culture filtrate (CneF) antigen have shown that the antigen is useful in detecting delayed-type hypersensitivity and that it is specific for *Cryptococcus*. This study further defined one more parameter of specificity, showing that the CneF antigen does not elicit delayed-type hypersensitivity responses in *Cryptococcus albidus*-sensitized guinea pigs. When the crude CneF antigen was subjected to ultrafiltration fractionation, the skin test active components were found to be in the 50,000 or greater molecular weight range fraction. The concentrated retentates of the XM50 ultrafiltration membrane were more sensitive antigens than the crude CneF antigens. Further fractionation of the XM50 retentate using 3% acrylamide gel electrophoresis separated the antigen into two bands. One band, the P fraction, migrated only a short distance into the gel; the fraction was carbohydrate-like and did not elicit significant skin test responses in sensitized guinea pigs. The other band, G fraction, appeared with the tracking dye, was glycoprotein-like, and elicited significantly positive skin tests in sensitized guinea pigs. G fractions prepared using three different serotypes of *Cryptococcus neoformans* elicited similar size indurations when used in skin testing guinea pigs sensitized with either the homologous serotype isolate of *C. neoformans* or the heterologous serotype isolate.

In a previous study, a cryptococcal culture filtrate antigen prepared according to the method of Murphy and Cozad (6) was shown to elicit positive delayed-type hypersensitivity (DTH) reactions in guinea pigs (7) and mice infected with *Cryptococcus neoformans* (1, 7). Furthermore, the antigen was shown to be specific for *C. neoformans* in that it did not stimulate DTH responses in guinea pigs sensitized to *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Sporothrix schenckii*, or *Candida albicans* (7).

A reliable, sensitive, and specific antigen for the detection of DTH respones or for use in in vitro correlates of DTH would be an extremely useful tool in gaining a greater understanding of the host-parasite relationships in cryptococcosis and in epidemiological studies on this disease. Since this culture filtrate antigen appeared to have the potential for reliability, sensitivity, and specificity, further studies were undertaken to purify and characterize it. The main objectives were: (i) to determine whether positive skin test reactions could be elicited with the culture filtrate antigen in guinea pigs sensitized to another species of *Cryptococcus* such as *Cryptococcus albidus*; (ii) to isolate the skin test reactive fraction(s) from the crude culture filtrate antigen; and (iii) to ascertain whether there was significant variation in skin test activity of antigens produced from different isolates of *C. neoformans*.

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MATERIALS AND METHODS

Animals. Hartley strain guinea pigs, approximately 15 weeks old of both sexes, were used in this study. All guinea pigs were born and raised in the animal facilities at the University of Oklahoma.

Organisms. Six different isolates of *C. neoformans* were used for sensitization of guinea pigs and in the preparation of the culture filtrate antigens (CneF): isolates 381A and 184A, serotype A; 381B and 687B, serotype B; and 381C and 139C, serotype C. Isolates 381A, 184A, 381B, and 381C were typed by Dexter Howard, Department of Medical Microbiology and Immunology, University of California, Los Angeles. Isolates 687B and 139C were serotyped by and obtained from John Bennett, National Institutes of Health, Bethesda, Md. Cultures were maintained on Sabouraud dextrose agar in the stock culture collection of the University of Oklahoma. To confirm the identity of each isolate, urease tests (8), carbohydrate assimilations (4), and nitrate utilization studies (8) were performed. All isolates demonstrated a typical *C. neoformans* profile.

C. albidus from the University of Oklahoma stock

culture collection was used in sensitizing guinea pigs and for preparing a culture filtrate antigen in the same manner that the CneF antigen was prepared.

Sensitization of guinea pigs. Guinea pigs were sensitized with *C. neoformans* or *C. albidus* emulsified in complete Freund adjuvant (CFA) (H37Ra) (Difco Laboratories, Inc., Detroit, Mich.). For preparation of inocula, organisms were grown for 72 h at 35°C, harvested, and washed with sterile 0.15 M NaCl (physiological saline) solution (SPSS). Total numbers of cells were determined by hemacytometer counts. Appropriate dilutions were made to give stock concentrations of 2×10^7 cells per ml for each sensitizing agent. A stable emulsion was made by mixing 1 volume of stock cell suspension with 1 volume of CFA. Guinea pigs were sensitized by injecting 0.2 ml of the emulsion into each of the front footpads and 0.6 ml of the emulsion, subcutaneously, into the neck region. Control guinea pigs were injected as indicated above with SPSS in equal amounts of CFA.

Skin testing of guinea pigs. Skin testing was done at 14 or 28 days after injection of sensitizing material. The abdomens of the guinea pigs were shaved the day before application of skin test. Animals were injected intradermally with 0.1 ml of antigen or control medium. Erythemas and indurations were measured at 6, 24, and 48 h after injection. Reactions were not present at 6 h on any guinea pigs, and 24- and 48-h data appeared very similar when plotted; therefore, only 48-h results will be indicated. To establish that guinea pigs injected with *C. albidus* in CFA were sensitized to *C. albidus*, the animals were skin tested with 0.1 ml of *C. albidus* culture filtrate antigen, which had been prepared in the same manner as the CneF, or with 2×10^6 Formalin-killed *C. albidus* cells.

Antigen preparation. The "crude" culture filtrate antigens were prepared according to the method of Murphy and Cozad (6). Briefly, this included adding 5×10^8 *C. neoformans* cells suspended in SPSS to 1-liter volumes of neopeptone dialysate medium. Cultures were grown for 72 h at 30°C before Formalin was added to give a final concentration of 2%. Cell-free filtrates were collected after centrifugation and filtration and dialyzed against four changes of physiological saline. The dialyzed culture filtrates were filter sterilized and stored at −20°C until employed as the crude antigens. An antigen control was prepared in the same manner; however, the neopeptone dialysate medium was not inoculated with *C. neoformans*.

Fractionation of CneF antigen. For initial fractionation, the constituents of the crude antigen were separated into different molecular weight ranges by using an Amicon TCF10 thin-channel system with three different membranes, XM50, PM30, and UM10. Two slightly different procedures were used (Fig. 1). In both procedures crude antigen, which had been previously dialyzed against distilled water, was successively passed through the series of Diaflo ultrafiltra-

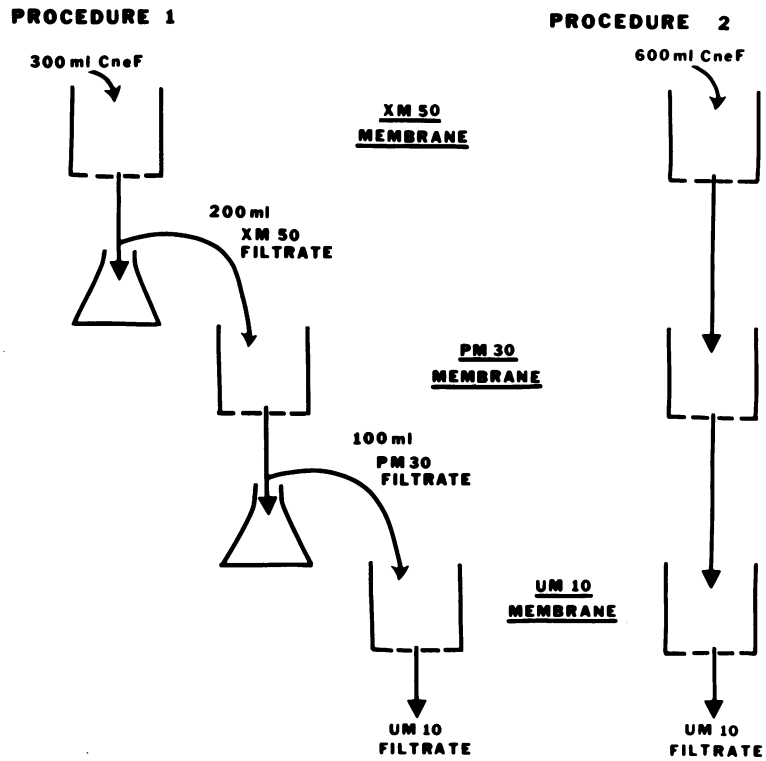


FIG. 1. *Two procedures used for ultrafiltration fractionation of the crude cryptococcal culture filtrate antigen (CneF).*

tion membranes. The first membrane, XM50, retained constituents having molecular weights of 50,000 or greater. The second, PM30, and the third membrane, UM10, retained molecular weights of approximately 30,000 and 10,000, respectively.

In the first procedure, fractionation of crude antigen was done in the following manner: 300 ml of crude antigen was placed on the XM50 membrane and the system was run until approximately a 25-ml working volume remained in the Diaflo cell. The XM50 filtrate was collected, and 200 ml of working volume was put through the PM30 membrane, again retaining approximately a 25-ml working volume in the cell. The PM30 filtrate was collected, and 100 ml was passed through the UM10 membrane, until only 25 ml remained in the cell. The retentate of each membrane was washed three times with 100 ml of glass-distilled water, and the washings were discarded. The washed retentates were lyophilized. Prior to skin testing, the lyophilized fractions were reconstituted with SPSS to 1/5 the original volume of material placed on each membrane. After hydration, the solutions were filter sterilized and used for skin testing.

The second procedure consisted of passing 600 ml of crude antigen through the XM50 membrane and collecting the filtrate and the membrane retentate. All of the XM50 filtrate was then passed through the PM30 membrane. The PM30 residue was collected, and the total volume of PM30 filtrate was then run through the UM10 membrane. The UM10 filtrate and retentate were collected. All membrane retentates and the UM10 filtrate were lyophilized. Each fraction was rehydrated with SPSS to a concentration of 1 mg (dry weight) per ml. The preparations were filter sterilized and used in skin testing. To determine the total amount of solids in the retentates of each membrane and the UM10 filtrate, another 600 ml of crude antigen was carried through procedure two (Fig. 1); however, after lyophilization the fractions were dried at 85°C until constant weights were obtained. Weighings were made on each fraction, and the percentage of total solids was calculated for each.

Gel electrophoresis. Disc gel electrophoresis was run in a Plexiglas cell similar to a commercial vertical electrophoresis apparatus, using a Buchler model 3-1014A power supply to provide a constant current of 2.5 mA per gel. Glass columns (5.5 by 100 mm) were filled with 3% chemically polymerized acrylamide prepared according to the procedure of Strauss and Kaesberg (9). All chemicals were obtained from BioRad Laboratories, Richmond, Calif. Samples were mixed with glycerol to give a final concentration of 10%. The volume of sample-glycerol mixture applied to each gel was adjusted between 20 and 65 μl, so that the amount of sugar per gel was approximately 20 to 40 μg and the amount of protein was between 40 and 200 μg per gel. Bromophenol blue was used as a tracking dye. Electrophoresis was performed in tris(hydroxymethyl)-aminomethane-glycine buffer (pH 8.3) (9) at room temperature with the cathode to the upper buffer reservoir. Eight gels were prepared for each antigen. Antigen and tracking dye were layered on tubes 1 and 2, antigen alone was placed on tubes 3 and 4, tracking dye was added to tubes 5 and 6, and nothing was put on tubes 7 and 8. Even-numbered tubes were stained for protein using 1% Amido Black in 7% acetic acid for 1 h and were destained electrophoretically. The odd-numbered gels were stained for carbohydrate using a modified periodic acid-Schiff (PAS) technique (10). This procedure allowed for comparison of the staining characteristics of the fractions and for determining how the rate of migration of each fraction related to the tracking dye migration.

Three percent acrylamide was chosen for these studies after preliminary experiments indicated that the distance moved by the PAS-staining fraction (P band) was inversely related to the acrylamide gel concentration used in electrophoresis. Four gel concentrations, (3, 4, 5, and 7%) were employed in the preliminary study. The Amido Black–PAS-staining fraction (G band) moved with the tracking dye in all gel concentrations.

Isolation of different electrophoretic bands. After determining how the antigens separated on the 3% gels, 120 gel tubes were prepared for each of four antigens, 184A, 381A, 381B, and 139C. Tracking dye was used in only one tube of each electrophoretic run, and that tube was stained with PAS after electrophoresis. Comparing the other seven gels with the stained gel, the different bands were cut from nonstained gels. Like bands were pooled, ground in a mortar with glass-distilled water, and allowed to stand overnight at 4°C. The pools were centrifuged, and the supernatants were collected and reduced to 1/4 volume by evaporation. A small volume of NaCl solution was added to each to give a final concentration of 0.15 M NaCl. Protein concentrations were measured, and then each fraction was adjusted so that the final concentration of protein was 400 μg/ml. The preparations were filter sterilized using Millipore 0.45-μm membrane filters. Protein and sugar concentrations were measured, and the solutions were stored at −20°C until used for skin testing.

To prepare the gel control solutions for this experiment, 3% acrylamide gel columns were subjected to electrophoresis after being loaded with 10% glycerol. Gel sections were cut from these control gels and extracted in the same manner as bands which contained the antigens. When the protein concentration of the control gel extract was measured, it was found to be 160 μg/ml. Since 400 μg/ml had been chosen as the concentration of protein to use for skin testing of the antigen fractions, the control extract was concentrated by evaporation; the protein concentration was measured, then adjusted to 400 μg/ml prior to being used for skin testing. Finding protein in the gel control extracts and the extracts of the P bands (those bands staining only with PAS) was unexpected; therefore, protein determinations were made on the tris(hydroxymethyl)aminomethane-glycine buffer used for electrophoresis. Since the buffer gave a positive test with the Lowry protein assay being employed, the protein in the control gel extract and P band extracts was considered to be contamination of the extracts with the tris(hydroxymethyl)aminomethane-glycine buffer used in electrophoresis.

Chemical analyses. Protein concentrations of the antigens, antigen fractions, and acrylamide gel extract were determined by the Lowry procedure (5). Bovine serum albumin solution was used as a standard and Monitrol I (Dade Division, American Hospital Supply

Corp., Miami, Fla.) as a control.

The phenol-sulfuric method (2) was used to determine the sugar concentrations of the preparations. A standard curve was run using mannose, and a known concentration of mannan served as a control.

Statistical analysis. Means, standard errors, and paired and unpaired *t* tests programmed on the Hewlett Packard calculator model 9810A were used in analyses of data.

RESULTS

Specificity of CneF antigen. Table 1 shows the results of skin testing guinea pigs injected 14 days previously with *C. neoformans*, *C. albidus*, or saline with culture filtrate antigens of *C. neoformans* and *C. albidus*. To demonstrate that the *C. albidus*-injected guinea pigs were sensitized to *C. albidus*, 10 animals were skin tested with a culture filtrate antigen prepared using *C. albidus*. The *C. albidus* antigen elicited strongly positive induration reactions in the *C. albidus*-sensitized guinea pigs, even though it showed some nonspecific activity on *C. neoformans*- or saline-injected guinea pigs. Five other *C. albidus*-injected animals were skin tested with 2×10^6 Formalin-killed *C. albidus* cells, and the mean induration elicited for that group was 10 mm, indicating they were sensitized to *C. albidus*. When the CneF antigen was used in skin testing the same guinea pigs, only 1 animal of the 15 tested demonstrated any induration, and that was only 4 mm in diameter, which was not considered a positive response. That animal also showed a 4-mm erythema. Two other animals responded with erythemas of 6 and 7 mm. The CneF antigen was very effective in eliciting DTH reactions (mean induration of 18.64 mm) in *C. neoformans*-injected animals.

Fractionation of CneF antigen into molecular weight ranges. The crude CneF was separated into four different molecular weight range fractions according to procedure 1 (Fig. 1). The fractions were lyophilized and reconstituted in 1/5 of the volume of the material used to prepare them. The reconstituted fractions were used to skin test guinea pigs which had been injected 14 days previously with *C. neoformans* in CFA or saline-CFA. No reactions were elicited by any of the fractions on 10 saline-CFA-injected guinea pigs. Mean skin test results from 10 *C. neoformans*-injected animals are shown in Fig. 2. The XM50 residue elicited the strongest responses, with a mean induration of 11.5 mm ± 2.4 mm (standard error of the mean). The PM30 and UM10 retentates and the UM10 filtrate elicited either no indurations or very weak indurated responses, indicating that they were significantly less effective than the XM50 residue as skin test antigens.

TABLE 1. *Skin test reactivity of C. neoformans and C. albidus culture filtrate antigens on C. neoformans-, C. albidus-, or saline-injected guinea pigs*

Skin test antigen	Protein concn per dose	Mean induration (mm) ± SE[a] in animals previously injected with:		
		C. neoformans (10)[b]	*C. albidus* (10)	Saline (10)
CneF	750 μg	18.64 ± 1.65	0.8 ± 0.8	0
C. albidus	775 μg	5.19 ± 2.21	21.4 ± 2.2	4.6 ± 1.3

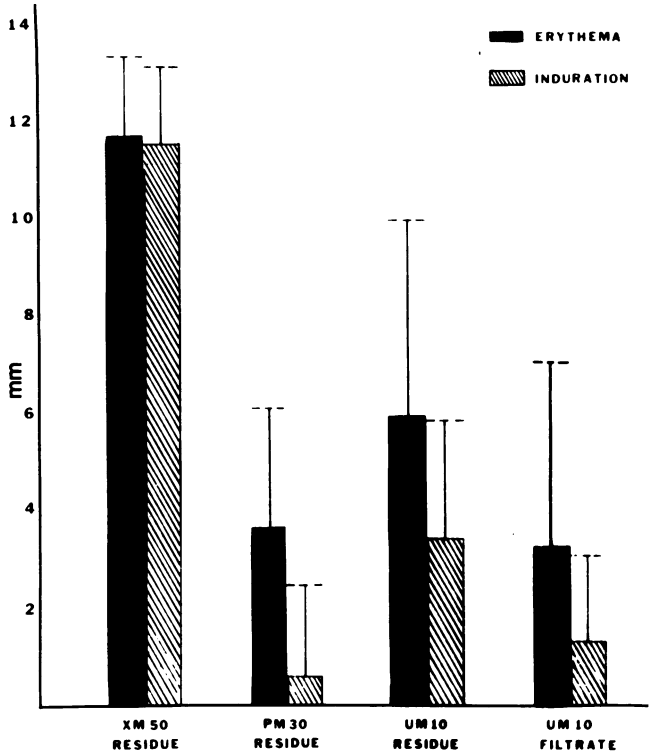
[a] SE, Standard error of the mean.

[b] Parentheses indicate number of animals.

When 600 ml of the crude antigen was serially passed through the series of Diaflo filters, and the fractions were collected, lyophilized, and reconstituted to 1 mg/ml prior to being used in skin testing, the results in Fig. 3 were obtained. In this case, both the XM50 and the PM30 residues elicited significant skin test reactions. However, only approximately 0.1% of the total solids of the crude antigen was collected in the 30,000 to 50,000 molecular weight range fraction, so the yield on the PM30 membrane was extremely small. Approximately 5% of the total solids had molecular weights greater than 50,000, thus giving a considerably better yield of solid material on the XM50 membrane. The UM10 residue fraction elicited only marginal skin test results and comprised approximately 6% of the total solids. The UM10 filtrate did not stimulate a reaction in any of the 20 sensitized animals on which it was used, even though the major part of the total solids (88.9%) was found to be in the UM10 filtrate fraction. Ten saline-CFA-injected animals did not respond with indurations when skin tested with any of the fractions.

Electrophoretic fractionation of the XM50 fraction of CneF. Figure 4 shows the typical pattern obtained when XM50 fractions of the various *C. neoformans* culture filtrate antigens were subjected to disc gel electrophoresis and staining. All six isolates of *C. neoformans* used in this study produced culture filtrate antigens which separated into two bands in 3% acrylamide gels. The fastest-moving component (G band) moved with the tracking dye and stained with protein and PAS stains. The other component (P fraction) appeared as a broad band, a short distance from the top of the gel and stained only with PAS. When XM50 fractions of various antigen preparations were mixed and subjected to electrophoresis and staining, only one P and one G band were observed, indicating that the P and G fractions of the various antigen preparations were not resolvable by this procedure.

New culture filtrate antigens prepared using *C. neoformans* isolates 381A, 381B, and 139C were subjected to ultrafiltration and electropho-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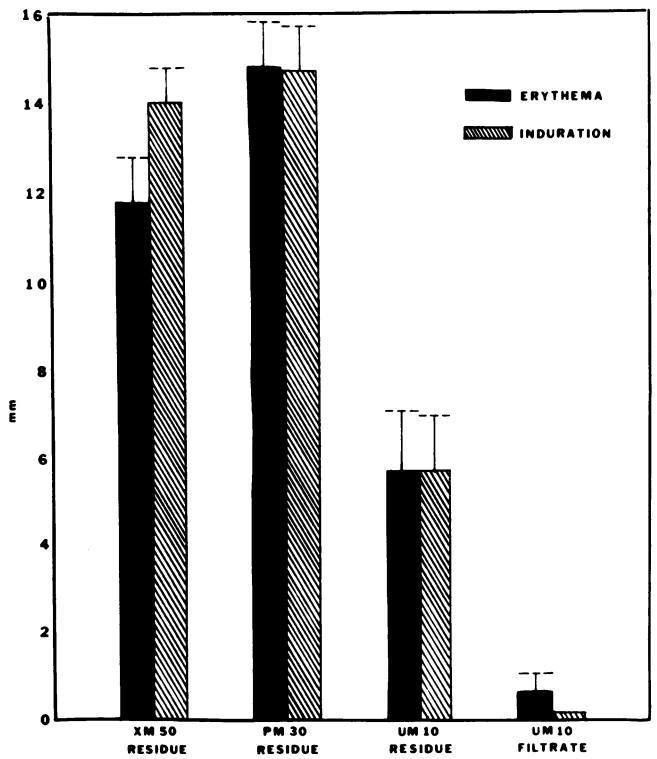
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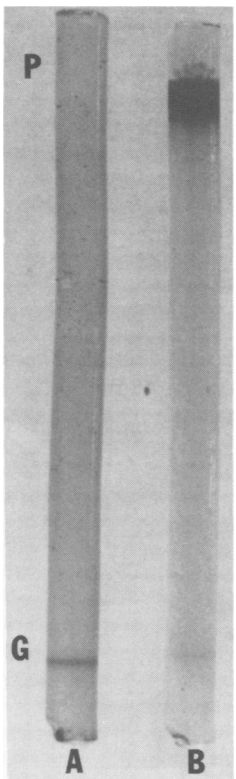

FIG. 4. *Typical electrophoretic pattern obtained when CneF XM50 retentates were subjected to electrophoresis on 3% acrylamide gels, then stained with 1% Amido Black for protein (A) or PAS for sugar (B). P and G bands are identified on the gels. Notches indicate the bottom of the gels.*

resis before the G and P fractions of each antigen were isolated. The protein concentration of each fraction was adjusted to 400 μg/ml, and the sugar concentrations varied as shown in Table 2. These fractions plus an acrylamide gel control preparation with a protein concentration of 400 μg/ml were used to skin test guinea pigs which had been injected 14 days previously with the homologous isolates of *C. neoformans* or saline in CFA. Four guinea pigs were sensitized with each *C. neoformans* isolate, and four were used as controls. Figure 5 shows the results of this experiment. The G fraction of each antigen elicited positive skin test responses on *C. neoformans*-injected animals; however, the P fractions of 381A, 381B, and the acrylamide gel control did not elicit positive responses. The P fraction of 139C produced marginal responses in 139C-injected animals. None of the preparations induced significant indurations in saline-injected guinea pigs; however, the 139C P fraction stimulated more activity than any of the other fractions in the control animals. When P and G fractions prepared from a 184A culture filtrate antigen were used in skin testing 184A-sensitized guinea pigs, results similar to those shown for the 381A fractions were obtained. The G fractions from all four antigen preparations induced significantly larger responses in sensitized guinea pigs than in control animals ($P < 0.005$). Furthermore, each G fraction elicited a greater induration response than did the homologous P fraction or gel control ($P < 0.025$, except 139C fractions with $P < 0.05$).

The P and G fractions of 381A, 381B, and 139C plus the gel control were used in skin testing a group of four guinea pigs sensitized with 381A *C. neoformans* cells (Fig. 6). Each fraction gave responses in this group of animals similar to the responses seen when the fractions were used to skin test homologously sensitized guinea pigs. Again the 139P fraction elicited marginally positive responses.

DISCUSSION

Data collected in studies using an antigen as an epidemiological tool or in disease diagnosis or

TABLE 2. *Protein and sugar concentrations of the various CneF antigen fractions separated by acrylamide gel electrophoresis*

Antigen fraction	Concn (μg/ml)	
	Protein	Sugar
381A		
G	400	280
P	400	420
381B		
G	400	580
P	400	524
139C		
G	400	740
P	400	641

FIG. 2. *Guinea pig skin test results using as antigens the XM50, PM30, and UM10 residues and the UM10 filtrate prepared according to procedure 1 (Fig. 1). Each antigen fraction was reconstituted with SPSS to 1/5 the original volume of the solution used in the preparation of the fraction. Ten guinea pigs had been sensitized 14 days prior to skin testing with 10^7 C. neoformans cells in CFA. Vertical bars denote standard errors of the means.*

FIG. 3. *Guinea pig skin test results using as antigens the XM50, PM30, and UM10 residues and the UM10 filtrate obtained according to procedure 2 (Fig. 1) Each antigen fraction was reconstituted with SPSS to a concentration of 1 mg (dry weight) per ml. Ten guinea pigs had been sensitized with 10^7 C. neoformans cells in CFA 14 days prior to skin testing. Vertical bars denote standard errors of the means.*

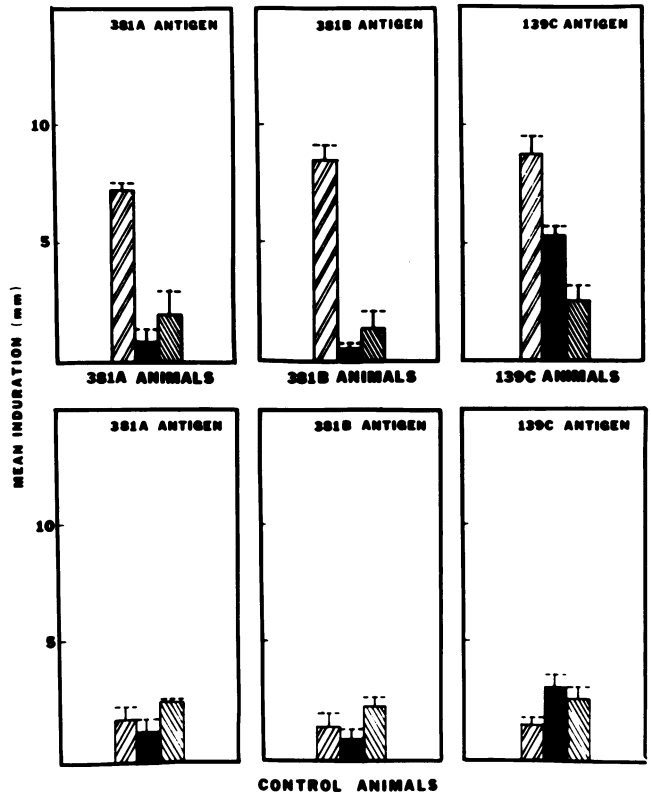

FIG. 5. *Mean indurations (mm) elicited by skin testing with (▨) the acrylamide gel control preparation and (▨) G (glycoprotein-like) and (■) P (carbohydrate-like) fractions prepared from XM50 retentates of antigens 381A, 381B, and 139C. Guinea pigs were sensitized, 14 days prior to skin testing, with 10^7 C. neoformans cells of homologous isolates used in preparation of the skin test antigens. Each bar represents the mean and standard error of the mean of results from four animals.*

prognosis can only be interpreted properly when the specificities of the antigen have been well defined. In an earlier study we have shown that the CneF antigen is specific for *Cryptococcus* (7); but we had not addressed the possibility that the CneF antigen might be able to detect a sensitivity developed against another *Cryptococcus* species. Since it is possible for a person to become sensitized to other *Cryptococcus* species such as *C. albidus* by harboring the organism or coming in sufficient contact with it, the question of whether the CneF antigen would detect this sensitivity was proposed. The data collected in this study clearly demonstrate that sensitization to *C. albidus* does not affect the results of skin testing with CneF antigen, thus defining one more parameter of specificity for the CneF antigen.

The next phase of this investigation was directed toward preparing a more sensitive antigen by isolating the skin test reactive fraction from the crude CneF. Ultrafiltration fractionation of the crude CneF established that the skin test active component(s) resided primarily in the fraction with molecular weight range of 50,000 and greater. The PM30 residue or the 30,000 to 50,000 molecular weight range fraction, when used at the same concentration as the XM50 residue (1 mg/ml), elicited skin test reactions comparable to those elicited by the XM50 residue. However, the amount of solid material recovered from the PM30 membrane was only about 0.1% of the total solids, whereas 5% of the total solids was recovered on the XM50 membrane. Sufficient shearing of the skin test active, 50,000 or greater molecular weight molecules could have occurred to account for the amount of material harvested from PM30 membrane and the biological activity associated with it. The results of the first fractionation experiment in which we found the XM50 fraction to be biologically active while the PM30 fraction lacked activity do not disagree with the results of our second experiment. When the fractions were reconstituted in the first experiment to 1/5 the original volume of material placed on

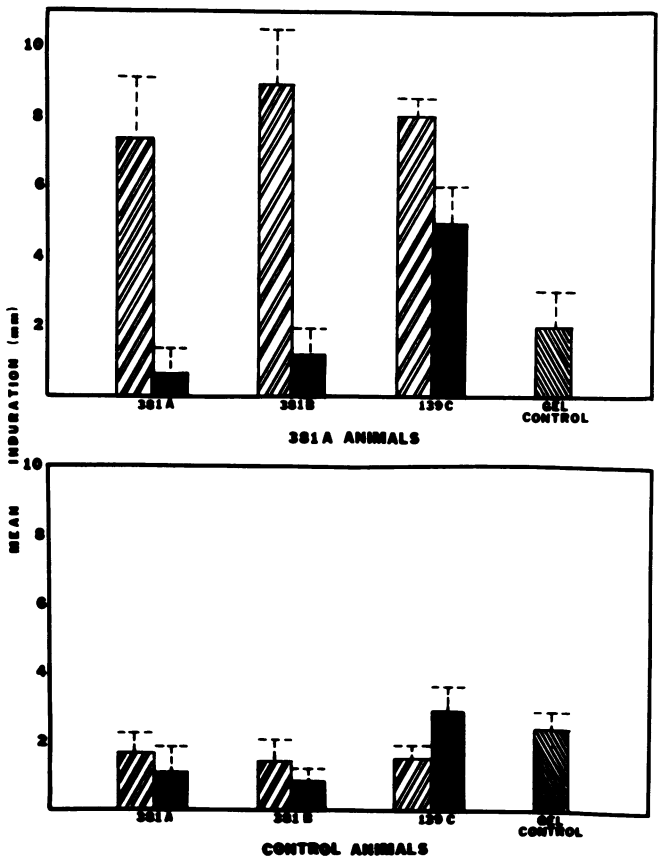

FIG. 6. *Mean values of indurations (mm) elicited on four C. neoformans isolate 381A-sensitized guinea pigs and four saline-CFA-injected animals, using as antigens (▨) the acrylamide gel control preparation and (▩) G (glycoprotein-like) and (■) P (carbohydrate-like) fractions prepared from XM50 retentates of antigen 381A, 381B, and 139C. Vertical bars denote the standard errors of the means.*

the membranes, the biologically active component(s) in the XM30 residue fraction was so diluted that significant indurations were not elicited.

Washed XM50 retentate of the crude CneF antigen is the material now being employed for all skin test procedures in our laboratory, and it has proven to be very effective in eliciting DTH responses in *C. neoformans*-infected mice (1). Dykstra and Friedman (3) prepared a cryptococcal culture filtrate antigen in the same manner used in preparing the crude CneF. Their preparation induced DTH responses in *C. neoformans*-sensitized guinea pigs; however, it did not elicit positive DTH responses in *C. neoformans*-injected mice. We have obtained similar results using unconcentrated crude CneF. However, an antigen with an ability to elicit DTH responses in mice can be prepared by concentrating the crude CneF. Therefore, the Dykstra and Friedman preparation probably was not sufficiently concentrated to elicit DTH responses in mice. This problem of insufficient sensitivity can be readily eliminated by isolation of the biologically active molecular species, concentration, and washing on an XM50 ultrafiltration membrane.

The XM50 fraction can be further separated into two components by electrophoresis. One fraction was a relatively high-molecular-weight polysaccharide possessing minimal if any biological activity. The other fraction, which migrated with the tracking dye on the 3% acrylamide gels and stained for protein and carbohydrate, possessed a significant amount of skin test activity. Most likely the active fraction is a glycoprotein; however, further studies must be done to prove this. Three different cryptococcal serotypes were used in producing culture filtrate antigens. All of the antigens prepared using the various *C. neoformans* isolates showed similar electrophoretic patterns, under the conditions employed, and the biological activity of each antigen preparation resided in equivalent fractions. This means that, in preparing materials for detection

of DTH, one does not have to be concerned about selecting a particular isolate or serotype of *C. neoformans* to obtain an active preparation.

Removal of the large-molecular-weight polysaccharide component from the XM50 retentates provided antigens which elicited equivalent responses on animals sensitized either with the homologous serotype isolate of *C. neoformans* used in antigen preparation or with a heterologous isolate. The same statement could not be made for the XM50 retentates prepared using the different serotype isolates of *C. neoformans* (J. E. Davis and J. W. Murphy, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, F28, p. 82). Therefore, ideally it would be better to isolate the glycoprotein-like material (G band fraction) and use it in all future studies. However, since the procedure for isolating the G fraction is slow and laborious, this fraction is not being used routinely for detection of DTH. Further experiments are now under way to develop a more efficient means of isolating the glycoprotein-like skin test active fraction from the crude CneF.

ACKNOWLEDGMENTS

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