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Specificity of *Cryptococcus neoformans* Factor Sera Determined by Enzyme-Linked Immunosorbent Assay and Dot Enzyme Assay

T. BELAY,[1] R. CHERNIAK,[1]* AND T. SHINODA[2]

Laboratory for Biological and Chemical Sciences, Department of Chemistry, Georgia State University, Atlanta, Georgia 30303,[1] and Department of Microbiology, Meiji College of Pharmacy, Tanashi, Tokyo 188, Japan[2]

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An indirect enzyme-linked immunosorbent assay (ELISA) and a dot enzyme assay (DEA) were used to determine the specificities of *Cryptococcus neoformans* factor sera to serotype type-specific capsular polysaccharides, glucuronoxylomannans (GXMs). Pure and chemically characterized GXMs were obtained from representative isolates of *C. neoformans* serotypes A, B, C, and D. Distinctive specificity patterns and quantitative differences were observed for each factor serum when the selected GXMs were studied by ELISA. The specificity patterns for each factor serum determined by DEA almost completely paralleled the ELISA results. The serotype specificities demonstrated by ELISA and DEA were similar to previously reported results that were obtained by slide agglutination studies of whole cells. On the basis of the ELISA and DEA activity patterns, factor sera 5, 6, and 8 were specific for serotypes B, C, and D, respectively; factor serum 1 was strongly reactive to all serotypes; factor serum 2 was specific for serotypes A, B, and D; factor serum 3 was specific for serotypes A and D; and factor serum 4 was specific for serotypes B and C. The specificity of factor serum 7 for serotype A was demonstrated by DEA only. Structural variation was indicated among the serotype C isolates studied because a unique activity pattern versus factor serum 6 was observed for each isolate. The quantitative differences in the activity of the GXMs from five serotype C isolates suggest that mannopyranoside residues substituted *O*-2 and *O*-4 with xylose are essential elements of the determinant responsible for the observed activity of factor 6. No significant differences in activity patterns and specificities of factor serum 6 were observed when O-deacetylated GXMs were substituted for the native GXMs. Our results show that ELISA and DEA are valuable techniques for the serological analysis of cryptococcal factor sera and GXMs.

Cryptococcus neoformans causes cryptococcosis, a major life-threatening opportunistic infection in individuals with an impaired immune system (15, 16, 25). The incidence of the disease has been steadily increasing, particularly in patients with AIDS (15, 16). *C. neoformans* has a major polysaccharide capsule, glucuronoxylomannan (GXM), that is a significant virulence factor (2, 18). In general, GXM consists of a linear (1→3)-α-D-mannopyranan bearing β-D-xylopyranosyl (Xyl*p*), β-D-glucopyranosyluronic acid (Glc*p*A), and 6-*O*-acetyl substituents (1, 22). A simple structural relationship between the model polysaccharides of the four serotypes exists. They are all composed of a core repeating unit,

β-D-Glc*p*A
1
↓
2
→3)-α-D-Man*p*-(1→3)-α-D-Man*p*-(1→3)-α-D-Man*p*-(1→

to which (1→2)-linked β-D-Xyl*p* and (1→4)-linked β-D-Xyl*p* units are added in increments of one to four residues. GXMs from serotypes A and D are mainly substituted at *O*-2, whereas GXMs from serotypes B and C are substituted with Xyl*p* at *O*-2 and at *O*-4. Precise molar Xyl/Man/GlcA ratios of serotypes D, A, B, and C of 1:3:1, 2:3:1, 3:3:1, and 4:3:1, respectively, were proposed (1). However, additional analytical data, obtained recently from the study of larger numbers of isolates of each serotype (6, 7, 22, 23), show that the precise molar ratio and substitution patterns as proposed in the original models of GXM structure are an oversimplification except in the case of serotype B (23). In addition, substituent dispositions previously thought to be characteristic of one serotype have been identified in heterologous isolates (22, 23).

Anti-capsular polysaccharide antibodies produced by immunization of rabbits with killed whole cells have been used to distinguish *C. neoformans* isolates. Five serotypes (A, B, C, D, and A-D) have been described on the basis of the specificity of anti-capsular antibodies to whole cells of *C. neoformans* (12–14, 24). In the United States and Canada, the majority of cryptococcal infections in AIDS patients are caused by *C. neoformans* var. *neoformans*. Serotype A is responsible for almost all of the infections reported, even in environmental areas where serotypes B and C are the most common (17). Although chemical analyses have been used extensively to explore the structure of GXM, the specific determinants responsible for serotype specificity have not been identified. The use of enzyme-linked immunosorbent assay (ELISA) to quantitate antibodies to cryptococcal capsular polysaccharides has been reported previously (4, 5, 11, 19). Because of the poor binding of capsular polysaccharides to polystyrene microtitration plates, researchers have introduced various schemes for improving capsular polysaccharide binding. For instance, coating ELISA wells with anti-cryptococcal immunoglobulin G (19), adipic acid hydrazide derivative of bovine serum albumin (AH-BSA [5]), or poly-L-lysine (11) prior to the addition of capsular antigen to the wells has been employed in ELISA procedures. Casade-

* Corresponding author.

vall et al. (4) developed an ELISA method that uses monoclonal antibody to immobilize GXM.

Specific antigenic patterns for *C. neoformans* serotypes have been proposed by Ikeda et al. (14). Eight factor sera, prepared by the reciprocal adsorption of rabbit antisera, were used in a slide agglutination assay to serotype individual isolates of *C. neoformans* on the basis of the distribution of the eight factors. Antigenic factor 1 is common to all serotypes; factor 2 is common to serotypes A, B, and D; factor 3 is common to serotypes A and D; and factor 4 is common to serotypes B and C. Antigenic factors 7, 5, 6, and 8 are specific to serotypes A, B, C, and D, respectively. The A-D serotype contains antigenic factors 7 and 8 as well as factors 1, 2, and 3. Insufficient data are available to permit the identification and assignment of specific structures to any of the specific antigenic factors. Herein, an indirect ELISA method (5) was used to evaluate the activity and specificity of the eight factor sera against purified GXM antigens. A semi-quantitative dot enzyme assay (DEA) was used in parallel to the ELISA method to confirm the specificity of the factor sera. Our ELISA and DEA results generally agree with the activity and specificity profile of the factor sera determined by slide agglutination of whole cells reported previously (14). The results show that characteristic determinants predominate in each of the serotype-specific GXMs. However, heterologous determinants characteristic of one or more of the other serotypes are also present, but at a considerably lower density.

MATERIALS AND METHODS

Factor sera. Eight cryptococcal factor sera, lot 910610, were provided by Iatron Laboratories, Inc., Tokyo, Japan. Samples were kept at 4 and −20°C for short- and long-duration storage, respectively.

Antigens. Pure GXM antigens for ELISA and DEA were prepared in our laboratory from the following isolates of *C. neoformans*: isolate 6, serotype A (T. Mitchell, Duke University Medical Center); isolate 3183, serotype C (E. Reiss, The Centers for Disease Control and Prevention); National Institutes of Health isolates 18, 34, 298, and 401, serotype C; isolates 409 and 444, serotype B (J. K. Kwon-Chung, National Institutes of Health); and isolate 9375, serotype D (H. Jean Shadomy, Medical College of Virginia). Isolation and purification of GXMs were performed as described previously (6, 9). A representative isolate for each serotype was selected on the basis of how well its GXM composition and structure (7, 8, 23) matched the classical chemical delineation originally proposed by Bhattacharjee et al. (1).

ELISA. The preparation of AH-BSA and activated GXM, prepared by controlled oxidation with sodium periodate, was described previously (5). Immulon-2 and Immulon Microbind-HZ microtitration plates were purchased from Dynatech Laboratories, Inc., Chantilly, Va. Immulon-2 microtitration plates were primed by adding 100 μl of AH-BSA (50 μg/ml of 0.01 M Na_2HPO_4–0.14 M NaCl, pH 7.2 [PBS]) per well. The plates were sealed and incubated at 37°C for 3 h and then were kept at 4°C overnight. After washing the plates four times with PBS–0.05% Tween 20 (PBS-T) 100 μl of oxidized GXM (50 μg in 1 ml of PBS) was added. The plates were incubated and washed as described above for the priming step. Factor sera, diluted in PBS-T containing 2% fetal bovine serum (Hyclone Lab), were added (100 μl per well), and the plates were incubated at 24°C for 3 h. Factor sera were examined in twofold dilutions from 1:200 to 1:204,800. Each assay was performed at least twice, with each dilution run in quadruplicate. After washing the plates with PBS-T three times, alkaline phosphatase-labeled anti-goat rabbit immunoglobulin G (whole molecule from Sigma Chemical Co., St. Louis, Mo.) was added (100 μl per well) and the plates were incubated at 24°C for 1 h. After three washes with PBS-T and one wash with PBS, 100 μl of 4-nitrophenyl phosphate (1 mg/ml of 0.95 M diethanolamine–9.5×10^{-4} M $MgCl_2$, pH 9.8) was added to each well; the plates were incubated at 24°C for 30 min. The enzyme reaction was terminated by adding 50 μl of 3 M NaOH to each well. Plates were read at 405 nm with a Microplate Reader (Molecular Devices Corp. Menlo Park, Calif.). The net absorbance for each serum dilution was determined by subtracting the mean absorbance value from four wells that lacked antigen and then repeating the net absorbance determination with four wells that lacked factor sera. Preimmune sera, when used in place of factor sera, gave ELISA titers that were below background values. Endpoint ELISA titers were reported as the final serum dilution with at least an A_{405} of 0.20 (about two times the mean absorbance value of the blank wells).

DEA. Rectangular strips (0.5 by 5.0 cm), cut from a nitrocellulose transfer membrane (Schleicher & Schuell), were spotted every centimeter with 1 μl of a freshly prepared solution of GXM (1 mg/ml of 0.06 M sodium carbonate buffer, pH 9.6). Desired dilutions of factor sera in PBS-T were added at a volume of 1 ml to each channel of an eight-trough incubation tray (Schleicher & Schuell) containing the antigen-coated strips. The tray was incubated at 24°C for 1 h with slow agitation on a rotator. Positive and negative control antisera were included in each assay. The strips were washed three times with PBS-T, and then 1 ml of peroxidase-labeled goat anti-rabbit immunoglobulin G (whole molecule from Sigma) was added (1:1,000) to each trough and the tray was incubated at 24°C for 30 min with slow agitation on a rotator. After the strips were washed three times with PBS-T and once with PBS, 1 ml of freshly prepared 3,3′-diaminobenzidine tetrahydrochloride (Sigma) (0.5 mg/ml in PBS containing 0.006% hydrogen peroxide) was added, and the trays were then incubated at 24°C for 15 min. Excess substrate was removed by aspirating the fluid, and the strips were washed four times with deionized water. The strips were finally dried with blotting paper.

RESULTS

Optimization of ELISA. The sensitivity of the ELISA procedure was optimized in a study in which the mode of immobilization of GXM was varied. Three configurations were used: (i) direct binding of GXM to Immulon-2 microtitration plates, (ii) binding of oxidized GXM to Immulon-2 microtitration plates primed with AH-BSA, and (iii) binding of oxidized GXM to Immulon Microbind-HZ microtitration plates. Immulon-2 plates bound the GXM poorly as revealed by the low sensitivity by ELISA. Microbind-HZ plates coated with oxidized GXM showed high levels of nonspecific absorbance. The Immulon-2 plates primed with AH-BSA and coated with oxidized GXM had the lowest level of background absorbance and the greatest sensitivity of the three configurations (data not shown). On the basis of these results, AH-BSA-primed microtitration plates were used exclusively in all subsequent ELISA studies. AH-BSA-primed microtitration plates, incubated with various concentrations (0.1 to 100 μg/ml) of native or oxidized GXMs, were used to determine the optimum conditions for the evaluation of factor serum specificities (Fig. 1). Controlled oxidation of

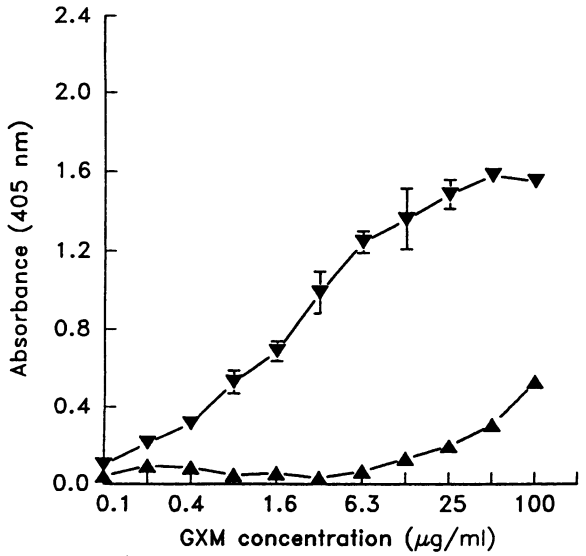


FIG. 1. Activity of factor serum 1 against various concentrations of either oxidized (▼) or native (▲) GXM from isolate CN6, serotype A, of *C. neoformans* as evaluated by indirect ELISA. Each point represents a mean absorbance value of four wells ± 1 standard deviation.

GXM in combination with AH-BSA-primed microtitration plates gave absorbancies that were four to eight times higher than those observed with native GXM and primed microtitration plates. A similar enhancement of activity was observed for all but factor 7 (data not shown). Factor serum 7 did not show the expected specificity to GXMs derived from *C. neoformans* serotype A isolates in ELISA; it reacted poorly, but equally, with GXMs of the four serotypes. The optimum concentration of oxidized GXM for coating the microtitration plates was chosen as 50 μg/ml on the basis of these initial trial observations.

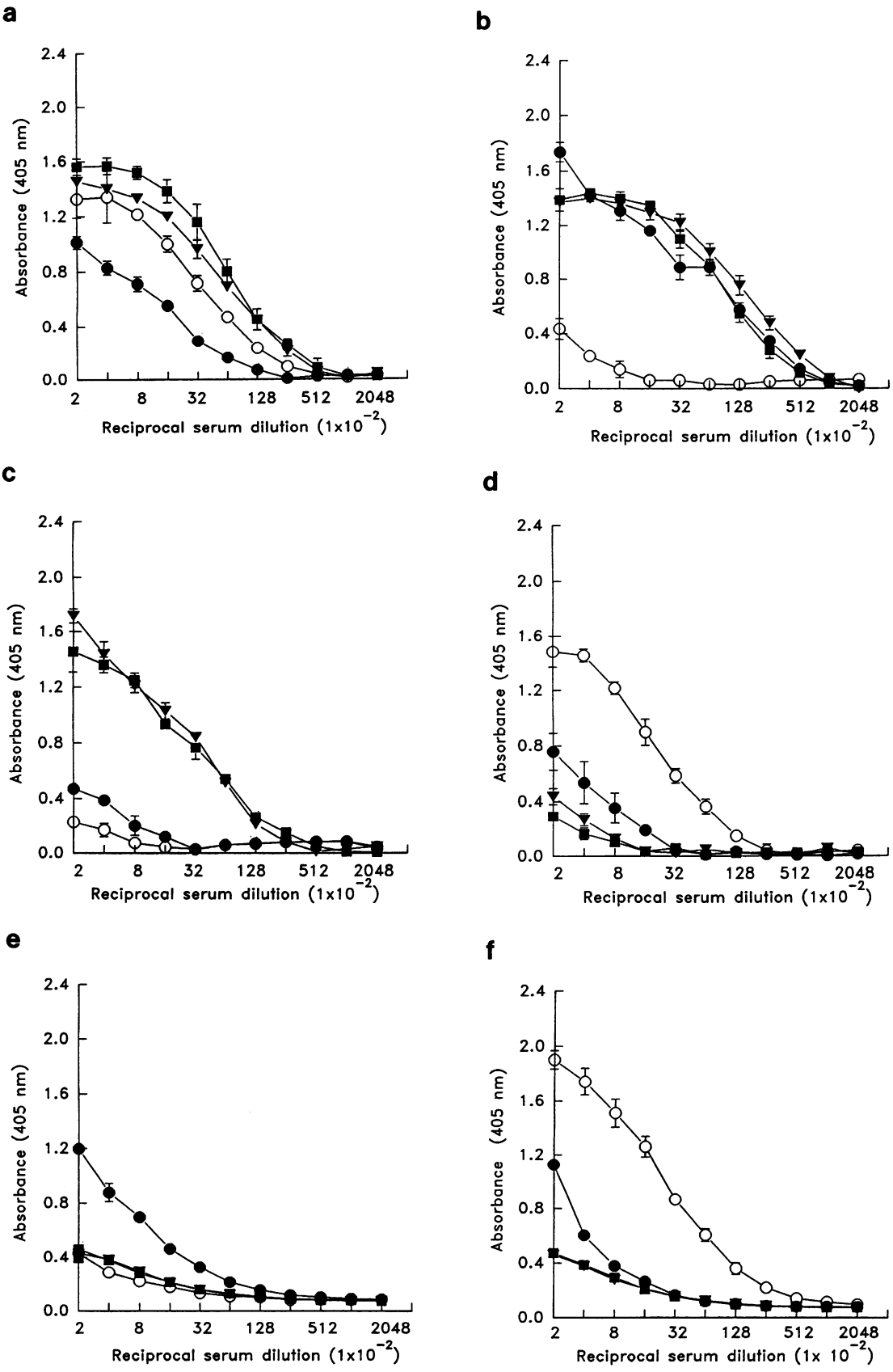
Factor serum titers. The titer of each factor serum (factors 1 to 8) was determined by ELISA with pure GXMs obtained from representative isolates of *C. neoformans* serotypes (A, B, C, and D) (Fig. 2a to h). The specificity of each factor serum generally paralleled the findings of Ikeda et al. (14) (Table 1). However, factor 7 showed only a low level of activity that was quantitatively equivalent to the level of activity found with the heterologous GXMs. The relative activity of factor serum 6 to five GXMs obtained from *C. neoformans* serotype C isolates (18, 3183, 401, 298, and 34) was investigated by ELISA. The carbohydrate composition and linkage assignments of this group of GXMs were defined in a previous study (7). The activity of the GXMs by ELISA increased progressively in the following order: 18, 3183, 401, 298, and 34 (Fig. 3). The observed increase in activity coincided with the increase in substitution of the GXMs with xylose at *O*-4 of mannose (7). The substitution of the chemically O-deacetylated GXMs derived from the same group of isolates gave the same relative activity data as those obtained for the O-acetylated polysaccharides (data not shown). Therefore, the *O*-acetyl groups are not part of the factor 6 determinant. Because a strong similarity exists in the structures of several of the serotype C isolates, particularly 18 and 3183, to that reported for the GXM from *C. neoformans* serotype B (23), the specificity of factor 5 for the five serotype C GXMs was examined by ELISA. The data showed that GXM of 3183 reacted with factor 5 at a relatively low serum dilution. However, the activity did not attain the value for the homologous serotype B GXM (isolate 409) even at low dilution. The level of activity decreased in the order 3183, 18, 34 (Fig. 4), as expected on the basis of their structure and the order of activity observed with factor 6 described above. The titer of the observed response in ELISA to background absorbance was determined by preincubation of the factor sera with a corresponding homologous antigen. The results of a comprehensive competitive binding study with cryptococcal antigens and related derivatives will be reported separately.

DEA. A DEA was used to determine the specificity of the factor sera against the same GXMs used in the ELISA. In the DEA, difference in color intensities was used as the basis for determining the specificities of the factor sera diluted 1:400. The observed specificity for each factor serum by DEA (Fig. 5) almost paralleled the results of ELISA titrations. The specificity of factor sera 5 and 6 for several native and O-deacetylated GXMs from select isolates of serotype B and C isolates was also examined by DEA (Fig. 6). The color intensities for the serotype C isolates with factor serum 6 decreased in the order 34, 298, 401, 3183, 18; 18 unexpectedly gave only background intensity. Factor serum 6 did not react with GXMs of serotype B. Factor serum 5 gave high-intensity dots with GXMs of serotype B but gave weak responses to native or O-deacetylated GXMs from serotype C isolates 401, 298, and 34 (Fig. 6). In the same experiment, GXMs from serotype C isolates 18 and 3183 gave dots with substantial intensity but lower intensity than those observed for the GXMs of serotype B isolates (Fig. 6). The reaction intensities for factors 5 and 6 for the corresponding native and O-deacetylated GXMs were indistinguishable when we consider the experimental limitations of the current DEA procedure (Fig. 6). These results correlate with those obtained in the ELISA studies described above.

DISCUSSION

Indirect ELISA and DEA were used to determine the specificity of cryptococcal factor sera against pure and chemically characterized GXMs from *C. neoformans* serotypes A, B, C, and D. Preliminary results showed that the priming of microtiter plates with AH-BSA, followed by the reaction with oxidized GXM (oxidized for 5 min with sodium periodate), formed an immobilized matrix that was superior to other methods and provided an ELISA of high sensitivity (5). However, the factor sera had to be diluted in PBS-T containing 2% fetal bovine serum to avoid nonspecific binding. The nonspecific binding was not observed with mouse monoclonal antibody, and the addition of fetal bovine serum was not required in our previous studies (5).

The ELISA titrations gave quantitative data that delineated the specificity of each factor serum. In general, the specificities and quantitative relationships for factor sera were in agreement with the slide agglutination specificities reported by Ikeda et al. (14) and summarized in Table 1. However, factor 7 gave a low titer with GXM of serotype A, and this value was coincident with the low cross-reactive titers obtained for the remaining three serotypes. This may be due to the masking of the serotype-specific determinant corresponding to factor 7 during antigen immobilization. Therefore, the specificity of factor 7 could not be determined by ELISA. A similar phenomenon was observed by Spiropulu et al. (20) in a study designed to characterize the activity of monoclonal antibodies specific for the GXM of *C. neoformans* by ELISA. Factor serum 1 reacted to all GXMs


a
b
c
d
e
f
Absorbance (405 nm)
Reciprocal serum dilution (1x10^{-2})
Reciprocal serum dilution (1x 10^{-2})
0.0
0.4
0.8
1.2
1.6
2.0
2.4
2
8
32
128
512
2048

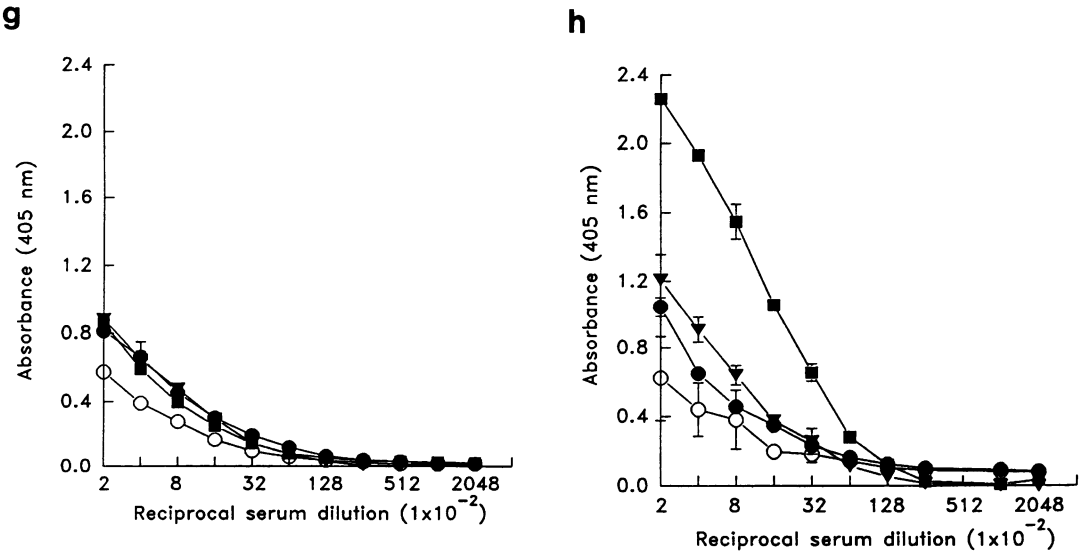


FIG. 2. ELISA titration of factor sera against GXMs of *C. neoformans* serotypes A, B, C, and D. Panels a through h are factor sera 1 through 8, respectively. Data shown were determined by subtracting the mean absorbance value from four wells that lacked antigen. ▼, serotype A, isolate CN6; ●, serotype B, isolate 409; ○, serotype C, isolate 34; ■, serotype D, isolate 9375. Each point represents a mean absorbance value of four wells ± 1 standard deviation.

of the four serotype isolates tested by ELISA. However, minor quantitative differences in activity were observed among the serotypes. This indicates that the determinant for antigenic factor 1 is shared by the other isolates. The high specific activity of factor serum 2 for GXMs of serotypes A, B, and D, but not for the GXM of serotype C, is evidence for the presence of antigenic factor 2 by serotypes A, B, and D. The high specific activity of factor 3 for GXMs of serotypes A and D is in accord with the expected specificity of this factor serum with serotypes A and D. Factor serum 4 reacted strongly with the GXM of serotype B and reacted less strongly to the GXM of serotype C. The presence of additional xylose residues linked *O*-4 to the mannopyrannan backbone of GXMs from typical serotype C isolates may be partially masking the determinant common to serotypes B and C. The results also provide evidence for the monospecificity of factor sera 5, 6, and 8 for GXMs of serotypes B, C, and D, respectively. However, quantitatively the specificities were not absolute; factor 6 showed activity with the GXM of serotype B (Fig. 2f) and factor 8 showed activity with GXMs of the other serotypes (D >> A > B > C; Fig. 2h).

The quantitative differences in the activities of the GXMs from five serotype C isolates indicated that mannopyranoside residues substituted *O*-2 and *O*-4 with xylose are essential elements of the determinant responsible for the observed activity of factor 6. GXM from isolate 18 was not reactive to factor serum 6 but reacted weakly with factor serum 5. On the basis of ^{13}C nuclear magnetic resonance spectrometry (6, 7, 22, 23), these two GXMs are identical in structure to the

TABLE 1. Factor sera to *C. neoformans*

Factor serum[a]	Antigen specificity[a,b]	Serotype specificity[a]	Relative ELISA activity[c]
1	1, 4, 6	A, B, C, D	D > A > C > B
2	2, 3, 7	A, B, D	A = B = D
3	3, 7	A, D	A = D
4	4, 6	B, C	B > C
5	5	B	B
6	6	C	C > B
7	7	A	—[d]
8	8	D	D >> A > B

[a] Data taken from reference 14.
[b] Polyclonal antibodies specific for these as yet undefined antigenic determinants are present.
[c] ELISA activity observed in this study (Fig. 2).
[d] —, expected activity was not observed (Fig. 2g).

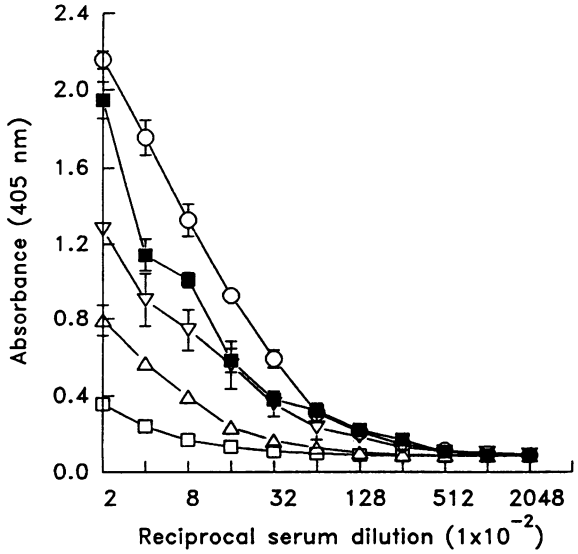


FIG. 3. Reactivity pattern of factor serum 6 against GXMs from different isolates of *C. neoformans* serotype C. ○, isolate 34; ■, isolate 298; ▽, isolate 401; △, isolate 3183; □, isolate 18. Each point represents a mean absorbance value of four wells ± 1 standard deviation.

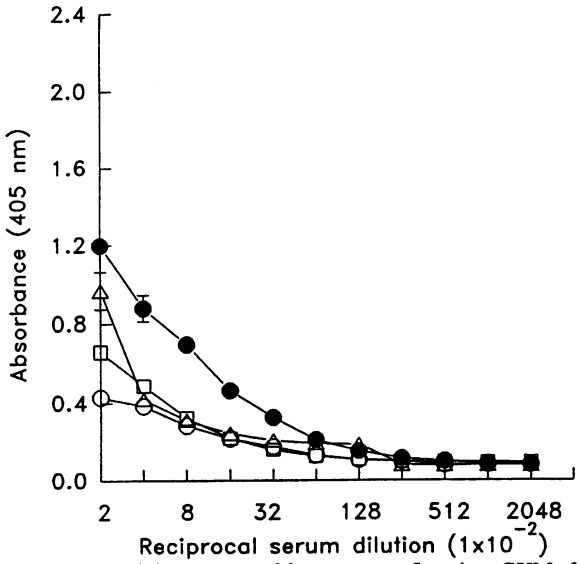


FIG. 4. Reactivity pattern of factor serum 5 against GXMs from an isolate of serotype B (isolate 409 [●]) and different isolates of *C. neoformans* serotype C. ○, isolate 34; △, isolate 3183; □, isolate 18. Each point represents a mean absorbance value of four wells ± 1 standard deviation.

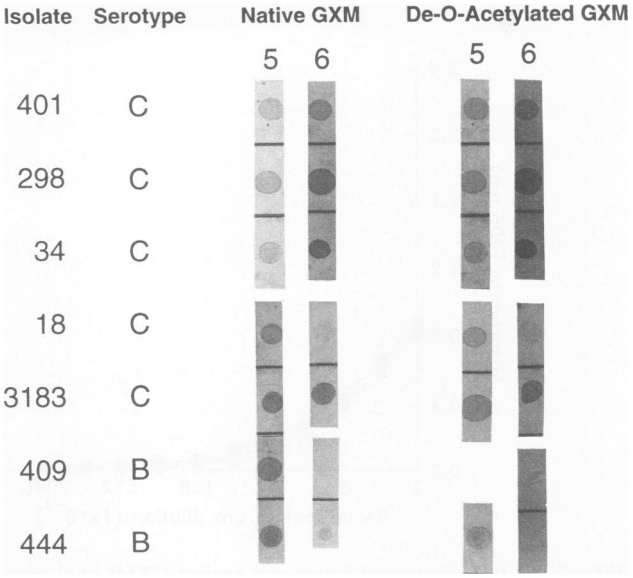


FIG. 6. Reactivity pattern of factor serum 5 or 6 against either native or de-O-acetylated GXMs of B or C isolates as evaluated with the DEA. GXMs from chemically well-studied isolates were spotted onto strips of nitrocellulose. Each assay was performed at least three times at a dilution of 1:400 of each factor serum.

GXMs from serotype B, except for their lower *O*-acetyl content. The DEA results mirrored those obtained by ELISA, the basic difference being that the ELISA gave quantitative data whereas DEA relied on visual comparisons of differences in color intensities. The activity of GXM 18 with factor 5 and its negative response to factor 6 are puzzling because it is this isolate that was used by Evans (12) to characterize serotype C. Thus, further immunochemical analysis of GXMs from serotype C isolates 18 and 3183 must be performed to resolve the discrepancy between current and past serological results. Although factor 7 did not give a satisfactory response in ELISA, it did show specificity for the GXM of serotype A in DEA.

On the basis of the ELISA and DEA results, there were no significant differences in the reactivity patterns of native and O-deacetylated GXMs of serotype B and C isolates. Therefore, O-acetylation does not influence the specific activity of factor sera 5 and 6.

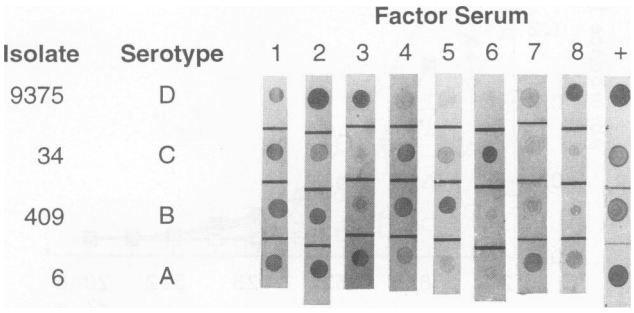


FIG. 5. Comparative specificity of cryptococcal factor sera against GXMs of *C. neoformans* serotypes as evaluated with the DEA. GXMs from chemically well-studied isolates were spotted onto strips of nitrocellulose. Each assay was performed at least three times at a dilution of 1:400 of each factor serum. +, unabsorbed rabbit antiserum.

Recently, the specificities of monoclonal antibodies have been compared with the antigenic specificities of the eight factor sera (3, 10, 11, 21). For example, three monoclonal antibodies described by Casadevall et al. (3) have serological specificities that correspond to factor sera 1, 2, and 3, and Dromer et al. have reported monoclonal antibodies with serological specificities that correspond to factor sera 2 and 7 (10). Eckert and Kozel have characterized monoclonal antibodies that have serological specificity for factor serum 1 (11).

In the present study, we demonstrated that ELISA and DEA provide reliable quantitative (ELISA) and semiquantitative (DEA) data that can be used to investigate the antigenic determinants of purified GXMs. Knowledge of the precise specificities of the factor sera will assist in defining the exact sequences and linkages comprising the eight antigenic determinants. The structures of the GXMs from within a particular serogroup can vary extensively, and this variation in structure was observed in the ELISA and DEA results presented herein with GXMs of serotype C. The excellent correlation between the structure of a particular GXM and its serological profile obtained in this study shows the importance of using *C. neoformans* isolates for which considerable chemical data are available. These results also demonstrate the utility of interdisciplinary approaches that merge serological and chemical expertise for the production of serotyping reagents and for the structural elucidation of the eight antigenic determinants of *C. neoformans*.

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