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Occurrences, Immunoglobulin Classes, and Biological Activities of Antibodies in Normal Human Serum That Are Reactive with *Cryptococcus neoformans* Glucuronoxylomannan

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Serum obtained from normal human subjects contains antibodies reactive in an enzyme-linked immunosorbent assay with the glucuronoxylomannan (GXM) of *Cryptococcus neoformans*. The frequency of occurrence of class-specific antibodies among normal subjects was 28% for immunoglobulin G (IgG), 98% for IgM, and 3% for IgA. Anti-GXM antibodies with kappa light chains occurred in 98% of normal subjects, while the occurrence of lambda light chains was 28%. Each of five subjects with high levels of anti-GXM IgG antibodies had readily detectable antibodies of the IgG2 isotype; two of the five subjects had readily detectable IgG1 antibody. An examination of sera from human immunodeficiency virus-infected patients showed that human immunodeficiency virus infection was accompanied by a significant decrease in the occurrence of IgM antibodies and anti-GXM antibodies with kappa light chains; these decreases occurred early in infection when CD4 counts were still ≥500 cells per μl. A slight but not statistically significant decrease in the occurrence of anti-GXM IgG antibodies was seen only in patients with CD4 levels of <200 cells per μl. Sera from normal subjects with high levels of anti-GXM IgG antibodies were examined to identify any contribution of the antibodies to complement activation or to opsonization of the yeast cells. An analysis of the kinetics for activation and binding of C3 to the yeast cell showed no pattern of quantitative or qualitative differences between sera with high or low levels of anti-GXM IgG antibodies. Phagocytosis studies showed that the naturally occurring IgG antibodies did not contribute to opsonization of the yeast cells.

Cryptococcus neoformans is an opportunistic yeast that is surrounded by a polysaccharide capsule. The primary structural component of the capsule is a polysaccharide, termed glucuronoxylomannan (GXM), which is composed of mannose, xylose, glucuronic acid, and *O*-acetyl groups. Anticapsular and/or anti-GXM antibodies occurring in cryptococcosis patients, raised in experimental animals or produced as monoclonal antibodies, have a variety of biological activities including complement fixation (3, 14), opsonization for phagocytosis by mouse resident peritoneal macrophages and human neutrophils (18, 25, 31), enhancement of anticryptococcal activity of human natural killer cells (27), and in vivo clearance of cryptococci via passive immunization (12, 30).

Several studies indicate that anticapsular antibody is found in the sera of normal subjects who have not been specifically immunized with *C. neoformans* antigens. Diamond et al. found that pooled sera from normal human donors is opsonic for phagocytosis of a weakly encapsulated strain of *C. neoformans* (10). The ability of the serum to opsonize the yeast was diminished if the serum was adsorbed at 0°C with encapsulated cryptococci. The opsonic activity was restored by the addition of purified human immunoglobulin G (IgG) to the adsorbed serum, suggesting the presence of anticryptococcal antibody in normal serum. Direct evidence for production of antibody to cryptococcal polysaccharide by normal subjects was provided by Henderson et al. (15). Results from a radioimmunoassay found that 89% of normal volunteers have detectable levels of antibody to serotype A polysaccharide. Finally, Dromer et al. studied anticapsular antibodies in normal subjects and in patients with AIDS-related complex or AIDS without a known history of cryptococcosis (11). Serum samples from most normal subjects contained anticapsular IgM (60%), and a lesser number (27%) contained anticapsular IgG. Anticapsular antibody occurred less frequently in patients with AIDS.

Some cryptococcosis patients develop anticryptococcal antibody during a cryptococcal infection. Diamond and Bennett found that approximately 40% of 81 cryptococcosis patients studied in the pre-AIDS era had detectable levels of antibody at the start of therapy (9). At least two studies showed that antibodies in sera from patients with cryptococcosis are predominantly of the IgG class (4, 32). In contrast, antibody produced in response to immunization of volunteers with cryptococcal polysaccharide is predominantly IgM (15).

The objectives of our study were (i) to characterize in greater detail the frequency and immunoglobulin class of anti-GXM antibodies among a group of normal adult volunteers, (ii) to determine the subclass of IgG antibodies found in the sera of normal volunteers, (iii) to confirm previous observations that the progression from human immunodeficiency virus (HIV) infection to AIDS is accompanied by a quantitative or qualitative effect on production of anti-GXM antibodies, (iv) to determine whether the presence of naturally occurring anti-GXM IgG antibody influences the ability of the serum to activate and bind C3 to encapsulated cryptococci, and (v) to determine if the presence of naturally occurring anti-GXM IgG enhances the opsonic activity of normal human serum. Our results showed that many normal subjects have high levels of anti-GXM IgG and essentially all subjects have readily detectable anti-GXM IgM. The anticapsular IgG is primarily of the IgG2 subclass. Finally, the presence of anti-

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GXM IgG in sera from normal donors did not affect the ability of the sera to support activation and binding of C3 to the yeast cells and had no effect on the opsonic activity of the sera.

MATERIALS AND METHODS

Sera. Serum samples were obtained from 40 normal adult volunteers after their informed consent was obtained. The normal volunteers were healthy medical students, graduate students, and laboratory personnel. Serum was also obtained from 40 HIV-positive subjects who were patients at the Early Intervention Clinic of the Washoe County District Health Department. Patients were categorized on the basis of CD4 levels of ≥500, 200 to 499, and <200 cells per μl.

Cryptococcal cells and GXM. *C. neoformans* 388 was used for all complement activation, immunoadsorption, and phagocytosis experiments. Strain 388 is a serotype A isolate with a capsule diameter of approximately 3.4 μm. Cryptococcal cells were grown in a liquid synthetic medium (8) on a gyratory shaker at 100 rpm for 72 h at 30°C. The yeast cells were killed with 1.0% formaldehyde and stored as a sterile suspension in PBS (phosphate [0.01 M]-buffered saline [127 mM; pH 7.3]). Yeast cells used for phagocytosis experiments were stained with rhodamine B isothiocyanate (RITC) to facilitate accurate identification of yeast cells in phagocytosis assays (25). Yeast cells were stained with RITC by incubation of 10^8 yeast cells for 30 min at room temperature with RITC solution (10 μg of RITC per ml of 20 mM borate [pH 9.0]). RITC-stained cells were washed 10 times with PBS before being used.

GXM was isolated by a modification of the procedure described by Cherniak et al. (7). Briefly, *C. neoformans* serotype A strain 24064 (American Type Culture Collection, Rockville, Md.) was grown in a liquid synthetic medium (8) on a gyratory shaker at 100 rpm for 4 days at 30°C. The yeast cells were killed by the addition of formaldehyde to a final concentration of 1%. Sodium acetate crystals and glacial acetic acid were added to final concentrations of 10 and 1%, respectively, and the yeast cells were removed by centrifugation at 10,000 × *g*. The polysaccharide was precipitated from the supernatant fluid by the addition of 2.5 volumes of 95% ethanol, and the precipitate was collected by centrifugation. The polysaccharide precipitate was dissolved at a concentration of approximately 5 mg/ml in 0.2 M NaCl, and the solution was clarified by centrifugation. Hexadecyltrimethylammonium bromide (CTAB) was added (3 mg of CTAB per mg of polysaccharide), and 2 volumes of 0.05% CTAB was added slowly with stirring. The GXM-CTAB complex was collected by centrifugation and redissolved at approximately 5 mg/ml of 0.2 M NaCl. The polysaccharide was reprecipitated with ethanol and CTAB as described above. Finally, the polysaccharide was precipitated with ethanol, dialyzed against deionized water, and lyophilized.

GXM was covalently linked to tyramine by using several modifications of the benzoquinone activation procedure (5, 19). The GXM was dissolved in deionized water (10 mg/ml) and mixed with an equal volume of 0.2 M sodium bicarbonate (pH 8.5). Benzoquinone (0.25 volume; 250 mM in absolute ethanol) was added to produce a final concentration of 50 mM benzoquinone in 20% ethanol and incubated for 60 min at room temperature. It is important that the pH not be above 8.5 or the incubation time extend beyond 60 min because the *O*-acetyl groups on the polysaccharide may be lost through alkaline hydrolysis (19). Sodium acetate crystals (10%, wt/vol) and glacial acetic acid (1%, vol/vol) were added, and the GXM was precipitated by the addition of 2 volumes of 95% ethanol. The precipitate was collected by centrifugation, dissolved in acetate buffer (10% sodium acetate crystals, 1% glacial acetic acid) at a concentration of approximately 2 mg of GXM per ml and reprecipitated by the addition of 2 volumes of ethanol. The precipitate was collected by centrifugation and dissolved at a concentration of approximately 4 mg of GXM per ml in 0.1 M sodium phosphate (pH 7.5) containing 0.5 M NaCl. The GXM was dialyzed against the same buffer and against 0.1 M sodium phosphate (pH 7.5).

Benzoquinone-activated GXM was coupled to tyramine by mixing the activated polysaccharide (approximately 4 mg/ml) with an equal volume of tyramine hydrochloride (1 mg/ml in 0.1 M sodium phosphate [pH 7.5]) and incubated overnight at room temperature. Sodium acetate crystals and glacial acetic acid were added as described above, and the tyramine-coupled GXM was precipitated by the addition of 2 volumes of 95% ethanol. The precipitate was collected by centrifugation, dissolved at a concentration of approximately 4 mg/ml in 0.1 M sodium phosphate (pH 7.5) containing 0.5 M NaCl, and dialyzed sequentially against 0.1 M sodium phosphate (pH 7.5) containing 0.5 M NaCl and 0.1 M sodium phosphate (pH 7.5). The GXM concentration was determined by the phenol-sulfuric acid assay (13), and the tyramine-coupled GXM was diluted to 2 mg/ml, sterilized by filtration, and stored at 4°C.

Enzyme-linked immunosorbent assays (ELISAs) for anti-cryptococcal antibody. Wells of Immulon I microtiter plates (Dynatech Laboratories, Inc., Chantilly, Va.) were coated overnight at 4°C with 100 ng of tyraminated GXM in 100 μl of coating buffer (0.05 M sodium phosphate [pH 7.4] containing 10 mM EDTA). The wells were washed three times with blocking buffer (0.05 M sodium phosphate [pH 7.4]) and blocked for 90 min with 300 μl of 0.05% Tween 20 in blocking buffer.

Plates coated with tyraminated GXM were washed three times with PBS containing 0.05% Tween 20 (PBS-Tween) and incubated for 90 min with serial twofold dilutions of human serum in PBS-Tween, beginning with a 1:5 dilution. As a negative control, all sera were diluted in PBS-Tween containing 4 μg of GXM per ml and incubated for 30 min prior to addition to wells coated with tyraminated GXM (GXM-neutralized serum). After incubation with serum or GXM-neutralized serum, the wells were washed three times with PBS-Tween and incubated for 90 min at room temperature with horseradish peroxidase (HRP)-labeled secondary antibody. The secondary antibodies were HRP-labeled, affinity-purified goat anti-human IgG heavy chains (1/16,000; Southern Biotechnology; catalog no. 2040-05), HRP-labeled, affinity-purified goat anti-human IgM heavy chains (1/15,000; Southern Biotechnology; catalog no. 2020-05), HRP-labeled, affinity-purified goat anti-human IgA heavy chains (1/12,000; Southern Biotechnology, catalog no. 2050-05), HRP-labeled, affinity-purified goat anti-human kappa chains (1/10,000; Southern Biotechnology; catalog no. 2060-05), or HRP-labeled affinity-purified anti-human lambda chains (1/5,000; Southern Biotechnology; catalog no. 2070-05). The secondary antibody was used at eight times the dilution of antibody producing an optical density at 450 nm (OD_{450}) of 2.5 with wells coated with purified IgG (anti-IgG, anti-kappa, or anti-lambda), IgM (anti-IgM), or IgA (anti-IgA). The plates were washed three times with PBS-Tween and incubated for 30 min with 100 μl of TMB Microwell peroxidase substrate solution (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.). The reaction was stopped by the addition of 100 μl of 1 M H_3PO_4. The A_{450} was read within 30 min with a Ceres 900 enzyme immunosorbent assay workstation (Bio-Tek Instruments, Inc., Winooski, Vt.).

All ELISAs, including the GXM-neutralized control, were done in duplicate. Averages were calculated from duplicate wells, and the specific OD_{450} was calculated by subtracting the

OD_{450} obtained from the GXM-neutralized serum from the OD_{450} obtained when sera were not preincubated with GXM. The data were analyzed with Kineticalc EIA Application Software version 2.12 (Bio-Tek Instruments) in a titer mode. The best fit for the data was determined with the four-parameter algorithm, and the titer threshold was set at 0.5. Under these conditions, the serum dilution producing an OD_{450}, corrected for background (GXM neutralized), of 0.5 was reported as the end point. Sera with an OD_{450} of less than 0.5 at a serum dilution of 1/5 were reported as negative. When a murine monoclonal IgG1 antibody was used as a standard, approximately 500 pg of antibody produced an OD_{450} of 0.5.

The ELISA described above was modified to determine the IgG subclass of anti-GXM IgG antibody. In this modified assay, plates were coated with GXM and incubated with serum dilutions or serum diluted in PBS-Tween containing GXM as described above. The wells were washed and incubated for 90 min at room temperature with optimally diluted murine monoclonal antibodies specific for human IgG1 (1/2,000; Calbiochem, La Jolla, Calif.; catalog no. 411451), IgG2 (1/8,000; Calbiochem; catalog no. 411461), IgG3 (1/40,000; Calbiochem; catalog no. 411481), or IgG4 (1/1,500; Calbiochem; catalog no. 411492). The plates were then washed with PBS-Tween, incubated with 100 μl of peroxidase-labeled goat anti-mouse IgG (1/4,500; Southern Biotechnology; catalog no. 1030-05), washed, and incubated with substrate as described above. The results are reported as the serum dilution, corrected for background (GXM neutralized), that produced an OD_{450} of 0.5.

Activation and binding of C3 to encapsulated cryptococci. The kinetics for activation and binding of C3 to encapsulated cryptococci were determined exactly as described previously (24) by using serum containing ^{125}I-labeled C3 (20). The key variable was the serum source; i.e., serum was selected on the basis of the presence or absence of anti-GXM antibody of the IgG class. All sera were diluted to 40% before use. Data are reported as the number of C3 molecules bound to the average yeast cell after 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 25, or 30 min of incubation.

Opsonization and phagocytosis of encapsulated cryptococci. Assays used to determine the opsonic activity of sera were based on procedures described by Levitz and Tabuni (26). Mononuclear cells were isolated from heparinized blood by centrifugation over Ficoll-Hypaque. Mononuclear cells (5×10^4) in Iscove's modified Dulbecco's medium (GIBCO-BRL, Grand Island, N.Y.) containing 10% homologous donor serum were added to each well of a 96-well tissue culture plate. The plates were incubated for 24 h at 37°C in 6% CO_2. Nonadherent cells were removed by gentle washing, the medium was replaced, and the cells were cultured for 14 to 21 days, during which time the monocytes matured to human monocyte-derived macrophages (MO-Mϕ).

Yeast cells were opsonized by preincubation of 2.5×10^6 cells with 1 ml of 40% human serum in Hanks balanced salt solution (HBSS) for 30 min at 37°C. The yeast cells were washed three times with HBSS and resuspended in 1 ml of HBSS. For phagocytosis experiments, tissue culture wells containing MO-Mϕ were washed two times with HBSS, and 200 μl of preopsonized yeast cells (5×10^5 yeast cells per well) was added. Opsonized yeast cells were incubated with MO-Mϕ for 30 min at 37°C. The wells were washed two times with PBS to remove nonadherent yeast cells, and the cells were stained with Uvitex and fixed with formaldehyde as described previously (26). Binding and phagocytosis were assessed by examination with a Leitz inverted microscope at ×400 with epifluorescence provided by a wide-band UV Leitz A filter block for use with Uvitex and by a fluorescence filter set prepared by Omega Optical, Inc. (Brattleboro, Vt.), for use with Texas red. Macrophages (200 per well) were randomly selected under bright-field illumination, and the numbers of bound but not internalized yeast cells (showing staining with both Uvitex and RITC) and internalized yeast cells (showing staining with RITC but not Uvitex) were determined. Data are reported as an attachment index (mean number of bound but not internalized yeast cells per MO-Mϕ) and as a phagocytic index (mean number of internalized yeast cells per MO-Mϕ). Four replicate wells were used for each experimental group. Both attachment and ingestion data passed tests for normality and equality of variance. As a consequence, data were analyzed by using analysis of variance (SigmaStat; Jandel Scientific, San Rafael, Calif.).

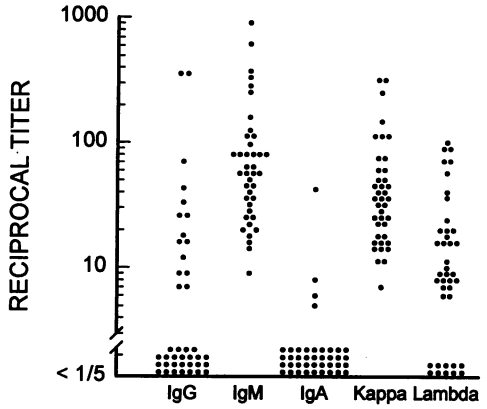

FIG. 1. Anti-GXM antibodies in serum samples from 40 normal adult donors. Each point represents an individual serum sample assayed for antibodies with IgG, IgM, or IgA heavy chains or kappa or lambda light chains.

RESULTS

Occurrence and immunoglobulin class of anti-GXM antibodies. Serum samples from 40 normal adults were examined to determine the levels of antibodies of the IgG, IgM, and IgA classes. The sera were also examined to assess the occurrence and levels of antibodies with kappa or lambda light chains. The results (Fig. 1) showed that 28% of the sera had IgG antibodies with titers of ≥1/10, 98% had IgM antibodies with titers of ≥1/10, and only one serum sample had IgA antibodies with a titer of≥1/10. Ninety-eight percent had antibodies with kappa chains with titers of ≥1/10, and 45% had antibodies with lambda chains with titers of ≥1/10. Data shown in Fig. 1 were analyzed by use of the Pearson product moment correlation in an effort to determine whether there was a correlation between the presence of high IgM titers and the presence of high titers of IgG or IgA. Data from all subjects were analyzed. In a second analysis, only subjects with IgG titers of ≥1/10 were considered. In all cases, there was no correlation ($P > 0.10$) between a high titer of anti-GXM antibodies of the IgG, IgM, or IgA classes and a high titer of one of the other immunoglobulin classes.

Five serum samples having IgG antibody titers of ≥1/50 were examined further to determine the IgG subclass of the anti-GXM IgG. The results (Fig. 2) showed that each of the serum samples contained detectable levels of anti-GXM antibody of the IgG2 subclass. Two of the serum samples also contained detectable levels of IgG1 antibody. None of the

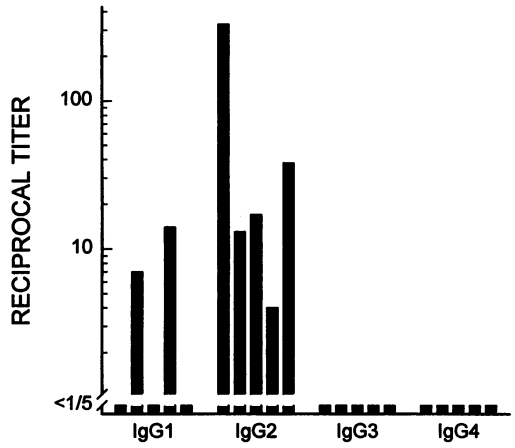

FIG. 2. Assay of normal human serum for anti-GXM antibodies of the IgG1, IgG2, IgG3, and IgG4 subclasses. Sera chosen for analysis had anti-GXM IgG antibody titers of ≥1/50.

serum samples contained detectable levels of anti-GXM antibody of the IgG3 or IgG4 subclass.

Occurrence and characteristics of anti-GXM antibodies in sera from HIV-positive donors. Dromer et al. reported a significant decrease in occurrence of anti-GXM IgG antibodies in patients with AIDS (11). As a consequence, we evaluated the frequency of occurrence and levels of anti-GXM antibodies with various heavy- and light-chain isotypes in sera of patients who were (i) HIV positive with CD4 counts of ≥500 cells per μl, (ii) HIV positive with CD4 counts of 200 to 499 cells per μl, and (iii) HIV positive with CD4 counts of <200 cells per μl. Positive titers were defined as antibody titers of ≥1/10. The results from an analysis of sera from HIV-positive donors are shown in Fig. 3. A comparison of the frequencies of positive titers among normal donors (Fig. 1) and HIV-positive donors (Fig. 3) is presented in Table 1. The results show that there is a trend toward less frequent occurrence of anti-GXM IgG in patients with CD4 levels below 200 cells per μl; however, the difference could not be confirmed by statistical means. Anti-GXM of the IgM class occurred significantly more frequently among normal subjects than among HIV-positive donors, regardless of CD4 levels. Similarly, anti-GXM antibodies with kappa light chains occurred significantly less frequently among HIV-positive donors than among normal subjects. It is possible that other differences might have been noted if actual antibody levels had been compared rather than the frequencies of subjects with titers of ≥1/10. As a consequence, individual titers in the various subject groups were compared by the Mann-Whitney rank sum test. With one exception, significant differences as determined by this latter test were exactly the same as those found when the data were evaluated in terms of the frequency of positive titers. The exception was a significant ($P = 0.01$) depression (relative to normal subjects) in the titers of antibodies with lambda light chains found in serum samples from HIV-positive patients with CD4 levels below 200 cells per μl.

Kinetics for activation and binding of C3 to encapsulated cryptococci. Previous studies showed that antibody plays no role in the activation and binding of C3 from pooled human sera onto the cryptococcal capsule (22, 23). In contrast, sera from normal donors contain anti-cell wall antibodies that markedly influence the kinetics for activation and binding of C3 to nonencapsulated cryptococci (33). Specifically, C3 binding initiated by the alternative pathway alone is characterized by a lag of 5 to 15 min before appreciable levels of C3 are bound to the yeast cells. Antibody-dependent activation of the classical pathway is characterized by the absence of a lag in initiation.

Pools of human sera used in previous studies were prepared without regard to the levels of anti-GXM IgG in sera from individual donors. The possibility remains that sera from donors selected for high levels of anti-GXM IgG might exhibit activation and binding kinetics characteristic of the classical pathway, e.g., an absence of a lag in initiation. As a consequence, we compared the kinetics for activation and binding of C3 from normal serum that had high levels of anti-GXM IgG (titers, ≥1/200) with the kinetics observed with sera that lacked anti-GXM IgG. The results (Fig. 4) showed that individual serum samples exhibited relatively minor differences (approximately twofold) in the maximum amount of C3 deposited onto the yeast cells. These differences were unrelated to the presence of anti-GXM antibody. There was no pattern of differences in the lag phase of the binding curve. The two serum samples with high levels of anti-GXM IgG required 4.0 and 5.7 min to deposit 5% of the maximum observable C3 onto the yeast cells. The two serum samples that lacked anti-GXM antibody required 3.5 and 5.3 min to deposit 5% of the maximum observable C3 onto the yeast cells. These results

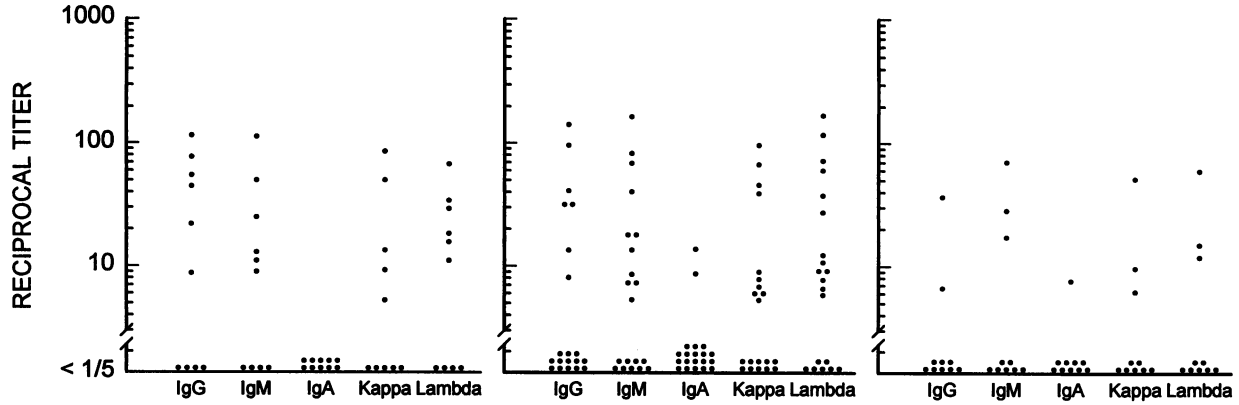

FIG. 3. Occurrences and characteristics of anti-GXM antibodies in sera obtained at various stages in the progression from HIV infection to AIDS. Serum samples were obtained from 10 subjects who were HIV positive with CD4 counts of ≥500 cells per μl (left), 20 subjects with CD4 counts of 200 to 499 cells per μl (center), and 10 subjects with CD4 counts of <200 cells per μl (right).

TABLE 1. Occurrence of anti-GXM antibodies of various immunoglobulin classes in sera obtained from normal donors or HIV-positive individuals with differing CD4 levels

Antibody class	Occurrence of anti-GXM antibodies in:						
	Normal sera	HIV-positive sera					
		CD4 ≥ 500 cells/μl		CD4 = 200–499 cells/μl		CD4 < 200 cells/μl	
	No. positive[a]/total	No. positive/total	*P* vs normal[b]	No. positive/total	*P* vs normal	No. positive/total	*P* vs normal
IgG	11/40	5/10	=0.27	6/20	>0.99	1/10	=0.42
IgM	39/40	5/10	<0.001	7/20	<0.001	4/10	<0.001
IgA	1/40	0/10	>0.99	1/20	>0.99	0/10	>0.99
Kappa	39/40	3/10	<0.001	2/20	<0.001	1/10	<0.001
Lambda	19/40	6/10	=0.51	8/20	=0.78	5/10	>0.99

[a] Serum samples with class-specific antibody titers of ≥1/10 were classified as positive.
[b] Statistical analysis by the Fisher exact test.

showed that the presence of anti-GXM IgG in sera obtained from normal, nonimmunized adult donors had no appreciable effect on the kinetics for activation and binding of C3 to encapsulated cryptococci.

Opsonic activity. Incubation of encapsulated cryptococci in human serum leads to the deposition of at least 10^7 fragments of C3 onto the capsule (21). These fragments are largely in the form of iC3b (20), and the fragments opsonize the yeast cells to facilitate phagocytosis by macrophages and other phagocytic cells (26). IgG is also a potent opsonin, often exhibiting synergy with opsonic fragments of C3. As a consequence, we compared the opsonic activity of sera with anti-GXM IgG antibodies having titers of ≥1/200 with the opsonic activity of sera that lacked detectable levels of anti-GXM IgG. The sera used in this experiment were the same sera used for the experiments shown in Fig. 4. Thus, there is clear evidence that the sera fully support activation and binding of C3 fragments onto the yeast cells. As a further means to assess the importance of naturally occurring anti-GXM antibody as an opsonin, the antibody was blocked by incorporation of GXM (10 μg/ml of 40% serum) into the serum used for opsonization (Table 2, experiment 1) or the antibody was removed by adsorption for 60 min at 0°C with whole encapsulated cryptococci (10^8 cells per ml of 40% serum) (Table 2, experiment 2). Previous studies have established that adsorption under these conditions has no effect on the kinetics for activation and binding of C3 fragments to the yeast cells, indicating that the alternative pathway is fully functional in the adsorbed serum (33).

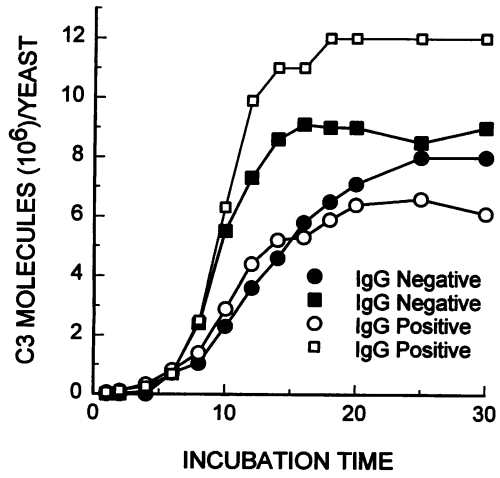

FIG. 4. Kinetics for activation and binding of C3 fragments to encapsulated cryptococci. Sera which either lacked anti-GXM IgG antibody or had anti-GXM IgG antibody titers of ≥1/200 were examined.

The results (Table 2) show that encapsulated cryptococci opsonized with normal human serum, regardless of the antibody status of the donor or the treatment of the serum, attached to MO-Mϕ at a relatively high level (2 to 6 yeast cells per MO-Mϕ), but there was very little ingestion (<0.1 yeast cell per MO-Mϕ). Encapsulated cells opsonized with serum containing high levels of anti-GXM IgG did not exhibit a consistent pattern of attachment or ingestion that was significantly ($P > 0.05$) greater than that of yeast cells opsonized with human serum that lacked detectable anti-GXM IgG. Moreover, neutralization of antibody by the addition of GXM or removal of antibody by adsorption had no significant ($P > 0.05$) effect on the ability of the sera to facilitate either attachment or ingestion of encapsulated cryptococci.

TABLE 2. Opsonization of encapsulated cryptococci by normal human serum containing high or low levels of anti-GXM IgG

Serum donor	Antibody titer (IgG)	Serum treatment	Attachment index[a]	Ingestion index[a]
Expt 1				
1	1/340	None	6.0 ± 0.2	0.05 ± 0.03
	1/340	GXM neutralized	6.3 ± 1.0	0.11 ± 0.04
2	1/370	None	7.5 ± 1.7	0.10 ± 0.02
	1/370	GXM neutralized	7.6 ± 1.3	0.07 ± 0.03
3	<1/5	None	5.5 ± 1.1	0.06 ± 0.04
	<1/5	GXM neutralized	7.2 ± 1.1	0.08 ± 0.07
4	<1/5	None	6.2 ± 1.7	0.01 ± 0.02
	<1/5	GXM neutralized	4.5 ± 2.1	0.03 ± 0.03
Expt 2				
1	1/340	None	3.3 ± 1.5	0.01 ± 0.01
	1/340	*C. neoformans* adsorbed	4.7 ± 2.0	0.02 ± 0.02
2	1/370	None	5.2 ± 1.4	0.05 ± 0.07
	1/370	*C. neoformans* adsorbed	4.8 ± 1.9	0.04 ± 0.02
3	<1/5	None	2.8 ± 0.8	0.01 ± 0.02
	<1/5	*C. neoformans* adsorbed	2.8 ± 1.7	0.01 ± 0.01
4	<1/5	None	2.9 ± 0.6	0
	<1/5	*C. neoformans* adsorbed	1.9 ± 0.8	0.01 ± 0.01

[a] Values are means ± standard deviations. For an explanation of the indices, see the text.

DISCUSSION

Our studies largely agree with previous studies, and they also provide new information regarding anti-GXM antibodies which occur in serum samples obtained from normal adult donors. Using a radioimmunoassay with tyraminated polysaccharide as an antigen, Henderson et al. found that 89% of serum samples obtained from normal volunteers demonstrated binding of cryptococcal polysaccharide (15). Polysaccharide used in the radioimmunoassay was not prepared in a manner that excludes cryptococcal galactoxylomannan or mannoprotein from the antigen. As a consequence, the radioimmunoassay may have also measured antibodies against these antigens. Nevertheless, the frequency of occurrence of anticryptococcal antibody was similar to our finding of antibody in serum samples from 98% of normal donors. The higher frequency in our study is likely due to the greater sensitivity of enzyme immunosorbent assays. Detection of antibody in serum samples from 98% of normal donors is considerably higher than the 69% frequency reported by Dromer et al. (11). Studies by Dromer et al. used an ELISA which differed in two important respects from the assay used in our study. First, microtiter wells were coated with untreated GXM. The strength of binding of serotype A GXM to microtiter plates is not known. It is notable that GXM of serotypes B and C bind poorly, if at all (6, 17a). Current studies in our laboratory have found that tyraminated GXM binds to standard microtiter plates with a remarkable tenacity. The second difference is the criterion used to classify sera as positive. Sera in the Dromer study were reported as positive if a 1/2 dilution of serum produced an absorbance that was two times the absorbance observed with buffer-coated wells. This rigorous criterion for classification of serum as positive may have introduced a bias against sera with high background absorbances, particularly at serum dilutions of 1/2.

Our finding of anti-GXM IgG in 28% of normal donors is in agreement with the report of IgG antibody in 27% of normal donors in the Dromer study. Examination of serum from five of the IgG-positive donors found that each donor had high levels of anti-GXM antibodies of the IgG2 subclass. In addition, two of the donors had readily detectable anti-GXM antibodies of the IgG1 subclass. Numerous previous studies have found that IgG antibodies directed against bacterial capsular polysaccharides are primarily of the IgG2 subclass. The occurrence of anti-GXM antibodies of the IgM class in 98% of normal donors is in good agreement with the report by Henderson et al. that 9 of 10 normal volunteers had anticryptococcal IgM antibodies that were readily detectable by ELISA (15). These results are higher than those of a study by Dromer et al. in which anticryptococcal IgM antibodies occurred in serum samples from 61% of a group of normal donors (11). Once again, this difference may be due to the manner in which positive sera were identified.

Studies of HIV-infected patients suggest that portions of the synthesis of antibodies reactive with GXM are lost through HIV infection and the progression to AIDS. Although not statistically significant, there was a trend toward a lower frequency of anti-GXM IgG when CD4 levels fell below 200 cells per μl. Anti-GXM IgG was found in 28% of the normal subjects and 10% of the AIDS group. A similar reduction was reported by Dromer et al. We found a striking reduction in the occurrence of anti-GXM antibodies of the IgM class in all categories of HIV-positive patients. A similar reduction was noted in the occurrence of antibodies with kappa light chains. In contrast, there was no reduction in the percentage of HIV-positive patients producing antibodies with lambda light chains. Decreased responses to type 2 T-independent antigens have been found to occur relatively early in the course of HIV infection (1, 17). Studies of the immune response to pneumococcal vaccines found impaired IgG2, IgM, and IgA responses in HIV-positive patients with persistent generalized lymphadenopathy; there was no loss of the IgG1 response (1). It is possible that the decrease in anti-GXM IgG antibodies is due to a selective loss of IgG2. This would be consistent with the occurrence of IgG1 in two of five normal serum samples that were examined. To our knowledge, a selective loss of the kappa light chain response in HIV-infected patients has not been reported previously.

Our study examined two possible functions of anti-GXM IgG that could be of importance in resistance to cryptococcosis, i.e., initiation of the classical complement pathway and Fc-mediated opsonization of encapsulated cryptococci. Previous studies from our laboratory showed that all normal subjects have relatively high levels of IgG antibodies directed against cryptococcal cell wall glucan (16). When nonencapsulated cryptococci are incubated in normal human serum, these anti-cell wall antibodies initiate the classical complement pathway, leading to deposition of C3 fragments at the cell wall (22, 33). This classical pathway initiation is characterized by an immediate accumulation of C3 fragments on the yeast cells. Incubation of encapsulated cryptococci in normal human serum leads to activation of the alternative pathway. Alternative pathway initiation is characterized by a 4- to 7-min lag before appreciable amounts of C3 can be detected on the yeast cells. The results in Fig. 4 show that the anticapsular IgG found in sera from normal donors with high levels of antibody is unable to alter the lag that characterizes alternative pathway initiation by encapsulated cryptococci. The amount of anti-GXM antibody needed to initiate the classical pathway is not known, but it is likely that levels of naturally occurring anti-GXM antibodies are not sufficient to significantly alter what is normally an alternative pathway process.

Despite the fact that naturally occurring anti-GXM IgG does not initiate the classical pathway, the possibility remained that this antibody could facilitate phagocytosis via direct interaction with phagocyte Fc receptors. In particular, this antibody could act synergistically with C3 fragments that were deposited via the action of the alternative pathway (28). This question was addressed in several ways. First, the opsonic activities of normal sera with high and low levels of anti-GXM IgG were compared. Second, anti-GXM antibody was blocked by incorporation of purified GXM into the buffer system. Finally, anti-GXM antibody was removed by adsorption at 0°C with encapsulated cryptococci. Results from all three approaches indicated no contribution to phagocytosis of encapsulated cryptococci by naturally occurring IgG. The failure to achieve antibody-mediated opsonization is most likely due to the concentration of anti-GXM IgG in the serum. If it is assumed that IgG concentrations of 500 pg of antibody per ml produce an OD_{450} of 0.5, then the two serum samples that were examined (ELISA titers, ~1/350) contained approximately 0.2 μg of anti-GXM IgG per ml. Previous studies from our laboratory have shown that at least 3 μg of murine monoclonal anti-GXM IgG per ml is required for minimal levels of opsonization and that appreciable opsonization requires approximately 30 μg of antibody per ml. Such calculations should be interpreted with caution because there is no certainty that human polyclonal and murine monoclonal antibodies will produce comparable dose-dependent effects. Nevertheless, the results of such calculations are consistent with the observed results. It is notable that approximately 2 μg of

human antibody per ml was required for opsonization of group B streptococci in a similar model (2).

Finally, the antigenic stimulus for the anti-GXM antibodies in normal human serum is unknown. The antibodies could be due to stimulation by cross-reactive antigens that are encountered in the environment. Natural antibodies reactive with several bacterial capsules are believed to be due to exposure to cross-reacting antigens of nonpathogenic enteric and nasopharyngeal organisms (29). Glucuronic acid and *O*-acetyl groups are common components of many polysaccharides. Thus, it is possible that the ubiquitous anti-GXM IgM antibody is reactive with one of these components. On the other hand, xylose is not characteristically found in bacterial capsules, although it is common among plants. A second explanation is the possibility that the anti-GXM antibody, particularly the IgG antibody, represents exposure to cryptococci either in nature or as a subclinical infection. This interpretation is consistent with our observation that high levels of anti-GXM antibodies of the IgG class are independent of the occurrence of high levels of IgM antibodies. If this latter explanation proves to be true, it raises the possibility that anti-GXM IgG could be a marker for latent cryptococcal infection and increased risk for cryptococcosis in HIV-infected or other immunocompromised patients.

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