INFECTION AND IMMUNITY, Oct. 1992, p. 4080–4087
0019-9567/92/104080-08$02.00/0

Vol. 60, No. 10

The Glucuronoxylomannan of *Cryptococcus neoformans* Serotype A Is a Type 2 T-Cell-Independent Antigen

J. B. SUNDSTROM† AND R. CHERNIAK*

Laboratory for Biological and Chemical Sciences, Department of Chemistry, Georgia State University, Atlanta, Georgia 30303

Received 28 April 1992/Accepted 8 July 1992

The humoral immune response of inbred mice to immunization with the glucuronoxylomannan (GXM) of *Cryptococcus neoformans* was investigated both serologically and in plaque-forming cells (PFCs). The T-helper-cell-independent quality of the GXM was demonstrated by using BALB/c *nu/nu* mice. Primary and secondary dose responses to three antigenic forms of GXM, (i) the native antigen, (ii) a GXM-bovine serum albumin protein conjugate, and (iii) a cryptococcal whole-cell vaccine, revealed a lack of isotype class switching and anamnestic responses. Both the levels of complement-fixing anti-GXM antibody in serum and the PFC responses in the athymic mice showed no significant differences from those in the wild-type controls. However, T cells are involved in the suppression of the primary response to GXM. When BALB/cBy mice were given rabbit anti-mouse thymocyte serum along with 0.5 μg of GXM, both antibody levels in serum and PFC responses were significantly increased over those of control mice that received GXM and normal rabbit serum. In addition, T cells were also shown to enhance the primary immune response to GXM. BALB/cBy mice were given GXM and anti-mouse thymocyte serum on day 1. On day 2, the experimental group was given anti-mouse thymocyte serum and the control group was given saline. On day 5, comparison of the PFC responses and anti-GXM antibody titers of the two groups revealed a significant increase in the immune response of the control over the experimental group. The type 2 T-cell-independent quality of GXM was also demonstrated in CBA/cHN *xid* mice. These mice lack the Lyb$^+$ subset of B cells and are unable to respond to type 2 T-independent antigens but respond normally to type 1 T-independent antigens. Type III pneumococcal polysaccharide, a type 2 T-independent antigen, was used as a negative control, and trinitrophenyl-lipopolysaccharide, a type 1 T-independent antigen, was used as a positive control. The CBA/cHN *xid* mice failed to respond to either type III pneumococcal polysaccharide or GXM but did respond to immunization with trinitrophenyl-lipopolysaccharide. BALB/cBy mice responded normally to all three antigens.

Cryptococcus neoformans is an opportunistic pathogen that can cause a fatal systemic mycosis in immunocompromised hosts. Among persons with AIDS, cryptococcosis has been reported to have a prevalence of 10% and to be the fourth leading cause of death (49). The main virulence factor of the yeast is its polysaccharide capsule (CnCAP) (29), which is tolerogenic and antiphagocytic (30, 35). CnCAP has also been described as a T-independent (T_{IND}) antigen with type 2 (T-2) characteristics (34). CnCAP is composed of at least three serologically distinct antigens: a major antigen, glucuronoxylomannan (GXM) (47), and two minor antigens, a galactoxylomannan complex (25) and a mannoprotein (48). Although all three antigens have been fractionated and studied independently, most immunologic studies have been performed with preparations consisting of a heterogeneous mixture of these subcomponents in undefined proportions. Therefore, the relative contributions of these three antigens to the immune response to the capsular polysaccharide of *C. neoformans* remain unclear.

To characterize the humoral response of BALB/c mice to the major antigen of CnCAP, this investigation examined the T-2 T_{IND} qualities of GXM. T_{IND} antigens are defined by their ability to induce a humoral response in the absence of T-cell help and by their inability to induce B-cell memory or isotype class switching in secondary immune responses. T-2 T_{IND} antigens are distinguished by their inability to induce humoral responses in CBA/N *xid* mice (33) and by their ability to recruit regulatory T cells that both suppress (T_S cells) and amplify (T_{AMP} cells) the specific B-cell response (9).

To assess the T independence of GXM, we used splenic plaque-forming cells (PFCs) and complement-fixing antibody titers in serum to measure primary and secondary dose-responses to three antigenic forms of GXM: the purified native antigen, a GXM-bovine serum albumin (BSA) conjugate, and a *C. neoformans* whole-cell vaccine (CnVAC). The PFC responses to GXM in BALB/c *nu/nu* and BALB/c ?/+ mice were also compared. T-2 T-cell independence was evaluated by comparing the PFC responses of GXM to a T-2 T_{IND} antigen (type III pneumococcal polysaccharide [SSS-III]) and to a T-1 T_{IND} antigen (trinitrophenyl-lipopolysaccharide [TNP-LPS]) in CBA/cHN *xid* mice. PFC responses to GXM in BALB/c mice coimmunized with rabbit anti-mouse thymocyte serum (ATS) provided evidence for two functionally separate subsets of T cells: one that suppresses PFC responses (T_S), and one that amplifies the PFC response (T_{AMP}).

The findings categorize GXM as a T-2 T_{IND} antigen. This classification of the purified major antigen of CnCAP may make possible analytical comparisons between the mechanisms of immune tolerance to GXM and the mechanisms of tolerance to other, better-characterized T-2 T_{IND} antigens.

* Corresponding author.
† Present address: Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, GA 30322.

MATERIALS AND METHODS

Mice. Adult female mice, 8 to 16 weeks of age and certified to be viral antigen free, were used exclusively. All inbred normal and mutant strains of mice were obtained from Jackson Laboratories (Bar Harbor, Maine). The normal inbred strains used were BALB/c and BALB/cBy. Two different athymic mutant strains were used, BALB/cBy *nu/nu* and CBYB6/F_1 *nu/nu*. The X-linked immunodeficient strain used was CBA/cHN *xid*. These mice have a mutation linked to the X chromosome that results in the lack of a subset of mature B lymphocytes expressing the LyB-3, LyB-5, and LyB-7 surface antigens (40). They are also unable to respond to T-2 T_{IND} antigens (33). All mice were maintained at the research animal facility at Georgia State University. They were given food (Purina Lab Chow) and water ad libitum. During the course of the experiments, all mice remained healthy and showed no overt signs of disease.

Antigens. (i) GXM. Native GXM from a large-capsule variant of *C. neoformans* serotype A (strain CDC 9759) was isolated and purified by the method described by Cherniak et al. (13). Working dilutions of 1 mg/ml were made up in 0.85% (wt/vol) physiological saline (NS) by dissolving the antigen overnight at 4°C with constant stirring. The GXM solution was then used immediately or stored at −20°C for no longer than 30 days.

(ii) GXM-BSA. A conjugate of GXM covalently bound to BSA with an adipic acid dihydrazide linkage was prepared in our laboratory by the following procedure. GXM (40 mg), dried overnight at 78°C in vacuo over P_2O_5, was dissolved in dry dimethyl sulfoxide (4 ml). 1-Cyano-4-dimethylaminopyridinium tetrafluoroborate (6 mg [Sigma Chemical Co.]) dissolved in dimethyl sulfoxide (4 ml) was added to the polysaccharide solution, and anhydrous triethylamine (10 μl) was introduced immediately. After being stirred for 15 min at 23°C, the activated polysaccharide was ready for direct coupling to protein. The solution of activated GXM was mixed with a solution of BSA-adipic acid dihydrazide (80 mg) in anhydrous dimethyl sulfoxide (8 ml) (41); the mixture was stirred overnight at 23°C. The precipitate that formed after the reaction was completed was removed by centrifugation. The supernatant fluid was dialyzed against 0.05 M Tris–0.1 M NaCl buffer, pH 7.6. The retentate was chromatographed on a column of Sepharose CL-6B (1.6 by 86 cm; Pharmacia) previously equilibrated with 0.05 M Tris–0.14 M NaCl (pH 7.6). The column was eluted at a flow rate of 15 ml/h. The column eluates were monitored continuously at 206 nm, and aliquots of the collected fractions were assayed for protein (8) (Bio-Rad dye-binding assay) and carbohydrate (20) content. The GXM-BSA conjugate eluted in the void volume, whereas the carrier protein (BSA-adipic acid dihydrazide) and unreacted GXM appeared in the included volume of the column (Fig. 1). The yield of conjugate, based on the initial amount of GXM, was 61%. The precipitate which formed during the coupling reaction was composed mostly of protein (85%). Gel filtration chromatography also showed the presence of some uncoupled polysaccharide. Lyophilized conjugates could not be reconstituted in buffer. However, conjugates could be concentrated by dialysis against polyethylene glycol 20,000 and stored at −20°C. The concentration of the conjugate (protein [8] plus carbohydrate [20]) was 372 μg/ml. The protein/carbohydrate ratio was 2.4 (wt/wt).

(iii) CnVAC. A *C. neoformans* whole-cell vaccine was made by first growing a 1-liter culture of strain 9759 as described by Cherniak et al. (13) and then adding Formalin to a final concentration of 0.5%. A dry-weight determination of the Formalin-treated yeast culture was made in the following manner. Three tared glass tubes (12 by 75 mm) were filled with 500 μl of well-suspended, precounted yeast cell suspension. The tubes were dried to a constant weight in a heat block at 100°C. The mean net weight of the three tubes along with the yeast cell concentration was then used to calculate the average number of yeast cells per milligram (2.81×10^7). With this value, dose equivalents of CnVAC were made for use in the PFC dose-response studies described below.

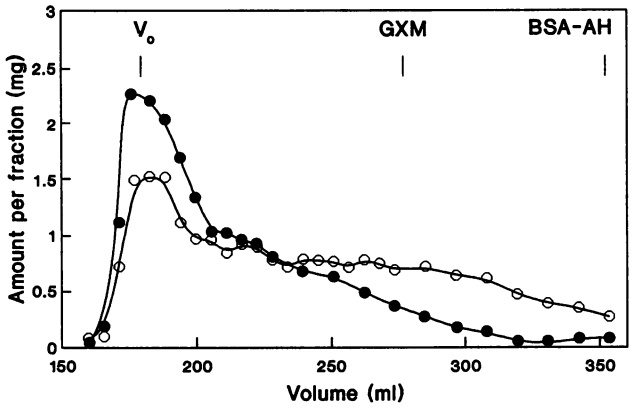

FIG. 1. Gel filtration chromatography of GXM-BSA conjugate on a column (1.6 by 86 cm) of Sepharose CL-6B. The GXM-BSA conjugate is found in the void volume (V_0), as shown. The protein (●) and carbohydrate (○) concentrations were determined colorimetrically. BSA-AH, BSA-adipic acid dihydrazide carrier protein.

(iv) SSS-III. SSS-III was a gift from Phillip J. Baker. The chemical purity of SSS-III was confirmed by ^{13}C-nuclear magnetic resonance spectroscopy (46). The storage arrangements and working dilutions for this antigen were the same as those for GXM.

(v) TNP-LPS. TNP hapten-conjugated LPS from *Escherichia coli* O55:B5 was obtained from Sigma. This antigen was stored in aliquots at −20°C until used.

Immunizations. All antigens were injected intraperitoneally. Antigens were diluted in NS so that the appropriate amount could be delivered in a total of 0.2 ml. In some experiments involving ATS, 0.3 ml of ATS, normal rabbit serum, or NS was injected along with 0.2 ml of antigen. In no instances were mice given an injection containing more than 0.5 ml.

Serological techniques. (i) Passive labeling of SRBC with purified antigens. The serological assays and the PFC assays relied on antibody-dependent complement-mediated lysis of antigen-labeled sheep erythrocyte (SRBC) targets. TNP groups were covalently bound to SRBC by using cacodylic acid and trinitrophenyl chloride (38). SRBC were passively labeled with the polysaccharide antigens GXM and SSS-III by using chromic chloride (6). Briefly, 1 ml of a 1-mg/ml solution of GXM or SSS-III was added to a 15-ml conical centrifuge tube. In this volume, 600 μl of packed SRBC were suspended and then washed twice at 4°C in modified borate buffer (39). The suspension was then vortexed and incubated at 24°C for 15 min. Next, 1 ml of a freshly prepared chromic chloride solution (1 mg/ml in modified borate buffer) was added dropwise with constant vortexing. This mixture was then incubated for another 15 min with constant agitation at 24°C. The treated SRBC were then washed twice in modified

borate buffer and tested in a hemagglutination assay for specific labeling. Mock-labeled SRBC were prepared in exactly the same way except that modified borate buffer was substituted for polysaccharide solution and only 0.5 ml of chromic chloride solution was added to the SRBC.

Detection of complement-fixing antibodies. Passively labeled SRBC were tested in a complement-mediated lysis assay with guinea pig serum complement (Sigma) and specific antisera. GXM was tested with rabbit anti-GXM-BSA serum; SSS-III was tested with mouse anti-SSS-III serum. TNP-labeled SRBC were tested with goat antidinitrophenyl serum, which cross-reacts with TNP (44). The specificity of the antigen labeling was demonstrated by inhibition of specific lysis after addition of the free antigen. Passive hemagglutination titers for antidinitrophenyl, anti-GXM, and anti-SSS-III could be reduced from 512 to 4 with the addition of 10 μg of free antigen.

Hemolytic plaque assay. The Cunningham and Szenberg variation (15) of the hemolytic plaque assay (26) was used to enumerate the number of PFC in the spleen. Mice were killed by CO_2 asphyxiation; their spleens were removed, and their splenocytes were teased into RPMI medium. Direct PFC responses were measured, in triplicate for each mouse, for both antigen-labeled and mock-labeled SRBC. The mock-labeled PFC response values (background number of nonspecific plaques) were subtracted from the mean antigen-specific response values (see below). Indirect plaques were developed in a similar manner except that goat anti-mouse immunoglobulin G (IgG) (whole molecule) diluted 1:400 in RPMI was used.

Statistical methods. Experimental and control groups routinely consisted of five randomly selected, female inbred mice of the same strain and age. Three spleen cell samples were measured for each mouse. Since PFC data have been shown to be log-normally distributed (24), all tests for statistical significance were performed on log-transformed data (50). The geometric net mean PFC responses were computed by subtracting the mean PFC responses obtained with mock-labeled cells from the antigen-specific PFC responses. Confidence intervals for geometric means were derived by using variances computed by model II analysis of variance (43). Back-transformed means represent the anti-ln of the geometric means. Levels of statistical significance between experimental and control groups were calculated by using a one-way mixed-model analysis of variance.

RESULTS

Primary immune dose-responses to GXM antigens. The primary immune responses to purified GXM, GXM-BSA, and CnVAC were investigated by measuring mean PFC responses and complement-fixing antibody titers in serum for groups of five BALB/c mice for a series of antigen doses (or dose equivalents) ranging from 0.005 to 500 μg (Table 1, Fig. 2). The responses in BALB/c mice were measured 5 days after immunization with the antigen, since this had been experimentally predetermined to be the optimal time for measurement of the PFC response (data not shown). The principal influence of the carrier in the primary response to GXM was on the immunogenic dose. The immunogenic dose was clearly defined for each antigen form: 0.5 μg for GXM, 5.0 μg for GXM-BSA, and 0.05 μg for CnVAC. Otherwise, the patterns of the primary responses to all three antigen forms of GXM had several common features. In each case, the serological dose-response paralleled the PFC response; the distribution of the data presented a single mode with a peak at the immunogenic dose; and there was pronounced unresponsiveness to antigen concentrations on either side of the immunogenic dose.

TABLE 1. Primary 5-day PFC dose-responses

Antigen and immunizing dose (μg)	Geometric mean[a] no. of PFCs/spleen	Back-transformed geometric mean
GXM		
0.005	6.69 ± 0.65	802
0.05	8.92 ± 0.27	7,500
0.5	10.47 ± 0.22	35,384
5.0	9.56 ± 0.55	14,194
50.0	7.13 ± 0.24	1,247
GXM-BSA		
0.005	3.32 ± 1.02	28
0.05	7.43 ± 0.47	1,686
0.5	8.26 ± 0.21	3,868
5.0	9.82 ± 0.27	18,313
50.0	8.12 ± 0.16	3,368
500.0	7.51 ± 0.44	1,829
CnVAC		
0.005	7.14 ± 0.33	1,257
0.05	9.63 ± 0.21	15,142
0.5	1.17 ± 0.66	3
5.0	2.17 ± 0.89	9
50.0	3.60 ± 0.53	38
500.0	3.20 ± 0.52	25

[a] Geometric means are given ± their corresponding 95% confidence limits.

Secondary immune dose-responses to GXM antigens. These experiments used the same series of antigen doses; however, groups of eight BALB/c mice were immunized on day 0. On day 21, five mice from each experimental group were boosted with the same dose of antigen, while the remaining three mice, designated the primary controls, were given an equal volume of NS (0.2 ml). Five days later, all the mice were killed, serum samples were collected, and the mean number of PFCs per spleen was determined. Direct and indirect plaque assays were performed for all eight mice in each group on day 26. The mean IgG versus IgM anti-GXM responses in serum were determined by measuring complement-fixing antibodies in sera with and without reduction by 2-mercaptoethanol.

(i) Measurement of B-cell memory. Anamnestic responses to the GXM antigens were measured by comparing secondary and primary PFC and serological responses. In the normal immune response to a T_{IND} antigen, B memory cells are not produced; the immune response does not increase upon a second exposure to the antigen. In this study, the secondary (boosted)-response and primary-response control groups for each antigen had similar dose-response patterns. A single immunogenic dose was determined, with general unresponsiveness to higher or lower antigen concentrations (Table 2). However, there were no significant differences in the magnitude of the direct PFC responses to antigen between the secondary-response groups and the primary-response control groups. The antigenic forms, or carrier effects, were not able to override the T independence of GXM. A significant difference between the two experimental groups is that while the secondary-response group received antigen 5 days before the plaque assay, the control group was last exposed to antigen 26 days before the assay was performed. When the PFC responses to the antigens in these two groups were compared with those of the 5-day primary-response groups (Table 1), the maximum response after 26 days was 4 to 18 times lower. This result suggests that a

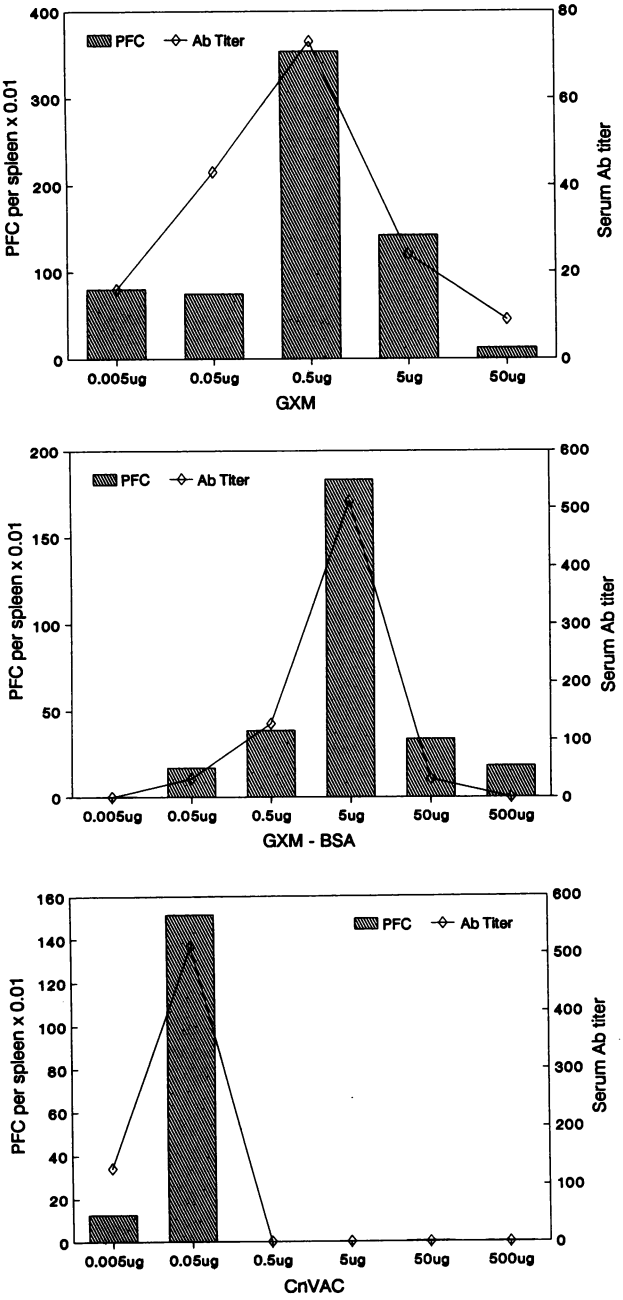

FIG. 2. Primary 5-day responses to GXM antigens. PFC responses and antibody (Ab) titers in serum are expressed as back-transformed geometric means on the left and right *Y* axes, respectively. Primary responses to GXM, GXM-BSA, and CnVAC are shown in separate panels.

second exposure to immunogenic doses suppresses the specific B-cell response.

(ii) Measurement of isotype class switching. Comparison of direct and indirect PFC responses revealed no class switching in the secondary responses to any of the three antigens. The PFC responses in the indirect assays that measured both IgG and IgM were no greater than those in the direct assays that measured only IgM responses. These results were confirmed by serological analysis. Treatment of the sera from the secondary-response groups with 2-mercaptoethanol resulted in complete elimination of detectable antibody titers, indicating that the anti-GXM antibodies in serum were mainly IgM.

PFC responses to GXM in athymic mice. The T-helper-cell-independent quality of GXM was also examined in two strains of athymic mice, CBYB6/F_1 and BALB/cBy. The mean PFC responses to 0.5 μg of GXM were measured in both homozygous *nu/nu* (athymic) mutants and ?/+ strains (Table 3). In the BALB/cBy strain, the PFC responses were higher. Also, the responses in the BALB/cBy ?/+ mice were higher than those in the BALB/cBy *nu/nu* mice. However, statistical analysis by analysis of variance showed that this difference was not significant at the $P = 0.05$ level (data not shown).

PFC responses in CBA/cHN *xid* mice. The PFC responses to 0.5 μg of GXM were compared with the responses to 10 μg of TNP-LPS (a T-1 T_{IND} antigen), 0.5 μg of SSS-III (a T-2 T_{IND} antigen), and 0.2 ml of NS (negative control) in CBA/cHN *xid* female mice. The inclusion of the NS negative control was necessary because it has been shown that murine antibodies formed against enteric *E. coli* β-galactosidase cross-react with TNP (23). Since it was expected that responses to T-2 T_{IND} antigens would be negative, the PFC responses to 0.5 μg of GXM and 0.5 μg of SSS-III were also measured in BALB/c mice as a positive control (data not shown).

There was virtually no PFC response to either GXM or the T-2 T_{IND} antigen SSS-III in the CBA/cHN *xid* mice (Table 4), whereas the PFC responses to these antigens in BALB/c mice were normal. The PFC response to 10 μg of the T-1 T_{IND} antigen TNP-LPS was strong, as expected. The number of background plaques formed after TNP-LPS treatment measured in the NS-primed mice was measurable but insignificant.

PFC responses to GXM with ATS. Evidence for the existence of antigen-specific T_S and T_{AMP} cells in the primary immune response to GXM was obtained in the following manner. Three groups of five BALB/cBy mice were immunized with 0.5 μg of GXM on day 0. Simultaneously, each mouse in two of the groups received 0.3 ml of ATS, while five mice in the third group received 0.3 ml of NS. On day 1, the mice in one of the two groups receiving ATS on day 0 were treated with another 0.3 ml of ATS; the mice in the remaining two groups received NS. On day 5, plaque assays were performed for all 15 mice. A separate control experiment was performed by comparing the PFC responses of BALB/cBy mice coimmunized with 0.5 μg of GXM and 0.3 ml of either normal rabbit serum that had been adsorbed with agarose (14) or NS. There was no significant difference between the mean PFC responses of the two groups (data not shown). Therefore, it was concluded that specific ATS antibody and not nonspecific serum effects was responsible for the modulation of the PFC response.

The rationale behind this experimental design was that ATS given on day 0 would eliminate putative T_S cells, resulting in a derepression (or amplification) of the immune response above the normal PFC response. ATS given again 24 h later should have eliminated putative T_{AMP} cells, which would not be ATS sensitive on day 0. The mean PFC response of the experimental group receiving ATS on day 0 was significantly greater than the mean PFC response of the control group (Table 5). On the other hand, the anti-GXM PFC response of the experimental group receiving ATS on days 0 and 1 was significantly lower than the response of the control group. Thus, these results provided evidence for at least two functionally distinct T-cell subsets involved in the

TABLE 2. Secondary versus primary 26-day direct and indirect PFC dose-responses

Antigen and immunizing dose (μg)	Boost[a]	Geometric mean[b] no. of PFCs/spleen		Back-transformed mean	
		Direct (IgM PFCs)	Indirect (IgM + IgG PFCs)	Direct	Indirect
GXM					
0.005	Same	4.40 ± 0.78	3.40 ± 0.80	81	30
	NS	3.62 ± 1.09	3.50 ± 1.26	38	33
0.05	Same	5.20 ± 0.70	5.11 ± 0.63	181	166
	NS	4.65 ± 1.41	3.36 ± 1.86	105	29
0.5	Same	7.55 ± 0.46	7.19 ± 0.44	1,891	1,325
	NS	7.36 ± 1.45	7.51 ± 1.02	1,569	1,826
5.0	Same	5.20 ± 0.59	4.72 ± 0.64	181	113
	NS	5.72 ± 0.71	5.58 ± 0.53	306	264
50.0	Same	4.43 ± 0.47	4.91 ± 0.43	84	136
	NS	4.32 ± 0.80	NR[c]	75	
500.0	Same	3.87 ± 0.82	2.67 ± 0.66	48	14
	NS	3.09 ± 1.51	NR	22	
GXM-BSA					
0.005	Same	4.17 ± 1.04	3.00 ± 0.61	65	20
	NS	3.19 ± 1.42	3.03 ± 1.81	24	21
0.05	Same	NR	NR		
	NS	NR	NR		
0.5	Same	6.26 ± 0.29	4.54 ± 0.78	523	94
	NS	5.47 ± 1.38	4.32 ± 1.49	238	75
5.0	Same	7.65 ± 0.31	7.54 ± 0.37	2,093	1,875
	NS	6.26 ± 1.62	4.83 ± 1.70	522	125
50.0	Same	6.21 ± 0.59	5.77 ± 0.73	500	320
	NS	4.37 ± 1.75	3.97 ± 1.51	79	53
CnVAC					
0.005	Same	5.78 ± 0.53	5.20 ± 0.58	324	182
	NS	4.99 ± 1.70	5.38 ± 1.02	147	216
0.05	Same	6.00 ± 0.50	5.71 ± 0.46	402	301
	NS	6.93 ± 1.64	5.77 ± 1.06	1,025	320
0.5	Same	7.69 ± 0.19	7.70 ± 0.29	2,185	2,199
	NS	8.34 ± 0.35	8.15 ± 0.52	4,195	3,453
5.0	Same	7.50 ± 0.59	7.20 ± 0.64	1,810	1,345
	NS	7.33 ± 0.32	7.22 ± 0.52	1,519	1,361
50.0	Same	3.11 ± 0.58	NR	23	
	NS	1.23 ± 1.14	1.61 ± 1.45	3	5
500.0	Same	2.42 ± 0.97	3.67 ± 0.73	11	39
	NS	4.60 ± 1.64	4.23 ± 1.62	99	69

[a] The boost consisted of either a second immunization with the same dose and antigen used for the first immunization (same) or mock immunization with 0.2 ml of NS. For each immunizing dose, the "same" row data are for the secondary response (5 days after the day 21 boost) and the "NS" row data are for the primary response (26 days after initial immunization).
[b] Geometric means are given ± their corresponding 95% confidence limits.
[c] NR, no response.

regulation of the PFC response to GXM: T cells that suppress the B-cell response (T_S) and that are ATS sensitive at the time of immunization with antigen, and T cells that amplify the B-cell response (T_{AMP}) and that are ATS sensitive 24 h after immunization with antigen.

TABLE 3. Five-day PFC responses to 0.5 μg of GXM in athymic mice

Mouse strain	Geometric mean[a] no. of PFCs/spleen	Back-transformed mean
BALB/c *nu/nu*	8.07 ± 0.14	3,188
BALB/c ?/+	8.85 ± 0.37	7,008
CBYB6/F_1 *nu/nu*	7.52 ± 0.37	1,846
CBYB6/F_1 ?/+	7.19 ± 0.46	1,326

[a] See Table 2, footnote *b*.

DISCUSSION

Previous studies have shown the importance of antibody against the capsular polysaccharide of *C. neoformans* in defining serotypes (7, 27, 42), in monitoring the course of disease (28), in antibody-dependent cellular cytotoxicity (32), and in protection against experimental infection (17). The antigenic heterogeneity of the capsular polysaccharide is a major problem in studying the humoral response, since preparations of CnCAP of known composition have not been standardized. The isolation and purification of the three antigenic components of CnCAP provide the opportunity to measure the humoral response to these antigens independently. This approach has led to the assignment of some of the immunologic properties of CnCAP to specific subcomponent antigens. For instance, the delayed-type hypersensitivity response of mice to CnCAP, which has been investigated extensively (34), is now known to be due exclusively to the mannoprotein antigen (36), whereas CnCAP serotype

TABLE 4. Five-day PFC response to T_{IND} antigens in CBA/cHN *xid* mice

Immunogen	Target antigen[a]	Geometric mean[b] no. of PFCs/ spleen	Back-transformed mean
TNP-LPS	TNP	10.51 ± 0.35	36,858
SSS-III	SSS-III	NR[c]	
GXM	GXM	NR	
None	TNP	6.95 ± 0.41	1,046

[a] Antigen (or hapten) used to label target SRBC in the hemolytic plaque assay.
[b] See Table 2, footnote *b*.
[c] NR, no PFC response detected with the appropriately labeled SRBC; however, normal PFC responses to this antigen were detected in BALB/c mice (data not shown).

specificities reside in the GXM (18, 21, 45). Our interest in understanding the molecular structures of epitopes that determine *C. neoformans* serotype specificities led to our selection of GXM for this investigation.

Earlier studies have reported that CnCAP is a T_{IND} antigen (12). Considering the antigenic complexity of CnCAP, this T independence could be due to any or all of the subcomponent antigens. We examined the T independence of GXM by comparing the primary and secondary PFC dose-responses to three forms of the antigen: the native antigen, a GXM-BSA protein conjugate, and a whole-cell vaccine. This comparison provided the opportunity to observe the relative contributions of the different carriers to two of the T_{IND} characteristics that were evaluated, induction of B-cell memory and isotype class switching.

First, primary dose-responses to the GXM antigens were measured. The PFC response pattern parallels that of the complement-fixing antibody titers in serum for all three antigenic forms (Fig. 2). This finding indicates that the splenic PFC response can be used as an estimate of the general humoral response to these antigens in the mouse. Also, with each antigen, there was a spike at a single immunogenic dose that was flanked by relative unresponsiveness at lower or higher doses. This result was similar to the dose-response pattern reported for SSS-III (5). However, the fact that GXM-BSA had a 10-fold-higher immunogenic dose and CnVAC had a 10-fold-lower immunogenic dose than GXM may only reflect minor stoichiometric differences in the GXM content of these antigens. These similarities in the primary dose-responses for the three antigens suggest weak carrier influences.

To determine whether isotype class switching and B-cell memory could be induced, mice were initially immunized with the same panel of doses for all three antigens and were then boosted with identical reinforcing doses 21 days later. On day 26, when the secondary responses of these mice were compared with the primary responses of the control groups of mice, which were secondarily challenged with NS instead, no difference was observed in the magnitude of the responses. This result was unexpected. The PFC responses of both the secondary-response and the 26-day primary-response control groups were 4 to 18 times lower than those of the primary-response groups measured 5 days postimmunization. These results suggested that a secondary immunization at 21 days results in suppression of the anti-GXM PFC response, regardless of the form or dose of the antigen. This finding differs from the results obtained by Murphy and Cozad (35), whose studies of the PFC responses to *C. neoformans* soluble culture filtrate antigen in CBA mice showed recovery from high-dose tolerance 14 days after initial immunization. However, the specific anti-GXM PFC response was not measured, nor was the GXM content of the soluble culture filtrate antigen determined.

Consistent with the lack of an anamnestic response, no isotype class switching was detected in the secondary responses to any of the three GXM antigens. There were no significant differences between direct and indirect PFC responses to GXM, even with the protein-conjugated form of the antigen (GXM-BSA). These results are in accord with the serological data obtained after 2-mercaptoethanol treatment of the serum. Mild reduction of the mouse serum with 2-mercaptoethanol effectively reduced the complement-fixing antibody titers to below detectable levels. Thus, IgM appeared to be the main isotype in the secondary response to GXM. Nevertheless, Devi et al. have been able to produce significant IgG responses by multiple subcutaneous injections of GXM-tetanus toxoid and complete Freund's adjuvant (16). Also, IgG-secreting hybridomas have been produced by Dromer et al. (18) and Eckert and Kozel (21) by using GXM conjugated to SRBC. Thus, the antigenic form and route of immunization are important considerations for creating the appropriate circumstances under which IgG responses to GXM can be elicited.

The T_{IND} qualities of GXM observed in the primary and secondary dose-responses to the antigens were confirmed by the PFC responses to the native GXM antigen in athymic mice. The PFC responses of the athymic and wild-type CBYB6/F_1 mice were comparable (Table 3). These mice express both *H-2*^{d} and *H-2*^{b} major histocompatibility complex haplotypes. However, the PFC responses in the BALB/c wild-type controls were much higher than those in the BALB/c *nu/nu* mice. Although this difference seems large, it was not statistically significant at the $P = 0.05$ level. Nevertheless, the fact that this difference was observed in BALB/c mice, which have been shown to be high responders to GXM (19), and not in the F_1 hybrid strain raises the

TABLE 5. Evidence for amplification and suppression of PFC responses to GXM by T cells

Observed effect	ATS treatment (day)	Geometric mean[a] no. of PFCs/10^7 splenocytes	Back-transformed mean	$MS_{groups}/MS_{subgroups}$[b]	P_{H_0}[c]
Suppression	0 + 1	4.62 ± 0.47	101	16.07	$0.01 > P > 0.005$
Amplification	0	7.67 ± 0.37	2,137	8.08	$0.05 > P > 0.02$
Normal (control)	None	6.61 ± 0.43	742		—[d]

[a] See Table 2, footnote *b*.
[b] The ratio of the mean squares for the experimental treatment groups to the mean squares for the subgroups (individual mice within groups [43]).
[c] Probability that the null hypothesis, H_0 (that there is no difference between experimental groups), is true (= *P* of committing a type I error) as measured by the statistic $F_{(2)1,8}$.
[d] —, probability of type II error was not computed when H_0 was rejected.

possibility that T cells are involved in regulating the response to GXM in a genetically restricted fashion.

Having established the T-cell independence of GXM, its T-2 T_{IND} characteristics were demonstrated in two lines of investigation. A comparison of the immune responses of CBA/cHN *xid* mice to GXM, SSS-III, and TNP-LPS revealed that, similar to the known T-2 T_{IND} antigen SSS-III and unlike the T-1 T_{IND} antigen TNP-LPS, GXM was unable to induce a PFC response (Table 4). An examination of the second T-2 T_{IND} quality showed that the primary PFC response to GXM is regulated both positively and negatively by T cells. It has been demonstrated that in the immune response to pneumococcal polysaccharide, the PFC counts increase dramatically when ATS is given along with antigen. The fact that no such amplification occurs in nude mice has led Baker et al. to propose functional T_{AMP} and T_S cell subsets that regulate the immune response to SSS-III (4). The same type of regulation has been observed with other T-2 T_{IND} antigens, e.g., polyvinylpyrrolidone and TNP-Ficoll (9). We observed a significant increase in the PFC response to GXM when ATS was given in vivo on day 0 (Table 5). These data are represented as PFC per splenocyte rather than PFC per spleen to compensate for splenomegaly caused by ATS treatment (31). In an earlier study by Breen et al. (11), ATS was used to demonstrate T_S cells in the primary response to GXM. However, since these observations were reported as PFC counts per spleen, it is unclear whether the enhancement was due to derepression or an increased number of lymphocytes.

This study presents evidence for both T_S and T_{AMP} cell regulation of the response to GXM. The T_S cell influence was ATS sensitive on day 0 (at the same time as immunization with antigen). However, the T_{AMP} cell effect was ATS sensitive on day 1 but not on day 0 or day 2 (data not shown). Elimination of T_S cells resulted in a significant increase in the PFC response. Elimination of T_S and T_{AMP} cells by consecutive ATS treatments on days 0 and 1 resulted in the basal PFC response. Some have proposed that T_{AMP} cells are actually T contrasuppressor (T_{CS}) cells (10). Both T_{CS} and T_{AMP} cells bind to the plant lectin from *Vicia villosa*. However, *V. villosa*-adherent T cells induced in BALB/c *nu*/+ mice with SSS-III can transfer their amplifier effect to BALB/c *nu/nu* mice (2). T_{CS} cells influence only T_S cells, not B cells. Thus, it has been concluded that amplification in the response to SSS-III is due to amplification of B-cell responses, not to contrasuppression by T-suppressor effects. However, identification of the specific targets of these regulatory T cells in the GXM system awaits further functional and phenotypic analyses.

This investigation presents evidence for the formal classification of the biochemically and structurally well defined GXM from *C. neoformans* serotype A with the group of T-2 T_{IND} antigens. However, growing evidence for the involvement of T cells and T-cell products in modulating the immune response to T_{IND} antigens may make the classification of these antigens as T-1 or T-2 an oversimplification (22, 37). Indeed, in our laboratory, we have discovered that T cells are involved not only in regulating the primary response to GXM at immunogenic doses but also in mediating low-dose antigen-specific tolerance to GXM (unpublished data). These findings parallel those reported for another well-characterized T-2 T_{IND} antigen, SSS-III (1). Nevertheless, the classification of GXM as a T-2 T_{IND} antigen may provide a basis for comparison of immune responses to these antigens at the cellular and molecular level. This new information can lead to the creation of improved protein conjugate or anti-idiotypic vaccines as well as to new strategies for the use of adjuvants for an improved directed immune response (3). The limiting size of the polysaccharide for the maximum immune response or the molecular structure of immunodominant groups can now be studied, as can the molecular structures of the antigenic determinants that define *C. neoformans* serotype specificities. Also, the mechanisms by which T cells specifically influence the immune response to this polysaccharide can be explored.

ACKNOWLEDGMENTS

We thank Phillip J. Baker for providing purified SSS-III and technical advice.

This research was supported in part by Public Health Service grant AI-31769 from the National Institutes of Health.

REFERENCES

1. **Baker, P. J., D. F. Amsbaugh, P. W. Stashak, G. Caldes, and B. Prescott.** 1982. Direct evidence for the involvement of T suppressor cells in the expression of low-dose paralysis to type III pneumococcal polysaccharide. J. Immunol. **128**:1059–1062.
2. **Baker, P. J., M. B. Fauntleroy, and B. Prescott.** 1988. Examination of the differential characteristics of amplifier and contrasuppressor T cells. Immunobiology **177**:438–448.
3. **Baker, P. J., J. R. Hiernaux, M. B. Fauntleroy, B. Prescott, J. L. Cantrell, and J. A. Rudbach.** 1988. Inactivation of suppressor T-cell activity by nontoxic monophosphoryl lipid A. Infect. Immun. **56**:1076–1083.
4. **Baker, P. J., N. D. Reed, P. W. Stashak, D. F. Amsbaugh, and B. Prescott.** 1973. Regulation of the antibody response to type III pneumococcal polysaccharide. I. Nature of regulatory cells. J. Exp. Med. **137**:1431–1441.
5. **Baker, P. J., P. W. Stashak, D. F. Amsbaugh, and B. Prescott.** 1971. Characterization of the antibody response to type III pneumococcal polysaccharide at the cellular level. I. Dose-response studies and the effect of prior immunization on the magnitude of the antibody response. Immunology **20**:469–480.
6. **Baker, P. J., P. W. Stashak, and B. Prescott.** 1969. Use of erythrocytes sensitized with purified pneumococcal polysaccharides for the assay of antibody and antibody-producing cells. Appl. Microbiol. **17**:422–426.
7. **Bennett, J. E., K. J. Kwong-Chung, and D. H. Howard.** 1977. Epidemiologic differences among serotypes of *Crytococcus neoformans*. Am. J. Epidemiol. **105**:582–586.
8. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
9. **Braley-Mullen, H.** 1982. Differential effect of activated T amplifier cells on B cells responding to thymus-independent type 1 and type 2 antigens. J. Immunol. **129**:484–489.
10. **Braley-Mullen, H.** 1986. Characterization and activity of contrasuppressor T cells induced by type III pneumococcal polysaccharide. J. Immunol. **137**:2761–2767.
11. **Breen, J. F., I. C. Lee, F. R. Vogel, and H. Friedman.** 1982. Cryptococcal capsular polysaccharide-induced modulation of murine immune responses. Infect. Immun. **36**:47–51.
12. **Cauley, L. K., and J. W. Murphy.** 1979. Response of congenitally athymic (nude) and phenotypically normal mice to *Cryptococcus neoformans* infection. Infect. Immun. **23**:644–651.
13. **Cherniak, R., L. C. Morris, B. C. Anderson, and S. A. Meyer.** 1991. Facilitated isolation, purification, and analysis of glucuronoxylomannan of *Cryptococcus neoformans*. Infect. Immun. **59**:59–64.
14. **Cohen, A., and M. Schlesinger.** 1970. Adsorption of guinea pig serum with agar. A method for elimination of its cytotoxicity for murine thymus cells. Transplantation **10**:130–132.
15. **Cunningham, A. J., and A. Szenberg.** 1968. Further improvements in the plaque-technique for detecting single antibody-forming cells. Immunology **14**:599–602.
16. **Devi, S. J. N., R. Schneerson, W. Egan, T. J. Ulrich, D. Bryla, J. B. Robbins, and J. E. Bennett.** 1991. *Cryptococcus neofor-*

mans serotype A glucuronoxylomannan-protein conjugate vaccines: synthesis, characterization, and immunogenicity. Infect. Immun. **59:**3700–3707.

17. **Dromer, F., J. Charreire, A. Contrepois, C. Carbon, and P. Yeni.** 1987. Protection of mice against experimental cryptococcosis by anti-*Cryptococcus neoformans* monoclonal antibody. Infect. Immun. **55:**749–752.
18. **Dromer, F., J. Salamero, A. Contrepois, C. Carbon, and P. Yeni.** 1987. Production, characterization, and antibody specificity of a mouse monoclonal antibody reactive with *Cryptococcus neoformans* capsular polysaccharide. Infect. Immun. **55:**742–748.
19. **Dromer, F., P. Yeni, and J. Charreire.** 1988. Genetic control of the humoral response to cryptococcal capsular polysaccharide in mice. Immunogenetics **28:**417–424.
20. **Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28:**350–356.
21. **Eckert, T. F., and T. R. Kozel.** 1987. Production and characterization of monoclonal antibodies specific for *Cryptococcus neoformans* capsular polysaccharide. Infect. Immun. **55:**1895–1899.
22. **Endres, R. O., E. Kushnir, J. W. Kappler, P. Marrack, and S. C. Kinsky.** 1983. A requirement for nonspecific T cell factors in antibody responses to "T cell independent" antigens. J. Immunol. **130:**781–784.
23. **Golding, B., G. Inghirami, E. Peters, T. Hoffman, J. E. Balow, and C. Tsokos.** 1987. In vitro generated human monoclonal trinitrophenyl-specific B cell lines. Evidence that human and murine anti-trinitrophenyl monoclonal antibodies cross-react with *Escherichia coli* β-galactosidase. J. Immunol. **139:**4061–4066.
24. **Gottlieb, C. F.** 1974. Application of transformations to normalize the distribution of plaque-forming cells. J. Immunol. **113:**51–57.
25. **James, P. G., and R. Cherniak.** 1992. Galactoxylomannans of *Cryptococcus neoformans*. Infect. Immun. **60:**1084–1088.
26. **Jerne, N. K., C. Henry, A. A. Nordin, H. Fuji, A. M. C. Koros, and I. Lefkovits.** 1974. Plaque forming cells: methodology and theory. Transplant. Rev. **18:**130–191.
27. **Kapend'a, K., L. Komichelo, D. Swinne, and J. Vandepitte.** 1987. Meningitis due to *Cryptococcus neoformas* biovar gattii in Zairean AIDS patient. Eur. J. Clin. Microbiol. **6:**320–321.
28. **Kaufman, L., and E. Reiss.** 1986. Serodiagnosis of fungal diseases, p. 440–466. *In* N. R. Rose, H. Friedman, and J. L. Fahey (ed.), Manual of clinical laboratory immunology, 3rd ed. American Society for Microbiology, Washington, D.C.
29. **Kozel, T. R., and J. Cazin, Jr.** 1971. Nonencapsulated variant of *Cryptococcus neoformans*. I. Virulence studies and characterization of soluble polysaccharide. Infect. Immun. **3:**287–294.
30. **Kozel, T. R., and R. P. Mastroianni.** 1976. Inhibition of phagocytosis by cryptococcal polysaccharide: dissociation of the attachment and ingestion phases of phagocytosis. Infect. Immun. **14:**62–67.
31. **Lake, J. P., and N. D. Reed.** 1976. Regulation of the immune response to polyvinylpyrrolidone: effect of antilymphocyte serum on the response of normal and nude mice. Cell. Immunol. **21:**364–370.
32. **Miller, G. P. G., and S. Kohl.** 1983. Antibody-dependent leukocyte killing of *Cryptococcus neoformans*. J. Immunol. **131:**1455–1459.
33. **Mosier, D. E., I. Scher, and W. E. Paul.** 1976. In vitro responses of CBA/N mice: spleen cells of mice with an X-linked defect that precludes immune responses to several thymus-independent antigens can respond to TNP-lipopolysaccharide. J. Immunol. **117:**1363–1369.
34. **Murphy, J. W.** 1989. Immunoregulation in cryptococcosis, p. 319–344. *In* E. Kurstak (ed.), Immunology of fungal infections. Marcel Dekker, Inc., New York.
35. **Murphy, J. W., and G. C. Cozad.** 1972. Immunological unresponsiveness induced by cryptococcal capsular polysaccharide assayed by the hemolytic plaque technique. Infect. Immun. **5:**896–901.
36. **Murphy, J. W., R. L. Mosley, R. Cherniak, G. H. Reyes, T. R. Kozel, and E. Reiss.** 1988. Serological, electrophoretic, and biological properties of *Cryptococcus neoformans* antigens. Infect. Immun. **56:**424–431.
37. **Pike, B. L., and G. J. V. Nossal.** 1984. A reappraisal of "T-independent" antigens. I. Effect of lymphokines on the response of single adult hapten-specific B lymphocytes. J. Immunol. **132:**1687–1695.
38. **Rittenberg, M. B., and A. A. Amkraut.** 1966. Immunogenicity of trinitrophenyl-hemocyanin: production of primary and secondary anti-hapten precipitins. J. Immunol. **97:**421–430.
39. **Rittenbereg, M. B., and K. L. Pratt.** 1969. Antitrinitrophenyl (TNP) plaque assay. Primary response of Balb/c mice to soluble and particulate immunogen. Proc. Soc. Exp. Biol. Med. **132:**575–581.
40. **Scher, I.** 1982. CBA/N immune defective mice: evidence for the failure of a B cell subpopulation to be expressed. Immunol. Rev. **64:**117–132.
41. **Schneerson, R., O. Barrera, A. Sutton, and J. B. Robbins.** 1980. Preparation, characterization, and immunogenicity of *Haemophilus influenzae* type b polysaccharide-protein conjugates. J. Exp. Med. **152:**361–376.
42. **Shimizu, R. Y., D. H. Howard, and M. N. Clancy.** 1986. The variety of *Cryptococcus neoformans* in patients with AIDS. J. Infect. Dis. **154:**1042.
43. **Sokal, R. R., and F. J. Rohlf.** 1981. Biometry, 2nd ed., p. 271–309. W. H. Freeman & Co., San Francisco.
44. **Steward, M.** 1989. Antigen recognition, p. 7.7. *In* I. Roit, J. Brostoff, and D. Male (ed.), Immunology. Gower Medical, New York.
45. **Todaro-Luck, F., E. Reiss, R. Cherniak, and L. Kaufman.** 1989. Characterization of *Cryptococcus neoformans* capsular glucuronoxylomannan polysaccharide with monoclonal antibodies. Infect. Immun. **57:**3882–3887.
46. **Turner, S. H., and R. Cherniak.** 1991. Glucuronoxylomannan of *Cryptococcus neoformans* serotype B: structural analysis by gas-liquid chromatography-mass spectrometry and ^{13}C-nuclear magnetic resonance spectroscopy. Carbohydr. Res. **211:**103–116.
47. **Turner, S. H., and R. Cherniak.** 1991. Multiplicity in the structure of the glucuronoxylomannan of *Cryptococcus neoformans*, p. 123–142. *In* J. P. Latagé and D. Boucias (ed.), Fungal cell walls and immune response (NATO ASI series, vol. H53). Springer-Verlag, New York.
48. **Turner, S. H., R. Cherniak, and E. Reiss.** 1984. Fractionation and characterization of galactoxylomannan from *Cryptococcus neoformans*. Carbohydr. Res. **125:**343–349.
49. **Wilek, R. M., E. T. Starcher, and J. W. Curran.** 1987. Opportunistic diseases reported in AIDS patients: frequencies, associations, and trends. AIDS **1:**175–182.
50. **Zar, J. H.** 1974. Biostatistical analysis, p. 182–189. Prentice-Hall, Englewood Cliffs, N.J.