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Antibody responses to *Cryptococcus neoformans* in Indian patients with cryptococcosis

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An important element of the host response to cryptococcosis is humoral immunity. Specific antibody responses in patients with cryptococcosis however, have not been extensively studied. We analyzed the antibody responses of 22 Indian patients with cryptococcosis, including both HIV+ and HIV− individuals. Sera from 10 Indian patients with AIDS and without cryptococcosis were studied as controls. Antibody responses to cryptococcal proteins were detected by immunoblot, while antibodies to glucuronoxylomannan (GXM), the main component of the cryptococcal capsular polysaccharide were measured by ELISA. Our results indicate that cryptococcosis elicits antibodies to a specific pattern of cytoplasmic proteins. Further, we find that antibody responses to both cytoplasmic proteins and GXM are less robust in HIV+ patients when compared with HIV− patients.

Keywords *Cryptococcus neoformans*, cryptococcosis, serology, AIDS

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Introduction

An important role for antibody in the host immune response to cryptococcosis has been hypothesized [1]. Antibody to the *Cryptococcus neoformans* capsule promotes phagocytosis of the yeast and subsequent killing by effector cells and enhances cellular immunity [2,3]. Animal studies demonstrate that both the capsular polysaccharide and protein antigens of *C. neoformans* elicit antibody during infection [4]. These studies further indicate that the type of antigen and the kinetics of the antibody response influence the effects of antibody on cryptococcosis. In mice, an early antibody response to cryptococcal proteins was associated with death [5], while an early antibody response to the capsular polysaccharide was associated with survival [6].

Antibody to the cryptococcal polysaccharide capsule has been detected in patients with cryptococcosis [7]. A strong antibody response against the cryptococcal capsular polysaccharide has been associated with a good prognosis in HIV− patients with cryptococcosis [8]. Antibody to a variety of cryptococcal proteins has also been detected in patients with cryptococcosis [9]. This antibody response to *C. neoformans* proteins is more complex in humans than in rodent models and is likely to be affected by both host and pathogen-related factors [4]. An alarmingly high rate of cryptococcosis has been described in India [10], especially among patients with AIDS from Northern India, including Delhi and Chandigarh [11]. In this study, we analyzed the antibody response to both cryptococcal proteins and capsular polysaccharide among Indian patients with cryptococcosis.

Methodology

Patients and sera

Sera were obtained from HIV+ and HIV− patients with cryptococcosis at the time of diagnosis. Cryptococcosis was defined as a positive culture for *C. neoformans,* or visualization of the organism by direct microscopy (India ink) and detection of cryptococcal antigen in the blood or CSF by ELISA and latex agglutination in a patient with a compatible clinical presentation. Sera from HIV+ individuals (CD4+T cells <200 cells/μl) without cryptococcosis were used as controls. All patients were cared for at the All India Institute of Medical Sciences. Consent was obtained

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from the patients prior to investigation. Specimens were left-over (extra) from routine clinical care and were obtained under IRB approval at the AIIMS and the Albert Einstein College of Medicine.

Clinical data

The following clinical data was collected: age, sex, form of cryptococcosis and acute mortality (defined as death within 1 week of diagnosis). The presence of serum and CSF cryptococcal polysaccharide was confirmed by the Premier Cryptococcal enzyme immunoassay (Meridian Diagnostics) on non-diluted specimens. Co-infections were also noted as were underlying immunosuppressive conditions.

Strains and growth conditions

C. neoformans strain 24067 (serotype D) and *Candida albicans* (BSMY 212) were obtained from American Type Culture Collection (Manassas, VA). This strain of *C. neoformans* was used because of our previous experience with it in an immunoblot assay [12]. Fungi were grown in Sabouraud's dextrose broth for 2 days at 30°C prior to protein isolation.

Cytosolic proteins

Cells were centrifuged at 4000 rpm for 20 min at 4°C and the pellet was washed twice with phosphate buffered saline (PBS). The pellet was re-suspended in PBS containing a protease inhibitor cocktail buffer without EDTA (Roche, Manheim, Germany) and 0.5 mm Zirconia/Silica beads (Sigma, St. Louis, MO). Cells were disrupted using a mini bead beater. The resulting suspension was centrifuged for 15 min at 4°C to obtain whole extracts. Whole extract was then centrifuged at 100,000 *g* for 1 h at 4°C to obtain cytosolic extracts. The membrane fractions were washed with PBS containing protease inhibitors and centrifuged at 100,000 *g* for 30 min at 4°C. The resulting supernatant was pooled with the previous supernatant as part of the cytosolic fraction. The same approach was used to obtain cytosolic proteins from *C. albicans*. Cytosolic mannoproteins of *C. neoformans* were isolated from total cytoplasmic proteins by column affinity chromatography using a Concanavalin A column, (Pierce, Rockford, IL), according to the manufacturer's instructions. Protein extracts were stored at −80°C prior to use.

Recombinant mannoproteins

Recombinant mannoproteins 115 and 84 were derived as described [13]. Briefly, plasmids for extracellular mannoproteins 115 and 84 were obtained from G. Teti (University of Messina, Italy) and expressed in *E. coli*. Following protein induction and cell lysis, histidine tagged proteins were purified by column affinity chromatography using a metal resin, BD TALON (BD, Biosciences, Clontec, Palo Alto, CA).

Immunoblot

Electrophoresis was done in the Bio-Rad Mini- Protean II system with a 10% resolving gel at 100V. Gels were transferred to nitrocellulose membranes, which were then blocked for 1 h at room temperature or overnight at 4°C in a buffer containing 5% milk in PBS with 0.1% Tween-20. Individual channels on a blotting frame were incubated with human (1:1600) sera for 1 h at room temperature or overnight at 4°C. Channels were washed three times with blocking buffer and then incubated with a horseradish peroxidase-conjugated goat antibody to human IgG, IgA, IgE and IgM. Immunoblots with recombinant proteins were performed in a manner similar to cytoplasmic proteins immunoblots, but reactive IgG was detected with alkaline phosphatase labeled anti-human IgG and color developed with nitroblue tetrazolium chloride-5 bromo-4-chloro-3-indolyl phosphate (Sigma). The substrate for HRP-conjugated antibodies was Luminol (Pierce, Rockford, IL).

Blots of cytoplasmic proteins were analyzed with respect to overall reactivity (i.e., to all proteins) and to a set of 9 designated proteins that were most commonly recognized by sera from patients with cryptococcosis. This included proteins with the following approximate masses: 133, 122, 106, 97, 82, 75, 71, 56, and 40 kDa.

ELISA

IgG to glucuronoxylomannan (GXM), the main component of the cryptococcal polysaccharide was measured with a previously described ELISA [14]. ELISA plates were coated with GXM, prepared by Cetyl trimethylammonium bromides precipitation [15], 5 ug/ml in PBS. ELISA plates were blocked with a solution containing 2% BSA, 1% fetal calf serum and 0.1M NaN_3. Patient sera were added to wells and serially diluted. GXM-specific antibodies were detected with alkaline phosphatase labeled Goat anti-human IgG (Southern Biotech). Mean absorbance readings at 405 nm for sera diluted 1:15 were compared between groups.

Statistical methods

The median number of proteins recognized by sera from different cohorts was compared by Kruskal-Wallis.

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Post-hoc analysis was done using the Dunn Test. The proportions of sera demonstrating reactivity to cryptococcal proteins were compared by either Fischer exact or Chi-square tests. Comparisons of ELISA absorbances were done using ANOVA. All statistics were done with Graph Pad InStat software (San Diego, CA).

Results

Patients

A total of 32 patients were studied, 22 had cryptococcosis (See Table 1). Twelve patients with cryptococcosis had AIDS (CN+ HIV+) and 10 patients with cryptococcosis did not have AIDS (CN+ HIV−). Sera from 10 patients with AIDS (all with CD4+T cell counts <200 cells/μl and without cryptococcosis (CN−HIV+) were studied as controls. Among HIV− patients the following underlying disorders were present: renal transplant recipient ($n = 4$), Lupus ($n = 1$), sarcoidosis ($n = 1$), diabetes mellitus ($n = 1$) and none ($n = 3$). All patients with AIDS had CD4+ T cell counts <200 cells/μl. Two patients in the CN+HIV+ group had active co-infection with *M. tuberculosis.* 11/12 CN+HIV+ patients had meningitis while 7/10 CN+HIV− patients had meningitis. The remaining patients had either disseminated disease or pulmonary cryptococcosis. Three patients died within 1 week of diagnosis. They were all CN+HIV+.

Antibody responses to cryptococcal proteins

Reactivity profile. Nine cytosolic proteins of the following molecular masses were most commonly recognized by sera from CN-infected individuals, including: 133, 122, 106, 97, 82, 75, 71, 56, and 40 kDa. These are referred to as the designated proteins. Con-affinity purification indicated that the 122, 106, 82 and 75 kDa proteins were mannosylated (not shown). Among the designated proteins, the proteins most commonly recognized by IgG from CN-infected individuals were of the following size: 133, 122, 82, 75, 56 and 40 kDa (Fig. 1A). Among the designated proteins, the proteins most commonly recognized by IgM from CN-infected individuals were of the following size: 82, 75, 56 and 40 kDa (Fig. 1B). Interestingly, differences in reactivity to specific proteins were present between CN+HIV− and CN+HIV+ sera. The percent of sera demonstrating IgG reactivity to each of the designated bands (except for the 106 kDa protein) was greater for the CN+HIV− group when compared to the CN+HIV+ group (Fig. 1). In addition, three proteins (approximate molecular masses, 30, 22 and 19 kDa) were all commonly (>80%) recognized by IgG from CN+HIV− patients, but uncommonly (<17%) recognized by IgG from CN+HIV+ patients ($p < 0.05$ for each comparison).

Table 1 Patients' characteristics.

	HIV−CN+	HIV+CN+	
n	10	12	
Median age/range (yrs)	35 (20–56)	31 (13–40)	NS
Male to female ratio	3:2	3:1	NS
Meningitis	70%	92%	NS
Acute mortality	0/10	3/12	NS

Recombinant mannoproteins. In previous studies, Teti *et al.* cloned 2 extracellular mannoproteins of *C. neoformans* and demonstrated that sera from infected patients commonly recognized these proteins [13]. When expressed in *E. coli*, these recombinant mannoproteins (115 and 84) were present as 26 and 42k kDa bands, respectively. Among CN+ sera, approximately 68% demonstrated IgG reactivity to both proteins, while among CN- sera approximately 20% demonstrated IgG reactivity to both proteins ($P = 0.02$). No difference in reactivity to these proteins was detected in patients with cryptococcosis based on HIV status.

IgG response. IgG reactivity to cryptococcal proteins was found in all groups. The greatest reactivity to total cytoplasmic proteins was observed in the CN+HIV− group (Fig. 2A and Fig. 3). Among patients with AIDS, there was no significant difference in IgG reactivity to total cytoplasmic proteins based on CN infection status. The median numbers of designated proteins recognized by CN+HIV−, CN+HIV+ and CN−HIV+ sera were as follows: 8, 5 and 0 (Fig. 2A). Overall 65% of CN+ sera (CN+HIV− 100% and CN+HIV+33%), regardless of HIV status exhibited extensive reactivity (≥6 bands) to designated proteins, while no CN− sera exhibited extensive reactivity. Approximately 75% of CN+HIV+ sera exhibited at least moderate reactivity (e.g., ≥4 bands) to designated bands, while 0% of CN−HIV+ sera exhibited at least moderate reactivity to designated bands (Fig. 4) ($P < 0.001$). Interestingly, sera from the 3 of the CN+HIV+ patients who died acutely with cryptococcosis exhibited minimal reactivity (i.e. 0–3 bands) to the designated proteins.

IgM response. Reactivity against total cytoplasmic proteins was found in all groups. The greatest reactivity was observed in the CN+HIV− group (Fig. 2B). The median number of total proteins recognized by

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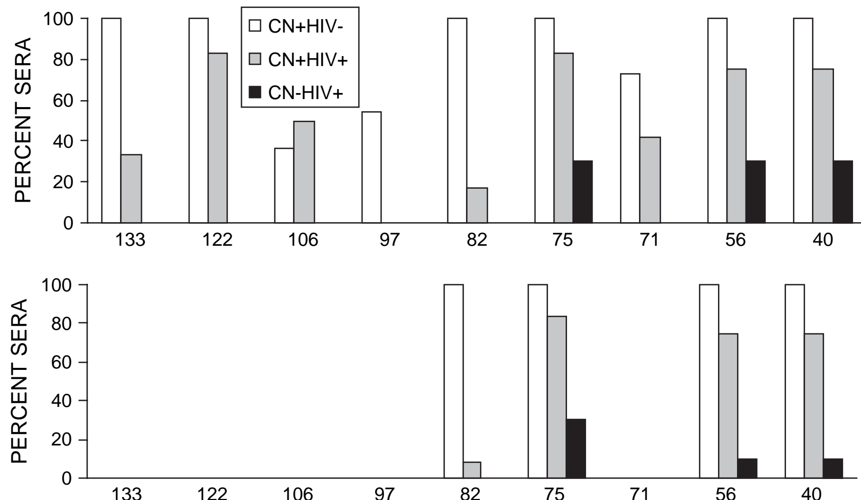

Fig. 1 Specific cytoplasmic proteins recognized by sera from patients. The percentages of sera from three cohorts (CN+HIV −, CN+HIV+, and CN −HIV+) with reactive IgG (A) and IgM (B) for individual cryptococcal cytoplasmic proteins are shown. These 9 cytoplasmic proteins were the most commonly recognized by CN+ sera.

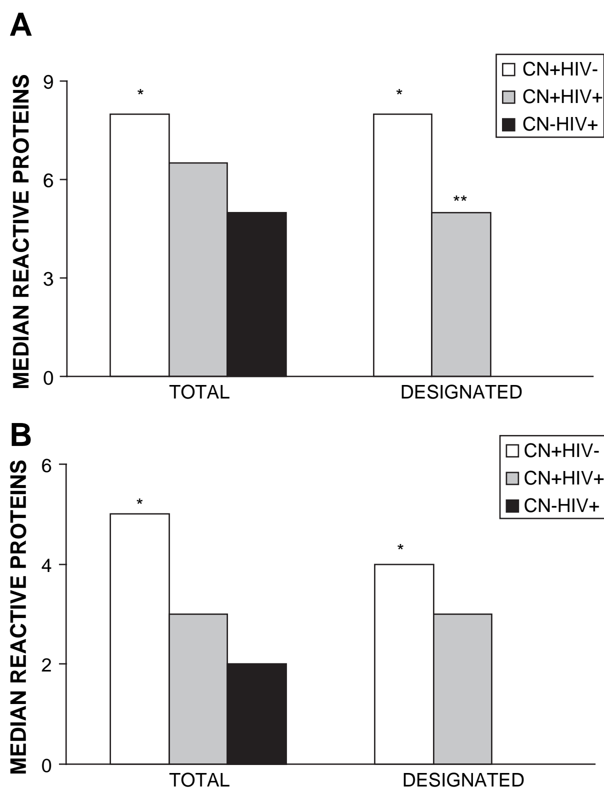

Fig. 2 Median number of proteins recognized by IgG (A) and IgM (B). Median number of cytoplasmic proteins recognized by different cohorts are shown. The left column (TOTAL) represents reactivity to all cytoplasmic proteins. The right column (DESIGNATED) represents reactivity to the 9 commonly recognized proteins. *$P < 0.05$ for comparisons between CN+HIV − vs. CN+HIV+ and CN −HIV+. **$P < 0.05$ for comparison between CN+HIV+ and CN −HIV+.

CN+HIV −, CN+HIV+, and CN −HIV+ sera were as follows: 5, 3 and 2 ($P < 0.005$ for overall comparison). Among patients with AIDS, there was no significant difference in IgM reactivity to total cytoplasmic proteins based on CN infection status. Overall, IgM reactivity to designated proteins was also greatest in the CN+HIV− group. The median number of designated proteins recognized by CN+HIV −; CN+HIV+ and CN −HIV+ sera were as follows: 4, 3 and 0 (Fig. 2B). A greater percent of CN+HIV− sera (100%) and CN+HIV+ sera (75%) exhibited IgM reactivity to at least three designated proteins when compared to CN −HIV+ sera (10%) ($P < 0.001$ for each comparison) (Fig. 4).

IgA and IgE response. No IgA or IgE reactivity to either total or designated proteins was found in any of the groups (not shown).

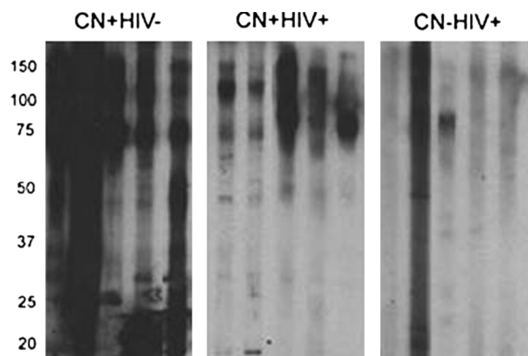

Fig. 3 IgG immunoblots. Representative IgG immunoblots against cytoplasmic proteins for sera from various cohorts are shown. Molecular markers in kDa are shown on the left.

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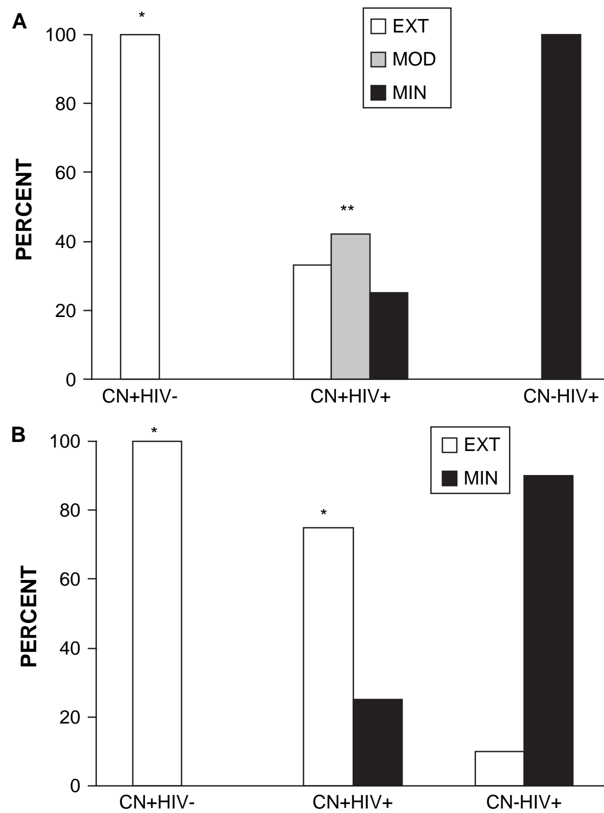

Fig. 4 Percent of sera with reactivity against cytoplasmic proteins. (A) Percentage of sera from various cohorts demonstrating extensive ($\geq$6 bands), moderate (4–5 bands) or minimal (0–3 bands) IgG reactivity to designated proteins (*$P<0.01$ for comparison of extensive reactivity between CN+HIV− vs. CN+HIV+ sera, **$P<0.01$ for comparison of at least moderate reactivity between CN+HIV+ and CN−HIV+ sera). (B) Percentage of sera from various cohorts demonstrating extensive (3–4 bands) or minimal (0–2 bands) IgM reactivity to designated bands. (*P value <0.05 for comparison against CN−HIV+).

IgG reactivity to cytoplasmic proteins of C. albicans. Sera from all three cohorts exhibited extensive IgG reactivity with *C. albicans* proteins. Differences in reactivity between the three cohorts were not detected. In addition, no correlation between reactivity to cytoplasmic proteins of *C. neoformans* and *C. albicans* was observed. Some sera with extensive reactivity to *C. neoformans* exhibited minimal reactivity to *C. albicans* (not shown).

Antibodies to Cryptococcal polysaccharide. IgG levels to cryptococcal polysaccharide were greatest for CN+HIV− sera as reflected in absorbance readings. There was no statistical difference in A_{405} readings between CN+HIV+ and CN−HIV+ sera (Fig. 5). The three patients who died acutely with cryptococcosis had minimal detectable antibody to GXM.

Discussion

Our study demonstrates that Indian patients with cryptococcosis developed specific antibody responses to cryptococcal proteins and GXM. Antibody responses to cryptococcal cytoplasmic proteins consisted of both IgG and IgM, though the complexity of the antibody response was greater for IgG. In analyzing antibody responses to proteins, we focused on reactivity to several proteins that were commonly recognized by sera from infected individuals. Antibody responses to these designated proteins was more sensitive in discriminating between infected and non-infected individuals when compared to reactivity to all cytoplasmic proteins, suggesting that these proteins are *C. neoformans* specific. This observation is consistent with the findings of other investigators who have noted antibody responses to cryptococcal proteins of similar molecular weights in their studies of patients and rodents [4,9,16,17].

Differences in the clinical and histopathologic features of cryptococcal meningoencephalitis between HIV+ and HIV− individuals have been described [18]. In particular, a lack of granulomatous inflammation and increased GXM deposition within the CNS appears to be characteristic of HIV infected patients [19,20]. In our study, we observed that sera from CN+HIV+ individuals consistently demonstrated less reactivity to cryptococcal proteins (with the exception of recombinant mannoproteins) and glucuronoxylomannan when compared to CN+HIV− individuals. While heterogeneity between the various cohorts could affect our analysis, our findings are consistent with those of Pitzurra *et al.* who observed impaired antibody

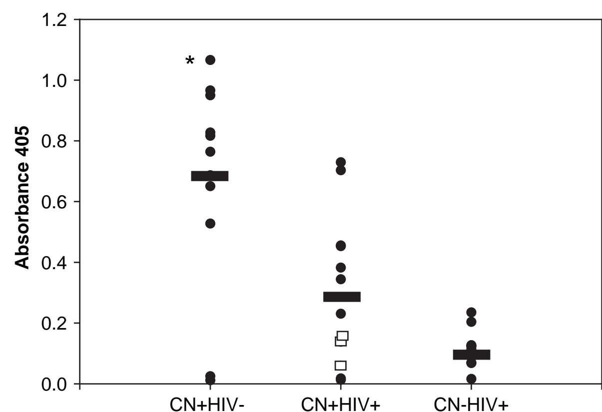

Fig. 5 Antibody to GXM. Individual A_{405} values for ELISA detecting IgG to GXM. Serum was diluted 1:15. Line represents the mean A_{405}. *P value <0.05 for comparison against CN−HIV+. Open squares represent sera from patients who died acutely with cryptococcal disease.

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responses to cryptococcal proteins among HIV infected patients [16]. In addition, several groups have demonstrated higher antibody titers to cryptococcal polysaccharide among HIV− individuals when compared to HIV+ individuals [21–23]. We note, however that circulating cryptococcal polysaccharide can confound the detection and interpretation of antibody titers to cryptococcal polysaccharide [24]. Taken together, these observations suggest patients with HIV infection have a global defect in the host immune response (involving cellular and humoral elements) to *C. neoformans*. The biologic significance of the relative deficiency in antibody response to CN among HIV infected individuals with cryptococcosis is unclear. As noted, antibodies to cryptococcal polysaccharide have protective activity in animals models of cryptococcosis [25,26]. Thus, it could be reasonably argued that specific antibody deficiency contributes to the increased severity and lack of inflammation typically associated with cryptococcosis in AIDS patients. Nonetheless, it is also possible that the lack of antibody reactivity is merely an epiphenomenon, reflecting the overall increased level of immunosuppression and immune dysregulation in AIDS patients.

Several lines of evidence indicate that symptomatic cryptococcosis represents the reactivation of a latent of focus of pulmonary infection in the setting of acquired immunosuppression. The diagnosis of latent pulmonary infection however is difficult given the non-specific nature and/or lack of respiratory symptoms together with the absence of serum polysaccharide, detectable by current methodologies. One potential application of the study of antibody responses to cryptococcal proteins relates to the identification of patients who are asymptomatically infected with *C. neoformans* and at risk for the development of symptomatic disease due to the presence of a co-existing immunosuppressive condition like AIDS and organ transplantation. Other serologic markers that have been linked with an increased risk for the development of cryptococcosis and could possibly be used in conjunction with this approach, expression of certain VH3 determinants in HIV+ patients [27] and GXM-specific IgM levels in transplant recipients [28]. At-risk patients could potentially be preemptively treated with anti-fungal agents or monitored for the development of cryptococcal polysaccharide antigenemia.

In summary, our study demonstrates that cryptococcosis in Indian patients elicits antibody responses to a characteristic profile of cryptococcal proteins and to cryptococcal polysaccharide. Further, we find that specific antibody responses to both the polysaccharide and protein antigens are relatively deficient in HIV+ individuals when compared to HIV− individuals. Additional study is necessary to define the utility of serologic assays in predicting those immunocompromised patients at risk for the development of symptomatic cryptococcosis.

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