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MAJOR ARTICLE

Comparative Analysis of Glucuronoxylomannogalactan and Glucuronoxylomannan Antibody Responses and Their Associations With Cryptococcal Disease Status in People With HIV

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Background. Cryptococcosis remains a major cause of mortality in people with HIV (PWH). While glucuronoxylomannan-binding immunoglobulin G (GXM-IgG) levels have been associated with disease status and survival, the clinical significance of glucuronoxylomannogalactan-binding IgG (GXMGal-IgG) has not been investigated.

Methods. We analyzed serological data from 2 previously reported cohorts of PWH: a prospective asymptomatic South African cohort (67 cryptococcal antigen [CrAg] positive, 130 CrAg negative), and a Vietnamese case-control cohort (30 with symptomatic cryptococcal meningitis [CM], 30 without), both followed for mortality for 6 months. Serum/plasma GXMGal-IgG levels were quantified by enzyme-linked immunosorbent assay and compared to previously reported GXM-IgG levels. Logistic regression adjusted for age, sex, and CD4 count examined associations between antibody levels and CrAg positivity or CM status, while Cox proportional hazards models adjusted for CD4 count estimated associations with time to mortality.

Results. Higher GXMGal-IgG was associated with CrAg positivity (odds ratio, 1.64; 95% confidence interval [CI], 1.14–2.36), not CM status. Among individuals with asymptomatic cryptococcal antigenemia, higher GXMGal-IgG trended toward higher survival (hazards ratio, 0.67; 95% CI, .41–1.09), but this was not statistically significant and no significant survival benefit was observed for those with CM.

Conclusions. GXMGal-IgG was associated with CrAg positivity and showed a modest trend toward survival for individuals with asymptomatic cryptococcal antigenemia but had limited predictive value for CM or mortality. These findings in antigenemia largely parallel previous observations for GXM-IgG, although associations observed were generally weaker. Further studies are needed to clarify the immune response to GXMGal and its potential diagnostic or prognostic significance.

Keywords. cryptococcosis; HIV; GXMGal-IgG; GXM-IgG; mortality; antibody-mediated immunity.

Cryptococcosis, caused by *Cryptococcus neoformans* and *Cryptococcus gattii* species complexes, remains a significant threat to people with human immunodeficiency virus (HIV). A recent clinical trial reported mortality rates up to 25% among individuals with cryptococcal meningitis (CM), despite receiving potent fungicidal therapy [1]. Our understanding of how immune responses beyond T-cell–mediated immunity, particularly antibody immunity, influence cryptococcal pathogenesis and disease outcomes remain limited, and has not been translated into clinical practice.

The main virulence factor of *Cryptococcus* spp is its polysaccharide capsule, composed predominantly of glucuronoxylomannan (GXM), which has a linear α(1,3)-mannan backbone, typically substituted with β(1,2)-glucuronic acid residues at approximately every third mannose unit, along with variable amounts of 6-*O*-acetylation [2, 3]. GXM has been extensively studied for its contribution to immunogenicity and virulence. Compared to controls, higher levels of GXM-binding immunoglobulin G (GXM-IgG) have been associated with CM status in

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cohorts of people with HIV [4, 5] and without HIV [6]. In cryptococcal antigen (CrAg)-positive individuals with HIV, GXM-IgG levels were significantly associated with survival [4], consistent with evidence that GXM-IgG enhances effector cell phagocytosis of *C. neoformans* in vitro and is protective in lethal experimental cryptococcal infection [7, 8].

In contrast, there is little information on human glucuronoxylomannogalactan (GXMGal)-binding antibodies. GXMGal is a structurally distinct polysaccharide composed predominantly of galactose, rather than mannose, but it also contains glucuronic acid, xylose, and *O*-acetyl groups [9]. Thus, it shares glucuronic acid, mannose, and *O*-acetyl groups with GXM. Although GXMGal only accounts for approximately 10% of the capsule by mass, it is the most abundant capsule polysaccharide on a molar basis, with about 2 to 3.5 moles of GXMGal per mole of GXM [10, 11], and can exhibit potent immunomodulatory properties [12]. However, studies on GXMGal immunomodulatory effects are conflicting; some show that it suppresses T and B cells and can induce immunological paralysis [13–15], whereas others demonstrate proinflammatory activities, including dendritic cell activation, increased peripheral blood mononuclear cell tumor necrosis factor-α (TNF-α) production [16, 17], and enhanced Th17 cytokine responses [18].

Early investigations of capsular polysaccharide (CPS) antibodies did not distinguish between GXM and GXMGal reactivity, characterizing detected antibodies as anti-CPS antibodies. In vitro studies showed that rabbit anti-CPS antibodies opsonized *C. neoformans* cells, enhanced antibody-dependent cellular cytotoxicity by human polymorphonuclear leukocytes [19, 20], and promoted *C. neoformans* growth inhibition by murine natural killer cells [21]. Notably, the absence of anti-CPS antibodies in a clinical cohort was associated with increased mortality [22] and passive immunization with a mouse anti-CPS monoclonal IgG protected mice against cryptococcosis [23].

It is currently unknown whether GXMGal elicits an antibody response in humans and, if so, if it also associates with cryptococcal disease status like GXM-IgG. To address this gap, we analyzed GXMGal-IgG in 2 cohorts of people with HIV, 1 from South Africa with and without asymptomatic cryptococcal antigenemia [4], and another from Vietnam with and without CM [6], and compared these findings with previously analyzed results for GXM-IgG. Cryptococcal antigenemia represents an early disease state, likely to reflect fungal reactivation, which can progress to CM. We compared GXMGal-IgG and GXM-IgG levels in these cohorts to seek state-specific antibody profiles and investigated their associations with mortality.

METHODS

Study Cohort

We analyzed serological data from 2 cohorts of people with HIV: (1) a prospective cohort in South Africa consisting of 67 asymptomatic CrAg-positive and 130 CrAg-negative individuals enrolled [4, 24]; and (2) a matched case-control cohort in Vietnam consisting of 30 individuals with symptomatic CM and 30 controls with HIV who were CrAg-negative and without CM (matched 1:1 by age, sex, and nadir CD4 count) [6]. Those with cryptococcal antigenemia were treated with fluconazole, while those with meningitis received an amphotericin B-based regimen. Both cohorts were followed for 6-month mortality (Supplementary Table 1).

Baseline clinical data, including demographic information, CD4 count, and serum CrAg titers (South Africa only), were available from the original studies. Detailed descriptions of each original cohort have been published previously [4, 25]. Serum (South Africa) and plasma (Vietnam) samples collected at baseline were available for laboratory assays. GXM-binding antibody levels reported here were originally measured and published separately for each cohort [4, 6], and are used herein for comparison with GXMGal-IgG.

Exposure Variables

GXMGal-IgG was the primary exposure variable, with GXM-IgG serving as the comparator. Covariables, selected based on their potential biological relevance in predicting outcomes in cryptococcal disease, included serum CrAg titers and CD4 count (measured at enrollment).

Outcome Measures

The outcomes were (1) CrAg positivity or CM status, and (2) all-cause mortality at 6 months among individuals with either positive CrAg or CM.

Cryptococcal Capsular Polysaccharides

Growth Conditions, and Isolation and Purification of C. neoformans *Capsular Polysaccharides*

GXMGal was isolated from a hypocapsular mutant (strain cap67, serotype D) as previously described [26]. Full details regarding strain growth conditions and the procedures for isolation and purification of CPS are provided in the Supplementary Material and have been published elsewhere [9].

Serum GXMGal Antibody Measurements

Antigen-capture enzyme-linked immunosorbent assays (ELISAs) were performed to determine serum/plasma GXMGal-IgM and -IgG binding based on previous methods [4, 6] adapted for GXMGal antibody detection. In brief, high-binding 96-well plates (Corning) were coated with purified GXMGal (10 μg/mL in phosphate-buffered saline [PBS]), blocked (1% bovine serum albumin in PBS), and then incubated with serially diluted serum or plasma (1:10 dilution) samples applied in duplicate. After incubation for 1 hour at 37°C and 3 washes with 0.05% Tween 20 in PBS, bound IgG or IgM was detected using isotype-specific mouse anti-human

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secondary antibodies conjugated to alkaline phosphatase (SouthernBiotech). Following incubation and washes, we used *p*-nitrophenyl phosphatase (Sigma-Aldrich) at 1 mg/mL for color development. Optical density (OD) was measured at 405 nm (SpectraMax, Molecular Devices), and titers were calculated by applying a positivity threshold defined as twice the background OD times the dilution factor.

Serum GXMGal-IgG and GXM-IgG Specificity

Inhibition ELISA assays were performed to investigate antibody specificity and cross-reactivity between GXMGal and GXM. Briefly, a fixed dilution of patient serum (determined by 50% binding to GXMGal or GXM) was preincubated with decreasing concentrations of soluble GXMGal (cap67, serotype D) or GXM (strain 24067, serotype D), then added to either GXMGal- or GXM (24067)-coated plates, followed by the ELISA procedure described above. The degree of competitive inhibition, defined as the decrease in antibody binding in the presence of soluble GXMGal or GXM was determined to assess specificity.

Statistical Analysis

Antibody levels were compared by CrAg or CM status using Mann-Whitney *U* tests. Correlations were assessed using Spearman rank correlation.

As previously done for GXM-IgG [4, 6], we estimated the association between log-transformed GXMGal-IgG and clinical outcomes (CrAg positivity in South Africa and CM status in Vietnam) using multivariable logistic regression in South Africa (adjusting for age, sex, and CD4 count) and conditional logistic regression in Vietnam (accounting for the matched design). Time to mortality analyses used multivariable Cox models adjusted for CD4 count. Goodness-of-fit was assessed for the logistic model, and the proportional hazards assumption was tested for the Cox model.

We additionally evaluated whether GXMGal-IgG provided incremental predictive value for survival beyond GXM-IgG by comparing a baseline Cox model (CrAg titer plus GXM-IgG) to one that also included GXMGal-IgG. Model fit was assessed by the Akaike information criterion (AIC). Discrimination was evaluated using the C statistic.

Odds ratios (ORs) and hazard ratios (HRs) are reported with 95% confidence intervals (CIs). A 2-sided P value of $< .05$ was considered statistically significant. All statistical analyses were performed using Stata/IC 16.1 (StataCorp) and graphs were generated using GraphPad Prism 10 (version 10.4.1, GraphPad Software).

Ethics

Ethical approval was obtained from relevant institutional review boards [24]. All patients provided written or witnessed verbal informed consent, and deidentified blood samples were studied under an Albert Einstein College of Medicine Institutional Review Board-approved protocol (1989–228).

RESULTS

Study Cohort

A total of 257 participants were included. Among South African participants (n = 197), the median age was 39 years (interquartile range [IQR], 32.4–47.1 years), 50.8% were male, and the median CD4 count was 33 cells/mm^3 (12–61 cells/mm^3). In the Vietnam cohort (n = 60), the median age was 35 years (30–39 years), 93.2% were male, and a median CD4 count was 12 cells/mm^3 (6–38 cells/mm^3). The median serum CrAg titer in South African participants was 40 (5–320). Of those with cryptococcosis, 17 (26.6%) in South Africa and 7 (23.3%) in Vietnam died by 6 months.

Bivariate Comparison of GXMGal Antibody Titers by Cryptococcal Disease States

GXMGal-IgG levels were numerically higher among CrAg-positive than CrAg-negative individuals (median, 389 [IQR, 159–1071] vs 351 [IQR, 146.7–486]) but the difference was not statistically significant ($P = .10$). GXMGal-IgG titers did not differ significantly by CM status (Figure 1). GXMGal-IgM titers also did not differ by CrAg status (limited samples; Supplementary Table 2), and no IgA binding to GXMGal was detected.

Correlation of GXMGal Antibodies With Other Markers

A moderate positive correlation was observed between GXMGal-IgG and GXM-IgG (ρ, 0.42; $P < .0001$; n = 253; Supplementary Figure 1). Among those with a positive CrAg, GXMGal-IgG was inversely correlated with serum CrAg titer

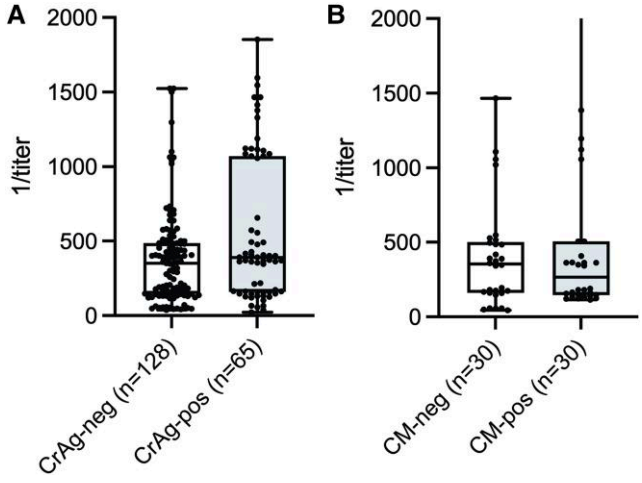


Figure 1. GXMGal-binding IgG titers measured by ELISA in (*A*) CrAg-negative versus CrAg-positive groups in South Africa, and (*B*) CM-negative versus CM-positive groups in Vietnam. Each box plot displays the median (horizontal line), interquartile range (box edges), and overall range (whiskers). Statistical comparisons were performed using the Mann-Whitney *U* test; no statistically significant differences were observed. Abbreviations: CM, cryptococcal meningitis; CrAg, cryptococcal antigen: ELISA, enzyme-linked immunosorbent assay: GXMGal, glucuronoxylomannogalactan: IgG, immunoglobulin G.

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(ρ, −0.27; $P = .03$, n = 65) and weakly positively correlated with CD4 count (ρ, 0.17; $P = .007$; n = 250) (Supplementary Table 3).

Cross-reactivity of GXM and GXMGal

To gain insight into the correlation between GXM-IgG and GXMGal-IgG titers, we determined the ability of soluble GXM to inhibit GXMGal binding and GXMGal to inhibit GXM binding of participant sera. On GXMGal-coated plates, the most inhibition occurred with soluble GXMGal (approximately 75%), whereas inhibition with soluble GXM was less (approximately 41%). On GXM-coated plates, the most inhibition occurred with soluble GXM (approximately 84%), while inhibition with soluble GXMGal was less (approximately 32%) (Supplementary Figure 2).

Association of GXMGal-IgG With CrAg Positivity and CM Status Adjusted for Age, Sex, and CD4 Count

Higher log-transformed GXMGal-IgG titers were significantly associated with CrAg positivity (OR, 1.64; 95% CI, 1.14–2.36; $P = .006$), but not with CM status (OR, 1.34; 95% CI, .76–2.38; $P = .31$) (Figure 2). Previously reported significant associations between log-transformed GXM-IgG titers and CrAg positivity [4] and CM status [6] are included in the figure for comparison.

Association of GXMGal-IgG With Mortality Adjusted for CD4 Count

Higher log-transformed GXMGal-IgG titers trended toward higher survival among individuals with asymptomatic cryptococcal antigenemia (HR, 0.67; 95% CI, .41–1.09; $P = .14$) and those with CM (HR, 0.61; 95% CI, .23–1.62; $P = .32$), although neither reached statistical significance (Figure 3).

For comparative purposes, we additionally estimated the association between log-transformed GXM-IgG and survival among those with CM in Vietnam, which was not reported previously. We observed a consistent, though not statistically significant, trend toward improved survival (HR, 0.58; 95% CI, .24–1.43; $P = .24$), aligning directionally with the significant protective effect of GXM-IgG previously reported in individuals with asymptomatic cryptococcal antigenemia (Figure 3) [4]. We did not include CrAg titers in these models due to potential causal interrelations and mediation but included both for predictive modeling, described below.

Predictive Model for Mortality in the South African Cohort

A Cox model incorporating serum CrAg titer (HR, 1.16; 95% CI, 1.02–1.32; $P = .03$) and GXM-IgG titer (HR, 0.59; 95% CI, .38–.91; $P = .02$) showed good discrimination (C statistic, 0.71; AIC, 129.9). Adding GXMGal-IgG titer further improved model fit (C statistic, 0.73; AIC, 112.7), but the change was not

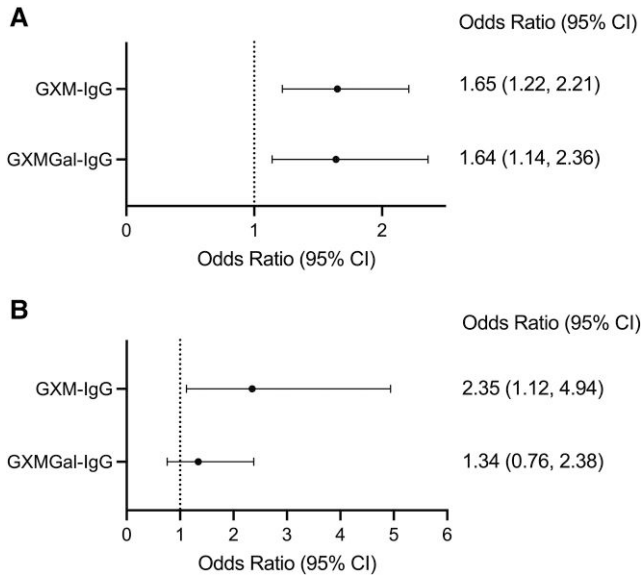


Figure 2. Forest plots showing associations of serum/plasma GXM-IgG and GXMGal-IgG with (*A*) odds of cryptococcal antigen positivity in the South Africa cohort (n = 197), and (*B*) odds of cryptococcal meningitis in the Vietnam cohort (n = 60). Odds ratios and 95% CIs were derived from multivariable logistic regression adjusted for age, sex, and CD4 count (South Africa), and from conditional logistic regression matched for age, sex, and CD4 count (Vietnam). Antibody titers and CD4 counts were log-transformed. The solid vertical line indicates an odds ratio of 1 (no effect), and each circle with error bars indicates the point estimate and corresponding 95% CI. Abbreviations: CI, confidence interval; GXM, glucuronoxylomannan; GXMGal, glucuronoxylomannogalactan; IgG, immunoglobulin G.

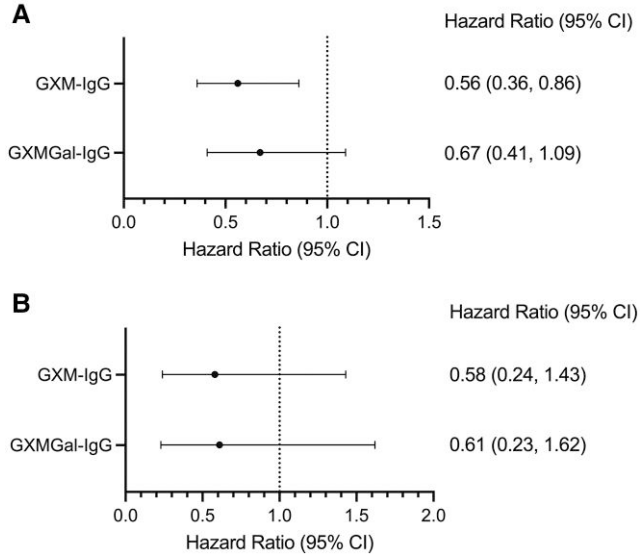


Figure 3. Forest plots showing associations of serum/plasma GXM-IgG and GXMGal-IgG levels with time to mortality among (*A*) cryptococcal antigen-positive individuals (n = 67), and (*B*) individuals with cryptococcal meningitis (n = 30). Hazard ratios and 95% CIs were derived from multivariable Cox proportional hazards models adjusted for CD4 count. Antibody titers and CD4 counts were log-transformed. The solid vertical line represents a hazard ratio of 1.0 (no effect), and each circle with error bars indicates the point estimate and corresponding 95% CI. Abbreviations: CI, confidence interval; GXM, glucuronoxylomannan; GXMGal, glucuronoxylomannogalactan; IgG, immunoglobulin G.

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statistically significant. No significant interaction was observed between the 2 antibody markers, suggesting neither synergy nor antagonism (Supplementary Table 4).

DISCUSSION

In this study, we measured serum/plasma antibody levels of GXMGal, which constitutes approximately 10% of the cryptococcal polysaccharide capsule, in 2 cohorts of people with HIV: those with and without asymptomatic cryptococcal antigenemia, and those with and without symptomatic CM. We found that GXMGal-IgG levels were associated with antigenemia status and showed a trend toward improved survival in individuals with antigenemia. This observation warrants study in additional cohorts, as it suggests that GXMGal-IgG may have diagnostic potential and possibly confer survival benefit. However, GXMGal-IgG levels did not reliably associate with CM status or survival in those with CM. In comparison, as previously reported, higher levels of GXM-IgG were significantly associated with both antigenemia [4] and CM [6] compared to controls, and among antigenemic individuals, higher GXM-IgG levels were significantly associated with survival [4].

The precise localization of GXMGal within the cryptococcal capsule is unclear [27, 28], but it is thought to be part of the innermost capsule layer [29], primarily functioning as an exopolysaccharide during capsule formation in budding, rather than as a main structural component of the mature capsule [28]. Thus, our data are biologically plausible, consistent with the possibility that expression of GXMGal epitopes may be more prominent during cryptococcal reactivation, leading to a higher GXMGal-IgG response to antigenemia, as we observed. However, as antigenemia progresses, fungal burden increases, and CM develops, capsule expansion and remodeling may mask GXMGal epitopes, while GXM becomes the dominant antigen for immune recognition [30]. Serum concentrations of GXM in people with HIV and CM range from 2100 to 6600 ng/mL [31], but GXMGal concentrations are unknown. Nonetheless, our data suggest that GXMGal-IgG may have promise as an early serological indicator of cryptococcal reactivation and antigenemia.

Independent of their clinical associations, GXMGal-IgG and GXM-IgG levels were moderately correlated. This is not unexpected because GXMGal and GXM have shared carbohydrate moieties. GXM and GXMGal contain glucuronic acid [32] and GXM, if isolated with cetyltrimethylammonium bromide (CTAB), may have galactose [33, 34]. GXMGal [9] and GXM serotypes A, B, C, and D, are all *O*-acetylated, albeit to varying degrees [35]. *O*-acetylation is a structural determinant of GXM serotype specificity and *O*-acetyl groups are immunodominant in the GXM antibody response [36, 37]. We found that serum GXMGal binding was inhibited by GXMGal and GXM, but GXMGal inhibition was greater. This is consistent with biochemical analyses revealing GXMGal and GXM share carbohydrate moieties, including *O*-acetyl groups. However, more extensive serological analyses are needed to determine the structure/structures governing cross-reactive GXMGal and GXM binding.

The greater concordance of GXMGal-IgG and GXM-IgG associations with antigenemia compared to CM status, along with trends or significant association with improved survival in antigenemia cases, suggests that a shared epitope (or epitopes) may elicit a beneficial response. The lesser concordance between GXM-IgG and GXMGal-IgG associations with CM status may indicate dominance of different epitopes in CM, reduced antibody production, or increased consumption by a high fungal burden.

An additional implication of our findings relates to diagnostic specificity. All participants with antigenemia were identified as positive by the CrAg lateral flow assay (IMMY), which was also positive in all CM cases [4, 6]. This assay employs 2 anti-GXM monoclonal antibodies: F12D2 (elicited by de-*O*-acetylated serotype A GXM) and 339 (elicited by serotype B GXM), and detects all 4 cryptococcal serotypes [35]. To our knowledge, this assay has not been validated for GXMGal detection. Its designed specificity for GXM may explain the stronger association between CrAg positivity and serum GXM-IgG compared to GXMGal-IgG. Nevertheless, our observation that GXMGal-IgG closely parallels GXM-IgG in cases of antigenemia suggests that the assay may also detect GXMGal. Therefore, our data call for further work to determine whether the IMMY platform may also detect GXMGal clinically and to investigate the hypothesis that the antibody response to GXMGal is state specific. Such studies may also reveal novel biological mechanisms modulating human cryptococcal immunity.

To our knowledge, this is the first study examining GXMGal antibodies in individuals with HIV or in any human cohort. Our data suggest a novel hypothesis, namely, that the antibody response to GXMGal may be more pronounced early in disease, for example, during antigenemia. Investigation of this idea may reveal that measurement of GXMGal-IgG has potential clinical utility as a state-specific serological marker. Importantly, our data also extend our prior observation that higher GXM-IgG levels predict improved survival in antigenemic individuals [4] to GXMGal-IgG, further highlighting the potential diagnostic and prognostic utility of GXM and GXMGal antibody measurements. Thus, our findings have the potential to bridge cryptococcal biology and antibody immunity via measurement of antibodies binding to capsular epitopes that may serve as surrogate markers of disease state and prognosis.

Our study also has limitations. We optimized and confirmed assay specificity, but we note that the GXMGal used in our assays was obtained from an acapsular serotype D *C. neoformans* strain (cap67) [38] and serotype D has more *O*-acetylation than serotype A [35], the dominant serotype in South Africa and Vietnam. Also, it remains uncertain whether GXMGal from acapsular strains

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fully represents wild-type strains [27], or if GXMGal obtained from a serotype D (parent) strain is similar to that of a serotype A strain. Our quantitative analyses do not provide mechanistic information, but functional antibody assays were beyond the scope of this study. Furthermore, our study was limited in being restricted to serological measurements from a single point in time. Peripheral blood cells were not available from the antigenemia cohort, precluding B-cell analysis or linking B-cell phenotypes or activation to serological findings. Nonetheless, the antibody levels we measured are likely to reflect recall responses from B-cell memory populations as expansion of prior antibody responses is a well characterized phenomenon in HIV [39].

Finally, statistical power (approximately 20% based on post hoc calculations) was limited for detecting GXMGal-IgG associations with CM status. Consequently, based on our current data, we remain cautious about overinterpreting GXMGal-IgG findings, even though distinct biological differences in antigen properties lend plausibility to our interpretation. Our findings should therefore be interpreted as exploratory and require validation in larger, longitudinal studies, incorporating mechanistic analyses to further clarify the role of GXMGal-IgG as a potential early indicator of cryptococcal reactivation.

CONCLUSION

Our data show that GXMGal-IgG and GXM-IgG responses are complementary, but GXMGal antibodies may serve as markers of cryptococcal reactivation and antigenemia. While GXM-IgG is a more robust correlate of cryptococcal disease status and survival in people with HIV, GXMGal-IgG might facilitate early identification of fungal reactivation, guiding risk stratification and early intervention.

These findings revisit a question first raised in 1990 by van de Moer and colleagues who produced GXMGal monoclonal antibodies (mAbs) in mice, proposing their potential utility for diagnostics and monitoring the clinical course of cryptococcal disease [40, 41]. Similar work with GXM mAbs has advanced to human trials [42] and preclinical studies suggest possible synergies between capsular-binding antibodies and antifungal therapies [43]. Although early investigations were promising, clinical applications of capsular-binding mAbs are still limited, underscoring the need for further research into the diagnostic and therapeutic potential of CPS-directed antibody responses.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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