

Antibody and B Cell Subset Perturbations in Human Immunodeficiency Virus-Uninfected Patients With Cryptococcosis

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The importance of antibody immunity in protection against *Cryptococcus neoformans* remains unresolved. We measured serum *C neoformans*-specific and total antibody levels and peripheral blood B cell subsets of 12 previously healthy patients with cryptococcosis (cases) and 21 controls. Before and after adjustment for age, sex, and race, cryptococcal capsular polysaccharide immunoglobulin G was higher in cases than controls, whereas total B and memory B cell levels were lower. These associations parallel previous findings in patients with human immunodeficiency virus-associated cryptococcosis and suggest that B cell subset perturbations may also associate with disease in previously normal individuals with cryptococcosis.

Keywords. antibody immunity; B cell subsets; *Cryptococcus neoformans*; HIV-associated cryptococcosis; memory B cells.

The importance of T-cell immunity in protection against *Cryptococcus neoformans* is well understood, but other factors must contribute, particularly in human immunodeficiency virus (HIV)-uninfected patients. In recent studies, macrophage defects, anticytokine autoantibodies, C-type lectin receptor deficiency, and genetic mutations [1–3] have been identified in some

such patients. However, there is a paucity of information on antibody immunity in apparently normal HIV-uninfected patients with cryptococcosis. In contrast, compared with HIV-uninfected individuals without cryptococcosis, HIV-infected individuals with and without cryptococcosis have higher serum cryptococcal capsular polysaccharide glucuronoxylomannan (GXM) immunoglobulin (Ig)G [4–7]. In addition, HIV-infected individuals who developed cryptococcosis were found to have lower peripheral blood IgM memory (CD19⁺CD27⁺IgM⁺) B cell levels than those who did not [7]. Going from bedside to bench, susceptibility to *C neoformans* is increased in mice that lack homologs of IgM memory B cells, B-1 cells [8], or their product, naive IgM (see [9]). This report describes an exploratory study to determine whether previously healthy HIV-uninfected persons with cryptococcosis also exhibit antibody repertoire and B cell subset perturbations previously identified in HIV-infected individuals.

METHODS

Twelve persons with cryptococcosis (cases) were recruited at the University of Alabama, Birmingham, Alabama (UAB). Inclusion criteria were as follows: (1) a current or past diagnosis of cryptococcosis confirmed by culture of *C neoformans* from blood, cerebrospinal fluid (CSF), or a positive cryptococcal antigen (CrAg) in blood or CSF; and (2) a compatible clinical history and/or radiographic abnormalities, in a previously healthy individual with no known underlying disease or immunodeficiency at presentation or upon subsequent evaluation. Exclusion criteria were HIV infection, other conditions of immunodeficiency, organ transplantation, and/or past or current treatment with cytotoxic agents or corticosteroids. Twenty-one healthy individuals (controls) with no history of cryptococcosis and no underlying condition, including HIV, other immunodeficiency, or history of cytotoxic or steroid therapy were recruited at the same center. The research protocol was approved by the institutional review boards of Albert Einstein College of Medicine (Einstein) and UAB. All participants gave written informed consent, after which whole blood samples were collected at UAB and shipped overnight to Einstein.

Serology was performed on sera prepared from whole blood and stored at 4°C until use. Immunoglobulin G, IgM, IgG1, and IgG2 concentrations were determined by radial immunodiffusion (RID, The Binding Site, UK) per manufacturer instructions. The GXM IgG and IgM titers were determined by antigen capture enzyme-linked immunosorbent assay using plates (Costar, Corning Inc., Kennebunk, ME) coated with 10 µg/mL GXM as described previously [5]. Titers were defined as the highest dilution to give a signal ≥2 times wells with all reagents except sera.

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Table 1. Demographic and Clinical Characteristics of Cases

| Cases | Age (y) | Sex | Race | Time From Diagnosis to Blood Draw | Disease Site | CrAg Time of Diagnosis (Latex Agglutination or Lateral Flow) | Serum CrAg When Sera Used (Lateral Flow) |
|-------|------------------|--------|--------|---|--------------|--|--|
| 1 | 46 | M | W | 9 d | CNS | ^a 1:40 (serum) | 1:1280 |
| 2 | 63 | M | W | 2 y, 7 m | CNS | NA | 1:2 |
| 3 | 40 | M | W | 5 y, 21 d | CNS | 1:64 (serum) | 1:2 |
| 4 | 68 | M | W | 9 m | CNS | 1:32 (serum) | 1:256 |
| 5 | 58 | M | W | 1 m | CNS | Unknown | Negative |
| 6 | 58 | F | W | 6 m | CNS | Unknown | 1:5120 |
| 7 | 41 | F | B | 23 d | CNS | ^a 1:80 CSF | 1:5120 |
| 8 | 65 | M | W | 5 m | CNS | 1:32 CSF | 1:5 |
| 9 | 36 | F | W | 27 d | CNS | 1:64 (serum) | Negative |
| 10 | 73 | M | B | 6 d | CNS | ^a 1:20 CSF | 1:10240 |
| 11 | 36 | M | B | 6 d | CNS | ^a 1:160 (serum) | 1:160 |
| 12 | 70 | M | W | 19 d | CNS | ^a 1:20 CSF | 1:5 |
| | 54.5 SD: 13.9 | M: 75% | W: 75% | 277 d (9 m, 7 d) Median: 39 d Range: 6–1846 d | | | |

Abbreviations: B, black; CNS, central nervous system; CSF, cerebrospinal fluid; CrAg, cryptococcal antigen; d, days; F, female; m, months; M, male; NA, not applicable; SD, standard deviation; W, white; y, years.

^aLateral flow.

Flow cytometry was performed with peripheral blood mononuclear cells, used immediately, prepared from anticoagulated whole blood by density gradient centrifugation in Ficoll paque (GE Healthcare Biosciences AB, Uppsala, SE). B- and T-cell subsets were determined with immunostaining and flow cytometry, as described previously [7], using mouse anti-human PE-Cy7 CD19, PerCP-Cy5.5 CD27, APC IgM, and PE IgD for B cells, and mouse anti-human PE-CF594 CD4, Alexa Fluor-700 CD8 (BD Biosciences, Franklin Lakes, NJ) for T cells. Fluorescence Minus One (FMO) and CompBeads (BD Bioscience) compensation controls were used for each reagent. Lymphocyte populations were identified as shown in Supplementary Figure S1 and analyzed using FlowJo software, version 10 (Tree Star).

Fisher's exact and exact Wilcoxon tests were used to analyze differences between cases and controls. Linear regression models were also used to adjust for age, sex, and race, because our sample size was small and the groups were heterogenous. For these analyses, data were checked for normality and log transformation was performed when data were not approximately normal.

RESULTS

The cohort is shown in Tables 1 and 2. Age, race, and sex distributions of cases and controls were not significantly different, but more cases were male ($P = .07$; Fisher test). Cases had higher GXM IgG than controls before and after adjustment, and titers were highest in samples obtained <1 month after diagnosis (Figure 1A, Supplementary Table S1). Acid treatment of (samples with ample remaining) sera to dissociate immune complexes, as described previously [10], did not result in a statistically significant increase in GXM-IgG or IgM, but GXM-IgG

increased in 3 of 4 samples obtained <1 month after diagnosis (Supplementary Figure S2A). There were no significant differences in GXM-IgM, IgM, IgG, IgG1, or IgG2 (Supplementary Figure S2B–D).

Among live lymphocytes, percentages of B (CD19⁺) and memory B (CD19⁺CD27⁺) cells were significantly lower for cases than controls before and after adjustment (Figure 1B and

Table 2. Demographic Characteristics of Controls

| Controls | Age (y) | Sex | Race |
|----------|------------------|-----------|-------------|
| 1 | 43 | F | W |
| 2 | 74 | F | W |
| 3 | 53 | F | B |
| 4 | 32 | F | B |
| 5 | 65 | F | W |
| 6 | 41 | F | W |
| 7 | 53 | F | W |
| 8 | 60 | M | W |
| 9 | 55 | M | W |
| 10 | 39 | F | W |
| 11 | 65 | F | W |
| 12 | 74 | F | W |
| 13 | 60 | F | W |
| 14 | 42 | F | B |
| 15 | 31 | M | W |
| 16 | 43 | M | W |
| 17 | 50 | M | W |
| 18 | 70 | M | W |
| 19 | 51 | M | W |
| 20 | 52 | F | W |
| 21 | 60 | M | W |
| | 53.1 SD: 12.7 | M: 38% | W: 80.9% |

Abbreviations: B, black; F, female; M, male; SD, standard deviation; W, white; y, years.

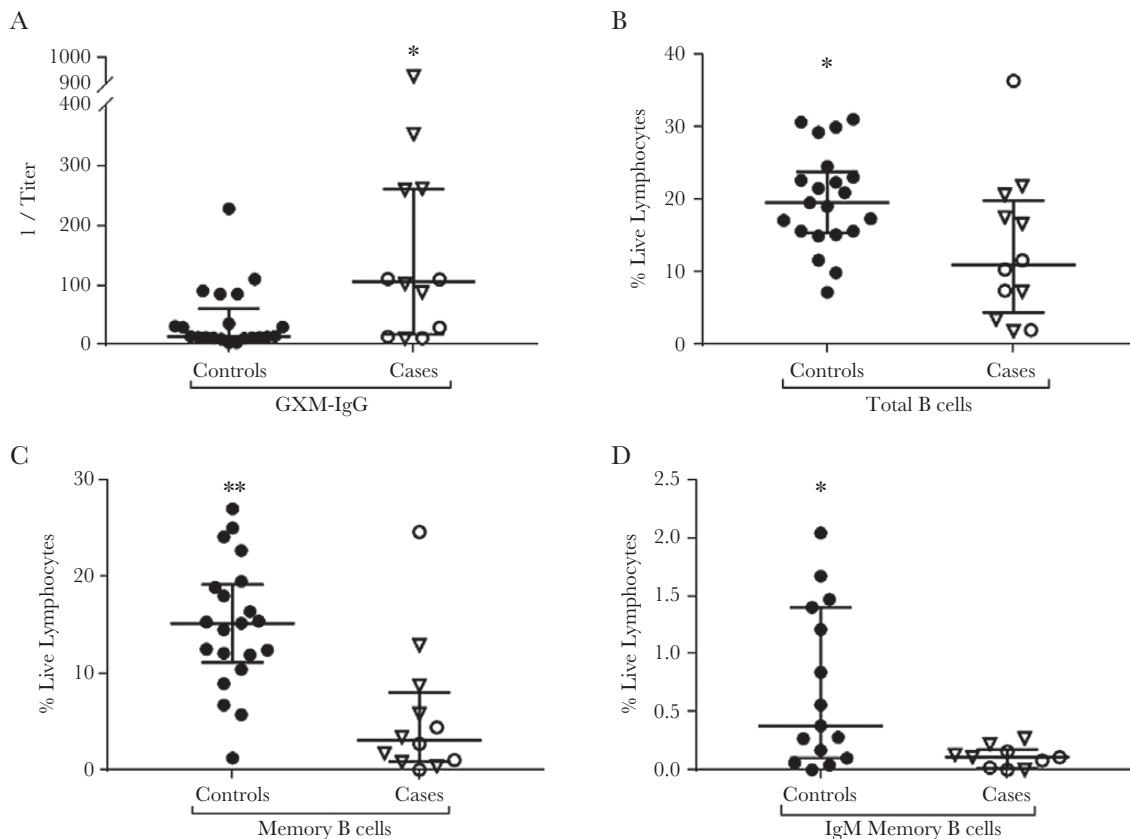


Figure 1. Glucuronoxylomannan (GXM) immunoglobulin (Ig)G and B cell levels. (A) GXM IgG titers (inverse, 1/titer) determined by antigen capture enzyme-linked immunosorbent assay are shown on the y-axis for the groups depicted on the x axis. For each group, the median and interquartile range are shown. Inverted triangles represent cases for whom samples were obtained <1 month after diagnosis, and open circles represent cases for whom samples were obtained ≥ 1 month after diagnosis. *P* values were determined using the Mann-Whitney test. (B–D) B cell subsets identified by flow cytometry are depicted as the percentage (%) of the indicated subset among live lymphocytes on the y axis. (B) Total B (CD19⁺) and (C) memory B (CD19⁺CD27⁺) cells are shown for 21 controls and 12 cases depicted on the x axis. (D) IgM memory B cells are shown for 15 controls and 10 cases depicted on the x axis. Medians with interquartile range are plotted. Inverted triangles represent cases for whom samples were obtained <1 month after diagnosis, and open circles represent cases for whom samples were obtained ≥ 1 month after diagnosis. *P* values were determined using the Mann-Whitney test. *, *P* < .05; **, *P* < .001.

C, Supplementary Table S1). Immunoglobulin M memory B (CD19⁺CD27⁺IgM⁺IgD⁻) cells were significantly lower for the first 10 cases than the first 15 controls enrolled before, but not after, adjustment (Figure 1D, Supplementary Table S1). There was no trend evident in the distribution of B cell levels in samples obtained less than (7 of 12) or greater than or equal to (5 of 12) 1 month of diagnosis (Figure 1B–D, Supplementary S3A–D). There was no significant difference in unswitched (CD19⁺CD27⁺IgM⁺IgD⁺) or switched (CD19⁺CD27⁺IgM⁻IgD⁻) memory B cells between the first 10 cases and 15 controls (Supplementary Figure S3A and B, Supplementary Table S1). These subsets and IgM memory B cells, which require IgD for analysis, could not be determined for 2 cases and 6 controls due to a problem with the IgD reagent. CD4 and CD8 T cells and CD4/CD8 ratios were not significantly different between cases and controls in the unadjusted analysis (Supplementary Figure S3C and D, Supplementary Table S1), but cases had a higher percentage of CD8 T cells after adjustment (Supplementary Table S1).

DISCUSSION

Our data show that HIV-uninfected patients with cryptococcosis and no known immunodeficiency exhibit the same B cell perturbations previously identified in HIV-infected patients with cryptococcosis. Cases also had higher serum GXM-IgG than controls, with the highest titers <1 month of diagnosis. Dissociation of immune complexes, presumably containing GXM, increased titers in 3 of 4 such samples. This suggests that GXM-IgG may reflect the fungal burden, but this cannot be established by our study because fungal burdens, which are not faithfully paralleled by CrAg, were not determined. Nonetheless, given that *C. neoformans* infection is asymptomatic and followed by latency in the setting of normal immunity [11], future studies to determine whether GXM-IgG titers reflect latent infection and/or fungal reactivation may be of clinical utility.

Compared to controls, cases had lower B, memory B, and IgM memory B cell levels. Given that our cohort included cases

with current and previous disease, we do not know whether these levels antedated, posing a risk for, or were an effect of cryptococcosis. We note that it would be impossible to assemble a prospective cohort because there are no known correlates of risk in healthy persons. Nonetheless, our findings mirror a previous study of patients with HIV-associated cryptococcosis in which IgM memory B cell levels were lower than HIV-infected and HIV-uninfected controls and similar in a pre- and postdisease cohort [7]. Immunoglobulin M memory B cell deficiency is a long-recognized, HIV-associated abnormality that has also been linked to poor pneumococcal polysaccharide responses (see [7]). However, we are not aware of data on the effect of active infectious diseases on IgM memory B cell repertoires.

Immunoglobulin M memory B cells, part of the “first-line” of defense against microbes [8], are peripheral blood homologs of splenic marginal zone B (MZB) cells. MZB cells respond to T-independent antigens [12], which include encapsulated pathogens such as *C neoformans*. It is noteworthy that mouse homologs of human IgM memory B cells, B-1 cells [8], bind *C neoformans* and reduce fungal dissemination to the brain [9, 13]. In humans, cryptococcosis has been reported in other conditions with memory B cell deficiency, including X-linked agammaglobulinemia, common variable immunodeficiency (CVID), and sarcoidosis (see [7, 14, 15]). Although CVID and sarcoidosis also feature T cell and/or other defects, current thought holds that cryptococcosis is likely to stem from multiple defects [3].

CD4 T-cell lymphocytopenia portends risk for cryptococcosis [14]. CD4 levels were not significantly different between cases and controls, but our cohort was likely too small to detect subtle differences. Similarly, our cohort was too small to tease out the factor(s) that may have led to higher CD8 levels in cases after adjustment. These points underscore several important limitations of our study. The cohort was small and heterogenous with respect to time of disease onset, the study design was retrospective, and, as an exploratory study, we did not examine B- or T-cell activation, subsets other than those studied previously, or the etiology of lower B cell levels in cases.

CONCLUSIONS

Our data show that total, memory (adjusted analysis), and IgM memory (unadjusted analysis) B cells were lower in previously healthy patients with cryptococcosis than controls. These findings reinforce previous reports of cryptococcosis in conditions with B cell deficiency, including HIV, and animal data linking B-1 cells to resistance to cryptococcosis. Together with the foregoing, this exploratory study illuminates possible associations between B cell subsets and protection against *C neoformans* that warrant further investigation in patients with active and previous cryptococcosis, those at high risk, including HIV-infected patients, solid organ transplant recipients, and those receiving biological response modifiers. We hope this report will encourage (1) clinicians to consider studies of antibody immunity

and B cells in evaluating patients with cryptococcosis and (2) researchers to delve further into the role that B cells may play in immunity to *C neoformans*.

Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

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