Serologic Evidence for Cryptococcus neoformans Infection in Early Childhood

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ABSTRACT. Objective. Cryptococcus neoformans is an important cause of central nervous system infection in adults with acquired immunodeficiency syndrome (AIDS) but an unusual cause of disease in children with AIDS. The basis for this age-related difference in incidence is not known but may be caused by differences in exposure or immune response. The objective of this study was to determine whether the low prevalence of cryptococcal disease among children is related to a lack of exposure to C neoformans.

Methods. Sera were obtained from 185 immunocompetent individuals ranging in age from 1 week to 21 years who were being evaluated in an urban emergency department. Sera were analyzed for antibodies to C neoformans and Candida albicans proteins by immunoblotting. Immunoblot patterns were compared with those obtained from sera of patients with cryptococcosis (n = 10) and workers in a laboratory devoted to the study of C neoformans. The specificity of our results was confirmed by several approaches, including antibody absorption and blocking studies. Sera were also analyzed for the presence of cryptococcal polysaccharide by both enzyme-linked immunosorbent assay and latex agglutination assays.

Results. Sera from children 1.1 to 2 years old demonstrated minimal reactivity to C neoformans proteins. In contrast, the majority of sera from children >2 years old recognized many (≥6) C neoformans proteins. For children between 2.1 and 5 years old, 56% of sera (n = 25) reacted with many proteins, whereas for children >5 years old (n = 120), 70% of samples reacted with many proteins. Reactivity was decreased by absorbing sera with C neoformans extracts or by preincubating blots with sera from experimentally infected but not from control rats. Reactivity to C neoformans proteins did not correlate with reactivity to C albicans proteins, which was common in sera from children between the ages of 1.1 and 2 years. Cryptococcal polysaccharide was detected at a titer of 1:16 (~10 ng/mL) in the sera of 1 child, a 5.6-year-old boy who presented to the emergency department with vomiting.

Conclusions. Our findings provide both indirect and direct evidence of C neoformans infection in immunocompetent children. Our results indicate that C neoformans infects a majority of children living in the Bronx after 2 years old. These results are consistent with several observations: the ubiquitous nature of C neoformans in the environment, including its association with pigeon excreta; the large number of pigeons in urban areas; and the increased likelihood of environmental exposure for children once they have learned to walk. The signs and symptoms associated with C neoformans infection in immunocompetent children remained to be determined. Primary pulmonary cryptococcosis may be asymptomatic or produce symptoms confused with viral infections and, therefore, not recognized as a fungal infection. Our results suggest that the low incidence of symptomatic cryptococcal disease in children with AIDS is not a result of lack of exposure to C neoformans. These findings have important implications for C neoformans pathogenesis and the development of vaccine strategies. Pediatrics 2001;107(5). URL: http://www.pediatrics.org/cgi/content/full/107/5/e66; Cryptococcus neoformans, fungal infection, human immunodeficiency virus, serology.

ABBREVIATIONS: AIDS, acquired immunodeficiency syndrome; HIV, human immunodeficiency virus; ELISA, enzyme-linked immunosorbent assay.

Cryptococcus neoformans is remarkable for its ability to cause disease in immunocompromised individuals. C neoformans disease occurs in 6% to 8% of adult patients with acquired immunodeficiency syndrome (AIDS), making it the most common central nervous system fungal infection associated with advanced human immunodeficiency virus (HIV) infection. In contrast, the prevalence of C neoformans infection in children with AIDS is ~1%. The basis for this discrepancy is not understood but could be caused by differences in exposure or in the immune response.

C neoformans infection is believed to be acquired via inhalation of aerosolized particles from the environment. Serologic studies using a polysaccharide-based enzyme-linked immunosorbent assay (ELISA) suggest that subclinical infection is very common among immunocompetent adults. Recently, we have used an immunoblot technique to confirm these findings. The potential advantages of this approach relate to the enhanced specificity of analyzing antibody responses to multiple proteins. Furthermore, polysaccharides are typically poor immunogens for children, which could limit the sensitivity of cryptococcal polysaccharide-based assays in pediatric studies. In this study, we used this approach to determine whether immunocompetent children are infected with C neoformans and to define the age at which infection occurs.

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METHODS

Study Population

One hundred eighty-five individuals ranging in age from 1 week to 21 years who underwent diagnostic blood studies while being evaluated at the pediatric emergency department at the Montefiore Medical Center in the Bronx from July 1998 to February 1999 had their excess blood collected from the laboratory for this seroprevalence investigation. Age, ethnicity, sex, chief complaint, and presence of chronic illnesses were recorded. Immuno-compromised children at risk for cryptococcosis were excluded. Sera from 10 patients with cryptococcosis, with and without AIDS, were also obtained. For the 5 patients with AIDS, sera were obtained from individuals in the Multi-Center AIDS Cohort Study who were found to have cryptococcal polysaccharide in their sera.7 Sera from patients with cryptococcosis but without HIV were donations. Sera were also obtained from individuals (n = 11) who worked for at least 2 months in a laboratory devoted to the study of C neoformans at the Albert Einstein College of Medicine (Bronx, NY). All samples were obtained according to the practices and standards of the institutional review boards at Albert Einstein College of Medicine and Montefiore Medical Center. Rats were endotracheally infected with C neoformans strain H-99 as described8 and sera were obtained at 3 months of infection.

Fungal Strains

C neoformans strains 24067 (serotype D), H-99 (serotype A), and CAP 67 (unencapsulated strain) were obtained from the American Type Culture Collection (Manassas, VA), John Perfect (Durham, NC), and Eric Jacobson (Richmond, VA), respectively. Candida albicans SC5314 was obtained from M. Ghannoum (Cleveland, OH).

Fungal Protein Extracts

Crude protein extracts of C neoformans and C albicans were obtained as described previously.6 Briefly, fungi were grown in Sabouraud dextrose broth for 1 to 2 days at 30°C or 37°C. Cells were collected by centrifugation, disrupted by serial vortexing with glass beads, and sonicated in a buffer containing protease inhibitors.

Immunoblotting

Immunoblotting of sera against fungal protein extracts was performed as described.6 Immunoglobulin G antibodies reactive to cryptococcal protein were detected with alkaline phosphatase-labeled goat anti-human immunoglobulin G (Southern Biotechnology Associates, Birmingham, AL). For all blots, serum from a laboratory worker was used as a positive control and omission of sera was used as a negative control. Several approaches were taken to ensure the specificity of our methods. Absorption studies were performed in which representative sera were incubated with C neoformans protein extracts, either in solution or immobilized on nitrocellulose membranes, and then blotted against C neoformans proteins. In addition, in some studies C neoformans blots were incubated with sera from uninfected rats or rats with experimental pulmonary cryptococcosis,6,9 before incubation with human sera. In previous studies, we have shown that the sera from uninfected rats exhibit no reactivity against cryptococcal proteins, whereas the sera from rats infected with C neoformans react with multiple proteins.6 For C albicans immunoblots, 81 representative serum samples were analyzed.

Serum Polysaccharide Levels

Sera were digested with pronase and screened for cryptococcal polysaccharide by latex agglutination using the CALAS system (Meridian Diagnostics, Cincinnati, OH). Specimens that were found to have polysaccharide on initial testing were retested to confirm initial findings using a previously described capture ELISA.10

Defining a Reactive Pattern

Western blot profiles of individuals with cryptococcosis and workers in a C neoformans laboratory were used to define a reactive pattern. Laboratory workers were used because of an increased likelihood of exposure and previous studies that have shown strong delayed-type hypersensitivity reactions to C neoformans extracts in these individuals.11 These sera most commonly recognized 9 proteins with approximate molecular weights of: 139, 116, 111, 106, 98, 85, 74, 64, and 46 kDa (Fig 1). Sera from 60% of patients with cryptococcosis (n = 10) and from 91% of individuals working in a C neoformans laboratory (n = 11) reacted with many (≥6) of the 9 designated proteins (Fig 1). Antibodies to these proteins in reactive samples were present regardless of which strain of C neoformans was used to prepare the extract (24067, H-99, or Cap 67) or the temperature at which C neoformans was grown (30°C vs 37°C). Most of these bands were of the same approximate size as those previously described in patients with cryptococcosis.12 Furthermore, 6 of these bands (116, 111, 106, 85, 74, and 64 kDa) were present regardless of serotype (D or A). The 24067 strain of C neoformans was used to prepare the extract.

Fig 1. Immunoblots against C neoformans extracts. Representative immunoblots of sera from: HIV+ and HIV− patients with cryptococcosis, individuals who work in a laboratory devoted to the study of C neoformans, and children without a defined exposure to C neoformans. Arrows point to bands that were studied.
kDa) were of the same approximate size as those recognized by the sera from rats with experimental cryptococcosis. 6

Data Analysis

The number of reactive bands in the immunoblots of sera from children of various ages were compared by the Mann-Whitney rank sum test. Sera reacting with ≥6 of the 9 designated bands (≥67%) were classified as reacting with many bands. Sera reacting with 3 to 5 bands were classified as reacting with some, and sera reacting with 0 to 2 bands were classified as reacting with few to no bands. χ² analysis was used to compare the reactivity between the various age groups according to this classification scheme. P values ≤.05 were considered significant.

RESULTS

Patient Demographics

Mean and median ages of children were 9.5 and 9.2 years, respectively. Fifty-two percent of the children were Hispanic, 31% black, 8% white, 3% Asian, and 6% were either nonspecified or listed as other.

Children

Variation among the reactivity of sera from children against C neoformans proteins was observed (Fig 1). Sera from children 1.1 to 2 years old demonstrated the least reactivity among the various age cohort groups (Fig 2). The majority of samples (64.3%) from this age group (n = 14) reacted with few or no (0–2) protein bands, with a median of 1 recognized protein. Sera obtained from older children demonstrated significantly more reactivity. For children between the ages of 2.1 and 5 years old, 56% of sera (n = 25) reacted with many proteins, with a median of 6 recognized proteins (Mann-Whitney rank sum test, P = .008; χ², P = .009). For children older than 5 years of age (n = 120), 70% of samples reacted with many proteins, with a median of 7 recognized proteins.

In general, the pattern of reactivity against individual proteins paralleled the overall increase in reactivity for sera from the various age groups (Fig 3). The percent of sera exhibiting reactivity against several proteins (139, 116, 111, 106, and 98 kDa) was particularly low for children between the ages of 1.1 and 2 years. Reactivity against these proteins was lower in this age group compared with sera from all age groups including children ages 0 to 1 year (Mann-Whitney rank sum, P = .036).

Immunoblotting Against C albicans

Children

Exposure to C albicans, another fungal pathogen, is common among children. We analyzed the reactivity of our study samples against C albicans to determine whether the observed reactivity against C neoformans was the result of antibodies against C albicans. The reactivity of sera from children for C albicans proteins was greater in both intensity and diversity than for C neoformans proteins. Many sera produced smears in certain regions of the blot, making a determination of individual bands in these regions not possible. For this analysis, these smears were considered as individual proteins, although they likely represent mul-

Fig 2. Reactivity of sera from children against C neoformans (A and B) and C albicans (C and D) extracts. The percentage of sera from children from the various ages demonstrating reactivity against many (≥6), some (3–5), or few to none (0–2) proteins for C neoformans extracts (A) or C albicans (C) extracts are shown. The median number of bands recognized by sera from children when blotted against C neoformans and C albicans are shown in B and D, respectively.
tiple reactive proteins. In contrast to the low level of reactivity observed for sera against \textit{C neoformans} in the 1.1- to 2-year-old age group, 75% of samples (\( n = 12 \)) from this age group recognized many (\( \geq 6 \)) \textit{C albicans} proteins (Fig 2). The median number of proteins recognized by sera in this age group was 8.

The least amount of reactivity against \textit{C albicans} was observed in children in the 0- to 1-year-old age group. In this age group, 39.1\% of samples (\( n = 23 \)) reacted with many proteins. Of the 11 sera that reacted with few to no \textit{C albicans} proteins, 3 samples recognized many \textit{C neoformans} proteins.

### Absorption Studies

Incubation of sera with \textit{C neoformans} protein in solution or by serial blotting reduced the reactivity of sera against \textit{C neoformans} proteins (data not shown). In addition, preincubation of \textit{C neoformans} blots with sera from rats with experimental cryptococcosis but not uninfected control rats resulted in a reduction in reactivity (Fig 4).

### Serum Polysaccharide

Of the 185 serum samples, 1 sample was found to contain polysaccharide by both latex agglutination and ELISA techniques. This serum came from a 5.6-year-old boy who presented to the emergency department with vomiting. The latex titer and antigen concentration for this sample were 1:16 and 10 ng/mL, respectively. The sera of this child reacted with 8 of the 9 designated bands.

### DISCUSSION

In this study, we identified 9 bands that were most commonly recognized by sera from individuals with cryptococcosis and individuals with a likelihood of exposure to \textit{C neoformans}. Most of these bands were of the same approximate size as those previously described in other human and animal studies.\textsuperscript{6,12} Interestingly, some patients with cryptococcosis (3/5 HIV\(^+\) and 1/5 HIV\(^-\)) exhibited a diminished antibody response to \textit{C neoformans} that likely reflects the great degree of immunosuppression in these patients. Among immunocompetent children without a defined exposure to \textit{C neoformans}, sera from children aged 1.1 to 2 years demonstrated minimal reactivity against \textit{C neoformans} proteins. We have previously shown a high prevalence of antibodies reactive to these bands in sera from HIV\(^-\) and HIV\(^+\) individuals without cryptococcosis.\textsuperscript{6} The absence of reactivity in the sera of these children served as a negative control for our assay. This decreased reactivity in the sera was particularly prominent for several bands (\( \sim 139, 46, 64, 74, 85, 98, 106, 111, 116, 139 \)).
116, 111, 106, and 98 kDa). Overall, the observed age-related pattern of reactivity against C neoformans resembles that described for infections caused by a variety of pediatric pathogens, where there is an initial decrease in sera reactivity attributable to waning maternal antibody followed by an increase in reactivity associated with subsequent infection.\textsuperscript{13,14} Reactivity against C albicans is increased in the sera from children ages 1.1 to 2 years relative to the first year of life. This presumably reflects acquisition of C albicans in the first year of life followed by an immune response to candidal antigens.

Our findings suggest that most children residing in the Bronx are infected with C neoformans after the second year of life. This age corresponds with increased mobility and independence of children and increased environmental exposure. C neoformans is ubiquitous in the environment and can be isolated from soil contaminated with bird excreta as well as the surrounding air.\textsuperscript{15} Regional differences in the incidence of symptomatic cryptococcal infection for both HIV\textsuperscript{+} and HIV\textsuperscript{−} individuals have been described.\textsuperscript{16} It is conceivable that the high density of people and pigeons in an urban environment like the Bronx contributes to the early age and high prevalence of C neoformans infection.

Although our findings could be interpreted to represent the acquisition of cross-reactive antibodies, several lines of evidence suggest that the observed antibody responses are the result of C neoformans infection. First, most of the proteins recognized by sera from children were of the same approximate size as those recognized by animals and humans with cryptococcosis.\textsuperscript{6,12} Second, the observed reactivity could be reduced by absorption with C neoformans proteins. Third, preincubation of blots with sera from rats with cryptococcosis but not controls reduced reactivity of pediatric sera. Fourth, the age distribution of antibody responses against C neoformans was remarkably different compared with the response against C albicans, which is a frequent cause of superficial skin infections in children. The increase in reactivity against C albicans in the 1.1- to 2-year old age group did not correlate with an in increase in reactivity against C neoformans, consistent with the notion that these responses are to independent microorganisms. Furthermore, sera from 3/11 children demonstrated minimal reactivity to C albicans proteins but reacted with many C neoformans proteins strongly arguing against cross-reactive antibodies as the explanation for our results. Finally, the detection of cryptococcal polysaccharide in the serum of 1 patient provided direct evidence of cryptococcal infection. Although rheumatoid factor reactions can cause false-positive latex agglutination tests, the use of pronase essentially eliminates this reaction.\textsuperscript{17} Furthermore, this is not a problem in the ELISA system that was used as a confirmatory method. False-positive latex agglutination tests have been reported in patients with disseminated trichosporon infection.\textsuperscript{18,19} Our patient, however, was immunocompetent and not at risk for this type of infection.

The findings of this study are consistent with the studies conducted by Abadi et al\textsuperscript{20} who found reactive antibodies to the cryptococcal polysaccharide in sera of children from the Bronx who did not have clinical cryptococcosis, regardless of HIV status. These antibodies were serogroup-specific and could not be absorbed by incubation with pneumococcal polysaccharide. In contrast, Speed and Kaldor\textsuperscript{21} found a low prevalence (~4\%) of antibodies reactive with cryptococcal polysaccharide in the sera of Australian children (>5 years old) compared with a prevalence of ~65\% in the sera obtained from adult blood donors. The basis for the discrepancy between these studies could be related to the differences in exposure and/or differences in methodology.

The diversity and persistence of the antibody profile in older children are consistent with either persistent or repeated C neoformans infections. We observed a similar pattern of antibody persistence in a rat model of chronic pulmonary infection.\textsuperscript{6,9} In this model, endotracheally infected rats developed small subpleural and interstitial granulomas in which C neoformans persisted for as long as 18 months after inoculation. Furthermore, an increasing amount of clinical and laboratory evidence supports the concept that C neoformans causes latent pulmonary infections that can be reactivated to produce central nervous system infection later in life. In the 1970s, Haugen and Baker\textsuperscript{22} described pulmonary granulomas containing C neoformans in a subset of autopsy studies performed on patients who lacked a history of C neoformans exposure. Recently, Fleuridor et al\textsuperscript{7} demonstrated the presence of antibodies to cryptococcal polysaccharide in HIV-infected patients who subsequently developed cryptococcosis. Finally, molecular typing studies have described significant discrepancies between environmental and clinical isolates. Molecular studies indicate that cryptococcosis in African individuals living in Europe are the result of reactivation of latent infection.\textsuperscript{23} In contrast, a case of apparent C neoformans infection from a pet cockatoo was recently reported\textsuperscript{24} suggesting that for some patients symptomatic C neoformans infection represents either a primary progressive infection or a re-infection.

**CONCLUSION**

Our findings indicate that children in an urban setting acquire antibodies reactive with C neoformans proteins after 2 years old and that these antibodies persist throughout childhood. These findings are consistent with either repeated or persistent C neoformans infection. A prospective study is needed to define the symptoms associated with primary C neoformans infection in children. Primary pulmonary infection could be asymptomatic or could produce symptoms confused with viral infections and, therefore, not recognized clinically, similar to infections caused by other endemic mycoses. The findings of this study suggest that the low incidence of symptomatic cryptococcal disease in children with AIDS is not the result of a lack of exposure to C neoformans; nevertheless, quantitative and qualitative differences in the exposure to C neoformans between children and adults may exist. Persistent C neoformans infection could have important implications. In a rat model,
persistent pulmonary cryptococcosis is associated with down-modulation of both humoral and cellular inflammatory responses. Furthermore, infection at an early age will have important implications on the approach to vaccination to prevent \textit{C. neoformans} infection.

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