Long-lasting, specific immunologic unresponsiveness associated with cryptococcal meningitis.

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Research Article

A sensitive radioimmunoassay and an antibody class-specific enzyme-linked immunosorbent assay were used to determine whether patients cured of cryptococcosis responded normally to immunization with cryptococcal capsular polysaccharide (CPS) and type III pneumococcal polysaccharide. 10 normal volunteers and 8 patients who had been cured of cryptococcal meningitis and who had been cured of cryptococcal meningitis and who had no serious underlying diseases were immunized with both antigens. Geometric mean titers to CPS measured by radioimmunoassay were 1:1 in both groups before vaccination, but were 1:3 in patients and 1:119 in controls following immunization (P less than 0.01, Student's t test). Analysis of the class-specific response to immunization with CPS found little anti-CPS IgG or IgA. Geometric mean postvaccination IgM titers were 1:31 in patients and 1:238 in controls (P less than 0.01). Responses to immunization with type III pneumococcal polysaccharide were similar in patients and controls, with IgA, IgM, and IgG mean titers of 1:1129, 1:369, and 1:158 in patients and 1:1504, 1:1039, and 1:163 in controls (P greater than 0.2 for each antibody class). Cured cryptococcal meningitis is often associated with prolonged specific immunologic unresponsiveness.

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Long-lasting, Specific Immunologic Unresponsiveness

Associated with Cryptococcal Meningitis

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each antibody class).

Cured cryptococcal meningitis is often associated
with prolonged specific immunologic unresponsive-
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INTRODUCTION

Although conditions that cause defects in cellular im-
munity have been associated with an increased risk of
developing cryptococcosis (1), the precise role of the
humoral immune system in host defense against *Cryp-
tococcus neoformans* remains conjectural. In 1974
Diamond and Bennett (2) reported that 60% of patients
with cryptococcal meningitis did not have detectable
levels of antibody against the type-specific capsular

polysaccharide of the yeast. This study, which used an
indirect fluorescent assay for IgG antibody, also found
that the absence of anticryptococcal antibody at the
initiation of therapy was a significant risk factor for
treatment failure.

The reason or reasons that patients with cryptococ-
cal meningitis fail to produce antibody against cryp-
tococcal polysaccharide (CPS)[1] remains uncertain.
Such a trait might be inherited; however no outbreaks
of cryptococcosis among kindred have been reported.
Whereas investigators have found that injections of
CPS induced a state of specific immunologic unre-
sponsiveness in animals (3, 4), no studies have been
conducted, to our knowledge, to determine whether
absence of detectable antibody to CPS in sera of cryp-
tococcosis patients is a result of specific immunologic
unresponsiveness. The study to be reported here used
a sensitive radioimmunoassay to address two different
questions. First, do cured cryptococcosis patients have
even less antibody to CPS than the normal population?
Secondly, will these patients make a subnormal re-
sponse when immunized with CPS?

METHODS

Normal volunteers. Sera from normal volunteers were
obtained from Dr. John Robbins (Bureau of Biologics, Food
and Drug Administration), from Dr. James C. Parke (Uni-
versity of North Carolina, Charlotte, NC), from Dr. Rafael
Dolin (University of Vermont, Burlington, VT), from Dr.
Paul Holland (Blood Bank, Clinical Center, National Insti-
tutes of Health), and from members of the Laboratory of
Clinical Investigation. The age of these volunteers ranged
from 2 to 72 yr of age.

In a separate study, college age volunteers were hospital-
ized at the Clinical Center, and, after providing informed
consent, were immunized with the polysaccharide vaccines
to be described below.

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[1] *Abbreviations used in this paper:* CPS, cryptococcal
polysaccharide; ELISA, enzyme-linked immunosorbent as-
say; RIA, radioimmunoassay; SSSIII, type III pneumococcal
polysaccharide.

Cryptococcosis patients. Sera from 51 cryptococcosis patients were selected because the patients met the following criteria: (*a*) All had culture-proven cryptococcosis. (*b*) All patients were infected with serotype A *C. neoformans.* (*c*) Sera were available that had been obtained both before therapy had been administered and 1 yr following the completion of therapy. (*d*) All had been patients of the Clinical Mycology Section, Laboratory of Clinical Investigation so that the complete records were available on each patient.

In addition, eight patients who were still being actively followed at the Clinical Center following therapy for cryptococcal meningitis were included in the immunization studies. These patients met the following criteria: (*a*) All had been cured of cryptococcal meningitis, and had been free of evidence of infection for a minimum of 1 yr following completion of therapy for cryptococcal meningitis. (*b*) All had been free of cryptococcal antigen in both serum and cerebrospinal fluid for at least 1 yr before immunization.

Other characteristics of the patients who were vaccinated were as follows. Their mean age was 46 yr (range, 32–70 yr). An average of 5.5 yr (range 1.5–11.5 yr) had elapsed since completion of therapy. All eight of these patients had cryptococcal antigen in CSF and four of eight had cryptococcal antigen in serum at the time of diagnosis as measured by the latex agglutination test (5). Antigen was not detectable in these patients, in either serum or CSF, for an average of 3.4 yr (range, 1.1–8.6 yr) at the time of CPS injection. One of the patients had serum antibody at the time of diagnosis and one patient developed antibody during treatment for his infection (titers 1:2 and 1:1, respectively). The remaining six patients had never produced detectable antibody in response to their infections. All eight patients were in good health. None had hematologic or reticuloendothelial malignancies, and none were receiving immunosuppressive chemotherapy.

Radioimmunoassay (RIA). Type A CPS was prepared from culture supernatant (6) and contained 0.17% N by microkjeldahl distillation and nesslerization. CPS was activated with CNBr using pH 11.0 and a weight ratio of CPS:CNBr of 5:1. Tyraminylation was carried out at pH 8.5. Free tyramine was removed by dialysis against 400 vol of toluene saturated phosphate-buffered saline with four changes over 4 d. Tyramine content of the tyraminyl-CPS was measured by Lowry (7) and CPS was measured by anthrone reagent (8) using a CPS standard. Assuming molar ratios of hexose:pentose of 2:1 (9), we calculated that one tyramine group was introduced per 139 monosaccharide units. After iodination with ^{125}I and chloramine-T, the ^{125}I-tyraminyl CPS (^{125}I-tyr-CPS) was dialyzed against four changes of NaCl (0.15 M), diluted with phosphate buffer (0.01 M, pH 7.4), and chromatographed on DEAE cellulose (Whatman DE 52, Reeve, Angel Company, Clifton, NJ) that had been equilibrated with the same buffer. CPS was eluted with 0.2 M NaCl in 0.01 M phosphate buffer (pH 7.4). Specific activity was calculated to be 2.0×10^7 cpm/μg CPS, assuming complete recovery of polysaccharide from the DEAE column. All samples in the study were counted in a gamma spectrometer (model 1185, Nuclear Chicago Corp., Chicago, IL) having an efficiency of 86% for ^{125}I. Assuming an average CPS molecular weight of one million (9), ~2 mol of ^{125}I were introduced per mole of CPS. Approximately 95% of the radioactivity could be precipitated by high titer antibody in the assay.

RIA for antibody to CPS was performed using the Farr test (10). Using 20% binding as the lower limit of positivity, this assay is sensitive to 136 ng/ml of anti-CPS antibody.

In the vaccination experiments, results were compared using log base 2 antibody titers. A least-squares line of best-fit was obtained for percent bound as a function of log dilution. The intercept of 20% bound and the least-squares line was used as the titer.

Enzyme linked immunosorbent assay (ELISA). ELISA were used to detect the class-specific antibody response to immunization with both CPS and type III pneumococcal polysaccharide (SSSIII). The procedures used were modifications of the original ELISA procedures described by Engvall and Perlmann (11). Wells in polystyrene microtiter substrate plates (Dynatech Laboratories, Alexandria, VA) were coated with 100 μl of solution containing either 12 μg/ml of CPS or 7 μg/ml of SSSIII in 0.05 M carbonate buffer (pH 9.6) for 18 h at 37°C. Serum dilutions were incubated in duplicate for 2 h at 37°C in antigen-coated wells.

Class-specific (IgA, IgG, and IgM) goat anti-human immunoglobulin that was conjugated to horseradish peroxidase (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was diluted in 0.45% sodium chloride, 3% bovine serum albumin (Pentex, Miles Research Laboratories, Elkhart, IN), and 0.05% Tween 20 (Sigma Chemical Co., St. Louis, MO).

O-phenylenediamine was used as substrate (Aldrich Chemicals, Milwaukee, WI). Reactions were stopped by the addition of 20 μl of 8 N sulfuric acid. Absorption was measured as 492 nm in an MR580 ELISA reader (Dynatech Laboratories). As a control each plate contained three dilutions in duplicate of a standard serum. Optical density was plotted as a function of log serum dilution for a minimum of six dilutions of each serum, and a best-fit line constructed by least-squares, using the linear part of the slope. The optical density of a specified dilution of the standard serum was derived from the curve of that serum. Then, the intercept of this optical density with curves of the unknown sera was used to determine titer. By convention, any serum that was negative in the lowest dilution tested (1:4) was assigned a titer of 2.

Quantitative determination of IgM levels. Postvaccination serum samples from each of the patients and volunteers who were immunized in the study were evaluated for quantitative levels of IgM by immunofluorescence (Immunofluor-IgM, Bio-Rad Laboratories, Richmond, CA) using a photofluorometer (American Instrument Co., Silver Spring, MD).

Vaccination studies. Serotype A *C. neoformans* CPS was obtained from Dr. K. J. Kwon-Chung, National Institute of Arthritis and Infectious Diseases (NIAID). This material had been purified as previously described (6) from culture supernatants, and contained 0.7% nitrogen by microkjeldahl analysis. CPS was suspended in sterile water, filtered with a 0.45-μm Millipore filter (Millipore Corp., Bedford, MA) and dispensed into sterile vials. SSSIII was produced by Eli Lilly & Co., Indianapolis, IN and obtained from Dr. James Hill, NIAID. The SSSIII preparation had been found to contain 0.2% nitrogen and 2% nucleic acid. After informed consent had been obtained, volunteers and patients were vaccinated with 0.5 ml of each preparation (37 μg of CPS; 50 μg of SSSIII).

Studies were conducted to determine the effect of vaccination with one polysaccharide on the antibody response to the other. Volunteers were vaccinated with either CPS or SSSIII then 2 wk later with the remaining antigen. Antibody responses were measured by RIA and ELISA and compared with those obtained when both antigens were administered simultaneously. No cross-reactions or antigenic competition between the two vaccines were found. Additionally, in other pilot studies, volunteers were vaccinated with various doses of CPS (doses used: 3.7, 7.4, 18.5, 37, and 74 μg). The optimal response to immunization occurred at the 37-μg dose of CPS.

Thus, for studies in which the normal volunteer response to immunization was compared with the response of patients who had been cured of cryptococcal meningitis, both the patients and the volunteers were immunized with 37 µg of CPS and 50 µg of SSSIII on the same day.

RESULTS

Prevalence of anti-CPS antibody in normal volunteers. 89% of the 185 serum samples obtained from normal volunteers demonstrated binding of CPS. The magnitude of the binding in these samples, however, was small. When three of the sera which, when undiluted, demonstrated nearly 100% binding of available antigen, were diluted 1:10, the binding in each serum fell to <50%. A small but statistically significant decrease (P < 0.05, Wilcoxon test) in the prevalence of antibody was noted with increasing age (Table I).

Prevalence of anti-CPS antibody in patients with cured cryptococcal meningitis. Only 21 of the 51 patients evaluated (41%) had detectable antibody against CPS on at least one occasion by RIA (Table I). The trend noted in the normal volunteers of decreasing prevalence of anti-CPS antibody with increasing age was also noted in the cryptococcosis patients. The prevalence of antibody in the entire group of patients was significantly different from the overall prevalence in the volunteer studies (41% vs. 89%) (P < 0.002, chisquare).

Immunization studies. When the patients' and volunteers' serologic responses to injection with CPS were measured by RIA, both the patients and the volunteers demonstrated significant increases in titers (P
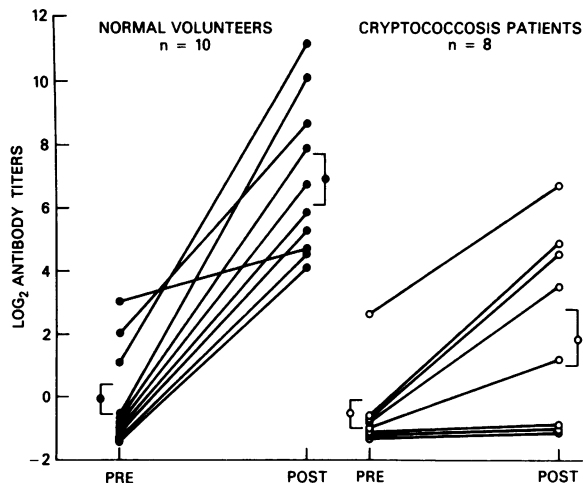

FIGURE 1 Responses of normal volunteers and cryptococcosis patients to immunization with CPS as measured by RIA. Post samples were obtained 2 wk following immunization. Each line represents an individual patient; the summary points represent the mean value plus and minus the standard error of the mean for each group before and after immunization. By convention, any serum sample that bound less than 20% of ^{125}I-tyraminyl-CPS was assigned a titer of 0.5.

< 0.01 for patients; P < 0.001 for volunteers: one-tailed, paired Student's t test) (Fig. 1). Both the normal volunteers and cured patients had geometric mean prevaccination titers of 1:1. Geometric mean anti-CPS antibody titers 2 wk following vaccination, however, were significantly different between the two groups. In the group of cryptococcosis patients, the geometric mean titer after CPS immunization was 1:3 (Fig. 1, right), whereas in the group of normal volunteers, the geometric mean postvaccination titer was 1:119 (Fig. 1, left). This difference was statistically significant (P < 0.01, one-tailed Student's t test). All 10 of the volunteers responded by elevating anti-CPS antibody titers, whereas only 5 of the 8 patients demonstrated any measurable rise in anti-CPS antibodies. 7 of 10 volunteers, and 1 of 8 patients developed postvaccination antibody titers of 1:32 or greater.

When the two groups were compared by ELISA for the class-specific response to immunization with CPS, the major response to immunization was found to be in the IgM class. Only the volunteers demonstrated a statistically significant increase in IgM anti-CPS antibody (Fig. 2) (P < 0.001 by the paired, one-tailed Student's t test). The volunteers' geometric mean prevaccination titer was 1:73 and the postvaccination titer was 1:238. In the group of patients, however, the geometric mean prevaccination titer was 1:38 and the postvaccination titer was 1:31. 9 of 10 volunteers responded to immunization with CPS by elevating IgM anti-CPS antibodies, whereas only 2 of 8 patients de-

TABLE I
Prevalence of Anticryptococcal Antibody in Normal Volunteers and Cryptococcosis Patients

Age	Volunteers		Patients	
	Positive* / Tested		Positive* / Tested	
yr	%		%	
2–17	$\frac{88}{95}$ (93)		$\frac{2}{3}$ (67)	
18–33	$\frac{36}{41}$ (88)		$\frac{6}{12}$ (50)	
34–49	$\frac{7}{8}$ (88)		$\frac{4}{14}$ (29)	
50–74	$\frac{33}{41}$ (81)		$\frac{9}{22}$ (41)	
Total	$\frac{164}{185}$ (89)		$\frac{21}{51}$ (41)	

* Positive sera defined as those undiluted sera that bind ≥20% of available antigen in RIA.

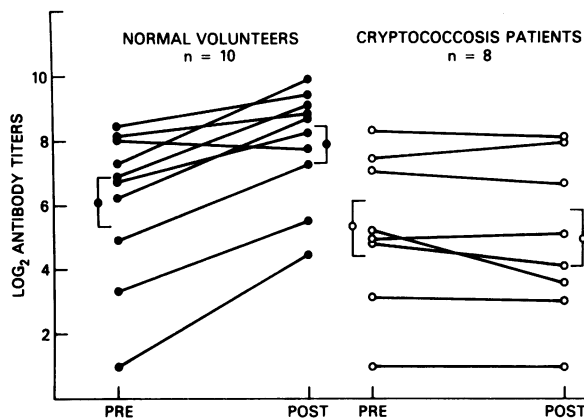*Immunologic Unresponsiveness in Cryptococcosis* 1187

FIGURE 2 IgM responses of normal volunteers and crypto-coccosis patients to immunization with CPS as measured by ELISA. Post samples were obtained 2 wk following immunization. Each line represents an individual patient; the summary points represent the mean value plus and minus the standard error of the mean for each group before and after immunization.

veloped modest elevations in IgM anti-CPS titers. The difference in IgM anti-CPS response between the patients and volunteers was again statistically significant ($P < 0.01$ by the one-tailed Student's t test). Neither patients nor volunteers demonstrated statistically significant elevations in either IgA or IgG titers to CPS when pre- and postvaccination samples were compared, nor was the geometric mean response different between the two groups.

Thus, patients cured of systemic cryptococcal infection, who had been free of CPS in serum and CSF for at least 1 yr responded poorly to immunization with CPS, whereas normal volunteers demonstrated a significantly greater response. A minor response was seen in five of the eight patients (Fig. 1). Of the five, only one had had serum antigen detected during infection. Thus, patients who have antigen in serum at the time of diagnosis may be more likely to demonstrate this immunologic unresponsiveness. The one patient who developed an antibody titer greater than 1:32 (Fig. 1) was also the only patient we studied who developed anti-CPS antibody during the course of his primary infection. Moderate titers of anti-CPS antibodies have persisted in his serum since his infection. He was also one of the two patients who, following immunization, developed elevated IgM anti-CPS antibodies as measured by ELISA (Fig. 2).

With respect to the response to immunization with SSSIII, both the patients and the controls responded similarly. Both the patients and the controls significantly elevated IgA, IgG, and IgM titers against SSSIII (Fig. 3). The titers in the cryptococcosis patients after immunization were not significantly different from the

normal volunteers ($P > 0.2$ by the paired, one-tailed Student's t test for each antibody class). Similarly, the preimmunization titers and fold rises of the two groups did not differ significantly.

Because of the apparent difference in IgM response to CPS, we studied the postimmunization sera for quantitative IgM levels. Mean values for the two groups were as follows: Volunteers: mean value 169.3±19.8 mg/100 ml, range 86–286 mg/100 ml; patients: mean value 143.4±17 mg/100 ml, range 70–221 mg/100 ml ($P > 0.4$ when compared by one-tailed Student's t test). No individual patient or volunteer had a value that fell below the normal range (normal for this test: 60–200 mg/100 ml).

DISCUSSION

Anti-CPS antibodies were detected in 88.6% of normal volunteers, though only in very low titers, represented by percent bound values between 21.2 and 89%. That the RIA measured binding by nonimmunoglobulin proteins appears unlikely. RIA on fractions obtained with one normal serum after Sephacryl chromatography or sucrose density gradient ultracentrifugation indicated molecular weights of the binding protein compatible with immunoglobulin (data not shown). Addition of calcium chelators did not alter binding, suggesting that C-reactive protein was not responsible (data not shown). The immunizing stimulus for these antibodies is unknown. Such antibodies may arise as a result of exposure to *C. neoformans* in nature (12, 13), or may have arisen by exposure to different immunologic stimuli that provoke cross-reacting anti-
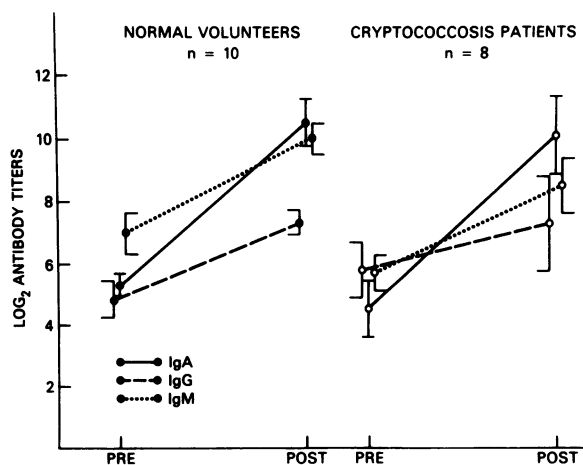

FIGURE 3 IgM, IgA, and IgG responses of normal volunteers and cryptococcosis patients to immunization with SSSIII measured by ELISA 2 wk after immunization. Lines represent the mean plus and minus the standard error of the mean for each group before and after immunization.

bodies (6, 14, 15). Similar experience has been encountered with normal humans having antibody to dextran (16), to melibiose (17), and to certain microbial polysaccharides (18).

In the group of cured cryptococcosis patients the prevalence of anti-CPS antibody as measured by RIA was 41.1% (Table I). This prevalence is similar to that encountered with other sensitive methods (2, 19–21), and is significantly lower than the control population studied by RIA ($P < 0.002$, chi-square).

Because of the reports of decreasing immunoglobulin levels with increasing age (22–24) and because of evidence that low preimmunization IgM and IgA levels in aged individuals correlated with blunted response to pneumococcal vaccination (25), we determined total serum IgM levels in both the patients and the volunteers. Whereas there was no difference detected when the two groups were compared using the Student's t test ($P > 0.4$), the type II error is large because of the small numbers of samples in each group. No patient, however, was detected as having a serum IgM value below the normal value for the test that was used.

Three distinct possibilities exist for the diminished prevalence of antibody in the patient population. First, the ability to mount an antibody response to infection with *C. neoformans* or to vaccination with CPS may be genetically determined. Thus, a subpopulation of patients who develop cryptococcal infection may be genetic hyporesponders. Human antibody response to bacterial flagellin (26), and polysaccharides of *Haemophilus influenzae* (27, 28), meningococcus group C (27), and *Streptococcus pneumoniae* (28) have been linked to immunoglobulin allotype. Such studies have not been conducted on patients with cryptococcosis. Alternately, patients who develop systemic infection with *C. neoformans* may have their antibody responses masked by excess CPS present in their body fluids. Although CPS may persist in the infected host for an undefined amount of time, the immunization studies carried out up to 8 yr after antigen had no longer been detectable in serum or CSF, make this explanation seem less likely. A final possibility is that patients who develop systemic cryptococcal infection may, during the course of their illness, develop a state of immunologic unresponsiveness.

Immunization of eight cryptococcosis patients with CPS produced a response that was heterogeneous, but blunted in comparison to the antibody response of normal volunteers. The two groups did not differ significantly with respect to their responses to immunization with SSSIII. To our knowledge, only one previous study, that of Norden and his colleagues (29), has documented that a prior infection can diminish antibody response to antigens from the same microbe. In their study, children who had had *H. influenzae* meningitis and orbital cellulitis failed to respond to immunization with the capsular polysaccharide of the infecting organism. This earlier study does not address the issue of the specificity of the immunologic unresponsiveness in these children with *H. influenzae* infections. In addition, although it seems likely that their patients had antigenemia previously, this was not documented.

The results of our study support the concept that long-term specific unresponsiveness to challenge with CPS also exists in humans in association with cryptococcosis.

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