Protection of Mice against Experimental Cryptococcosis by Anti-
Cryptococcus neoformans Monoclonal Antibody

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Received 28 July 1986/Accepted 2 December 1986

Humoral immunity does not play a prominent role during experimental cryptococcosis. However, previous
studies have shown that immunoglobulin G (IgG) anti-Cryptococcus neoformans antibodies can mediate
cell-dependent yeast killing in vitro. Therefore, the protective effect of a previously described monoclonal IgG1
anti-C. neoformans antibody (E1) administered intraperitoneally 24 h before intravenous infection with a C.
neoformans serotype A strain was evaluated in mice. Heavily infected (3 × 10⁶ cells) untreated mice died in 2.9
± 0.5 (standard deviation) days. Survival time was 17.9 ± 1.6 days for mice treated with 100 µg of E1 and 3.0
± 0.7 days for mice treated with 100 µg of a monoclonal IgG1 anti-thyroglobulin antibody used as a control.
Protection was dose dependent and required at least 10 µg of E1 (mean antibody concentration in serum ±
standard deviation, 6.6 ± 2.3 µg/ml). Insufficient concentrations of IgG anti-C. neoformans antibody could explain
previous negative results obtained with polyclonal immune serum. After infection with a smaller
inoculum (5 × 10⁵ to 5 × 10⁴), the protective effect of E1 was confirmed by the presence of fewer CFUs in the
spleens and brains of treated mice than in those of controls. CFU were still detected in the brains of protected
mice 5 days after infection, although soluble antigen was negative in sera. These results suggest that passive
serotherapy with monoclonal IgG antibodies could participate in the prevention or treatment of experimental
cryptococcosis.

Cryptococcus neoformans is a yeastlike fungus responsible for disseminated cryptococcosis, a fatal infection unless
treated. Amphotericin B, with or without 5-fluorocytosine, is the treatment of choice, but treated cases still have a 20%
mortality rate. Therefore, other forms of therapy deserve to be evaluated in association with amphotericin B. Enhancement
of specific humoral immunity could be a logical approach, because in contrast with studies showing very low levels of anti-C.
neoformans antibodies in serum during cryptococcosis (2, 6), many in vitro studies suggest that anti-C.
neoformans antibodies, essentially immunoglobulin G (IgG), could, if present, participate in yeast killing in vivo
(5, 15, 19, 21). However, previous attempts to protect mice against experimental cryptococcosis by hyperimmunization
or passively transferred immune serum led to controversial but rather negative results (8), which could be explained by
low levels of circulating IgG anti-C. neoformans antibodies. We therefore decided to investigate whether passive
serotherapy performed with the monoclonal IgG anti-C. neoformans antibody described in the accompanying paper
(7) would be more protective against experimental murine cryptococcosis.

MATERIALS AND METHODS

Organism. The virulent strain (271) of C. neoformans
serotype A used in this study was kindly donated by J. E.
Bennett, National Institute of Allergy and Infectious Dis-
ases, Bethesda, Md. The culture was maintained by bi-
monthly transfer on Sabouraud agar slants (Diagnostic
Pasteur, Paris, France). Yeast cells for animal inoculation
were harvested from a 48-h culture at 37°C on the same
medium and washed three times in sterile physiological
saline solution (SPSS). The washed cells were counted in a
hemacytometer and adjusted to give the desired concentra-
tion in a 0.1-ml volume. The precise amount of viable
inoculated cells was retrospectively determined by dupli-
cate-plate counts on Sabouraud-chloramphenicol agar.

Monoclonal antibody. The monoclonal anti-C. neoformans
capsular polysaccharide (CNPS) antibody used for passive
immunization has been previously described (7). This IgG1
(E1) reacts strongly with CNPS serotype A. It was diluted in
0.2 ml of SPSS and injected intraperitoneally (i.p.) 24 h
before the infectious challenge. A mouse IgG1 anti-
thyroglobulin monoclonal antibody (anti-Tg) (J. Salamero,
in press) unable to bind to CNPS serotype A (data not shown)
served as a negative control throughout the study.

Animals. DBA/2 male mice were obtained from IFFA-
CREDO, Lyon, France, and were maintained in the animal
facilities of our institute until use at 8 to 10 weeks of age.

Survival study. Mice (9 or 10 animals) were injected
intravenously (i.v.) in a lateral tail vein with approximately
5 × 10⁶ C. neoformans 24 h after an i.p. injection of 100 µg of
E1 or 0.2 ml of SPSS (experiment A). In experiment B, the
effects of 100 µg of E1 and 100 µg of anti-Tg were compared
in groups of 10 mice infected with 3 × 10⁶ yeast cells. In
experiment C, groups of 7 mice each received 0.1 to 100 µg
of E1 24 h before infection with 5 × 10⁶ C. neoformans to
define the minimal dose of E1 capable of modifying the
course of infection. Cages were monitored at least once daily
to evaluate the viability of the animals. Brains and spleens
were cultured on Sabouraud-chloramphenicol agar to con-
firm that death was due to C. neoformans.

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antibody levels were measured by enzyme-linked immunosorbent assay (7) 6 days after passive i.p. immunization (100 μg of E1 or 100 μg of anti-Tg) in sera collected individually from uninjected mice, as well as from mice infected with 5 × 10⁴, 5 × 10⁵, or 5 × 10⁶ C. neoformans 24 h after immunization. Antibody levels were expressed in microliters per milliliter by using a logit-log formulation of a standard calibration curve. The latex agglutination test to measure circulating CNPS levels (Meridian Diagnostics Inc., Cincinnati, Ohio; 25) was performed with sera from all mice 6 days after passive immunization. Results were expressed in microliters of CNPS per milliliter after comparison with a standard CNPS A solution.

Effects of E1 on C. neoformans tissue counts 5 days after infection. The protective effect of E1 for an inoculum size of 5 × 10⁴ or 5 × 10⁵ was evaluated by counting C. neoformans CFU in the spleen and brain 5 days after infection. Therefore, groups of five mice each were injected i.p. with 100 μg of either E1 or anti-Tg and challenged 24 h later with 5 × 10⁴ or 5 × 10⁵ C. neoformans injected i.v. The mice were killed 5 days later by cervical dislocation, and the CFUs were counted by 10-fold dilutions of spleen and brain tissue homogenates plated in duplicate on Sabouraud-chloramphenicol agar. Fewer than 10 CFU per organ could not be detected.

Statistical analysis. The results of the experiments on survival time are expressed as mean survival times in days ± the standard deviations. The Student’s t test for unequal values was used to compare mean tissue counts and mean survival times.

RESULTS

Effect of anti-CNPS antibody on survival. Deaths occurred in less than 2 days in 100% of the DBA/2 mice infected i.v. with 5 × 10⁶ C. neoformans. Mice injected i.p. with 100 μg of E1 24 h before infection with 5 × 10⁶ CN survived longer than mice injected with 0.2 ml of sterile saline (10 mice; 1) 24 h before infection.

Serum CNPS antigen and antibody levels. Anti-CNPS antibody levels were measured by enzyme-linked immunosorbent assay (7) 6 days after passive i.p. immunization (100 μg of E1 or 100 μg of anti-Tg) in sera collected individually from uninjected mice, as well as from mice infected with 5 × 10⁴, 5 × 10⁵, or 5 × 10⁶ C. neoformans 24 h after immunization. Antibody levels were expressed in microliters per milliliter by using a logit-log formulation of a standard calibration curve. The latex agglutination test to measure circulating CNPS levels (Meridian Diagnostics Inc., Cincinnati, Ohio; 25) was performed with sera from all mice 6 days after passive immunization. Results were expressed in microliters of CNPS per milliliter after comparison with a standard CNPS A solution.

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**DISCUSSION**

Cellular immunity is believed to be the most important specific host defense mechanism involved in cryptococcosis. Nude mice (3, 10) are more susceptible to infection than normal mice, and cryptococcosis is more frequent in patients with cellular immunodeficiencies (17), including acquired immunodeficiency syndrome (25). Conversely, the role of humoral immunity is not prominent; the course of infection is not altered in B-cell-deficient mice (20), and cryptococcosis is rare in patients with humoral immunodeficiencies. The following evidence favors a quantitative defect of humoral immunity. Patients with cryptococcosis have little or no serum anti-C. neoformans antibodies (6), and low levels of antibody are produced in mice (14) and humans (12) after active immunization with C. neoformans or CNPS. However, in vivo, the presence of serum anti-C. neoformans antibodies is correlated with a favorable outcome in patients with cryptococcosis (6). In vitro experiments suggest that anti-CNPS antibodies and especially IgG could, if present, play a major role in host defenses. The antibodies enhance phagocytosis of C. neoformans by macrophages (15) and polymorphonuclear leukocytes (16), and an antibody-dependent cell-mediated cytotoxicity has been demonstrated with human peripheral blood lymphocytes (4, 5) and polymorphonuclear leukocytes (19). Finally, Nabavi and Murphy (21) recently showed that C. neoformans growth inhibition mediated by natural killer cells is augmented by IgG anti-cryptococcal antibody.

Given the above considerations, it is tempting to speculate that a high level of anti-CNPS antibody might be protective during experimental cryptococcosis. Previous studies dealing with active or passive immunization gave conflicting results, on the whole, rather negative results. Hyperimmunization of mice with CNPS-bovine gammaglobulin conjugates (9) or of rabbits with Formalin-killed C. neoformans (22) elicits significant antibody production (serum titer, <1:1,000 by agglutination) but is not protective. The effect of passive serotherapy with polyclonal immune serum is also controversial; protected mice have fewer CFU in their spleens compared with unprotected mice when serotherapy precedes infection (1), whereas no effect on survival was obtained by Louria and Kaminski (18) when mice were treated 24 h after infection. Graybill et al. (11) showed that a partial protection is demonstrated only if the antibodies are present at the time of challenge and injected at the site of challenge. Taken together, the findings suggest that protection conferred by immune serum is at least difficult to obtain. However, it should be noted that the precise concentration of circulating anti-CNPS antibodies and the proportion of IgG are unknown in the above-mentioned studies. In our hands, less than 1 μg of IgG antibody per ml of serum was obtained in high-responder strains of mice after an optimal immunization. We therefore decided to reassess the protective effect of passively transferred anti-CNPS antibodies by using a monoclonal IgG antibody at various concentrations.

The monoclonal IgG1 anti-CNPS antibody (E1) used throughout this study has previously been described (7). E1 binding to CNPS was detected by enzyme-linked immunosorbent assay in the range of nanograms per milliliter. Its ability to protect mice against experimental cryptococcosis was compared with that of anti-Tg (25), which did not bind to CNPS. DBA/2 mice were chosen for protective studies because they have been shown to be extremely sensitive to experimental cryptococcosis owing to a complement deficiency (24). Moreover, we found (unpublished data) that

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### Table 3. C. neoformans CFU counted 5 days after infection in spleens and brains of mice treated i.p. with 100 μg of either E1 or anti-Tg 24 h before i.v. infection

<table>
<thead>
<tr>
<th>C. neoformans inoculum</th>
<th>Organ</th>
<th>CFU/organ after treatment with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E1</td>
<td>Anti-Tg</td>
</tr>
<tr>
<td>5 × 10^4</td>
<td>Spleen</td>
<td>236 ± 137b</td>
</tr>
<tr>
<td>5 × 10^4</td>
<td>Spleen</td>
<td>&lt;10^6</td>
</tr>
<tr>
<td>5 × 10^5</td>
<td>Brain</td>
<td>37,000 ± 13,000b</td>
</tr>
<tr>
<td>5 × 10^6</td>
<td>Brain</td>
<td>6,000 ± 2,000</td>
</tr>
</tbody>
</table>

* Mean values are the means ± the standard deviations for five mice in each group.

* P < 0.05 versus the control group treated with anti-Tg.

* P < 0.02 versus the control group treated with anti-Tg (10 CFU was the limit of sensitivity).

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DBA/2 mice had low levels of anti-CNPS antibodies after priming with 1 μg of CNPS given i.v. (mean concentration in serum ± the standard deviation on day 7, 22 ± 11 ng/ml), auguring that infection by itself would not lead to a high anti-C. neoformans antibody response in this strain of mice. Results reported in Table 2 show that this deduction is correct.

Survival studies performed in DBA/2 mice infected i.v. with 5 × 10^6 C. neoformans 24 h after an i.p. injection of 100 μg of E1 showed that anti-CNPS antibody exerted a strong protective effect. It should be noted that protection occurred despite the use of a highly virulent C. neoformans challenge (100% mortality in 1.5 days for mice infected with 5 × 10^6 C. neoformans) and did not require the simultaneous injection of antibody and yeast cells through the same route. A dose-response study indicated that protection was dose dependent and necessitated an injection of at least 10 μg of E1, giving an IgG anti-CNPS concentration in serum (6.6 μg/ml) higher than that obtained with immune serum.

Studies on the relative concentrations of CNPS antigens and antibody in the sera of infected mice showed that in the case of a large inoculum (5 × 10^6 C. neoformans), antigen and antibody were simultaneously detected 5 days after infection. The same situation has been described (2, 13) during human disseminated cryptococcosis. With a 5 × 10^4 C. neoformans challenge, antigen was clearly detected in the sera of unprotected mice in the absence of a significant amount of antibody, whereas only antibody was detected in the sera of protected mice. Both serum patterns of CNPS antigen and antibody can be observed during human cryptococcosis, in which both a rapidly negated latex agglutination test for CNPS and the presence of antibody are considered elements of a favorable outcome (2, 17). No circulating antigen was detected in mice, protected or unprotected, infected with 5 × 10^3 C. neoformans or in protected mice infected with 5 × 10^4 C. neoformans. Therefore, to assess whether protected mice were totally resistant to infection, CFU were counted in the spleens and brains 5 days after challenge with 5 × 10^3 and 5 × 10^4 C. neoformans; protected mice infected with 5 × 10^3 or 5 × 10^4 C. neoformans still had more than 10^3 CFU in their brains. The results show that the absence of antigen from serum does not imply eradication of yeast cells.

In conclusion, this monoclonal anti-CNPS antibody is protective against experimental cryptococcosis, but protection was transient in host inoculated mice. Our experiments are under way to assess the effects of various protocols of serotherapy with or without amphotericin B in normal and immunodeficient mice. Owing to their cellular
immunodeficiency, patients with acquired immunodeficiency syndrome are particularly prone to cryptococcosis. Treatment with amphotericin B often fails to cure these patients, and cryptococcosis returns after withdrawal of the drug (26). Extrapolation of our results to human cryptococcosis is difficult because experimental and human diseases differ in many respects (e.g., route of infection, inoculum size, and cause of death [23]). However, the results suggest that passive serotherapy with a monoclonal anti-C. neoformans antibody would be of value in treating acquired immunodeficiency syndrome patients if it could prevent disseminated cryptococcosis or improve the results obtained with amphotericin B.

ACKNOWLEDGMENTS

The work was supported in part by grants from the Conseil Scientifique, Université Paris VII Paris, France, and the Association pour la Recherche sur le Cancer, Villejuif, France.

The authors are indebted to J. E. Bennett, National Institutes of Health, Bethesda, Md., for his generous gift of C. neoformans 271 used throughout the study and to Janet Jacobson for helpful comments.

LITERATURE CITED


