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# Opsonic Activity of Cerebrospinal Fluid in Experimental Cryptococcal Meningitis

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**The role of antibody in protection against infection with *Cryptococcus neoformans* is undefined. In this paper we describe the development of opsonic activity in the cerebrospinal fluid (CSF) of rabbits in response to cryptococcal meningitis. The opsonin appeared to be immunoglobulin G (IgG); the activity was heat stable, copurified with the IgG fraction during protein A separation, and could be absorbed by encapsulated cryptococci. Immunosuppression with cyclosporine could be administered to prevent or allow in vivo deposition of IgG on the polysaccharide capsule of yeasts in the CSF. Both early and late cyclosporine regimens resulted in prolonged, severe meningeal infections corresponding to the complete absence of in vitro opsonic activity in the CSF. While the production of opsonic antibody is part of the successful host response against *C. neoformans* in the central nervous system of rabbits, the presence of specific immunoglobulin by itself is insufficient for complete protection.**

Strong evidence indicates that the primary host defenses against *Cryptococcus neoformans* are cell mediated (9, 11, 23-25). The role of antibody in protection from cryptococcal disease is less clear. Antibodies are not protective in experimental animals (3, 7, 20, 26). In patients, the prognosis is better if anticryptococcal antibody is present (6), and disseminated cryptococcal infections have been described in association with hypogammaglobulinemia (12). In addition, antibody-dependent cell-mediated killing of *C. neoformans* has been demonstrated (5, 21). *C. neoformans* has a well-known but unexplained predilection for dissemination to the central nervous system (CNS) (27). There have been reports of intrathecal antibody responses to *C. neoformans* (17) and other CNS pathogens, including *Treponema pallidum* (22), herpes simplex virus (28), and *Mycobacterium tuberculosis* (15). In this paper we describe the production of anticapsular opsonic antibody in the CNS of rabbits with experimental cryptococcal meningitis. Opsonic antibody was detected in the cerebrospinal fluid (CSF) of infected animals and on the surface of yeasts isolated from the CSF. Immunosuppression with cyclosporine profoundly suppressed the ability of rabbits to eliminate *C. neoformans* from the CNS, which correlated with a failure to produce opsonic antibody in the CSF. Delayed administration of cyclosporine relative to inoculation with cryptococci allowed anticryptococcal antibody production yet did not alter the prolonged course of infection in these animals. While anticryptococcal antibody production may be necessary for a successful host response to cryptococcal meningitis, it is not sufficient.

## MATERIALS AND METHODS

**Cryptococcal meningitis model.** Male New Zealand White rabbits (weight, 2 to 3 kg) were sedated with ketamine (Bristol Laboratories, Syracuse, N.Y.) and xylazine (Cutter Laboratories, Shawnee, Kans.) and inoculated intracisternally with approximately $5 \times 10^7$ CFU of *C. neoformans* H99 (serotype A) in 0.3 ml of 0.015 M phosphate-buffered saline (PBS). This model has been described previously in detail (25). Beginning on day 3 of infection, CSF was aspirated daily from the subarachnoid space of anesthetized animals and pooled for analysis. Cells were removed by centrifugation, and CSF was stored at −70°C for in vitro determinations of opsonic activity. Rabbits treated with cyclosporine received daily intravenous injections of 30 mg starting 1 day prior to inoculation with yeasts or beginning on day 3 of infection. Quantitative yeast cultures were performed by using serial dilutions of CSF in PBS on Sabouraud agar containing 100 μg of chloramphenicol per ml.

**Phagocytosis assay.** The opsonic activity of CSF was gauged by the ability of a sample to mediate phagocytosis of *C. neoformans* in vitro. *C. neoformans* clone C3D from strain H99 is thinly encapsulated under physiological concentrations of $CO_2$ and is easily phagocytized in the presence of opsonins (8). Yeasts were labeled overnight with 50 μCi of $Na_2{}^{35}SO_4$ (specific activity, 43 Ci/mg of S; ICN Pharmaceuticals Inc., Plainview, N.Y.) per ml in Dulbecco modified Eagle medium (GIBCO Laboratories, Grand Island, N.Y.) containing 24 mM $NaHCO_3$, 25 mM sodium 3-(*N*-morpholino)propanesulfonic acid (pH 7.3), additional glucose (final concentration, 25 mM), 100 U of penicillin G per ml, and 100 μg of streptomycin per ml (DMEM). Yeasts were washed three times with PBS, and $8 \times 10^3$ CFU was added to confluent monolayers established from $4 \times 10^5$ oil-elicited rabbit peritoneal macrophages in microdilution wells. Phagocytosis was allowed to proceed for 90 min at 37°C with 5% $CO_2$ in 0.2 ml of DMEM alone (negative control), DMEM containing 20% fresh rabbit serum (positive control), or CSF diluted 1:2 in DMEM. The extracellular yeasts were removed from the macrophages with three PBS washes. The phagocytes were then lysed with 0.2 ml of 0.5% sodium deoxycholate. The cryptococci were suspended, and 0.1 ml of the suspension was mixed with Aquasol 2 (New England Nuclear Corp., Boston, Mass.) and counted with a liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.). The percentages of uptake of *C. neoformans* by macrophages were calculated as follows: 2(sample counts per minute/total counts per minute added) × 100. Pooled samples from each experiment were tested in triplicate.

Three observations suggested that this assay measured internalization and not merely attachment. First, multiple

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transmission electron micrographs showed cryptococci within phagocytic vacuoles bounded by unit membranes through 360°. Second, phagocyte-associated cryptococci did not stain with fluorescein-conjugated goat anti-rabbit immunoglobulin G (IgG) antibodies when intact macrophages were used but did stain in the same preparations fixed with glutaraldehyde, which allowed penetration of macromolecules into the phagocytes. Third, phase-contrast microscopy showed clear morphological differences between phagocytized and extracellular cryptococci; this was not a function of addition of protein opsonins alone but required phagocytes for internalization and loss of phase-contrast highlighting of the fungal cell outer border.

**IgG purification.** Two methods were used to isolate CSF IgG from opsonic samples. A 1-ml protein A–Sepharose CL-4B (Sigma Chemical Co., St. Louis, Mo.) column was prepared according to the directions of the manufacturer. One milliliter of CSF or serum was allowed to enter the column, followed by approximately 12 ml of PBS. The protein peak from the sample, excluding bound IgG, was collected in approximately 4 ml, dialyzed for 24 h against PBS (pH 7.4) at 4°C, and concentrated to the original sample volume of 1 ml with a Minicon concentrator (Amicon Corp., Danvers, Mass.). The bound IgG from the sample was eluted from the column with citrate buffer (pH 2) (2 ml), dialyzed, and concentrated as described above for the original sample. The samples were then tested for opsonic activity in the phagocytosis assay.

For purification of IgG from CSF for subsequent sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Western blot analysis to confirm the location of CSF IgG on gels, 0.5 ml of a sample was absorbed with 3.3 mg of preswollen protein A–Sepharose CL-4B for 15 min at room temperature in a Microfuge tube placed on a rotary mixer. The samples were centrifuged in a Microfuge B centrifuge (Beckman Instruments, Inc., Palo Alto, Calif.) for 2 min; the supernatant CSF was removed and stored at 4°C for analysis. To remove unbound but trapped CSF proteins, the protein A–Sepharose CL-4B pellet was suspended in 0.5 ml of PBS and centrifuged, and the resulting supernatant was discarded. CSF IgG was eluted from the pellet by suspension followed by centrifugation with two 0.5-ml volumes of citrate buffer (pH 2). Supernatants containing IgG were pooled for each sample, and proteins were precipitated by adding 1.0 ml of a saturated ammonium sulfate solution, followed by incubation for 60 min at 0°C. The protein precipitate was centrifuged at 48,000 × *g* for 30 min in a Sorval centrifuge (Du Pont Co., Newton, Conn.), the supernatant was discarded, and the pellet was suspended in treatment buffer for SDS-PAGE.

**SDS-PAGE.** CSF samples were purified with protein A–Sepharose CL-4B as described above or were diluted directly with an equal volume of treatment buffer containing 0.125 M Tris chloride (pH 6.8), 4% SDS, and 20% glycerol (see Fig. 4). Samples were electrophoresed on gradient slab gels prepared from 5 to 20% acrylamide-bisacrylamide (30:0.8) by using the method of Hames (13) and 4% polyacrylamide stacking gels. Gels were polymerized with ammonium persulfate and *N,N,N′,N′*-tetramethylethylenediamine. Electrophoresis was performed with a model SE600 apparatus (Hoefer Scientific Instruments, San Francisco, Calif.) at 15 A and full power. Phenol red was used as an indicator, and it reached the bottom of the gels in approximately 3.5 h. Protein bands were stained with 0.125% Coomassie blue R-250 by using methods described by Hames (13). Molecular weight markers, a rabbit IgG standard, and CSF samples were run concurrently on each gel to identify the bands. The staining intensities of protein bands from some gels were quantified with a model GS300 scanning densitometer (Hoefer Scientific Instruments).

**Western blot confirmation of purified CSF IgG.** Protein bands from a gradient SDS-polyacrylamide gel were transferred to a nitrocellulose membrane with a model Transphor TE52 apparatus (Hoefer Scientific Instruments). The bands were then stained by using the immunoenzymatic technique of the Biotin-Streptavidin System (Amersham Corp., Arlington Heights, Ill.). Briefly, a nitrocellulose membrane was incubated with biotinylated anti-rabbit IgG and then with a peroxidase-biotin-avidin complex and finally stained with 4-chloro-1-naphthol in 0.03% $H_2O_2$.

**CSF absorption with yeasts.** Pooled opsonic CSF from several experiments was absorbed three times with a solution containing $10^7$ CFU of various yeasts per ml for 30 min in a rotating mixer at room temperature. The yeasts were removed by centrifugation, and CSF samples were tested with the phagocytosis assay to determine opsonic activity.

**Immunofluorescent staining.** Pooled daily samples of CSF were centrifuged at 1,000 × *g* for 10 min. The resulting pellets containing CSF cells and cryptococci were suspended in fluorescein-conjugated goat anti-rabbit IgG and incubated at 0°C for 30 min. Following three washes with PBS, cytospin preparations were made with CSF cells and yeasts and were examined with a fluorescence microscope equipped with an appropriate filter.

**Antigen detection.** Cryptococcal polysaccharide antigen titers were determined for CSF samples by using the Crypto-LA test (International Biological Laboratories, Cranbury, N.J.) according to the directions of the manufacturer. Serial 1:2 dilutions of samples were incubated with latex beads coated with anticryptococcal antibody. Agglutination was scored, and titers were determined as the highest dilutions yielding unequivocal positive results.

## RESULTS

The opsonic activities of CSF from rabbits infected with *C. neoformans* and from cyclosporine-treated, infected rabbits are shown in Fig. 1. These activities, which peaked at day 7 in infected rabbits, were not removed by heating CSF at 56°C for 30 min and, therefore, were not dependent upon intact complement. For comparison, the level of uptake of radiolabeled *C. neoformans* by rabbit peritoneal macrophages in the presence of 20% fresh rabbit serum was 81% ± 5% ($n = 24$); in medium alone, the level of uptake was 11% ± 2% ($n = 24$). Heat-inactivated rabbit serum (20%) did not increase the level of uptake compared with media alone.

Animals treated with cyclosporine prior to infection failed to develop opsonic activity in their CSF. This drug also blocked the mechanism(s) responsible for killing *C. neoformans* in the CSF in vivo (Fig. 2). The time of peak opsonic activity in CSF from infected rabbits not receiving cyclosporine corresponded with significant in vivo fungicidal activity in the CNS of these animals. To assess the importance of the timing of immunosuppression relative to inoculation with *C. neoformans* on the development of opsonic activity, cyclosporine administration was delayed until day 3 of infection in some animals. CSF from these rabbits was not opsonic in the phagocytosis assay, and the quantitative yeast counts were indistinguishable from those of completely suppressed animals.

To identify the opsonin in CSF from rabbits infected with *C. neoformans*, we removed IgG from a pool of opsonic samples by passing them over a protein A–Sepharose col-

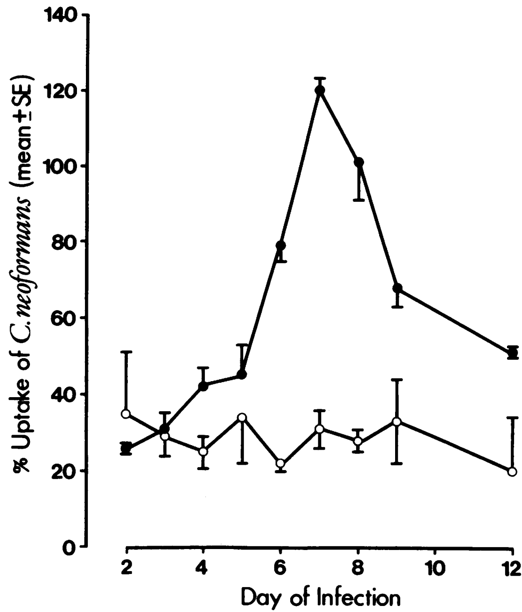


FIG. 1. Phagocytosis of radiolabeled cryptococci by rabbit peritoneal macrophages in the presence of CSF from infected rabbits. Uptake was measured after 90 min of incubation with CSF diluted 1:2 in DMEM. Symbols: ●, CSF from untreated rabbits; ○, CSF from cyclosporine-treated rabbits.

umn. Removal of IgG, which represented 9% of the total protein in the CSF, resulted in the complete abrogation of opsonic activity; 81% of the original opsonic activity was recovered in the eluted IgG fraction.

The specificity of the immunoglobulin present in opsonic CSF was determined by absorption with three strains of *C. neoformans* serotype A, an unencapsulated strain of this organism, and *Candida albicans* (Carter strain). A sham absorption with an equivalent volume of PBS was performed as a control. The opsonic activity in CSF remaining after absorption is shown in Fig. 3. Absorption with encapsulated

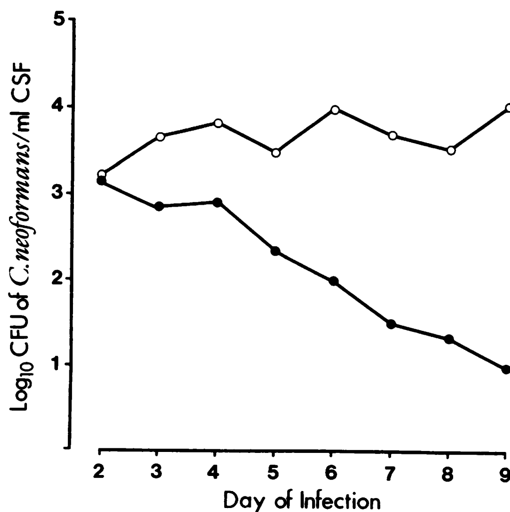


FIG. 2. Quantitative CSF yeast counts in rabbits receiving no treatment (●) or in rabbits receiving 30 mg of cyclosporine per day (○) from 1 day prior to infection with *C. neoformans*. Samples from five to eight rabbits per group were pooled daily.

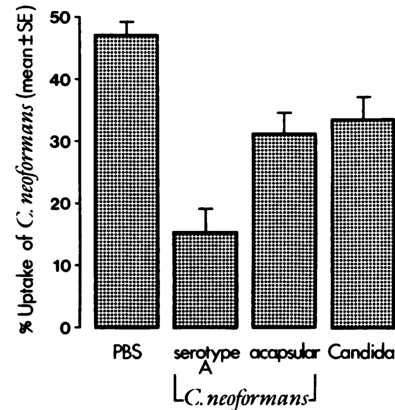


FIG. 3. Absorption of activity from opsonic CSF from untreated rabbits infected with *C. neoformans*. Uptake of radiolabeled yeasts in the presence of CSF was determined after preincubation with PBS ($n = 6$), three strains of *C. neoformans* serotype A ($n = 18$), an acapsular *C. neoformans* strain ($n = 6$), or *Candida albicans* ($n = 6$).

serotype A strains of *C. neoformans* removed 68% of the opsonic activity. After absorption with the unencapsulated *C. neoformans* strain or *Candida albicans*, 67% of the opsonic activity was still present.

The lack of opsonic activity in CSF from cyclosporine-treated rabbits with meningitis might have been explained by an excess of soluble antigen present in the samples. The cryptococcal polysaccharide antigen titers in CSF, as determined by latex agglutination on day 7 of infection, were 1:128 for cyclosporine-treated animals and 1:8 for untreated rabbits. Antigen-antibody complexes could have made the immunoglobulin unavailable for opsonization in the phagocytosis assay. To study the differences in immunoglobulins present in the CSF from cyclosporine-treated and untreated infected animals, we analyzed samples by SDS-PAGE. CSF samples were boiled with 10% SDS to dissociate any soluble immune complexes. The results of an SDS-PAGE analysis of CSF from infected animals with and without cyclosporine treatment are shown in Fig. 4. The identities of the CSF IgG bands were confirmed by (i) a Western blot analysis in which anti-rabbit IgG was the primary antibody and an examination of the CSF purified by protein A-Sepharose CL-4B electrophoresis on an SDS-PAGE gel (data not shown) and (ii) use of a rabbit IgG standard (Fig. 4, lane 1). The 150-kilodalton IgG band in CSF from normal, infected rabbits was heavier at all times of infection (Fig. 4, lanes 3, 5, and 7) than the band in uninfected CSF (lane 2) or in samples from cyclosporine-treated, infected rabbits (lanes 4, 6, and 8). Thus, the lack of opsonic activity in the CSF of rabbits treated with cyclosporine was not due to the removal of antibody by immune complex formation with soluble antigen.

The staining intensities of the IgG bands shown in Fig. 4 were quantified by scanning densitometry. The area under the absorbance curve for an IgG band represented the amount of IgG present in the sample. Since equal volumes of CSF were used for all samples, relative IgG concentrations could be inferred from these values. The intensities of staining of the IgG bands from cyclosporine-treated rabbits on days 3, 7, and 12 were 18, 65, and 17%, respectively, compared with CSF samples from untreated infected animals at the same times. Therefore, there seemed to be a specific defect in the production of IgG in these immunosuppressed animals.

Alternatively, decreased opsonic activity in CSF from

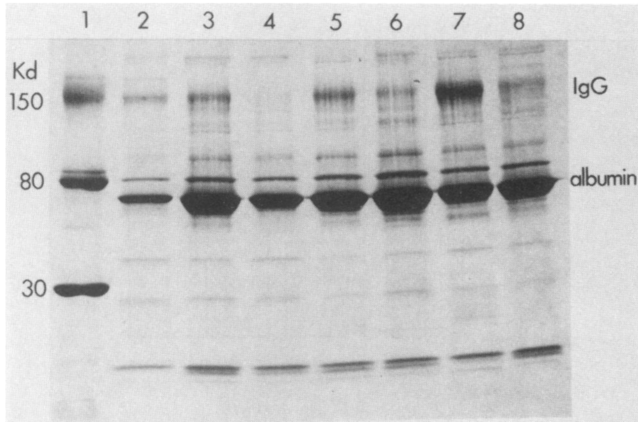


FIG. 4. SDS-PAGE of CSF from rabbits with cryptococcal meningitis. Lane 1, Rabbit IgG (150 kilodaltons), transferrin (80 kilodaltons), and carbonic anhydrase (30 kilodaltons); lane 2, uninfected rabbit CSF; lanes 3, 5, and 7, CSF from untreated rabbits removed on days 3, 7, and 12 of infection, respectively; lanes 4, 6, and 8, CSF from cyclosporine-treated rabbits removed on days 3, 7, and 12 of infection with *C. neoformans*, respectively. The gradient gel was prepared from 5 to 20% acrylamide-bisacrylamide (30:0.8). Samples were run under nonreducing conditions. Kd, Kilodaltons.

immunosuppressed rabbits could have resulted from simple adsorption of antibody onto the yeasts, which were present in much greater numbers than in untreated infected CSF. The in vivo deposition of IgG onto *C. neoformans* in the CSF of untreated and cyclosporine-treated animals was examined by direct immunofluorescent staining of IgG-coated yeasts. The capsules of budding yeasts from the pooled CSF of untreated rabbits on day 6 of infection were heavily stained with fluoresceinated anti-rabbit IgG, indicating the presence of immunoglobulins (Fig. 5). This IgG binding to the cryptococci in vivo occurred as early as 5 days after inoculation. When yeasts from rabbits treated with cyclosporine prior to inoculation were examined on day 6 of infection, there was no capsular staining. On the other hand, delayed cyclosporine treatment (beginning on day 3) allowed anticryptococcal antibody production. By day 6, deposits of IgG were observed on yeasts in the CSF of these rabbits. However, CSF from these animals did not facilitate phagocytosis of *C. neoformans* in vitro.

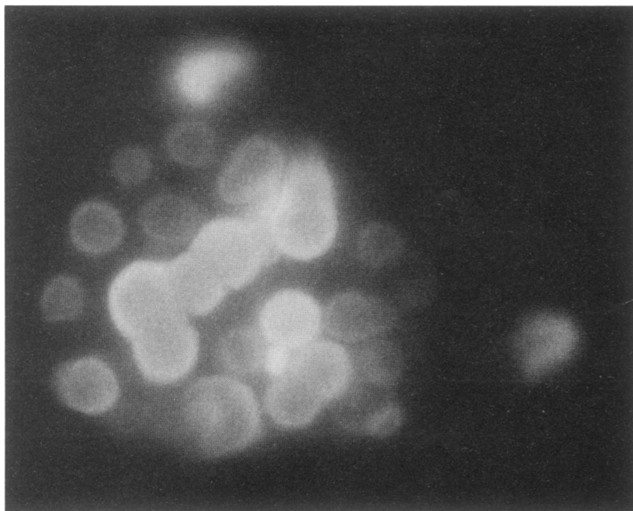

FIG. 5. *C. neoformans* cells aspirated directly from the CSF of untreated rabbits after 6 days of infection and incubated with fluorescein-conjugated goat anti-rabbit IgG.

To determine the relationship between the development of opsonic activity in the CSF and the development of opsonic activity in the serum of untreated infected rabbits, we examined purified IgG preparations from simultaneous serum and CSF samples removed during cryptococcal infection. In the in vitro phagocytosis assay, daily CSF and serum samples collected from days 4 to 9 of infection displayed equal opsonic activities, with peak activity occurring on day 7.

## DISCUSSION

*C. neoformans* infections of the CNS are usually associated with underlying immune defects. While cellular immune mechanisms are of primary importance, there is conflicting evidence regarding the role of antibody production against cryptococci. The results of one previous study (10, 20) indicated that anticapsular antibody in mice does not confer protection from infection, while in another study workers found benefit only if the protective antibody was given at the time of initial infection (20). Workers in our group have demonstrated previously that the presence of preformed agglutinating antibody has no effect on resistance to cryptococcal meningitis in rabbits (26). However, observations of humans with cryptococcal meningitis suggest that the presence of antibody in the serum may contribute to an improved prognosis (6). Gupta et al. (12) reported disseminated cryptococcosis in a patient with normal T-cell immunity but moderately severe hypogammaglobulinemia. Antibody-dependent killing of *C. neoformans* by human peripheral blood monocytes and polymorphonuclear cells (5, 21) also suggests that antibody may play a role in host defense against cryptococcal infection.

The results reported above provide evidence of production of anticryptococcal antibody in the CSF of rabbits with experimental cryptococcal meningitis. Infected rabbits developed opsonic activity in the CSF in response to *C. neoformans* infection, corresponding temporally in the efficient elimination of yeasts from CSF. Previous studies demonstrated the opsonization of *C. neoformans* by alternative complement components in serum (16). When opsonic CSF was heated at 56°C for 30 min, there was no reduction in uptake of *C. neoformans* by rabbit peritoneal macrophages. While we did not specifically eliminate the activity of heat-stable complement components, the removal of opsonic activity by absorption with encapsulated cryptococci combined with copurification of activity with IgG during protein A separation strongly suggests that the CSF opsonin is IgG. The opsonin was also found in the serum at the same time with an opsonic activity similar to that found in the CSF. In this model the humoral response is not limited to the CNS. This is not surprising, since we have shown previously that this infection disseminates to other organs from the subarachnoid space (25). Absorption of opsonic activity by encapsulated strains of *C. neoformans* but not by unencapsulated strains of *C. neoformans* indicated that the antibody was directed toward the polysaccharide capsular material. Other data indicated that absorption of CSF with *C. neoformans* serotypes B and C removed opsonic activity as well as absorption of CSF with the serotype A strain against which the antibody was formed (data not shown). The epitopes recognized by the opsonin(s) produced in this model may be different from those conferring serospecificity.

The experimental model of cryptococcal meningitis de-

scribed above allows comparison of the effects of relatively selective immunosuppressive agents with the natural resistance mechanisms of normal rabbits. Cyclosporine is a fungal metabolite with reportedly selective immunosuppressive effects on T lymphocytes (1, 2, 14, 18, 19, 29) which result in progressive, fatal cryptococcal meningitis in rabbits (23). Unlike animals treated with cortisone, which causes a marked reduction in leukocytes in the CSF (25), cyclosporine-treated animals develop CSF pleocytosis with functional suppression of the T cells which are present (23, 24). Activated CSF macrophages from infected rabbits treated with cyclosporine exhibit depressed cytotoxicity for tumor cell targets, but are fully capable of producing hydrogen peroxide in vitro (24). The most striking effect of immunosuppression with cyclosporine in our model was the total elimination of opsonic activity in the CSF of infected animals at the time when their ability to eliminate *C. neoformans* from CSF was also suppressed.

The production of opsonic antibody in the CSF of rabbits during cryptococcal meningitis is an early event in response to infection. When immunosuppression with cyclosporine was delayed from 1 day prior to inoculation with yeasts to day 3 of infection, organisms isolated from the CSF were coated with anticryptococcal IgG. However, these rabbits maintained high yeast counts and showed no opsonic activity in the CSF.

Differences in CSF IgG concentration detected by a SDS-PAGE analysis of samples from infected animals and animals receiving cyclosporine prior to inoculation were not affected by the presence or absence of soluble immune complexes. The observed differences in soluble CSF immunoglobulin were not due to simple adsorption onto yeast cells. Yeasts isolated from the CSF of cyclosporine-treated rabbits prior to infection lacked detectable anticapsular IgG. Therefore, it seems reasonable to suggest that immunosuppression with cyclosporine resulted in an IgG production defect rather than increased consumption of antibody by more numerous organisms or additional soluble polysaccharide antigen present in the CSF. This defect in opsonic antibody production may be related to T-cell-mediated events or a B-cell subset(s) responsible for the generation of IgG that is both specific for cryptococcal polysaccharide material and opsonic as defined by potentiation of phagocytosis of *C. neoformans* by macrophages in vitro. While our results indicate that there is a role for anticryptococcal antibody in cryptococcal meningitis in rabbits, they reaffirm the notion that successful defense against this opportunistic fungal pathogen requires other host responses.

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