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Cerebrospinal Fluid Macrophage Response to Experimental Cryptococcal Meningitis: Relationship between In Vivo and In Vitro Measurements of Cytotoxicity

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The functional abilities of macrophages from cerebrospinal fluid (CSF) have so far been little studied. We examined the acquisition of activation characteristics by CSF macrophages during the course of experimental cryptococcal meningitis. CSF macrophages developed the ability for increased reactive oxidative intermediate (H_2O_2) production and tumor and fungal cytotoxicity. Despite having been activated, CSF macrophages could not inhibit the growth of *Cryptococcus neoformans* in vitro. Immunosuppression with cyclosporine, which eliminates the natural resistance of rabbits to cryptococcal meningitis, did not prevent or diminish H_2O_2 production by CSF macrophages but did reduce their tumoricidal activity. Activation of CSF macrophages appears to be an integral part of the central nervous system immune response to *C. neoformans* in this model, but alone is insufficient to eliminate *C. neoformans* from the central nervous system.

The central nervous system (CNS) represents an immunologically sequestered site. That metastatic and recurrent neoplasms can be sheltered from chemotherapy and immunotherapy in this separate compartment is well known. Certain chronic infections can persist in the CNS even when evidence of active infection cannot be found in other organs. These include infections with tuberculosis, *Cryptococcus neoformans*, *Toxoplasma gondii*, *Coccidioides immitis*, *Histoplasma capsulatum*, and *Treponema pallidum*. Most functional studies of CNS immune responses have focused on antibody and complement activity in the cerebrospinal fluid (CSF) during infection; there have also been a few investigations of CNS lymphocytes (21, 37). Despite the paucity of information on immune mechanisms in the CNS, this site appears to be an important area for the examination of dynamic cellular functions during inflammation. The subarachnoid compartment in particular allows access to cells and CSF, which can be used to study the dynamics of local immune responses.

The CSF macrophage is commonly found in the subarachnoid space during inflammation of the CNS. Resident mononuclear phagocytes constitute approximately 30% of the total "circulating" CSF cells of normal, healthy humans (29) and 24% of normal rabbit CSF cells (7). The origin of these cells is unclear. They may arise by differentiation from mesothelial cells of the arachnoid membrane or from microglial cells or may derive directly from blood monocytes (29, 30). The function of these cells is even more obscure than their origin. Resident cells may be important in immune surveillance or may be scavengers of debris from the subarachnoid space. During trauma to the CNS which disrupts the blood-CSF barrier, cell radiolabeling studies indicate a heterogeneous population; approximately two-thirds of the mononuclear phagocytes derive from the blood (18, 24, 38). Nevertheless, many CSF macrophages presumably arise from local microglial cells. The multinucleated giant cell is also involved in many inflammatory processes occurring in the subarachnoid space and contributes to the heterogeneous population of CSF macrophages. During certain pathological conditions, CSF macrophages have been described which are histologically similar to both blood monocytes and tissue histiocytes (10, 15, 29). These observations suggest that CSF mononuclear phagocytes present during inflammation have multiple origins. They presumably carry out a variety of functions. Previous investigations have emphasized the importance of mononuclear phagocyte location in effector cell responses. For instance, peritoneal mononuclear phagocytes behave differently from alveolar macrophages in response to infection with *Aspergillus* (40), *Nocardia* (3), *Pasturella* (6), and *Candida* (20) spp. This reinforces the concept that generalization of macrophage function from studies on cells from a particular body site may be inappropriate.

In the following studies, we examine CSF mononuclear phagocytes as they acquire effector functions during an experimental CNS fungal infection. CSF mononuclear phagocytes become rapidly activated during fungal meningitis by several criteria: (i) increased secretion of a reactive oxygen intermediate product, and (ii) acquisition of tumor and fungal cytotoxicity. Nevertheless, they remain unable to kill *Cryptococcus neoformans* in vitro.

MATERIALS AND METHODS

Cryptococcal meningitis model. Prior to inoculation, yeast cells were grown for 3 to 4 days on Sabouraud agar at 37°C. After sedation with ketamine (Bristol Laboratories, Syracuse, N.Y.) and xylazine (Cutter Laboratories, Shawnee, Kan.), New Zealand White rabbits (2 to 3 kg) from a local supplier were inoculated intracisternally with approximately 5×10^7 CFU of *C. neoformans* (H99 serotype A strain) in 0.3 ml of 0.015 M phosphate-buffered saline (PBS). This model has been described in detail previously (35). Beginning on day 3 of infection, CSF was aspirated daily on 6 consecutive days from five to eight sedated rabbits and pooled daily for experiments. Rabbits treated with cyclosporine received the drug daily by intravenous injection of 30 mg/day, starting 1 day prior to intracisternal yeast inoculation. Quantitation of yeast cells in the CSF during infection was performed from

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pooled CSF daily by serial dilutions in PBS plated on Sabouraud agar containing 100 μg of chloramphenicol per ml. After 48 to 72 h of incubation at 37°C, colonies were counted and the number of yeast cells per milliliter of CSF was calculated. Rabbits were fed Purina rabbit chow and water ad libitum.

Macrophage monolayers. CSF containing host cells was removed from infected rabbits by intracisternal aspiration. Samples were pooled and centrifuged at 800 × *g* to pellet cells. CSF was carefully removed and stored at −70°C, and cells were suspended in Dulbecco modified Eagle media (DMEM; Gibco Laboratories, Grand Island, N.Y.). Cells were allowed to adhere for 60 min onto Falcon microtiter plates (Becton Dickinson Co., Oxnard, Calif.) at a concentration of 4×10^5 cells per well. Monolayers were washed three times with PBS, and nonadherent cells were collected and counted by hemacytometer. Differential counts of CSF cells were performed by Giemsa stain morphology and nonspecific esterase-stained cells from cytospin preparations.

H_2O_2 measurements. H_2O_2 production was measured by the method of Pick and Mizel, with phenol red as an oxidizable substrate (36). Microtiter wells containing 4×10^5 CSF cells received a solution containing phenol red (0.3 mg/ml), horseradish peroxidase (19 U/ml), and 6 mM glucose in PBS with or without 200 nM phorbol myristate acetate. Absorbance of the phenol red following the addition of 0.01 ml of 1 N NaOH to terminate the reaction was measured in a Titertek Multiskan automatic photometer (Flow Laboratories, McLean, Va.) at 620 nm. Concentrations of H_2O_2 were calculated by using linear regression coefficients from a standard curve prepared from known concentrations of fresh reagent hydrogen peroxide.

Tumoricidal assay. A subconfluent inoculum of murine fibrosarcoma cells (3T12) was labeled for 24 h in a 75-cm^2 tissue culture flask (Costar, Cambridge, Mass.) containing 4 μCi of tritiated thymidine (specific activity, 2 Ci/mmol; New England Nuclear Corp., Boston, Mass.) in DMEM plus 10% fetal bovine serum (FBS) at 37°C in 5% CO_2. Tritiated-thymidine-labeled 3T12 cells were trypsinized and washed, and 3×10^3 cells were added to CSF macrophage monolayers in DMEM with 10% FBS in a final volume of 0.2 ml/well. After 72 h of incubation, thymidine release was measured in 0.1-ml portions of supernatant from wells containing macrophages plus tumor cells. Spontaneous release was determined from wells containing tumor cells alone. Samples were counted in a liquid scintillation counter (Packard Instrument Co., Downers Grove, Ill.). Total counts added to each well were determined from samples of tumor cell inoculum lysed with 1% sodium dodecyl sulfate. The percentage of tumor cells killed was calculated as follows: % tumoricidal activity = 2 × (cpm in supernatant from macrophage − cpm of spontaneous release)/total cpm added. Each assay was performed in triplicate. Three separate groups of animals were examined. The thymidine release assay for tumor cell killing was correlated with visual counting of Giemsa-stained tumor targets remaining after exposure to macrophages.

Fungistasis and fungicidal assay. The strain of *C. neoformans* used as a target organism in vitro was C3D, a clone from the H99 strain, which is unresponsive to CO_2-induced increase in polysaccharide capsule synthesis. Both clone C3D and strain H99 have been characterized previously (16). A laboratory strain of *Candida parapsilosis* was also used as a target organism. The yeasts were grown overnight on a Sabouraud agar slant at 37°C in air. Yeast cells (10^3) were added to wells with and without macrophage monolayers in modified DMEM containing 3-(*N*-morpholino)propane sulfuric acid (26 mM), dextrose (21 mM), bicarbonate (53 mM), and 10% FBS at a final pH of 7.4. When C3D was used as the test organism, 20% fresh normal rabbit serum and 2% anticryptococcal rabbit serum containing specific antibody were substituted for FBS in the medium to maximize the intracellular ingestion of these yeasts. In addition, the monolayer was seeded a second time with 4×10^5 CSF cells, which were allowed to adhere and then washed as in the original monolayer preparation. This procedure served to minimize empty spaces and avoid extracellular proliferation of yeast cells not in direct contact with macrophages. Although precise quantitation of macrophages within this double monolayer is impossible, the effector-to-target ratio for assays in which this method was used is greater than 200:1. After incubation for 18 to 24 h, supernatant medium from wells containing yeast cells plus macrophages and from yeast growth control wells was removed. Intracellular yeast cells were harvested from remaining monolayers by cell lysis with 0.5% deoxycholate, which was added to the supernatants. Yeast growth control wells were also rinsed with 0.5% deoxycholate. The contents of the wells were serially diluted in PBS and plated on Sabouraud agar. After 48 to 72 h of incubation at 37°C, the number of CFU per milliliter was determined.

Statistical methods. The significance of differences in values between groups was determined by a one-way analysis of variance.

RESULTS

We examined the monolayers from each day of infection to determine the variability in mononuclear phagocyte composition in the monolayer as infection progressed. The number of CSF macrophages in the washed monolayer before target cells were added approximated 2×10^5 macrophages per well. There was generally less than a 10% loss of macrophages from the monolayer with washing. The numbers of macrophages did not significantly vary between days 3 and 9. These macrophages were identified by Giemsa stain and nonspecific esterase stain morphology, and numbers in the monolayer were calculated by determining the number of nonadherent cells in the washes and subtracting them from the original number plated. The number of heterophils in the monolayer prior to washing varied little over days 3 to 9 of infection, with a range from 1.2×10^4 to 5.6×10^4 cells per well, and washing removed from 3 to 30% of these cells.

The production of hydrogen peroxide in vitro by CSF macrophages isolated during infection is shown in Fig. 1. There was an increase in the production of this reactive oxygen intermediate by the CSF macrophages over time. Peak CSF macrophage hydrogen peroxide production occurred between days 4 and 8 of infection. This increase correlated with a significant in vivo reduction of yeast cells in the CSF of animals not receiving immunosuppressive agents (Fig. 2). After 8 days of infection, hydrogen peroxide production stabilized at a lower level. Few detectable yeast cells were left in the CSF of normal rabbits during this later time of infection. The addition of phorbol myristate acetate in vitro further stimulated oxidative metabolism by the CSF macrophages on each day of infection, and although the level of hydrogen peroxide production increased by two- to threefold, the pattern of secretion paralleled that of unstimulated cells. In Fig. 1, the broken line shows the oxygen metabo-

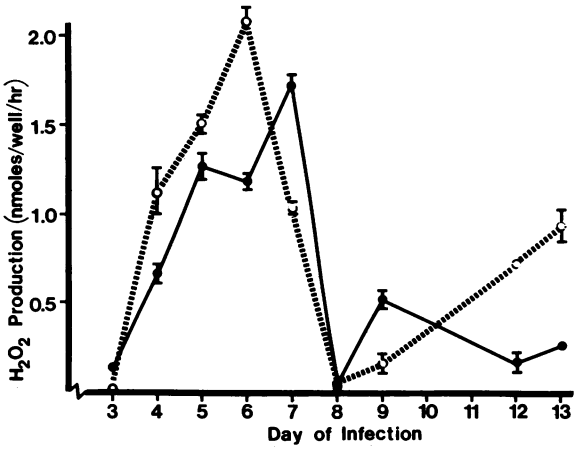

FIG. 1. CSF macrophage hydrogen peroxide production. The hydrogen peroxide production by CSF macrophages from normal infected rabbits (●) is compared with that by CSF macrophages from cyclosporine-treated infected rabbits (○) during the first 2 weeks of cryptococcal meningitis. The measurement was performed on microtiter wells containing 4×10^5 CSF cells.

lism of CSF macrophages from cyclosporine-treated rabbits. Hydrogen peroxide production by CSF macrophages from cyclosporine-treated animals followed the same pattern as in untreated animals. Despite the active respiratory burst from their CSF macrophages, cyclosporine-treated animals did not effectively kill *C. neoformans* in vivo (Fig. 2).

To further characterize the activation of CSF macrophages in response to cryptococcal infection, we examined the tumor cytotoxicity of these cells. Figure 3 shows rabbit CSF macrophage tumoricidal activity against tritiated thymidine-labeled 3T12 cells during infection. Early in the infection, low-grade cytotoxicity was produced by CSF macrophages, but within 24 to 48 h of the increase in hydrogen peroxide production, there was an increase in CSF macrophage tumor cytotoxicity, suggesting in vivo activation of these cells. This CSF macrophage activation resulting in increased tumoricidal activity also correlated with the

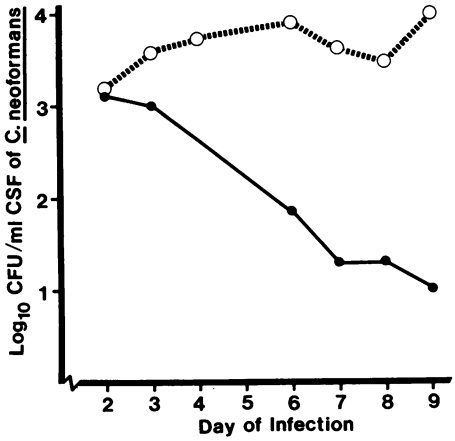

FIG. 2. Yeast cell counts in CSF during meningitis. The concentration of yeast cells in the CSF from days 2 through 9 of experimental infection was monitored with quantitative plate counts. Symbols: ●, CSF yeast cell counts from a pool of five to eight normal rabbits infected with *C. neoformans*; ○, CSF yeast cell counts from a pool of five to eight cyclosporine-treated infected rabbits.

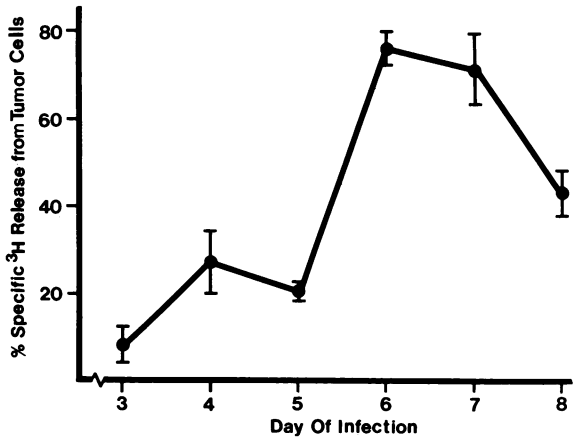

FIG. 3. CSF macrophage tumor cytotoxicity. Acquisition of CSF macrophage tumor cytotoxicity, shown by release of [^{3}H]thymidine from labeled 3T12 cells cultured with CSF macrophages from normal, infected animals.

time of significant in vivo killing of *C. neoformans* as shown in Fig. 2. In contrast to the pattern of tumor cytotoxicity developed by CSF macrophages from normal infected rabbits, cells from immunosuppressed animals receiving cyclosporine showed significantly reduced cytotoxicity. There was no difference in macrophage cytotoxicity between untreated and cyclosporine-treated animals on day 3 of infection. The CSF macrophage cytotoxicity (untreated versus cyclosporine treated) on days 4 to 8 of infection (± standard error of the mean) was as follows: day 4, 60 ± 6% versus 22 ± 3%; day 5, 54 ± 12% versus 36 ± 5%; day 6, 44 ± 7% versus 27 ± 4%; day 7, 69 ± 14% versus 28 ± 8%; and day 8, 49 ± 11% versus 30 ± 12%. The mean tumor cytotoxicity for CSF macrophages from untreated animals when averaged from days 4 through 8 of infection was 56.6 ± 5.1% (40 observations), compared with 29.4 ± 3.1% (38 observations) for cells from cyclosporine-treated animals ($P < 0.001$).

To examine the in vitro fungicidal potential of these macrophages, we exposed them to *Candida parapsilosis*, a nonpathogenic yeast strain which is susceptible to macrophage killing (34). Figure 4 shows the ability of CSF macrophages to kill *Candida parapsilosis*. The pattern of fungicidal activity of these CSF macrophages mimicked those of hydrogen peroxide production and tumor cytotoxicity. This

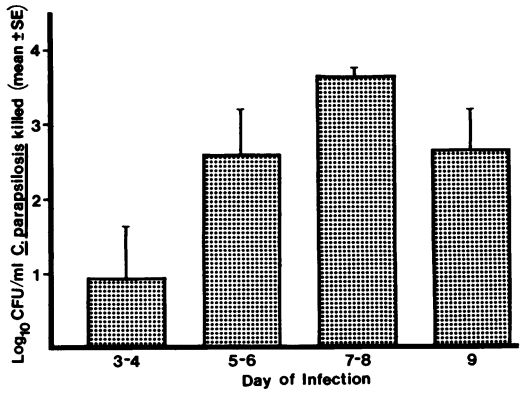

FIG. 4. CSF macrophage fungicidal activity. Fungicidal activity of CSF macrophages against *Candida parapsilosis* was measured during days 3 through 9 of meningitis.

parameter of macrophage effector response peaked at about day 5 of infection. By this time, CSF macrophages have acquired the ability to kill between 1,000 and 10,000 yeast cells in vitro over a 24-h period. Cultures of yeast cells with CSF macrophages examined at 2, 8, 12, 18, and 24 h showed that the majority of killing occurred progressively during the first 12 h of contact. The addition of 20,000 U of catalase per ml to the macrophage-yeast monolayers to block extracellular hydrogen peroxide had no effect on macrophage killing of yeast cells over the first 8 h of exposure.

We examined in vitro anticryptococcal activity of CSF macrophages during this cryptococcal infection. When the C3D clone of strain H99 was added to the CSF macrophage monolayers, the host cells were neither fungicidal nor fungistatic. There was no effect on yeast growth by CSF cells from days 4 through 8 of infection. Wells with macrophages contained the same number of yeast cells as did growth control wells after 24 h. Adding serum with anticryptococcal antibodies or fresh complement to wells did not improve the antifungal activity of CSF macrophages. To examine further the effect of these CSF macrophages on the intracellular growth of cryptococci, we plated CSF macrophages which had ingested the wild-type strain H99 in the subarachnoid space. One group of CSF cells containing cryptococci were immediately frozen at −70°C for 30 min and then thawed. This procedure killed the host cells without affecting the viability of the yeast cells, as determined by trypan blue exclusion and quantitative culture. This method provided a growth control for the initial number of intracellular yeast cells in the monolayer. The freeze-treated monolayers were compared with those not subjected to freezing. After a 24-h incubation in 5% CO_2 at 37°C, CSF monolayers were examined for viable yeast cells. Growth of H99 occurred intracellulary over 24 h in the monolayers with live activated macrophages in vitro. Viable CSF macrophages from each day between 3 and 7 of infection contained at least 10 to 100 times more yeast cells than monolayers of freeze-killed macrophages after incubation for 24 h in vitro. The number of H99 cells in a monolayer ($\log_{10}$ CFU per milliliter) with live versus killed macrophages was: day 3, 4.22 versus 3.20; day 4, 3.73 versus 2.68; day 5, 3.13 versus 1.30; day 6, 1.78 versus <1.0; and day 7, 2.20 versus <1.

DISCUSSION

Most of our knowledge about macrophage biology has been gathered from the study of peritoneal and alveolar macrophages. CSF macrophage effector functions have seldom been studied. It has previously been shown that the site at which a macrophage differentiates and interacts with a fungal pathogen may profoundly influence effector responses. Illustrating this concept is the observation that alveolar macrophages have more effective fungicidal mechanisms against *Aspergillus* spores which they have encountered naturally than do peritoneal macrophages (40). Our study focused on the CSF macrophage and its effects on *C. neoformans*, a primary pathogen for the CNS. Using an experimental animal model and various functional in vitro assays, we were able to examine some effector aspects of this heterogeneous population of mononuclear phagocytes during fungal meningitis.

During experimental cryptococcal meningitis, the CSF macrophage dynamically acquired characteristics of activation (28). Biochemical activation was indicated by increased production of H_2O_2, a reactive oxygen intermediate. The secretion of hydrogen peroxide in our assays reflected primarily the oxidative metabolism of CSF macrophages. Monolayers were not significantly contaminated by CSF polymorphonuclear cells (heterophils), as shown by the morphology of stained cells and the fact that polymorphonuclear cells will not produce H_2O_2 in vitro without a phagocytic or chemical stimulus. The spontaneous H_2O_2 production by these macrophages could be due to phagocytosis of opsonized yeast cells occurring in vivo prior to harvest or may be mediated by lymphokines such as gamma interferon released into the CSF during infection. The correlation between increased H_2O_2 production and microbicidal activity by macrophages has been shown previously for several microorganisms, such as *Toxoplasma gondii* (26), *Candida* species (39), *Trypanosoma cruzi* (27), and *Listeria monocytogenes* (21). An in vivo study particularly relevant to our own showed that during a liver infection with *L. monocytogenes*, the increased production of hydrogen peroxide by inflammatory macrophages infiltrating the liver correlated with elimination of bacteria from this site (21). Similarly, we found a rise in the production of this reactive oxygen intermediate from cells at the site of infection during the period of most active in vivo fungicidal activity by the host.

A second criterion for macrophage activation is cytotoxicity potential. The rabbit CSF macrophages demonstrated increased tumor cytotoxicity during meningitis. CSF macrophage tumor cytotoxicity increased concomitantly with the cells' ability to produce H_2O_2. A second signal such as endotoxin or amphotericin B was not necessary for tumor cytotoxicity by these macrophages, as is the case with murine peritoneal macrophages (34).

A third criterion for macrophage activation in this study was fungicidal activity. With *Candida parapsilosis* as the target, CSF macrophages acquired fungicidal activity over the first 10 days of infection in a pattern similar to that for tumor cytotoxicity. We have previously shown that activated murine peritoneal macrophages kill *Candida parapsilosis* in vitro (34). The mechanism(s) for macrophage killing of *Candida parapsilosis* remains unclear. Previous investigations have demonstrated the importance of both reactive oxygen intermediates and mechanisms not requiring the respiratory burst (19). Although the mechanism of fungicidal activity of these CSF macrophages was not determined in this study, several lines of evidence suggest, but do not prove, that a toxic oxygen intermediate such as H_2O_2 is not primarily involved. First, catalase did not inhibit fungicidal activity in our assay. This finding shows that extracellular release of H_2O_2 is not important, but it remains possible that H_2O_2 inside the phagocytic vacuoles which may not be reached by catalase could be important. Second, the time course of killing by the CSF macrophages was measured over 8 to 12 h rather than in minutes, as would be expected for the cytotoxic activity of oxygen intermediates in many of the classic assays for respiratory burst-induced antimicrobial activity.

Previous investigations into the role of the host macrophages in the control of cryptococcosis have been inconclusive. Macrophages have been shown to inhibit *C. neoformans* under some in vitro conditions (13, 17, 25) and yet to allow its growth under others (8). Despite conflicting indications of macrophage importance from in vitro assays with *C. neoformans*, histologically the macrophage is often a prominent cell at the site of infection in both human and animal infections. Frequently, ingested or attached yeast cells can be seen. These observations have made this professional phagocyte a candidate for the primary effector cell in host

response to many fungi. In previous work, we demonstrated that potentially confusing results could occur if the activation state of the macrophage was not controlled (13, 34). We found that only activated murine macrophages produced consistent and profound fungistasis when challenged with cryptococci. Thus, the state of macrophage activation was crucial to its inhibitory effects on *C. neoformans* growth. CSF macrophages, despite activation by the several criteria described here, not only did not kill *C. neoformans* in vitro, they did not even inhibit growth. Neither intracellular nor extracellular yeast cells were inhibited by these activated cells. This poor performance by CSF macrophages in vitro could not be improved by opsonins and did not correlate with their apparent activity seen histologically in the subarachnoid space during infection (35). However, it should be emphasized that these assays were performed under static conditions with host cells adherent to plastic plates. This artificial assay does not provide the dynamic turnover of cells present in the CSF or the same geometric constraints present in the host. These in vivo factors may be important to macrophage functions.

Cyclosporine is an immunosuppressive agent used primarily in transplantation. It can have either enhancing or inhibiting influences on certain infections. In our model, it produces a profound depressive effect on killing of *C. neoformans* in the subarachnoid space (33). Its selective immune suppression of lymphocyte functions such as production of interleukin-2 and interleukin-2 receptors has been viewed as an advantage over other clinically useful immunosuppressive agents (4). Prior studies have shown the effects of cyclosporine on macrophage functions to be limited to direct suppression of oxidative mechanisms when cells were directly exposed in vitro and supression of macrophage chemotactic response on direct exposure to the drug (9). However, others have found no impairment of macrophages from cyclosporine-treated animals in the production of reactive oxygen intermediates (9), activation in response to a parasitic infection (23), or response to lymphokines (1, 42, 43). In our experiments with cyclosporine, we found that it can depress the cytotoxic potential of CSF macrophages as measured by tumoricidal activity, but there was no effect on H_2O_2 production by these cells compared with those from untreated animals. Interestingly, neoplasms can develop in patients receiving immunosuppressive agents, including cyclosporine (32). If macrophages are important to the host immune response, the findings for cyclosporine-treated animals also suggest that classic oxidative mechanisms are not sufficient by themselves for CSF macrophage anticryptococcal activity. Either other cells must interact with macrophages, or other macrophage effector responses must be important in vivo. Macrophages do contain other effector systems (5, 23), including the production and release of certain peptides, known as defensins, which have been shown to have potent anticryptococcal activity (11, 31). Also, a new and potentially important biochemical mechanism(s) for macrophage tumoricidal and fungistatic capacity has been identified which correlates with the production of nitrates and/or nitrites from activated murine macrophages (14, 16). These alternative mechanisms require further study in CSF macrophages to determine their significance.

The activated CSF macrophage clearly plays an important role in host cytotoxicity within the CNS of rabbits with fungal meningitis beyond a simple scavenger function. However, effective defense against *C. neoformans* undoubtedly requires dynamic cooperation among other host cells with these macrophages.

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