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Effects of Cyclosporine in Experimental Cryptococcal Meningitis

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We studied the effects of cyclosporine on experimental cryptococcal meningitis. Like cortisone, cyclosporine depressed the highly effective defense mechanisms of normal rabbits against inoculated *Cryptococcus neoformans*, causing them to develop progressive, fatal cryptococcal meningitis. Unlike cortisone, which causes a striking reduction in leukocytes in cerebrospinal fluid, cyclosporine depressed mononuclear cell function rather than numbers. Interleukin 2, a primary target for the immunodepressive action of cyclosporine, appears to be of central importance in central nervous system defenses against cryptococci. The findings suggest that humans receiving cyclosporine are likely to suffer increased incidence of cryptococcal infection.

Cyclosporine, a cyclic endecapeptide produced by fungi, is a potent immunosuppressive agent widely used for organ transplantation (4, 11, 18, 30). Associated opportunistic infections have been reported (10). Such opportunistic infections probably occur less frequently with cyclosporine than with other standard immunosuppressive drugs such as azathioprine, cyclophosphamide, and corticosteroids (34), but further studies are needed to confirm this impression.

Cyclosporine can be used as an experimental probe to dissect the roles of immune mechanisms operating against infection. Although further mechanisms of action may yet be discovered, it is clear that cyclosporine has a primary, selective effect on T lymphocytes (1, 20, 21, 24, 37). It blocks the proliferation of T lymphocytes induced by antigens or mitogens, both by inhibition of synthesis of interleukin 2 (IL-2) and by making T cells unresponsive to IL-2 (5). These actions can prevent transplant rejection in humans and experimental animals, including rabbits (17).

Cell-mediated immunity is a crucial factor in host defenses against many fungi, including *Cryptococcus neoformans* (9, 10, 14, 34, 36). For example, athymic mice have been used to demonstrate the need for functioning T lymphocytes to control cryptococcal infection (5, 16). We have used cyclosporine to produce a selective block in T-lymphocyte function in rabbits inoculated with cryptococci. Unlike in athymic mice and animals treated with lympholytic doses of corticosteroids, T cells are still present (although functionally suppressed) in cyclosporine-treated rabbits. Their ability to eradicate *C. neoformans* from the central nervous system was profoundly suppressed by cyclosporine.

MATERIALS AND METHODS

Production of meningitis. New Zealand White rabbits, each weighing 2 to 3 kg, were housed in separate cages and given Purina rabbit chow and water ad libitum. A 4- to 5-day growth of *C. neoformans* (DP strain, serotype A) (32) on Columbia blood agar base (Difco Laboratories, Detroit, Mich.) with 100 μg of chloramphenicol per ml was suspended in 0.015 M phosphate-buffered saline at approximately 5×10^7 CFU/ml. Rabbits were sedated with 50 mg of Ketaject (Bristol Laboratories, Syracuse, N.Y.) per ml and 5 mg of Rompun (Cutter Laboratories, Shawnee, Kans.) per kg intramuscularly. After sedation, individual rabbits were inoculated intracisternally with a 0.3-ml yeast suspension through a 25-gauge needle on a 3-ml syringe. Rabbits were sedated on days 4 and 7 after inoculation, and cerebrospinal fluid (CSF) was withdrawn. Quantitative yeast cultures were performed by plating serial dilutions of CSF in phosphate-buffered saline onto Columbia blood agar.

CSF cell populations. CSF leukocytes were counted in Turk solution by using a hemocytometer. Differential leukocyte counts were performed on cytocentrifuged CSF with Giemsa stain. To analyze for lymphocyte subpopulations and phagocytic cells, samples of CSF and blood mononuclear cells were centrifuged at $400 \times g$ for 10 min at 4°C. The supernatant was discarded, and the pellet was resuspended in 200 μl of RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.). The suspension was incubated with latex beads for 1 h at 37°C and centrifuged again. The pellet was resuspended in 100 μl of RPMI 1640 supplemented with fetal calf serum (10%) and sodium azide (0.04 g/dl). A 100-μl portion of 9AE10, a monoclonal mouse immunoglobulin M antibody directed against a rabbit T-lymphocyte antigen (26), and 10 μl of rhodamine-conjugated goat anti-rabbit immunoglobulin (Cappel Laboratories, Cochranville, Pa.) were added, and the mixture was kept on ice for 30 min. The cells were washed twice and suspended in a solution of fluorescein-conjugated goat anti-mouse immunoglobulin M (Meloy Laboratories, Springfield, Va.). The cells were washed three times and examined with a fluorescence microscope equipped with appropriate filters. One hundred cells were counted with each sample. T lymphocytes were identified as cells with a fluorescent rim; B lymphocytes stained positively with rhodamine. Null cells were defined as cells which were nonphagocytic and did not stain with T or B cell markers.

Blood lymphocyte transformation studies. Rabbits from each treatment group were bled on day 4 or 5 of infection, immediately after receiving their daily dose of cyclosporine. Blood mononuclear cells were separated from heparinized blood by centrifugation over lymphocyte separation medium (Bionetics Inc., Kensington, Md.). Blood mononuclear cells from individual rabbits were suspended in RPMI 1640 with 10% fetal calf serum, and 100 μl (2×10^5 cells) was added to 96-well Linbro tissue culture plates (Flow Laboratories, Inc., McLean, Va.). Various concentrations of concanavalin A (1 to 5,000 μg/ml) (Con A; Sigma Chemical Co., St. Louis, Mo.) were added to appropriate wells in triplicate. Peak activity for lymphocyte transformation was found for Con A at concentrations of 1,000 μg/ml. Purified human interleukin

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TABLE 1. Cyclosporine in serum and CSF of three rabbits with cryptococcal meningitis 1, 6, and 24 h after a single 30-mg intravenous dose

Time postinfection (h)	Amt of Cyclosporine (mean ± SE) (ng/ml) in:	
	Serum	CSF
1	606 ± 187	<5
6	34 ± 17	ND
24	<5	<5

2 (IL-2; Electronucleonics Inc., Silver Spring, Md.) was added to wells at a 1:2 dilution. Plates were incubated for 72 h in 5% CO_2 at 37°C; 18 h before harvest of cells they were pulsed with 0.5 μCi of tritiated thymidine. Cells were harvested with a MASH cell harvester (M. A. Bioproducts, Walkersville, Md.), and filters were counted in Aquasol 2 (New England Nuclear Corp., Boston, Mass.) with a scintillation counter. A lymphocyte stimulation index for each rabbit was calculated as the quotient of the counts per minute for cells treated with Con A over the counts per minute for untreated cells.

Treatment groups. Rabbits were randomized to one of three treatment groups. The first group received daily intravenous injections of cyclosporine (a gift from Sandoz Pharmaceuticals, East Hanover, N.J.), 30 mg, in cremophor EL (Blagden Campbell Chemicals LTB, Surrey, U.K.), starting 1 day before inoculation of *C. neoformans* and continuing for 5 to 7 days. The second group received daily intramuscular injections of cortisone acetate (Merck Sharpe & Dohme, West Point, Pa.), 2.5 mg/kg, starting 1 day before inoculation of *C. neoformans* and continuing for 7 days. The final group received no drug treatment, but was inoculated with cryptococci at the same time as the first two groups.

Cyclosporine levels. Three rabbits with cryptococcal meningitis at day 11 received an intravenous bolus of cyclosporine, 30 mg, in cremophor EL. Serum and CSF were drawn 1, 6, and 24 h and 1 and 24 h later, respectively. Cyclosporine was assayed in these fluids with a commercial radioimmunoassay kit (Sandoz, Inc., East Hanover, N.J.). The sensitivity of the assay was 5 ng/ml or greater.

Statistics. Student's *t* test for unpaired means was used to compare results from the treatment groups. For results with a nonnormal distribution, the nonparametric Wilcoxon rank sum test was used. Fisher's exact test was used for comparing mortality between groups.

RESULTS

Cyclosporine concentrations in serum and CSF of rabbits with cryptococcal meningitis are shown in Table 1. The mean serum concentration at 1 h was 606 ng/ml, with a range from 233 to 784 ng/ml. Cyclosporine was eliminated during 1 day; less than 5 ng/ml was present at 24 h. No cyclosporine was detectable in CSF at either 1 or 24 h after a single dose.

TABLE 3. Total number of leukocytes and percentage of polymorphonuclear heterophils, monocytes, and lymphocytes in CSF of rabbits with cryptococcal meningitis receiving cyclosporine, cortisone, or no treatment 4 days after inoculation of *C. neoformans*[a]

Treatment	Total no. of leukocytes per mm^3	% PMNs[b]	% MNCs[b]	% LYMPHs[b]
None	2,086 ± 410	17 ± 7	31 ± 8	52 ± 4
Cyclosporine	1,119 ± 361	11 ± 3	38 ± 4	50 ± 5
Cortisone	74 ± 33	16 ± 6	41 ± 9	43 ± 5

[a] Results are mean ± standard error; there were 6 to 11 rabbits per group.
[b] Abbreviations: PMN, polymorphonuclear heterophil; MNC, monocyte; LYMPH, lymphocyte.

To detect any possible direct antifungal effect of cyclosporine on *C. neoformans* DP strain, we used a standard method for in vitro broth antifungal susceptibility testing. The inoculum was approximately 5×10^3 CFU/ml in buffered yeast nitrogen base medium incubated at 30°C for 18 to 24 h. There was no effect on yeast growth at up to 100 μg of cyclosporine per ml.

Table 2 shows the effects of cyclosporine and cortisone on the eradication of cryptococci from the CSF of rabbits. Both drugs profoundly inhibited the normal capacity of these rabbits to clear yeasts from CSF. Yeast counts in CSF were significantly higher on days 4 and 7 after inoculation in cyclosporine- and cortisone-treated animals than in untreated animals ($P < 0.01$). Mortality was much higher in rabbits treated with cyclosporine: 16 of 20 cyclosporine-treated animals died over 4 weeks while none of 18 normal rabbits died ($P < 0.001$) (one rabbit was killed by CSF withdrawal and was therefore not included in the survival data). The median time between inoculation and death was 11 days, with a range of 5 to 27 days. These deaths could not be attributed to cyclosporine toxicity alone, because only one of five rabbits died within 4 weeks after receiving seven daily treatments of 30 mg of cyclosporine intravenously ($P < 0.01$). The four cyclosporine-treated rabbits that did survive the infection eventually eradicated *C. neoformans* from their CSF.

The differential leukocyte counts in peripheral blood on day 3 were similar for the rabbits receiving cyclosporine and those receiving no treatment. Rabbits receiving cortisone had approximately one-half the total number of blood lymphocytes compared with the other two groups. The effects of cyclosporine and cortisone on leukocyte counts in CSF are presented in Table 3. The characteristic CSF leukopenia found in cortisone-treated rabbits did not occur in

TABLE 2. Quantitative CSF yeast counts in rabbits receiving cyclosporine, cortisone, or no treatment after 4 and 7 days of infection with *C. neoformans*

Treatment	No. of rabbits	Log_{10} cfu for *C. neoformans* per ml of CSF (mean ± SE) on:			
		Day 4		Day 7	
Cyclosporine	21	5.03 ± 0.31	$P > 0.05$	4.25 ± 0.42	$P > 0.05$
Cortisone	11	4.46 ± 0.37	$P < 0.01$	4.50 ± 0.45	$P < 0.01$
None	18	3.12 ± 0.29		0.89 ± 0.30	

TABLE 4. Total number of lymphocytes and lymphocyte subpopulations in CSF of rabbits receiving cyclosporine, cortisone, or no treatment 4 days after inoculation of *C. neoformans*[a]

Treatment	Total no. of lymphocytes per mm^3	Lymphocyte subpopulations (%)		
		T cells	B cells	Null cells
None	889 ± 204	45 ± 8	24 ± 5	31 ± 7
Cyclosporine	548 ± 190[b]	56 ± 4	15 ± 4	29 ± 5
Cortisone	19 ± 7[c]	62 ± 7	15 ± 5	23 ± 3

[a] Results are expressed as mean ± standard error; there were three to seven rabbits per group.
[b] $0.05 < P < 0.1$ versus no treatment.
[c] $P < 0.001$ versus cyclosporine or no treatment.

cyclosporine-treated animals. Although the median cell count was somewhat lower after cyclosporine treatment ($0.05 < P < 0.10$ by the Wilcoxon rank sum test compared with normal rabbits), there was considerable overlap between CSF cell counts in the cyclosporine-treated and untreated groups. This finding contrasts with the effect of cortisone treatment, which reduces all lymphocyte subpopulations, including the predominant T lymphocyte, to very low numbers in the CSF. Examination of lymphocyte subpopulations on day 4 showed that cyclosporine did not alter their relative proportions in CSF (Table 4).

Because there was little or no effect on the quantitative host cellular response in the CSF during cyclosporine treatment, we examined lymphocyte function. Functional assays of rabbit CSF lymphocytes are technically difficult because of the small number of cells available, so we examined some functional capabilities of blood lymphocytes. Figure 1 shows the mitogenic lymphocyte stimulation in vitro of peripheral blood cells in individual rabbits infected with *C. neoformans* and receiving either cyclosporine for 4 to 5 days or no treatment. Con A acted as a strong mitogen for all rabbits studied. Treatment with cyclosporine in vivo significantly reduced the response to Con A. There was no effect on spontaneous lymphocyte transformation: median counts were 208 cpm (range, 97 to 1,134) for cyclosporine-treated animals compared with 210 cpm (77 to 832) for untreated animals. However, there was a significant reduction in the response to Con A: median counts were 395 cpm for cyclosporine-treated animals and 1,996 cpm for those receiving no treatment ($P < 0.01$). For the median stimulation index, which was 2.1 (1.1 to 3.9) versus 11.8 (5.8 to 32.9), respectively, there was no overlap between individual rabbits in each group. However, lymphocytes from rabbits receiving cyclosporine or no treatment responded equally well to exogenous human IL-2 in vitro. There was an increase in spontaneous lymphocyte transformation in all rabbits when IL-2 was incubated with the blood mononuclear cells in vitro. There was no significant difference between cyclosporine-treated animals (mean, 2,636 ± 861 cpm) versus normal animals (mean, 1,828 ± 456 cpm). Thus, the lymphocytes of cyclosporine-treated animals remained responsive to exogenous IL-2.

DISCUSSION

The extent to which cyclosporine causes opportunistic infection during clinical use in humans remains to be defined. Experience after organ transplantation suggests that serious infections caused by a variety of opportunistic pathogens can occur in patients receiving cyclosporine (4, 10, 18, 30). A few patients have developed serious disseminated mycoses, including cryptococcosis, while receiving cyclosporine (10). However, some reports suggest that the incidence of such infections is much lower than with older immunosuppressive regimens (34). The true relationship between cyclosporine treatment and opportunistic infection is further confused by the frequent practice of using cyclosporine and corticosteroids together (4, 30).

The immune response to *C. neoformans* is incompletely understood. For example, the importance or otherwise of the humoral response to this infection remains unproven (7, 15, 25, 27, 33). On the other hand, the cellular immunity limb has been emphasized as an extremely important factor in cryptococcosis (10, 17, 37). Most patients have underlying diseases that could depress cellular immunity or have received corticosteroids or other immunosuppressive drugs (8). Even apparently normal patients who develop cryptococcal meningitis may have abnormal lymphocyte function (9, 14, 36). Cyclosporine-treated rabbits provide a model in which the mitogenic responses of blood lymphocytes are likewise reduced. Previous in vitro and in vivo studies have shown the importance of NK cells (28), antigen-induced suppressor T cells (29), and activated macrophages in the eradication of *C. neoformans* from the animal host (12). The athymic mouse model has demonstrated the primary importance of T-cell function in the immune response of the host to *C. neoformans* (5, 16). We have shown that a selective immune suppressant can block the efficient mechanisms for killing cryptococci in the CSF of intact animals.

Although additional actions of cyclosporine may yet be

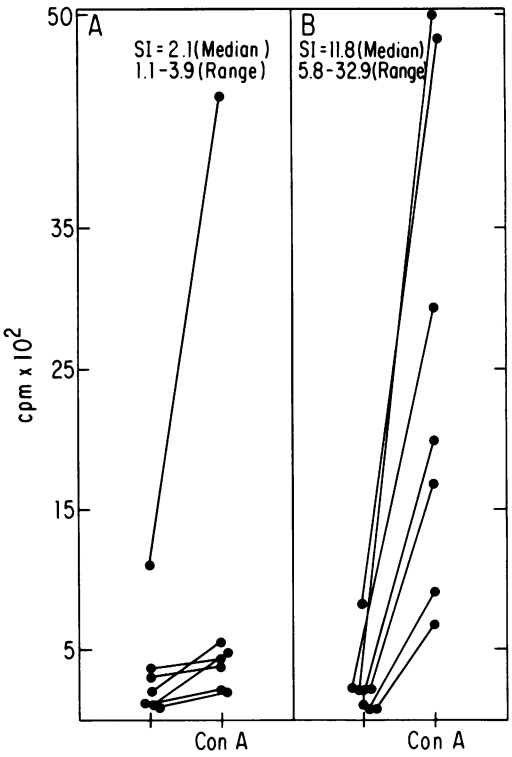

FIG. 1. Proliferative responses induced by the mitogen Con A in rabbit lymphocytes from individual animals with cryptococcal meningitis receiving cyclosporine treatment (A) or no immunosuppressive treatment (B), as measured by incorporation of [^{3}H]thymidine are shown. SI, Median stimulation index with range.

defined, the major effect of this agent seems to be its ability to block production of IL-2 by T lymphocytes; it probably blocks the receptors on host cells for IL-2 as well (3). Patients with the acquired immune deficiency syndrome have a severe deficiency of IL-2 (35). Patients with the acquired immune deficiency syndrome are susceptible to overwhelming *C. neoformans* infection. These observations suggest that IL-2 (13) may be an important mediator in the immune response to *C. neoformans* in humans. Our experiments are consistent with a central role for IL-2 in the eradication of yeast from the CSF. The rabbit model could be used to study whether treatment with exogenous IL-2, injected intrathecally, can cure the immune defect in these animals. If so, this experimental treatment could be tried in humans. Our data suggest that lymphocytes from cyclosporine-treated rabbits will respond in vitro to human IL-2. The availability of purified IL-2 allows further studies of its effect on host responses at the site of infection. It should also be noted that cyclosporine can depress the production of gamma interferon (21). This action may be another component of its immunosuppressive effect in rabbits.

Cyclosporine and corticosteroids have different immunosuppressive action on cells in the CSF. Corticosteroids produce a dramatic reduction in CSF leukocyte counts, associated with lowered peripheral blood leukocyte counts and reduced CSF chemotactic activity (31). The CSF leukopenia seen with cortisone treatment in the rabbit model mimicks the findings seen in some human cryptococcal meningitis patients with a poor prognosis (8). In contrast, cyclosporine treatment has little or no effect on the number of cells at the site of infection. Thus, while cyclosporine induces a definable functional abnormality, cortisone causes multiple quantitative and qualitative changes which make any functional defects much more difficult to define (7, 33). Cryptococcal meningitis progressed more rapidly to death in rabbits treated with cyclosporine than in those treated with cortisone. Median time to death was approximately 2 weeks with cyclosporine at the doses used in this study, compared with approximately 4 weeks with cortisone (33).

Although we have shown a striking suppression of the immune response to *C. neoformans* in the central nervous system of rabbits, cyclosporine may be less detrimental for the host response to other species of fungi and protozoa. Cyclosporine has direct inhibitory actions on schistosomes (2) and malaria parasites (38). The growth of certain fungal species, including *Coccidioides imitis*, can also be inhibited by this drug both in vitro and in vivo (23). Thus for some mycotic infections there may be a complicated balance between the effects of the immune suppression and the direct antifungal activity of this agent. We found no direct effect of cyclosporine on growth of the *C. neoformans* strain used in our experiments. Therefore, the primary action of cyclosporine in our model must be immune suppression. We were not able to measure cyclosporine in CSF after a single dose, but serum concentrations were comparable to those found in humans, where the therapeutic range is 100 to 1,000 ng/ml and trough levels are 100 to 400 ng/ml. Levels of 1,000 ng/ml have been shown to suppress cellular proliferative and cytotoxic T-cell generation completely in mixed lymphocyte culture (22). Peak concentrations of cyclosporine in rabbits during treatment approached this level, and it was able to block the effective killing mechanisms for *C. neoformans* in rabbits. The absence of detectable cyclosporine in the CSF suggests that it acts on immunocytes before they reach the actual site of infection in the central nervous system.

The observed differences in effects on the host response by these two immunosuppressive agents against *C. neoformans* suggest that a combination of the agents would greatly potentiate the degree of immune dysfunction. It is likely that the use of cyclosporine and corticosteroids together will increase the frequency and severity of infection with *C. neoformans* or other pathogens, such as *Pneumocystis carinii*, normally controlled by cellular immunity. Cyclosporine, like corticosteroids, increases the frequency of *Pneumocystis* infections in rats (19).

Cyclosporine represents a great advance in prevention of graft rejection, despite side effects such as nephrotoxicity, defective tumor surveillance, and infections (22). Our studies on cryptococcosis in cyclosporine-treated rabbits help to identify the mechanisms which kill these yeasts efficiently in normal rabbits. Further studies will define these mechanisms more fully, perhaps leading to interventions which could reduce the risk of opportunistic infections or help in management of those which do occur. Meanwhile, we predict that more cases of cryptococcosis will occur in patients receiving cyclosporine, especially if it is combined with corticosteroid therapy.

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