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Cyclosporin A Inhibits *Coccidioides immitis* In Vitro and In Vivo

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BALB/c mice infected intraperitoneally with *Coccidioides immitis* were treated with cyclosporin (CyA) subcutaneously. CyA prevented infection when treatment was started at day zero. When treatment was delayed until day 6 after infection, the mice that received either 75 or 25 mg/kg per day survived, but those treated with 7.5 mg/kg per day had the same mortality rate as controls. The higher doses of CyA prevented dissemination of the fungus from the peritoneum to the lung but did not eliminate the peritoneal infection. In vitro, CyA inhibited the growth of the mycelial phase of eight test strains of *C. immitis* at a concentration of 1.0 μg/ml. One or two strains of 10 other fungi were tested for susceptibility to CyA; only *Aspergillus niger* was inhibited, at a concentration of 0.1 μg/ml. CyA is structurally unrelated to the polyenes and imidazoles and has a very restricted spectrum of antifungal activity. CyA may represent a new class of antifungal agents with a novel mechanism of antifungal activity.

Infections due to *Coccidioides immitis* are very refractory to treatment with the limited number of antifungal drugs currently available. Although amphotericin B is uniformly active against *C. immitis* in vitro (6), it is not always clinically effective and is very toxic (11). Miconazole, an imidazole drug initially thought to be useful for the treatment of coccidioidomycosis, has less nephrotoxicity than amphotericin B but is of limited therapeutic value (11). Ketoconazole, a newly licensed imidazole derivative, is active against a variety of fungi, including *C. immitis* (10). Ketoconazole is relatively nontoxic, is orally absorbed, and represents a significant addition to our therapeutic armamentarium. However, in clinical trials of ketoconazole in coccidioidomycosis, this drug is also not uniformly effective, and there is a high relapse rate after treatment is stopped (4). Thus, there is still a need for additional nontoxic antifungal drugs which are effective against *C. immitis*.

In this paper we report the discovery that cyclosporin A (Cya), a drug noted for its immunosuppressive activity (2), is an antibiotic that inhibits the growth of *C. immitis* in vitro and is effective therapy for experimental murine coccidioidomycosis.

MATERIALS AND METHODS

Drug. CyA was the gift of J. Borel of Sandoz Ltd., Basel, Switzerland. For in vivo use, the drug was dissolved in pure olive oil at a final concentration of 15 mg/ml by heating to 50°C. For in vitro use, the drug was dissolved in absolute ethanol at 28 mg/ml, and an equal volume of Tween 80 (J. T. Baker Chemical Co., Phillipsburg, N.J.) was added with stirring. Saline (0.90%) was slowly added to this mixture with stirring, to achieve a final concentration of 2 mg of CyA per ml. This procedure is necessary because CyA is poorly soluble in water.

Plasma levels of CyA were determined 24 h after the last of three injections of the drug. Mice were matched for weight and age to the experimental animals. The plasma levels of drug were determined in competitive radioimmunoassay (materials supplied by Sandoz) as previously described (7).

MIC determinations. An agar dilution method was used for minimal inhibitory concentration (MIC) determinations. CyA was incorporated into Mycophil agar (BBL Microbiology Systems, Cockeysville, Md.) using 10-fold dilutions with final concentrations of 0.01 μg/ml. A solvent control consisting of agar with the highest concentration of ethanol-Tween 80-saline had no antifungal activity. One hundred-microliter suspensions of arthroconidia, at a density of 2×10^3 to 5×10^3 CFU/ml, were spread over the surface of the agar. The plates were incubated at 25°C, and the results were read after 5 days. The MIC was defined as the lowest drug concentration which completely inhibited fungal growth.

Infection. BALB/c female mice (Simonsen Laboratories, Gilroy, Calif.) were used at 3 to 4 months of age. Groups of mice were infected intraperitoneally with 5×10^4 arthroconidia of the R.S. strain of *C. immitis* (13), 1,000 times the 50% lethal dose for this mouse strain (9). One group of mice was treated with daily subcutaneous injections of 1.5 mg of CyA in 0.1 ml of olive oil; mice in the control group received injections of olive oil starting on the day of infection. All animals were sacrificed on day 14, and quantitative cultures of omentum, spleen, and lungs were done. In

another experiment, treatment with CyA was delayed until 6 days after infection with 5×10^2 arthroconidia. The mice were treated for 11 days and then sacrificed 4 days after the last dose of CyA. Quantitative cultures were done of the lungs, spleen, and omentum of all surviving mice.

Quantitative cultures. Omenta, spleens, and lungs were aseptically removed from animals and homogenized in sterile water containing 1% Triton X-100. Serial dilutions were made with sterile water, and the appropriate dilutions were spread over the surface of Mycosel agar (BBL) containing 50 μg of gentamicin per ml. The culture plates were incubated at 25°C, and the number of CFU was determined on day 3.

Fungi. *C. immitis* R.S., the kind gift of H. Walch, San Diego State University, was used to infect the mice. For in vitro testing, eight recent clinical isolates of *C. immitis* were obtained from the microbiology laboratory of our institution. The other fungi and yeasts tested were also clinical isolates. All strains were maintained by passage on Mycophil agar.

RESULTS

In vivo activity. We initially observed that CyA was active against *C. immitis* in vivo in an experiment in which mice were given CyA to evaluate the effect of ablating the primary T-lymphocyte immune response on the course of murine coccidioidomycosis. The mice were treated daily with CyA, beginning on the day of infection. All mice in both the CyA and control groups survived for 2 weeks. Surprisingly, none of the CyA-treated mice had any viable organisms in any organ, whereas the control mice were heavily infected (Table 1).

To determine whether CyA was also effective treatment for an established infection, treatment with CyA was delayed until 6 days after intraperitoneal infection. Previous studies have shown that on day 6 there is an established intraperitoneal infection but infection is still confined to the abdomen (9). Mice were treated for 11 days with three different doses of CyA or with olive oil as a control. Four days after treatment was stopped (20 days after infection), the surviving mice were sacrificed, and quantitative cultures of the omentum, spleen, and lungs were done. Table 2 shows that all mice treated with 75 or 25 mg/kg per day survived, whereas only four of nine control mice survived. The 7.5-mg/kg dose did not improve survival. Treatment with the highest dose also prevented spread of the infection from the peritoneum to the spleen and lungs in most animals. Treatment with 25 mg/kg per day prevented spread of the organism to the lungs but not the spleen. Treatment with 7.5 mg/kg per day had no effect on the dissemination of the infection. The levels of CyA in the plasma of weight-matched mice treated for 3 days with the three doses of CyA are also presented in Table 2. The plasma was collected 24 h after the last dose, so they are trough levels.

TABLE 1. Prevention of infection with *C. immitis* by CyA[a]

Treatment group	CFU/organ[b]		
	Omentum	Spleen	Lungs
Control[c]	6.6×10^5	5.0×10^4	3.3×10^2
CyA[d]	<10	<10	<10

[a] Inoculum, 5×10^4 CFU in the form of arthroconidia. There were eight mice in each group.
[b] Determined on day 14 of treatment.
[c] Olive oil (0.1 ml) was given subcutaneously for 14 days.
[d] CyA (1.5 mg/day) was given subcutaneously for 14 days.

In vitro activity. To learn whether CyA had direct antifungal activity, we cultured the mycelial phase of *C. immitis* in the presence of CyA in agar and determined the MIC for each strain tested. The MIC for other molds and yeasts was also determined in a similar manner. All eight strains of *C. immitis* were completely inhibited by 1.0 μg/ml but not by 0.1 μg/ml (Table 3). CyA did not inhibit the mycelial phase of two other pathogenic fungi, *Histoplasma capsulatum* and *Blastomyces dermatitidis*, even at concentrations of up to 10 μg/ml. At a concentration of 1.0 μg/ml, CyA inhibited the growth of one strain of *Aspergillus niger*. CyA had no activity at concentrations of up to 10 μg/ml against *Aspergillus fumagatus*, *Aspergillus flavus*, or a variety of yeasts.

DISCUSSION

CyA, a metabolite of the fungus *Tolypocladum inflatum* Gamms, was initially developed as an antibiotic. However, this cyclic endecapeptide had no antibacterial activity and was believed to have no important antifungal activity (8). Instead, it was found to selectively inhibit lymphocytes and to inhibit primary T-dependent immune responses (2). Although most of the current interest in CyA is as an immunosuppressive agent, recently it was discovered that CyA directly inhibits *Plasmodium berghei* and *Plasmodium chabaudi*, the agents of rodent malaria (12). It has also been shown that CyA reduces the worm burden in mice experimentally infected with *Schistosoma mansoni*, although whether this is a direct effect on the worm is unclear (3).

CyA has not been previously reported to inhibit medically important fungi either in vitro or in vivo. We have found that CyA inhibits the growth of the mycelial phase of *C. immitis* in vitro and is effective therapy for murine coccidioidomycosis. Of the other fungi we have tested in vitro, CyA is active at low concentrations only against *A. niger*, as has been previously reported (8). Thus, compared to amphotericin B

TABLE 2. Treatment of *C. immitis* peritonitis in mice with CyA[a]

Dose (mg/kg per day)	CyA level (μg/ml)	No. surviving/ total no.	% Positive cultures[b]		
			Omentum	Spleen	Lungs
75.0	0.553 ± 0.178[c]	10/10	90	20	10
25.0	0.094 ± 0.027	10/10	90	80	0
7.5	<0.062	3/9	100	100	66
0	ND[d]	4/9	100	100	75

[a] Inoculum, 5×10^2 arthrocondidia.
[b] All surviving animals in each group were autopsied 4 days after the last dose.
[c] Mean ± standard error of the mean.
[d] ND, Not done.

and the imidazole antifungal drugs, CyA has a very limited spectrum of antifungal activity. The reason for its selective activity remains to be determined.

The in vivo activity of CyA was demonstrated in two ways. CyA was completely effective at preventing infection when treatment was started on the day of infection. This result is particularly impressive because we infected the mice with 1,000 times the 50% lethal dose of arthroconidia (9) and because CyA, a potent immunosuppressive agent, almost certainly prevented the mice from mounting an effective immune response.

We also tested CyA by delaying treatment until peritonitis was established, but before dissemination had occurred. All mice survived that were treated with 75 or 25 mg/kg per day. Both those doses resulted in low but measurable blood levels of CyA that were slightly below the MIC for the mycelial form of *C. immitis*. Therefore, it is possible that higher doses might effect a cure rather than just limiting the spread of infection. However, no other antifungal drug has been shown to cure mice of established coccidioidomycosis (10), so that may not be a realistic goal.

TABLE 3. MIC of CyA[a]

Organism	No. of strains tested	MIC (μg/ml)
Molds		
Coccidioides immitis	8	1.0
Aspergillus flavus	1	>10
Aspergillus fumagatus	1	>10
Aspergillus niger	1	0.1
Petriellidum boydii	1	>10
Histoplasma capsulatum	2	>10
Blastomyces dermatitidis	2	>10
Yeasts		
Cryptococcus neoformans	2	>10
Candida albicans	2	>10
Candida tropicalis	2	>10
Cryptococcus glabrata	2	>10

[a] CyA was incorporated in Mycophil agar at concentrations of 0.01, 1.0, and 10.0 μg/ml. Plates were inoculated with 2×10^2 to 5×10^2 CFU.

CyA and polymyxins are both cyclic peptides which inhibit the growth of *C. immitis* (5). However, polymyxins differ significantly from CyA. Polymyxin B, which is the most active polymyxin, is a highly charged basic molecule that includes an ester-linked medium-chain fatty acid. The basic charge is necessary for activity (5). Since CyA contains only neutral amino acids and no fatty acids, we do not think it likely that CyA acts as a membrane detergent, as does polymyxin.

Because CyA is an immunosuppressive drug which affects T lymphocytes, the component of immunity thought to be crucial for resistance to *C. immitis* infection (1), it is unlikely that CyA will be a clinically useful drug for coccidioidomycosis. However, the structure of CyA is quite different from the structure of amphotericin B or the imidazoles. Therefore, CyA may have a different mechanism of antifungal activity and may be a prototype for new antifungal agents. It may be possible to develop congeners of CyA which are not immunosuppressive but retain antifungal activity. For these reasons, the activity of CyA against *C. immitis* deserves further study.

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