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In Vitro and Ex Vivo Effects of Cyclosporin A on Phagocytic Host Defenses against *Aspergillus fumigatus*

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Because cyclosporin A (CsA) is extensively used as an immunosuppressive agent, its effects on phagocytic defenses against *Aspergillus fumigatus* were studied in vitro and ex vivo. After incubation with 10 to 250 ng of CsA per ml at 37°C for 60 min, polymorphonuclear leukocytes (PMNs) exhibited unaltered superoxide anion (O_2^-) production in response to phorbol myristate acetate and *N*-formylmethionyl leucyl phenylalanine, whereas ≥500 ng/ml significantly suppressed it ($P < 0.01$). Moreover, at <250 ng of CsA per ml, PMNs exhibited no change in their capacity to damage unopsonized hyphae of *A. fumigatus* compared with controls, whereas at ≥250 ng/ml, CsA suppressed the function ($P < 0.01$). Although neither CsA (250 ng/ml) nor hydrocortisone (10 μg/ml) suppressed PMN O_2^- production in response to phorbol myristate acetate and *N*-formylmethionyl leucyl phenylalanine, combination of the two agents reduced the function compared with that at the baseline ($P < 0.05$). Incubation of monocytes with 100 ng of CsA per ml for 1 or 2 days suppressed their antihyphal activity. No essential change in phagocytic activity of monocyte-derived macrophages (MDMs) against *A. fumigatus* conidia, tested as the percentage of phagocytosing MDMs and average number of MDM-associated conidia, was detected after 2 or 4 days of incubation with 10 to 1,000 ng of CsA per ml. Furthermore, in rabbits treated with CsA (up to 20 mg/kg of body weight per day intravenously for 7 days), neither O_2^- production and hyphal damage caused by PMNs or monocytes against hyphae nor phagocytosis of conidia by pulmonary alveolar macrophages was significantly suppressed. Thus, these results demonstrated that CsA within therapeutically relevant concentrations does not suppress antifungal activity of phagocytes except that of circulating monocytes. However, it may induce significant immunosuppression of phagocytes' antifungal function at relatively high concentrations in vitro, especially when combined with corticosteroids.

Cyclosporin A (CsA) is a fungal metabolite with potent immunosuppressive properties. It down-regulates the production of interleukin-2 by T lymphocytes, affecting both T helper and T effector cell functions (7, 14, 15). Because of this action, CsA has been extensively used for the prevention of organ rejection in transplant patients as well as for the treatment of autoimmune diseases.

Serious infections, including several cases of aspergillosis, have occurred in patients receiving immunosuppressive therapy with CsA (4, 10, 12, 29), including recipients of kidney (10, 12), heart (4, 10, 12), and liver (29) transplants. In comparison to patients with neutropenia, however, episodes of invasive aspergillosis have been less frequently encountered in patients with organ transplants or autoimmune diseases who are treated with CsA-containing immunosuppressive regimens. The most common *Aspergillus* species isolated has been *A. fumigatus*. The immunologic impairment underlying susceptibility to *Aspergillus* infection in these patients is unknown.

The major host defenses against invasive aspergillosis are pulmonary alveolar macrophages (PAMs) and peripheral blood polymorphonuclear and mononuclear phagocytes. Macrophages have been shown to constitute the first line of defense by ingesting inhaled conidia of *Aspergillus* spp. and inhibiting their germination (17, 26, 30). As a second line of defense, polymorphonuclear leukocytes (PMNs) damage escaping hyphae by secreting microbicidal oxidative and nonoxidative metabolites and preventing invasive disease (5, 17, 26).

Previous investigators have demonstrated that hyphae and pseudohyphae of fungi, such as *Candida albicans*, are killed by PMNs and monocytes (6, 19). Furthermore, PMNs and monocytes respond to stimulation with activation of an oxidative burst and subsequent release of microbicidal metabolites, such as hydrogen peroxide, hypochlorous acid, and chloramines (19). Hyphae of *Aspergillus* spp. are also susceptible to these microbicidal substances (17) and can elicit similar responses from the attacking PMNs. To assess the effects of CsA on the activity of circulating phagocytes and macrophages against hyphae and conidia of *A. fumigatus*, we undertook a series of in vitro and ex vivo studies.

MATERIALS AND METHODS

Source of effector cells. Twenty-five healthy adult volunteers served as donors for testing the effects of CsA on the function of phagocytes in vitro. Six of them donated twice during these studies. In addition, 15 pathogen-free female New Zealand White rabbits (Hazleton, Rockville, Md.) weighing 2.0 to 3.0 kg each were used for the ex vivo experiments. Rabbits were intravenously treated with CsA obtained from Sandoz Ltd., Basel, Switzerland, at dosages of either 10 mg/kg of body weight per day (plus methylprednisolone [5 mg/kg/day] in some experiments) for 4 days or 20 mg/kg/day for 7 days. The plasma CsA levels achieved with these dosages were approximately 40 to 50 or 100 to 120 ng/ml, respectively. At the end of CsA treatment of the rabbits, PMNs and mononuclear leukocytes

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(MNCs) were obtained from venous blood and PAMs were obtained from lungs by bronchoalveolar lavage as described below.

Preparation of effector cells. (i) PMNs. Human or rabbit PMNs were isolated from heparinized (5 to 10 U/ml) venous blood by dextran sedimentation followed by centrifugation over Ficoll and hypotonic lysis of erythrocytes as previously described (3, 22). They were then resuspended in Hanks' balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+}. The resulting cell preparations consisted of more than 95% viable PMNs by trypan blue exclusion and modified Wright-Giemsa stain (Diff-Quick; Diagnostic Systems, Inc., Gibbstown, N.J.).

(ii) Monocytes. Human monocytes were purified from venous blood of adult volunteers by use of a previously described two-step procedure that consisted of automated leukapheresis and counterflow centrifugal elutriation at the Transfusion Medicine Department, Clinical Center, National Institutes of Health, Bethesda, Md. (1). Monocytes were counted on a hemocytometer and resuspended in HBSS without Ca^{2+} and Mg^{2+}. The viability of cells was higher than 95% by trypan blue exclusion, and more than 90% of elutriated cells were monocytes by modified Wright-Giemsa stain and/or nonspecific esterase stain (Sigma Chemical Co., St. Louis, Mo.). Rabbit MNCs were separated by centrifugation of venous blood over Ficoll. By modified Wright-Giemsa stain, the proportion of monocytes in the rabbit MNC suspensions was 28.2% ± 2.4% (mean ± standard error).

(iii) MDMs. Human MNCs were isolated from heparinized venous blood of adult volunteers by centrifugation over Ficoll. They were washed twice with HBSS free of Ca^{2+} and Mg^{2+} and resuspended in complete medium (CM) consisting of RPMI 1640, 25% human serum (Gibco Laboratories, Grand Island, N.Y.), 100 U of penicillin per ml, and 100 μg of streptomycin per ml at an adjusted concentration of 5×10^6 monocytes per ml after calculating the percentage of monocytes by using modified Wright-Giemsa stain.

One million estimated monocytes in CM (200 μl of suspension) were placed on 18-mm-diameter sterile round glass coverslips in 12-well plates (Costar, Cambridge, Mass.) and were incubated at 37°C with 5% CO_2 for 1 h. The coverslips were then washed two times with warm HBSS not containing Ca^{2+} and Mg^{2+}. Fresh CM was added to the wells, and the glass-adherent monocytes were further incubated at 37°C with 5% CO_2 for 2 to 4 days to allow differentiation to monocyte-derived macrophages (MDMs).

(iv) PAMs. Rabbits were sacrificed with an excessive dose of pentobarbital, and their lungs were excised. The edges of the lobes were cut, and phosphate-buffered saline (PBS) was forcefully injected through the trachea and bronchi. The lavage washings were filtered through sterile gauze pads and pelleted at 300 × *g* for 10 min at 4°C. Supernatants were removed, pellets were resuspended in HBSS without Ca^{2+} and Mg^{2+}, and erythrocytes were lysed by hypotonic lysis. PAMs were washed and resuspended in CM containing 10% fetal bovine serum (Gibco) instead of human serum at a concentration of 5×10^6/ml.

Reagents and pretreatment of effector cells. CsA used in vitro was a gift of Gene Shearer, National Institutes of Health, who had originally received it from Sandoz. It was dissolved in ethanol to a stock concentration of 100 mg/ml. Hydrocortisone sodium succinate (HCS) was purchased from Upjohn Co. (Kalamazoo, Mich.). It was dissolved in sterile distilled H_2O to a stock concentration of 100 mg/ml. Both agents were further diluted in HBSS without Ca^{2+} and Mg^{2+}.

For the in vitro experiments, effector cells were pretreated with CsA (and/or HCS in the case of PMNs). Thus, 2×10^7 PMNs were incubated with the agent(s) in 1 ml of HBSS without Ca^{2+} and Mg^{2+} at 37°C for 1 h before the assays. After this, the antifungal activity of PMNs was evaluated without further washing. Human monocytes at a concentration of 10^6/ml of CM were incubated with CsA at 37°C and 5% CO_2 for 1 or 2 days before the assays. Coverslip-adherent monocytes were incubated with CsA in 1 ml of CM for 2 or 4 days during their differentiation to MDMs. Both monocytes and MDMs were washed twice after treatment with the agent and before the assays.

Organism. A strain of *A. fumigatus*, 4215, isolated from a cancer patient with invasive aspergillosis, was used in these studies. The strain was preserved on frozen potato dextrose agar slants at −70°C. Conidia were harvested by scraping the surface of the slants, suspended in PBS, filtered through sterile gauze, washed, and kept in PBS at 4°C as previously described in detail (23).

Superoxide anion production assay. Production of superoxide anion (O_2^-) in response to the synthetic tripeptide *N*-formylmethionyl leucyl phenylalanine (FMLP) and phorbol myristate acetate (PMA) was assessed spectrophotometrically by superoxide dismutase-inhibitable reduction of cytochrome *c* (23). One million phagocytes, which had been incubated with buffer-CM only or with CsA in vitro or ex vivo, were mixed with 50 μM cytochrome *c* (Sigma). As a stimulus, 500 nM FMLP or 500 ng of PMA per ml (both from Sigma) was added to the phagocytes in 1 ml of HBSS, and the mixtures were incubated on a shaker at 37°C for 30 min. Control tubes containing all of the constituents described above plus superoxide dismutase (40 μg/ml) were also included. After this incubation, O_2^- production was assessed as the difference in A_{550} from the control as measured on a Gilford 260 spectrophotometer (Ciba-Corning Diagnostics Corp., Oberlin, Ohio). Superoxide produced by 10^6 phagocytes was then calculated with the millimolar extinction coefficient for reduced cytochrome *c*.

Hyphal damage assay. The colorimetric MTT assay (18, 23) was employed. Briefly, 1 day before the experiment, a suspension of 10^5 conidia per ml was made in yeast nitrogen base supplemented with 2% glucose, and 1-ml aliquots were plated in the wells of 24-well plates (Costar). The plates were incubated for approximately 15 h at 30°C, by which time more than 95% of the conidia had germinated to hyphae approximately 150 to 200 μm in length. Hyphae were either used immediately or stored at 4°C for no longer than 1 to 2 h.

The supernatants were aspirated, and phagocytes that had been incubated with or without CsA in vitro or had been derived from CsA-treated or untreated animals were added to the wells in final effector/target cell (E/T) ratios of 5/1 and 10/1. After 2 h at 37°C and 5% CO_2, supernatants were aspirated, effector cells were lysed by adding 300 μl of 0.5% sodium deoxycholate, and live hyphae were washed three times with sterile water. Subsequently, 1 ml of RPMI 1640 without phenol red containing 0.5 mg of MTT per ml was added to each well, and the plates were further incubated at 37°C and 5% CO_2 for 3 h. The wells were then aspirated dry, 200 μl of isopropanol was used to extract the dye in each well, volumes of 150 μl were transferred into the wells of a 96-well plate, and the color was measured on a Titertek Multiscan microplate spectrophotometer (Flow Laboratories, McLean, Va.) at 570 nm, with 690 nm used as the reference wavelength. A well containing only isopropanol was used as a blank. Control wells containing hyphae seeded and buffer only but not effector cells were included in each experiment. Anti-

fungal activity (hyphal damage) was calculated with the formula % of hyphal damage =

$$\frac{\text{OD of control wells} - \text{OD of test wells}}{\text{OD of control wells}} \times 100$$

where OD is optical density and control wells were those containing hyphae only. Each condition was tested in duplicate or quadruplicate, and the results were averaged. CsA at the highest concentration used (1,000 ng/ml) was included in some wells with hyphae only seeded and was found not to have a direct effect on the hyphae of *Aspergillus* spp.

Assays of phagocytosis of conidia by MDMs and PAMs. At the end of pretreatment, supernatants were removed and 1 ml of CM containing 10^6 *A. fumigatus* conidia per ml was added to each well. After 1 h of incubation at 37°C with 5% CO_2 for phagocytosis, coverslips were washed three times with warm HBSS without Ca^{2+} and Mg^{2+} to remove the extracellular conidia and cells were fixed and stained with modified Wright-Giemsa stain. The percentage of phagocytosis and the phagocytic index were determined by light microscopy. The percentage of phagocytosis was the percentage of MDMs or PAMs that had one or more conidia phagocytosed or attached among 100 of them counted. The phagocytic index was the average number of conidia that had been phagocytosed or attached to each phagocytosing MDM or PAM.

Statistics. Differences between values at individual CsA concentrations and baseline values were assessed with unbalanced analysis of variance (ANOVA). The paired *t* test was used to compare numbers of PAMs recovered from CsA-treated rabbits with those recovered from untreated rabbits. Standard errors of means were used to present the data. All *P* values reported are two sided.

RESULTS

Effect of CsA on PMN-released superoxide anion. The oxidative burst of human PMNs in response to stimulation with soluble stimuli (PMA and FMLP) is shown in Fig. 1. At concentrations of 10 to 250 ng/ml, CsA did not have any significant effect on O_2^- production. At concentrations equal to or higher than 500 ng/ml, however, CsA significantly suppressed PMN function after stimulation with PMA ($P < 0.01$ [Fig. 1A]). A similar pattern of significant suppression of O_2^- production by high concentrations of CsA (>250 ng/ml) was observed when FMLP was used as a stimulus of PMN function instead of PMA ($P < 0.01$ [Fig. 1B]).

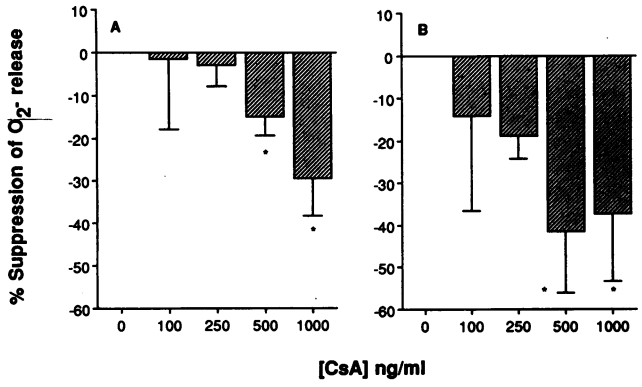

FIG. 1. Effect of CsA on the O_2^- released by human PMNs in response to PMA (A) and FMLP (B). Means ± standard errors of means derived from 4 to 10 experiments performed with each concentration of CsA are shown. The PMNs were pretreated with the concentrations of CsA shown on the horizontal axis for 60 min at 37°C in HBSS without Ca^{2+} and Mg^{2+}. The baseline O_2^- release from 10^6 buffer-pretreated PMNs in 10 min was 14.7 ± 2.0 nmol for PMA and 11.2 ± 1.7 nmol for FMLP. An asterisk indicates that the differences between the CsA-treated PMNs and the buffer-pretreated PMNs (percentage of suppression) are significant ($P < 0.01$).

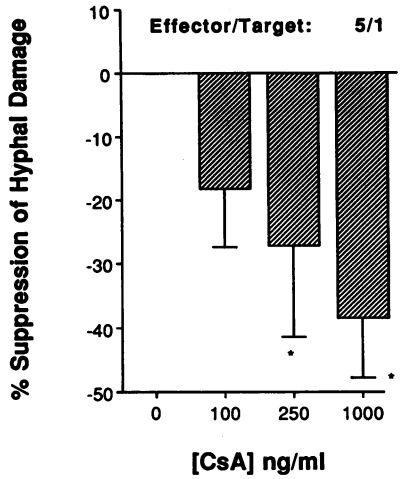

FIG. 2. Effect of CsA on the damage to unopsonized hyphae of *A. fumigatus* caused by PMNs. Human PMNs were pretreated with the indicated concentrations of CsA and were then added to wells containing 10^5 hyphae per well and incubated at 37°C for 2 h. The E/T ratio used in these experiments was 5/1. The data presented were derived from 4 to 10 experiments performed. The vertical bars indicate standard errors of means. The baseline hyphal damage induced by buffer-pretreated PMNs was 73.5% ± 5.5%. An asterisk indicates that the differences between the CsA-treated PMNs and the buffer-pretreated PMNs (percentage of suppression) are significant ($P < 0.01$).

Effect of CsA on PMN-caused damage of *A. fumigatus* hyphae. Human PMNs were pretreated with buffer or CsA at 100 to 1,000 ng/ml and then were incubated with unopsonized hyphae at 37°C for 2 h. By using the MTT assay to detect damage to hyphae and an E/T ratio of 5/1, CsA at a relatively low concentration (100 ng/ml) did not significantly alter PMN antifungal activity (Fig. 2). The lowest concentration of CsA that showed significant suppression of PMN function compared with that of the control (untreated PMNs) was 250 ng/ml ($P < 0.01$). A higher concentration (i.e., 1,000 ng/ml) showed further suppression of PMN antifungal activity. A similar effect of CsA was observed at an E/T ratio of 10/1 (data not shown).

Effect of combination of CsA and HCS on PMN-released superoxide anion. To search for a combined effect of clinically relevant concentrations of CsA and HCS on oxidative burst, human PMNs were incubated with the two agents alone or in combination and then O_2^- production in response to PMA or FMLP was assessed. While neither CsA at 250 ng/ml nor HCS at 10 μg/ml exerted significant suppression of O_2^- production in response to either PMA (Fig. 3, left panel) or FMLP (Fig. 3, right panel), the combination of the two agents significantly suppressed this PMN function in response to both stimulants compared with baseline O_2^- production ($P < 0.05$). However, the differences between O_2^- production for the combined treatment and that for treatment with each agent alone were not significant. At the high concentration of 500 ng/ml, CsA alone significantly suppressed O_2^- production in response to both stimuli.

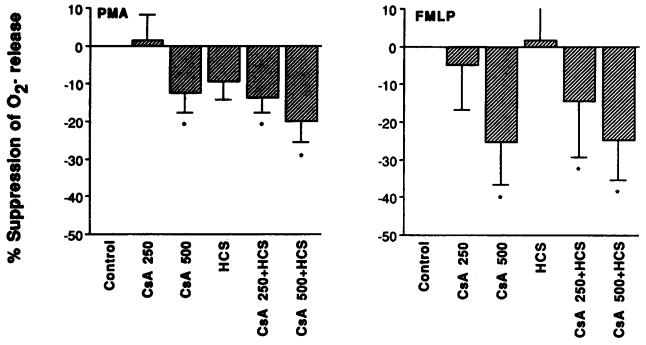

FIG. 3. Effect of CsA (250 or 500 ng/ml) and HCS (10 μg/ml) alone or in combination on the O_2^- released by human PMNs in response to PMA (left panel) and FMLP (right panel). Means ± standard errors of means derived from 13 experiments performed with each concentration of CsA and/or HCS are shown. The PMNs were pretreated with the two agents as indicated on the horizontal axis for 60 min at 37°C in HBSS without Ca^{2+} and Mg^{2+}. The baseline O_2^- release from 10^6 buffer-pretreated PMNs in 10 min was 12.2 ± 1.2 nmol for PMA and 7.4 ± 1.6 nmol for FMLP. An asterisk indicates that the differences between CsA-treated PMNs and buffer-pretreated PMNs (percentage of suppression) are significant ($P < 0.05$).

Effect of CsA on monocyte-caused damage of *A. fumigatus* hyphae. In addition, using the MTT assay and an E/T ratio of 10/1, it was found that treatment with 100 ng of CsA per ml significantly suppressed the antifungal activity of human monocytes against *A. fumigatus* hyphae (Fig. 4 [$P = 0.05$]). CsA at 1,000 ng/ml also showed a suppressive effect on antifungal activity, but this effect did not reach statistical significance. Incubation for either 1 or 2 days showed the same trend with regard to effect. However, incubation for only 1 h showed no effect (data not shown).

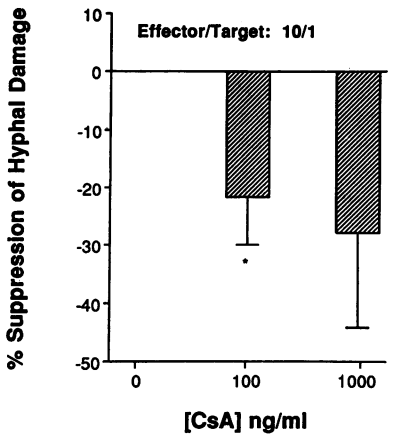

FIG. 4. Effect of CsA on damage to unopsonized hyphae of *A. fumigatus* caused by human monocytes incubated with either CM alone or with various concentrations of CsA. Because there were no differences, the results obtained after incubation for 1 or 2 days were pooled and the cumulative results are presented in this figure. Monocytes that were either preincubated with CsA or with CM alone were added to wells containing 10^5 hyphae per well and were incubated at 37°C for 2 h. The E/T ratio used in these experiments was 10/1. The baseline hyphal damage induced by buffer-pretreated PMNs was 68.0% ± 5.8%. The asterisk indicates that the difference between CsA (100 ng/ml)-treated monocytes and controls (percentage of suppression) is significant ($P = 0.05$).

TABLE 1. MDM-induced phagocytosis of *A. fumigatus* conidia[a]

CsA concn (ng/ml)	No. of expts	% of phagocytosis (mean ± SE)[b]	Phagocytic index (mean ± SE)[c]
Control	3	23.7 ± 1.86	1.37 ± 0.07
10	4	26.3 ± 4.89	1.55 ± 0.08
100	4	33 ± 4.64	1.58 ± 0.14
250	3	25 ± 2	1.44 ± 0.15
500	3	23.3 ± 1.67	1.85 ± 0.18
1,000	4	25 ± 5	1.5 ± 0.09

[a] Monocytes were cultured in the presence of CM only or CM containing various concentrations of CsA at 37°C for 2 or 4 days, and their phagocytic function was then tested against *A. fumigatus* conidia. Because there was no difference between them, data from the two incubation times were combined.

[b] The percentage of phagocytosis was calculated as (number of phagocytosing MDMs divided by the total number of MDMs counted) × 100.

[c] The phagocytic index was calculated as the total number of MDM-associated conidia divided by the number of MDMs with conidia phagocytosed or attached to them.

Effect of CsA on MDM phagocytic activity against *A. fumigatus* conidia. Human monocytes were incubated at 37°C for either 2 or 4 days in the presence of CsA (10 to 1,000 ng/ml) or CM alone. Within this range of concentrations, CsA did not exert any significant suppressive effect on the percentage of phagocytosis of *A. fumigatus* conidia caused by MDMs (Table 1). Similarly, no effect of CsA, at the same range of concentrations, was detected by the phagocytic index. No particular difference in the results between 2 and 4 days of incubation was seen.

Ex vivo effects of CsA on phagocytic host defenses. To investigate whether the in vitro effects of CsA on phagocytic host defenses described above are associated with ex vivo effects, rabbits were treated with CsA and their phagocytes were isolated and studied. Treatment with CsA at 10 mg/kg/day intravenously for 4 days did not result in a suppressive effect on either O_2^- production or hyphal damage caused by PMNs of three animals, whereas there was a trend toward lower O_2^- production by PMNs of three animals treated with CsA at 20 mg/kg/day intravenously for 7 days (from 5.0 ± 2.0 nmol of O_2^- for controls to 3.34 ± 1.96 nmol of O_2^- for CsA-treated rabbits; $P = 0.1$). This trend, however, was not associated with a similar decrease in PMN-induced hyphal damage (at an E/T ratio of 10/1, from 35.9% ± 10.3% for controls to 36.4% ± 11.6% for CsA-treated rabbits).

Tentative decreases in both O_2^- production and hyphal damage caused by PMNs were also observed after treatment of rabbits with CsA (10 mg/kg/day) plus methylprednisolone (5 mg/kg/day) for 4 days. For example, O_2^- production by PMNs from three control rabbits was 5.7 ± 0.5 nmol compared with 5.3 ± 0.4 nmol for three CsA-treated rabbits and 3.3 ± 1.4 nmol for three CsA-methylprednisolone-treated animals ($P > 0.05$). In addition, hyphal damage caused by PMNs from three control rabbits at an E/T ratio of 5/1 was 40.1% ± 0.7% compared with 47.3% ± 12.1% for three CsA-treated rabbits and 23.9% ± 3.1% for three CsA-methylprednisolone-treated animals ($P > 0.05$ for all of the differences).

No suppressive trend with regard to monocyte-induced O_2^- production or hyphal damage was observed after treatment of three rabbits with CsA at 20 mg/kg/day for 7 days. No effect on the PAM percentage of phagocytosis or the phagocytic index of *Aspergillus* conidia was observed after treatment of three rabbits with 20 mg of CsA per kg per day for 7 days compared with that of three control rabbits.

Treatment with CsA did not alter the PMN or monocyte counts of any of the animals. In contrast, treatment with either

CsA regimen diminished the number of PAMs recovered by total lung bronchoalveolar lavage (from an average of 48.9×10^6 PAMs recovered from each of four controls to 24.5×10^6 PAMs recovered from each of four CsA-treated animals [paired t test; $P = 0.018$). The viabilities of the PAMs, as tested by trypan blue exclusion, were similar (>90%) in CsA-treated and control animals.

DISCUSSION

This study demonstrated that CsA at concentrations above therapeutic levels exerted a suppressive effect on oxidative burst and on the antifungal activity of peripheral blood phagocytes against hyphae but not on the phagocytic activity of macrophages against conidia of *A. fumigatus*.

The ability of CsA to inhibit T helper responses to alloantigens of transplanted organs or in autoimmune diseases made the compound a valuable immunosuppressor with selective activity. Frequently, however, it is administered in combination with corticosteroids and/or other broad-spectrum immunosuppressive agents. Thus, it was important to search for suppressive effects of CsA in areas of the immune system, such as the phagocytic compartment, suppression of which would increase the susceptibility of patients to opportunistic fungi. Invasive aspergillosis has been encountered in patients undergoing transplantation (4, 10, 12, 29), although these patients have been treated with other immunosuppressive agents as well. In addition, other immunosuppressive agents are known to interfere with phagocytic function (9, 24), thereby rendering the host more susceptible to infection.

Although a number of studies have previously examined the impact of CsA on PMNs and other phagocytes, somewhat controversial results have been obtained (8, 9, 13, 16, 20, 28, 31, 32). Moreover, no data were available regarding the effects of CsA on phagocytic host defenses in invasive aspergillosis. Our study is, to our knowledge, the first to report the effects of CsA on antifungal activities of phagocytes against *Aspergillus* infection both in vitro and ex vivo. The therapeutic serum CsA concentrations are targeted to trough levels below 250 ng/ml during immunosuppressive therapy. Our study was designed to test concentrations below and above this clinically relevant level. In general, only concentrations higher than this level that are otherwise toxic for the patient suppressed the antifungal activity of phagocytes significantly. Given the high percentage of CsA bound to serum and tissue proteins, such suppressive effects seen in vitro are unlikely to occur in vivo, and this is supported by the finding of an overall lack of suppressive activity observed in rabbits ex vivo.

CsA has been reported to induce immunosuppressive effects on fungicidal activity of murine PMNs against blastoconidia of *C. albicans* in vivo and has rendered the animals susceptible to subsequent challenge with this organism (28). In addition, CsA has been found to have suppressive effects on certain antimicrobial functions of pulmonary macrophages and PMNs in vitro and ex vivo (8, 9). These findings are somewhat different from our observations, as well as those of others (16, 20), and may be due to an organism-dependent or function-dependent specificity of CsA's suppressive properties.

Our findings that there is no suppression of the PMN oxidative burst and antifungal activity against *A. fumigatus* by therapeutically relevant concentrations of CsA are consistent with previous findings of no effect of CsA on certain functions of PMNs (13, 16, 31). In addition, Kharazmi et al. (16) found that there were no effects on monocyte chemotaxis and phagocytosis at clinically relevant levels. Of note, supratherapeutic concentrations of CsA increase the prostaglandin levels from human monocytes (32), rendering them hyporesponsive to microbial stimuli. Lack of suppression of macrophage responses to lymphokines has been found by other investigators (27). The findings presented above suggest that there are diverse effects of CsA on certain functions of mononuclear phagocytes.

These in vitro results showing only mild immunosuppression induced by CsA on PMNs and complete lack of immunosuppression induced on anti-*Aspergillus* macrophages are consistent with the findings of a recent in vivo study in which CsA or corticosteroids were administered to rabbits and the rabbits were subsequently challenged with intratracheal instillation of *Aspergillus* conidia. That study showed a better outcome of pulmonary aspergillosis in CsA-treated animals than in corticosteroid-treated animals (2). It appears that CsA exerts milder suppression on phagocytic host defenses than other immunosuppressive agents. The suppressive property of CsA that was found in these in vitro and ex vivo studies may be related to the inhibitory effects of this compound on the production of tumor necrosis factor alpha and gamma interferon by mononuclear cells, cytokines that are useful in the activation of phagocytes (11). This hypothesis, however, remains to be further studied.

In the ex vivo part of the study, of note was the finding of consistently lower numbers of PAMs that were recovered from lungs of CsA-treated animals. This phenomenon does not appear to be due to direct toxicity of CsA on PAMs and may have been associated with certain factors that inhibit differentiation and/or migration of circulating monocytes to pulmonary alveoli, contributing to increased susceptibility to *Aspergillus* infection.

Corticosteroids, including hydrocortisone, alone have either a moderate or a profound suppressive effect on activities against *Aspergillus* infection, depending on whether the effector cells are PMNs (24), monocytes (21), or pulmonary macrophages (25). Because in previous studies a hydrocortisone concentration of 10 μg/ml was shown to be unable to induce a significant suppressive effect on PMN antifungal function in vitro (24), this concentration was chosen in the present study. The findings that the combination of therapeutic concentrations of CsA and HCS showed a significant suppression in vitro and that the combination of CsA plus methylprednisolone showed a trend toward a decrease in PMN function not shown by CsA alone ex vivo correlate with the results of the in vivo study mentioned above (2). Indeed, these findings may contribute to understanding the heightened susceptibility to aspergillosis of patients on long-term therapy with both agents.

Because phagocytes are critical effector cells against conidia and hyphae of opportunistic fungi, including *Aspergillus* spp., the lack of significant suppression of antifungal activities of PMNs and macrophages within the therapeutic range of CsA is important for the usage of the agent. However, the observation that phagocytic defenses against *Aspergillus* infection may be altered when CsA is combined with corticosteroids has potential implications for patients receiving these immunosuppressive agents.

In summary, the results of our study demonstrated that CsA does not suppress the antifungal activity of phagocytes, except for that of monocytes, within therapeutically relevant concentrations. However, it may cause significant inhibition of phagocytes' antifungal function at relatively high concentrations, and it may induce significant immunosuppression of these effector cells when combined with corticosteroids.

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