INFECTION AND IMMUNITY, Apr. 1989, p. 1158–1164
0019-9567/89/041158-07$02.00/0

Vol. 57, No. 4

Effects of Cyclosporin A on the Cells Responsible for the Anticryptococcal Cell-Mediated Immune Response and Its Regulation

PAUL L. FIDEL, JR., AND JUNEANN W. MURPHY†*

Department of Botany and Microbiology, University of Oklahoma, Norman, Oklahoma 73019

Received 24 October 1988/Accepted 5 January 1989

Cyclosporin A (CsA), a potent immunosuppressive drug, was used to explore further the induction, expression, and regulation of lymphoid cells involved in the delayed-type hypersensitivity (DTH) response to cryptococcal antigen(s). We found that the induction of the cells responsible for DTH (T_{DH} cells) was not affected by CsA, but their expression was inhibited in CsA-treated mice. The inhibition of expression of the T_{DH} cells could not be attributed to the *Cryptococcus neoformans*-specific suppressor T (Ts) cells, even though the Ts cells were induced in CsA-treated mice. Instead, the suppressed expression of the T_{DH} cells in CsA-treated mice was a direct effect of CsA or its products. Our studies with CsA also resulted in the first identification of a population of cells that significantly amplify the anticryptococcal DTH response. The amplifier cells were induced in mice that were given a primary immunizing dose of cryptococcal antigen in complete Freund adjuvant, and they amplified the anticryptococcal DTH response in recipient mice when they were transferred at the time of immunization of the recipient. The amplifier cell population was distinct from the T_{DH} cells in that CsA inhibited the production of the amplifying cells but did not affect the induction of T_{DH} cells. Amplification of the DTH response was a cell-mediated event, since cells but not serum from immunized mice mediated the amplified response in recipient mice. Thus, CsA enabled us to characterize anticryptococcal T_{DH} and Ts cells further and to add to the immune cell circuit of the cryptococcal system a distinct population of cells that amplifies the anticryptococcal DTH response.

Induction and regulation of cell-mediated immunity (CMI), the primary protective immune response in cryptococcosis, has been a major interest to our laboratory (19, 30, 32–34). The delayed-type hypersensitivity (DTH) reaction to antigens of *Cryptococcus neoformans* correlates well with protection against this yeastlike pathogen in the murine model and is used as a measure of the level of anticryptococcal CMI (5, 19, 23, 24, 30, 32–34). The anticryptococcal DTH response can be induced by immunizing mice subcutaneously with a soluble cryptococcal culture filtrate (CneF) antigen preparation in complete Freund adjuvant (CFA) (32), and this response is down-regulated by a series of *C. neoformans*-specific suppressor T (Ts) cells (19, 30, 32–34). The cascade of Ts cells is initiated by injecting cryptococcal antigen (CneF or serum from *Cryptococcus*-infected mice with high cryptococcal antigen titers) intravenously (i.v.) into mice. A suppressive dose of cryptococcal antigen induces cyclophosphamide (Cy)-sensitive T cells to develop into first-order Ts (Ts1) cells (34). Ts1 cells or the soluble factor derived therefrom are bifunctional in that they suppress the induction of the cells responsible for DTH (T_{DH} cells) (30) and induce second-order Ts (Ts2) cells (34). The Ts2 cells or the soluble factor produced by these cells functions with third-order Ts (Ts3) cells to suppress the expression of T_{DH} cells (19, 33). Ts3 cells develop from Cy-sensitive precursor cells following an immunization with CneF-CFA (19).

Cyclosporin A (CsA), a fungal metabolite and potent immunosuppressive drug that inhibits cytokines involved in interleukin-2-dependent proliferation of T cells (4, 6, 8, 10–12, 15–18, 21, 27, 36, 37, 41), has been shown to affect the various T-cell subpopulations in different ways. For instance, CsA suppresses the induction of cytotoxic T cells (4, 12, 14, 41, 44) and helper T cells (9, 20, 41). On the other hand, the T cells mediating DTH responses to dinitrofluorobenzene (3), ovalbumin (43), and sheep erythrocytes (28, 35, 40, 42) are induced when CsA is present but are not functional in the presence of CsA. Conflicting results have been published regarding the effects of CsA on the induction of Ts cells (1–3, 13, 22, 45, 47). The majority of reports indicate that CsA does not affect Ts cells (2, 13, 22, 46, 47); however, tolerizing doses of dinitrofluorobenzene (3), sheep erythrocytes (45), or herpes simplex virus (1) given to CsA-treated mice result in positive DTH responses, suggesting that Ts cells are inhibited by CsA in those models. Thus, it appears that CsA does not necessarily affect each system of antigen-specific immunity in the same manner. In spite of this, CsA can be a useful tool in differentiating functional cell populations within antigen-specific cellular pathways, and we used CsA in this study to gain more information about the cells that are involved in the anticryptococcal DTH response and its regulation.

A series of experiments was performed in which mice were treated with CsA throughout the induction of the CMI response or the induction of the various Ts cells that regulate the anticryptococcal DTH response. We found that anticryptococcal T_{DH} cells, as in other antigen models, were produced in mice that were treated with CsA but were inhibited in their expression in the presence of CsA. The induction of the three cryptococcal-specific Ts-cell populations was not affected by CsA. In addition, with the use of CsA we identified a yet undefined cell population in the spleens of CneF-CFA-immunized mice. This newly identified cell population in the cryptococcal model significantly amplified the

* Corresponding author.
† Present address: Department of Microbiology/Immunology, P.O. Box 26901, Oklahoma City, OK 73190.

DTH response in recipient mice when the population was transferred to the recipients at the time of immunization. CsA blocked the induction of the amplifying cell population yet did not affect induction of the T_{DH}-cell population, providing evidence that the two populations of cells are distinct. The amplified DTH response was not evident in recipient mice following injection of serum from immunized mice, so the amplification was considered to be cell mediated and not a result of humoral factors in immune mouse serum.

MATERIALS AND METHODS

Mice. Female CBA/J mice (*H-2*^{k}) purchased from Jackson Laboratory, Bar Harbor, Maine, were used for this study when they were 7 to 10 weeks of age.

Antigen. CneF used for the immunization, footpad challenge, and induction of Ts cells was prepared by the procedure of Cauley and Murphy (5) with *C. neoformans* 184-A (31). The lot of CneF used in this study had a protein concentration of 4.1 mg/ml, based on the procedure of Lowry et al. (25) as modified by Miller (26), and a carbohydrate concentration of 3.8 mg/ml, as determined by the phenol-sulfuric acid assay (7).

CsA. CsA was a generous gift from Sandoz Ltd., East Hanover, N.J. It was diluted in Labrifil (Sandoz Ltd.)–12.5% ethanol or in Cremophor EL (Sigma Chemical Co., St. Louis, Mo.)–33% ethanol. CsA (60 mg/kg per day in 30 μl of solvent) or, as a control, the solvent (Labrifil with 12.5% ethanol or Cremophor EL plus 33% ethanol) alone (30 μl) was administered intramuscularly (i.m.) to mice.

Assessment of CsA treatment on the anticryptococcal DTH response. In addition to being immunized subcutaneously at the base of the tail with CneF-CFA on day 0, mice were either untreated (positive control) or treated i.m. every 24 h with CsA (60 mg/kg per day) or solvent (solvent control) beginning on day −1 and continuing through day 5. On day 6 the mice were challenged in one hind footpad with 30 μl of CneF, and 24 h later their DTH responses were measured (32). Negative controls were naive mice that were challenged with CneF. Early in these studies we found that footpad reactions of solvent-treated, immunized animals were not significantly different from the footpad reactions of untreated, immunized animals, so in subsequent experiments, solvent-treated, immunized animals were used as the sole control group.

In specific experiments in which Cy was used to abrogate the induction of Ts1 and Ts3 cells, Cy (Sigma) was administered to the mice 1 day before CsA or solvent treatment and 2 days before immunization with CneF-CFA (34).

Induction of *C. neoformans*-specific Ts cells in CsA-treated mice. In experiments designed to assess the effect(s) of CsA on *C. neoformans*-specific Ts1, Ts2, and Ts3 cells, each respective suppressor cell population was induced in donor mice as described previously (19, 30, 32–34). Designated groups of donor mice were given daily i.m. injections of CsA (60 mg/kg per day) or solvent beginning on day −1 and continuing through day 6 of the Ts-cell induction protocol, which began on day 0 in each case. The ability of the specific Ts-cell populations to suppress the anticryptococcal DTH response was assayed in recipient mice as described previously (19, 32, 33).

Induction and function of anticryptococcal T_{DH} cells in mice treated with CsA. To study separately the induction and function of T_{DH} cells, one group of mice (donor mice) was used to induce T_{DH} cells and another group of mice (recipient mice) was used to determine the presence of T_{DH} cells in the spleens of the donor mice. In these experiments, the donor and recipient mice were subjected to daily i.m. injections of CsA or solvent beginning on day −1 and continuing through day 5. The donor mice were immunized with CneF-CFA on day 0, and then on day 6, spleen cells (SPCs) from these mice were harvested and transferred i.v. (19, 30) to untreated, solvent-treated, and CsA-treated recipient mice. One hour after the cells were transferred, the groups of recipient mice were challenged in the footpads with CneF, and 24 h later the footpads were measured.

Reversibility of the inhibitory action of CsA on the anticryptococcal DTH response. To determine the reversibility of the inhibitory action of CsA on the anticryptococcal DTH response, CsA treatments were terminated at various times before and during the induction period of the anticryptococcal DTH response. Mice were subjected to daily i.m. injections of CsA or solvent for various time periods beginning 1 week to 1 day prior to immunization with CneF-CFA and terminating 1 day before immunization or between 3 and 6 days after immunization. The mice were challenged in the footpads with CneF 6 days after immunization, and the footpads were measured 24 h later.

Effect of CsA on induction of cells which amplify the DTH response. Groups of mice were either untreated or treated i.m. with CsA or solvent beginning on day −1 and continuing every 24 h through day 5 and were immunized with CneF-CFA on day 0. On day 6 SPCs from these groups of mice (immune SPCs) were collected as a source of putative T_{DH} cells (19, 30) and were transferred i.v. to naive, syngeneic, recipient mice. In specific experiments, serum from immunized mice was collected and injected i.v. into naive recipients. Immediately following the transfer of immune SPCs or serum, the recipient mice were immunized with CneF-CFA. Six days after the immunization of the recipients (day 12), they were challenged in the footpads, and 24 h later their footpads were measured for DTH reactivity. Because we had not characterized such CMI responses previously, stringent control groups were included. They were (i) negative and positive immune controls; (ii) a group of naive recipient mice injected i.v. on day 6 with immune SPCs, but remained unimmunized to assess the amount of DTH transferred; and (iii) groups of naive recipient mice injected on day 6 with SPCs from mice injected with sterile physiological saline solution (SPSS) in CFA (SPSS-CFA) on day 0. For the latter control, one group of recipient mice remained untreated, whereas another group was immunized immediately with CneF-CFA. This particular control served to demonstrate the effects of CFA on DTH responses.

Group	Treatment of Mice		Mean increase in footpad thickness ±SEM (10^{-3} in) day 7	Compared to group 2 p<
	CsA or solvent day −1→5	Immunize with CneF-CFA day 0	10 20	
1	–	–		0.0005
2	–	+		–
3	solvent	+		NS
4	CsA	+		0.0005

FIG. 1. Effects of CsA on the anticryptococcal DTH response. Designated groups of mice were treated i.m. with 60 mg of CsA per kg per day or with the same volume of solvent. Experiments were performed at least twice with five mice per group. SEM, Standard error of the mean; NS, not significant, when $P > 0.05$.

TABLE 1. Effects of CsA on *C. neoformans*-specific Ts cells

Group	Treatment of donor mice during Ts-cell induction[a]	Ts cell type induced	% Suppression of DTH response in recipient mice	CsA-treated compared with solvent-treated mice (*P*)
1		Ts1	45	
2	Solvent	Ts1	39	
3	CsA	Ts1	48	NS[b]
4		Ts2	49	
5	Solvent	Ts2	65	
6	CsA	Ts2	54	NS
7		Ts3	100	
8	Solvent	Ts3	100	
9	CsA	Ts3	100	NS

[a] Ts-cell induction was performed in naive or CsA- or solvent-treated donor mice, and then Ts cells were transferred into recipient mice and assayed in recipient mice. CsA (60 mg/kg per day) or the same volume of solvent was administered i.m. Experiments were repeated twice with five recipient mice per group.

[b] NS, Not significant, when $P > 0.05$.

Statistical analysis. The unpaired Student's *t* test was used to analyze the data.

RESULTS

Effects of CsA on the anticryptococcal DTH response. The anticryptococcal DTH response in immunized mice treated with CsA (Fig. 1, group 4) was inhibited relative to the DTH response of untreated, immunized mice (Fig. 1, group 2). The inhibition was the result of CsA treatment rather than the solvent in which the CsA was dissolved, because the DTH responses of animals given solvent and immunized (Fig. 1, group 3) were comparable to the DTH responses of the positive control animals (Fig. 1, group 2).

Effect of CsA on the induction of *C. neoformans*-specific Ts cells. *C. neoformans*-specific Ts1-, Ts2-, and Ts3-cell populations were induced in untreated, solvent-treated, and CsA-treated mice. The ability of Ts1, Ts2, and Ts3 cells from CsA-treated animals to suppress the anticryptococcal DTH response (Table 1, groups 3, 6, and 9, respectively) was similar to the suppressive ability of Ts1, Ts2, and Ts3 cells, respectively, obtained from untreated mice (Table 1, groups 1, 4, and 7, respectively) or solvent-treated mice (Table 1, groups 2, 5, and 8, respectively). These data indicate that CsA has no demonstrable effect on the induction of Ts1, Ts2, or Ts3 cells of the cryptococcal suppressor cell circuit.

Effect of Ts cells on the inhibition of DTH in CsA-treated mice. Mice were pretreated with Cy (100 mg/kg), a drug that effectively inhibits the induction of Ts1 and Ts3 cells (19, 34), 1 day before CsA treatments were begun (60 mg/kg per day for 7 days) and 2 days before immunization with CneF-CFA. The data in Fig. 2 show that pretreating mice with Cy, a procedure which inhibits Ts-cell production, did not reverse the inhibition of the anticryptococcal DTH response in CsA-treated mice, since the mean footpad thickness in the Cy- and CsA-treated, immunized mice (Fig. 2, group 5) was comparable to the mean footpad thickness of CsA-treated mice which were not pretreated with Cy (Fig. 2, group 4). In mice treated with Cy and solvent prior to immunization, Ts-cell induction was inhibited, as indicated by the fact that their DTH responses were augmented (Fig. 2, group 3) compared with the DTH responses of solvent-treated, immunized mice (Fig. 2, group 2). It should be noted that pretreatment of mice with Cy prior to immunization consistently increased the mean DTH response above the mean DTH response of mice that were immunized and not pretreated with Cy; however, the differences in DTH responses of the two groups of mice were not always significantly different, as can be seen with groups 2 and 3 in Fig. 2. In experiments in which we did get a statistically significant difference in DTH responses between the Cy-treated and untreated groups, we obtained responses in mice in groups 4 and 5 that were similar to the responses shown in Fig. 2 for those respective groups. We regarded the modest increase in DTH responses in the Cy-treated, immunized mice to be caused by Cy blocking the development of Ts1 and Ts3 cells. Since it is likely that only a small number of Ts cells were induced by an immunizing injection of cryptococcal antigen, we would expect to obtain only a modest increase in the DTH responsiveness when the Ts cells were ablated by the Cy treatment. The abrogation of the Ts cells did not affect the responses in CsA-treated mice in any of the experiments we performed, irrespective of whether we had a statistically significant increase in the DTH responsiveness in the Cy-treated, immunized group over that in the immune control group.

Differential effects of CsA on the induction and expression of anticryptococcal T_{DH} cells. To determine whether CsA was inhibiting the induction of anticryptococcal T_{DH} cells or

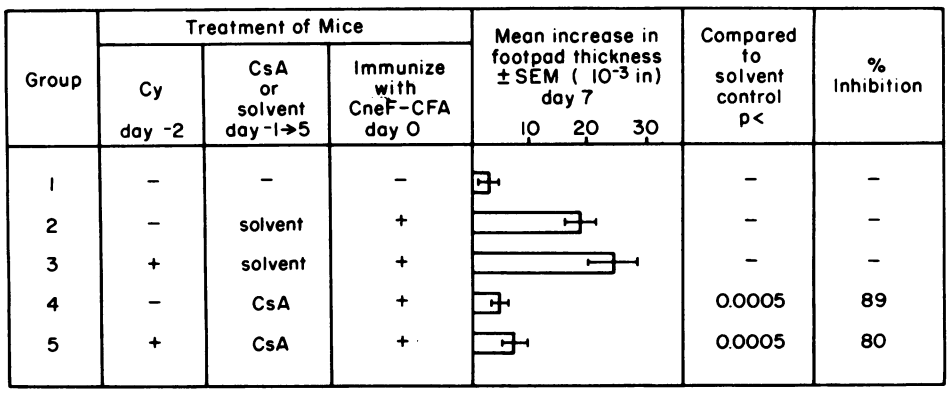

FIG. 2. Abrogation of Ts cells by Cy treatment does not alter the DTH response in CsA-treated mice. Designated groups of mice were treated intraperitoneally with 50 mg of Cy per kg. Additionally, CsA (60 mg/kg per day) or the same volume of solvent was administered i.m. Experiments were performed with five mice per group. Abbreviations are the same as those in the legend to Fig. 1.

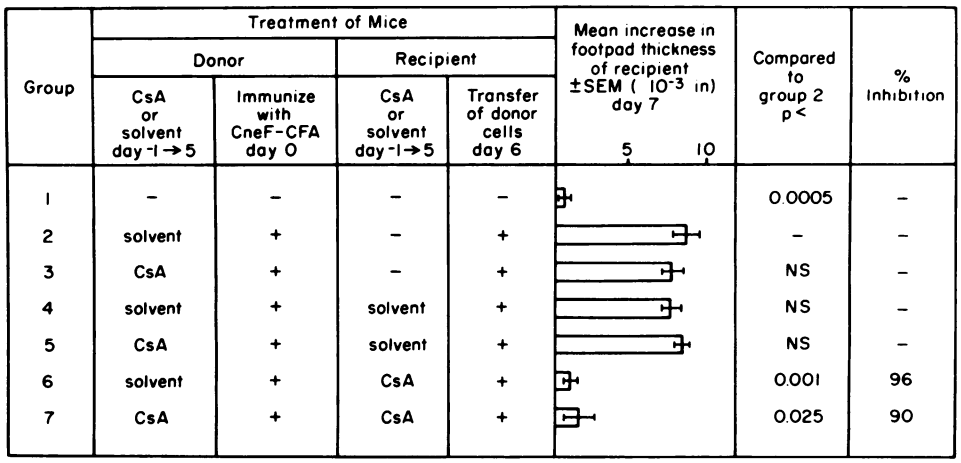

FIG. 3. Differential effects of CsA on induction and function of anticryptococcal T_{DH} cells. Designated donor and recipient mice were treated i.m. with 60 mg of CsA per kg per day or with the same volume of solvent. Experiments were repeated twice with five recipient mice per group. Abbreviations are the same as those in the legend to Fig. 1.

whether CsA was affecting the expression of T_{DH} cells 6 days after immunization, SPCs from CsA-treated donor mice were collected as a source of putative T_{DH} cells and transferred to syngeneic recipient mice. The recipient mice were challenged in the footpads 1 h after cell transfer, and DTH reactivity was assessed 24 h later. The results from a representative experiment (Fig. 3) show that SPCs from solvent- or CsA-treated, CneF-CFA-immunized donor mice transfer DTH responses to untreated recipient mice (Fig. 3, groups 2 and 3) or to solvent-treated recipient mice (Fig. 3, groups 4 and 5, respectively). However, when the same SPC populations were transferred to CsA-treated recipients (Fig. 3, groups 6 and 7, respectively), anticryptococcal DTH responses were not demonstrable in the recipients.

Effect of discontinuing CsA treatments on the expression of T_{DH} cells. CsA or solvent treatments were initiated and terminated at various times before and during the induction phase of the anticryptococcal DTH response. DTH responses of CsA-treated, immunized mice that received CsA treatments from day −1 until the day of footpad challenge (day 6) were inhibited 79% relative to the responses of immunized mice that were treated with solvent for the same time period (Fig. 4, group 2). In contrast, DTH responses of immunized mice that received CsA treatments from days −1 to 3 were inhibited only 22% compared with the responses of immunized mice that were treated with solvent for the same 5-day period (Fig. 4, group 3). A level of inhibition of DTH (38%) comparable to that of this latter group was observed in mice in which CsA treatments were begun 7 days before immunization and terminated on the day of immunization (Fig. 4, group 4).

Effect of CsA on the induction of cells capable of amplifying the DTH response. Mice were treated with CsA and immunized as described above, but instead of challenging the animals with antigen in the footpads 6 days after immunization, their SPCs were transferred to naive syngeneic recipient mice, and the recipients were immunized immediately with CneF-CFA. The recipients were challenged in the footpads 6 days after the transfer of cells and immunization. Footpad thicknesses were measured 24 h after challenge.

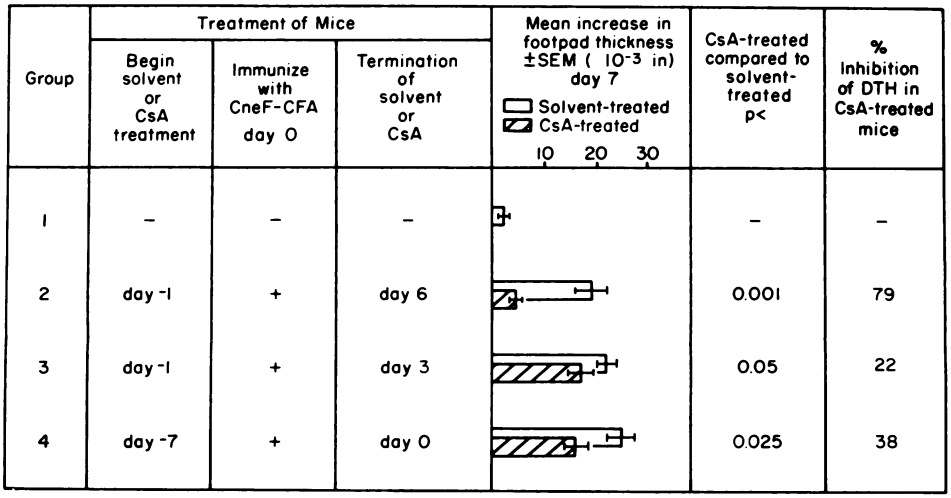

FIG. 4. Effects of terminating CsA treatments during the induction period of the anticryptococcal DTH response. Designated mice were treated i.m. with 60 mg of CsA per kg per day or with the same volume of solvent. Experiments were repeated twice with five mice per group. SEM, Standard error of the mean.

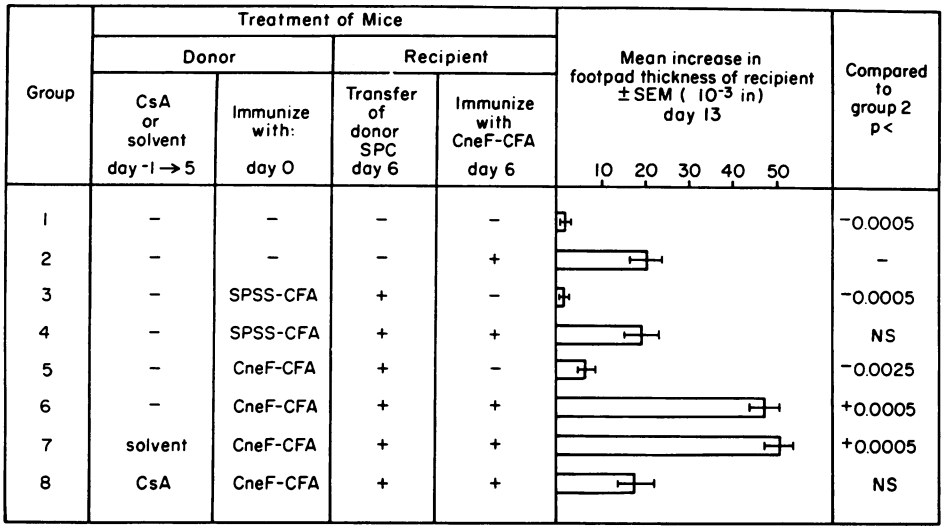

FIG. 5. Effects of CsA on an amplified anticryptococcal DTH response. Designated donor mice were treated i.m. with 60 mg of CsA per kg per day or with the same volume of solvent. Experiments were performed twice with five mice per group. SEM, Standard error of the mean; NS, not significant, when $P > 0.05$.

Data from a representative experiment are shown in Fig. 5. Recipient mice that received SPCs from untreated (Fig. 5, group 6) or solvent-treated (Fig. 5, group 7) mice responded to an immunization with CneF-CFA with an amplified anticryptococcal DTH response when compared with (i) the DTH responses of mice given a primary immunizing dose of CneF-CFA (Fig. 5, group 2), (ii) the DTH responses of unimmunized recipient mice that received SPCs from the immunized donors (Fig. 5, group 5), and (iii) the additive DTH responses of groups 2 and 5. Amplified DTH responses were not evident, however, when SPCs from CsA-treated, immunized mice were transferred to recipient mice at the time of immunization of the recipients (Fig. 5, group 8). The cell(s) responsible for the amplified DTH response was induced by CneF and not by CFA, as demonstrated by the fact that the SPCs transferred from mice that were injected with SPSS-CFA did not mediate amplified DTH responses in mice that were not immunized (Fig. 5, group 3) or in mice that were immunized with CneF-CFA (Fig. 5, group 4).

Evidence indicating the amplification of the anticryptococcal DTH response was cell mediated. To confirm that the amplified DTH response was cell mediated, 6 days after mice were immunized with CneF-CFA, their SPCs or sera were collected and transferred to recipient mice at the time of immunization of the recipients. The recipient mice were challenged in the footpads 6 days after cell or serum transfer and immunization. The results of the DTH assay show that serum collected from immunized mice did not amplify the anticryptococcal DTH response in immunized recipient mice (Fig. 6, group 4), since the mean of their DTH responses was not significantly different from that of the positive control animals (Fig. 6, group 2). As expected, SPCs from the immunized mice significantly amplified the DTH responses in the immunized recipient group (Fig. 6, group 3) compared with the responses of the positive immune controls (Fig. 6, group 2; $P < 0.0005$) or the responses of the serum recipients (Fig. 6, group 4; $P < 0.0005$).

DISCUSSION

CsA, a drug that is used in patients who have received transplants to suppress the CMI response, has been reported to inhibit the induction or function of certain T-cell populations, leaving others unaffected. Thus, we considered CsA to be a useful reagent for dissecting the CMI response to *C. neoformans* antigen(s). In this study, we investigated the effects of a continual treatment of CsA on various T-cell populations that are responsible for the anticryptococcal DTH response and its regulation. We found that seven successive injections of CsA (60 mg/kg per day) during the induction phase of the anticryptococcal DTH response resulted in a significant inhibition of DTH reactivity (Fig. 1).

Suppression of the DTH response in CsA-treated mice could have been due to (i) CsA inhibiting the induction of T_{DH} cells or (ii) CsA inhibiting the expression of T_{DH} cells following the normal induction of T_{DH} cells. If the latter was the case, inhibition of expression of T_{DH} cells could have resulted from the action of Ts cells which may have been preferentially modulated in CsA-treated, immunized mice, or the inhibition could have been caused by the direct action of CsA or its products on T_{DH} cells. Based on what is known regarding the effects of CsA on the induction of T_{DH} cells in other antigen models of CMI (3, 28, 35, 40, 42, 43), it seemed most likely that the anticryptococcal T_{DH} cells were induced in the presence of CsA but their expression was inhibited. Since Ts cells are readily induced in mice injected with

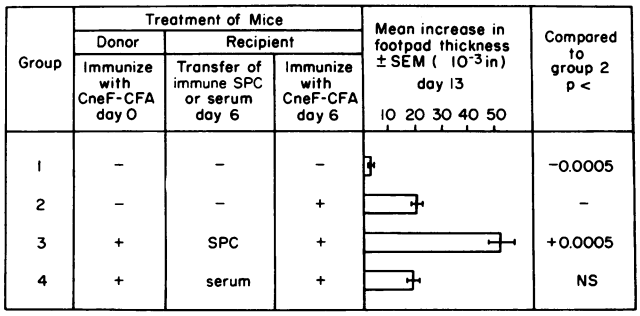

FIG. 6. Inability of serum from immune mice to amplify the anticryptococcal DTH response. Experiments were performed twice with five recipient mice per group. SEM, Standard error of the mean; NS, not significant, when $P > 0.05$.

cryptococcal antigens (19, 30, 32–34), it also seemed reasonable to suspect that Ts cells may be contributing to the reduced DTH responses observed in CsA-treated, immunized mice. Furthermore, we demonstrated through a series of experiments that CsA does not inhibit the induction of the three different Ts-cell populations that specifically suppress the anticryptococcal DTH response (Table 1), supporting the feasibility of Ts-cell-mediated inhibition of T_{DH} cells in CsA-treated mice. This possibility was eliminated, however, by showing that the inhibition of the DTH response in CsA-treated mice was not reversed when the mice were pretreated with Cy to block the production of Ts1 and Ts3 cells (Fig. 2).

Since our data indicate that Ts cells do not contribute to the reduced DTH responses in the CsA-treated, immunized mice, the two other possibilities for the inhibition were examined. To determine whether CsA inhibited the induction of T_{DH} cells or whether CsA inhibited the expression of T_{DH} cells, we transferred SPCs from CsA-treated, immunized mice to naive mice, solvent-treated mice and CsA-treated mice. Following challenge of the recipient animals in the footpads, positive DTH responses were demonstrable in naive and solvent-treated recipient mice (Fig. 3, groups 3 and 5). These data demonstrate that T_{DH} cells are induced in CsA-treated, immunized mice but are not functional. In contrast, when T_{DH} cells induced in solvent- or CsA-treated mice were transferred to recipient mice that were treated with CsA, the expression of T_{DH} cells was abrogated (Fig. 3, groups 6 and 7). These data suggest that CsA or its products are responsible for inhibiting the expression of T_{DH} cells. We confirmed this by showing that the suppressed DTH response obtained in CsA-treated, immunized mice could be reversed if CsA treatments were terminated at least 72 h prior to footpad challenge (Fig. 4). The findings that anticryptococcal T_{DH} cells are induced in CsA-treated mice but do not function in the presence of CsA or its products are consistent with results obtained with other antigen models of DTH in which CsA has been used (3, 28, 35, 40, 42, 43).

The clinical relevance of the CsA-mediated effects on the anticryptococcal DTH response is questionable. From our results, one might expect the incidence of cryptococcosis to be higher in transplant patients given CsA than in the normal population. However, this is not the case; in fact, opportunistic infections with *C. neoformans* occur less frequently in patients who are treated with CsA than in patients who are given other immunosuppressant drugs (39). This is not surprising considering that CsA has been reported to inhibit the growth of *C. neoformans* cells in vitro as well as in vivo in the mouse (29). Although CsA-treated patients have reduced CMI and thus reduced immune protection against cryptococci, the toxicity of the drug on the organism could be sufficient to limit the infection. This explanation, however, may not be satisfactory for all situations. For example, when rabbits, animals that are normally very resistant to *C. neoformans*, are treated with CsA and infected with *C. neoformans*, they succumb to lethal cryptococcal meningitis very rapidly (38).

Despite the possibility that CsA-mediated effects on anticryptococcal CMI may not have clinical relevance, CsA has proven to be an extremely valuable tool in defining functional cells in the CMI response to cryptococcal antigen(s). Through the use of this immunosuppressive drug, we have provided evidence for the existence of a functional cell population that induces an amplified DTH response when it is transferred to syngeneic recipient mice at the same time that the recipients are immunized with CneF-CFA (Fig. 5). The amplified DTH response is specific for cryptococcal antigen, as indicated by the fact that the cells that transfer the response are induced by CneF and not by CFA (Fig. 5); and the amplified DTH response is mediated by cells and not serum, as demonstrated by our data in Fig. 6. We interpreted our data to indicate that there are two functionally distinct cell populations involved in the amplified DTH response. One is the T_{DH} cell, which is induced but is not functional in the presence of CsA, and the other is this newly defined amplifier cell, which is not induced in CsA-treated mice. The possibility exists, although we consider it remote, that there is only one cell population involved in the amplified response, but the one cell population has two functions. If the amplified DTH response was mediated by a single cell population with two functions, then CsA would have to permanently affect the amplifier function of the cell population and temporarily affect the function which contributes to the DTH response. To date, CsA has not been shown to have permanent cellular effects, since it must be given to animals continuously in order to affect a given cell population (2, 13, 22, 28, 40, 43, 46, 47). Thus, we favor the concept of two functionally distinct cell populations: one that mediates DTH and one that amplifies the DTH response. Prior to this investigation in which we used CsA, we were not able to distinguish between the cells which function to mediate the anticryptococcal DTH response and the cells that amplify that response. Results of our preliminary studies to characterize this newly defined cell population indicate that it is a T cell since it is nylon wool nonadherent and Thy-1^+ (unpublished data). Thus, we refer to it as an amplifier T cell.

In summary, our findings demonstrate that CsA can be a valuable tool in dissecting cellular pathways of immune responses. A prime example is our demonstration that a previously undefined cell population, distinct from T_{DH} cells, exists in the anticryptococcal DTH model and is responsible for amplifying the anticryptococcal CMI response.

ACKNOWLEDGMENT

This work was supported by Public Health Service grant AI-15716 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. **Altmann, D. M., and W. A. Blyth.** 1985. The effects of cyclosporin A on the induction, expression and regulation of the immune response to herpes simplex virus. Clin. Exp. Immunol. **59**:17–22.
2. **Araujo, J. L., J. W. Kupiec-Weglinski, D. Araneda, E. Towpik, C. Heidecke, J. M. Williams, and N. L. Tilney.** 1985. Phenotype, activation status, and suppressor activity of host lymphocytes during acute rejection and after cyclosporine-induced unresponsiveness of rat cardiac allografts. Transplantation **40**:278–284.
3. **Braida, M., and J. Knop.** 1986. Effect of cyclosporin A on the T-effector and T-suppressor response in contact sensitivity. Immunology **59**:503–507.
4. **Bunges, D., C. Hardt, M. Roellinghof, and H. Wagner.** 1981. Cyclosporin A mediates immunosuppression of primary cytotoxic T cell responses by impairing the release of interleukin 1 and interleukin 2. Eur. J. Immunol. **11**:657–661.
5. **Cauley, L. K., and J. W. Murphy.** 1979. Response of congenitally athymic (nude) and phenotypically normal mice to *Cryptococcus neoformans* infection. Infect. Immun. **23**:644–651.
6. **Colombani, P. M., and A. D. Hess.** 1986. Calmodulin, cyclophilin, and cyclosporin A. Science **233**:987–989.
7. **Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorimetric method for the determination of sugars and related substances. Anal. Chem. **28**:350–356.
8. **Elliott, J. F., Y. Lin, S. B. Mizel, R. C. Bleakley, D. G. Harnish,**

and V. Paetkau. 1984. Induction of interleukin-2 messenger RNA inhibited by cyclosporin A. Science **226**:1439–1441.
9. **Hall, B. M., M. E. Jelbart, and S. E. Dorsch.** 1985. Function of W3/25+ Th cells in rats with prolonged cardiac allograft survival after treatment with cyclosporine. Transplant. Proc. **17**:1372–1373.
10. **Hess, A. D., and P. M. Colombani.** 1986. Mechanism of action of cyclosporine: role of calmodulin, cyclophilin, and other cyclosporine-binding proteins. Transplant. Proc. **18**:219–237.
11. **Hess, A. D., and P. M. Colombani.** 1987. Cyclosporin-resistant and sensitive T-lymphocyte subsets. Ann. Immunol. (Paris) **138**:606–611.
12. **Hess, A. D., P. J. Tutschka, and G. W. Santos.** 1983. Effect of cyclosporine on the induction of cytotoxic T lymphocytes: role of interleukin-1 and interleukin-2. Transplant. Proc. **15**:2248–2258.
13. **Hutchinson, I. F., C. A. Shudur, J. S. A. Duarte, T. B. Strom, and N. L. Tilney.** 1981. Cyclosporin A selectively spares lymphocytes with donor-specific suppressor characteristics. Transplantation **32**:210–216.
14. **Kabelitz, D., C. Zanker, K. Heeg, and H. Wagner.** 1987. Human cytotoxic T lymphocytes. II. Frequency analysis of cyclosporin A-sensitive alloreactive cytotoxic T-lymphocyte precursor. Immunology **61**:57–62.
15. **Kahan, B. D., N. Yoshimura, N. R. Pellis, T. Burstain, C. T. Van Buren, P. Leinikki, R. D. Schreiber, T. R. Roesel, and R. K. Kerman.** 1986. Pharmacodynamics of cyclosporine. Transplant. Proc. **18**:238–251.
16. **Kalman, V. K., and G. R. Klimpel.** 1983. Cyclosporin A inhibits the production of gamma interferon (IFN-γ), but does not inhibit production of virus-induced IFN-γ. Cell. Immunol. **78**: 122–129.
17. **Kay, J. E.** 1987. The cyclosporin-A-sensitive event in lymphocyte activation. Ann. Immunol. (Paris) **138**:622–625.
18. **Kermani-Arab, V., S. Salehmoghaddam, G. Danovitch, K. Hirji, and A. Rezai.** 1985. Mediation of the antiproliferative effect of cyclosporine on human lymphocytes by blockade of interleukin-2 biosynthesis. Transplantation **39**:439–442.
19. **Khakpour, F. R., and J. W. Murphy.** 1987. Characterization of third-order suppressor T cell (Ts3) induced by cryptococcal antigen(s). Infect. Immun. **55**:1657–1662.
20. **Klaus, G. G. B., and A. Kunkl.** 1983. Dissociation of T helper-cell function and helper-cell priming by cyclosporine. Transplant. Proc. **15**:2321–2322.
21. **Kronke, M., W. J. Leonard, J. M. Depper, S. K. Arya, F. Wong-Stall, R. C. Gallo, T. A. Waldman, and W. C. Greene.** 1984. Cyclosporin A inhibits T-cell growth factor gene expression at the level of mRNA transcription. Proc. Natl. Acad. Sci. USA **81**:5214–5218.
22. **Kupiec-Weglinski, J. W., P. A. Lear, T. B. Strom, and N. L. Tilney.** 1983. Population of cyclophosphamide-sensitive T suppressor cells maintain cyclosporin-induced allograft survival. Transplant. Proc. **15**:2357–2363.
23. **Lim, T. S., and J. W. Murphy.** 1980. Transfer of immunity to cryptococcosis by T-enriched splenic lymphocytes from *Cryptococcus neoformans*-sensitized mice. Infect. Immun. **30**:5–11.
24. **Lim, T. S., J. W. Murphy, and L. K. Cauley.** 1980. Host-etiological agent interactions in intranasally and intraperitoneally induced cryptococcosis in mice. Infect. Immun. **29**:633–641.
25. **Lowry, O. H., N. H. Rosenbrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
26. **Miller, G. L.** 1959. Protein determination for large numbers of samples. Anal. Chem. **31**:964–966.
27. **Miller, G. P. G., and D. E. Lewis.** 1987. In vitro effect of cyclosporine on interleukin-2 receptor expression stimulated by *Cryptococcus neoformans*. J. Infect. Dis. **155**:799–802.
28. **Milon, G., P. Truffa-Bachi, B. Shidani, and G. Marchal.** 1984. Cyclosporin A inhibits the delayed-type hypersensitivity effector function of T lymphocytes without affecting their clonal expansion. Ann. Immunol. (Paris) **135**:237–245.
29. **Mody, C. H., G. B. Towes, and M. F. Lipscomb.** 1988. Cyclosporin A inhibits the growth of *Cryptococcus neoformans* in a murine model. Infect. Immun. **56**:7–12.
30. **Murphy, J. W.** 1985. Effects of first-order *Cryptococcus*-specific T suppressor cells on induction of cells responsible for delayed-type hypersensitivity. Infect. Immun. **48**:439–445.
31. **Murphy, J. W., and G. C. Cozad.** 1972. Immunological unresponsiveness induced by cryptococcal capsular polysaccharide assayed by the hemolytic plaque assay technique. Infect. Immun. **5**:896–901.
32. **Murphy, J. W., and J. W. Moorhead.** 1982. Regulation of cell-mediated immunity in cryptococcosis. I. Induction of specific afferent T suppressor cells by cryptococcal antigen. J. Immunol. **128**:276–282.
33. **Murphy, J. W., and R. L. Mosley.** 1985. Regulation of cell-mediated immunity in cryptococcosis. III. Characterization of second-order T suppressor cells (Ts2). J. Immunol. **134**:577–584.
34. **Murphy, J. W., R. L. Mosley, and J. W. Moorhead.** 1983. Regulation of cell-mediated immunity in cryptococcosis. II. Characterization of first-order T suppressor cells and induction of second-order suppressor cells. J. Immunol. **130**:2876–2881.
35. **Naor, D., and A. Langer.** 1985. Analysis of the cyclosporine mechanism in an immunological autoreactive model of delayed-type hypersensitivity. Transplant. Proc. **17**:2706–2708.
36. **Palacious, R., and G. Moller.** 1981. Cyclosporin A blocks receptors for HLA-DR antigens on T cells. Nature (London) **290**:792–794.
37. **Palay, D. A., S. W. Cluff, P. A. Wentworth, and H. K. Ziegler.** 1986. Cyclosporine inhibits macrophage-mediated antigen presentation. J. Immunol. **136**:4348–4353.
38. **Perfect, J. R., and D. T. Durak.** 1985. Effects of cyclosporine in experimental cryptococcal meningitis. Infect. Immun. **50**:22–26.
39. **Peterson, P. K., R. Ferguson, S. S. Fryd, H. H. Balfour, J. Rynasiewicz, and R. L. Simmons.** 1982. Infectious diseases in hospitalized renal transplant recipients: a prospective study of a complex and evolving problem. Medicine **61**:360–372.
40. **Schiltknecht, E., and G. L. Ada.** 1986. In vivo effects of cyclosporine in the generation and expression of effector cell function. Transplant. Proc. **18**:357–359.
41. **Shevach, E. M.** 1985. The effect of cyclosporin A on the immune system. Annu. Rev. Immunol. **3**:397–423.
42. **Shidani, B., I. Motta, and P. Truffa-Bachi.** 1987. Cyclosporin A does not affect the in vitro induction of antigen-specific delayed-type hypersensitivity-mediating T cells. Eur. J. Immunol. **17**: 291–294.
43. **Thomson, A. W., D. K. Moon, Y. Inque, C. L. Geczy, and D. S. Nelson.** 1983. Modification of DTH to ovalbumin in CsA-treated guinea pigs. Immunology **48**:301–308.
44. **Wang, B. S., E. H. Heacock, Z. Chang-Xue, N. L. Tilney, T. B. Strom, and J. S. Mannick.** 1982. Evidence for the presence of suppressor T lymphocytes in animals treated with cyclosporin A. J. Immunol. **128**:1382–1385.
45. **Webster, L. M., and A. W. Thomson.** 1988. Augmentation of delayed-type hypersensitivity to high dose sheep erythrocytes by cyclosporin A in the mouse: influence of drug dosage and route of administration and analysis of spleen cell populations. Clin. Exp. Immunol. **71**:149–154.
46. **Yoshimura, N., and B. D. Kahan.** 1985. Nature of the suppressor cells mediating prolonged graft survival after administration of extracted histocompatibility antigen and cyclosporine. Transplantation **39**:162–168.
47. **Yoshimura, N., and B. D. Kahan.** 1985. The immunosuppressive action of suppressor cells from antigen-cyclosporine-treated hosts on renal allograft survival. Transplantation **40**:384–389.