Effects of Cyclosporin A on Susceptibility and Interferon-Generating Capacity in Mice Infected with *Toxoplasma gondii*

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ABSTRACT. Cyclosporin A (Cs-A), a potent immunosuppressant, was administered to mice to evaluate the role of T lymphocytes for the development of a protective immunity to an obligate intracellular protozoan parasite, *Toxoplasma gondii*. Although daily administration of various amounts of Cs-A for 7 days enhanced the host susceptibility at all doses employed, a dose-dependent manner of Cs-A treatment was not observed as far as the dosage regimens applied here are concerned; mice died at the same rate (40%) among the groups receiving various amounts of Cs-A. Cs-A treatment had a differential effect on the course of disease depending on how it was given in relation to infection. All mice receiving 50 mg of Cs-A per kg per day for 10 days from the beginning of infection eventually died of toxoplasmosis. Cs-A did not suppress the production of interferon (IFN)- α/β that was induced shortly after the infection, whereas it reduced greatly the ability of *Toxoplasma*-infected mice to produce IFN- γ induced by stimulation with *Toxoplasma* lysate antigen (TLA). Moreover, the decrease in IFN- γ production correlated with an increase in the parasitic growth in the peritoneal cavities of Cs-A-treated mice. These results suggest that the immunosuppressive effect of Cs-A on the primary *Toxoplasma* infection in mice is expressed by inhibiting the development of effector T cells responsible for the production of IFN- γ .—**KEY WORDS:** Cyclosporin A, gamma-interferon, *Toxoplasma gondii*.

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Acquired resistance to *Toxoplasma* (*T.*) *gondii* infection is mediated by T lymphocytes. Several studies have clearly demonstrated that protective immunity can be transferred with T cells or T cell subsets from the donors infected chronically with *Toxoplasma* to the recipient animals [2, 13, 20]. Although the precise mechanisms whereby T cells play a key role for protection against *Toxoplasma* infection have not been fully elucidated, recent evidence has strongly suggested that interferon-gamma (IFN- γ) released from sensitized T cells is the major mediator that influences host defense mechanisms [8, 19].

Cyclosporin A (Cs-A) is a potent immunosuppressive drug which acts in various T cell-mediated immune responses. Previous papers suggested that the immunosuppression induced by Cs-A was due to its

ability to inhibit the expression of genes for lymphokines including IFN- γ [3, 15]. There have been a few reports showing that the administration of Cs-A aggravated the infection in mice infected with certain obligate intracellular protozoan parasites such as *T. gondii* and *Trypanosoma cruzi* [9, 10]. However, so far, comparatively little is known about the effect of Cs-A on immune responses, especially in relation to the lymphokine production in mice infected with *Toxoplasma*. Here, we present data to show that Cs-A suppresses the production of IFN- γ in the bloodstreams of mice infected with *T. gondii*, in parallel with a decrease of acquired resistance to the primary infection.

MATERIALS AND METHODS

Mice: Female, 6-week-old ICR mice were

purchased from a commercial breeder (Clea Japan Inc., Tokyo) and maintained in our laboratory under conventional condition throughout the experiment.

Parasite and infection: The low-virulent S-729 strain (Swine origin) of *T. gondii* was provided by Dr. Ohshima of Tanabe Pharmaceutical Co., Ltd., Saitama, and maintained by intraperitoneal (ip) passages of a suspension of brain containing cysts from ICR mice with chronic infection. For infection, mice (8 to 10 weeks old) were inoculated ip with 5×10^3 tachyzoites of the S-729 strain, which were obtained from the peritoneal fluids of the cyst-inoculated mice as described previously [16].

Toxoplasma lysate antigen (TLA): Water-extracted TLA was prepared as described previously by collecting the tachyzoites of the virulent RH strain from the peritoneal cavities of 3-day infected mice [18]. The total protein content of TLA was estimated by the Lowry method [6].

Treatment with Cs-A: The commercially available formula (Sandimmun^R; 50 mg Cs-A per ml solvent) was purchased from Sankyo Pharmaceutical Co., Ltd., Tokyo and dissolved in 0.01 M phosphate-buffered saline (PBS, pH 7.2). Mice were injected ip with different doses of Cs-A in a volume of 0.2 ml PBS for various days starting before or after infection with *Toxoplasma*. Control mice were injected ip with solvent (olive oil) alone.

Induction of IFN- γ : The induction of IFN- γ in the bloodstream was carried out as reported previously [17]. Briefly, mice infected with *Toxoplasma* were injected ip with 200 μ g of TLA in 0.2 ml saline. Five to 6 hr after the injection of TLA, mice were anesthetized with ether and blood was collected by cutting the axillary artery. Serum was separated individually and stored at -80°C until assayed for IFN activity.

Assay for IFN: The IFN assay was carried

out by plaque reduction method using vesicular stomatitis virus (VSV) and L-929 cells [17]. One unit in this assay was equivalent to 1.2 U of the reference mouse IFN- α/β (Lot No., G-002-904-511, National Institute of Allergy and Infectious Disease, NIH, U.S.A.). IFN titer was expressed in international units (IU) per ml.

Neutralization test of IFN: The procedure used for IFN neutralization test was described elsewhere [17]. Rabbit anti-mouse IFN- α/β globulin and rat monoclonal IgG₁ antibody to mouse IFN- γ (Lee Laboratories, San Diego, U.S.A.) were purchased from Funakoshi Pharmaceutical Co., Ltd., Tokyo. These antisera were diluted in 1:40 with Eagle's minimum essential medium supplemented with 1% heat-inactivated fetal calf serum (1% EMEM) to obtain an IFN-neutralizing titer of 250 U per ml. One neutralizing U of these antisera can neutralize 8 to 10 IU of mouse IFN- α/β or IFN- γ .

Enumeration of tachyzoites: Mice infected with *Toxoplasma* were injected ip with 5 ml of 0.01 M PBS containing heparin (10 U/ml) and the resulting peritoneal fluids were collected by syringe. After washing by centrifuge, the peritoneal exudates were resuspended in 2.5 ml of PBS and the total numbers of extracellular tachyzoites in the peritoneal cavity were determined by counting with Neubauer hemocytometer.

Statistical analysis: Student's *t* test was used to calculate the statistical significance. P values of less than 0.05 were considered significant.

RESULTS

Effect of Cs-A on survival of mice infected with *Toxoplasma*: Daily administrations of various amounts of Cs-A for 7 days from the beginning of infection (day 0) enhanced the host susceptibility to *Toxoplasma* infection at all doses employed (Fig. 1). However, no dose-dependent manner of Cs-A treatment

Fig. 1. Effect of Cs-A dose on resistance in mice infected with *Toxoplasma gondii*. Ten ICR mice per group were infected with 5×10^3 tachyzoites of the S-729 strain. Mice received daily 12.5 mg(★), 25 mg(●), 50 mg(■) or 100 mg(▲) of Cs-A per kg per day from day 0 to day 6 of infection. The controls(□) received olive oil only from day 0 to day 6.

Fig. 2. Effect of Cs-A treatment in various days on resistance in mice infected with *Toxoplasma gondii*. Ten ICR mice per group were infected with 5×10^3 tachyzoites of the S-729 strain. Mice received daily 50 mg of Cs-A per kg per day from day -6 to day 0(○), day 0 to day 6(▲), day -6 to day 3(■) or day 0 to day 9(●). The controls(☆) received olive oil only from day 0 to day 9.

was observed, since mice died at the same mortality rate (40%) among the groups receiving various amounts of Cs-A. In contrast, all mice treated daily with 50 mg per kg of Cs-A for 10 days from day 0 died of toxoplasmosis by day 16 postinfection (Fig. 2). Only 30% of mice receiving 50 mg per kg per day of Cs-A from day -6 to day 3 died, while 40% died when Cs-A was given for 7 days starting on day 0. All controls and mice treated daily with the same amount of Cs-A from day -6 to day 0 survived to develop the chronic infection. Thus, Cs-A showed more suppressive effect on host resistance to *Toxoplasma* when treatment was started immediately after infection and continued for 10 days. There was no death in noninfected mice treated with a daily dose of 50 mg per kg of Cs-A for 10 days (data not shown).

Effect of Cs-A on parasitic growth in peritoneal cavities of mice infected with *Toxoplasma*: The numbers of extracellular tachyzoites of *T. gondii* in the peritoneal cavities of mice treated with Cs-A were determined on days 5, 10 and 15 postinfection (Table 1). There was no significant difference in the number of tachyzoites

between Cs-A-treated mice and controls at 5 days after infection. However, remarkable increases in the numbers of organisms were noted in the drug-treated mice at 10 and 15 days postinfection, when mice were entering the moribund phase. In contrast, the numbers of organisms in controls diminished gradually after the 5th day of infection and all mice survived.

Effect of Cs-A on IFN production in mice infected with *Toxoplasma*: In the early phase of infection, the titers of IFN produced in the bloodstream of Cs-A-treated mice were comparable with that produced in the controls (Table 2). The antiviral activities produced by live *Toxoplasma* were neutralized with anti-mouse IFN- α/β antibody. The effect of Cs-A on IFN production in the late phase of infection was next examined upon stimulation with TLA. As shown in Table 3, mice infected with *Toxoplasma* and treated with solvent alone produced considerable amounts of IFN in the bloodstreams in response to TLA. In contrast, the titers of IFN induced in mice treated with Cs-A for 7 to 10 days from day 0 were significantly lower than that induced in the controls at

Table 1. Effect of Cs-A on number of tachyzoites in the peritoneal cavities of mice infected with *Toxoplasma*

Cs-A treatment ^{a)} (days)	Days after infection	No. of tachyzoites ^{b)} ($\times 10^4$ /mouse)
0 to 5	5	28.8 $\pm$ 4.6
0 to 9	10	104.6 $\pm$ 11.2
0 to 9	15	538.2 $\pm$ 35.9
(-)	5	32.5 $\pm$ 3.6
(-)	10	8.6 $\pm$ 0.6
(-)	15	1.0 $\pm$ 0.6

a) Fifteen ICR mice per group were infected with 5×10^3 tachyzoites of the S-729 strain. Cs-A (50 mg/kg/day) was injected daily for 6 or 10 days from day 0 of infection.

b) Results were mean $\pm$ S. D. from 5 mice.

Table 2. Effect of Cs-A on IFN- α/β production in the bloodstream of mice infected with *Toxoplasma*

Group ^{a)}	Cs-A treatment ^{b)} (days)	Days after infection	IFN titer(IU/ml)		
			Induced by <i>Toxoplasma</i> ^{c)}	After neutralization with ^{d)}	
				anti-IFN- α/β	1%EMEM
I	0 to 1	1	52.6 $\pm$ 12.8	<10	48
	0 to 2	2	69.8 $\pm$ 11.8	<10	60
	0 to 3	3	42.8 $\pm$ 8.2	<10	45
II	-6 to 1	1	46.7 $\pm$ 10.3	<10	40
	-6 to 2	2	67.6 $\pm$ 11.0	<10	55
	-6 to 3	3	44.2 $\pm$ 9.8	<10	48
III	(-) ^{e)}	1	52.8 $\pm$ 12.6	<10	55
	(-)	2	58.3 $\pm$ 17.3	<10	53
	(-)	3	43.6 $\pm$ 12.2	<10	38

a) Fifteen ICR mice per group were infected with 5×10^3 tachyzoites of the S-729 strain.

b) Cs-A (50mg/kg/day) was injected daily for indicated periods.

c) Mean titer of IFN measured individually from 5 mice.

d) The neutralization test was carried out using pooled sera from 5 mice.

e) Control mice were injected with solvent alone.

any days examined ($P < 0.05$). There were also significant differences between the titers of IFN induced in mice treated with Cs-A from day -6 to day 3 and those induced in the controls at 10 and 12 days postinfection ($P < 0.05$). The antiviral activities induced by TLA were neutralized with monoclonal anti-mouse IFN- γ , but not with polyclonal anti-mouse IFN- α/β antibody.

DISCUSSION

The results presented in this paper show

that the administration of Cs-A to mice enhance the host susceptibility to *Toxoplasma* infection. Daily injection of Cs-A during the first week or 10 days of infection converted subclinical infection into clinical disease with pronounced morbidity and high mortality. McCabe *et al.* [9] reported that Cs-A treatment (150 mg/kg/day) of mice infected with 2×10^4 tachyzoites of the C-56 strain shortened their survival days but it prolonged the time to death in a higher dose of the organisms (5×10^4 /mouse), although the treatment strongly suppressed both IgG

Table 3. Effect of Cs-A on IFN- γ production induced by specific antigen in the bloodstream of mice infected with *Toxoplasma*

Group ^{a)}	Cs-A treatment ^{b)} (days)	Days after infection	IFN titer(IU/ml)		
			Induced by TLA ^{d)}	After neutralization with ^{e)}	
				anti-IFN- α/β	anti-IFN- γ
I	0 to 6	6	38.2 $\pm$ 14.6	35	<10
	0 to 8	8	41.4 $\pm$ 15.9	42	<10
	0 to 9	10	37.4 $\pm$ 17.9	30	<10
	0 to 9	12	40.2 $\pm$ 10.9	48	<10
II	-6 to 3	6	64.8 $\pm$ 24.9	NT ^{g)}	NT
	-6 to 3	8	91.6 $\pm$ 18.1	NT	NT
	-6 to 3	10	89.0 $\pm$ 23.4	65	<10
	-6 to 3	12	83.4 $\pm$ 21.0	78	<10
III	(-) ^{f)}	6	117.0 $\pm$ 38.5	110	<10
	(-)	8	192.1 $\pm$ 52.5	165	<10
	(-)	10	221.4 $\pm$ 40.6	205	<10
	(-)	12	304.1 $\pm$ 98.8	260	<10

a) Twenty ICR mice per group were infected with 5×10^3 tachyzoites of the S-729 strain.

b) Cs-A (50mg/kg/day) was injected daily for indicated periods.

c) At the indicated day, blood was collected at 5 to 6 hr after injection of TLA (200 μ g/mouse).

d) Mean titer of IFN measured individually from 5 mice.

e) The neutralization test was carried out using pooled sera from 5 mice.

f) Control mice were injected with solvent alone.

g) Not tested.

and IgM antibody production in all the mice. Thus, their result was somewhat different from those observed in our experiment. The cause of this discrepancy might be the difference in the virulence of organisms used for the challenge or the dosage of Cs-A employed. In this study, the effect of Cs-A on survival of *Toxoplasma*-infected mice was not dose dependent, since mice died at the same rate among the groups receiving daily various amounts of Cs-A for 7 days (Fig. 1). This is not compatible with the recent findings observed in other models of infection wherein the dose dependent immunosuppressive effect of Cs-A was clearly demonstrated [12, 21].

Although the reason why the effect of Cs-A on mortality was not dose-dependent in our system cannot be explained adequately at present, the results in Fig. 2 suggest that Cs-A exerts a different immunosuppressive effect depending on either the length of administration or the time at which

treatment was started. Huegin *et al.* [4, 5] reported that Cs-A was able to suppress bacterial elimination more efficiently from the organs of *Listeria*-infected mice when it was given starting at 3 to 4 days after infection, whereas it must be administered before or up to 12 hr after infection to suppress the cytotoxic T cell activity against vaccinia virus or choriomeningitis virus. Hence, it is likely that the effect of Cs-A on immunity to *T. gondii* is dependent on how it was given in relation to infection, although the possibility that the effect of Cs-A on protective immunity to *Toxoplasma* might be dose dependent cannot be excluded.

There is a possible limitation to the use of Cs-A in a model of immunodeficiency in toxoplasmosis, since *in vitro* experiments have shown that this drug possesses antitoxoplasmic activity when it was added simultaneously with the extracellular tachyzoites to cell monolayers [7, 9]. However, the

infecting organisms did not appear to be sensitive to Cs-A at the concentrations used in the present study, because the tachyzoites in the peritoneal cavities of Cs-A-treated mice increased significantly in number and persisted for extended periods of time as compared with that of nontreated mice (Table 1).

In this study, Cs-A was used to define the involvement of T cell-dependent mechanisms in murine resistance to *Toxoplasma* infection, since this drug has been shown to affect many T cell functions by inhibiting release of lymphokines [3, 14, 15]. Actually, the administration of Cs-A to mice during *Toxoplasma* infection resulted in the significant decrease in IFN- γ production induced by stimulation with *Toxoplasma* lysate antigen (TLA) in the bloodstreams, in parallel with the aggravation of infection. This result suggests that specifically sensitized T cells play an essential role for the induction of antitoxoplasmic resistance by secreting IFN- γ . Thus, the defect of IFN- γ production is presumed to result in the exacerbation of disease in the Cs-A-treated mice, because this lymphokine is known to play an important role in resistance to *Toxoplasma* infection by activating macrophages [1, 11, 19].

Recently, Nakane *et al.* [12] and Takashima and Collins [21] have reported that Cs-A treatment caused the suppression of both IFN- γ production induced by specific antigen and antibacterial resistance in mice infected with either *Listeria monocytogenes* or *Mycobacterium bovis*, BCG. Additionally, Nakane *et al.* [12] have demonstrated that the administration of recombinant murine IFN- γ restored the impaired antilisterial resistance and IFN- γ -generating capacity in the Cs-A-treated mice. Hence, it is reasonable to presume that immunosuppressive effect of Cs-A on primary *Toxoplasma* infection is expressed by inhibiting the development of effector T cells responsible for the production of IFN- γ , thus result-

ing in the prevention of macrophage activation.

REFERENCES

1. Black, C. M., Catterall J. R., and Remington, J. S. 1987. *In vitro* and *in vivo* activation of alveolar macrophages by recombinant interferon- γ . *J. Immunol.* 134: 491-495.
2. Frenkel, J. K. 1967. Adoptive immunity to intracellular infection. *J. Immunol.* 98: 1309-1319.
3. Granelli-Piperno, A., Andrus, L., and Steinman, R. M. 1986. Lymphokine and monokine mRNA levels in stimulated human T cells. Kinetics, mitogen requirements, and effect of cyclosporin A. *J. Exp. Med.* 163: 922-937.
4. Huegin, A. W., Cerny, A., Hengartner, H., and Zinkernagel, R. M. 1985. Suppression by cyclosporin A of murine T-cell-mediated immunity against viruses *in vivo* and *in vitro*. *Cell. Immunol.* 90: 464-473.
5. Huegin, A. W., Cerny, A., Wrann, M., Hengartner, H., and Zinkernagel, R. M. 1986. Effect of cyclosporin A on immunity to *Listeria monocytogenes*. *Infect. Immun.* 52: 12-17.
6. Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J. 1951. Protein measurement with Folin phenol reagent. *J. Biol. Chem.* 193: 265-275.
7. Mack, D. G. and McLeod, R. 1984. New micromethod to study the effect of antimicrobial agents of *Toxoplasma gondii*: Comparison of sulfadoxine and sulfadiazine individually and in combination with pyrimethamine and study of clindamycin, metronidazole, and cyclosporin A. *Antimicrob. Agents Chemother.* 26: 26-30.
8. McCabe, R. E., Luft, B. J., and Remington, J. S. 1984. Effect of murine interferon gamma on murine toxoplasmosis. *J. Infect. Dis.* 150: 961-962.
9. McCabe, R. E., Luft, B. J., and Remington, J. S. 1986. The effects of cyclosporin on *Toxoplasma gondii* *in vivo* and *in vitro*. *Transplantation* 41: 611-615.
10. McCabe, R. E., Remington, J. S., and Araujo, F. G. 1985. *In vivo* and *in vitro* effects of cyclosporin A on *Trypanosoma cruzi*. *Am. J. Trop. Med. Hyg.* 34: 861-865.
11. Murray, H. W., Spitalny, G. L., and Nathan, C. F. 1985. Activation of mouse peritoneal macrophages *in vivo* and *in vitro* by interferon- γ . *J. Immunol.* 134: 1619-1622.
12. Nakane, A., Minagawa, T., Yasuda, I., Yu, C., and Kato, K. 1988. Prevention by gamma interferon of fetal infection with *Listeria monocytogenes*.

- togenes* in mice treated with cyclosporin A. *Infect. Immunol.* 56: 2011–2015.
13. Pavia, C. S. 1986. Protection against experimental toxoplasmosis by adoptive immunotherapy. *J. Immunol.* 137: 2985–2990.
  14. Reem, G. H., Cook, L. A., and Vilcek, J. 1983. Gamma interferon synthesis by human thymocytes and T lymphocytes inhibited by cyclosporin A. *Science* 221: 63–65.
  15. Shebach, E. M. 1985. The effect of cyclosporin A on the immune system. *Annu. Rev. Immunol.* 3: 397–427.
  16. Shimizu, K., Kito, S., and Shirahata, T. 1967. Experiments on mouse passage of the cyst type strain of *Toxoplasma gondii* for enhancement of virulence. *Jpn. J. Vet. Sci.* 29: 71–78.
  17. Shirahata, T., Mori, A., Ishikawa, H., and Goto, H. 1986. Strain differences of interferon-generating capacity and resistance in *Toxoplasma*-infected mice. *Microbiol. Immunol.* 30: 1307–1316.
  18. Shirahata, T., Shimizu, K., and Suzuki, N. 1976. Effects of immune lymphocyte products and serum antibody on the multiplication of *Toxoplasma* in murine peritoneal macrophages. *Z. Parasitenkd.* 49: 11–23.
  19. Suzuki, Y., Orellana, N. A., Schreiber, R. D., and Remington, J. S. 1988. Interferon- γ : The major mediator of resistance against *Toxoplasma gondii*. *Science* 240: 516–517.
  20. Suzuki, Y. and Remington, J. S. 1988. Dual regulation of resistance against *Toxoplasma gondii* infection by Lyt-2^+ and Lyt-1^+ , L_3T_4^+ T cells in mice. *J. Immunol.* 140: 3943–3946.
  21. Takashima, T. and Collins, F. M. 1987. Immunosuppressive effect of cyclosporin A on *Mycobacterium bovis* BCG infection in mice. *Infect. Immun.* 55: 1701–1706.

要 約

トキソプラズマ感染マウスの感受性とインターフェロン産生におよぼすサイクロスポリンAの影響：白幡敏一・勝田 賢・太田千佳子・後藤 仁(帯広畜産大学獣医学科家畜微生物学教室)——トキソプラズマ(Tp)感染マウスにサイクロスポリンA(Cs-A)を投与し、生存率とインターフェロン(IFN)の産生性を指標にして、本剤の免疫抑制効果を検討した。感染当日から種々の濃度のCs-Aを7日間連続投与されたマウスの死亡率は、投与濃度に比例せず、いずれの投与群においても40%であった。一方、50mg/kg/日量のCs-Aを感染当日から10日間連続投与されたマウスは感染16日目までに全例が死亡したが、感染6日前から同量のCs-Aを7日間および10日間投与した場合の死亡率は、それぞれ0%と30%で、Cs-Aの免疫抑制効果はその投与時期や期間により異なることが示された。また、Cs-A投与マウスにおける血中IFNの産生状況を検討したところ、感染に伴って出現するIFN- α/β の産生性には特に影響を与えないが、抗原特異的に誘発されるIFN- γ の産生は顕著に抑制された。従って、Tp感染マウスでは、Cs-A投与により、IFN- γ 等のマクロファージ活性化に関与するリンフォカイン産生性T細胞の機能が障害され、感染の悪化を招くものと考えられた。