PARADOXICAL EFFECTS OF CYCLOSPORIN A ON COLLAGEN ARTHRITIS IN RATS

By NOBUHIRO KAIBARA,* TAKAO HOTOKEBUCHI, KENJI TAKAGISHI, AND ICHIRO KATSUKI

From the Department of Orthopaedic Surgery, Faculty of Medicine, Kyushu University, Fukuoka, Japan 812

Collagen arthritis in rats is an animal model of polyarthritis that can be produced by an intradermal injection of homologous or heterologous native type II collagen emulsified in incomplete Freund's adjuvant (ICFA)[1] (1). The disease, which resembles human rheumatoid arthritis in many aspects (2), is not completely understood but appears to be the result of an autoimmune response to type II collagen (3, 4). It has recently been reported that the development of arthritis and the immune response to type II collagen are suppressed by pretreating the rats with immunosuppressive agents such as cyclophosphamide, azathioprine, and steroids (5–7).

Cyclosporin A (CS-A), a new antilymphocytic drug, has been described as a potent immunosuppressive agent. Several lines of evidence suggest that the action of CS-A is predominantly or exclusively limited to T cell-mediated immune responses (8–11). Its usefulness is currently being actively investigated in various in vivo and in vitro conditions, and great expectations have been placed on CS-A because its remarkable immunosuppressive potency is associated with a very low degree of myelotoxicity (12).

In the present study, we evaluated CS-A for its effectiveness in preventing the development of collagen arthritis by treating the rats for the first 14 d with CS-A. In an attempt to gain further insight into the mode of action of CS-A, we also investigated the effects of CS-A treatment in three different regimens: (*a*) only during the induction phase of immunity; (*b*) only during the immediate preclinical phase of arthritis; (*c*) on the established disease. This report describes some preliminary results of CS-A in this animal model of polyarthritis.

Materials and Methods

Rats. Outbred female Sprague-Dawley rats (Japan Charles River Breeding Laboratories, Kanagawa, Japan), 6–8 wk old and weighing 130–200 g, were used. They were housed in groups of five or six in metal cages and fed *ad libitum* with standard laboratory chow and water.

Collagen Preparation and Analysis. Type II collagen was isolated and purified from bovine articular cartilage, as described by Trentham et al. (1). Purity was assessed by

* To whom correspondence should be addressed at the Department of Orthopaedic Surgery, Faculty of Medicine, Kyushu University, 3-1-1, Maidashi, Higashi-ku, Fukuoka, Japan 812.

[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; CS-A, cyclosporin A; DTH, delayed-type hypersensitivity; ICFA, incomplete Freund's adjuvant; PBS, phosphate-buffered saline.

J. Exp. Med. © The Rockefeller University Press · 0022-1007/83/12/2007/09 $1.00 2007
Volume 158 December 1983 2007–2015

amino acid analysis, which showed 96 residues of hydroxyproline per 1,000 total amino acid residues of the collagen preparation. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate, under conditions of urea denaturation (13), revealed a band pattern characteristic of type II collagen. Proteoglycan contamination was below the limits of detection by uronic acid assay (14). The collagen was lyophilized and stored at −20°C.

Immunization Procedures. Lyophilized type II collagen was dissolved in 0.1 M acetic acid at a concentration of 6 mg/ml. The collagen solution was clarified by centrifugation (20,000 *g*, 90 min) and diluted when necessary with 0.1 M acetic acid. Equal volumes of collagen solution and ICFA (Difco Laboratories, Inc., Detroit, MI) were emulsified using a homogenizer (Polytron PT 10-35; Kinematica, Lucerne, Switzerland) and kept cold with an ice bath. A total volume of 1 ml of the cold emulsion was injected intradermally at several sites on the back and at 1 or 2 sites into the base of the tail.

Treatment with CS-A. CS-A (OL 27-400; Sandoz Ltd., Basel, Switzerland) was provided in powder form and dissolved in pure olive oil at concentrations of 5 or 10 mg/ml by heating in a water bath to 65°C. It was prepared fresh every 3 d. CS-A was administered orally by gastric intubation under light ether anesthesia on the days and at the doses indicated in the text. The dose was adjusted according to the daily body weight. Control rats, while immunized with the same amount of type II collagen, received oral administrations of olive oil alone. CS-A-treated and control rats were handled identically for administration of the agent and the solvent.

Assessment of Arthritis. Rats were examined daily for 4–5 wk after immunization with type II collagen to record the day of onset and the severity of arthritis. The lesions of the four paws were each graded from 0 to 4 according to increasing extent of periarticular erythema and swelling as well as joint deformity, as described previously (15). The maximum possible score was 16.

Immune Responses to Type II Collagen. Blood was collected by cardiac puncture under light ether anesthesia. Sera were removed, heat inactivated at 56°C for 30 min, and stored at −20°C until used. Serum antibodies to type II collagen were measured by the enzyme-linked immunoassay technique. The methods used were adapted from those of Voller et al. (16). Briefly, disposable polystyrene cuvettes were coated by incubation with 100 μl of type II collagen solution (25 μg/ml in coating buffer, pH 9.6). After overnight incubation at 4°C and washing three times with phosphate-buffered saline (PBS) containing 0.05% Tween 20, the remaining protein-binding sites were blocked by the addition of coating buffer containing 1% bovine serum albumin (BSA) and incubation continued at room temperature for 1 h. The cuvettes were washed as described above. Serum was diluted with PBS-Tween containing 1% BSA and 100-μl aliquots of each dilution were placed in duplicate or triplicate cuvettes. After incubation at room temperature for 2 h and washing with PBS-Tween, 100 μl of a 1:500 dilution of alkaline phosphatase-conjugated anti-rat IgG (Miles Laboratories, Inc., Elkhart, IN) was added and incubation continued at room temperature for 3 h. Excess conjugate was washed out and the amount of conjugate specifically bound was determined by adding 100 μl of *p*-nitrophenyl phosphate solution (1 mg/ml in 10% diethanolamine buffer, pH 9.8). After incubation at room temperature for 30 min, the reaction was stopped by adding 20 μl of 3 N NaOH to each well and the reaction product was measured by absorbance at 410 nm using an automated device (Dynatech Laboratories, Inc., Alexandria, VA). All of the experiments reported here used a single batch of alkaline phosphatase conjugate and included appropriate positive and negative controls. Antibody levels were expressed in absorbance values based on preliminary studies of serial dilutions of the reference samples under the actual assay system. Delayed-type hypersensitivity (DTH) skin testing was performed on day 25, as described by Griffiths et al. (17), and the responses were read at 48 h.

Statistics. Continuous variables were analyzed by their group means (Student's *t* test) and dichotomous variables by their proportionate group frequencies (chi-square test). *P* values <0.05 were considered to be statistically significant.

Results

Effect of the Immunizing Dose of Type II Collagen on the Production of Arthritis. To find the optimal dose of type II collagen for the production of a high incidence of arthritis, rats were injected with various doses of type II collagen emulsified in ICFA. The results are summarized in Table I. 15 of 30 rats (50%) injected with 0.5 mg of type II collagen developed arthritis. When larger doses of type II collagen (1.5 mg or more) were used, 86% of the rats developed arthritis and the peak incidence of the disease occurred between 9 and 14 d after immunization. In our hands, consistently 80–90% of the rats developed arthritis when 1.5 mg of type II collagen in ICFA was injected intradermally not only at several sites on the back but also at 1 or 2 sites into the base of the tail. Therefore, in the subsequent studies, we routinely used this regimen of immunization.

Dose-Response Studies of CS-A on the Development of Collagen Arthritis in Rats. Dose-response studies of CS-A were carried out in a 14-d course of treatment in which the rats received daily administration of CS-A or olive oil for 14 d, starting on the same day as the type II collagen immunization. An inflammatory polyarthritis was induced in 12 of 14 rats treated with olive oil for 14 d. Daily treatment with CS-A in doses of 15 or 25 mg/kg per day for 14 d gave complete suppression of arthritis induction during an observation period of 4 wk, while a daily dose of 5 mg/kg was without effect (Table II).

Serum antibody levels to type II collagen were measured on day 21, and DTH skin testing was performed on day 25. Olive oil-treated control rats showed high antibody levels and strong positive skin test responses to type II collagen, whereas very weak or no antibody responses and negative to weak skin test responses to type II collagen could be detected in the rats treated with CS-A at doses of 15 or 25 mg/kg per day. These results indicate that CS-A blocks the helper T cell function necessary for the antibody responses to type II collagen, because CS-A does not directly affect B lymphocytes.

Timing Studies of CS-A Treatment on Collagen Arthritis in Rats. In the preceding section it was demonstrated that CS-A treatment at doses of 15 mg/kg per day or more for the first 14 d suppressed the development of arthritis as well as humoral and skin test responses to type II collagen. In this section, we wished to see if CS-A given only during the induction phase of immunity or only during the immediate preclinical phase of arthritis would have similar effects. Also, the

TABLE I
Effect of the Immunizing Dose of Type II Collagen on the Production of Arthritis

Type II collagen	Incidence of arthritis*	Number of rats with polyarthritis scoring:			
		0	1–5	6–10	11–16
mg					
0.5	15/30 (50%)	15	10	5	0
1.5	28/33 (85%)	5	15	12	1
3.0	9/10 (90%)	1	6	3	0

* Number of rats with arthritis per number of rats tested.

TABLE II

Effect of CS-A Treatment (Days 0–13) on the Development of Collagen Arthritis

	CS-A dose (mg/kg)			Olive oil
	25	15	5	
Incidence of arthritis	0/14*	0/14*	11/14 (79%)	12/14 (86%)
Arthritic index‡	—	—	4.9 ± 0.9	5.5 ± 0.8
Day of onset§	—	—	11.0 ± 0.6	10.4 ± 0.4
Antibody level‖	0.01¶	0.01**	0.66 ± 0.13	0.90 ± 0.14
DTH skin reaction‡‡	3.3 ± 0.6§§	3.2 ± 0.9‖‖	5.4 ± 0.5	5.7 ± 0.4

* $P < 0.002$ vs. the oil group.
‡ Expressed as the mean of maximum arthritic indices ± SEM.
§ Based on arthritic rats only (mean ± SEM).
‖ Antibody levels to type II collagen were measured using enzyme-linked immunoassay system on day 21 and expressed in absorbance values at 410 nm (mean ± SEM). A serum dilution of 1:30,000 was used for detection of antibodies.
¶ $P < 0.001$ vs. the oil group.
** $P < 0.005$ vs. the oil group.
‡‡ On day 25; expressed as the mean ± SEM diameter of induration (mm).
§§ $P < 0.01$ vs. the oil group.
‖‖ $P < 0.02$ vs. the oil group. For all the other parameters depicted in this table, the differences between the groups were not significant.

TABLE III

*Effect of CS-A Treatment (Days 0–6) on the Development of Collagen Arthritis**

	CS-A dose (mg/kg)		Olive oil
	25	15	
Incidence of arthritis	2/16 (13%)‡	4/18 (22%)‡	14/15 (93%)
Arthritic index	0.3 ± 0.2§	1.0 ± 0.5§	6.1 ± 0.8
Day of onset	13.5 ± 2.5	15.0 ± 2.8	11.4 ± 1.0
Antibody level	0.15 ± 0.04§	0.22 ± 0.08§	1.04 ± 0.10
DTH skin reaction	3.6 ± 0.4‖	4.2 ± 0.4¶	5.7 ± 0.2

* Parameters and units are identical to those described in Table II.
‡ $P < 0.002$ vs. the oil group.
§ $P < 0.001$ vs. the oil group.
‖ $P < 0.005$ vs. the oil group.
¶ $P < 0.02$ vs. the oil group. For all the other parameters depicted in this table, the differences between the groups were not significant.

response of the established disease to CS-A treatment was studied. In other words, groups of rats were treated with CS-A or solvent for 7-d periods only starting on days 0, 7, and 14. The results are given in Tables III–V. The development of arthritis was suppressed only if CS-A treatment was started on the same day as type II collagen immunization. The results showed that a 7-d course was nearly as effective as a 14-d course. When CS-A treatment was started 7 d after immunization, all of the rats developed arthritis, though the incidence and onset day of arthritis in CS-A-treated rats were not significantly different from those of olive oil-treated control rats, statistically. However, the clinical signs of arthritis in CS-A-treated rats were significantly enhanced in a dose-dependent manner (Table IV). In particular, forepaw involvement was more frequently observed in CS-A-treated rats. 13 of 19 arthritic rats treated with a 50 mg/kg per day dose of CS-A developed swelling and inflammation of digits

TABLE IV
Effect of CS-A Treatment (Days 7–13) on Collagen Arthritis

	CS-A dose (mg/kg)			Olive oil
	50	25	15	
Incidence of arthritis	19/19	20/20	8/8	14/16 (88%)
Arthritic index	9.7 ± 0.7*	9.3 ± 1.0‡	7.6 ± 1.1	5.8 ± 0.7
Day of onset	9.9 ± 0.3	9.8 ± 0.3	11.5 ± 1.0	10.1 ± 0.4
Antibody level				
Day 15	0.47 ± 0.06§	0.57 ± 0.07‖	ND¶	0.84 ± 0.08
Day 21	0.51 ± 0.07*	0.72 ± 0.08‖	0.59 ± 0.11‖	1.03 ± 0.12
DTH skin reaction	7.0 ± 0.4‡	7.0 ± 0.2‡	7.1 ± 0.4‖	5.5 ± 0.4

Parameters and units are identical to those described in Table II.
* $P < 0.001$ vs. the oil group.
‡ $P < 0.02$ vs. the oil group.
§ $P < 0.005$ vs. the oil group.
‖ $P < 0.05$ vs. the oil group. For all the other parameters depicted in this table, the differences between the groups were not significant.
¶ Not done.

TABLE V
Effect of CS-A Treatment (Days 14–20) on the Established Lesions of Collagen Arthritis

	CS-A dose (mg/kg)		Olive oil
	50	25	
Number of rats tested	14	15	15
Arthritic index	10.4 ± 1.1*	9.3 ± 1.1	7.5 ± 0.7
Antibody level	0.88 ± 0.11	0.96 ± 0.11	1.07 ± 0.12
DTH skin reaction	7.6 ± 0.4‡	7.5 ± 0.4‡	5.3 ± 0.6

Parameters and units are identical to those described in Table II.
* $P < 0.05$ vs. the oil group.
‡ $P < 0.02$ vs. the oil group. For all the other parameters depicted in this table, the differences between the groups were not significant.

and wrists, grossly evidenced within 6 d of disease onset. In contrast, only 1 of 14 arthritic rats in the control group developed forepaw inflammation during an observation period of 4 wk. This enhancement of the disease was maintained for the period of CS-A dosing and as long as 7 d after withdrawal of CS-A. In addition, this regimen caused an augmentation of DTH skin reactions to type II collagen and concomitantly suppressed antibody responses measured on days 15 and 21.

In the next experiment, the response of the established disease to CS-A treatment was studied. Groups of 16 rats were immunized with type II collagen on day 0. One or 2 animals in each group did not develop arthritis by day 14 and were removed from the experiment. The remaining 14 or 15 rats per group were then given CS-A in doses of 25 or 50 mg/kg per day or olive oil alone from day 14–20. The results are shown in Table V. CS-A treatment from day 14 to 20 also caused a dose-related enhancement of the disease and an augmentation of DTH skin reactions without affecting antibody responses.

Discussion

Although the precise mechanism of action of CS-A is not completely understood, current evidence suggests that it may exert its action on the early events of T lymphocyte proliferation by interfering with the production and/or release of interleukin 2, and possibly also by abrogating the responses of T cells to interleukin 2 (18–21). The main target for the action of CS-A appears to be the helper T cells (8–11, 21–23).

The results described in this report clearly demonstrate that CS-A completely prevented the development of arthritis in type II collagen-immunized rats when the agent was given prophylactically. They also demonstrate the suppression of anti-type II collagen antibody formations and DTH skin reactions to type II collagen. These results accord with the data obtained in several in vivo systems (8, 9, 12, 23), indicating that CS-A has a suppressive effect on various aspects of T cell-mediated immune responses, possibly by interfering with helper T cells.

Since evidence was presented that CS-A impairs T cell functions by interfering with an early stage of antigenic triggering (8–11), almost invariably, treatment with CS-A has been started at the beginning or soon after the antigenic stimulation both in vitro and in vivo. However, in a model such as experimental autoimmune uveitis, treatment with CS-A starting only during the effector phase proved to be successful (24). Moreover, therapeutic treatment with CS-A demonstrated a pronounced reduction of the inflammatory symptoms in adjuvant arthritis (8) and an impressive improvement of the conditon of experimental allergic encephalomyelitis in the rat (25).

Based on these findings, one might expect a therapeutic effect of CS-A on an ongoing or established disease of collagen arthritis in rats. Therefore, in the present study, we also investigated the effects of CS-A on collagen arthritis in the three experimental protocols; i.e., the time of CS-A administration was varied in respect to the time of immunization. In the first, CS-A was given only during the induction phase of immunity, which proved to be successful. It was confirmed that a 7-d course of CS-A treatment was nearly as effective as a 14-d course.

In the second experiment, CS-A treatment was started only during the immediate preclinical phase of arthritis. The results of this experiment were unexpected. In contrast to the olive oil-treated control rats, CS-A-treated rats showed a marked enhancement of the disease in a dose-dependent manner. In addition, this regimen caused an augmentation of DTH skin reactions, and concomitantly depressed antibody responses. These results are in marked contrast to those seen with experimental autoimmune uveitis in the rat, where CS-A treatment begun 7 d after immunization efficiently prevented the disease process (24). The reasons for this apparent discrepancy are unclear, but the enhancing effect of CS-A in the present experiment seems to be operational only in immunologically triggered inflammatory responses. A possible explanation is that CS-A treatment in a late stage of immunization inhibited a clonal expansion of a population of suppressor T cells while permitting the activation of helper T cells and caused severe arthritis. In support of this, recent data from Palacios (20) show that CS-A inhibits the generation of suppressor T cells in an autologous mixed lymphocyte culture system. Further in vivo circumstantial evidence was presented by Thomson et al. (26), in which CS-A administration to guinea pigs on days 0–4

after ovalbumin immunization caused dose-related potentiation of 14-d skin responses. Wick et al. (27) demonstrated that CS-A administration to Obese strain chicken embryos resulted in the development of significantly more severe spontaneous autoimmune thyroiditis and suggested a suppressive effect of CS-A on precursors of suppressor T cells.

Of much greater interest to us was the associated augmentation of DTH skin reactions and concomitant suppression of antibody responses. A similar reciprocal relationship between humoral and DTH responses produced by cyclophosphamide treatment is explained by Diamantstein et al. (28), in which various T lymphocyte subpopulations, involved in mediation and regulation of DTH and of humoral responses, differ in their sensitivity to cyclophosphamide. The precise mechanism of the inverse action of CS-A on humoral and cell-mediated immune responses in the present study is unclear, but it is conceivable that an inhibitory effect of CS-A on a population of suppressor T cells is at least in part responsible. These results further indicate that antibody is not the sole regulatory factor and that cell-mediated immune responses measured by DTH skin reactions play an important role in influencing the course of the disease.

In the third experiment, we investigated the effect of CS-A on the established disease. CS-A also showed an enhancing effect on the established disease and this enhancement was accompanied by an augmentation of DTH skin reactions and unaffected antibody responses. Although the enhancing effect of CS-A in the present experiment and in its preclinical use was rapidly reversible upon cessation of its administration, we are concerned by the therapeutic use of CS-A since the clinical relevance of this finding is that CS-A may not be effective for the treatment of human arthritides. In fact, the studies by Graf et al. (29) indicate that the effect of CS-A on rheumatoid and psoriatic arthritis is not satisfactory.

In conclusion, CS-A can either suppress or enhance the clinical symptoms of collagen arthritis in rats depending on the treatment regimen. The paradoxical effects of CS-A on collagen arthritis might be caused by the altering of the sensitive balance of the two regulatory subpopulations of T lymphocytes, helper and suppressor cells. The results also suggest that cell-mediated immune responses may play an important role in influencing the course of the disease. If further experiments in other situations bear these out, CS-A will provide an extremely useful tool for the investigation of functionally different T cell subpopulations.

Summary

The effect of the immunosuppressive agent cyclosporin A (CS-A) on collagen arthritis in Sprague-Dawley rats is investigated. A 14-d course of CS-A treatment at doses of 15 mg/kg per day or more, begun on the same day as type II collagen immunization, suppressed the development of arthritis as well as humoral and delayed-type hypersensitivity (DTH) skin test responses to type II collagen, possibly by interfering with helper T cells. Additional studies demonstrated that CS-A treatment only during the induction phase of immunity proved to be successful. When CS-A treatment was started only during the immediately preclinical phase of arthritis or after the disease onset, a significant enhancement of the disease was obtained in a dose-dependent manner. This enhancement was

accompanied by an augmentation of DTH skin reactions, while antibody responses were either suppressed or unaffected. These results appear to be attributable at least in part to a suppressive effect of CS-A on a population of suppressor T cells, thus resulting in a T cell-mediated helper effect. It is therefore reasonable to assume that the paradoxical effects of CS-A on collagen arthritis in rats might be caused by an altering of the sensitive balance of the two regulatory subpopulations of T cells. It is also possible that cell-mediated immune responses may play an important role in influencing the course of the disease.

We are very grateful to Dr. J. F. Borel and Dr. E. Wiskott of Sandoz Ltd., Basel, Switzerland, for supplying cyclosporin A and also for much helpful advice.

Received for publication 8 July 1983 and in revised form 13 September 1983.

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