SUPPRESSIVE EFFECT OF CYCLOSPORIN A ON DELAYED-TYPE HYPERSENSITIVITY TO SYNGENEIC TESTICULAR CELLS

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We have investigated the immunosuppressive effect of cyclosporin A (CsA) on delayed-type hypersensitivity (DTH) to syngeneic testicular cells (TC). DTH to syngeneic TC was induced by subcutaneous (sc) immunization with viable syngeneic TC and was augmented by a high dose of cyclophosphamide (CY)-pretreatment. DTH was suppressed by administration of CsA in a dose-dependent manner. When the mice were immunized alone or with 100 mg/kg of CY-pretreatment, 30 mg/kg or more of CsA suppressed DTH to TC. In mice immunized with 200 mg/kg of CY-pretreatment, 50 mg/kg of CsA was needed to suppress DTH.

DTH is thought to play a key role in the induction and/or maintenance of experimental autoimmune orchitis (EAO). Our data show that DTH to syngeneic TC induced by immunization is suppressed by administration of CsA. Pretreatment of mice with immunization and administration of CsA suppressed DTH significantly when the mice were challenged with immunization with CY-pretreatment. However, DTH was rather enhanced significantly in mice pretreated with administration of CsA alone without preimmunization. Therefore, even though administration of CsA with immunization suppresses DTH, administration of CsA alone might rather eliminate suppressive mechanism resulting in augmentation of DTH.

Cyclosporin A (CsA), a fungal metabolite, is a potent immunosuppressive agent that has specific actions on T cells[1]. It is clinically quite useful for prevention of allograft rejection, suppression of graft-*vs*-host disease after allogeneic bone marrow transplantation, and treatment of certain autoimmune diseases[2~4]. Many studies have been made, but still there is some uncertainty about the mechanism by which CsA exerts its effect.

Studies on testicular autoimmunity are significant for a better understanding of human male infertility. Delayed-type hypersensitivity (DTH) has been detected in animals showing experimental autoimmune orchitis (EAO) and is thought to play a key role in the development of the lesion[5~7].

YOSHIDA *et al.*[8] and SAKAMOTO *et al.*[9] have reported that delayed footpad reaction (DFR) against syngeneic testicular cells (TC) was elicited in male mice immunized subcutaneously (sc) with syngeneic viable TC without using adjuvants and the reaction could be augmented by the pretreatment of a high dose of cyclophosphamide (CY). C3H/He mice solely immunized with syngeneic TC developed a slight damage of seminiferous tubules and a moderate degree of EAO was induced by immunization with 100 mg/kg of CY pretreatment[6]. Severer EAO was induced by a booster, reimmunization with TC two weeks later[10]. Therefore, this kind of immunization model might reflect a clinical immunological testis lesions.

In the present study, we have determined the suppressive effect of CsA on DTH to syngeneic TC induced by immunization with viable syngeneic TC. We also tried to induce the tolerance by preimmunization and administration of CsA.

Materials and Methods

Animals

Male mice of inbred C3H/He strain were supplied from the Institute for Animal Experiment, Faculty of Medicine, Kyushu University. Three- to 4-month-old mice were utilized for the experiments.

Antigens

The viable TC of syngeneic mice were used as the immunizing antigen, and for the tolerance experiment, the liver cells of syngeneic mice were used as an example of irrelevant antigens. The testes were removed from mice after complete bleeding and squeezed with a plunger in cold HANKS' balanced salt solution (HBSS). Cell suspensions were passed through a Cell Strainer (Becton Dickinson & Company, New Jersey, U.S.A.) to remove residual large fragments. After washing, the cells were resuspended in HBSS and adjusted to designed concentrations after counting viability by the trypan blue exclusion method. The testicular cell suspension contained 20~30% of sperm and spermatids and 70~80% of round-shaped cells. The liver cells were prepared by the same methods as those used for the preparation of TC.

Treatment with CY

CY (Endoxan, Shionogi & Co., Japan) was dissolved in phosphate buffered saline (PBS) at a concentration of 20 mg/ml and injected intraperitoneally (ip) in a dose of 100 or 200 mg/kg 2 days prior to immunization.

Immunization

Mice were injected sc into the right flank with 1×10^7 of antigenic cells in 0.1 ml of HBSS.

Evaluation of DFR

An eliciting dose of 1×10^6 viable syngeneic TC in a volume of 0.05 ml of HBSS was injected into the sc tissue of the plantar surface of the right hind footpad 6 days after immunization. As a control, 0.05 ml of HBSS was injected into the left footpad. The degree of swelling was measured 24 hours later with a dial thickness gauge (Peacock, Ozaki Mfg. Co. Ltd., Japan). Reactions were expressed as the difference in thickness between the right and left footpads. The results were expressed as the mean $\pm$ S.D. of 5 to 10 animals.

Administration of CsA

CsA (Sandimmun, Sandoz Ltd., Japan) was dissolved in PBS and injected ip in a dose of 1, 2, 5, 10, 30 or 50 mg/kg for 7 consecutive days starting on the day before immunization.

Tolerance Induction

Mice were preimmunized sc with 1×10^7 viable syngeneic TC or liver cells (irrelevant antigen) or not preimmunized and 50 mg/kg of CsA was administered for 7 consecutive days starting on the day before immunization. Then, the mice were given 100 mg/kg of CY on the final day of CsA administration, followed by sc immunization with syngeneic TC 2 days later. DFR was measured 6 days after immunization.

Statistics

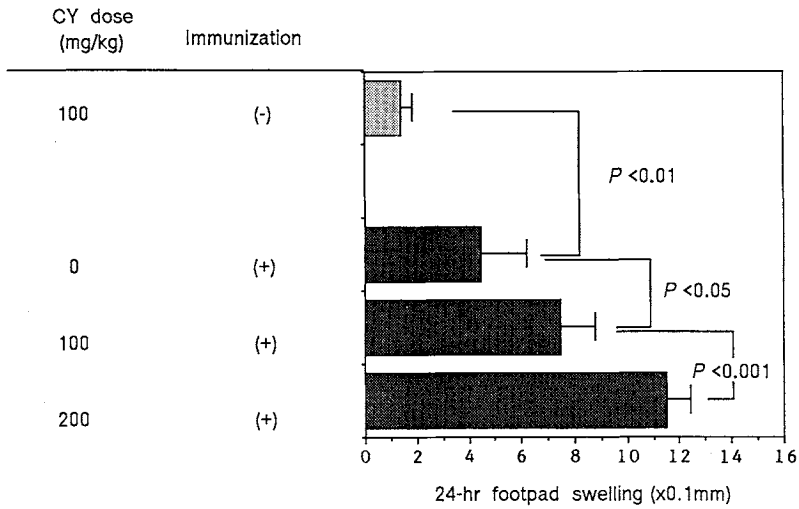
The statistical significance of the data was determined according to STUDENT's *t*-test using the values for footpad swelling. A P value of less than 0.05 was taken as significant.

Results

Induction of DFR by sc Immunization and its Augmentation by CY-pretreatment

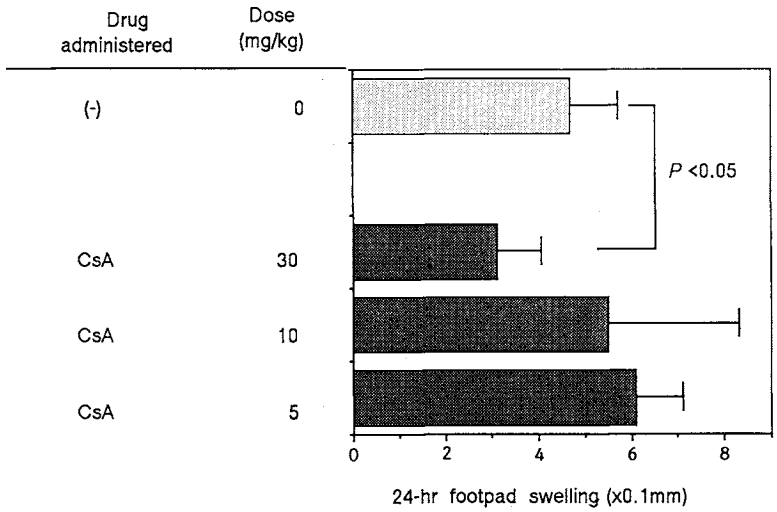
When mice were immunized sc with syngeneic TC, significant DFR was detected ($P<0.01$). When mice were pretreated with CY of 100 or 200 mg/kg, DFR was enhanced significantly in a dose-dependent manner ($P<0.05$ and $P<0.001$, respectively) (Fig. 1).

Fig. 1. Induction of delayed footpad reaction to syngeneic testicular cells (TC) by sc immunization with and without cyclophosphamide (CY)-pretreatment.



Mice were immunized sc with 1×10^7 viable syngeneic TC with or without pretreatment of 100 or 200 mg/kg of CY 2 days before or not immunized at all. Footpad elicitation was carried out with 1×10^6 syngeneic TC 6 days after immunization.

Fig. 2. Dose-dependent suppressive effect of cyclosporin A (CsA) on delayed footpad reaction to syngeneic testicular cells (TC) induced by immunization alone.

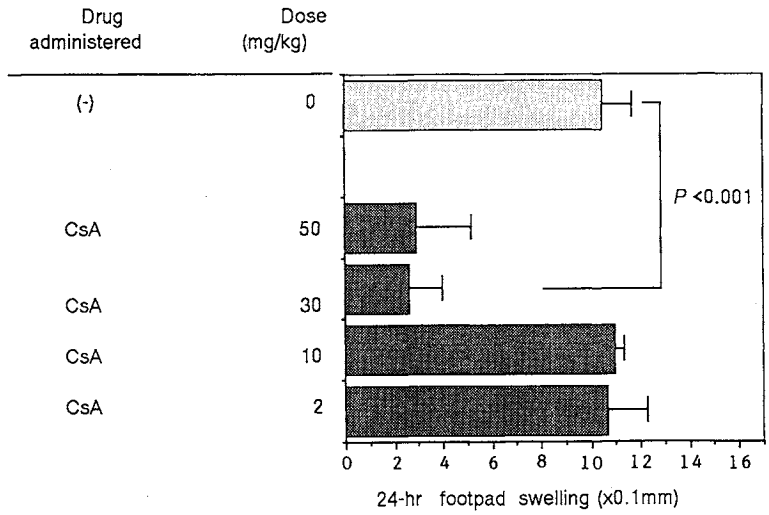


Mice were immunized sc with 1×10^7 viable syngeneic TC without CY-pretreatment. CsA of 5, 10 or 30 mg/kg was administered for 7 consecutive days starting on the day before immunization. Footpad elicitation was carried out with 1×10^6 syngeneic TC 6 days after immunization.

Suppressive Effect of CsA on DFR Induced by Immunization Alone

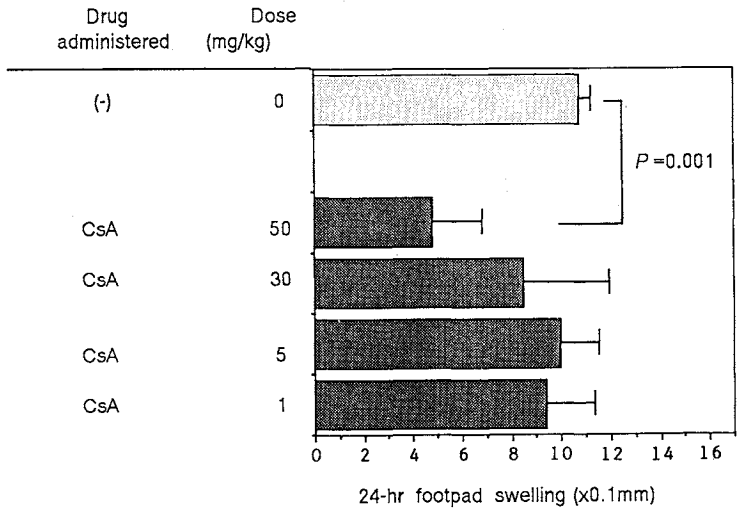
When the immunized mice were injected with 30 mg/kg of CsA for 7 consecutive days, DFR was suppressed significantly ($P<0.05$). However, administration of 10 or 5 mg/kg of CsA did not affect DFR (Fig. 2).

Fig. 3. Dose-dependent suppressive effect of cyclosporin A (CsA) on delayed footpad reaction to syngeneic testicular cells (TC) induced by immunization with 100 mg/kg of cyclophosphamide (CY)-pretreatment.



Mice were immunized sc with 1×10^7 viable syngeneic TC with pretreatment of 100 mg/kg of CY 2 days before. CsA of 2,10, 30 or 50 mg/kg was administered for 7 consecutive days starting on the day before immunization. Footpad elicitation was carried out with 1×10^6 syngeneic TC 6 days after immunization.

Fig. 4. Dose-dependent suppressive effect of cyclosporin A (CsA) on delayed footpad reaction to syngeneic testicular cells (TC) induced by immunization with 200 mg/kg of cyclophosphamide (CY)-pretreatment.

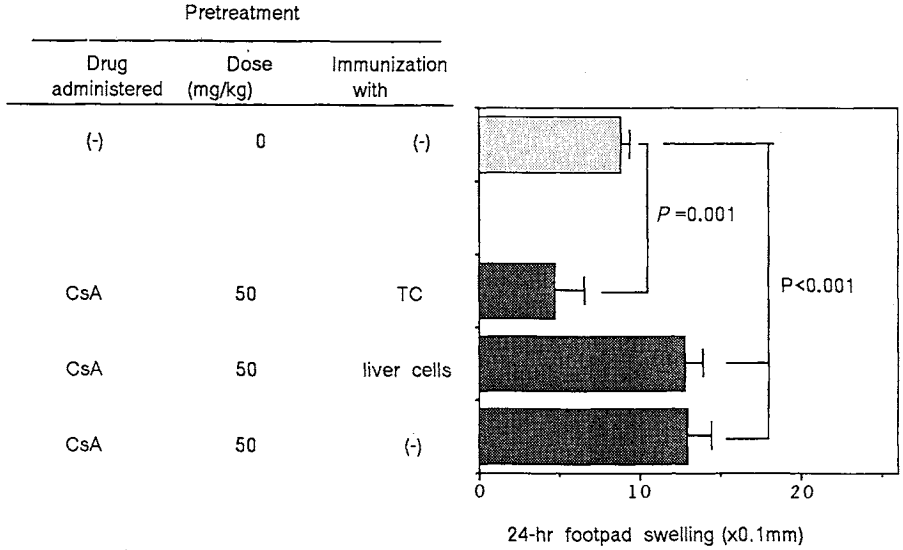


Mice were immunized sc with 1×10^7 viable syngeneic TC with pretreatment of 200 mg/kg of CY 2 days before. CsA of 1, 5, 30 or 50 mg/kg was administered for 7 consecutive days starting on the day before immunization. Footpad elicitation was carried out with 1×10^6 syngeneic TC 6 days after immunization.

Suppressive Effect of CsA on DFR Induced by Immunization with 100 mg/kg of CY-pretreatment

Mice were immunized with 100 mg/kg of CY-pretreatment. Administration of CsA of 50 or 30 mg/kg significantly suppressed DFR ($P<0.001$), however, CsA of 10 or 2 mg/kg were not effective (Fig. 3).

Fig. 5. Immunoregulatory effect of pretreatment with immunization and/or administration of cyclosporin A (CsA) on the induction of delayed footpad reaction to syngeneic testicular cells (TC) by immunization with 100 mg/kg of cyclophosphamide (CY)-pretreatment.



Mice were preimmunized sc with 1×10^7 viable syngeneic TC or liver cells or not preimmunized and 50 mg/kg of CsA was administered for 7 consecutive days starting on the day before preimmunization. Then, the mice were given 100 mg/kg of CY on the final day of CsA administration, followed by sc immunization with syngeneic TC 2 days later. Footpad elicitation was carried out with 1×10^6 syngeneic TC 6 days after immunization.

Suppressive Effect of CsA on DFR Induced by Immunization with 200 mg/kg of CY-pretreatment

When mice were immunized with 200 mg/kg of CY-pretreatment, only administration of 50 mg/kg of CsA suppressed DFR significantly ($P=0.001$). Administration of 30 mg/kg of CsA were not effective on DFR suppression as well as 5 or 1 mg/kg of CsA (Fig. 4).

Tolerance Induction by Pretreatment with Immunization and/or Administration of CsA

Mice were preimmunized with syngeneic TC and 50 mg/kg of CsA was administered for 7 consecutive days. Then, the mice were given 100 mg/kg of CY, followed by immunization with syngeneic TC 2 days later. DFR was suppressed significantly in such-treated mice ($P=0.001$). However, when mice were preimmunized with syngeneic liver cells (irrelevant antigen) or not preimmunized at all and CsA was administered, DFR was rather enhanced significantly in such-treated mice ($P<0.001$) (Fig. 5).

Discussion

Recently, studies on EAO have been advanced thanks to induction of several murine EAO models[10,11]. We have also reported a simple method for inducing the lesion in C3H/He mice by sc immunization with viable syngeneic TC, especially when the mice were pretreated with 100 mg/kg of CY[6]. Many reports imply that DTH plays an important role in the onset and maintenance of orchitis[5,12] In our model, significant DFR was induced and was augmented by a high dose of CY-pretreatment (Fig. 1). We have already shown that this augmentation is due to elimination of CY-sensitive suppressor T cells for DTH[13].

CsA is already widely used for prevention of allograft rejection[2]. CsA is also expected to be very effective in the treatment of organ-specific autoimmune diseases. Hojo *et al.* have reported that EAO can

be abrogated by CsA in guinea pigs[14]. It is quite promising that a pilot study was partly successful in treating autoimmune infertile men[15]. However, there are some questions to this report. First, the authors defined "autoimmune infertility" based on the presence of serum antisperm antibodies (ASA), ignoring the cellular immunity. Second, even if three out of nine successful pregnancies occurred in the CsA-treated patients, successful conceptions with CsA were unrelated to falls in ASA titer. Based on the more significance of cellular immunity than humoral immunity in the research of EAO, and also on the more powerful suppressive effect of CsA on cellular immunity than humoral immunity, it may be logical that discrepancy occurred between successful treatment and ASA titer. Therefore, it seems that more basic studies such as our present one are necessary and significant. We should add, however, that it is very promising for the potential clinical use of CsA that there were very few and minor side effects in the CsA-treated patients compared with the report of some serious side effects of corticosteroids-treated patients[16].

In the present report, DTH to syngeneic TC induced by sc immunization was suppressed by administration of CsA in a dose-dependent manner. When the mice were immunized with syngeneic TC alone or with 100 mg/kg of CY-pretreatment, 30 mg/kg or more of CsA was needed (Figs. 2 and 3). However, when the mice were immunized with pretreatment of 200 mg/kg of CY, 50 mg/kg of CsA was necessary (Fig. 4). Further studies are under way to examine the immunosuppresive effect of CsA on the induction of EAO.

We have tried to induce antigen-specific immune tolerance by immunization and administration of CsA. Pretreatment of mice with immunization and administration of CsA suppressed DTH significantly but not inhibited completely when the mice were challenged with immunization with CY-pretreatment (Fig. 5). However, it was surprising that DTH was rather enhanced significantly by pretreatment of mice without immunization or immunization with the third party antigen and administration of CsA. Our tentative explanation for this phenomenon is that administration of CsA without the relevant antigen might rather eliminate suppressor T cells, even though administration of CsA with the relevant antigen eliminate effector T cells for DTH. In regard to this, CsA caused organ-specific autoimmune disease in mice when the drug was administered daily for 1 week to newborns[17] and CsA treatment elicited diabetes in a diabetes-resistant substrain of BB rats[18]. Currently, studies are under way to elucidate this mechanism by our local passive transfer system[9] and systemic suppression transfer system[13].

Acknowledgments

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