THYMUS AND AUTOIMMUNITY

Transplantation of the Thymus from Cyclosporin A–Treated Mice Causes Organ-specific Autoimmune Disease in Athymic Nude Mice

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The fungal metabolite cyclosporin A (CsA)[1] is a potent immunosuppressant that has specific effects on T cells and can induce permanent acceptance of allografts in certain species (reviewed in reference 1). However, CsA is not only able to induce tolerance to non-self, but also to break tolerance to self. We recently demonstrated that CsA caused organ-specific autoimmune disease in mice when the drug was administered to newborns even for a limited period (Sakaguchi, S., and N. Sakaguchi, submitted for publication).

It was previously shown that various organ-specific autoimmune diseases could be produced in mice by depleting Lyt^+ T cells, including $L3T4^+$ ($CD\text{-}4^+$) T cells as well as $Lyt\text{-}2^+$ ($CD\text{-}8^+$) T cells, leaving $Thy\text{-}1^+$, Lyt^- T cells in the immune system (2). CsA is known to affect thymopoiesis (3, 4). We recently showed that CsA selectively abrogated $L3T4^+$ T cells and $Lyt\text{-}2^+$ T cells in the murine thymus (Sakaguchi, S., and N. Sakaguchi, submitted for publication).

The issues then addressed are: (*a*) whether CsA causes autoimmune disease by primarily affecting the thymus and interfering with the thymic production of certain T cells that are keeping the expansion of self-reactive T cells in check; and (*b*) why administration of CsA to newborns can cause autoimmune disease, but CsA treatment of adult mice can not. We showed in this report that engrafting of the thymus from CsA-treated euthymic (*nu/+*) mice, either newborns or adults, into syngeneic, athymic nude (*nu/nu*) mice produced various organ-specific autoimmune diseases.

Materials and Methods

Mice. BALB/c *nu/+* and *nu/nu* mice (6 wk old) were purchased from Life Science Associates, St. Petersburg, FL. To obtain newborn mice, *nu/+* mice were mated in our animal facility.

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[1] *Abbreviations used in this paper*: CsA, cyclosporin A; IF, immunofluorescence; 00, olive oil; PC, parietal cells.

CsA Treatment. CsA, a gift from Sandoz, Co. Ltd., Basel, Switzerland, was dissolved in olive oil (OO) (Sigma Chemical Co., St. Louis, MO) at a concentration of 5 mg/ml, then further diluted to the appropriate concentrations for intraperitoneal injection at a volume of 50 μl for newborn mice or 100 μl for adult mice.

Transplantation of Thymus into nu/nu Mice. Mice were anesthesized by Nenbutal and the kidney was exposed by retroperitoneal incision; two thymic lobes from neonatally CsA- or OO-treated mice, or one lobe, cut into three to five pieces, from adult CsA- or OO-treated mice were engrafted under the renal capsule of bilateral kidneys with fine forceps.

ELISA for Detection of Autoantibodies. For detecting autoantibodies against the gastric parietal cells (PC), the microsome/plasma membrane fraction of gastric mucosa was prepared according to the method of Mariotti et al. (5). Details of the method were described elsewhere (Sakaguchi, S., and N. Sakaguchi, submitted for publication). Briefly, the fundal portion of BALB/c mice stomachs was homogenized with an Ultraturax TP/18 homogenizer with 4 vol of 0.25 M sucrose, centrifuged at 9,000 *g* for 30 min; the supernatant was centrifuged again at 105,000 *g* for 60 min. The pellet was washed with PBS and solublized with 1.0% Triton X-100 in 0.01 M PBS. The solution was diluted 1:1,000 with PBS (final protein concentration 25 μg/ml) and used for overnight coating of 96-well ELISA plates (Flow Laboratories, Inc., McLean, VA). For detection of anti-thyroglobulin antibodies, ELISA plates were coated overnight with purified mouse thyroglobulins (2 μg/ml). ELISA were performed according to the method of Voller et al. (6). Briefly, the plates were incubated for 1 h at room temperature with the diluted test sera (twofold serial dilutions starting from 1:10 dilution); after washing, they were incubated for 1 h with 1 μg/ml of alkaline phosphatase–conjugated anti–mouse IgG (Sigma Chemical Co.); and the absorbance at 405 nm was measured by an ELISA reader (MR580; Dynatech Laboratories, Inc., Alexandria, VA) after 30-min color development with 1 mg/ml of *p*-nitrophenyl disodium hexahydrate (Sigma Chemical Co.) in 10% diethanolamine buffer, pH 9.8. In this anti-PC autoantibody assay, the absorbance with the 1:10 diluted sera from 3–4-mo-old normal BALB/c mice was 0.07 ± 0.028 (n = 50). The titer of anti-PC autoantibody was, therefore, defined as the highest dilution at which the absorbance was >0.1. There was a good correlation between the titer and specificity of anti-PC antibodies assessed by indirect immunofluorescence (IF) tests with tissue sections and those by ELISA (Sakaguchi, N., and S. Sakaguchi, manuscript in preparation).

Other Methods. Tissue-specific autoantibodies were surveyed by indirect IF test with cryostat sections of normal mouse tissues, as previously described (2). Histology was prepared by paraffin embedding and stained with hematoxylin and eosin.

Results and Discussion

Development of Autoimmune Disease in nu/nu Mice after Engrafting Thymus from CsA-Treated nu/+ Mice. 6-wk-old female *nu/nu* mice were engrafted with either one thymus from 7-d-old female *nu/+* mice treated daily with CsA (10 mg/kg body weight per day) for 1 wk from the day of birth, or one thymic lobe from the adult female *nu/+* mice administered daily with CsA (20 mg/kg per day) for 2 wk. When examined histologically 3 mo later, some of the recipient *nu/nu* mice developed organ-specific autoimmune diseases, such as gastritis, oophoritis, thyroiditis, or insulitis whether CsA-treated donor mice were newborn or adult (Table I). Each autoimmune disease was accompanied by circulating autoantibodies specific for gastric parietal cells, oocytes (zona pellucida and/or ooplasm), thyroglobulin, or cell surface of Langerhans' islet cells, respectively. Histological and serological characteristics of each autoimmune disease were similar to those produced in euthymic mice by neonatal CsA-treatment (Sakaguchi, S., and N. Sakaguchi, submitted for publication) or those induced in *nu/nu* mice by the transfer of T cell subsets from *nu/+* mice (2) (see refer-

TABLE I

Induction of Autoimmune Disease in nu/nu Mice by Engrafting the Thymus from CsA-treated nu/+ Mice

Group	Treatment of thymus donor *nu/+* mice‡	Incidence of autoimmune disease in *nu/nu* mice*			
		Gastritis	Oophoritis	Thyroiditis	Insulitis
A	CsA from day 0 to 6	5/6	2/6	0/6	1/6
B	OO from day 0 to 6	0/6	0/6	0/6	0/6
C	CsA for 2 wk in adult	4/7	4/7	1/7	0/7
D	OO for 2 wk in adult	0/6	0/6	0/6	0/6

* Incidence of autoimmune disease was assessed histologically. See reference 2 for histopathology of each autoimmune disease. See Fig. 1 for the titers of autoantibodies.

‡ BALB/c *nu/+* mice, newborn or adult (6 wk old), were inoculated daily with CsA (10 or 20 mg/kg body weight per day, respectively) or OO from the day of birth (day 0) to day 6 or for 2 wk in adults. Thymus was removed the day after the last inoculation of CsA and transplanted under the kidney capsule of 6-wk-old female *nu/nu* mice. The recipient *nu/nu* mice were killed 3 mo later for examination of histology and check of autoantibodies.

ence 2 for histology and autoantibodies demonstrable by indirect IF; a manuscript is in preparation for pathology of insulitis and anti–islet cell autoantibodies). Three *nu/nu* mice also developed mild systemic vasculitis (data not shown). All *nu/nu* mice transplanted with the thymus showed elevated level of γ-globulins (two to four times higher than the nontransplanted *nu/nu* mice). However, significant difference was not found between the groups transplanted with CsA-treated or OO-treated thymus in terms of serum IgG level, the autoantibody titer against single-stranded DNA, or spontaneous development of antibodies against non-self antigens, such as trinitrophenyl haptens (data not shown). Fig. 1 shows the titer and time course of the development of autoantibodies specific

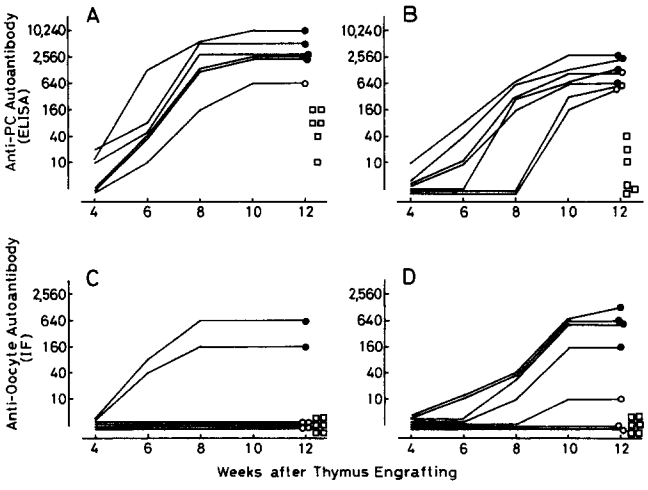

FIGURE 1. BALB/c female athymic *nu/nu* mice (6 wk old) were engrafted with the thymus from the 7-d-old mice treated with CsA from day 0 to 6 (*A* and *C*), or for 2 wk in adult mice (*B* and *D*). Anti-PC autoantibodies were assayed by ELISA (*A* and *B*). Anti-oocyte autoantibodies were assessed by indirect IF (*C* and *D*). (●) Gastritis or oophoritis macroscopically and histologically evident (see reference 2 for details of pathological criteria); (○) intact gastric mucosa or ovaries; (□), control *nu/nu* mice engrafted with the thymus from OO-treated 7-d-old mice (*A* and *C*), or from adult *nu/+* mice treated with OO for 2 wk (*B* and *D*). Histologically, no pathological changes were observed in these groups of mice.

TABLE II

Prevention of Autoimmune Disease in nu/nu Mice by Inoculation of Thymocytes from Normal nu/+ Mice

Group	Treatment of *nu/nu* mice	Incidence of autoimmune diseases in *nu/nu* mice*		
		Gastritis	Oophoritis	Thyroiditis
A	Transplantation of CsA-treated *nu/+* thymus‡	5/6	3/6	0/6
B	Transplantation of CsA-treated *nu/+* thymus plus inoculation of normal *nu/+* thymocytes§	1/6	0/6	0/6
C	Transplantation of CsA-treated *nu/+* thymus plus inoculation of thymocytes from CsA-treated *nu/+* mice‖	4/5	2/5	1/5

* See Table I.

‡ Thymus obtained from 8-wk-old *nu/+* mice treated with CsA (20 mg/kg body weight per day) for 2 wk was transplanted.

§ Thymocyte suspensions (10^8) were intravenously inoculated at the time of thymus transplantation.

‖ Thymocyte suspensions (10^8) were prepared from 8-wk-old *nu/+* mice CsA treated for 2 wk.

for gastric parietal cells or oocytes in the thymus-engrafted *nu/nu* mice. Control *nu/nu* mice transplanted with the thymuses from OO-treated mice did not show histological evidence for autoimmune disease, but some mice developed a low titer of anti-PC autoantibodies (Fig. 1). The mice with histologically evident gastritis or oophoritis generally showed high titer of anti-PC or anti-oocyte autoantibodies, respectively. Some of the recipients of CsA-treated thymus, however, developed high titer of anti-PC autoantibodies of IgG isotype, but little histological lesions. It is likely in these mice that helper T cells for autoantibody production were active, but the activity of effector T cells of cell-mediated immunity might be insufficient to elicit tissue damage, since our previous study (7) on similar organ-specific autoimmune disease induced by early neonatal thymectomy showed the definitive role of cell-mediated immune reactions in destruction of the target organs.

Inoculation of spleen cell suspensions from CsA-treated adult mice did not produce any histological or serological autoimmunity. Transfer of thymocyte cell suspensions from the CsA-treated adult mice, however, induced anti-PC autoantibodies (positive at dilution of 320–640 by ELISA), although histological lesions were not evident.

At the time of sacrifice, the transplanted thymuses were histologically populated with massive lymphocytes. The composition of thymocyte subsets was similar to that of a normal adult thymus. No significant difference was found in the number of peripheral Thy-1^+ cells and the composition of T cell subsets between *nu/nu* mice transplanted with CsA or OO-treated thymus, i.e., Thy-1^+ cells, L3T4^+ cells, and Lyt-2^+ cells in the lymph nodes were, ~50, ~40, and ~10%, respectively.

Prevention of Autoimmune Disease by Inoculation of Thymocytes. When thymocytes were inoculated immediately after transplantation of CsA-treated thymus, thymocyte suspensions (10^8) from normal *nu/+* mice could suppress the devel-

TABLE III

Prevention of Autoimmune Disease in nu/nu Mice by Transplanting the Thymus from Normal nu/+ Mice

Group	Treatment of *nu/nu* mice	Incidence of autoimmune disease in *nu/nu* mice*		
		Gastritis	Oophoritis	Thyroiditis
A	Transplantation of CsA-treated *nu/+* thymus plus normal *nu/+* thymus‡	0/6	0/6	0/6
B	Transplantation of CsA-treated *nu/+* thymus and transplantation of normal *nu/+* thymus 2 wk later§	3/6	2/6	0/6

* See legend to Table I.

‡ Thymus from the CsA-treated adult *nu/+* mice (8 wk old) was transplanted in the right kidney, and the thymus from normal adult *nu/+* mice (8–10 wk old) was transplanted in the left kidney.

§ Thymus from the CsA-treated adult *nu/+* mice was transplanted in the right kidney, and normal *nu/+* thymus was transplanted in the left kidney 2 wk later.

opment of autoimmune disease, while those from CsA-treated *nu/+* mice could not (Table II). When the composition of thymocyte subsets was examined by two-color cytofluorometric analysis, $L3T4^+$, $Lyt\text{-}2^-$ and $L3T4^-$,$Lyt\text{-}2^+$ thymocytes were found to be absent from the thymus of CsA-treated mice (Sakaguchi, S., and N. Sakaguchi, submitted for publication). Thus, it is highly likely that CsA caused autoimmune disease by interfering selectively with the thymic production of certain suppressor T cells controlling self-reactive T cells.

Prevention of Autoimmune Disease by Transplanting Normal Thymus with CsA-Treated Thymus. Cotransplantation of the thymus from the normal adult *nu/+* mice with the thymus from CsA-treated mice prevented autoimmune disease; however, transplantation of normal thymus 2 wk after engrafting CsA-treated thymus could not (Table III). We suggested earlier (8) that suppressor T cells are regulating the early stage of proliferation/differentiation of effector T cells, but they can not suppress the T cells engaging in ongoing effector functions. It is plausible that a number of effector precursors generated through the CsA-treated thymus in an unchecked manner might have differentiated by 2 wk into active effector T cells, which the suppressor T cells in the normal thymus transplanted 2 wk later were unable to control. Furthermore, the result suggests that depletion of suppressor T cells in the thymus does not need to be permanent to induce autoimmune disease. When suppressor T cells eventually recover in the CsA-treated thymus, they seem to be incapable of controlling the self-reactive T cells that have proliferated/differentiated by then into effector T cells of autoimmune disease.

Prevention of Autoimmune Disease by Inoculation of Spleen Cells from Adult nu/+ Mice, but Not from nu/+ Newborns. Inoculation of spleen cell suspensions (4×10^7 nucleated cells with 25–30% of $Thy\text{-}1^+$ cells) prepared from normal, 10–12-wk-old *nu/+* mice immediately after engrafting the thymus from CsA-treated *nu/+* mice completely inhibited the occurrence of autoimmune disease (Table IV). In contrast, transfer of spleen cell suspensions (4×10^7 nucleated cells with $<5\%$ of $Thy\text{-}1^+$ cells) prepared from 1–3-d-old *nu/+* or *+/+* newborn mice

TABLE IV

Prevention of Autoimmune Disease in nu/nu Mice by Inoculation of Spleen Cell Suspensions from nu/+ Mice

Group	Treatment of *nu/nu* mice	Incidence of autoimmune disases in *nu/nu* mice*		
		Gastritis	Oophoritis	Thyroiditis
A	Transplantation of CsA-treated *nu/+* thymus‡	4/5	3/5	0/5
B	Transplantation of CsA-treated *nu/+* thymus plus inoculation of spleen cells from adult *nu/+* mice§	0/6	0/6	0/6
C	Transplantation of CsA-thymus plus inoculation of spleen cells from newborn *nu/+* mice‖	5/5	3/5	0/5

* See Table I.

‡ Thymus obtained from 8-wk-old *nu/+* mice treated with CsA (20 mg/kg body weight per day) for 2 wk was transplanted.

§ Spleen cell suspensions (4×10^7) from 10–12-wk-old *nu/+* mice were intravenously inoculated immediately after transplanting the thymus from the CsA-treated adult *nu/+* mice.

‖ Spleen cell suspension (4×10^7) from 1–3-d-old *nu/+* mice were inoculated immediately after transplanting the thymus from CsA-treated adult *nu/+* mice.

could not prevent the disease. In the previous study, we demonstrated that CsA could deplete thymocytes irrespective of the age of mice. However, administration of CsA to euthymic mice caused organ-specific autoimmune diseases only when the drug was administered to newborns; CsA treatment of adult mice failed to induce autoimmune disease even with higher doses of CsA and longer treatment periods (Sakaguchi, S., and N. Sakaguchi, submitted for publication). These results taken together suggest the following: (*a*) normal thymus is continuously producing potential self-reactive (autoimmune) clones of T cells as well as suppressor T cells, the latter dominantly controlling the former; (*b*) CsA can selectively abrogate the thymic production of such suppressor T cells in mice at any age, allowing unchecked production of self-reactive T cells through the thymus; and (*c*) however, for self-reactive T cells to proliferate/differentiate into effector T cells of autoimmune disease, active suppressor T cells must be absent in the periphery or have not yet migrated to the periphery. Thus, engrafting of the thymus from CsA-treated mice into T cell–deficient *nu/nu* mice or CsA administration to euthymic newborn mice can cause autoimmune disease, but CsA administration to euthymic adult mice can not.

Summary

Organ-specific autoimmune diseases such as gastritis, oophoritis, thyroiditis, or insulitis developed in athymic *nu/nu* mice after engraftment of the thymus from euthymic *nu/+* mice treated with cyclosporin A (CsA), a potent immunosuppressant. The development of autoimmune disease in the *nu/nu* mice was prevented by inoculation of thymocyte suspensions prepared from normal *nu/+* mice, but not by thymocyte suspensions from CsA-treated *nu/+* mice. Cotransplantation of normal *nu/+* mouse thymus with CsA-treated thymus also suppressed the development of autoimmune disease. Inoculation of spleen cell

suspensions prepared from normal adult *nu/+* mice prevented autoimmune disease, but inoculation of those from newborn *nu/+* mice did not. Thus, CsA appears to interfere selectively with the thymic production of certain suppressor T cells controlling self-reactive (autoimmune) T cells, allowing the latter to expand and cause autoimmune disease.

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