CYCLOSPORINE-INDUCED AUTOIMMUNITY.

Conditions for Expressing Disease, Requirement for Intact Thymus, and Potency Estimates of Autoimmune Lymphocytes in Drug-Treated Rats

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Originally discovered as a fungal extract (1), cyclosporine A (CSA)[1] has proven to be an important and interesting drug for immunologists in both clinical and experimental settings (2). Recipients of allografted tissues and cells treated over a limited period with CSA show a profound degree of graft survival (3–6). Used both experimentally in several animal models (7–13) and in some clinical situations (14–16) in humans, CSA has been shown to be highly effective in preventing or reversing several autoimmune diseases. Considerable effort over the past decade has lead to the accumulation of much information about CSA, but at present its mechanism of action on the immune system remains largely unknown, and some enigmas remain.

As an example of one such enigma, CSA is known to be a potent suppressor of acute allogeneic GVHD in rodent models (17). Nevertheless, recent studies of Glazier et al. (18) have produced the unexpected finding that irradiated rats, reconstituted with syngeneic or autologous marrow and maintained on CSA for 6 wk, develop a fatal autoimmune syndrome soon after the drug is discontinued. This autoimmune disease shows the same histopathologic features as allogeneic GVHD, is mediated by T cells, and can be transferred adoptively to secondary recipients (18).

In the present study, we confirm the unusual findings of Glazier et al. (18) concerning CSA-induced autoimmunity in Lewis rats. In addition, (a) we explore the role of the thymus in this drug-induced disease process; (b) we demonstrate that adoptive transfers of this disease to secondary recipients can be accomplished with relatively few T cells from autoimmune donors, and that onset of disease from such transfers can be blocked by cotransfers of lymphoid cells from normal donors; and (c) we show that the potencies of syngeneic and allogeneic reactivity of cells from autoimmune donors are similar in popliteal lymph node assays. Thus, aside from the potent suppressive effect CSA exerts on the immune system in some circumstances, these data demonstrate that prolonged treatment with CSA can lead to profound T cell–mediated autoimmunity when the drug is

[1] *Abbreviations used in this paper:* BM, bone marrow; CSA, cyclosporine A; Tx, thymectomy.

TABLE I

CSA-induced Autoimmunity

Treatment	Mortality (dead/total) and survival time (days)			
	+CSA*		−CSA	
x-irradiation (1000 rad), BM (3.0–5.0 × 10⁷)	11/11	18, 19 (3 animals), 20, 21 (3 animals), 24, 25, 30	1/7	27, >100 (6 animals)
x-irradiation (750 rad)	5/6	25, 27, 28, 30, 46	0/6	>100 (6 animals)

* 7.5 mg/kg/d for 40 d.

discontinued. This may provide the basis for a useful animal model for more definitive studies of the cellular events involved in the onset and inhibition of at least some autoimmune diseases.

Materials and Methods

Rats. Lewis (L, RT1^1) and (L × DA)F$_1$ hybrids (RT1$^{1/a}$) rats were purchased from The Trudeau Institute (Saranac Lake, NY). These animals were used at ~8 wk of age. Where required, they were irradiated at 83 rad/min (Philadelphia) or 133 rad/min (La Jolla). Thymectomies were performed on adult animals using standard procedures (19, 20).

Autoimmune rats were prepared as described by Glazier et al. (18). This included irradiation (1,000–1,050 rad), marrow reconstitution (2.0–5.0 × 10⁷ cells) and CSA treatment (7.5 mg/kg/d, diluted in olive oil) subcutaneously for 6 wk. Cells were obtained from these animals for transfer studies 10 d to 2 wk after CSA was discontinued. The authors are grateful to Sandoz, Inc. (East Hanover, NJ) for supplies of CSA.

Cells. Suspensions of lymph node cells were prepared according to standard procedures (21).

Antibodies. Monoclonal antibodies that identify various lymphocyte subsets in rats were kind gifts of Dr. D. W. Mason, Medical Research Council Cellular Immunology Unit (Oxford, UK), or were purchased from Accurate Chemical and Scientific Corp. (Westbury, NY). These included W3/25 for Th cells (22). OX8 for Tk/s cells (23), and Ox12, Igk (24). Fluorescein-conjugated rabbit F(ab')$_2$ anti–mouse Ig and affinity-purified sheep F(ab')$_2$ anti–mouse Ig antibodies were purchased from Cappel Laboratories (Cochranville, PA). These latter two exhibit some crossreactivity on rat Ig; this was minimized by using 5% rat serum in the binding reactions.

Cell Fractionation. Lymph node cells were depleted of selected subpopulations using modifications (25) of rosetting procedures described by Parish and Hayward (26), and by Mason (27). Contamination of purified subpopulations was monitored by FACS analysis (25).

Local GVH Reactions. These were measured by the quantitative popliteal lymph node weight assay (28).

Results

CSA-induced Autoimmunity. Results of the first series of experiments (Table I) confirmed in a striking way the report of Glazier et al. (18). Lewis rats, 8 wk of age, lethally irradiated (1,000 rad), reconstituted with marrow (5.0 × 10⁷ cells), and given daily subcutaneous inoculations of CSA (7.5 mg/kg) for 6 wk, developed a fatal syndrome very similar in appearance to acute GVHD. These animals developed erythroderma of the ears, eyelids, and feet; they had swollen digits, diarrhea, raised fur, and a hunched appearance soon (8–10 d) after the 6-wk

TABLE II

CSA-induced Autoimmunity: Effects of Thymectomy

Group	Treatment	Mortality (dead/total) +CSA	Mortality (dead/total) −CSA	Survival time (d)
I	Sham Tx (day 1), x-irradiation, BM, ±CSA*	5/5	0/3	18–30
II	Tx (day 1) x-irradiation, BM, ±CSA	1/12	0/5	—
III	x-irradiation, BM, ±CSA, Tx (day 14)	3/5		25–60 (all animals showed disease)
IV	x-irradiation, BM, ±CSA, Tx (day 28)	5/5		25–60
V	x-irradiation, BM, ±CSA, Tx (day 42)	5/5	0/5	25–60
VI	x-irradiation, BM, ±CSA, sham Tx (day 42)	5/5	0/4	18–30

* In each case, x-irradiation was 1,000 rad; 3.0×10^7 BM cells were transferred; and CSA was given at 7.5 mg/kg/d for 42 days, as indicated.

course of CSA injections was discontinued. Later (14–21 d), they displayed extensive weight loss and partial alopecia. In experiments conducted with the rat colony maintained in Philadelphia, death routinely occurred within a month, usually early in the third week.

Table I also shows that rats given sublethal irradiation (750 rad) without marrow reconstitution develop lethal autoimmune disease after discontinuing CSA treatment, although somewhat more slowly.

In experiments conducted more recently in La Jolla (with rats from the same vendor), there are some marked differences in the timing and expression of these disease symptoms (data not shown). The early features noted above occur in all animals (70 to date), and 70% of the animals die within a 6-wk period. The remaining 30% of animals develop total alopecia, appear to pass a crisis point in terms of their vitality, and then remain quite healthy, except for total hair loss for several months, at which time this study was discontinued. Evidently, the time of death and some of the symptoms of autoimmune disease are determined by various conditions prevailing in different animal colonies. Thus it may be somewhat difficult to compare results from different laboratories with endpoints based on mortality, but it does seem clear that the early classic symptoms (erythroderma of the ears, dermatitis, diarrhea, etc.) of acute systemic GVHD in adult animals occur routinely and reliably within the first 2 wk after CSA treatment is discontinued.

Effects of Thymectomy. Table II shows the results of a study designed to explore the thymus dependency of CSA-induced autoimmunity. All groups were treated the same: irradiation (1,000 rad), marrow reconstitution, with and without CSA treatment daily for 6 wk; the various groups differ, however, with respect to the timing of thymectomy. Rats thymectomized immediately before irradiation and CSA treatment (group II) do not develop any symptoms whatsoever of autoimmune disease, but rats thymectomized 2 wk (group III), 4 wk (group IV), or 6 wk (on the day that CSA is discontinued, group V) develop disease, although it is somewhat delayed in terms of onset and time of death.

These results imply that the CSA-induced autoimmune cell population originates in the thymus, and moreover, that these cells mature and exit the thymus during the course of CSA treatment.

Source of Autoimmune Cell Precursors. Results of the previous study provide the basis for an assay to assess the presence of CSA-inducible autoimmune

TABLE III

CSA-induced Autoimmunity: Host Modifications

Group	Treatment	Mortality (dead/total)	
		+CSA	–CSA
I	Tx, x-irradiated, BM*	0/8	0/5
	Sham Tx, x-irradiated, BM	4/4	
II	Tx, x-irradiated, BM, thoracic duct lymphocytes (5.0 × 10⁸)	0/5	
	Tx, x-irradiated, BM, spleen and lymph node cells (5.0 × 10⁸)	0/6	0/5
	Tx, x-irradiated, BM, thymocytes (9.0 × 10⁸ i.v. + 9.0 × 10⁸ i.p.)	0/5	
	Tx, x-irradiated, BM, thymus lobes (three per left axilla)	4/5	
	Sham Tx, x-irradiated, BM, thoracic duct lymphocytes (5.0 × 10⁸)	3/3	

Deaths occurred in 18–30 d.
* In each case, x-irradiation was 1,000 rad; 3.0–5.0 × 10⁷ BM cells were transferred; and CSA was given at 7.5 mg/kg/d, as indicated.

precursors from various sources. Animals lacking a thymus during CSA treatment fail to develop autoimmune disease. Thus, in the experiment shown in Table III, thymectomized, irradiated, and marrow-reconstituted animals were also given lymphoid cells from various sources or grafted in the axilla with intact thymus lobes from newborn syngeneic donors, and then treated with CSA as before; only the animals given intact thymus lobes developed the disease. These results imply that the cell population that develops autoreactivity during CSA treatment (*a*) is either absent or present in insufficient numbers both in the peripheral lymphoid pool and in normal thymocyte populations; and (*b*) requires some element of an intact thymus not present in thymocyte suspensions. Evidently, the autoreactive population arises from precursors that traffic to the postirradiated or engrafted newborn thymus, and their subsequent maturation in that organ is influenced by CSA.

Adoptive Transfer of CSA-induced Autoimmunity. The data in Fig. 1 provide a comparison of various cell populations obtained from CSA-induced autoimmune donors in transferring this disease adoptively to irradiated secondary recipients. These transfers involved various numbers of whole spleen and lymph node cells, T cells (negatively depleted by rosetting procedures of class I–positive and Ig⁺ cells), Th cells (depleted of class II–positive, Ig⁺, and Tk/s cells), Tk/s cells (depleted of class II–positive, Ig⁺, and Th cells), and B cells depleted of Th and Tk/s cells. These cell populations were obtained from the primary autoimmune donors 14–21 d after CSA treatment was stopped.

Several points seem clear from these data. First there is a dose dependence in the transfer of the disease. Animals receiving large numbers of whole spleen and lymph node cell populations die within 3 wk. However, transfers of as few as 10⁶ cells are also effective, although the recipients survive longer (4–6 wk). Second, populations enriched for both Th and Tk cells or Th cells only are as effective quantitatively as unseparated populations, and B cell–enriched and Tk/s-enriched populations are much less effective. The marginal effectiveness of the B cell population most likely reflects its contamination with T cells; as few as 0.3 ×
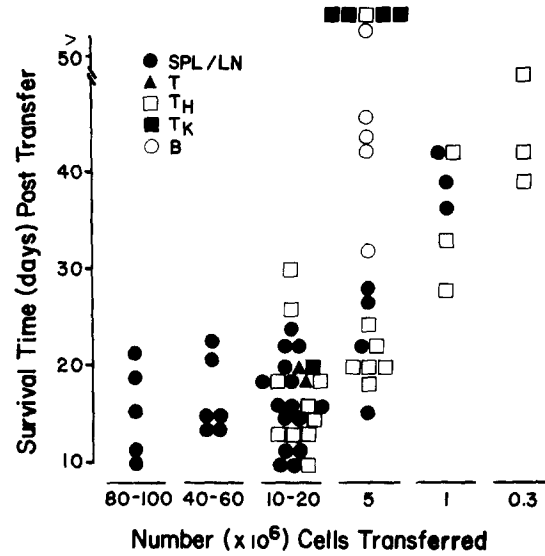
FIGURE 1. Adoptive transfer of lethal autoimmune disease with various numbers of unseparated spleen and lymph node cells (●), T cells depleted of OX12⁺ cells (▲), Th cells depleted of OX8⁺ and OX12⁺ cells (□), Tk/s cells depleted of W3/25⁺ and OX12⁺ cells (■), and B cells depleted of W3/25⁺ and OX8⁺ cells (O). All the cell populations were monitored by FACS analysis for contamination by depleted cell subsets; this was usually <2% and did not exceed 4%.

TABLE IV

CSA-induced Autoimmunity: Inhibition of Adoptive Transfer with Normal SPL/LN

Cells transferred (×10⁶)		Survival time of secondary recipients (d)
Normal donor	CSA donor	
100		>100 (3 animals)
	10	10, 12, 14, 18, 31
100	10	>100 (6 animals)

10^6 Th cells are effective, and this could represent a 6% contamination of the B cell preparation.

Inhibition of Autoimmunity by Cotransfer with Normal Lymphocytes. Table IV shows that cotransfers of 10-fold excess of spleen and lymph node cells from normal donors effectively block the onset of autoimmune disease following adoptive transfer of 10^7 cells from autoimmune donors. Thus, if autoimmune donors contain a significant number of T cells (Fig. 1), it appears that spleen and lymph node cell populations from normal donors contain a substantial number of some cell type that inhibits this syngeneic immune reactivity.

Comparison of Syngeneic and Alloreactivity. The finding that a lethal autoimmune reactivity can be adoptively transferred with relatively few T cells (Fig. 1) implies that the relevant cell involved must be a prevalent one. In Table V, we compare the potency of syngeneic and alloreactivity of cells form autoimmune donors in quantitative popliteal lymph node assays. While cells from control, oil-

TABLE V

CSA-induced Autoimmunity: Auto- and Alloreactivity of Lymphocytes from CSA-treated Lewis Donors

Group	Donor/recipient combination*	Donors (n)	Recipients (n)	Popliteal lymph node weights (mg)
I	L normal → L	5	14	4.6 ± 1.6
II	L oil → L	5	15	6.0 ± 1.9
III	L CSA → L	9	26	19.4 ± 7.1
IV	L normal → L/DA	4	10	69.5 ± 38.2
V	L oil → L/DA	5	13	33.8 ± 14.5
VI	L CSA → L/DA	6	13	25.5 ± 9.8

* 10^7 donor cells injected per recipient footpad; donors in groups II, III, V, and VI were irradiated (1,050 rad), marrow reconstituted, and given CSA 7.5 mg/kg in olive oil, or olive oil only daily for 6 wk. Donor cells were obtained 10–14 d after CSA treatment was discontinued.

treated donors show no syngeneic reactivity (6.0 ± 1.9 mg), they show substantial alloreactivity (33.8 ± 14.5 mg) in L/DA recipients. By comparison, cells from CSA-treated donors show elevated reactivity in both syngeneic (19.4 ± 7.1 mg) and allogeneic (25.5 ± 9.8 mg) recipients of similar magnitude.

Discussion

These studies explore further the somewhat unexpected and enigmatic finding (18) that when irradiated, marrow-reconstituted rats are given daily maintenance doses for 6 wk of CSA, a drug usually used to suppress alloreactivity and some autoimmune conditions (3–17), they develop an acute, usually fatal, autoimmune syndrome soon after the drug is discontinued. We report here several findings concerning the onset of the CSA-induced autoimmunity. First, it does not require lethal irradiation and marrow reconstitution; sublethal irradiation and drug treatment is also effective (Table I). This finding rules out any possibility of genetic heterogeneity in the colony as a contributing factor, i.e., GVHD based on inadvertent antigenic disparity between bone marrow donor and irradiated host.

Second, the thymectomy experiments clearly indicate that development of CSA-induced autoreactivity requires the presence of a radioresistant thymic component (Table II, group I vs. group II) present also in intact thymus lobes from newborn donors (Table III). Autoimmune precursors appear in the postirradiated thymus, mature, then exit within 2 wk to the peripheral lymphocyte pool during the course of CSA treatment (Table II, group III). There, present in significant numbers, they evidently remain dormant in the presence of CSA until the drug is discontinued, at which time they become activated, probably by self-MHC class II molecules (29), and then they initiate an as yet unknown cascade of events leading to fatal autoimmune disease.

Just how CSA alters event in the thymus leading to appearance of autoimmune precursors, or to the failure to delete them, is not clear, and this remains a key question in this interesting model. Cheney and Sprent (30) have suggested that

CSA inhibits the expression of class II molecules on macrophages and/or dendritic cells in the thymus, thereby interfering with the normal process of self-tolerance. The normal elimination of self-reactive clones fails to occur, allowing the production of a significant population of nontolerant T cells that peripheralize and eventually cause disease. Although there are some attractive features to this model, it may not be correct. It fails to account for the finding that autoimmunity does not develop in CSA-treated animals if the thymus is shielded during irradiation (18, 31).

Cheney and Sprent (30) do indicate, however, that while normal, nonirradiated animals treated with CSA fail to show any symptoms of autoimmunity, cells from these donors are able to cause fatal disease when adoptively transferred to irradiated secondary recipients. Evidently, autoimmune precursors can develop in CSA-treated animals without irradiation, but the activity of these cells seems to be held in check, perhaps by some kind of suppressor population.

Third, the findings in Table III deal with the question of whether CSA-inducible autoimmune precursors occur in the normal peripheral lymphocyte pool or in suspensions of normal thymus cells. Animals thymectomized (and irradiated) before CSA exposure show no evidence of autoimmunity whatsoever. Reconstitution with thymocyte suspensions from three to four donors, splenic and lymph node cells from two donors, or thoracic duct lymphocytes drained from two donors fails to reveal the presence of CSA-inducible autoimmune precursors in these populations.

Fourth, although the thymectomy studies (Tables II and III) indicate the importance of the thymus in CSA-induced autoimmunity, adoptive transfer studies (Fig. 1) using enriched T or B cell populations from autoimmune donors indicate the involvement of T cells, particularly those of the helper subset, in the effector phases of this disease. In view of the effectiveness of rather small numbers of cells ($0.3-1 \times 10^6$) in transferring this disease, the frequency of autoreactive cells in CSA-treated animals must be considered to be quite high.

Fifth, adoptive cotransfer experiments with lymphocytes from normal and autoimmune donors indicate the presence of some cell type among normal lymphocyte populations that effectively inhibits the transfer of autoreactivity in secondary recipients. If the frequency of cells with autoreactivity is high (see above), the proportion of suppressive cells present in the normal lymphocyte pool that block autoreactivity must also be considered to be significant. Preliminary experiments currently in progress indicate that this suppressive cell in normal lymph node suspensions is a T cell (Wilson, D. B., D. H. Wilson, K. Schroder, and H. Kimura, unpublished data), but we cannot yet determine whether it belongs to the Th or Tk/s subset.

Finally, Table V provides a comparison of auto- and alloreactivity of cells in CSA-treated animals. Lymphocytes from autoimmune donors provoke enlargement of popliteal lymph nodes in quantitative local GVH assays in syngeneic and F_1 recipients that are of the same magnitude (group III vs. group VI). Evidently, cells with antiself reactivity are as prevalent as those with alloreactivity in autoimmune animals.

There are marked similarities in the CSA-induced autoimmunity model and in the systemic GVHD model. Both involve T cells of the $W3/25^+$ subset, and

they display very similar histopathologic features (22). It is therefore tempting to consider the possibility that they share common underlying mechanisms. If this is so, then some of our current understanding of the details of the interaction of MHC gene products with anti–MHC receptors of alloreactive T cells in GVHD may be directly applicable to consideration of the interaction of self MHC gene products with autoreactive T cells in CSA-induced autoimmunity.

Previous studies with the GVH model in rats have generated several key findings that may be pertinent to consideration of the mechanisms of CSA-induced autoimmunity and self-tolerance. First, anti–MHC receptors of alloreactive T cells are themselves potent immunogens. Nonirradiated A/B F_1 rats immunized with 3.0×10^7 A-strain T cells recover from very slight GVH reactions and become specifically resistant to GVHD caused by further inoculation with A-strain T cells, but they remain vulnerable to GVHD caused by B-strain T cells (32, 33).[2] The immunogenic markers appear to be anti–MHC receptors on T cell clones having a particular allospecificity.

Second, immune A/B animals, resistant to anti-MHC^b alloreactivity, remain susceptible to anti-MHC^a GVH reactions. Thus, anti-MHC T cell receptors seem idiotypically diverse. But A/B F_1 rats immunized with A-strain T cells, and thus resistant to A anti-b alloreactivity, also resist anti-b reactivity by T cells from third-party rat strains. Surprisingly, therefore, the idiotypic markers of T cell receptors specific for a particular MHC antigen (e.g., MHC^b) behave as if they were monomorphic in different strains (34).

Third, the GVH resistance mechanism is mediated by F_1 T cells; it can be adoptively transferred to secondary F_1 recipients (32), and these same transferred cell populations contain cytotoxic T cells that can be stimulated to lyse anti-MHC^b blasts from any strain, but not anti-MHC^b blasts nor cells of other specifications (35).

These findings with the GVH model may relate to the autoimmune model in two ways. First, as discussed above, some portion of receptors for MHC alloantigens appears to be highly conserved in different rat strains; it follows that crossreactive idiotypic determinants expressed on anti–MHC^b receptors of alloreactive T cells from strains A, C, D, . . . , may also be expressed on self-reactive T cells of autoimmune (e.g., strain B) rats, and that these receptors may be immunogenic in the appropriate setting.

The second feature of T cell receptors in the GVH model relevant to the autoimmune model is pertinent to the preceeding argument; it concerns the surprising ease and rapidity with which parental T cells can induce GVH resistance in F_1 animals. F_1 rats immunized with parental T cells and then irradiated (750 rads), even as soon as 1 d later, demonstrate specific GVH resistance to subsequent challenge with T cells from the same parental strain. This finding suggests that a normal A/B F_1 rat already has clones of T cells with specificity for anti–MHC^b receptors on donor A anti-b T cells. Evidently these can be rapidly primed; they behave as a population already expressing immunologic memory. If this is so, what is the internal antigen present in the A/B F_1 that drives them? It seems likely that these internal "self" antigens are the

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[2] For simplicity, we refer to various involved strains as A, B, C, etc. having different MHC haplotypes a, b, c, etc.

receptor molecules present on anti–self-MHC^b–reactive T cells in the F_1 rat (36); the same cells that are somehow deregulated in the CSA-treated autoimmune rat. Our current studies are directed at this issue.

Summary

These studies explore the phenomenon of cyclosporine-induced autoimmunity in irradiated Lewis rats. We show that (a) the presence of a thymus is required, and autoimmune precursors develop in and exit from this organ to the peripheral lymphocyte pool within a 2-wk period after the initiation of cyclosporine treatment; (b) adoptive transfers of drug-induced autoimmunity to irradiated secondary recipients can be accomplished with relatively few cells of the Th subset, and these transfers of autoimmunity can be blocked by cotransfer of normal lymphoid cells; and (c) potency estimates, using popliteal lymph node assays in syngeneic and F_1 recipients indicate similar levels of auto- and alloreactivity by cells from drug-induced autoimmune donors. These various findings indicate that this particular animal model may be useful for studies of the onset and control of autoimmunity, and they raise the possibility that the lack of autoimmunity in normal animals and its induction with cyclosporine may involve similar cellular mechanism as have been found to be operative in GVH reactions and specifically induced immunologic resistance to GVHD.

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