Brief Definitive Report

An In Vitro Model for Cyclosporin A–induced Interference of Intrathymic Clonal Elimination

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Summary

The effects of cyclosporin A (CsA) on influencing the intrathymic clonal deletion were investigated by using our established thymic stromal cell clone with capacities to express Ia antigens and to produce a unique T cell growth factor. The following were revealed: (*a*) T cell clone with a given specificity was killed on the Ia^+ stromal cell monolayer in the presence of the relevant antigens, a process depending on T cell receptor (TCR) stimulation; and (*b*) CsA allowed the T cell clone to continuously proliferate even during TCR stimulation by virtue of the stromal cell–derived T cell growth factor. This paper describes an in vitro model of a mechanism by which CsA is responsible for the generation of normally "forbidden" T cell clones.

Cyclosporin A (CsA) is a powerful immunosuppressive drug that is widely used in alleviating allograft rejection by inhibiting lymphokine production of Th cells (1). CsA is also effective in preventing GVHD secondary to allogeneic marrow transplantation (2), as well as several autoimmune diseases (3, 4). Paradoxically, irradiated hosts transplanted with syngeneic or autologous bone marrow and then treated with, and withdrawn from, CsA develop autoimmunity as characterized by a GVHD-like syndrome (5–8). These studies have suggested that CsA interferes with the processes of intrathymic T cell development, allowing the escape of autoreactive T cells (6).

There have been advances in the delineation of roles of the thymic microenvironment in T cell maturation and repertoire selection (9, 10). We have also established a thymic stromal cell clone MRL104.8a (11) exhibiting two major features: potential to express class I and class II H-2 antigens and production of a unique T cell growth factor designated as thymic stroma–derived T cell growth factor (TSTGF) (11–13). We observed that: (*a*) MRL104.8a monolayer can promote the growth of antigen-specific Th clones by producing the TSTGF when the relevant antigen is absent in cultures; and (*b*) lethal growth inhibition of a Th clone is induced when its TCRs are activated by adding the relevant antigen to the Ia-expressing monolayer (14). Thus, the dual function of this thymic stromal cell clone represented a model for selecting the T cell repertoire in the thymus that depends on whether the antigen receptor for each T cell clone is triggered. This also permitted us to directly examine the effect of CsA on the interaction between T lymphocytes and thymic stromal cells.

Materials and Methods

Thymic Stroma–derived Cell Clone. A thymic stroma–derived cell clone (MRL104.8a) that has been recently established from MRL-*lpr/lpr* mouse thymuses (11) was used.

T Cell Clones. KLH-specific, I-E^k-restricted Th clone 9-16 and allo-I-E^k-reactive Th clone 2-13 were kindly provided by Dr. Y. Asano (Tokyo University School of Medicine, Tokyo, Japan) and Dr. M. Kimoto (Saga Medical School, Saga, Japan), respectively.

Reagents and mAbs. Recombinant murine IFN-γ was provided by Shionogi Pharmaceutical Co. Ltd., Osaka, Japan. The supernatant containing TSTGF was obtained from cultures of the MRL104.8a thymic stromal cell clone. A semipurified sample of TSTGF that was free of activities for IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, and CSFs was prepared as described (11, 12). CsA was from Sandoz Pharmaceuticals Division (Sandoz Inc., Basel, Switzerland). 5 mg CsA was dissolved in 0.4 ml of ethanol and 0.1 ml of Tween 80, and RPMI 1640 medium was added to give a total volume of 1 ml. This stock solution was further diluted with the complete culture medium.

Gamma globulin fractions of hybridoma culture supernatants producing mAbs against K^k (11-4.1), I-A^k (10-2.16), and I-E^k (14-4-4s) were prepared as described (14).

Culture Media. DMEM supplemented with 10% FCS, 5×10^{-5} M 2-ME, and gentamycin (50 μg/ml) was used throughout this study.

Proliferation Assay. Th clones were cultured on MRL104.8a monolayer (3,000 rad, X-irradiated) or C3H/He splenic feeder cells (2,000 rad X-irradiated) in wells of flat-bottomed, 96-well microplates at 37°C in a CO_2 incubator for 2 d (14). The cells were then pulsed with 0.5 μCi/well of [^{3}H]TdR for 6 h, and the incorporated radioactivity was measured. Results are shown as the mean cpm ± SE of triplicate cultures.

Immunofluorescence Staining and Flow Microfluorometry (FMF). The

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cell preparation and staining procedures were essentially the same as described previously (14).

Results and Discussion

The MRL104.8a thymic stromal monolayer was allowed to express class I and II H-2 antigens by exposure to IFN-γ (14). A KLH-specific Th clone, 9-16, was cultured on Ia-expressing MRL104.8a monolayer in the absence or presence of the relevant KLH antigen (Table 1). The 9-16 Th clone was supported for the growth in the absence of KLH, whereas its growth was markedly inhibited in the presence of KLH, which confirmed the previous observation (14). This growth inhibition was ascribed not to a mere reduction of [^{3}H]TdR uptake by Th clone, but to a striking decrease of Th clone cell number (cell death) (Fig. 1, *A* and *B*).

The addition of CsA to cultures resulted in complete rescue of the Th clone from the lethal effect (Table 1). This is also illustrated in Fig. 1, *C* and *D*. Similar killing-rescue patterns of results were obtained by using the anti-I-E^k-alloreactive Th clone 2-13 (Table 1). This clone, which is capable of proliferating on IFN-γ–unexposed MRL104.8a monolayer to a limited but detectable extent, was killed on the Ia-expressing monolayer. Such a lethal effect was also prevented by CsA. It should also be noted that semipurified TSTGF sample could promote the growth of the 9-16 Th clone irrespective of whether CsA was included in cultures (Table 1).

We additionally investigated the effects of various concentrations of CsA on TSTGF-mediated growth promotion and lethal growth inhibition of Th clone. Fig. 2 *A* shows that growth promotion of the 9-16 Th clone on the MRL104.8a monolayer containing no KLH antigen is not affected by the presence of a wide range of CsA doses. These data are compatible with the results that CsA exhibited no suppressing effect on the TSTGF activity (line 2 of Table 1), as well as that the production of the TSTGF activity in culture supernatants was not influenced by the presence of CsA (data not shown).

In the presence of a KLH antigen, lethal growth inhibition of the 9-16 Th clone was again observed, and its lethal effect was prevented by CsA in a CsA dose-dependent way

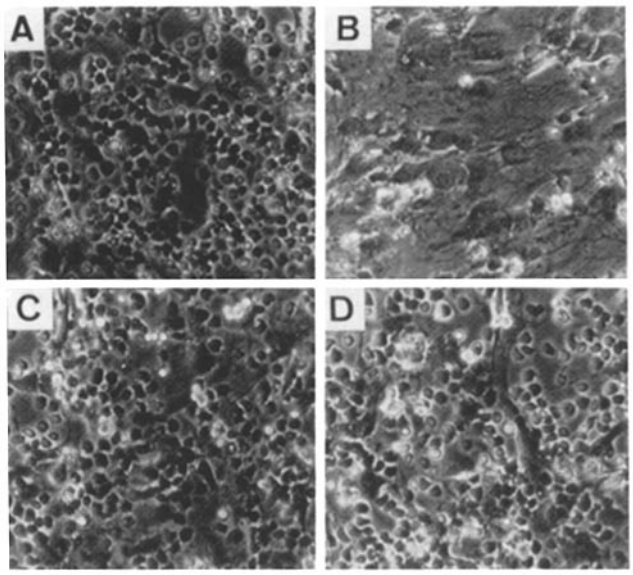

Figure 1. Phase-contrast micrographs of Th clone cells on an MRL104.8a monolayer. MRL104.8a monolayers were prepared in 24-well culture plates and allowed to express Ia antigens. 9-16 Th clone (2×10^5/well) was cultured for 4 d at the same concentrations of KLH and CsA as described at the footnotes of Table 1. (*A*) KLH(−) and CsA(−); (*B*) KLH(+) and CsA(−); (*C*) KLH(−) and CsA(+); (*D*) KLH(+) and CsA(+). Note that Th are seen as dark spots since they are present under stromal cells.

Table 1. *CsA Prevents Antigen-stimulated Clonal Elimination on Thymic Stromal Cell Monolayer*

Th clone	Stimulation* with:	[^{3}H]TdR uptake (cpm)‡ CsA (−)	CsA (+)
9-16	−	200(1.11)	299(1.10)
	TSTGF	11039(1.02)	10395(1.03)
	MRL104.8a(IFN-γ)	9378(1.21)	10395(1.03)
	MRL104.8a(IFN-γ) + KLH	1661(1.21)	14793(1.17)
2-13	−	316(1.15)	551(1.23)
	TSTGF	801(1.06)	772(1.02)
	MRL104.8a	2736(1.06)	2416(1.05)
	MRL104.8a(IFN-γ)	899(1.17)	2456(1.03)

* MRL104.8a monolayers prepared in 96-well microculture plates were untreated or exposed to 100 U/ml IFN-γ[MRL104.8a (IFN-γ)]. After washing, the monolayers were 3,000 rad X-irradiated before culturing with Th clone (2×10^4/well). KLH, 40 μg/ml; TSTGF, 1 U/ml.

‡ Th clone was cultured in the absence (solvent control) or presence of 100 ng/ml CsA for 2 d.

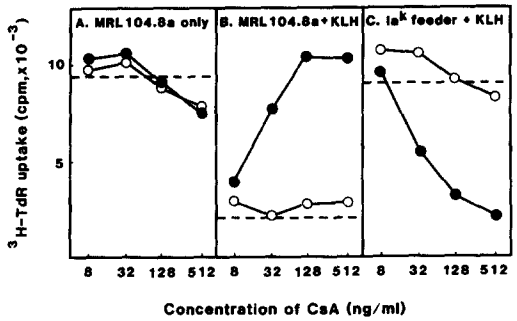

Figure 2. CsA dose-dependent influences on T cell growth inhibition or stimulation induced by the respective thymic stromal cells or splenic APC. (*A* and *B*) 9-16 Th clone (2×10^4) was cultured with or without 20 μg/ml KLH antigen on MRL104.8a monolayers in 96-well microplates. (*C*) 9-16 Th clone was cultured with 2×10^5/well of Ia^k (C3H/He) spleen cells plus KLH. Each culture contained various concentrations of CsA (●) or solvent control (○). Dashed lines indicate [^{3}H]TdR uptake of Th clone in cultures containing neither control solvent nor CsA.

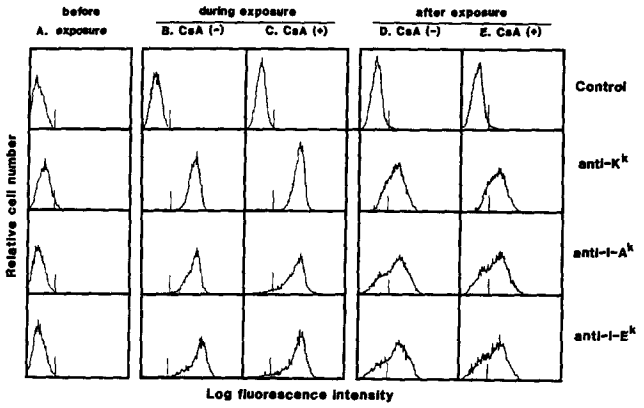

Figure 3. Effect of CsA on MHC expression of MRL104.8a cells. MRL104.8a cells were exposed to 50 U/ml rIFN-γ in the absence (*B*) or presence (*C*) of 200 ng/ml CsA for 2 d. MRL104.8a cells that had been exposed to IFN-γ were cultured in the absence (*D*) or presence (*E*) of CsA for an additional 2 d. Cells were stained with anti-K^k, anti-I-A^k, or anti-I-E^k antibody as described (10).

(Fig. 2 *B*). In contrast, proliferation of this Th clone was induced by TCR stimulation with antigen plus I-E^k on C3H/He spleen cells, and CsA induced a dose-dependent inhibition of TCR-mediated activation (Fig. 2 *C*). These results suggest that the rescue of the Th clone from lethal growth inhibition depends on CsA-induced inhibition of TCR-mediated activation events.

The inhibitory effect of CsA on TCR-mediated activation events might be explained by considering the possibility that CsA alters the expression of H-2 molecules on thymic stromal cells required for forming TCR-stimulating complex (7). To test this, CsA was added to either of two phases of culturing, in which H-2 molecules are induced on MRL104.8a cells by exposure to IFN-γ or in which they decrease after removal of IFN-γ. Fig. 3 illustrates that CsA does not exert its inhibiting effect on either the induction or reduction phase of H-2 antigens.

Thus, our results demonstrate that: (*a*) CsA blocks the deletion of Th clone with a given specificity that is otherwise induced on thymic stromal monolayer; and (*b*) CsA does not inhibit either the production of TSTGF activity by stromal cells or implementation of its growth-promoting effect.

CsA exerts many of its effects on T cells via specific inhibition of TCR-mediated activation (1). In the present model, CsA induced the rescue of Th clone from lethal growth inhibition. This rescue observed in the presence of CsA is provocatively similar to that induced in the presence of antibody to class II MHC or CD3 to block TCR stimulation (14). Thus, it is likely that CsA prevents the Th clone from lethal growth inhibition through interference with TCR-mediated signals. It has also been reported that CsA reduces the expression of class II MHC on cells in the medullary region of the thymus (7). Whether CsA affects in vivo the expression of class II MHC on thymic stromal cells responsible for negative selection is unclear. Irrespective of this, it is evident that the lack of TCR-mediated signals leads to the failure of deletion of the relevant T cell clones.

Regarding the effects of CsA on T cell development in the thymus, two major observations have been reported (15, 16). First, CsA interferes with the differentiation of immature $CD3^{-\text{ or low}}\ 4^+8^+$ thymocytes into mature $CD3^{high}\ 4^+8^-$ or $CD3^{high}\ 4^-8^+$ cells. Second, CsA induces incomplete deletion of potentially autoreactive T cells expressing a high density of TCR. Taken together, it is likely that CsA blocks the deletion of autoreactive T cell clones among the small number of α/β T cells that can mature in the presence of CsA, preventing negative selection (15, 16).

We have proposed that: (*a*) thymic stromal cells with specialized functions (MRL104.8a) are capable of negatively regulating the growth of the Th clone that is used here as a model of α/β TCR^{high} maturing or mature thymocytes and (*b*) that such regulation is dependent on TCR stimulation (14). The present results, obtained by using a minimal unit of the thymic stroma that is lethal to antigen-specific T cells and results in clonal elimination, provide an in vitro model for explaining the paradox of CsA-induced autoimmunity. Since our results demonstrate that CsA does not interfere with the capacity of the thymic stroma to contribute to T cell proliferation, this model could also account for survival of thymocytes including autoreactive T cell precursors during the presence of CsA in the thymic microenvironment of CsA-treated recipients.

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