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

By Hiroshi Kosaka, Hidetoshi Matsubara, Shinji Sogoh, Masato Ogata, Toshiyuki Hamaoka, and Hiromi Fujiwara

From the Biomedical Research Center, Osaka University Medical School, Fukushima-ku, Osaka 553, Japan

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 Paradoxically, irradiated host transplanted with syngeneic or autologous bone marrow and then treated with, and withdrawn from, CsA develop autoimmunity as characterized by a GVHD-like syndrome (5–8).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) that has been recently established from MRL/lpr/lpr mouse thymuses (11) was used.

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-Ek-restricted Th clone 9-16 and allo-I-Ek-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-6, and CSF 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 Kk(I-4.1), I-Ak(10-2.16), and I-Ek(14-4-4s) were prepared as described (14).

Culture Media. DMEM supplemented with 10% FCS, 5 x 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 CO2 incubator for 2 d (14). The cells were then pulsed with 0.5 μCi/well of [3H]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
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 [3H]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-Ek-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.

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

| Th clone | Stimulation* with: | [3H]TdR uptake (cpm)† |
|----------|--------------------|-----------------------|
|          | CsA (-) | CsA (+) | 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 | 166(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 × 10⁴/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 × 10⁴) 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 × 10⁶/well of Ia⁺ (C3H/He) spleen cells plus KLH. Each culture contained various concentrations of CsA (●) or solvent control (○). Dashed lines indicate [3H]TdR uptake of Th clone in cultures containing neither control solvent nor CsA.
during exposure
after exposure

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).

We thank Dr. G.M. Shearer for his critical reviewing of this manuscript and Miss N. Kawai for her expert secretarial assistance.

This work was supported in part by Grant-in-Aids for Scientific Research from the Ministry of Education, Science and Culture, Japan.

Address correspondence to Dr. Hiromi Fujiwara, Biomedical Research Center, Osaka University Medical School, 1-1-50 Fukushima, Fukushimaku, Osaka, 553, Japan.

Received for publication 3 January 1990 and in revised form 15 March 1990.
References

1. Shevach, E.M. 1985. The effects of cyclosporin A on the immune system. *Annu. Rev. Immunol.* 3:397.

2. Tutschka, P.J., W.E. Beschorner, A.C. Allison, W.H. Burns, and G.W. Santos. 1979. Use of cyclosporin A in allogeneic bone marrow transplantation in the rat. *Nature (Lond.)* 280:148.

3. Bolton, C., G. Allsopp, and M.L. Cuzner. 1982. The effect of cyclosporin A on the adoptive transfer of experimental allergic encephalomyelitis in the Lewis rat. *Clin. Exp. Immunol.* 47:127.

4. Stiller, C.R., J. Dupre, M. Gent, M.R. Jenner, P.A. Keown, A. Laupacis, R. Martell, N.W. Rodger, B.V. Graffenried, and B.M.J. Wolfe. 1984. Effects of cyclosporin immunosuppression on insulin-dependent diabetes mellitus of recent onset. *Science (Wash. DC).* 223:1362.

5. Glazier, A., P.J. Tutschka, E.R. Farmer, and G.W. Santos. 1983. Graft-versus-host disease in cyclosporin A-treated rats after syngeneic and autologous bone marrow reconstitution. *J. Exp. Med.* 158:1.

6. Sorokin, R., H. Kimura, K. Schroder, D.H. Wilson, and D.B. Wilson. 1986. Cyclosporin-induced autoimmunity. Conditions for expressing disease, requirement for intact thymus, and potency estimates of autoimmune lymphocytes in drug-treated rats. *J. Exp. Med.* 164:1615.

7. Cheney, R.T., and J. Sprent. 1985. Capacity of cyclosporine to induce auto-graft-versus-host disease and impair intrathymic T cell differentiation. *Transplant. Proc.* 17:528.

8. Sakaguchi, S., and N. Sakaguchi. 1988. Thymus and autoimmunity. Transplantation of the thymus from cyclosporin A-treated mice causes organ-specific autoimmune disease in a thymic nude mice. *J. Exp. Med.* 167:1479.

9. Kappler, J.W., N. Roehm, and P. Marrack. 1987. T cell tolerance by clonal elimination in the thymus. *Cell.* 49:273.

10. Kistemol, P., H. Bluthmann, U.D. Staerz, M. Steinmetz, and H. Von Boehmer. 1988. Tolerance in T-cell-receptor transgenic mice involves deletion of nonmature CD4-8+ thymocytes. *Nature (Lond.)* 333:742.

11. Ogata, M., H. Matsubara, Y. Takai, H. Kosaka, T. Katagiri, H. Sano, K. Ishimura, H. Fujita, T. Hamaoka, and H. Fujiwara. 1989. Capacities of a newly established thymic stromal cell clone to express Ia antigens and to produce interleukin-6, colony-stimulating factor, and thymic stroma-derived T cell growth factor. *J. Leukocyte Biol.* 45:69.

12. Sato, S., M. Ogata, H. Sano, Y. Mizushima, M. Muramatsu, H. Doi, T. Itoh, T. Hamaoka, and H. Fujiwara. 1988. Thymic stroma-derived T cell growth factor (TSTGF). I. Biochemical and functional characterization. *J. Leukocyte Biol.* 44:149.

13. Mizushima, Y., M. Saitoh, M. Ogata, H. Kosaka, Y. Tatsumi, C. Kiyotaki, T. Hamaoka, and H. Fujiwara. 1989. Thymic stroma-derived T cell growth factor (TSTGF). IV. Capacity of TSTGF to promote the growth of L3T4-Lyt-2 thymocytes by synergy with phorbol myristate acetate or various IL. *J. Immunol.* 142:1195.

14. Kosaka, H., M. Ogata, I. Hikita, S. Maruo, S. Sugihara, H. Matsubara, Y. Takai, T. Hamaoka, and H. Fujiwara. 1989. Model for clonal elimination in the thymus. *Proc. Natl. Acad. Sci. USA.* 86:3773.

15. Jenkins, M.K., R.H. Schwartz, and D.M. Pardoll. 1988. Effects of cyclosporine A on T cell development and clonal deletion. *Science (Wash. DC).* 241:1655.

16. Gao, E.K., D. Lo, R. Cheney, O. Kanagawa, and J. Sprent. 1988. Abnormal differentiation of thymocytes in mice treated with cyclosporine A. *Nature (Lond.)* 336:176.