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Effect of Cyclosporin A on rat thymus: time course analysis by immunoperoxidase technique and flow cytofluorometry

M. TANAKA, K. SHINOHARA, T. FUKUMOTO*, H. TANAKA & T. KANEKO *Third Department of Internal Medicine, * First Department of Anatomy, Yamaguchi University School of Medicine, Ube, Japan*

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SUMMARY

The effect of Cyclosporin A (CyA) administration 20 mg/kg body weight i.p. for 3 or 7 days on rat lymphoid tissues, especially on the thymus, and the recovery after stopping CyA treatment were investigated by both immunoperoxidase technique and flow cytofluorometry using monoclonal antibodies against rat lymphocytes: OX6, OX7, OX8, OX18 and W3/25. The marked reduction of thymic medulla with CyA treatment was clearly demonstrated by staining with OX18. This change was maximal 7 to 10 days after the start of CyA administration. The obvious restitution of the thymic medulla occurred about 7 days after stopping CyA and was almost completed within 14 days. Flow-cytofluorometric analysis of the thymus showed that the percentages of positive cells labelled with OX7, OX8, OX18 and W3/25 appeared to be not changed except for OX18 during and after CyA treatment. However, the expression of each antigen per cell changed in the amount; the peak of fluorescence intensity of $OX7^+$ cells showed a temporary shift to the right during CyA treatment. Bright positive cell populations for each OX8 and W3/25 increased relatively during CyA treatment, and reverted to the normal levels soon after stopping the CyA treatment. On the other hand, bright $OX18^+$ cells decreased with CyA treatment, but this change recovered gradually after stopping the CyA treatment. Treatment with CyA gave no significant changes in the flow-cytofluorometric analyses for these antibodies on lymph node cells. Natural killer cell activity and the ability to cause local graft-versus-host reaction were not inhibited with CyA treatment. These results suggest that CyA inhibits the proliferation and differentiation of thymocytes, or that CyA makes thymocytes migrate rapidly from cortex to periphery.

Keywords Cyclosporin A thymus monoclonal antibodies immunohistochemistry flow cytofluorometry

INTRODUCTION

Cyclosporin A (CyA) has immunosuppressive effects, which are predominantly or exclusively limited to T lymphocyte dependent immune responses (Borel *et al.*, 1977). In relation to the toxicological and histopathological effects of CyA, there was one study which showed lymphocytic depletion of the thymic cortex in the mouse (Ryffel, Deyssenroth & Borel, 1981). According to subsequent studies, however, a striking reduction in the size and cellularity of thymic medulla with little effect on the cortex is observed after relatively short CyA treatment in rodents (Bladwin *et al.*, 1981; Blair *et al.*, 1982). After CyA treatment was stopped, the atrophic medulla rapidly reformed and normal thymic architecture was restored within 10 days (Glazier, Tutschka & Farmer, 1983).

T cells develop in the thymus and may mature as they migrate from the cortex to the medulla and then to the periphery, acquiring OX18 antigen and selectively losing and acquiring W3/25 and OX8 antigens. Monoclonal antibodies (MoAb) against these surface antigens have been useful for distinguishing thymocyte subpopulations in rats (Mason *et al.*, 1983).

We have investigated more closely the effect of CyA on thymus and its recovery after the stopping of CyA, using MoAb. We have also analysed the effect of CyA on peripheral lymphoid tissues in relation to those on thymus, studying by the flow-cytofluorometric analysis of the lymphoid tissues and the graft-versus-host reaction (GVHR).

The effect of CyA on natural killer (NK) activity is controversial (Introna *et al.*, 1981; Shao-Hsien *et al.*, 1983).

Correspondence: M. Tanaka MD, Third Department of Internal Medicine, Yamaguchi University School of Medicine, Ube-shi, Yamaguchi-ken 755, Japan.

Thus, we have investigated the NK activity in order to make this point clear and discussed the results in relation to the changes of the lymphoid tissues.

MATERIALS AND METHODS

Animals

For the analysis of thymus and cervical lymph nodes, inbred female DA (RT-1^a) rats, 7 to 12 weeks of age, were used. For local GVHR assay, male DA rats, 7 to 12 weeks of age, and male (Lou/M × DA)F_1 (RT-$1^{u \times a}$) hybrids, 4 to 8 weeks of age, were used. For NK cell analysis, male DA rats, 7 to 12 weeks of age, were used. These rats were kept in our animal colony under routine laboratory conditions with free access to food and water.

Cyclosporin A (CyA) administration

The first day of administration represented day 0 of the experiment. DA rats received CyA (20 mg/kg body weight) by daily intraperitoneal injection for 7 (or 3) days, from day 0 to day 6 (or day 2). CyA stock solution (in ethanol and polyethoxylated castor oil; Sandoz Ltd, Basel, Switzerland) was diluted in 1 ml normal saline before use.

Experimental protocol

Three separate experiments were conducted. In the first experiment (analysis of the lymphocyte subpopulations with MoAb), the thymuses from CyA-treated rats were analysed on days 3, 7, 10, 14 and 21 by immunohistochemistry and flow cytofluorometry. The cervical lymph nodes from these CyA-treated rats were also analysed on days 7 and 14 by flow cytofluorometry. In the second experiment, the assay for local GVHR were performed. CyA-treated rats were used on days 7 and 14 as donors, and normal F_1 hybrids were used as recipients. In the third experiment, NK cell assay was performed using the spleen cells from CyA-treated rats on days 7 and 14. In these experiments, CyA-untreated normal DA rats were used as control rats.

Antibodies

Monoclonal antibodies (MoAb) that identified the lymphocyte surface antigens of rats were W3/25 (CD4), T helper cells(Th) (Williams, Galfrè & Milstein, 1977) and macrophages (Jefferies, Green & Williams, 1985); OX8 (CD8), T killer/suppressor cells(Tk/s) (Brideau *et al.*, 1980); OX18, class I MHC gene products (Fukumoto, McMaster & Williams, 1982); OX6, class II MHC gene products (McMaster & Williams, 1979) and OX7, Thy-1 antigen (Mason & Williams, 1980). These specific MoAb (from ascites fluid) were purchased from Sera Labs (Sussex, England) or were gifts from Dr Alan F. Williams, MRC Cellular Immunology Unit, Oxford. FITC-conjugated sheep $F(ab')_2$ anti-mouse IgG and horseradish peroxidase (HRP) conjugated rabbit $F(ab')_2$ anti-mouse IgG were obtained from Cappel Labs (Cochranville, USA).

Indirect immunohistochemical staining

CyA-treated rats were sacrificed by anaesthesia, and the thymus was frozen. Immunohistochemical staining of the thymus was performed following the method described previously (Fukumoto, McMaster & Williams, 1982), using MoAb at 1/10 dilution in phosphate buffered saline (PBS).

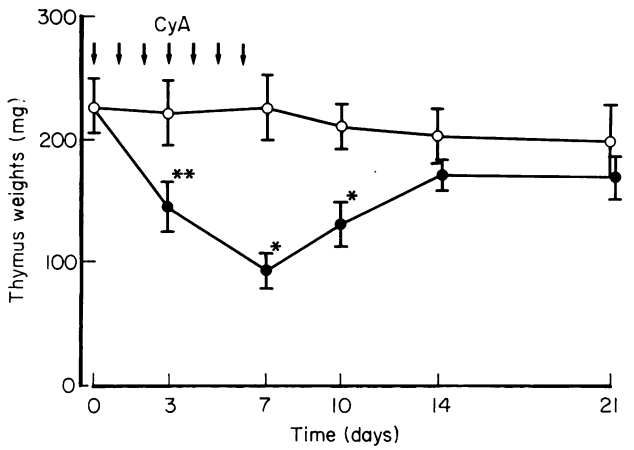

Fig. 1. Thymus weights of CyA-treated rats (●) or control rats (○) on various days. Thymus weights (mg) are plotted against days after the start of CyA treatment. The mean of four thymus weights (±s.d.) is shown. Arrows show days on which CyA was administered. Control and CyA-treated groups were compared to each other with Student's *t*-test. The decrease in mean thymus weights on days 3, 7 and 10 is statistically significant. * $P < 0.01$; ** $P < 0.05$.

Flow cytofluorometry

Cells from the thymus or the cervical lymph nodes were labelled with MoAb and FITC-conjugated secondary antibody, and analysed by a fluorescence-activated cell sorter (FACS-III; Becton-Dickinson Electronic Laboratories, Mountain View, USA), following the method described by Fukumoto, McMaster & Williams (1982). The labelled cells were freshly analysed or fixed by 1% paraformaldehyde. Cell analysis of the fixed and stored samples was performed on the same day.

Assay for local GVHR

The assay has been described elsewhere (Ford, Burr & Simonsen, 1970). In brief, $5–40 \times 10^6$ viable pooled lymph node cells (cervical and mesenteric) were injected subcutaneously into the hind foot pad of F_1 hybrids. The animals were killed 7 days later and popliteal lymph nodes (PLN) were weighed.

Natural killer (NK) cell assay

The NK cell assay was performed as described by Reynolds, Timonen & Herberman (1981). Briefly, spleen cells were used as effector cells. Cells from a rat myeloma cell line (Y3) were labelled with ^{51}Cr and served as targets for assessment of NK activity (Dallman, Mason & Webb, 1982). Percent specific release was calculated by the following formula: % specific ^{51}Cr release = (ct/min experimental − ct/min spontaneous) × 100/(ct/min maximum − ct/min spontaneous).

Statistical analysis

Control and CyA-treated groups were compared using Student's *t*-test.

RESULTS

Change of thymus weight

CyA treatment resulted in significant decrease of thymus weights on days 3, 7 and 10, compared with controls. After stopping the CyA treatment, the thymus weight recovered gradually (Fig. 1).

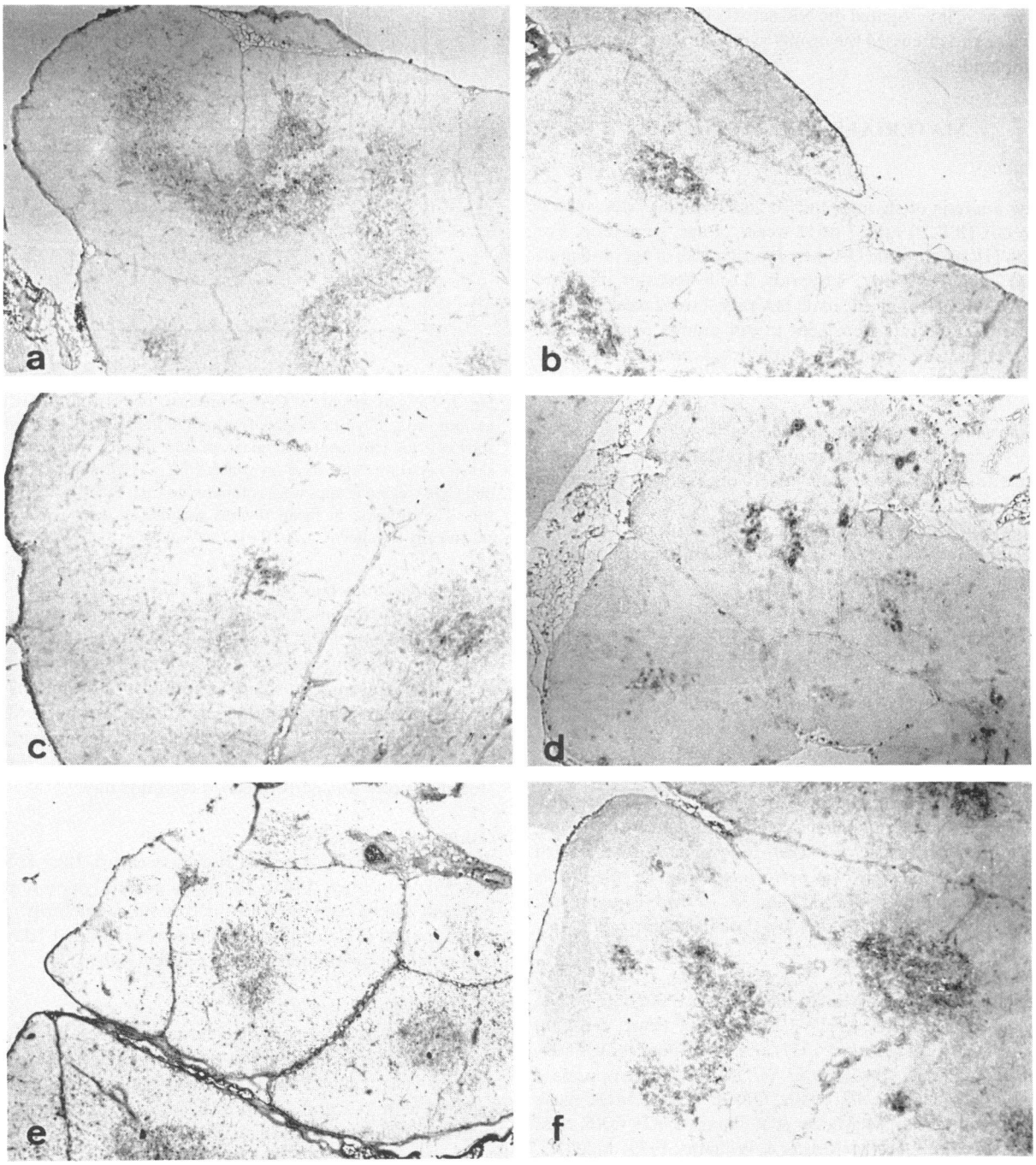

Fig. 2. Immunoperoxidase-staining patterns of frozen thymus sections from CyA-treated rats labelled with OX18 on day (a) 0 (control), (b) day 3, (c) day 7, (d) day 10, (e) day 14 and (f) day 21. Frozen sections were prepared and fixed in methanol, then reacted with 100 μl of the MoAb at 1/10 dilution in PBS. The sections were then washed, stained with 100 μl of HRP-conjugated rabbit (F (ab')$_2$ anti-mouse IgG at 1/10 dilution in PBS, and washed again before development of the histochemical staining. Note that OX18$^+$ cells were located in the medulla (a). Atrophy of the thymic medulla was observed (b-d), which was maximal on days 7 and 10. Reformed thymus was observed on days 14 and 21 (e,f). ($\times$40).

Immunohistochemical analysis of thymus

The majority of medullary thymocytes of normal adult rat were strongly stained with OX18 (Fig. 2a). The cortical thymocytes were almost negative for OX18, but a small number of OX18$^+$ cells were observed in the cortex. The strongly stained cells with OX6 were observed in the medulla and the weakly stained cells with OX6 were observed in the cortex. Most of the OX6$^+$ cells seemed to be reticular cells. Virtually all the thymocytes were positively stained with OX7, which gave stronger staining in the cortex than in the medulla. OX8$^+$ cells were distributed in the same way as OX7$^+$ cells. Most of thymocytes were W3/25$^+$ and were uniformly distributed in the thymus (data not shown).

CyA treatment resulted in a marked reduction of thymic medulla which were examined by the specific staining with

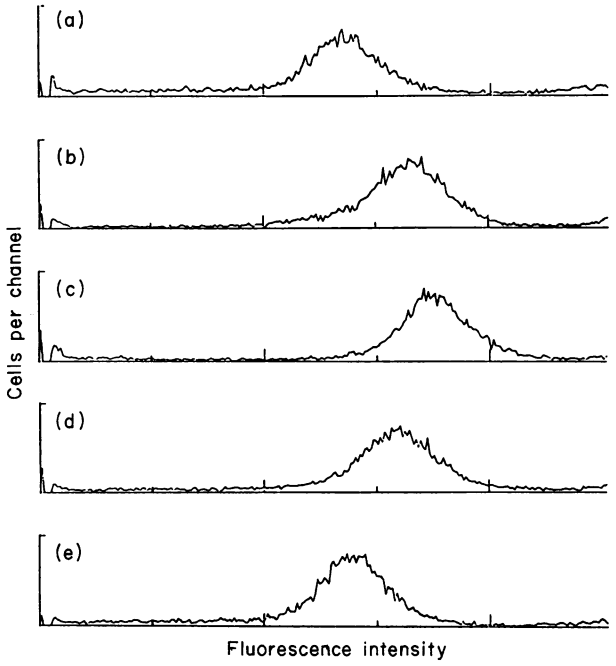

Fig. 3. Fluorescence profiles of thymocytes labelled with OX7. Profiles (a), (b), (c), (d) and (e) represent the specific staining of the thymocytes of CyA-treated rats on days 0 (control), 3, 7, 14 and 21, respectively.

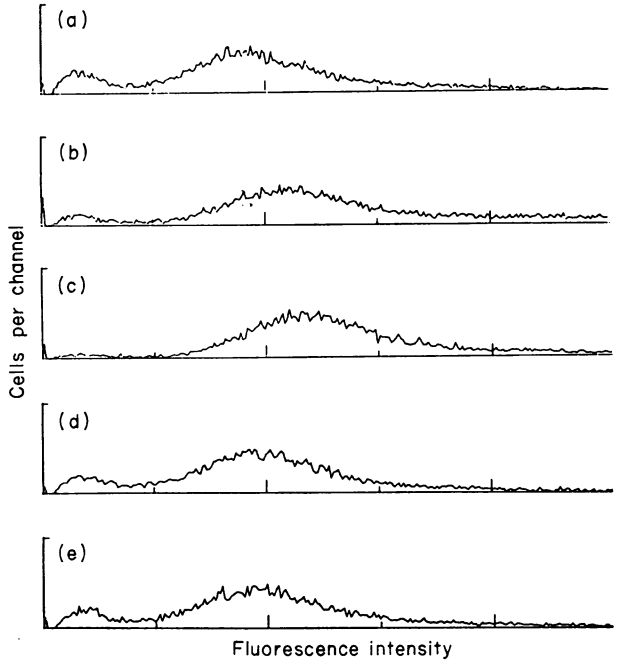

Fig. 4. Fluorescence profiles of thymocytes labelled with OX8. Details as in Fig. 3.

OX18 (Fig. 2b–d). The medulla had already begun to reduce on day 3 (Fig. 2b). On day 7, the medulla was almost depleted, whereas the cortex was comparatively preserved (Fig. 2c). The marked reduction of the medulla continued until day 10 (Fig. 2d). On day 14, which is 7 days after stopping the CyA treatment, the thymic reconstitution began with a marked increase of the $OX18^+$ cells in the medulla (Fig. 2e). Until day 21, the thymic reconstituion was almost completed (Fig. 2f).

The stained patterns of the thymus labelled with OX6, OX7, OX8 and W3/25 were not affected with CyA treatment (data not shown).

Flow-cytofluorometric analysis of thymus

In the scatter analysis, the size of each thymocyte was invariable among the samples from day 3 to day 21 (data not shown).

The fluorescence profile of thymocytes from normal adult rat, stained with OX7, showed a wide range of fluorescence intensity (Fig. 3a). The peak of the fluorescence intensity shifted to the right with CyA treatment on day 3 (Fig. 3b). On day 7, the labelling pattern was more evident (Fig. 3c). However, after the stopping of CyA, the shift of the peak was restored to the peak of the control until day 21 (Fig. 3d,e).

Analysis of $OX8^+$ cells of thymocytes from normal adult rat showed a major $OX8^+$ population (Fig. 4a). CyA treatment for 3 or 7 days resulted in an increase of bright $OX8^+$ cells (Fig. 4b,c), which returned soon after the stopping of CyA (Fig. 4d,e). The percentages ($\pm$s.d.) of $OX8^+$ cells on days 3, 7, 10, 14 and 21 were $94{\cdot}0 \pm 1{\cdot}5$, $95{\cdot}5 \pm 1{\cdot}7$, $94{\cdot}6 \pm 2{\cdot}1$, $93{\cdot}9 \pm 1{\cdot}8$ and $93{\cdot}4 \pm 1{\cdot}0$, respectively, compared with $93{\cdot}1 \pm 0{\cdot}4$ of controls. These changes were not significant.

The fluorescence profiles of W3/25 in normal adult rat showed that the majority of thymocytes were $W3/25^+$ (Fig. 5a). CyA treatment also resulted in a slight increase of the bright

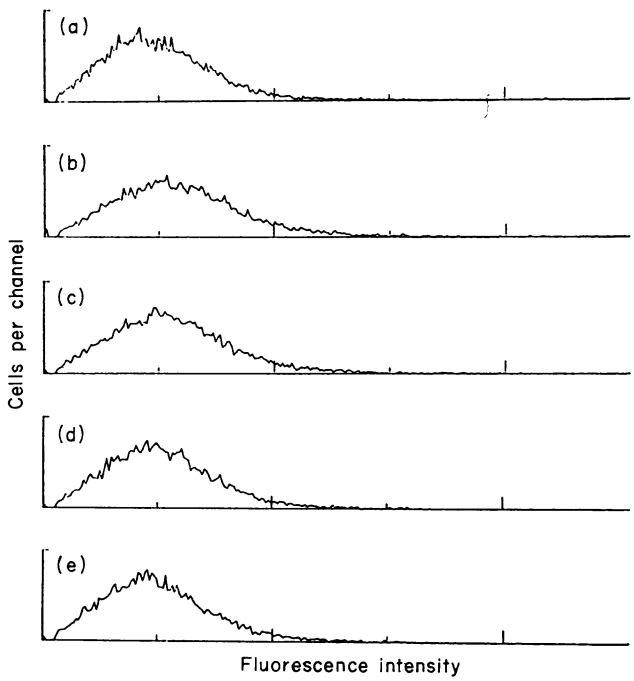

Fig. 5. Fluorescence profiles of thymocytes labelled with W3/25. Details as in Fig. 3.

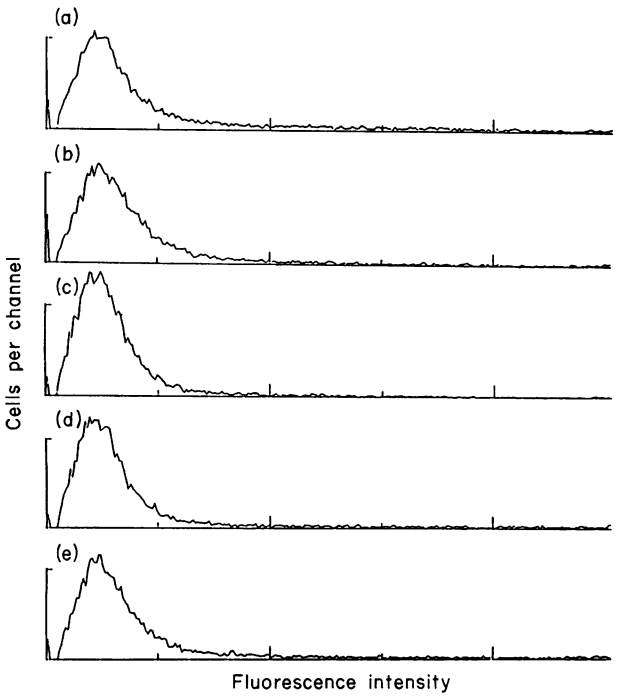

Fig. 6. Fluorescence profiles of thymocytes labelled with OX18. Details as in Fig. 3.

W3/25$^+$ cells on days 3 and 7 (Fig. 5b,c). On days 14 and 21, the labelling patterns were similar to those of the control (Fig. 5d,e). The percentages (±s.d.) of W3/25$^+$ cells on days 3, 7, 10, 14 and 21 were 93·7±2·1, 94·2±0·9, 93·3±1·1, 93·3±1·4 and 92·6±2·0, respectively, compared with 93·5±0·9 of controls. These changes were not significant.

About 30% of the thymocytes of normal adult rat were OX18$^+$ and showed a wide range of fluorescence intensity (Fig. 6a). CyA treatment resulted in a decrease of the bright OX18$^+$ cells on days 3 and 7 (Fig. 6b,c). After stopping the CyA treatment, the bright OX18$^+$ cells was gradually restored to those of control (Fig. 6d,e). The percentages (±s.d.) of OX18$^+$ cells on days 3, 7, 10, 14 and 21 were 26·6±2·7, 24·6±6·5, 25·8±2·0, 25·6±4·0 and 31·0±4·9, respectively, compared with 30·3±0·2 of controls. The percentage of OX18$^+$ cells appeared to be decreased with CyA treatment from day 3 to day 14, which was restored on day 21.

About 10% of normal adult rat thymocytes were OX6$^+$ with dull fluorescence intensity. CyA treatment did not affect the fluorescence intensity of OX6$^+$ cells (data not shown). The percentages (±s.d.) of OX6$^+$ cells on days 3, 7, 10, 14 and 21 were 13·3±3·0, 10·2±1·0, 12·4±0·7, 9·5±1·3 and 11·1±1·0, respectively, compared with 10·2±0·1 of controls. These changes were not significant.

The effect of CyA on lymph nodes of donor rat

The lymphocyte subpopulations of lymph node cells from CyA-treated rats were analysed. On days 7 and 14, the mean (±s.d.) of the percentages of W3/25$^+$ cells were 36·6±1·6, 41·5±4·7, respectively, as compared with 39·4±0·2 of controls. The percentages (±s.d.) of OX8$^+$ cells on days 7 and 14 were 19·4±0·2 and 18·2±2·9, respectively, as compared with 18·3±0·1 of controls. Thus, the ratios of the W3/25$^+$ cells/OX8$^+$ cells were 1·89 and 2·28 on days 7 and 14, respectively, as compared with 2·15 of controls. The percentages (±s.d.) of OX6$^+$ cells on days 7 and 14 were 39·5±3·8 and 40·6±2·1, respectively, as compared with 38·9±0·7 of controls. There were no differences between the lymphocyte subpopulations from CyA-treated rats and those from control rats.

The effect of CyA on the ability to cause local GVHR

The mean (±s.d.) of three PLN weights from F_1 hybrids on days 7 and 14, and those of controls were 44·3±12·1, 25·2±4·4 and 22·4±5·8, respectively, when 5×10^6 donor lymph node cells were injected. Similarly, these values were 69·5±13·2, 44·7±17·7 and 45·3±4·8 for 1×10^7 of donor cells, 95·4±14·1, 61·7±26·5 and 70·0±13·7 for 2×10^7 of donor cells, and 115·7±16·9, 76·8±24·7 and 88·3±17·2 for 4×10^7 of donor cells. These changes were not significant.

The effect of CyA on NK activity

Following one representative result, percent specific release of days 7 and 14, and that of control were 0·5, 1·9 and 1·7, respectively, when the E/T ratio was 5. Similarly, these values were 2·8, 2·6 and 3·9 (E/T ratio 10), 5·5, 4·5 and 6·5 (E/T ratio 20), 9·0, 10·4 and 10·8 (E/T ratio 40), and 10·4, 14·9 and 13·7 (E/T ratio 80), respectively. Thus, CyA treatment had little effect on NK activity of the spleen cells on days 7 and 14.

DISCUSSION

We observed immunohistochemically that CyA treatment induced the marked reduction of thymic medulla, and this change continued until day 10 (Fig. 2), while the thymus weight decreased during CyA treatment, and started to increase on day 10, 3 days after stopping CyA treatment (Fig. 1). Thus, these two findings appeared discrepant to each other, suggesting that the recovery of the thymic cortex may occur earlier than that of the medulla.

Marked reduction of OX18$^+$ cells was observed by immunohistochemistry (Fig. 2), although only the slight reduction in this population was seen by flow cytofluorometry (Fig. 6). Three possible mechanisms might explain this discrepancy: (a) even the weakly stained OX18$^+$ cells in the cortex may be detected by flow cytofluorometry, but may not be observed by immunohistochemistry; (b) the marked depletion of medullary epithelia which are strongly OX18-positive may make the relatively weak staining pattern of OX18 in the thymus from CyA-treated rats, as there was a report that the medullary epithelium is essentially absent in CyA-treated rats (Beschorner *et al.*, 1987); (c) some proportions of cortical immature cells which are supposed to be OX18-negative may die when preparing cell suspension, and this may let the percentage of OX18$^+$ cells less in flow-cytoflurometric analysis.

The relative increase of cortical thymocytes were observed in the atrophic thymus by immunohistochemistry (Fig. 2). Since cortical thymocytes are considered to be less maturated and less differentiated than the medullary thymocytes (Mason *et al.*, 1983), thymus during CyA treatment may mainly consist of immature cells. In fact, a relative increase of bright OX7$^+$ cells during CyA treatment was repeatedly observed by flow cytofluorometry (Fig. 3). And this increase of the bright OX7$^+$ cells

may suggest the increase of Thy-1 antigens per thymocyte, because the size of the thymocytes during CyA treatment was not remarkably changed (data not shown). Subsequently, this may suggest the increase of immature thymocytes (Williams, 1982). Furthermore, the bright OX18$^+$ cells decreased during CyA treatment (Fig. 6). This may also support the above conclusion, as MHC class I antigens per cell are supposed to increase following the maturation of thymocytes (Fukumoto, McMaster & Williams, 1982).

On the other hand, the relative increases of the bright OX8$^+$ cells and W3/25$^+$ cells (Figs 4 & 5), and the existence of more than 20% of OX18$^+$ cells in the thymus during CyA treatment (Fig. 6) suggest the existence of some mature thymocytes and the occurrence of thymocyte maturation in the thymic cortex of the CyA-treated rats.

Thus, the increased proportion of cortical thymocytes observed during CyA treatment may be explained by three kinds of mechanisms: (a) the thymocyte proliferation and differentiation in the thymus might be interfered with by CyA treatment, resulting in the depletion of mature T cells in the peripheral lymphoid organs; (b) the cortical thymocytes might mature in the cortex during CyA treatment and rapidly enter the periphery; (c) the cortical thymocytes might leave the thymus without maturation and enter the periphery, where such immature cells may mature. The effect of CyA on the lymphocyte subpopulations of the cervical lymph node cells and on the NK activity of the spleen cells was not significant, and CyA treatment of donor rats was ineffective in suppressing the local GVHR of the F_1 hybrids. From these results, hypothesis (b) or (c) is more likely. These hypotheses are supported by current thinking that the cortex and medulla have independent generation kinetics (Scollay, 1983). In this study, we could not conclude which hypothesis was the most probable.

The CyA pretreatment of donor rats may be ineffective for the suppression of GVHR, as far as the number of injected dose was kept. W3/25$^+$ cells are supposed to be a responsible cell subset for local GVHR in the donor's factor (Mason *et al.*, 1983), and this population did not decrease in the CyA-treated rats (Table 2). With respect to further considerations of the effect of CyA on local GVHR, in a preliminary study we observed that treatment *in vitro* with CyA of donor cells or pretreatment of the F_1 hybrid recipients *in vivo* did not affect GVHR, and that the suppression of local GVHR were only induced by the treatment of F_1 recipients during the assay for local GVHR.

We found no effect of CyA on NK activity. This might suggest that the maturation of NK cells is independent of the thymic changes which were observed in this experiment, or that even no direct or indirect effect of CyA on the peripheral NK cells is proved.

Following the stopping of CyA, medullary regions rapidly reformed, and normal thymic architecture was restored within 14 days (Fig. 2d–f). The reformed thymic medulla was similar to that of the control thymus in the antigen expressions detected by several MoAB such as OX6, OX7, OX8, OX18 and W3/25. The changes on the fluorescence intensity during CyA treatment almost disappeared until day 21, which were consistent with the findings of the immunohistochemistry. These results show that the effect of CyA on thymus is reversible and that the altered lymphocyte generation kinetics with CyA treatment may be restored to those of normal rat.

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