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Effects of Cyclosporin A on Popliteal Lymph Node Graft-versus-Host Reaction in Rats

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Abstract The immunosuppressive effect of cyclosporin A (CYA) was studied in the popliteal lymph node graft-versus-host (GvH) reaction. Viable lymph node cells from DA rats were injected into foot pad of (DA×Lou/M)F_1 hybrids and popliteal lymph nodes were weighed 7 days later. *In vivo* treatment of F_1 hybrids with 7-days course of CYA, which was given intraperitoneally at the dose of 20 mg/kg body weight, suppressed this local GvH reaction when treatment started at the time of cell transfer. However, neither *in vivo* treatment of F_1 hybrids with CYA for 7 days before cell transfer, *in vitro* incubation of the cellular inoculum with CYA, nor the inoculation of lymph node cells from CYA-treated donors affected the local GvH reaction.

Examination of the popliteal lymph nodes undergoing a GvH reaction by immunoperoxidase-staining revealed a disorganization of the lymph node architecture with disappearance of the follicles, and the increase of Ia^+ ($OX6^+$) cells in both paracortex and medulla. In the flow cytofluorometric analysis, the relative increase of Ia^+ cells was observed, and the percentage of T helper (Th ; $W3/25^+$) cells relatively decreased and that of T suppressor (Ts ; $OX8^+$) cells increased as compared with that of normal cervical lymph nodes, resulting in the decrease of Th/Ts ratio. When CYA was given to F_1 hybrids during the assay for local GvH reaction, the architecture of popliteal lymph nodes was similar to that of popliteal lymph nodes undergoing a GvH reaction except for the comparatively preserved cortex with follicles and the decreased distribution of Ia^+ cells in the paracortex and medulla. Flow cytofluorometry showed the almost unchanged Th/Ts ratio and the decrease of the percentage of Ia^+ cells.

These findings indicate that the effect of CYA is variable, depending on the schedule of the treatment, and that only *in vivo* CYA treatment of F_1 hybrid recipients affects GvH reaction.

Key Words : Cyclosporin A, Local GvH reaction, Monoclonal antibody, Flow cytofluorometry, Immunohistochemistry

Introduction

The fungal metabolite cyclosporin A (CYA) is known to exert immunosuppressive effects[1]. CYA suppresses both humoral and cell-mediated immunity[2)–4)], inhibits adjuvant arthritis, experimental allergic encephalomyelitis and graft-versus-host (GvH) reaction[1], including the local GvH reaction[5)–7)], and prolongs survival of organ

grafts in a variety of species in laboratory animals[8].

The GvH reaction that developed in F_1 hybrid rats has been a useful experimental model both for the study of mechanism of cell interaction in immune responses, and for the study of the pathogenic mechanism behind certain immunological diseases[9]. Local GvH reaction is induced by donor T cells[10], more specifically of the helper T subset (in rat, $W3/25^+$ lymphocytes)[11)-13], and causes a massive enlargement of the popliteal lymph node. It is reported that *in vivo* and *in vitro* treatments with CYA suppress this local GvH reaction[7].

This study have been concentrated on the effects of CYA on a local GvH reaction, using the popliteal lymph node weight assay described by Ford, *et al*[14] as a model. We first have demonstrated *in vivo* effect of CYA on host or donor lymph node cells, depending on the different schedules of administration, and *in vitro* effect of CYA on the capacity of the cell inocula to induce local GvH reaction. Secondly, we have examined the influences of CYA on the lymph node architecture and the lymphocyte subpopulations taking place in the GvH nodes, using immunohistochemical and flow cytofluorometric analyses.

Materials and methods

Animals Inbred male DA (RT-1^a) rats, 7 to 12 weeks of age, and male (DA×Lou/M) F_1 (RT-1^{axu}) hybrids, 4 to 8 weeks of age, were used in the assay for local GvH reaction. All rats were maintained on a standard diet and given water *ad libitum*.

Cyclosporin A (CYA) CYA (concentrate for intravenous infusion; Sandoz Ltd., Basel, Switzerland), which is a water-soluble preparation, was diluted in 1ml normal saline and intraperitoneally administered to DA donors or F_1 hybrids with concentration of 20 mg/kg body weight for 7 days. In *in vitro* incubation study of lymph node cells with CYA, the concentrate of CYA was properly diluted in RPMI 1640 medium.

Experimental protocol The first day of the assay for the local GvH reaction represented day 0 of the experiment. Three separate experiments were conducted (Fig. 1). In the first experiment, CYA was daily administered to F_1 hybrids

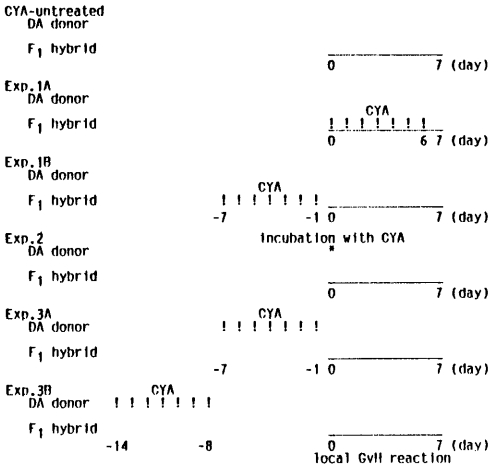

Fig. 1 Experimental protocols conducted in this study. (!) shows the day of CYA administration by intraperitoneal route, and (*) shows the incubation of DA donor cells with CYA.

for 7 days after or before parental cell transfer (Exp. 1A or 1B).

In the second experiment, DA donor lymph node cells were incubated *in vitro* with CYA and then used for the local GvH reaction (Exp. 2). In the third, CYA was daily given to DA donors for 7 days before cell transfer into F_1 hybrids from day −7 to day −1 (Exp. 3A) or from day −14 to day −8 (Exp. 3B).

In these experiments, CYA-untreated F_1 hybrid recipients were used as the control (Fig. 1).

Assay for local GvH reaction The assay has been described elsewhere[14]. In brief, 5 to 40×10^6 viable lymph node cells from DA donors were subcutaneously injected into the hind foot pads of (DA×Lou/M)F_1 hybrids on day 0. The animals were killed on day 7 and their popliteal lymph nodes were weighed to an accuracy of 0.1mg.

The *in vitro* incubation of the cell inoculum with CYA was performed as follows. Approximately 2.5×10^7 lymph node cells from DA donors were washed twice and suspended in a final volume of 2ml of RPMI 1640 medium containing 100 μg/ml of CYA. Immediately after incubation for 60 min at 37°C, the cells were washed twice and viability was assessed by trypan blue dye exclusion. Five to 20×10^6 viable cells resuspended in phosphate-buffered saline (PBS) were inoculated into F_1 hybrids.

Preparation of the cells All F_1 hybrid recipients were sacrificed by anesthesia on day 7, and their popliteal lymph nodes were removed. DA donor lymph nodes were also prepared on day 0. A part

of the tissues was frozen for immunohistochemistry or fixed in Carnoy solution for methyl green -pyronin stain, and the rest was used for the preparation of their cell suspension in PBS.

Antibodies Monoclonal antibodies (mAb) that identify the lymphocyte surface antigens of rats were W3/25 (CD4 ; T helper cells, Th)[15], OX8 (CD8 ; T suppressor cells, Ts)[11] and OX6 (class II MHC gene products, Ia)[16]. These specific mAb (from ascites fluid) were purchased from Sera Labs. (Accurate Chemical and Scientific Corp., Westbury, USA). 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. (Cochraville, USA).

Histology Paraffin sections from each specimen were stained with methyl green-pyronin. Selected sections were stained with mAb by the immunoperoxidase technique. Briefly, 5μm frozen sections of the tissues were fixed in 95% methanol and washed three times in cold PBS. The sections were incubated at room temperature with 100μl of the diluted mAb, rinsed three times in cold PBS and followed by the incubation at 4℃ with 100μl of HRP-conjugated rabbit $F(ab')_2$ anti-mouse IgG at 1/10 dilution in PBS, containing 2% heat-inactivated normal rat serum. The peroxidase activity was demonstrated, using a solution of 0.5% diaminobenzidine hydrochloride (Sigma Chemical Company, St louis, USA) and 0.01% hydrogen peroxide. Control sections in which mAb was omitted were examined for nonspecific staining.

Flow cytofluorometry Cell suspensions (5 to 10×10^6) were incubated with 5μl of the mAb at 4℃ for 40 min, washed three times in cold PBS, and then incubated at 4℃ for 60 min with 100μl of the FITC-conjugated secondary antibody at 1/10 dilution in PBS, containing 2% heat-inactivated normal rat serum. FITC-labeled cells were washed twice and resuspended in cold PBS. Cell analysis was performed with a fluorescence-activated cell sorter (FACS-III ; Becton-Dickinson Electronic Laboratories, Mountain View, USA).

Statistical analysis CyA-untreated and CyA-treated groups were compared using Student's *t*-test.

Results

1. GvH lymph node without CYA treatment

The mean popliteal GvH lymph node weight of CYA-untreated F_1 hybrid recipients was almost linearly related to the dose of parental donor cells injected on a double log scale (Fig. 2).

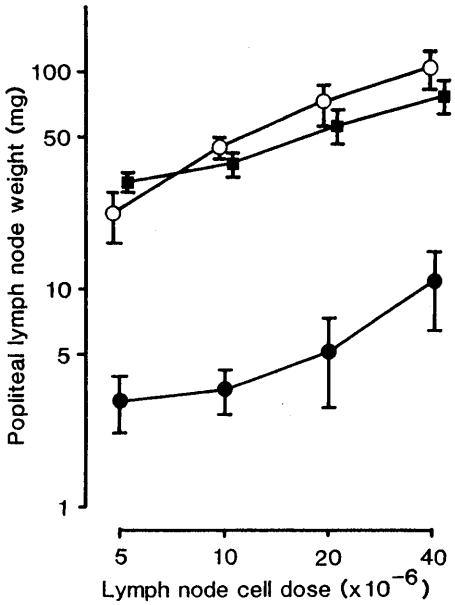

Fig. 2 Popliteal lymph node weights in $(DA\times Lou/M)F_1$ hybrid recipients inoculated with parental lymph node cells. Recipients were treated with 20mg of CYA per kg^{-1} administered for 7 days after cell transfer (●) or before cell transfer (■), or not treated with CYA (○). Values are means ± SD obtained from groups of four or five rats.

The popliteal GvH lymph nodes from CYA-untreated F_1 hybrids showed a massive enlargement of the organ, an increase of the cellularity in both paracortex and medulla, and no distinct cortex (Fig. 3a). The numerous large pyroninophilic cells in the paracortex and medulla were depicted in the GvH nodes (Fig. 3a), comparing with the normal cervical lymph nodes (data not shown). In the immunohistochemical analysis, many $OX6^+$ cells were seen in the deep cortical layer and medulla of the nodes (Fig. 3b). Furthermore, $OX8^+$ cells were densely represented in the enlarged paracortical area of the GvH nodes, while the $W3/25^+$ cells were

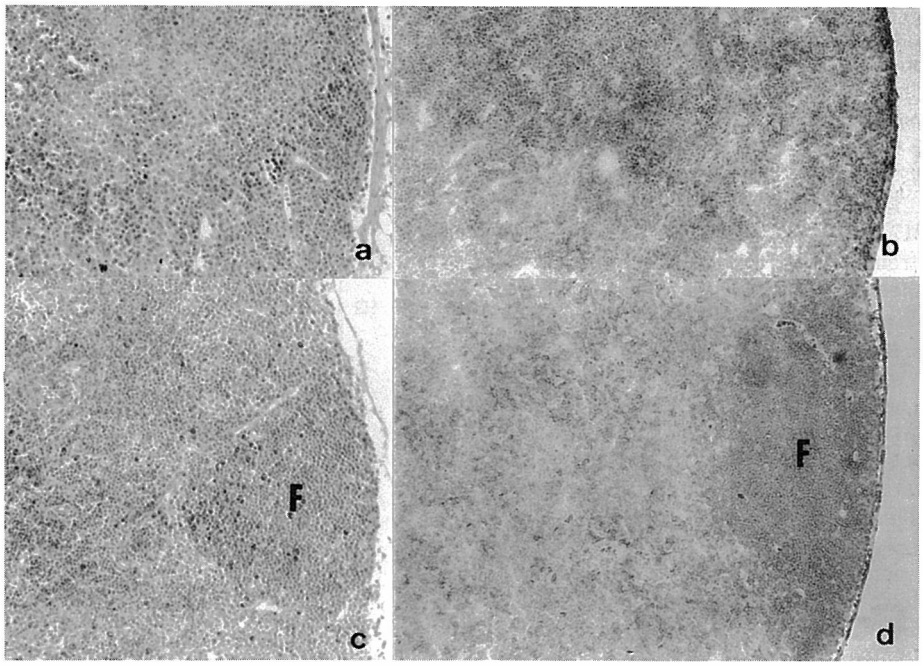

Fig. 3 Sections of popliteal GvH lymph nodes from a CYA-untreated F_1 hybrid recipient (3a, b) or a CYA-treated F_1 hybrid recipient (3c, d) 7 days after the inoculation of parental lymph node cells. 3a, b) Note a disorganizaiton of the lymph node architecture with disappearance of the follicles. Paracortex and medullary cords develop well and a marked proliferation of large pyroninophilic cells are observed in paracortex and medulla (3a). Ia^+ cells are relatively evenly distributed in paracortex and medulla of the GvH nodes (3b). 3c, d) Note the comparatively preserved cortex with follicles and the decrease of large pyroninophilic cells in the paracortex and medulla. However, paracortex and medulla of a popliteal lymph node develop as much as those from a CYA-untreated F_1 hybrid rat (3c). Ia^+ cells decrease in density in the paracortex and medulla (3d). F=follicle. Methyl green-pyronin stain (3a, c) or immunoperoxidase staining sections labeled with OX6 (3b, d). Magnification, ×77 (3a, c) or ×53 (3b, d)

Table I Frequencies of various lymphocyte subpopulations in the cervical lymph node cells of normal rats and in the popliteal GvH lymph node cells of CYA-untreated F_1 hybrids and CYA-treated F_1 hybrids at the maximum of the GvH response

Staining	W3/25	OX8	$\frac{W3/25}{OX8}$	OX6
Normal	39.7±0.5	19.7±2.3	2.02	39.1±0.6
Untreated*	24.9±2.9	24.4±1.3	1.02	66.7±1.7
Exp.1A**	28.6±3.0	23.3±1.9	1.23	41.4±1.1

The mean percentages and standard deviations of the cells binding to antibodies are given.

*Popliteal GvH lymph nodes from CYA-untreated (DA×Lou/M) F_1 hybrids 7 days after inoculation of $1\text{-}4\times10^7$ DA rat lymph node cells.

**F_1 hybrids were injected intraperitoneally with CYA of 20 mg/kg body weight/day for 7 days after parental cell transfer.

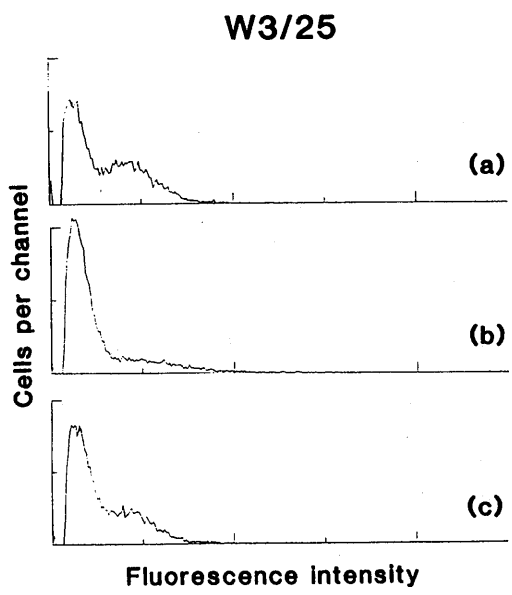

Fig. 4 Fluorescence profiles for W3/25. Profile (a), (b) and (c) represent the specific staining of normal cervical lymph node cells, popliteal GvH node cells from a CYA-untreated F_1 hybrid recipient and those from a CYA-treated F_1 hybrid recipient, respectively.

as dense as those in cervical lymph nodes from normal rats (data not shown).

The representation of lymphocyte subpopulations was determined in popliteal lymph nodes removed 7 days after cell transfer when the nodes were at maximum size (Table I). The percentage of W3/25$^+$ cells was relatively decreased and that of OX8$^+$ cells increased as compared with that of normal cervical lymph nodes, resulting in the decrease of the W3/25 /OX8 ratio. However, the fluorescence profiles for W3/25 and OX8 revealed the relative increase of bright positive cells (Fig. 4, 5). Although a decrease in the percentage of W3/25$^+$ cells was observed, the absolute number of these cells increased because of the enlargement of popliteal GvH lymph nodes in CYA-untreated F_1 hybrids. An increase of OX6$^+$ cells, especially, that of the dull OX6$^+$ cells was also noted (Table I, Fig. 6).

2. GvH lymph node treated with CYA

The extent of GvH reactivity was significantly reduced by CYA administration to F_1

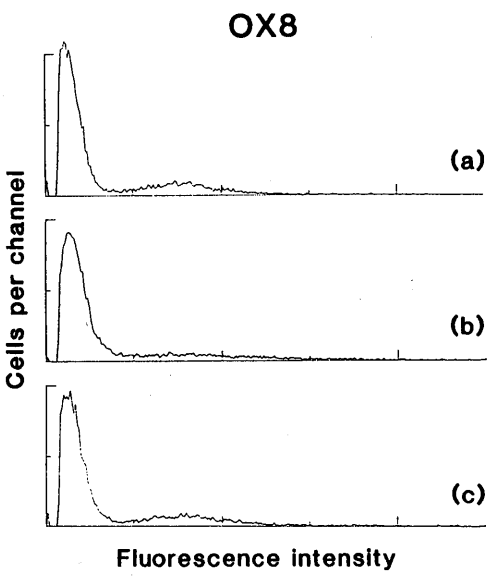

Fig. 5 Fluorescence profiles for OX8. See Fig. 4 for the detail.

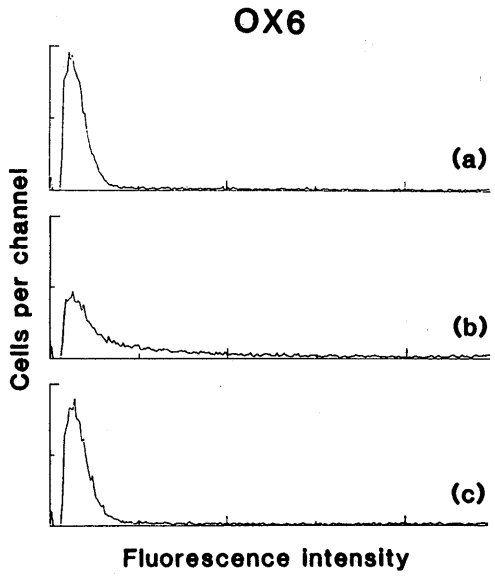

Fig. 6 Fluorescence profiles for OX6. See Fig. 4 for the detail.

hybrid recipients at each cell dose when the treatment started at the time of cell transfer (Fig. 2). As the inoculated cell dose increased from 10 to 40×10^6 lymph node cells, the gradual increase of mean popliteal lymph node weight of CYA-treated F_1 hybrids was clearly observed. However, CYA administration to F_1 hybrids before cell transfer did not suppress GvH reactivity (Fig. 2).

The popliteal GvH lymph nodes from CYA-treated F_1 hybrids resembled the GvH nodes from CYA-untreated F_1 hybrids, although a decrease in size of popliteal lymph nodes and the comparatively preserved cortex were observed (Fig. 3c). Paracortical region showed a decreased number of large pyroninophilic cells as compared with those of CYA-untreated F_1 hybrids. Immunohistochemically, $OX6^+$ cells in both paracortex and medulla decreased (Fig. 3d), but the distribution of the $W3/25^+$ cells and the $OX8^+$ cells changed little, comparing with the GvH nodes from CYA-untreated F_1 hybrids (data not shown).

CYA treatment of F_1 hybrids during the assay for local GvH reaction did not influence on the percentages of $W3/25^+$ cells and $OX8^+$ cells, and the W3/25 /OX8 ratio. However, the proportion of $OX6^+$ cells remained as that in normal cervical lymph nodes (Table I). Fluorescence profiles for W3/25, OX8 and OX6 in the GvH nodes from CYA-treated rats were not like typical fluorescence profiles observed in the GvH nodes from CYA-untreated rats, but rather similar to those of normal cervical lymph nodes (Fig. 4, 5 and 6).

3. In vitro treatment of donor cells with CYA

In vitro incubation of parental donor

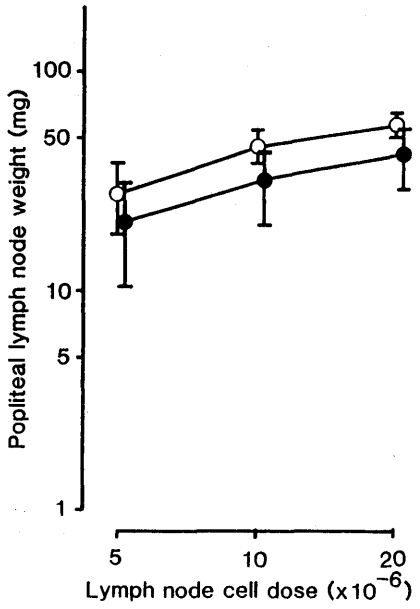

Fig. 7 Popliteal lymph node weights in $(DA\times Lou/M)F_1$ hybrid recipients inoculated with parental lymph node cells. Prior to cell transfer, 2×10^6 parental cells/ml were incubated at 37℃ for 60 min with RPMI 1640 medium alone (○), or with that containing of CYA at the concentration of 100 μg/ml (●). Values are means ± SD obtained from groups of three rats.

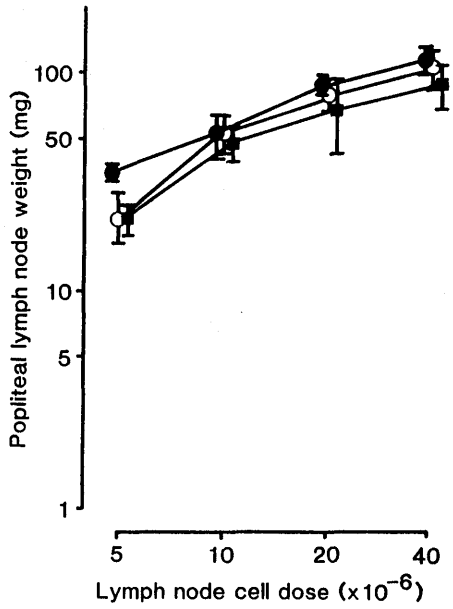

Fig. 8 Popliteal lymph node weights in $(DA\times Lou/M)F_1$ hybrid recipients inoculated with parental donor lymph node cells. DA donors were treated with 20mg of CYA per kg^{-1} given for 7 days before cell transfer from day −14 to day −8 (●), or from day −7 to day −1 (■), or was not treated with CYA (○). Values are means ± SD obtained from groups of four or five rats.

Table II Frequencies of various lymphocyte subpopulations in the cervical lymph node cells of normal rats and CYA-treated DA donor rats

Staining	W3/25	OX8	W3/25 / OX8	OX6
Normal	39.4±0.3	18.5±0.6	2.13	39.1±0.7
Exp. 3A*	36.6±1.6	19.4±1.2	1.89	39.5±3.8
Exp. 3B*	40.4±4.7	17.8±2.4	2.27	39.4±1.0

The mean percentages and standard deviations of the cells binding to antibodies are given.
*DA donors were injected intraperitoneally with CYA of 20 mg/kg body weight/day for 7 days from day −7 to −1 (Exp. 3A) or from day −14 to −8 (Exp. 3B).

lymph node cells with CYA prior to cell transfer into F_1 hybrids did not reduce the local GvH reaction (Fig. 7).

4. Pretreatment of donor rats with CYA

When CYA was given to DA donors before cell transfer, no suppression of GvH reactivity was observed in spite of the different administration schedules (Fig. 8).

To examine the effect of CYA pretreatment of DA donor rats on lymph node cells, the cervical lymph nodes from CYA-treated DA donors were analyzed by flow cytofluorometry. The lymphocyte subpopulations did not alter significantly as compared with those of normal rats (TableII).

Discussion

When CYA was given to F_1 hybrids after parental cell transfer, a marked decrease of mean popliteal lymph node weight was seen, indicating the suppression of local GvH reaction (Fig. 2). However, local GvH reaction was never completely abolished, since slight popliteal lymph node enlargement was induced (Fig. 2). This residual enlargement in the popliteal lymph node from the CYA-treated rats was previously observed by Markwick, *et al*[5].

When CYA was given to F_1 hybrids for 7 days before parental cell transfer, no significant reduction was noted in the popliteal lymph node weight (Fig.2). Indeed, the immunosuppressive effects of CYA are dependent on time of drug administration with respect to immunization[1]. The strongest inhibition of hemagglutinin production was obtained when CYA treatment was started on the time of investigation or up to two days before it, and the treatment received before the immunizing antigen was ineffective[3].

The GvH reactivity was not suppressed when the donor cells were incubated *in vitro* with CYA (Fig. 7). Ryffel and coworkers reported that the local GvH reaction was almost totally suppressed when spleen cells were incubated with CYA[7]. In that experiment, however, a small number of incubated parental cells (3×10^6) were inoculated. Our results indicate that GvH reactivity is not reduced by *in vitro* incubation of parental cells with CYA when a sufficient number of parental cells are inoculated (Fig. 7).

The lymph node cells from CYA-treated donors were able to induce the local GvH reaction in the different schedules of CYA administration (Fig. 8). The lymphocyte subpopulations of the cervical lymph nodes of these donors showed little change as compared with those of normal cervical lymph nodes (Table II). $W3/25^+$ cells which are supposed to be a responsible cell subset for local GvH reaction in the donor's factor did not decrease. This result indicates that the CYA treatment of donors is ineffective for the suppression of GvH reactivity.

In the popliteal lymph node undergoing a GvH reaction, host cells are induced to proliferation in numbers[9]. And there were also distortions of the lymphocyte subpopulations, so that Ts ($OX8^+$) cells were at an advantage

over Th(W3/25$^+$) cells resulting in the decrease of Th/Ts ratio, and the increase of Ia$^+$(OX6$^+$) cells were also noted (Table I). The increase of Ia$^+$ cells may involve not only the huge proliferation of B cells, but also the induction of Ia on Ts cells in GvH nodes[17]. This hypothesis would fit the staining patterns in day 7 GvH nodes with OX6 (Fig. 3b) and OX8 (data not shown), and account for the maintenance of the high OX8$^+$ frequency in GvH nodes (Table I). The increase of Ia expression in the popliteal GvH lymph nodes seems to be a result of the recognition of alloantigen by the inoculated parental donor cells. During GvH disease, Ia expression can occur on cells of the epidermis and the epithelium of the small and large intestine that are not normally considered parts of the immune systems[18),19)]. Their expression of Ia during GvH reaction indicates that the Ia$^+$ cells may take on an antigen-presenting or processing function.

The effects of CYA on distribution of varied lymphocyte subpopulations in the GvH node were the decrease of Ia$^+$ cells in the paracortex and medulla, and the comparative preservation of cortex. Except for these findings, however, the histological distribution in the GvH node from a CYA-treated F_1 hybrid was almost similar to those of GvH nodes from CYA-untreated F_1 hybrid rats (Fig. 3d). Flow cytofluorometric analysis showed that the effect of CYA on the GvH nodes resulted in the decrease of Ia$^+$ cells and the almost unchanged Th/Ts ratio, comparing with those of the normal cervical lymph nodes (Table I). The decrease of Ia$^+$ cells reflects that, in addition to the effect on B cells, CYA may inhibit the development of GvH - induced Ia-positive Ts cells. In the fluorescence profiles for W3/25, OX8 and OX6 (Fig. 4～6), the similar patterns observed in the normal cervical lymph nodes and the GvH nodes from CYA-treated rats may indicate that the activation of both Th cells and Ts cells was suppressed by CYA treatment. However, the relative increase of Ts cells and the change of Th/Ts ratio were not influenced by CYA treatment. These findings of immunohistochemical and flow cytofluorometric analyses indicate the existence of GvH reaction. In conclusion, in rats CYA can suppress the GvH reaction when it is administered to F_1 hybrid recipients at the time of cell transfer, but it can not stop the occurrence of GvH reaction. In clinically transplanted patients, further investigations into the mechanism of action of CYA should be made for the prevention of GvH disease.

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