Histol Histopathol (1997) 12: 337-342

Histology and Histopathology

# Analysis of allogenic lymphocytes in rat thymus following sublethal irradiation

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**Summary.** The effects of allogeneic lymphocytes on the rat thymus following sublethal irradiation were investigated using immunofluorescence. The recovery of thymus weight following irradiation was delayed in rats 6 days after receiving lymphocytes compared to controls. Allogeneic cells forming colonies were detected by immunofluorescence in both the cortex and medulla of the host thymus, most frequently on day 15 when an appropriate number ($3x10^6$) was injected. The allogeneic cells detected in the host thymus, presumably T lymphocytes, appeared to disturb thymic reconstitution following irradiation.

However, double-immunofluorescence staining revealed that allogeneic cells did not affect the thymic stromal microenvironment. Allogeneic cells may have subsequently affected thymic tissue via cytokines.

It is important to investigate not only the character of allogeneic cells in the host thymus but also the interactions of donor allogeneic cells, host immature lymphocytes and thymic epithelial cells because of the possibility that these allogeneic cells in the host thymus could prevent the rejection of allogeneic transplants.

**Key words:** Allogeneic cells, Immunohistochemistry, GVHR, Irradiation, Rat thymus

## Introduction

It is known that rat bone marrow-derived cells injected into irradiated mice remain in the mouse thymus (Surh and Sprent, 1991; El-Ezz et al., 1992; Prakapas et al., 1993) and induce tolerance to xenografts (Ildstad et al., 1992). However, the tissue distribution of allogeneic lymphocytes in the host thymus and their effects on thymic reconstitution following sublethal irradiation of the host have not been well characterized. Regarding this, it would be interesting to determine whether transplanted peripheral lymphocytes can differentiate in the host thymus and induce donor-specific unresponsiveness to allografts.

We have analysed the effects of cyclosporin A (Tanaka et al., 1988), FK506 (Takai et al., 1990, 1991, 1992; Konishi et al., 1994; Tsuchida et al., 1994b,c) and glucocorticoids (Tsuchida et al., 1994a,b) on rat thymus and investigated the process of intrathymic maturation of T cells, using immunoperoxidase staining and flow cytofluorometry. In addition, we have analysed changes induced by the thymic stromal environment in rats following sublethal irradiations, using immunohistochemical techniques (Kuniki et al.,, 1995). We have also reported that lymphocyte infiltration occurs in some areas of the brain during graft-versus-host reactions (GVHR), a finding which may be important in the analysis of autoimmune brain disease (Kajiwara et al., 1991). In this study, we investigated changes in donor allogeneic lymphocytes in the host thymus, their effects on thymic reconstitution in rats undergoing GVHR following sublethal irradiation and whether transplanted peripheral lymphocytes can differentiate in the host thymus.

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## Materials and methods

### Animals

Eight-week-old inbred Lewis (RT-$1^l$) and DA (RT-$1^a$) female rats were obtained from Seiwa Experimental Animals (Fukuoka, Japan). They were maintained in the Institute of Laboratory Animals (Yamaguchi University, School of Medicine) using standard laboratory conditions with free access to food and water.

### Antibodies

We used murine monoclonal antibodies directed against rat MHC-encoded antigens and leukocyte surface markers as shown in Table 1. HAM2 (Fukumoto et al., 1984) is directed against MHC class I antigens. I1.69 cross-reacts with Lewis-specific MHC class I antigens, but not with those of DA rats (Kimura and Willson, 1984). OX6 is directed against MHC class II antigens (McMaster and Williams, 1979). OX19 is directed against CD5 (Dallman et al., 1984), OX8 against CD8

**Table 1.** Monoclonal antibodies used in this study.

| ANTIBODY | SPECIFICITY |
|---|---|
| HAM2 | MHC class I (Fukumoto et al., 1984) |
| I1.69 | MHC class I (positive for Lewis, negative for DA) (Kimura and Willson, 1984) |
| OX6 | MHC class II, Ia-A (McMaster and Williams, 1979) |
| OX19 | CD5, peripheral T, thymocytes (Dallman et al., 1984) |
| OX8 | CD8 (Brideau et al., 1980) |
| OX35 | CD4 (Jefferies et al., 1985) |
| HAM4 | Hepato-renal membrane antigen (Tamakoshi et al., 1985) |
| W3/13 | Leukosialoglycoproteins (CD43), peripheral T, thymocytes (Williams et al., 1977) |
| OX2 | Thymocytes, B cells, dendritic cells (Barclay, 1981) |
| OX7 | Thy-1.1 (Mason and Williams, 1980) |
| OX39 | CD25, IL-2 receptor (Paterson et al., 1987) |
| R73 | TCRαß (Hünig et al., 1989). |

(Brideau et al., 1980), OX35 against CD4 (Jefferies et al., 1985) and W3/13 against leukosialoglycoprotein which has recently been designated CD43 (Williams et al., 1977). HAM4 is directed against hepato-renal membrane antigen (Tamakoshi et al., 1985), OX2 against thymocytes, B cells and dendritic cells (Barclay, 1981), and OX7 against Thy-1.1 (Mason and Williams, 1980). OX39 is directed against the α chain of interleukin-2 (IL-2) receptor, which has recently been designated CD25 (Paterson et al., 1987). R73 is directed against αß T-cell antigen receptors (TCRαß) (Serotec, Oxon, UK). In addition to these monoclonal antibodies, anti-laminin rabbit polyclonal antibodies (Medac, Gesellschaft für klinische Spezialpräparate mbH, Humburg) and anti-keratin rabbit polyclonal antibodies (Medac, Gesellschaft für klinische Spezialpräparate mbH, Humburg) were used.

As secondary antibodies, horseradish peroxidase (HRP)- or fluorescein isothiocyanate (FITC)-conjugated goat $F(ab')_2$ anti-mouse IgG (Cappel Laboratories, Inc., West Chester, PA, USA) and rhodamine isothiocyanate (RITC)-conjugated goat anti-rabbit IgG (Amersham, U.K.) were used at concentrations of 10-20 µg/ml.

### Preparation of lymphocytes

Cervical and mesenteric lymph nodes from Lewis rats were gently teased apart on a stainless steel mesh, and cells were suspended in saline after washing three times. Viability of cells was assessed by trypan blue exclusion; more than 90% of the cells obtained were viable.

### Induction of graft-versus-host reactions (GVHR)

Lymphocytes ($3x10^6$) from Lewis rats were injected intravenously into sublethally irradiated DA rats (6 Gy, X-ray irradiation system, MBR-1520R, Hitachi Medical Co. Ltd., Tokyo, Japan). The development of GVHR was confirmed by observation of weight loss, decreased activity, diarrhea, alopecia, irritability, and erythema in the ears and paws.

### Experimental protocol

Rats were killed by ether anaesthesia. The thymuses were removed from three rats for immunohistochemical staining on days 0, 2, 4, 6, 8, 10 and 15 following induction of GVHR. Irradiated DA rats of the same age were examined on the same days as controls. Day 0 rats were killed prior to lymphocyte infusion.

### Immunoperoxidase staining

Frozen sections were air-dried for 30 min and fixed in methanol at 4 °C for 10 min. After rinsing in phosphate-buffered saline (PBS), the specimens were exposed to 100 µl of primary antibody for 60 min at room temperature. After further rinsing in PBS, the sections were then incubated with HRP-conjugated goat $F(ab')_2$ anti-mouse IgG for 60 min at 4 °C. After another rinse in PBS, sites binding HRP-conjugated antibody were developed with 0.05% diaminobenzidine tetrahydrochloride in 0.025M Tris-HCl (pH 7.6) containing 0.01% $H_2O_2$.

### Immunofluorescence staining

Methanol-fixed sections were reacted with primary antibody for 60 min at room temperature. After rinsing in PBS, the sections were incubated with FITC-conjugated goat $F(ab')_2$ anti-mouse IgG for 60 min at 4 °C. Double immunofluorescence staining was performed using I1.69 and anti-laminin rabbit antibody followed by FITC-conjugated goat $F(ab')_2$ anti-mouse IgG and RITC-conjugated goat anti-rabbit IgG. FITC and RITC were detected using a fluorescence microscope (Nikon XF-EF, Tokyo, Japan). Normal rabbit serum was used as a control for primary antibodies.

## Results

### Clinical signs to determine GVHD

The recipients gradually lost activity and their skin developed erythema, seen best in the ears and paws on days 7-9.

### Changes in thymus mass

A marked decreased in thymus mass was observed in irradiated and GVHR rats on day 2 (Fig. 1), after which thymus mass gradually increased. In GVHR rats, thymus mass did not increase after day 6.

### Relationship between the number of allogeneic cells and host response

When $3x10^6$ allogeneic cells were injected, two out of three rats were alive on day 10 (Table 2), and

**Table 2.** Relationship between number of allogeneic cells injected and host response on day 10.

| RAT No. | INJECTED CELL NUMBER | SURVIVAL STATUS | BW (g) | TW (mg) | ALLOGENEIC CELLS PRESENT IN THYMUS |
|---|---|---|---|---|---|
| 1 | $2x10^7$ | dead | 128 | NE | NE |
| 2 | $2x10^7$ | dead | 133 | NE | NE |
| 3 | $3x10^6$ | dead | 132 | NE | NE |
| 4 | $3x10^6$ | alive | 131 | 39 | (+) |
| 5 | $3x10^6$ | alive | 142 | 136 | (+) |
| 6 | $2x10^6$ | alive | 142 | 191 | (-) |
| 7 | $2x10^6$ | alive | 146 | 140 | (-) |
| 8 | $1x10^6$ | alive | 140 | 152 | (-) |
| 9 | $1x10^6$ | alive | 146 | 155 | (-) |

BW: body weight; TW: thymus weight; NE: not examined.

allogeneic cells were detected in their thymuses. Allogeneic cells were not detected on day 10 when $2x10^6$ or $1x10^6$ cells were injected (Table 2). In contrast, host rats died prior to day 10 when $2x10^7$ cells were injected (Table 2).

### Immunohistochemical analysis of the thymus

Frozen thymus sections from irradiated and GVHR rats examined at different times were stained with I1.69 (anti-donor specific MHC class I). In GVHR rats, the size and cellularity of both the medulla and cortex were greatly reduced and no I1.69$^+$ (allogeneic) cells were detected on day 2 (Fig. 2A). Allogeneic cells were detected on day 2 only in the parathymic lymph nodes (Fig. 2B); these were also HAM4-positive when compared on serial sections. Allogeneic cells forming clusters were detected in the cortex and perivascular areas of the thymus from GVHR rats on day 4 and were detected more frequently on day 6. On day 8, allogeneic

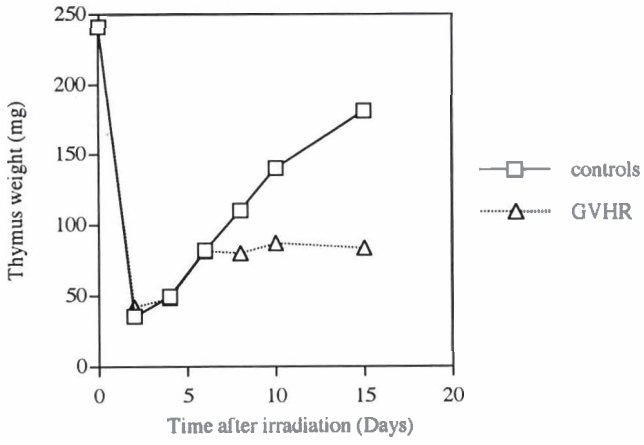


**Fig. 1.** Changes in thymus mass of irradiated control and GVHR rats. Thymus weights (mg) of control (square) and GVHR (triangle) rats were plotted against time after irradiation (days). Thymus mass was markedly decreased in both groups on day 2, after which it gradually increased in controls but did not increase after day 6 in GVHR rats.

cells were detected in the thymic cortex, cortico-medullary zone and perivascular areas, with a frequency similar to that for day 6. On day 10, allogeneic cells forming colonies frequently appeared in the thymic medulla and cortico-medullary zone and less frequently in the cortex (data not shown). On day 15, allogeneic cells were detected more frequently than on day 10. Colony formation on day 15 was observed in the thymic medulla (Fig. 2C) and more frequently in the cortex (Fig. 2D). In contrast, no I1.69$^+$ cells were detected at any time-point in thymuses from control irradiated rats that had not received donor lymphocytes (data not shown).

Double immunofluorescence staining with I1.69 and anti-laminin antibody (Fig. 2C,D) or anti-keratin antibody revealed that allogeneic cells did not affect reticular epithelial cells at any time-point examined when compared to controls (Fig. 2E).

In GVHD rat thymus, OX35$^+$ thymocytes were observed in both medulla and cortex and OX8$^+$ thymocytes were observed mainly in the cortex, but less in the medulla. The staining with OX2 was observed mainly in the medulla and less in the cortex, but there was no apparent difference in staining between control and GVHR rat thymuses using these antibodies as well as OX6, OX19, W3/13, OX7, OX39 and R73 (data not shown).

## Discussion

In general, the blood-thymic barrier prevents intrathymic invasion by foreign cells (Marshall and White, 1961). In this study, however, allogeneic cells were detected in the host thymus by immunofluorescence. This invasion of allogeneic cells was probably due to destruction of the blood-thymic barrier by irradiation and/or GVHR, similar to the mechanism of cerebral invasion by allogeneic lymphocytes due to destruction of the blood-brain barrier by GVHR (Kajiwara et al., 1991). Normally, entrance of cells through the named transcapsular route occurs (Nieuwenhuis et al., 1988). Subsequently, another hypothesis is that entrance of the allogeneic cells through the named transcapsular route might frequently occur under GVHR.

We have analysed the effects of irradiation on cell-surface antigen expression by rat thymocytes and found that the percentage of CD4-CD8-TCR$\alpha\beta^{high}$ cells increases during thymic reconstitution following irradiation. We have also observed defects in the maturation of CD4$^+$CD8$^+$ cells to CD4$^+$CD8$^-$ cells and of TCR$\alpha\beta^{low}$ MHC class I$^+$ to TCR$\alpha\beta^{high}$ cells following FK506 administration (Konishi et al., 1994). We have observed significant increases in the percentages of CD4$^-$CD8$^-$TCR$\alpha\beta^-$ cells and CD4$^-$CD8$^-$ TCR$\alpha\beta^-$ cells following glucocorticoid treatment by three-color flow cytofluorometry analysis (Tsuchida et al., 1994a). Furthermore, we have observed extensive UB13 antigen expression, localized to reticular epithelial

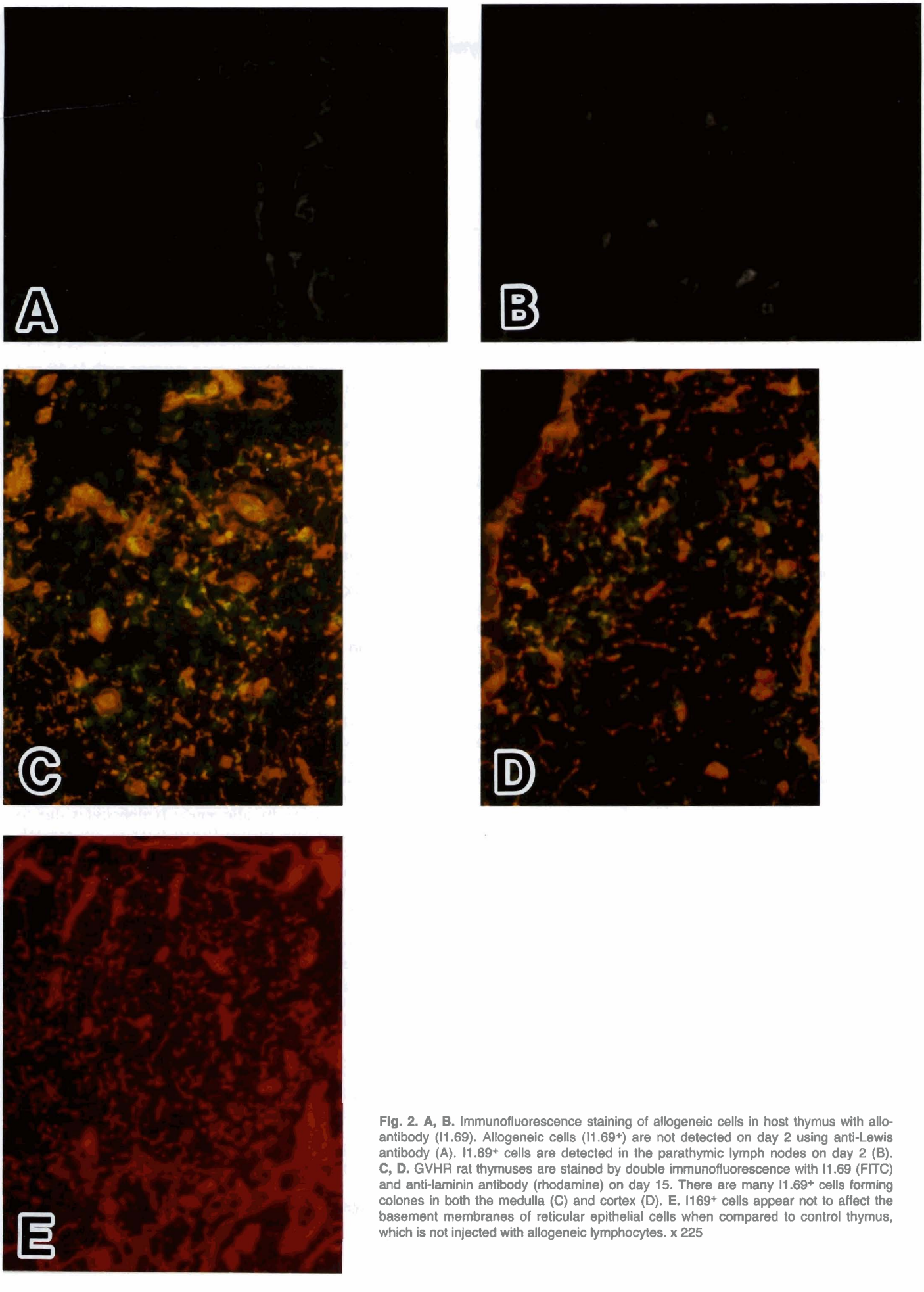


**Fig. 2. A, B.** Immunofluorescence staining of allogeneic cells in host thymus with alloantibody (I1.69). Allogeneic cells (I1.69+) are not detected on day 2 using anti-Lewis antibody (A). I1.69+ cells are detected in the parathymic lymph nodes on day 2 (B). **C, D.** GVHR rat thymuses are stained by double immunofluorescence with I1.69 (FITC) and anti-laminin antibody (rhodamine) on day 15. There are many I1.69+ cells forming colones in both the medulla (C) and cortex (D). **E.** I169+ cells appear not to affect the basement membranes of reticular epithelial cells when compared to control thymus, which is not injected with allogeneic lymphocytes. x 225

tissues, in the thymuses of recovering irradiated rats, with reduction and massive proliferation of thymocytes (Kuniki et al., 1995).

Thus, we have reported that glucocorticoids and FK506 influenced the expression of surface markers of regenerating thymocytes. Although the allogeneic cells did not influence the expression of surface markers of regenerating thymocytes, our data suggest that they apparently influenced regenerating thymocytes and the stromal environment during thymic reconstitution following sublethal irradiation, because the recovery of thymus weight following irradiation was slowed in GVHR rats when compared to irradiated controls. However, expression of surface markers of allogeneic cells was not distinguishable from host thymocytes. Similarly, thymic cellularity of allogeneic cells was not distinguishable from that of host thymocytes, even though the change of total thymic cellularity in both groups paralleled the change of thymus mass. Subsequently, allogeneic cells and host thymocytes should be examined separately for expression of surface marker and cellularity. Further detailed examination is required in the future.

Double immunofluorescence staining revealed that the allogeneic cells did not affect reticular epithelial cells. Nevertheless, it has been reported that GVHR results in damage to the epithelial and lymphoid compartments of the thymus, and abnormal maturation and function of thymocytes in the host (Seemayer et al., 1977; Potworowski et al., 1979; Seddik et al., 1980). Thus, donor lymphocytes might disturb thymic recovery by secreting Th2 cytokines, such as IL-4 and IL-13 (Saoudi et al., 1995; Stumbles and Mason, 1995), in addition to decreasing IL-2 production in the GVHR-immunosuppressed host (Mendes et al., 1985).

There are some reports on the events occurring during reconstitution of the SCID mouse thymus following xenogeneic bone marrow cell transplantation (Surh and Sprent, 1991; El-Ezz et al., 1992; Prakapas et al., 1993). According to these studies, irradiated thymic lobes allowed the development of bone marrow-derived cells, mainly in the medulla, with a few positive cells scattered in the cortex. These could differentiate in the host thymus. In our study, allogeneic cells were detected in both the cortex and medulla, with a higher frequency in the former. If allogeneic cells detected in the host thymus originate from bone marrow cells, they could proliferate in the host thymus only when an appropriate number ($3x10^6$) were injected, since these cells were not detected when less than $2x10^6$ were injected and all host rats died when $2x10^7$ cells were injected (Table 2).

Because there are only a few stem cells in peripheral lymph nodes (12 cells/mouse) (Metcalf and Moore, 1971), and we injected peripheral lymphocytes rather than allogeneic bone marrow cells into irradiated hosts, the possibility exists that the allogeneic cells detected in the host thymus originated from mature lymphocytes. If allogeneic lymphocytes were allowed to develop in the irradiated host thymus, rejection of allogeneic transplants may not take place, as immunological unresponsiveness to transplanted allogeneic cells could be induced by intrathymic injection of these cells (Oluwole et al., 1993; Sherburne et al., 1993). We are now undertaking the analysis of the immune responsiveness of allogeneic cells to the host, and phenotypic characterization of the allogeneic cells detected in the host thymus by flow cytofluorometric and immunoelectron microscopic methods. It would also be interesting to investigate interactions between donor allogeneic cells, immature host cells and thymic reticular cells.

Acknowledgements. The authors thank Dr. Kimura (Department of Research Surgery, National Children's Medical Research Center, Tokyo, Japan) for donation of several monoclonal antibodies, and Dr. Konishi and Dr. Tsuchida (Department of Urology, Yamaguchi University School of Medicine, Ube, Japan) for serious discussion.

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Accepted September 30, 1996