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Differentiation and maturation of functional T lymphocyte subsets in the thymus. II. Generation of T cell specificities and functions from a single stem cell.

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SUMMARY

Functional differentiation of T cells in the thymus from a single stem cell was investigated by transferring bone marrow cells dirrectly into the thymus (i.t.) of irradiated and reconstituted mice, and assaying the immunologic function of donor drived T cells. To assure the clonality of donor derived T cells, bone marrow cells from two mutually indentifiable strains were 1 : 1 mixed and the limited number of the mixture was transferred i.t. into the recipient mice. As the donor of bone marrow cells, C57BL/6 (H-2^b, Thy-1.2, Lyt-2.2) and B6. Lyt-2.1 (H-2^b, Thy-1.2, Lyt-2.1) mice were used, and B10. Thy-1.1 (H-2^b, Thy-1.1, Lyt-2.2) mice were used as the recipients. Recipient mice were either thymus-shielded and irradiated or whole-body irradiated and reconstituted with 10^7 syngeneic bone marrow cells. The recipient's thymus which contained Thy-1.2^+ cells on day 28 were regarded as positive for donor derived cells, and among these positive thymuses those which contained either Lyt-2.1^+ cells or Lyt-2.2^+ cells were regarded as seeded by a single stem cell (single positive thymus cells). Such single positive thymus cells were treated with anti-Thy-1.1 plus complement to remove recipient-type T cells and the recovered cells were assayed for proliferative response and cytotoxic response in mixed lymphocyte culture against allogeneic lymphocytes. Cells were also assayed for concanavalin A-mediated polyclonal helper T cell (Th) and cytotoxic T cell (CTL) functions.

Results indicated that proliferative response to alloantigens was observed only in thymus-shielded recipients, whereas CTL to alloantigens was generated in both thymus-shielded recipients and whole-body irradiated recipients. No general rule of preference for any allogeneic cells was found. Both Th and CTL activities, detectable by polyclonal assays, were generated from a single stem cell in thymus-shielded recipients. However, only CTL but not Th was generated in whole-body irradiated recipients. Moreover, a clone of T cells which responded with CTL to a given allogeneic cells did not necessarily respond with a marked proliferation to the same stimulator cells.

Abbreviations C; complement Con A; concanavalin A CTL; cytotoxic T cells FCS; fetal calf serum FITC; fluorescein isothiocyanate MEM; Eagle's minimum essential medium MLC; mixed lymphocyte culture MLR; mixed lymphocyte response PE; phycoerythrin PHA-M; phytohemagglutinin-M TCGF; T cell growth factor Th; helper T cells

INTROCUCTION

The thymus has been implicated as the site where clonal diversification of T cells occurs and where the T cell functions first appear (1-4). Self-tolerance and major histocompatibility complex (MHC)-restriction of T cells are shown to be mainly attributed to the selective processes in the thymus (1, 5-8). Helper T cells (Th) recognize antigen in the context of self Ia molecules and carry the $L3T4^{+}Lyt\text{-}2^{-}$ phenotype, while cytotoxic T cells (CTL) recognize antigen in association with H-2K/D molecules and possess the $L3T4^{-}Lyt\text{-}2^{+}$ phenotype (9-12). Although it is known that T lymphocytes are derived from extrathymic precursors or stem cells (13-16), it is unclear whether these subpopulations of T cells are produced from a common precursor or whether all T cells covering whole repertoire of antigen specificity are generated from a single precursor. In order to elucidate these problems, it is necessary to establish an experimental system which permits a single precursor of T cells to enter the thymus and undergo a series of differentiational and maturational events in the thymic microenvironment.

Using intrathymus (i.t.) transfer of stem cell source to irradiated recipients, we have recently established a novel experimental system that allows a quantitative analysis of stem cell sctivity for T cell lineage (17). By using this system together with in vivo limiting dulution assay, we could quantitatively estimate the frequencies of stem cells for T cell lineage in various organs (manuscript in preparation). These analyses revealed that the frequency of stem cells in the adult bone marrow was calculated to be about $1/8\times10^{3}$ cells.

In the present study, taking advantage of such i.t. transfer of limiting number of bone marrow cells, we attempted to investigate the generation of T cell functions from a single stem cell. To ensure the clonality of the donor-type T cells, limiting numbers of an 1 : 1 mixture of bone marrow cells from B6.Lyt-2.1 and B6(Lyt-2.2) mice were i.t. transferred into Thy-1 congeneic B10.Thy-1.1 recipient mice, which enabled us to discriminate the origin of donor type cells in recipient's thymus. For functional assay, highly sensitive Con A-mediated polyclonal helper and cytotoxic activity assays (18, 19) as well as conventional mixed lymphocyte culture (MLC)-induced proliferative and cytotoxic responses were used. As the recipients of donor cells, thymus-shielded and irradiated mice as well as whole-body irradiated mice were used. The results showed that both helper and cytotoxic T cell subsets were generated from a single stem cell. Consistent with the results obtained in i·v. transfer system (19), generation of Th subsets in the thymus was shown to be markedly retarded by the irradiation on the thymus. Our results also suggest that generation of T cell specificity repertoire from a single precursor seems to occur at random.

MATERIALS AND METHODS

Mice

C57BL/6(B6) ($H\text{-}2^{b}$, Thy-1.2, Lyt-2.2) and BLAB/c ($H\text{-}2^{d}$) mice were purchased from Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). AKR/J($H\text{-}2^{k}$) and SJL/J($H\text{-}2^{s}$) mice were obtained from Funabashi Farms Co. (Chiba, Japan). B10.Thy-1.1 ($H\text{-}2^{b}$, Thy-1.1, Lyt-2.2) strain (20) was established and kindly donated by Dr. T. Sado (National Institute of Radiological Sciences, Chiba, Japan). B6.Lyt-2.1($H\text{-}2^{b}$, Thy-1.2,

Lyt-2.1) mice were a gift from Dr. K. Kuribayashi (Kyoto University, Kyoto, Japan). These strains were bred and maintained in our animal facility, and mice were used at 10 to 12 weeks of age. Both male and female mice were used, and cell transfer was carried out only in sex-matched combinations. Irradiated mice were given acidified, chlorinated water (PH 3.0).

Reagents

Concanavalin A (Con A), phytohemagglutinin-M (PHA-M), and peanut agglutinin (PNA) were obtained as described in the previous paper(19). Fluorescein isothiocyanate (FITC) was purchased from Nordic Immunology (Tilburg, Netherland) and R-phycoerythrin(PE) was from Molecular Probes, Inc. (Junction City, OR).

Antibodies

Hybridoma cell lines secreting anti-Thy-1.1(T-11/D7) and anti-Thy-1.2(HO-13-4) antibodies were donated by H. Ishikawa (Keio University, Tokyo, Japan). The ascitic fluid of monoclonal anti-Lyt-2.1 antibody producing hybridoma (HO-2.2) (21) was donated by Dr. K. Kuribayashi (Kyoto University). Hybridoma cell line producing anti-Lyt-2.2 antibody was established by Dr. E. Nakayama (Nagasaki University, Nagasaki, Japan) and the ascitic form of this antibody was donated to us. The methods for purification of these antibodies and the conjugation of fluorescein isothiocyanate(FITC) to the antibodies were perfomed as previously described (22). Conjugation of R-phycoerythrin (PE) to anti-Thy-1.2 antibody was perfomed according to the method by Oi et al. (23), and PE-conjugated antibody was further purified from unconjugated antibody and free PE by high-pressure liquid chromatography. Goat anti-mouse μ-chain specific antibody was prepared as previously descrived (24).

Cell suspensions

Bone marrow cells from B6, B6.Lyt-2.1 or B10.Thy-1.1 mice were prepared as described in the previous paper(22). These cells were routinely treated with appropriate anti-Thy-1 antibody (anti-Thy-1.2 for cells from B6 and B6.Lyt-2.1 mice and anti-Thy-1.1 for cells from B10.Thy-1.1 mice) and complement (C) to remove T cells from these cell sources as previously described (22). The preparation of thymus cell suspension and the fractionation of thymus cells with PNA were described in the previous paper (18). For functional assay, thymus cells from the recipient mice were treated with 1 : 400 dilution of ascitic fluid of anti-Thy-2.2 antibody plus C (1 : 10 dilution of selected rabbit serum) and the same treatment was repeated once in order to ensure the killing of host derived cells. Spleen cell suspension and B cell enriched fraction of spleen cells were prepared as described previously (18).

Irradiation of mice and cell transfer

Whole-body irradiation (800R) of mice with ^{60}CO γ-ray, and X-ray irradiation (800R) of thymus-shielded mice were performed as described previously (22). The intrathymus (i.t.) injection of donor bone marrow cells was performed as follows. Whole body irradiated mice or thymus-shielded and irradiated mice were anesthetized by intraperitoneal injection of sodium pentobarbital (10 μg/g body weight) and were settled on a board with rubber bands. The mediastinum was opened and 12.5 μl of cell suspension was injected into each lobe of the thymus by using a tuberculin syringe with a 1/6 mm needle. The whole-body irradiated recipients were transferred i.v. with 10^7 syngeneic bone marrow cells to protect from the radiation death. The

thymus-shielded and irradiated mice were not transferred with host bone marrow cells, since these mice can survive without reconstitution (22).

Fluorescence staining and fluorescence-activated cell analysis

For staining thymus cells, all treatments were carried out in ice-cold Eagle's minimum essential medium (MEM, Nissui Seiyaku Co. Tokyo, Japan) containing 2% of fetal calf serum (FCS, M. A. Bioproducts, Walkersville, MA). Staining thymocytes with FITC conjugated anti-Thy-1.2 (FITC-anti-Thy-1.2) antiboy and flow cytofluorometric analysis of cells on a Ortho Spectrum III Laser Flow Cytometry System (Ortho Diagnostic Systems, Westwood, MA) were as described in the previous paper (22).

Two color staining of thymus cells with PE-anti-Thy-1.2 plus FITC-anti-Lyt-2.1 or with PE-Thy-1.2 plus FITC-anti-Lyt-2.2 was performed as follows. Samples of thymus cell suspensions (5×10^5 cells in 50 μl MEM) were mixed with 10 μl of PE-anti-Thy-1.2 (100 μg/ml) plus 10 μl of FITC-anti-Lyt-2.2 (100 μg/ml). They were incubated for 30 min at 4°C, washed twice, and the cells were suspended in 1 ml MEM. The stained cells were analysed on a Ortho Spectrum III equipped with Model 2140 Data Handling System (Ortho Diagnostic systems Inc.). Stained cells were excited by 488 nm Argon-ion laser beam. To assure data collection only on viable cells, dead cells were excluded by the gating window on a two-parameter (forward vs side scatter) histogram. Fluorescence emitted from FITC (green) or PE (red) was detected by photomultipilier-tube after passing through a 515–530 nm band pass filter (for green fluorescence) and by a 562–580 nm band pass filter (for red fluorescence). Fluorescence intensity was converted to logarithmic scale by the log amplifiers, and leakage of fluorescent light to the other dye region was electrically compensated. Two parameter (green vs red) histogram of sample cells was obtained.

Limiting dilution analysis of stem cells for T cell lineage

In vivo limiting dilution analysis of stem cells for T cell lineage was performed as follows. Bone marrow cells from B6 mice were mixed 1 : 1 with bone marrow cells from B6.Lyt-2.1 mice. Three or six thousand cells of this mixture were transferred i.t. into thymus-shielded and irradiated B10.Thy-1.1 mice or whole-body irradiated B10.Thy-1.1 mice. Four weeks later, thymuses of the recipiet mice were assayed for the Thy-1.2 bearing cells. Cell samples which contained Thy-1.2$^+$ cells more than 0.1% were regarded as positive for donor-derived cells as described previously (22). The estimation of stem cell frequency was based on the Poisson probability distribution, that is $u=-\ln Fo$, where Fo is the fraction of negative recipients and u is the average number of stem cells in the transferred cell suspension. According to this formula, the inoculum that produces 37% negative recipients can be expected to contain one stem cell.

Functional assay of thymus cells

Thymus cells of the recipients which contained Thy-1.2$^+$ T cells derived from only one of the two donors were selected and were treated two times with anti-Thy-1.1 plus C to remove the host derived cells.

Proliferative response to allogeneic spleen cells (mixed lymphocyte reaction, MLR) was measured as follows. Thymus cells (2×10^5) were cultured with 7.5×10^5 3300R X-irradiated spleen cells of B6, BALB/c, AKR/J or SJL/J mice. On day 5, 0.5 μCi tritiated thymidine

(^{3}H-TdR) was added to the culture and cells were harvested after 10 hours of incubation. Incorporation of ^{3}H-TdR was determined by a liquid scincillation counter.

CTL activity of tymus cells against allogeneic targets was measured as follows. Responder thymus cells (5×10^4) were stimulated with 3300R X-irradiated syngeneic (B6) as well as allogeneic (BALB/c, AKR/J or SJL/J) spleen cells (5×10^5) in the presence of rat TCGF (10%) as a source of helper factor. On day 7, 10^4 of ^{51}Cr-labeled target cells (Con A-induced blasts of the spleen cells from the same strain as used for the stimulators) were added to each well and further incubated at 37°C. After 5 hours of incubation, aliquots of culture supernatant were harvested and counted in a γ-counter. Percent specific cytotoxicity was calculated using the formula: % specific cytotoxicity = (experimental cpm-back ground cpm)/(total release Cpm-back ground cpm) $\times100$.

Highly sensitive and efficient polyclonal Th and CTL assays mediated by Con A was performed as described in the previous paper (19). In Th assay, polyclonal immunoglobulin (Ig) production was assayed as the index of helper activity. For detection of Ig production, solid phase radioimmunoassay was used to measure the IgM production by B cells. Briefly, serialy diluted samples of culture supernatant were added to wells of polyvinyl chloride plates (Falcon 3911, Becton Dickinson Labwre, Oxnard, CA) which was precoated with goat anti-mouse μ chain specific antibody. Wells were then developed with ^{125}I-labeled goat anti-mouse μ chain antibody and the radioactivity bound was counted in a γ-counter. Concentration of IgM in culture supernatant was determined by comparing with a standard curve obtained in myeloma protein 267 ($\mu\lambda$).

RESULTS

Estimation of stem cell frequency and selection of the thymuses which contain T cells probably derived from a single stem cell of donor origin

We have established a method to determine the frequency of stem cell for T cell lineage, and by using this method estimation of the stem cell frequency in various organs has been carried out (paper in preparation). In this work, B10.Thy-1.1 mouse was used as the donor, and the cells from this mouse was transferred i.t. into B6 recipient mice which had been 800R whole-body irradiated and marrow reconstituted. Stem cell frequency in the donor bone marrow cells was estimated to be $1/8\times10^3$. In the present study, 1 : 1 mixture of bone marrow cells from B6 and B6.Lyt-2.1 mice was used as the source of stem cells. All experiments were carried out by assuming that stem cell frequency in this mixture was the same as that in B10.Thy-1.1 bone marrow cells. Thus, 3×10^3 or 6×10^3 of the mixture was used for i.t. transfer into B10.Thy-1.1 mice, expecting that very limited number of stem cells were included in the transferred cell suspension. Recipient mice were either thymus-shielded and irradiated or whole-body irradiated and marrow reconstituted. Four weeks after the transfer, thymuses were removed and cell suspensions were made. A small portion of cell suspension was assayed for Thy-1.2 antigen. Accumulated data for proportion of negative mice, which do not contain Thy-1.2$^+$ cells, were plotted in Figure 1. This Figure shows a shape of limiting dilution assay, and it is noticeable that the irradiation or non-irradiation on the thymus affected little on the

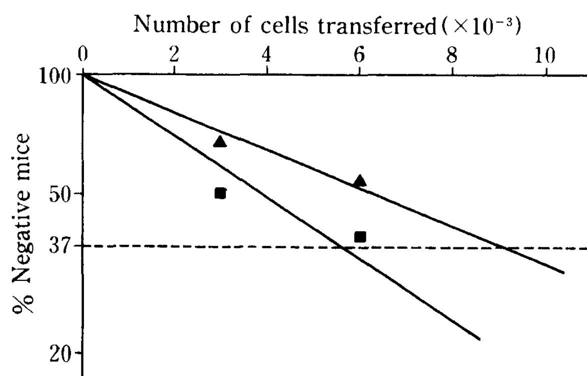

Fig. 1. Limiting dilution analysis of stem cells for T cell lineage. Bone marrow cells from B6 and B6.Lyt-2.1 mice were mixed 1 : 1, and 3×10^3 or 6×10^3 cells of this mixture were transferred i. t. into B10. Thy-1.1 recipients which were either thymus-shielded and 800R irradiated (▲) or whole-body 800R irradiated and reconstituted with 10^7 syngeneic bone marrow cells (■). Thymocytes of the recipient mice were harvested 28 days later, stained with FITC-anti-Thy-1.2, and analysed on a flow cytofluorometer. Thymocytes which contained Thy-1.2$^+$ cells more than 0.1% of total thymocytes were regarded as positive, and accumulated data are shown. In thymus-shielded group, 6 out of 19 mice given 3×10^3 cells and 5 out of 11 mice given 6×10^3 cells were positive for donor-type Thy-1.2$^+$ cells. In whole-body irradiated group, 4 out of 8 mice given 3×10^3 cells and 3 out of 5 mice given 6×10^3 were positive for donor derived T cells. The fractions of negative mice were calculated and semilogarithmically plotted. The best filtting lines were calculated by the least square method. The numbers of cells at 37% negative line were cultulated as 9.0×10^3 in thymus-shielded group and 5.7×10^3 in whole-body irradiated group.

frequency of stem cells. The estimated frequencies, $1/9.0\times10^3$ and $1/5.7\times10^3$in thymus-shielded group and thymus-irradiated group, respectively, are quite similar to the value in B10.Thy-1.1 bone marrow cells (see above).

All the recipients which were shown to be positive for donor-type (Thy-1.2$^+$) T cells were further assayed for Lyt-2.1 and Lyt-2.2 antigens. If a thymus contained T cells positive for either of these antigens but not both, Thy-1.2 positive cells in such a thymus were regarded to be derived from a single stem cell. Samples of thymus cells which were shown to be positive for Thy-1.2$^+$ cells were selected, and a portion of the cell suspension was stained with PE-anti-Thy-1.2 and FITC-anti-Lyt-2.1 or with PE-anti-Thy-1.2 and FITC-anti-Lyt-2.2. These cells were analysed on a flow cytofluorometer, and three examples showing different staining patterns are shown in Figure 2. These recipient mice were recipeient No. 1, 2 and 3 in Table 1, which were thymus-shielded and 800R irradiated, and transferred i.t. with an 1 : 1 miture of bone marrow cells. Cells distributed in lower regions of all panels are the recipient-type T cells which were derived from stem cells saved in the bones of shielded portion. A large proportion of Thy-1.2$^+$ cells of recipient No. 1 (Figure 2, A and B) was also positive for Lyt-2.1 (A) but negative for Lyt-2.2 (B), inidcating that Thy-1.2$^+$ thymus cells in this mouse were derived from B6.Lyt-2.1 stem cell. Reverse was true for recipient No. 2 (C and D), indicating that Thy-1.2$^+$ cells in this mouse were B6 bone marrow origin. On the other hand, Thy-1.2$^+$ cells of recipient No. 3 were stained with both anti-Lyt-2.1 (E) and anti-Lyt-2.2 (F) antibodies,

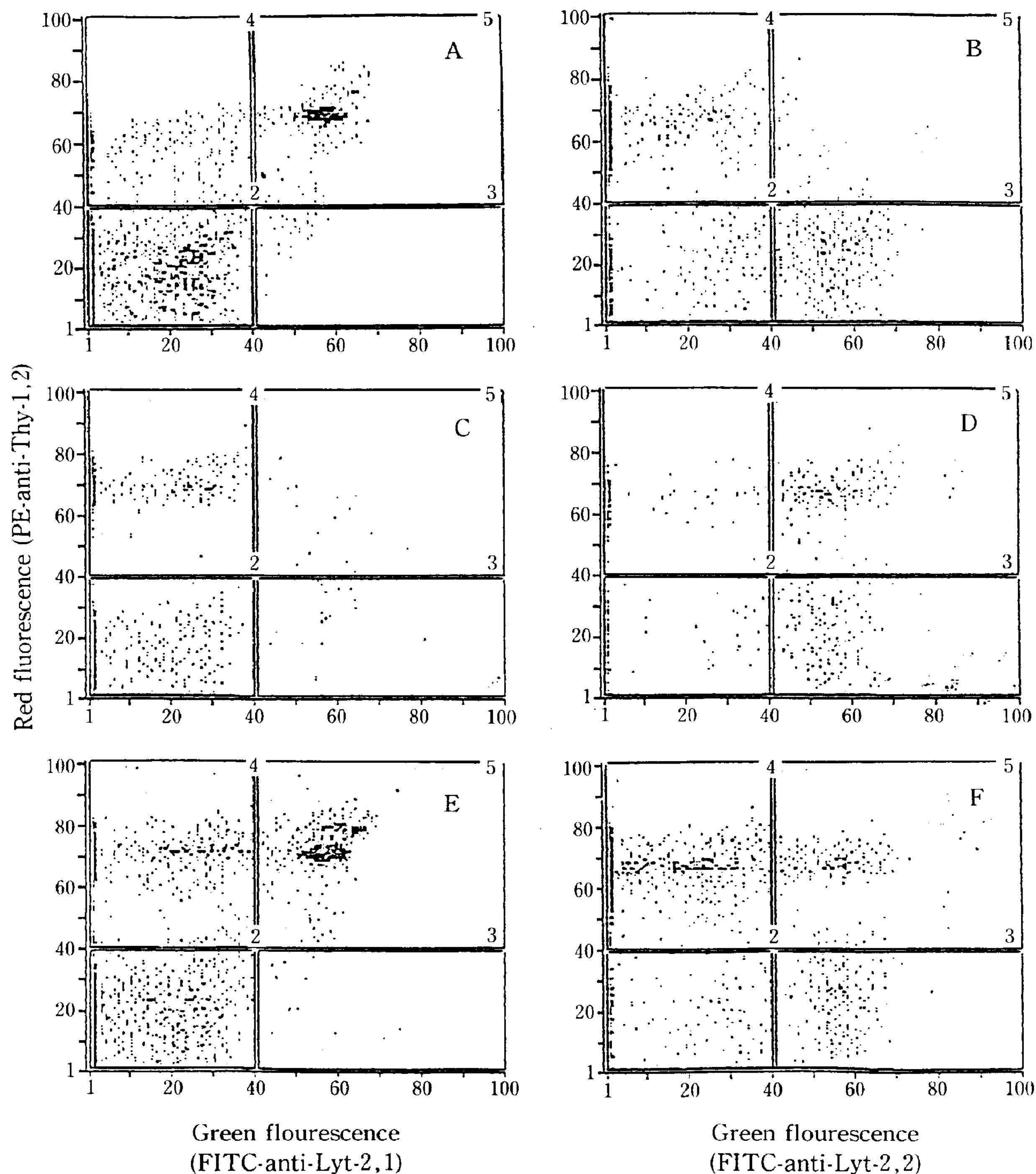

Fig. 2. Two color flow cytofluorometry of donor-type thymus cells which were generated from a limited number of bone marrow cells. Bone marrow cells from B6 and B6.Lyt-2.1 mice were mixed 1:1, and 3×10^3 cells of this mixture were transferred i.t. to thymus-shielded and 800 R-irradiated B10. Thy-1.1 mice. Thymus cells were assayed for Thy-1.2$^+$ cells 28 days later, and cells of positive mice were stained with PE-anti-Thy-1.2 and FITC-anti-Lyt-2.1 or with PE-anti-Thy-1.2 and FITC-anti-Lyt-2.2. Two color analysis of stained cells were performed on a flow cytofluorometer. In this experiment, 5 out of 13 mice were positive for donor type Thy-1.2$^+$ cells, and the characterization of Lyt-2 phenotype of these Thy-1.2$^+$ cells is shwon in Experiment 1 in Table 1. Two parameter histograms of three representative mice (recipient NO. 1-3 in Table 1) are shown. A and B; recipient No. 1, C and D; recipient No. 2, E and F; recipient No. 3.

Table 1. Characterization of donor derived thymus cells in thymus-shielded and 800 R irradiated recipients[a)]

Experiment	Recipient[b)] No.	No. of cells transferred	% donor-type Thy-1.2+ cells	Lyt-2 phenotype of Thy-1.2+ cells	Origin of donor cells	Functional[c)] assay
I	1	3×10³	14.4	2.1+, 2.2−	B6. Lyt-2.1	Yes
	2	〃	16.8	2.1−, 2.2+	B6	Yes
	3	〃	37.4	2.1+, 2.2+	Both	No
	4	〃	12.7	2.1−, 2.2+	B6	Yes
	5	〃	5.3	2.1+, 2.2+	Both	No
	6	6×10³	0.2	n.d.[d)]		No
	7	〃	26.4	2.1−, 2.2+	B6	Yes
	8	〃	0.5	2.1+, 2.2−	B6. Lyt-2.1	No
II	9	3×10³	2.1	2.1−, 2.2+	B6	Yes
	10	6×10³	22.1	2.1−, 2.2+	B6	Yes
	11	〃	10.1	2.1−, 2.2+	B6	Yes

a) Thymus-shielded and 800R irradiated B10.Thy-1.1 mice were transferred i.t. with 3×10^3 or 6×10^3 cells of 1:1 mixture of B6 and B6.Lyt-2.1 bone marrow cells. Four weeks later, thymus cells of recipient mice were assayed for donor derived Thy-1.2+ cells. In Experiment I, 6 out of 13 mice given 3×10^3 cells and 3 out of 7 mice given 6×10^3 cells were positive for donor Thy-1.2+ cells, and in Experiment II, 1 out of 6 mice given 3×10^3 cells and 2 out 4 mice given 6×10^3 cells were positive for donor Thy-1.2+ cells.

b) Recipient number which contained donor Thy-1.2+ cells more than 0.1% of thymocytes.

c) See Table 3 and 4.

d) Not done.

indicating that stem cells from both B6.Lyt-2.1 and B6 donors seeded in the thymus of this mouse.

Other individuals' thymuses in thymus-shielded group and in whole-body irradiated group which contained Thy-1.2+ cells were similorly assayed for Lyt-2.1 and Lyt-2.2 antigens, and the results are summarized in Table 1 and Table 2. In thymus-shielded group, 6 out of 19 recipient mice given 3×10^3 cells and 5 out of 11 mice given 6×10^3 cells were positive for Thy-

Table 2. Characterization of donor derved thymus cells in whole-body 800R irradiated recipients[a)]

Recipient No.[b)]	No. of cells transferred	% donor-type Thy-1.2+ cells	Lyt-2 phenotype of Thy-1.2+ cells	Origin of donor cells	Functional assay[c)]
1	3×10³	7.1	2.1+, 2.2−	B6. Lyt-2.1	Yes
2	〃	18.0	2.1+, 2.2+	Both	No
3	〃	12.1	2.1+, 2.2−	B6. Lyt-2.1	Yes
4	〃	0.3	n.d.[d)]		No
5	6×10³	5.0	2.1−, 2.2+	B6	Yes
6	〃	9.4	2.1+, 2.2−	B6. Lyt-2.1	Yes
7	〃	27.3	2.1+, 2.2−	B6. Lyt-2.1	Yes

a) Whole-body 800R irradiated B10. Thy-1.1 mice were transferred i.t. with 3×10^3 or 6×10^3 cells of 1:1 mixture of B6 and B6.Lyt-2.1 bone marrow cells. Four weeks later, thymus cells of recipient mice were assayed for donor derived Thy-1.2+ cells. In this experiment 4 out of 8 mice given 3×10^3 cells and 3 out of 5 mice given 6×10^3 cells were positive for donor Thy-1.2+ cells.

b) Number of recipient which contained docor Thy-1.2+ cells more than 0·1% of thymocytes.

c) See Table 5 and 6.

d) Not done.

1.2$^+$ cells (Figure 1). As shown in Table 1, among 10 recipient mice which contained a considerable number of donor type cells, 8 were shown to be seeded with the stem cell of either one of two donors. The situation was similar to whole-body irradiated group (Figure 1 and Table 2). These results strongly suggest that the Thy-1.2$^+$ T cells of the recipients which showed the phenotype of either B6 or B6.Lyt-2.1 are the progeny of a single stem cell. Thus, only Thy-1.2$^+$ cells of such recipients were used for functional assays in the following sections.

Generation of functional subsets of T cells in the nonirradiated thymus

Among 11 thymus-shielded and irradiated recipients which were shown to contain Thy-1.2$^+$ cells, 2 were omitted because their Thy-1.2$^+$ cells comprised T cells derived from both B6 and B6.Lyt-2.1 donors, and other 2 were also omitted because the number of thier Thy-1.2$^+$ cells were too small to carry out functional assays (Table 1). Thymocytes of remaining 7 mice were treated twice with anti-Thy-1.1 plus C to remove host derived T cells. The recovered cells were assayed for MLC-induced proliferation (MLR) and CTL responses, and Con A-mediated polyclonal Th and CTL activities. These results are shown in Table 3 and 4. Experiment I in Table 3 indicated that Thy-1.2$^+$ cells of all 4 recipients showed CTL response to all 3 different allogeneic cells, though the reactivity of cells in recipient No. 4 to BALB/c and SJL/J was considerably lower than that of cells from other recipient mice. On the other hand, MLR activity was not observed in all recipients. Recipient No. 1, 4 and 10 showed virtually no resonse to all 3 stimulators used in this experiment. Recipient No. 2 responded to BALB/c and

Table 3. MLR and CTL activities of donor derived thymus cells in thymus-shielded and irradiated recipients[a)]

Experiment	Source of thymus cells	MLR activity (^{3}H-TdR uptake, CPM/well)[b)]					CTL activity (% cytotoxicity)[c)]			
		None	B6	BALB/c	AKR/J	SJL/J	B6	BALB/c	AKR/J	SJL/J
I	None	—	166	274	90	53	—	—	—	—
	Normal thymus	117	137	1206	1265	1479	0	42.3	38.5	25.2
	PNA$^-$ thymus	351	107	1983	3385	2489	0	36.5	53.5	28.8
	Recipient No. 1	450	170	369	211	222	0	38.1	34.2	24.2
	〃 2	501	710	2080	480	2562	0	32.5	67.2	21.5
	〃 4	38	165	160	108	621	0	10.6	28.3	7.1
	〃 7	289	184	201	1213	1754	0	37.7	33.9	26.5
II	None	—	322	458	253	210				
	Normal thymus	104	172	2563	2284	2530				
	PNA$^-$ thymus	327	267	3057	20252	21664				
	Recipient No. 9	335	617	25826	17252	10365				
	〃 10	159	464	407	235	221				
	〃 11	321	2471	4992	2305	1810				

a) Recipient thymus cells are the same as shown in Table 1.
b) Thymus cells (2×10^5) were cultured with 7.5×10^5 3300R X-irraidated spleen cells from B6, BALB/c, AKR/J or SJL/J mice, and 5 days later ^{3}H-TdR uptake was determined.
c) Responder thymus cells (5×10^4) were stimulated with 3300R X-irradiated B6, BALB/c, AKR/J or SJL/J spleen cells (5×10^5) in the presence of rat TCGF (10%). On day 7, % specific cytotoxicity was determined against the same target cells as stimulators.

Table 4 Con A-mediated polyclonal Th and CTL activities of donor derived thymus cells in thymus-shielded and 800 R irradiated recipients[a]

Experiment	Source of thymus cells	Th activity[b] IgM concentration (μg/ml)	CTL activity[c] % specific cytotoxicity
I	None	0.2	—
	Normal thymus	5.1	77.4
	PNA⁻ thymus	6.4	70.2
	Recipient No. 1	5.7	60.0
	〃 2	4.4	81.3
	〃 4	5.2	78.7
	〃 7	5.6	74.9
II	None	0.1	—
	Normal thymus	7.5	57.1
	PNA⁻ thymus	10.1	43.8
	Recipient No. 9	7.3	52.0
	〃 10	0.2	1.3
	〃 11	6.8	32.1

[a] Recipient thymus cells are the same as shown in Table 1.
[b] Thymus cells (10^5) were cultured with 5×10^4 B6 splenic B cells in the presence of Con A (5μg/ml). On day 7, IgM concentration of culture supernatant was measured by solid-phase radioimmunoassay.
[c] Responder thymus cells (5×10^4) were stimulated with Con A (3 μg/ml) in the presence of 3300R X-irradiated B6 spleen cells (5×10^5) and 10% rat TCGF. After 7 days of culture, PHA-dependent polyclonal cytotoxicity against P815 target cells was assayed.

SJL/J but not to AKR/J, whereas recipient No. 7 responded to AKR/J and SJL/J but not to BALB/c. Recipient No. 9 responded to all 3 allogeneic stimulators. Interestingly, cells of recipient No. 11 showed the proliferative response agalinst all stimulator cells including syngeneic one. This may suggest tht self-reactive cells were not eliminated or suppressed in this mouse.

Results of polyclonal Th and CTL assays are shown in Table 4. Cells from all but one recipient mice were active for both Th and CTL, and the levels of activities in these mice were comparable to those of normal thymus cells. Thus, the failure of recipient No. 1 and 4 to respond with proliferation to stimulators used in the experiments shown in Table 3 may not mean the defect in helper T cells. On the other hand, cells of recipient No. 10 did not show any activities even in polyclonal assay.

Absence of helper and proliferative activities in donor-type T cells developed in the irradiated thymus

Among 7 whole-body irradiated and marrow reconstituted recipients which were shown to contain Thy-1.2^+ cells, one was omitted because its Thy-1.2^+ cells comprised with T cells derived from both B6 and B6.Lyt-2.1 donors, and another was also omitted because the number of its Thy-1.2^+ cells was to small (Table 2). Thymocytes of remaining 5 mice were treated twice with anti-Thy-1.1 plus C to remove host derived T cells. The recovered cells were assayed for MLC-induced proliferative and CTL responses, and Con A mediated polyclonal Th and CTL activites. Results are shown in Table 5 and 6. Different from T cells developed in nonirradiated thymus, the Tulls developed in irradiated thymus did not show any MLR activities against 3

Table 5. MLR and CTL activities of donor derived thymus cells in whole-body irradiated recipients[a)]

Source of Thymus cells	MLR activity (^{3}H-TdR uptake, CPM)[b)]					CTL activity (% cytotoxicity)[c)]			
	None	B6	BALB/c	AKR/J	SJL/J	B6	BALB/c	AKR/J	SJL/J
None	—	117	119	191	148	—	—	—	—
Normal thymus	236	391	1541	1267	2483	0	60.9	70.9	46.8
PNA$^-$ thymus	252	324	6261	6338	4563	0	52.9	66.0	46.8
Recipient No. 1	141	141	394	172	408	0	49.1	61.2	23.9
〃 3	139	349	142	96	97	0	13.1	25.4	0.7
〃 5	573	298	832	299	676	0	61.8	71.1	54.9
〃 6	376	176	529	258	312	0	35.7	58.5	14.6
〃 7	179	164	255	81	210	0	0.3	9.7	0

a) Recipient thymus cells are the same as shown in Table 2.
b) Thymus cells (2×10^5) were cultured with 7.5×10^5 3300R X-irradiated spleen cells from B6, BALB/c, AKR/J or SJL/J mice, and 5 days later ^{3}H-TdR uptake was determined.
c) Responder thymus cells (5×10^4) were stimulated with 3300R X-irradiated B6, BALB/c, AKR/J or SJL/J spleen cells (5×10^5) in the presence of rat TCGF (10%). On day 7, % specific cytotoxicity was determined against the same target cells as stimulators.

strains of allogeneic mice (Table 5). Moreover, these T cells showed only marginal, if any, helper activity in Con A-mediated polyclonal assay (Table 6). In contrast, development of CTL was comparable to that in thymusshielded recipients when compared in polyclonal assay (Table 6). However, CTL was not induced against all stimulator cells in MLC. As shown in Table 5, Thy-1.2$^+$ cells of recipient No. 3 responded to BALB/c and AKR/J but not to SJL/J, and cells of recipient No. 7 responded only to AKR/J. These results strongly suggest that a stem cell seeded in irradiated thymus generated only CTL subset in the earlier stage, and that the repertoire diversification of CTL subset is retarded compared to that in non-irradiated thymus.

Table 6. Con A-mediated polyclonal Th and CTL activities of donor derived thymus cells in whole-body 800R irradiated recipients[a)]

Source of thymus cells	Th activity[b)] IgM concentration (μg/ml)	CTL activity[c)] % specific cytotoxicity
None	0.2	—
Normal thymus	2.7	59.6
PNA$^-$ thymus	3.8	52.4
Recipient No. 1	0.5	54.6
〃 3	0.4	56.8
〃 5	0.4	54.9
〃 6	0.4	54.8
〃 7	0.4	55.4

a) Recipient thymus cells are the same as shown in Table 2.
b) Thymus cells (10^5) were cultured with 5×10^4 B6 splenic B cells in the presence of Con A (5μg/ml), On day 7, IgM concentration of culture supernatant was measured by solid-phase radioimmunoassay.
c) Responder thymus cells (5×10^4) were stimulated with Con A (3μg/ml) in the presence of 330R X-irradiated B6 spleen cells (5×10^5) and 10% rat TCGF. After 7 days of culture, PHA-dependent polyclonal cytotoxicity against P815 target cells was assayed.

DISCUSSION

Almost all studies on the development of T cell function published so far were undertaken in radiation chimera which had been transferred i.v. with a large number of bone marrow cells (25–27). In such an experimental system, however, it is difficult to investigate the detailed mechanism involved in the development of T cells in the thymus, since the bone marrow cells contain heterogeneous populations of stem cells and since it is not known how many stem cells in the bone marrow cells transferred i.v. immigrate into the thymus. Present study aimed at investigating the function of thymic environment in the differentiation of Th and CTL subsets from a single stem cell. For this purpose, an experimental system was designed to ensure that the thymus was seeded with only one stem cell.

We have already shown that the frequency of the stem cells for T cell lineage can be determined by transferring limited numbers of cells from marker bearing strain of mice directly into the thymus of H-2 syngeneic mice whih had been whole-body irradiated and reconstituted with bone marrow cells of host strain (paper in preparation). This work was carried out by using B10·Thy-1.1 mouse and B6 mouse as the donor and recipient, respectively. It was also shown that in transferring a limited number of bone marrow cells the progeny was derived from a single stem cell. The minimal frequency of stem cells in the bone marrow of B10.Thy-1.1 mouse was estimated to be $1/8\times10^3$. In the present work, small numbers (3×10^3 or 6×10^3) of the 1 : 1 mixture of bone marrow cells from B6 and B6.Lyt-2.1 mice were used as the source of stem cells, and the mixture was transferred i.t. into B10.Thy/1.1 mice. The use of the mixture of bone marrow cells from mutually identifiable donors enabled us to determine the origin of the stem cell, and only the donor-type thymic T cells which were shown to be derived from one of the two donors were used for functional assays. Since only a limited number of stem cells are included in the donor cell suspenstion, it is most probable that the donor-type Thy-1.2^+ T cells which comprised either B6 or B6.Lyt-2.1 derived cells are the progeny of a single stem cell. In the thymus-shielded and irradiated recipients, it was shown that both helper and CTL subsets of T cells were derived from a single stem cell. This is consistant with the recent finding of Kingston et al. (28) that three distinct phenotypes of T cells, $L3T4^+Lyt\text{-}2^+$, $L3T4^+Lyt\text{-}2^-$ and $L3T4^-Lyt\text{-}2^+$, were generated from a single thymic stem cell in organ cultured fetal thymus. Prsent study further showed that T cells developed from a single stem cell in the shielded-thymus responded with proliferation and production of CTL to three different allogeneic cells in MLC. On the other hand, only CTL subset developed in irradiated thymus, no polyclonal helper activity or MLR activity being observed, although the proportion of $L3T4^+Lyt\text{-}2^-$ cells were similar between whole-body irradiated recipients and thymus-shielded recipients (unpublished data). These results confirmed our previous finding done in i.v. transfer system that intact thymus was required for development of Th but not of CTL (19).

Of interest is the observation that functional maturation and reactivity in MLC of donor derived T cells are heterogeneous among individual recipients. In thymus-shielded recipients, donor derived T cells of 1 out 7 recipients examined did not show any activities even in polyclonal assays (recipient No. 10 in Table 3 and 4), despite the fact that 22.1% of the thymus cells were donor derived cells (Table 1). Among remaining 6 recipients, T cells of two mice did

not show proliferative response to BALB/c, AKR/J or SJL/J, and two clones of T cells responded to two of these allogeneic cells tested (Table 3). Only one clone of T cells (recipient No. 9) responded to all three stimulators without responding to H-2 syngeneic B6 cells. The other clone (recipient No. 11) was unique in that it responded not only to allogeneic cells but to syngeneic B6 cells. Similar heterogeneity of reactivity was also seen in MLC-induced CTL response in whole-body irradiated recipients (Table 5). These results strongly suggest that the clones of T cells investigated in this work are in the sage of functional maturation, repertoire diversification, and self-tolerance. The experimental system used in this paper may provide a hopeful strategy to further investigation of T cell differentiation.

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