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TOLEROGENIC ABILITY OF THYMOCYTES IN ORGAN-CULTURED THYMUS LOBES

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It is generally believed that macrophages and dendritic cells are the major cell populations that present tolerogenic self antigens to developing thymocytes. However, it is still controversial whether self antigens expressed on thymocytes themselves work as tolerogens in the thymus. To evaluate this possibility, Thy-1 bright cells were sorted out from fetal thymus cells on the 15th gestation day, and were colonized into 2′-deoxyguanosine-treated allogeneic thymus lobes. The repopulated thymus lobes were organ-cultured, and the allo-specific killer activity of thymocytes recovered from the lobes was examined. These cells were tolerant to class I but not to class II-MHC of the donor haplotype, indicating that class I molecules expressed on the thymocytes worked as tolerogen. Tolerogenic ability of Thy-1$^+$ cells was also demonstrated in another system. Upon intimate contact with allogeneic thymus lobes on a polycarbonate filter, thymus lobes fused with each other and Thy-1$^+$ cells co-migrated (*Eur. J. Immunol. 19:1525–1530, 1989*). In thymus lobes rendered parabiotic from day 5, CTL tolerance was achieved against class I but not to class II MHC. These data indicate that thymocyte-thymocyte interaction is sufficient to induce class I CTL tolerance in developing thymocytes.

Accumulating evidence has demonstrated that interaction between TCR on developing thymocytes and MHC molecules on thymic stromal cells is a key element in both positive and negative selections of thymocytes (1, 2). Experiments using thymic chimeras transplanted with dGuo[2] -treated fetal thymuses have demonstrated that cells responsible for positive selection are different from those for negative selection (3–5); dGuo-resistant thymic epithelial cells are involved in positive selection, whereas dGuo-sensitive hemopoietic cells, macrophages, and dendritic cells are involved in negative selection. However, this notion may be an over-simplified version of the reality. Thymic epithelial cells demonstrated tolerogenic ability under certain experimental conditions (6–8). Moreover, another population of dGuo-sensitive cells in the thymus, thymocytes, or their descendants, has been suggested to work as tolerogenic cells in vivo and in vitro (9–13). To evaluate the tolerogenic ability of thymocytes in the thymus, we colonized Thy-1 bright developing thymocytes into allogeneic thymuses in vitro and examined whether pCTL developing in the lobes were tolerant to class I MHC of the donor haplotype.

MATERIALS AND METHODS

Mice. C57BL/10 (B10, H-2^b), B10.BR/Sn (H-2^k), BALB/c (H-2^d), B10.D2/new-Sn (H-2^d), and RFM/Ms (H-2^f) mice were bred in our colony at the National Institute of Radiological Sciences, Chiba, Japan. The gestation day of the fetuses was determined by counting the day of vaginal plug as gestation day 0.

Medium. α-MEM was supplemented with penicillin G (100 U/ml), streptomycin (100 μg/ml), 2 mM L-glutamine, 15 mM HEPES, 5×10^{-5} M 2-ME, 1% anti-pleuropneumonia-like organisms agent (GIBCO, Chagrin Falls, OH), and 10% heat-inactivated FCS.

Reagents. dGuo was purchased from Sigma Chemical Co. (St. Louis, MO). mAb M5/114, reactive with I-A^b and I-E^k (14), and 10-2.16, anti-I-A^k (15), were used in an ascitic form at the dilution of 1:200. Culture supernatant of K24.199 was used as an anti-I-A^f reagent (16). FITC-conjugated anti-Thy-1.2 were from the Meiji Institute of Health Science, Tokyo, Japan. For cytotoxic treatment of spleen cells, anti-brain-associated-θ antibody (ascitic form, at the dilution of 1:400; a generous gift from Dr. Tomio Tada, Department of Immunology, Faculty of Medicine, University of Tokyo) and Low Tox Guinea Pig Complement (Cedarlane Laboratories, Ontario, Canada) were used.

Fetal thymus organ culture. Fetal thymus organ culture was performed according to the method of Jenkinson et al. (17) with slight modifications (18). In brief, fetal thymus lobes of GD15 were cultured on polycarbonate filters (0.8 μm pore size; Nuclepore, Pleasanton, CA) floated on 4 ml of culture medium in a 10-mm tissue culture dish. Medium was exchanged every 5 days. To produce parabiosis of thymuses, thymus lobes were organ-cultured independently for 5 days and transferred onto another filter to make close contact with allogeneic thymus lobes (19). Pre-culture period of 5 days was critical, since CTL tolerance could not be induced in thymus-parabiosis after 7-day pre-culture (19).

Another kind of chimeric state was introduced into fetal thymus organ culture by colonizing allogeneic thymus cells into host thymus lobes. The host lobes from GD15 BALB/c fetuses were organ-cultured in the presence of 1.35 mM dGuo for 3 days to diminish hemopoietic cells, and thereby to increase recolonization efficacy. After incubation without dGuo for 24 h, the dGuo-treated thymuses were colonized with Thy-1-sorted or nonsorted cells (2×10^4/lobe) from GD15 fetus thymuses by an overnight-hanging drop method (20). Recolonized lobes were organ-cultured, and 13 days later, pCTL developing in the lobes were evaluated for their tolerance state.

Sorting. Thymocytes were stained with FITC-anti-Thy-1.2, and brightly stained thymocytes were sorted under gating for lymphocyte fraction on FACStar (Becton Dickinson, Mountain View, CA). Purity of the sorted cells was re-analyzed on FACStar, which showed that more than 99% of them were Thy-1 bright (data not shown).

CTL assay. Responder thymocytes (1 to 2×10^4/well) were cultured with 30-Gy-irradiated spleen cells (3×10^6/well) in a 24-well tissue culture plate for 6 days in the presence of 1.25% EL-4 supernatant as a source of exogenous IL-2. Four-h ^{51}Cr-release assay was performed as reported previously (19). Splenic Con A blasts and LPS blasts were used as target cells. LPS blasts were elicited from spleen cells that had been cytotoxically treated with anti-brain-associated θ antibody and guinea pig complement, by stimulating with 10 μg/

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[2] Abbreviations used in this paper: dGuo, 2′-deoxyguanosine; dGuo-BALB/c↔B10.BR, dGuo-treated fetal thymus lobes repopulated with B10.BR fetal thymocytes; GD15, 15th gestational day; pCTL, precursor CTL; SP, single positive.

ml LPS for 48 h. B10.D2 LPS blasts were used as H-2^d class II-expressing targets, because BALB/c LPS blasts did not develop well. Con A blasts were prepared by stimulating spleen cells with 3 μg/ml Con A for 48 h.

RESULTS

Induction of CTL tolerance in organ-cultured thymuses by repopulating with allogeneic thymus cells. BALB/c fetal thymuses were treated with 1.35 mM dGuo and were repopulated with unfractionated thymus cells from GD15 B10 and/or B10.BR fetuses (dGuo-BALB/c←B10, etc.). As shown in Table I, thymocytes from dGuo-BALB/c←B10 or from dGuo-BALB/c←B10.BR killed B10.BR or B10 Con A blasts, respectively (lines 2 and 3), which showed that allo-MHC specific CTL against a third party could be induced from the dGuo-treated BALB/c thymuses repopulated with allogeneic thymus cells. However, they were tolerant to the donor MHC (lines 1 and 4). When dGuo-treated BALB/c lobes were repopulated with B10 and B10.BR thymus cells at the same time, cytotoxic activities against B10.BR and B10 were gone (lines 5 and 6). In short, unfractionated thymus cells had tolerogenic ability upon repopulating allogeneic thymuses.

Since unfractionated thymus cells are composed of many kinds of cells including Ia-expressing macrophages and dendritic cells, it was expected that class II tolerance would be achieved in such chimeric thymuses. Therefore, class I and class II reactivities of thymocytes from dGuo-BALB/c←B10.BR were examined (Fig. 1). They could not kill B10.BR T cell blasts nor B cell blasts, though did kill a third party, B10 cells. Thus, they were tolerant not only to class I, but also to class II MHC on the donor cells.

Thy-1-bright cells repopulated into allogeneic lobes can induce class I, but not class II tolerance. To evaluate the tolerogenic ability of thymocytes, Thy-1-bright cells from GD15 B10.BR fetal thymuses were sorted out and were colonized into dGuo-treated BALB/c lobes. As shown in Figure 2, allo-MHC specific CTL against a third party was inducible from the chimeric thymuses (Fig. 2, *C* and *F*). When CTL was induced against the donor (B10.BR) MHC, they failed to kill B10.BR T cell blasts (Fig. 2*A*). However, they did kill B10.BR B cell blasts (Fig. 2*D*). Furthermore, this killing was specifically blocked by adding anti-I-A^k and anti-I-E^k antibodies in combination into the effector phase of the CTL assay, but not by anti-I-E^k alone. The killing activity was MHC specific, as B10.D2 and B10 LPS blasts were not lysed (Fig. 2, *E* and *F*). Finally, anti-B10 CTL cross-reacted with B10.BR B cell but not T cell blasts (Fig. 2, *A* and *D*), demonstrating that class I tolerance against the donor haplotype was valid even in a cross-priming condition.

These split reactivities against Con A blasts and LPS blasts were in sharp contrast to the previous result, in which CTL generated from dGuo-BALB/c←B10.BR failed to kill both B10.BR T and B cell blasts (Fig. 1). These results indicate that Thy-1-bright cells and/or their descendants have a tolerogenic ability to induce class I tolerance, but not class II tolerance.

Class I tolerance induced in parabiotic thymus lobes. Inasmuch as most of the thymocytes expressed the donor type MHC molecules in the thymus cell-repopulation experiment shown in Figure 2, we wished to introduce a small number of thymocytes into allogeneic lobes to examine their tolerogenic ability. In a previous report, we produced parabiosis between allogeneic thymus lobes in an organ culture. This maneuver resulted in mutual CTL tolerance against class I of the parental MHC haplotypes (19). Since Thy-1-bright cells, but not Ia$^+$ cells, migrated rapidly between parabiotic lobes, it was supposed that these migrating Thy-1-bright cells were responsible for the CTL tolerance induction. If this is the case, split tolerance between class I and class II MHC of the parental haplotypes is expected. Indeed, as demonstrated in Figure 3, pCTL that were generated in the parabiotic thymuses between B10.BR and RFM lobes were tolerant to class I (Fig. 3*A*), but not to class II-MHC of the RFM haplotype (Fig. 3*B*). In addition, the class II killing was blocked by anti-I-A^f antibody. Thus, class I, but not class II tolerance, was induced in thymuses that had been made parabiotic from GD15+5.

TABLE I

Allospecific CTL activity of thymocytes from dGuo-treated BALB/c thymic lobes repopulated with allogeneic fetal thymus cells[a]

BALB/c Thymuses Colonized with	Stimulator in MLC	% Specific CR Releases from	
		B10-T	B10.BR-T
1. B10	B10	0	20
2.	B10.BR	3	70
3. B10.BR	B10	70	11
4.	B10.BR	ND[b]	0
5. B10 + B10.BR	B10	4	0
6.	B10.BR	2	10

[a] Killer assay was performed at the E/T ratio of 10:1.
[b] ND, not done.

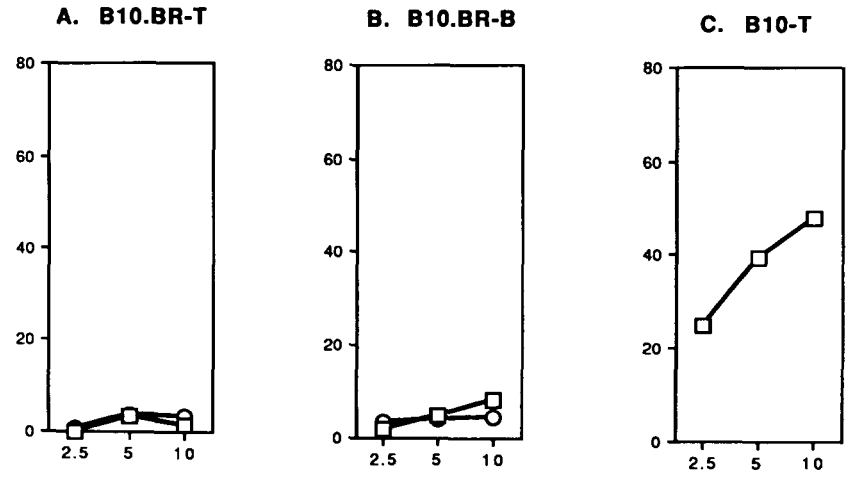

Figure 1. Tolerogenic ability of unfractionated fetal thymus cells upon colonizing into allogeneic fetal thymuses. Thymocytes developing in dGuo-BALB/c←B10.BR were stimulated with B10 (□) or B10.BR (○) spleen cells, and the resultant cells were examined for their killer activity against B10.BR-T, B10.BR-B, or B10-T cell blasts. The data are representative of three similar experiments.

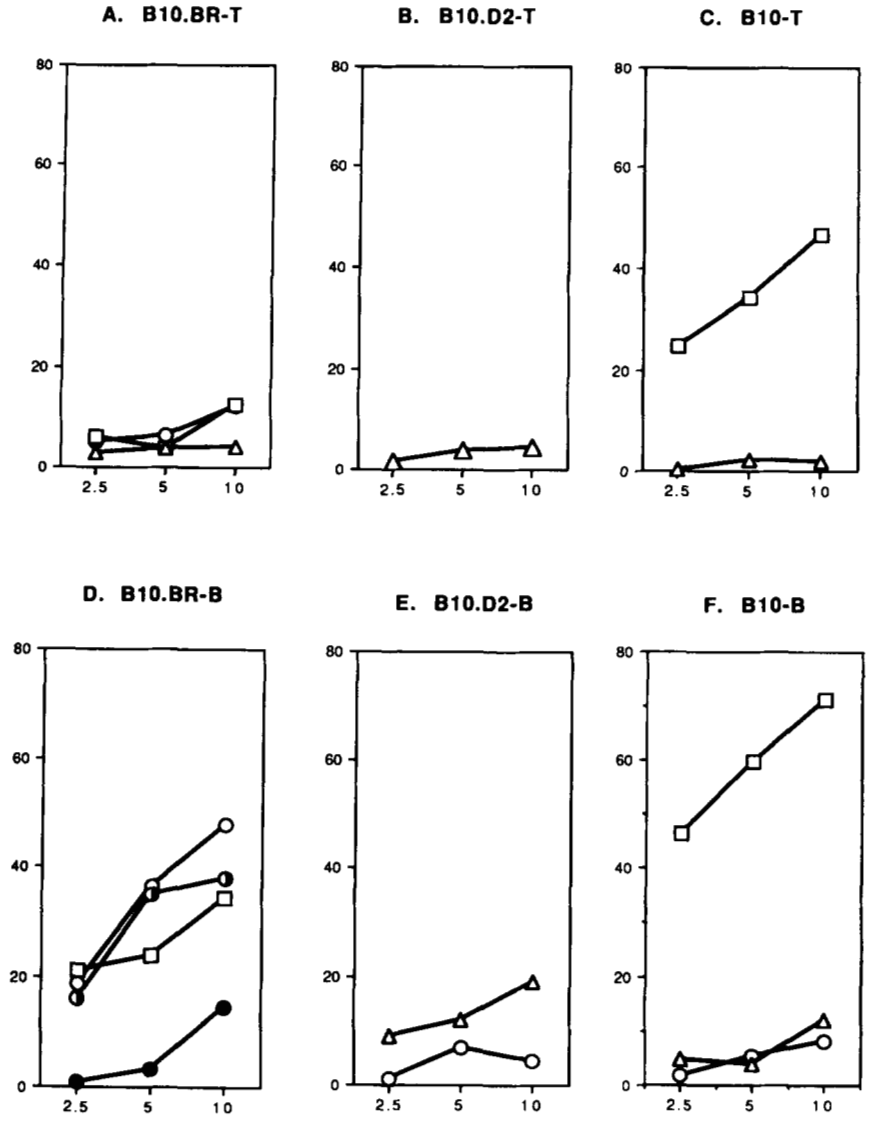

Figure 2. Tolerogenic ability of Thy-1 bright fetal thymus cells upon colonizing into allogeneic fetal thymuses. Thymocytes developing in dGuo-treated BALB/c thymuses repopulated with Thy-1 bright B10.BR fetal thymocytes were stimulated with B10 (□), B10.BR (○), or B10.D2 (△) spleen cells, and were examined for their killer activity against T or B cell blasts of B10, B10.BR, or B10.D2. In *D*, anti-I-A^k plus anti-I-E^k (●) or anti-I-E^k alone (◑) was added to the wells containing B10.BR-stimulated responder cells at the effector phase of the killer assay. The data are representative of three similar experiments.

DISCUSSION

Mature T cells do not react with self Ag. This phenomenon, self tolerance, is thought to be carried out by several mechanisms including deletion of self-reactive clones, induction of anergy to such clones, or suppression (1, 2, 21, 22). To inquire into the mechanisms of self tolerance, the tolerizing ability of thymus cell populations was examined in this study. It has been demonstrated that hematopoietic cells, especially macrophages/dendritic cells, can delete self-reactive clones in the thymus (5), whereas thymic epithelial cells can mediate positive selection (23–26). However, the possibility that another cellular component of the thymus, thymocytes themselves, may induce tolerance has not been ruled out. The present study was aimed at elucidating this issue.

Figure 1 shows that unfractionated thymus cells colonized into allogeneic fetal thymuses induced class I and class II tolerance in an allo-CTL assay. This tolerance induction may be attributed to Ia-positive cell populations including macrophages/dendritic cells. In contrast, in Figure 2, Thy-1 bright thymus cells induced class I, but not class II CTL tolerance when colonized into allogeneic thymuses. Since thymocytes are the sole Thy-1-bright cell population, the result listed above indicates that thymocytes can act as tolerizing cells to their class I MHC. In this experiment, it might be assumed that a small number of class II dull-positive macrophages have been responsible for the class I tolerance. However, this is not likely, since repeated experiments always showed only class I tolerance in chimeric thymus constructed with Thy-1-sorted cells. Since Thy-1 marker was used in the cell sorting, it is difficult to see how the contaminating cell population was always only the Ia dull cells. Thus, Thy-1 bright cells, thymocytes, did tolerize thymocytes themselves to the donor's class I MHC in the absence of Ia positive cells of the donor haplotype.

It has been shown that in parabiotic thymuses, tolerance induction is achieved by cells migrating between fused thymus lobes (19). In this system, the timing of parabiosis is critical for tolerance induction, because T cell maturation proceeds in situ in organ-cultured thymus lobes (19). Mutual class I tolerance can be induced in GD15 fetal thymuses fused with allogeneic thymus lobes after 5 days of culture (GD15+5; Fig. 3), but not after 7 days (GD15+7; Ref. 19). In contrast, parabiosis started from GD15+0-induced class I and class II tolerance (data not shown), which may have been due to co-migration of

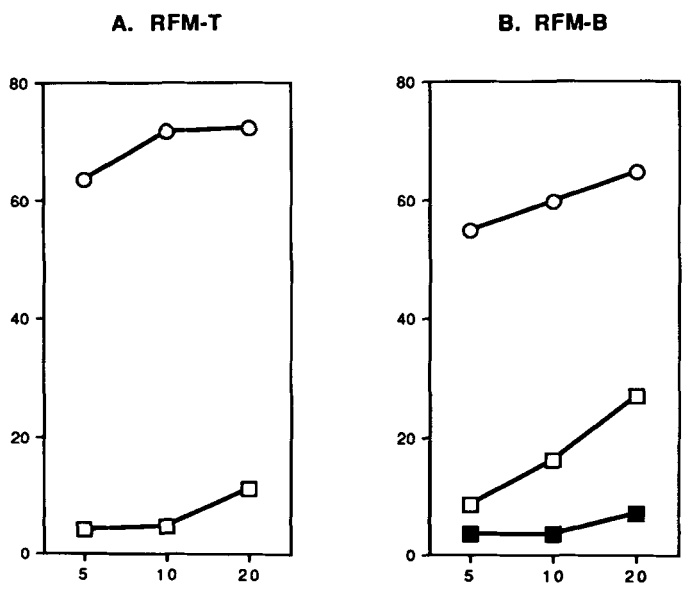

Figure 3. Tolerogenic ability of thymocytes developing in parabiotic thymuses. Responder cells from B10.BR (control, ○) or B10.BR/RFM parabiotic thymuses (made parabiotic from GD15+5, □) were stimulated with RFM spleen cells, and the CTL activity against RFM T or B cell targets was assayed. Anti-I-A^r antibody was added at the effector phase (■). The data are representative of three similar experiments.

Ia positive cells such as macrophages or dendritic cells.

These data do not clarify the mechanism of tolerance. In a previous study, it has been shown that tolerance in parabiotic thymuses was not induced by suppression (19). Since only thymocytes can migrate between two parabiotic lobes within a 2-day period (from day 5 to day 7; Ref. 19), it is likely that the small number of thymocytes did induce the class I tolerance. It is noteworthy that this tolerance was demonstrated in GD15+5 thymuses, in which SP cells have already emerged (18). Considering that both GD15+5 and GD15+7 thymus lobes contain SP cells, it may be that recently developed SP cells are not yet immunocompetent. Another possibility is that the number of SP cells is too small in GD15+5 cells to produce enough lymphokines for inducing functionally competent effector cells.

Thus it has been demonstrated that thymocytes, which express class I, but not class II MHC, can induce class I tolerance even in SP cells. However, the precise mechanism of self tolerance mediated by thymocytes themselves is not known. It has been demonstrated that clonal deletion is the major mechanism of self tolerance (1, 2). It requires the presence of the thymus and takes place at the double-positive cell stage (27–29). It is thought that clonal deletion is accomplished by apoptosis of thymocytes upon reaction with self Ag presented by macrophages/dendritic cells (30–32). It is not clear if Ag presentation by thymocytes results in the induction of apoptosis of the thymocytes.

In contrast, a major part of peripheral tolerance is supposed to be induced by clonal anergy (21, 22, 33–39). It has been suggested that Ag presentation by cells incapable of producing second signals results in the induction of clonal anergy, instead of the activation of T cells (40). Such phenomena have also been demonstrated in T cell clones upon recognition of Ia-Ag complexes on fixed APC or on artificial membranes (41, 42). Moreover, in one experiment, Ag presentation by HLA-DR positive human T cells was accompanied by clonal anergy (10). Perhaps a part of the veto cell phenomenon may be explained in this manner (11–13). In this context, if thymocytes cannot produce such a second signal, it is possible that they can induce anergy to thymocytes themselves.

Additional experiments will be necessary to determine if the tolerance induction of SP cells by thymocytes is mediated by a clonal deletion or by a clonal anergy mechanism.

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