

TOLERANCE OF THYMOCYTES TO ALLOGENEIC I REGION  
DETERMINANTS ENCOUNTERED PRETHYMICALLY

Evidence for Expression of Anti-Ia Receptors by  
T Cell Precursors before Their Entry into the Thymus

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Self-recognition and self-tolerance are two closely related yet distinct aspects of the T cell repertoire. Because the thymus is the organ within which T cells differentiate, it has been suggested that the thymus plays an important role in determining both aspects of the T cell repertoire. Indeed, there is evidence that the T cell repertoires for both self-recognition and allorecognition are influenced by the major histocompatibility complex (MHC)<sup>1</sup> determinants expressed by the thymus within which T cells mature (1-4). However, the mechanism by which the thymus exerts its influence on the developing T cell repertoire remains obscure. It is conceivable that the thymus either positively or negatively selects clones of T cells based on their expression of a predetermined receptor repertoire (5). Alternatively, it is conceivable that the thymus induces somatic mutations that result in the generation of new receptor specificities (6). One way of distinguishing between thymic selection of a predetermined T cell repertoire and the generation of a novel thymically induced T cell repertoire would be to first analyze T cell precursors for expression of MHC-specific receptors and then to compare the receptor repertoires expressed by precursor T cells before thymic processing with that expressed by T cells that have undergone thymic processing.

Consequently, to eventually accomplish this sort of repertoire comparison, it is imperative to first know whether precursor T cells express MHC-specific receptors before thymic processing. It was recently observed that cytotoxic T lymphocytes (CTL) that were differentiating within a syngeneic thymus graft were tolerant to the allogeneic K/D determinants their precursors encountered on prethymic elements (7). Such results suggested that pre-T cells do express anti-MHC receptors specific for the recognition of K/D region-encoded determinants before their entry into the thymus. These results were consistent with observations that T cells from nude mice that have

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<sup>1</sup> *Abbreviations used in this paper:* CTL, cytotoxic T lymphocytes; FACS, fluorescence-activated cell sorter; FIGAM<sub>1</sub>g, fluorescein conjugated affinity-purified F(ab')<sub>2</sub> goat anti-mouse Ig; FIRAM<sub>2</sub>, fluorescein-conjugated affinity-purified F(ab')<sub>2</sub> rabbit anti-mouse IgG<sub>2</sub>; FMF, flow microfluorometry; IF, immunofluorescence; MHC, major histocompatibility complex; MLR, mixed lymphocyte reactions; RAMB, rabbit anti-mouse brain serum; Tx, thymectomized.

not undergone thymic processing do, nonetheless, express anti-K/D region receptors (8–10). However, there exists no information whether T cells express anti-MHC receptors specific for the recognition of I region-encoded determinants before thymic processing.

The aim of the present study was to determine whether or not T cell precursors express receptors for the recognition of allogeneic I region determinants before their entry into the thymus. To address this question, thymocytes were assayed for mixed lymphocyte reactivity (MLR), a response that is stimulated by recognition of allogeneic I region-encoded determinants. The influence of either thymic or extra-thymic allogeneic MHC determinants upon the subsequent repertoire of competent intrathymic T cells was assessed using thymus-engrafted radiation bone marrow chimeras and thymus-engrafted nude mice. The results of this study suggest that T cell precursors do express anti-I region receptors before their entry into the thymus and that novel anti-Ia receptor specificities are not generated intrathymically.

### Materials and Methods

*Animals.* C57BL10/Sn (B10), B10.A, B10.D2, B10.BR, (B10.BR × B10.D2)<sub>F1</sub> A/J, (B6 × A/J)<sub>F1</sub> (B6AF<sub>1</sub>), BALB/c, C3H.SW, (BALB/c × B6)<sub>F1</sub> (CB6F<sub>1</sub>) adult mice and A/J, B6, and CB6F<sub>1</sub> neonates were obtained from The Jackson Laboratory, Bar Harbor, ME. BALB/c nude mice were obtained from Charles River Breeding Laboratories, Inc., Wilmington, MA. B10.A(2R), B10.AQR, and B10.T(6R) adult mice were provided by Dr. David Sachs, National Institutes of Health, Bethesda, MD. Thymocytes and splenocytes were obtained from normal mice at least 4 and 8 wk, respectively, after birth.

*Radiation Bone Marrow Chimeras.* Radiation bone marrow chimeras are designated as bone marrow donor → irradiated recipient and were tested individually. An extensive description of the production and typing of such chimeras has been given elsewhere (11, 12). Briefly, recipient mice were irradiated with 950 rad cesium and reconstituted 4–6 h later with  $1.5 \times 10^7$  bone marrow cells that had been depleted of T cells by pretreatment with a rabbit anti-mouse brain serum (RAMB) and complement. The RAMB reagent used is specifically cytotoxic for T cells (11, 12). Thymocytes from chimeras were used at least 4 wk after irradiation. Typing of thymocytes by indirect immunofluorescence (IF) and flow microfluorometry (FMF) on a fluorescence-activated cell sorter (FACS) showed they were (>98%) of donor bone marrow origin (13).

*Thymus-engrafted Nude Mice.* Thymus grafting of nude mice was carried out by subcutaneously implanting three individual thymic lobes from donors <24 h old. Thymocytes from the grafts were used 4–6 wk after grafting. At this time, thymic tissue was visible in >70% of such mice. The number of thymocytes recovered from the grafts varied from 60–100 × 10<sup>6</sup> cells. Typing of thymocytes by FMF on the FACS verified that the engrafted thymi were fully repopulated by cells of nude host origin (14).

*Thymus-engrafted Radiation Bone Marrow Chimeras.* B6AF<sub>1</sub> mice were thymectomized (Tx) at 4–6 wk of age; 10 d later they were subcutaneously engrafted with 3–4 thymic lobes from A/J or B6 neonates; 4 d after graft implantation, the animals were lethally irradiated (950 rad cesium) and then reconstituted with  $1.5 \times 10^7$  bone marrow cells that had been pretreated with RAMB plus complement. B6AF<sub>1</sub> hosts with A/J thymus grafts received B10.A bone marrow; B6AF<sub>1</sub> hosts with B6 thymus grafts received B10 bone marrow, so that thymus and bone marrow were always syngeneic at the MHC. Such mice are designated as bone marrow donor → irradiated recipient (Tx plus thymus donor), so that the two groups of thymus-engrafted radiation bone marrow chimeras constructed for this study were B10.A → B6AF<sub>1</sub>(Tx + A/J Thy) and B10 → B6AF<sub>1</sub>(Tx + B6 Thy). Survival of such A → A × B(Tx + A Thy) chimeras was >80%. Thymocytes were obtained from these mice 4–5 wk after irradiation, yielding 15–75 × 10<sup>6</sup> cells per thymus graft. In addition, nonthymectomized B6AF<sub>1</sub> mice were engrafted with neonatal B6 thymus grafts, irradiated, and reconstituted with C3H.SW bone marrow that had been pretreated with RAMB plus complement and are designated C3H.SW → B6AF<sub>1</sub>(+ B6

Thy). Thymocytes from individual grafts were typed by FMF on the FACS, verifying that each thymus graft had been fully repopulated by cells of bone marrow donor origin.

*Inoculation of Neonates with Allogeneic Cells.* B10.BR neonates <24 h old were injected intravenously with  $2 \times 10^7$  spleen cells from B10.BR  $\times$  B10.D2 adult mice and rested 2-3 mo before use.

*Typing Reagents.* Anti-Ly-9.1 and anti-Ly-9.2 sera were prepared as previously described and were the generous gift of Dr. Bonnie Mathieson, NIH, Bethesda, MD (15). Monoclonal anti-I-A<sup>k</sup> antibodies were culture supernatants of the clone 10-2.16 described by Oi et al. (16). Anti-I-A<sup>b</sup> was a mixture of two monoclonal anti-I-A<sup>b</sup> culture supernatants from clones 25-5-16S and 25-9-17S, described by Ozato and Sachs (17). Anti-H-2<sup>d</sup> was a mixture of three monoclonal anti-H-2<sup>d</sup> culture supernatants from the clones 34-1-2, 34-2-12, and 34-7-23, described by Ozato and Sachs (18). Fluorescein-conjugated, affinity-purified F(ab')<sub>2</sub> goat anti-mouse Ig (FIGAMIg) was the kind gift of Dr. B. J. Fowlkes and Dr. R. J. Asofsky, NIH, Bethesda, MD. Fluorescein-conjugated affinity-purified rabbit F(ab')<sub>2</sub> anti-mouse IgG<sub>2</sub> (FIRAMG<sub>2</sub>) was prepared under contract NCI-CB-53912-31.

*Immunofluorescence Staining and Flow Microfluorometry.* The preparation of cells and staining procedures were as described previously (13). Briefly,  $1 \times 10^6$  thymus cells were incubated at 4°C for 45 min with anti-Ly-9 or anti-Ia reagents in amounts predetermined to be saturating, washed twice by centrifugation, incubated at 4°C for 45 min with saturating amounts of FIGAMIg or FIRAMG<sub>2</sub>, washed twice, and analyzed for fluorescence. These procedures were performed in Hanks' balanced salt solution without phenol red and containing 0.1% bovine serum albumin and 0.1% NaN<sub>3</sub>.

FMF analysis was performed using an FACS II cell sorter (B-D FACS Systems, Becton, Dickinson & Co., Sunnyvale, CA), as previously described (13). Data were collected on  $5 \times 10^4$  viable cells, as determined by forward light scatter intensity. Data are displayed either as a graph of fluorescence (abscissa) vs. cell number (ordinate) or as the integration of the area under this curve. A background value obtained from cells incubated with the fluorescent reagent alone is subtracted from experimental values obtained from cells stained with specific antisera plus the fluorescent reagent.

*Thymic Mixed Lymphocyte Reaction.* Mixed lymphocyte cultures of thymocyte responder cells and spleen stimulator cells were performed in Dulbecco's modified Eagle's medium containing 4.5 g/liter glucose (Gibco, Grand Island Biological Co., Grand Island, NY) and supplemented with additional amino acids (19),  $5 \times 10^{-5}$  M 2-mercaptoethanol, 10% FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, and 10 mM Hepes.  $1 \times 10^6$  responding thymocytes (or as otherwise noted) were cultured with  $2.5 \times 10^5$  1,000 rad cesium-irradiated stimulator spleen cells in a total volume of 200 µl and incubated for 6 d in a 7.5% CO<sub>2</sub>-humidified air atmosphere. Cultures were pulsed with 1 µCi [<sup>3</sup>H]thymidine 16-20 h before harvest.

## Results

*Thymic MLR Reflects Expression of Receptors for I Region Products.* To first determine that T cells that are resident in the thymus are able to recognize allogeneic I region determinants, normal thymocytes were assayed for their ability to specifically proliferate in response to allogeneic stimulator cells. The results in Table I demonstrate that thymocytes were indeed competent to generate proliferative responses to allogeneic stimuli, in agreement with the recent findings of Wu and Bach (20). The magnitude of responses stimulated by syngeneic spleen stimulator cells and by medium alone were not statistically different; nonetheless, both are presented as background controls in most tables. To document that such responses reflected the expression of receptors specific for I determinants by T cells within the thymus, the reactivity of thymocytes to stimulator cells differing at defined regions of the H-2 complex was examined (Table I). As can be seen, the stimulation of thymocyte proliferation predominantly mapped to the I region of H-2 because a genetic difference

TABLE I  
Stimulation of Thymic MLR Maps to the I Region of the MHC

Responder thymo- cytes (K I D)	Stimulator spleen		Genetic differences	cpm $\times 10^{-3}$
	Strain	Genotype K I D		
B10.A (k k/d d)	B10	b b b	K, I, D	31.7 $\pm$ 8.9
	B10.D2	d d d	K, I	35.4 $\pm$ 6.5
	B10.T(6R)	q q d	K, I	20.8 $\pm$ 2.1
	B10.AQR	q k/d d	K	1.9 $\pm$ 0.2
	B10.A(2R)	k k/d b	D	1.3 $\pm$ 0.1
	B10.A	k k/d d	—	0.7 $\pm$ 0.1
	Medium	—	—	0.5 $\pm$ 0.1
B10.T(6R) (qqd)	B10	b b b	K, I, D	64.8 $\pm$ 2.9
	B10.A	k k/d d	K, I	63.6 $\pm$ 11.5
	B10.D2	d d d	K, I	69.3 $\pm$ 10.5
	B10.AQR	q k/d d	I	61.1 $\pm$ 4.5
	B10.T(6R)	q q d	—	2.2 $\pm$ 0.8
	Medium	—	—	0.9 $\pm$ 0.2
B10.A(2R) (k k/d d)	B10.T(6R)	q q d	K, I, D	18.4 $\pm$ 5.9
	B10.D2	d d d	K, I, D	19.1 $\pm$ 1.0
	B10	b b b	K, I	20.0 $\pm$ 2.0
	B10.AQR	q k/d d	K D	2.7 $\pm$ 0.1
	B10.A	k k/d d	D	3.0 $\pm$ 0.8
	B10.A(2R)	k k/d b	—	1.1 $\pm$ 0.2
	Medium	—	—	1.6 $\pm$ 0.2

between responder and stimulator cells in the I region alone [B10.T(6R) anti-B10.AQR] generated a response fully comparable to a whole H-2 difference [B10.T(6R) anti-B10]. In contrast, K or D region differences individually or together [B10.A anti-B10.AQR or anti-B10.A(2R); B10.A(2R) anti-B10.AQR] gave significantly less stimulation than a whole H-2 difference, though the response was measurable above background. Taken together, these data demonstrate that an I region difference is both necessary and sufficient for the maximum induction of a thymic MLR. It can be concluded that T cells resident within the thymus express receptors for allogeneic I region products.

*Tolerance of Thymocytes to Allogeneic Ia Determinants in Radiation Bone Marrow Chimeras.* We next asked what effect the presence of allogeneic Ia determinants within their differentiation environment would have upon thymocyte expression of I region-specific alloreactivity. To address this question, we examined the reactivity of thymocytes from A  $\rightarrow$  B radiation bone marrow chimeras. Thymocytes from such chimeras are genotypically strain A but undergo differentiation within a strain B host (12). As can be seen in Table II, although thymocytes from B10.A  $\rightarrow$  B10 chimeras were fully capable of generating a response to third party I<sup>d</sup> (BALB/c) determinants, these cells were specifically tolerant to allogeneic host I<sup>b</sup> (B10) determinants as well as syngeneic I<sup>k</sup> (B10.A) determinants. The B10 and B10.A stimulator cells, though not stimulatory for B10.A  $\rightarrow$  B10 thymocytes, were effective stimulators for thymocytes from appropriate control mice (Table II). Thus, these experiments demonstrate that

TABLE II  
Tolerance of Thymocytes from Radiation Bone Marrow Chimeras to Allogeneic Host Ia Determinants

Experiment	Responder thymocytes	Stimulator cells			
		B10	B10.A	BALB/c	Medium
<i>cpm</i> × 10 <sup>-3</sup>					
1	B10.A → B10*	0.9 ± 0.1	0.5 ± 0.1	16.4 ± 1.2	0.5 ± 0.1
	B10.A	8.8 ± 1.0	0.4 ± 0.1	5.6 ± 0.4	0.3 ± 0.1
	B10	0.3 ± 0.1	20.4 ± 1.8	14.5 ± 2.3	0.3 ± 0.1
2	B10.A → B10*	3.4 ± 0.1	2.0 ± 0.3	33.8 ± 0.3	2.3 ± 0.5
	B10.A	12.6 ± 1.7	0.7 ± 0.1	13.1 ± 0.7	0.6 ± 0.1
	B10	0.6 ± 0.1	18.1 ± 0.6	16.0 ± 2.2	0.4 ± 0.1

\* Chimeras were constructed as described in Materials and Methods.

TABLE III  
Tolerance of Thymocytes from Thymus-engrafted Nude Mice to Intrathymic Allogeneic Ia Determinants

Experiment	Responder thymocytes*		Stimulator cells			
	Nude host	Thymus	B10	BALB/c	B10.A	Medium
<i>cpm</i> × 10 <sup>-3</sup>						
1	BALB/c	B6 graft	1.2 ± 0.3	0.9 ± 0.1	20.9 ± 2.2	0.2 ± 0.1
	BALB/c	CB6F <sub>1</sub> graft	0.6 ± 0.1	1.2 ± 0.2	13.9 ± 2.1	0.9 ± 0.1
	—	BALB/c control	42.5 ± 2.4	1.5 ± 0.1	62.6 ± 8.5	0.1 ± 0.1
	—	B10 control	0.6 ± 0.1	25.3 ± 3.0	18.1 ± 2.2	0.5 ± 0.1
2	BALB/c	B6 graft	2.6 ± 0.7	0.9 ± 0.1	24.2 ± 0.3	0.4 ± 0.1
	BALB/c	CB6F <sub>1</sub> graft	1.7 ± 0.3	3.3 ± 0.7	20.8 ± 2.2	1.1 ± 0.1
	—	BALB/c control	14.4 ± 0.8	1.1 ± 0.1	20.8 ± 2.8	0.5 ± 0.1
	—	B10 control	0.6 ± 0.1	17.4 ± 0.6	17.3 ± 1.1	0.3 ± 0.1

\* Thymocytes were cells of BALB/c nude origin that had repopulated the indicated engrafted neonatal thymic lobes.

exposure of T cells to allogeneic Ia determinants before or concomitant with their acquisition of functional competence in the thymus results in specific tolerization to those allogeneic Ia determinants.

*Tolerance of Thymocytes to Allogeneic Ia Determinants Expressed Only on Thymic Elements.* To examine the possibility that T cells can be tolerized to allogeneic Ia determinants that they first encounter within the thymus in which they are differentiating, thymocytes were obtained from nude mice that had been engrafted with allogeneic or semiallogeneic thymic lobes. The cells repopulating the engrafted thymi of such mice were typed by immunofluorescence and FMF analysis and were shown to be of nude host origin with no cells of thymic origin detected (data not shown). The proliferative responses of thymocytes from BALB/c nude mice engrafted with either an allogeneic B6 or a semiallogeneic CB6F<sub>1</sub> thymus are shown in Table III. Because these cells were BALB/c in origin and had differentiated within an environment that was totally BALB/c except for the thymus, these H-2<sup>d</sup> T cells first encountered allogeneic I<sup>b</sup> determinants within the engrafted thymus. It can be seen that nude T cells that have differentiated within either an allogeneic I<sup>b</sup> or semiallo-

genic I<sup>d/b</sup> thymus were fully reactive against third party I<sup>k</sup> (B10.A) but were specifically tolerant to both self I<sup>d</sup> (BALB/c) and thymic I<sup>b</sup> (B10) (Table III). Although neither B10 nor BALB/c spleen cells were stimulatory to nude thymocytes, both were stimulatory to the appropriate control thymocytes. Therefore, the thymus itself is capable of tolerizing T cells maturing within it to the I region determinants that the thymus expresses.

*Tolerance of Thymocytes to Allogeneic Ia Determinants Expressed Only on Extrathymic Elements.* The next series of experiments was designed to address the possibility that developing T cells express anti-Ia receptors prethymically. (A × B)F<sub>1</sub> mice were sequentially thymectomized, engrafted with strain A neonatal thymic lobes, lethally irradiated, and finally reconstituted with strain A bone marrow [A → A × B (Tx + A Thy)]. Theoretically, in these animals strain A T cells are differentiating within an H-2 syngeneic strain A thymus but have potentially encountered strain B Ia determinants on prethymic host elements. If T cells first express anti-Ia receptors intrathymically, thymocytes from A → A × B(Tx + A Thy) chimeras should exhibit anti-B reactivity, just as thymocytes from a normal strain A mouse do. On the other hand, if T cells express anti-Ia receptors prethymically, thymocytes from A → A × B(Tx + A Thy) chimeras would have had the opportunity to recognize and be tolerized to the allogeneic strain B Ia antigens expressed on prethymic F<sub>1</sub> host elements.

Clearly, it was important to determine the origin of the cells repopulating these engrafted thymi before determining their functional reactivity. Consequently, the strain combinations used in the construction of A → A × B(Tx + A Thy) chimeras were specifically chosen so that cells of donor bone marrow origin could be distinguished from cells of host and thymus origins by their expression of allelic non-MHC (e.g., Ly) antigens. Hence, A → A × B(Tx + A Thy) thymocytes were typed for Ly-9 by FMF on the FACS 4-5 wk after irradiation (Table IV). In B10.A → B6AF<sub>1</sub> (Tx + A/J Thy) chimeras, essentially all cells in the engrafted thymi were of bone marrow donor origin, as evidenced by the fact that they only expressed the Ly-9 phenotype of the B10.A bone marrow donor (Ly-9.2) but not the Ly-9 phenotype of either the A/J thymus (Ly-9.1) or the B6AF<sub>1</sub> host (Ly-9.2/9.1). Similarly, in the B10 → B6AF<sub>1</sub>(Tx + B6 Thy) chimera, all cells in the engrafted thymi only expressed the Ly-9 phenotype of the bone marrow donor (Ly-9.2) but not that of the B6AF<sub>1</sub> host (Ly-9.1/9.2).

The alloreactive proliferative responses of the cells within the thymic grafts of A → A × B(Tx + A Thy) chimeras were next examined (Table V). H-2<sup>a</sup> thymocytes from B10.A → B6AF<sub>1</sub>(Tx + A/J Thy) chimeras were normally alloreactive to third party I<sup>d</sup> (B10.D2) determinants and were tolerant to syngeneic I<sup>k</sup> (B10.A) determinants. Importantly, they were also tolerant to allogeneic I<sup>b</sup> (B10) determinants, which could only have been derived from nonthymic host elements. Similarly, H-2<sup>b</sup> thymocytes from B10 → B6AF<sub>1</sub>(Tx + B6 Thy) chimeras were normally alloreactive to third party I<sup>d</sup> (B10.D2) determinants and were tolerant to both syngeneic I<sup>b</sup> and allogeneic host I<sup>a</sup> determinants. Such tolerance was not because of a failure of the B10.A and B10 irradiated spleen cells to be stimulatory, because they were stimulatory to appropriate control thymocyte populations (Table V). Also included as controls were thymocytes from B10.A → B10.A and B10 → B10 syngeneic chimeras, which had been constructed at the same time and with the same bone marrow populations as the A → A × B(Tx + A Thy) chimeras. As can be seen from the response of

TABLE IV  
*Phenotype of Cells Repopulating the Engrafted Thymi of Thymus-engrafted Radiation Bone Marrow Chimeras*

Thymocytes	Percent cells staining positive with reagents specific for*	
	Ly-9.1	Ly-9.2
B10 → B6AF <sub>1</sub> (Tx + B6 Thy)‡	2§	94
B10.A → B6AF <sub>1</sub> (Tx + A/J Thy)‡	0§	95
Control thymocytes		
B10	10	94
A/J	93	5
B6AF <sub>1</sub>	68	69

\* Thymocytes were stained with either anti-Ly-9.1 or anti-Ly-9.2 followed by FlGAM1g.

‡ B6AF<sub>1</sub> mice were thymectomized, engrafted with B6 or A/J thymic lobes, lethally irradiated (950 rad), and reconstituted with B10 or B10.A bone marrow (depleted of T cells), as indicated.

§ Percent Ly-9.1-positive thymocytes were calculated relative to negative control thymocytes (B10) as [(percent cells positive from experimental mice - percent cells positive from negative control mice)/100 - percent cells positive from negative control mice] × 100.

|| Percent Ly-9.2-positive thymocytes were calculated relative to negative control thymocytes (A/J) according to the above formula.

TABLE V  
*Tolerance of Thymocytes from Thymus-engrafted Radiation Bone Marrow Chimeras to Extrathymic Allogeneic Ia Determinants*

Experiment	Responder thymocytes	Stimulator cells			
		B10.A	B10	B10.D2	Medium
		<i>cpm × 10<sup>-3</sup></i>			
1	B10.A → B6AF <sub>1</sub> (Tx + A/J Thy)*	0.9 ± 0.4	1.6 ± 0.3	17.7 ± 4.4	0.5 ± 0.2
	B10 → B6AF <sub>1</sub> (Tx + B6 Thy)‡	0.6 ± 0.3	2.1 ± 0.6	32.0 ± 1.1	0.3 ± 0.2
	A/J	0.8 ± 0.3	8.7 ± 1.9	13.0 ± 3.9	0.9 ± 0.5
	B10	20.5 ± 4.4	0.8 ± 0.1	14.2 ± 2.3	0.5 ± 0.1
2	B10.A → B6AF <sub>1</sub> (Tx × A/J Thy)*	1.6 ± 0.5	1.3 ± 0.5	20.6 ± 2.9	2.9 ± 0.4
	B10 → B6AF <sub>1</sub> (Tx + B6 Thy)‡	3.7 ± 0.2	1.8 ± 0.4	40.4 ± 3.9	2.3 ± 0.7
	A/J	2.3 ± 0.2	19.1 ± 3.6	33.0 ± 8.0	2.2 ± 0.1
	B10	15.8 ± 1.3	3.3 ± 0.4	31.6 ± 1.0	3.3 ± 0.3
	B10.A → B10.A	1.5 ± 0.1	17.7 ± 3.2	13.2 ± 1.4	1.1 ± 0.1
	B10 → B10	19.4 ± 3.3	1.7 ± 0.5	53.0 ± 14.6	0.4 ± 0.1

\*‡ See footnote (‡) to Table IV.

thymocytes from these syngeneic chimeras, the bone marrow populations used in the construction of A → A × B(Tx + A Thy) chimeras were capable of generating T cells that could become alloreactive to I<sup>b</sup> and I<sup>a</sup>, respectively (Table V). Hence, these results demonstrate that strain A thymocytes from A → A × B(Tx + A Thy) chimeras were tolerized to extrathymic strain B Ia determinants and suggest that the tolerization events might have occurred prethymically.

*Tolerance of Thymocytes to Extrathymic Allogeneic Ia Determinants Is Not Because of a Post-thymic Suppressor Mechanism.* One alternative explanation for the observation that

thymocytes from  $A \rightarrow A \times B(\text{Tx} + A \text{ Thy})$  chimeras were tolerant to the extrathymic allogeneic Ia determinants of the host was that it resulted from an active suppressive mechanism operating within the thymus and mediated by the recirculation of suppressor T cells generated in the post-thymic compartment. If such a post-thymically generated suppressor mechanism existed, thymocytes from  $B10 \rightarrow B6AF_1(\text{Tx} + B6 \text{ Thy})$  chimeras should be suppressive to the response of normal B10 thymocytes to B10.A ( $I^k$ ) alloantigens. As can be seen in Table VI, normal B10 thymocytes were alloreactive to  $I^k$  (B10.A), whereas  $B10 \rightarrow B6AF_1(\text{Tx} + B6 \text{ Thy})$  thymocytes were unreactive to  $I^k$ . Addition of  $B10 \rightarrow B6AF_1(\text{Tx} + B6 \text{ Thy})$  thymocytes to cultures containing normal B10 thymocytes did not significantly affect the response of the B10 thymocytes to stimulator  $I^k$  (B10.A). These results fail to support the existence of a suppressor mechanism as the explanation for the tolerance of  $A \rightarrow A \times B(\text{Tx} + A \text{ Thy})$  thymocytes to allogeneic host Ia antigens.

*Tolerance of Thymocytes to Extrathymic Allogeneic Ia Determinants Is Probably Not Because of the Intrathymic Presence of Those Allogeneic Determinants.* Another possible alternative explanation for the observed tolerance of  $A \rightarrow A \times B(\text{Tx} + A \text{ Thy})$  chimeric thymocytes to nonthymic allogeneic host Ia determinants was that the tolerization actually occurred intrathymically, because the allogeneic strain B Ia antigens of the host might have been present within the strain A thymus.

To address this possibility, thymocytes were assessed for expression of allogeneic I-A determinants using immunofluorescence and FMF analysis. The validity of FMF analysis of thymocytes as a method for detecting the intrathymic presence of allogeneic Ia determinants derives from the fact that thymocytes acquire genotypically inappropriate (i.e., allogeneic) I-A determinants that are present within their thymic differentiation environment (13). To illustrate this point,  $C3H.SW \rightarrow B6AF_1(+ B6 \text{ Thy})$  radiation bone marrow chimeras were constructed from  $B6AF_1$  mice that had not been thymectomized and so possessed two thymi, a B6 thymus graft, and an *in situ*  $B6AF_1$  thymus. Both thymi were fully repopulated by cells of donor  $C3H.SW$  ( $H-2^b$ )

TABLE VI  
*Tolerance of Thymocytes to Extrathymic Allogeneic Ia Determinants Is Not the Result of Demonstrable Suppression*

Experiment	Responder thymocytes		Stimulation index‡	
	Normal B10	B10 $\rightarrow$ B6AF <sub>1</sub> (Tx + B6 Thy)*	Stimulator cells	
			B10.A	B10.D2
1	$1 \times 10^6$	—	10.4	33.9
	—	$1 \times 10^6$	1.1	44.9
	$0.5 \times 10^6$ +	$0.5 \times 10^6$	11.5	38.3
2	$1.5 \times 10^6$	—	13.6	—
	—	$1 \times 10^6$	0.5	—
	$1.5 \times 10^6$ +	$0.5 \times 10^6$	16.3	—

\* See footnote to Table IV.

‡ Stimulation index is experimental cpm/medium control cpm. Medium control cpm was <1,000 cpm in all groups.



origin, as assessed by Lyt-1 typing, because in both thymi all cells were Lyt-1.1-positive (the phenotype of C3H.SW but not the phenotype of either B6 or B6AF<sub>1</sub>) (data not shown). As can be seen in Fig. 1, the C3H.SW (H-2<sup>b</sup>) thymocytes found in the *in situ* B6AF<sub>1</sub> thymus expressed I-A<sup>k</sup> determinants, whereas the C3H.SW (H-2<sup>b</sup>) thymocytes found in the B6-engrafted thymus of the same animal did not. Because both thymocyte populations were genotypically H-2<sup>b</sup> and derived from the identical bone marrow stem cell population and because both thymocyte populations were exposed to the identical prethymic environment, the only difference between the H-2<sup>b</sup> thymocyte populations derived from the B6-engrafted thymus and those derived from the B6AF<sub>1</sub> *in situ* thymus was the presence intrathymically of allogeneic H-2<sup>a</sup> determinants. Thus, the presence of allogeneic I-A<sup>k</sup> determinants on H-2<sup>b</sup> thymocytes resident within the B6AF<sub>1</sub> *in situ* thymus, but not on H-2<sup>b</sup> thymocytes resident within the B6-engrafted thymus, demonstrates that immunofluorescence and FMF analysis of thymocytes specifically reflects the intrathymic presence of allogeneic Ia determinants.

Consequently, the same thymocyte populations from A → A × B(Tx + A Thy) chimeras, which were shown to be functionally tolerant to allogeneic host Ia determinants, were also assessed for the intrathymic presence of allogeneic host I-A determinants by immunofluorescence and FMF. As can be seen in Fig. 2, cells from the thymus of the same B10.A → B6AF<sub>1</sub>(Tx + A/J Thy) chimeras that were tolerant to I-A<sup>b</sup> (Table V) were, by FMF, negative for I-A<sup>b</sup>. Similarly, cells from the thymus of the B10 → B6AF<sub>1</sub>(Tx + B6 Thy) chimeras that were tolerant to I-A<sup>k</sup> (Table V) were negative for I-A<sup>k</sup> (Fig. 2). These data demonstrate that the thymocyte populations from both types of chimeras, although tolerant to the allogeneic Ia determinants of the nonthymic host, neither contained detectable numbers of host cells nor had acquired on their surface detectable quantities of the allogeneic I-A determinants of the nonthymic host. Because the acquisition of allogeneic I-A determinants by thymocytes reflects the presence intrathymically of those determinants (Fig. 1), these

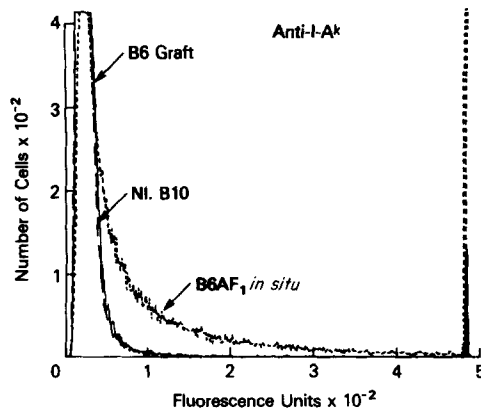


FIG. 1. Detection of allogeneic Ia determinants present intrathymically by immunofluorescence and FMF analysis. C3H.SW (H-2<sup>b</sup>) bone marrow cells were injected into an irradiated B6AF<sub>1</sub> mouse that had been engrafted with an additional parental B6 thymus. Both the B6AF<sub>1</sub> *in situ* thymus as well as the B6-engrafted thymus were fully repopulated with cells derived from the C3H.SW (H-2<sup>b</sup>) bone marrow inoculum. The H-2<sup>b</sup> cells repopulating each thymus as well as control B10 thymocytes were stained with anti-I-A<sup>k</sup> reagents followed by FIGAM1g and assessed by FMF for their cell surface expression of allogeneic I-A<sup>k</sup> determinants.

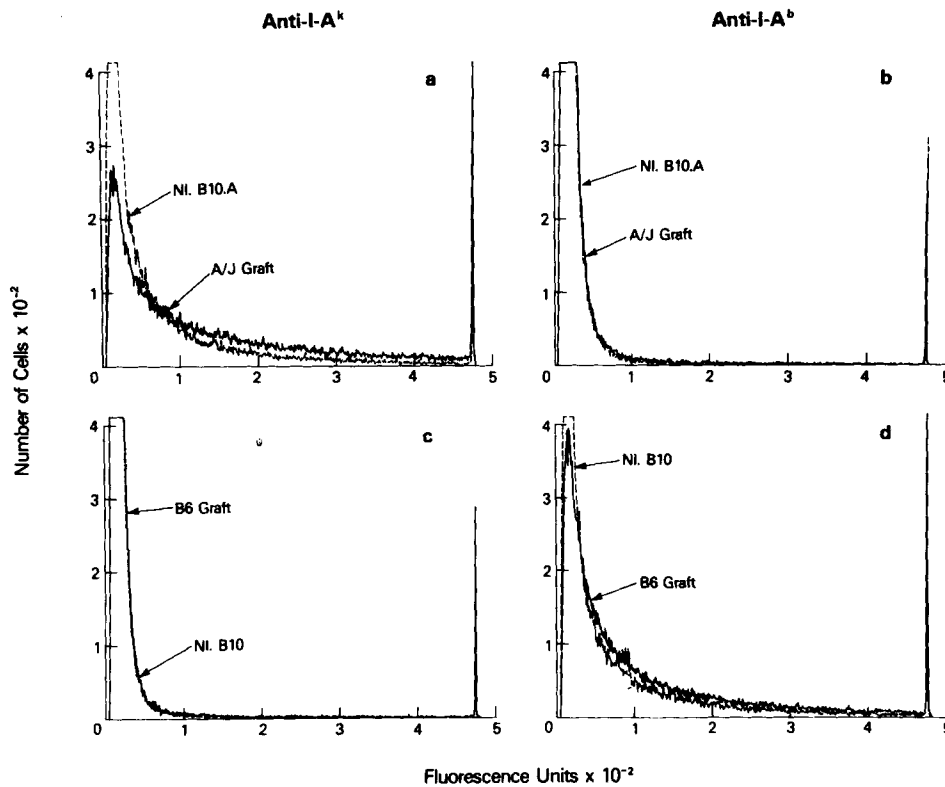


FIG. 2. Failure to detect the intrathymic presence of allogeneic host Ia determinants in thymus-engrafted radiation bone marrow chimeras [ $A \rightarrow A \times B(Tx + A \text{ Thy})$ ]. Panels a and b, IF profiles of thymocytes from normal B10.A and B10.A  $\rightarrow$  B6AF<sub>1</sub>(Tx + A/J Thy) chimeric mice stained with anti-I-A<sup>k</sup> or anti-I-A<sup>b</sup> followed by FIGAM1g. Panels c and d, IF profiles of thymocytes from normal B10 and B10  $\rightarrow$  B6AF<sub>1</sub>(Tx + B6 Thy) chimeric mice stained with anti-I-A<sup>k</sup> or anti-I-A<sup>b</sup> followed by FIGAM1g.

results demonstrate that the allogeneic I-A determinants of the nonthymic host were not present in detectable quantities within these chimeric thymi.

However, these results do not exclude the possibility that cells or alloantigen from the nonthymic host were present intrathymically in numbers insufficient to be detected by FMF, but were present in numbers sufficient to induce tolerance intrathymically. To determine whether or not flow microfluorometry was sufficiently sensitive to detect the numbers of allogeneic cells that are capable of tolerizing intrathymically, we examined thymocytes from mice that had been neonatally injected with allogeneic lymphoid cells, because the maintenance of long-term tolerance in such mice has been reported to parallel the persistence of the alloantigen in these mice (21). B10.BR (H-2<sup>k</sup>) neonates were injected with B10.BR  $\times$  B10.D2 (H-2<sup>b</sup>  $\times$  H-2<sup>d</sup>) lymphoid cells, and their thymocytes were assayed 2-3 mo later for functional reactivity against H-2<sup>d</sup> by MLR as well as for the intrathymic presence of H-2<sup>d</sup> by flow microfluorometry. The results of one such experiment are shown in Table VII. Of the five individual thymocyte populations assayed, only thymocytes from animal five were tolerant to H-2<sup>d</sup>, and only thymocytes from animal five contained detectable numbers of cells expressing H-2<sup>d</sup>. Indeed, of the 13 individual animals of this type

TABLE VII

*Correlation between the Intra-Thymic Presence of Cells Expressing H-2<sup>d</sup> Alloantigen, as Determined by Flow Microfluorometry, and the Functional Tolerance to H-2<sup>d</sup> Expressed by Thymocytes from H-2<sup>k</sup> Mice That Had Been Neonatally Injected with H-2<sup>d</sup> Allogeneic Cells*

Animal*	Responder thymocytes	Strain of tolerizing inoculum	Percent thymus cells <sup>‡</sup> positive for H-2 <sup>d</sup>	Stimulator cells			
				B10.BR	B10.D2	B10	Medium
				<i>cpm × 10<sup>-3</sup></i>			
1	B10.BR	B10.BR × B10.D2	0	0.6 ± 0.1	10.9 ± 0.6	6.6 ± 0.7	0.6 ± 0.1
2	B10.BR	B10.BR × B10.D2	0	0.5 ± 0.1	3.5 ± 0.7	4.5 ± 0.6	0.3 ± 0.1
3	B10.BR	B10.BR × B10.D2	0	0.6 ± 0.1	4.2 ± 0.4	4.3 ± 0.4	0.4 ± 0.1
4	B10.BR	B10.BR × B10.D2	0	0.6 ± 0.1	4.7 ± 0.1	6.9 ± 0.5	0.4 ± 0.1
5	B10.BR	B10.BR × B10.D2	2.5	0.3 ± 0.1	0.3 ± 0.1	1.5 ± 0.1	0.2 ± 0.1

\* B10.BR neonates were injected with  $2 \times 10^7$  spleen cells from adult B10.BR × B10.D2 mice. 2-3 mo later their thymocytes were assessed for the presence of H-2<sup>d</sup> and for MLR reactivity against H-2<sup>d</sup>.

<sup>‡</sup> See Table IV footnote (§). Thymocytes were stained with anti-H-2<sup>d</sup> reagents followed by F1RAMG<sub>2</sub>.

that we have examined so far, thymocytes from only two mice were fully tolerant by MLR to H-2<sup>d</sup>, and their thymocyte populations were the only ones that contained detectable numbers of cells expressing either H-2<sup>d</sup> or I<sup>d</sup> alloantigens. It should be noted that the majority of mice did not contain detectable numbers of H-2<sup>d</sup>-positive cells in their thymi, even though they did contain detectable numbers of H-2<sup>d</sup>-positive cells in their spleens, confirming that they had been successfully injected with H-2<sup>d</sup> allogeneic cells. Thus, in such mice a concordance exists between intrathymic tolerance to allogeneic Ia determinants, as measured by MLR, and the detection by FMF of cells within the thymus that express allogeneic determinants. These results, which will be presented in greater detail elsewhere, argue that FMF analysis of thymocyte populations is, in fact, sufficiently sensitive to detect the numbers of allogeneic cells that are required to induce tolerance intrathymically.

Taken together, all of these results suggest that the tolerance of A → A × B(Tx + A Thy) chimeric thymocytes to the allogeneic Ia determinants of the nonthymic host did not result from the intrathymic presence of those allogeneic determinants, because they should have been detected by flow microfluorometry. However, it is important to emphasize that our failure to detect such determinants intrathymically, even by flow microfluorometry, does not necessarily exclude their presence. Nevertheless, in the absence of any evidence to support either an intrathymic or post-thymic tolerization mechanism as the explanation for the specific tolerance of thymocytes from thymus-engrafted radiation bone marrow chimeras to the allogeneic Ia determinants of the nonthymic host, it seems reasonable to conclude that such thymic nonreactivity resulted from a prethymic tolerization mechanism.

### Discussion

To gain insights into the mechanism by which the thymus influences the MHC-specific T cell repertoire, the present study was performed to ascertain whether precursor T cells first express receptors specific for I region-encoded determinants before their entry into the thymus. Because such cells do not yet exhibit functional competence, it was not possible to directly assess the ability of pre-T cells to recognize

and react against alloantigen. However, experiments with thymocytes from allogeneic chimeras suggested that T cells can be specifically tolerized to allogeneic Ia determinants, which they encounter during their differentiation toward functional competence. Indeed, the ability to induce specific tolerance in immature T cell precursors to allogeneic Ia determinants suggested that immature T cell precursors were able to specifically recognize allogeneic Ia determinants, even though they were unable to directly react against them. We found that T cell precursors were tolerized to allogeneic Ia determinants encountered on intrathymic elements of the host animal. More importantly, we also found that T cell precursors were specifically tolerized to allogeneic Ia determinants encountered on extrathymic host elements as well. Indeed, the tolerance of thymic T cells to extrathymic Ia determinants suggests that the tolerization events occurred extrathymically but are reflected by the subsequent intrathymic reactivity pattern of the developing thymocytes.

The tolerization of strain A thymocytes from  $A \rightarrow A \times B(T_x + A \text{ Thy})$  chimeras to extrathymic allogeneic host  $I^B$  determinants could theoretically have occurred post-thymically, intrathymically, or prethymically. A post-thymic mechanism could account for our results if suppressor cells were generated post-thymically and recirculated to the thymus to specifically suppress anti-host  $I^B$  reactivity of the T cells developing within the thymus. However, when cell-mixing experiments were performed to test this possibility, no evidence for suppression was found. An intrathymic mechanism could explain our results if allogeneic host  $I^B$  determinants were present in the engrafted strain A thymus, so that pre-T cells were actually tolerized to allogeneic  $I^B$  determinants within the thymus itself. However, when the engrafted thymi were typed for the presence of allogeneic extrathymic Ia determinants by flow microfluorometry, a method shown to be capable of detecting the intrathymic presence of allogeneic I-A determinants, no such determinants were found. Clearly, we cannot exclude the possibility that the thymus contained quantities of allogeneic host Ia determinants that were sufficient to tolerize but insufficient to be detected. However, experiments designed to determine the sensitivity of FMF analysis revealed that it, in fact, appeared to be sufficiently sensitive to detect the numbers of allogeneic cells that result in intrathymic tolerance. Although our failure to detect the intrathymic presence of either allogeneic host Ia determinants or post-thymically induced suppressor cells cannot completely exclude their presence, our failure to detect either one argues against an intrathymic or post-thymic tolerization process as the explanation for our results. Thus, our results suggest that T cell tolerance can be specifically induced prethymically, and, by extension, that T cell precursors express receptors specific for allogeneic Ia determinants before their entry into the thymus.

The present results also have implications for the role of the thymus in the generation of new T cell receptor specificities that were not expressed prethymically. If novel alloreactive T cell specificities were generated within the thymus, anti-B receptors should have arisen anew within the strain A thymi of  $A \rightarrow A \times B(T_x + A \text{ Thy})$  chimeras, regardless of the prethymic tolerization of the strain A T cells to allogeneic host B MHC determinants. However, little if any anti-B reactivity was observed in this situation (see Table V). Thus, these results argue that, within the sensitivity of the MLR to detect the presence of alloreactive cells, T cells expressing novel anti- $I^B$  receptor specificities were not generated in the thymus. Because much, if not all, alloreactivity is thought to derive from cross-reactions of the T cell repertoire

for self + X (22–24), it might be speculated that the failure to detect the intrathymic generation of novel anti-allo Ia receptor specificities also implies that novel self Ia + X receptor specificities were not generated intrathymically. In any case, the present results argue against the concept that novel anti-allo Ia receptor specificities that were not expressed prethymically are generated intrathymically. Instead, these results suggest that the function of the thymus in determining the MHC specificity of the T cell repertoire is to select from a population of precursor cells that express a prethymically determined receptor repertoire.

Several mechanisms for the prethymic tolerization of developing T cells to Ia determinants can be imagined. An early step leading to tolerization is presumably specific recognition and binding of prethymic Ia determinants via anti-Ia receptors expressed by pre-T cells. Such pre-T cells might never migrate to the thymus, either because they are physically deleted or because they remain irreversibly bound to prethymic Ia determinants. Alternatively, having bound prethymic Ia determinants, pre-T cells might be triggered to mutate their receptor specificity so that they no longer bind prethymic Ia determinants with high affinity, allowing them to migrate to the thymus and become functionally competent but expressing an altered receptor specificity. Our results do not distinguish among these or other possible mechanisms of prethymic tolerization but do suggest a possible preliminary role for the prethymic environment in the selection and determination of the T cell repertoire.

In conclusion, the present results argue against the concept that pre-T cells first express anti-Ia receptors intrathymically. Rather, these experiments are most consistent with the concept that pre-T cells express anti-Ia receptors before their entry into the thymus and that the recognition by pre-T cells of allogeneic Ia determinants in the prethymic compartment results in their functional inactivation.

### Summary

The present study has assessed whether precursor T cells express receptors specific for the recognition of allogeneic I region-encoded determinants before their entry into the thymus. Because the ability of thymocytes to proliferate in response to allogeneic stimulator cells was shown to primarily result from the recognition of allogeneic I region determinants, thymocytes must already express anti-Ia receptors. In contrast, the expression of anti-Ia receptors by functionally immature thymocyte precursors could not be directly assessed by mixed lymphocyte reaction reactivity. However, expression of anti-Ia receptors by thymocyte precursors could be assessed by their ability to be specifically tolerized by the allogeneic Ia determinants that they encountered during their differentiation.

To determine whether T cell precursors could specifically recognize and be tolerized to allogeneic Ia determinants expressed prethymically, thymus-engrafted radiation bone marrow chimeras were constructed [ $A \rightarrow A \times B(Tx + A Thy)$ ] such that strain A T cells would be differentiating within a syngeneic strain A thymus but would have been previously exposed to the allogeneic strain B Ia determinants of the irradiated  $A \times B$  host. The strain A thymocytes from these experimental animals were indeed tolerant to the extrathymic allogeneic strain B Ia determinants expressed by the irradiated host. Such tolerance was not mediated by detectable suppression and was not explained by the presence intrathymically of extrathymic allogeneic Ia determinants. Thus, these results suggest that T cell precursors can be specifically tolerized

prethymically, and, consequently, that they do express anti-Ia receptors before their entry into the thymus. In addition, the failure to detect the generation of thymocytes with specificity for the allogeneic Ia determinants of the irradiated host, which were not deleted prethymically, argues that novel anti-allo Ia receptor specificities are not generated intrathymically.

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