T CELL TOLERANCE TO NON–H-2-ENCODED STIMULATORY ALLOANTIGENS IS INDUCED INTRATHYMICALLY BUT NOT PRETHYMICALLY

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Self-recognition and self-tolerance are two characteristics of the receptor repertoire expressed by functionally reactive T cells. Both of these facets of the T cell receptor repertoire are importantly influenced by the environment in which immature T cell precursors differentiate. For example, T cells specifically recognize as self the major histocompatibility complex (MHC)[1]-encoded determinants expressed by cellular elements in their differentiation environment, and fail to recognize as foreign the non–MHC-encoded determinants that are also present in their differentiation environment. It is now known that the nonlymphoid cells present within the thymus perform a unique role in determining the MHC specificities that T cells recognize as self-restriction elements (1, 2). In contrast, the precise role of the thymus in establishing self-tolerance is less clear. From experiments performed in mice engrafted with allogeneic thymuses, it is known that thymic nonlymphoid cells can induce tolerance to antigens that only they express (3). However, such experiments could not assess the possible role of the prethymic environment in the induction of T cell tolerance. For instance, if T cell precursors express a receptor repertoire before their entry into the thymus, it is possible that the prethymic compartment would be the initial site in which T cell tolerance is induced. Alternatively, if T cells first express their receptor repertoire within the inductive environment of the thymus, the thymus would be the initial site in which T cell tolerance to any antigen could be induced. Consequently, determining the initial site in which T cell tolerance to MHC and non–MHC-encoded alloantigens is induced has important implications for our understanding of the mechanisms of self-tolerance as well as for the role performed by the thymus in generating and processing the T cell receptor repertoire.

It was recently observed that intrathymic T cells were tolerant to the MHC alloantigens expressed only by the extrathymic host through which their precursors had migrated (3, 4). These findings could be envisioned as resulting from the deletion of T cell precursors with receptors specific for MHC alloantigens in the prethymic compartment. Thus, these results suggested that the inductive environment of the thymus was not required for T cell precursors to express

[1] *Abbreviations used in this paper*: BSA, bovine serum albumin; MHC, major histocompatibility complex; MLS, minor lymphocyte-stimulating locus; Tx, thymectomized, thymectomy.

receptors specific for MHC alloantigens. It should be noted that in these studies the MHC alloantigens of the irradiated extrathymic host were not detected intrathymically (3, 4), but it is not yet technically possible to prove with certainty that such alloantigens were not present in the thymus in quantities sufficient to have induced tolerance.

In the present report, the ability of T cell precursors to be tolerized prethymically to non–MHC-encoded alloantigens was assessed. The non–MHC-encoded alloantigens chosen were those that stimulate primary proliferative responses in MHC-identical T cell populations and that have been reported to be encoded in the minor lymphocyte-stimulating locus (MLS) (5). The proliferative responses of unprimed T cells to MLS alloantigens are similar to their responses to MHC alloantigens in that there exists a high frequency of MLS-specific as well as MHC-specific responding T cells (6, 7), and that the cells which stimulate both proliferative responses are Ia^+ (8, 9). Consequently, since thymocytes are tolerant to the MHC alloantigens expressed by the extrathymic host through which their precursors had migrated, it might be expected that thymocytes would also be tolerant to the MLS alloantigens expressed by the same host. Surprisingly, however, this was not the case. It was observed that intrathymic T cells were not tolerant to the MLS alloantigens expressed extrathymically, but were only tolerant to the MLS alloantigens expressed on intrathymic elements. Thus, the present results indicate that the thymus is the initial site in which T cell tolerance to MLS alloantigens is induced and represents the first example in which T cell tolerance to an antigen occurs intrathymically but not prethymically.

Materials and Methods

Animals. C3H/HeN mice were obtained from the Small Animal Section, National Institutes of Health. All other mice used in this study were obtained from The Jackson Laboratory, Bar Harbor, ME. The strains and genetic haplotypes of these mice are shown in Table I.

Thymus-engrafted Radiation Bone Marrow Chimeras. Normal and thymectomized adult mice were grafted subcutaneously with 3–4 thymic lobes from 1-d-old B6 mice (4). 3–4 d after grafting, these mice were exposed to 980 rad from a ^{137}Cs source and subsequently injected with 1.5×10^7 B10 bone marrow cells that had been depleted of T cells by treatment with rabbit anti-mouse brain sera plus complement. These mice were studied individually 5–14 wk after irradiation. The genetic phenotype of the bone marrow, host, and thymus of these experimental chimeras is shown in Table I.

Mixed Lymphocyte Reaction. Mixed lymphocyte cultures were performed in flat-bottomed microtiter plates using 10^6 responder thymocytes and 0.5×10^6 stimulator cells or 0.5×10^6 responder spleen cells and 0.2×10^6 stimulators. Macrophage-enriched spleen cells were used as stimulator cells for these assays. The stimulator cells were isolated on bovine serum albumin (BSA) gradients by suspending the spleen cells in 5 ml of 35% BSA (Sigma Chemical Co., St. Louis, MO) and overlaying this with 5 ml of 11% BSA. The cells that banded at the interface after centrifugation (22,000 *g*) for 30 min were enriched in macrophages and were potent stimulators of proliferation (10). These cells were irradiated (2,000 rad) before culture.

Culture Conditions. Cells were cultured in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) which was supplemented with nonessential amino acids, 5×10^{-5} M 2-mercaptoethanol, 2 mM glutamine, 1 mM sodium pyruvate, and antibiotics. In addition, for cultures in which thymocytes were the responding cells, the medium contained 10% fetal calf serum; for cutures in which spleen cells were the responding cells, the medium contained either 5% fetal calf serum or normal mouse serum. No exogenously generated

TABLE I
Genetic Haplotypes of the Mice Used in this Study

Normal mice	H-2, MLS
AKR/J	k, a
B10, B6	b, b
B10.BR	k, b
B10.D2	d, b
CBA/J	k, d
C3H/HeN	k, c
DBA/2	d, a
B6 × DBA/2 (B6D2F$_1$)	b/d, b/a
(B6 × CBA/J)F$_1$	b/k, b/d

Experimental mice	Bone marrow	Host	Engrafted thymus	In situ thymus
	H-2, MLS	H-2, MLS	H-2, MLS	H-2, MLS
B10 → (B6 × CBA/J)F$_1$ (Tx + B6 thymus)	b, b	b/k, b/d	b, b	—
B10 → B6D2F$_1$ (Tx + B6 thymus)	b, b	b/d, b/a	b, b	—
B10.BR → C3H/HeN (Tx + B10.BR thymus)	k, b	k, c	k, b	—
B10 → (B6 × CBA/J) (+ B6 thymus)	b, b	b/k, b/d	b, b	b/k, b/d

growth factors were added to these cultures. Cultures were incubated for 4 d and pulsed with [^{3}H]thymidine (New England Nuclear, Boston, MA) 8–12 h before harvest.

Results

Experimental Strategy. The purpose of the present study was to determine where T cell tolerance to MLS alloantigens is induced. The experimental strategy used to approach this question was: (*a*) to introduce MLS alloantigens in the extrathymic host through which T cell precursors would migrate on their way to the thymus, and (*b*) to then determine the anti-MLS reactivity of intrathymic T cells after they had attained functional competence. The experimental model devised was one in which T cells or their precursors could, in theory, be selectively exposed to alloantigens either in their extrathymic or intrathymic differentiation environments. Neonatal thymic lobes were transplanted into thymectomized (Tx) adult mice that were subsequently irradiated (980 rad ^{137}Cs) and reconstituted with T cell-depleted bone marrow cells as a source of hematopoietic stem cells. Experimental animals of this type are designated as bone marrow donor → irradiated recipient followed by parentheses in which additional manipulations such as thymectomy (Tx) and the origin of the engrafted thymus are indicated. For example, an animal of the designation B10 → B6 × CBA/J(Tx + B6 Thy) is a B6 × CBA/J mouse that had been thymectomized, engrafted with neonatal B6 thymic lobes, subsequently irradiated, and reconstituted with T cell-depleted B10 bone marrow cells. In this combination, B10 (H-2^b, MLS^b) bone marrow-derived pre-T cells would potentially encounter both MLS^d and H-2^k alloantigens of the irradiated B6 × CBA/J host during their circulation and migration to the thymus, but would ultimately differentiate within the irradiated syngeneic B6 (H-2^b, MLS^b) thymus graft.

The origin of the lymphocytes present in these experimental animals has been

extensively examined and reported previously (3, 4). It was shown that the lymphoid cells which repopulate both the thymuses and spleens of these experimental animals are of donor bone marrow origin (3, 4). Additionally, it was shown that the engrafted thymus of most animals contained neither cells nor quantities of MHC alloantigens from the irradiated F_1 host that could be detected by immunofluorescence and flow microfluorometry. For clarity, the genetic haplotypes of the normal mice, as well as the genetic composition of the experimental animals constructed for this study, are shown in Table I.

Thymocytes Proliferate in Response to MLS-encoded Alloantigens. MLS-encoded alloantigens stimulate primary proliferative T cell responses between MHC-identical spleen cell populations that differ in their non-MHC genes (5). The MLS locus maps to chromosome 1, and four alleles (a, b, c, d) of unequal antigenic strength have been described (5, 11). Of the four alleles, MLS^a and MLS^d stimulate strong primary responses, MLS^c stimulates weakly, and MLS^b does not stimulate at all. For the purposes of the present study, it was necessary to first determine if thymocytes, like splenic T cells, were able to proliferate in response to MLS alloantigens. This possibility was assessed by stimulating normal thymocytes with irradiated BSA gradient-purified spleen cells from MHC-identical but MLS-disparate strains of mice. Gradient-purified spleen cells are enriched in macrophages and stimulate proliferative responses to both MHC and MLS alloantigens significantly better than unfractionated spleen cell populations. In Table II, it is shown that thymocytes from B10.BR ($H\text{-}2^k$, MLS^b) responded well to stimulators from CBA/J ($H\text{-}2^k$, MLS^d) and AKR/J ($H\text{-}2^k$, MLS^a), but responded weakly to stimulators from C3H/HeN ($H\text{-}2^k$, MLS^c). In contrast, stimulator cells from B10.BR ($H\text{-}2^k$, MLS^b) essentially failed to provoke a proliferative response from any of the MHC-identical responder thymocytes, but did stimulate an MHC-specific proliferative response from B10 thymocytes. This thymocyte response pattern coincides with the reported spleen cell response pattern against each of the four MLS alleles. Thus, these data demonstrate that thymocytes can generate primary proliferative responses against stimulatory non-H-2 alloantigens which are probably identical to the MLS alloantigens that stimulate spleen T cell responses.

TABLE II

Thymocytes Proliferate in Response to MLS- and MHC-Encoded Alloantigens

Responder thymocyte	Strain	Stimulator cells			
	(H-2, MLS)	AKR/J (k, a)	CBA/J (k, d)	C3H/HeN (k, c)	B10.BR (k, b)
		Δ *cpm* $\times$ 10^{-3}*			
AKR/J	(k, a)	0.7 ± 0.1	17.9 ± 2.3	7.6 ± 0.7	2.2 ± 0.2
CBA/J	(k, d)	6.6 ± 0.2	0.4 ± 0.2	1.2 ± 0.2	0.7 ± 0.1
B10.BR	(k, b)	10.5 ± 0.2	36.7 ± 6.1	5.6 ± 0.5	0.1 ± 0.1
C3H/HeN	(k, c)	44.5 ± 2.3	32.3 ± 2.7	0.8 ± 0.2	1.7 ± 0.1
B10	(b, b)	27.3 ± 1.9	38.7 ± 3.6	23.8 ± 1.9	26.1 ± 3.1

* These values are the mean ± the standard error of quadruplicate cultures containing stimulator cells minus the mean ± the standard error of quadruplicate cultures without stimulator cells. The [^{3}H]thymidine uptake for the cultures without stimulator cells (medium control) was <1,100 cpm for all groups.

Failure to Prethymically Tolerize T Cell Precursors to MLS Alloantigens. To examine whether T cell precursors are initially tolerized to MLS alloantigens prethymically, B10 → B6 × CBA/J(Tx + B6 Thy) experimental animals were constructed. These chimeras were rested for at least 5 wk, at which time the anti-MLS reactivity of the cells that had repopulated the B6 thymus graft was assessed. It can be seen in Table III that the thymocytes from individual B10 → B6 × CBA/J(Tx + B6 Thy) responded well to third party H-2^d alloantigens expressed by B10.D2 stimulators, and were tolerant to self-H-2^b determinants expressed by B10 stimulators. In addition, these chimeric thymocytes were also tolerant to the allogeneic H-2^k determinants of the B6 × CBA/J host as shown by their failure to respond to B10.BR stimulators. These results confirm previous findings (3, 4) that thymocytes are tolerant to the MHC-encoded alloantigens expressed by the extrathymic host and are consistent with the concept that T cell precursors can be tolerated to allogeneic MHC determinants before their entry into the thymus. In contrast, the same chimeric thymocyte populations that were tolerant to the allogeneic H-2^k determinants of the B6 × CBA/J host were reactive to the allogeneic MLS^d determinants of the B6 × CBA/J host as demonstrated by their response to CBA/J stimulators (Table III). The anti-MLS^d reactivity of the chimeric thymocytes cannot simply be due to the reactivity of undetectable numbers of residual radiation-resistant B6 thymocytes in the B6 thymus graft that had never encountered the alloantigens of the B6 × CBA/J host, because such B6 "passenger" thymocytes would be reactive against H-2^k as well as MLS^d alloantigens. In addition, the anti-MLS^d reactivity of the chimeric thymocytes cannot be due to the possibility that the CBA/J stimulators provoke nonspecific responses, since they did not stimulate proliferative responses in control CBA/J or B6 × CBA/J thymocyte populations. Finally, it should be noted that the design of these experiments has circumvented the possibility that tolerance to CBA/J stimulators is H-2 restricted, and thus requiring the simultaneous exposure of T cell precursors to both H-2^k and MLS^d determinants, since any such requirements would have been fulfilled by the B6 × CBA/J extrathymic host. Thus, these results demonstrate that T cell precursors are not

TABLE III

Failure of T Cell Precursors to Be Tolerized Prethymically to MLS^d Alloantigen

Responder thymocytes	Stimulator cells			
	B10.D2	B10	B10.BR	CBA/J
	Δ *cpm* × 10^{-3}*			
B10 → (B6 × CBA/J)F_1 (Tx + B6 thy)				
Chimera 1	45.4 ± 3.5	0.5 ± 0.1	0.1 ± 0.1	21.5 ± 2.3
Chimera 2	34.3 ± 1.0	0.1 ± 0.4	0.7 ± 0.2	95.4 ± 4.3
Chimera 3	21.1 ± 1.5	1.0 ± 0.3	2.0 ± 0.2	19.2 ± 1.1
Chimera 4	ND	0.6 ± 0.1	0.2 ± 0.1	23.7 ± 0.8
B10	15.8 ± 2.9	0.5 ± 0.1	12.2 ± 0.3	69.1 ± 3.6
B10.BR	19.6 ± 2.5	8.0 ± 1.0	0	35.3 ± 3.1
CBA/J	30.7 ± 6.7	23.5 ± 4.3	0.8 ± 0.3	3.3 ± 2.7
(B6 × CBA/J)F_1	25.3 ± 1.8	0.5 ± 0.1	0.5 ± 0.4	1.5 ± 1.9

* See footnote to Table II. The medium control was <1,000 cpm in all groups.

tolerized to allogeneic MLS determinants before their entry into the thymus.

Irradiated F_1 Host Expresses Sufficient Quantities of Allogeneic MLS Determinants to Tolerize Peripheral T cells. The anti-MLS^d reactivity of H-2^b thymocytes from B10 → B6 × CBA/J(Tx + B6 Thy) experimental animals might have been due to the lack of sufficient amounts of MLS^d alloantigen in the irradiated B6 × CBA/J host to induce T cell tolerance. If this were the case, spleen cells as well as thymocytes from these experimental mice should be reactive against MLS^d determinants. To assess this possibility, spleen cell responses were evaluated from the same experimental animals whose thymocyte reactivities were shown in Table III. It is evident in Table IV that even though the chimeric spleen cells responded well against third party H-2^d alloantigens expressed by B10.D2 stimulators, they failed to respond against either the H-2^k or the MLS^d alloantigens of the B6 × CBA/J host as demonstrated by their failure to respond against either B10.BR or CBA/J stimulators (Table IV). Thus, in the same individual experimental animals, intrathymic T cells were reactive against MLS^d determinants whereas extrathymic T cells were tolerant. These results indicate that the irradiated B6 × CBA/J host expressed sufficient quantities of MLS^d determinants to tolerize postthymic T cells. It should be noted that these results also strongly argue against the possibility that the anti-MLS reactivity expressed by intrathymic T cells was due to the recirculation of postthymic T cells back to the thymus.

Reactivity of Intrathymic T Cells Against Extrathymic MLS Alloantigens Is Observed for All Three Stimulatory MLS Alleles. Since it was possible that the intrathymic reactivity against extrathymic MLS alloantigens was unique to MLS^d determinants expressed by H-2^k cells, other experimental mice were studied which involved a different combination of MHC and MLS alloantigens. Chimeras were constructed using thymectomized (B6 × DBA/2J)F_1 mice which were engrafted with B6 thymic lobes, irradiated, and reconstituted with B10 bone marrow stem cells. In these mice, H-2^b pre-T cells would be exposed to the MLS^a and H-2^d alloantigens of the B6 × DBA/2J host but would ultimately differentiate in a syngeneic B6 thymus. The results obtained from these experimental mice are

TABLE IV

Spleen Cells from B10 → (B6 × CBA/J)F_1 (Tx + B6 thymus) Chimeras Are Tolerant to the MLS^d Alloantigen

Responder spleen cells	Stimulator cells			
	B10.D2	B10	B10.BR	CBA/J
	Δ *cpm* × 10^{-3}*			
B10 → (B6 × CBA/J)F_1 (Tx + B6 thy)				
Chimera 1	30.1 ± 5.3	3.8 ± 1.2	0.5 ± 1.2	2.6 ± 2.4
Chimera 2	27.2 ± 2.1	4.8 ± 1.6	2.8 ± 0.9	3.1 ± 0.8
Chimera 3	36.1 ± 2.8	9.6 ± 1.6	4.6 ± 1.0	2.5 ± 2.1
Chimera 4	21.6 ± 2.6	6.0 ± 1.0	0.6 ± 1.1	2.4 ± 1.2
B10	85.8 ± 2.1	7.2 ± 1.5	69.8 ± 8.9	51.7 ± 4.5
B10.BR	78.5 ± 7.3	60.5 ± 4.9	1.9 ± 2.5	51.9 ± 4.2
(B6 × CBA/J)F_1	151.9 ± 9.9	7.0 ± 1.5	9.2 ± 3.3	7.6 ± 4.9

* See footnote to Table II. The medium controls range from 5,729 to 12,660 cpm.

shown in Table V. Similar to what was observed above, the H-2^b thymocytes that had repopulated the B6 thymus graft in these animals were tolerant to the H-2^d alloantigens of the B6 × DBA/2J host as indicated by their failure to react against B10.D2 stimulators, but were strongly reactive against the MLS^a alloantigens of the B6 × DBA/2J host as indicated by their responses against DBA/2 stimulators. Also similar to the results obtained above, the spleen cells from these animals were tolerant to MLS^a alloantigens of the F_1 host since they failed to react significantly against DBA/2J stimulators. Thus, these results demonstrate that the failure of intrathymic T cells to be prethymically tolerized to MLS alloantigens is not unique to a single combination of H-2 and MLS.

The next possibility considered was that prethymic exposure to both MHC and MLS alloantigens simultaneously might have interfered with the induction of MLS-specific T cell tolerance. To examine this, experimental mice were constructed of the type B10.BR → C3H/HeN(Tx + B10.BR Thy). This com-

TABLE V

T Cell Precursors Are Tolerized Postthymically but Not Prethymically to MLS^a Alloantigen

Responder cells	Stimulator cells			
	B10.BR	B10	B10.D2	DBA/2J
	Δ *cpm* × 10^{-3}*			
Thymocytes				
B10 → B6D2F$_1$(Tx + B6 thy)				
Chimera 1	10.7 ± 0.7	0.2 ± 0.1	1.3 ± 0.1	38.7 ± 2.9
Chimera 2	15.4 ± 0.4	0	1.1 ± 1.1	19.6 ± 1.8
B10	8.7 ± 0.1	0.5 ± 0.2	12.6 ± 1.1	53.8 ± 1.2
B10.D2	13.2 ± 1.3	12.4 ± 1.1	1.4 ± 0.1	36.5 ± 3.7
B6D2F$_1$	24.7 ± 2.3	0.7 ± 0.4	0.9 ± 0.3	2.9 ± 0.7
Spleen Cells				
B10 → B6D2F$_1$ (Tx + B6 thy)				
Chimera 1	31.7 ± 0.7	0.6 ± 0.2	1.2 ± 0.2	4.9 ± 0.4
Chimera 2	15.1 ± 0.3	0	0	2.5 ± 0.6
B10	145.5 ± 11.2	3.0 ± 0.8	158.9 ± 9.4	150.6 ± 8.1
B10.D2	138.0 ± 14.3	98.6 ± 8.5	9.5 ± 0.5	169.3 ± 10.3
B6D2F$_1$	112.7 ± 8.1	0.8 ± 0.5	5.0 ± 1.7	5.0 ± 0.8

* See footnote to Table II. The medium controls for the thymocyte responses were <1,000 cpm for all groups and the medium controls for the spleen cell groups ranged from 1,272 to 10,380 cpm.

TABLE VI

In an MHC-Identical Chimera, T Cell Precursors Are Not Prethymically Tolerized to the MLS^c Alloantigen

Responder thymocytes	Stimulator cells		
	B10.BR	C3H/HeN	CBA/J
		Δ *cpm* × 10^{-3}*	
B10.BR → C3H/HeN (Tx + B10.BR thy)	0.3 ± 0.1	6.9 ± 0.4	14.2 ± 0.2
B10.BR	0	6.2 ± 0.9	9.3 ± 0.6

* See footnote to Table II. The medium control for the chimeric thymocytes was 236 cpm; for the B10.BR thymocytes, 570 cpm.

bination permitted the evaluation of both the induction of tolerance to MLS alloantigens in the absence of foreign MHC alloantigens and the induction of tolerance to the weakly stimulatory MLS^c allele. It can be seen in Table VI that even in this situation, expression of MLS^c alloantigens in the extrathymic environment had no effect on intrathymic T cell reactivities, as indicated by the similar responses of the chimeric and normal thymocyte populations to C3H stimulators. Thus, the failure to prethymically tolerize T cell precursors to MLS alloantigens is a general phenomenon that was observed for all three stimulatory MLS alleles.

Thymocytes Are Tolerant to MLS Alloantigens Expressed Intrathymically But Are Not Tolerant to MLS Alloantigens Expressed Extrathymically. We considered that the failure of intrathymic T cells to be tolerized to MLS alloantigens which their precursors had potentially encountered in the prethymic environment might simply reflect the possibility that thymocytes can never be tolerized to MLS alloantigens. Such a possibility seemed highly unlikely since thymocytes from normal mice are not reactive to MLS determinants expressed by syngeneic cells. Indeed, the failure of chimeric thymocytes to be tolerized to MLS determinants expressed prethymically suggested that the tolerance of normal thymocytes to syngeneic MLS determinants was induced during their differentiation within the thymus, presumably because they encountered and recognized MLS determinants expressed on intrathymic elements. Therefore, to determine if tolerance to MLS determinants can be induced intrathymically, it was necessary to determine (*a*) if intrathymic elements do express MLS determinants, and (*b*) whether thymocytes can be tolerized to MLS alloantigens expressed by intrathymic elements.

To determine if intrathymic cells bear MLS determinants, BSA gradient-purified irradiated stimulator cells were obtained from the thymuses of normal B10.BR, AKR/J, and CBA/J mice. It can be seen in Table VII that all three stimulator cell populations were able to stimulate proliferative responses by H-2 allogeneic B10 responder spleen cells. More importantly, it can also be seen that AKR/J and CBA/J stimulator cells did stimulate MLS-specific proliferative responses in H-2 identical B10.BR responder spleen cells. Thus, MLS determinants are expressed on intrathymic cells.

Next, to determine if T cell precursors are capable of being tolerized to MLS alloantigens intrathymically, experimental animals were constructed that contained two thymuses, one of which expressed allogeneic MLS determinants and

TABLE VII

Intrathymic Cells Express MLS Determinants

Responder spleen cells	Strain (H-2, MLS)	Thymic stimulator cells			
		AKR/J (k, a)	CBA/J (k, d)	B10.BR (k, b)	B10 (b, b)
		Δ *cpm* × 10^{-3}*			
B10	(b, b)	101.2 ± 3.4	92.4 ± 6.5	63.8 ± 3.6	6.1 ± 0.7
B10.BR	(k, b)	38.4 ± 5.6	32.5 ± 2.3	3.5 ± 0.2	43.9 ± 3.1

* See footnote to Table II. The medium control for the B10 responder spleen cells was 5,594 cpm; for the B10.BR responder spleen cells, 9,094 cpm.

one of which did not. Experimentally, nonthymectomized B6 × CBA/J mice were engrafted with B6 thymuses, irradiated, and reconstituted with B10 bone marrow. These mice were identical to those studied earlier except that they contained both an engrafted B6 thymus and an in situ B6 × CBA/J thymus. Both thymuses were fully repopulated with cells of B10 bone marrow origin so that both populations of thymocytes had been exposed to the identical extrathymic environment. As can be seen in Table VIII, H-2^b thymocytes from the B6 thymus graft were strongly reactive against MLS^d expressed by CBA/J stimulators; in contrast, H-2^b thymocytes from the B6 × CBA/J in situ thymus were markedly depleted of anti-MLS^d reactivity and were not significantly more reactive against the CBA/J stimulators than were the genetically tolerant CBA/J and B6 × CBA/J control thymocytes. Thus, the B10 cells that repopulated the MLS^d-negative thymus were reactive to the MLS^d antigen whereas the B10 cells that repopulated the MLS^d-positive thymus were tolerant to that antigen. It should be emphasized that both thymocyte populations (*a*) were derived from the same B10 stem cell source, (*b*) had been exposed to the identical extrathymic MLS^d- and H-2^k-containing environment, and (*c*) were equally tolerant to the H-2^k alloantigens of the B6 × CBA/J host. Thus, the difference in anti-MLS^d reactivity between these two thymocyte populations demonstrates that the anti-MLS reactivity of intrathymic T cells is only influenced by intrathymic elements and is not at all influenced by extrathymic elements. It can be concluded that in these experimental mice the thymus is the primary site in which T cell tolerance to MLS alloantigens is induced.

TABLE VIII

Thymocytes Are Tolerant to MLS Alloantigens Only When these Alloantigens Are Expressed Intrathymically

Responder thymocytes	Stimulator cells			
	B10.D2	B10	B10.BR	CBA/J
	Δ *cpm* × 10^{-3}*			
B10 → (B6 × CBA/J)F_1 (+ B6 thy)‡				
Chimera 1				
B6 graft	24.8 ± 1.7	0.4 ± 0.2	0.4 ± 0.3	108.8 ± 7.1
F_1 in situ	15.6 ± 0.9	0	0	5.9 ± 2.4
Chimera 2				
B6 graft	9.3 ± 0.5	0	0	31.9 ± 2.4
F_1 in situ	10.9 ± 0.3	0	0	3.9 ± 1.4
B10	15.8 ± 2.0	0.5 ± 0.1	8.0 ± 0.2	69.7 ± 3.1
B10.BR	9.7 ± 1.5	18.8 ± 1.3	0	35.5 ± 0.3
CBA/J	30.1 ± 5.8	23.4 ± 0.9	0.8 ± 0.1	3.2 ± 1.7
(B6 × CBA/J)F_1	25.6 ± 1.9	0.9 ± 0.4	0.5 ± 0.2	1.5 ± 0.3

* See footnote to Table II. The medium controls for all groups were <1,000 cpm.

‡ Flow microfluorometric analysis of the thymocytes stained with anti-Ly-1.1 monoclonal antibody and fluorescinated developing reagent showed that >90% of the thymocytes repopulating the F_1 in situ thymuses from these chimeras were of donor bone marrow origin.

Discussion

The T cell differentiation environment can be considered as consisting of three compartments: a prethymic compartment through which T cell precursors migrate on their way to the thymus, an intrathymic compartment in which T cell precursors differentiate into functional competence, and a postthymic compartment to which competent T cells emigrate after thymic processing. In the present study, it was demonstrated that T cell precursors are not tolerized in the prethymic compartment to MLS alloantigens, but are in both the intrathymic and postthymic compartments. These experiments demonstrate that the thymus can induce T cell tolerance to MLS alloantigens and indicate that the thymus is the initial site in which tolerance to MLS alloantigens occurs.

The observation that T cell precursors are tolerized intrathymically but not prethymically can be understood in one of two ways; either (*a*) T cell precursors cannot be tolerized to MLS alloantigens until they have undergone some degree of intrathymic differentiation, or (*b*) cells present in the prethymic compartment cannot induce tolerance in pre-T cells to MLS-encoded antigens. The first possibility, that T cell precursors are incapable of being tolerized to MLS alloantigens prethymically, implies that prethymic T cells either do not express receptors capable of binding MLS alloantigens or are unaffected by their receptors having bound MLS alloantigens. In either case, an intrathymic differentiation step would be required for T cell precursors to express functional MLS-specific receptors that, having bound MLS alloantigens, are able to induce tolerance. The alternative possibility, that the prethymic compartment is composed of cells unable to induce MLS-specific tolerance, implies that either all cells bearing MLS alloantigens, or a specialized subset of tolerance-inducing cells such as veto cells (12), are absent from the prethymic compartment. Since elements able to induce MLS-specific tolerance are present in the postthymic compartment, this possibility suggests that the prethymic and postthymic compartments are composed, at least in part, of different cellular elements. Nevertheless, the possibility that prethymic T cells cannot be tolerized to MLS alloantigens and the possibility that cells in the prethymic compartment cannot induce MLS-specific tolerance have important implications for the mechanisms by which T cell tolerance is induced. However, we feel that the failure of prethymic T cells to be tolerized to MLS alloantigens most likely reflects their failure to express MLS-specific receptors until they have undergone further differentiation in the thymus.

In all of the experimental animals examined in this study splenic T cells were tolerant to extrathymic MLS alloantigens even when intrathymic T cells present in the same mice were reactive against those MLS alloantigens. While this difference indicates that the irradiated extrathymic host does express MLS alloantigens, it also reflects the existence in these experimental animals of a postthymic tolerance mechanism. It is interesting to speculate that the mechanism by which immature T cell precursors are tolerized intrathymically may not be the same mechanism by which mature T cells are tolerized postthymically. For instance, it is conceivable that immature T cell precursors are tolerized intrathymically by a clonal deletion mechanism whereas mature T cells are tolerized extrathymically by an active suppressor mechanism.

Another striking disparity observed throughout the present study was that intrathymic T cells were consistently tolerant to the allogeneic MHC determinants expressed by the irradiated extrathymic host whereas the same intrathymic T cell populations were reactive to the allogeneic MLS determinants expressed by the same host. For example, H-2^b thymocytes from B10 → B6 × CBA/J(Tx + B6 Thy) animals were tolerant to the H-2^k alloantigens but reactive to the MLS^d alloantigens of the B6 × CBA/J hosts. This disparity cannot easily be dismissed by suggesting that MLS alloantigens, in contrast to MHC alloantigens, are expressed on only a subpopulation of extrathymic cells. It has previously been demonstrated that MHC-specific tolerance as assessed by thymocyte proliferation reflects tolerance to Ia-encoded alloantigens (3) that are also expressed on only a subpopulation of extrathymic cells, indeed largely the same subpopulation which expresses MLS alloantigens. If the observation that T cell precursors are initially tolerized in the thymus to MLS alloantigens can be generalized to all antigens including MHC alloantigens, the disparity might be explained by postulating that extrathymic Ia alloantigens, but not extrathymic MLS alloantigens, are shed and penetrate the thymus. However, efforts to detect allogeneic Ia determinants from the irradiated F_1 host in the engrafted thymuses have been unsuccessful (3). If our failure to detect extrathymic MHC alloantigens in the thymuses of these experimental mice reflects its absence, the present data would indicate that T cell precursors are tolerized to MHC alloantigens at an earlier point in their differentiation than they are tolerized to MLS alloantigens. Such a possibility has important implications for understanding receptor expression and repertoire generation in developing T cells. For example, it would suggest that the receptors that T cell precursors express initially are specific for MHC determinants and not for non–MHC-encoded MLS determinants. However, two points should be emphasized. First, since T cell proliferative responses to MLS alloantigens are not typical of T cell responses to other non–MHC-encoded alloantigens, the point in their differentiation that T cells are initially tolerized to MLS alloantigens may not necessarily reflect the point in their differentiation that T cells are initially tolerized to more conventional non–MHC-encoded alloantigens. Second, definitive evidence that T cell precursors recognize and are tolerized to MHC alloantigens prethymically has not yet been obtained and may have to await the development of more sensitive assay systems.

Streilen et al. (13) have also reported experiments that suggest that MHC-specific T cell precursors can be tolerized prethymically. However, we are aware of results from only one experimental system which suggest that T cell precursors can be tolerized prethymically to antigens other than MHC alloantigens. Using bone marrow cells from in vivo tolerized mice as a source of T cell precursors, Cohn and Scott (14) and Sanfilippo et al. (15) have suggested that carrier-specific T helper cell precursors can be tolerized prethymically. If this conclusion is correct, it suggests that the failure of T cell precursors to be prethymically tolerized to non–MHC-encoded MLS alloantigens cannot be generalized to all non–MHC-encoded antigens. This point is currently under investigation.

In conclusion, the present report demonstrates that T cell tolerance to a foreign antigen is induced intrathymically but not prethymically, indicating that the thymus is the initial site in which T cell tolerance to non–MHC-encoded

MLS alloantigens is induced. In addition, this study also suggests that there exists a mechanism for tolerizing functionally competent MLS-specific postthymic T cells. And finally, the present report has demonstrated a striking disparity in the reactivity of intrathymic T cells to the MHC and MLS alloantigens expressed by the extrathymic host through which their precursors had migrated, suggesting that T cell precursors may be tolerized to MHC alloantigens at an earlier point in their differentiation than they are tolerized to MLS alloantigens.

Summary

The present report has evaluated the differentiation compartment in which T cells are tolerized to non–major histocompatibility complex (MHC)-encoded minor lymphocyte-stimulating locus (MLS) alloantigens. It was observed that T cell precursors are not tolerized prethymically to MLS alloantigens but are tolerized intrathymically and postthymically to MLS alloantigens. The failure of prethymic T cells to be tolerized indicates either that T cell precursors are unable to be tolerized to MLS alloantigens or that cells in the prethymic compartment are unable to induce MLS-specific tolerance. In either case, these results demonstrate that the thymus is the initial site in which T cell tolerance to MLS alloantigen is induced.

The present results also demonstrate a striking disparity in the reactivity of thymocytes to MHC and MLS alloantigens expressed in the extrathymic host through which their precursors had migrated. In the experimental mice constructed for these studies, intrathymic T cells were tolerant to the MHC alloantigens but were reactive to the MLS alloantigens expressed by the extrathymic host. This observation is consistent with the concept that T cell precursors may be tolerized to MHC alloantigens at an earlier point in their differentiation than they are tolerized to non–MHC-encoded MLS alloantigens.

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