

# Tolerance of thymic cytotoxic T lymphocytes to allogeneic H-2 determinants encountered prethymically: Evidence for expression of anti-H-2 receptors prior to entry into the thymus

(T-cell receptor/major histocompatibility complex/chimeras)

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**ABSTRACT** This study has assessed the possibility that anti-H-2 receptors are expressed on T-cell precursors prior to their entry into the thymus. Parental strain A thymus was transplanted into either normal or thymectomized (A×B)<sub>1</sub>F<sub>1</sub> mice which were then irradiated and reconstituted with strain A bone marrow. The cells repopulating the engrafted strain A thymus were shown to be of donor bone marrow origin. Thus, strain A thymocytes were differentiating within a syngeneic thymus, after exposure to allogeneic strain B major histocompatibility complex (MHC) determinants of the irradiated F<sub>1</sub> host. The cells repopulating the engrafted thymus were assessed for their ability to generate alloreactive cytotoxic T lymphocyte responses and were found to be specifically tolerant to allogeneic strain B MHC determinants. This tolerance existed in the absence of detectable suppression and in the absence of detectable strain B MHC determinants intrathymically. These data are most consistent with the concept that precursor T cells express anti-MHC receptors prior to their entry into the thymus and that exposure to MHC determinants prethymically results in their functional inactivation.

A unique aspect of the antigen receptor of T cells is its parallel specificity for determinants encoded by the major histocompatibility complex (MHC) (1, 2). The specificity of the receptor repertoire for polymorphic MHC determinants is dictated by the MHC determinants that the T cells encounter at critical points during their differentiation and maturation (3–5). The process of receptor development and T-cell maturation has been largely attributed to the inductive environment of the thymus (3, 4). However, recent evidence has suggested that the T-cell repertoire may not be determined entirely within this organ. For instance, the self-specificity of cytotoxic T lymphocyte (CTL) precursors in the spleen of thymus-engrafted *nude* mice is not entirely dictated by the H-2 phenotype of the engrafted thymus (6). In addition, the ability to generate allo-MHC specific and trinitrophenyl-modified self-specific CTLs from athymic *nude* mice implies that *K/D*-region restricted T cells can express receptors specific for MHC determinants without thymic processing (7–9).

It is important to our understanding of the mechanisms that influence the generation of the T-cell receptor repertoire to determine at which point in their differentiation precursor T cells first express receptors specific for the recognition of MHC determinants. For example, if precursor T cells do not express receptors until they enter the thymus, then it is possible that the thymus alone determines the specificity of the T-cell repertoire. Indeed, it was first proposed by Jerne that the T-cell

receptor repertoire for antigen recognition is derived from a finite set of anti-MHC gene products which mutate somatically upon binding thymic MHC determinants (10). However, if precursor T cells express receptors prior to entering the thymus, then it is possible that the MHC determinants encountered prethymically might alter the receptor repertoire which enters the thymus and is available for thymic processing.

To investigate the possibility that precursor T cells first express anti-MHC receptors prior to their entry into the thymus, T-cell precursors were exposed to allogeneic MHC determinants in the prethymic environment. Specifically, (A×B)<sub>1</sub>F<sub>1</sub> mice were grafted with strain A thymus, irradiated, and reconstituted with strain A bone marrow. The thymocytes from these mice were then assessed for their alloreactivity to A, B, and unrelated MHC determinants. The results obtained in this study demonstrate that such thymocyte populations were specifically tolerant to the B MHC determinants of the F<sub>1</sub> host. These findings suggest that precursor T cells do express receptors for allogeneic MHC determinants prior to their entry into the thymus and that exposure to alloantigen in the prethymic environment results in the induction of specific tolerance.

## MATERIALS AND METHODS

**Animals.** C57BL/10Sn (B10), B10.A, B10.D2, B6AF<sub>1</sub>, B6, A/J, and C3H.SW males and timed-pregnant B6 and A/J females were obtained from The Jackson Laboratory.

**Chimeras.** Normal and thymectomized B6AF<sub>1</sub> mice were grafted subcutaneously with three or four thymic lobes from 1-day-old B6 or A/J mice. Three days after thymic grafting the mice were irradiated [950 R (1 R = 2.58 × 10<sup>-4</sup> coulomb/kg)] and injected with 1.5 × 10<sup>7</sup> bone marrow cells that had been depleted of T cells by treatment with a rabbit anti-mouse brain antiserum plus complement. At 4–8 weeks after irradiation the mice were studied individually. The number of cells recovered from the engrafted thymi ranged between 1.6 and 7.5 × 10<sup>7</sup>. Chimeras are designated as bone marrow donor → irradiated recipient followed by parenthesis in which additional manipulations such as thymectomy (Tx) and the origin of the thymus graft are indicated.

**In Vitro Generation of CTLs.** Five-day mixed lymphocyte cultures were established with 4 × 10<sup>6</sup> thymocytes as responder cells and 4 × 10<sup>6</sup> irradiated (2000 R) spleen cells from normal mice as stimulators (11). Because the generation of CTLs from

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Abbreviations: MHC, major histocompatibility complex; Tx, thymectomy; CTL, cytotoxic T lymphocytes; FMF, flow microfluorometry; FIGAMlg, fluorescein-conjugated affinity-purified F(ab')<sub>2</sub> goat anti-mouse immunoglobulin; IL-2, interleukin 2; Con A, concanavalin A.

thymocytes has been shown to require interleukin 2 (IL-2) (12), all cultures contained 25% (vol/vol) of the supernatant from concanavalin A (Con A)-stimulated BALB/c spleen cells and sufficient  $\alpha$ -methyl D-mannoside to neutralize the residual Con A (13). The cytotoxic activity of the cultured cells was assessed in a standard 4-hr  $^{51}\text{Cr}$  release assay using Con A-stimulated spleen cells as targets (11). % specific Cr release =  $100 \times (\text{experimental} - \text{spontaneous release}) / (\text{maximum} - \text{spontaneous release})$ .

**Reagents.** Monoclonal anti-Lyt 1.1 was the kind gift of U. Hammerling (Sloan-Kettering Institute for Cancer Research). Anti-Ly 9.1 and anti-Ly 9.2 antisera were prepared as described (14) and were the generous gift of B. J. Mathieson (National Institutes of Health, Bethesda, MD). Monoclonal anti-H-2K<sup>k</sup> antibodies were mixtures of culture supernatants of previously described hybrid cell lines 11-4.1 (15) and 16-3-22 and 16-3-1 (16). Anti-H-2K<sup>b</sup> antiserum [(B10.AKM  $\times$  C3H.OH)<sub>F</sub><sub>1</sub> anti-B10.MBR] was the kind gift of David Sachs (National Institutes of Health). Fluorescein-conjugated affinity-purified F(ab')<sub>2</sub> goat anti-mouse Ig (FIGAMlg) was used as the fluoresceinated sandwich reagent and was the kind gift of B. J. Fowlkes and R. Asofsky (National Institutes of Health).

**Immunofluorescence Staining and Flow Microfluorometry (FMF).** Thymocytes ( $1 \times 10^6$ ) were stained with reagents in amounts predetermined to be saturating as described (17). FMF analysis of the thymocytes was performed with a fluorescence-activated cell sorter (FACS II, Becton Dickinson, Mountain View, CA). Data were collected on  $5 \times 10^4$  viable cells and are displayed as a graph of fluorescence intensity versus cell number or expressed as percentage positive cells.

## RESULTS

**Thymocytes Are Tolerant to Extrathymic Allogeneic MHC Determinants.** The possibility that specific recognition of allogeneic MHC determinants can occur prethymically was approached by the use of experimental animals in which bone marrow-derived precursor T cells would encounter allogeneic MHC determinants prior to their entry into a syngeneic thymus. To accomplish this, chimeras were constructed by transplanting bone marrow from strain A mice into an irradiated (A $\times$ B)<sub>F</sub><sub>1</sub> host that had previously been engrafted with strain A thymic lobes. In this situation, bone marrow-derived strain A thymocytes would have been exposed to allogeneic strain B MHC determinants of the F<sub>1</sub> host during circulation and migration to the thymus but would then have matured in the MHC syngeneic environment of the engrafted strain A thymus.

Before evaluating the functional specificity of the cells that repopulated the thymi of such chimeric mice, it was important to document that they were of donor bone marrow origin. It was shown previously that analysis of the MHC phenotype of chimeric thymocytes by immunofluorescence does not determine their genetic origin because thymocytes acquire the MHC determinants of the chimeric host. However, the non-MHC determinants that thymocytes express are only those of their own genotype (17). Consequently, the strains utilized in their construction differed in background (non-MHC) genes at either the *Lyt 1* or the *Ly 9* locus (Table 1).

In the first series of chimeras, normal B6AF<sub>1</sub> mice were grafted subcutaneously with neonatal B6 thymus lobes. Three days later they were irradiated (950 R) and injected with C3H.SW bone marrow. Thus, each chimera had two separate thymi—i.e., an *in situ* B6AF<sub>1</sub> thymus and an engrafted B6 thymus. After 4 weeks had elapsed, for the repopulation of the thymi with bone marrow-derived cells, immunofluorescence typing and mixed lymphocyte cultures were performed on the cells from both the engrafted B6 thymus and the F<sub>1</sub> *in situ* thymus of individual mice.

Table 1. Genetic haplotype of mice used in this study

Strain	H-2	Lyt 1	Ly 9
A/J	a	1.2	9.1
B10, B6	b	1.2	9.2
B10.A	a	1.2	9.2
B6AF <sub>1</sub>	b/a	1.2	9.2/9.1
C3H	k	1.1	9.1
C3H.SW	b	1.1	9.1

Fig. 1a shows the fluorescence profile of thymocytes from control mice stained with the anti-Lyt 1.1 antiserum. From this figure it is apparent that C3H thymocytes were positive for this allele whereas B10 and B6AF<sub>1</sub> thymocytes were negative. More than 95% of the cells that repopulated the *in situ* B6AF<sub>1</sub> thymus and the engrafted B6 thymus from the chimera were positive for Lyt 1.1 and therefore must have been derived from the injected C3H.SW bone marrow population (Fig. 1b and c; Table 2). The cytotoxic responses of these thymocyte populations were also determined and are shown in Table 2. All the C3H.SW thymocyte populations were immunocompetent as evidenced by their responsiveness to trinitrophenyl-modified H-2<sup>b</sup> stimulator cells. As expected, the C3H.SW (H-2<sup>b</sup>) thymocytes differentiating within the *in situ* H-2<sup>a/b</sup> F<sub>1</sub> thymus were tolerant to both H-2<sup>a</sup> and H-2<sup>b</sup> MHC determinants. However, H-2<sup>b</sup> cells differentiating within the engrafted B6 (H-2<sup>b</sup>) thymus were also tolerant to both H-2<sup>a</sup> and H-2<sup>b</sup> MHC determinants.

The tolerance of H-2<sup>b</sup> cells differentiating within the engrafted H-2<sup>b</sup> thymus to H-2<sup>a</sup> determinants would not be expected if T cells first expressed receptors for MHC determinants within the thymus and if there were no H-2<sup>a</sup> determinants present within this thymic environment to make them tolerant. In order to evaluate the possibility that nonthymic MHC determinants (H-2<sup>a</sup>) were present within the thymic differentiation environment of these chimeras, thymocytes were also stained with MHC-specific reagents and analyzed. C3H.SW (H-2<sup>b</sup>, *Lyt 1.1*) thymocytes from the F<sub>1</sub> *in situ* thymus stained positively for H-2K<sup>k</sup> even though they were genetically H-2<sup>b</sup> (Fig. 1e). This result is consistent with previous data that thymocytes acquire on their surface the MHC determinants that are present within their differentiation environment (17). Consequently, if H-2K<sup>k</sup> determinants were present within the B6 thymus as they were within the *in situ* F<sub>1</sub> thymus, then it would be expected that C3H.SW cells which repopulated the B6 thymus graft would also stain positively. The C3H.SW thymocytes differentiating within the engrafted B6 thymus were negative for H-2K<sup>k</sup>, demonstrating that there were no detectable quantities of H-2K<sup>k</sup> within the B6 thymus graft (Fig. 1f). This finding demonstrates that, in an experimental situation in which genotypically inappropriate MHC determinants could be detected on the bone marrow-derived C3H.SW thymocytes from the F<sub>1</sub> *in situ* thymus, they were not observed on the C3H.SW thymocytes from the engrafted B6 thymus. These data as well as the results of staining and analysis of the thymocyte populations with anti-H-2K<sup>b</sup> are also shown in Table 2. There was no evidence that the tolerance to allogeneic H-2<sup>a</sup> MHC of the H-2<sup>b</sup> thymocytes was due to the presence of H-2<sup>a</sup> determinants within the B6 thymus graft.

However, it is possible that the functional characteristics of the thymocytes differentiating within the parental thymus graft were somehow influenced by the presence of the H-2<sup>a/b</sup> F<sub>1</sub> *in situ* thymus. In order to eliminate this possibility, similar experiments were performed with chimeras constructed in the same manner but with thymectomized F<sub>1</sub> hosts. Immunofluorescence staining with reagents specific for Ly 9 and FMF

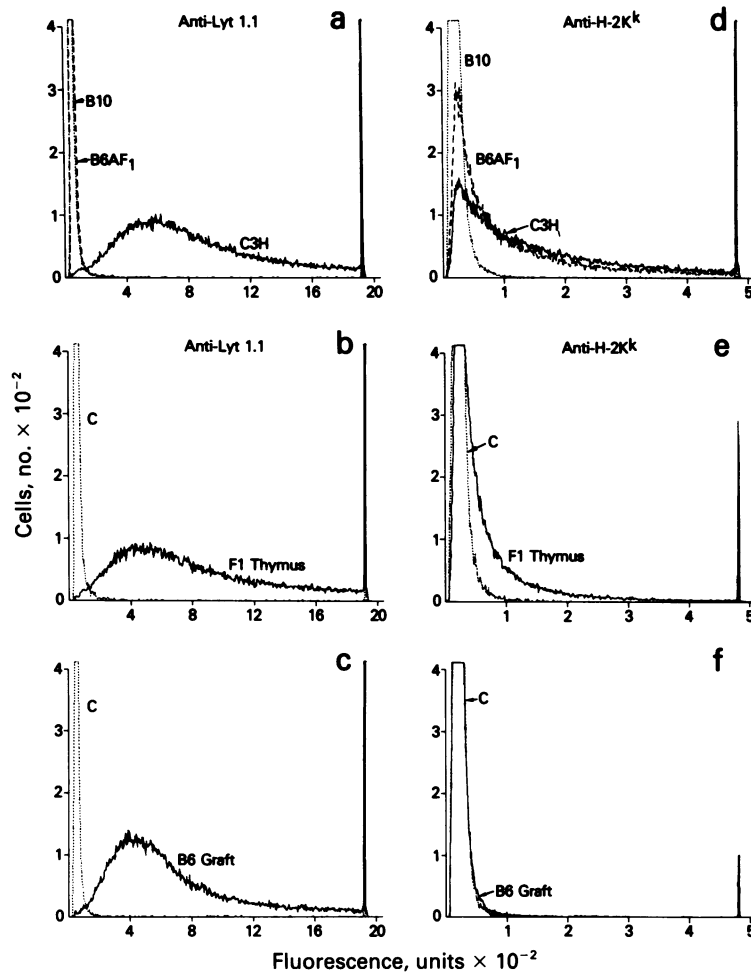


FIG. 1. Immunofluorescence profiles of normal and chimeric thymocytes. (a and d) The profiles of normal thymocytes from B10, B6AF<sub>1</sub>, and C3H strains of mice stained with anti-Lyt 1.1 or anti-H-2K<sup>k</sup> and FIGAM1g, respectively. (b and c) The profiles obtained by staining the *in situ* F<sub>1</sub> thymus or the B6 engrafted thymus from a C3H.SW → B6AF<sub>1</sub> (+ B6 thymus) chimera with anti-Lyt 1.1 plus FIGAM1g. (e and f) The profiles of these same chimeric cell populations stained with anti-H2K<sup>k</sup> plus FIGAM1g. The curves designated "C" indicate the profile of the cells stained only with FIGAM1g.

analysis of the cells that had repopulated the engrafted thymus from these mice demonstrated that they were not of host origin for the B10 → B6AF<sub>1</sub> (Tx + B6 thymus) chimeras and that they were of donor bone marrow origin for the B10.A → B6AF<sub>1</sub> (Tx + A/J thymus) chimeras (Table 3). In addition, staining and analysis of the cells with reagents specific for MHC determinants demonstrated no detectable levels of the nonthymic haplotype in the parental thymus graft. Thymectomies were judged to be technically complete both by macroscopic examination of the thoracic cavity for thymic remnants and by studying the spleen cell reactivity of irradiated and bone marrow reconstituted F<sub>1</sub> mice that had not been grafted with a thymus. Spleen cells from these thymectomized mice never showed significant reactivity even in the presence of IL-2.

Functional analysis of the thymocytes differentiating within

the thymus graft revealed a failure to develop CTL reactivity against the nonthymic haplotype. For example, thymocytes from a B10 → B6AF<sub>1</sub> (Tx + B6 thymus) chimera were alloreactive to third-party stimulators (H-2<sup>d</sup>) but were tolerant to both H-2<sup>a</sup> as well as H-2<sup>b</sup> (Table 3). Similarly, thymocytes from B10.A → B6AF<sub>1</sub> (Tx + A/J thymus) were also alloreactive to third-party H-2<sup>d</sup> but tolerant to both H-2<sup>a</sup> and H-2<sup>b</sup>. The potential of the transplanted bone marrow cells to develop alloreactivity to the nonthymic host haplotype was documented by assaying the thymocyte responses of lethally irradiated B10 and B10.A mice that had been reconstituted with the same bone marrow cell populations used for the reconstitution of the experimental chimeras. The results of experiments with all such chimeras are summarized in Table 4. The observation that exposure of precursor T cells to extrathymic allogeneic MHC de-

Table 2. Thymocytes of H-2<sup>b</sup> origin prethymically exposed to H-2<sup>a</sup> are tolerant to H-2<sup>a</sup>

Animal	Source of responder cells	% of thymocytes positive* with reagents specific for:			E/T ratio	% specific <sup>51</sup> Cr release <sup>†</sup>		
		H-2K <sup>k</sup>	H-2K <sup>b</sup>	Lyt 1.1		Stimulator/target		
					B10	B10.A	B10-TNP	
B6AF <sub>1</sub>	<i>In situ</i> F <sub>1</sub> thymus	59	69	0	60	2	-1	56
					20	-1	0	42
C3H.SW → B6AF <sub>1</sub> (+ B6 thymus)	<i>In situ</i> F <sub>1</sub> thymus	33	82	96	60	1	-2	50
					20	0	-2	33
	Engrafted B6 thymus	0	78	97	60	-2	1	42
					20	-1	-1	28

\* % positive cells =  $\frac{\% \text{ of experimental cells above FIGAM1g background} - \% \text{ of negative control cells above FIGAM1g background}}{100 - \% \text{ of negative control cells above FIGAM1g background}}$

† These values represent the mean of triplicate determinations; SEM was <5%. All cultures contained IL-2.

Table 3. Thymocytes prethymically exposed to MHC alloantigen are specifically tolerant to those alloantigens

Animal	Source of responding cells	% of thymocytes positive* with reagents specific for:				% specific <sup>51</sup> Cr release†				
		H-2K <sup>k</sup>	H-2K <sup>b</sup>	Ly 9.1	Ly 9.2	E/T ratio	Stimulator/target			
							B10	B10.A	B10.D2	
B6AF <sub>1</sub>	Spleen					40	4	6	76	
						20	4	5	64	
	<i>In situ</i> F <sub>1</sub> thymus		57	71	69	70	40	3	2	58
							20	-1	0	56
B10 → B6AF <sub>1</sub> (Tx)	Spleen					40	1	6	10	
						20	3	5	4	
B10 → B6AF <sub>1</sub> (Tx + B6 thymus)	Engrafted B6 thymus		1	75	0	94	40	-1	8	40
							20	0	3	36
B10 → B10	<i>In situ</i> thymus					40	2	41	62	
						20	5	38	60	
B10.A → B6AF <sub>1</sub> (Tx + A/J thymus)	Engrafted A/J thymus		50	0	0	94	40	4	0	24
							20	4	0	12
B10.A → B10.A	<i>In situ</i> thymus					40	83	0	55	
						20	72	-1	50	

\* Calculated as in Table 2.

† These values represent the mean of triplicate determinations; SEM was &lt;5%. All cultures contained IL-2.

terminants depleted the engrafted thymus of reactivity against those determinants was a consistent finding at all time points tested.

**Tolerance to the Nonthymic Haplotype Is Not Due to Suppression.** The observed tolerance to allogeneic MHC determinants could have reflected the generation of cells specifically suppressive for T cells alloreactive against those determinants. It was possible that thymocyte tolerance reflected the generation of suppressor cells in the post-thymic periphery which migrated back into the thymus and specifically suppressed alloreactivity against the host nonthymic MHC determinants. If such a suppressor mechanism functioned in these animals, it would be expected that chimeric thymocytes would contain haplotype-specific suppressor cells which would suppress the activation of normal thymocytes alloreactive toward chimeric host MHC determinants. In order to examine this possibility, thymocytes from a B10 → B6AF<sub>1</sub> (Tx + B6 thymus) chimera were tested for their ability to suppress the alloreactivity of syngeneic normal B10 thymocytes against H-2<sup>a</sup> MHC determinants. In Table 5, the results of mixing the chimeric B10 thymocytes with normal B10 thymocytes in cultures stimulated by irradiated B10.A spleen cells are shown. The presence of the chimeric thymocytes had no effect on the development of CTLs against the H-2<sup>a</sup> haplotype even when cultured in a 2:1 excess. Thus, the absence of alloreactivity to the nonthymic haplotype in the thymi of these chimeras is not due to demonstrable suppression.

## DISCUSSION

The results of the present study demonstrate that bone marrow-derived thymocytes differentiating within a syngeneic thymus are tolerant to extrathymic allogeneic MHC determinants. The tolerance to these determinants theoretically could have resulted from a post-thymic, an intrathymic, or a prethymic induction mechanism. If post-thymic development of tolerance occurred, there would have to have been some retrograde influence by mature T cells on the alloreactivities expressed by the developing thymocytes. The finding that the tolerant thymocyte populations from the chimeras could not suppress an alloreaction by syngeneic thymocytes directed toward the extrathymic haplotype is strong evidence against a post-thymic mechanism. If intrathymic development of tolerance occurred, the presence within the thymus of F<sub>1</sub> host allogeneic MHC determinants would have been expected. However, no extrathymic host determinants were observed by FMF of the thymocyte population, a method that is capable of detecting allogeneic MHC determinants on thymocytes. It should be emphasized that the presence within the engrafted thymus of F<sub>1</sub> host allogeneic MHC determinants at concentrations sufficient to induce tolerance but insufficient to be detected cannot be excluded. Nonetheless, because none of the evidence supports a post-thymic or intrathymic mechanism of tolerance induction, the most straightforward explanation for the findings of the present study is that the thymocyte populations became tolerant before they reached the thymus. This directly implies

Table 4. Summary of results from thymus-engrafted thymectomized chimeras

Animal	No. tested	% of thymocytes stained positive* with reagents specific for:				% specific <sup>51</sup> Cr release		
		H-2K <sup>k</sup>	H-2K <sup>b</sup>	Ly 9.1	Ly 9.2	Stimulator/target†		
						B10	B10.A	B10.D2
B10 → B6AF <sub>1</sub> (Tx + B6 thymus)	9	0.7 ± 0.8	70 ± 2.3	4.5 ± 2.8	92 ± 0.7	1 ± 0.4	8 ± 3.5	56 ± 3
B10.A → B6AF <sub>1</sub> (Tx + A/J thymus)	4	54 ± 3.1	3.4 ± 2.8	2 ± 3.4	91 ± 2.4	5 ± 4.5	0.3 ± 0.3	54 ± 10

\* These values represent the arithmetic mean ± SEM of the % positive cells of the individual animals tested.

† These results represent the arithmetic mean ± SEM of the % specific lysis values obtained at a 40:1 effector cell/target ratio of the individual animals tested.

Table 5. Lack of alloreactivity to the nonthymic haplotype is not due to suppression

Thymocytes, no. $\times 10^{-6}$ /culture		% specific $^{51}\text{Cr}$ release*
B10 $\rightarrow$ B6AF <sub>1</sub> (Tx + B6 thymus)	Normal B10	Stimulator/target B10.A
None	2	59
4	None	6
2	2	59
4	2	52

\*These values represent the mean of triplicate determinations at a 13:1 effector/target cell ratio.

that T-cell precursors express anti-MHC receptors prior to their entry into the thymus.

Considerations of the mechanisms by which thymocytes might become tolerant to prethymic MHC determinants have significant implications concerning the possible mechanisms by which the T-cell receptor repertoire for self + X might be somatically generated from a genetically encoded anti-MHC repertoire. If precursor T cells with specificity for prethymic MHC determinants are prevented from reaching the thymus because they bind to these MHC determinants, then the receptors are not available for the development of the self + X T-cell receptor repertoire in the thymus. This possibility conflicts with the requirements of the Jerne model for the generation of the T-cell self + X repertoire because this model proposes that it is from these receptors that the anti-self + X repertoire is generated within the thymus (8). Alternatively, if binding to prethymic MHC determinants leads to somatic mutation in the prethymic environment, then the receptors that enter the thymus have been altered such that they no longer bind this MHC with high affinity. These possibilities suggest that the prethymic environment may be important in the generation of the T-cell repertoire for self + X because it would have a significant influence on the repertoire that enters the thymus and is available for further thymic processing (18). It should be emphasized that, although the results of this study place constraints on models that suggest that the T-cell repertoire is generated from a genetically determined anti-MHC repertoire, they are completely compatible with the concept that the T-cell receptor repertoire for self + X is genetically encoded (19, 20).

In conclusion, the observation that thymocytes are tolerant to extrathymic MHC determinants that could not be detected within the thymus is most consistent with the concept that T-cell precursors express anti-MHC receptors prior to their entry into the thymus. Whether precursor T cells only express anti-MHC receptors or express receptors for other specificities as well remains to be determined.

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