INTRATHYMIC CLONAL DELETION OF $V_{\beta 6}^+$ T CELLS IN CYCLOPHOSPHAMIDE-INDUCED TOLERANCE TO H-2-COMPATIBLE, Mls-DISPARATE ANTIGENS

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Three possible mechanisms have been proposed to explain development and maintenance of T cell tolerance to self antigens: the inactivation of self-reactive lymphocytes (clonal anergy), their chronic suppression (clonal suppression), and their elimination (clonal deletion) (1, 2). Experimental attempts to prove these three possibilities have been severely limited because of the technical difficulties (2). A more direct approach to assess the cellular basis of tolerance induction, however, is now available by using the fact that the usage of a certain TCR $V\beta$ domain is strongly correlated with the reactivity to specific antigens (3–5). By exploiting such correlations, it has been demonstrated that self-tolerance to the products encoded by the Mls-1^a allele (4, 5) or the Mls-2^a allele (6, 7) is mediated via the clonal deletion of the self-reactive T cell clones.

As to tolerance induction to allogeneic antigens, many attempts have been experimentally made. Those include neonatally induced tolerance (8), irradiation-induced tolerance (9–11), and antilymphocyte serum–induced tolerance (12, 13). Recently, MacDonald et al. (14) have indicated that neonatally induced tolerance to Mls-1^a-encoded antigens was likewise accompanied by the intrathymic elimination of $V\beta 6$-bearing cells, which are capable of recognizing Mls-1^a-encoded antigens bound to MHC class II molecules, thus supporting the clonal deletion model. On the other hand, Qin et al. (15) have raised the possibility that tolerance to allograft induced by combining bone marrow transplantation (BMT)[1] together with administration of CD4 and CD8 mAb in adult mice is, at least in part, due to clonal anergy rather than deletion. Thus, the underlying mechanisms in the development and maintenance of the donor-specific transplantation tolerance still remain controversial.

We have previously reported a method of allo-tolerance induction in adult mice

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[1] *Abbreviations used in this paper:* BMT, bone marrow transplantation; CP, cyclophosphamide; CRT, cortisone-resistant thymocytes; MLR; mixed lymphocyte reaction; MST, mean survival time; PE, phycoerythrin.

that comprises intravenous injection of 10^8 allogeneic spleen cells followed, usually 2 d later, by administration of 200 mg/kg of cyclophosphamide (CP) by the intraperitoneal route. Using this method, we have readily established a minimal degree of mixed chimerism associated with a long-lasting skin allograft tolerance in a variety of H-2 haplotype-identical strain combinations in mice (16). Possible mechanisms of this tolerance induction have been considered to be destruction of antigen-stimulated and then proliferating reactive cells (17–19) followed by establishment of a minimal degree of mixed chimerism (16, 20).

In the present article, in order to address this issue more directly, we have investigated the cellular basis of the transplantation tolerance in a model system of C3H (Mls-1^b) mice rendered CP-induced tolerant to AKR (Mls-1^a) skin allografts by assessing T cells bearing $V_{\beta 6}$ reactive to Mls-1^a-encoded antigens. Our results indicated that both destruction of donor antigen-stimulated T cells in the periphery and intrathymic clonal elimination of donor-reactive T cells were essential mechanisms of CP-induced tolerance.

Materials and Methods

Animals. Inbred mice of C3H/HeSlc (C3H; H-2^k, Mls-1^b), AKR/JSea (AKR; H-2^k, Mls-1^a), B10.BR SgSnSlc (B10.BR; H-2^k, Mls-1^b), BALB/cCrSlc (BALB; H-2^d, Mls-1^b), DBA/2CrSlc (DBA; H-2^d, Mls-1^a), and B10.D2 nSnSlc (B10.D2; H-2^d, Mls-1^b) strains were obtained from the Seiwa Experimental Animal Center (Nakatsu, Oita, Japan) and the Japan SLC Inc. (Hamamatsu, Shizuoka, Japan). Female mice, 8–10 wk old, were used.

Cell Preparation. Mice were killed by decapitation. The thymus, spleen, and lymph nodes (axillary, inguinal, and mesenteric) were collected and kept on ice in HBSS. They were disrupted in the medium by pressing their fragments between two glass slides. Cell suspensions were filtered through cotton gauze and washed three times in HBSS. Viable nucleated cells were counted. Viability of the cells was evaluated using trypan blue dye exclusion in a standard way.

In some experiments (Table III), cortisone-resistant thymocytes were obtained 48 h after a single intraperitoneal injection of 4 mg of hydrocortisone acetate (see reference 21).

Drug and Tolerance Induction. CP (Endoxan, Shionogi, Tokyo, Japan) in PBS (20 mg/ml) was injected intraperitoneally at a dose of 200 mg/kg. The day of CP injection is called day 0. Recipient mice were primed (on day -2) intravenously with 10^8 spleen cells of donor mice in 0.5 ml of HBSS and were given 200 mg/kg CP intraperitoneally 2 d later (on day 0).

Skin Grafting. Using the procedure we have reported previously (22), skin grafting was carried out usually 2 wk after CP treatment (on day 14). Graft beds, measuring 1 × 1 cm, were prepared on the right lateral thoracic wall. The panniculus carnosus was kept intact in the graft beds. Full-thickness square grafts, 1 × 1 cm, were sutured to the graft beds by eight interrupted sutures and were covered with protective tape. The first inspection of skin grafts was done 7 d after grafting, followed by daily inspections for 3 wk and weekly inspections thereafter. In the tolerant state, luxurious hair growth was demonstrated with the growth of hair of a different color from the normal hair of the recipient strain. Grafts were considered as rejected when the grafts formed dry scabs or when all of the normal epithelium was lost. Double-blinded observation was not performed. Graft survival was expressed in days as mean survival time (MST) ± SD.

Flow Microfluorometry. The direct immunofluorescent method using FITC-conjugated anti-Thy-1.2, anti-Lyt-1 (Becton Dickinson & Co., Mountain View, CA), anti-Thy-1.1, and anti-Lyt-1.1 (Meiji, Tokyo, Japan) mAbs was used to stain lymph node cells and thymus cells for the assay of chimerism. Undiluted culture supernatant of the rat hybridoma, 44-22-1, was used as a mAb that recognizes all TCRs using $V_{\beta 6}$ gene segments (4) in order to stain lymph node cells and thymus cells followed by FITC-conjugated goat anti-rat IgG (Tago Inc., Burlingame, CA). mAb KJ16-133, which recognizes TCRs using products of the $V_{\beta 8}$ gene family ($V_{\beta 8.1/8.2}$) (23), was also used. Fluorescence-positive cells were measured mainly by means

of a FACS 440 flow cytometer (Becton Dickinson & Co.), and in some experiments (Table IV and Fig. 5) by a FACScan (Becton Dickinson & Co.). The scatter was used to exclude dead cells, and fluorescence histograms (representing 10^4–10^5 viable cells) were accumulated on a logarithmic scale. Data were presented as percent positive cells after subtracting the number of cells stained with the fluorescent conjugate alone.

In some experiments, a cell suspension was incubated with both FITC-conjugated mAb and phycoerythrin (PE)-conjugated mAb for two-color flow cytometry. PE-conjugated anti-CD4 mAb and anti-CD8 mAb were purchased from Becton Dickinson & Co. All data were displayed on a log scale of increasing green and red fluorescence intensity. To obtain percentage of a T cell subpopulation, a total count was integrated in a selected area of the contour plots.

Mixed Lymphocyte Reaction (MLR). Spleen cells were used as both responders and stimulators in MLR. Spleen cells were washed and reconstituted in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY), which was supplemented with 10% Nu-serum (Collaborative Research, Lexington, MA), 5×10^{-5} M 2-ME, and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). The responder cells (5×10^5 cells/0.1 ml) and 3,000-rad-irradiated stimulator cells (10^4–10^6 cells/0.1 ml) were cocultured in a flat-bottomed microplate (25860; Corning Glass Works, Corning, NY) in a humidified atmosphere containing 5% CO_2 at 37°C for 2, 3, 4, or 5 d, and were pulsed on each last day with [^{3}H]thymidine (1 μCi/well) followed by harvesting 12 h later.

Statistics. Group data in each experiment were compared with the analysis of variance and then with student's t test when the data seemed to be parametric. However, when the data seemed to be nonparametric, the Mann-Whitney U test was used; $p = <0.05$ was considered significant.

Results

Long-lasting Skin Allograft Tolerance in H-2 Haplotype-identical Strain Combinations and Specificity of Tolerance. As shown in Table I, the recipient mice (C3H, H-2^k, Mls-1^b; and BALB, H-2^d, Mls-1^b) were primed intravenously with 10^8 spleen cells of H-2 haplotype-identical donors (AKR, H-2^k, Mls-1^a; B10.BR, H-2^k, Mls-1^b; and DBA, H-2^d, Mls-1^a) on day -2, and treated intraperitoneally with 200 mg/kg of CP on day 0. Skin grafting with AKR, B10.BR, DBA, or B10.D2 was carried out on day 14. In all of the experiments, three control groups were set up as follows: group 1, untreated (untreated controls); group 2, CP treatment alone (CP controls); and group 3, priming with donor spleen cells alone (primed controls).

In all of the combinations, a long-lasting skin allograft tolerance was produced in the groups given both viable cells and CP (groups 4; Exp. 1–3) compared with the other three control groups. In the tolerant groups, normal hair grew in the grafted skin. In the CP control groups (groups 2) and the primed control groups (groups 3), however, the skin graft survival was not prolonged compared with the untreated control groups (groups 1; Exp. 1–3).

When the C3H mice made tolerant of AKR were grafted with the third-party skin (B10.BR), the skin graft survival was not prolonged compared with the CP control mice (group 5 vs. 6; Exp. 1). The same results were obtained when the C3H mice made tolerant of B10.BR were grafted with AKR skin (group 6; Exp. 2), and when the BALB mice made tolerant of DBA were grafted with B10.D2 skin (group 6; Exp. 3).

Chimeric Analysis in C3H Mice Made Tolerant of AKR. As had been done with the foregoing reports in other strain combinations (16), the existence of mixed chimerism in the C3H mice made tolerant of AKR with 10^8 AKR spleen cells plus CP was examined (Table II).

TABLE I

Long-lasting Skin Allograft Tolerance in H-2 Haplotype-identical Strain Combinations and Specificity of Tolerance

Exp.	Recipient	Group	Treatment		Donor of skin graft§ (on day 14)	No. of mice	Skin graft survival	Mean survival time ± SD
			SC* (on day −2)	CP‡ (on day 0)				
							d	*d*
1	C3H	1	–	–	AKR	5	11, 11, 12, 12, 12	11.6 ± 0.5
		2	–	+	AKR	5	11, 12, 12, 12, 13	12.0 ± 0.7
		3	AKR	–	AKR	5	9, 9, 10, 11, 11	10.0 ± 1.0
		4	AKR	+	AKR	10	29, >120 × 9‖	>110.9 ± 28.8¶
		5	–	+	B10.BR	5	10, 11, 11, 12, 12	11.2 ± 0.8
		6	AKR	+	B10.BR	5	11, 12, 12, 13, 13	12.2 ± 0.8**
2	C3H	1	–	–	B10.BR	5	10, 11, 11, 12, 12	11.2 ± 0.8
		2	–	+	B10.BR	5	10, 11, 11, 12, 12	11.2 ± 0.8
		3	B10.BR	–	B10.BR	5	9, 10, 10, 11, 11	10.2 ± 0.8
		4	B10.BR	+	B10.BR	10	>120 × 10	>120.0 ± 0.0¶
		5	–	+	AKR	5	11, 12, 12, 12, 13	12.0 ± 0.7
		6	B10.BR	+	AKR	5	11, 12, 12, 13, 14	12.4 ± 1.1**
3	BALB	1	–	–	DBA	5	11, 11, 12, 12, 13	11.8 ± 0.8
		2	–	+	DBA	5	11, 12, 12, 13, 14	12.4 ± 1.1
		3	DBA	–	DBA	5	10, 11, 11, 11, 12	11.0 ± 0.7
		4	DBA	+	DBA	10	33, 45, >80 × 8	>71.8 ± 17.5¶
		5	–	+	B10.D2	5	12, 12, 13, 13, 14	12.8 ± 0.8
		6	DBA	+	B10.D2	5	12, 12, 13, 13, 13	12.6 ± 0.5**

* The recipient mice were primed intravenously with 10^8 viable AKR, B10.BR, or DBA spleen cells on day −2.

‡ 200 mg/kg CP was given intraperitoneally on day 0.

§ Skin grafting was carried out on day 14.

‖ The skin graft survival was >120 d in nine mice.

¶ $p < 0.01$ compared with group 1 in each experiment.

** Not significant compared with group 5 in each experiment.

TABLE II

Chimeric Analysis in the Thymus and Lymph Node of Each Tolerant Mouse

Group	Recipient* (treatment)	Timing‡ of the assay (on day *X*)	Thymus (n = 5): Percent positive cells ± SD)		Lymph node (n = 5): Percent positive cells ± SD)	
			Thy-1.1	Thy-1.2	Thy-1.1	Thy-1.2
1	C3H (naive)	-	0.1 ± 0.1	97.8 ± 1.5	0.1 ± 0.1	74.4 ± 2.3
2	C3H (CP alone)	35	0.2 ± 0.2	98.0 ± 0.2	0.1 ± 0.2	76.7 ± 1.5
3	C3H (AKR-SC alone)	35	0.2 ± 0.1	97.8 ± 0.8	0.1 ± 0.2	74.6 ± 1.8
4-a	C3H (AKR-SC + CP)	14	0.2 ± 0.2	97.4 ± 0.6	6.0 ± 1.7§	70.0 ± 1.2‖
4-b		35	4.9 ± 4.3‖	94.7 ± 3.9‖	6.6 ± 1.3§	96.0 ± 1.4§
4-c		70	32.8 ± 10.5§	69.5 ± 11.3§	9.1 ± 2.8§	65.5 ± 2.0§
5	AKR (naive)	-	98.0 ± 0.8	0.2 ± 0.2	70.2 ± 2.6	0.2 ± 0.2
			Lyt-1.1	Lyt-1 (Lyt-1.1 + Lyt-1.2)	Lyt-1.1	Lyt-1 (Lyt-1.1 + Lyt-1.2)
6	BALB (naive)	-	0.4 ± 0.3	97.9 ± 0.8	0.5 ± 0.2	75.6 ± 2.0
7	BALB (DBA-SC + CP)	35	3.4 ± 2.3¶	97.8 ± 0.6	5.3 ± 2.0**	75.8 ± 2.4
8	DBA (naive)	-	97.8 ± 0.6	98.1 ± 0.5	70.8 ± 2.3	71.2 ± 1.8

* See first two footnotes of Table I.

‡ The day of CP treatment is called day 0.

§ $p < 0.01$ compared with group 1.

‖ $p < 0.05$ compared with group 1.

¶ $p < 0.05$ compared with group 6.

** $p < 0.01$ compared with group 6.

In the C3H (Thy-1.2) mice made tolerant of AKR (Thy-1.1), a small number of Thy-1.1$^+$ cells of donor (AKR) origin were detected in the recipient C3H lymph node cells, but not in the thymus cells on day 14 (group 4-a). On day 35, Thy-1.1$^+$ cells of donor origin were detected more clearly both in the thymus and in the lymph nodes of the recipient C3H mice, and the proportion of Thy-1.2$^+$ cells of recipient origin decreased (group 4-b). Furthermore, on day 70, the proportion of Thy-1.1$^+$ cells of donor origin reached 32.8% in the thymus (group 4-c). In the C3H mice primed with AKR spleen cells alone, such mixed chimerism was not established (group 3).

Similarly, in the BALB (Lyt-1.2) mice made tolerant of DBA (Lyt-1.1), a small number of Lyt-1.1$^+$ cells of donor (DBA) origin were also detected in the thymus and lymph nodes of the recipients (BALB) as well (group 7).

In vitro Unresponsiveness of Spleen Cells from C3H Mice Made Tolerant of AKR. Two segregating loci are found in the Mls trait. One locus on chromosome 1 is known to stimulate the proliferation of a high percentage of T cells from the mice that are identical in H-2 complex but differing at Mls loci. The locus has two alleles; Mls-1^a and Mls-1^b. Lymphocytes from the mice carrying Mls-1^a, such as AKR mice, are a potent stimulator of T cells from the mice carrying Mls-1^b, such as C3H mice. On the other hand, lymphocytes from the mice carrying Mls-1^b are incapable of stimulating T cells from the mice carrying Mls-1^a (24). There are some reports (25–27), however, describing that Mls determinants cannot either act as transplantation antigens or cause graft-vs.-host reactions. These results may implicate that skin tolerance and Mls tolerance can be independent. To determine whether tolerance to Mls-1^a was established in our CP-induced tolerance system, we have examined in vitro MLR of spleen cells from the C3H mice that had been made tolerant of AKR 5 wk earlier and had been carrying AKR skin for 3 wk.

As shown in Fig. 1, the spleen cells from the C3H mice made tolerant of AKR skin were unresponsive in vitro to AKR stimulation, whereas they normally responded to the third-party BALB alloantigens. The spleen cells from the C3H mice treated with CP alone (CP controls), however, responded normally to both the AKR and third-party alloantigens in vitro. Thus, a specific tolerance to Mls-1^a antigens was confirmed in the C3H mice made tolerant of AKR skin.

Expression of TCR Vβ6 in Tolerant Mice. Recently, several reports (4–7, 28) have shown that the expression of certain TCR Vβ domains correlates with the reactivity to Mls antigens. For example, T cell reactivity to Mls-1^a antigens in vitro has been shown to be strongly correlated with the expression of TCR Vβ6 (4). Thus, mature T cells expressing TCR Vβ6 are absent in Mls-1^a strains of mice, whereas these cells constitute 10–15% of lymph node cells in Mls-1^b strains of mice. In Mls-1^a mice, the lack of Vβ6$^+$ cells is considered to be ascribed to the clonal deletion during thymocyte maturation. Such Vβ6-bearing receptors can be readily quantitated by their specific reactivity with the mAb 44-22-1 (4).

To investigate the cellular basis of our CP-induced tolerance to Mls-1^a antigens, we examined the expression of TCR Vβ6 in the C3H (Mls-1^b) mice that had been made tolerant of AKR (Mls-1^a) and were carrying AKR skin, or in the BALB (Mls-1^b) mice that had been made tolerant of DBA (Mls-1^a) and were carrying DBA skin. Thymocytes and lymph node cells from the tolerant mice were stained with the anti-Vβ6 mAb 44-22-1. As consistent with an earlier report (29), only mature T cells ex-

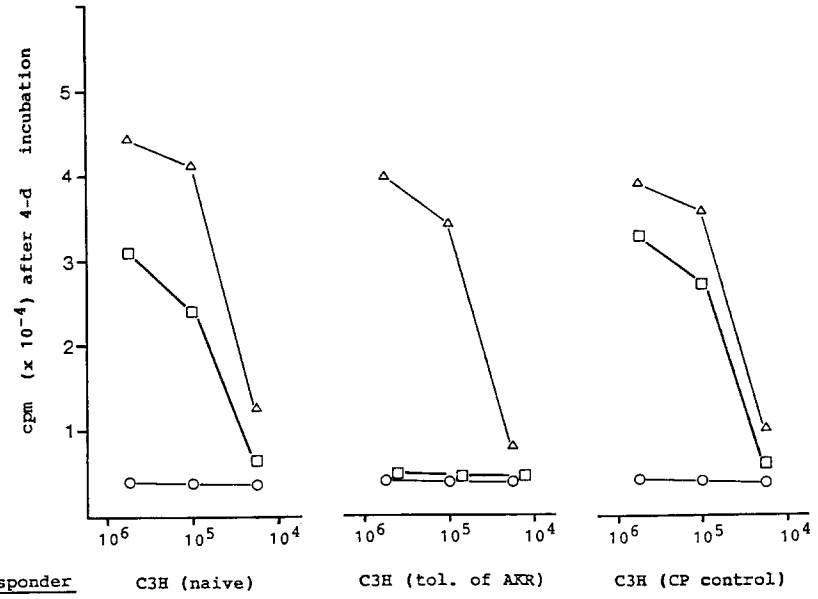

FIGURE 1. In vitro unresponsiveness of spleen cells from C3H mice made tolerant of AKR skin. 5×10^5 spleen cells from the naive C3H mice (naive), the C3H mice that had been made tolerant of AKR 5 wk earlier with AKR spleen cells plus CP and were carrying AKR skin for 3 wk (tolerant of AKR), or the C3H mice that had received CP treatment alone (CP control), were incubated with 10^4-10^6 3,000-rad-irradiated C3H (○), AKR (□), or BALB (△) spleen cells for 2, 3, 4, or 5 d. Proliferation was represented by [^{3}H]thymidine incorporation after the optimal 4-d incubation.

pressing TCR Vβ6 in the thymus were strongly stained with the mAb 44-22-1, while immature thymocytes expressing Vβ6 were stained in a low intensity. In some experiments we used cortisone-resistant thymocytes (CRT), since the CRT were reported to represent a thymic subpopulation phenotypically and functionally equivalent to mature T cells (21).

A representative immunofluorescence result was shown in Fig. 2, and all of the data were summarized in Table III. An appreciable number of thymocytes in the the naive C3H mice were strongly stained with the Vβ6-specific mAb 44-22-1 (Fig. 2 *A* and Table III, group 1). In the C3H mice that had been made tolerant of AKR and were carrying AKR skin, the Vβ6$^+$ cells were not detected in the thymocytes on day 35, as compared with those in the naive C3H mice (Fig. 2, *A* vs. *E*, Table III, group 1 vs. 4-b), although the Vβ6$^+$ T cells remained at a normal level in the thymocytes on day 14 (Table III, group 4-a). The same deletion was observed when the CRT were examined (Table III, group 1 vs. 4-b). On the other hand, in the lymph node cells of the tolerant C3H mice on day 35, a small but significant number of Vβ6$^+$ cells were detected, although the expression of Vβ6 was obviously decreased as compared with that in the naive C3H mice (Fig. 2, *B* vs. *F*, and Table III, group 1 vs. 4-b). The obvious decrease of Vβ6$^+$ T cells in the periphery was already observed on day 14, whereas the number of those remained at a normal level in the thymus (Table III, group 4-a). The number of the Vβ6-bearing T cells further decreased in these mice on day 70 (Table III, group 4-c). In the C3H mice treated

with CP alone (Fig. 2, *C* and *D*, and Table III, group 2) and in the C3H mice primed with AKR spleen cells alone (Table III, group 3), the $V_{\beta 6}$-bearing T cells remained at normal levels both in the thymus and in the lymph nodes. Furthermore, in the C3H mice that had been made tolerant of B10.BR (Mls-1[b]) and were carrying B10.BR skin, TCR $V_{\beta 6}$ was normally expressed both in the thymus and in the lymph nodes (Fig. 2, *G* and *H*, and Table III, group 5).

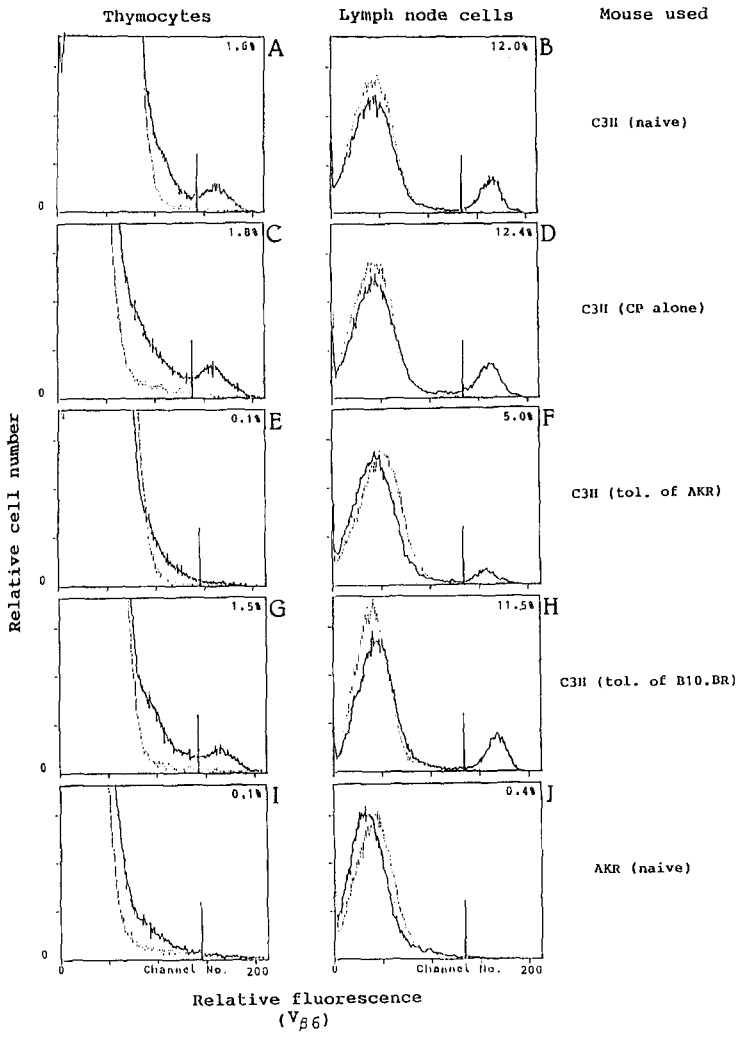

FIGURE 2. $V_{\beta 6}^+$ T cells are deleted in thymocytes but no in lymph node cells of C3H mice made tolerant of AKR. The thymocytes and lymph node cells were stained with the mAb 44-22-1 (specific for TCR $V_{\beta 6}$), followed by fluoresceinated goat anti-rat Ig. Thin lines represent the fluorescent conjugate alone. (*A* and *B*) Naive C3H mice; (*C* and *D*) C3H mice given CP treatment alone; (*E* and *F*) C3H mice that had been made tolerant 5 wk earlier with AKR spleen cells plus CP and were carrying AKR skin; (*G* and *H*) C3H mice that had been made tolerant 5 wk earlier with B10.BR spleen cells plus CP and were carrying B10.BR skin; and (*I* and *J*) naive AKR mice. Data represent one of the five animals tested. All the other four showed similar staining patterns in each panel.

TABLE III

Expression of TCR Vβ6 in Recipient Mice Made Tolerant of Mls-1^a Antigens

Group	Recipient* (treatment)	Timing‡ of the assay (on day *X*)	Percent Vβ6 strongly positive cells§ ± SD (*n* = 5)		Percent Vβ6 positive cells ± SD (*n* = 5) (Lymph node cells)
			Thymocytes	CRT‖	
1	C3H (naive)	-	1.7 ± 0.2	12.0 ± 1.1	11.8 ± 0.8
2	C3H (CP alone)	35	1.9 ± 0.3	ND	12.2 ± 0.9
3	C3H (AKR-SC alone)	35	1.8 ± 0.3	ND	10.9 ± 1.1
4-a	C3H (AKR-SC + CP)	14	1.5 ± 0.3	ND	6.8 ± 0.8¶
4-b		35	0.2 ± 0.1**	0.5 ± 0.3	5.6 ± 0.5¶
4-c		70	0.1 ± 0.1**	ND	3.1 ± 0.7¶
5	C3H (B10.BR-SC + CP)	35	1.6 ± 0.2‡‡	ND	11.6 ± 0.6‡‡
6	AKR (naive)	-	0.1 ± 0.1	0.4 ± 0.2	0.5 ± 0.3
7	BALB (naive)	-	1.4 ± 0.2	ND	11.0 ± 0.7
8	BALB (DBA-SC + CP)	35	0.1 ± 0.1§§	ND	5.1 ± 1.2‖‖
9	DBA (naive)	-	0.0 ± 0.1	ND	0.4 ± 0.2

* See first two footnotes of Table I.
‡ The day of CP treatment is called day 0.
§ Mature T cells expressing TCR Vβ6 in the thymus are strongly stained with the mAb 44-22-1.
‖ CRT were obtained 48 h after a single intraperitoneal injection of 4 mg hydrocortisone acetate.
¶ $p < 0.01$ compared with groups 1 and 6.
** $p < 0.01$ compared with group 1.
‡‡ Not significant compared with group 1.
§§ $p < 0.01$ compared with group 7.
‖‖ $p < 0.01$ compared with groups 7 and 9.

In the BALB mice that had been made tolerant of DBA and were carrying DBA skin, the same results were obtained as described above in the C3H mice made tolerant of AKR. In the thymocytes of the tolerant BALB mice, Mls-1^a-reactive Vβ6-bearing T cells were eliminated. In the lymph nodes, however, Vβ6$^+$ cells were also clearly detected despite the obvious decrease (Table III, group 8).

On the other hand, we also examined, as a control of the TCR Vβ6, the expression of TCR Vβ8 in the C3H mice that had been made tolerant of AKR and were carrying AKR skin. Thymocytes and lymph node cells from the tolerant mice were stained with the Vβ8-specific mAb KJ16-133 (23). As shown in Table IV, expression of TCR Vβ8 was only marginally reduced both in the thymus and in the periphery of the tolerant C3H mice, as compared with that in the naive C3H mice (Table IV, group 1 vs. 4-a or 4-b).

Subset Analysis of Residual Vβ6$^+$ Cells in the Lymph Nodes of the Tolerant Mice. As described above, the tolerance to Mls-1^a antigens was established, as well as the skin tolerance to AKR in the C3H mice given AKR spleen cells followed by CP (Fig. 1). In the lymph nodes of these tolerant C3H mice, however, Vβ6$^+$ cells were clearly detected despite the obvious decrease (Fig. 2, *F*). To further analyze the lymph node cells from the C3H mice made tolerant of AKR 5 wk earlier, these cells were stained on day 35 with the Vβ6-specific mAb 44-22-1 together with the mAb to CD4 or CD8 antigen for two-color flow cytometry.

As shown in Fig. 3, 8–9% of the lymph node cells in the normal C3H mice belonged to CD4$^+$-Vβ6$^+$ subset, whereas 4–5% of those belonged to CD4$^-$-Vβ6$^+$ subset.

TABLE IV

Expression of TCR $V_{\beta 8}$ in Recipient Mice Made Tolerant of Mls-1^a Antigens

Group	Recipient* (treatment)	Timing‡ of the assay (on day *X*)	Thymus (n = 5) Percent $V_{\beta 8}$ strongly positive cells ± SD§	Lymph node (n = 5) (Percent $V_{\beta 8}$ positive cells ± SD)
1	C3H (naive)	-	2.5 ± 0.3	18.9 ± 1.0
2	C3H (CP alone)	35	2.3 ± 0.2	18.2 ± 0.8
3	C3H (AKR-SC alone)	35	2.4 ± 0.3	17.7 ± 1.1
4-a	C3H (AKR-SC + CP)	35	2.0 ± 0.3	16.5 ± 0.7
4-b		70	1.8 ± 0.2	16.0 ± 0.9
5	AKR (naive)	-	1.7 ± 0.2	11.2 ± 0.7

* See first two footnotes of Table I.

‡ The day of CP treatment is called day 0.

§ Mature T cells expressing TCR $V_{\beta 8}$ in the thymus are strongly stained with the mAb KJ16-133.

In the lymph nodes of the C3H mice made tolerant of Mls-1^a antigens, however, CD4$^+$ T cells using TCR $V_{\beta 6}$ were eliminated (<1%), whereas the percentage of CD4$^-$-$V_{\beta 6}^+$ T cells remained at a normal level (Fig. 3, *A* vs. *B*). The same results were obtained when two-color analysis was done in the lymph nodes of the BALB mice made tolerant of DBA (Fig. 3, *C* vs. *D*). Moreover, the same was the case when the assay was done on day 14 using lymph node cells from the C3H mice made tolerant of AKR 2 wk earlier (data not shown).

On the other hand, the percentage of the CD8$^+$-$V_{\beta 6}^+$ T cells in the lymph nodes of the C3H mice made tolerant of AKR was unchanged or marginally reduced as compared with that in the naive C3H mice (Fig. 4, *A* vs. *B*). In turn, the CD8$^-$-$V_{\beta 6}^+$ T cells were hardly detected in the lymph nodes of the tolerant C3H mice (Fig. 4, *A* vs. *B*).

Furthermore, the lymph node cells from the same tolerant C3H mice were stained with the $V_{\beta 8}$-specific mAb KJ16-133 together with the mAb to CD4 antigen for two-color flow cytometry. In the lymph nodes of the C3H mice made tolerant of Mls-1^a antigens, CD4$^+$ T cells using TCR $V_{\beta 8}$ were only marginally reduced as compared with those in the naive C3H mice (Fig. 5, *A* vs. *B*). Such selective disappearance as seen in the T cells using TCR $V_{\beta 6}$ was not observed in the T cells using TCR $V_{\beta 8}$ in the C3H mice made tolerant of AKR.

Discussion

We have demonstarted here one of the mechanisms of CP-induced skin allograft tolerance in mice by examining the expression of TCR $V_{\beta 6}$ that is exclusively used for the T cells reactive against Mls-1^a-encoded antigens (4). At the relatively early phase of mice rendered tolerant to Mls-1^a-encoded antigens, neither CD4$^+$CD8$^-$ nor CD4$^-$ CD8$^+$ thymocytes bearing a high density of $V_{\beta 6}$ were detected in the thymus of these mice in which mixed chimerism was observed (Fig. 2 *E* and Table III, group 4-b). These results were considered to be Mls-1^a specific, since the expression of TCR $V_{\beta 6}$ was at a normal level in the thymus of the C3H mice (Mls-1^b) made tolerant of B10.BR (Mls-1^b) (Fig. 2), and since the expression of TCR $V_{\beta 8}$ was only marginally reduced in the thymus of C3H mice rendered tolerant to Mls-1^a-encoded antigens (Table IV). This marginal reduction of the expression of TCR

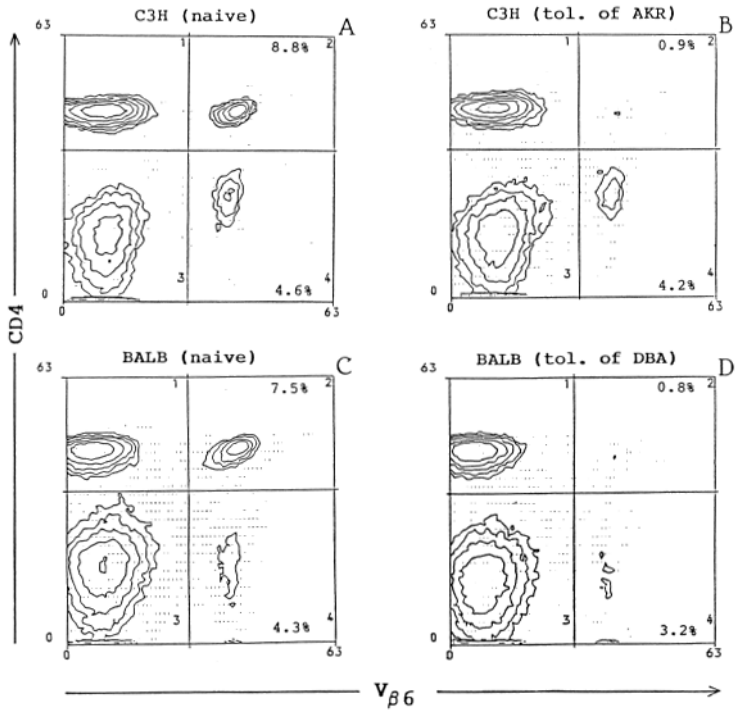

Figure 3. $CD4^+$-$V_{\beta 6}^+$ T cells are selectively eliminated in lymph node cells of recipient mice made tolerant of the Mls-1^a strain. The lymph node cells were double stained with PE-conjugated anti-CD4 antibody and the mAb 44-22-1 (specific for TCR $V_{\beta 6}$) followed by FITC-conjugated anti-rat IgG. The percentage of each positive population is given in the respective regions. (*A*) Naive C3H mice; (*B*) C3H mice that had been made tolerant 5 wk earlier with AKR spleen cells plus CP and were carrying AKR skin; (*C*) naive BALB mice; (*D*) BALB mice that had been made tolerant 5 wk earlier with DBA spleen cells plus CP and were carrying DBA skin. Data represent one of the five animals tested. All the other four showed similar staining patterns in each panel.

$V_{\beta 8}$ in the thymus may be ascribed to the deletion of TCR $V_{\beta 8.1}$, as described by Kappler et al. (5), which is also used for the T cells reactive to Mls-1^a-encoded antigens. The apparent disappearance of $V_{\beta 6}^+$ T cells in the thymus was not due to dilution of $V_{\beta 6}^+$ (C3H) T cells by $V_{\beta 6}^-$ (AKR) T cells, since the proportion of Thy-1.1^+ cells of donor (AKR) origin was only small in the thymus of the recipient C3H on day 35 (Table II), and the elimination was also found in two-color flow cytometry analysis of the thymocytes stained with mAb Thy-1.2 together with the anti-$V_{\beta 6}$ mAb 44-22-1 (data not shown). The elimination was more clearly shown when the CRT were examined (Table III). Here, the CRT represent a thymic subpopulation that are phenotypically and functionally equivalent to mature ($CD4^+$ or $CD8^+$) T cells (21). Taken together, these data strongly suggest that the clonal elimination of Mls-1^a-reactive $V_{\beta 6}^+$ cells in the thymus is actually occurring in the C3H mice made tolerant of AKR by the CP-induced tolerance method.

Another remarkable feature of the present study is the selective disappearance of $CD4^+$-$V_{\beta 6}^+$ T cells in the periphery of the tolerant mice (Figs. 3 and 4), because Qin et al. (15) reported that neither $CD4^+$-$V_{\beta 6}^+$ nor $CD8^+$-$V_{\beta 6}^+$ T cells were deleted

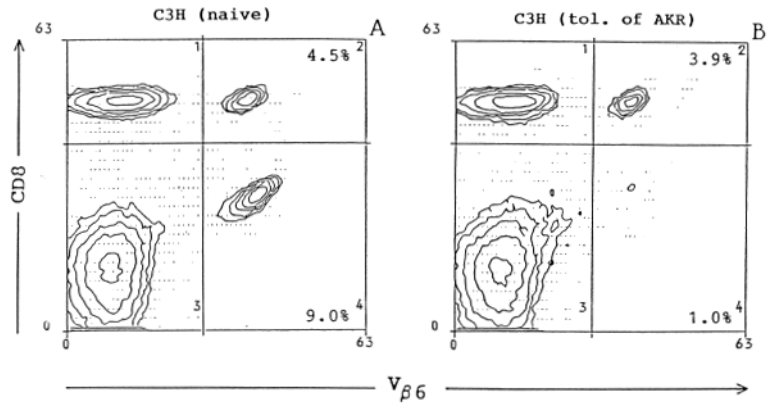

FIGURE 4. CD8⁺-Vβ6⁺ T cells remain at normal levels in lymph node cells of recipient mice made tolerant of the Mls-1ª strain. The lymph node cells were double stained with PE-conjugated anti-CD8 antibody and the mAb 44-22-1 followed by FITC-conjugated anti-rat IgG. The percentage of each positive population is given in respective regions. (*A*) See Fig. 3 *A*; (*B*) see Fig. 3 *B*. Data represent one of the five animals tested. All the other four showed similar staining patterns in each panel.

in the periphery of the adult mice that had been treated with the mAb followed by allogeneic BMT, and because MacDonald et al. (14) reported that both CD4⁺-Vβ6⁺ and CD8⁺-Vβ6⁺ T cells were deleted in the periphery of the adult mice that had been neonatally made tolerant to Mls-1ª-encoded antigens. The preferential disappearance of CD4⁺-Vβ6⁺ T cells in the present study was considered to be Mls-1ª

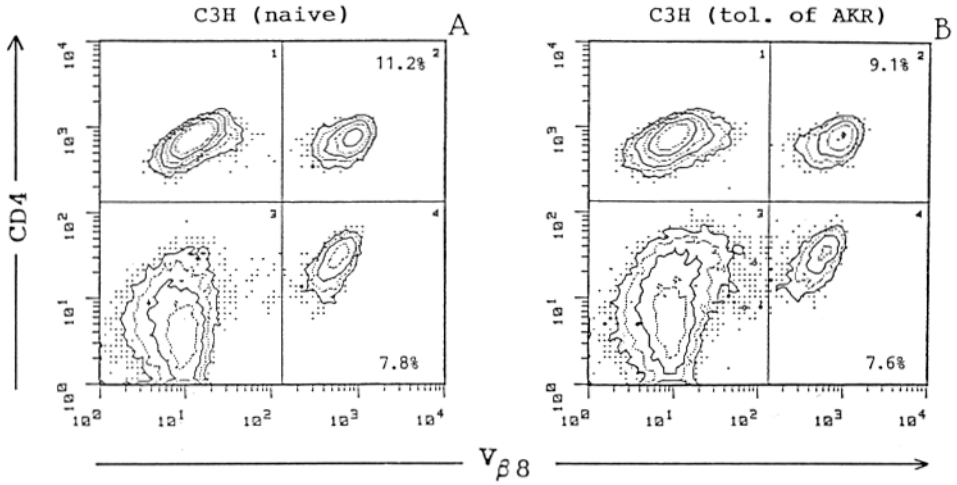

FIGURE 5. CD4⁺-Vβ8⁺ T cells are only marginally reduced in lymph node cells of recipient C3H mice made tolerant of the Mls-1ª strain. The lymph node cells were double stained with PE-conjugated anti-CD4 antibody and mAb KJ16-133 followed by FITC-conjugated anti-rat IgG. The percentage of each positive population is given in respective regions. (*A*) See Fig. 3 *A*; (*B*) see Fig. 3 *B*. Data represent one of the five animals tested. All the other four showed similar staining patterns in each panel.

specific (Figs. 3 and 5), and this state had been established before the intrathymic clonal deletion occurred (Table III). $CD4^+$-$V\beta6^+$ T cells have been considered to be mainly responsible for MLR to the Mls-1^a-encoded antigens (30, 31). At the induction phase of the CP-induced tolerance in the C3H mice, the Mls-1^a-reactive $CD4^+$-$V\beta6^+$ T cells proliferating after the AKR antigen stimulation may be totally abolished by CP treatment (Fig. 3). In contrast, most of the $CD8^+$-$V\beta6^+$ T cells may not be abolished with CP because of their resting cell cycle (17) and remain in the periphery (Fig. 4), although a small number of the $CD8^+$ T cells, whether or not $V\beta6^+$, which are responsible for AKR skin graft rejection, appear to be destroyed by the AKR antigen stimulation plus CP, because such tolerant C3H mice were always bearing AKR skin (Table I). Since there was no recruitment of $V\beta6^+$ mature T cells from the thymus to the peripheral lymph nodes in the recipient mice rendered tolerant to Mls-1^a-encoded antigens, these residual $CD8^+$-$V\beta6^+$ T cells appeared to decrease gradually in number by day 70 after the CP treatment (Table III, group 4-c).

A similar selective disappearance of $V\beta$ expression in $CD4^+$ but not $CD8^+$ T cells in the periphery has previously been reported by Gao et al. (32) for T cells using TCR $V\beta11$, which is reactive to I-E molecules. They reported that $CD4^+$-$V\beta11^+$, but not $CD8^+$-$V\beta11^+$, T cells were selectively deleted in the periphery of I-E^+ mice, such as (B6 × CBA/Ca)F_1 mice. Although detail mechanisms of this selective deletion of $V\beta11^+$ T cells in the I-E^+ F_1 mice have not been clarified, Gao et al. (32) explained that the deletion appeared to occur during negative selection in the thymus. Therefore, the mechanisms of the selective deletion reported by them are considered to be quite different from those of the present system, because the selective destruction of the $CD4^+$-$V\beta6^+$ mature but not immature T cells is considered to be achieved usually outside of the thymus in the present system by destructing the proliferating T cells that are reactive against the tolerogenic antigens. The fact that $V\beta6^+$ T cells in the periphery had obviously decreased on day 14 before the clonal deletion mechanisms started to work in the thymus (Table III, group 4-a) may support this explanation.

From their results showing that neither $CD4^+$-$V\beta6^+$ nor $CD8^+$-$V\beta6^+$ T cells were deleted in the periphery of the tolerant mice, Qin et al., (15) concluded that the mechanisms of their antibody-induced tolerance to Mls-1^a-encoded antigens may be clonal anergy but not clonal deletion. Although Qin et al. (15) did not show the status in the thymus in their study, it became clear from the present study that residual $V\beta6^+$ T cells can exist in the periphery of the tolerant mice even if the clonal deletion mechanism is working in the thymus. Some delay may be required to establish a completely $V\beta6^+$ cell-deleted state in the periphery of the adult tolerant mice. The present result (Table III, group 4-c) indicating gradual decrease of the $V\beta6^+$ T cells in the periphery in the chronic stage of tolerance may support this conclusion. Moreover, the results obtained by MacDonald et al. (14) in the neonatally induced tolerance system may be, thus, compatible with our present results, because they induced the tolerance in the neonatal stage and tested the $V\beta6^+$ T cells in the periphery of the adult mice.

As to the mechanism responsible for the intrathymic elimination of Mls-1^a-reactive T cells, thymic chimerism seemed to be essential in CP-induced tolerance. MacDonald et al. (31) have shown that dendritic cells and/or macrophages could

induce tolerance accompanied by the elimination of $V\beta6^+$ cells in radiation bone marrow chimeras. Another report stated that significant chimerism of bone marrow-derived nonlymphoid cells was detected in the thymus of neonatally tolerant mice (33). Such chimerism may be important in the development and/or maintenance of the tolerant state. As we have emphasized in the previous reports (16, 20), the establishment of mixed chimerism in the thymus of the recipient mice made tolerant of Mls-1^a strain with the donor spleen cells followed by CP was also confirmed in the present study (Table II). The precise correlation between the duration of these mixed chimerisms in the thymus and the intrathymic elimination of Mls-1^a-reactive T cells using TCR $V\beta6^+$, however, has not been clear. In the present study using the C3H mice made tolerant of AKR, neither mixed chimerism nor clonal deletion of $V\beta6^+$ T cells was detected in the thymus on day 14 (Tables II and III). In the tolerant C3H mice, however, intrathymic clonal deletion of $V\beta6^+$ T cells was observed on day 35, associating with mixed chimerism in the thymus (Tables II and III). From our preliminary results, moreover, the duration of mixed chimerism appears to correlate with the maintenance of the intrathymic clonal deletion mechanism in the chronic stage of the CP-induced tolerance (Eto et al., unpublished data). These results strongly suggest the close correlation between the intrathymic chimerism and the clonal deletion mechanism.

From our previous (16–20) and present studies, the two major mechanisms that are essential to our CP-induced tolerance system have been elucidated and clearly segregated. The first mechanism is important to destroy specific mature T cells in the periphery and may be called destruction of anitgen-stimulated and then proliferating cells. Namely, the reactive cells in the recipient mice are stimulated with injection of allogeneic lymphoid cells. During the subsequent cell proliferation, these reactive cells are specifically destroyed with CP, whereas nonreactive cells survive after the CP treatment because of their resting cell cycle (17). The second mechanism is the clonal deletion in the thymus as presented in this study. Although a further detailed mechanism in deleting immature T cells in the thymus has not been made clear yet (4, 5), the most important point is that the mechanism works in the thymus only to delete immature (usually CD4 CD8 double-positive) T cells but not mature T cells (29, 34).

The third major mechanism in our CP-induced tolerance system may be generation of Ts cells (35), especially in the late stage of the tolerance. This aspect of the CP-induced tolerance was further analyzed by our colleagues (36). Although precise relationships among these three major mechanisms of CP-induced tolerance have not been made clear, all of them appear to be indispensable in inducing and maintaining a long-lasting tolerance to skin allograft in mice.

Summary

When C3H (H-2^k, Mls-1^b) mice were primed intravenously with 10^8 viable spleen cells from AKR (H-2^k, Mls-1^a) and treated intraperitoneally with 200 mg/kg of cyclophosphamide (CP) 2 d later, not only a long-lasting skin allograft tolerance but also a tolerance in mixed lymphocyte reaction to Mls-1^a-encoded antigens was established. The cellular mechanisms of CP-induced tolerance were examined by assessing the $V\beta6$-bearing T cells that are strongly correlated with reactivity to Mls-

1[a]-encoded antigens bound to MHC class II molecules. At the relatively early stage (2 or 5 wk) after the CP treatment, $CD4^+$-$V\beta6^+$ T cells of C3H origin were preferentially eliminated in the lymph nodes of the tolerant mice, whereas $CD8^+$-$V\beta6^+$ T cells remained. On the other hand, neither $CD4^+CD8^-$ nor $CD4^-CD8^+$ thymocytes bearing a high density of $V\beta6$ was detected in the chimeric thymus. Namely, in the thymus of the tolerant C3H mice, neither mixed chimerism nor the clonal deletion of the $V\beta6$-bearing T cells was observed on day 14, whereas both of them were observed on day 35. The clonal deletion and mixed chimerism in the thymus were lasting for >10 wk after the CP treatment. Expression of $V\beta6$ on the peripheral T cells in the tolerant C3H mice gradually reduced in the process of time. These results strongly suggested that the clonal deletion in the thymus was one of the essential mechanisms in the CP-induced tolerance system.

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