Distinct Fates of Self-specific T Cells Developing in Irradiation Bone Marrow Chimeras: Clonal Deletion, Clonal Anergy, or In Vitro Responsiveness to Self-Mls-1^a Controlled by Hemopoietic Cells in the Thymus

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Summary

Elimination of potentially self-reactive T lymphocytes during their maturation in the thymus has been shown to be a major mechanism in accomplishing self-tolerance. Previous reports demonstrated that clonal deletion of self-Mls-1^a-specific Vβ6$^+$ T lymphocytes is controlled by a radiosensitive I-E$^+$ thymic component. Irradiation chimeras reconstituted with I-E$^-$ bone marrow showed substantial numbers of mature Vβ6$^+$ T cells despite host Mls-1^a expression. Analysis of the functional properties of such chimeric T cells revealed a surprising variability in their in vitro reactivity to host Mls-1^a, depending on the H-2 haplotype of stem cells used for reconstitution. In chimeras reconstituted with B10.S (H-2^s) stem cells, mature Vβ6$^+$ lymphocytes were present but functionally anergic to host-type Mls-1^a in vitro. In contrast, in chimeras reconstituted with B10.G (H-2^q) bone marrow, nondeleted Vβ6$^+$ cells were highly responsive to Mls-1^a in vitro. These findings suggest that clonal anergy of Vβ6$^+$ cells to self-Mls-1^a may be controlled by the affinity/avidity of T cell receptor interactions with bone marrow–derived cells in the thymus depending on the major histocompatibility complex class II molecules involved. Furthermore, chimeras bearing host (Mls-1^a)-reactive Vβ6$^+$ cells did not differ clinically from those with anergic or deleted Vβ6$^+$ cells and survived more than one year without signs of autoimmune disease. Interestingly, their spleen cells had no Mls-1^a stimulatory capacity in vitro. Therefore, regulation at the level of antigen presentation may be an alternative mechanism for maintenance of tolerance to certain self-antigens such as Mls-1^a.

Immunological tolerance to self determinants is thought to be accomplished by several distinct mechanisms. Recently, it has been shown that clonal deletion of self-specific T cells in the thymus is a major factor controlling autoreactivity of the immune system (1–11). For example, mice of Mls-1^a genotype have been found to clonally delete mature T cells expressing TCR Vβ6, Vβ8.1, Vβ9, and Vβ7, all four of which confer specificity to Mls-1^a (2, 3, 11, 12). The cellular basis of clonal deletion was further studied in irradiation bone marrow chimeras. Vβ6$^+$ T cells have been shown to be deleted independent of Mls-1^a being expressed by donor or host tissue (6, 13). This indicates that irradiated recipient mice express sufficient Mls-1^a to tolerize immature lymphocytes. However, we (14, 15) and others (16–18) have demonstrated that deletion of Vβ6$^+$ T cells in chimeras required MHC class II (I-E) expression on donor bone marrow–derived cells, thus suggesting an important role for presentation of self-Mls-1^a by hemopoietic cells in the thymus.

Besides clonal deletion, other mechanisms have been implicated in the maintenance of self-tolerance. Recently, T cells specific for (but not reactive to) self-antigens have been identified in immunized (19), chimeric (16, 17, 20), and transgenic (21–25) mice. This unresponsiveness (frequently referred to as anergy) was found among thymic and/or peripheral T lymphocytes; however, the molecular basis of anergy remains obscure.

To investigate the mechanism(s) of unresponsiveness in more detail, we took advantage of the fact that effective deletion of Vβ6$^+$ (Mls-1^a-specific) T cells in irradiation bone marrow chimeras depends upon the H-2 haplotype of donor stem cells used for reconstitution (14–18). In particular, the functional properties of Vβ6$^+$ T cells that escaped clonal deletion were investigated and compared with T cells from chimeras where clonal deletion was effective. Lymphocytes from chimeras reconstituted with B10.S (I-E$^-$) bone marrow cells responded very weakly to host-type Mls-1^a stimulator cells,

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confirming other recent examples of clonal anergy (16, 17, 19, 20, 25). In contrast, T cells from similar recipients reconstituted with B10.G (I-E$^-$) bone marrow reacted vigorously to host-type Mls-1^a stimulator cells in vitro. These data indicate first that clonal anergy (at least in the Mls-1^a system) is controlled by hemopoietic cells, and second, that T cell unresponsiveness in vivo may in some cases depend on mechanisms other than clonal anergy, e.g., absence of appropriate antigen presentation in vivo.

Materials and Methods

Animals. Inbred DBA/2 (H-2^d), BALB/c (H-2^d), B10.D2 (H-2^d), B10.BR (H-2^k), and C57BL/6 (H-2^b) mice were purchased from the Institute für Zuchthygiene, Tierspital, University of Zürich, Switzerland. B10.G (H-2^q) and B10.S (H-2^s) mice were obtained from Olac, Bicester, Oxon, UK. BALB.D2-Mls^a (26) breeders were kindly provided by Dr. Hilliard Festenstein, London Hospital Medical College, UK. BALB.D2-Mls^a (also referred to as BALB.D2) and hybrid F_1 mice were bred locally. Characteristics of these strains relevant to the present study are summarized in Table 1.

Chimeras. Bone marrow recipients were lethally radiated (950 rad, 117 rad/min, ^{137}Cs source) and reconstituted 1 d later with 2×10^7 T cell–depleted bone marrow cells (27). For the following 8 wk, chloramphenicol (0.4 g/liter) was added to the drinking water. The transplanted mice had a survival rate of 85–100%. Analysis was performed between 7 and 16 wk after reconstitution. Chimerism was monitored by FACS analysis of lymph node or spleen cells with H-2 haplotype–specific mAbs.

Cytofluorographic Analysis. Aliquots of thymocytes or lymph node cells were stained at 4°C with rat mAbs 44-22-1 (Vβ6 specific) or KJ16-133 (Vβ8.1/Vβ8.2 specific) followed by FITC-conjugated goat anti-rat IgG (Tago Inc., Burlingame, CA). PE-conjugated CD4-specific mAb GK 1.5 (Becton Dickinson & Co., Mountain View, CA) was used for double staining. To assess the chimerism of transplanted mice, haplotype-specific mouse IgG2a mAbs 100-5.28 (K^{k}D^k specific, crossreactive with H-2^q), 34-2-12 (D^d specific), or 14-4-4 (I-E^{d,k} specific), respectively, were used followed by a fluorescent goat anti–mouse IgG2a reagent (Southern Biotechnology Associates, Inc., Birmingham, AL). Viable cells (20,000/sample) were analyzed by flow cytometry on a Epics Profile Analyzer (Coulter Electronics, Inc., Hialeah, FL) with logarithmic scales. Percentages after subtraction with the fluorescein conjugate alone are indicated.

Mixed Lymphocyte Cultures. Responder lymphocytes (1.5×10^6) were incubated with irradiated (1,000 rad) anti-Thy-1.2 (mAb AT-83) plus rabbit complement–depleted splenic stimulator cells (4.5×10^6) in 2 ml IMDM supplemented with 19 mM L-glutamine, 10^5 U/liter penicillin-streptomycin solution, 5×10^{-5} M 2-ME, and 10% heat-inactivated FCS in 24-well plates at 37°C in 5% CO_2 plus air. Cultures used for the assessment of [^{3}H]thymidine uptake and IL-2 secretion contained 5×10^5 responder cells and $1–10 \times 10^5$ stimulator cells in 96-well flat-bottomed plates. In some experiments, responder populations were depleted of CD8$^+$ cells by treatment with rat IgM mAb 3.168.1 (CD8 specific) plus rabbit complement before culture.

IL-2 Measurement. IL-2 contents of 48-h MLC supernatants were assayed on CTLL-2 cells as described (28). IL-2 values were calculated using OD 405-nm measurements after background subtraction. rIL-2 was used to calibrate a standard curve where 50% of the maximal OD 405-nm value was arbitrarily defined as 100 U of IL-2/ml. Consequently, IL-2 values were calculated as follows: 100 U/ml (dilution factor of sample supernatant at 50% max. OD)/(dilution factor at 50% max. OD of rIL-2 standard). Growth of CTLL-2 cells of MLC supernatants was completely blocked by the IL-2-specific mAb S4B6, proving that the only factor measured was IL-2 (some CTLL-2 cells also show a minor sensitivity to IL-4).

Blast Purification. 3-d MLC-responding T cell blasts were isolated on a Percoll density gradient and resuspended (2×10^5 viable cells/ml) in fresh medium supplemented with human rIL-2 (200

Table 1. *Characteristics of Mouse Strains Used in this Study*

Mouse strain	Mls-1	MHC class II		Percent Vβ6$^+$/CD4$^+$ cells
		I-A	I-E	
DBA/2	a	d	d	0.4
BALB.D2-Mls^a (BALB.D2)	a	d	d	0.4
BALB/c	b	d	d	12.4
B10.D2	b	d	d	9.3
B10.G	b	q	–	3.8
B10.S	b	s	–	8.2
B10.BR	b	k	k	8.8
C57BL/6	b	b	–	7.4
(DBA/2 × B10.G)F_1	a/b	d/q	d/–	0.8
(BALB.D2-Mls^a × B10.G)F_1	a/b	d/q	d/–	0.6
(BALB.D2-Mls^a × B10.S)F_1	a/b	d/s	d/–	1.1

Percentages of Vβ6$^+$/CD4$^+$ lymph node cells (means of three individual mice) were determined by two-color immunofluorescence and are normalized to the total CD4$^+$ populations.

ng/ml). After 2 d, cells were recovered, counted, and analyzed for Vβ expression.

Assessment of Mls-1^a Stimulatory Capacity. Spleen cells (1–10 × 10^5) from chimeras and control mice were cocultured with the Mls-1^a-specific T cell hybrid RG17.16 (2 × 10^4 cells) in 96-well flat-bottomed plates (29). In some experiments, the stimulating spleen cells were irradiated with 1,000 rad and/or T cell depleted. No significant difference in stimulatory capacity was seen between these treatment protocols. IL-2 contents of supernatants were determined as described above.

Results

Chimeras Reconstituted with I-E$^-$ Bone Marrow Show Impaired Clonal Deletion of Vβ6$^+$ T Cells. We constructed semiallogeneic parent → F_1 chimeras using either B10.D2 (H-2^d), B10.S (H-2^s), or B10.G (H-2^q) mice as donors of T cell–depleted bone marrow. Recipients were either (BALB.D2-Mls^a × B10.S)F_1 or (BALB.D2-Mls^a × B10.G)F_1 mice. As shown in Table 2, both types of transplanted animals that received I-E$^-$ stem cells from B10.S or B10.G mice, respectively, revealed impaired clonal deletion of Vβ6$^+$ (Mls-1^a-specific) cells. In contrast, chimeras transplanted with B10.D2 (I-E$^+$) bone marrow showed low levels of Vβ6$^+$ cells. Comparable results have been obtained in irradiated (DBA/2 × B10.G)F_1 mice reconstituted with either B10.G or B10.D2 bone marrow cells; recipients reconstituted with DBA/2 stem cells served as positive controls. The fact that ~5% of Vβ6$^+$ cells were constantly found in the chimeras reconstituted with I-E$^-$ cells may indicate that host-derived hemopoietic cells persisting at low but variable amounts did not significantly alter differentiation of Mls-1^a-specific T cells. Vβ8$^+$ cells were monitored as an internal positive control that revealed efficient maturation of T cells in all the chimeras tested.

T Cells from Fully Allogeneic Chimeras Reconstituted with I-E$^-$ (H-2^q) Bone Marrow Show In Vitro Reactivity to Host Mls-1^a. We have previously demonstrated (14, 15) that fully allogeneic B10.G (I-E$^-$) → DBA/2 (I-E$^+$) chimeras contained 6.8% of Vβ6$^+$ T cells among their peripheral CD4$^+$ lymphocytes. Surprisingly, in initial analyses of the functional properties of chimeric T cells, we found strong host-specific reactivity. After stimulation with host-type DBA/2 (Mls-1^a) spleen cells, ~30 U of IL-2/ml were produced (Table 3). This response was specific for Mls-1^a as shown by comparison of BALB.D2-Mls^a vs. congenic BALB/c (Mls-1^b) stimulator cells. In contrast, lymphocytes from B10.D2 → DBA/2 chimeras containing very low levels of Vβ6$^+$ cells did not produce significant amounts of IL-2 when stimulated with Mls-1^a spleen cells. The control response after stimulation with third-party allogeneic MHC antigens was comparable for all cell populations. Thus, T cells from fully allogeneic chimeras reconstituted with I-E$^-$/H-2^q bone marrow cells exhibited reactivity to host Mls-1^a.

Mls-1^a-specific Reactivity of Chimeric Lymphocytes In Vitro Depends on the H-2 Haplotype of the Bone Marrow Donor. While our studies were in progress, others published data from semiallogeneic parent → F1 chimeras using H-2^s bone marrow cells (16, 17). They demonstrated impaired clonal deletion of Vβ6$^+$ cells similar to our findings, but reported on functional anergy of Mls-1^a-specific T cells in their chimeras. Because this unresponsiveness of Vβ6$^+$ cells was in contrast to the Mls-1^a-specific reactivity in our system, we analyzed Mls-1^a responsiveness of lymphocytes from parent → F1 chimeras using either B10.S (H-2^s) or B10.G (H-2^q) donors. As demonstrated above (Table 2), both chimeras made with I-E$^-$ bone marrow cells, i.e., B10.S → (BALB.D2-Mls^a × B10.S)F_1 and B10.G → (BALB.D2-Mls^a × B10.G)F_1 chimeras, revealed relatively high percentages of Vβ6$^+$ cells, whereas in control chimeras reconstituted with B10.D2 (I-E$^+$) bone marrow, most of the Vβ6$^+$ cells were deleted.

Fig. 1 demonstrates IL-2 responses to titrated stimulator cells by chimeric lymphocytes, and their [^{3}H]thymidine uptake is shown in Table 4. In all cases, lymphocytes from chimeras or control mice proliferated and secreted IL-2 after stimulation with third-party allogeneic MHC antigens. How-

Table 2. *Clonal Deletion of Vβ6$^+$ Cells in Semiallogeneic Parent → F_1 Bone Marrow Chimeras Requires Donor I-E Expression*

Donor		Host	Percent lymph node cells expressing: Vβ6/CD4	Vβ8/CD4	I-E	H-2D^d	H-2D^q
B10.D2	→	(BALB.D2 × B10.S)F_1	1.4	25.9	33.6	ND	ND
B10.D2	→	(BALB.D2 × B10.G)F_1	1.7	23.0	40.2	ND	ND
B10.S	→	(BALB.D2 × B10.S)F_1	4.8	20.5	2.1	ND	ND
B10.G	→	(BALB.D2 × B10.G)F_1	5.6	20.7	4.5	ND	ND
B10.D2	→	(DBA/2 × B10.G)F_1	0.6	18.3	36.3	97.3	1.9
B10.G	→	(DBA/2 × B10.G)F_1	5.3	13.2	2.4	1.8	99.6
DBA/2	→	(DBA/2 × B10.G)F_1	0.6	17.9	ND	96.5	3.0

Mls-1^a mice were irradiated with 950 rad and reconstituted with T cell–depleted bone marrow from the indicated strains. Analysis was performed between 7 and 16 wk later. Means of three to four individual chimeras for Vβ expression are given as a proportion of the total CD4$^+$ cells, respectively. Chimerism was assessed by H-2 expression. SDs ranged between 0.2 and 4.7%.

Table 3. *Self-Mls-1ª-specific IL-2 Production of Spleen Cells from Fully Allogeneic Chimeras*

Responder spleen cells			Percent	IL-2 produced after stimulation with spleen cells from:					
Donor		Host	Vβ6⁺/CD4⁺	BALB.D2	BALB/c	DBA/2	B10.D2	B10.BR	C57BL/6
						U			
B10.D2	→	DBA/2	0.4	<2	<2	<2	<2	22.1 ± 6.1	13.7 ± 3.2
B10.G	→	DBA/2	6.8	23.6 ± 4.4	<2	29.9 ± 5.6	<2	28.9 ± 1.5	16.9 ± 3.7
B10.D2			9.3	31.1 ± 4.5	<2	25.4 ± 3.6	<2	14.4 ± 5.0	14.4 ± 2.6
DBA/2			0.4	<2	2.8	<2	<2	29.2 ± 3.1	25.3 ± 2.6

5 × 10⁵ T cell–depleted stimulator spleen cells were incubated with 5 × 10⁵ responder spleen cells from the chimeras or control mice indicated in 96-well plates for 48 h. Supernatants were assayed for IL-2 content. Data are means ± SD of three to five individual mice and of triplicate cultures. Percentages of expresison of Vβ6 by responder lymphocytes have been published previously (15).

ever, Mls-1ª-specific responses were variable. As expected, T cells from chimeras reconstituted with B10.D2 donor cells (which clonally deleted Vβ6⁺ cells) were not reactive to Mls-1ª stimulators Furthermore, lymphocytes from B10.S → (BALB.D2-Mls^a × B10.S)F$_1$ chimeras did not secrete IL-2 after stimulation with host-type Mls-1ª (BALB.D2-Mls^a × B10.S)F$_1$ spleen cells, suggesting anergy of the Vβ6⁺ cells found at levels of ~5% in these animals. Interestingly, when lymphocytes from the same chimeras were stimulated with parental BALB.D2-Mls^a spleen cells, we detected weak proliferation and ~7 U of IL-2/ml MLC supernatant. As observed previously in fully allogeneic chimeras (Table 3), B10.G → (BALB.D2-Mls^a × B10.G)F$_1$ chimeras showed reactivity to (BALB.D2-Mls^a × B10.G)F$_1$ stimulators and were strongly reactive to BALB.D2-Mls^a spleen cells. In summary, T cell responsiveness to host Mls-1ª was absent in chimeras reconstituted with B10.D2 (I-E⁺) bone marrow, most probably due to clonal deletion of Mls-1ª-reactive cells. Furthermore, the Mls-1ª-specific response of chimeras reconstituted with B10.S (I-E⁻) bone marrow was very low against BALB/D2-Mls^a stimulators, and no reactivity was found after stimulation with host-type (BALB.D2-Mls^a × B10.S)F$_1$ stimulator cells. In contrast, lymphocytes derived from chimeras reconstituted with B10.G (I-E⁻) bone marrow cells were clearly reactive to Mls-1ª presented by parental or F$_1$ stimulators.

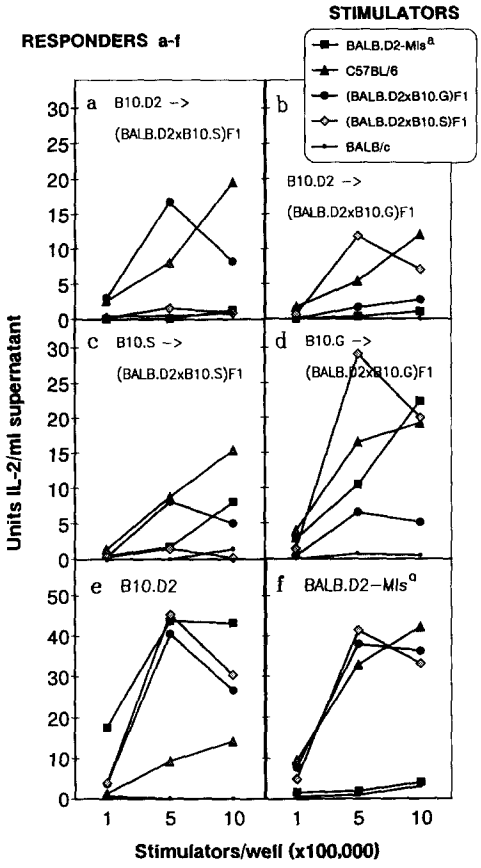

Figure 1. Mls-1ª-specific IL-2 production of lymphocytes from semiallogeneic parent → F$_1$ bone marrow chimeras. Titrated T cell–depleted stimulator spleen cells of the indicated genotype were incubated with 5 × 10⁵ responder lymphocyte from the indicated chimeras (*a–d*) or control mice (*e* and *f*) in 96-well plates for 48 h. Supernatants were assayed for IL-2 content. Data are means of triplicate cultures from two individual mice.

MHC (I-E) Homozygous Mls-1ª Stimulator Cells Induce Stronger T Cell Responses than Heterozygous Stimulators. As demonstrated in Fig. 1, the Mls-1ª-specific activation of chimeric T cells was clearly higher when parental stimulator cells were used as compared with spleen cells derived from F$_1$ hybrid mice. We reasoned that this discrepancy may have been due to a reduced Mls-1ª stimulatory capacity of F$_1$ hybrid cells. To test this, the Mls-1ª-reactive Vβ6⁺ T cell hybrid RG17.16 was incubated with the various stimulator spleen cells. As shown in Table 5, the response to homozygous BALB.D2-Mls^a cells was significantly higher when compared with heterozygous stimulators. In addition, we confirmed by flow cytometry that homozygous spleen cells expressed more I-E molecules than cells heterozygous at the MHC locus. Therefore, it is suggested that the decreased Mls-1ª stimulatory capacity of spleen cells from F$_1$ mice is due to their lower I-E expression. Additional experiments ruled out that heterozygosity at the Mls-1 locus by itself would

Table 4. *Self-Mls-1^a-specific Proliferation of Lymphocytes from Semiallogeneic Parent → F_1 Chimeras*

Responder lymphocytes				[^{3}H]Thymidine uptake after stimulation with spleen cells from:		
Donor (H-2, Mls-1)		Host (H-2, Mls-1)	Tissue	BALB.D2	BALB/c	C57BL/6
					cpm × 10^{-3}	
B10.D2	→	(BALB.D2 × B10.S)F_1	Thymus	0.1	<0.1	0.6
(d, b)		(d/s, a/b)	Thymus	<0.1	<0.1	0.4
			Lymph nodes	<0.1	<0.1	6.6
			Lymph nodes	<0.1	<0.1	8.0
B10.D2	→	(BALB.D2 × B10.G)F_1	Thymus	<0.1	<0.1	0.7
(d, b)		(d/q, a/b)	Thymus	<0.1	<0.1	0.3
			Lymph nodes	<0.1	<0.1	9.3
			Lymph nodes	<0.1	<0.1	3.8
B10.S	→	(BALB.D2 × B10.S)F_1	Thymus	1.1	<0.1	1.4
(s, b)		(d/s, a/b)	Thymus	1.0	<0.1	0.9
			Lymph nodes	3.8	<0.1	8.5
			Lymph nodes	5.6	<0.1	7.6
B10.G	→	(BALB.D2 × B10.G)F_1	Thymus	4.4	<0.1	0.7
(q, b)		(d/q, a/b)	Thymus	3.6	<0.1	0.6
			Lymph nodes	13.1	<0.1	6.0
			Lymph nodes	11.7	<0.1	3.6
B10.D2			Thymus	5.9	<0.1	0.3
(d, b)			Lymph nodes	26.3	<0.1	7.4
BALB.D2			Thymus	<0.1	<0.1	3.5
(d, a)			Lymph nodes	<0.1	1.6	27.4

5 × 10^5 irradiated (1,000 rad) T cell–depleted stimulator spleen cells were cultivated with 5 × 10^5 responder CD8$^-$ thymocytes or lymph node cells from the chimeras or control mice indicated in 96-well plates and pulsed for the last 12 h of a 3-d incubation. The data (mean cpm × 10^{-3} of three to four cultures after subtraction of background counts from cultures with responder cells alone) are from two chimeras of each group measured in a representative experiment. Underlined values are significantly ($p < 0.05$; student's t test) above background levels. SDs ranged between ±3 and ±32% for thymocyte responders, and between ±4 and ±37% for responders from lymph nodes. IL-2 values measured in supernatants of similar MLC are shown in Fig. 1.

Table 5. *Mls-1^a Stimulatory Capacity of Parental vs. F_1 Hybrid Spleen Cells of Different H-2 Haplotypes*

Stimulators from:	IL-2 produced by RG17.16 cells after stimulation with titrated spleen cells			Mean fluorescence intensity of I-E$^+$ spleen cells
	10^5	5 × 10^5	10^6	
		U		
BALB.D2-Mls^a	25.0	57	75	133.2
(BALB.D2-Mls^a × B10.S)F_1	6.9	31	18	45.6
(BALB.D2-Mls^a × B10.G)F_1	6.2	31	35	62.8

The Mls-1^a-reactive T cell hybrid RG17.16 (2 × 10^4 cells/well) was incubated with titrated T cell–depleted stimulator spleen cells in 96-well flat-bottomed plates for 48 h. Supernatants were assayed for IL-2 activity. Data are mean U IL-2/ml supernatant of triplicate cultures. Mean fluorescence intensity of cells positively stained with mAb 14-4-4 specific for I-E$^{d/k}$ is given from analyses of similar T cell–depleted spleen cells (from the mice indicated), which were 91–95% I-E$^+$.

significantly reduce antigenicity of stimulator cells, because spleen cells from (BALB.D2-Mls^a × BALB/c)F_1 were similarly Mls-1^a stimulatory compared with BALB.D2-Mls^a cells (data not shown).

Self-Mls-1^a-specific In Vitro Proliferation of Vβ6$^+$ T Cells after Addition of Exogenous IL-2. We further investigated responsiveness of chimeric T cells by analyzing Vβ6 expression in MLC after Mls-1^a stimulation. Since functional anergy in some systems appears to reflect defective IL-2 production (30), we applied the following protocol. Responding T cell blasts were isolated from 3-d MLC and re-incubated in IL-2 containing medium for an additional 2 d. Vβ6$^+$/CD4$^+$ lymphocytes from chimeras that received B10.G bone marrow expanded ~10-fold when cocultured with BALB.D2-Mls^a stimulator cells (Fig. 2). This increased proportion of Vβ6$^+$/CD4$^+$ cells was specific for Mls-1^a. Lymphocytes from chimeras reconstituted with B10.S stem cells showed approximately fivefold expansion of the Vβ6$^+$/CD4$^+$ subset in response to Mls-1^a. Control stimulation with spleen cells from third-party C57BL/6 mice (H-2^b) resulted in a proliferative response in all cases, but no specific expansion of Vβ6$^+$ T cells. In conclusion, these data indicate that "anergic" Vβ6$^+$ cells from irradiated mice reconstituted with B10.S bone marrow responded to strong Mls-1^a stimulation and addition of exogenous IL-2.

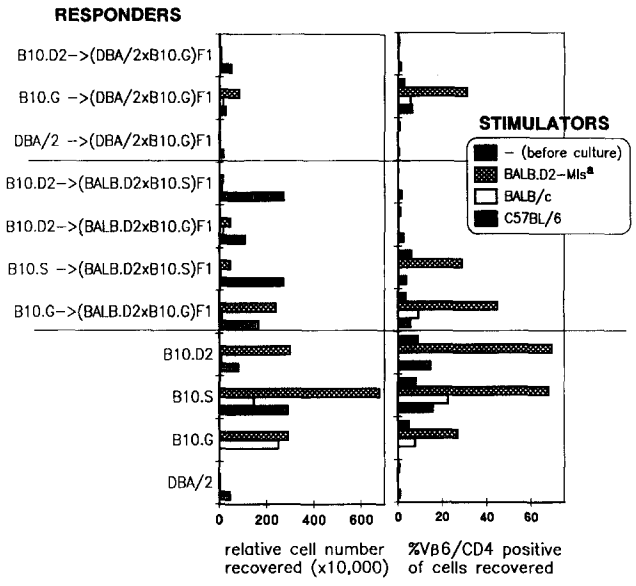

Figure 2. Mls-1^a-specific proliferation (*left panel*) and expansion of Vβ6$^+$/CD4$^+$ cells (*right panel*) of lymphocytes from semiallogeneic chimeras in the presence of IL-2. Pooled lymph node and spleen cells from two individual chimeras were stimulated with irradiated T cell–depleted spleen cells. After 3 d, responding blasts were isolated and cultured for an additional 48 h in IL-2. Recovered cells were counted (*left panel*) and stained with Vβ6-specific mAb. Data shown in the right panel are percent Vβ6$^+$ cells normalized to total CD4$^+$ cells. Measurement of Vβ6$^+$/CD4$^+$ were only done in cultures where numbers of viable cells recovered allowed appropriate analyses. Numbers and proportions of Vβ6$^+$/CD4$^+$ cells in the starting population (before culture) are indicated with black bars.

Mls-1^a Stimulatory Capacity of Chimeric Spleen Cells. The finding that chimeras made with I-E$^-$ donor cells remained healthy despite the presence of in vitro self-Mls-1^a-reactive T cells raised the question of reactivity of Vβ6$^+$ T cells in vivo. Because B cells responsible for Mls-1^a stimulation (31–33) are very radiosensitive (34), we reasoned that the potentially reactive T cells may not be activated in vivo because the chimeras might lack Mls-1^a stimulatory capacity, unless they had been reconstituted with bone marrow cells expressing both Mls-1^a and I-E determinants. To test this hypothesis, the Mls-1^a reactive Vβ6$^+$ T cell hybrid RG17.16 was incubated with stimulator spleen cells from the various chimeras (Table 6). IL-2 secretion was observed when stimulator cells originated from DBA/2 (I-E$^+$, Mls-1^a) mice or chimeras reconstituted with DBA/2 bone marrow. In contrast, chimeric spleen cells from Mls-1^a hosts reconstituted with bone marrow from B10.G or B10.S (both I-E$^-$ and Mls-1^b), or B10.D2 (I-E$^+$, Mls-1^b) donors were not stimulatory. Thus, no Mls-1^a stimulatory capacity was found in spleens of chimeras containing Vβ6$^+$ T cells that had escaped clonal deletion.

Discussion

The data presented herein confirm and extend recent studies (14–18) indicating that clonal deletion of self-reactive TCR Vβ domains in irradiation bone marrow chimeras requires expression of I-E by donor-derived bone marrow cells. Thus, Vβ6$^+$ (Mls-1^a-specific) cells derived from H-2^q or H-2^s (I-E$^-$) bone marrow were not efficiently deleted in irradiated H-2^{dxq} or H-2^{dxs} hosts of Mls-1^a genotype. This contrasts with H-2^d (I-E$^+$) bone marrow chimeras that effectively deleted Vβ6$^+$ cells in both F_1 recipients. Similarly, Ramsdell et al. (16) and Roberts et al. (17) have recently shown that H-2^s (I-E$^-$) bone marrow is not effective in inducing deletion of Vβ6$^+$ or Vβ17a$^+$ cells in irradiated I-E$^+$ recipients expressing the appropriate self-antigen (Mls-1^a or I-E plus an unknown ligand for Vβ17a). Collectively, these studies are compatible with the notion that in these systems clonal deletion is the result of contact between TCR on developing immature lymphocytes and self-antigens presented by I-E$^+$ hemopoietic cells (such as B cells, dendritic cells, or macrophages). In this model, radioresistant (presumably epithelial) cells in the thymus apparently do not contribute significantly to the deletion process.

Clonal anergy has been proposed to maintain tolerance to self-antigens in cases when clonal deletion does not (or cannot) occur (21–25); e.g., anergy of Vβ6$^+$ cells to Mls-1^a has recently been demonstrated in several different experimental systems (16, 17, 19, 20). Using irradiation bone marrow chimeras, both Ramsdell et al. (16) and Roberts et al. (17) have shown that Vβ6$^+$ T cells in P → F_1 chimeras derived from H-2^s (I-E$^-$) donors are selectively unresponsive to Mls-1^a in vitro. The present study largely confirms these findings (in s → d × s chimeras), although a weak response of chimeric T cells could be observed when Mls-1^a was presented on MHC homozygous (d × d) stimulator cells. In striking contrast, Vβ6$^+$ T cells in congenic chimeras of

Table 6. *Mls-1^a Stimulatory Capacity of Chimeric Spleen Cells*

Stimulators from:			IL-2 produced by RG17.16 cells after stimulation with titrated spleen cells		
Donor		Host	10^6	5×10^5	10^5
				U	
B10.D2	→	DBA/2	<2	<2	ND
B10.G	→	DBA/2	<2	<2	ND
B10.D2	→	(DBA/2 × B10.G)F_1	<2	<2	<2
B10.G	→	(DBA/2 × B10.G)F_1	<2	<2	<2
DBA/2	→	(DBA/2 × B10.G)F_1	51.3	48.2	13.7
B10.D2	→	(BALB.D2 × B10.S)F_1	<2	<2	ND
B10.D2	→	(BALB.D2 × B10.G)F_1	<2	<2	ND
B10.S	→	(BALB.D2 × B10.S)F_1	<2	<2	ND
B10.G	→	(BALB.D2 × B10.G)F_1	<2	<2	ND
B10.D2			<2	<2	<2
DBA/2			48.4	45.3	14.1

The Mls-1^a-reactive T cell hybrid RG17.16 (2×10^4) was incubated for 48 h with stimulator spleen cells from the chimeras or control mice as indicated. Supernatants were assayed for IL-2 activity. Data are means of triplicate cultures from two individual mice.

H-2^q donor bone marrow type (q → d × q) responded strongly to host Mls-1^a antigens in vitro, as assessed by proliferation and IL-2 production. The pattern of responsiveness of these chimeric T cells to a panel of stimulator cells indicated further that they were tolerant to host MHC (H-2^d) but reacted to third-party allogeneic MHC. Failure of tolerance induction to host Mls-1^a therefore markedly contrasts with successful tolerance induction to host MHC products. This dichotomy of reactivity to Mls-1^a vs. tolerance to MHC, first demonstrated by Morrissey et al. (35), is difficult to interpret without knowledge of the molecular structure of Mls-1^a gene products. One possible explanation might be that MHC products presented by radioresistant (epithelial) thymic cells may preferentially induce clonal deletion (or functional anergy) because of their high level of expression, or because of some intrinsic property of the MHC molecules themselves. The ability of thymic epithelium to tolerize across MHC barriers is consistent with a number of tissue-grafting studies (36–38). However, other studies on tolerance to MHC products expressed by the radioresistant component of the thymus have revealed tolerance in vivo but some reactivity in vitro (see references 39–48 and 49 for review).

Induction of anergy of Vβ6$^+$ T cells to self Mls-1^a in s → d × s but not q → d × q chimeras is surprising. In terms of I-E expression and ability to present Mls-1^a to the Mls-1^a-specific hybrid RG17.16, no quantitative differences could be found between spleen cells from the H-2 congenic d × s and d × q F_1 hybrid hosts utilized. Assuming these properties apply also to cells in the thymus, it seems unlikely that the differential induction of clonal anergy to Mls-1^a in these chimeras can be explained by interaction of developing T cells with host components. We therefore favor the notion that donor-derived hemopoietic cells are involved in the clonal anergy phenomenon. It is noteworthy that several studies (50), including our own (29), have recently revealed that there is a hierarchy of I-A alleles with respect to their capacity to clonally delete Vβ6$^+$ cells in MHC congenic, recombinant inbred, and transgenic mouse strains of Mls-1^a genotype that lack I-E expression. Thus, I-A^s gene products can support a significant (although limited) deletion of Vβ6$^+$ cells, whereas I-A^q gene products seem to be ineffective. Given these results, it is possible that Mls-1^a is differentially "presented" on radiosensitive (hemopoietic) thymic APC expressing I-A^s or I-A^q. Potential differences in the affinity of Vβ6$^+$ TCR for Mls-1^a in association with I-A^s and I-A^q may lead to anergy in one case (I-A^s) but not in the other (I-A^q). Accordingly, the fate of developing immature thymocytes expressing potentially autoreactive TCR (at least for certain antigens such as Mls-1^a) would be largely (if not exclusively) determined by the relative affinity of the TCR interaction with self-antigen presented on MHC class II–positive hemopoietic-derived cells. Thus, deletion, anergy, or no detectable effect would result from high, intermediate, or low affinity interactions (corresponding to H-2^d, H-2^s, or H-2^q presenting cells, respectively, in the case of self-Mls-1^a). In contrast to similar studies (16, 17), the results summarized here suggest that interactions of TCR on immature thymocytes with radioresistant (presumably epithelial) cells would not be relevant for negative selection (manifested either as

deletion or anergy) in the Mls-1^a system. It should be emphasized, however, that thymic epithelial cells play a role in negative selection in other antigenic systems, as suggested convincingly by several reports (39–49, 51).

Despite the potential for in vitro reactivity to Mls-1^a, Mls-1^a recipients reconstituted with H-2^q bone marrow cells survived >1 yr without clinical symptoms of disease. Also, the chimeric spleen cells when adoptively transferred to irradiated Ml-1^a recipients failed to provoke obvious graft-vs.-host disease (not shown). This parallels results from similar chimeras reported by Ramsdell et al. (16). The lack of reactivity may be interpreted as a form of "anergy" of chimeric Vβ6$^+$ cells in vivo. However, this may not necessarily be so, since Korngold and Sprent (52) have shown that adoptive transfer of Mls-1^b spleen cells into irradiated Mls-1^a recipients does not always cause lethal graft-vs.-host disease. An alternative mechanism to explain lack of pathogenic activity of Mls-1^a-specific T cells in vivo may simply be the absence of appropriately presented Mls-1^a in these chimeras. This is suggested by the inability of chimeric spleen cells to stimulate IL-2 production by the Mls-1^a-specific T cell hybrid RG17.16 shown in Table 6. Possibly, Vβ6$^+$ cells may not be harmful in the chimeras because of inadequate Mls-1^a presentation in vivo.

It is noteworthy that spleen cells from d → d × q and d → d × s chimeras, likewise, did not stimulate the Mls-1^a-specific hybrid RG17.16, despite the fact that effective clonal deletion of Vβ6$^+$ cells occurred in these animals. This finding is reminiscent of the situation observed for Vβ11$^+$ cells in normal mice, where clonal deletion occurs in I-E$^+$ strains, but no in vitro reactivity to I-E$^+$ cells can be shown (9). Such a dissociation could reflect differential presentation of self-antigen in thymus vs. peripheral lymphoid tissue; alternatively, it is possible that the affinity threshold for clonal deletion of immature T cells is lower than that required for mature T cell activation. A third possibility to be considered in our model is that Mls-1^a expression may rapidly disappear after irradiation of recipient mice, thereby still fulfilling requirements of immature T cells to be tolerized but not of mature peripheral lymphocytes to be activated. Final discernment between these models most likely depends on a molecular definition of the Mls-1^a gene product.

In summary, irradiation chimeras revealed distinct fates of Vβ6$^+$ T cells specific for the self-antigen Mls-1^a. In the thymus, immature lymphocytes expressing Vβ6 may have differential affinities/avidities for and/or receive differential signals from hemopoietic Mls-1^a-presenting cells depending on the MHC class II molecules involved. Such interactions may apparently result in either clonal deletion, clonal anergy, or clonal responsiveness.

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