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Neonatal tolerance to Mls-1^a determinants: deletion or anergy of V$_\beta$6$^+$ T lymphocytes depending upon MHC compatibility of neonatally injected cells

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Abstract

Recent investigations in mice revealed that natural immunological tolerance to endogenous minor lymphocyte-stimulating locus 1^a (Mls-1^a) antigen correlates primarily with deletion of Mls-1^a-specific V$_\beta$6$^+$ T lymphocytes in the thymus. Similar mechanisms account for acquired tolerance in some instances since the neonatal injection of Mls-1^a-expressing MHC compatible cells in neonatal mice within the first 24 h of life causes clonal deletion of V$_\beta$6$^+$ T cells. Here we demonstrate that V$_\beta$6$^+$ T cells are not deleted in mice neonatally treated with Mls-1^a spleen cells expressing allogeneic H-2 molecules. However, when such non-deleted V$_\beta$6$^+$ T cells were tested *in vitro*, no interleukin 2 (IL-2) secretion or proliferation was observed after Mls-1^a stimulation. This non-responsive state could be overcome by addition of exogenous IL-2, consistent with the fact that V$_\beta$6$^+$ cells enlarged and expressed IL-2 receptors upon Mls-1^a stimulation. Furthermore, the same neonatally treated mice showed *in vitro* functional unresponsiveness of cytotoxic T cells but not of IL-2-secreting cells specific for the tolerated allogeneic MHC antigens. Taken together, our data indicate that neonatal tolerance to Mls-1^a can be accomplished by either clonal deletion or clonal anergy, and that it does not necessarily correlate with tolerance to MHC determinants.

Introduction

Antigen-specific lymphocytes capable of responding to pathogens must simultaneously be unresponsive to self antigens. Tolerization of T cell precursors may take place by their physical elimination during differentiation in the thymus (1). Although clonal deletion has been well described in both normal (2 – 4) and transgenic (5 – 8) mouse models, it is conceivable that alternative tolerizing mechanisms exist. Possible mechanisms include clonal inactivation or anergy, suppression, and lack of induction of autoantigen specific T cells because self determinants are expressed in anatomically 'privileged sites' or because they are expressed only on non-lymphoid cells that are incapable of effective antigen presentation. Recently, *in vitro* clonal anergy has been characterized in detail for certain antigen-specific T cell clones (9 – 11) as well as for T cells obtained from normal (12 – 16) or transgenic (17 – 20) mice. However, it is not clear whether 'anergic' T lymphocytes exist *in vivo* and how this functional anergy is regulated.

The minor lymphocyte-stimulating 1^a (Mls-1^a) antigen (21 – 24), although molecularly not defined, provides a useful model system for studying self tolerance. Because Mls-1^a-specific T cells express particular TCR V$_\beta$ domains such as V$_\beta$6 (3), V$_\beta$7 (25), V$_\beta$8.1 (4), and V$_\beta$9 (26), their fate can be followed by serological means using V$_\beta$-specific monoclonal antibodies. In Mls-1^b mice, Mls-1^a-specific V$_\beta$6$^+$ cells range between 4 and 15% of the total T cell pool, whereas most Mls-1^a mice show $<1\%$ V$_\beta$6$^+$ cells due to clonal deletion. Interestingly, recent studies in immunized (13) and chimeric (12,14 – 16) mice have shown that V$_\beta$6$^+$ cells need not be deleted to achieve unresponsiveness to Mls-1^a *in vitro*. Instead, V$_\beta$6$^+$ cells were functionally unresponsive, indicating that clonal inactivation or

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anergy was an alternative means for maintaining tolerance to Mls-1^a.

Ever since Billingham *et al.* initially showed that the injection of allogeneic cells into a newborn mouse induces specific immunological tolerance for the donor's tissues and organs (27), this approach has been widely used to study antigen-specific immunological unresponsiveness. It was reasoned that neonatal mice may recognize foreign molecules as 'self' because of the immaturity of their immune systems. Accordingly, we recently demonstrated that Mls-1^b mice clonally deleted their $V_\beta6^+$ T cells after neonatal injection of Mls-1^a spleen cells (28), thus exhibiting a phenotype of acquired tolerance similar to natural tolerance to self Mls-1^a.

In this study we show that deletion of $V_\beta6^+$ cells occurred only when the Mls-1^a spleen cells injected were mouse MHC (H-2) compatible with the newborn Mls-1^b mouse. In contrast, when H-2-incompatible Mls-1^a spleen cells were inoculated, practically no or only limited deletion of $V_\beta6^+$ T cells was found. Nevertheless, functional unresponsiveness to Mls-1^a *in vitro* was detected, indicating that the $V_\beta6^+$ cells present in mice neonatally treated with H-2-incompatible spleen cells were anergic. Possible mechanisms involved in deletion or anergy of Mls-1^a-specific cells depending upon MHC compatibility of neonatally injected cells are discussed.

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 Institut für Zuchthygiene, Tierspital, University of Zürich, Switzerland. B10.G (H-2^q) and DBA/1 (H-2^q) mice were obtained from Olac, Bicester, Oxon, UK. BALB.D2-Mls^a (29) 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.

Neonatal tolerization

Spleen cells (10^8) from untreated donor mice were washed and injected i.p. in 100 µl Hank's balanced salt solution within 24 h of birth.

Cytofluorographic analysis

Aliquots of thymocytes or lymph node cells were stained at 4°C with rat mAb 44-22-1 ($V_\beta6$-specific) (30) or KJ16-133 ($V_\beta8.1/V_\beta8.2$-specific) (4) followed by fluorescein isothiocyanate-conjugated goat anti-rat IgG (Tago Inc., Burlingame, CA). The PE-conjugated, CD4-specific mAb GK 1.5 (31) (Becton-Dickinson, Mountain View, CA) was used for double staining. To assess the chimerism of neonatally transfused mice, haplotype-specific mouse IgG2a mAbs K7-309 (K^b-specific) (32) or 34-2-12 (D^d-specific) (33) was used followed by a fluorescent goat-anti mouse IgG2a reagent (Southern Biotechnology Associates Inc., Birmingham, AL). Viable cells (10,000 per sample) were analyzed by flow cytometry on a Epics Profile Analyzer (Coulter Electronics Inc., Hialeah, FL) with logarithmic scales. Percentages after subtraction of backgrounds (0.0 – 1.4%) obtained with the fluorescein conjugate alone are indicated.

Mixed lymphocyte reactions

Responder lymphocytes (3×10^6) were incubated with irradiated (1000 rad) anti-Thy-1.2 mAb (AT-83) (34) plus rabbit complement-treated splenic stimulator cells (5×10^6) in 2 ml Iscove's modified Dulbecco's medium supplemented with 19 mM L-glutamine, 10^5 U/l penicillin – streptomycin solution, 5×10^{-5} M 2-mercaptoethanol, 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 interleukin 2 (IL-2) secretion contained 5×10^5 responder cells and $1 - 10 \times 10^5$ stimulator cells in 96-well flat-bottomed plates. As controls, responder cells and stimulator cells respectively were cultured alone, in the presence of concanavalin A (5 µg/ml), or together with the Mls-1^a-specific T cell hybrid RG17.16 (22). In cultures with exogenous IL-2 either human recombinant (r)IL-2 (200 U/ml; corresponding to 60 ng/ml) or 10% supernatant of concanavalin A-stimulated rat spleen cells was added.

IL-2 measurement

Il-2 contents of 48 h mixed lymphocyte reaction (MLR) supernatants were assayed on CTLL-2 cells (35) as described (36). 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: (Dilution factor of sample supernatant at 50% max. OD) ÷ (Dilution factor at 50% max. OD of rIL-2 standard) × 100 U/ml. In representative control experiments growth of CTLL-2 cells in MLR supernatants was always 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).

^{51}Cr release assay

Mice were killed and responder spleen cells stimulated in mixed lymphocyte cultures with irradiated (2000 rad) stimulator spleen cells at a ratio of 4×10^6:1×10^6 cells in 24-well plates. After 5 days effector cells were harvested and tested for cytotoxic activity on ^{51}Cr-labeled target fibroblasts as described in detail elsewhere (37,38). MC57G (H-2^d), D2 (H-2^d), or DBA/1 (H-2^q) target cells (established methylcholanthrene induced or SV40 transformed murine cell lines) were placed (10^4/well) in round-bottomed microtiter plates (Flacon Labware, Division of Becton-Dickinson, Oxnard, CA) and co-incubated with titrated effector cells for 4.5 h.

Results

Mls-1^b mice neonatally treated with H-2-compatible but not those treated with H-2-incompatible Mls-1^a spleen cells deleted $V_\beta6^+$ T cells

In this study we compared two different regimes for neonatal tolerance induction. The first was the injection of spleen cells which only express H-2 molecules compatible with the recipient's MHC (referred to as 'H-2-compatible cells'); the second was the injection of spleen cells expressing foreign MHC molecules (referred to as 'H-2 incompatible cells'). Newborn BALB/c (H-2^d/Mls-1^b) mice were treated i.p. with spleen cells from the various donor strains within 24 h of birth. Two to six weeks later,

cortisone-resistant thymocytes were analyzed by flow cytofluorometry. As expected, injection of syngeneic (Mls-1^b) spleen cells did not affect $V_\beta6$ expression (Table 2A). However, as described earlier (28), strongly reduced $V_\beta6$ percentages were observed when the spleen cells injected were from DBA/2 (H-2^d/Mls-1^a) mice. Efficient deletion of $V_\beta6^+$ cells was also found in BALB/c mice neonatally treated with Mls-1^a spleen cells from H-2^{dxd} F_1 mice [i.e. from (BALB/c × DBA/2)F_1 or from (BALB/c × BALB.D2-Mls^a)F_1 respectively]. Furthermore, comparable deletion of $V_\beta6^+$ cells occurred when (BALB/c × B10.G)F_1 (H-2^{dxq}) mice were neonatally transfused from Mls-1^a spleen cells from H-2-compatible (B10.D2 × DBA/1)F_1 mice or from (BALB/c × DBA/2)F_1 (H-2^{dxd}) mice. Concerning the latter combination, earlier studies (39) had shown that Mls-1^a spleen cells from donors not tolerant to MHC determinants of the neonatal host were capable of inducing clonal deletion of $V_\beta6^+$ cells despite overt graft versus host disease. Finally, control treatment with syngeneic Mls-1^b spleen cells did not alter $V_\beta6$ expression significantly when compared to untreated controls (Tables 1 and 2A).

We next injected H-2-incompatible spleen cells and made the following surprising observation: the injection of Mls-1^a spleen cells from fully allogeneic DBA/1 (H-2^q) mice or from F_1 mice heterozygous at the H-2 locus did not induce elimination of $V_\beta6^+$ lymphocytes. For example, BALB/c mice neonatally treated with (BALB/c × DBA/1)F_1 or (DBA/2 × DBA/1)F_1 (both HB-2^{dxq}/Mls-1^a) spleen cells exhibited 9.7 or 8.1% $V_\beta6^+$ lymphocytes respectively. Newborn (BALB/c × B10.G)F_1 mice which received H-2-semi-allogeneic (B10.BR × DBA/1)F_1 (H-2^{kxq}) spleen cells showed some deletion but still had quite high percentages (6.0%) of $V_\beta6^+$ mature thymocytes. Thus, Mls-1^b mice neonatally treated with H-2-(semi)allogeneic Mls-1^a spleen cells showed much impaired deletion of $V_\beta6^+$ cells. As positive controls, normal lymphocytic maturation in the mice studied was documented by stainings with monoclonal antibody KJ16-133, specific for a population of lymphocytes which developed largely (but not entirely) independently of Mls-1^a.

Similar analyses were performed by injection of H-2^{bxd} F_1 spleen cells in neonatal C57/BL/6 (H-2^b) or BALB/c (H-2^d) mice, respectively. Expression of $V_\beta6$ and $V_\beta8$ by CD4$^+$CD8$^-$ thymocytes or lymph node cells are shown in Table 3. In parallel to the findings described above, C57BL/6 or BALB/c mice (both Mls-1^b) treated with H-2-incompatible Mls-1^a spleen cells from (C57BL/6 × DBA/2)F_1 mice showed practically no or only partially reduced percentages of $V_\beta6^+$ cells when compared to controls transfused with Mls-1^b F_1 spleen cells (Table 3B). Furthermore, lymph node cells from BALB/c mice treated with Mls-1^a F_1 or Mls-1^b F_1 spleen cells, respectively, did not differ significantly in $V_\beta6$ fluorescence intensity, suggesting that their TCRs were not specifically down-regulated in response to Mls-1^a.

Since tolerogen-specific lymphocytes showed reduced levels of accessory molecules in some systems (40), the expression of CD4 on peripheral T cells was analyzed. No decrease in either the number of CD4$^+$ cells or the surface density of CD4

Table 1. Characteristics of mouse strains used in this study

Mouse strain	Mls-1	H-2	%$V_\beta6^+$/CD4$^+$
BALB/c	b	d	12 4
B10.D2	b	d	9.3
BALB.D2-Mls^a(BALB.D2)	a	d	0.4
DBA/2	a	d	0.4
C57BL/6	b	b	7.4
B10 G	b	q	3.8
DBA/1	a	q	4.2
B10 BR	b	k	8.8
(C57BL/6 × BALB/c)F_1	b/b	b/d	12.0
(C57BL/6 × DBA/2)F_1	b/a	b/d	0.5
(BALB/c × B10 G)F_1	b/b	d/q	11.3
(BALB/c × DBA/1)F_1	b/a	d/q	0.7

Lymph node cells were analyzed by two-color immunofluorescence. Data (means of three individual mice; SEM < 0.8) represent %$V_\beta6^+$ cells of the total CD4$^+$ population, calculated as follows: (%$V_\beta6^+$CD4$^+$ ÷ %CD4$^+$) × 100.

Table 2. Expression of $V_\beta6$ and $V_\beta8$ in mice neonatally treated with H-2$^{d/q}$ spleen cells

Neonatally treated mice		No. of mice	% positive cortisone-resistant thymocytes	
Recipient (all Mls-1^b)	Cells injected (Mls-1)		$V_\beta6$	$V_\beta8$
(A) *Mice treated with H-2-compatible spleen cells*				
BALB/c	BALB/c (b)	3	12.0 ± 0.6	21.1 ± 0.7
BALB/c	DBA/2 (a)	3	1.0 ± 0.3	18.0 ± 0.9
BALB/c	(BALB/c × DBA/2)F^1 (b/a)	3	1.7 ± 0.8	19.4 ± 1.1
BALB/c	(BALB/c × BALB.D2)F_1 (b/a)	3	0.8 ± 0.7	17.6 ± 1.1
(BALB/c × B10.G)F_1	(BALB/c × B10.G)F_1 (b/b)	2	9.8 ± 1.3	17.7 ± 1.3
(BALB/c × B10 G)F_1	(B10.D2 × DBA/1)F_1 (b/a)	3	2.6 ± 0.7	16.5 ± 0.9
(BALB/c × B10.G)F_1	(BALB/c × DBA/2)F_1 (b/a)	3	0.8 ± 0.8	17.8 ± 2.9
(B) *Mice treated with H-2-incompatible spleen cells*				
BALB/c	DBA/1 (a)	3	12.4 ± 0.3	19.3 ± 1.0
BALB/c	(DBA/2 × DBA/1)F_1 (a/a)	2	8.1 ± 1.1	18.8 ± 1.4
BALB/c	(BALB/c × DBA/1)F_1 (b/a)	3	9.7 ± 0.9	19.9 ± 0.1
(BALB/c × B10.G)F_1	(B10.BR × DBA/1)F_1 (b/a)	3	6.0 ± 1.2	16.8 ± 1.6

Mice were treated with 10^8 spleen cells i.p. within 24 h of birth and assayed after 2–6 weeks. Indirect immunofluorescence with mAbs 44-22-1 ($V_\beta6$-specific) or KJ16-133 ($V_\beta8$-specific) was performed. The values represent mean ± SEM of cortisone-resistant thymocytes (obtained 48 h after a single i.p. injection of 4 mg hydrocortisone acetate).

Table 3. Expression of $V_\beta 6$ and $V_\beta 8$ in mice neonatally treated with H-$2^{d/b}$ spleen cells

Neonatally treated mice		No. of mice	Tissue	% positive lymphocytes	
Recipient (all Mls-1^b)	Cells injected (Mls-1)			$V_\beta 6$/CD4	$V_\beta 8$/CD4
(A) *Mice treated with H-2-compatible spleen cells*					
(C57BL/6 × BALB/c)F_1	(C57BL/6 × BALB/c)F_1 (b/b)	3	thymus	8.7 ± 1 0	13.5 ± 0.8
			lymph node	12.0 ± 0 6	16.2 ± 0.1
(C57BL/6 × BALB/c)F_1	(C57BL/6 × DBA/2)F_1 (b/a)	5	thymus	0.8 ± 0.0	11.7 ± 1.4
			lymph node	0.9 ± 0.4	12.7 ± 0.5
BALB/c	DBA/2 (a)	3	thymus	0.8 ± 0.2	14.3 ± 2.0
			lymph node	0.9 ± 0.1	15.9 ± 1.3
(B) *Mice treated with H-2-incompatible spleen cells*					
C57BL/6	(C57BL/6 × BALB/c)F_1 (b/b)	3	thymus	7.9 ± 1.2	14.3 ± 1.7
			lymph node	8.4 ± 1.0	15.5 ± 1.4
C57BL/6	(C57BL/6 × DBA/2)F_1 (b/a)	3	thymus	6.5 ± 0.7	14.4 ± 1.0
			lymph node	8.2 ± 0.6	14.8 ± 1.6
BALB/c	(C57BL/6 × BALB/c)F_1 (b/b)	8	thymus	6.1 ± 0.7	13.1 ± 1.2
			lymph node	12.5 ± 0.8	17 8 ± 0 5
BALB/c	(C57BL/6 × DBA/2)F_1 (b/a)	11	thymus	4.7 ± 0.9	13.1 ± 0.5
			lymph node	8.9 ± 0 7	16.9 ± 0.5

Mice were treated with 10^8 spleen cells i.p. within 24 h of birth and assayed after 2 – 6 weeks. Thymocytes treated with CD8-specific rat IgM mAb 3.168.1 plus complement or untreated lymph node cells were stained with mAbs 44-22-1 ($v_\beta 6$-specific) or KJ16-133 ($V_\beta 8$-specific), respectively, plus a CD4-specific mAb. Data are mean ± SEM and represent percentages of the total $CD4^+$ population. Chimerism determined with MHC class I-specific mAbs in mice treated with H-2-incompatible cells was as follows: immunofluorescence on lymph node cells stained with donor MHC-specific mAb revealed mean values per group between 7.6 and 10.5% positive cells (SEM < 5.0), those stained with host MHC-specific mAb revealed means between 97 3 and 98.3% positive cells (SEM < 0 9).

Table 4. IL-2 response of lymphocytes from neonatally tolerant BALB/c mice

Neonatally treated mice		Stimulators	Response to:	IL-2 (U/ml)
Recipient	Cells injected (Mls-1)			
BALB/c	–	BALB.D2-Mls[a]	Mls-1^a	33.0 ± 3.3
		BALB/c	–	2.5 ± 0.7
		C57BL/6	H-2^b	32.5 ± 0.5
		B10.G	H-2^q	20.1 ± 1.1
DBA/2	–	BALB.D2-Mls[a]	–	1.8 ± 0 9
		BALB/c	–	3.2 ± 1.4
		C57BL/6	H-2^b	29.2 ± 1 2
		B10.G	H-2^q	32.8 ± 1.6
BALB/c	DBA/2 (a)	BALB.D2-Mls[a]	Mls-1^a	0.0
		BALB/c	–	0.0
		C57BL/6	H-2^b	20.5 ± 0.5
		B10.G	H-2^q	12.7 ± 0.8
BALB/c	(C57BL/6 × BALB/c)F_1 (b/b)	BALB D2-Mls[a]	Mls-1^a	20.3 ± 1.2
		BALB/c	–	1.7 ± 0.1
		C57BL/6	H-2^b	9.5 ± 1.1
		B10.G	H-2^q	15.3 ± 3.5
BALB/c	(C57BL/6 × DBA/2)F_1 (b/a)	BALB D2-Mls[a]	Mls-1^a	1.9 ± 0.7
		BALB/c	–	2.2 ± 0.5
		C57BL/6	H-2^b	10.8 ± 1.6
		B10.G	H-2^q	13.0 ± 2.3

Spleen cells (5×10^5) from 2- to 3-week-old neonatally treated or control mice respectively were stimulated with 5×10^5 irradiated T cell-depleted spleen cells in 96-well plates. Supernatants were harvested after 48 h and assayed for IL-2 content. Data are mean ± SEM of U IL-2/ml culture supernatant of three individual mice and of triplicate cultures.

molecules on $V_\beta6^+$ T cell was observed in lymphocytes from BALB/c mice neonatally treated with Mls-1^a F_1 cells when compared to those transfused with Mls-1^b F_1 (data not shown).

Lymphohemopoietic chimerism in mice treated with H-2-incompatible cells was between 6.9 and 17.2% donor H-2 class I-expressing lymph node cells when analyzed in 2- or 3-week-old mice and between 0.6 and 5.1% in 4- to 6-week-old mice (data not shown). Furthermore, the degree of chimerism appeared to be independent of Mls-1^a expression by the neonatally injected cells.

Finally, in control experiments Mls-1^a heterozygous (C57BL/6 × DBA/2)F_1 spleen cells were injected neonatally into H-2-syngeneic (C57BL/6 × BALB/c)F_1 hosts. As expected, this resulted in deletion of $V_\beta6^+$ T cells (Table 3A). In conclusion, Mls-1^b mice neonatally treated with Mls-1^a spleen cells efficiently deleted $V_\beta6^+$ T cells if H-2-compatible but not if H-2-incompatible spleen cells were used.

Non-deleted $V_\beta6^+$ T cells from BALB/c (Mls-1^b) mice neonatally treated with H-2-incompatible Mls-1^a spleen cells neither secreted IL-2 nor proliferated after Mls-1^a stimulation in vitro

Since BALB/c mice neonatally transfused with (C57BL/6 × DBA/2)F_1 Mls-1^a spleen cells did not delete the majority of their $V_\beta6^+$ lymphocytes, the question arose whether these lymphocytes would respond to an Mls-1^a stimulus *in vitro*. Analyses of lymphocytes from 2- to 3-week-old neonatally treated BALB/c mice revealed practically no proliferation (data not shown) and very low levels of IL-2 secretion after Mls-1^a stimulation *in vitro* (Table 4). In contrast, control untreated mice or mice neonatally treated with (C57BL/6 × BALB/c)F_1 Mls-1^b spleen cells responded strongly to BALB.D2-Mls-1^a stimulator cells. This latter response was Mls-1^a-specific since only background IL-2 levels were found upon stimulation with syngeneic BALB/c spleen cells. All the responder populations tested were responsive to third-party H-2-incompatible B10.G stimulator cells. As expected, lymphocytes from DBA/2 (Mls-1^a) mice or BALB/c mice neonatally given H-2-compatible Mls-1^a spleen cells (both lacking $V_\beta6^+$ cells due to clonal deletion) revealed only very low levels of IL-2 secretion after Mls-1^a stimulation *in vitro*. In summary, these data show that Mls-1^b mice neonatally treated with H-2-semi-allogeneic Mls-1^a F_1 spleen cells contained substantial numbers of $V_\beta6^+$ T cells, which were unresponsive to Mls-1^a stimulation *in vitro*. However, it has to be mentioned that this Mls-1^a-specific *in vitro* unresponsiveness was only observed in mice up to the age of 3 weeks; non-deleted $V_\beta6^+$ lymphocytes from neonatally tolerized mice 4 or more weeks old expressed only slightly reduced or even normal IL-2 and proliferative responses to Mls-1^a *in vitro* (data not shown).

Anergic $V_\beta6^+$ T cells responded to Mls-1^a in vitro by blastogenesis and expression of IL-2 receptors but did not proliferate unless IL-2 was added to the cultures

Lymphocytes from thymus or lymph nodes of BALB/c mice neonatally transfused with (C57BL/6 × DBA/2)F_1 or (C57BL/6 × BALB/c)F_1 spleen cells respectively were comparable in size as indicated by their mean values of forward light scatter (thymocytes: 23.0 ± 0.7 or 22.7 ± 1.0 respectively; lymph node cells: 25.9 ± 0.3 or 27.0 ± 0.4 respectively). These values were not significantly different from untreated or syngeneically treated control mice (data not shown), suggesting that no detectable *in vivo* blastogenesis of tolerogen-specific T cells occurred. However, after 3 day *in vitro* stimulation with irradiated T cell-depleted spleen cells, as described above (without addition of growth factors), many cells enlarged. In cultures stimulated with Mls-1^a cells this subset of large lymphocytes contained ~40% $V_\beta6^+$ cells (data not shown). Furthermore, up to 66% of lymphoblasts stained positive with the IL-2R-specific mAb PC61.51 (41) after *in vitro* Mls-1^a stimulation. Similar observations were made in lymphocyte populations containing responsive or anergic $V_\beta6^+$ T cells, indicating that blastogenesis and expression of IL-2R (but not IL-2 secretion or proliferation) of anergic $V_\beta6^+$ cells occurred after Mls-1^a stimulation *in vitro*.

Since functional anergy in some systems appears to reflect defective IL-2 production (11,13 – 16), we further analyzed $V_\beta6$ expression in cultures after addition of exogenous IL-2: responding T cell blasts from 3 day MLRs were re-incubated in IL-2-containing medium for an additional 2 days. As shown in Table 5, responder populations from BALB/c mice, whether untreated or neonatally treated with (C57BL/6 × DBA/2)F_1 or (C57BL/6 × BALB/c)F_1 spleen cells, respectively, revealed an increased proportion of $V_\beta6^+$/CD4$^+$ cells (to ~40%) following Mls-1^a stimulation. Since viable cell recoveries were ~10-fold greater in response to Mls-1^a than in syngeneic controls (Table 5), these data indicate that actual expansion (rather than preferential survival) occurred. In contrast, no significant expansion of the $V_\beta6^+$ subset was detected in CD4$^+$ lymphocytes from mice rendered tolerant to Mls-1^a by clonal deletion (i.e. DBA/2 mice or BALB/c mice neonatally treated with DBA/2 spleen cells). In conclusion, non-deleted $V_\beta6^+$ T cells from BALB/c mice neonatally treated with (C57BL/6 × DBA/2)F_1 Mls-1^a spleen cells generated blasts and expressed IL-2R after Mls-1^a stimulation *in vitro* but were defective in IL-2 production and therefore apparently did not proliferate unless exogenous IL-2 was added to the cultures.

In-vitro unresponsiveness of allospecific cytotoxic T cell precursors but not of allospecific IL-2-secreting cells from BALB/c mice neonatally treated with H-2^{bxd} spleen cells

In MLRs such as described above, the response to donor allogeneic (H-2^b) stimulators was investigated in parallel. The data in Table 4 show that lymphocytes from H-2^d mice neonatally treated with H-2^{bxd} spleen cells secreted significant amounts of IL-2 after H-2^b stimulation *in vitro*, although this response was lower than the response of controls (untreated or treated with H-2^d spleen cells). In contrast, spleen cells from BALB/c mice neonatally treated with H-2^{bxd} F_1 spleen cells did not give rise to H-2^b-specific cytotoxic T lymphocytes (CTLs) in standard mixed lymphocyte cultures, but generated CTLs specific for third-party H-2^q determinants (Table 6). Thus, neonatal injection of semi-allogeneic spleen cells expressing foreign H-2^d molecules rendered allospecific CTLs unresponsive but only partially reduced allospecific IL-2 secretion. In conclusion, tolerance induction to MHC determinants did not parallel Mls-1^a tolerance described above.

Discussion

Actively acquired tolerance to MHC-incompatible grafts can be achieved by neonatal injection of the relevant histoincompatible

Table 5. Mls-1^a-specific *in vitro* proliferation of $V_\beta6^+$ cells from neonatally tolerant mice after addition of exogenous IL-2

Neonatally treated mice		Stimulators	Response to:	Cells recovered	
Recipient	Cell injected (Mls-1)			Relative no. ($\times10^{-5}$/ml)	% $V_\beta6^+$/CD4$^+$
BALB/c	–	before culture			12 8 ± 0.7
		BALB.D2-Mls^a	(Mls-1^a)	15	40.8 ± 0.9
		(C57BL/6 × DBA/2)F$_1$	(Mls-1^a/H-2^b)	ND	27.6 ± 4.0
		BALB/c		2.3	7.2 ± 3.9
DBA/2	–	before culture			<3
		BALB.D2-Mls^a	(Mls-1^a)	4.2	<3
		BALB/c		3.0	<3
BALB/c	DBA/2 (a)	before culture			<3
		BALB.D2-Mls^a	(Mls-1^a	2 8	<3
		(C57BL/6 × DBA/2)F$_1$	(Mls-1^a/H-2^b)	ND	<3
		BALB/c		2.5	<3
BALB/c	(C57BL/6 × BALB/c)F$_1$ (b/b)	before culture			12 3 ± 0.9
		BALB.D2-Mls^a	(Mls-1^a)	17 6	40 5 ± 5.2
		(C57BL/6 × DBA/2)F$_1$	(Mls-1^a/H-2^b)	ND	26.7 ± 5 6
		BALB/c		1 8	9 1 ± 0.2
BALB/c	(C57BL/6 × DBA/2)F$_1$ (b/a)	before culture			9.6 ± 0.7
		BALB.D2-Mls^a	(Mls-1^a	16.1	37.2 ± 1 6
		(C57BL/6 × DBA/2)F$_1$	(Mls-1^a/H-2^b)	ND	31.4 ± 3 1
		BALB/c		1.5	4.4 ± 2 0

Spleen cells (3 × 10^6) from 2- to 3-week-old neonatally treated or control mice respectively were stimulated with 5 × 10^6 irradiated T cell-depleted spleen cells in 24-well plates. After 3 days, cells were harvested, washed, and re-cultured for an additional 48 h in IL-2-containing medium. Recovered cells were counted and double-stained with $V_\beta6$ and CD4-specific mAbs Measurements are given as percentages of the total CD4$^+$ cells. The cultures stimulated with BALB.D2-Mls^a contained <3% H-2^{b+} cells (mAb K7-309) and between 45 and 55% IL-2R$^+$ cells (mAb PC61.51) with the exception of the cultures with responder lymphocytes from untreated DBA/2 mice or BALB/c mice treated with DBA/2 cells where <17% cells were IL-2R$^+$ Data are mean (±SEM) of three mice per group. SEM of numbers of recovered cells ranged between 0.8 and 2 8. ND, not done

Table 6. Allo-H-2-specific cytotoxic T cell response of neonatally tolerant BALB/c mice

Neonatally treated mice		% specific lysis of target cells (E:T ratio 25/8/2.5/0.8)		
Recipient	Cells injected	B10.D2 (H-2^d)	MC57G (H-2^b)	DBA/1 (H-2^q)
BALB/c	–	0/1/0/0	78/56/29/7	62/42/23/9
DBA/2	–	22/17/5/2	91/91/58/23	73/57/32/18
BALB/c	(C57BL/6 × BALB/c)F$_1$	0/0/0/0	0/0/0/0	81/72/56/19
BALB/c	(C57BL/6 × BALB/c)F$_1$	0/0/0/9	6/0/0/0	83/69/62/18
BALB/c	(C57BL/6 × DBA/2)F$_1$	22/7/2/0	0/0/0/0	84/62/34/14
BALB/c	(C57BL/6 × DBA/2)F$_1$	2/5/8/2	0/0/0/0	83/52/34/17

Representative data for two experiments with effector spleen cells from six individual neonatally treated or control mice are shown. Spleen cells (3 × 10^6) were stimulated in 24-well plates with 5 × 10^6 irradiated (2000 rad) spleen cells of BALB/c (H-2^d), C57BL/6 (H-2^b), or DBA/1 (H-2^q) mice respectively, according to the H-2 haplotype of the target fibroblasts. After 5 days, cells were harvested and tested in a ^{51}Cr-release assay Test duration was 4.5 h; spontaneous ^{51}Cr-release of target cells was <25%. E:T ratio, effector:target ratio.

lymphoid cells (27). More recently, the same protocol has been used to induce specific functional tolerance to Mls-1^a determinants (42,43). With the realization that T cell reactivity to Mls-1^a correlates with usage of particular V_β segments (3,4), it has now become possible to investigate directly whether neonatal tolerance to Mls-1^a is obligatorily associated with clonal deletion of the relevant Mls-1^a-specific T cells. We show here that neonatal tolerance to Mls-1^a may be accomplished by either clonal deletion or clonal unresponsiveness, depending on the MHC molecules co-expressed on the injected cells. Thus, neonatal injection of MHC-compatible Mls-1^a-bearing cells results in virtually complete clonal deletion, in agreement with earlier reports (28,44). In contrast, inoculation of MHC-incompatible cells expressing Mls-1^a does not lead to efficient clonal deletion but, rather, causes a transient non-responsiveness of T cells to Mls-1^a determinants *in vitro*.

In this latter case, where H-2 semi-allogeneic Mls-1^a spleen cells were injected in neonatal mice, it could be argued that only $V_\beta6^+$ T cells with low affinity/avidity for Mls-1^a have survived. However, because Mls-1^a-specific blastogenesis and IL-2R

expression were inducible in the otherwise unresponsive $V_\beta6^+$ lymphocytes, it appears that their TCR affinity was sufficient for Mls-1^a-specific interactions. Therefore it seems likely that the neonatal injection of H-2 semi-allogeneic Mls-1^a spleen cells induced a state of clonal anergy in host $V_\beta6^+$ cells.

Previous studies indicated that antigen-specific clonal paralysis may be induced *in vivo* in adult mice by injection of chemically fixed accessory cells (10) or MHC class II-bearing L cell transfectants (45). Anergy of $V_\beta6^+$ T cells to Mls-1^a was subsequently demonstrated directly by Qin *et al.* (12), who showed specific *in vitro* unresponsiveness of $V_\beta6^+$ lymphocytes from adult mice after *in vivo* treatment with T cell-specific mAbs plus Mls-1^a-bearing hemopoietic cells. Similarly, Rammensee *et al.* (13) described *in vitro* anergic $V_\beta6^+$ cells from adult Mls-1^b mice after *in vivo* immunization with Mls-1^a spleen cells. Furthermore, *in vitro* unresponsive $V_\beta6^+$ T cells were observed in irradiated Mls-1^a mice reconstituted with certain ($I-E^-$) bone marrow stem cells (14 – 16). Mechanisms of T cell unresponsiveness in these models may be similar to the neonatally treated mice described in this study. The generation of antigen-specific blasts expressing IL-2R in the absence of IL-2 secretion and proliferation are common features of these systems (11,13 – 16) However, the cellular and molecular interactions responsible for this anergic state remain to be elucidated (11).

The limited data on MHC tolerance obtained in this study do not allow detailed conclusions except that there was no direct correlation with Mls-1^a tolerance. The differential induction of allospecific functional tolerance in CTLs (presumably MHC class I-specific) but not in IL-2 producers (presumably MHC class II-specific) observed in mice treated neonatally with semi-allogeneic spleen cells is consistent with some examples of split tolerance (43,46), whereas other studies revealed successful neonatal tolerance induction to both allogeneic MHC class I and II determinants (47,48). In any event, interpretation of split tolerance is difficult since allogeneic MHC responses do not correlate with usage of particular TCR V_β segments and hence lack of responsiveness (as observed for MHC class I) may result from clonal deletion, clonal anergy, or other unspecified mechanisms.

It has been suggested that neonatal tolerance depends upon persistence of antigen *in vivo* (49,50). Our evidence that peripheral lymphocytes of neonatally treated mice contained ~10% donor MHC class I-expressing cells when analyzed after 2 weeks and still ~3% of such cells when tested at the age of 5 weeks may indicate long-lasting persistence of neonatally administered MHC class I-expressing cells. Unfortunately, persistence of donor Mls-1^a expressing cells is more difficult to monitor *in vivo* and it is possible that such cells may have been rejected more rapidly, thus causing the observed transient tolerance to Mls-1^a.

In conclusion, the present results emphasize the complexity inherent in establishing neonatal T cell tolerance to foreign Mls-1^a and MHC determinants. Nevertheless, the availabiltiy of a model system in which several distinct tolerogenic mechanisms (i.e. clonal deletion and clonal anergy) operate for a single antigen (Mls-1^a) should facilitate further experimentation.

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Abbreviations

APC	antigen-presenting cell
CTL	cytotoxic T lymphocyte
H-2	mouse MHC
rIL-2	recombinant interleukin 2
IL-2R	interleukin 2 receptor
MLR	mixed lymphocyte reaction
Mls-1	minor lymphocyte-stimulating locus 1

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