INDUCTION OF NEONATAL TOLERANCE TO H-2^k IN B6 MICE DOES NOT ALLOW THE EMERGENCE OF T CELLS SPECIFIC FOR H-2^k PLUS VACCINIA VIRUS*

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Most reports have indicated that T cells are restricted to self major histocompatibility complex (MHC)[1] determinants by radiation-resistant thymic epithelium (reviewed in 1 and 2), or by intrathymic bone marrow-derived cells (3–5). However, others (6–8) have presented evidence that this self-restriction is, at best, a relative phenomenon largely induced by peripheral priming. A major problem in the experimental analysis of this issue is the strong allo-response elicited by cross-haplotype stimulation. Three general methods have been used to remove alloreactive precursors prior to stimulation with non-MHC antigens in an H-2-different environment: (*a*) acute depletion of alloreactive cells by negative selection in vivo through allogeneic irradiated hosts, adsorption in vitro on allogeneic monolayers, or "suicide" of proliferating cells that have been stimulated with alloantigen; (*b*) construction of radiation and embryo-fusion chimeras; and (*c*) neonatal tolerance induction. The latter two approaches yield chronically tolerized cell populations.

Acute depletion protocols have given conflicting results in the trinitrophenyl (TNP) system (9–12). Using a virus model, Bennink and Doherty (13, 14) showed that potent H-2K^k-restricted, vaccinia-specific cytotoxic T lymphocytes (CTL) could be generated by H-2^d or H-2^b T cells that had been filtered through appropriate semiallogenic recombinant or F_1 hosts and then stimulated with virus in the presence of H-2K^k. However, the reciprocal negative selections did not result in comparable "aberrant" recognition; H-2^k T cells could not be stimulated with H-2^b-vaccinia virus (15). Looking at the response to sheep erythrocytes, Sprent and von Boehmer (16) found no collaboration with allogeneic B cells by T cells that had been depleted of alloreactivity by negative selection in vivo. The situation for (k × b)F_1 →b chimeras is also complex (17, 18). Thymocytes from such animals show fairly precise restriction to H-2^b in their vaccinia-specific CTL response after stimulation in irradiated (k × b)F_1 mice. However, with time, splenocytes from some of these chimeras show a marked increase in frequency of H-2^k-restricted anti-vaccinia CTL precursors (CTL-P), emerging between 2 and 3 mo after reconstitution with bone marrow. In a series of experiments with neonatally tolerized mice, Forman et al. (19, 20) showed that

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[1] *Abbreviations used in this paper:* CTL, cytotoxic T lymphocyte; CTL-P, cytotoxic T lymphocyte precursors; E/T, effector target; FCS, fetal calf serum; i.v., intravenously; MHC, major histocompatibility complex; MR, maximum release; N, uninfected; PBS, phosphate-buffered saline; SR, spontaneous release; TNP, trinitrophenyl; VACC, virus-infected.

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TNP and minor histocompatibility antigens could be recognized in the context of the tolerated H-2 haplotype. Conversely, Waldmann et al. (21) and Zinkernagel et al. (22) were unable to demonstrate, respectively, allorestricted T-B help or anti-vaccinia CTL responses in mice made tolerant as neonates.

We have now analyzed the capacity of H-2^b mice neonatally tolerized to H-$2K^k$ to make an H-$2K^k$-restricted vaccinia response. These experiments are similar in design to those of Zinkernagel et al. (22), the differences being that we used strain combinations which have been shown to be associated with aberrant recognition by adult negatively selected T cells, and have tested both thymus and spleen at much earlier time points than were previously examined.

Materials and Methods

Mice. The C3H/He (H-2^k) mice were obtained from the Institute for Cancer Research, Fox Chase, Philadelphia, PA. Adult male and female C57BL/6J (B6, H-2^b), (B6 × CBA)F_1 (H-2^b × H-2^k), B10.A(2R) (H-$2^{k/b}$), and (BALB/c × B6)F_1 [(CB6)F_1, (H-2^d × H-2^b)] mice were purchsed from The Jackson Laboratory, Bar Harbor, ME. The adult (B6 × C3H)F_1 [(B6C3)F_1, H-2^b × H-2^k)] mice were bred at The Wistar Institute or obtained from pregnant mothers purchased from Lab Supply Co., Indianapolis, IN.

Neonatal Tolerance Induction. Newborn mice (≤24 h) were injected intravenously (i.v.) via the orbital branch of the anterior facial vein with 2–4 × 10^7 allogeneic or semiallogeneic cells suspended in 0.1 ml of Puck's medium or phosphate-buffered saline (PBS).

Alloreactive CTL. A standard protocol was used (23). The culture medium was RPMI 1640 (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY), supplemented with 10% fetal calf serum (FCS), 2 mercaptoethanol (5 × 10^{-5}), penicillin (100 U/ml), streptomycin (100 mg/ml), gentamycin (0.1 mg/ml), minimal essential medium, nonessential amino acids, sodium pyruvate, and essential amino acids. Basically, 1.0–1.5 × 10^7 spleen cells were mixed with 2.0 × 10^7 irradiated (1,100 rad from a Cesium source) semiallogeneic or allogeneic stimulator cells, using 10.0 ml of medium in 25-cm^2 plastic tissue culture flasks. These cultures were incubated for 5–6 d at 37°C in 8% CO_2, and lymphocytes were then asayed for 4–6 h at 37°C on ^{51}Cr-labeled, uninfected L cells (H-2^k), MC57G (H-2^b), and KD2SV (H-2^d) or P815 (H-2^d) targets. The effector/target (E/T) ratio used was calculated from the number of cells present in the flask at the end of the 5- to 6-d culture period. At the end of incubation, a 100-μl aliquot of supernatant was removed for counting in a Beckman Gamma 400 spectrometer (Beckman Instruments, Inc., Fullerton, CA). Groups were assayed in quadruplicate, and results are expressed as mean percent specific ^{51}Cr-release relative to spontaneous release (SR) in media alone, or maximum release (MR) in cetramide saline alone, calculated as: ([percent experimental release − SR]/[MR − SR]) × 100. SR never exceeded 25% of MR, and standard errors of mean percent specific ^{51}Cr-release were always <5%.

Thymus cells used as responders were mixed at 2–3 × 10^7 cells with 2 × 10^7 irradiated stimulators and, in certain experiments, 1 × 10^7 irradiated (1,100 rad) spleen cells syngeneic with responder thymus and/or interleukin 2 (Collaborative Research Inc., Waltham, MA) at 5 half-U/ml were added on day 0 of culture.

Generation and Assay of Virus-specific CTL. Single-cell suspension of thymus and spleen (at least 2 × 10^7) were injected i.v. into mice lethally irradiated (850 rad) 24 h previously (24). The recipients were then inoculated i.v. with 5 × 10^6 plaque-forming units of vaccinia virus after 3 h, and spleen cells from these mice were assayed for CTL activity 6 d later. The virus-infected (VACC) and uninfected (N) target cells used were: L cells (H-$2K^kD^k$), MC57G (H-$2K^bD^b$), and KD2SV (H-$2K^dD^d$). The assays were incubated for 7–8 h at 37°C, and results are expressed as specific ^{51}Cr release.

Reagents. Monoclonal anti-Thy-1.2 (Jlj) IgM antibody was made as previously reported (25) and used at a 1:40 dilution of ascites fluid in a one-step protocol with 1:25 dilution of guinea pig complement (Flow Laboratories, Inc., Rockville, MD) in 7% CO_2 at 37°C.

Anti-H-2^k antiserum was raised in (CB6)F_1 mice primed with C3H spleen cells and used at 1:10 dilution in a two-step protocol: 5 × 10^7 cells/ml were mixed with antibody on ice for 30

min, washed, and incubated for 30 min at 5×10^7 cells/ml with guinea pig complement (1:5 dilution) plus rabbit complement (1:60). Treatments were carried out in RPMI 1640 plus 10% FCS, and cells were washed twice with RPMI before culture or with PBS bfore i.v. injection.

Results

Normal B6 spleen and thymus cells recovered 6 d after transfer into irradiated, infected (k × b)F_1 hosts often do not kill normal H-2^k targets, even though they are clearly capable of generating an anti-H-2^k CTL response when simultaneously stimulated in vitro (data not shown). Thus, it is possible that an ongoing allo-response could suppress a virus-specific T cell response restricted to that foreign haplotype in vivo, even if the anti-allo CTL were undetectable in the spleen at time of assay. Therefore, only neonatally tolerized animals whose cells did not generate an anti-H-2^k CTL response in vitro were considered tolerant. We have shown elsewhere that this tolerance is due to a central deletion of alloreactive CTL-P rather than positive suppression or absence of help.[2]

Typical data for in vitro CTL allo-responsiveness are presented in Table I. The same animals were used for the experiment presented in Table II, which shows that spleen cells, but not thymocytes, from 24-d-old B6 mice neonatally tolerized with untreated (B6C3)F_1 spleen cells made a significant response to H-2^k + vaccinia when transferred into irradiated infected C3H hosts. However, when spleen cells from these mice were pretreated with anti-H-2^k + complement(C′) before transfer, they no longer generated H-2K-restricted virus-specific CTL, whereas the response to H-2^b + vaccinia was retained. The cells that responded to H-2^k + virus must, therefore, have originated from the inoculum used to induce tolerance. Maintenance of neonatally induced tolerance requires low level chimerism (26), generally estimated to be <10% (27, 28). Nossal and Pike (29) used fluoresceinated inocula to demonstrate 0.1% donor cells in thymus and 4% in spleen 24 h after injection. Our own estimates for chimerism in spleens at various ages range from 0 to 20% using pooled antisera in a standard two-step protocol.[2]

Hence, to avoid the possibility that even a small number of (k × b)F_1 T cells may be sufficiently expanded in the adoptive host to give a detectable virus-specific H-2^k-restricted response, a second approach used anti-Thy-1-treated F_1 cells as the tolerizing inoculum. The kinetics of vaccinia-specific T lymphocyte reconstitution of lethally irradiated mice by cells of donor origin has been reported by us previously (18): such CTL-P do not appear in thymus until 4 wk after reconstitution with anti-Thy-1 + C′-depleted bone marrow. Spleen cells from B6 mice tolerized at birth by i.v. inoculation of anti-Thy-1 + C′ (k × b)F_1 cells 12, 16, 25, and 35 d previously generate an H-2^b-restricted vaccinia-immune CTL when transferred to syngeneic or (k × b)F_1 hosts, but do not make an allorestricted response when primed in F_1 or C3H (H-2^k) hosts (Table III). By day 31 post-injection of these anti-Thy + C′-treated F_1 cells, a 1,000-rad irradiated B6 host begins to show a T cell response to vaccinia (group G, Table III), but cells from neonatally tolerized 25- or 35-d-old mice still do not show allorestricted killing 6 d after transfer to infected hosts (groups F, H, and I, Table III).

In an effort to uncover possible synergistic interactions between spleen and thymus cells of neonatally tolerized mice, we injected mixtures of splenocytes and thymocytes

[2] Schwartz, D. H., and P. C. Doherty. Mechanisms of tolerance in mice exposed to alloantigens as neonates. Manuscript submitted for publication.

TABLE I

In Vitro Generation of Alloreactive CTL for Spleen Cells from Normal and Neonatally Tolerized Animals

1.5×10^7 responder cells*	Neonatally tolerized with:	1,100-rad stimulators	Percent specific lysis (E/T = 30:1)‡	
			L ($H\text{-}2^k$)	KD2 ($H\text{-}2^d$)
Normal day-18 B6 spleen	—	$(B6C3)F_1$ [b × k]	70	15
		$(CB6)F_1$ [d × b]	19	61
Day-24 B6 spleen	3×10^7 $(B6C3)F_1$ spleen	$(B6C3)F_1$	0	3
		$(CB6)F_1$	0	59
		$(B6C3)F_1 + (CB6)F_1$	4	55

* Pooled cells from three animals were stimulated in vitro for 6 d.

‡ The specific lysis on syngeneic MC57 targets was ≤4% for all groups. The E/T ratio is calculated for numbers of cells present at the end of the 6-d culture period.

TABLE II

Virus-immune CTL Response Patterns for Neonatally Tolerized H-2^b Spleen Cells

2×10^7 donor cells from 24-d-old B6 mice neonatally tolerized with 3×10^7 $(B6C3)F_1$ spleen cells*	850-rad infected recipient	E/T	L ($H\text{-}2^k$)				MC57G ($H\text{-}2^b$)				KD2 ($H\text{-}2^d$)	
			VACC		N		VACC		N		VACC	
			30:1	15:1	30:1	15:1	30:1	15:1	30:1	15:1	30:1	15:1
Spleen	B6		2	1	1	0	20	11	3	0	−1	2
	C3H		21	13	1	1	2	2	2	1	6	3
Thymus	B6		4	2	0	2	42	29	3	4	1	1
	C3H		—‡	6	—	6	—	9	—	7	—	6
Spleen, anti-$H\text{-}2^k$ + C′ treated	$(B6C3)F_1$		5	2	4	1	22	12	2	5	3	0
	C3H		7	4	2	2	5	3	3	2	2	3
Thymus, anti-$H\text{-}2^k$ + C′ treated	B6		2	—	−1	—	25	—	−2	—	−3	—
	C3H		4	—	4	—	7	—	3	—	4	—

* Lymphocytes from neonatally tolerized mice were adoptively transferred into irradiated recipients, with or without treatment with anti-$H\text{-}2K^k$ + C′, and stimulated with vaccinia virus for 6 d.

‡ Not done.

from tolerized animals (as judged by absence of alloreactive cell-mediated lympholysis in vitro) into irradiated $(B6C3)F_1$ hosts. Again, we found significant virus-specific killing in the context of the syngeneic, but not the tolerated, H-2 haplotype (Table IV).

The $H\text{-}2D^k$ allele is invariably associated with CTL nonresponsiveness to vaccinia virus (1). Thus any "aberrant" recognition by B6 T cells stimulated in a k- or (k × b)F_1-stimulating environment would presumably be restricted to K^k, just as in the acute negative selection experiments where K^kD^b recombinants were used as irradiated filter and stimulator hosts (14, 17). However, in light of the explanation originally put forth for the aberrant recognition phenomenon (14), namely that $H\text{-}2K^k$ + vaccinia "looked like" $H\text{-}2^b$ + X, it seemed possible that tolerance to $H\text{-}2D^k$ alloantigens and/or C3H minor histocompatibility antigens could eliminate a significant portion of the $H\text{-}2K^k$ + vaccinia reactive clones. We therefore considered it important to use a

TABLE III

Adoptive Transfer of Cells from H-2^b Mice Tolerized with Anti-Thy-1-Treated or Untreated H-2$^{k \times b}$ F_1 Cells

Experiment	Donor population	850-rad irradiated host	Group	E/T ratio	Percent specific lysis: L (H-2^k) VACC	L (H-2^k) N	MC57G (H-2^b) VACC	MC57G (H-2^b) N
1	Tolerized* day 12 B6							
	2×10^7 spleen	B6C3 F_1	A	30:1	3	0	18	1
	2×10^7 thymus	B6C3 F_1	B	30:1	8	4	21	4
		B6	C	30:1	0	1	30	0
		C3H	D	30:1	7	1	5	2
	Spleen cells from 1,000-rad irradiated B6 mice reconstituted with 3×10^7 Thy-1-depleted (B6C3)F_1 spleen cells used as tolerizing inoculum, infected 12 d later and assayed after another 6 d		E	100:1	1	2	0	−2
2	Tolerized* day 25 B6							
	2×10^7 spleen	B6C3 F_1	F	100:1	2	1	16	0
	Spleen cells from 1000-rad irradiated B6 mice reconstituted with 3×10^7 Thy-1-depleted (B6C3)F_1 spleen cells used as tolerizing inoculum, infected 25 d later and assayed after another 6 d		G	100:1	5	1	15	5
	Tolerized‡ day 35 B6							
	2×10^7 spleen	B6C3 F_1	H	100:1	5	0	21	0
	2×10^7 thymus	B6C3 F_1	I	25:1	9	0	33	1
	Normal (B6C3)F_1 6 d post-infection		J	25:1	41	6	48	0
3	Neonatally tolerized§ day 16 B6							
	2×10^7 spleen	(CBA × B6)F_1	K	30:1	0	0	17	2
	2×10^7 thymus	(CBA × B6)F_1	L	15:1	0	0	19	1
	Normal day 16 (B6C3)F_1							
	2×10^7 spleen	(CBA × B6)F_1	M	30:1	24	0	25	8
	2×10^7 thymus	(CBA × B6)F_1	N	15:1	21	0	21	1

* Newborn mice (<24 h) were injected with 3×10^7 anti-Thy-1 + C′-treated (B6C3)F_1 spleen cells.

‡ Newborn mice (<24 h) were injected with 3×10^7 untreated (B6C3)F_1 spleen cells.

§ Newborn mice (<24 h) were injected with 2.5×10^7 untreated (CBA × B6)F_1 spleen cells.

(K^kD^b) recombinant strain to rigorously compare neonatally and acutely tolerized repertoires with respect to aberrant recognition. Table V shows that spleen cells from 4-mo-old B6 mice tolerized to B10.A(2R) do not exhibit vaccinia-specific activity restricted to K^k when stimulated in a B10.A(2R) environment (groups B and D) although they respond well in syngeneic hosts (C and E). Next we used completely allogeneic anti-Thy-1 + C-treated C3H spleen cells to tolerize B6 neonates. The spleens of these animals were then tested 7 wk later in vitro for CTL allo-response, and stimulated with virus in irradiated C3H hosts as well. Again, the B6 cells were specifically tolerant to H-2^k, but no H-2K^k-restricted vaccinia-specific CTL activity was observed (Table V, experiment 2). These results, along with those presented in Table II, formally rule out objections that the use of F_1 or k/b recombinant hosts might favor a syngeneic H-2^b-restricted response.

The possibility of positive suppression operating on the H-2^k-restricted vaccinia response in the virus-infected H-2$^{k \times b}$ environment was considered. We looked for suppression after injection of 2×10^7 spleen or thymus cells from neonatally tolerized B6 animals along with equal numbers of normal (CBA × B6)F_1 spleen or thymus cells, into (k × b)F_1 hosts. As shown in Table VI, thymus cells from neonatally

TABLE IV

Cotransfer of Tolerized Spleen and Thymus from Tolerized Mice

Donor population	850-rad irradiated recipient	E/T ratio	Percent specific lysis			
			L cells		MC57G	
			VACC	N	VACC	N
Day 14 neonatally tolerized B6*						
1.5×10^7 spleen + 1.5×10^7 thymus	$(B6C3)F_1$					
Mouse 1		100:1	1	−2	22	−1
Mouse 2		100:1	7	1	17	3
Mouse 3		100:1	−3	1	24	−2
Contols						
Day 14 normal $(B6C3)F_1$						
2×10^7 spleen	$(B6C3)F_1$	100:1	19	0	12	3
	B6	100:1	9	4	26	7
2×10^7 thymus	$(B6C3)F_1$	20:1	20	1	—	—
Day 14 normal B6						
1.5×10^7 spleen + 1.5×10^7 thymus	B6	100:1	—	—	34	2

* Newborn (<24 h) B6 mice were injected i.v. with 3×10^7 anti-Thy-1 + C′-treated $(B6C3)F_1$ cells.

TABLE V

Absence of K^k + Vaccinia Response by B6 Cells Tolerant of Recombinant or Fully Allogeneic H-2

Experiment	Donor spleen (2×10^7 cells)	Group	850-rad irradiated infected host	Percent specific lysis*			
				L (K^kD^k)		MC57G (K^bD^b)	
				VACC	N	VACC	N
1	4-mo-old normal B10.A(2R)	A	B10.A(2R)	21	3	11	10
	4-mo-old neonatally tolerized B6‡	B	B10.A(2R)	8	6	18	9
	pooled cells (three mice)	C	B6	5	2	31	9
	Single mouse	D	B10.A(2R)	7	5	13	13
		E	B6	4	7	34	18
2	7-wk-old normal C3H	F	C3H	23	−5	−6	−4
	7-wk-old neonatally tolerized B6§	G	C3H	2	−1	−1	0
		H	B6	−1	−3	24	0

* E/T = 100:1 and 30:1 in experiments 1 and 2, respectively.
‡ Newborn (<24 h) mice were injected i.v. with 3×10^7 anti-Thy-1 + C′-treated B10.A(2R) spleen cells.
§ Newborn (<24 h) mice were injected i.v. with 3×10^7 anti-Thy-1 + C′-treated C3H spleen cells.

tolerized B6 mice have no suppressive effect on normal, transferred $(CBA \times B6)F_1$ thymus (compare groups M and N), whereas neonatally tolerized B6 splenocytes have an enhancing effect on cotransferred $(CBA \times B6)F_1$ spleen (compare group G with H–J).

Discussion

The capacity of allotolerant CTL populations from $H\text{-}2^b$ mice to respond to vaccinia-infected $H\text{-}2^k$ cells varies among different experimental protocols. At one end

TABLE VI

Stimulation of Normal (CBA × B6)F_1 T cells in the Presence of B6 Spleen and Thymus Cells Tolerant of H-2^k

Transferred donor cells				Percent specific lysis*					
						L cells		MC57G	
Organ source	Mouse	2×10^7 neonatally tolerized B6‡	2×10^7 normal adult (CBA × B6)F_1	850-rad host	Group	VACC	N	VACC	N
Spleen	1	+	−	B6	A	−6	2	19	1
	2	+	−	B6	B	−4	2	18	2
	3	+	−	B6	C	−1	3	20	3
	1	+	−	(CBA × B6)F_1	D	1	6	13	6
	2	+	−	(CBA × B6)F_1	E	3	4	9	6
	3	+	−	(CBA × B6)F_1	F	1	9	21	9
	4	−	+	(CBA × B6)F_1	G	40	3	29	3
	1 + 4	+	+	(CBA × B6)F_1	H	56	3	67	11
	2 + 4	+	+	(CBA × B6)F_1	I	75	5	74	9
	3 + 4	+	+	(CBA × B6)F_1	J	78	8	77	15
Thymus									
	1	+	−	B6	K	−1	ND§	25	2
	1	+	−	(CBA × B6)F_1	L	1	4	8	4
	4	−	+	(CBA × B6)F_1	M	75	12	78	11
	1 + 4	+	+	(CBA × B6)F_1	N	85	11	73	12

* E/T ratio was 30:1 for groups A–J, 15:1 for groups K–N. Assays performed 6 d after injection of adoptive host with cells and virus.

‡ Donor mice 1–3 were neonatally tolerized at birth (<24 h) by i.v. injection of 2.0×10^7 anti-Thy-1 + C′-treated (CBA × B6)F_1 spleen cells, and killed at 3 wk.

§ Not done.

of the spectrum, it seems that acutely depleted negatively selected adult H-2^b cells make at least as strong a response to H-2K^k + vaccinia as to H-2^b + vaccinia (14, 17). At the other, H-2^b animals made tolerant at birth to H-2^k are unable to generate CTL-specific for H-2^k + vaccinia. The intermediate situation is found with (k × b) →b chimeras, where H-2^b-restriction of thymocytes seems to hold, but spleen cells of older (several months) animals exhibit variable responsiveness to vaccinia in the context of H-2^k (18). Analysis of the similarities and differences among these protocols may reveal clues to explain the apparent discrepancies.

We have demonstrated elsewhere that our neonatally tolerized B6 animals are functionally depleted of anti-H-2^k CTL-P and are not actively suppressed in vitro.[2] Their hematopoietic systems contain 80–99% self (H-2^b) cells, which have differentiated in the context of self (H-2^b) radiation-resistant thymic epithelium and a self (H-2^b) -dominated peripheral stimulating environment. The B6 T cells used for negative selection through irradiated H-2$^{k \times b}$ mice have also differentiated in the context of H-2^b, but only encounter H-2^k after post-thymic maturation. In contrast, lymphocytes from B6 mice tolerized as neonates are associated with at least some cells bearing H-2^k throughout ontogeny, and depletion of alloreactive cells is chronic and central (thymic or prethymic). The (k × b) →b chimera shares this feature of chronic central allotolerance but the lymphocytes and hematopoietic presenting environment are, in the long-term, of the H-2$^{k \times b}$ phenotype. These points are summarized in Table VII,

TABLE VII
Ontogenetic History of Tolerant Cells

Method of inducing tolerance	T lymphocyte H-2 haplotype	Thymic epithelial H-2 haplotype	Peripheral stimulating H-2 haplotype	H-2 restriction in (k × b)F_1 irradiated vaccinia infected host	
				Thymus	Spleen
Negative selection: acute, peripheral, post-thymic	b	b	b	NT*	k + b
(k × b)→b chimera: chronic, central, preintrathymic, or intrathymic	k × b	b	k × b	b	k + b
Neonatal tolerance: chronic, central, preintrathymic, or intrathymic	b > > k	b	b > > k	b	b

* Not tested, as thymocytes do not recirculate through irradiated mice.

which shows that H-2^b thymic epithelium is the single common feature of all three experimental systems.

It is at least arguable that central elimination of high affinity anti-self MHC clones occurs before encounter with the thymic epithelium, whereas low affinity anti-self clones proliferate intrathymically before leaving and alloreactive clones with no crossreactivity for self (if such cells exist as a separate set) may simply pass through the thymus. We further suggest that the majority of anti-K^k + vaccinia CTL-P in a normal B6 animal are derived by somatic mutation and expansion from anti-H-$2K^k$ progenitor clones, whereas relatively few are derived from low-affinity anti-H-2^b (anti-"altered self") clones having no cross-affinity for H-2^k. Acute depletion (negative selection) of high-affinity anti-H-2^k CTL-P from the adult B6 repertoire does not remove all of the anti-H-2^k + vaccinia reactive clones, as the latter have only low affinity for H-2^k. Theoretically, reactivity for any allo-MHC + antigen X is permissible but not necessarily present or predictable in any T cell population that is operationally restricted to a particular MHC type.

In contrast, neonatally tolerized animals have had a central deletion of both the high-affinity anti-H-2^b clones, and the anti-H-2^k clones. The latter are therefore unavailable for somatic mutation and expansion in the thymus or periphery to give rise to anti-K^k + vaccinia CTL-P. Low numbers of anti-H-$2K^k$ + vaccinia clones in neonatally tolerized mice might be revealed by limiting dilution or in vitro restimulation but are not numerous enough to cause observable lysis in bulk assays of cells from in vivo primed mice.

The (k × b) → b chimera is similar to the neonatally tolerized animal in that, again, there has been a central deletion of high affinity anti-H-2^k and H-2^b clones. The clones potentially available for H-$2K^k$ + vaccinia recognition are low-affinity anti-H-2^b clones and perhaps, as discussed below, low-affinity anti-H-2^k clones. The thymi and spleens of young chimeras would thus contain few anti-H-$2K^k$ + vaccinia CTL-P. However, unlike the situation for normal or neonatally tolerized H-2^b animals, the presenting accessory cell population in the peripheral lymphoid tissue of the F_1 → P chimeras is H-$2^{k \times b}$ This could provide stimulation by H-2^k + X antigens, which cross-stimulate for K^k + vaccinia, and over time, the peripheral pool of aberrantly reactive CTL-P would increase. The prediction would be that the anti-K^k

+ vaccinia CTL from spleens of older (k × b) → b chimeras would express idiotypes different from those of anti-K^k + vaccinia CTL present in normal negatively selected spleen populations.

One of us has previously suggested (30) that low- as well as high-affinity anti-H-2^k clones would be eliminated intrathymically if they encountered H-2^k on nonepithelial elements, i.e., other thymocytes. This idea was put forward to account for the lack of aberrant recognition seen in (k × b) → b chimeras tested up to that time. Subsequent findings (31) of anti-H-2^k + vaccinia reactivity in adoptively transferred spleens of some older (k × b)→b chimeras is accommodated by postulating that these cross-reactive clones can be derived via somatic mutation from low-affinity anti-H-2^b progenitors. To account for the data from neonatally tolerized mice, we would have to argue that the very low level of H-2^k chimerism in these animals is sufficient to delete low affinity anti-H-2^k clones capable of responding to H-$2K^k$ + vaccinia. This places even more emphasis on the premise of extreme sensitivity on the part of these low affinity clones to intrathymic, but not pre- or post-thymic, deletion (an idea originally invoked to explain the ability of K^k + vaccinia restricted clones to escape negative selection in allogeneic hosts).

An alternative explanation for the absence of H-2^k-restricted vaccinia-specific CTL-P in neonatally tolerized mice is that high- rather than low-affinity anti-H-2^k progenitor clones comprise the major source of H-$2K^k$ + vaccinia reactive progeny (either by virtue of originally greater frequencies or selective somatic mutation and expansion). Elimination of these high-affinity clones by long-term contact with very low levels of H-2^k antigen would give the neonatally tolerized animal a nonresponder phenotype to H-$2K^k$ + vaccinia, even with persistence of low affinity anti-H-2^k clones.

Considerable experimental evidence from other laboratories is in accord with our results. Wagner et al. (6) found allorestricted anti-Sendai and anti-TNP CTL-P in mice of several strains when tested in vitro with limiting dilution assays after removal of alloreactive cells on monolayers. There was a numerical bias towards self-restricted vs. allorestricted CTL-P (3–10:1) present in both spleen and thymus cells of normal animals. These authors also present evidence that this central thymic bias for self-restriction is a function of syngeneic interactions between CTL-P and bone-marrow-derived elements in the thymus. The radiation-resistant thymic epithelium seems not to influence this self-preference, as completely allogeneic thymus-stem cell chimeras revealed the same skewing of restriction to stem-cell MHC. They argue persuasively for the idea, originally formulated by Finberg et al. (32) that Ly-$2,3^+$ CTL-P in the periphery are really derived from Ly-$1,2,3^+$ self-restricted precursors (33, 34). This idea is supported by the age-related increase in frequencies of alloreactive and Sendai-specific allorestricted CTL-P found in spleen. No such increase was observed in the thymus. Kruisbeek et al. (7) found that in allogeneic thymus-engrafted nude mice it is the thymic epithelium rather than host-derived elements which determines self-restriction of thymocytes. This would appear to conflict with the finding of Wagner et al. (6). However, spleen cells from thymus-grafted nudes were restricted to host as well as to thymus haplotype, an observation that is in agreement with the concept of peripherally imposed self-restriction.

Experiments recently reported by Mullbacher (35) showed that neonatally induced tolerance to H-2^b eliminated the ability of adult CBA (H-2^k) mice to make an H-2^k-restricted anti-H-Y response. This was true for 100% of neonatally tolerized animals, even though the anti-H-Y response is crossreactive with H-2^b in only 10–20% of

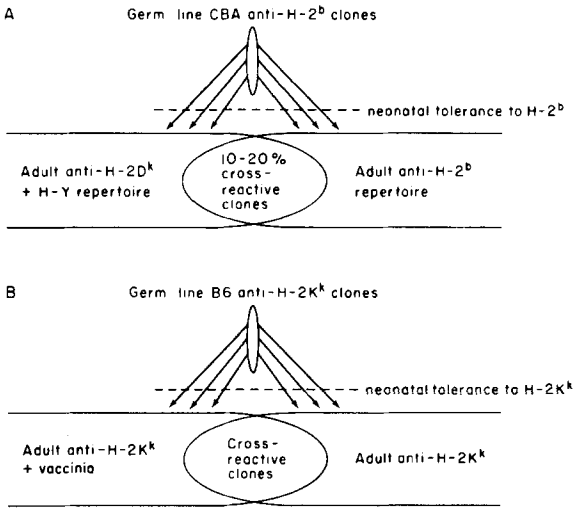

FIG. 1. Schematic model illustrating the generation of only partially overlapping adult repertoires from a common germline progenitor pool. Neonatal allotolerance induction (-----) eliminates non-cross-reactive as well as cross-reactive adult CTL-P. Model can apply to data of Mullbacher (36) (A) or the present experiments (B).

normal adult CBA mice. Mullbacher (36) favors a peripheral anti-idiotype suppression model which he has previously detailed. However, the analogies to our present findings are striking, and it seems likely that for H-2^k + H-Y (which is actually limited to H-$2D^k$ + H-Y) as for H-$2K^k$ + vaccinia, chronic central elimination of a progenitor (germ line?) repertoire eliminates potentially reactive daughter clones. Fig. 1 illustrates how our model could account for our data and Mullbacher's (35).

Summary

Thymocytes and spleen cells from C57BL/6 mice (H-2^b) neonatally tolerized to H-2^k alloantigens do not generate an anti-vaccinia response restricted to H-$2K^k$ when adoptively transferred to appropriate irradiated hosts. This is in sharp contrast to the case for negatively selected C57BL/6 spleen cells acutely depleted of alloreactivity. No evidence for suppression was found in cell mixture experiments.

We have shown elsewhere that our neonatally tolerized animals have a centrally induced deletion-type tolerance in the absence of obvious suppression.[2] We now suggest that in the neonatally tolerized mouse, chronic, central deletion of anti-H-2^k clones during early T cell ontogeny eliminates the major source of cells able to give rise, via somatic mutation and expansion, to anti-H-$2K^k$ + vaccinia specific cytotoxic T lymphocyte precursors (CTL-P) in the adult. A similar mechanism may operate in the (k × b) → b chimera; however, the presence of H-$2^{k \times b}$ accessory and presenting cells may permit the eventual generation (via cross-stimulation) of an H-2^k-restricted vaccinia-specific repertoire. This would account for our observation of such "aberrant recognition" CTL-P emerging in the spleens of older (k × b) → b chimeras.

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