

# NEONATAL TOLERANCE OF MAJOR HISTOCOMPATIBILITY COMPLEX ANTIGENS ALTERS Ir GENE CONTROL OF THE CYTOTOXIC T CELL RESPONSE TO VACCINIA VIRUS

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The strength of the immune response to certain antigens is controlled by immune response (Ir)<sup>1</sup> genes, the majority of which are linked to the major histocompatibility complex (MHC) (1). In the murine MHC, the *I* region of the *H-2* gene complex contains genes coding for class II MHC antigens that are recognized by T cells of the helper (Th) and delayed hypersensitivity-mediating (Td) classes; these genes are apparently the Ir genes that control immune responses dependent upon Th and Td cells (1, 2). Similarly, the *K* and *D* regions of *H-2* contain genes coding for class I antigens that are recognized by cytotoxic T cells (Tc) and act as Ir genes controlling Tc cell responses (3).

The mechanisms by which these *H-2*-coded Ir genes work have been a matter of intense speculation based on the knowledge that T cells responding to a foreign antigen must simultaneously recognize a self-MHC antigen (MHC restriction) (1-3). Three basic hypotheses have been proposed. First, a combination of a certain foreign antigen and a self-MHC antigen may not be able to associate on the surface membrane of an antigen-presenting cell in a manner required for precursor T cell stimulation (4, 5). Second, somatic generation of diversity in genes coding for T cell antigen-receptors may involve a selection process that precludes certain germline genes from undergoing variation, thus leaving gaps in the repertoire (6, 7). Third, a large repertoire of T cell antigen receptors may be generated, but those T cell clones expressing receptors that react with self-antigens are suppressed. Cross-reactivity of these suppressed clones with some foreign antigens would also result in gaps in the T cell response repertoire (8-10). The first two of these hypotheses can explain apparently simple Ir gene effects in which weak responses to a foreign antigen are associated with a particular MHC allele. However, when tested in a number of examples (10) these hypotheses have been shown to be invalid. Furthermore, they cannot cope with more complex effects.

Here we describe investigations into the murine Tc cell response to vaccinia virus in which alleles of the *K* region of the *H-2* complex influence the strength of the response associated with the *D<sup>b</sup>* allele (11, 12). The results exclude the first two hypotheses outlined above, but are consistent with the third.

<sup>1</sup> *Abbreviations used in this paper:* Ir, immune response; LCMV, lymphocytic choriomeningitis virus; MHC, major histocompatibility complex; MLC, mixed lymphocyte culture; PFU, plaque-forming units; Tc, cytotoxic T cells; Td, hypersensitivity-mediating T cells; Th, helper T cells.

## Materials and Methods

*Animals.* Mouse strains used were obtained from Olac Ltd., Oxfordshire, United Kingdom, or obtained from the breeding establishment of the John Curtin School. Only females older than 6 wk were used. The strains of mice, their *H-2* haplotypes, and the abbreviations used in this paper are given in Table I.

*Viruses.* WR vaccinia virus, ectromelia virus, and Armstrong strain of lymphocytic choriomeningitis virus (LCMV) were grown and titrated as described elsewhere (13, 14).

*Immunization.* Mice were immunized with either  $10^7$  plaque-forming units (PFU) of vaccinia virus,  $10^4$  PFU of ectromelia virus, or  $5 \times 10^5$  PFU LCMV, intravenously.

*Generation of Effector Cells.* Spleen cells from mice immunized 6 or 7 d previously were used for primary vaccinia-, ectromelia-, or LCMV-immune Tc cells, respectively (13, 14).

Cultures for the generation of secondary vaccinia-immune Tc cells have been described in detail elsewhere (15). Briefly,  $8 \times 10^7$  spleen cells from mice immunized with vaccinia at least 21 d previously were co-cultured for 5 d with  $1 \times 10^7$  vaccinia-infected stimulator spleen cells infected at a multiplicity of 1 PFU/cell. The generation of alloreactive Tc cells has been described in detail (16). In all experiments only spleen cells from individual mice were used.

*Target Cells.* Thioglycollate-induced peritoneal macrophages, BW cells (*H-2<sup>k</sup>*), L929 cells (*H-2<sup>b</sup>*), and EL-4 cells (*H-2<sup>b</sup>*), and methylcholanthrene-induced fibrosarcoma cell lines (12), MC57 (*H-2<sup>b</sup>*), 2R (*H-2K<sup>k</sup>,D<sup>b</sup>*), D2 (*H-2<sup>d</sup>*), 5R (*H-2K<sup>b</sup>,D<sup>d</sup>*), and BYR (*H-2K<sup>a</sup>,D<sup>b</sup>*) were infected with vaccinia, ectromelia, or LCMV virus and labeled with  $^{51}\text{Cr}$ , as described in detail elsewhere (12, 17).

*Preparation of Neonatally Tolerant Mice.* B10 mice were injected intraperitoneally with  $5 \times 10^7$  adult (B10  $\times$  4R)F1 or (B10  $\times$  BYR)F1 spleen cells not later than 24 h after birth. Spleen cells of all adult tolerant mice were tested for persisting F1 cells, using appropriate anti-*H-2* sera plus complement, before in vitro assays for antiviral Tc cells. They were found to be >95% of recipient phenotype.

*Preparation of Chimeras.* The method used has been described (18). Briefly, 950-rad-irradiated host mice were reconstituted with a total of  $2 \times 10^7$  fetal liver cells from 15–16-d embryos and used in experiments after at least 8 wk. In the case of (CBA  $\times$  B6)F<sub>1</sub> mice reconstituted with a 50:50 mixture of CBA and B6 fetal liver cells, the spleen cells were tested at the time of antiviral response assays with anti-*H-2* sera and complement. Results cited are from chimeras with <60:40 imbalance of CBA and B6 cells in their spleen cell populations.

*Cold Target Competition.* Macrophage target cells were labeled with  $^{51}\text{Cr}$ , infected with vaccinia virus as described above, and distributed at  $5 \times 10^4$  cells/well in 0.05-ml aliquots. Unlabeled macrophages were either left uninfected or were infected with 2 PFU/cell vaccinia virus ( $2 \times 10^6$  cells/ml) for 1 h. They were washed twice with complete medium and added in

TABLE I  
Strains of Mice Used

Strain	Abbreviation	K	A <sub>α</sub>	A <sub>β</sub>	E <sub>β</sub>	J	E <sub>α</sub>	D
C57BL/10	B10	b	b	b	b	b	b	b
C57BL/6	B6	b	b	b	b	b	b	b
B10.A(2R)	2R	k	k	k	k	k	k	b
B10.A(4R)	4R	k	k	k	k	b	b	b
B10.A(5R)	5R	b	b	b	b	k	k	d
HTG	HTG	d	d	d	d	d	d	b
B10.BYR	BYR	q	k	k	k	k	k	b
B10.BR	BR	k	k	k	k	k	k	k
BALB/c	B/c	d	d	d	d	d	d	d
D2.GD	D2.GD	d	d	d	d/b	b	b	b
CBA/H	CBA	k	k	k	k	k	k	k
A/J	A/J	k	k	k	k	k	k	d

0.1-ml aliquots/well at ratios of 1, 2, 4, and 8 times the number of labeled targets. Vaccinia-immune Tc cells were added in 0.1-ml aliquots/well. The assay was run for 6 h.

<sup>51</sup>Cr-release Cytotoxicity Assay. The methods used for the cell lines and macrophage targets have been described in detail elsewhere (12, 17). The duration of the assays was 6–12 h. The percent specific lysis was calculated using the formula: percent specific lysis = [(experimental release – medium release)/(maximum release – medium release)] × 100. Data given are the means of triplicates. Standard errors of the means were always <5% and are omitted for clarity. Significance was determined by Student's *t* test.

## Results

*Presence of K<sup>k</sup> Affects D<sup>b</sup>-restricted Anti-Vaccinia Responses.* Four strains of mice, each carrying the H-2D<sup>b</sup> allele but possessing different H-2K alleles [B10 (K<sup>b</sup>), HTG (K<sup>d</sup>), BYR (K<sup>q</sup>), and 4R (K<sup>k</sup>)] were tested on a panel of vaccinia-infected macrophage targets for their ability to generate primary D<sup>b</sup>-restricted vaccinia-immune Tc cells in vivo. Table II illustrates a typical example of such an experiment and confirms earlier reports (11, 12). B10, BYR, and HTG Tc cells lysed vaccinia-infected targets efficiently when H-2D<sup>b</sup> was shared by donors of Tc cells and targets. In contrast, 4R Tc cells caused very little lysis of vaccinia-infected targets such as B10, HTG, and BYR although they also share H-2D<sup>b</sup>. Nonetheless, 4R effectors strongly lysed 4R or BR vaccinia-infected targets, demonstrating that 4R animals are immunocompetent in respect to vaccinia and H-2K<sup>k</sup>. The low responsiveness of 4R Tc cells in relation to vaccinia-D<sup>b</sup> varied in magnitude between individual mice and ranged from 3- to 20-fold lower lysis of vaccinia-D<sup>b</sup> targets compared with B10 Tc cells (data not shown).

*Stimulation of F1 Lymphocytes in Parental Recipients.* The possibility that the defect in the anti-vaccinia-D<sup>b</sup> Tc cell response in mice expressing K<sup>k</sup> and D<sup>b</sup> antigens is caused by low numbers of vaccinia-D<sup>b</sup>-reactive precursor T cells was investigated by transfer

TABLE II  
Influence of H-2K on Primary H-2D-restricted Anti-Vaccinia Tc Cell Response In Vivo

Mouse strain	H-2K/H-2D	Killer/target ratio	Percent specific lysis of vaccinia-infected macrophage targets*				
			B10 (K <sup>b</sup> D <sup>b</sup> )	BR (K <sup>k</sup> D <sup>k</sup> )	4R (K <sup>k</sup> D <sup>b</sup> )	HTG (K <sup>d</sup> D <sup>b</sup> )	BYR (K <sup>q</sup> D <sup>b</sup> )
B10	b/b	45:1	75	2	47	51	49
		15:1	57	4	42	34	38
		5:1	32	0	15	12	27
HTG	d/b	45:1	61	0	51	86	64
		15:1	41	0	52	75	51
		5:1	34	0	37	49	39
BYR	q/b	45:1	48	2	50	39	87
		15:1	34	0	29	38	73
		5:1	24	7	22	18	52
4R	k/b	45:1	12	67	75	18	25
		15:1	6	52	61	17	15
		5:1	2	18	34	11	16

\* Mean percent <sup>51</sup>Cr-release from vaccinia-infected targets over a 6-h period. Spontaneous release ranged from 17 to 29%. Means of triplicates are given; SE of the means were never >2.7%. Lysis of uninfected targets was not significant.

of (B10 × 4R)F1 spleen cells into 950-rad irradiated parental recipients. The recipient mice were inoculated intravenously with vaccinia virus 3 h post-transfer of spleen cells. Cytotoxicity in spleens was tested 6 d after infection on macrophage target cells. Table III shows two representative examples of such experiments. The main points are: F1 cells transferred into B10 recipients responded to vaccinia-D<sup>b</sup> (D2.GD targets) with strength comparable to that of B10 cells in B10 recipients. When F1 spleen cells were transferred into 4R or F1 recipients, the anti-vaccinia-D<sup>b</sup> response was relatively weak. These results indicate that (B10 × 4R)F1 mice do not lack the precursors to respond to vaccinia-D<sup>b</sup>, but that the environment determines whether or not the full potential of these precursors is expressed, as shown previously by others (11).

*Generation of Secondary Vaccinia-immune Tc Cells In Vitro in the Presence or Absence of K<sup>k</sup>.* The previous experiments (Table III) showed that generation of anti-vaccinia-D<sup>b</sup> effector Tc cells from their precursors was inefficient in an environment containing K<sup>k</sup>, but did not distinguish between two possibilities. First, vaccinia-D<sup>b</sup> "complexes" may not be displayed immunogenically in the presence of K<sup>k</sup>. This possibility is not refuted by the fact that anti-vaccinia-D<sup>b</sup> effector Tc cells can recognize and lyse infected 4R (K<sup>k</sup>,D<sup>b</sup>) targets (Table II) because we have reported two previous examples of antigenic patterns that were recognized by effector T cells, but were inefficient at inducing precursors to respond (19, 20).

An alternative possibility is that mice with K<sup>k</sup> may possess a mechanism that suppresses generation of anti-vaccinia D<sup>b</sup> effector Tc cells. The first of these alternatives was tested by stimulating vaccinia-immune memory spleen cells of B10 or 4R

TABLE III  
*Stimulation of F1 Lymphocytes in Irradiated, Vaccinia-infected Parental Recipients*

Donor lymphocytes	Recipients*	Percent specific lysis of vaccinia-infected macrophage targets‡				
		BR (K <sup>k</sup> D <sup>k</sup> )	B10 (K <sup>b</sup> D <sup>b</sup> )	4R (K <sup>k</sup> D <sup>b</sup> )	5R (K <sup>b</sup> D <sup>d</sup> )	D2.GD (K <sup>d</sup> D <sup>b</sup> )
Experiment 1						
B10	B10	-2	31	22	34	29
4R	4R	11	3	16	-8	9
(B10 × 4R)F1	(B10 × 4R)F1	23	14	24	12	9
(B10 × 4R)F1	B10	1	25	13	NT§	23
(B10 × 4R)F1	4R	10	5	12	-4	8
Experiment 2						
B10	B10	0	24	10	20	14
4R	4R	9	-3	18	-1	2
(B10 × 4R)F1	(B10 × 4R)F1	15	24	24	26	1
(B10 × 4R)F1	B10	7	17	11	15	10
(B10 × 4R)F1	4R	12	6	21	8	2

\* Animals were irradiated with 950 rad from a <sup>60</sup>Co source, given 5 × 10<sup>7</sup> spleen cells 18 h later, and immunized with WR vaccinia after an additional 3 h. Spleen cells were assayed for cytotoxicity after 6 d. Irradiated, infected controls that received no spleen cells died before the 6th d.

‡ Mean percent specific <sup>51</sup>Cr-release over a 6-h period. Spontaneous release ranged from 22 to 28%. The values given are from titration curves with regression values between 0.9 and 1.1 and were solved at a killer/target cell ratio of 15:1. The assay was performed in triplicates. Lysis of uninfected targets was not significant.

§ Not tested.

origin *in vitro* with vaccinia-infected spleen cells of either B10 ( $K^b, D^b$ ), 4R ( $K^k, D^b$ ), or BYR ( $K^q, D^b$ ) type (Table IV).

Vaccinia-infected 4R cells were similar to infected B10 or BYR cells in their ability to stimulate vaccinia- $D^b$  Tc cells from either B10 or 4R memory cells, as shown by lysis of infected BYR targets. Thus, immunogenic display of vaccinia- $D^b$  antigens takes place on infected cells that also express  $K^k$ . Simultaneous generation of allo-reactive Tc cells in allogeneic mixtures did not detract from this result. 4R responder cells gave about threefold lower lysis on vaccinia-infected  $D^b$  targets (B10, BYR) than did B10 responders, irrespective of whether the stimulator cells were infected 4R, B10, or BYR spleen cells. Presumably this result reflects relatively weaker generation of anti-vaccinia- $D^b$  memory cells in 4R mice, in keeping with their weaker primary responses.

*Tc Cell Responses to Vaccinia in Neonatally Tolerant B10 Mice.* The proposition that the presence of  $K^k$  induces suppression of Tc cell responses to vaccinia- $D^b$  through a mechanism of cross-reactive self-tolerance was tested in experiments using B10 ( $K^b, D^b$ ) animals tolerized neonatally to  $K^k$  or  $K^q$ .

TABLE IV  
*In Vitro Cross-Stimulation of Vaccinia-primed Spleen Cells*

Vaccinia-immune memory spleen cell responders	Vaccinia-infected stimulators*	Killer/target ratio	Percent specific lysis of macrophage targets‡					
			B10 ( $K^b, D^b$ )		4R ( $K^k, D^b$ )		BYR ( $K^q, D^b$ )	
			uninfected	vaccinia	uninfected	vaccinia	uninfected	vaccinia
B10	B10	30:1	14	41	14	59	8	47
		10:1	14	37	18	56	5	46
		3:1	7	37	5	28	-2	31
B10	4R	30:1	14	46	44	64	9	57
		10:1	10	46	34	60	3	47
		3:1	6	31	32	38	-2	28
B10	BYR	30:1	9	43	18	62	32	46
		10:1	11	39	16	57	14	47
		3:1	9	23	10	27	1	34
4R	B10	30:1	40	49	-8	56	15	42
		10:1	33	27	7	54	9	24
		3:1	15	13	-5	37	0	16
4R	4R	30:1	22	41	-7	64	8	45
		10:1	11	21	-3	55	1	26
		3:1	5	7	-8	46	0	9
4R	BYR	30:1	20	42	-6	56	25	46
		10:1	10	28	-5	58	14	33
		3:1	1	13	-10	48	3	12

\* Spleen cells were infected for 1 h with 1 PFU vaccinia virus and irradiated with 2,000 rad from a  $^{60}\text{Co}$  source.

‡ Mean percent specific  $^{51}\text{Cr}$  release over a 6-h period. Spontaneous release ranged from 14 to 16%. Assays were performed in triplicates with SE of the mean never  $>3.7\%$ .

B10 neonates were injected intraperitoneally with  $5 \times 10^7$  spleen cells of (B10  $\times$  4R)F1, (B10  $\times$  CBA)F1, or (B10  $\times$  BYR)F1 type no later than 24 h after birth. At 6 wk old the mice were infected with vaccinia virus and tested for vaccinia-immune Tc cells 6 d later. Table V shows a representative experiment using cell line targets. B10 mice tolerant of 4R ( $K^k$ ) or CBA ( $K^k, D^k$ ) gave impaired responses to vaccinia- $D^b$  (HTG-infected targets) similar to the (B10  $\times$  4R)F1 control and inferior to the B10 and BYR controls. Their responses to vaccinia- $K^b$  were similar to the B10 and (B10  $\times$  4R)F1 controls. In contrast, B10 mice tolerant of BYR ( $K^a$ ) gave unimpaired responses to vaccinia- $D^b$ . The lack of an anti-vaccinia- $K^k$  response in B10 mice tolerized to 4R or CBA indicated no significant numbers of (B10  $\times$  4R)F1 or (B10  $\times$  CBA)F1 spleen cells in these mice.

Further examples of the effect of tolerance of  $K^k$  upon the anti-vaccinia- $D^b$  response in B10 mice are given in the experiment in Table VI in which macrophage targets were used, and in which the B10 and 4R controls were injected as neonates with syngeneic cells. Two out of the three B10 mice injected neonatally with (B10  $\times$  4R)F1 spleen cells gave impaired anti-vaccinia- $D^b$  responses (infected 4R and HTG targets),

TABLE V  
*Anti-Vaccinia- $D^b$  Responses of Neonatally Tolerized B10 Mice \**

Responders	Tolerance treatment	Killer/target Ratio	Percent specific lysis of cell line targets‡					
			L929 ( $K^k, D^k$ )		5R ( $K^b, D^b$ )		HTG ( $K^d, D^b$ )	
			uninfected	vaccinia	uninfected	vaccinia	uninfected	vaccinia
B10	(B10 $\times$ BYR)F1	45:1	12	8	10	46	2	72
		15:1	6	5	1	33	3	59
		5:1	2	3	4	20	4	25
B10	(B10 $\times$ CBA)F1	45:1	4	1	10	39	3	19
		15:1	1	1	0	14	2	5
		5:1	0	0	2	10	0	4
B10	(B10 $\times$ 4R)F1	45:1	3	1	4	27	3	4
		15:1	3	0	3	16	0	3
		5:1	0	1	3	3	3	3
B10	None	45:1	10	7	8	38	1	73
		15:1	3	3	2	11	0	65
		5:1	1	0	1	4	0	35
BYR	None	45:1	11	5	8	6	2	71
		15:1	0	6	1	0	2	53
		5:1	0	0	0	0	1	24
(B10 $\times$ 4R)F1	None	45:1	7	56	15	46	0	4
		15:1	1	39	8	29	5	4
		5:1	0	15	7	7	2	4

\* Neonates were given  $5 \times 10^7$  spleen cells intraperitoneally <24 h after birth. Animals were infected with virus at >6 wk old and spleens removed 6 d post-immunization.

‡ Mean percent specific  $^{51}\text{Cr}$ -release from targets over a 10-h period. Spontaneous release ranged from 20 to 25%. Means of triplicates are given with SE of the mean never >3.7%.

TABLE VI  
*Anti-Vaccinia-D<sup>b</sup> Responses of Neonatally Tolerized B10 Mice\**

Re- sponders	Tolerance treatment	Percent specific lysis of macrophage targets‡						
		Vaccinia-immune effectors					Alloge- neic ef- fectors§	
		B10- Vaccinia (K <sup>b</sup> ,D <sup>b</sup> )	BR- Vaccinia (K <sup>k</sup> ,D <sup>k</sup> )	5R- Vaccinia (K <sup>b</sup> ,D <sup>d</sup> )	4R- Vaccinia (K <sup>k</sup> ,D <sup>b</sup> )	HTG- Vaccinia (K <sup>d</sup> ,D <sup>b</sup> )	4R	B/c
B10	B10	25	4	51	17	26	86	91
	B10	23	6	38	21	24	84	87
4R	4R	0	31	0	27	0	9	54
	4R	0	59	0	43	0	8	58
B10	(B10 × 4R)F1	14	0	23	1	6	7	68
	(B10 × 4R)F1	14	0	25	6	2	9	41
	(B10 × 4R)F1	23	0	28	26	25	97	87

\* Same procedure in Table V.

‡ Mean percent specific <sup>51</sup>Cr-release over a 6-h period. Spontaneous release ranged from 21 to 32%. Values given are from titration curves with regression values between 0.9 and 1.1 and were solved at a killer/target ratio of 15:1. The assay was performed in triplicates. Lysis of uninfected targets by vaccinia-immune effectors was not significant.

§ See text for details.

whereas the third animal was similar to the B10 controls. Samples of spleen cells from the same mice were set up in one-way mixed-lymphocyte culture (MLC) with either 4R or B/c irradiated stimulator cells and tested after 5 d on target cells syngeneic with the stimulators (last two right-hand columns, Table VI). The two mice with impaired anti-vaccinia-D<sup>b</sup> responses were apparently tolerant of K<sup>k</sup> (little lysis of 4R targets), whereas the third animal with a normal anti-vaccinia-D<sup>b</sup> response was clearly not tolerant of K<sup>k</sup>. All mice gave substantial lysis of third-party B/c targets.

However, tolerance to K<sup>k</sup> did not always produce an impaired anti-vaccinia-D<sup>b</sup> response. Overall, 29 individual B10 animals treated with (B10 × 4R)F1 cells as neonates have been evaluated. 3 out of 29 were not tolerant to K<sup>k</sup>, as tested by MLC against 4R stimulators, and also exhibited normal anti-vaccinia-D<sup>b</sup> Tc cell responses. The remaining 26 mice were tolerant of K<sup>k</sup>. 19 of these showed depressed anti-vaccinia-D<sup>b</sup> responses and 7 gave an appreciable anti-vaccinia-D<sup>b</sup> response (data not shown).

*Virus Specificity of Cross-reactive Tolerance.* Because tolerance of K<sup>k</sup> could suppress anti-vaccinia-D<sup>b</sup> Tc cell responses, but not K<sup>k</sup>-restricted responses, it was of interest to ask if other H-2D<sup>b</sup>-restricted antiviral responses were affected. Table VII shows one experiment using LCMV in which B10 mice tolerized by neonatal injection of (B10 × 4R)F1, (B10 × CBA)F1, or (B10 × BYR)F1 spleen cells gave similar anti-LCMV-D<sup>b</sup> responses (lysis of 2R-LCMV targets) to B10 controls. All of these mice were tolerant of K<sup>k</sup> or K<sup>q</sup>, as tested by MLC against 2R, CBA, or BYR stimulators. Because 19 of 26 K<sup>k</sup>-tolerant mice showed depressed anti-vaccinia-D<sup>b</sup> responses, the finding of no depression of anti-LCMV-D<sup>b</sup> responses in 4 K<sup>k</sup>-tolerized animals indicates that the phenomenon probably does not occur with LCMV ( $P = 0.012$  with Fisher's exact test).

*Investigation of Possible Cross-Reactivity Between K<sup>k</sup> and Vaccinia-D<sup>b</sup>.* The evidence

TABLE VII  
*Anti-*LCMV-D<sup>b</sup>* Responses of Neonatally Tolerized B10 Mice\**

Re- sponders	Tolerance treat- ment	Percent specific lysis of targets‡					
		LCMV-immune effectors		Allogeneic effectors§			
		2R- LCMV (K <sup>k</sup> ,D <sup>b</sup> )	5R- LCMV (K <sup>b</sup> ,D <sup>d</sup> )	2R (K <sup>k</sup> ,D <sup>b</sup> )	BYR (K <sup>q</sup> ,D <sup>b</sup> )	L929 (K <sup>k</sup> ,D <sup>k</sup> )	D2 (K <sup>d</sup> ,D <sup>d</sup> )
B10	None	78	86	NT	23	59	52
B10	None	53	68	NT	31	40	48
B10	(B10 × 4R)F1	50	42	3	NT	NT	23
B10	(B10 × 4R)F1	49	40	4	NT	NT	26
B10	(B10 × CBA)F1	78	72	NT	NT	8	24
B10	(B10 × CBA)F1	50	75	NT	NT	7	33
B10	(B10 × BYR)F1	55	89	NT	2	NT	24

\* Same procedure in Table V.

‡ Mean percent specific <sup>51</sup>Cr-release from cell line targets over a 10-h period for the anti-*LCMV* and 6 h for the allogeneic response. Spontaneous release ranged from 17 to 27%. The assays were performed in triplicate and titrated. Values are given for a killer/target ratio of 15:1 for the anti-*LCMV* and 3:1 for the antiallogeneic response. SE of the means were never >5%. Lysis of uninfected targets was insignificant in the *LCMV* assay.

§ See text for details.

presented here suggested the possibility that natural or induced tolerance to *K<sup>k</sup>* causes suppression of Tc cells that react with *vaccinia-D<sup>b</sup>*, but does not impair responses to *vaccinia-K<sup>b</sup>*, *vaccinia-K<sup>k</sup>*, *LCMV-D<sup>b</sup>*, *LCMV-K<sup>b</sup>*, *LCMV-K<sup>k</sup>*, or *H-2<sup>d</sup>*. This evidence implicates a suppressive mechanism with some degree of antigen specificity. There would seem to be only two ways by which *vaccinia-D<sup>b</sup>*-reactive Tc cells could be identified: (a) by the ability of their antigen receptors to bind to antigen, or (b) by an antiidiotype that binds to the antigen receptors. Therefore, we have looked for Tc cell cross-reactivity between *K<sup>k</sup>* and *vaccinia-D<sup>b</sup>* that would be a corollary of a and b above. Tables II–VI show no indication of significant cross-reactivity of anti-*vaccinia-D<sup>b</sup>* Tc cells on *K<sup>k</sup>* targets. Table VIII shows two representative examples of many experiments that showed no cross-reaction of MLC-generated anti-*K<sup>k</sup>* T cells on *vaccinia*-infected *H-2<sup>b</sup>* targets. However, over the course of many experiments it was noted that occasional individual BYR and HTG mice generated *vaccinia*-immune effector cell populations that cross-lysed uninfected *H-2<sup>k</sup>* targets (data not shown). Because cross-lysis by an effector cell population does not establish that the same individual effector cell kills two different target cells, cold target competition experiments were performed to test this point. Fortunately, one such experiment gave evidence of varying degrees of cross-reactivity of anti-*vaccinia D<sup>b</sup>* Tc cells with uninfected *K<sup>k</sup>* competitors in three individual responders (Table IX). The first BYR (*K<sup>q</sup>,D<sup>b</sup>*) mouse gave anti-*vaccinia-D<sup>b</sup>* effectors (lysis of *vaccinia*-infected B10 [*K<sup>b</sup>,D<sup>b</sup>*] targets) that were strongly inhibited by unlabeled *vaccinia*-infected B10 (*K<sup>b</sup>,D<sup>b</sup>*) competitors but showed little cross-inhibition by unlabeled, uninfected 4R (*K<sup>k</sup>,D<sup>b</sup>*) or BR (*K<sup>k</sup>,D<sup>k</sup>*) competitors. In contrast, the second BYR mouse gave effectors that were strongly cross-inhibited by unlabeled, uninfected 4R or BR competitors. The HTG mouse was intermediate between the 2 BYR animals.

Taken together, the data in Tables II–VI, VIII, and IX indicate that Tc cell clones

TABLE VIII  
*No Apparent Cross-reactivity of Anti-K<sup>k</sup> Tc Cells\* on  
 Vaccinia-infected H-2<sup>b</sup> Targets*

Responder	Stimulator	Killer/tar- get ratio	Percent specific lysis of targets‡		
			BW (K <sup>k</sup> ,D <sup>k</sup> ) uninfected	MC57 (K <sup>b</sup> ,D <sup>b</sup> ) uninfected    vaccinia	
Experiment 1					
B10(K <sup>b</sup> ,D <sup>b</sup> )	4R(K <sup>k</sup> ,D <sup>b</sup> )	30:1	67	10	5
		10:1	55	5	3
		3:1	32	3	-1
		1:1	12	2	1
Experiment 2					
B10(K <sup>b</sup> ,D <sup>b</sup> )	4R(K <sup>k</sup> ,D <sup>b</sup> )		CBA (K <sup>k</sup> ,D <sup>k</sup> ) uninfected	B10 (K <sup>b</sup> ,D <sup>b</sup> ) uninfected    vaccinia	
		9:1	52	10	12
		3:1	52	4	6
		1:1	29	-1	-2

\* Tc cells were generated in MLC as described in Materials and Methods.

‡ Mean percent specific <sup>51</sup>Cr-release from cell line targets over a 6-h period (experiment 1) or from macrophage targets over a 16-h period (experiment 2). Spontaneous release ranged from 14 to 18% (experiment 1) and from 14 to 17% (experiment 2). SE of means were never >4.5%.

TABLE IX  
*Cold Target Competition of Anti-Vaccinia-D<sup>b</sup> Effector Cells*

Effectors	Percent specific lysis of B10-vaccinia-infected macrophage targets*				
	Cold competitors				
	None	B10-Vacci- nia (K <sup>b</sup> ,D <sup>b</sup> )	B10 (K <sup>b</sup> ,D <sup>b</sup> )	4R (K <sup>k</sup> ,D <sup>b</sup> )	BR (K <sup>k</sup> ,D <sup>b</sup> )
BYR(K <sup>a</sup> ,D <sup>b</sup> )	33	7	33	28	27
BYR(K <sup>a</sup> ,D <sup>b</sup> )	30	3	31	19	13
HTG(K <sup>d</sup> ,D <sup>b</sup> )	20	4	22	15	10

\* Mean percent specific <sup>51</sup>Cr-release from B10-vaccinia-infected target cells in the presence or absence of unlabeled competitors over a 6-h period. Killer/target ratio was 15:1. The ratio of competitors to labeled targets was 4:1 for the values given. Spontaneous release was 19%. All values are the means of triplicates with the SE of the mean never >1.9%.

that react with both K<sup>k</sup> and vaccinia-D<sup>b</sup> antigens do exist, but constitute a variable and often insignificant part of the anti-K<sup>k</sup> and anti-vaccinia-D<sup>b</sup> Tc cell repertoires in mice expressing the H-2D<sup>b</sup> antigen.

*Comparison of Strengths of Responses to Vaccinia-D<sup>b</sup> and K<sup>k</sup> in Individual B10 Mice.* If a suppressive mechanism operates upon the developing T cell repertoire to induce or maintain self-tolerance in normal animals, then it might be expected that Tc cell subsets such as anti-K<sup>k</sup> and anti-vaccinia-D<sup>b</sup> that can be suppressed in a cross-reactive manner (Tables V and VI) would exhibit simultaneous fluctuation in different

individuals. Therefore, spleen cells from individual B10 ( $K^b, D^b$ ) mice immunized 6 d previously with vaccinia virus were tested for anti-vaccinia- $D^b$  activity, and samples of the same spleen cells were cultured for 5 d in one-way MLC with irradiated stimulator cells of 4R ( $K^k, D^b$ ) or B/c ( $H-2^d$ ) type. Most of the 40 B10 mice tested were similar in their anti- $K^k$  and anti-vaccinia- $D^b$  responses. Table X shows two selected examples of experiments in which the anti-B/c response was also similar in all three mice, thus indicating no general defect in immunocompetence in any individual. However, in experiment I, all three individual B10 animals gave significantly different anti-vaccinia- $D^b$  responses, of 30, 21, and 6% specific lysis, respectively. This variability was mirrored in the anti-4R ( $K^k$ ) response with 50, 12, and 7% lysis, respectively. The very low response of animal 3 to vaccinia- $D^b$  was the only example seen in 50–60 B10 mice tested. In experiment II, animals 2 and 3 gave similar levels of lysis in their anti-vaccinia- $D^b$  and anti-4R responses. Animal 1 showed lower anti-vaccinia- $D^b$  and anti-4R responses when compared with animals 2 and 3.

*Responses to Ectromelia- $D^b$  in Chimeric Mice.* The foregoing data are consistent with the idea that tolerance of  $K^k$  results in suppression of anti-vaccinia- $D^b$  responses, but do not exclude the possibility that other mechanisms may also contribute to the phenomenon. Previous work showed that antiviral Tc cell responses are subject to simple feedback regulation, i.e., the effector T cells mediate destruction of the virus-infected cells that present the antigenic patterns responsible for stimulating the generation of effector T cells (21). It follows that if the effector Tc cell response to vaccinia- $K^k$  was generated more quickly than the response to vaccinia- $D^b$ , the latter may be suppressed as a consequence of elimination of the antigenic stimulus by the former. Ectromelia virus was used to test this hypothesis because Tc cells do not distinguish between vaccinia- and ectromelia-infected cells (13), because it was used

TABLE X  
*Comparison of Anti-Vaccinia- $D^b$  Responses with Anti- $K^k$  Responses in Individual B10 Mice*

Mouse number	Percent specific lysis of macrophage targets*		
	Vaccinia-immune effectors‡	Allogeneic effectors‡	
	4R-Vaccinia ( $K^k, D^b$ )	4R ( $K^k, D^b$ )	B/c ( $K^d, D^d$ )
Experiment 1			
1	30	50	64
2	21	12	67
3	6	7	66
Experiment 2			
1	31	11	32
2	50	27	37
3	59	29	29

\* Mean percent specific  $^{51}\text{Cr}$ -release from macrophage targets over a 6-h period. Spontaneous release ranged from 20 to 24%. All values given were determined from a four point regression curve and solved at 15:1 killer/target cell ratio for the antiviral response, and 3:1 for the allogeneic responses. SE of the means were not >3.2%.

‡ See text for details of effector cell generation.

TABLE XI  
*Responses to Ectromelia-D<sup>b</sup> in (CBA + B6) → (CBA × B6)F1 Chimeras*

Animals*		Killer/Target Ratio	Percent specific lysis of ectromelia-infected targets‡	
			A/J (K <sup>k</sup> ,D <sup>d</sup> )	D2GD (K <sup>d</sup> ,D <sup>b</sup> )
(CBA + B6) → (CBA × B6)F1 mixed chimeras	1	9:1	35	22
		3:1	28	8
		1:1	6	3
	2	9:1	39	25
		3:1	42	10
		1:1	22	0
(CBA × B6)F1 → (CBA × B6)F1 chimeras	1	9:1	39	25
		3:1	33	11
		1:1	26	2
	2	9:1	46	23
		3:1	44	9
		1:1	16	13
(CBA × B6)F1	1	9:1	37	25
		3:1	36	9
		1:1	17	2
	2	9:1	23	17
		3:1	11	2
		1:1	12	6

\* Chimeras were produced as described in Materials and Methods. Data given are from primary responses in the spleen 6 d after intravenous injection of 10<sup>4</sup> PFU of ectromelia virus.

‡ Mean percent specific <sup>51</sup>Cr-release from ectromelia-infected macrophage targets over a 16-h period. Spontaneous release ranged from 30 to 38%. SE of the means were never >5%. Lysis of uninfected targets was not significant.

for the earlier feedback regulation work (21), and because the phenomenon of weak anti-ectromelia-D<sup>b</sup> responses in mice expressing K<sup>k</sup> antigen was obtained (Table XI).

Feedback suppression of this type would only operate if both K<sup>k</sup> and D<sup>b</sup> antigens were expressed on the same individual virus-infected antigen-presenting cell. It should not operate in chimeric mice produced by reconstituting lethally irradiated (CBA × B6)F1 hybrids with a 50:50 mixture of fetal liver stem cells from CBA (K<sup>k</sup>,D<sup>k</sup>) and B6 (K<sup>b</sup>,D<sup>b</sup>) mice, in which K<sup>k</sup> and D<sup>b</sup> are expressed on separate cell populations. However, anti-ectromelia-D<sup>b</sup> responses were just as weak (relative to anti-ectromelia-K<sup>k</sup> responses) in 22 such chimeras as they were in normal F1 mice or in F1 → F1 chimera controls, thus indicating that feedback regulation was an insignificant factor. Data from two representative examples of chimeric mice are given in Table XI.

### Discussion

In this paper we have described investigations of several hypotheses that might account for weak anti-vaccinia-D<sup>b</sup> Tc cell responses in mice expressing K<sup>k</sup> and D<sup>b</sup> H-2 antigens. These responses varied from 3-fold to 20-fold lower than anti-vaccinia-D<sup>b</sup> responses in B10 (K<sup>b</sup>,D<sup>b</sup>), HTG (K<sup>d</sup>,D<sup>b</sup>), and BYR (K<sup>q</sup>,D<sup>b</sup>) mice, and were also lower than anti-vaccinia-K<sup>k</sup> responses in (K<sup>k</sup>,D<sup>b</sup>) mice. First, it was apparent that the defect was not at the level of antigen presentation. Vaccinia-D<sup>b</sup> antigens were displayed on

vaccinia-infected,  $K^k$ -expressing cells in a manner that allowed recognition and lysis by vaccinia- $D^b$ -reactive effector Tc cells (Table II) and stimulation of their precursors to generate responses (Table IV). Second, the defect was not explained by a relative lack of anti-vaccinia- $D^b$  precursors in mice expressing  $K^k$  and  $D^b$  antigens, because T cell populations from (B10  $\times$  4R)F1 ( $K^k, K^b, D^b$ ) mice could give anti-vaccinia- $D^b$  responses comparable to those of B10 ( $K^b, D^b$ ) cells if they were stimulated by vaccinia-infected B10 cells in lethally irradiated B10 mice (Table III).

Thus, the simultaneous expression of  $K^k$  and  $D^b$  antigens in a mouse in some way results in impaired responses by its anti-vaccinia- $D^b$  reactive precursors. Two hypotheses that might account for this phenomenon were explored further. One hypothesis was based on feedback regulation of Tc cell responses (21). If anti-vaccinia- $K^k$  effector Tc cells were generated more quickly (for some reason) than anti-vaccinia- $D^b$  cells, then the latter may be stimulated only weakly because of destruction of virus-infected, antigen-presenting cells by the anti-vaccinia- $K^k$  response. This proposition was tested using chimeric mice in which the lymphomyeloid system consisted of a 50:50 mixture of CBA ( $K^k, D^b$ ) and B6 ( $K^b, D^b$ ) cells in (CBA  $\times$  B6)F1 hosts. Because the  $K^k$  and  $D^b$  antigens would now be on different virus-infected, antigen-presenting cells, the feedback regulation outlined above would not operate. However, this maneuver did not produce stronger anti-ectromelia- $D^b$  responses<sup>2</sup> than those seen in (CBA  $\times$  B6)F1 mice or F1  $\rightarrow$  F1 control chimeras, thus refuting the hypothesis. Feedback regulation has been similarly excluded as an explanation for the phenomenon of parental preference in anti-H-Y Tc cell responses of F1 hybrid mice by using mixed parental strain cells as stimulators (22).

We also tested the hypothesis that natural self-tolerance to  $K^k$  caused suppression of responses by anti-vaccinia- $D^b$  Tc cell precursors, following the precedent for regulation of anti-H-Y Tc cells (17). Considerable evidence consistent with this idea was obtained. First, anti-vaccinia- $D^b$  precursor Tc cell populations from (B10  $\times$  4R)F1 mice gave good responses in irradiated, vaccinia-infected B10 ( $K^b, D^b$ ) hosts (comparable to anti-vaccinia- $K^k$  responses from the same F1 T cell population and to anti-vaccinia- $D^b$  responses of B10 precursors), whereas they gave weaker anti-vaccinia- $D^b$  responses in 4R ( $K^k, D^b$ ) hosts. One interpretation of these data is that the anti-vaccinia  $D^b$  Tc cell precursors in the F1 donor spleens were released from suppression by the process of producing single-cell suspensions and their injection into B10 hosts, and that suppression could be reimposed in irradiated 4R hosts. This interpretation might be subject to the qualification that an allogeneic effect could improve anti-vaccinia- $D^b$  responses of (B10  $\times$  4R)F1 Tc cells in B10 hosts. However, this possibility seems small, because such an effect should be nonspecific, and there was no evidence of it on anti-vaccinia- $K^b$  responses (Table III).

The most convincing evidence in favor of the cross-tolerance hypothesis was obtained using B10 mice that were neonatally tolerized to  $K^k$  antigens. Of 26 such mice, 19 gave anti-vaccinia- $D^b$  responses that were significantly lower than responses of normal B10 controls, or B10 mice tolerant to  $K^q$  (Tables V and VI). Natural or induced tolerance to  $K^k$  did not affect responses to vaccinia- $K^b$ , vaccinia- $K^k$ , LCMV- $D^b$ , LCMV- $K^b$ , LCMV- $K^k$ , and H-2<sup>d</sup> (Tables V-VII).

Taken together, the results discussed thus far can be explained by a reversible

<sup>2</sup> Ectromelia and vaccinia are closely related pox viruses that are antigenically indistinguishable by Tc cells (13).

suppressor mechanism that cross-reacts between anti- $K^k$  and anti-vaccinia- $D^b$  Tc cell precursors, but does not affect the other responses listed above. Suppression with this property of partial antigen specificity could be mediated either through cross-reactive binding of anti- $K^k$  and anti-vaccinia- $D^b$  antigen-receptors to  $K^k$  antigen, or by cross-reactive antiidiotypes. It follows that effector Tc cells that cross-react between  $K^k$  and vaccinia- $D^b$  targets should be found. Bidirectional tests of B10 effector Tc cells failed to demonstrate such cross-reactivity by target lysis (Tables II-VI, VIII), but cold target competition experiments using some BYR and HTG anti-vaccinia- $D^b$  Tc cells showed that  $K^k$ -expressing competitors inhibited lysis of vaccinia- $D^b$  targets (Table IX), thus establishing that the predicted cross-reactivity can exist.

Although these reactive Tc cells are obviously an insignificant part of the effector Tc cell population generated by the majority of  $D^b$ -expressing mice, it could be that cross-reactive precursors were generated during T cell ontogeny, and then suppressed or deleted as a consequence of self-tolerance mechanisms. If this process took place to a variable extent in different individual mice, then anti- $K^k$  and anti-vaccinia- $D^b$  response capabilities might exhibit simultaneous variation. This phenomenon was found (Table X) by screening individual B10 mice that were similar in their Tc responses to a third-party alloantigen (H-2<sup>d</sup>).

In conclusion, the data presented here are consistent with the idea that the effect of  $K^k$  on anti-vaccinia- $D^b$  Tc cell responses is a result of a self-tolerance mechanism that cross-reactively suppresses both anti- $K^k$  and anti-vaccinia- $D^b$  Tc cell precursors. That cross-reactivity between anti- $K^k$  and anti-vaccinia- $D^b$  effector Tc cells is rarely demonstrable does not deny the possibility that anti-vaccinia- $D^b$  precursor Tc cells that never give rise to effector progeny are suppressed through cross-reactive binding either to  $K^k$  antigen or to an anti- $K^k$  antiidiotype. In any case, there are at least two examples of antigenic patterns that are inefficient at stimulating precursor Tc cells, but which can be recognized by effector Tc cells, the generation of which was stimulated by cross-reacting antigens (19, 20). There is also the precedent in the H-Y system in which neonatal tolerance caused suppression of a Tc cell response that was more cross-reactive than the relevant effector Tc populations (17). This work thus constitutes the second investigation consistent with the idea that self-tolerance mechanisms can mediate Ir gene effects, and illustrates the power of neonatally induced tolerance as a tool for elucidating the processes that shape the MHC-restricted T cell repertoire.

### Summary

The  $K$  region of  $H-2$  controls the Tc cell response to vaccinia- $D^b$ . The  $K^b$ ,  $K^d$ , and  $K^q$  alleles allow good Tc cell responses against vaccinia- $D^b$ . In contrast, the presence of  $K^k$  in  $H-2$  recombinants 2R ( $K^k, D^b$ ) and 4R ( $K^k, D^b$ ) or in F1 hybrids greatly reduces the anti-vaccinia- $D^b$  response. The defect does not lie in antigen presentation, as infected 4R cells can stimulate anti-vaccinia- $D^b$  Tc cells in vitro. Furthermore, nonresponder animals possess Tc cell precursors for vaccinia- $D^b$ , as transfer of F1 nonresponder spleen cells into infected, lethally irradiated responder recipients allowed generation of anti-vaccinia- $D^b$  effector Tc cells. Secondary responses to vaccinia- $D^b$  can also be obtained in vitro from T cells of 4R animals. Feedback inhibition was excluded in experiments with mixed chimeras in which  $K^k$  and  $D^b$  were expressed on separate cell populations.

Neonatal tolerance of B10 animals to  $K^k$  suppressed the anti-vaccinia- $D^b$  response but did not affect anti-vaccinia- $K^b$ , anti-lymphocytic choriomeningitis virus, or anti-H-2<sup>d</sup> responses. In cold target competition experiments, H-2<sup>k</sup> competitors inhibited vaccinia- $D^b$ -specific target cell lysis by Tc cells, which suggests that anti-vaccinia- $D^b$  and anti-H-2 $K^k$  Tc cells may cross-react. Therefore, we propose that the suppressive influence of  $K^k$  on anti-vaccinia- $D^b$  Tc cell responses is a consequence of self-tolerance and that suppression of anti- $K^k$  Tc cells results in cross-reactive suppression of anti-vaccinia- $D^b$  Tc cells.

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