

Recognition of endogenous ecotropic murine leukaemia viruses by anti-AKR/Gross virus cytotoxic T lymphocytes (CTL): epitope variation in a CTL-resistant virus

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AKR/Gross virus-specific cytotoxic T lymphocytes (CTL) from C57BL/6 (B6) mice are H-2K^b-restricted and recognize epitopes encoded by the prototype endogenous ecotropic murine leukaemia virus (Emv) AKR623. Four CTL epitopes have been identified by the use of synthetic peptides corresponding to AKR623-encoded amino acid sequences. Here we present both functional and nucleotide sequence data indicating that three closely related Emv share all of these CTL epitopes. We also found that one other murine leukaemia virus (MuLV) was not susceptible to lysis by these CTL. This is the ecotropic component of the LP-BM5 virus complex that causes murine AIDS. Nucleotide sequencing revealed that three of the four epitopes, including the immunodominant peptide, are altered in

this virus. The other epitope was unchanged. These data implied that the inability of anti-AKR/Gross virus CTL to lyse cells infected with the LP-BM5 ecotropic (BM5eco) MuLV was due to the functional loss of three of the four CTL epitopes. Using recombinant vaccinia and Sindbis virus vectors, we have shown that the BM5eco-encoded form of the immunodominant epitope, which differs only by an arginine for lysine substitution at the N-terminal residue, fails to induce a CTL response in B6 mice. Immunization with BM5eco-infected cells also failed to induce MuLV-specific CTL. In light of the long *in vivo* passage history of the LP-BM5 complex in B6 mice, our results are consistent with a contribution of CTL-mediated immune selection to the evolution of the BM5eco MuLV.

Introduction

Many inbred mouse strains carry stably integrated ecotropic murine leukaemia virus (MuLV) proviruses in their germline genomes (Jenkins *et al.*, 1982). Some of these proviruses encode replication-competent viruses that can be expressed from shortly after birth, resulting in a life-long viraemia which can be pathogenic. The AKR mouse strain, for example, has a high incidence of leukaemia that depends on the early expression of endogenous ecotropic murine leukaemia virus (Emv; Lilly *et al.*, 1975). AKR/Gross virus is a highly leukaemogenic isolate which is derived from the endogenous viruses of AKR mice. Other Emv-derived viruses are associated with different pathologies. The LP-BM5 MuLV complex induces a progressive immunodeficiency in certain mouse strains that exhibits many disease features similar to the clinical symptoms dis-

played by patients infected with human immunodeficiency virus (HIV). LP-BM5 is a mixture of retroviruses including ecotropic, recombinant mink cytopathic focus-inducing (MCF) and replication-defective MuLV (Chattopadhyay *et al.*, 1991). The defective virus is thought to be the primary cause of murine AIDS (MAIDS), while the ecotropic LP-BM5 (BM5eco) serves as a helper virus for the defective genome (Aziz *et al.*, 1989; Chattopadhyay *et al.*, 1991). Restriction mapping and partial nucleotide sequence data indicate a close relationship between this virus and endogenous MuLV. Since LP-BM5 was originally isolated from an X-ray-irradiated B6 mouse (Latarjet & Duplan, 1962), it seems likely that BM5eco originated from *Emv-2*, the lone Emv of B6 mice (Jenkins *et al.*, 1982).

B6 mice can generate H-2K^b-restricted anti-AKR/Gross virus cytotoxic T lymphocytes (CTL) that lyse tumour cells expressing viral antigens (Green *et al.*, 1979). These antiviral CTL can also lyse SC-1 fibroblasts transfected with the H-2K^b gene and infected with AKR623 virus, derived from *Emv-11* (White *et al.*, 1990). Recently, four synthetic peptides representing potential AKR623-encoded, H-2K^b-restricted CTL epitopes, based on the motif described by Falk *et al.* (1991), were

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found to be capable of sensitizing virus antigen-negative target cells for lysis by anti-AKR/Gross virus CTL (White *et al.*, 1994a). These peptides could also stimulate the *in vitro* production of cytolytic effectors that lysed AKR623-infected cells and AKR/Gross MuLV-induced tumours (White *et al.*, 1994b). One peptide (KSPWFRTL), located in the p15E transmembrane (TM) region of the Env protein (amino acid position TM134–141), appears to represent an immunodominant epitope, based on competitive inhibition assays with AKR 623-infected target cells and its ability to sensitize uninfected targets for lysis by antiviral CTL at very low (10 µg/ml) concentrations (Sijts *et al.*, 1994; White *et al.*, 1994a). In contrast, a peptide from the matrix (MA) protein (MA125–132, RSALYPAL) and two from the reverse transcriptase (RT; RT142–149, SHRWYTVL; RT456–463, RMTHYQAM) may represent minor CTL epitopes; significantly higher concentrations of these peptides are needed to sensitize uninfected targets and the resulting levels of lysis are generally lower than for peptide TM134–141.

To extend these observations and to determine whether other Emv are also susceptible to anti-AKR/Gross virus CTL-mediated lysis, the *Emv-3D*, *Emv-3R* and *Emv-15* proviruses and the BM5eco virus were examined. Cells expressing B6-derived class I MHC genes were infected or transfected with these MuLV in order to determine their susceptibility to B6 anti-AKR/Gross virus CTL. We subsequently determined the nucleotide sequence of selected regions of these MuLV proviral clones to determine the CTL epitope-coding potential of these viruses. Finally, we have used recombinant viral vaccines to directly assess the immunogenicity of allelic forms of the immunodominant epitope.

Methods

Cell lines. SC-1 cells stably transfected with the class I MHC genes H-2K^b and H-2D^b (SC.Kb and SC.Db cells, respectively) have been described (White *et al.*, 1990). These cell lines were subsequently transfected with *Emv-3D*, *Emv-3R* or *Emv-15* proviral DNA to yield SC.KbEmv3D, SC.KbEmv3R, SC.KbEmv15, SC.DbEmv3D, SC.DbEmv3R and SC.DbEmv15. In addition, infection with the BM5eco virus yielded SC.Kb/BM5eco and SC.Db/BM5eco cells. Virus expression in these cell lines was confirmed by indirect immunofluorescence, XC plaque and RT assays (data not shown).

The H-2^b, AKR/Gross virus-positive T cell tumour lines E β G2, AKR.H-2^bSL1 (SL1) and B.GV, and the H-2^b, AKR/Gross virus-negative lymphoma E β K1, as well as their culture and use in the generation and assay of AKR/Gross virus-specific CTL, have all been described (Green *et al.*, 1979, 1980; Green, 1982). HuTK-143B cells (CRL 8303) were obtained from the ATCC and maintained in MEM supplemented with 10% fetal bovine serum, L-glutamine and antibiotics.

CTL assays. ⁵¹Cr-release assays were performed as previously described (Green *et al.*, 1979; White *et al.*, 1990). The plates were incubated at 37 °C for 4 h or 6 h for tumour or fibroblast target cells,

respectively. For the experiments with recombinant vaccinia viruses (rVV), HuTK-143B cells were infected at an m.o.i. of 10 with K^bVac (rVV expressing H-2K^b), either alone or in combination with one of the two epitope-encoding rVV (see below), for 1 h at 37 °C. After excess virus was washed out, the infected cells were cultured overnight in the presence of ⁵¹Cr and washed extensively prior to use as targets.

Emv proviral plasmids. The *Emv-3D* plasmid was kindly provided by Dr N. Copeland [National Cancer Institute (NCI), Frederick, Md., USA; Copeland *et al.*, 1984]. Although this clone encodes a replication-defective virus, all viral proteins are expressed in transfected cells. The revertant (replication-competent) *Emv-3R* molecular clone was a generous gift from Dr P. Jorgensen (Institute of Molecular Biology and Plant Physiology, Aarhus University, Denmark; Jorgensen *et al.*, 1988). The molecular clone of *Emv-15* was kindly provided by Dr L. Siracusa (Department of Life Science, Jefferson University, Philadelphia, Penn., USA; Siracusa *et al.*, 1987). Infectious BM5eco (clone 12) virus and proviral plasmid were generous gifts from Dr H. Morse III, (NIAID/NIH, Bethesda, Md., USA; Chattopadhyay *et al.*, 1991).

Mice, immunizations and spleen cell cultures. Seven-week-old male C57BL/6 (B6) mice were purchased from the NCI (Bethesda, Md., USA). Polyclonal B6 AKR/Gross virus-specific CTL were generated as previously published (Green, 1982). Briefly, B6 mice were immunized by intraperitoneal injection of 1 × 10⁶ live B.GV cells. Primed responder spleen cells were obtained 11–14 days later and cultured with irradiated (80 Gy) E β G2, E β K1 or SL1 tumour cells or with synthetic peptides as a source of recall antigen *in vitro*. Synthetic peptides were included in the culture medium at a final concentration of either 10 µg/ml (for control and minor epitope peptides) or 0.1 µg/ml (for the immunodominant peptide). Peptides from the capsid (CA) and surface envelope (SC) proteins, CA114–121, SU158–165 and SU319–326, were previously shown to be unable to sensitize SC.Kb cells for antiviral CTL lysis (White *et al.*, 1994a) and therefore served as negative controls in these experiments. Immunization with rVV was by intravenous injection of 10⁷ p.f.u. Mice were immunized at least 3 weeks prior to spleen cell culture. *In vitro* restimulation of rVV-primed spleen cells with recombinant Sindbis viruses was accomplished by infecting irradiated (80 Gy) or mitomycin C-treated E β K1 cells at an m.o.i. of 50 for 1 h at 37 °C before culture. The responder to stimulator cell ratio was 50:1. We employed the Sindbis virus recombinants rather than the rVV as *in vitro* stimulators to avoid the generation of an overwhelming VV-specific CTL response.

Oligonucleotide primers and DNA sequencing. Four oligonucleotide primers corresponding to either the coding (5' primer) or non-coding (3' primers) strand of the AKR623 provirus were synthesized by the Pennsylvania State University Protein and DNA Chemistry Facility or by the Dartmouth Medical School Molecular Biology Core Facility, as follows: for peptide MA125–132, 3' gag gattccaccacctctcgggct; for peptide RT142–149, 3' RT ggtaatctgggcaaggcact; for peptide TM134–141, 3' env cagaaagaggggggaatgaaaga; for peptide RT456–463, 5' RT tggataacaccctgctaccgc. Nucleotide sequences were determined by thermal cycle dideoxynucleotide DNA sequencing using a kit from New England Biolabs and [α -³⁵S]dATP (DuPont NEN).

Recombinant viral expression vectors. The rVV K^bVac was kindly provided by Dr J. Bennink (NIAID/NIH). Recombinant vaccinia and Sindbis viruses engineered to express the AKR623-encoded H-2K^b-restricted immunodominant epitope represented by peptide TM134–141 (KSPWFRTL), or the allelic form of this peptide encoded by the BM5eco MuLV (RSPWFRTL) have been described (Coppola & Green, 1994). These recombinant vaccines are designated TM134K-141Vac, SIN: TM134K-141, TM134R-141Vac and SIN: TM134R-141, respectively. The epitope-coding sequences in these vectors are preceded by an initiator methionine codon and followed by two consecutive termination codons.

Synthetic peptides. The synthetic peptides used in these studies have been described (White *et al.*, 1994a) and were synthesized either by Research Genetics (Huntsville, Ala., USA) or by the Dartmouth Medical School Molecular Biology Core Facility.

Results

To assess whether distinct Emv are susceptible to lysis by polyclonal anti-AKR/Gross virus CTL, SC.Kb cells infected or transfected with various MuLV were employed as targets in ⁵¹Cr-release assays. Table 1 shows that these CTL, which lysed Gross virus-induced tumours and AKR623-infected SC.Kb cells as previously shown (Green *et al.*, 1980; White *et al.*, 1990), also lysed SC.Kb cells expressing *Emv-3D*, *Emv-3R* and

Emv-15 MuLV. On the other hand, lysis of SC.Kb cells infected with the BM5eco virus was comparable to that of uninfected cells. Similar patterns of lysis of Emv-infected SC.Kb cells to those shown were obtained in several repeat experiments. We have not observed lysis of SC.Db cells infected with any of these viruses by anti-AKR/Gross virus CTL (White *et al.*, 1990; data not shown).

We next addressed whether or not synthetic peptides representing individual CTL epitopes could stimulate specific subpopulations of anti-AKR/Gross virus-specific CTL that could lyse these Emv-expressing SC.Kb target cells. Table 2 shows a representative experiment in which peptides MA125–132, RT142–149,

Table 1. Specificity of C57BL/6 anti-AKR/Gross virus CTL*

Stimulation		Effector: target ratio	Percentage specific lysis of target cells								
<i>In vivo</i>	<i>In vitro</i>		Tumour cells			SC.Kb cells infected with:					
			E β G2	E ϕ K1	SL1	None†	BM5‡	623	<i>Emv-3D</i>	<i>Emv-3R</i>	<i>Emv-15</i>
B.GV	None	100:1	2	0	9	5	2	11	4	4	4
	E ϕ K1	100:1	7	9	10	8	5	14	7	4	6
	E β G2	100:1	88	2	68	10	11	60	46	50	60
		20:1	72	2	47	6	6	50	25	30	36
	SL1	100:1	67	3	62	9	0	48	43	49	46
		20:1	49	4	41	6	2	40	28	31	29

* Spontaneous release values for all targets were 20% or lower.

† Uninfected SC.Kb cells.

‡ BM5eco MuLV clone 12.

Table 2. Specificity of C57BL/6 anti-AKR/Gross virus CTL stimulated by H-2K^b motif synthetic peptides*

Stimulation		Effector: target ratio	Percentage specific lysis of target cells								
<i>In vivo</i>	<i>In vitro</i>		Tumour cells			SC.Kb cells infected with:					
			E β G2	E ϕ K1	SL1	None†	BM5‡	623	<i>Emv-3D</i>	<i>Emv-3R</i>	<i>Emv-15</i>
B.GV	None	100:1	8	11	5	4	5	7	6	9	6
	CA114-121										
	HLVLYRQL	100:1	4	7	10	6	7	9	13	8	6
	SU158-165										
	SSWDYITD	100:1	12	9	10	11	5	13	12	11	15
	SU319-326										
	VKGAYQAL	100:1	6	10	4	8	10	11	7	3	4
	MA125-132										
	RSALYPAL	100:1	50	14	41	10	11	50	46	53	66
		20:1	32	7	31	6	8	34	31	31	42
	RT142-149										
	SHRWYTVL	100:1	17	13	16	19	10	24	22	19	30
		20:1	9	4	11	13	7	20	18	11	19
	RT456-463										
	RMTHYQAM	100:1	51	6	58	7	11	60	36	25	62
		20:1	32	2	37	3	6	40	20	15	41
	TM134-141										
	KSPWF TTL	100:1	58	10	69	12	10	66	59	56	76
		20:1	36	2	31	4	8	37	45	40	49

* Spontaneous release values for all targets were 18% or lower.

† Uninfected SC.Kb cells.

‡ BM5eco MuLV clone 12.

	Nucleotide sequence	Amino acid sequence
(a) TM134-141		
AKR623	aagtcaccttggttcaccaccctg	KSPWFTTL
<i>Emv-3D</i>
<i>Emv-3R</i>
<i>Emv-15</i>
BM5eco	.g.....	R.....
(b) MA125-132		
AKR623	cgatctgccctttaccctgctctt	RSALYPAL
<i>Emv-3D</i>
<i>Emv-3R</i>
<i>Emv-15</i>
BM5ecoc...t..F
(c) RT142-149		
AKR623	tcccaccggtggtatactgtgctt	SHRWYTVL
<i>Emv-3D</i>
<i>Emv-3R</i>
<i>Emv-15</i>
BM5ecoa.....c.....c	..Q.....
(d) RT456-463		
AKR623	cgcatgaccactaccaggcaatg	RMTHYQAM
<i>Emv-3D</i>
<i>Emv-3R</i>
<i>Emv-15</i>
BM5eco

Fig. 1. Nucleotide and predicted amino acid sequences of CTL epitopes encoded by various *Emv* proviruses.

RT456-463 and TM134-141 stimulated CTL that lysed E δ G2 and SL1 (AKR/Gross virus-positive) tumour targets, as well as SC.Kb cells expressing any of the MuLV tested except BM5eco, which were not lysed by any of the peptide-stimulated effectors. Although RT142-149-stimulated lysis was usually higher than in the experiment shown, this peptide was generally somewhat less active than the others, particularly with respect to SC.KbEmv3R targets. Three other peptides fitting the H-2K^b motif and derived from the AKR623 capsid or surface proteins were unable to restimulate AKR/Gross virus-specific CTL *in vitro*. This inability of peptides CA114-121, SU158-165 and SU319-326 to stimulate CTL was consistent with their inability to sensitize uninfected target cells for lysis by anti-AKR/Gross virus CTL (White *et al.*, 1994a).

These functional studies suggested that peptides MA125-132, RT142-149, RT456-463 and TM134-141 were encoded by all *Emv* tested, but not the BM5eco virus. To test this hypothesis, relevant regions of the viral genomes were sequenced and compared to the published sequence of AKR623 (Herr, 1984). Fig. 1(a) shows that the region encoding the immunodominant epitope, TM134-141, of AKR623 was found to be identical in *Emv-3D*, *Emv-3R* and *Emv-15*. In contrast, the BM5eco genome contained a single nucleotide difference within codon 1, resulting in the substitution of arginine for lysine. With respect to MA125-132, DNA sequencing revealed a single amino acid substitution in the BM5eco MuLV, changing a leucine to a phenylalanine at position

8 (Fig. 1b). All other *Emv* examined encoded the AKR623 form of this peptide. We also found that all of these *Emv*, except BM5eco, were identical to AKR623 in the region encoding RT142-149 (Fig. 1c). The BM5eco genome carried an altered codon 3, resulting in an arginine to glutamine change. The nucleotide sequence of the region encoding RT456-463 was found to be the same for all *Emv* proviruses examined (Fig. 1d). The data in Fig. 1 suggest that the lack of lysis of BM5eco-infected cells by anti-AKR/Gross virus CTL (Tables 1 and 2) may have resulted from the alteration of the immunodominant CTL epitope and two of three minor epitopes, and that the expression of these epitopes is conserved among many *Emv*.

We have further analysed the immunogenicity of the BM5eco virus in several ways: firstly, several attempts to generate either K^b- or D^b-restricted anti-viral CTL by *in vivo* immunization and *in vitro* restimulation with cells infected with BM5eco have failed, although K^b-restricted anti-viral CTL could be generated by this protocol when AKR623-infected cells were used (T. Lam & W. Green, unpublished observations). Secondly, because the immunodominant CTL epitope is derived from the MuLV envelope protein, we determined the nucleotide sequence of the entire BM5eco envelope gene (R. Strawbridge & M. Coppola, unpublished data). When the deduced amino acid sequence was searched for potential CTL epitopes using the published K^b and D^b motifs (Falk *et al.*, 1991), TM134-141 was the only potential K^b-restricted epitope found to differ from AKR623. Furthermore, no K^b or D^b motif peptides not present in the AKR623 envelope protein were found. We did find changes at non-anchor positions in two potential D^b epitopes. The relevance of these changes is questionable, however, since we have never observed D^b-restricted CTL responses to this type of MuLV (Green *et al.*, 1980; White *et al.*, 1990), although others have reported, but not extensively characterized, such CTL (Plata *et al.*, 1987). Thirdly, we have directly tested the immunogenicity of the BM5eco-encoded version of the immunodominant epitope using recombinant viral vaccines.

Fig. 2 shows that spleen cells primed *in vivo* with TM134R-141Vac and stimulated *in vitro* with SIN:TM134R-141 did not lyse targets infected with TM134R-141Vac (and K^bVac) any more efficiently than the negative control effectors (VV-primed and Sindbis virus-stimulated) lysed negative control targets (K^bVac only). The low level of activity that we did see may be VV-specific, since CTL from all of the rVV-primed cultures lysed targets infected with only K^bVac to a similar extent (data not shown). Thus, the allelic form of the immunodominant AKR623 epitope encoded by BM5eco did not appear to be immunogenic under these

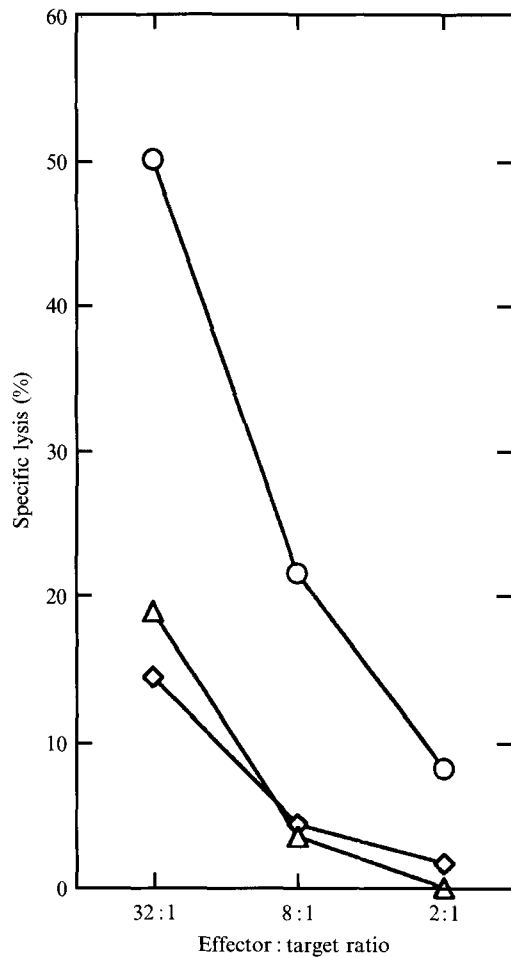


Fig. 2. Recombinant virus vectors encoding the peptide RSPWF^TTLL fail to induce epitope-specific CTL from B6 mice. '○', Cytolytic activity of TM134K-141Vac-primed, SIN:TM134K-141-stimulated cultures against ⁵¹Cr-labelled HuTK-143B targets infected with TM134K-141Vac and K^bVac; '△', activity of TM134R-141Vac-primed, SIN:TM134R-141-stimulated cultures against targets infected with TM134R-141Vac and K^bVac; '◇', activity of control VV-primed, control Sindbis virus-stimulated cultures against targets infected with K^bVac only. Two similar experiments also gave the same pattern of lysis.

conditions. The vectors expressing KSPWF^TTLL, however, are immunogenic. That is, TM134K-141Vac-primed, SIN:TM134K-141-stimulated spleen cells were able to lyse targets doubly infected with TM134K-141Vac and K^bVac. Mice immunized with any of the rVV generated vigorous secondary VV-specific responses upon *in vitro* restimulation with VV, indicating that *in vivo* priming was effective even in the TM134R-141Vac-primed group (data not shown).

Discussion

The experiments reported here show that anti-AKR/Gross virus CTL, whether stimulated by viral antigen-positive tumours or by synthetic peptides representing

four CTL epitopes, can lyse cells infected with several distinct Emv. Because the *Emv* loci are independent proviral integrations of closely related MuLV, it may well be that expression of these CTL epitopes is conserved among most of the Emv. However, we have previously reported that at least one member of this family, *Emv-14*, encodes a virus that is not recognized by AKR/Gross virus-specific CTL (Green & Graziano, 1986; White *et al.*, 1990).

We found that the genomes of CTL-susceptible Emv all encode four K^b-restricted CTL epitopes identical to those of AKR623 (Fig. 1). Conversely, three of the four epitopes are altered in the CTL-resistant BM5eco virus. The leucine to phenylalanine change found at position 8 of the peptide MA125-132 (Fig. 1*b*) probably prevents the presentation of the BM5eco MuLV form of this peptide by H-2K^b, since high-affinity binding of peptides to K^b is primarily determined by 'anchor' residues at positions 5 and 8 (Falk *et al.*, 1991). Although peptides containing methionine, valine and isoleucine, as well as leucine have been reported to bind K^b, C-terminal amino acids with aromatic side chains have not been observed. The arginine to glutamine change at position 3 of RT142-149 (Fig. 1*c*) may also affect presentation of the peptide, since this position is considered by some to be a 'secondary' anchor for K^b binding (Falk *et al.*, 1990; Matsumura *et al.*, 1992).

In contrast to these altered CTL epitopes, we found no changes in the BM5eco-encoded RT456-463 (Fig. 1*d*). It is not clear why this apparently intact epitope does not result in lysis of BM5eco-infected cells by AKR/Gross virus-specific CTL. It may be that BM5eco-infected cells fail to process and present this epitope with sufficient efficiency to trigger T cell recognition. However, other interpretations are also possible and further experiments are needed to address this seemingly paradoxical result.

Unlike the changes at either dominant or secondary anchor residues seen in the other altered peptides, the arginine for lysine substitution that we found at position 1 of TM134-141 in BM5eco (Fig. 1*a*) would not be expected to prevent binding to K^b. In fact, an immunodominant K^b-restricted epitope from vesicular stomatitis virus also has an arginine at its N terminus (VanBleek & Nathenson, 1990). Moreover, Sijs *et al.* (1994) have reported that the binding of KSPWF^TTLL and RSPWF^TTLL to K^b on the surface of live cells is similar. Position 1 is, however, considered to be important for T cell receptor (TCR) interaction with K^b-peptide complexes (Matsumura *et al.*, 1992) and even this relatively conservative change could prevent recognition of BM5eco virus-infected cells by AKR/Gross virus-specific CTL. We have also recently shown that these CTL lyse cells infected with the highly leukaemogenic recombinant MCF247, but not MCF13 MuLV (Coppola

& Green, 1994). The nucleotide sequence of MCF13 was determined and, like BM5eco, encodes arginine at position 1 of this epitope, while the published sequence of MCF247 is identical to those of AKR623, *Emv-3* and *Emv-15* in this region (Kelly *et al.*, 1983).

The lack of immunogenicity of RSPWF^bTTL in our experiments (Fig. 2) appears to be in contrast with the recent report of Sijts *et al.* (1994), who showed that immunization with either peptide KSPWF^bTTL or RSPWF^bTTL in incomplete Freund's adjuvant followed by *in vitro* restimulation with the same peptide yielded epitope-specific, K^b-restricted CTL. Interestingly, although CTL generated in this way recognize both peptides equally well and lyse cells expressing MuLV that encode KSPWF^bTTL, they fail to lyse a tumour induced by Rauscher MuLV, which encodes the RSPWF^bTTL peptide (Sijts *et al.*, 1994). These authors therefore argue that the arginine to lysine substitution interferes with processing of the peptide. Alternatively, it is possible that H-2^b mice lack CTL precursors with sufficient affinity for K^b-RSPWF^bTTL to be activated by the levels of these complexes present on infected cells and that lower affinity T cells are activated only after peptide immunization. However, in accordance with Sijts *et al.* (1994), our data can also be viewed as supporting the hypothesis that RSPWF^bTTL expressed inside the cell is not efficiently presented at the cell surface.

Most peptides recognized by CTL arise from degradation of proteins on cytosolic proteosomes which generate short peptides that are translocated into the lumen of the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP) (for review see Monaco, 1992). The viral vectors we employed should express the peptides with only a single amino acid (the initiator methionine) needing to be removed to generate the minimal epitope. This could presumably occur in the cytoplasm or in the ER, either before or after binding to class I MHC. In any case, the approach we have used here obviates the need for extensive proteolysis to generate the antigenic peptide. Thus, although the lack of recognition of RSPWF^bTTL by CTL raised against MuLV-induced tumours is clearly explained for the most part by the specificity of TCR recognition (Sijts *et al.*, 1994; White *et al.*, 1994a), the present results raise the possibility that the lack of immunogenicity of RSPWF^bTTL expressed within the cell may result from a failure of the peptide to reach the ER and complex with nascent K^b molecules. At the very least, our data suggest that if RSPWF^bTTL is, in fact, not presented efficiently, the 'defect' lies in a post-proteosome step in antigen processing, possibly involving TAP. It is also worth noting that the immunodominant peptide in this system is one of only two CTL epitopes known to be derived from the membrane-spanning portion of a

protein (the other was described in Braciale *et al.*, 1987), so a possible role for alternative pathways of MHC class I antigen presentation (Henderson *et al.*, 1992; Hammond, *et al.*, 1993) must also be considered.

Our findings also indicate that the resistance of BM5eco to lysis by K^b-restricted, *Emv*-specific CTL may be the result of immune selection of CTL epitope-variant viruses, since LP-BM5 was passaged *in vivo* in mice of the H-2^b haplotype for many years before its component viruses were biologically and molecularly cloned (Yetter *et al.*, 1988; Chattopadhyay *et al.*, 1991). B6 and other H-2^b strains are highly susceptible to MAIDS (Makino *et al.*, 1990), while H-2^d or H-2^a mice are resistant. The correlation between disease resistance and class I MHC genes (Makino *et al.*, 1990) suggests that H-2^d and H-2^a, but not H-2^b, mice may generate protective LP-BM5 virus-specific CTL responses. In keeping with this hypothesis, our laboratory has recently shown that MAIDS-resistant BALB/cByJ and C57BL/KsJ (H-2^d), but not MAIDS-susceptible B6 or BALB/B (H-2^b), mice generate CTL that lyse target cells expressing the defective or BM5eco Gag antigen (Schwarz & Green, 1994). Thus, MAIDS-susceptible H-2^b strains may be non-responders to both Gag and Env antigens of the LP-BM5 viruses. This failure to mount CTL responses to the BM5eco virus may contribute significantly to the sensitivity of B6 mice to LP-BM5-induced MAIDS. Whatever the precise mechanisms underlying the markedly reduced immunogenicity of BM5eco, the potential role of CTL-mediated immunoselection may now be addressed directly in *in vivo* studies using MuLV engineered to differ only within a single CTL epitope.

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References

- AZIZ, D., HANNA, Z. & JOLICOEUR, P. (1989). Severe immunodeficiency disease induced by a defective murine leukaemia virus. *Nature* **338**, 505-508.
- BRACIALE, T. J., BRACIALE, V. L., WINKLER, M., STROYNOWSKI, I., HOOD, L., SAMBROOK, J. & GETHING, M.-J. (1987). On the role of the transmembrane anchor sequence of influenza hemagglutinin in target cell recognition by class I MHC-restricted, hemagglutinin-specific cytolytic T lymphocytes. *Journal of Experimental Medicine* **166**, 678-692.
- CHATTOPADHYAY, S., SENGUPTA, D., FREDRICKSON, T., MORSE, H. & HARTLEY, J. (1991). Characteristics and contributions of defective, ecotropic and mink cell focus-inducing viruses involved in a

- retrovirus-induced immunodeficiency syndrome of mice. *Journal of Virology* **65**, 4232–4241.
- COPELAND, N., BEDIGIAN, H., THOMAS, C. & JENKINS, N. (1984). DNAs of two molecularly cloned endogenous ecotropic proviruses are poorly infectious in DNA transfection assays. *Journal of Virology* **49**, 437–444.
- COPPOLA, M. A. & GREEN, W. R. (1994). Cytotoxic T lymphocyte responses to the envelope proteins of endogenous ecotropic and mink cytopathic focus forming murine leukemia viruses in H-2^b mice. *Virology* **202**, 500–505.
- FALK, K., ROTZSCHKE, O., STEVANOVIC, S., JUNG, G. & RAMENSEE, H. (1991). Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. *Nature* **351**, 290–296.
- GREEN, W. R. (1982). The *in vitro* generation of H-2-restricted cytotoxic T cells to AKR/Gross leukemia virus-induced tumors. I. Requirement for stimulation with allogeneic leukemia cells *in vivo*. *Journal of Immunology* **128**, 1043–1049.
- GREEN, W. R., NOWINSKI, R. & HENNEY, C. (1979). The generation and specificity of cytotoxic T cells raised against syngeneic tumor cells bearing AKR/Gross murine leukemia virus antigens. *Journal of Experimental Medicine* **150**, 51–66.
- GREEN, W. R., NOWINSKI, R. & HENNEY, C. (1980). Specificity of cytolytic T cells directed against AKR/Gross virus-induced syngeneic leukemias: antibodies directed against H-2K, but not against viral proteins, inhibit lysis. *Journal of Immunology* **125**, 647–655.
- GREEN, W. R. & GRAZIANO, R. (1986). Cytolytic T lymphocyte-defined retroviral antigens on normal cells encoding by the Akv-1 proviral locus. *Immunogenetics* **23**, 106–110.
- HAMMOND, S. A., BOLLINGER, R. C., TOBERY, T. W. & SILICIANO, R. F. (1993). Transporter-independent processing of HIV-1 envelope protein for recognition by CD8⁺ T cells. *Nature* **364**, 158–161.
- HENDERSON, R. A., MICHEL, H., SAKAGUCHI, K., SHABANOWITZ, J., APPELLA, E., HUNT, D. F. & ENGELHARD, V. H. (1992). HLA-A2.1-associated peptides from a mutant cell line: a second pathway of antigen presentation. *Science* **255**, 1264–1266.
- HERR, W. (1984). Nucleotide sequence of AKV murine leukemia virus. *Journal of Virology* **49**, 471–478.
- JENKINS, N. A., COPELAND, N. G., TAYLOR, B. A. & LEE, B. K. (1982). Organization, distribution, and stability of endogenous ecotropic murine leukemia virus DNA in chromosomes of *Mus musculus*. *Journal of Virology* **43**, 26–36.
- JORGENSEN, E. C., KJELDGAARD, N. O., PEDERSEN, F. S. & JORGENSEN, P. (1988). A nucleotide substitution in the gag N-terminus of the endogenous ecotropic DBA/2 virus prevents Pr65gag myristylation and virus production. *Journal of Virology* **62**, 3217–3223.
- KELLY, M., HOLLAND, C. A., LUNG, M. L., CHATTOPADHYAY, S. K., LOWY, D. R. & HOPKINS, N. H. (1983). Nucleotide sequence of the 3' end of MCF247 murine leukemia virus. *Journal of Virology* **45**, 291–298.
- LATARJET, R. & DUPLAN, J. F. (1962). Experiment and discussion on leukemogenesis by cell-free extracts of radiation-induced leukemia in mice. *International Journal of Radiation Biology* **5**, 223–233.
- LILLY, F., DURAN-REYNALS, M. L. & ROWE, W. P. (1975). Correlation of early MuLV titer and H-2 type with spontaneous leukemia in mice of the BALB/c × AKR cross: a genetic analysis. *Journal of Experimental Medicine* **141**, 882–889.
- MAKINO, M., MORSE, H. C. M., III, FREDRICKSON, T. N. & HARTLEY, J. W. (1990). H-2-associated and background genes influence the development of a murine retrovirus-induced immunodeficiency syndrome. *Journal of Immunology* **144**, 4347–4352.
- MATSUMURA, M., FREMONT, D. H., PETERSON, P. A. & WILSON, I. A. (1992). Emerging principles of the recognition of peptide antigens by MHC class I molecules. *Science* **257**, 927–934.
- MONACO, J. J. (1992). A molecular model of MHC class-I-restricted antigen processing. *Immunology Today* **13**, 173–179.
- PLATA, F., LANGLADE-DEMOYEN, P., ABASTADO, J. P., BERBAR, T. & KOURILSKY, P. (1987). Retrovirus antigens recognized by cytolytic T lymphocytes activate tumor rejection *in vivo*. *Cell* **48**, 231–240.
- SCHWARZ, D. A. & GREEN, W. R. (1994). CTL responses to the gag polyprotein encoded by the murine AIDS defective retrovirus are strain dependent. *Journal of Immunology* **153**, 436–441.
- SIJTS, A. J. A. M., OSSENDORP, F., MENGEDÉ, E. A. M., VAN DEN ELSEN, P. J. & MELIEF, C. J. M. (1994). Immunodominant mink cell focus-inducing murine leukemia virus (MuLV)-encoded CTL epitope, identified by its MHC class I-binding motif, explains MuLV-type specificity of MCF-directed cytotoxic T lymphocytes. *Journal of Immunology* **152**, 106–116.
- SIRACUSA, L., RUSSELL, L., JENKINS, N. & COPELAND, N. (1987). Allelic variation within the *Emv-15* locus defines genomic sequences closely linked to the agouti locus on mouse chromosome 2. *Genetics* **117**, 85–92.
- VANBLEEK, G. M. & NATHENSON, S. G. (1990). Isolation of an endogenously processed immunodominant viral peptide from the class I H-2K^b molecule. *Nature* **348**, 213–216.
- WHITE, H. D., ROBBINS, M. R. & GREEN, W. R. (1990). Mechanism of escape of endogenous murine leukemia virus Emv-14 from recognition by anti-AKR/Gross virus cytolytic T lymphocytes. *Journal of Virology* **64**, 2608–2619.
- WHITE, H. D., ROEDER, D. A. & GREEN, W. R. (1994a). An immunodominant K^b-restricted peptide from the p15E transmembrane protein of endogenous ecotropic murine leukemia virus (MuLV). AKR623 that restores susceptibility of a tumor line to anti-AKR/Gross MuLV cytotoxic T lymphocytes. *Journal of Virology* **68**, 897–904.
- WHITE, H. D., ROEDER, D. A., LAM, T. & GREEN, W. R. (1994b). Major and minor K^b-restricted epitopes encoded by the endogenous ecotropic murine leukemia virus AKR623 that are recognized by anti-AKR/Gross MuLV CTL. *Viral Immunology* **7**, 51–59.
- YETTER, R. A., BULLER, R., LEE, J. S., ELKINS, K., MOSIER, D. E., FREDRICKSON, T. N. & MORSE, H. C. (1988). CD4⁺ T cells are required for the development of a murine retrovirus-induced immunodeficiency syndrome (MAIDS). *Journal of Experimental Medicine* **168**, 623–635.

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