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

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H-2^b tumor cells expressing the endogenous ecotropic murine leukemia virus (EMV) induce an anti-AKR/Gross murine leukemia virus (MuLV) cytotoxic T-lymphocyte (CTL) response in the C57BL/6 mouse strain. The EMV clone AKR623 has been used to infect SC.K^b/623 fibroblast cells, resulting in SC.K^b/623 targets that are lysed by bulk anti-AKR/Gross MuLV CTL with a profile that is similar to that for the EMV⁺ AKR.H-2^b SL1 tumor target. Anti-AKR/Gross MuLV CTL are restricted by the class I K^b antigen and do not cross-react with Friend-Moloney-Rauscher virus-positive targets. The AKR623 genome was searched by computer for coding sequences that fit the motif XXXX(FY)XX(VIML) for peptides that bind K^b . Of 30 octameric peptides identified, 12 that were unique to AKR623 and different from published Friend-Moloney-Rauscher sequences were synthesized and bound to EMV-negative SC.K^b cells, which were then assayed as targets against anti-AKR/Gross MuLV CTL. One peptide, peptide 12 (KSPWF^TTTL) from the p15E transmembrane protein, sensitized SC.K^b target cells to lysis by anti-AKR/Gross MuLV CTL with a profile similar to those seen for AKR.H-2^b SL1 tumor targets and SC.K^b/623 fibroblast targets. Low concentrations of peptide were sufficient, the half-maximal lysis occurring at 10 to 100 pg/ml. SC.K^b/peptide 12 targets were recognized by the *H-2^b*-restricted bulk CTL in a conventional class I K^b -restricted fashion. Unlabeled SC.K^b/peptide 12-pulsed targets were effective in competing with radiolabeled SC.K^b/623 targets for lysis by anti-AKR/Gross MuLV CTL. This finding is consistent with the notion that peptide 12 represents the dominant endogenously processed epitope recognized by these antiviral CTL. In addition, peptide 12 is immunogenic in that it could stimulate the *in vitro* generation of an anti-AKR/Gross MuLV CTL response from tumor-primed C57BL/6 responder spleen cells. Finally, the physiological relevance of peptide 12 was suggested by its ability to fully restore the recognition and lysis of AKR.H-2^b SL1 clone 18-5 tumor cells, a naturally occurring variant tumor clone that is insusceptible to lysis by anti-AKR/Gross MuLV CTL. These data indicate that a virus-encoded antigen, represented by peptide 12, and not a nonviral tumor antigen, is the immunodominant epitope responsible for the recognition of EMV⁺ tumor cells by C57BL/6-derived anti-AKR/Gross MuLV CTL.

The AKR mouse strain has a high spontaneous incidence of T-cell lymphoma that correlates with the early expression of endogenous ecotropic murine leukemia virus (AKR/Gross murine leukemia virus [MuLV] or EMV), despite the proximal etiologic agent being acknowledged as the mink cell cytopathic focus-inducing (MCF) MuLV, a recombinant between EMV and endogenous xenotropic and polytropic MuLV sequences. Previous studies from our laboratories have demonstrated the ability to raise type-specific AKR/Gross MuLV-specific cytotoxic T lymphocytes (CTL) that do not recognize tumors induced by or cells infected with the Friend-Moloney-Rauscher (FMR) group of MuLVs (8). The generation of anti-AKR/Gross MuLV CTL was readily accomplished in mouse strains of the *H-2^b* haplotype, which are relatively resistant to Gross virus-induced leukemogenesis. In contrast, in *H-2^k* strains, including AKR and others with a high incidence of spontaneous leukemia and/or high susceptibility to Gross

virus-induced leukemia, little or no antiviral CTL activity could be generated (10, 26). In addition, the induction of anti-AKR/Gross MuLV CTL is correlated with protection against EMV-associated tumor growth in the AKR.H-2^b and AKR.H-2^b:Fv-1^b congenic strains of mice (2). *H-2K^b* has been defined as the class I restriction element mediating recognition by these CD8⁺ anti-AKR/Gross MuLV CTL (13), and the *emv-11* locus is known to be important as well (11).

To better understand the leukemogenic process associated with EMV, it would be helpful to know the immunologic epitope(s) presented by *H-2K^b* that are required to induce an anti-AKR/Gross MuLV CTL response. Furthermore, it has not been clear whether the epitopes bound within the K^b groove are virus encoded or are nonviral tumor-associated antigens.

The identification of various class I binding antigens has been made easier by the elucidation of the corresponding peptide binding motifs (4). Using the K^b motif XXXX(Y F)XX(VIML), we now identify and immunologically characterize an octameric peptide, KSPWF^TTTL (peptide 12), encoded within the p15E region of the *emv-11*-derived clone AKR623 (20) that confers high levels of lysis by anti-AKR/Gross MuLV CTL at low concentrations (half-maximal lysis at

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TABLE 1. AKR623-encoded peptides (peptides 1 to 12) that fit the K^b motif but differ from Moloney MuLV

Peptide	Sequence	Location ^a
1	RSALYPAL	p15/p12 (MA/?) ^b
2	HLVLYRQL	p30 (CA)
3	SHRWYTVL	p80 (RT)
4	RMTHYQAM	p80 (RT)
5	DQFVFELL	p46 (IN)
6	THLGYSQM	p46 (IN)
7	GESPYMYL	p46 (IN)
8	WEIDFTEV	p46 (IN)
9	SSWDYITV	gp70 (SU)
10	VKGAYQAL	gp70 (SU)
11	QQGWFEGL	p15E (TM)
12	KSPWFTTL	p15E (TM)
13 (Mo) ^c	RSPWFTTL	p15E (TM)
K^b		
Consensus ^d	XXXX(FY)XX(VIML)	

^a Retrovirus protein designations: MA, matrix; CA, capsid; RT, RTase; IN, integration; SU, surface; TM, transmembrane.

^b The two-letter designation for p12 has not been given, since protein function has not been elucidated.

^c From reference 28. Mo, Moloney.

^d As determined by Falk et al. (4).

10 to 100 pg/ml) on EMV-negative $H-2K^b$ target cells. In addition to acting at the target level, peptide 12 is immunogenic, stimulating an EMV-specific CTL response when added to in vitro cultures of primed C57BL/6 spleen cells. Furthermore, peptide 12 is able to restore the lysis of AKR.H-2^b SL1 clone 18-5 tumor targets, a CTL-insusceptible variant line cloned from the AKR.H-2^b SL1 tumor line which is highly susceptible to lysis by anti AKR/Gross MuLV CTL. Taken together, the data strongly support the contention that peptide 12 represents an immunodominant epitope recognized by anti-AKR/Gross MuLV CTL and that the predominant response mediated by these CTL is directed against a virus-encoded antigen, not a nonviral tumor antigen.

MATERIALS AND METHODS

Peptide synthesis. Peptides 1 to 13 (Table 1) were synthesized by Research Genetics (Huntsville, Ala.), using an Advanced Chemtech model 350 synthesizer. The amino acid content of each peptide was analyzed by high-pressure liquid chromatography and by mass spectroscopy using the electrospray technique. All peptides were stored desiccated at -20°C in the solid phase, small aliquots being weighed out on the day of the experiment. This was done to minimize the degradation of unstable residues such as tryptophan in peptide 12, for example.

Cells. B.GV (25) and E δ G2 (12) are $H-2^b$ Gross MuLV cell surface antigen (GCSA)-positive tumor cell lines derived from a BALB.B congenic mouse tumor and a C57BL/6 tumor, respectively, both induced with Gross passage A virus. E δ K1 is a GCSA⁻ $H-2^b$ tumor cell line derived from a C57BL/6 tumor induced with AKR virus and obtained from E. Gomard, Laboratoire d'Immunologie et de Virologie des Tumeurs, Hôpital Cochin, Paris, France (7). AKR.H-2^b SL1 (SL1) is a GCSA⁺ tumor cell line derived from a spontaneous leukemia from an AKR.H-2^b congenic mouse (9, 12). AKR.H-2^b SL1 clone 18-5 (cl.18-5) is a naturally occurring CTL-insusceptible variant clone derived from the SL1 line by limiting dilution (9, 12). YAC-1 (YAC) is an $H-2^a$ tumor cell line that is used here as a highly natural killer cell-sensitive target cell and as an $H-2$ haplotype-specific control for anti- $H-2^b$ allogeneic CTL. The

hybrid cell line LB27.4 (ATCC HB99) was produced by fusing a BALB/c lymphoma line ($H-2^d$) to T-cell-depleted spleen cells of a C57BL/10 ($H-2^b$) mouse. 1710.5 and 1153 are $H-2^b$ murine AIDS (MAIDS) virus-positive tumor cell lines (19). All tumor cell lines (B.GV, E δ G2, E δ K1, SL1, cl.18-5, YAC, LB27.4, 1710.5, and 1153) were grown in supplemented medium (RPMI plus 5% fetal bovine serum, 2 mM L-glutamine, antibiotics, and 50 mM 2-mercaptoethanol). The CTL clonal lines G8 (1) and 0.3.1 (3) are AKR/Gross virus specific and MAIDS virus specific, respectively, and were cultured in supplemented medium at 10% fetal bovine serum plus 100 mM nonessential amino acids solution (GIBCO), 1 mM sodium pyruvate, and 15 U of interleukin-2 per ml. Every 2 weeks, the CTL clones were fed and restimulated in a 5-ml volume with 2×10^6 C57BL/6 spleen cells irradiated at 2,000 rads and 10^6 E δ G2 tumor cells (for G8) or 1710.5 tumor cells (for 0.3.1) irradiated at 8,000 rads. SC.K^b is a fibroblast line derived from the feral mouse embryo-derived SC-1 cell line and transfected with the K^b gene (31). SC.K^b/623 was generated by infecting SC.K^b cells (31) with the AKR623 virus (20). Fibroblast cell lines (SC.K^b and SC.K^b/623) were grown in supplemented Dulbecco modified Eagle medium except with 10% fetal bovine serum and without 2-mercaptoethanol. All cells were maintained at 37°C in a 5% CO_2 atmosphere.

CTL assay. Anti- $H-2^d$ allogeneic CTL were generated by culturing C57BL/6 spleen responders with irradiated LB27.4 ($H-2^{bxd}$) or BALB/c spleen stimulator cells at a responder-to-stimulator cell ratio of 50:1 or 2:1 for 6 days. For the generation of anti-AKR/Gross MuLV CTL, C57BL/6 mice were immunized intraperitoneally with 10^6 B.GV cells. Ten days later, the spleen cells were harvested and cultured with media (negative controls) or with irradiated (8,000 rads) E δ G2 or SL1 stimulator cells at a responder-to-stimulator cell ratio of 50:1. Peptides were used at the stimulation level by culturing with an excess of the indicated peptide (final concentration of 10 $\mu\text{g/ml}$ or as indicated) in the presence or absence of E δ K1-presenting cells. In other experiments using E δ K1 plus peptide as stimulators, the excess peptide was washed away before culturing, with similar results. For both the standard CTL and the allogeneic CTL, after 6 days in culture, effector cells were harvested and incubated with ^{51}Cr -labeled target cells at various effector-to-target cell (E:T) ratios at 37°C , 6 h for fibroblast targets or 4 h for tumor targets. The fibroblast targets were used as adherent cells, being plated the day before the chromium release assay. Peptides were added at the time of fibroblast target plating and excess peptide was washed out the next day, or (for some fibroblast targets and for all experiments with E δ K1 targets) the final concentration of peptide was added the day of the chromium release assay. In the latter case, the peptides were preincubated with the targets for 30 to 60 min before the effectors were added.

Unlabeled target competition experiments. Radiolabeled SC.K^b/623 targets were plated the day before the chromium release assay for a final density of 2×10^3 cells per well. On the day of the assay, unlabeled SC.K^b cells were incubated with an excess of peptide (10 $\mu\text{g/ml}$) at 37°C for 30 min in a 5% CO_2 atmosphere. The unbound peptide was then washed out before addition of the unlabeled target in various numbers to the radiolabeled SC.K^b/623 target. Finally, standard bulk antiviral effectors (described above) were added at an E:T ratio of 50:1.

Negative peptide competition experiments. Peptides that were unable to sensitize SC.K^b targets for lysis by anti-AKR/Gross MuLV CTL at concentrations as high as 10 $\mu\text{g/ml}$ were added at decreasing concentrations either to adherent (pre-plated) SC.K^b radiolabeled targets in some experiments or to E δ K1 radiolabeled targets in other experiments. Following

incubation for 30 to 40 min at 37°C in a 5% CO₂ atmosphere, peptide 12 was added to a final concentration of 100 pg/ml, and the target cells were incubated at 37°C in a 5% CO₂ atmosphere for a further 30 min before addition of the standard bulk antiviral CTL effectors (described above) at an E:T ratio of 50:1.

RESULTS

Low concentrations of a peptide (peptide 12) encoded by the p15E region of the *env* gene sensitize SC.K^b targets for lysis by anti-AKR/Gross MuLV CTL. The approach used to define the molecular specificity of K^b-restricted anti-AKR/Gross MuLV CTL was based on definition of the K^b binding motif XXXX (FY)XX(VIML) by Falk et al. (4). Anti-AKR/Gross MuLV CTL are specific for EMV⁺ tumor targets and do not recognize targets that express the FMR MuLV (8). SC.K^b/623 target cells express virus derived from the sequenced *env*-11-like clone AKR623 and are recognized by anti-AKR/Gross MuLV CTL, similar to EMV⁺ tumor targets. It was therefore reasonable to first consider those AKR623 sequences that fit the K^b motif but differed from the Moloney sequences. A computer search revealed that AKR623 (14) encodes 30 peptides potentially capable of binding to K^b: 2 within *gag*, 19 within *pol*, and 9 within *env*. Twelve peptides, numbered 1 to 12 (Table 1) and representing the 12 AKR623-unique motifs, were synthesized and tested for the ability to sensitize SC.K^b (EMV⁻) targets for lysis by anti-AKR/Gross MuLV CTL.

Figure 1 shows the sensitivity of targets to CTL after incubation with various concentrations of these peptides. Of the 12 peptides, only peptide 12, encoded by the p15E region of the *env* gene, conferred high levels of lysis at low concentrations of peptide. The concentration of peptide effecting half-maximal lysis was 10 to 100 pg/ml. It is noteworthy that the levels of lysis achieved with peptide 12 were similar to those seen with SC.K^b/623 or SL1 tumor targets. This experiment was performed five times, and similar results were obtained regardless of whether the *in vitro* stimulation was with E δ G2 or SL1 tumor cells. Positive and negative control tumor targets (SL1, cl.18-5, and YAC) were always used to verify that the CTL were typical of anti-AKR/Gross MuLV CTL and not NK cells, for example.

We then tested whether the peptide encoded by the analogous region of the p15E portion of the *env* gene of the Moloney MuLV was recognized under the same assay conditions. As expected, the Moloney peptide (peptide 13), which differs only at position 1 (Lys→Arg; Table 1), required peptide concentrations approximately 3 orders of magnitude greater than those required for the AKR623-derived sequence to obtain significant, although minimal, levels of lysis (Fig. 1B). The difference in susceptibility to lysis conferred by the Moloney peptide relative to peptide 12 is consistent with the earlier observation that FMR MuLV-positive tumors are not recognized by polyclonal anti-AKR/Gross MuLV CTL generated as we have previously described (8).

Peptide 12 is a dominant peptide recognized at the target level by anti-AKR/Gross MuLV CTL. Although peptide 12 uniquely sensitizes EMV⁻ targets for lysis and at low concentrations, it was possible that this peptide is not the major determinant recognized by anti-AKR/Gross MuLV CTL. For example, there are reports of peptides that confer lysis by CTL but do not fit the relevant class I motif (30); therefore, it is possible that the search strategy used to find peptides 1 to 12 within the AKR623 genome failed to identify some other peptide that is important for anti-AKR/Gross MuLV CTL recognition. We considered this possibility unlikely, since

FIG. 1. Peptide-induced sensitization of SC.K^b cells for lysis by anti-AKR/Gross MuLV CTL. As described in Materials and Methods, the bulk anti-AKR/Gross MuLV CTL were generated as follows. C57BL/6 mice were primed with B.GV tumor cells *in vivo*. Ten days later, the responder cells were harvested and stimulated in culture with irradiated SL1 tumor cells (for the data shown). The radiolabeled chromium release assay was performed 6 days later, using adherent SC.K^b target cells with peptide titrated in at concentrations as indicated in the figure. The peptides used are defined in Table 1. An E:T ratio of 100:1 was used for the titrations. (A) Titrations using AKR623-specified peptides; (B) titrations comparing peptide 12 with the Moloney (Mo) virus equivalent peptide 13. The chromium release assay was performed as specified in Materials and Methods.

peptide 12 confers lysis at such low concentrations (half-maximal lysis at 10 to 100 pg/ml) and the levels of lysis are similar to those seen for SC.K^b/623 targets, in which the viral antigen(s) are endogenously synthesized, processed, and presented. Nonetheless, it was important to determine the relevance of peptide 12, in comparison with the endogenously synthesized, processed, and transported K^b-bound antigen(s) found on AKR623 positive targets, by measuring the ability of unlabeled SC.K^b/peptide 12 (or, as a control, peptide 2)-pulsed targets to compete with radiolabeled SC.K^b/623 targets for standard polyclonal anti-AKR/Gross MuLV CTL.

As can be seen in Fig. 2, over a range of unlabeled/labeled target cell ratios, unlabeled SC.K^b/peptide 12 targets are essentially as effective as unlabeled SC.K^b/623 targets in competing against radiolabeled SC.K^b/623 targets for recognition by anti-AKR/Gross MuLV CTL. Similar results were obtained in three of three experiments. That the competition is specific for peptide 12 is shown by the inability of SC.K^b targets bound with a representative negative peptide (peptide 2, shown in

FIG. 2. Competition of unlabeled peptide 12- or peptide 2-bound targets with radiolabeled AKR623-infected SC.K^b targets. Radiolabeled SC.K^b/623 targets were plated so they were at a density of 2×10^3 per well the day of the chromium release assay. Unlabeled competing targets consisted of SC.K^b (negative control), SC.K^b bound with an excess of peptide 2 or 12, or SC.K^b/623 (positive control). Peptide was bound at 10 μ g/ml for 30 min, and the excess was washed out before plating onto the radiolabeled targets at the labeled/unlabeled target cell (T:T*) ratios indicated. Anti-AKR/Gross MuLV effectors (data from SL1-stimulated effectors are shown) were then added at an effector/labeled target cell ratio of 50:1. Details for generating the anti-AKR/Gross MuLV CTL and performing the chromium release assay are given in the legend to Fig. 1 and Materials and Methods.

Fig. 1 not to directly sensitize targets for recognition) to compete. Evidence that peptide 2 binds to the K^b groove is provided in Fig. 3. Similar data were obtained regardless of whether E δ G2 or SL1 in vitro-restimulated effectors were used to generate anti-AKR/Gross MuLV CTL, as would be expected. The results from the unlabeled target competition experiments along with the ability of peptide 12 to sensitize targets at very low concentrations indicated that peptide 12 represents a dominant epitope encoded by the AKR623 virus and recognized by C57BL/6-derived anti-AKR/Gross MuLV CTL.

FIG. 3. Ability of negative peptides to compete with peptide 12. E ϕ K1 targets were plated on the day of the chromium release assay and bound with the concentrations of negative peptide as indicated. After incubation at 37°C for 40 min in a 5% CO₂ atmosphere, peptide 12 was added at 100 pg/ml. Anti-AKR/Gross MuLV effectors (Fig. 1 and Materials and Methods) were then added at an E:T ratio of 50:1, and the chromium release assay was performed as specified in Materials and Methods.

Ability of peptides not capable of sensitizing targets to lysis by anti-AKR/Gross MuLV CTL to bind to the K^b antigen. To determine whether the peptides that failed to confer lysis on SC.K^b targets were binding to the K^b groove but not being recognized by bulk antiviral CTL or simply failing to bind to K^b, we performed a peptide competition study in which various negative peptides were assayed for the ability to inhibit the CTL-mediated lysis of SC.K^b targets conferred by peptide 12. Figure 3 shows data obtained for a sampling of peptides from a representative experiment. Peptides 2, 5, 6, 9, and to a lesser degree 10 inhibited the CTL-mediated lysis conferred by 100 pg of peptide 12 per ml, consistent with their having the ability to bind to the class I K^b molecule on SC.K^b cells. In contrast, peptide 11 competed poorly if at all under these conditions, although it may be possible to demonstrate binding to K^b by peptide 11 in the absence of peptide 12. Similar profiles were obtained in four experiments (although in one experiment, peptide 11 did inhibit peptide 12-induced lysis to a small extent). Thus, the inability of at least some peptides such as 2, 5, 6, and 9 to sensitize SC.K^b targets to lysis is likely due not to a failure to bind K^b but rather to a failure to be recognized by the bulk anti-AKR/Gross MuLV CTL.

Specificity of K^b peptide 12 CTL recognition. Because peptide 12 is so effective in conferring lysis to EMV⁻, K^b-expressing target cells by anti-AKR/Gross MuLV CTL, it was important to ensure the specificity of this activity. If the interaction of peptide 12 with SC.K^b cells is specific as governed by conventional class I major histocompatibility complex-restricted recognition of peptide, peptide 12 should only sensitize H-2^b cells for lysis by the H-2^b-restricted anti-AKR/Gross MuLV CTL. As seen in one of two similar experiments, Fig. 4A shows that irrelevant C57BL/6 anti-H-2^d allogeneic CTL do not recognize SC.K^b/peptide 12 targets, similar to their lack of recognition of SC.K^b (or SC.K^b/623; not shown) control targets. The activity of these anti-H-2^d allogeneic effectors was confirmed by the lysis of LB27.4 (H-2^{bxd}) target cells. Thus, H-2^b-restricted anti-AKR/Gross MuLV CTL, but not irrelevant CTL, recognize SC.K^b/peptide 12 targets. In addition, AKR/Gross MuLV-specific bulk CTL fail to lyse NIH 3T3/peptide 12 (H-2^a) targets (Fig. 4B), as expected for H-2^b-restricted CTL and consistent with a specific presentation of peptide 12.

Evidence for the specificity of sensitization of SC.K^b target cells for lysis was also obtained with clonal CTL. Figures 4C and D show that H-2^b-restricted CTL clone G8, with specificity for AKR/Gross virus-positive cells but not the FMR class of MuLV (1), lyses SC.K^b/peptide 12 targets at levels similar to those seen for the SL1-positive control tumor target. In contrast, only low levels of lysis of SC.K^b/peptide 13 (the analogous p15E peptide encoded by Moloney MuLV) were observed with the G8 clone. On the other hand, an irrelevant H-2^b-restricted CTL clone 0.3.1, specific for the C57BL/6-derived 1710 tumor that harbors the MAIDS virus complex (unpublished data), failed to recognize SC.K^b/peptide 12 target cells (Fig. 4E and F).

Peptide 12 is immunogenic. Although it is clear that peptide 12 is highly antigenic, in that low concentrations effect a high percentage of specific lysis of SC.K^b/peptide 12 cells by bulk CTL at the target cell level, we wished to know whether peptide 12 was immunogenic as well, with respect to stimulating the generation of anti-AKR/Gross MuLV CTL in vitro. To address this question, peptide 12 was used at the secondary in vitro stimulation step to effect a response. C57BL/6-derived anti-B.GV immune splenocytes were cultured in vitro either with irradiated E δ G2 stimulators as a positive control, with the EMV⁻ H-2K^b tumor line E ϕ K1 bound with an excess of

FIG. 4. The recognition of peptide 12 by AKR/Gross virus CTL is EMV specific and *H-2^b* restricted. (A) Anti-BALB/c allogeneic CTL were generated as specified in Materials and Methods and assayed in the standard chromium release assay against SC.K^b targets (negative control), SC.K^b targets with peptide 12 (100 pg/ml) added on the day of the chromium release assay, or LB27.4 targets (positive control). The E:T ratios were as indicated. (B) Peptide 12 was added at the indicated concentrations to NIH 3T3 targets that had been plated the day before. Anti-AKR/Gross MuLV effectors (Fig. 1 and Materials and Methods) were then added at an E:T ratio of 100:1, and the chromium release assay was performed as specified in Materials and Methods. (C) *H-2^b*-restricted EMV-specific G8 clonal effectors were assayed at an E:T ratio of 3.6:1 against SC.K^b targets to which peptide 12 or the Moloney (Mo) peptide 13 were added at the concentrations indicated. The chromium release assay was performed as specified in Materials and Methods. (D) The same G8 effectors as those used for panel C were assayed (within the same experiment as for panel C) against SL1 or cl.18-5 targets at the E:T ratios indicated. (E) *H-2^b*-restricted anti-MAIDS 0.3.1 clonal effectors were assayed at an E:T ratio of 10:1 against SC.K^b targets to which peptide 12 or the Moloney peptide 13 were added at the concentrations indicated. The chromium release assay was performed as specified in Materials and Methods. (F) Controls for panel E. 0.3.1 effectors (same batch as used for panel E) were assayed against 1710.5 or 1153 tumor targets (both *H-2^b*). The chromium release assay was performed as specified in Materials and Methods.

peptide 2 as a negative control, or with E $\varnothing$ K1 bound with an excess of peptide 12. As in the experiments described above, the positive control (E δ G2-stimulated) effectors lysed SC.K^b/623 and SC.K^b/peptide 12 targets efficiently (Fig. 5). Demonstrating the immunogenicity of peptide 12, the effector cells restimulated with E $\varnothing$ K1/peptide 12 (but not E $\varnothing$ K1/peptide 2) were as cytolytic as the E δ G2-stimulated effectors, specifically lysing both the SC.K^b/623 and SC.K^b/peptide 12 targets but not SC.K^b/peptide 2 (not shown) or SC.K^b targets. Peptide 12-stimulated effectors also lysed SL1 but failed to lyse cl.18-5 or YAC targets, just as our standard tumor-stimulated anti-AKR/Gross MuLV CTL do (data not shown). This type of experi-

ment was repeated three times with similar results, including one experiment in which peptide 12 was used in the culture by itself without having first been bound to E $\varnothing$ K1, indicating that sufficient antigen-presenting cells were present in the heterogeneous responder cell population.

Peptide 12 restores the ability of cl.18-5 to be killed by anti-AKR/Gross MuLV CTL. Cl.18-5 is a naturally occurring variant tumor line that is insensitive to lysis by anti-AKR/Gross MuLV CTL. This line was subcloned from the CTL-susceptible SL1 tumor line. Because the molecular basis for the insensitivity of cl.18-5 was not known, nor was the issue of whether anti-AKR/Gross MuLV CTL recognize viral anti-

FIG. 5. Peptide 12 is strongly immunogenic. Anti-B.GV immune spleen cells were cultured for 6 days with the following stimulators: E δ G2 (circles) and E δ K1 in the presence of peptide 12 (10 μ g/ml) (triangles) or peptide 2 (10 μ g/ml) (squares). The resultant effectors were then assayed against SC.K^b/623 targets (unbroken lines) or SC.K^b targets (dashed lines) at the E:T ratios indicated in a standard chromium release assay as described in Materials and Methods.

gen(s) per se or nonviral tumor-associated antigens, it was of particular interest to determine whether peptide 12, added exogenously to the cl.18-5 target, could restore the ability of this tumor variant to be recognized by bulk anti-AKR/Gross MuLV CTL. Representative data (Fig. 6) demonstrate that peptide 12 can indeed fully restore the lysis of the cl.18-5 target. The resulting lysis profile is similar to those obtained when peptide 12 is added to either the SC.K^b fibroblasts (Fig. 1) or the EMV⁻ tumor line E δ K1 (data not shown). The ability of peptide 12 to restore the lysis of cl.18-5 targets by anti-AKR/Gross MuLV CTL strongly suggests that (i) cl.18-5 is functionally missing the major immunodominant epitope recognized by anti-AKR/Gross MuLV CTL and represented by peptide 12 and (ii) the primary antigen on SL1 targets that is recognized by these bulk antiviral CTL is the virus-encoded p15E epitope represented by peptide 12 rather than some other viral or nonviral epitope.

FIG. 6. Peptide 12 restores the ability of cl.18-5 to be killed by anti-AKR/Gross virus CTL. Peptide 12 was added to cl.18-5 targets at the concentrations indicated. Anti-AKR/Gross MuLV effectors (Fig. 1 and Materials and Methods) were then added at an E:T ratio of 100:1, and the chromium release assay was performed as specified in Materials and Methods. The percentage of specific lysis for the negative control, cl.18-5 to which no peptide was added, is indicated by an isolated circle.

DISCUSSION

The data presented here demonstrate the identification of immunodominant peptide 12 (KSPWFTTL), located in the p15E region, which confers lysis of EMV⁻ targets by anti-AKR/Gross MuLV CTL. There are other examples of a single dominant epitope from an entire pathogen being found (e.g., reference 29), and in at least one other case, a particular H-2 class I motif has been used to identify a class I major histocompatibility complex-restricted immunodominant epitope for a bacterium (24). The data presented here, characterizing the ability of peptide 12 to confer CTL function, indicate that the CTL response to EMV-associated tumors is directed primarily against the viral antigen represented by peptide 12. It is interesting that the sequence represented by peptide 12 is located within the p15E region as opposed to the highly variable gp70 region. One might think to first look for an epitope within the gp70 region, where the coding sequence is highly variable among MuLVs, including the FMR MuLV, which are not recognized by these AKR/Gross MuLV-specific CTL. Yet peptide 12 is located within the highly conserved p15E (transmembrane) region at a site which differs from the known sequences of the FMR MuLV, which all have an Arg at position 1 instead of the Lys present in AKR623.

Cl.18-5 is a naturally occurring CTL-insusceptible variant tumor line that was subcloned from the CTL-susceptible line SL1. Using antibodies against gp70 (the surface receptor antigen), radioimmunoprecipitation assays and fluorescence-activated cell sorting analyses have shown that cl.18-5 expresses an altered gp70 envelope antigen (21). There is no defect in the class I K^b expression of these cells. Treatment of cl.18-5 with iododeoxyuridine, known to activate latent proviruses, resulted in the restoration of normal gp70 expression levels and susceptibility to lysis by anti-AKR/Gross MuLV CTL. Furthermore, AKR.H-2^b:Fv-1^b mice were more susceptible to tumor growth and mortality resulting from inoculation with cl.18-5 tumor cells than with SL1 tumor cells. This increased mortality correlated with the inability of cl.18-5 to induce CTL in these mice (2). We show here that peptide 12 restores the lysis of cl.18-5 target cells to levels equivalent to those seen for the susceptible parent target SL1, indicating that the viral epitope represented by peptide 12 is indeed the predominant tumor antigen recognized by the bulk CTL generated as described here.

The sequence of peptide 12 fits the XXXX(FY)XX(VIML) motif determined by acid elution of peptides from the class I K^b groove (4). X-ray crystallographic data for K^b (6, 23, 32) indicate that the binding of peptides to class I molecules occurs primarily through the peptide backbone via hydrogen bonding, via the main chain atoms of the peptide termini, and via a dominant peptide anchor residue(s) (with a dominant residue at position 5 for the K^b-binding peptides). Recognition by the T-cell receptor (TCR) appears to occur primarily via solvent accessible side chain interactions. In particular for K^b-binding peptides, the side chain at peptide position 1 is available for TCR recognition rather than binding to the class I molecule (6, 23, 32). Consistent with the X-ray crystallographic data, the variation of only one side chain at position 1 within EMV peptide 12 markedly diminishes the ability of this peptide to confer lysis of EMV⁻ target cells by anti-AKR/Gross MuLV CTL. There are other examples of a change in a single exposed amino acid being sufficient to abrogate TCR recognition (18, 27) without interfering with binding of peptide to class I (15, 22).

Finally, regarding whether the peptide held within the K^b groove is a virus-encoded or some nonviral tumor antigen, two

lines of evidence have previously suggested that anti-AKR/Gross MuLV CTL recognize virus-encoded antigens. First, the EMV AKR623 can be expressed in the nontumor fibroblast line SC.K^b and confer full recognition of these targets by bulk populations of anti-AKR/Gross MuLV CTL, with levels of lysis similar to those seen for EMV⁺ tumor targets such as SL1 or E δ G2. It is still possible, however, that a nonviral antigen is being induced or altered in response to the presence of AKR623 virus within the fibroblast cell. Second, the restoration of the variant tumor subclone cl.18-5 to lysis by anti-AKR/Gross MuLV CTL by peptide 12 argues against the involvement of a nonviral epitope.

It is interesting to compare our results with those of Jiang and Flyer (16, 17). These investigators presented evidence that for Moloney MuLV-induced tumors, a nonviral tumor antigen accounts for approximately 60% of the Moloney MuLV-specific CTL-mediated lysis, being non-cross-reactive with EMV⁺ tumor targets. A virus-negative variant tumor line (MBLv) of the Moloney MuLV-induced lymphoma line MBL-2 retained the expression of the tumor-associated antigen (TAA) and its susceptibility to the TAA-specific CTL in vitro but was not susceptible to virus-specific CTL. Immunization with the variant, shown to give rise to only TAA-specific CTL capable of lysing both MBL-2 and MBLv in vitro, failed to protect mice from challenge with either MBL-2 or MBLv. The virus-specific CTL were less specific, being cross-reactive with EMV MuLV (5), and immunization protocols inducing the antiviral response protected against tumor development after challenge with MBL-2 but not MBLv. Thus, whereas the basis for the ability of CTL raised against Moloney MuLV to distinguish between FMR and AKR/Gross MuLV-induced tumors may be due to recognition of a nonviral antigen, this does not appear to explain the fine specificity of C57BL/6-derived anti-AKR/Gross MuLV CTL which poorly recognize FMR MuLV-induced tumors. Clearly in the latter case, AKR/Gross MuLV-specific CTL are primarily directed to a virally encoded immunodominant epitope that appears to be important for tumor recognition.

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