JOURNAL OF VIROLOGY, Nov. 1996, p. 7773–7782 Vol. 70, No. 11
0022-538X/96/$04.00+0

Identification of a *gag*-Encoded Cytotoxic T-Lymphocyte Epitope from FBL-3 Leukemia Shared by Friend, Moloney, and Rauscher Murine Leukemia Virus-Induced Tumors

WEI CHEN,[1]* HUILIAN QIN,[1] BRUCE CHESEBRO,[2] AND MARTIN A. CHEEVER[1]

Division of Oncology, Department of Medicine, University of Washington, Seattle, Washington 98195,[1] and Laboratory of Persistent Viral Diseases, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, Hamilton, Montana 59840[2]

Received 16 April 1996/Accepted 5 August 1996

FBL-3 is a highly immunogenic murine leukemia of C57BL/6 origin induced by Friend murine leukemia virus (MuLV). Immunization of C57BL/6 mice with FBL-3 readily elicits $CD8^+$ cytotoxic T lymphocytes (CTL) capable of lysing FBL-3 as well as syngeneic leukemias induced by Moloney and Rauscher MuLV. The aim of this current study was to identify the immunogenic epitope(s) recognized by the FBL-3-specific $CD8^+$ CTL. A series of FBL-3-specific $CD8^+$ CTL clones were generated from C57BL/6 mice immunized to FBL-3. The majority of CTL clones (32 of 38) were specific for F-MuLV *gag*-encoded antigen. By using a series of recombinant vaccinia viruses expressing full-length and truncated F-MuLV *gag* genes, the antigenic epitope recognized by the FBL-3 *gag*-specific CTL clones, as well as by bulk-cultured CTL from spleens of mice immune to FBL-3, was localized to the leader sequence of $gPr80^{gag}$ protein. The precise amino acid sequence of the CTL epitope in the leader sequence was identified as CCLCLTVFL (positions 85-93) by examining lysis of targets incubated with a series of synthetic leader sequence peptides. No evidence of other CTL epitopes in the $gPr80^{gag}$ or $Pr65^{gag}$ core virion structural polyproteins was found. The identity of CCLCLTVFL as the target peptide was validated by showing that immunization with the peptide elicited CTL that lysed FBL-3. The CTL elicited by the Gag peptide also specifically lysed syngeneic leukemia cells induced by Moloney and Rauscher MuLV (MBL-2 and RBL-5). The transmembrane peptide was shown to be the major *gag*-encoded antigenic epitope recognized by bulk-cultured CTL derived from C57BL/6 mice immunized to MBL-2 or RBL-5. Thus, the CTL epitope of FBL-3 is localized to the transmembrane anchor domain of the nonstructural Gag polyprotein and is shared by leukemia/lymphoma cell lines induced by Friend, Moloney, and Rauscher MuLV.

The role of T-cell-mediated immunity in the elimination of transformed cells has been extensively studied by using a group murine leukemia and lymphoma cell lines induced by Friend, Moloney, and Rauscher (FMR) murine leukemia viruses (MuLV). Early transplantation studies demonstrated that immunization of mice with syngeneic FMR MuLV-induced leukemia cells induces resistance to transplantation of the immunizing leukemia and cross-resistance to the transplantation of other FMR MuLV-induced leukemias (15). Resistance to FMR MuLV-induced leukemias is primarily T-cell mediated (2). FBL-3, a Friend MuLV (F-MuLV)-induced erythroleukemia of C57BL/6 (B6) origin, has been the focus of extensive studies by ourselves and others for learning how to manipulate host immunity to eradicate advanced disseminated malignancy (16). Immunization of B6 mice with FBL-3 protects against the growth of subsequently transplanted FMR MuLV-induced tumors and elicits T cells therapeutically effective for eradicating disseminated FBL-3 in adoptive transfer regimens (3).

Previous evaluation of the antigenic determinants detected on FBL-3 cells after immunization with FBL-3 tumor cells demonstrated that the majority of the FBL-specific $CD8^+$ cytotoxic T lymphocytes (CTL) recognize F-MuLV *gag*-encoded antigens, whereas FBL-reactive $CD4^+$ helper T cells recognize F-MuLV *env*-encoded antigens (23). In contrast, spontaneous recovery of $H\text{-}2^b$ mice from Friend virus infection appears to induce primarily CTL with specificity for F-MuLV *env*-encoded antigens (30), and these CTL have also been shown to be capable of lysing FBL-3 cells in vitro (7). In this infectious virus model, $CD8^+$ CTL, $CD4^+$ helper T cells, and virus-neutralizing humoral antibodies specific for viral envelope protein are all required for successful recovery (17, 30), whereas in the FBL-3 leukemia cell system, $CD4^+$ and $CD8^+$ T cells may be sufficient for eliminating tumor cells in vivo (16). Thus, the type of host exposure to retrovirus-induced antigens may play a large role in the immune mechanisms required for resistance and the immunodominance of particular retrovirus-encoded antigenic epitopes. More recently, several epitopes derived from the FMR MuLV gp70 envelope glycoprotein and recognized by the $CD4^+$ helper T cells have been identified (21, 33, 34). The current study was designed to identify the F-MuLV *gag*-encoded CTL epitope(s) expressed by FBL-3 and shared by syngeneic tumors induced by Moloney and Rauscher MuLV.

The single F-MuLV *gag* gene open reading frame codes for two alternative translational products, $Pr65^{gag}$ and $gPr80^{gag}$ (10). Transcription of $gPr80^{gag}$ is initiated upstream from $Pr65^{gag}$, yielding a polyprotein that has the same amino acid sequence as $Pr65^{gag}$ but with the addition of a 98-amino-acid leader sequence (29). As a result, $Pr65^{gag}$ and $gPr80^{gag}$ polyproteins are processed differently and have markedly different functions. The $Pr65^{gag}$ polyprotein is myristylated on the N-terminal glycine, nonglycosylated, and proteolytically cleaved into four virion proteins (p15, p12, p30, and p10) from the N terminus to the C terminus (Fig. 1). By marked contrast, the $gPr80^{gag}$ polyprotein is the precursor to nonstructural cell surface pro-

* Corresponding author. Present address: The Cleveland Clinic Foundation, FF-50, 9500 Euclid Ave., Cleveland, OH 44195. Phone: (216) 445-3824. Fax: (216) 445-3805. Electronic mail address: wchen@cesmtp.ccf.org.

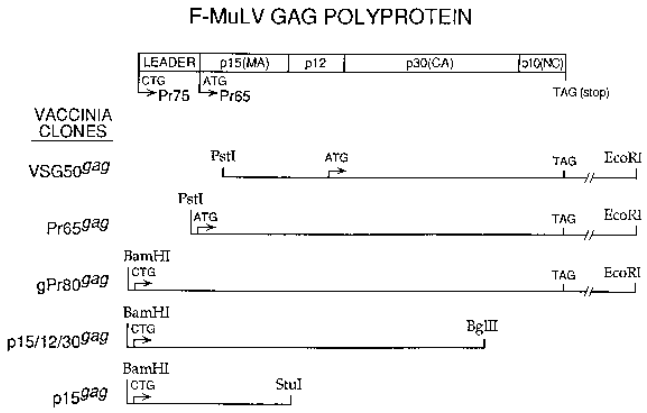

FIG. 1. Recombinant vaccinia virus clones expressing various portions of the F-MuLV *gag* gene. Two polyproteins, Pr75 and Pr65, are translated from the F-MuLV *gag* gene. Pr75 starts at a CTG codon at position 355 and includes an additional 88-amino-acid leader sequence. This protein is glycosylated to produce gPr80^{gag}, which appears in the plasma membrane of infected cells but is not a component of virus particles themselves. Pr65 starts at an ATG codon at position 618 and is incorporated into virus particles, where it is further processed by proteolytic cleavage into p15 (MA), p12, p30 (CA), and p10 (NC) proteins. The generation of the recombinant vaccinia virus clones and the positions of the restriction sites used in F-MuLV clone FB29 were described previously (27).

teins. The gPr80^{gag} precursor polyprotein is transported through the endoplasmic reticulum, glycosylated, and expressed on the cell surface with an outward orientation (10). The gPr80^{gag} polyproteins appear to have no discernible function for MuLV replication in vitro but are necessary for efficient spreading and pathogenesis of MuLV in vivo (9, 29).

The current studies showed that the F-MuLV *gag*-encoded epitope recognized by the FBL-3 specific CTL is present in the transmembrane region of the gPr80^{gag} leader sequence. The leader sequence epitope (gPr80^{gag} 85-93; CCLCLTVFL [p85-93]) was identified by using a series of synthetic peptides. A previous study has independently localized a different CTL epitope to the same gPr80^{gag} leader sequence by testing a single CTL clone (24). However, the identity of the epitope (CCLCLTVFL) identified in the current studies was validated by showing that immunization of B6 mice with the peptide elicited CTL that lysed FBL-3, as well as syngeneic leukemias induced by Moloney and Rauscher MuLV. Moreover, the transmembrane peptide proved to also be a major epitope for bulk-cultured CTL from B6 mice immunized with Moloney and Rauscher MuLV-induced leukemias. No evidence of other CTL epitopes in the gPr80^{gag} or Pr65^{gag} core virion structural polyproteins was found. Thus, the gPr80^{gag} leader sequence epitope (p85-93) may account for the majority of the *gag*-specific CTL cross-reactivity between FMR MuLV-induced tumors in B6 mice. Identification of the CTL epitope of FBL-3 leukemia will allow subsequent studies using FMR MuLV tumor models to elucidate the principles involved in the use of peptide-based vaccine therapy and T-cell therapy to eradicate antigenic malignancies.

MATERIALS AND METHODS

Mice. Six- to eight-week-old B6 mice and BALB/c mice were obtained from the Jackson Laboratory (Bar Harbor, Maine) and maintained in the animal care facilities of the University of Washington.

Tumor. FBL-3 is an F-MuLV-induced leukemia of B6 (*H-2^b*) origin that expresses tumor-associated antigens that cross-react with other FMR MuLV-induced tumors. MBL-2 is a Moloney MuLV-induced lymphoma cell line that possesses tumor-associated antigens that cross-react with other FMR MuLV-induced tumors. RBL-5 is a Rauscher MuLV-induced leukemia of B6 (*H-2^b*) origin that possesses tumor-associated antigens that cross-react with other FMR MuLV-induced tumors. EL-4 is a dimethylbenzanthrene-induced lymphoma of B6 origin that is antigenically distinct from FBL-3 leukemia. LSTRA is a Moloney MuLV-induced lymphoma of BALB/c (*H-2^d*) origin (15). RMA-S is a genetically defective Rauscher MuLV-induced leukemia of B6 (*H-2^b*) origin that expresses low levels of H-2 D^b and K^b molecules and does not process endogenous antigens (38).

Fibroblast cell lines. The transfectant fibroblast cell lines expressing the class I-restricting antigen D^b and/or F-MuLV proteins were established as previously described (20). Briefly, plasmids containing the D^b gene and the selectable marker pSV2neo were transfected into Fisher rat embryo fibroblasts cells and cultured with 400 μg of G418 (GIBCO Laboratories, Grand Island, N.Y.) per ml, and stable G418-resistant transfectants were isolated (cell line B2). Plasmids consisting of the F-MuLV provirus (pF-MuLV clone 57) or F-MuLV *gag* or F-MuLV *env* gene were constructed and cotransfected into Fisher rat embryo fibroblasts along with D^b in pSV2neo. Stable G418-resistant cell lines that express D^b plus F-MuLV *gag* and *env*, D^b plus F-MuLV *gag*, or D^b plus F-MuLV *env* were isolated and termed FB2, Ps6, and N34, respectively.

Construction of recombinant vaccinia viruses expressing the F-MuLV *gag* genes. Recombinant vaccinia viruses containing the entire F-MuLV *gag* gene or its shorter fragments were described previously (27). Recombinant vaccinia viruses expressing the F-MuLV *env* gene (11) or influenza virus hemagglutinin gene (37) have been described previously.

Peptides. The amino acid sequences of synthetic F-MuLV Gag peptides used in this study are shown in Tables 2 and 3. The peptide sequences were selected from F-MuLV gPr80^{gag} protein on the basis of the peptide binding motifs of the D^b or K^b molecules (12). An H-2 K^b-specific ovalbumin p257-264 (SIINFEKL) peptide (31) and an H-2 D^b-specific influenza virus NP 1934 p366-374 (ASNENMETM) peptide (32) were constructed. All peptides were synthesized by Patrick S. H. Chou (Biopolymer Facility, University of Washington) by using 9-fluorenylmethoxycarbonyl chemistry in an automated peptide synthesizer (model 433A; Applied Biosystems, Inc., Foster City, Calif.). All peptides synthesized were purified by high-performance liquid chromatography using a reverse-phase semipreparative C_{18} column and were shown to have purity of greater than 99%. Mass spectroscopy analysis was used to confirm that the peptides had the exact predicted molecular weight. The peptides were first dissolved either in water or in dimethyl sulfoxide (Sigma Chemical Co., St. Louis, Mo.), depending on the water solubility of individual peptides. The peptide solutions were then diluted either in phosphate-buffered saline (PBS) or in medium. The concentration of dimethyl sulfoxide was less than 0.1% in the final dilution used.

Peptide binding assay. The ability of synthetic F-MuLV Gag peptides to bind to D^b or K^b class I molecules was detected by using the leukemia cell line RMA-S as described previously (13, 25), with slight modification. In brief, RMA-S cells were cultured for 7 h at 26°C in complete medium supplemented with 1% fetal calf serum (FCS). A total of 10^6 RMA-S cells were added into each well of a 24-well plate and incubated either alone or with the designated peptide (25 μg/ml) for 16 h (overnight) at 26°C and additional 3 h at 37°C in complete medium. Cells were then washed three times and stained with fluorescein isothiocyanate-conjugated anti-D^b or anti-K^b antibody (PharMingen, San Diego, Calif.). Labeled cells were again washed twice, resuspended and fixed in 500 μl of PBS with 1% paraformaldehyde, and analyzed for fluorescence intensity by

TABLE 1. Cytotoxicity of FBL-3-specific CD8$^+$ CTL clones to syngeneic or allogeneic tumors induced by FMR MuLV[a]

Effector	Effector/target cell ratio	% Specific lysis of targets						
		Expt 1				Expt 2		
		FBL-3	RBL-5	EL-4	LSTRA	MBL-2	RMA	RMA-S
5D5	25:1	63	64	1	0	86	71	3
	1:1	45	42	2	0	63	67	3
5C2	25:1	62	69	0	0	99	83	9
	1:1	40	36	0	0	75	64	4
5C6	25:1	64	55	0	0	83	65	13
	1:1	34	30	0	0	72	69	8
2G3	25:1	61	69	4	0	73	70	11
	1:1	50	47	0	0	49	57	7
3B12	25:1	38	40	4	0	98	69	4
	1:1	33	37	0	0	92	65	5
BALB/c anti-B6	80:1	61	49	50	4	67	75	11
	20:1	50	25	21	5	49	46	5

[a] The CD8$^+$ CTL clones generated from FBL-3-immunized B6 mice were assessed for lysis of syngeneic (FBL-3, MBL-2, RBL-5, RMA, and RMA-S) or allogeneic (LSTRA) tumors induced by FMR MuLV in a standard 4-h chromium release assay. EL-4 is a B6 lymphoma antigenically distinct from the FMR MuLV-induced tumors. Alloreactive T cells (BALB/c anti-B6) were used as positive control effectors and were generated by standard 5-day mixed lymphocyte culture.

TABLE 2. Recognition by the majority of FBL-3-specific $CD8^+$ CTL clones of F-MuLV *gag*-encoded antigen[a]

Effector	Effector/ target cell ratio	% Specific lysis of targets				
		FBL-3	B2	FB2	Ps6	N34
5D5	25:1	59	8	41	23	2
	5:1	57	6	39	21	0
2G3	25:1	65	7	48	24	2
	5:1	61	0	47	19	0
3B12	25:1	51	4	7	6	4
	5:1	43	0	2	3	2
BALB/c anti-B6	80:1	72	60	64	35	52
	20:1	67	45	44	26	38

[a] FBL-3-specific CTL clones were assessed in a standard 4-h chromium release assay with ^{51}Cr-labeled target cells, including FBL-3 cells and rat fibroblasts transfected with the class I-restricting element D^b alone (B2), D^b and F-MuLV *env* and *gag* (FB2), D^b and F-MuLV *gag* (Ps6), or D^b and F-MuLV *env* (N34). Alloreactive T cells (BALB/c anti-B6) were used as a positive control and were generated by standard 5-day mixed lymphocyte culture.

flow cytometry in a flow cytometer (Coulter). The percentage of increase of D^b or K^b molecules on the surface of the RMA-S cells was measured by increased mean fluorescent intensity of cells incubated with peptide compared with that of cells incubated in medium alone.

Generation of FMR MuLV-induced tumor-specific bulk-cultured CTL lines and clones. T cells specific for FMR MuLV-induced tumors were derived from spleens of B6 mice previously immunized with FBL-3, MBL-2, or RBL-5. Mice were immunized with the FMR MuLV-induced tumors in vivo by two intraperitoneal inoculations, separated by 2 weeks, of 10^7 irradiated (10,000 rads of γ radiation) tumor cells, and immune cells were obtained 2 weeks later. Bulk-cultured tumor-specific CTL were generated under conditions described previously (5). Briefly, 4×10^6 immune splenocytes were specifically activated by culture for 5 days with 5×10^5 irradiated (10,000 rads of γ radiation) immunizing tumor cells and 2×10^6 irradiated (3,000 rads of γ radiation) B6 spleen cells as accessory cells in wells of a 24-well plate in medium consisting of a 1:1 mixture of RPMI 1640 medium (GIBCO Laboratories) and EHAA medium (Biofluids Inc., Rockville, Md.) with 5×10^{-5} M 2-mercaptoethanol, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 10 mM L-glutamine, and 10% FCS. The cells were then expanded in number by 7 to 9 days of culture with medium containing 10 U of human recombinant interleukin-2 (rIL-2; provided by Hoffmann-La-Roche, Inc., Nutley, N.J.) per ml. The resultant tumor-specific bulk T-cell lines were propagated by repeated cycles of restimulation with irradiated tumor cells plus syngeneic antigen-presenting cells and expanded in number in culture with 10 U of rIL-2 per ml every 2 weeks.

FBL-3-specific T-cell clones were derived from the FBL-3 tumor-reactive bulk T cells (12 days in culture) by the limiting-dilution method. Briefly, T cells were placed at 5, 1, and 0.5 cells per well in 96-well microtiter plates with 10^4 irradiated FBL-3 tumor cells plus 10^6 irradiated B6 splenic cells per well in the presence of 100 U of rIL-2 per ml. T-cell clones were expanded only from the positive wells, plated at 0.5 cells per well, propagated by repeated cycles of restimulation with antigen plus irradiated syngeneic antigen-presenting cells, and expanded in number in culture with low-dose rIL-2 (10 U/ml) every 2 weeks. The

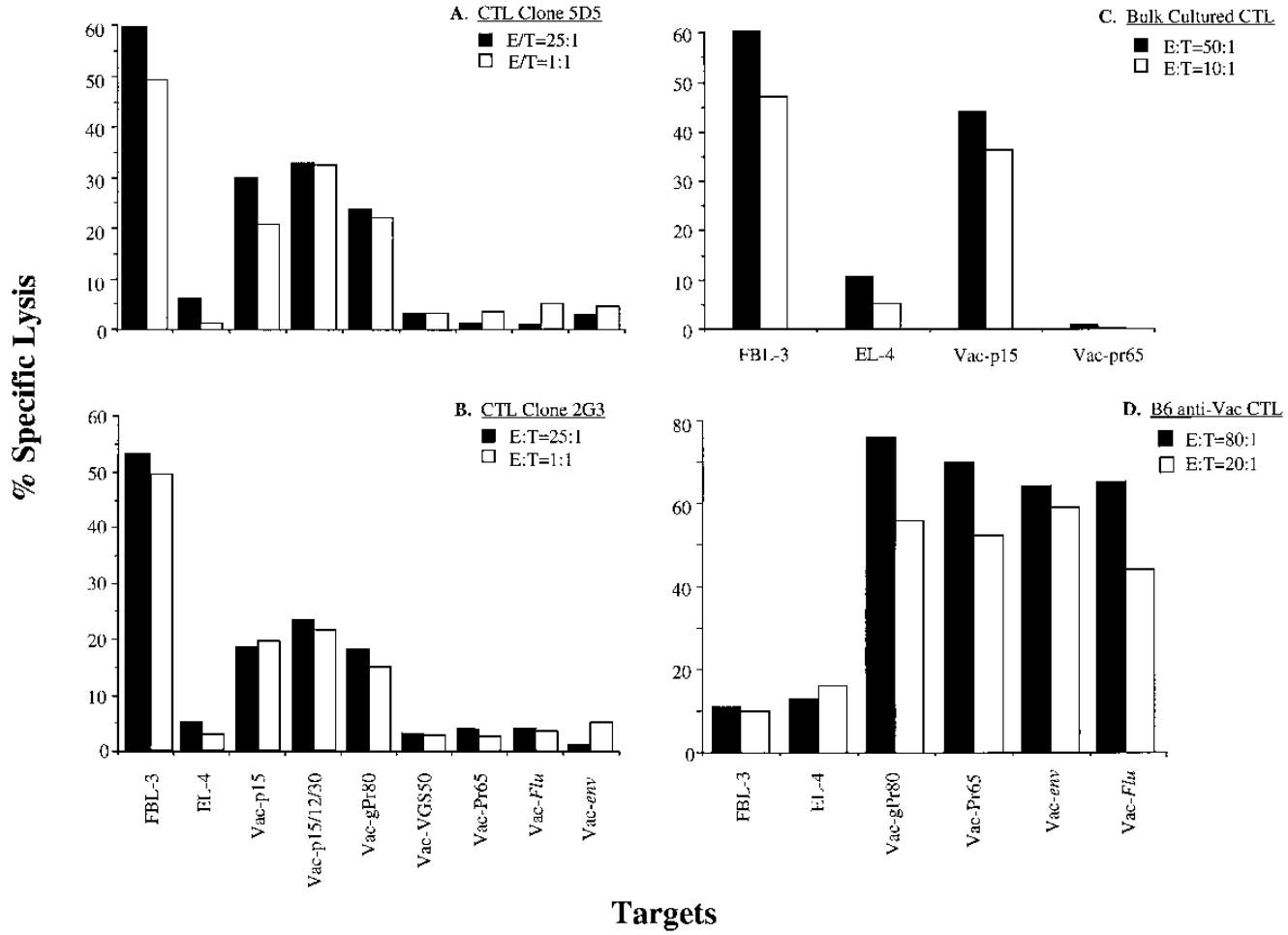

FIG. 2. FBL-3-specific $CD8^+$ CTL clones recognize only targets infected with vaccinia viruses containing genes encoding the leader sequence of F-MuLV $gPr80^{gag}$ protein. ^{51}Cr-labeled EL-4 cells were infected with a panel of recombinant vaccinia viruses (Vac) expressing overlapping regions of the F-MuLV *gag* or *env* gene (Vac-*env*) or influenza virus HA gene (Vac-*Flu*) and used as targets. The FBL-3-specific CTL clones and bulk-cultured CTL from spleens of FBL-3-immunized B6 mice were assessed for the ability to recognize and lyse the recombinant vaccinia virus-infected EL-4 targets in a standard 4-h ^{51}Cr release assay. B6 anti-vaccinia virus CTL were used as positive control effectors. E/T and E:T, effector/target cell ratio.

phenotype of T-cell clones was determined by fluorescence-activated cell sorting (FACS) analysis following staining with fluorescein-conjugated anti-Thy 1.2 (1%), fluorescein-conjugated anti-Lyt2 (2%), or phycoerythrin-conjugated anti-L3T4 (3%) monoclonal antibody (Becton Dickinson Immunocytochemistry Systems, Mountain View, Calif.).

Generation of F-MuLV Gag peptide-specific CTL lines. B6 mice were inoculated on day 0 by subcutaneous injection in the rear flank with various F-MuLV Gag peptides (100 μg in 50 μl) emulsified with 50 μl of complete Freund's adjuvant (Sigma) or with complete Freund's adjuvant (50 μl) emulsified with PBS as a control. Two weeks later, mice were boosted by subcutaneous injection with the immunizing Gag peptides (100 μg in 50 ml) emulsified with 50 μl of incomplete Freund's adjuvant (Sigma) or with incomplete Freund's adjuvant emulsified with PBS as a control. Spleens were harvested 10 days after the final immunization. Immune splenic lymphocytes were stimulated with the immunizing peptides at 25 μg/ml in 24-well culture plates (total volume, 2 ml) at 4×10^6 cells per well with 2×10^6 irradiated syngeneic spleen cells. The plates were cultured in a humidified atmosphere under 5% CO_2 tension at 37°C for 5 days and then split 1:2. After 10 days, lymphocytes were restimulated with 25 μg of the Gag peptides per ml at 10^6 lymphocytes per well in 24-well plates with 5×10^6 irradiated syngeneic spleen cells. On days 12 and 15, cells were split 1:2 with medium containing 10 U of rIL-2 per ml. Peptide-specific cytotoxic T-cell lines were maintained by periodic restimulation with peptide (5 μg/ml) plus irradiated syngeneic spleen cells and then subjected to expansion with rIL-2 (10 U/ml) every 2 weeks.

Cytotoxicity assay. Target cells (10^7) were incubated at 37°C with 250 μCi of ^{51}Cr (New England Nuclear, Boston, Mass.) in 1 ml of 20% FCS–RPMI 1640 for 45 min. Labeled targets were washed three times and resuspended in 20% FCS–RPMI 1640 at 10^6 cells per ml. For peptide loading, ^{51}Cr-labeled cells were incubated with various concentrations of Gag peptide for 60 min at 37°C and then diluted with 20% FCS–RPMI 1640 to 10^5 cells per ml. Effector cells in 100 μl were added to 96-well plates, and then 10^4 labeled target cells were added. Plates were incubated for 4 h at 37°C; then 100 μl of supernatant was harvested from each well and counted in a gamma counter. The percent specific lysis was calculated. All determinations of cytotoxicity were carried out in triplicate with a minimum of three effector-to-target cell ratios.

For antibody blocking assays, ^{51}Cr-labeled target cells at 10^4 cells in 50 μl were added to each well of 96-well plates. Various concentrations (0.02 to 20 μg/ml) of antibodies to H-2 D^b, K^b, or Ia or to Fc receptor (PharMingen) in 50 μl were added to appropriate wells and incubated for 45 min at room temperature. FBL-3-specific CTL clones were used as effector cells and added in 100 μl to each well of 96-well plates. After 4 h of incubation at 37°C, 100 μl of supernatant was harvested from each well and counted in a gamma counter.

For assays using vaccinia virus-infected targets, EL-4 cells were infected (10 PFU per cell) with various vaccinia virus recombinants in 24-well plates (10^6 cells per well) at 37°C for 1 h with frequent shaking. Chromium (100 μl, 100 μCi) was then added to each well, and the plates were cultured at 37°C for 16 h. The cells were then harvested, washed twice with Hanks balanced salt solution, resuspended in RPMI 1640 with 20% FCS, and used as targets in a standard 4-h chromium release assay. FBL-3-specific $CD8^+$ CTL clones and bulk-cultured CTL were used as effector cells. Spleen cells from B6 mice immunized in vivo with wild-type vaccinia virus (10^7 PFU, intravenous injection) were used as positive control effector cells.

TABLE 3. FBL-3-specific CTL recognize a single peptide from the leader sequence of the F-MuLV gPr80^{gag} polyprotein

Target	Amino acid sequence	H-2 D^b binding[a]	% Specific lysis of targets[b]		
			5D5	2G3	Bulk CTL
Expt 1					
FBL-3			52	58	53
EL-4			0	0	6
EL-4 plus:					
gPr80^{gag} 13-21	DVPGTSGAI	−	5	1	5
gPr80^{gag} 25-32	RPESNHPDR	−	2	3	6
gPr80^{gag} 34-42	FGLFGAPPL	+	6	2	2
gPr80^{gag} 41-49	PLEEGYVVL	−	3	4	2
gPr80^{gag} 47-55	VVLVGDRGL	−	0	0	1
gPr80^{gag} 57-65	RFPPPSEFL	+	0	7	0
gPr80^{gag} 66-74	LSVWNRSRA	+	4	3	2
gPr80^{gag} 74-82	AARLVCCSI	+	1	1	2
gPr80^{gag} 76-84	RLVCCSIVL	−	2	2	2
gPr80^{gag} 81-89	SIVLCCLCL	−	0	1	5
gPr80^{gag} 85-93	CCLCLTVFL	+	58	59	65
gPr80^{gag} 87-95	LCLTVFLYL	−	0	1	7
gPr80^{gag} 94-102	YLSENMGQT	−	3	3	4
Expt 2					
FBL-3			59	57	60
EL-4			3	4	0
EL-4 plus:					
gPr80^{gag} 81-89	SIVLCCLCL	−	5	0	0
gPr80^{gag} 81-89+KK	SIVLCCLCLKK	−	0	0	0
gPr80^{gag} 85-92	CCLCLTVF	−	2	4	0
gPr80^{gag} 85-93	CCLCLTVFL	+	54	68	65
gPr80^{gag} 86-93	CLCLTVFL	+	41	36	38
gPr80^{gag} 87-95	LCLTVFLYL	−	3	4	7
gPr80^{gag} 85-93+KK	CCLCLTVFLKK	+	58	57	61

[a] The gPr80^{gag} peptides were tested for binding to H-2 D^b molecules in a binding assay as detailed in Materials and Methods. Positive peptide sequences were capable of binding to D^b and induced upregulation of at least 50% above background levels of the D^b molecules on RMA-S cells at a peptide concentration of 25 μg/ml.

[b] ^{51}Cr-labeled EL-4 cells were preincubated with designated gPr80^{gag} peptides (25 μg/ml) for 1 h at 37°C before used as targets. Cytotoxicity were assessed in a standard 4-h ^{51}Cr release assay with three effector/target cell ratios. The data represent the lysis of targets by representative FBL-3-specific CTL clones (effector/target cell ratio of 5:1) and bulk-cultured CTL from FBL-3-immunized B6 mice (effector/target cell ratio of 20:1).

RESULTS

The majority of FBL-3-specific $CD8^+$ CTL clones generated from mice immunized to FBL-3 are specific for F-MuLV *gag*-encoded antigen. Thirty-eight FBL-3-specific $CD8^+$ CTL clones were generated from spleens of B6 mice immunized to FBL-3 and tested for cytolytic activity against syngeneic and allogeneic tumors induced by FMR MuLV. All CTL clones tested lysed FBL-3 as well as syngeneic MBL-2, RBL-5, and RMA, induced by Moloney and Rauscher MuLV, respectively. The FBL-3-specific $CD8^+$ CTL clones failed to lyse FMR-MuLV-negative EL-4 and Moloney MuLV-induced allogeneic LSTRA tumors. RMA-S, a subline of RMA that is defective in transporter associated with antigen processing (TAP) and subsequently expresses low levels of *H-2^b*, was lysed to a low degree. Results with five representative CTL clones are presented in Table 1. The D^b restriction of the FBL-3-specific $CD8^+$ CTL clones was confirmed in antibody blocking assays showing that CTL activity against FBL-3 was blocked by antibody to D^b but not by antibody to K^b, Ia, or Fc receptor (data not shown).

The majority (32 of 38) of FBL-3-specific CTL clones are specific for antigen determinants encoded by the F-MuLV *gag* gene, as determined by lysis of fibroblast lines cotransfected with genes encoding D^b molecules plus either F-MuLV *gag* and *env* genes (FB2) or the F-MuLV *gag* gene (Ps6), as represented by studies with clones 5D5 and 2G3 in Table 2. The CTL clones failed to lyse the fibroblast line transfected with genes encoding D^b molecules alone (B2) or a fibroblast line cotransfected with genes encoding D^b molecules and the F-MuLV *env* gene (N34). A minority of FBL-3-specific CTL clones (6 of 38) did not recognize either Gag or Env, as represented by studies with clone 3B12 in Table 2. The antigen(s) recognized by these CTL clones has not yet been determined. These results demonstrating that the majority of FBL-3-specific CTL clones generated by immunization to FBL-3 are specific for antigen determinants encoded by the F-MuLV *gag* gene confirmed previous findings with bulk-cultured CTL (23).

FBL-3-specific CTL recognize the leader sequence of the gPr80^{gag} polyprotein. The F-MuLV *gag*-encoded CTL epitope was localized by using recombinant vaccinia viruses expressing F-MuLV *gag* gene fragments. F-MuLV codes for two different but highly related Gag precursor polyproteins, Pr65^{gag} and gPr80^{gag}. Pr65^{gag} is cleaved into four virion proteins, p15, p12, p30, and p10. The gPr80^{gag} glycoprotein contains the entire 539-amino-acid sequence of Pr65^{gag} proteins plus a leader se-

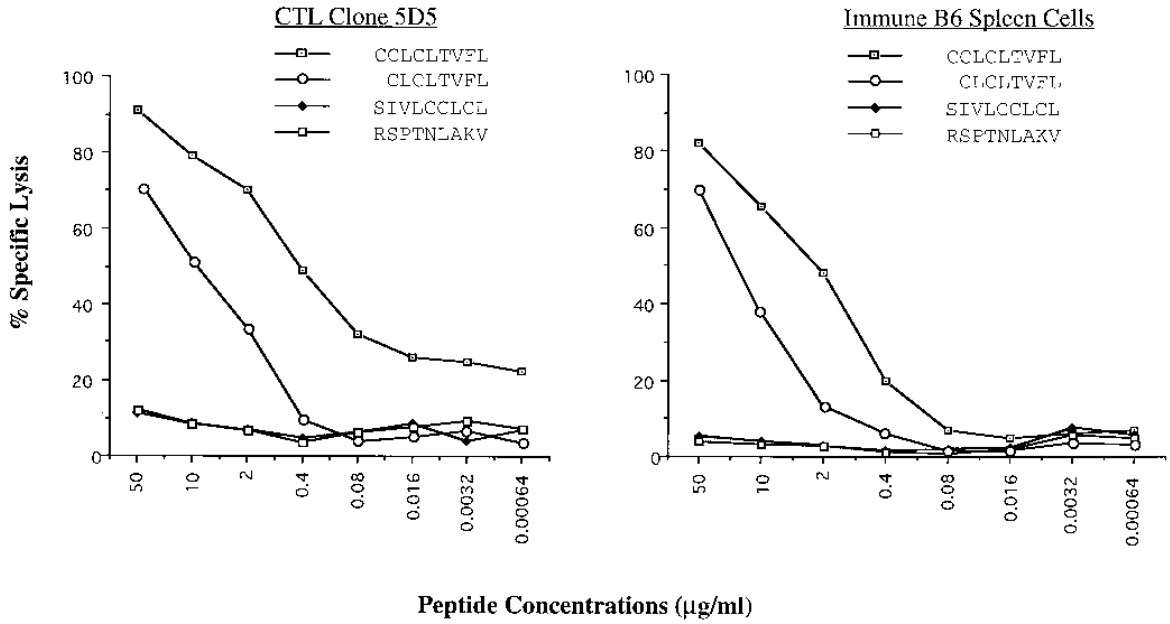

FIG. 3. FBL-3-specific CTL clones and bulk-cultured CTL recognize the leader sequence Gag peptide CCLCLTVFL in a peptide dose-dependent manner. Various concentrations of four F-MuLV gPr80^{gag} peptides were preincubated with ^{51}Cr-labeled EL-4 cells and used as targets. The peptide-pulsed targets were assessed for lysis by the FBL-3-specific CTL clones and bulk-cultured T cells from FBL-3-primed B6 mice in a standard 4-h ^{51}Cr release assay. The data represent the specific lysis of targets by FBL-3-specific CTL clones (effector/target cell ratio of 25:1) and bulk-cultured CTL from immune spleens of FBL-3 primed B6 mice (effector/target cell ratio of 50:1).

quence. To localize the F-MuLV *gag*-encoded CTL epitope, five vaccinia virus recombinants which expressed the F-MuLV *gag* gene or truncated fragments were tested in CTL assays (Fig. 1). FBL-3-specific CTL clones were shown to lyse EL-4 cells infected with Vac-p15^{gag}, Vac-p15/12/30^{gag}, and Vac-gPr80^{gag} but failed to lyse EL-4 cells infected with Vac-Pr65^{gag}, Vac-VGS50^{gag}, Vac-*env*, or Vac-*Flu* (Fig. 2A and B). Similarly, the bulk-cultured FBL-3-specific CTL lysed EL-4 cells infected with Vac-p15^{gag} but failed to lyse EL-4 cells infected with Vac-Pr65^{gag} (Fig. 2C). The results suggested that the main CTL epitope recognized by these clones is in the leader sequence of the gPr80^{gag}, which is the common region contained in Vac-p15^{gag}, Vac-p15/12/30^{gag}, and Vac-gPr80^{gag} recombinants but is not contained in Vac-Pr65^{gag} and Vac-VGS50^{gag} (Fig. 1).

FBL-3-specific CTL recognize a single epitope in the leader sequence of the gPr80^{gag} polyprotein. The leader sequence of gPr80^{gag} protein is 98 amino acids in length. Thirteen nonamer gPr80^{gag} peptides with amino acid sequence motifs for possible binding to H-2 D^b molecules were selected and constructed as detailed in Materials and Methods. Three peptides had the dominant anchor residues for H-2 D^b of N at position 5. Ten peptides had the dominant anchor residues of an aliphatic residue (I or L) at position 9. Five peptides were capable of binding to the D^b molecules and increased the mean fluorescence intensity on the surface of RMA-S cells at least 50% above the background levels as measured by FACS analysis (Table 3). None of the 13 nonamer gPr80^{gag} peptides were capable of binding to the K^b molecules. The FBL-3-specific CTL clones as well as bulk-cultured CTL from FBL-3-primed mice were tested for lysis of EL-4 cells incubated with individual peptides. Only 1 of 13 candidate peptides, CCLCLTVFL (gPr80^{gag} 85-93), was recognized by FBL-3-specific CTL clones and by the FBL-3-induced bulk-cultured CTL (Table 3).

To determine whether the nonamer peptide, CCLCLTVFL (gPr80^{gag} 85-93), was the minimum-length peptide epitope recognized by FBL-3-specific CTL, two octamer peptides, CLCL TVFL (p86-93) and CCLCLTVF (p85-92) from the gPr80^{gag} sequence were constructed and tested. CTL recognized both the nonamer (CCLCLTVFL) and octamer (CLCLTVFL) peptides but failed to recognize the octamer (CCLCLTVF) peptide (Table 3). CTL recognized the nonamer peptide CCLC LTVFL (p85-93) at a much lower peptide concentration (0.00064 μg/ml) than the octamer (CLCLTVFL) peptide (0.4 μg/ml) (Fig. 3). The peptide CCLCLTVFL has a very hydrophobic sequence and is insoluble in water and buffers. It had to be dissolved in dimethyl sulfoxide. Adding two lysine residues to the C terminus greatly improved the solubility of the peptide in water and buffers. This longer peptide (CCLCLTVFLKK) was found to be able to bind to H-2 D^b molecules and be recognized by the FBL-3-specific CTL (Table 3). The gPr80^{gag} 81-89 peptide (SIVLCCLCL), which was shown by Kondo et al. (24) to be recognizable by FBL-3 CTL clone B413-4, was also tested with and without C-terminus lysines, and neither form was recognized by our CTL clones or bulk-cultured CTL (Fig. 3 and Table 3).

The finding that all of the FBL-3 Gag-specific CTL clones tested recognized only the unique leader sequence peptide of gPr80^{gag} protein was striking and raised the question as to whether any other FBL-3-specific CTL recognize Pr65^{gag}-encoded proteins. Eighteen nonamer peptides with amino acid sequence motifs for possible binding to D^b molecules and three octamer peptides with amino acid sequence motifs for possible binding to K^b molecules were selected from Pr65^{gag} protein and constructed. The Pr65^{gag} peptides were tested for binding to D^b or K^b molecules in a peptide binding assay as detailed in Materials and Methods. Three nonamer peptides were capable of binding to the D^b molecules, and one octamer peptide (p15^{gag} 128-136) was capable of binding to the K^b molecules as measured by FACS analysis. However, when bulk-

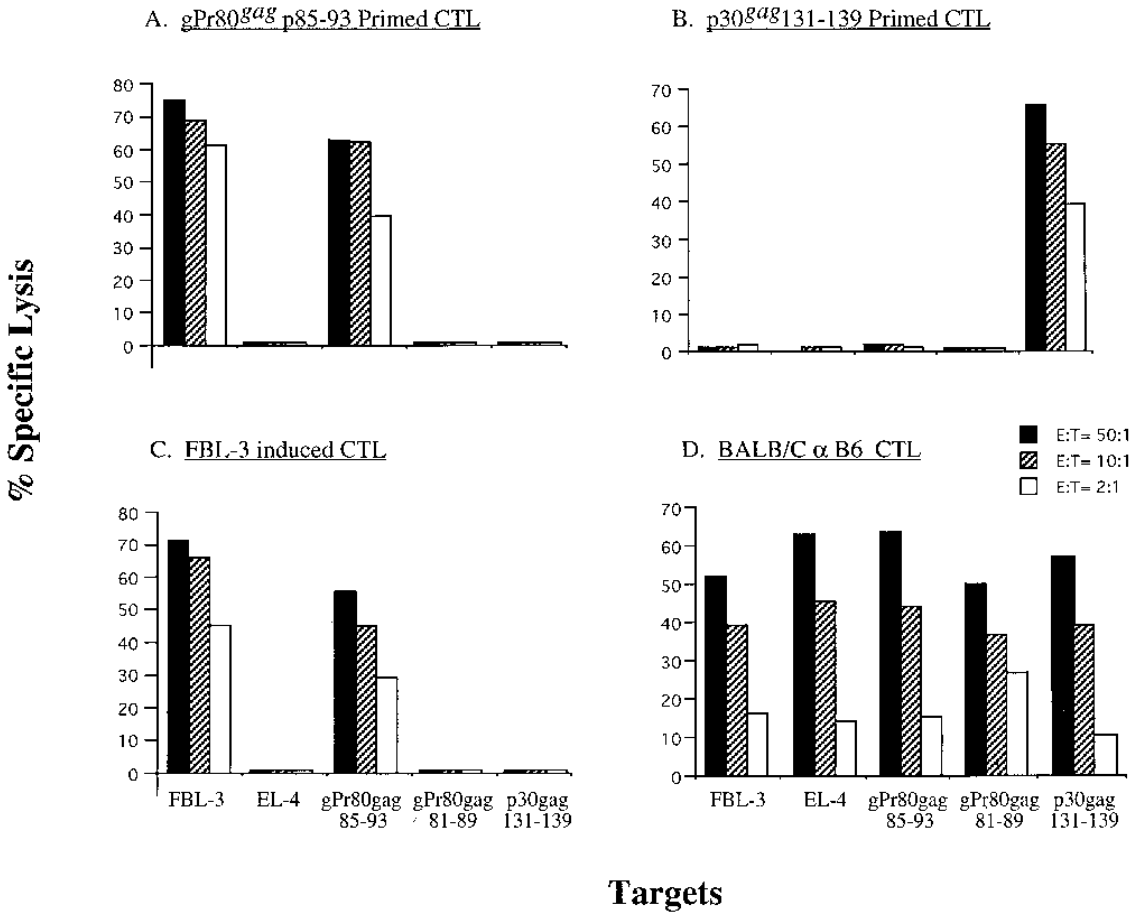

FIG. 4. Immunization of B6 mice with FBL-3 Gag p85-93 leader sequence elicits peptide-specific CTL that lyse FBL-3. B6 mice were immunized with one of two nonamer FBL-3 Gag peptides, CCLCLTVFL (gPr80^{gag} 85-93) or RSPTNLAKV (p30^{gag} 131-139). The peptide-primed splenic lymphocytes from each group of mice were restimulated in vitro with the immunizing peptides and assessed in a standard 4-h ^{51}Cr release assay for cytotoxicity against FBL-3 or EL-4 cells with or without preincubation with the immunizing Gag peptides or a control Gag peptide, SIVLCCLCL (gPr80^{gag} 81-89), at 25 μg/ml. BALB/c anti-B6 alloreactive T cells were generated by standard 5-day mixed lymphocyte culture and used as positive control effector cells. E:T, effector/target cell ratio.

cultured FBL-3-specific CTL (Table 3) and FBL-3 Gag-specific CTL clones were tested for lytic activity against a panel of 21 synthetic peptides from the Pr65^{gag} protein, none of the 21 candidate peptides was recognized.

Immunization of B6 mice with the leader sequence Gag peptide, CCLCLTVFL, elicits peptide-specific CTL that lyse FBL-3. To investigate whether the gPr80^{gag} 85-93 CTL epitope peptide was immunogenic, B6 mice were immunized with CC LCLTVFL (gPr80^{gag} 85-93) or with an alternative D^{b}-binding Gag peptide, RSPTNLAKV (p30^{gag} 131-139), from the p30^{gag} protein. The bulk-cultured CTL elicited from mice immunized with CCLCLTVFL specifically lysed EL-4 cells pulsed with CCLCLTVFL but did not lyse EL-4 cells alone or EL-4 cells pulsed with the gPr80^{gag} 81-89 (SIVLCCLCL) or p30^{gag} 131-139 (RSPTNLAKV) peptide (Fig. 4A). Most importantly, CCLCLTVFL peptide-specific CTL lysed FBL-3. The bulk-cultured CTL elicited from mice immunized with RSPTNL AKV lysed EL-4 cells pulsed with RSPTNLAKV but did not lyse FBL-3, EL-4 cells alone, or EL-4 cells pulsed with either gPr80^{gag} 85-93 or gPr80^{gag} 81-89 peptides (Fig. 4B). The failure of CTL specific for the p30^{gag} 131-139 peptide to lyse FBL-3 was expected, as this was not an epitope recognized by FBL-3-induced CTL (Table 4). The failure of the D^{b}-restricted CTL specific for the p131-139 peptide to lyse EL-4 cells pulsed with CCLCLTVFL demonstrated that this peptide did not nonspecifically sensitize target cells to lysis by irrelevant CTL. As controls, bulk-cultured FBL-3-specific CTL were shown to lyse FBL-3 and EL-4 cells pulsed with CCLCLTVFL but failed to lyse EL-4 cells pulsed with the alternate peptides (Fig. 4C). All targets were lysed by alloantigen-specific CTL (Fig. 4D). These results show that the gPr80^{gag} 85-93 peptide was capable of priming FBL-3-specific CTL by in vivo immunization.

The FBL-3 leader sequence peptide CCLCLTVFL is a major CTL epitope shared by leukemia/lymphoma cell lines induced by FMR MuLV. The F-MuLV gPr80^{gag} leader sequence peptide CCLCLTVFL is also present in the leader sequences of the gPr80^{gag} polyproteins encoded by Moloney and Rauscher MuLV. To determine whether immunity to CCLCLTVFL might account for the cross-reactivity between FMR MuLV-induced tumors, CTL generated by immunization with CCLC LTVFL peptide were tested for lysis of other FMR MuLV-induced tumors. The CCLCLTVFL peptide-induced CTL lysed FBL-3 as well as Moloney MuLV-induced MBL-2 and Rauscher MuLV-induced RBL-5 but failed to lyse EL-4 and LSTRA (Fig. 5). The CCLCLTVFL peptide-specific CTL lysed both RMA-S and EL-4 pulsed with the CCLCLTVFL peptide. The CCLCLTVFL peptide also proved to be an antigenic epitope of other FMR MuLV-induced tumors, as bulk-cultured CTL derived from B6 mice immunized to MBL-2 or RMA could effectively recognize and lyse EL-4 pulsed with

TABLE 4. FBL-3-specific CTL fail to recognize synthetic peptides from the amino acid sequence of the Pr65^{gag} structural polyprotein[a]

Target	Amino acid sequence	H-2 D^b binding	% Specific lysis
FBL-3			86
EL-4			4
EL-4 plus:			
gPr80^{gag} 85-93	CCLCLTVFL	+	66
p15^{gag} 53-61	DGTFNPDII	−	3
p15^{gag} 58-66	PDIITQVKI	−	3
p15^{gag} 75-83	GHPDQVPYI	+	4
p15^{gag} 81-89	PYIVTWEAI	−	8
p15^{gag} 128-136	QSSLYPAL	−	2
p12^{gag} 4-12	TSPLNTKPR	+	5
p12^{gag} 41-49	SSDGNGGSG	−	3
p12^{gag} 62-72	PMVSRLRGR	−	2
p30^{gag} 3-11	RQGGNGQFQ	−	1
p30^{gag} 19-26	SDLYNWKNN	−	8
p30^{gag} 87-95	TQLPNDIND	−	8
p30^{gag} 114-121	HLVHYRQL	−	2
p30^{gag} 124-132	AGLQNAGRS	−	3
p30^{gag} 131-139	RSPTNLAKV	+	0
p30^{gag} 143-151	TQGPNESPS	−	2
p30^{gag} 172-180	PGQETNVAM	−	2
p30^{gag} 175-183	ETNVAMSFI	−	5
p30^{gag} 192-200	RKLERLEDL	−	3
p30^{gag} 207-215	DLVREAEKI	−	2
p30^{gag} 221-229	TPEEREERI	−	4
p30^{gag} 255-262	RHREMSKL	−	4

[a] Pr65^{gag} peptides were tested for binding to H-2 D^b molecules and were assessed for recognition by FBL-3-specific CTL clones or bulk-cultured CTL in assays similar to those described in the footnotes to Table 3. The cytotoxicity assay data represent the specific lysis of targets by bulk-cultured CTL from FBL-3 immunized B6 mice (effector/target cell ratio of 20:1).

CCLCLTVFL peptide (Fig. 6). Thus, the FBL-3 leader sequence peptide CCLCLTVFL is a major CTL epitope shared by leukemia/lymphoma cell lines induced by FMR MuLV.

DISCUSSION

Previous studies established that CD8$^+$ CTL from B6 mice immunized with FBL-3 recognize F-MuLV *gag*-encoded antigens (23). The present experiments indicate that the main CTL response of these mice is directed against a single transmembrane epitope, CCLCLTVFL (p85-93), within the leader sequence of the gPr80^{gag} polyprotein. The use of CTL clones might conceivably have skewed the response toward epitopes that preferentially stimulate T-cell replication; however, the use of bulk-cultured CTL confirmed that the majority of the response was directed against this same epitope. Mouse endogenous retroviruses are primarily members of the xenotropic and polytropic families, which have Gag and Env structural proteins which are quite distinct from those of ecotropic MuLV such as FMR MuLV. FMR MuLV strains are exogenous ecotropic retroviruses which are genetically similar but immunologically distinct from the endogenous ecotropic viruses such as AKV and Gross MuLV. The current results provide a possible molecular explanation at the level of CTL for the lack of cross-protection observed many years ago between MuLV-induced leukemias induced by the exogenous ecotropic FMR MuLV and the endogenous ecotropic AKV and Gross MuLV (7, 15, 19). In AKV MuLV, the peptide from the portion of the transmembrane region of the gPr80^{gag} leader region observed to be a major Gag CTL epitope in FMR MuLV-induced leukemias has the sequence SFVCSLLFW, which has only two positions identical to the CTL epitope peptide, CCLCLTVFL, identified in this study.

Others have independently identified an FBL-3-specific CTL epitope in the transmembrane region of gPr80^{gag} (24). This epitope, identified by a single CTL clone (B413-4), consisted of amino acids SIVLCCLCL at positions 81 to 89 and thus at five positions overlapped the epitope CCLCLTVFL at positions 85 to 93 identified in the present study. However, the FBL-3-specific CTL clones as well as FBL-3-induced bulk CTL tested in our studies recognized and lysed only EL-4 cells incubated with peptide CCLCLTVFL (p85-93) but not the peptide SIVLCCLCL (p81-89) (Table 3, Fig. 3, and Fig. 4). Our results also showed that deletion of one amino acid at either the C or the N terminus of the Gag p85-93 peptide resulted in complete or significant reduction of the recognition of the peptide by our FBL-3-specific CTL. At this time we have no satisfactory explanation for this discrepancy in results; however, it is possible that the CTL clone used by Kondo et al. (24) is different in specificity from our CTL clones or bulk-cultured CTL from FBL-3-immunized mice. Importantly, the current study showed that immunization of B6 mice with the Gag p85-93 peptide can elicit peptide-specific CTL capable of lysing FBL-3 as well as syngeneic tumors induced by Moloney and Rauscher MuLV, which provides substantial evidence that CCLCLTVFL is a major *gag*-encoded CTL epitope. Sequenc-

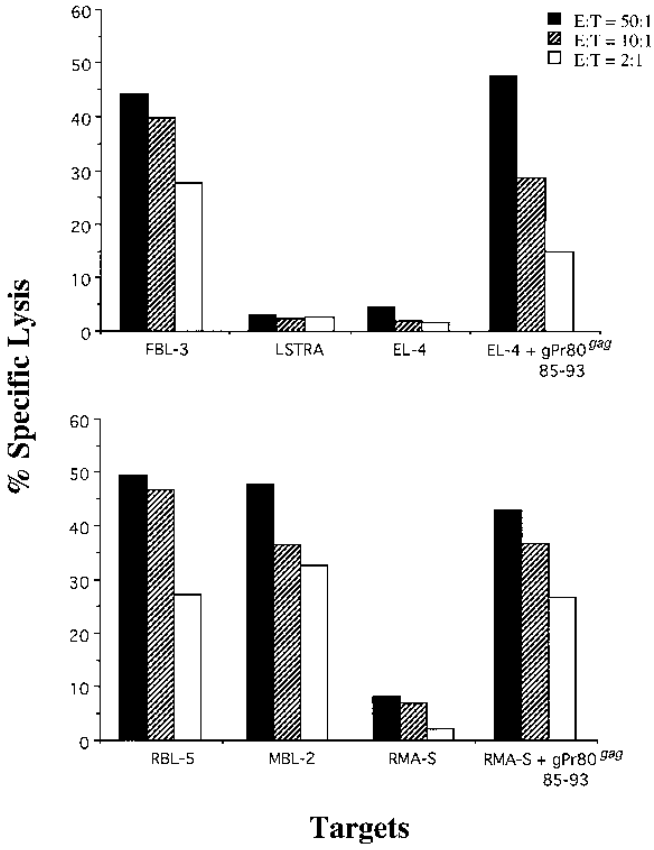

FIG. 5. CTL specific for FBL-3 leader sequence epitope CCLCLTVFL can lyse syngeneic leukemias induced by FMR MuLV. The CD8$^+$ CTL generated from FBL-3 gPr80^{gag} 85-93 peptide-immunized B6 mice were tested in a standard 4-h ^{51}Cr release assay with ^{51}Cr-labeled syngeneic (FBL-3, MBL-2, RBL-5, RMA, and RMA-S) or allogeneic (LSTRA) tumors induced by FMR MuLV as targets. Peptide-pulsed EL-4 or RMA-S targets were preincubated with gPr80^{gag} 85-93 peptide (25 μg/ml) for 1 h before used. E:T, effector/target cell ratio.

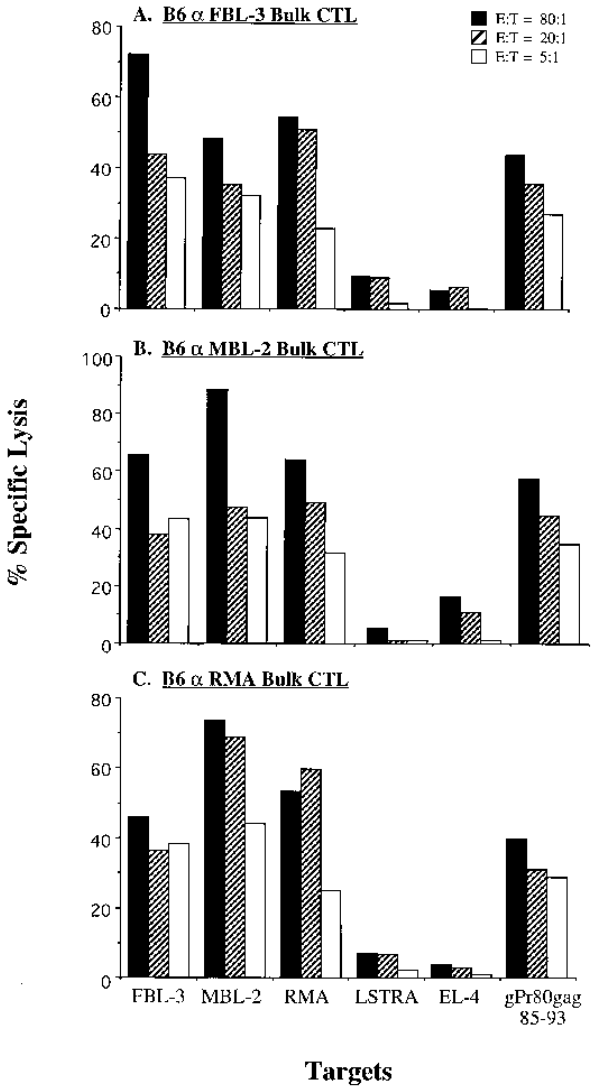

FIG. 6. CTL from B6 mice immunized with Moloney or Rauscher MuLV-induced syngeneic leukemias can recognize the immunodominant FBL-3 leader sequence epitope CCLCLTVFL. Bulk-cultured CTL derived from B6 mice immunized with MBL-2 or RMA leukemia cells were assessed in a standard 4-h ^{51}Cr release assay for cytolytic activity against ^{51}Cr-labeled FMR MuLV-induced tumors (FBL-3, MBL-2, RMA, and LSTRA) and EL-4 cells with or without preincubation with the FBL-3 gPr80^{gag} 85-93 peptide (25 μg/ml). E:T, effector/target cell ratio.

ing of peptides stripped from purified H-2 D^b will be required to validate the identity of the naturally processed immunodominant target peptide. The demonstration that immunization to CCLCLTVFL can effectively induce FBL-3-reactive CTL in mice will now allow use of the FBL-3 model to examine the principles of peptide-based vaccine and T-cell-mediated immunotherapy (26).

The presence of the *gag*-encoded CTL epitope in the leader sequence of the gPr80^{gag} polyprotein raises two interconnected questions: (1) Why is the $CD8^+$ T-cell-mediated immunity directed to an epitope in the transmembrane region? (2) How do *gag*-encoded structural virion proteins escape recognition by CTL? Dominance of CTL responses is related to the abundance of particular peptides in the binding cleft of class I major histocompatibility complex (MHC) molecules as well as to the abundance of T cells with antigen receptors specific for the particular peptides. A lack of response can mean that the particular epitope is not processed to be presented by class I MHC molecules, or a hole in the T-cell receptor repertoire may preclude T-cell reactivity. Peptides presented in the context of MHC class I molecules on the cell surface are usually processed fragments of internal proteins in cytosol transported by TAP1-TAP2 heterodimer proteins into the endoplasmic reticulum. The peptides presented need to be able to bind to MHC class I with a great enough affinity to compete with numerous other peptides for binding to nascent class I MHC molecules. One possibility for the CCLCLTVFL epitope is that the transmembrane peptide binds with exceedingly high affinity and competitively precludes binding of other Gag peptides. This possibility is unlikely, given the demonstration that the binding affinity of CCLCLTVFL is only moderate, as determined by competitive binding of synthetic peptides representing the epitope (data not shown). However, the binding affinity assays for synthetic peptides representing highly hydrophobic transmembrane regions might not be accurate because of the great difficulty in solubilizing the peptide. Moreover, methods to increase the solubility of the peptide such as dissolving it in polar compounds or adding flanking amino acids may have altered affinity characteristics.

While the MHC class I-restricted antigen-processing pathway may have evolved primarily to enable presentation of cytoplasmic proteins, accumulating evidence has shown that a variety of membrane proteins can be processed for class I-restricted recognition. Specific CTL epitopes have been identified and localized in the transmembrane domain and even within the signal peptide (reviewed in reference 36). The presentation of peptides derived from membrane proteins on class I molecules is suggested to involve both TAP-dependent and TAP-independent mechanisms (36). A second possibility for the CCLCLTVFL epitope is that leader sequence peptides may enter into the class I MHC processing pathway via an alternative pathway (1, 18, 40). In cells with deficient peptide transport, class I MHC molecules are present at the cell surface in only small amounts as a result of a lack of adequate MHC-binding peptides needed for MHC stability. The alternative pathway might be preferential for particular peptide MHC combinations, particularly if the peptide binds to the MHC molecule with moderate or greater affinity. It is possible that entry of the transmembrane CCLCLTVFL peptide into class I by the alternate pathway accounts for its importance despite only moderate affinity. The only experimental evidence supporting this possibility is the observation that CCLCL TVFL peptide-specific CTL lyse RMA-S to a low level. RMA-S has deficient peptide transport and low-level class I MHC expression. It is possible that a portion of the small number of class I MHC molecules expressed by RMA-S contain the CCLCLTVFL peptide.

A third possibility is that all T cells capable of recognizing structural Pr65^{gag} polyproteins have been deleted and that true immunological tolerance exists. Mouse DNA is known to contain numerous endogenous retroviral genes, and many of these are expressed at various times during ontogeny. Such early expression would be expected to influence selection of the T-cell repertoire and thus influence of the MuLV-encoded epitopes recognized by both $CD4^+$ and $CD8^+$ T cells. However, this is unlikely since mouse B cells producing monoclonal antibodies specific for p15, p12, and p30 F-MuLV Gag proteins have been described (6, 8). We have not yet examined whether immunization with cells expressing the Pr65^{gag} polyprotein without the gPr80^{gag} leader sequence can induce Gag-specific CTL. Moreover, the transmembrane region has been shown to

contain a CTL epitope only in B6 mice. Since individual allelic class I MHC molecules have unique peptide binding characteristics, it would be of interest to determine whether the *gag*-encoded CTL epitopes in other mouse strains are also present in the transmembrane domain of the Gag leader sequence.

A fourth possibility is that F-MuLV Pr65^{gag} polyproteins are blocked from entry into the class I MHC processing pathway. For some complex viruses, particular proteins, e.g., Epstein-Barr virus nuclear antigen 1 protein (22, 28) and cytomegalovirus-encoded IE1 protein (14), are blocked from entry into the class I MHC processing pathway. However, F-MuLV is considered to be a simple virus and has only structural proteins with the exception of the alternatively transcribed Gag glycopolyproteins. A class I MHC K^b molecule-restricted CTL epitope derived from the MCF1233 MuLV envelope (Env)-p15E region and shared by all endogenous AKV types of MuLV expressed on MCF1233 MuLV-induced lymphomas of B6 mice has recently been reported (35, 39). However, the MCF MuLV-Env peptide-induced CTL do not recognize B6 leukemias/lymphomas induced by FMR MuLV.

Although the majority of FBL-3-specific $CD8^+$ CTL recognize F-MuLV *gag*-encoded antigen, cytotoxicity assays using $CD8^+$ FBL-3-specific CTL clones showed that 6 of the 38 clones lysed FBL-3 but did not lyse cells expressing either F-MuLV Gag or Env antigen. The antigen(s) recognized by the non-F-MuLV Gag or Env antigen-specific CTL clones has not yet been determined. One possibility would be these CTL clones are also directed to F-MuLV *gag*-encoded antigen on FBL-3 but possess lower-affinity T-cell receptors and thus failed to exhibit detectable cytotoxicity in CTL assays using F-MuLV *gag*-transfected or vaccinia virus-*gag*-infected targets. Alternatively and more likely, such clones may recognize proteins with altered expression as a result of the upstream virus-induced transforming events. Altered expression of cancer-related proteins, such as oncogenic proteins with aberrant amino acid sequences, may offer the opportunity to serve as tumor antigens (4).

ACKNOWLEDGMENTS

We gratefully acknowledge V. Reese and K. Slaven for excellent technical assistance, A. Fefer and C. Öhlén for helpful discussions, and K. Whitham for manuscript preparation.

This work was supported by grants CA57851, CA54561, and CA30558 from the National Cancer Institute and by grant IRG-185 from the American Cancer Society.

REFERENCES

1. **Bacik, I., J. H. Cox, R. Anderson, J. W. Yewdell, and J. R. Bennink.** 1994. TAP (transporter associated with antigen processing)-independent presentation of endogenously synthesized peptides is enhanced by endoplasmic reticulum insertion sequences located at the amino- but not carboxyl-terminus of the peptide. J. Immunol. **152:**381–387.
2. **Berenson, J. R., A. B. Einstein, Jr., and A. Fefer.** 1975. Syngeneic adoptive immunotherapy and chemoimmunotherapy of a Friend leukemia: requirement for T cells. J. Immunol. **115:**234–238.
3. **Cheever, M., P. Greenberg, and A. Fefer.** 1980. Specificity of adoptive chemoimmunotherapy of established syngeneic tumors. J. Immunol. **125:**711–714.
4. **Cheever, M. A., M. L. Disis, H. Bernhard, J. R. Gralow, S. L. Hand, E. S. Huseby, H. L. Qin, M. Takahashi, and W. Chen.** 1995. Immunity to oncogenic proteins. Immunol. Rev. **145:**33–59.
5. **Chen, W., V. A. Reese, and M. A. Cheever.** 1990. Adoptively transferred antigen-specific T cells can be grown and maintained in large numbers in vivo for extended periods of time by intermittent restimulation with specific antigen plus IL-2. J. Immunol. **144:**3659–3666.
6. **Chesebro, B., W. Britt, L. Evans, K. Wehrly, J. Nishio, and M. Cloyd.** 1983. Characterization of monoclonal antibodies reactive with murine leukemia viruses: use in analysis of strains of Friend MCF and Friend ecotropic murine leukemia virus. Virology **127:**134–148.
7. **Chesebro, B., and K. Wehrly.** 1976. Studies on the role of the host immune response in recovery from Friend virus leukemia. II. Cell-mediated immunity. J. Exp. Med. **143:**85–99.
8. **Chesebro, B., K. Wehrly, M. Cloyd, W. Britt, J. Portis, J. Collins, and J. Nishio.** 1981. Characterization of mouse monoclonal antibodies specific for Friend murine leukemia virus-induced erythroleukemia cells: Friend-specific and FMR-specific antigens. Virology **112:**131–144.
9. **Corbin, A., A. C. Prats, J. L. Darlix, and M. Sitbon.** 1994. A nonstructural *gag*-encoded glycoprotein precursor is necessary for efficient spreading and pathogenesis of murine leukemia viruses. J. Virol. **68:**3857–3867.
10. **Dickson, C., R. Eisenman, H. Fan, E. Hunter, and N. Teich.** 1982. Protein biosynthesis and assembly, p. 513–648. *In* R. Weiss, N. Teich, H. Varmus, and J. Coffin (ed.), RNA tumor viruses. Molecular biology of tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
11. **Earl, P. L., B. Moss, R. P. Morrison, K. Wehrly, J. Nishio, and B. Chesebro.** 1986. T-lymphocyte priming and protection against Friend leukemia by vaccinia-retrovirus env gene recombinant. Science **234:**728–731.
12. **Falk, K., O. Rotzschke, S. Stevanovíc, G. Jung, and H. G. Rammensee.** 1991. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. Nature (London) **351:**290–296.
13. **Feltkamp, M. C., M. P. Vierboom, W. M. Kast, and C. J. Melief.** 1994. Efficient MHC class I-peptide binding is required but does not ensure MHC class I-restricted immunogenicity. Mol. Immunol. **31:**1391–1401.
14. **Gilbert, M. J., S. R. Riddell, C. R. Li, and P. D. Greenberg.** 1993. Selective interference with class I major histocompatibility complex presentation of the major immediate-early protein following infection with human cytomegalovirus. J. Virol. **67:**3461–3469.
15. **Glynn, J. P., J. L. McCoy, and A. Fefer.** 1968. Cross-resistance to the transplantation of syngeneic Friend, Moloney, and Rauscher virus-induced tumors. Cancer Res. **28:**434–439.
16. **Greenberg, P. D.** 1991. Adoptive T cell therapy of tumors: mechanisms operative in the recognition and elimination of tumor cells. Adv. Immunol. **49:**281–355.
17. **Hasenkrug, K. J., D. M. Brooks, and B. Chesebro.** 1995. Passive immunotherapy for retroviral disease: influence of major histocompatibility complex type and T-cell responsiveness. Proc. Natl. Acad. Sci. USA **92:**10492–10495.
18. **Heemels, M. T., and H. Ploegh.** 1995. Generation, translocation, and presentation of MHC class I-restricted peptides. Annu. Rev. Biochem. **64:**463–491.
19. **Herberman, R. B., T. Aoki, M. Nunn, D. H. Lavrin, N. Soares, A. Gazdar, H. Holden, and K. S. Chang.** 1974. Specificity of ^{51}Cr-release cytotoxicity of lymphocytes immune to murine sarcoma virus. J. Natl. Cancer Inst. **53:**1103–1111.
20. **Holt, C. A., K. Osorio, and F. Lilly.** 1986. Friend virus-specific cytotoxic T lymphocytes recognize both gag and env gene-encoded specificities. J. Exp. Med. **164:**211–226.
21. **Iwashiro, M., T. Kondo, T. Shimizu, H. Yamagishi, K. Takahashi, Y. Matsubayashi, T. Masuda, A. Otaka, N. Fujii, A. Ishimoto, A. Ishimoto, M. Miyazawa, M. N. Robertson, B. Chesebro, and K. Kuribayashi.** 1993. Multiplicity of virus-encoded helper T-cell epitopes expressed on FBL-3 tumor cells. J. Virol. **67:**4533–4542.
22. **Khanna, R., S. R. Burrows, M. G. Kurilla, C. A. Jacob, I. S. Misko, T. B. Sculley, E. Kieff, and D. J. Moss.** 1992. Localization of Epstein-Barr virus cytotoxic T cell epitopes using recombinant vaccinia: implications for vaccine development. J. Exp. Med. **176:**169–176.
23. **Klarnet, J. P., D. E. Kern, K. Okuno, C. Holt, F. Lilly, and P. D. Greenberg.** 1989. FBL-reactive CD8+ cytotoxic and CD4+ helper T lymphocytes recognize distinct Friend murine leukemia virus-encoded antigens. J. Exp. Med. **169:**457–467.
24. **Kondo, T., H. Uenishi, T. Shimizu, T. Hirama, M. Iwashiro, K. Kuribayashi, H. Tamamura, N. Fujii, R. Fujisawa, M. Miyazawa, and H. Yamagishi.** 1995. A single retroviral Gag precursor signal peptide recognized by FBL-3 tumor-specific cytotoxic T lymphocytes. J. Virol. **69:**6735–6741.
25. **Ljunggren, H.-G., N. Stam, C. Öhlén, J. Neefjes, P. Höglund, M.-T. Heemels, J. Bastin, T. Schumacher, A. Townsend, K. Kärre, and H. Ploegh.** 1990. Empty MHC class I molecules come out in the cold. Nature (London) **346:**476–480.
26. **Melief, C. J., and W. M. Kast.** 1994. Prospects for T cell immunotherapy of tumours by vaccination with immunodominant and subdominant peptides. Ciba Found. Symp. **187:**97–104.
27. **Miyazawa, M., J. Nishio, and B. Chesebro.** 1992. Protection against Friend retrovirus-induced leukemia by recombinant vaccinia viruses expressing the *gag* gene. J. Virol. **66:**4497–4507.
28. **Murray, R. J., M. G. Kurilla, J. M. Brooks, W. A. Thomas, M. Rowe, E. Kieff, and A. B. Rickinson.** 1992. Identification of target antigens for the human cytotoxic T cell response to Epstein-Barr virus (EBV): implications for the immune control of EBV-positive malignancies. J. Exp. Med. **176:**157–168.
29. **Prats, A. C., G. De-Billy, P. Wang, and J. L. Darlix.** 1989. CUG initiation codon used for the synthesis of a cell surface antigen coded by the murine leukemia virus. J. Mol. Biol. **205:**363–372.
30. **Robertson, M. N., G. J. Spangrude, K. Hasenkrug, L. Perry, J. Nishio, K. Wehrly, and B. Chesebro.** 1992. Role and specificity of T-cell subsets in

spontaneous recovery from Friend virus-induced leukemia in mice. J. Virol. **66:**3271–3277.

31. **Rotzschke, O., K. Falk, K. Deres, H. Schild, M. Norda, J. Metzger, G. Jung, and H. G. Rammensee.** 1990. Isolation and analysis of naturally processed viral peptides as recognized by cytotoxic T cells. Nature (London) **348:**252–254.
32. **Rotzschke, O., K. Falk, S. Stevanovíc, G. Jung, P. Walden, and H. G. Rammensee.** 1991. Exact prediction of a natural T cell epitope. Eur. J. Immunol. **21:**2891–2894.
33. **Ruan, K. S., and F. Lilly.** 1991. Identification of an epitope encoded in the env gene of Friend murine leukemia virus recognized by anti-Friend virus cytotoxic T lymphocytes. Virology **181:**91–100.
34. **Shimizu, T., H. Uenishi, Y. Teramura, M. Iwashiro, K. Kuribayashi, H. Tamamura, N. Fujii, and H. Yamagishi.** 1994. Fine structure of a virus-encoded helper T-cell epitope expressed on FBL-3 tumor cells. J. Virol. **68:**7704–7708.
35. **Sijts, A. J. M., F. Ossendorp, E. A. M. Mengedé, P. J. van den Elsen, and C. J. M. Melief.** 1995. 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. J. Immunol. **152:**106–116.
36. **Siliciano, R. F., and M. J. Soloski.** 1995. MHC class I-restricted processing of transmembrane proteins: mechanism and biologic significance. J. Immunol. **155:**1–5.
37. **Smith, G. L., B. R. Murphy, and B. Moss.** 1983. Construction and characterization of an infectious vaccinia virus recombinant that expresses the influenza hemagglutinin gene and induces resistance to influenza virus infection in hamsters. Proc. Natl. Acad. Sci. USA **80:**7155–7159.
38. **Townsend, A., C. Ohlén, J. Bastin, H. G. Ljunggren, L. Foster, and K. Karre.** 1989. Association of class I major histocompatibility heavy and light chains induced by viral peptides. Nature (London) **340:**443–448.
39. **White, H. D., D. A. Roeder, and W. R. Green.** 1994. An immunodominant Kb-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. J. Virol. **68:**897–904.
40. **Zweerink, H. J., M. C. Gammon, U. Utz, S. Y. Sauma, T. Harrer, J. C. Hawkins, R. P. Johnson, A. Sirotina, J. D. Hermes, B. D. Walker, et al.** 1993. Presentation of endogenous peptides to MHC class I-restricted cytotoxic T lymphocytes in transport deletion mutant T2 cells. J. Immunol. **150:**1763–1771.