Resistance to Polyoma Virus-Induced Tumors Correlates with CTL Recognition of an Immunodominant H-2D<sup>k</sup>-Restricted Epitope in the Middle T Protein

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Resistance to Polyoma Virus-Induced Tumors Correlates with CTL Recognition of an Immunodominant H-2D<sup>k</sup>-Restricted Epitope in the Middle T Protein<sup>1</sup>

Aron E. Lukacher<sup>2</sup> and Christopher S. Wilson

The natural mouse pathogen polyoma virus is highly oncogenic in H-2<sup>k</sup> mice carrying the endogenous superantigen encoded by the mouse mammary tumor provirus Mtv-7. This superantigen results in deletion of V<beta>6 TCR-expressing polyoma-specific CD8<sup>+</sup> CTL, which appear to be critical effectors against polyoma tumorigenesis. Here we have isolated cloned lines of CD8<sup>+</sup> T cells from resistant (i.e., Mtv-7<sup>-</sup>) H-2<sup>k</sup> mice that specifically lyse syngeneic polyoma virus-infected cells and polyoma tumor cells. Nearly all these CTL clones express V<beta>6 and are restricted in their recognition of virus-infected cells by H-2D<sup>k</sup>. Screening a panel of synthetic peptides predicted to bind to D<sup>k</sup>, for which no consensus peptide binding motif is known, we identified a peptide corresponding to a nine-amino acid sequence in the carboxyl-terminus of the middle T (MT) protein (amino acids 389–397) that was recognized by all the V<beta>6<sup>+</sup>CD8<sup>+</sup> CTL clones. The inability of MT<sub>389–397</sub>-reactive CTL to recognize cells infected with a mutant polyoma virus encoding a MT truncated just proximal to this sequence indicates that MT<sub>389–397</sub> is a naturally processed peptide. The frequencies of precursor CTL specific for polyoma virus and MT<sub>389–397</sub> peptide were similar, indicating that MT<sub>389–397</sub> is the immunodominant epitope in H-2<sup>k</sup> mice. In addition, polyoma-infected resistant mice possess a 10- to 20-fold higher MT<sub>389–397</sub>-specific precursor CTL frequency than susceptible mice. This highly focused CTL response to polyoma virus provides a valuable animal model to investigate the in vivo activity of CTL against virus-induced neoplasia.


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<sup>4</sup>Abs abbreviations used in this paper: HPV, human papillomavirus; ST, small T protein; MT, middle T protein; LT, large T protein; SAG, superantigen; Mtv, mouse mammary tumor provirus; mos, multiplicity of infection; BMK, primary baby mouse kidney cell cultures; IMDM, Iscove’s modified Dulbecco’s medium; pCTL, CTL precursor.

Specific mutations in a class I MHC CTL viral epitope in cervical cancers (7). A primary obstacle to dissecting the components of immune responsiveness to papovaviruses and their induced tumors is the absence of good animal models that mimic natural infection.

Polyoma virus is a mouse papovavirus capable of inducing a broad spectrum of epithelial and mesenchymal cell-derived tumors (8). The 5.3-kb circular polyoma DNA genome encodes three early region nonstructural proteins, termed small T (ST), middle T (MT), and large T (LT), and three late region virion capsid proteins. Cellular transformation, tumor induction, and virion assembly require constitutive expression of MT (9–11), the oncoprotein of polyoma virus. A number of studies using athymic mice and adoptive transfer of virus-immune T cells clearly document that anti-viral T lymphocytes are responsible for conferring resistance to polyoma tumors (12, 13). A protective anti-tumor effector function for anti-polyoma class I MHC-restricted CTL is suggested by a recent report showing that mice immunized with a synthetic peptide corresponding to an LT protein sequence that binds to a class I MHC molecule reject syngeneic polyoma tumors (14).

In immunocompetent mice, high susceptibility to polyoma virus-induced tumors is manifested only by neonatal inoculation of certain inbred strains of H-2<sup>b</sup> mice (15, 16). We recently presented evidence indicating that this susceptibility is conferred by the endogenous superantigen (SAG) encoded by the mouse mammary tumor provirus-7 (Mtv-7). By deleting thymocytes whose TCR use specific β-chain variable domains (Vβ) (17), Mtv-7SAG creates a hole in the peripheral T cell repertoire for T cells required for polyoma tumor immunosurveillance (18). Biased usage of the Mtv-7SAG-reactive Vβ domain, Vβ6, was found among CD8<sup>+</sup> T cells infiltrating a polyoma tumor implanted in a virus-immune, resistant (i.e., Mtv-7-negative) H-2<sup>b</sup> mouse, and CD8<sup>+</sup>Vβ6<sup>+</sup> T cells expressing virus-specific cytotoxicity dominated bulk cultures of T cells derived from neonatally infected, resistant mice (18). These findings strongly implicate polyoma virus-specific
CD8$^+$ V$\beta$6$^+$ CTL as essential anti-polyoma tumor effectors in vivo.

A long-standing difficulty in defining the effector T cell(s) that mediate anti-polyoma tumor activity and identifying T cell epitopes has been an inability to establish lines of polyoma-specific T cells. One early study reported induction of specific lytic activity against virus-infected and -transformed cells by CD8$^+$ T cells from virus-infected mice after coculture with polyoma-transformed syngeneic cells (19). We recently showed that short term cultures of virus-immune T cells restimulated in vitro with infected syngeneic stimulator cells exhibited anti-viral and anti-tumor cytotoxicity (18). Here, we report the isolation of polyoma-specific CD8$^+$ CTL cloned lines from virus-infected H-2$^b$ mice. As expected from the predominant usage of V$\beta$6 by anti-viral CTL in bulk cultures, most CTL clones express a V$\beta$6 TCR. Moreover, nearly all the clones are restricted by the H-2D$^k$ molecule in their recognition of virus-infected cells. In the absence of a consensus motif for peptides bound to D$^k$, potential anchor residues for D$^k$ binding peptides were predicted from crystallographically solved and molecularly modeled class I MHC:peptide structures. Using this deduced D$^k$ peptide binding motif, we identified a nine-amino acid sequence in the MT protein as the immunodominant anti-viral CTL epitope. CTL directed to this epitope recognize both virus-infected cells and cells derived from polyoma-induced tumors. Polyoma tumor-susceptible mice were found to possess a 10- to 20-fold lower frequency of precursor CTL to this epitope than resistant mice. Possible explanations for the inability of a weak anti-viral CTL response to prevent the outgrowth of virus-transformed cells are discussed.

Materials and Methods

Animals

C57BR/cdJ and C3H/HeSnJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). C3H/HeNcr and C3H/BiDaCr mice were purchased from the Frederick Cancer Research and Development Center of the National Cancer Institute (Frederick, MD). Pregnant ICR outbred mice and female, 4-wk-old, Sprague-Dawley rats were purchased from Harlan Sprague-Dawley, Inc. (Indianapolis, IN).

Virus and virus inoculation

The polyoma virus strain A2 was molecularly cloned and plaque purified. Virus stocks were prepared by inoculating primary kidney cells from 10-day-old ICR mice at a multiplicity of infection (moi) of 0.1 and carrying cultures in the presence of DMEM (Mediatech, Herndon, VA) containing 2% FBS (HyClone, Inc., Logan, UT) to complete the viral cytopathic effect. Virus stocks used for inoculating mice were similarly prepared, except that kidney cells were obtained from 10-day-old C57BR/cdJ mice, and virus infection was conducted in the absence of serum; these stocks are designated serum-free A2 virus. Cell lysates were titrated by plaque assay on UC1B cells (obtained from American Type Culture Collection, Rockville, MD) and typically contained 2 $\times$ 10$^7$ plaque-forming units/mL. Newborn mice (<18 h of age) were injected s.c. in hindfootpads with 20 to 30 $\mu$L of serum-free A2 virus.

The polyoma virus strain PTA1387T encodes a MT protein lacking the carboxyl-terminal 37 amino acids (11). The parental PTA virus, like the A2 virus, is a highly tumorigenic, large plaque, polyoma strain (8); the MT protein sequences of PTA and A2 are identical. PTA1387T virus stocks, is a highly tumorigenic, large plaque, polyoma strain (8); the MT protein sequences of PTA and A2 are identical. PTA1387T virus stocks, derived from molecularly cloned virus on primary baby mouse kidney cells were obtained from American Type Culture Collection. Cell lines were maintained in DMEM containing 10% FBS.

Cell lines

AG014A cells were derived from a spontaneous tumor of C3H/HeN (H-2$^b$) origin (20) (provided by Dr. H. Schreiber, University of Chicago, Chicago, IL, SVCOL, SVB10.A, and SVD2 are SV40-transformed cell lines from C3H/OL (H-2K$^D$), B10.A (H-2K$^D$), and DBA/2 (H-2$^d$) mice, respectively (21) (provided by Dr. L. Gooding, Emory University, Atlanta, GA). 6215 cells were derived from a polyoma virus-induced salivary tumor arising in a gamma-irradiated (900 rad), virus-infected adult C57BR/cdJ (H-2$^b$) mouse (18). L929 cells were obtained from American Type Culture Collection. Cell lines were maintained in DMEM containing 10% FBS.

P1.HTR.D$^d$ and P1.HTR.K$^d$ are D$^d$- and K$^d$-expressing transfectants, respectively, of P1.HTR (H-2$^d$) cells (provided by Dr. A. Van Pel, Ludwig Institute for Cancer Research, Brussels, Belgium) and were maintained in Iscove’s modified Dulbecco’s medium (IMDM; Life Technologies, Gaithersburg, MD) supplemented with 10% FBS, 1,029, 6215, P1.HTR.D$^d$, and P1.HTR.K$^d$ are nonpermissive, and AG104A, SVCOL, SVB10.A, and SVD2 are highly permissive for productive infection by polyoma virus (data not shown).

Pepitides

Peptides were synthesized by the solid phase method on a Symphony/Multiplex Peptide Synthesizer (Rainin, Woburn, MA) with F-moc chemistries. HPLC analysis showed that peptides were >95% pure. Peptide stock solutions were prepared in water at a concentration of 3 mM and stored at $-20^\circ$C. Peptides were diluted in IMDM and 10% FBS immediately before use in cytotoxicity assays.

Establishment of bulk and cloned lines of polyoma virus-specific CTL

Bulk cultures of polyoma-immune T cells were established as previously described (18). Briefly, 1 x 10$^6$ mononuclear cells from draining popliteal and inguinal lymph nodes of 12- to 14-day-old virus-infected mice were cocultured with 10 x 10$^5$ A2 virus-infected syngeneic splenocytes, gamma-irradiated (2000 rad) 24 h after infection, in 24-well cluster plates (Costar, Cambridge, MA) in 2 mL of IMDM complete medium (IMDM supplemented with 10% FBS, 8% medium of Con A-pulsed rat splenocytes (peptide-reactive T cells described in Ref. 22), 4 mM glutamine, 5 mM 2-mercaptoethanol, 50 mM 2-ME, penicillin, and streptomycin). In vitro tertiary cultures were established by restimulating 2 to 5 x 10$^5$ viable mononuclear cells from these day 7 cultures using the same protocol. All cultures were maintained in a humid atmosphere of 7% CO$_2$ at 37°C.

T cells were cloned by limiting dilution from day 7 in vitro secondary or tertiary cultures. Viable cells were cocultured in 96-well flat-bottom microtiter plates (Costar) with 1 x 10$^6$ virus-infected, gamma-irradiated (2000 rad) syngeneic splenocytes in 0.2 mL/well IMDM complete medium. T cells were cultured in replicates of 48 or 96 wells at dilutions ranging from 1 to 20 cells/well. Clusters of proliferating cells appeared at 5 to 7 days of incubation. To insure that expanded T cells were derived from individual precursor clones, proliferating cell clusters were selected for expansion when the frequency of positive wells at a given responder dilution was <15%, and individual wells contained only one proliferating cell cluster. Typically, 10 of 96 replicate wells from in vitro tertiary cultures plated at 10 cells/well were positive for proliferating cell clusters. T cells were initially expanded for 7 days in 48-well cluster plates containing 5 x 10$^5$ virus-infected, irradiated, syngeneic stimulators in 1 mL/well IMDM complete medium and subsequently expanded to 24-well dishes under the same conditions as those used to established the bulk cultures.

$^{51}$Cr release assay

Polyoma virus-infected AG104A target cells were prepared as follows. AG104A monolayers harvested with trypsin-EDTA (Life Technologies) were plated at 5 x 10$^5$ cells/well into six-well cluster plates (Nunc, Naperville, IL) in 0.2 mL of DMEM containing polyoma virus at an moi of 5, incubated at 37°C for 1.5 to 2 h, overlaid with 3 mL of DMEM containing 10% FBS and murine rIFN-γ (100 U/mL), and incubated at 37°C for another 16 h. Uninfected cells were either treated with lysates of uninfected BMK cells or simply overlaid with medium. To radiolabel adherent target cells, medium was removed, and 150 to 200 μCi of Na$_2$CrO$_4$ (400–1200 Ci/g; New England Nuclear, Boston, MA) with 5% FBS was added to cells. After incubating at 37°C for 1.5 h, $^{51}$Cr was removed, then replaced with 1 mL of DMEM and 10% FBS, and incubated at 37°C for 1 h. For experiments using peptide-pulsed targets, proteases at the indicated concentration were prepared in DMEM and 10% FBS, and added at this second incubation step. Cells were then rinsed with DMEM three times, harvested with trypsin-EDTA, and washed twice with DMEM and 10% bovine calf serum (Spectrum Biotechnology, Ft. Collins, CO). $^{51}$Cr-labeled target cells were aliquoted at 5000 cells/well into either V-bottom or U-bottom 96-well microtiter plates (Costar). In certain experiments, 50 μL of peptides were added at 3 times their final concentration to wells containing 50 μL of $^{51}$Cr-labeled cells, then after 1-h incubation at 37°C, 50 μL of T cells were added. For experiments using virus-infected or peptide-pulsed targets, 100 μL of target cells and 100 μL of T cells were cocultured in each well. The assay medium was IMDM and 10% FBS. After a 4- to 6-h incubation at 37°C, half the volume of each well was removed and counted in a Beckman Gamma-4000 counter (Beckman, Fullerton, CA).Spontaneous $^{51}$Cr release from target cells in all assays was 10

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to 20% of the total lysis. The percent specific lysis was calculated as follows: ([106Cr released with effector cells) − (spontaneous 106Cr release) / (total 106Cr released with 1% Triton X-100) − (spontaneous 106Cr release)] × 100. The percent specific lysis values represent the means of four replicate wells. SEMs were always <5% of the mean values and are omitted. Each 106Cr release assay was performed a minimum of three times with similar results; representative experiments are shown.

Limiting dilution assay for polyoma-specific CTL precursor frequency

Viable, nucleated spleen cells from polyoma virus-immune mice were treated in replicates of 16 wells into U-bottom 96-well microtiter plates (Costar) containing 5 × 105 virus-infected, irradiated (2000 rad) C3H/HeN spleen cells in a total volume of 0.2 ml/well IMDM complete medium. Seven days later, each well received 5 × 103 infected, irradiated C3H/HeN spleen cells to further expand Ag-specific T cells and increase sensitivity in detecting anti-polyoma CTL. Five days later, murine rIL-12 (PharMingen, San Diego, CA) was added at a final concentration of 10 U/ml. IL-12 boosts the Ag-specific cytotoxicity of mature, anti-polyoma CD8+ CTL (A. Lukacher, manuscript in preparation). After an additional 2 days of incubation, each well was split into three wells of U-bottom 96-well microtiter plates, to which were added 2500 3H-Cr-labeled, murine rIFN-γ (PharMingen)-treated (100 U/ml for 24 h) AG104A target cells that were untreated, pulsed with 10 μM MT383-390 peptide, or infected with polyoma virus. Positive wells were defined as wells for which 3H release values exceeded the mean release values of uninfected target cells by >3 times the SD. The CTL precursor frequencies were determined according to the method of Quintans and Lefkovits (23).

Cytofluorometric analysis

Viable T cells were isolated on LSM (Organon Teknika Corp., Durham, NC) step gradients and indirectly stained with FITC-conjugated goat anti-hamster IgG or FITC-conjugated goat anti-rat IgG F(ab)2 secondary Abs (Caltag Laboratories, South San Francisco, CA) and culture supernatants of the following hybridomas: H57-597 (anti-TCR-β), B20.6 (anti-Vβ2), 44-22-1 (anti-Vβ6), and KJ16 (anti-Vβ1/8.2). Phycoerythrin-conjugated anti-CD4, anti-CD8α, and IgG2a isotype control Abs were also purchased from Caltag Laboratories. Flow cytometry was performed on a Becton Dickinson (Mountain View, CA) FACSort using LYSIS II software, and data were analyzed using CellQuest software.

Results

Isolation of polyoma virus-specific CTL cloned lines

Virus-specific CTL from two polyoma tumor-resistant H-2k inbred mouse strains, C57BR/cdJ and C3H/HeSnJ (18), were cloned by limiting dilution from in vitro tertiary cultures of T cells harvested from draining lymph nodes of approximately 2-wk-old mice inoculated with polyoma virus at birth. Each round of in vitro restimulation consisted of weekly coculture of T cells with syngeneic target cells, the spleen cell stimulators were irradiated at 24 h after polyoma virus infection (A. Lukacher, unpublished observations). Because H-2L genes are absent in H-2k mice (27), only H-2K and D molecules are available to present viral Ag to these anti-polyoma CTL clones was determined using as targets SV40-transformed fibroblasts from B10.A (Kk Dd), C3H.OL (Kd Dk), and B10.D2 (Kd Dk) mice. These target cells are sensitive to CTL-mediated lysis (21) and are highly permissive for productive polyoma virus infection (A. Lukacher, unpublished observations). Because H-2L genes are absent in H-2k mice (27), only H-2K and D molecules are available to present viral peptides to anti-polyoma CTL. As shown in Figure 2, all 10 CTL clones specifically recognized virus-infected cells bearing H-2Dk.

Identification of an H-2Dk-bound peptide epitope for anti-polyoma CTL

The highly biased usage of Vβ6 and common Dk restriction pattern of the anti-polyoma CTL clones led us to hypothesize that polyoma virus-specific CTL recognize very few epitopes. To determine whether these CTL are directed to an epitope derived from an early region viral protein, a cell line derived from a syngeneic polyoma virus-induced tumor, designated 6215, was assayed for recognition by anti-polyoma CTL. 6215 cells express full-length genes that encode the early region viral protein polyomavirus middle T antigen (Fig. 1). The middle T antigen is a trans-activator of the virus that expresses a protein, p64, which is a transcriptional activator for the viral genome. The middle T antigen is also a transforming protein that is required for tumorigenesis. Therefore, the polyomavirus middle T antigen is a key player in the life cycle of the virus.

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Each of these T cell clones lysed polyoma virus-infected, but not uninfected, syngeneic target cells (Fig. 1). Identification of the class I MHC molecule that restricts viral Ag presented to CTL was determined using class I MHC-deficient tumor targets. The polyomavirus middle T antigen is a key player in the life cycle of the virus.

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ST and MT, a carboxyl-truncated LT, and no viral capsid proteins (18). We had previously shown a preferential infiltration of CD8⁺ Vβ6⁺ T cells into 6215 tumors implanted in virus-immune C57BR/cdJ mice (18). As shown in Table II, representative Vβ6⁺ CTL clones 11-1 and 13-2 as well as an in vitro tertiary culture of virus-primed C3H/HeSnJ mice composed nearly entirely of CD8⁺ Vβ6⁺ T cells lysed 6215 cells but not the syngeneic, spontaneous tumor cell line AG104A. The Vβ8.1⁺ CTL clone, 16-5, also specifically lysed this polyoma tumor cell target. The ability of anti-polyoma CTL clones to lyse 6215 cells shows that CTL induced in polyoma-infected mice and passed in vitro with infected syngeneic stimulators recognize Ags shared by syngeneic virus-infected and -transformed cells, and that these epitopes are derived from an early region viral protein(s).

No consensus motif has been described for peptides bound to Dk to permit prediction of CTL epitopes from polyoma early region proteins presented by this class I MHC molecule. Only one Dk-bound peptide has been reported (28). This nonapeptide, gag88–96, is encoded by a transposed element within the gene of an endogenous defective retrovirus selectively expressed in a spontaneous leukemia. To deduce a plausible Dk peptide binding motif, we examined the polymorphic residues of the α1/α2 domains of the Dk heavy chain (29) predicted by MHC class I:peptide structures to occupy peptide binding pockets and to interact with specific peptide side chains (reviewed in Ref. 30). Prediction of Dk peptide anchor residues was gauged against the sequence of gag88–96. Among class I MHC molecules, the collection of polymorphic residues whose side chains point into the B pocket of HLA-B27 (H9, T24, E45, C67, Y99) most closely approximates the corresponding residues in Dk (E9, S24, D45, A67, S99) in charge and/or polarity. Because threonine 24 and glutamic acid 45 in HLA-B27 are particularly important in stabilizing binding to peptides with arginine at position 2 (31), we predict a basic residue at this position (arginine in gag88–96) for peptides binding to Dk. Peptides bound to murine class I molecules invariably have a hydrophobic carboxyl-terminal anchor residue (32), with pocket F residue 116 primarily determining the identity of this peptide anchor (33). Like Kk, which accommodates peptides with a carboxyl-terminal leucine, Dk also has a phenylalanine 116; leucine occupies the carboxyl-terminal position of Dk (34). Finally, a basic anchor residue at position 5 (lysine in gag88–96) is predicted based on molecular modelling of peptides bound to Dk, where aspartic acid 156 in the D pocket, a polymorphism shared by Dk and Dd, interacts with a position 5 arginine in Dk binding peptides (34).

Table II. Anti-polyoma virus CTL specifically lyse a polyoma tumor cell line

<table>
<thead>
<tr>
<th>CTL</th>
<th>Vβ</th>
<th>% Specific Lysis</th>
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<tbody>
<tr>
<td>11-1</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>13-2</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>16-5</td>
<td>8.1</td>
<td>4</td>
</tr>
<tr>
<td>Bulk culture</td>
<td>6</td>
<td>0</td>
</tr>
</tbody>
</table>

The AG104A and 6215 cell lines were used as targets in a 4-h ⁵¹Cr-release assay at an E:T ratio of 10:1.

The Vβ phenotyping was performed as described in Table I.

The 6215 cell line is derived from a polyoma virus-induced tumor in an irradiated C57BR/cdJ mouse (see Materials and Methods).

The bulk culture is a >98% CD8⁺ Vβ6⁺ T cell in vitro tertiary culture of lymph node cells from a polyoma virus-immune C3H/HeSnJ mice.

![FIGURE 2. MHC restriction mapping of polyoma virus-specific CTL clones. CTL clones were assayed for cytolytic activity against uninfected or virus-infected SV40-transformed target cells of DBA/2 (KdDk), C3H.COL (KdDk), and B10.A (KkDd) origin for 4 h at an E:T cell ratio of 10:1.](Image)
Vβ8.1 (data not shown). An MT389–397 nonreactive CTL clone, 26-2 (Fig. 4C), was restricted by Kk in its recognition of virus-infected cells (data not shown). The MHC and peptide specificities of the MT389–397 peptide-specific CTL clones were confirmed by their capacity to specifically lyse MT389–397-pulsed allogeneic target cells expressing transfected Dk, but not Kk, molecules (data not shown).

Titration of the early region peptides revealed cross-reactivity by MT389–397-reactive CTL clones with the MT236–244 peptide at high peptide concentrations. MT389–397 peptide concentrations as low as 10−8 to 10−6 M were able to sensitize L929 (H-2Kk) targets for CTL lysis, with maximal levels of lysis seen at 0.1 to 1 μM (Fig. 4A); this concentration range is similar to that reported for CTL recognition of peptide-pulsed L929 targets by Kk-restricted CTL clones (35). At concentrations 3 to 4 logs greater than that required for MT389–397 target cell sensitization (100 μM), the MT236–244 peptide rendered target cells sensitive to CTL-mediated lysis (Fig. 4A). No target cell lysis was seen with the four other peptides tested in this concentration range. The Dk-restricted presentation of MT236–244 was confirmed using peptide-pulsed P1.HTR.Dk and P1.HTR.Kk cells as CTL targets (data not shown). Of the other MT389–397-reactive CTL clones, only clone 14-1 lysed target cells pulsed with MT389–397 but not MT236–244 (Fig. 4B), indicating that recognition of MT389–397 is sufficient for killing polyoma-infected cells. To determine whether cross-reactive recognition of MT236–244 contributes to the overall lysis of infected cells by MT389–397-reactive CTL, CTL clones were tested for their ability to recognize target cells infected with the mutant polyoma virus PTA1387T, whose MT is truncated at amino acid 384. As shown in Figure 4C, the MT389–397 nonreactive CTL clones 15-5 and 26-2 lysed cells infected with either wild-type virus or PTA1387T virus, while the MT389–397-reactive CTL clones 11-1 and 13-2 efficiently lysed only wild-type virus-infected targets. The low level lysis of PTA1387T-infected targets by clone 11-1, but not by clone 13-2, may indicate a minor contribution of the MT236–244 peptide to recognition of polyoma-infected cells by some MT389–397-reactive CTL. Taken together, these results demonstrate that the MT389–397 epitope is a naturally processed peptide in polyoma-infected cells, and that CTL recognition of MT389–397 is necessary and sufficient for lysis of infected cells.

MT389–397 is the core peptide epitope

Because the MT389–397 peptide has repetitive amino acids at both its amino and carboxyl termini, we asked whether truncations at either end affect CTL recognition. Peptides lacking the arginine at position 1, lacking either one or both carboxyl-terminal leucines, or lacking the first and last amino acids were assayed over a 5-log concentration range for their efficiency in sensitizing syngeneic targets. The low level lysis of PTA1387T-infected targets by MT389–397-nonreactive CTL clones 11-1 and 13-2 efficiently lysed only wild-type virus-infected targets. The low level lysis of PTA1387T-infected targets by clone 11-1, but not by clone 13-2, may indicate a minor contribution of the MT236–244 peptide to recognition of polyoma-infected cells by some MT389–397-reactive CTL. Taken together, these results demonstrate that the MT389–397 epitope is a naturally processed peptide in polyoma-infected cells, and that CTL recognition of MT389–397 is necessary and sufficient for lysis of infected cells.

The predicted motif for peptides binding to H-2Dk molecules is a basic amino acid at positions 2 and 5 and a carboxyl-terminal leucine; X designates nonanchor residues. Peptide residue positions are numbered starting at the amino terminus.

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| Table III. Polyoma virus early region amino acid sequences that satisfy a predicted Dk peptide-binding motif* |
|-----------------------------------------|-------------------------------------------------|
| 1           | X | Basic | X | Basic | X | X | X | L |
| gag88–96    | R | R | K | G | K | Y | T | G | L |
| T16-14      | S | R | A | D | K | E | R | L | L |
| MT236–244   | S | R | R | L | L | R | L | P | S | L |
| MT389–397   | R | R | L | G | R | T | L | L | L | L |

* The predicted motif for peptides binding to H-2Dk molecules is a basic amino acid at positions 2 and 5 and a carboxyl-terminal leucine; X designates nonanchor residues. Peptide residue positions are numbered starting at the amino terminus.

FIGURE 3. Identification of a Dk-bound peptide by polyoma virus-specific CTL clones. Anti-polyoma virus CTL clones described in Table I were assayed for cytolytic activity against 51Cr-labeled L929 targets at an E:T cell ratio of 5:1 for 4 h. Peptides were added to the assay at a final concentration of 1 μM.
MT<sub>389–397</sub> is the immunodominant epitope for anti-polyoma CTL in H-2<sup>k</sup> mice

Since MT<sub>389–397</sub> is recognized by the majority of the anti-polyoma CTL clones, we asked whether this epitope is immunodominant in polyoma tumor-resistant H-2<sup>k</sup> mice. Short-term cultures of polyoma virus-immune T cells established from neonatally infected C3H/HeSnJ mice were evaluated for cytolytic activity against infected or MT<sub>389–397</sub> peptide-pulsed syngeneic target cells. As shown in Figure 6, virus-immune T cells from four individual mice lysed MT<sub>389–397</sub>-pulsed and virus-infected targets, but not gag<sub>88–96</sub>-coated and uninfected targets. The level of killing of MT<sub>389–397</sub>-coated targets tended to exceed that of infected targets, suggesting that this epitope may be inefficiently processed and/or presented by cells productively

**FIGURE 4.** Cross-reactive recognition of MT<sub>236–244</sub> by MT<sub>389–397</sub>-reactive CTL clones. A, CTL clones 8-1 and 13-2 were assayed for 4 h at an E:T cell ratio of 5:1 for cytolytic activity against <sup>51</sup>Cr-labeled L929 target cells in the presence of the indicated concentration of peptide. The flu NP<sub>50–57</sub> (SDYEGRLL) peptide binds to H-2K<sup>k</sup> (72). B, CTL clones 11-1 and 14-1 were assayed for 4 h at the indicated E:T cell ratios for cytolytic activity against <sup>51</sup>Cr-labeled L929 target cells pulsed with MT<sub>389–397</sub> (1 μM), MT<sub>236–244</sub> (100 μM), or gag<sub>88–96</sub> (100 μM) peptides. C, Anti-polyoma CTL clones 15-5 and 26-2 (MT<sub>389–397</sub>-nonreactive), and clones 11-1 and 13-2 (MT<sub>389–397</sub>-reactive) were assayed for 7 h at an E:T cell ratio of 10:1 for their capacity to lyse <sup>51</sup>Cr-labeled AG104A cells infected with the wild-type virus, A2 strain, or the mutant polyoma virus, PTA1387T, whose MT ends at amino acid 384.

**FIGURE 5.** Determination of the minimum epitope optimally recognized by MT<sub>389–397</sub>-reactive CTL. The ability of MT<sub>389–397</sub>-reactive CTL clones 11-1 and 13-2 to recognize titrated concentrations of peptides lacking amino- and/or carboxyl-terminal residues of the MT<sub>389–397</sub> sequence were tested in a 4-h <sup>51</sup>Cr release assay against L929 target cells at an E:T cell ratio of 5:1.
infected with polynoma, as described in other anti-viral CTL systems (36–38).

In our previous report (18), the resistant H-2^k mouse strain C57BR/cdJ was used to assess the anti-polyoma CTL response. C57BR/cdJ is a VB^6^ genotype mouse strain in which approximately 40% of the germ-line VB^6^ gene segments are deleted (39). We wanted to determine whether the highly biased usage of VB^6^ by anti-polyoma CTL in C57BR/cdJ mice also extended to the CTL response elicited by polynoma in C3H/HeSnJ mice, a polynoma tumor-resistant H-2^k^ strain possessing a complete repository of VB^6^ genes (i.e., VB^6^ genotype). Single-color flow cytometric analysis for VB^6^, CD8, and CD4 was therefore conducted on each of the C3H/HeSnJ anti-viral CTL cultures shown in Figure 6. As expected based on the impact of Mtv-7SAG deletion of VB^6^ expressing thymocytes on polynoma susceptibility (18), T cells in each of these in vitro tertiary cultures predominantly expressed VB^6^ and CD8 (Fig. 6). Moreover, in contrast to T cell Ag unresponsiveness incurred by the VB^6^-truncated TCR repertoire in other systems (40, 41), C57BR/cdJ and C3H/HeSnJ mice both mounted an immunodominant CTL response to the MT_{389–397} epitope.

Limiting dilution analysis was performed to quantitate the numbers of CTL precursors (pCTL) in neonatally infected C3H/HeSnJ that were directed toward polynoma virus-infected and MT_{389–397} peptide-pulsed cells. pCTL analysis was conducted separately on two virus-primed mice. As shown in Figure 7A, the precursor frequency for CTL recognizing polynoma-infected syngeneic targets is approximately 1/40,000 spleen cells, and that for pCTL recognizing MT_{389–397}-pulsed targets is approximately 1/20,000 spleen cells. This virus-specific pCTL frequency is similar to that reported in other virus-mouse systems (42, 43). Given that each well in the limiting dilution assay containing cytotoxic activity against MT_{389–397}-pulsed targets also exhibited specific killing, although generally at lower levels, of virus-infected targets (data not shown), the higher precursor frequency for MT_{389–397}-reactive CTL than for anti-viral CTL probably reflects higher sensitivity in detecting specific CTL recognition using peptide-pulsed targets.

**Polyoma tumor-susceptible mice have a lower MT_{389–397} specific precursor CTL frequency than resistant mice**

Since the MT_{389–397} peptide is the immunodominant epitope for anti-polyoma CTL in resistant (i.e., Mtv^-^-^-) mice, we asked whether the precursor frequencies for MT_{389–397}-reactive CTL differed between resistant and susceptible (i.e., Mtv^-^-^-) mice. Spleen cells from neonatally immunized C3H/HeSnJ (resistant) and C3H/BiDa (susceptible) mice were plated in limiting dilution cocultures with virus-infected, irradiated syngeneic stimulators.
Fig. 8. As expected given their derivation from a Mtv-7 provirus-susceptible and -resistant H-2k mice is directed to the same immunodominant epitope in MT.

Discussion

In this report we provide the first identification of an epitope for polyoma virus-specific CTL. Derived from the viral oncprotein, MT, the MT389–397 nonapeptide was predicted to bind to H-2Dk based on a three-residue MHC anchor motif deduced from crystallographic and molecular modelling analyses of class I MHC:peptide complexes. Comparison of this CTL epitope with the only reported Dk-associated CTL epitope (28) lends support to the prediction that peptides with basic amino acids at positions 2 and 5 and a carboxyl-terminal leucine interact with pockets B, D, and F, respectively, in the peptide binding groove of Dk. Additional support for this Dk peptide consensus motif comes from the finding that two other polyoma early region peptides (T6–14 and MT102–109), selected on the basis of this putative motif, compete with MT389–397 in sensitizing cells for lysis by anti-polyoma CTL clones, while a Kk-binding peptide (Flu NP50–57) did not compete (data not shown). A third peptide from the polyoma early region sequence that satisfies the Dk binding motif, MT236–244, also associates with Dk, as evidenced by its weak agonist activity for MT389–397-reactive CTL clones (Fig. 4A). Studies are in progress to directly demonstrate that peptide positions 2, 5, and 9 are Dk anchor residues and to determine their relative contributions to the overall binding of peptides to Dk.

A central question is whether CTL mediate protection from polyoma virus tumorigenesis by eradicating virus-infected cells, virus-transformed cells, or both. Adoptive transfer of virus-immune spleen cells into infected nude mice has been shown to eliminate replicating viral DNA, which, in turn, is associated with the absence of polyoma virus-induced tumors (44). That polyoma-transformed cells may also serve as in vivo targets for virus-immune T cells is illustrated by the inability of CD4+ and/or CD8+ T cell-depleted, virus-infected mice to reject a polyoma tumor cell challenge (45). The MT389–397 sequence spans the region of MT immediately amino-terminal to and extending into the hydrophobic membrane-anchoring region of this type II-integral membrane, 421-amino acid protein. Because localization of MT to membranes is essential for its capacity to transform cells and induce tumors (11), and full-length MT is constitutively expressed in polyoma-induced tumors of all histotypes (46) (A. Lukacher, unpublished observations), MT389–397 is an ideal target epitope for a host CTL response against polyoma tumors. Furthermore, as an early region viral protein, MT is expressed by cells that are nonpermissive for viral DNA replication (24) in addition to its expression by productively infected cells. CTL surveillance for Dk-MT389–397 complexes, then, should control polyoma tumors not only by destroying virus-transformed cells but also by eliminating permissively and nonpermissively infected cells, both of which have the potential to undergo neoplastic transformation in vivo.

The marked immunodominance of the MT389–397 epitope in the anti-polyoma CTL response is also notable given that this CTL reactivity is directed toward a nonstructural target protein in a system permissive for polyoma replication. Class I MHC-restricted CTL epitopes from proteins of oncogenic viruses are typically identified using CTL isolated from virus-inoculated nonnatural hosts (i.e., nonpermissive for viral replication) and/or by in vitro culture with virus-transformed cells (47–49). Under these experimental conditions, potential CTL epitopes from viral proteins only expressed in productively infected cells are excluded. In the present study, polyoma-specific CTLs are isolated from virus-infected mice by in vitro restimulation with virus-infected spleen cells, which support productive polyoma virus replication (D. Drake and A. Lukacher, unpublished observations).

While high MHC binding affinity is likely to be a prerequisite for the immunodominance of MT389–397 as for other class I MHC epitopes (50), virus-host cell interactions may also guide selection of MT389–397 as the immunodominant CTL epitope. The MT389–397 epitope would be expected to be presented by productively infected, nonpermissive cells and transformed cells. Such widespread Ag presentation as well as persistent presentation by nonlytically infected cells may preferentially drive expansion of CTL directed to MT389–397 over, for example, CTL recognizing...
epitopes from viral capsid proteins, which are expressed in lytically infected cells and variably expressed in polyoma tumors (46). Also, since the anti-polyoma CTL described here are derived from mice 2 wk postinfection, the dominant anti-polyoma virus CTL response to MT_{389–397} may reflect a narrowing of the repertoire of anti-viral CTL specificities elicited during acute infection to those found in the memory CTL population (43, 51).

A number of scenarios can be invoked to account for the failure of the weak anti-polyoma CTL response to control polyoma virus tumorigenesis. A low precursor frequency anti-polyoma CTL response may provide sufficient time for transformed cells to acquire mutations that enable them to evade CTL surveillance by any of a number of mechanisms (52). Although rare immune escape polyoma tumor variants emerge in polyoma-tumor-resistant mice (18), in vivo selection of such tumor cell variants by anti-polyoma CTL is unlikely to serve as a general mechanism for neoplasia in susceptible mice given their high incidence of tumors, multiplicity of tumor types (8, 15). We, instead, favor the hypothesis that Mtv-7SAG-mediated deletion of V{beta}_{6} CTL in neonatally infected mice, which include the predominant polyoma-specific CTL response in Mtv-7{sup *} (resistant) H-2{sup b} mice, leads to a persistently high viral Ag state that interferes with the expansion and/or the function of the residual non-V{beta}_{6} anti-polyoma CTL. In tumor-susceptible mice, high levels of integrated, often defective, replicating viral DNA are present in tissues destined to develop tumors as well as in the tumors themselves, and only low amounts of infectious virus can be recovered from tumors (46, 53, 54). In contrast, little replicating viral DNA is detectable in virus-infected immunocompetent adult mice (53), which are uniformly resistant to polyoma virus tumorigenesis (15).

In this regard, it is interesting to note that polyoma-infected adult Mtv-7{sup *}H-2{sup b} mice mount a vigorous anti-viral CTL response directed primarily to the MT_{389–397} epitope (D. Drake and A. Lukacher, unpublished observation). Experiments are underway to compare the levels of viral DNA and viral protein expression in tumor-prone tissues in neonatally infected susceptible mice to those sites in neonatally infected resistant mice. Selective loss of anti-viral CTL in the setting of high systemic viral loads is seen in mice neonatally infected with lymphocytic choriomeningitis virus (55, 56), and high doses of antigenic peptides can induce apoptosis of CD8{sup +} CTL in vitro (57, 58). In light of the regulatory effect of Ag dose on Th1 vs Th2 differentiation (59), it is also conceivable that a persistently elevated Ag state could indirectly limit differentiation and/or clonal expansion of anti-polyoma CD8{sup +} CTL by promoting Th2 over Th1 differentiation of polyoma-specific CD4{sup +} T cells. Studies are in progress to investigate these possibilities.

A striking feature of the anti-polyoma CTL response in resistant H-2{sup b} mice is its strongly biased usage of V{beta}_{6} (Fig. 6) (18). The recently solved structures of two TCRs with class I MHC:peptide complexes show the V{beta} CDR1 domain positioned over the peptide’s carboxyl end (60, 61). Because all V{beta}_{6}{sup +} polyoma virus-specific CTL clones examined to date (n = 20) recognize the D^{k}: MT_{389–397} ligand, it is conceivable that the V{beta} CDR1 may directly contact common residues in this peptide. The distinct profiles of recognition of truncated MT_{389–397} peptides by V{beta}_{6}^{+} CTL clones (Fig. 5) further suggest a level of diversity in their CDR3s to accommodate interactions with different residues in the peptide. This conclusion is supported by preliminary TCR sequence analysis of the anti-polyoma V{beta}_{6}{sup +} CTL clones, indicating considerable length and sequence heterogeneity in their CDR3B regions and diverse V{beta} gene usage (C. Wilson and A. Lukacher, manuscript in preparation). Diverse TCR repertoires to single immunodominant class I MHC-restricted CTL epitopes have been described in other viral systems (62–64). Although strongly preferred, the V{beta} domain is not essential for TCR interaction with D^{k}:MT_{389–397} complexes, as rare MT_{389–397}reactive V{beta}8.1{sup +} CTL clones have been isolated from resistant mice, and Mtv-7{sup +} susceptible mice (which lack V{beta}_{6} and V{beta}8.1-bearing T cells; data not shown and Ref. 65) also recognize this MHC:peptide complex. Whether MT_{389–397}reactive thymocytes expressing the V{beta}6 domain are preferentially positively selected by polyoma-infected/-transformed thymic epithelial cells (8) or an endogenous D{sup 8} peptide ligand, or naive V{beta}6^{+} MT_{389–397}-specific T cells are preferentially activated upon contact with virus-infected cells remains to be determined.

The in vivo relevance of MT_{236–244} cross-reactivity by MT_{389–397}-reactive CTL is unclear. The expression of two functional TCRs is unlikely given the high frequency of MT_{236–244} cross-reactivity among the MT_{389–397}-reactive CTL clones (66, 67). In light of recent studies showing that a given TCR can interact with several unrelated peptides in the context of the same MHC molecule (68–70), we favor the concept that MT_{389–397}-reactive CTL express a single TCR that can recognize the MT_{236–244} peptide. In this regard, it is interesting to note that MT_{236–244} shares only the three putative D{sup 8} anchor residues with MT_{389–397}, with the corresponding residues at the other positions having nonconservative side chains (Table III). Studies are in progress to distinguish between single and dual TCR expression by these cross-reactive CTL. In vivo CTL recognition of the MT_{236–244} Peptide is also suspect given the high concentrations of MT_{236–244} peptide required to sensitize target cells for lysis by MT_{389–397}-specific CTL clones. In addition, attempts to expand T cells from virus-immune mice with MT_{236–244}-pulsed splenic stimulators have been unsuccessful (data not shown). This dissociation between target cell sensitization and proliferation is in line with a report that higher ligand concentrations are required to drive CD8^{+} CTL proliferation than to trigger target cell lysis (71). In contrast, MT_{389–397}-pulsed splenic stimulators efficiently promoted the outgrowth of T cells from polyoma-immune mice; these T cells specifically killed not only virus-infected and MT_{389–397}-pulsed target cells, but, notably, MT_{236–244} pulsed targets as well (data not shown). We were also unable to detect TCR antagonism of target cell lysis by MT_{236–244} or by the nonagonist peptides T_{6–14} and MT_{102–109} of MT_{389–397}-specific CTL clones (data not shown). Another possibility is that D{sup 8} binding peptides from polyoma proteins compete intracellularly with MT_{389–397} for binding to D{sup 8}; the consequent reduction in cell surface MT_{389–397} epitope density could contribute to the apparent weaker CTL recognition of virus-infected than MT_{389–397}-pulsed targets.

In conclusion, we have identified a D^{k}-bound nonamer peptide derived from the polyoma virus oncoprotein, MT, as the immunodominant anti-polyoma CTL epitope in H-2{sup b} mice. To the best of our knowledge, this is the first identification of an epitope for CTL specific for polyoma virus-infected/transformed cells. Importantly, CTL directed to the MT_{389–397} epitope were detected in mice susceptible to developing polyoma virus-induced tumors, but their precursor frequency was at least 10-fold lower than that of syngeneic resistant mice. This raises interesting questions concerning the inability of these CTL to control outgrowth of polyoma tumors. Rare polyoma virus-specific CTL clones that do not recognize MT_{389–397} have also been isolated, and studies to map their epitopes are in progress. Understanding the contributions of CTL directed to the dominant MT_{389–397} epitope and to subdominant viral epitopes in immunity to polyoma tumorigenesis has general importance in the development of vaccination strategies against virus-induced tumors.
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