# Cytotoxic T Lymphocytes (CTL) Against a Transforming Gene Product Select for Transformed Cells with Point Mutations within Sequences Encoding CTL Recognition Epitopes

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## Summary

The 94-kD large tumor (T) antigen specified by simian virus 40 (SV40) is sufficient to induce cell transformation. T antigen contains four H-2D$^b$-restricted cytotoxic T lymphocyte (CTL) recognition epitopes that are targets for CTL clones Y-1, Y-2, Y-3, and Y-5. These epitopes have been mapped to T antigen amino acids 207–215 (site I), 223–231 (sites II and III), and 489–497 (site V), respectively. Antigenic site loss variant cells that had lost one or more CTL recognition epitopes were previously selected by coculturing SV40-transformed H-2D$^b$ cells with the site-specific D$^b$-restricted CTL clones. The genetic bases for T antigen CTL recognition epitope loss from the variant cells were identified by DNA amplification and direct sequencing of epitope-coding regions from variant cell DNAs. Cells selected for resistance to CTL clone Y-1 (K-1; K-1,4,5; K-3,1) carry deleted SV40 genomes lacking site I, II, and III coding sequences. Point mutations present within the site II/III coding region of Y-2-/Y-3-resistant cell lines specify the substitution of asparagine for lysine as T antigen amino acid 228 (K-2) or phenylalanine for tyrosine at position 230 (K-3). Point mutations identified within independently selected Y-5 resistant populations (K-5 and K-1,4,5) direct the substitution of isoleucine for asparagine at position 496 (K-5) or the substitution of phenylalanine for isoleucine at position 491 (K-1,4,5) of T antigen. Each substitution causes loss of the relevant CTL recognition epitope, apparently by compromising CTL T cell receptor recognition. These experiments identify specific amino acid changes within a transforming protein that facilitate transformed cell escape from site-specific CTL clones while allowing maintenance of cellular transformation. This experimental model system provides unique opportunities for studying mechanisms of transformed cell escape from active immunosurveillance in vivo, and for analysis of differential host immune responses to wild-type and mutant cell-transforming proteins.

Tumors induced by DNA viruses acquire specific antigens that provide targets for T lymphocyte-mediated immune responses modulating tumor cell growth in vivo and abrogating cellular transformation in vitro (1–4). Specific CTL recognition of tumor cells requires the presentation by MHC class I molecules of processed tumor antigen to the T lymphocyte antigen receptor. Tumor cell variants that escape CTL immunosurveillance have been described (5–18). In most instances, the emergence of CTL escape variant cells is associated with the decrease or absence of MHC class I antigens required for antigen recognition by CTL (11–16). In other cases, a complete loss of tumor antigen from the variants has been reported (6, 10, 17–19).

Molecular studies indicate that mutations within individual cellular genes can induce the formation of new CTL recognition epitopes (20). In the P815 mouse mastocytoma tumor system, nontumorigenic variants (tum$^-$)[1] of tumorigenic P815 cells (tum$^+$) were isolated after chemical mutagenesis of the tum$^+$ cells (21). The decrease in tumorigenicity observed for tum$^-$ cells correlated with the acquisition of genomic point mutations creating new CTL antigenicities recognized by tum$^-$-specific CTL (21–25). Antigen-loss variant cells were obtained by cocultivating tum$^-$ "antigen-gain" variant cells with CTL specific for the new tum$^-$

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CTL epitopes. In each of three antigen-loss cell lines, a deletion within a gene encoding a tum⁻ CTL recognition epitope was demonstrated (26). However, the loss of CTL recognition sites from antigen-loss variant tumor cells because of spontaneous point mutations in CTL recognition epitope coding sequences has not been reported.

The simian virus 40 (SV40) large tumor (T) antigen, a 94-kD protein that is sufficient to induce transformation of mammalian cells to malignancy (27), serves as a tumor rejection antigen (28, 29). In context, portions of T antigen form epitopes recognized by T antigen-specific CTL. The T antigen contains four H-2D^b-restricted CTL recognition sites recognized by site-specific CTL clones Y-1, Y-2, Y-3, and Y-5 (28, 30), and one H-2K^b-restricted site recognized by CTL clone Y-4 (28). These CTL recognition epitopes are designated sites I, II, III, V, and IV, respectively. CTL recognition site I maps to T antigen amino acids 207–215 (31); sites II and III lie within T antigen amino acids 223–231 (31); and site V was first mapped within amino acids 489–503 (30). Recent experiments have delimited site V within T antigen amino acids 489–497 (31). Site IV lies within T antigen amino acids 369–511.

To define the mechanisms of spontaneous loss of functional CTL recognition sites from tumor antigens, we have analyzed the T antigen CTL recognition epitope coding sequences of SV40 T antigen-transformed antigenic site loss variant cells. Variant cells were previously selected by cocultivating SV40-transformed C57BL/6 primary kidney cells (K-0) with site-specific CTL clones recognizing T antigen epitopes (32, 33). Variant cells selected by coculture with CTL clone Y-1 were designated K-1. Those selected by coculture with CTL clone Y-2 or Y-3 were designated K-2 and K-3, respectively. Coculture of K-0 with CTL clone Y-5 selected the variant cell population designated K-5. It was demonstrated that loss of variant cell susceptibility to lysis by T antigen-specific CTL was not due to a loss of T antigen or MHC class I antigen (32).

The purpose of this study was to determine the genetic basis and molecular mechanism of spontaneous functional loss of CTL recognition sites from antigenic site loss variant cells. Changes in primary amino acid sequences corresponding to the CTL recognition epitopes of SV40 T antigen are shown to disrupt the ability of T antigen to function as a target for site-specific CTL clones.

## Materials and Methods

*CTL Clones.* The generation and maintenance of SV40 T antigen-specific, H-2D^b-restricted CTL clones Y-1, Y-2, Y-3, and Y-5, and of H-2K^b-restricted CTL clone Y-4, have been described (28, 30).

*SV40 T Antigen Epitope Loss Variants and Additional Cells.* Antigenic site loss variant cells were derived by coculture of SV40-transformed C57BL/6 primary kidney cells (K-0) with SV40 T antigen-specific CTL clones (32, 33). For example, K-1 cells were selected by coculture with CTL clone Y-1, K-2 cells with CTL clone Y-2, and K-1,4,5 cells with Y-1, then Y-4, and then Y-5. Cell lines B6/PY derived from a tumor induced by polyoma virus (34), and MC57G derived from a tumor induced by methylcholanthrene

in a C57BL/6 mouse (35) have been described. All cell lines were maintained in DME containing 5 or 10% fetal bovine serum.

*Cytotoxicity Assays.* Target cell susceptibility to lysis by T antigen-specific CTL clones was evaluated in chromium release assays as described (28, 36).

*Amplification of SV40 DNA from Variant Cell Genomes.* Sequences were amplified from variant cell genomic DNA (37) according to the specifications of the GeneAmp DNA Amplification Kit (Perkin-Elmer Cetus Corp., Norwalk, CT). Supplemental MgCl₂ was added to 3.5 mM. The thermal cycler program (Step-cycle; Perkin-Elmer Cetus Corp.) used consisted of one cycle denaturation at 94°C for 2 min; 29 cycles of annealing at 51°C for 2 min, extension at 72°C for 3 min, and denaturation at 94°C for 1 min; and 1 cycle of annealing at 51°C for 2 min and extension at 72°C for 10 min. Primary amplification products were isolated by gel electrophoresis, band excision, and DNA extraction (GeneClean, BIO 101, Inc., La Jolla, CA). Asymmetric secondary amplification (38) was performed on 100–200 ng of primary amplification product, with one primer present at 10 nM and the second at 1 µM. Reaction products were extracted with chloroform, and purified by ultrafiltration (Ultrafree MC 30,000 NMW polysulfone, Millipore Corp., Bedford, MA) in 1× TE.

*PCR Primers.* Orientations of oligonucleotides used are indicated by "s" (indicates same sense as T antigen mRNA) or "o" (indicates opposite sense). Primers for site I, II, and III primary amplifications (spanning SV40 nucleotide (nt) 4760–3233) were s⁵'GGGTTGATTGCTACTGCTTC³' and o⁵'TCATGGTGACTA-TTCCAGGGGG³'. For site I, II, and III secondary amplifications (spanning nt 4571–3749), primers s⁵'GGGTTGATTGCTACTGC-TTC³'/o⁵'TAGTTAATTGTAGGCTATCAACCCGC³' were used. For some secondary amplifications of sites II and III, (spanning nt 4316–3749), s⁵'GCTGCACTGCTATACAAGAA³' and o⁵'TAGTT-AATTGTAGGCTATCAACCCGC³' were used. For site V primary amplifications, (spanning nt 4196–3011), s⁵'ATTAATAACTA-TGCTCAAGAATTGTGTACCTTTAGC³' and o⁵'ATCTCTCTT-TCCACTCCACA³', or (spanning nt 3850–3011) s⁵'ATGCTGCTA-TATTTGCTGACAGC³' and o⁵'ATCTCTCTTTCCACTCCACA³' were used. For site V secondary amplifications, (spanning nt 3850–3137) s⁵'ATGCTGCTATATTTGCTGACAGC³' and o⁵'CAC-TGCGTTCCAGGCAATGC³' were used.

*DNA Sequence Analysis.* Sequencing reactions were carried out using Taq DNA polymerase (TaqTrack Sequencing Core System, DEAZA; Promega Corp., Madison, WI). Reaction products were electrophoresed through 6% acrylamide gels (SequaGel system; National Diagnostics, Manville, NJ) in a buffer gradient (39). PCR products from separate amplifications were used to sequence the complementary strands of variant cell DNA.

*Oligonucleotide-directed In Vitro Mutagenesis.* Variant cell-specified mutant coding sequences were generated using the Oligonucleotide-directed in vitro Mutagenesis System, version 2 (Amersham International, Amersham, Bucks, UK). Parent phagemids pGC2ESV and pGC1ESV40pro were constructed by transferring SV40 sequences into the GC-clamp plasmids (40). For plasmid pGC2ESV, the SV40 DNA NcoI/BamHI restriction fragment comprising nt 37–2533, and encompassing the viral origin of replication, early region promoter, and complete T antigen coding region, was ligated to the NcoI/BamHI pGC2 vector fragment. Construct pGC2ESV was used for mutagenesis, but not for expression of T antigen in mammalian cells (due to the lack of SV40 enhancer sequences). Mutated sequences were either subcloned from pGC2ESV into pGC1ESV40pro, or were obtained by direct mutagenesis of pGC1-ESV40pro. The plasmid pGC1ESV40pro was generated by converting the SV40 NaeI site (nt 345) to a SalI site, and inserting the SV40 SalI/BamHI fragment into the plasmid pGC1.

*Generation of Cell Lines Expressing Mutant T Antigens.* Mutant plasmids were transfected into adult C57BL/6 mouse primary kidney cells (B6/K) as described (41). Transformed colonies were expanded for analysis.

*Synthetic Peptides.* Peptides were synthesized as described previously (31). Synthetic peptides used for these studies and corresponding to wild-type or mutant T antigen CTL recognition sites are described in the Results section. Additional peptides used as control peptides included the H-2D$^b$-restricted influenza virus nucleoprotein CTL recognition epitope NP 365–380 (IASNENM-DAMESSTLE) (42) and the T antigen peptide LT 195–209 (LFF-LTPHRHRVSAIN). Neither control peptide is recognized by any of the SV40 T antigen-specific CTL clones used in this study.

## Results

### Characterization of Antigenic Site Loss Variant Cell Lines

Clonally derived cell lines were generated from the K-1, K-2, K-3, and K-5 variant cell populations. The susceptibility of each variant cell line to lysis by site-specific CTL clones mirrored that of the variant cell population from which it was isolated (32, 33). Data for representative cell lines from each variant group (Table 1) show that K-1 cell lines were resistant to lysis by CTL clones Y-1, Y-2, and Y-3. In this experiment, lysis of the K-1 cell lines by CTL clone Y-5 was depressed relative to Y-5 lysis of K-0. In another experiment, CTL clone Y-5 lysed the K-1 cell lines by CTL clone Y-5 lysis of K-0. In another experiment, CTL clone Y-5 lysed the K-1 cell lines as well as it did the K-0 cells, while the K-1 cell lines were not lysed by CTL clone Y-1 (data not shown). K-2 and K-3 cell lines were resistant to lysis by CTL clones Y-2 and Y-3, and K-5 cell lines were resistant to CTL clone Y-5. Immunoprecipitation of K-0 and variant cell line T antigens using T antigen-specific mAbs PAb 419 (43) and PAb 901 (44, 45) indicated that the amino acid and carboxyl termini of the proteins were intact (data not shown). As demonstrated previously (28, 32, 33), the K-2, K-3, and K-5 cell lines and the K-0 population expressed apparently full-length T antigen. A 79-kD T antigen was immunoprecipitated from K-1 cell lines (not shown).

### Identification of Genetic Lesions Involving CTL Recognition Site Coding Sequences of Variant Cells

To determine whether gross deletions or rearrangements of SV40 DNA sequences within variant cells could explain the loss of CTL recognition sites, DNA/DNA hybridization experiments were performed on variant cell line genomic DNA. K-1 cell line DNA cleaved with HindIII was shown to carry a deletion within the SV40 HindIII B band specifying T antigen amino acids 1–272. This region incorporates CTL recognition sites I, II, and III. No deletions were detected within K-2, K-3, and K-5 cell line T antigen coding sequences. Sequence analysis of viral DNA present within the variant cells was conducted. Integrated viral sequences were amplified by the PCR, and the amplification reaction products were sequenced.

DNA of the five K-1 cell lines lacked SV40 nt 4421–4030, corresponding to the loss of T antigen amino acid residues 134–263 (Fig. 1 A). Therefore, loss of CTL recognition sites
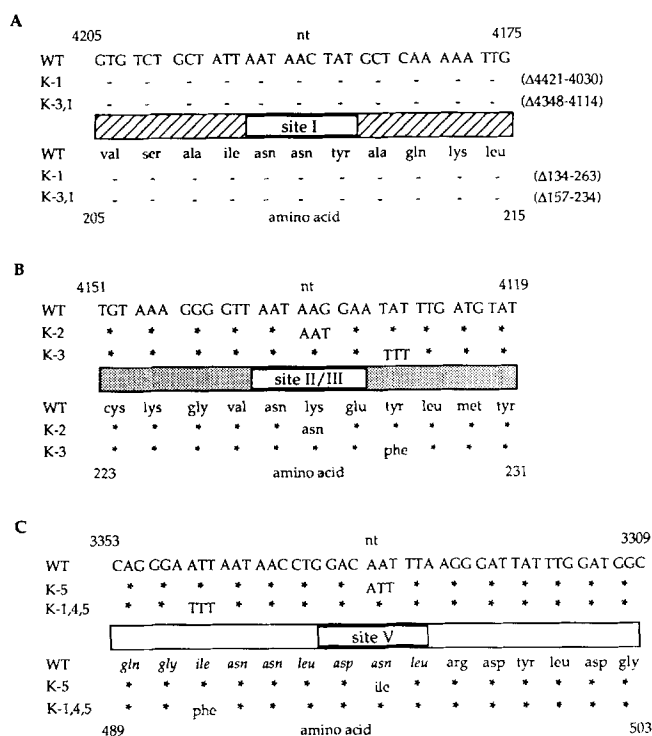
**Table 1.** *Lysis of Variant Cells by T Antigen-specific CTL Clones*

| Target cell | Percent specific lysis by CTL clone | | | | |
|---|---|---|---|---|---|
| | Y-1 | Y-2 | Y-3 | Y-4 | Y-5 |
| **A.** K-0 | 99.0 | 71.5 | 70.4 | 90.4 | 63.3 |
| K-1 CL3 | 2.7 | 0.5 | 0.3 | 68.7 | 34.9 |
| K-1 CL4 | 1.3 | 1.9 | 1.9 | 77.0 | 17.4 |
| K-1,4,5 | 1.7 | 3.7 | 1.3 | 3.2 | 1.0 |
| **B.** K-0 | 93.0 | 59.7 | 69.0 | 55.9 | 61.0 |
| K-2 CL1 | 79.2 | 2.4 | 1.1 | 19.9 | 38.7 |
| K-2 CL4 | 77.5 | 0.0 | 0.0 | 17.8 | 33.2 |
| K-1,4,5 | 1.0 | 1.4 | 0.9 | 0.7 | 0.5 |
| **C.** K-0 | 91.4 | 20.0 | 33.0 | 76.6 | 25.0 |
| K-3 CL1 | 96.5 | 0.0 | 0.0 | 79.1 | 29.2 |
| K-3 CL3 | 86.9 | 0.0 | 0.0 | 59.4 | 32.6 |
| K-5 CL2 | 88.2 | 74.8 | 46.4 | 81.3 | 0.0 |
| K-5 CL3 | 78.9 | 28.4 | 33.2 | 53.5 | 0.3 |
| K-1,4,5 | 3.5 | 0.0 | 0.0 | 1.8 | 0.0 |

Target cells were treated with γ-IFN before assay. For these experiments, a CTL-to-target cell ratio of 9:1 was used.

I, II, and III from K-1 variants was due to the deletion of the DNA sequences encoding them. DNA amplification and sequencing also were performed for the population K-1,4,5. K-1,4,5 had been selected by sequential coculture of K-1 with Y-4 and then Y-5 (33), and was resistant to lysis by CTL clones Y-1, Y-2, Y-3, Y-4, and Y-5. The deletion present within the K-1 cell lines was also present in cells of the K-1,4,5 population. The basis for CTL recognition site loss was determined for an independently selected CTL Y-1-resistant variant cell population. K-3,1 cells had been selected by coculture of CTL clone Y-3 selected cells (K-3) with Y-1 (32), and lacked CTL recognition sites I, II, and III. The K-3,1 T antigen was of apparent size 81 kD (32), suggesting that the genetic basis for antigenic site loss in K-3,1 differed from that of the K-1 cells. Amplified K-3,1 DNA was sequenced. A deletion of SV40 nt 4348–4114, with a C-to-A transversion at nt 4113, was detected (Fig. 1 A). The K-3,1 T antigen lacks amino acids 157–234, with the substitution of aspartic acid for alanine at position 235. Coding sequences for CTL recognition sites I, II, and III are absent.

Sequences for the five K-2 cell lines showed a single point mutation at SV40 nt 4134 (Fig. 1 B). The G-to-T transversion substitutes asparagine for lysine as T antigen amino acid 228. The sequence from each K-3 cell line DNA indicated an A-to-T transversion at SV40 nt 4129 (Fig. 1 B), substituting phenylalanine for tyrosine as T antigen amino acid 230. Both the K-2 and K-3-substituted amino acids lie within the defined minimal site II/III CTL recognition epitope.

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**A**

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    4205                          nt                        4175
WT  CTG TCT GCT ATT AAT AAC TAT GCT CAA AAA TTG
K-1  -   -   -   -   -   -   -   -   -   -   -    (Δ4421-4030)
K-3,1 -  -   -   -   -   -   -   -   -   -   -    (Δ4348-4114)
        [//////////// site I //////////////]
WT  val ser ala ile asn asn tyr ala gln lys leu
K-1  -   -   -   -   -   -   -   -   -   -   -    (Δ134-263)
K-3,1 -  -   -   -   -   -   -   -   -   -   -    (Δ157-234)
    205                     amino acid            215
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**B**

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    4151                          nt                        4119
WT  TGT AAA GGG GTT AAT AAG GAA TAT TTG ATG TAT
K-2  *   *   *   *   *  AAT  *   *   *   *   *
K-3  *   *   *   *   *   *   *  TTT  *   *   *
            [///////// site II/III /////////]
WT  cys lys gly val asn lys glu tyr leu met tyr
K-2  *   *   *   *   *  asn  *   *   *   *   *
K-3  *   *   *   *   *   *   *  phe  *   *   *
    223                     amino acid            231
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**C**

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    3353                          nt                        3309
WT  CAG GGA ATT AAT AAC CTG GAC AAT TTA AGG GAT TAT TTG GAT GGC
K-5  *   *   *   *   *   *   *  ATT  *   *   *   *   *   *   *
K-1,4,5 *  *  TTT  *   *   *   *   *   *   *   *   *   *   *   *
                    [//////// site V //////]
WT  gln gly ile asn asn leu asp asn leu arg asp tyr leu asp gly
K-5  *   *   *   *   *   *   *  ile  *   *   *   *   *   *   *
K-1,4,5 *  *  phe  *   *   *   *   *   *   *   *   *   *   *   *
    489                     amino acid                        503
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**Figure 1.** Mutations identified within T antigen CTL recognition site coding sequences of variant cells. The "WT" SV40 nucleotide and amino acid numbers indicate K-0 sequences encoding or comprising, respectively, minimal or functional CTL recognition sites (32, 33). (*) Variant cell sequences identical to those of the K-0 population. (-) Sequences deleted from variant cells, but present in K-0 cells. Amino acid substitutions or deletions (Δ) specified by variant cell DNA mutations are shown beneath the WT amino acid sequence. Deletions within K-1 cell line and K-3,1 population DNA (A) extend beyond the minimal site I sequences. Mutations present within variant cells lacking site II/III (B) or site V (C) are shown. Minimal site V sequences, as defined by synthetic peptide studies (32), comprise T antigen amino acids 489–497. These residues are indicated in italics within the WT site V amino acid sequence 489–503 used for these experiments.

K-5 cell line DNA sequences showed a point mutation within the coding sequences for CTL recognition site V. An A-to-T transversion at SV40 nt 3331 specifies the substitution of isoleucine for asparagine as T antigen amino acid 496 (Fig. 1 C). Sequence data for the site V coding region from K-1,4,5 amplifications indicated a point mutation at SV40 nt 3347 (A-to-T), specifying the substitution of phenylalanine for isoleucine as T antigen amino acid 491 (Fig. 1 C). Amino acids 496 and 491 both lie within the mapped recognition site for CTL clone Y-5.

### Effects of Mutations Identified by PCR Amplification and Sequence Analysis on CTL Recognition

*Generation of Cell Lines Expressing T Antigens Carrying the K-2, K-3, K-5, or K-1,4,5 Mutations and Effect of these Mutations on CTL Recognition.* To confirm that each substitution was sufficient to disrupt CTL recognition sites II and III or V, the point mutations identified by PCR amplification and sequence analysis were introduced individually into an expression construct carrying the coding sequences for wild-type T antigen. Mutant constructs were used for transfection of primary C57BL/6 kidney cells. Foci of immortalized cells were expanded and screened for the presence of the appropriate wild-type or substituted nucleotides by DNA sequencing of PCR amplified products. In all cases, the sequences corresponded to those of the transfected construct (data not shown). Cells were then screened for expression of T antigen by immunofluorescence and immunoprecipitation. Each transformant produced a nuclear T antigen of ~94 kD (data not shown). Cell lines expressing wild-type or mutant T antigen were designated B6/SV-T wt, B6/SV-T228(lys→asn), B6/SV-T230(tyr→phe), B6/SV-T496(asn→ile), or B6/SV-T491-(ile→phe) (Table 2).

The cell lines were assayed for their susceptibility to CTL clones Y-1, Y-2, Y-3, and/or Y-5. As shown in Fig. 2 A, B6/SV-T228(lys→asn) cells were not lysed by either CTL clone Y-2

**Table 2.** *Mutant DNA Constructs Carrying Variant-specified Substitutions and Synthetic Peptides*

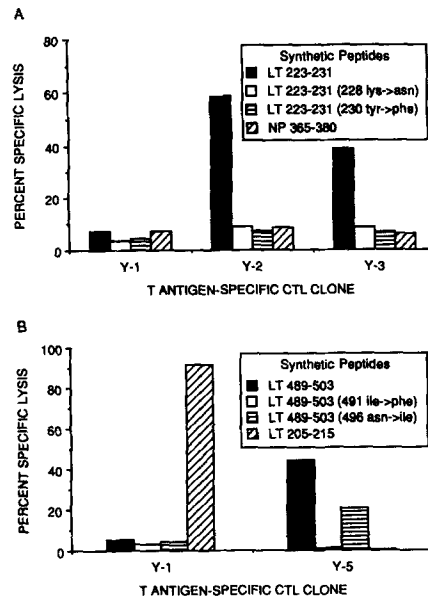| Cell population/line | Mutation identified within CTL recognition site coding sequences | Cell line reconstructing the identified mutation | Corresponding CTL recognition site synthetic peptide | Synthetic peptide amino acid sequence |
|---|---|---|---|---|
| K-0 | none | B6/SV-T wt | LT 205-215 | VSAINNYAQKL |
| K-0 | none | B6/SV-T wt | LT 207-215 | AINNYAQKL |
| K-0 | none | B6/SV-T wt | LT 223-231 | CKGVNKEYL |
| K-2 | G→T substitution, nt 4134 | B6/SV-T228(lys→asn) | LT 223-231 (228 lys→asn) | CKGVNNEYL |
| K-3 | A→T substitution, nt 4129 | B6/SV-T230(tyr→phe) | LT 223-231 (230 tyr→phe) | CKGVNKEFL |
| K-0 | none | B6/SV-T wt | LT 489-503 | QGINNLDNLRDYDG |
| K-1,4,5 | A→T substitution, nt 3347 | B6/SV-T491 (ile→phe) | LT 489-503 (491 ile→phe) | QGFNNLDNLRDYDG |
| K-5 | A→T substitution, nt 3331 | B6/SV-T496 (asn→ile) | LT 489-503 (496 asn→ile) | QGINNLDILRDYDG |
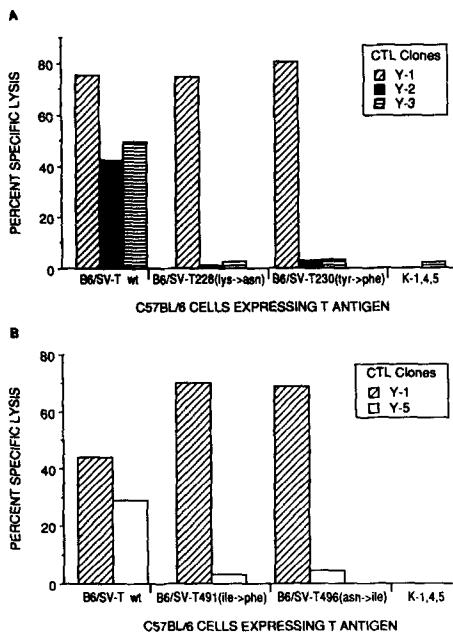
**Figure 2.** CTL recognition site losses caused by introduction of variant cell T antigen mutations into wild-type T antigen. Cell lines carrying individual K-2 or K-3 cell line mutations (A) or K-5 cell line or K-1,4,5 population mutations (B) in a wild-type T antigen background were tested for susceptibility to lysis by T antigen-specific CTL clones. A standard cytotoxicity assay was used, with a CTL-to-target cell ratio of 9:1.
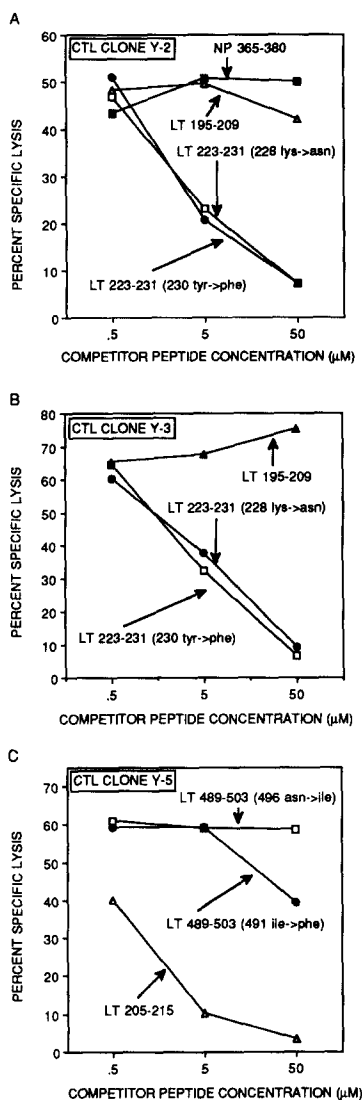
**Figure 3.** Wild-type and substituted synthetic peptides as targets for CTL recognition. MC57G cells pulsed with site II/III wild-type or substituted peptides were used as target cells for CTL clones Y-1, Y-2, and Y-3 (A). Recognition of site V wild-type and mutant peptides by CTL clones Y-1 and Y-5 (B) was also evaluated. For peptide pulsing, 100 μM peptide solutions were used. The CTL-to-target cell ratio was 5:1.

or Y-3. B6/SV-T230(tyr→phe) cells also were not lysed by either Y-2 or Y-3. However, both cell lines were lysed by CTL clone Y-1, indicating that H-2D^b class I antigen in these cell lines was functional. Thus, either of the point mutations identified within K-2 or K-3 cell lines was sufficient to cause loss of recognition by both CTL Y-2 and Y-3.

The results for the B6/SV-T496(asn→ile) cell line, carrying a mutation in the coding region for CTL recognition site V, are shown in Fig. 2 B. B6/SV-T496(asn→ile) cells were not lysed by CTL clone Y-5, however, they were lysed by CTL clone Y-1. Therefore, resistance to Y-5 could be attributed directly to the substitution at T antigen amino acid 496.

B6/SV-T491(ile→phe) cell line, which contains the mutation identified in the independently derived CTL clone Y-5-resistant population K-1,4,5, was resistant to lysis by CTL clone Y-5, yet maintained susceptibility to lysis by CTL clone Y-1 (Fig. 2 B). These results indicate that amino acid substitution at position 491 also leads to loss of site V recognition.

*Loss of CTL Recognition Site Function by Synthetic Peptides Representing Mutant CTL Recognition Sites.* To determine whether synthetic peptide reconstruction of CTL recognition sites (42) could be used to define mechanisms of variant cell CTL recognition site loss, cytotoxicity assays were carried out on peptide-pulsed H-2^b target cells (MC57G) that did not express endogenous T antigen. The mutant peptides (Table 2) used for analysis of K-2 and K-3 T antigen substitutions (LT 223-231 [228 lys→asn] and LT 223-231 [230 tyr→phe]) were not recognized by either CTL clones Y-2 or Y-3 (Fig. 3 A).

Peptides corresponding to T antigen amino acids 489-503 were used for analysis of site V substitutions, as minimal site V had not been delimited within amino acids 489-497 (31) when these experiments were initiated. CTL clone Y-5-mediated lysis of MC57G cells pulsed with LT 489-503 (496 asn→ile) was suppressed relative to lysis of cells pulsed with the wild-type (LT 489-503) peptide (Fig. 3 B). Mutant peptide LT 489-503 (491 ile→phe) failed to provide a target for CTL clone Y-5.

*Presentation of Mutant Peptides by H-2D^b Class I Antigens.* To determine whether the mutant peptides can be presented by the H-2D^b class I antigen, peptide competition assays (46, 47) were undertaken. $^{51}$Cr-labeled target cells were incubated with a constant amount of wild-type peptide mixed with increasing amounts of mutant peptide before exposure to CTL clones. Increasing concentrations of site II/III mutant peptides (LT 223-231 [228 lys→asn] and LT 223-231 [230 tyr→phe]) progressively decreased the ability of the wild-type peptide LT 223-231 to provide a target for CTL clone Y-2 (Fig. 4 A) and CTL clone Y-3 (Fig. 4 B). This indicated that the mutant peptides were able to bind to class I MHC H-2D^b. As shown in Fig. 4 A, the influenza virus NP peptide failed to compete efficiently against the site II/III wild-type peptide in this experiment at the concentrations indicated.

The ability of the mutant site V peptides to associate with the class I H-2D^b molecule also was assessed through a standard competition assay. Peptide LT 489-503 (491 ile→phe) was able to bind to class I H-2D^b antigen, as reflected by the reduction in target cell lysis by CTL clone Y-5 at increasing

**Figure 4.** Binding to MHC class I H-2D$^b$ antigen of substituted synthetic peptides corresponding to variant cell mutant T antigen CTL recognition sites. Binding of amino acid-substituted peptides to class I MHC H-2D$^b$ antigen was evaluated in a peptide competition assay. Target cells were pulsed simultaneously with wild-type peptides at constant concentration and substituted peptides at variable concentrations (see figure axes). Target cells and wild-type peptide concentrations used were (A) K-1,4,5 cells with 1 μM LT 223–231, and (B) MC57G cells with 1.57 μM LT 489–503. Additional peptides included NP 365–380, LT 195–209, and LT 205–215 (site I peptide). The CTL-to-target cell ratio was 5:1.

concentrations of mutant competitor peptide (Fig. 4 C). The ability of LT 489–503 (496 asn→ile) to compete with wild-type peptide could not be addressed. As shown in Fig. 3 B, this peptide is recognized by CTL clone Y-5, albeit with a much lower efficiency than the wild-type peptide.

These studies show that the mutant peptides bind to class I H-2D$^b$ molecules. It seems likely, therefore, that CTL recognition site loss in the variant cells is effected at the level of epitope interaction with the TCR.

## Discussion

The SV40 transforming protein, T antigen, is a multifunctional gene product possessing an array of biochemical activities that map to discrete structural domains of the protein (27). Domains involved in the transformation of primary mouse embryo fibroblasts (B6/MEF) in culture and in conferral of tumorigenicity in vivo have been defined (44, and references therein). Cells transformed in vitro by T antigen acquire the phenotype of tumor cells, and are transplantable in athymic nude mice (44). The T antigen is novel as a transforming gene product in that it has been shown to provide a target for host immune rejection of the transformed cell. The protein is processed intracellularly into CTL recognition elements, which are presented at the cell surface in the context of class I MHC antigens and induce class I-restricted CTL responses in responder hosts. Since T antigen is required for maintenance as well as for initiation of the transformed phenotype, and as its tumorigenic function is modulated by the cellular immune response, it is an ideal experimental oncogene product for evaluating how mutations within transforming protein CTL epitopes enhance or disrupt antitumor immunity. The T antigen domains constituting CTL recognition epitopes are defined, the regions required for transformation are broadly mapped, and T antigen-transformed cells carrying point mutations disrupting CTL recognition sites have been characterized in this study. Therefore, the genetic information and biological reagents for dissecting the contribution of individual CTL recognition sites to host rejection of transformed cells, and for analysis of differential host ability to respond to mutant transforming proteins, are readily available in this system.

The results reported here demonstrate that CTL specific for a transforming protein select for survival of transformed cells bearing disrupted CTL epitopes. Deletions have been shown to inactivate a subset of CTL recognition sites. However, single point mutations inducing amino acid substitutions within recognition epitopes are sufficient for the elimination of most sites. Each of the mutant CTL recognition epitopes identified within the T antigen epitope loss variant cells retains the ability to associate with MHC class I H-2D$^b$ antigen. Preliminary experiments designed to compare the H-2D$^b$-binding efficiencies of substituted CTL recognition sites with those of the wild-type sites (48) indicate that the substituted synthetic peptides used in this study bind to MHC class I H-2D$^b$ as efficiently as, or better than, their wild-type counterparts (N. Lill and S. Tevethia, unpublished data). This suggests that the variant-specified amino acid substitutions disrupt target cell recognition at the level of epitope interaction with the TCR.

CTL recognition of tumor antigens has been shown to inhibit tumor development and progression (1, 29, 49, 50). Loss of tumor cell susceptibility to CTL may contribute to the progressive growth of tumor cells. Five immunologically distinct H-2$^b$-restricted CTL recognition sites within T antigen have been mapped (28, 30, 31). This multiplicity of CTL recognition sites provides a backup to guard against the complete loss of transformed cell susceptibility to CTL.

It is unlikely that, under active immune surveillance in vivo, transformed cells lacking multiple sites will accumulate before they can be eliminated by CTL reactive against at least one of the recognition sites. However, it must be emphasized that a transformed cell population (K-1,4,5) expressing T antigen and resistant to CTL clones specific for each of the five mapped epitopes, has been selected (33). Even if such cells arise with low frequency, their appearance would have significant implications for tumor progression.

The present study has dealt with variants selected from a population of cells transformed by wild-type SV40. Based on our results, we hypothesize that point mutations within CTL epitope coding sequences of transforming proteins may allow escape of tumor cells from active immunosurveillance in vivo. SV40 T antigen contains domains required for immortalization of primary cells and for conferral of other aspects of the transformed phenotype, including loss of contact inhibition, decreased growth factor dependence, anchorage-independent growth, and conferral of cellular tumorigenic potential (44). The presence of T antigen containing these domains is required continuously for maintenance of the fully transformed phenotype (27, 44). The antigenic site loss variant cells analyzed in this study, and the corresponding "reconstruction" cell lines generated by transfection of murine primary cell cultures with appropriate mutant coding sequences, respectively maintained and acquired the immortalized phenotype characteristic of SV40-transformed cells. Although the tumorigenicity of the variant cells used in this study has not been tested, the functions of T antigen that are sufficient to immortalize B6/MEF and those that are involved in conferring tumorigenicity colocalized to T antigen amino acids 250–625 (44). Three of the four H-2D$^b$-restricted CTL epitopes are located outside of the immortalization–tumorigenicity domain. It is anticipated, therefore, that the variants and reconstruction cell lines will form tumors efficiently in nude mice. The T antigen residues altered by point mutations in variant CTL recognition epitope V lie within the immortalization–tumorigenicity domain. The site V reconstruction plasmids immortalize B6/MEF as efficiently as wild-type, and the resulting cell lines appear fully transformed in cell culture. We infer, therefore, that they also will form tumors in nude mice.

Targeted immune therapeutic approaches against tumor cells would ideally be directed against antigens that must be expressed by all of the tumor cells. Transforming proteins required for maintenance of the transformed phenotype and containing CTL epitopes represent ideal targets for such

therapy. In the SV40 variant cell model system, T antigen provides the target for CTL, but also confers a selective cellular survival advantage (cellular immortalization). SV40-transformed cells acquiring spontaneous mutations that compromise cellular transformation would not survive during the CTL selection period because of loss of immortalization. In this sense, the T antigen model system differs from other model systems in which the CTL target antigens are probably not required for maintenance of cellular transformation (26). Immune responses targeted against nontransforming antigens would be ineffective in preventing malignant growth of transformed cells that had lost expression of those antigens. In the case of transforming proteins such as T antigen, loss of entire protein expression would render the cell nonsusceptible to antigen-specific immune responses, but that cell would concurrently lose potential for tumorigenicity, as the domains of T antigen are continuously required for the maintenance of transformed state. For this reason, T antigen and other CTL epitope-bearing transforming proteins such as the activated ras oncogene (51, 52) may represent the preferred targets for immune therapy. CTL epitope loss from these proteins may have a significant impact on transformed cell survival and tumor progression in vivo.

Spontaneous mutations resulting in the loss of CTL recognition sites from antigens have significant implications for the development and persistence of diseases states. CTL recognition site loss variants have been demonstrated in the lymphocytic choriomeningitis (LCMV) model system in vivo (53) and in vitro (54). In these cases, epitope loss was caused by point mutations within antigen-coding sequences, and led to virus persistence in vivo (53). It has also been shown that T cell-resistant variants of the HIV arise in vivo during the course of infection (55). The accumulation of escape variants may, in this case, contribute to HIV escape from immunosurveillance. We have determined through the analysis of SV40-transformed antigen loss variant cells that single amino acid substitutions within CTL recognition sites are sufficient to abrogate transformed cell lysis by site-specific CTL clones. Our results suggest that, in principle, cumulative minimal genetic mutations may result in sequential CTL recognition epitope loss with concomitant escape of the transformed cell from immune surveillance. This study extends the number of potential mechanisms whereby SV40-transformed cells can escape lysis by CTL, and provides information required for further dissection of the relative contribution that individual CTL recognition sites make toward the host defense against tumor cells.

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