A Tumor Escape Variant that Has Lost One Major Histocompatibility Complex Class I Restriction Element Induces Specific CD8$^+$ T Cells to an Antigen that No Longer Serves As a Target

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Summary

After loss of expression of a major histocompatibility complex class I K^k allele, the escape variant of an immunogenic tumor grows progressively in normal mice. This progressor variant is resistant to killing by cytotoxic T lymphocytes (CTLs) directed against the A and B antigens presented by K^k. Although the variant retains the expression of the D^k allele and is sensitive to CTLs directed against the C antigen presented by D^k, the variant failed to induce CTLs to this antigen in vivo. Instead, the variant induced CD8$^+$ T cells directed to the A antigen. This was shown at the molecular level by T cell receptor β chain sequence analysis of the responding cells. Further evidence for the presence of A antigen in the variant came from the finding that spleen cells of mice injected intraperitoneally with the variant tumor cells were primed for an anti-A CD8$^+$ CTL response in vivo. Thus, in contrast to other variants that lost a target antigen and induced a CTL response to remaining target antigens, the K^k loss variant continued to induce an immune response to a tumor antigen that is no longer presented on the tumor cell surface. Even though the variant escapes in a single step because an effective CTL response to secondary antigens is prevented, these secondary antigens remain as potential targets of immunotherapy on the variant's cell surface.

Murine ultraviolet (UV)–induced tumors can possess multiple antigens that are not found on other tumors or normal autologous control cells (1, 2), and several studies have suggested that human cancers also commonly express multiple antigens (for a review see reference 3). Multiple antigens on tumors display a hierarchy of antigenicity such that at any one time the host usually responds to only one of several antigens displayed simultaneously on the tumor surface (4, 5). If the tumor loses the immunodominant antigen, the variant becomes a potential target for immune destruction by the host because one of the remaining antigens on the variant takes over the dominant role as the target antigen (5). Thus, immunodominance leads to a pecking order with sequential targeting and sequential loss of multiple tumor-specific antigens which may result at the end in highly malignant cancer variants that lack CTL-recognized target antigens. Therefore, loss of a single antigen may not lead to permanent immune escape since the selection process is repeated for consecutive variants until antigens that induce CTL responses are no longer present.

This paper shows that immunodominance leads to a quite different course of events when tumor cells retain tumor-specific antigens but lose expression of a MHC class I restriction molecule that presents the dominant antigen. Loss of expression of all MHC class I from murine or human cancers would result in complete tumor cell resistance to CTL-mediated lysis (6), but such changes are rare. By contrast, selective loss of one restriction element is frequently found (7–11), but so far there has been no model of how a single allelic loss might enable a tumor to escape immune destruction since it is unlikely that presentation of multiple antigens is restricted by a single allele. In this study, we find that loss of a single allele of MHC class I may be sufficient for the tumor to permanently escape destruction by the host in a single step.

Using an H-2K^k loss variant of a UV-induced murine tumor expressing multiple antigens, we demonstrate that the variant that has lost only the restriction element for the immunodominant antigen continues to induce a CD8$^+$ T cell response to that antigen, even though the variant cannot be lysed by CTLs specific for this antigen. This immunodominance impedes responsiveness to antigens on the tumor presented by other restriction elements and thereby allows the tumor to escape the host's immune response in a single step despite retaining other tumor-specific antigens as potential targets for immune destruction.

Materials and Methods

Mice. 5–10-wk-old germ-free–derived C3H/HeN/MTV$^-$ females were purchased from the National Cancer Institute Frederick Cancer Research Facility (Bethesda, MD) and were maintained as

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/93/09/0933/08 $2.00
Volume 178 September 1993 933–940

previously described (1). Mice were housed in the FMI Animal Care Facility at the University of Chicago in laminar flow hoods, given sterile acidified water, and fed autoclaved food (5010 Rodent laboratory diet; Purina, Inc., St. Louis, MO).

Tumor Cell Lines. Derivation of UV-induced and spontaneous tumors in C3H/HeN mice has been described (1, 2). The regressor tumors used in this study are strongly immunogenic in that these tumors, when transplanted into young syngeneic mice, grow during the first 10 d and then regress. These tumors grow progressively in nude mice and kill by infiltrative growth without macroscopic evidence of distant metastases. VAR1, VAR3, and VAR4 are antigen loss variants derived from the parental 6130 regressor fibrosarcoma (2). These variants can grow progressively in normal mice. All cell lines were cultured in MEM (Gibco Laboratories, Grand Island, NY) supplemented with 10% heat inactivated FCS (Gibco Laboratories) and 1% penicillin/streptomycin (600–5140AG; Gibco Laboratories) (CMEM)[1].

Induction of Peritoneal Exudate Lymphocytes (PELs). 3×10^6–1×10^7 tumor cells were injected four times intraperitoneally at 3-d intervals as described (12). 2 d after the final injection, the mice were killed by cervical dislocation. 5 ml of CMEM or PBS with 1% FCS was injected intraperitoneally and cells were removed by peritoneal lavage.

Mixed Lymphocyte–Tumor Cell Cultures (MLTC). Mice were immunized by injecting 10^7 tumor cells intraperitoneally. Spleens from naive and immunized mice were aseptically removed 7–10 d after the injection and dispersed into single cell suspensions with sterile glass tissue grinders. RBCs were lysed with 0.83% (wt/vol) Tris-ammonium chloride and washed twice with CMEM. Tumor cells were treated with mitomycin C (Sigma Chemical Co., St. Louis, MO) for 45 min at 37°C and washed three times. Culture consisted of 8×10^6 responder spleen cells and 4×10^4 stimulators. These cells were cocultured in 3.5 ml of RPMI 1640 (Gibco Laboratories) supplemented with 10% non-heat inactivated FCS (prescreened from Gibco Laboratories), 1% penicillin/streptomycin, 0.1% gentamycin (Gibco Laboratories) and 5×10^{-5} M 2-ME (Calbiochem Novabiochem, La Jolla, CA). Culture vessels were 16 × 125 mm round-bottomed tissue culture tubes (Falcon Labware, Oxnard, CA). Cultures were incubated for 6 d at 37°C, 5% CO_2, humidified atmosphere as has been described (1).

Generation and Maintenance of Cytolytic T Cell Lines and Clones. Methods for generating and maintaining CTL lines and clones have been described (1). Briefly, mice were primed with 10^7 tumor cells intraperitoneally. Spleens from these immune mice were used as responder cells in MLTC with the immunizing tumor cells as the stimulators. The CTLs were tested in a ^{51}Cr-release assay for cytotoxicity and specificity. Long-term CTL lines and clones were passaged in V-bottomed 96-well nontissue culture treated plates (Linbro, McLean, VA) or 24-well tissue culture plates (Costar Corp., Cambridge, MA). To pass CTLs in 96-well plates, 10^6 irradiated (2,000 rad) syngeneic spleen filter cells, 2×10^4 CTL, and 2×10^3 mitomycin C–treated stimulator cells were mixed and added to each well in 200 μl of CTL passage medium. For passage of CTLs in 24-well plates, 10^5 CTLs, 5×10^6 irradiated (2,000 rad) syngeneic spleen cells, and 2.5×10^4 mitomycin C–treated tumor cells were mixed in 1.5 ml of CTL passage medium (RPMI 1640, 33% [vol/vol] supernatant from a secondary MLC as a source of T cell growth factor [13], 10% non-heat inactivated FCS, 1% penicillin/streptomycin, 0.1% gentamycin, and 5×10^{-5} M 2-ME). BS1 and PD4 are two anti-A specific CTL clones derived from immunizations with the parental 6130 tumor (kind gifts from B. Starr and P. Dubey, The University of Chicago).

^{51}Cr-release Assay. Cytotoxicity of CTLs was determined in a 4.5-h ^{51}Cr-release assay, as described (14). Briefly, effectors from MLTCs, CTL clones and PELs were serially diluted in V-bottomed, flexible 96-well plates (Dynatech Laboratories, Inc., Chantilly, VA) in 100 μl of CMEM. Tumor cells (5×10^5) in 100 μl of CMEM were mixed with 100 μl of ^{51}Cr (sodium chromate at 1 mCi/ml) (Amersham Corp., Arlington Heights, IL) for 1 h. Labeled tumor targets were washed four times with CMEM, and resuspended in CMEM at 5×10^4 cells/ml. 100 μl of this suspension was added to each well of effectors. Effectors and labeled targets were incubated for 4.5 h at 37°C, 7.5% CO_2 in a humidified incubator. After incubation, 100 μl of supernatant from each well was collected and analyzed for radioactivity by a gamma counter (Micromedic Systems Inc., Horsham, PA). The percent specific lysis was calculated by the formula: 100× [(experimental release − spontaneous release)/(maximum release − spontaneous release)]. Spontaneous release was <15% of maximum release.

Staining and Cell Sorting. FITC-conjugated rat anti–mouse L3T4 (anti-CD4; RM 4-5), and rat anti–mouse Ly-2 (anti-CD8; 53-6.7), and PE-conjugated mouse anti–mouse Vβ13 (MR-12-4), were purchased from PharMingen (San Diego, CA). 5×10^5–1×10^6 PELs were incubated with FITC-anti-Ly-2 and PE-anti-Vβ13 or FITC-anti-L3T4 and PE-anti-Vβ13 for 30 min on ice. Cells were washed three times with PBS containing 1% BSA albumin and 0.1% sodium azide (PBSA). Tumor cells were incubated with anti-K^k (16-1-11) or anti-D^k (15-5-5) culture supernatant (generously provided by Dr. A. Sant, The University of Chicago) or with PBSA for 30 min on ice and washed three times with PBSA. Tumor cells were then incubated with 1:10 dilution of FITC goat anti–mouse Ig (Hyclone Laboratories, Logan, UT) and washed three times with PBSA. Cells were analyzed on a FACScan flow cytometer (Becton Dickinson & Co., Mountain View, CA). Stained PELs were sorted on a Coulter Epics 753 (model 2566; Argon laser run at 488 nm, Coulter Electronics, Inc., Hialeah, FL). Sorted cells were incubated for 16 h in 24-well plates (model 3424, Costar Corp.), each well containing 10^5 sorted cells, 5×10^6 irradiated (2,000 rad) syngeneic spleen cells, and 2.5×10^4 mitomycin C–treated tumor cells in 1.5 ml of CTL passage medium.

Preparation of cDNA. 1–2 × 10^6 PELs were lysed in RNAzol ™B (Tel-Test, Inc., Friendswood, TX) and total RNA was prepared as prescribed by the manufacturer. First-strand cDNA was synthesized using cDNA Synthesis System Plus (Amersham Corp.) according to the manufacturer's recommendations. The cDNA reaction was heated at 90°C for 10 min before PCR amplification.

PCR Conditions and Primers Used. The sequence for the Vβ13 primer was as follows: 5′-GAGCTAGCGAATTCGTACTGTCTGAAGCTGGAGTCACCC-3′; the Cβ primer sequence was as follows: 5′-GAGCTAGCGAATTCAGCCTTTTGTTTGTTTGCAATCTCT-3′. The The upstream K^k-specific primer and downstream K^k-specific exon 2 primer sequences were as follows: 5′-GAGCTAGCGAATTCAAGTCTCGAATCGCCGACCGGTGCG-3′ and 5′-GAGCTAGCGAATTCGCCCGCGCTCTGGTTGTAGTAGCGC-3′, respectively. Underlining indicates an EcoRI restriction site. The upstream and downstream primers for β-actin were as follows: 5′-TGGAATCCTGTGGCATCCATGAAAC-3′ and 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′, respectively (15). PCR reactions were performed using 20 pmoles of each primer pair, 25 nmoles of dNTPs (Pharmacia, Piscataway, NJ), 5% of the cDNA reaction, and 0.5 U of Taq polymerase (Boehringer Mann-

[1] *Abbreviations used in this paper:* CMEM, MEM supplemented with 10% FCS, 1% penicillin/streptomycin; MLTC, mixed lymphocyte tumor cell culture; PEL, peritoneal exudate lymphocyte.

heim, Indianapolis, IN) in a total volume of 100 μl. All reactions were subjected to denaturation at 94°C for 2 min, followed by 30–60 cycles consisting of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min on DNA thermal cycler (model 480; Perkin Elmer Corp., Norwalk, CT) (16).

Sequencing. PCR products were electrophoresed on low melting point agarose gel (Bethesda Research Laboratories, Gaithersburg, MD), isolated by excision, and purified by phenol/chloroform extractions. The fragments were digested with EcoRI (New England Biolabs Inc., Beverly, MA) in 40 μl volume at 37°C for 2–3 h, heat inactivated at 68°C for 5 min and ethanol-precipitated in 2 M ammonium acetate. The pellet was resuspended in dH_2O and ligated into EcoRI-digested M13mp19 (0.1 μg, New England Biolabs, Inc.) with T4 DNA ligase and buffer (New England Biolabs Inc.) in a volume of 20 μl for 16 h at 15°C. 2–5 μl of the ligation mixture was used to transform 100 μl of competent JM101 strain of *Escherichia coli*. The transformation mixture was plated onto a lawn of overnight culture of JM101 with isopropyl β-D-thiogalactoside (IPTG) and 5-Bromo-4-chloro-3-indoxyl β-D-galactoside (X-gal) (Sigma Chemical Co.). White, well isolated plaques were picked and inoculated into 2 ml of an exponential phase culture of JM101. After 5–16 h, the viral DNA was purified from the culture supernatant. The isolated DNA was resuspended in 25 μl of dH_2O. Sequencing was performed by dideoxy chain termination method (17) using Sequenase DNA sequencing kit (United States Biochemical Corp., Cleveland, OH) with ^{35}S-labeled dATP (SJ.1304; Amersham Corp.).

Results

VAR1 Is H2-K-negative and Is Insensitive to Anti-A CTL Clones. The parental 6130 regressor tumor which is rejected by normal mice after transient growth has at least three CTL-defined antigens designated as "A", "B", and "C" (2). The progressor variant VAR1 is a variant of 6130 that was negatively selected using anti-A and anti-B CTL clones. Phenotypically, this variant was previously shown to be H2-K^k-negative and insensitive to lysis by both the anti-A and anti-B CTL clones (2). To confirm this phenotype, immunofluorescent staining and cytolysis assays were performed. Unlike the parental 6130 tumor, VAR1 was not detectably stained by an anti-K^k mAb, even when pretreated with IFN-γ, but did retain expression of the H-2D^k molecule (Fig. 1). We also looked for transcription of the K^k gene by PCR. First-strand cDNA was synthesized from total RNA extracted from 6130 and VAR1. PCR performed with K^k-specific primers showed no detectable message for the K^k molecule in VAR1 cells (Fig. 2). Furthermore, VAR1 is insensitive to cell-mediated killing by each of two anti-A specific CTL clones, BS1 and PD4, and is also insensitive to anti-B CTL (2). However, VAR1 is sensitive to lysis by the anti-C clone SS1 (Fig. 3). These data indicate that either the A antigen and the K^k

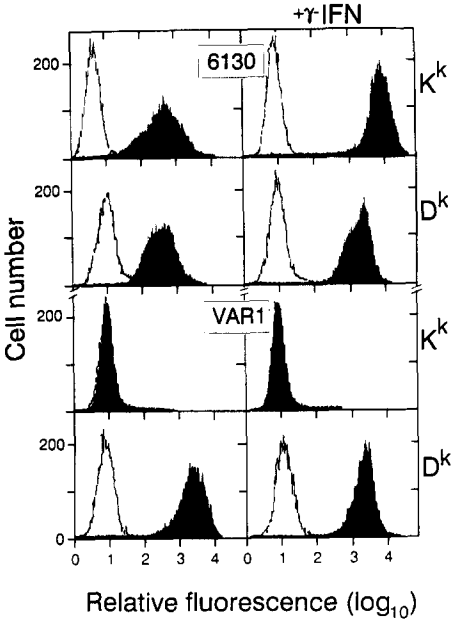

Figure 1. VAR1, the $A^-B^-C^+$ variant of the 6130 tumor, fails to express K^k on the cell surface. 6130 and VAR1 were stained with second step antibody alone, 16-1-11 hybridoma culture supernatant (anti-K^k) plus second step antibody, or 15-5-5 hybridoma culture supernatant (anti-D^k) plus second step antibody as described in Materials and Methods. Cells were grown with or without IFN-γ (30 μ/ml, 48 h). (*Unfilled curves*) Fluorescence after staining with second step antibody alone; (*filled curves*) fluorescence after anti-K^k or anti-D^k staining.

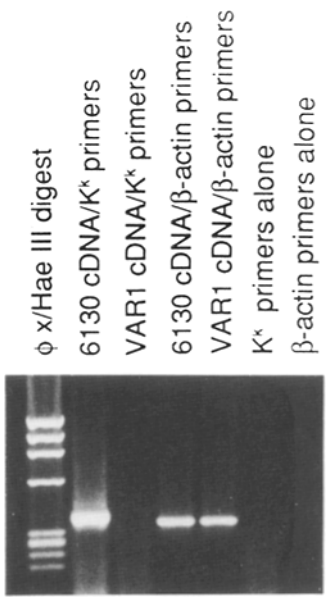

Figure 2. VAR1 tumor cells fail to express detectable amounts of K^k mRNA, consistent with the lack of K^k protein expression. RNA from 6130 and VAR1 were reverse transcribed into cDNA and PCR performed with K^k-specific or β-actin specific primers. No K^k-specific band is visible for VAR1.

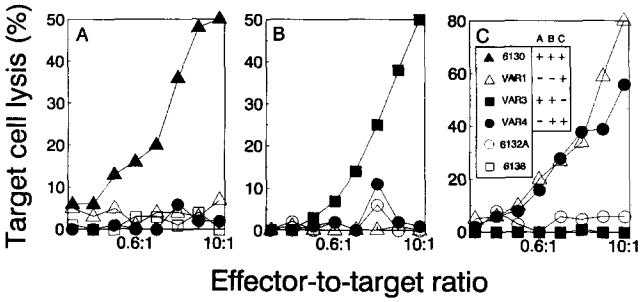

Figure 3. VAR1 tumor cells are insensitive to anti-A CTL clones but still retain sensitivity to lysis by an anti-C clone. Both BS1 (*A*) and PD4 (*B*) CTL clones specifically lyse A antigen-positive targets, but not A antigen-negative VAR1, VAR4, or 6138 cells. The SS1 clone (*C*) lyses C antigen-positive VAR1 and VAR4, but not the C antigen-negative VAR3 cells.

molecule are concomitantly lost, or that the K^k molecule is required for presentation of the A and B antigens. It is unlikely that VAR1 can revert to K^k expression and thereby present the A antigen on its cell surface since the VAR1 tumor cells we used in this study repeatedly failed to express surface K^k or message for K^k even after IFN-γ stimulation during a 4-mo period. Also, VAR1 tumor cells reisolated from a mouse remained K^k-negative (data not shown). Nevertheless, we do not know the molecular basis of the K^k molecule loss and whether all of the abnormalities described for VAR1 can be reversed by making VAR1 express K^k after transfection. So far, we have not succeeded in generating K^k-expressing VAR1 cells.

VAR1 Primes for a Response against the A Antigen. To determine if VAR1 still expresses the A antigen, mice were immunized with 6130, VAR1, and VAR4 ($A^-B^+C^+$, K^{k+} 6130 variant [2]) tumor cells intraperitoneally. After 7–10 d, spleens were harvested from the immunized mice, and MLTC cultures set up with 6130 tumor cells as the stimulators. Responder cells from both 6130 and VAR1-immunized mice showed a strong secondary anti-A response, whereas responder cells from VAR4-immunized mice showed an anti-B response (Fig. 4, *A–C*). Thus, although VAR1 cannot present the A antigen on its surface, presumably because of the lack of K^k molecules, it still primes for an immune response to the A antigen. To confirm that the apparent A-specific priming requires immunizations with a tumor of the 6130 tumor lineage, mice were immunized with 6132A, a K^{k+} UV-induced tumor with noncrossreacting CTL-defined antigens. MLTC cultures were established with 6130 tumor cells as stimulators. As seen in Fig. 4 *D*, no anti-A cytolytic activity was evident.

To determine if presentation of the A antigen on the target surface is required to induce an effective secondary response, spleen cells from 6130 and VAR1-immunized mice were used in MLTC cultures with VAR1 tumor cells as stimulators. This resulted in the absence of an effective secondary response to the A antigen (Fig. 4, *E* and *F*). Surprisingly, no anti-C response was evident. Anti-C clones could, however, be isolated after cloning anti-VAR1 CTL lines (P. Ward, unpublished data).

6130 and VAR1, but Not VAR4, Induce a Dominant $V\beta13^+$ PEL Proliferation. Whereas our priming assays indicated that VAR1 cells stimulate a response against the A antigen, we analyzed further the nature of the response by characterizing the TCR of the responding T cells, since we have recently determined that the A antigen induces T cells with unique β chain sequences (12).

TCR genes encoding β chains elicited in the response to 6130 tend to employ Vβ13, Dβ1.1, and Jβ1.1 gene segments. In addition, the genes encode characteristic CDR3 amino acids that are not employed by T cells responding to other tumors with noncrossreacting antigens (12). We found that VAR1 ($A^-B^-C^+$) induces T cells expressing similar TCR β chains to those associated with the A antigen–specific response to 6130 ($A^+B^+C^+$). Specifically, VAR1 induces a $CD8^+$ PEL response that is predominantly $V\beta13^+$ as assessed by flow cytometry (Fig. 5, Table 1). DNA sequence analysis revealed that the VAR1-induced $V\beta13^+$ cells were also oligoclonal. 18 of 19 PCR isolates derived from VAR1-induced T cells utilized one of four CDR3 sequences (E1-E3, E4-E9, D1-D5, and D6-D9; Fig. 6 *A*). Two of these sequences, those shared by isolates D6-D9 and D10, are identical except for a single nucleotide, which could represent

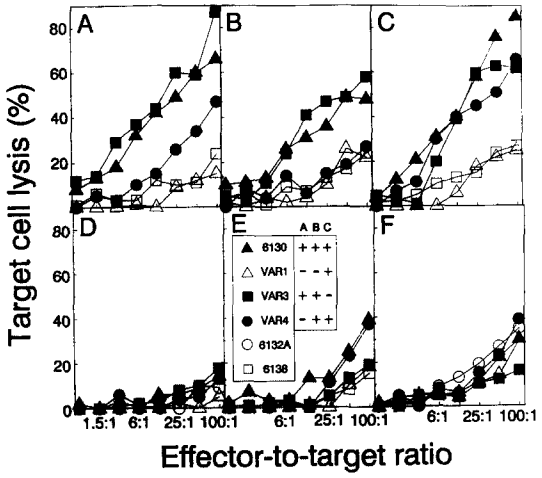

Figure 4. Although insensitive to killing by anti-A CTLs, VAR1 tumor cells prime in vivo for an anti-A response in vitro. Mice were immunized with 6130 (*A* and *E*), VAR1 (*B* and *F*), VAR4 (*C*), or 6132A tumor cells (*D*). Spleen cells of these mice were tested in an MLTC. (*A–D*) Responders stimulated with 6130 tumor cells. (*E* and *F*) Responders stimulated with VAR1 tumor cells. 6130 and VAR1 immunized mice demonstrate a strong anti-A response (*A* and *B*). VAR4-immunized mice show anti-B response (*C*), whereas 6132A immunized mice show no significant killing (*D*). 6130 and VAR1 immune responders stimulated with VAR1 tumor cells fail to generate an anti-A response (*E* and *F*).

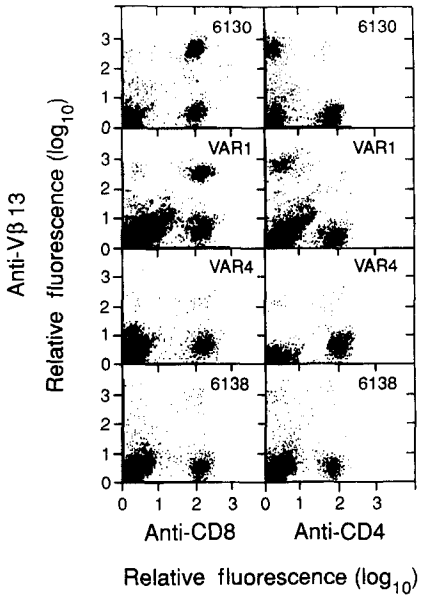

Figure 5. 6130 and VAR1, but not VAR4 or other tumor cells induce a strong $V\beta13^+$ PEL response among the $CD8^+$ T cell population. PELs were induced and stained as described in Materials and Methods. The response to 6138 tumor cells is shown as being representative for a non-6130 lineage tumor.

Table 1. *Significant Increase of Vβ13⁺ CD8⁺ T Lymphocytes Induced by 6130 and VAR1 but Not other Tumor Cells*

Antigen	Percent Vβ13⁺ of CD8⁺	No. mice tested	Percent Vβ13⁺ of CD4⁺	No. mice tested
6130*	42.3 ± 4.7‡	10	1.9 ± 0.3§	7
VAR1‖	28.3 ± 2.2‡	5	4.0	2
VAR4*¶	8.2 ± 0.4	3	1.7	2
6138*	6.9 ± 2.8	3	2.3	2
Normal spleen	4.4 ± 0.2	3	1.0 ± 0.4	3

* From reference 12. The data is presented here for comparison.
‡ These values are significantly different from other values at $p < 0.05$, as determined by a 2-tailed Student t test.
§ The values are the mean ± SEM.
‖ A⁻B⁻C⁺, Kᵏ⁻ variant of 6130.
¶ A⁻B⁺C⁺, Kᵏ⁺ variant of 6130.

PCR error. All used the Dβ1.1 gene segment, and 8 out of 19 used the Jβ1.1 gene segment, which was also prevalent among the 6130-induced Vβ13⁺ cells (18 out of 26) (12). At the amino acid level, the CDR3 regions of the Jβ1.1⁺ β chains (D and E isolates) were also similar to those of Jβ1.1⁺ T cells induced by 6130. In each case, a characteristic CDR3 length of 9 amino acids was employed (Fig. 6 *B*). Isolates D1-D5 and E1-E3 each share seven out of nine amino acids with the CDR3 sequence of the 6130-specific anti-A CTL clone BS1, which had been sequenced previously

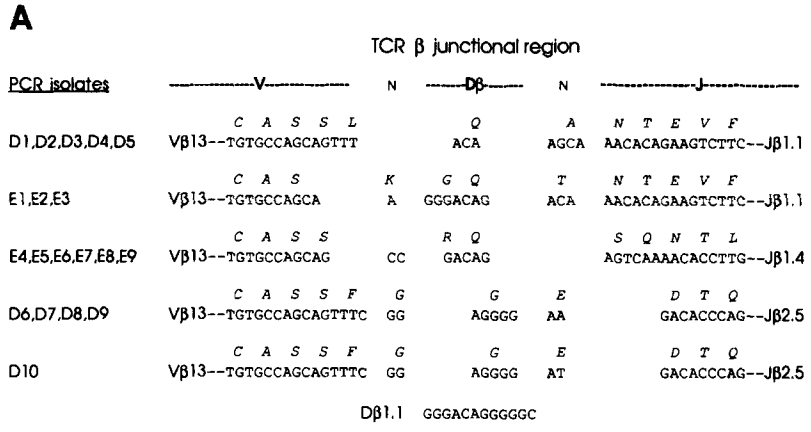

Figure 6. Sequence analysis of Vβ13⁺ TCR β chains obtained by PCR of mRNA from T cells responding in vivo to the VAR1 tumor. (*A*) Sequences of nucleotides and translated amino acids in single letter code. Only the junctional sequences are shown. Sequences upstream of those shown were identical to those previously published for the Vβ13 gene segment (39) and those downstream of those shown were identical to those previously published for the indicated Jβ gene segments (40) and for a portion of the Cβ segment (40, 41). The 3′ boundary of the Vβ13 gene segment is arbitrary and based on comparison between these and previously published Vβ13 sequences. The 5′ boundaries of the Jβ gene segments are based on comparison to their known germline sequences (40, 41). All isolates employed a portion of the Dβ1.1 segment (sequence below [42]) and are aligned accordingly. Nucleotides between the V and D gene segments and between the D and J gene segments are presumed N region insertions. (*B*) Comparison of the translated amino acid sequences of the CDR3 regions of PCR isolates utilizing Jβ1.1 (D and E isolates) from this paper (*A*) with the CDR3 sequence of the anti-A CTL clone BS1 (12). (*Light shading*) Similarity with the CDR3 regions of 18 Jβ1.1⁺ PCR isolates amplified from PEL responding to the 6130 tumor in vivo (12). (*Dark shading*) Increased similarity. The numbers below each CDR3 amino acid indicate the percentage of the 18 anti-6130 PCR isolates sharing that particular amino acid at each position with the anti-A clone BS1, derived the 6130-immune mice, and with clones E1-E3 and D1-D5 from VAR1-immune mice.

(12). The numbers below each amino acid in Fig. 6 *B* indicate the degree of identity to previously sequenced PCR isolates amplified from PEL reacting to the A antigen of the parental 6130 (12). The values are expressed as the percentage of 6130-induced $J\beta1.1^+$ isolates (18 total) possessing the indicated amino acid at each position. The similarities in CDR3 sequences listed above, in addition to the cellular data, suggest that the T cells responding to the different tumors are recognizing similar, if not the same, antigen. Thus, VAR1, which fails to display the A antigen on its surface, nonetheless induces T cells bearing TCR which in many ways mimic those of the anti-A $V\beta13^+$ cells induced by the 6130 tumor.

$V\beta13^+$ PEL Induced by VAR1 Are Not Cytolytic. PELs induced by 6130 respond specifically to the A antigen, despite the presence of the other independent B and C antigens (Fig. 7 *A*). Although VAR1 induces a strong $V\beta13^+$ PEL response with TCR sequences indicative of A-antigen specificity (Figs. 5 and 6), A-specific killing was not evident by these PELs (Fig. 7 *C*). Therefore, we sought to determine the specific cytolytic activity of VAR1-induced $V\beta13^+$ PELs. $V\beta13^+$ cells from anti-6130 and anti-VAR1 PELs were sorted to 99% purity (data not shown). The sorted cells were tested for their cytolytic activity. The VAR1-induced $V\beta13^+$ CTLs displayed no significant cytolytic activity (Fig. 7 *D*). 6130-induced $V\beta13^+$ CTLs, on the other hand, killed VAR3 ($A^+B^+C^-$ 6130 variant [2]) but not VAR4 ($A^-B^+C^+$) (Fig. 7 *B*) (12).

VAR1 may either inhibit or reverse the acquisition of the cytolytic phenotype. The ability of VAR1 cells to restimulate cytolytic anti-C CTL clones in vitro (data not shown) argues against the notion that VAR1 reverses the cytolytic phenotype. However, the finding of VAR1-induced expansion of $V\beta13^+$ $CD8^+$ PELs that lack cytolytic activity is consistent with the possibility that VAR1 cells inhibit the acquisition of the cytolytic phenotype. An alternative possibility is that a target presenting the A antigen was required to allow anti-A T cells to become fully cytolytic (Fig. 4).

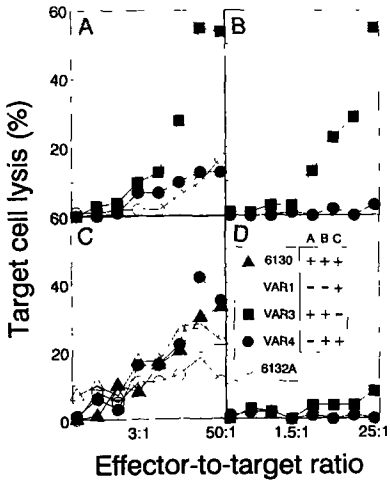

Figure 7. CTLs induced by VAR1 in vivo do not lyse A antigen-positive targets. 6130 induced PELs, non-sorted (*A*) and $V\beta13^+$ $CD8^-$ sorted (*B*), are specific for the A antigen (12). VAR1-induced PELs, nonsorted (*C*) or $V\beta13^+$ $CD8^+$ sorted (*D*), fail to exhibit anti-A specific lysis.

Discussion

In this study, we show that loss of expression of one MHC class I restriction element by a tumor expressing multiple antigens can allow escape from immune destruction by the host, even though the variant tumor remains sensitive to CTLs obtained exogenously which are directed against other antigens presented by retained MHC class I molecules. The response to the remaining target antigens seems to be prevented because the variant continues to induce a clonally restricted $CD8^+$ immune response to a dominant antigen (A) that no longer serves as a target for CTL-mediated destruction. The evidence for this dominance of the immune response to the A antigen comes from two observations. First, VAR1, which is completely resistant to anti-A CTLs but is susceptible to anti-C CTLs (Fig. 3), could prime for the response to the A antigen (Fig. 4 *B*) as effectively as the parental 6130 tumor (Fig. 4 *A*). This priming was specific for the A antigen since the unrelated 6132A tumor failed to prime for an anti-A response (Fig. 4 *D*). Furthermore, anti-A priming by VAR1 was probably not caused by a lineage-specific helper antigen (18) since VAR4 ($A^-B^+C^-$) failed to prime for an anti-A response (Fig. 4 *C*). Second, VAR1, like 6130, induced a significant increase in $V\beta13^+$ $CD8^+$ PELs in vivo whereas VAR4 did not (Fig. 5, Table 1). Sequence analyses of the $V\beta13$ CDR3s from VAR1-induced PELs revealed striking similarities with those of the A antigen–specific CDR3s of 6130-induced PELs and anti-A CTL clone (Fig. 6). Since the CDR3 is closely correlated with antigen fine specificity (19–23), the similarities observed in the CDR3s collectively support the notion that the $V\beta13^+$ $CD8^+$ cells induced in the peritoneal cavity by VAR1 bear TCRs specific for the A antigen. These two observations (A-specific priming and induction of clonally restricted $V\beta13^+$ PELs characteristic of anti-A $CD8^+$ response) strongly suggest that VAR1 continues to express the A antigen even though it can no longer serve as a target, presumably because of loss of the K^k molecule.

The A antigen may be released from K^k-negative VAR1 cells and picked up by K^k-positive host APCs which subsequently induce A antigen–specific $CD8^+$ T cells. Generally, antigens in the extracellular fluid are taken up by APCs, processed and presented in association with MHC class II molecules, and induce $CD4^+$ T cells (24). In contrast, endogenously synthesized antigens are processed in an intracellular compartment distinct from the MHC class II pathway, presented in association with MHC class I molecules, and induce $CD8^+$ CTLs (25, 26). Thus, the two different pathways largely determine whether $CD4^+$ or $CD8^+$ T cells are utilized in an immune response (25–27), e.g., $CD8^+$ CTLs selectively respond to cells expressing viral or tumor-specific antigens. However, these two mechanisms of antigen presentation may not be absolutely segregated. The existence of an APC that can process and present exogenous antigens in association with MHC class I molecules has been postulated (28), and described (29). It is not clear how an antigen

can impede the response to a different coexisting antigen even if the antigens are restricted by different restriction elements. For example, we have shown previously (12) and here that a given antigen presented in the context of H-2 K^k can block responses to another antigen presented by H-2 D^k. Since the antigen remains immunodominant despite the loss of its restricting element from the tumor, it appears that the host APCs are critically involved in the establishment of immunodominance. Immunodominance is also observed in response to the parental 6130 tumor (12), to other tumors with multiple antigens (4), and to multiple minor H antigens (30).

Whereas tumor variants become resistant to anti-A CTL whether the A antigen or the K^k molecule is lost, the immune response to the two types of variants and the effects of this response are quite different. The A antigen loss variant can induce immune responses to the remaining target antigens followed by stepwise selection of further antigen loss variants until the tumor lacks a target molecule. By contrast, the K^k loss variant VAR1 continues to induce a response to the immunodominant A antigen, even though that antigen is no longer a target on the tumor cell surface. Thus, other target antigens remain on the tumor cell surface for potential specific immune therapy even though there is no host response to these antigens.

Overall, this study suggests a previously undescribed pathway for a tumor with multiple antigens to escape from immune destruction. In this pathway, a heritable variant causes the host to focus on responding to a tumor antigen that is no longer a target for destruction on the variant tumor. This focusing may also prevent the host from mounting an immune response to secondary antigens, thereby rendering an antigenic tumor unrecognizable to the host. For this mechanism of escape, loss of a single allele was apparently sufficient to convert an immunogenic regressor tumor to a progressor variant, despite the existence of another antigen presented by the progressor variant on a remaining MHC allele. Loss of single MHC class I alleles occurs rather frequently in common human tumors, and more so than the loss of all MHC expression (7–11). Therefore, the described pathway may be commonly used for immune escape of human tumors. Our results may have important implications for interpreting the significance of the reported attraction of restricted T cell populations to certain progressively growing human cancers (31). Such attraction may occur even though the infiltrated cancer may not be a sensitive target for the attracted T cells. Finally, transfecting low MHC-expressing tumors with genes encoding the MHC allele that present tumor-associated antigens (32–35) or IFN-γ (36–38) have yielded effective immunogens against the untransfected parental tumors since they remained susceptible to lysis by the induced CTLs. Such protocols may not be feasible with tumors, such as VAR1, which are completely resistant to lysis by the dominant antigen-specific CTLs even after stimulation with IFN-γ. However, it may be feasible to override the effects of the dominant antigen in MHC class I allelic loss variant by depleting the dominant T cell subsets that are induced (e.g., the Vβ13 population in the case of the VAR1 tumor) or by immunization with variants that have lost the immunodominant antigen rather than just its restricting element. Such treatment may well enhance the CTL response to antigens retained by the escape variants.

We wish to thank Dr. A. Sant for the anti-K^k and anti-D^k mAb; Dr. D. Rowley for reviewing the manuscript, and Ms. Cheryl Small for typing the manuscript.

This study was supported by National Institutes of Health grants R37 CA-22677, PO1 CA-19266, and RO1 CA-37156. Steven Seung was supported by the Lederer Foundation and partially by Immunology Training grant 5-T32-GM07281.

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Received for publication 17 November 1992 and in revised form 20 April 1993.

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