IDENTIFICATION OF A UNIQUE TUMOR ANTIGEN AS REJECTION ANTIGEN BY MOLECULAR CLONING AND GENE TRANSFER

By HANS J. STAUSS, CARTER VAN WAES, MARY ANN FINK, BARBARA STARR, and HANS SCHREIBER

From the Department of Pathology, University of Chicago, Chicago, Illinois 60637

The most convincing evidence for the existence of tumor-specific antigens comes from the studies of chemically induced tumors in mice (1–3). Immunization with these tumors, but not with normal donor tissues, caused strong resistance to a subsequent challenge with the same tumor but no resistance to a challenge with any other independently induced tumor (4, 5). Thus, these transplantation antigens were tumor specific as well as unique, i.e., individually distinct for each independently isolated tumor. Unique antigens were seen even when the tumors were induced with the same carcinogen in the same organ system in the same strain of mice (4, 5). This finding of unique tumor specificity suggested that the diversity of these tumor-specific antigens is very large.

Identifying the molecular nature of these unique tumor-specific antigens that cause tumor rejection has proven to be extremely difficult in the past. Serological probes with unique tumor specificity are difficult to obtain (6, 7), and the serologically recognized antigens may not be the target for tumor rejection (8, 9) that is primarily T cell–mediated (10).

Like other neoplasias, UV light–induced tumors display unique tumor-specific transplantation antigens (11, 12), and we used such tumors for studying the nature of these antigens for the following reasons: (*a*) the unique tumor-specific rejection antigens on UV-induced tumors are stronger than those on chemically induced tumors in that UV-induced tumors often regress after transplantation into normal mice even without prior immunization (13); (*b*) several of the tumor-specific rejection antigens on one such UV-induced regressor (RE) tumor, called 1591-RE, have been defined by cytolytic T cell clones (14–16); (*c*) Philipps et al. (17) have generated mAbs with unique specificity for this UV-induced RE tumor that reacted with novel MHC class I molecules on this tumor; and (*d*) the genes encoding these novel class I molecules have been cloned (18), identified by transfection (18), and their DNA sequence has been determined (19).

In this study, we determine the relationship between the novel MHC class I

This work was supported by National Cancer Institute grants P01 CA-19266, R01 CA-22677, and R01 CA-37156. H. J. Stauss was supported by a Fellowship of the National Cancer Cytology Center and a Fellowship of the Deutsche Forschungsgemeinschaft. C. Van Waes was supported by grant PHS 5T32 GMO-7281 from the National Institute of General Medical Sciences. Address correspondence to Dr. Hans J. Stauss, La Rabida-University of Chicago Institute, East 65th Street at Lake Michigan, Chicago, IL 60649.

 J. Exp. Med. © The Rockefeller University Press · 0022-1007/86/11/1516/15 $1.00
Volume 164 November 1986 1516–1530

TABLE I
1591 Cell Lines Used in This Study

Designation	Growth in normal mice*	Phenotype‡				Previous designation	Reference
		A	B	C	D		
1591-RE	RE	+	+	−	−	1591-RE2	15
1591-RE1	RE	+	+	+	+	1591-RE1	15
1591-VAR1	PRO	−	+	−	ND	1591-UVS7	21
1591-VAR2	PRO	−	+	−	ND	1591-PRO-2	14
1591-VAR3	PRO	−	+	−	+	1591-AS1	16
1591-VAR4	PRO	−	+	−	ND	1591-PRO-3	14
1591-VAR5	PRO	−	+	−	+	1591-AS4	16
1591-VAR6	PRO	−	+	−	+	1591-AS5	16
1591-VAR7	RE	+	−	−	−	1591-BS1	16
1591-VAR8	PRO	−	+	−	−	1591-PRO-4	14
1591-VAR9	PRO	−	−	−	−	1591-HM‡	22

* RE, regressor; PRO, progressor.
‡ A, B, C, and D represent 1591 tumor-specific antigens that are recognized by 1591-specific CTL clones (15, 16).

molecules encoded by the cloned genes and the rejection antigens of the 1591 tumor. We positively identify one of the novel 1591 class I genes as encoding an antigen that causes immunological tumor rejection in normal mice. Furthermore, we show that transfection of this novel class I gene into a 1591 progressor (PRO) tumor variant reverts the progressive growth behavior of the variant and leads to the rejection of the gene-transfected progressor tumor by normal mice.

Materials and Methods

Animals and Tumor Lines. We obtained C3H/HeN (MTV^-) mice from the National Cancer Institute Cancer Research Facility (Bethesda, MD). The fibrosarcoma 1591-RE was induced by UV irradiation (20) and regresses when transplanted into normal syngeneic C3H/HeN mice. Electrophoretic analysis of seven isoenzymes revealed that the 1591 tumor displays a pattern characteristic of the C3H strain (17). The designation and the antigenic phenotype of the 1591 cell lines used in this study are shown in Table I. 1591-RE1 ($A^+B^+C^+D^+$) and 1591-RE2 ($A^+B^+C^-D^-$) are two cloned tissue culture cell lines of the 1591-RE tumor (15). For convenience, 1591-RE2 is referred to as 1591-RE in this paper. The cell lines 1591-VAR3, -VAR5, and -VAR6 are progressor variants of 1591-RE2, selected in vitro with anti-A CTL and previously referred to as 1591-AS1, -AS4, and -AS5, respectively (16). 1591-VAR2, -VAR4 and -VAR8 are three of the progressor variants seen in 5 of 100 animals that were challenged with fragments of the 1591-RE tumor (14). 1591-VAR1 is a progressor variant that has been isolated from a UV-irradiated animal that was challenged with 1591-RE tumor fragments; it was previously referred to as 1591-UVS7 (21). 1591-VAR7 is a variant of 1591-RE that has been isolated after in vitro selection with anti-B CTL (16). 1591-VAR9 is a tumor variant that was isolated from a C3H nude mouse injected with a mixture of 1591-VAR8 cells and spleen cells from mice immunized with sublethal doses of 1591-VAR8 (22).

Chromium-release Assay. The cytotoxicity of cloned CTL lines (15) or of uncloned mixed lymphocyte-tumor cell cultures (14) was determined as described. The percentage of specific lysis was calculated by the formula: specific release = [(experimental release-spontaneous release)/(total release-spontaneous release)] × 100. Spontaneous release was <15% of total release.

Immunological Analyses. The properties of the antibodies CP28 and CP3F4 and the anti-A 1591 T cell lines have been described (17, 23). The anti-MHC class I mAbs were

gifts from K. Ozato (National Institute of Childhood and Human Health Development, Bethesda, MD) and their specificities have been described (24). The FACS IVB (Becton Dickinson & Co., Mountain View, CA) was used to quantify the degree of MHC class I antigen expression of tumor cells and of class I gene transfectants. Cells were incubated with the MHC class I–specific antibodies first, and then with fluorescein-coupled goat anti–mouse Ig antibodies. The binding ratio was determined as the amount of fluorescence after staining with both antibodies, divided by the amount of fluorescence after staining with the second antibody alone.

DNA-mediated Gene Transfer. The isolation of the novel 1591 class I genes 149, 166, and 216 and the transfection of these genes into mouse L cells has been described in detail (18). In this paper, the gene-transfected L cells are referred to as $L149^+$, $L166^+$, and $L216^+$, respectively. 1591 progressor variants were cotransfected with the cloned 1591 class I genes and with the plasmid pZipNeo (25), which contains the gene encoding the aminoglycoside phosphotransferase (26), by the calcium phosphate precipitation method as described (27). The cells that aquired aminoglycoside phosphotransferase activity were selected by their ability to grow in the presence of 500 μg/ml of the antibiotic G418 (untransfected cells consistently died after 1 wk under those conditions). The G418-resistant cell population was cloned by limiting dilution and the individual clones were analyzed with the FACS IVB for class I antigen expression.

Southern Blotting. DNA was extracted from tumor cell lines grown in vitro or from spleen cells of inbred mouse strains and was digested to completion using an excess of restriction enzyme (New England BioLabs, Beverly, MA). The digested DNA was separated on a 0.9% agarose gel and blotted onto zetabind membrane (AMF-Cuno, Meriden, CT) by capillary flow using 20 × SSC as transfer buffer. The conditions of the hybridization and the origin of the MHC class I–specific probe 149.6-6 have been described (18).

Results

Loss of the Novel MHC Class I Genes from Tumor Variants that Grow Progressively in Normal Mice. The 1591 tumor contains three novel class I genes that account for the abnormal reactivity of the tumor cells with MHC class I–specific mAbs (18). In addition, the 1591-RE1 tumor expresses multiple, independent, CTL-defined antigens (15), each of which can independently cause tumor rejection. In the first part of our study we determined whether there is a correlation between the presence of the novel class I genes and the expression of a CTL-defined antigen. We have previously described (16) a 1591-RE tumor cell line, designated 1591-RE2, which expresses in addition to the novel class I antigens only one known rejection antigen, namely the one previously defined as "A" by cytolytic T cell clones. For convenience 1591-RE2 is referred to in this paper as 1591-RE. We used this 1591-RE tumor for analyzing the biological significance of the novel class I gene products and to determine their possible role in tumor rejection. 1591-RE tumor variants that had acquired progressor phenotype either after selection in vitro with the anti-A CTL line (16) or after selection in vivo by the normal host (14) were tested for the presence of the three novel class I genes. Since these genes contain novel restriction enzyme sites, they are detectable as restriction enzyme fragment length polymorphisms in the genomic 1591-RE DNA (18). Fig. 1 shows a Southern blot of DNA from 1591-RE and from six progressor variants, and it shows that all the progressor variants had lost a polymorphic band (*arrow*) that, as we have shown previously (18), represents the novel 1591 class I genes 216, 166, and 149. All progressor variants display a class I restriction fragment pattern identical to normal C3H DNA. Thus, our

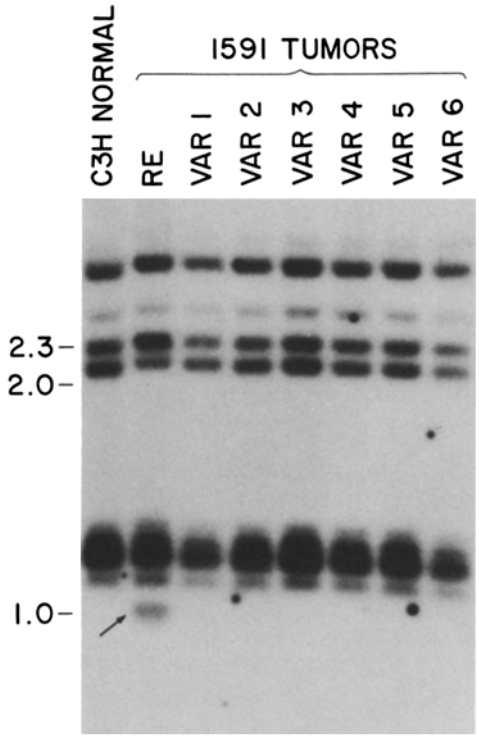

FIGURE 1. Loss of the novel MHC class I genes 216, 166, and 149 in 1591 progressor variants. DNA from the parental regressor tumor, 1591-RE (lane *2*) and from six progressor variants (lanes *3–8*) derived from this tumor was digested with the restriction enzyme Pst I and analyzed by Southern blotting with the class I–specific probe 149.6-6 derived from gene 149 (18). Spleen DNA from the C3H HeN MTV$^-$ strain (lane *1*) is used as a control. VAR1, VAR2, and VAR4 are progressor variants of 1591-RE derived in vivo by immune selection, by UV-irradiated, or by normal mice (14, 21) and VAR3, VAR5, and VAR6 are progressor variants of 1591-RE selected in vitro with anti-A CTL (16). For details of the variants see Table I. The positions of the size markers are indicated by kb numbers.

results indicate that progressively growing variants of the 1591-RE regressor tumor always lose all three novel class I genes: 216, 166, and 149.

The Novel MHC Class I Gene 216 Encodes the 1591 Tumor-specific Antigen Defined by Anti–A CTL. We have shown previously (16) that tumor variants selected for the loss of the anti–A CTL–defined antigen are no longer rejected by normal mice, implicating a close linkage between (or even identity of) the A antigen and the antigen leading to tumor rejection. However, careful attempts to block A antigen–specific CTL clones with antibodies specific for any one of the three novel class I MHC antigens encoded by the 216, 166, or 149 genes failed (23). Therefore, the relationship of the serologically defined novel class I antigens to the CTL-defined antigen remained uncertain. In the following experiments, we used gene-transfected L cells to determine whether any of the three novel MHC class I genes (149, 166, or 216) encoded the antigen recognized by anti–A CTL. Because of the commonly seen instability of the expression of the introduced genes after DNA-mediated gene transfer, we recloned the gene-transfected cell lines. The analysis of the recloned gene transfectants with a panel of MHC class

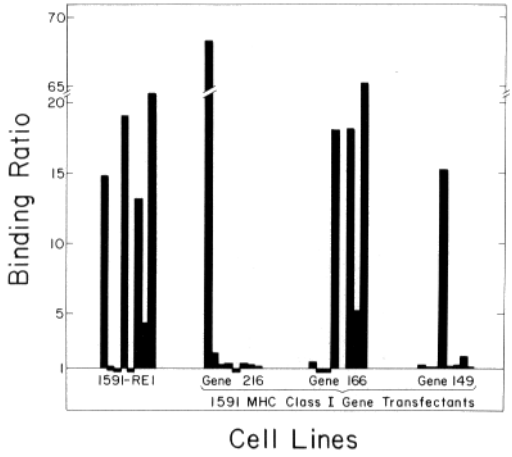

FIGURE 2. The 1591 class I genes 216, 166, and 149 account for all the novel MHC class I antigens that have previously been detected on 1591-RE using MHC class I–specific antibodies. Furthermore, the novel class I gene 216 selectively encodes for the antigen reactive with the syngeneic tumor-specific mAb CP28 (17). The FACS IVB was used to determine the indirect fluorescence listed as binding ratio, i. e., fluorescence of the tumor cells stained with the first and second antibody over the fluorescence of the tumor cells stained with the second antibody alone for background (for details see Materials and Methods). The bars from left to right indicate respectively the binding of CP28, 34-2-12 (D^d), 34-5-8 (D^d), 28-14-8 (L^dD^b, q), 34-4-20 (L^dD^d), 30-5-7 ($L^dD^qL^qL^b$), 23-5-21 ($D^bD^dD^{s,q,p}$), and CP3F4 to the parental 1591-RE tumor or the gene-transfected L cells.

I–specific antibodies confirmed that the transfectants had the same reactivity as described previously (18). Fig. 2 summarizes the results of a FACS analysis of 1591 tumor cells and of gene-transfected L cells using eight distinct mAbs. The L cells transfected with the gene 216 react only with the CP28 antibody and not with any of the other MHC class I–specific antibodies. The gene 149–transfected L cells react only with the 28-14-8 antibody, while L cells transfected with the 166 gene react with the 28-14-8, the 30-5-7, the 23-5-21, and the CP3F4 antibodies. Together, the molecules encoded by the genes 216, 166, and 149 contain all the novel class I determinants that are detectable on the 1591 tumor (17). The analysis of the fluorescence histograms of the recloned transfected L cells indicated that there was no detectable level (<1%) of antigen loss variants in the cell population (data not shown). These gene-transfected cell lines were then labeled with ^{51}Cr and used as targets for the 1591 tumor-specific CTL lines. Fig. 3 shows that only the 216 gene–transfected L cell line was killed by the anti–A CTL line, while L cells transfected with the 166 gene or the 149 gene were not affected by the anti–A CTL clone. The $A^-B^+C^-D^-$ or the $A^-B^-C^-D^-$ variants of 1591 were not killed. As expected, however, the $A^+B^-C^-D^-$ variant of 1591 was killed by the anti–A CTL, as was the $A^+B^+C^-D^-$ parental 1591-RE tumor line. Anti–B, anti–C, or anti–D CTL did not kill any of the L cells transfected with the novel MHC class I genes (not shown). Together, our data clearly indicate that the 216 gene-encoded novel class I antigen is recognized by both the CP28 mAb (Fig. 2) as well as the anti–A CTL clone (Fig. 3).

The Cytolytic T Cell Response Against the 1591-RE Tumor Is Directed Primarily Against the 216 Gene Product. Since the 1591-RE tumor expresses three novel

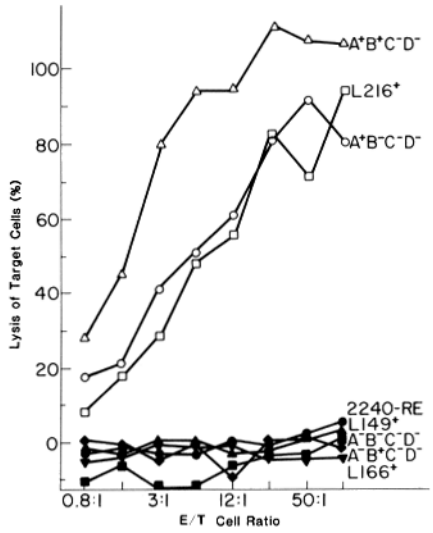

FIGURE 3. Selective recognition of the 216 gene-encoded, CP28-recognized antigen by the tumor-specific T cell clone anti-A. This T cell line defines the A antigen on the 1591 tumor as rejection antigen because it selects in vitro from the parental regressor tumor for antigen-loss variants that grow progressively in normal mice. The 1591-RE ($A^+B^+C^-D^-$) and the 1591-VAR7 ($A^+B^-C^-D^-$) tumor cells, and the 216 gene-transfected L cells are lysed by this CTL clone. L cells transfected with the 1591 class I genes 149 or 166, or the A^- variants of the 1591 tumor VAR8 and VAR9 (see Table I), or an unrelated UV-induced C3H tumor (2240-RE) are not lysed in a 4.5-h ^{51}Cr release assay.

class I antigens encoded by gene 216, 166, and 149, we next determined the individual role of the different gene products in a syngeneic anti-tumor CTL response. T cells from syngeneic animals that had been immunized with the 1591-RE tumor were restimulated in vitro with 1591-RE1 cells for 6 d. Fig. 4 shows the lytic activity of this uncloned T cell population. L cells transfected with gene 216 are lysed most efficiently, while L cells transfected with gene 166 or 149 are lysed to a lesser extent. L cells transfected with the thymidine kinase (tk)[1] gene alone are not lysed by these effector T cells. This suggests that the 1591-RE1 tumor induces a CTL response in the syngeneic host that is mainly directed against the 216 gene-encoded antigen, although the antigens encoded by the genes 149 and 166 are also recognized by these anti–1591 CTL.

Immunization with 216 Gene-transfected Cells Leads to Radioresistant A Antigen-Specific Tumor Rejection. We next tested whether the 216 gene-encoded antigen would alone be sufficient to induce radioresistant transplantation immunity to protect against a challenge with the 1591 tumor. Syngeneic C3H mice were immunized with 1591-VAR7 (A^+B^-) regressor tumor cells, L cells transfected with gene 216 (A^+B^-), or L cells transfected with the tk gene only (A^-B^-). After sublethal irradiation these animals were challenged with the A^+B^+ 1591-RE or the A^-B^+ 1591-VAR8 tumor, respectively. Fig. 5 shows that immunization with the 216 gene transfectants protected against the growth of the 1591-RE tumor but not against growth of the 1591-VAR8 tumor that does not express the 216 gene-encoded A antigen. As expected, immunization with the 1591-VAR7

[1] *Abbreviation used in this paper:* tk, thymidine kinase.

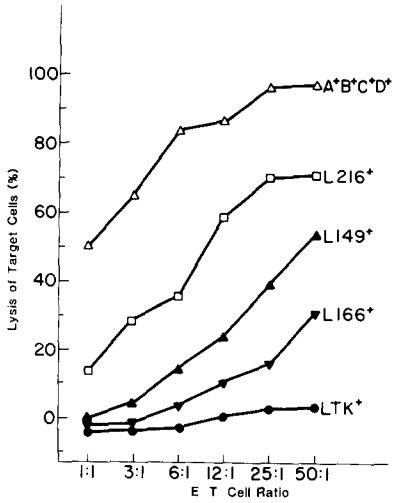

FIGURE 4. Lytic reactivity of spleen cells from 1591-RE1 ($A^+B^+C^+D^+$)-immunized mice with L cells transfected with 1591 class I genes 216, 166, or 149. The spleen cells were restimulated in vitro with 1591-RE1 cells for 6 d before they were used as effectors in a 4.5-h ^{51}Cr release assay. The 216 gene transfectant is lysed most effectively, while L cells transfected with the 149 or the 166 class I gene are lysed to a lesser degree. L cells transfected with the tk gene only are not lysed.

variant that shares a 1591 tumor lineage–specific antigen (designated H) with the 1591-RE and 1591-VAR8 tumors (22), leads to transplantation resistance to challenge with 1591-RE as well as 1591-VAR8. Immunization with L cells transfected with the tk gene only does not prevent the growth of either the 1591-RE or the 1591-VAR8 tumor, since the C3H-derived L cells do not express any of the known unique tumor-specific antigens found on tumor cells of the 1591 lineage. These data indicate that 216 gene–transfected L cells can be used to induce an A antigen–specific radioresistant immunity that prevents the outgrowth of A antigen–positive tumor cells.

Transfection of an A^- 1591 Progressor Tumor with the Novel Tumor Class I Gene 216 Abolishes the Progressive Growth Behavior of this Tumor. In the previous section, we have shown that the 216 gene–encoded molecule is the most immunogenic antigen expressed on the 1591-RE tumor and that the 216 gene–encoded antigen can induce a radioresistant immunity. However, since all the in vivo– or in vitro–derived progressor variants of the 1591-RE tumor had lost all three novel class I genes (216, 166, and 149) simultaneously, it was not clear if the presence of the 216 gene would alone be sufficient to establish the regressor phenotype. To test this, we introduced by transfection the 216 gene into a progressively growing A^- 1591 variant, designated 1591-VAR8, which had lost all 3 novel class I genes. This progressor tumor was cotransfected with the 216 gene and the gene encoding the enzyme aminoglycoside phosphotransferase, which confers resistance to the drug G418. The G418-resistant cell population was cloned, and 24 of 77 clones expressed the 216 gene–encoded antigen as determined by their reactivity with the CP28 antibody. Two 216 gene–expressing, A antigen–positive clones, designated $TR216^+.1$ and $TR216^+.2$, and two negative clones, designated $TR216^-.3$ and $TR216^-.4$, which were G418-

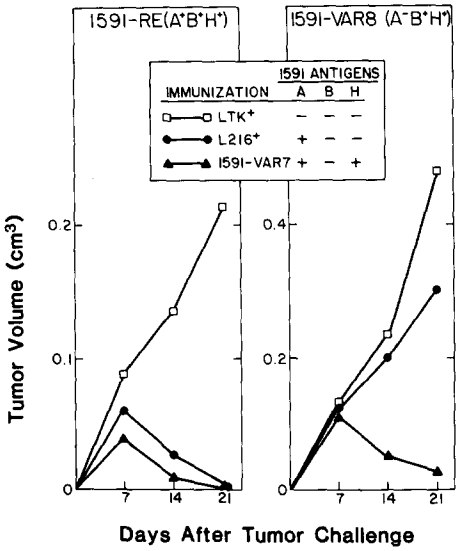

FIGURE 5. Immunization with 216 gene-transfected L cells protects against A antigen–positive 1591 tumors. Mice were immunized with 10^7 $A^+B^-C^-D^-$ 1591-VAR7 tumor cells, with 216 gene transfectants, or with L cells transfected with the tk gene only. 1 mo later, the animals received a sublethal irradiation (600 rad) and were challenged with three 1-mm^3 fragments of 1591-RE (*left*) or 1591-VAR8 (*right*) and tumor growth was measured in weekly intervals. The antigenic composition of the cells used for immunization and for tumor challenge is shown. A and B represent two independent 1591-specific antigens defined by CTL clones and H represents an antigen recognized by Th cells on tumor cells of the 1591 lineage (22).

resistant but did not express the 216 gene–encoded antigen, were analyzed further. Cells of these four clones were injected into nude mice and fragments of the growing tumors were used to challenge normal animals. The use of tumor fragments grown in nude mice ensured that the cloned transfectants could still grow as a malignant tumor in nude mice. Table II shows that the 216 gene–expressing $TR216^+.1$ clone grew out in only one of five animals, and that the 216 gene–expressing $TR216^+.2$ clone was rejected in all animals despite the fact that the mice were challenged with a large tumor dose ($>10^8$ cells). In contrast, the clones $TR216^-.3$ and $TR216^-.4$, which do not express the 216 gene–encoded antigen, grew in five of five and four of five mice, respectively, and all these mice died of progressive tumor growth. The single tumor that grew in one of the animals challenged with the 216 gene–expressing $TR216^+.1$ clone was readapted to culture and was analyzed with the FACS for the expression of the 216 gene–encoded antigen. All cells of the reisolate were negative for the 216 antigen, indicating that the cells either lost the transfected 216 gene or that its expression was prevented by some other mechanism (Fig. 6). This variant that had lost 216 gene expression was injected into five normal animals and tumor growth resulted in all the mice (Table II). Together, these data indicate that the stable expression of the 216 gene–encoded antigen is sufficient to change the phenotype of a progressor tumor so that it is rejected by the normal animal. Furthermore, the loss of the expression of this 216 antigen in transfected tumor cells allows these cells to regain the progressor phenotype characteristic of the untransfected parental progressor tumor.

TABLE II

Reversal of Malignant Growth in Normal Mice by Transfection of the Novel Class I Gene 216

Cell line*	Expression of the 216 gene product‡	Tumor incidence (%)§	
		Normal mice	Nude mice
1591-VAR8 TR216$^+$.1	+	1/5¶	2/2
1591-VAR8 TR216$^+$.2	+	0/5	2/2
		Total 1/10 (10)	
1591-VAR8 TR216$^-$.3	−	5/5	2/2
1591-VAR8 TR216$^-$.4	−	4/5	2/2
		Total 9/10 (90)	
1591-VAR8 TR216.1 Reisolate¶	−	5/5 (100)	ND
1591-VAR8	−	8/10 (80)	ND
1591-RE	+	0/10 (0)	2/2

* A clone of the 1591-VAR8 tumor (clone VAR8.7) was transfected with the 216 gene and the neomycin resistance gene. The G418 drug-resistant cell population was cloned and two clones that expressed the 216 gene-encoded antigen and two clones that did not express the 216 gene-encoded antigen were used to challenge five normal mice with tumor fragments (each animal was injected with a total of 10 tumor fragments ~1 mm^3 in size) or two nude mice (with five 1-mm^3 tumor fragments each).

‡ Expression of the 216 gene product was determined by FACS IVB analysis using the mAb CP28 that specifically recognizes this gene product and a fluoresceinated second antibody. Cell lines designated positive for expression of the 216 gene product stained at least two times above background (binding ratio, >2), while all cell lines designated negative for 216 gene expression stained <1.5-fold above background (binding ratio, <1.5).

§ Number of mice with progressively growing tumors per number of mice challenged. Mice receiving the 216$^-$ clones died within ~6 wk due to the large tumor burden. The mice that were challenged with the 216$^+$ transfectants did not develop tumors even after 4 mo except for one mouse, which grew out an antigen loss variant ~2.5 wk after injection. All cell lines used in this experiment readily formed tumors in nude mice.

¶ One of the mice injected with the transfected 1591-VAR8 TR216$^+$.1 cell line developed a progressively growing tumor that was reisolated (designated 1591-VAR8 TR216.1 Reisolate) and reanalyzed for expression of the 216 gene by FACS IVB and for tumor incidence in normal mice.

Discussion

Many years ago, studies (4, 5) clearly showed that tumor-specific antigens that are distinct (unique) for each individual tumor can lead to a complete immunological destruction of experimental cancers. However, the molecules eliciting (and being the target of) these immune responses have remained completely obscure. Our present study not only gives a first example of a molecularly defined, unique tumor-specific rejection antigen, it also points out several important problems that can be encountered in defining these rejection antigens.

Several lines of evidence clearly indicate that the novel MHC class I gene, designated 216, encodes an antigen that elicits 1591 tumor-specific rejection and is the target molecule of tumor rejection: (*a*) The most conclusive evidence comes from the fact that transfection of the 216 gene into progressively growing 1591 tumor variants leads to the expression of the 216 gene–encoded antigen on the tumor and to complete rejection of all cells expressing this antigen. This was seen even though extremely large doses (i. e., 10 1-mm^3 fragments having ~10^8–10^9 tumor cells) were used, and even though the mice had not been

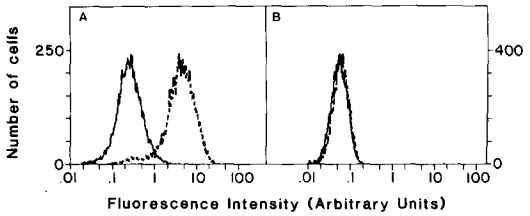

FIGURE 6. Loss of expression of the transfected 216 gene in a reisolated 1591-VAR8 tumor that grew progressively despite transfection with the 216 gene. The 216 gene-transfected tumors analyzed are the same as those used for the experiments described in Table II. *A* shows the histogram of the 1591-VAR8 TR216$^+$.1 cell line and *B* shows the histogram of the 1591-VAR8 TR216.1 reisolate. Cells were incubated with the mAb antibody CP28 followed by incubation with fluorescein-coupled goat anti-mouse Ig (--) or incubated with only the goat anti-mouse Ig (—). 10^4 cells were analyzed with the FACS IVB.

immunized before they were challenged with the transfected tumor. Thus, the strength of the observed transplantation resistance is the same as reported before (11) for the parental tumor 1591-RE or other UV-induced regressor tumors; (*b*) our present study clearly shows that the 216 gene–encoded antigen must be lost before the tumor can grow progressively in a normal immunocompetent mouse. Southern blot analysis showed that all of the in vivo– or in vitro–derived progressor variants analysed had lost the 216 gene. In agreement with this, we also found that the one 216 gene–transfected progressor tumor that failed to be rejected by one of the normal mice had lost completely the expression of the 216 gene–encoded molecule when reisolated from the tumor-bearing mouse; (*c*) further important evidence comes from our finding that the rejection antigen on the 1591 tumor that is encoded by the 216 gene is identical to that recognized by cloned 1591 A antigen–specific CTL. We had previously shown (16) that this anti–A CTL clone defines a 1591 tumor-specific rejection antigen because this clone selected in vitro for A$^-$ variants that acquired malignant growth behavior in normal mice; (*d*) finally, we have transfected the 216 gene into a cell line not of the 1591 origin and we can, thereby, show that even in this different cellular background the 216 gene–encoded antigen can induce transplantation immunity that is 1591 tumor-specific. The immunity is also radioresistant, as would be expected from unique tumor-specific antigens on UV-induced tumors (11). At present, we do not know whether the novel MHC genes 166 and 149 of the 1591-RE tumor also encode antigens that elicit and are targets of tumor rejection. Furthermore, the three MHC class I genes 216, 166, and 149 appear to be distinct from the genes encoding the CTL-defined B, C, and D antigens, because the analysis of a B$^+$C$^+$D$^+$ 1591 tumor variant revealed that this variant had lost the three novel class I genes (18) that we have identified. However, the genetic origin(s) of the B, C, and D antigen is at present unknown, and it is possible that these antigens are encoded by other, yet unidentified 1591 class I genes or by genes that do not belong to the MHC class I gene family.

Previous studies have failed to correlate the loss of the novel class I genes with tumor progression since a variant of the 1591 tumor that had lost the genes encoding the novel class I antigens (18) was still rejected by normal mice (16) and, therefore, retained the regressor phenotype. This might be due to the fact that this variant still expresses the CTL-defined B, C, and D antigens (23), some

of which are highly immunogenic and can cause tumor rejection in the normal host. Our study clearly shows that all of the progressor variants derived from the 1591-RE tumor either by selection by the normal host in vivo or by selection in vitro using anti-A CTL clones have lost the gene encoding the unique tumor-specific A antigen. As would be expected, the variants did not aquire a general resistance to lysis by CTL since they remained susceptible to lysis by tumor-specific CTL clones that recognize less immunogenic tumor antigens that are still retained on the progressor variants (not shown). Furthermore, we found that the variants expressed similar levels of normal MHC class I molecules as the parental regressor tumor (data not shown). Thus, one mechanism by which the 1591 tumor escapes immune destruction involves loss of the gene encoding a tumor-specific class I antigen, and this is distinctly different from another previously described mechanism that involves loss of expression of normal MHC class I antigens (28). The latter mechanism has recently been reported (29–32) to account for the progressive growth behavior of certain malignant tumors, although the nature of the tumor-specific antigens on the tumors analyzed has not been determined.

Unique tumor-specific transplantation antigens are antigenically distinct for independently induced tumors. These different antigens may, therefore, be encoded either by numerous different unrelated genes or by a single gene that underwent multiple different mutational changes. Alternatively, these antigens might be encoded by the members of a gene family such as the Ig genes, the T cell receptor genes, the MHC class I and class II genes, or the genes of the multiple retroviral proviruses that are present in the murine genome. Some of these gene families are known to contain the coding information for a large variety of distinct molecules and could therefore account for the observed remarkable antigenic polymorphism among tumor-specific transplantation antigens. It is interesting to notice that even a single malignant cell can express multiple unique tumor-specific antigens, as has been shown for the tumor P815 (33) or 1591-RE (15). Thus, to resolve multiple unanswered questions concerning the origin of these antigens and to determine if they are encoded by a family of related genes, it is necessary to analyze more tumors and to identify, molecularly and genetically, more unique tumor-specific transplantation antigens.

Another important and still unresolved question regarding the origin of unique tumor-specific antigens is whether the genes encoding such antigens are preexisting in the genome or whether these genes appear as the result of somatic mutation and as such represent the product of the mutagenic action of carcinogens. Previous studies (34, 35) showing unique antigenicity of each of the independent transformants which were all derived from one single parental cell seemed to suggest somatic carcinogen-induced mutations as a likely mechanism. However, it was not excluded by these studies that the carcinogen treatment activated heritably, but at random, different preexisting, previously silent genes. Such a mechanism could also account for the observed immunogenicity of tumors in the autochthonous host (5). Furthermore, Bailey (36) has pointed out the problems of residual heterozygosity in inbred mouse strains, which might then be mistaken for tumor-specific antigens on tumors. Although this possibility has not been formally excluded it is difficult to conceive how such residual hetero-

zygosity could be prevalent enough to account for the fact that up to 90% of UV-induced tumors in a given batch of mice can be highly immunogenic regressor tumors in normal mice and express individually tumor-specific antigens. In addition, the electrophoretic isoenzyme analysis of several C3H-derived, UV-induced regressor tumors revealed that all these tumors, including 1591, displayed an isoenzyme pattern characteristic for the C3H strain (17). Furthermore, the fact that tumors experimentally induced with a known carcinogen appear to be more immunogenic than "spontaneous" tumors (37) might suggest that a high dosage of a carcinogen more frequently leads to the expression of immunogenic tumor-specific antigens than a low dose of a carcinogen. The appearance of such antigens might be a consequence of the known mutagenic action of most carcinogens, including UV (38). Such an explanation would be consistent with the observed lower immunogenicity of spontaneous tumors that presumably arise as a consequence of exposure to low dose of environmental carcinogens. However, whether the genes encoding these antigens preexisted and are simply activated, or whether they are structural genes mutated by carcinogens cannot be decided at present.

The DNA sequence of the 1591 genes 216, 166, and 149 has been determined (19) and will be compared with the sequence of the potential parent genes that have been isolated using gene-specific DNA probes. However, even though these studies may identify a probable mechanism by which the novel tumor-specific class I genes arose, it will be impossible to determine whether these genes originated as a result of a somatic or a germ line mutation. This is because normal somatic cells from the original mouse that gave rise to the 1591 tumor are not available. Since such important autochthonous control cells are also not available for other tumors whose tumor-specific antigens have been carefully analyzed in the past (39), we are generating a new bank of UV-induced tumors in which normal control cells are concurrently isolated from each tumor-bearing mouse. Several of these new tumors are as highly immunogenic as 1591-RE or other tumors previously induced by UV-irradiation. The new tumors will be very useful to compare the molecular nature of other unique tumor-specific transplantation antigens with our present findings and to determine whether or not such antigens are encoded by related genes. Furthermore, the analysis of these new tumors will also allow us to clearly distinguish between somatic and germ line mutation and to understand the possible role that these molecules might play in the establishment of malignant behavior.

Summary

Tumor-specific transplantation antigens are antigens that can lead to complete immunological destruction of a transplanted cancer by the syngeneic host. When such antigens are expressed on cancers induced by chemical or physical carcinogens, then they are usually unique, i. e., antigenically different for each independently induced tumor. In this study, we show that the product of a gene encoding a novel MHC class I molecule and isolated from the murine UV light-induced regressor tumor 1591 represents one such unique tumor-specific transplantation antigen that causes tumor rejection. The major evidence comes from our finding that 1591 progressor variants regularly lost the gene encoding this

antigen that is expressed in the parental tumor that regresses in normal mice; furthermore, reintroduction of this gene into a 1591 progressor variant by DNA transfection caused the progressor variant to regress in normal immunocompetent mice. Thus, the progressor tumor reverted to the parental regressor phenotype following transfection. Consistent with the conclusion that the expression of the novel MHC class I gene following transfection was responsible for the regressor phenotype is also our finding that a variant of the transfected tumor that had lost expression of the transfected gene resumed its progressive growth behavior. Finally, we show that the molecule encoded by the novel class I gene is specifically recognized by a syngeneic tumor-specific cytolytic T cell clone that we have previously shown to select in vitro for progressor variants from the parental regressor tumor cell line. It remains to be determined to what extent unique tumor-specific rejection antigens of other highly immunogenic regressor tumors are encoded by novel MHC class I genes and whether these genes represent germline mutations or somatic mutations caused by the carcinogen treatment.

The authors thank Dr. D. A. Rowley for carefully reviewing the paper, Mr. John Hartley for cytofluorometric analyses, and Ms. Carolyn Hence for typing the manuscript.

Received for publication 11 June 1986 and in revised form 18 July 1986.

References

1. Gross, L. 1943. Intradermal immunization of C3H mice against a sarcoma that originated in an animal of the same line. *Cancer Res.* 3:326.
2. Foley, E. J. 1953. Antigenic properties of methylcholanthrene-induced tumors in mice of the strain of origin. *Cancer Res.* 13:835.
3. Baldwin, R. W. 1955. Immunity to methylcholanthrene-induced tumours in inbred rats following implantation and regression of implanted tumours. *Br. J. Cancer.* 9:652.
4. Prehn, R. T., and J. M. Main. 1957. Immunity to methlcholanthrene-induced sarcomas. *J. Natl. Cancer Inst.* 18:769.
5. Klein, G., H. O. Sjögren, E. Klein, and K. E. Hellstrom. 1960. Demonstration of resistance against methylcholanthrene-induced sarcomas in the primary autochthonous host. *Cancer Res.* 20:1561.
6. Old, L. J. 1982. Cancer immunology: The search for specificity. *Natl. Cancer Inst. Monogr.* 60:193.
7. Carbone, G. M., M. Boicchi, and G. Parmiani. 1981. Lack of anti-TSTA antibodies in mice immunized with a methylcholanthrene-induced fibrosarcoma. *Cancer Immunol. Immunother.* 11:31.
8. Davey, G. C., G. A. Currie, and P. Alexander. 1979. A serologically detected tumor-specific membrane antigen of murine lymphomas which is not the target for syngeneic graft rejection. *Br. J. Cancer.* 40:168.
9. Brown, J. P., J. M. Klitzman, I. Hellström, R. C. Nowinski, and K. E. Hellström. 1978. Antibody response of mice to chemically induced tumors. *Proc. Natl. Acad. Sci. USA.* 75:955.
10. Rouse, B. T., M. Roellinghoff, and N. L. Warner. 1972. Anti-θ serum induced suppression of cellular transfer of tumor-specific immunity to a syngeneic plasma cell tumor. *Nature (Lond.).* 238:116.
11. Kripke, M. L. 1974. Antigenicity of murine skin tumors induced by ultraviolet light. *J. Natl. Cancer Inst.* 53:1333.

12. Daynes, R. A., M. K. Schmidt, L. K. Roberts, and C. W. Spellman. 1979. Phenotypic and physical characteristics of the lymphoid cells involved in the immunity to syngeneic UV-induced tumors. *J. Immunol.* 122:2458.
13. Kripke, M. L. 1981. Immunologic mechanisms in UV radiation carcinogenesis. *Adv. Cancer Res.* 34:69.
14. Urban, J. L., R. C. Burton, J. M. Holland, M. Kripke, and H. Schreiber. 1982. Mechanisms of syngeneic tumor rejection. Susceptibility of host-selected progressor variants to various immunological effector cells. *J. Exp. Med.* 155:557.
15. Wortzel, R. D., C. Philipps, and H. Schreiber. 1983. Multiple tumor-specific antigens expressed on a single tumor cell. *Nature (Lond.).* 304:165.
16. Wortzel, R. D., J. L. Urban, and H. Schreiber. 1984. Malignant growth in the normal host after variant selection *in vitro* with cytolytic T cell lines. *Proc. Natl. Acad. Sci. USA.* 81:2186.
17. Philipps, C., M. McMillan, P. M. Flood, D. B. Murphy, J. Forman, D. Lancki, J. E. Womack, R. S. Goodenow, and H. Schreiber. 1985. Identification of a unique tumor-specific antigen as a novel class I major histocompatibility molecule. *Proc. Natl. Acad. Sci. USA.* 82:5140.
18. Stauss, H. J., R. Linsk, A. Fischer, D. Banasiak, A. Haberman, I. Clark, J. Forman, M. McMillan, H. Schreiber, and R. S. Goodenow. 1986. Isolation of the MHC genes encoding the tumor-specific class I antigens expressed on a murine fibrosarcoma. *J. Immunogenet. (Oxf.).* In press.
19. Linsk, R., H. Stauss, J. Forman, and R. S. Goodenow. 1986. Structure and function of three novel MHC class I antigens derived from a C3H ultraviolet-induced fibrosarcoma. *J. Exp. Med.* 164:794.
20. Fisher, M. S., and M. L. Kripke. 1978. Systemic alteration induced by mice by ultraviolet light irradiation and its relationship to ultraviolet carcinogenesis. *Proc. Natl. Acad. Sci. USA.* 74:1688.
21. Urban, J. L., J. M. Holland, M. L. Kripke, and H. Schreiber. 1982. Immunoselection of tumor cell variants by mice suppressed with ultraviolet radiation. *J. Exp. Med.* 156:1025.
22. Van Waes, C., J. L. Urban, J. L. Rothstein, P. L. Ward, and H. Schreiber. 1986. Highly malignant tumor variants retain tumor-specific antigens recognized by T helper cells. *J. Exp. Med.* 164:1547.
23. Philipps, C., H. J. Stauss, R. D. Wortzel, and H. Schreiber. 1986. A novel MHC class I molecule as tumor-specific antigen: correlation between the antibody-defined and the CTL-defined target structure. *J. Immunogenet. (Oxf.).* In press.
24. Evans, G. A., D. M. Margulies, B. Shykind, J. G. Seidman, and K. Ozato. 1982. Exon shuffling: mapping polymorphic determinants on hybrid mouse transplantation antigens. *Nature (Lond.).* 300:755.
25. Cepko, C. L., B. E. Roberts, and R. C. Mulligan. 1984. Construction and applications of a highly transmissible retrovirus shuttle vector. *Cell.* 37:1053.
26. Colbere-Garapin, F., F. Horodniceanu, P. Kourilsky, and A.-C. Garapin. 1981. A new dominant hybrid selective marker for higher eukaryotic cells. *J. Mol. Biol.* 150:1.
27. Parker, B. A., and G. R. Stark. 1979. Regulation of simian virus 40 transcription: sensitive analysis of the RNA species present early in infections by virus or viral DNA. *J. Virol.* 31:360.
28. Schrier, P. I., R. Bernards, R. T. M. J. Vaessen, A. Houweling, and A. J. Van der Eb. 1983. Expression of class I major histocompatibility antigens switched off by highly oncogenic adenovirus 12 in transformed rat cells. *Nature (Lond.).* 305:771.
29. Bernards, R., P. I. Schrier, H. Houweling, J. L. Bos, A. J. Van der Eb, M. Zijstra, and C. J. M. Melief. 1983. Tumorigenicity of cells transformed by adenovirus type 12 by evasion of T cell immunity. *Nature (Lond.).* 305:776.

30. Hui, K., F. Grosveld, and H. Festenstein. 1984. Rejection of transplantable AKR leukaemia cells following MHC DNA-mediated cell transformation. *Nature* (*Lond.*). 311:750.
31. Wallich, R., N. Bulbuc, G. J. Hämmerling, S. Katzav, S. Segal, and M. Feldman. 1985. Abrogation of metastatic properties of tumour cells by *de novo* expression of H-2K antigens following H-2 gene transfection. *Nature* (*Lond.*). 315:301.
32. Tanaka, K., K. J. Isselbacher, G. Khoury, and G. Jay. 1985. Reversal of oncogenesis by the expression of a major histocompatibility complex class I gene. *Science* (*Wash. DC*). 228:26.
33. Uyttenhove, C., J. Maryanski, and T. Boon. 1983. Escape of mouse mastocytoma P815 after nearly complete rejection is due to antigen-loss variants rather than immunosuppression. *J. Exp. Med.* 157:1040.
34. Basombrio, M. A., and R. T. Prehn. 1972. Studies on the basis for diversity and time of appearance of antigens in chemically-induced tumors. *Natl. Cancer Inst. Monogr.* 35:117.
35. Embleton, M. J., and C. Heidelberger. 1972. Antigenicity of clones of mouse prostate cells transformed *in vitro*. *Int. J. Cancer* 9:8.
36. Bailey, D. W. 1982. How pure are inbred strains of mice. *Immunol. Today.* 3:210.
37. Klein, G., and E. Klein. 1977. Immune surveillance against virus-induced tumors and nonrejectability of spontaneous tumors: contrasting consequences of host versus tumor evolution. *Proc. Natl. Acad. Sci. USA.* 74:2121.
38. Ames, B. N. 1979. Identifying environmental chemicals causing mutations and cancer. *Science* (*Wash. DC*). 204:587.
39. Pravtcheva, D. D., A. B. DeLeo, F. H. Ruddle, and L. J. Old. 1981. Chromosome assignment of the tumor-specific antigen of a 3-methylcholanthrene-induced mouse sarcoma. *J. Exp. Med.* 154:964.