Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 5485–5489, August 1985
Immunology

Molecular characterization of novel H-2 class I molecules expressed by a C3H UV-induced fibrosarcoma

(major histocompatibility complex/unique tumor-associated antigens/immunoprecipitation/two-dimensional gel electrophoresis/high-pressure liquid chromatography tryptic peptide mapping)

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Communicated by Ray D. Owen, April 12, 1985

ABSTRACT **Two novel class I-like molecules expressed on tumor 1591, a C3H UV-induced fibrosarcoma, are biochemically characterized using two-dimensional gel electrophoresis, a cross-blocking RIA, and tryptic peptide mapping. One novel molecule that reacts with CP28, a syngeneic tumor-specific monoclonal antibody, appears mosaic because it possesses characteristics of both K^k and D^k class I molecules. The second molecule is closely related but not identical to the bona fide L^d molecule expressed on BALB/c spleen. Thus 1591 expresses at least two novel class I molecules and is vigorously rejected by normal C3H mice, while a variant tumor derived from 1591, termed AS7, does not express these two class I molecules although it still expresses K^k and D^k. The significance of these observations to the immunobiology and genetics of the UV-induced fibrosarcoma system is discussed. Speculations on the role that the major histocompatibility complex may play in the immunosurveillance of neoplasms are also presented.**

The recognition of target structures expressed by aberrant cells and the subsequent destruction of those cells by the immune system is central to the hypothesis of immune surveillance (1, 2). On virus-infected cells the target structures recognized by cytotoxic T lymphocytes (CTLs) have been shown clearly to be viral antigen in association with class I molecules that are encoded within the major histocompatibility complex (MHC), denoted *H-2*, in the mouse (3). This discovery, together with the elucidation of the structure of the *MHC* and the gene products encoded therein, has had a dramatic effect on our understanding at a molecular level of the role that class I molecules play in T lymphocyte recognition. A class I molecule is composed of a class I polypeptide, molecular weight approximately 43,000–47,000. It is a highly polymorphic, integral membrane glycoprotein, noncovalently associated with β_2-microglobulin, a polypeptide of molecular weight 12,000 (4). Amino acid sequence data have demonstrated that class I protein molecules have three external domains, $\alpha1$, $\alpha2$, and $\alpha3$, although only the two amino-terminal domains, $\alpha1$ and $\alpha2$, are recognized by CTLs (5, 6). Two types of class I genes are located in the *H-2* complex (7). One category mapping to the *Qa* and *Tla* regions contains at least 25–30 genes (8, 9). Their function to date is unclear (10–14). The second category encodes the classical transplantation antigens H-2K and H-2D (also H-2L in some mouse strains) (15). They are expressed in various concentrations on most cells of the mouse. For a mouse of the $H\text{-}2^k$ haplotype the molecules encoded by the two different loci are denoted as K^k and D^k.

In contrast to virus-infected cells, the molecular nature of the potential target structures on neoplasms remains elusive. Perhaps the most precisely characterized unique tumor-associated antigen is one expressed on a murine methylcholanthrene-induced fibrosarcoma in which expression has been closely linked to the immunoglobulin heavy chain gene family on chromosome 12 (16).

The murine UV-induced fibrosarcoma system is attractive to study because the carcinogenic process clearly depends on the immunological status of the host (17). Tumors induced by UV irradiation can be transplanted to immunologically defective, old, nude, or UV-irradiated syngeneic mice with lethal result (18–20). In contrast, when the tumor is transplanted into young, healthy adult mice the tumor is vigorously rejected. However, a variant tumor occasionally arises, termed a progressor variant, that results in death of the host animal (21).

One such tumor, 1591, generated in a C3H ($H\text{-}2^k$) mouse, has been the subject of an elegant analysis using cloned CTLs (22, 23). Schreiber and his colleagues have shown that 1591 expresses at least four different epitopes ($A^+B^+C^+D^+$) that are recognized by different T-cell clones and that in a variant, AS7, derived from 1591, the A^+ epitope is not expressed. Schreiber also generated a syngeneic monoclonal antibody, CP28, that appears to react with a unique tumor-specific antigen expressed only on 1591. Thirty-seven other syngeneic tumors tested were negative. Using CP28, we immunoprecipitated a class I-like molecule from 1591 cell lysates; microcytotoxicity assays and fluorescence-activated cell sorting showed that CP28 cross-reacted with the two amino-terminal domains of the D^d class I molecule. However, the 1591 tumor itself reacted, not with D^d-specific antibodies but with three out of four L^d-specific antibodies and with two out of four L^d-specific cloned CTL lines (24). In summary, the CP28 antibody is 1591-specific and cross-reacts with the D^d molecule while 1591 itself appears to express L^d-like epitopes.

Our studies resolve this apparent paradox and show that the A^+ epitope correlates with the expression of at least two novel class I molecules. We show that one of these molecules is remarkably similar but not identical to the L^d molecule while the other CP28-specific molecule appears mosaic, exhibiting both K^k- and D^k-specific characteristics. We discuss the relevance of these findings to the immunobiology and genetics of the UV-induced fibrosarcoma system and to the role that the MHC may play in immunosurveillance in tumors.

MATERIALS AND METHODS

Mice, Tumor Lines, and Monoclonal Antibodies. C3H/HeN (mammary tumor virus-negative) mice were purchased from the National Cancer Institute Frederick Cancer Research Facility while BALB/cJ mice were obtained from The Jackson Laboratories. The 1591-RE1 and AS7 tumor lines (22, 23)

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Abbreviations: CTL, cytotoxic T lymphocyte; MHC, major histocompatibility complex; H142, H142-23.3; H100, H100-5.28.

together with monoclonal antibody CP28 (24) were gifts from H. Schreiber (University of Chicago).

H142-23.3 (anti-K^k and -D^k; H142), H100-5.28 (anti-K^k; H100), 34-5-8 (anti-D^d), 28-14-8 (anti-L^d), and 30-5-7 (anti-L^d) monoclonal antibody reagents were generously sent to us by R. S. Goodenow (University of California, Berkeley) (25–27).

Isolation of Class I Molecules. The procedures for radiolabeling and isolation of proteins have been described (28). Briefly, splenic lymphocytes or tumor cells were cultured for 6 hr in Hanks' balanced salt solution with 10 mM Hepes, 5% dialyzed fetal calf serum, and 2.5 mCi (1 Ci = 37 GBq) of [^{3}H]phenylalanine (Amersham). The cells were lysed with TST buffer (0.01 M Tris·HCl/0.15 M NaCl, pH 7.3/0.5% Triton X-100) containing 1 mM phenylmethylsulfonyl fluoride. After centrifugation, the cell lysate was stored at −100°C.

Antigen–antibody complexes were isolated as described in (29). The radiolabeled class I molecules were eluted either by boiling for 2 min in elution buffer (0.063 M Tris·HCl, pH 6.8/2% 2-mercaptoethanol/2% $NaDodSO_4$) (for HPLC) or by incubating at room temperature for 10 min with lysis buffer (9.5 M urea/2% Triton X-100/1.6% ampholines, pH 5–7/ 0.4% ampholines, pH 3.5–10/5% 2-mercaptoethanol) (for two-dimensional gels).

Two-Dimensional Gel Electrophoresis. The protocol used has been described (28, 30). Briefly, samples were loaded at the anode and subjected to isoelectric focusing for 6 hr at 500 V; 10% $NaDodSO_4$/polyacrylamide slab gels were used for the second dimension.

HPLC Tryptic Peptide Mapping. Class I polypeptides in elution buffer were separated on 10% polyacrylamide disc gels (31), eluted from gel slices using 0.01% $NaDodSO_4$, mixed with 0.25 mg of bovine immunoglobulin, and lyophilized. The protein was then reduced, alkylated, and digested with trypsin as described (28). The resulting tryptic peptides were analyzed on a Perkin-Elmer series 3B HPLC system using a Waters Z module system fitted with a reverse-phase Radial-PAK CN cartridge at room temperature and a 0.01 M sodium phosphate/0.04 M NaCl buffer, pH 2/acetonitrile solvent system. One-half-milliliter fractions were collected every 30 sec. The reproducibility of the maps, ascertained by monitoring the separation of immunoglobulin peptides at 210 nm, proved to be within 1 min (two fractions) for all the maps.

Cross-Blocking RIA. The procedure used is described in ref. 32. Spleen lymphocytes and tumor cells were lysed without radiolabeling. Monoclonal antibodies were affinity-purified using protein A-Sepharose 4B (Pharmacia) and eluted with buffer, 0.05 M glycine/0.15 M NaCl, pH 3.0. Antibodies were equilibrated with phosphate-buffered saline. Iodination was carried out using Iodo-Beads (Pierce) and Na^{125}I (ICN). Briefly, the wells of Immunolon Removawell strips (Dynatech, Alexandria, Va) were incubated overnight at 4°C with the first antibody. After removal of excess reagent, unlabeled cell lysate was incubated in the wells for 1 hr at room temperature. The wells were washed and then incubated for 1 hr at room temperature with an ^{125}I-labeled second antibody. Excess radiolabel was removed by washing and the radioactivity in the wells was determined. Each experiment was repeated three times.

RESULTS

Two-Dimensional Gel Electrophoresis. To characterize the target molecules on 1591 that CP28 and the L^d-specific antibodies, 30-5-7 and 28-14-8, recognize, we used these reagents in sequential immunoprecipitations of biosynthetically radiolabeled cell lysates and analyzed the resulting immune complexes by two-dimensional gel electrophoresis. This technique has proved to be very powerful in the analysis of class I gene products. It appears that each transplantation antigen gives a complex but distinctive two-dimensional gel pattern which is virtually characteristic of haplotype and locus (33, 34). We have shown that even molecules encoded by genes transfected into L cells yield patterns almost identical to proteins encoded by germ line genes (35, 36). Fluorographs of the two-dimensional gels are shown in Fig. 1. Clearly, two distinct molecules can be isolated from 1591 by using CP28 and 30-5-7. Lysates from which one molecule has been removed by immunoprecipitation—e.g., CP28—can be used to isolate the second 30-5-7-specific molecule and vice versa. These two molecules are not expressed on the AS7 tumor variant or on normal C3H spleen cells.

We wished to determine whether the 1591 and AS7 lines also express bona fide K^k and D^k molecules as does the C3H

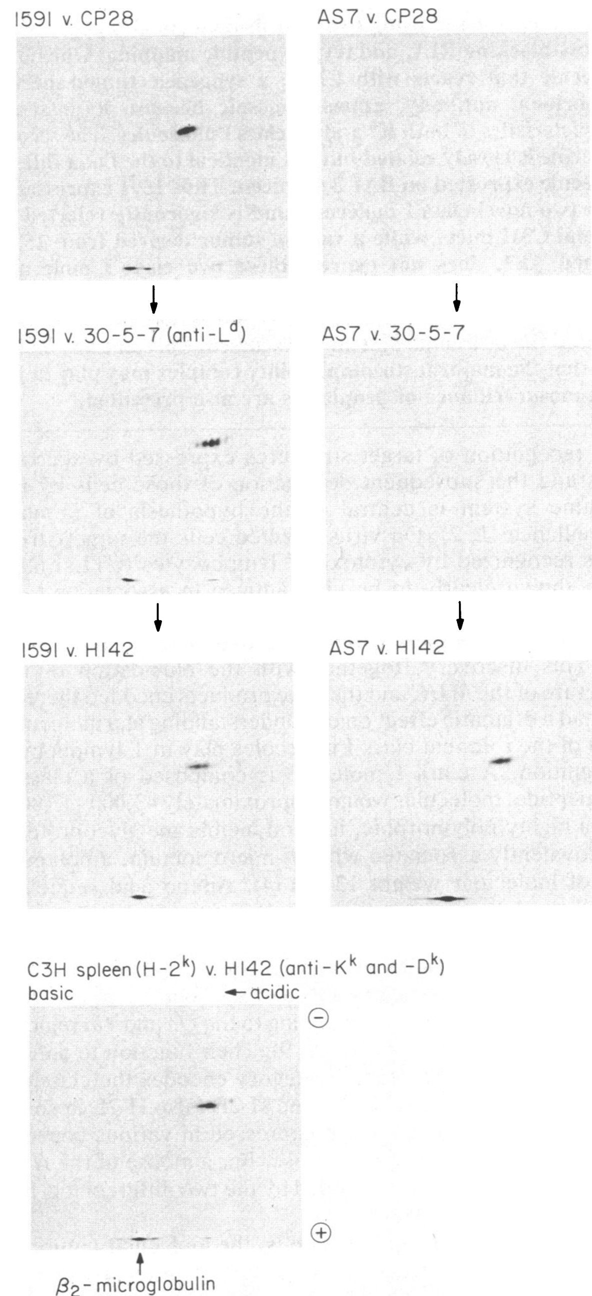

FIG. 1. Fluorographs of two-dimensional gels of immunoprecipitates from tumors 1591 and AS7 and from C3H spleen cell lysates. Target versus (v.) monoclonal antibody is indicated above each fluorograph. Arrows indicate the sequence of immunoprecipitations.

mouse strain from which the tumor was derived. Fig. 1 shows two-dimensional gels using a K^k/D^k-specific monoclonal antibody (H142) on cell lysates from which the tumor-associated antigens had previously been removed. The patterns obtained from both cell lines are virtually identical to those obtained from the radiolabeled C3H spleen cells. We conclude that K^k and D^k class I molecules are indeed expressed on both 1591 and AS7.

Fig. 2 shows partial fluorographs of two-dimensional gels contrasting the CP28-tumor specific molecule to the D^d molecule with which CP28 has been shown to cross-react. They are clearly different. Fig. 2*C* shows that the 30-5-7-reactive tumor molecule is also different from the bona fide L^d molecule expressed on BALB/c spleen. We also immunoprecipitated 1591 cell lysate using a second L^d-specific monoclonal antibody, 28-14-8, and obtained a virtually identical pattern, suggesting that these two antibodies are recognizing the same molecule. We conclude from these two-dimensional gel analyses that the 1591 tumor expresses two novel class I molecules that are not expressed on the variant, AS7, in addition to the normal $H-2^k$ class I molecules expressed by C3H spleen cells.

Cross-Blocking RIA. Because the CP28-specific tumor molecule has a very similar if not identical isoelectric point to K^k, we wished to determine whether it bears any other characteristic of the endogenous class I $H-2^k$ molecules. This is a difficult question to address because we have shown that the tumor expresses bona fide K^k and D^k molecules.

A cross-blocking RIA has been used to show that the K^k class I molecule possesses at least two epitopes that are recognized by two different categories of monoclonal antibody (clusters A and B). A cluster A-specific antibody, on binding to a K^k molecule, blocks the binding of a second cluster A-specific antibody because they recognize identical or almost identical epitopes. A cluster A antibody does not block the binding of a cluster B-specific antibody, which recognizes a different epitope (37).

In our experiments, we coated plastic RIA wells with CP28 and then incubated them with 1591 cell lysate. Excess lysate was washed off, leaving CP28-reactive molecules bound to the plastic. ^{125}I-labeled $H-2^k$-specific antibodies known to

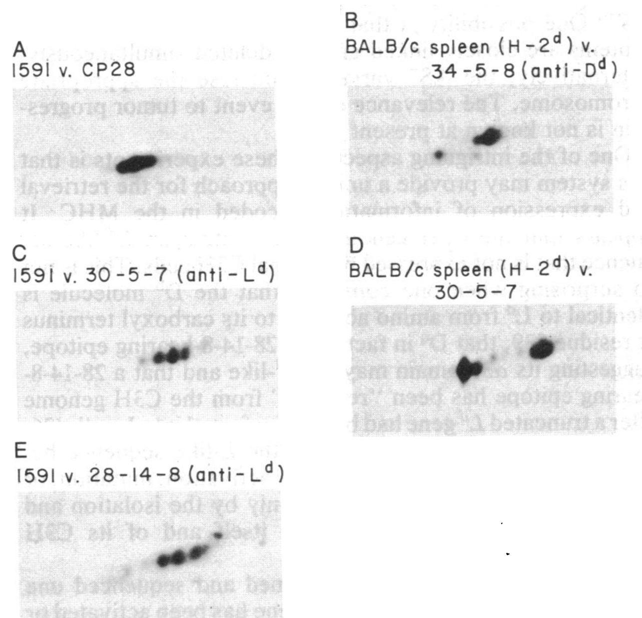

FIG. 2. Partial fluorographs of two-dimensional gels of class I molecules of M_r 36,000–60,000 and pI 4.5–6. Target versus (v.) monoclonal antibody is indicated above each photograph. Actin and β_2-microglobin were used as markers to align the fluorographs before cutting. The most acidic spots are to the right.

react with cluster A (H100) and cluster B (H142) were then added to the wells and, after 1 hr, unbound radioactive antibodies were removed by washing. Since we have shown by immunoprecipitation that H100 does not react with the CP28-specific molecule and therefore does not cross-block the CP28 antibody (data not shown), the results in Table 1 demonstrate that the CP28-specific molecule possesses cluster B but not cluster A epitopes. In a reciprocal fashion, some molecules expressing cluster B epitopes also express CP28-specific epitopes. The positive controls show, as would be expected, that endogenous $H-2^k$ molecules have both cluster A and cluster B on the same molecule. We conclude, therefore, that the CP28-reactive molecule on tumor 1591 possesses the cluster B epitope characteristic of $H-2^k$ class I molecules.

HPLC Tryptic Peptide Mapping. To determine the relationships of the CP28-reactive molecule to K^k and D^k and of the 30-5-7-reactive tumor molecule to the L^d molecule expressed on BALB/c spleen, we used HPLC tryptic peptide mapping analysis. This technique is particularly suited to comparing closely related molecules because it exaggerates the differences between homologous molecules (28). We chose to use [^{3}H]phenylalanine because it is easily incorporated into cells, because phenylalanine is distributed in all three external domains of the L^d molecule (38), and because we did not wish to obtain too many radiolabeled peptides in case small differences in peptides were obscured by the overlapping of too many peptides. However, small dissimilarities may go undetected if the structural diversity resides only in unlabeled peptides.

Tryptic peptide maps of the 30-5-7-reactive tumor molecule are compared with that of the bona fide L^d molecule in Fig. 3 *Right*. We were astonished to find that the four major peaks of radioactivity appeared identical and that only the peaks after fraction 130 differed in retention time. Three peptides were observed in each case. We interpret this difference in peptide elution to reflect a subtle change in protein structure because there are no phenylalanine-containing peptides in L^d molecules that have carbohydrate-asparagine linkages and because the molecular weights of these two molecules are approximately the same, suggesting that there are no extra carbohydrate attachment sites.

The CP28-reactive molecule has an isoelectric point virtually identical to that of K^k molecules. Therefore we compared its peptide map to that of the K^k and D^k molecules expressed by 1591 and by C3H spleen cells. To do this we cleared the 30-5-7- and CP28-specific molecules from the cell lysate and then immunoprecipitated with the H100 monoclonal antibody (anti-K^k). The resulting lysate was then treated with H142, a D^k/K^k-specific monoclonal antibody. C3H spleen cell lysate was also treated with H100 and then with H142. The precipitates were then subjected to our HPLC peptide mapping protocol. The results are shown in Fig. 3.

The CP28 tumor molecule appears to share at least five peptides with D^k molecules (H142) whereas it shares only two

Table 1. Cross-blocking RIA demonstrating that the CP28-reactive molecule expressed on tumor 1591 possesses an epitope recognized by monoclonal antibody H142

	^{125}I cpm of labeled mAb bound				
	Unlabeled 1591 lysate			Unlabeled C3H spleen lysate	
Unlabeled mAb bound	CP28	H142	H100	H142	H100
CP28	3843	8,320	1,834	ND	ND
H142	9148	1,643	49,746	1431	10,446
H100	4385	26,304	1,549	6193	772

mAb, monoclonal antibody; ND, not determined.

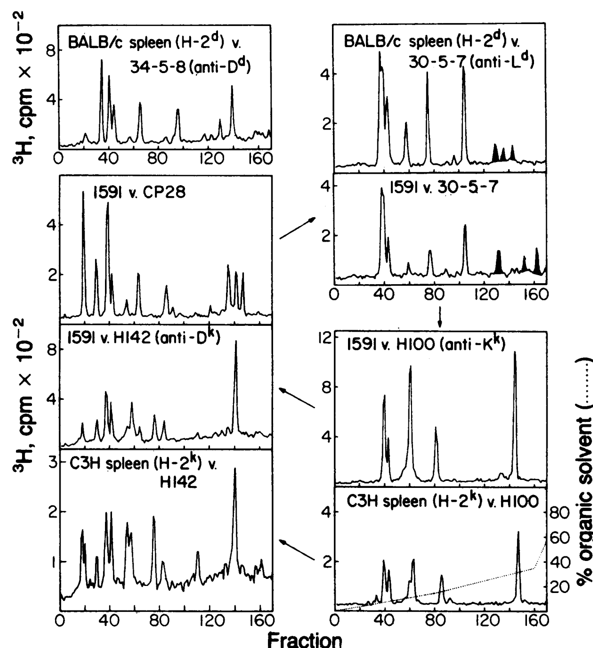

FIG. 3. HPLC tryptic peptide maps of [^{3}H]phenylalanine-labeled peptides of class I polypeptides. Target versus (v.) monoclonal antibody is indicated at the top of each map. Arrows indicate the sequence of immunoprecipitations. Black peaks represent peptides unique to a particular molecule.

peptides (at fractions 38–45) with K^k molecules (H100). The most striking difference between the CP28-specific molecule and D^k is that the peptide at fraction 140 in D^k is replaced by three peptides in the tumor molecule. In addition, the peptide at fraction 76 in D^k is replaced by a peptide at fraction 64 in the tumor molecule and the peptide at fraction 20 is more intense in the tumor than in D^k. We conclude that although the D^k molecule and the CP28-specific molecule have peptides in common they differ from each other more than do the 30-5-7-reactive molecules; also in this analysis the CP28-specific molecule more closely resembles D^k than it resembles K^k.

Comparison of D^k molecules from 1591 and spleen also shows minor differences. We believe that the 1591 vs. H142 map contains trace amounts of the CP28 molecule (it possesses a cluster B epitope); the peptide at fraction 110 in the spleen may represent a partial cleavage since it is present in small amounts in the 1591 vs. H142 map. Comparison of K^k molecules (H100) from 1591 and C3H spleen shows they are virtually identical.

Because the CP28 antibody has been shown to cross-react with D^d molecules we also compared the map of the CP28-reactive molecule with that of the D^d molecule from spleen (24). They appear to have peptides in common at fractions 64–66 and 139–141 plus a peptide cluster at 40 that is present in all the maps. However, to conclude these peptides are truly identical would require further evidence.

From our peptide mapping data we conclude that the 30-5-7-reactive molecule is closely related to the L^d molecule while the CP28 reactive molecule appears to bear more resemblance to D^k molecules than to K^k ones.

DISCUSSION

Our biochemical analyses unequivocally show that the UV-induced fibrosarcoma 1591 expresses a minimum of two novel class I molecules. One, though closely related to L^d, is clearly different from it while the other appears mosaic and possesses some properties of both K^k and D^k molecules. A trivial explanation of these data would be genetic contamination resulting from uncontrolled outcrossing of breeding stocks. However, we believe this is not correct for the following reasons: (*i*) electrophoretic analyses of seven isoenzymes located on six different chromosomes are completely consistent with C3H; (*ii*) Southern blot hybridization analysis of *Bam*HI-digested fragments from 1591 shows a normal pattern when compared with C3H spleen DNA; (*iii*) 1591 expresses normal K^k and D^k class I molecules expected of a C3H-derived tissue; and (*iv*) the tumor was raised in the well-controlled, germ-free, specific pathogen-free colony at the National Cancer Institute Frederick Cancer Research Center. We therefore believe our findings are not due to genetic contamination, although a subtle germ line mutation in the original tumor-bearing mouse cannot be excluded with absolute certainty.

The two molecules that we have characterized on 1591 appear unique to this tumor in that they are not expressed on normal C3H spleen or on 37 other UV-induced tumors. We have shown that both of these molecules are not expressed on the variant, AS7, and therefore one or both of these molecules appear to comprise the A^+ epitope defined by CTL clones. Our findings shed no light on the molecular nature of the $B^+C^+D^+$ epitopes. We have looked for further class I molecules by using a rabbit anti-β_2-microglobulin antiserum but could detect no further molecules. We conclude that either the B^+, C^+, and D^+ epitopes are not β_2-microglobulin-associated class I molecules or that they are expressed in such small quantities that although they are detected by CTLs they cannot as yet be detected biochemically. A crucial question to address is whether these two novel class I molecules are in fact the target molecules for the CTLs. This can be answered unequivocally only by cloning the appropriate genes, transfecting them into L cells, and determining whether the transfectants are indeed targets for the CTLs.

Other questions concerning the genetics of this system remain to be answered. (*i*) Are these novel class I molecules encoded by the *H-2* complex on chromosome 17 as would be expected (1591 cells are tetraploid so there is sufficient DNA to ensure the presence of normal copies of K^k and D^k genes) or has a translocation taken place? (*ii*) By what mechanism is the expression of these two novel class I molecules lost on AS7? One possibility is that the two genes encoding these proteins are closely linked and are deleted simultaneously. Alternatively, the AS7 variant could lose the appropriate chromosome. The relevance of this event to tumor progression is not known at present.

One of the intriguing aspects of these experiments is that this system may provide a unique approach for the retrieval and expression of information encoded in the MHC. It appears that the C3H genome may contain an L^d-like sequence that is not expressed by normal C3H cells. This is not so surprising when one considers that the D^b molecule is identical to L^d from amino acid 158 to its carboxyl terminus at residue 339, that D^q in fact has a 28-14-8-bearing epitope, suggesting its $\alpha3$ domain may be L^d-like and that a 28-14-8-bearing epitope has been "rescued" from the C3H genome after a truncated L^d gene had been transfected into L cells (26, 39, 40). The mechanism by which the *L*-like sequence becomes expressed in 1591, whether by activation, mutation, or recombination can be determined only by the isolation and characterization of the 1591 gene itself and of its C3H homologue.

Until these genes have been cloned and sequenced one cannot determine whether a silent gene has been activated or whether UV irradiation, a potent mutagen, has caused the creation of some hybrid or mosaic molecule. One is reminded that a B10.D2 mouse treated with diethyl sulfate produced a molecule having characteristics of both D^d and L^d; the "cross-over" event is in $\alpha2$ (41, 42). For the *CP28* gene,

information encoded by two loci, *H-2K* and *H-2D*, appears to be involved, but there is evidence that *H-2K*-like information can in fact be located in the *H-2D* and *Qa* regions. Flavell and co-workers (43) have shown that a DNA sequence that changes the K^b gene product into a mutant molecule K^{bm1} appears to be encoded by a gene located in the *Qa* region. A second observation also provides evidence that epitopes previously thought to be unique to *H-2K* may in fact also be found on molecules encoded in the *H-2D* or *Qa* regions. The monoclonal antibody 20-8-4 has been shown to react not only with K^d but also with a molecule CR encoded to the right of the *H-2D* locus (44). Undoubtedly the isolation and characterization of the genes encoding these novel class I molecules will shed light on the genetic events leading to their expression.

In the case of 1591 we have shown that tumor regression correlates with the presence of at least one of these novel class I molecules while variant progression correlates with a lack of expression of this unique phenotype. Thus, the expression of novel class I molecules by tumor cells may play an important part in the rejection of a tumor particularly since UV-induced fibrosarcomas are exceedingly immunogenic. Analyses of other tumors will define the generality of this hypothesis. Such studies will also demonstrate whether UV irradiation can be used as a general agent to create novel class I molecules.

These results raise the intriguing question of whether novel class I molecules mediate the rejection or progression of other types of tumors and thereby play a general role as target structures for the recognition and destruction of neoplasms. If immunosurveillance of tumors indeed operates by such a mechanism then the creation and expression of these novel class I molecules on cancer cells represents a new and important role for the MHC in the functioning of the immune response.

We wish to express our gratitude to Dr. H. Schreiber for the gifts of the CP28 monoclonal antibody and the 1591 and AS7 tumor cells. We thank Dr. R. S. Goodenow for his enthusiastic support and for communicating his data to us prior to publication. We also acknowledge the helpful advice of Dr. B. Malissen on the cross-blocking RIA technique and Mr. K. Choy for his work on HPLC gradients. This work was supported by Grants R01 AI 19297 and 2T32 NS 07149 from the National Institutes of Health.

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