TUMOR ANTIGENS DEFINED BY CLONED IMMUNOLOGICAL PROBES ARE HIGHLY POLYMORPHIC AND ARE NOT DETECTED ON AUTOLOGOUS NORMAL CELLS

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One of the most important but most difficult goals of cancer biology has been to demonstrate the existence of tumor-specific antigens. The first strong evidence for their existence was obtained about 30 years ago from transplantation studies using chemically induced fibrosarcomas of mice (1–5). But despite decades of extensive research, there is still no convincing molecular evidence for the existence of tumor-specific antigens, i.e., for molecules that are recognized by the immune system and encoded by tumor-specific mutant genes not present in nonmalignant cells. The identification of these antigens has been hampered by the lack of control cells from the host from which the tumor was obtained and by the lack of cloned probes specific for the putative tumor antigens. The fact that cloned immunological probes have been generated for only a few tumors suggests the possibility that the particular tumors may have arisen in mice not fully syngeneic with mice from which nonmalignant control cells were obtained, i.e., the assumed tumor antigens (6–8) may have been alloantigens (9).

In the present paper we have, therefore, derived UV light–induced and spontaneous mouse tumors, and we have isolated from each original tumor-bearing animal nonmalignant cells and DNA. We were successful in consistently generating cloned immunological probes to a large number of these tumors. These probes did not react with normal autologous control cells, but showed exquisite specificity for the tumor used for immunization. These tumors, immunological probes, and control materials will, therefore, allow a reliable search for tumor-specific mutant genes encoding tumor-specific antigens.

Materials and Methods

Mice. 5–10-wk-old germ free–derived, pathogen-free female C3H/HeN mammary tumor virus–negative (MTV⁻),[1] BALB/cAnN or nude NCR/Nu mice were purchased from the Na-

tional Cancer Institute, Frederick Cancer Research Facility Animal Production (Frederick, MD), and were maintained in laminar flow units as described (10). The original stock of nude C3H mice was in its 23rd backcross generation when obtained from a colony at the Biology Division of the Oak Ridge National Laboratory, Oak Ridge, TN.

Derivation of Tumor Bank. The general method for induction of tumors in mice with UV light has been described (11). Briefly, the backs of 4–5-mo-old mice were shaved weekly and exposed three times per week to a source of UV light consisting of a bank of six Westinghouse FS40 sunlamps mounted in parallel 10.5 cm apart and 20 cm above the bottoms of the animal cages (see footnote to Table I). A colony of >2-yr-old C3H/HeN (MTV⁻) mice was maintained for the appearance of spontaneous tumors. Induced or spontaneous tumors were excised from the original host under sterile conditions and fragments were (*a*) placed in vitro for establishing a tumor cell line, (*b*) transplanted into syngeneic nude and normal mice to determine growth behavior in vivo, and (*c*) stored in liquid nitrogen for reference and for obtaining DNA from the original tumor. Cells and DNA from nonmalignant tissue were isolated from each tumor-bearing host.

Tumor cells were adapted to growth in vitro by adding finely minced tumor fragments to culture flasks containing MEM (410-1100; Gibco Laboratories, Grand Island, NY) supplemented with 10% FCS (29-161-54; Flow Laboratories Inc., McLean, VA), 1% penicillin/streptomycin (600-5140; Gibco Laboratories), 1% gentamycin (600-5750AD; Gibco Laboratories), and 1% nystatin (600-5340; Gibco Laboratories). Fibroblasts from the heart and lungs of each tumor-bearing mouse were established. Briefly, the heart and lungs were removed en bloc, minced with scissors and placed in MEM supplemented as outlined above. Flasks with adapted tumor cells or normal fibroblasts were usually subconfluent with cells within 1–2 wk of culture, and cells were then frozen in liquid nitrogen to allow later reference to the first culture of the original tumor or normal tissue. DNA was extracted from single cell suspensions of normal spleen and kidney tissues from each host; cells prepared with a glass tissue grinder were lysed with 2% SDS in 0.4 M EDTA and DNA was extracted using standard procedures (12). For use in experiments, aliquots of the original tumor cell or fibroblast batch were expanded in vitro for ∼2 wk and cryopreserved in aliquots. Whenever tumor cells were required for experiments, aliquots were thawed and used 24–48 h later without further passage.

Tumor Transplantation. For tumor challenge an equal number (3–6) of 1-mm³ tumor fragments were implanted subcutaneously with a trocar into one inguinal region of nude and normal mice. The nude mice were always injected last to verify that the tumor tissue remained viable until the end of the transplantation procedure. When transplanted into young syngeneic mice, strongly immunogenic tumors grow during the first 10 d and then regress. These tumors can grow, however, in nude or immunosuppressed mice (13) and eventually kill these animals by infiltrative growth without macroscopic evidence of distant metastases.

Generation of CTL. For immunization with UV-induced regressor tumors, mice were immunized either subcutaneously with tumor fragments, or intraperitoneally with 10⁷ tissue culture cells. For immunization against poorly immunogenic spontaneous tumors, mice were immunized intraperitoneally two to four times with 10⁷ irradiated (10,000 rad) tumor cells at 2-wk intervals. Extensive preliminary experiments showed that the following procedure and culture conditions, modified from a previously described method (10), were most consistently successful for generating CTL to the various tumors used. 10 d after immunization, spleens were removed from immune animals and 8×10^6 immune spleen cells were restimulated in 6-d mixed lymphocyte tumor cell cultures (MLTC) with 4×10^4 mitomycin C–treated tumor cells in 3 ml of RPMI 1640 (430-1800; Gibco Laboratories), supplemented with 10% FCS, 1% penicillin/streptomycin, and 5×10^{-5} M 2-ME (CRPMI). Cultures were prepared in upright 16×125 mm round-bottomed tissue culture tubes (3033; Falcon Labware, Becton Dickinson & Co., Lincoln Park, NJ), which were found to be superior to upright culture flasks. We select lots of FCS that do not induce lymphocytes with promiscuous cytolytic activity, but do support the growth of specific T cells; at least four different lots of FCS have worked in the past (selected from ∼20 lots tested). Long-term CTL lines and clones were maintained by passage in V-bottomed 96-well microtiter plates (76-223-05; Linbro, McLean, VA) at 37°C and 5% CO₂ in a fully humidified incubator using a modification

of a previously described method (14). T cells from several wells were pooled and counted. Irradiated (2,000 rad) syngeneic fillers were added at a 1:100 ratio of T cells to filler cells. The mixture was washed, and mitomycin C–treated tumor cells were added as stimulators at a 10:1 responder-to-stimulator ratio in CRPMI supplemented with 33% (vol/vol) supernatant from a secondary MLC as a source of T cell growth factor (15, 16). The cell suspension was then dispensed in 0.2-ml aliquots per well containing 10^4 T cells, 10^3 stimulators, and 10^6 filler cells. Specific CTL were cloned by limiting dilution in flat-bottomed microculture wells (76-003-05; Linbro) and aliquots were stored in liquid nitrogen as a permanent reference. Briefly, aliquots of 10^6 CTL in CRPMI containing 15–20% FCS and 10% DMSO were placed on ice for 30 min before transfer to a −80°C freezer overnight and long-term storage in liquid nitrogen. Frozen cells were recovered by culturing cells at an increased density (2 × 10^4 CTL/well) and then passaged as outlined above.

^{51}Chromium-release Assay. Cytotoxicity of CTL was determined in a 4–6 h ^{51}chromium-release assay as previously described (17). The percentage of specific lysis was calculated by the formula: percent specific lysis = 100 × [(experimental release − spontaneous release)/(total release − spontaneous release)]. Spontaneous release was always 25% of the total maximum release and usually <15%. For some experiments, 30 U/ml of murine rIFN-γ (lot 4407-47; 1.97 × 10^7 U/mg; Genentech, South San Francisco, CA) was added to cultured cells 48 h before their use as targets in a ^{51}chromium-release assay. We have found that this dose of IFN-γ increases the level of expression of MHC class I antigens in tumors as determined by FACS analysis.

Generation of mAbs. Syngeneic mAbs were generated by fusing SP2/0 mouse myeloma cells (18) with spleen and lymph node cells from mice immunized with 10^7 irradiated (10,000 rad) AG-104A tumor cells intraperitoneally, followed 1 mo later by a subsequent immunization of 2 × 10^6 tumor cells intravenously. The fusion was done 3 d after the intravenous boost. Supernatants of growing hybrids were screened by FACS IVB analysis (Becton Dickinson & Co., Mountain View, CA) for reactivity with the immunizing tumor (AG-104A) and control tumors. Tumor cells were first incubated with supernatants from growing hybrids followed by incubation with fluorescein-coupled goat anti–mouse Ig. 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. Hybridomas secreting specific mAb were cloned in soft agar (19), expanded in tissue culture, and stored in liquid nitrogen.

Selection of Antigen Loss Variants In Vitro. Tumor cells (2–3 × 10^6) in 20 ml CMEM were added to 75-cm^2 tissue culture flasks that were incubated at 37°C for 3 h to allow cells to attach. CTL were added to the flask at an E/T ratio of 1:1. 24 h later, medium was aspirated from the flask to remove most of the CTL and killed tumor cells, and was replaced with fresh CMEM. After 2–3 wk, the flask was ∼50-75% confluent and was retreated with CTL. Three to four selections were necessary to ensure stability of the variant phenotype. Variant cell lines were then cloned, expanded, and cryopreserved.

Results

Transplantation Characteristics of UV-induced and Spontaneous Tumors. During the years of 1983 to 1987, five series of UV-induced or spontaneous tumors were isolated as outlined in Materials and Methods. We first determined whether these newly isolated tumors were as immunogenic as UV-induced tumors that have been previously described (20). Indeed, we observed that a significant percentage of UV-induced tumors in C3H mice (44–78%), and BALB/c mice (19%) were rejected by normal mice but grew in nude mice (Table I). These results are consistent with the previous observation that primary UV-induced tumors of C3H/HeN (MTV⁻) mice exhibited a greater degree of antigenicity or a greater percentage of regressor tumors than those of BALB/cAnN mice (11). To establish whether these transplantation characteristics were a heritably stable trait of the isolated tumors upon subsequent trans-

TABLE I

Transplantation Characteristics of 121 UV-induced and 7 Spontaneous Tumors
that Have Been Isolated with Autologous Normal Control Cells

Mode of tumor induction*	Designation (series)	Strain	Year of experiment	Growth behavior in normal mice[‡]		
				Regressor phenotype		Progressor phenotype
				Stable (%)	Unstable (%)	
Ultraviolet light	UV-(4000)	C3H/HeN	(1983-84)	4 (45)	3 (33)	2 (22)
	UV-(5000)	BALB/cAn	(1984-85)	5 (19)	0 (0)	22 (81)
	UV-(5000)	C3H/HeN	(1984-85)	0 (0)	4 (44)	5 (56)
	UV-(6000)	C3H/HeN	(1986-87)	31 (41)	19 (25)	26 (34)
Unknown (spontaneous aging)	AG-(100)	C3H/HeN	(1983-86)	0 (0)	0 (0)	7 (100)
Total				40	26	62

* For induction of tumors of the 4000 and 5000 series, mice were irradiated for 1 h three times per week on a Monday, Wednesday, Friday schedule until a tumor of a sufficient size ($\sim$10 mm diameter) developed. For tumors of the 6000 series, mice were irradiated 1 h three times per week for 15 wk and then 30 min three times per week for 6 wk. All mice developed tumors between 20 and 40 wk after start of UV irradiation. A total of 121 UV-induced tumors were isolated from 93 mice (28 mice had 2 to 3 tumors) and 7 spontaneous tumors were isolated from 6 mice (1 mouse had 2 tumors).

‡ Three 1-mm^3 fragments of each original primary tumor were implanted subcutaneously into one nude and three normal mice (first transplantation, see Materials and Methods). Tumors were reisolated from the nude mice and subsequently transplanted into a second nude mouse and three additional normal mice (second transplantation, also see Fig. 1).

plantation, we reisolated the tumors of the first transplant generation from the nude mice and transplanted them again into normal syngeneic and nude mice (Fig. 1 and Table I). At this second transplantation, 35 tumors were rejected by all of the normal mice, and 5 additional tumors were rejected by 2 of the 3 normal mice challenged. These 40 tumors were defined as "stable regressors." "Unstable regressors" were defined as tumors that grew in the majority of mice at the second transplant generation when transplanted tissue from the nude rather than the original tumor was used. Testing of 31 of the "stable regressors" in 1–4 subsequent transplantation experiments showed that 27 of these tumors continued to be rejected by the majority of normal mice. 17 of the "unstable regressors" that had showed progressive growth behavior in the second transplantation maintained this phenotype when similarly tested in subsequent transplantation experiments. In some instances, it was attempted to break the resistance of normal mice to challenge with these regressor tumors by injecting increasing numbers of tumor fragments. However, even the largest numbers of tumor fragments, e.g., >20 1-mm^3 fragments transplanted subcutaneously were rejected by normal mice. Thus, as far as we could test, regressor tumors that had a stable regressor phenotype seemed to be as immunogenic as the UV-induced tumor 1591-RE (21) we had previously studied (10). In fact, upon several occasions we have tried to derive in vivo progressor variants from these regressor tumors, and found that these variants were as infrequent as we had previously observed in the UV-induced tumor 1591 (10). Progressor tumors usually grow in two-thirds or more of normal mice. All of the spontaneous tumors grew in normal mice in the first transplant generation consistent with the previously reported lower immunogenicity of spontaneous tumors (2, 3). For example AG-104A, one of the tumors we tested extensively, grows progressively at a dose as low as 10^4 cells. Multiple tumors (up to
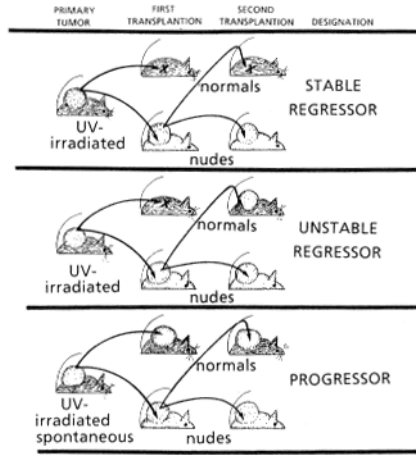
FIGURE 1. Types of growth behavior of UV-induced or spontaneous tumors when transplanted into normal mice and T cell-deficient nude mice (also see Table I).

3) were observed in several (28/93) of the UV-irradiated mice and in one of the mice developing spontaneous tumors as has been observed previously in UV-irradiated (12) and aging normal MTV⁻C3H mice (22). However, with regard to immunogenicity, these tumors showed the same distribution pattern as that observed in mice developing single tumors. Similarly, there was no apparent correlation between histologic type and immunogenicity of the tumors, even though some tumors appeared to be squamous cell carcinomas, while others had the appearance of undifferentiated spindle cell tumors. The latter histologic type appears to be a common final pattern of a variety of tumors often inappropriately referred to by experimental pathologists as fibrosarcomas irrespective of whether the tumors were of mesenchymal or epithelial origin. Recent histochemical studies suggested that >95% of UV-induced murine tumors of the C3H strain are squamous cell carcinomas (23), but we have not yet analyzed our recently induced tumors with keratin-specific probes.

Individual Tumor Specificity of CTL-defined Antigens on UV-induced Regressor Tumors. The UV light–induced regressor tumors were used as immunogens to generate cytolytic T cell clones. Fig. 2 indicates that each of three tumors tested elicited CTL that killed only cells used for immunization but not cells of the other tumors. The other tumors could be used, however, to elicit their own specific CTL. Since the degree of diversity of CTL-defined antigens had not been rigorously analyzed in the past, we determined next how commonly the same CTL-defined epitope is expressed by tumors of independent origin. Fig. 3 shows that a CTL clone raised against the C3H-
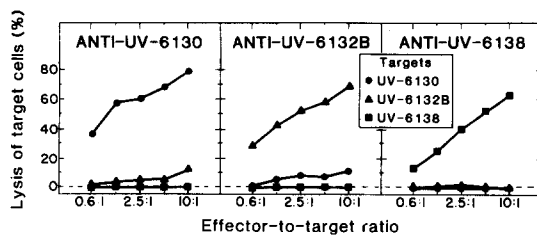

FIGURE 2. Specificity of CTL clones generated in reciprocal experiments to three different UV-induced tumors. Each of these C3H tumors elicits CTL that are specific for the tumor used for immunization and do not lyse cells of the other two tumors in a 4.5-h ⁵¹Cr-release assay.
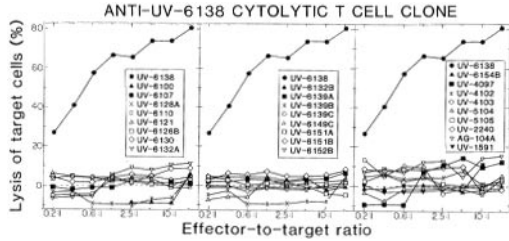
FIGURE 3. Uniqueness of a CTL-defined antigen on the UV-induced tumor 6138. None of 25 other syngeneic C3H tumor cell lines are lysed by this CTL clone in a 4.5-h ^{51}Cr-release assay.

derived UV-6138 regressor tumor killed UV-6138 tumor cells but not cells of any of the other 25 tumors tested. Similar results were obtained using a CTL clone specific for the BALB/cAnN-derived UV-induced regressor tumor UV-5117 (Fig. 4 *a*) and using another CTL clone specific for the C3H-derived UV-induced regressor tumor UV-6130 (Fig. 4 *b*). In several instances, two or more tumors arose in UV-irradiated animals. This allowed us to test whether tumors originating in the same animal displayed different antigenic specificities. Fig. 5, *a* and *b*, shows that anti-UV-6132A CTL did not kill UV-6132B tumor cells while in the reciprocal experiment anti-UV-6132B CTL did not kill UV-6132A tumor cells. Furthermore, anti-UV-6139B CTL (Fig. 5 *c*) did not kill either of the other two tumors that arose in the same mouse.
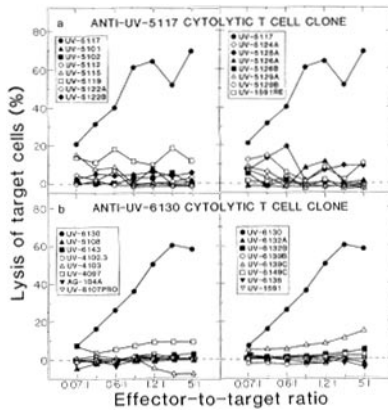

FIGURE 4. Generality of the finding that unique tumor antigens are expressed in UV-induced tumors. (*a*) Unique antigens are also detected in a UV-induced tumor of another strain, BALB/cAnN; (*b*) a second C3H tumor tested expresses such antigens in addition to the one analyzed in Fig. 3. Targets were tested in a 4.5-h ^{51}Cr-release assay.
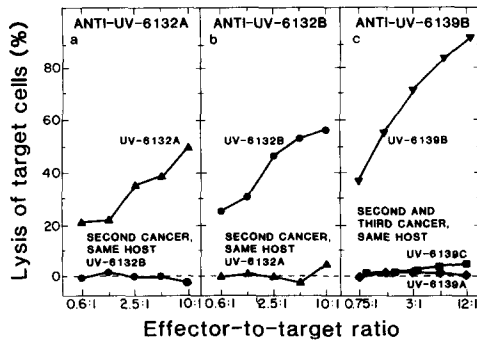

FIGURE 5. Multiple tumors isolated from a single host have individually distinct CTL-defined antigens. All five tumors are poorly differentiated spindle cell tumors of very similar histologic appearance. (*a*, *b*) Tumors UV-6132A and UV-6132B were isolated from one individual mouse. (*c*) Tumors UV-6139A,B and C were isolated from one individual mouse. Targets were tested in a 4.5-h ^{51}Cr-release assay.

All of these five tumors are poorly differentiated spindle cell tumors of very similar histologic appearance.

CTL Clones Reactive with Unique Tumor Antigens Do Not Lyse Nonmalignant Autologous Control Cells. The above experiments showing that multiple tumors arising in the same animal have individual antigenic specificities are consistent with previous transplantation assays (24) that have been taken to suggest that the unique tumor antigens could not be due to genetic impurity in the mouse strain. However, because of the genetic instability of cancer cells (25), genes encoding an alien antigenic specificity (8) could be lost during continuous tumor growth, particularly when tumors are serially transplanted in immunocompetent mice (10, 11, 26). Therefore, we also tested whether CTL clones with unique tumor specificity would kill nonmalignant fibroblasts derived from the same mouse that gave rise to the tumor. Fig. 6 (*a*, *b*, *d*, *e*, *f*, *h*) shows that none of six CTL clones killed autologous normal fibroblasts. Cytolytic effector T cells generated in MLTCs against two other tumors (Fig. 6, *c*, *g*) also did not lyse autologous normal fibroblasts. The resistance of the normal fibroblasts was not reversed by pretreatment of the target cells with IFN-γ (Fig. 6 *h* and Table II) at doses that increased the level of MHC class I expression at least fivefold (data not shown). However, all of eight autologous fibroblast lines tested could clearly be lysed by an anti-H2^k alloreactive CTL line (Table II) indicating that the nonmalignant fibroblasts were not inherently resistant to the lytic mechanism by which CTL kill target cells. Four of the eight effector populations (Fig. 6) were analyzed for surface phenotype and were found to be CD8$^+$ (data not shown).

A Single Malignant Cell Can Express Multiple, Independent, Unique Tumor Antigens. Using the 1591 tumor model, we have previously found that a single malignant cell can express multiple, unique tumor antigens (27). However, the CTL clones used for this analysis may have detected alloantigens (such as minor histocompatibility antigens), since autologous normal control cells were not available for this tumor which was later found to express two MHC class I molecules whose α1 and α2 coding sequences are identical to those of another mouse strain (9). Therefore, we re-examined the possibility that multiple, independent, unique antigens can be expressed by a single malignant cell using a similar approach as was used previously for such antigenic dissection (27), but with a tumor for which autologous normal fibroblasts are available. Thus, the parental UV-6139B tumor was used to generate cytolytic T cells
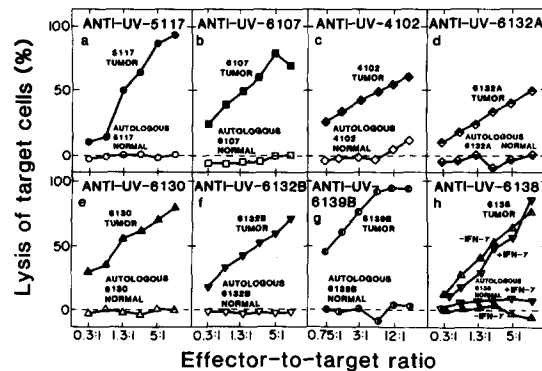

FIGURE 6. Absence of CTL-defined tumor antigens on autologous normal control cells. (*a–g*) Untreated targets. (*h*) Tumor and control cells were pretreated with 30 U/ml IFN-γ for 48 h before testing in a 4.5-h ^{51}Cr-release assay. (*c* and *g*) Effector cells are from bulk cultures generated in MLTCs (See Materials and Methods). In all other panels, effector cells are from cloned CTL lines.

TABLE II

Autologous Nonmalignant Fibroblasts Are Killed by Alloreactive CTL
but not by Tumor-specific CTL

Targets	IFN-γ*	E/T	Lysis of targets by	
			Tumor-specific CTL	Anti-H-2^{k†} CTL
			%	
6130	–	5:1	41	65
	–	2.5:1	33	63
6130 HLF	+	5:1	– 2	36
	+	2.5:1	– 2	26
	–	5:1	ND	35
	–	2.5:1	ND	23
6132A	–	5:1	39	22
	–	2.5:1	24	15
6132 HLF	+	5:1	– 2	44
	+	2.5:1	– 1	39
	–	5:1	ND	49
	–	2.5:1	ND	40
6138	–	5:1	43	28
	–	2.5:1	33	27
6138 HLF	+	5:1	0	31
	+	2.5:1	0	27
	–	5:1	ND	17
	–	2.5:1	ND	14
6139B	–	5:1	61	63
	–	2.5:1	63	60
6139 HLF	+	5:1	0	28
	+	2.5:1	1	21
	–	5:1	ND	8
	–	2.5:1	ND	7

* 30 U/ml of murine rIFN-γ (lot 4407-47, 1.97 × 10^7 U/mg; Genentech, South
San Francisco, CA) was added to heart-lung fibroblast (HLF) cells 48 h be-
fore their use as targets. We have found that this dose of IFN-γ raises the level
of expression of MHC class I antigens (at least fivefold) as determined by FACS
analysis.
$\dagger$ The anti-H-2^k CTL line of B10.D2 origin was generously provided by Dr.
Jeffrey Bluestone, Chicago, IL.

in an MLTC (Fig. 7 a) from which a CTL clone was isolated that was designated
anti-A (Fig. 7 b). This clone was used to select an antigen-loss variant resistant to
the anti-A CTL clone (Fig. 7 c) as described in Materials and Methods. This A⁻
antigen-loss variant was used to generate cytolytic T cells that killed the A⁻ variant
as well as the parental UV-6139B tumor cells (Fig. 7 d). A CTL clone with this reac-
tivity was designated anti-B (Fig. 7 e) and used to select a B⁻ variant from the pa-
rental tumor (Fig. 7 f). This B⁻ variant still retained the A antigen (and, therefore,
had an A⁺B⁻ phenotype) while the A⁻ variant was still killed by the anti-B CTL
and, therefore, had an A⁻B⁺ phenotype (Fig. 8). The CTL used in these experi-
ments had unique reactivity with the UV-6139B tumor and did not lyse autologous
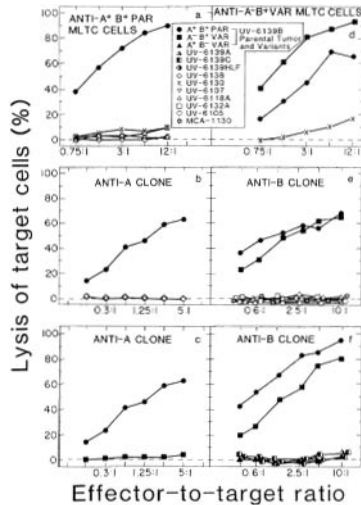
FIGURE 7. Generation of two different CTL clones reactive to the same tumor cell. A CTL clone (designated anti-A) against the parental UV-6139B tumor was generated (a, b) to select an A⁻ variant in vitro (c). This A⁻ variant was then used as an immunogen to generate an independent CTL clone (designated anti-B; d, e) for selection of a B⁻ variant from the parental UV-6139B tumor (f). All targets were tested in a 4.5-h ^{51}Cr release assay.

normal fibroblasts (Fig. 7). Together, these experiments clearly indicate that a single tumor can express multiple, independent, unique tumor antigens.

A Spontaneous Tumor also Expresses Unique Antigens not Detected on Autologous Controls. Because of the high doses of carcinogen used to induce cancer in experimental animals, unique tumor antigens could represent laboratory curiosities not observed when cancers are tested that arose without deliberate exposure to carcinogens, i.e., in tumors that are thought by many investigators to more closely resemble human cancer (28). Therefore, we tested whether CTL or antibody probes could detect on a spontaneous tumor unique tumor antigens that were absent from autologous normal control cells. The AG-104A tumor seemed to be particularly useful for such studies, as two tumors arose independently in the same animal, allowing additional tests for unique tumor specificity. As previously mentioned, this tumor like many other spontaneous tumors, is poorly immunogenic, and therefore, it required multiple immunizations to elicit tumor-specific CTL. These CTLs were clearly tumor specific in that they killed the AG-104A tumor, but not a second spontaneous tumor AG-104B that arose in the same host, nor autologous normal control cells (Fig. 9). Even
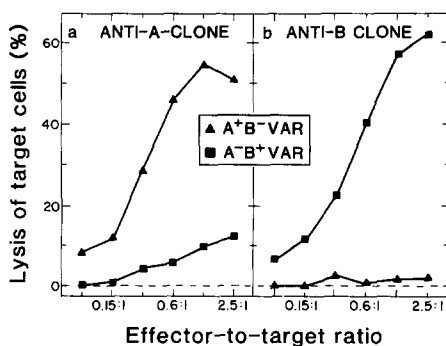

FIGURE 8. Demonstration of independence of two unique CTL-defined antigens expressed by a single tumor cell. The A⁻ and the B⁻ variants show a reciprocal sensitivity pattern to anti-A and anti-B CTL clones, indicating that the A⁻ variant had an A⁻B⁺ and the B⁻ variant an A⁺B⁻ phenotype. The specificity of these CTL was analyzed in Fig. 7. Targets were tested in a 4.5-h ^{51}Cr-release assay.
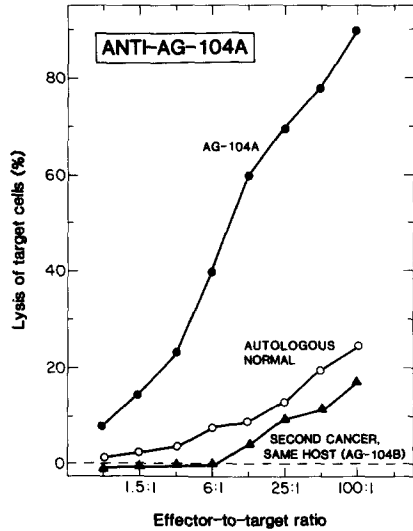
FIGURE 9. Unique tumor specificity of cytolytic T cells generated in an MLTC to the spontaneous tumor AG-104A. Neither a second tumor from the same host nor autologous fibroblasts are lysed in a 4.5-h ^{51}Cr-release assay.

though CTL with such specificity could be generated consistently in several independent experiments, we were unable to establish CTL clones specific for this tumor for reasons that are unclear. Interestingly, however, we did succeed in generating an AG-104A-reactive hybridoma after a single intraperitoneal injection of C3N/HeN mice with irradiated tumor cells and a single intravenous boost 3 d before fusion. This hybridoma, designated PW237, secreted an IgG2a mAb. Cytofluorometric analysis using hybridoma supernatant showed that the antibody was specific for the AG-104A tumor (Fig. 10). 25 other tumors that included UV-induced, MCA-induced,
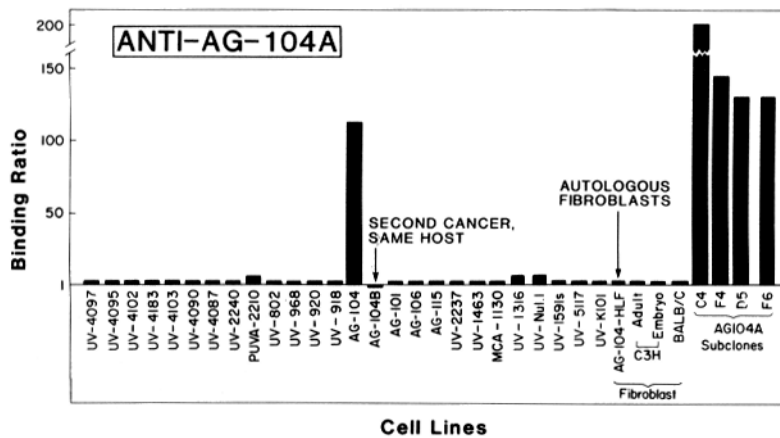

FIGURE 10. A mAb defines an antigen with unique tumor specificity on a murine tumor of spontaneous origin. The syngeneic mAb, PW237, did not react with a second spontaneous tumor or autologous fibroblasts isolated from the same mouse, or 24 other syngeneic tumors tested irrespective of whether they were UV-induced, MCA-induced, or of spontaneous origin (AG). All subclones of this tumor expressed high levels of the antigen recognized by this mAb.

or other spontaneous tumors including the one other spontaneous tumor that arose in the same host as AG-104A did not react with the PW237 antibody. In addition, autologous nonmalignant fibroblasts as well as other adult or embryonic fibroblasts of C3H origin or BALB/c origin were not recognized, while high levels of antigen expression were detected on all subclones derived from the AG-104A tumor. We are now in the process of analyzing the molecular nature of the PW 237-defined antigen and its relationship to the CTL-defined unique antigen(s) on the AG-104A tumor.

Discussion

In this study, we have generated cloned immunological probes that define individually distinct tumor antigens on UV-induced and spontaneous tumors. These probes did not react with nonmalignant fibroblasts derived from the same host. CTL clones and mAbs generated previously to putative unique tumor antigens (27, 29, 30) have lacked critical controls required to exclude that similar antigens were widely expressed on normal tissues of the autologous host. It might be considered trivial to require that normal control cells and DNA be obtained from the same host since the tumors were induced in inbred mice. Presumably cells from syngeneic, i.e., genetically identical hosts should be acceptable. While this is correct under idealized conditions, experimental practice has shown repeatedly that allogenicity can be introduced insidiously through errors that can occur during breeding (31).

Allogenicity did not influence the results of the early tumor transplantation studies of chemically induced tumors. These studies provided a firm basis for tumor immunology because (a) transplantation of autologous control tissues from the hosts of tumor origin did not protect mice against challenge with autologous cancer cells (3), and (b) immunity to tumors could be generated in the autologous host (4). Furthermore, allogenicity as a common source of such transplantation antigens can be excluded, because certain carcinogens induce strongly antigenic tumors at a very high frequency (11), while with proper breeding precautions introduction of allogenicity is a rare event. However, the generation of cloned immunological probes for the transplantation antigens of MCA-induced tumors has been unsuccessful and there are only rare instances in which transplantation antigens have been defined with specific in vitro probes in other tumor models (27, 29, 30). The difficulty in generating cloned immunological probes may, therefore, have selected for exceptional tumors. In this paper, however, we show that individually distinct tumor antigens can be defined with CTL probes in the majority of UV-induced regressor tumors.

Previous studies have suggested that a single cancer cell can display multiple, independent, unique tumor antigens (27, 29). Such antigens could be ideal targets for combination therapy because the possibility that a cancer cell will lose several independent antigens simultaneously is likely to be remote. However, allogenicity resulting from multiple, unlinked, contaminating genes may mimic such multiple unique tumor antigens. We, therefore, re-examined the possibility that a single cancer cell can express several unique, independent antigens using a tumor model for which autologous control cells were available. Indeed, we find that a single tumor cell can express at least two tumor antigens, both of which are uniquely expressed on the particular tumor tested but lost independently of each other upon immune selection.

In agreement with previous studies using transplantation assays (32, 33), our cloned in vitro probes demonstrate a very large diversity of unique tumor antigens on ex-

perimentally induced and spontaneous tumors. Our present experiments do not determine conclusively whether the CTL-defined antigens are responsible for transplantation resistance and whether CTL are essential for tumor rejection. Nevertheless, we have shown previously that progressor variants can be selected in vitro using cytolytic T cell clones (34), and by this method of in vitro selection we have recently found that at least some of the unique antigens defined here by in vitro CTL probes appear also to be the targets of tumor rejection in vivo (Ward, P. L., and H. Schreiber, unpublished observations). In addition, several progressor variants that arose in vivo were found to have lost a CTL-defined antigen upon analysis in vitro (Ward, P. L., and H. Schreiber, unpublished observations). Thus, loss of a CTL-defined antigen appears to correlate in several instances with tumor progression in vivo and the antigens defined by transplantation assays and by CTL clones may, therefore, be closely related.

It should be acknowledged that there is no evidence that the antigens described here are truly tumor specific in the sense that each is encoded by a tumor-specific mutant gene. If the antigens are not the result of somatic mutations, then they are normal antigens, either syngeneic or allogeneic. Neither this study nor previous studies have excluded the possibility that these antigens are expressed on normal cells. In the previous studies (35, 36), a nonmalignant fibroblast line was cloned and expanded, and the subclones were malignantly transformed. Immunological analyses indicated that all transformants had individually distinct antigens even though all tumors had been derived from the same precursor cell. These experiments seemed to indicate that the appearance of the antigens followed the exposure to carcinogen and that these are new antigens, i.e., "neoantigens" that were not previously expressed on the normal precursor cell. However, normal cells can generate considerable diversity of surface molecules during clonal expansion from a single precursor (37, 38) and the transformation event caused by the carcinogen may simply fix a particular antigenic phenotype (39). Alternatively, it is possible that normal previously nonexpressed genes are randomly activated by the carcinogen (40). Obviously, both mechanisms could produce considerable antigenic diversity with apparent tumor specificity even though these antigens are expressed in normal cells.

By the use of autologous normal fibroblasts as control target cells, our experiments make it unlikely that the tumor antigens defined here by CTL probes are widely expressed on normal tissues and recognized by the host as a result of residual heterozygosity. However, we did not test the exact autologous normal tissue counterpart of the tumor, or better still, the particular normal cell that underwent malignant transformation and gave rise to the particular tumor. Therefore, our experiments cannot exclude that these CTL probes recognized normal tissue-specific differentiation antigens or normal, clonally restricted antigens. However, if these antigens represented normal tissue-specific differentiation antigens they would have to be highly polymorphic to account for the observed diversity of the CTL-defined antigens on tumors of similar differentiation type. Our experiments showed that three CTL clones (Fig. 5) did not crossreact with autologous control tumor cells that were of the same histologic type and arose as second or third independent tumors in the same animals. These findings do not support the notion that such CTL-defined antigens are recognized as tissue-specific antigens because of residual heterozygosity, even though this possibility is not excluded. Even more difficult to exclude is the

possibility that these antigens are found on a clonally restricted population of non-malignant cells because cells expressing this antigen may not be found even after extensive testing (41). Together, our experiments cannot prove that the so-called tumor-specific antigens are tumor specific in the strictest sense, since they might be encoded by normal genes and could be expressed on an unrecognized normal cell population. On the other hand, there are new methods to use the cloned CTL (42, 43) or antibody probes (44) for identifying the genes that encode the antigens defined by these probes. The tools (tumors, immunological probes, and autologous control cells and DNA), are now available to answer conclusively whether these antigens are encoded by normal genes, or are truly tumor specific in that they are encoded by tumor-specific mutant genes.

Experimental cancers are often considered laboratory curiosities because of the large doses of carcinogens used to induce these cancers (28). The unique tumor antigens expressed on these cancers may not be representative of human cancer which may be induced by lower doses of carcinogens. Some investigators, therefore, consider "spontaneous" cancers of mice that arise without any known experimental intervention as the most appropriate model of human cancer (28). Since spontaneous murine cancers have been found by several investigators to be very poorly immunogenic by transplantation assays (2, 3), this might imply that human cancers also do not express tumor-specific target antigens useful for diagnosis and therapy. In fact, it has been suggested that the previously reported immunogenicity of spontaneous tumors may have been caused by residual heterozygosity in the inbred animal strains (45), since after more generations of inbreeding spontaneous tumors were found to be much less immunogenic (46). However, our experiments clearly indicate that an individually distinct tumor antigen can be expressed on a spontaneous cancer in a controlled tumor model where the antigen is not detected on autologous normal fibroblasts or on a second cancer isolated from the same host. Our results are consistent with the early studies that demonstrated the presence of individually distinct antigens on several spontaneous tumors by transplantation assays (47–49). Even though the spontaneous tumors were much less immunogenic than UV-induced tumors, our results strongly suggest that spontaneous cancers in mice can express unique tumor antigens.

Summary

We have isolated UV light–induced and spontaneous tumors along with nonmalignant cells and tissues from each host. CD8$^+$ CTL clones generated to a number of highly immunogenic UV-induced tumors did not react with autologous normal fibroblasts nor with autologous second tumors. Using up to 25 independently induced tumors as targets, these CTL clones were found to be uniquely specific for the particular tumor used for immunization even when multiple tumors isolated from the same animals were used as targets. In addition to this extensive antigenic diversity of independently induced tumors, we found that a single cancer cell can express multiple independent antigens that were uniquely expressed on the tumor but were not detectable on autologous nonmalignant fibroblasts. A poorly immunogenic spontaneous tumor was also found to express an antigen that was uniquely specific for the immunizing tumor in that it was absent from any of 25 other tumors

tested. This antigen was recognized by a mAb and not detected on autologous non-malignant fibroblasts or on an autologous second spontaneous tumor. These findings demonstrate that syngeneic CTL clones or mAbs can define unique antigens on UV-induced or spontaneous tumors. The use of autologous nonmalignant fibroblast targets made it unlikely that these antigens were widely expressed on normal cells. The availability of cloned immunological probes to antigens on tumors isolated with autologous normal cells will allow a reliable identification of the genetic origins of unique antigens on experimentally induced and spontaneous tumors and permit a decisive answer to whether these unique antigens are encoded by normal genes or by genes that have undergone somatic mutations; i.e., whether these antigens are truly tumor specific.

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