

ANIMALS BEARING MALIGNANT GRAFTS REJECT NORMAL GRAFTS THAT EXPRESS THROUGH GENE TRANSFER THE SAME ANTIGEN

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Tumor-free hosts often develop strong resistance to transplanted tumor cells after active immunization (1). However, immunotherapy of hosts with well-established cancers, i.e., cancers that have been growing progressively for several weeks or longer, is rarely effective whether in patients or experimental animals (2). For example, it has been shown that immunological resistance can be induced to an autologous methylcholanthrene-induced murine tumor if the tumor is first completely removed and the tumor-free mouse is "rested." The removed tumor, "stored" by transplantation in another mouse and then transplanted back into the original host, is rejected (3). However, tumor rejection has not been induced in these mice while they were bearing the established autologous primary tumors.

Even though the presence of strong tumor-specific rejection antigens on a particular primary tumor may be suggested by rejection of a tumor transplant in the tumor-free secondary hosts, the presence of such strong rejection antigens may have neither prevented nor inhibited the development of this tumor in the original (primary) host. For example in UV-induced tumors of mice, the distinction between a regressor tumor and progressor tumor phenotype can only be made after the original tumor has been transplanted into tumor-free hosts (4).¹ How antigenic tumor cells escape immune destruction in the original host is not fully understood, but once they have escaped and become established as a tumor then the host may be immunosuppressed due to the tumor burden (5-8; for review see reference 2).² While the mechanisms

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¹ In these transplant recipients, either progression or spontaneous regression of the tumor may occur, while in the original host, both regressor and progressor tumors show similar malignant behavior and eventually kill. Thus, so-called "regressor" tumors do not undergo spontaneous regression in the primary host, and may therefore be equally important as "progressor" tumors from a therapeutic point of view. In addition, regressor tumors may be important for immunotherapy, because they may have suitable target antigens when they grow as the primary tumor in the host.

² This is indicated for example by the observation that mice with a progressively growing regressor or progressor tumor graft fail to reject a second challenge with highly antigenic UV-induced regressor tumor cells (8). The established tumor and the tumor cells used for second challenge do not have to share individually distinct tumor antigens, which points at a more generalized defect in the immune response of these tumor-bearing animals to syngeneic highly immunogenic tumor cells.

for the defect of tumor-bearing mice are not well understood, breaking the state of immunological unresponsiveness of tumor-bearing individuals to cancer is a prerequisite for active or passive immunotherapy. It is known that tumor cells can produce immunosuppressive substances or induce immune suppression. If this were important, then removing the tumor antigen from the tumor cell environment and presenting it on nonmalignant normal cells might induce an immunity specific for this antigen even in the tumor-bearing animal (TBA).³

To test this possibility, mice bearing progressively growing regressor or progressor tumors (RE-TBA or PRO-TBA) received transplants of either normal or malignant cells expressing the same highly immunogenic MHC class I antigen, designated K²¹⁶. We show that mice bearing the K²¹⁶-negative progressor tumor (PRO-TBA) do not respond to highly immunogenic K²¹⁶-positive regressor tumor cells, but do respond to nonmalignant transgenic cells or tissue grafts expressing the same K²¹⁶ antigen. Furthermore, mice bearing the progressively growing K²¹⁶-positive regressor tumor (RE-TBA) also rejected K²¹⁶-positive skin, but this response had no measurable effect on the established tumor even though it expressed the same K²¹⁶ target antigen as the rejected skin.⁴ These findings are consistent with the idea that immune responses may be induced in the TBA more effectively by presenting a tumor antigen on normal rather than malignant tissue but that such manipulation alone will not cause immunologic rejection of an established tumor.

Materials and Methods

Animals and Tumor Lines. C3H/HeN (MTV⁻), BALB/cAn, and C57BL/6 mice were obtained from the National Cancer Institute Frederick Animal Production Facility, Bethesda, MD. The skin tumor 1591-RE (9) was induced by UV irradiation and regresses when transplanted into normal C3H/HeN mice. Even though this tumor was reported to have originated in C3H/HeN (MTV⁻) (H-2^k) mice (9) and expresses normal K^k and D^k MHC class I antigens, this tumor also expresses three immunogenic MHC class I antigens designated L^q, D^q, and K²¹⁶ (10, 11). The coding regions of the genes for the first two antigens are 100% homologous to L^q and D^q genes (11). The third, K²¹⁶, does not represent K^q as determined by comparison of a partial DNA sequence of K^q made available to us by Dr. Gilbert Jay (National Cancer Institute, Bethesda, MD); the precise origin of the K²¹⁶ gene is still unknown, but it may well encode a normal alloantigen like the other two normal MHC class I genes found in the 1591-RE tumor. Gene K²¹⁶ carries the number 216 because it was designated gene 216 when isolated from a genomic library of the 1591 regressor tumor (10). It is designated as a K gene because this MHC class I gene contains the 27 extra base pairs (bp) in the intracytoplasmic domain characteristic for K genes. Furthermore, K²¹⁶ lacks the L- and D-specific nucleotides in the leader sequence of exon I and is >99% homologous to K^k from a point 300 bp 5' of exon 4 continuing 1,800 bases into the 3' untranslated region (12). The K²¹⁶ gene encodes an antigen that alone is sufficient for tumor rejection by normal C3H/HeN mice (13). This gene, along with the other two immunogenic class I antigens, D^q and L^q of the 1591-RE tumor, is always lost when 1591-PRO progressor variants of the tumor develop (13). The 1591-PRO tumor (also designated 1591-PRO4L or 1591-VAR8 [13]) used in this study is one of the progressor variants observed in 5 out of 100 animals that were challenged with fragments of the 1591-RE tumor (14). 1591-PRO will grow progressively in ~80% of normal mice after subcutaneous transplantation. This progressor variant when

³ Abbreviations used in this paper: PRO, progressor; RE, regressor; TBA, tumor-bearing animal.

⁴ This method for generating regressor TBA provides a model for studying primary highly antigenic UV-induced tumors which exhibit a regressor phenotype upon transplantation into tumor-free mice.

transfected with the K²¹⁶ gene (designated K²¹⁶ tumor) is always rejected by normal mice unless the K²¹⁶ gene is lost (13). 5128-PRO and 5117-RE are recently described UV-induced BALB/c tumors (15). P815 is a mastocytoma that arose spontaneously in DBA/2 mice. All tumors were cultured in vitro in minimum essential medium containing 10% heat-inactivated FCS (CMEM) (14).

Transgenic Mice. The λ phage clone K²¹⁶ was restricted with Hind III and Sal I and the fragment containing the K²¹⁶ gene was isolated from an agarose gel by electroelution. Approximately 200 copies were injected into the nuclei of C3H/HeN zygotes as previously described (16). Founder mice and offspring were confirmed as containing the transgene by Southern blot analysis of DNA isolated from tails.

Southern Blotting. DNA was extracted from tumor cell lines grown in vitro or from tail cells; DNA was digested to completion using an excess of restriction enzyme (New England Biolabs, Beverly, MA). The digested DNA was then separated on a 0.9% agarose gel and blotted onto Zetabind membrane (AMF Cuno, Meriden, CT) by capillary flow using 20 \times 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 (10).

Tumor Transplantation, Removal, and Readaptation to Culture. For tumor challenges, solid tumors grown in nude C3H mice that had been inoculated with cultured cells were implanted subcutaneously as 1-mm³ fragments with a 13-gauge trocar. Deep anesthesia was induced by inhaled ethylether and chloral hydrate (240 mg/kg body weight) intraperitoneally. Fine needle biopsy aspirations of the tumors were done on anesthetized mice using a 10-ml syringe fitted with a 20-gauge, 1.5-inch needle. The plunger was withdrawn to the 9-ml mark to create maximal suction and two or three different locations of the tumor were sampled this way without withdrawing the needle tip from the initial insertion site in the tumor. The aspirates were expelled into CMEM containing gentamycin and cultured for a few days before analysis.

Transplantation of Normal Tissues. Skin for grafting was obtained from the ventral surface of the donor mouse and applied to the dorsal thoracic wall according to an adaptation of the method of Billingham and Medawar (17). Bandages were removed on day 7 and grafts were scored daily until rejection (defined as loss of at least 80% of grafted tissue) or the end point of the experiment. Tissues for fetal gut or heart transplants were removed from 16-20-d-old embryos. Fetal gut tissue was minced with a curved scissors into 1-2-mm³ sections, and three to four fragments of fetal gut or whole fetal heart were implanted through an incision 5 mm caudal to the base of the ear and moved close to the tip of the ear to allow easy inspection. Rejection or acceptance of graft was confirmed histologically 3-5 wk after transplantation.

Induction of Cytolytic T Cells In Vivo and In Vitro. Polyurethane sponge matrix grafts (0.5 cm³; Future Foam Co., Chicago, IL) were transplanted as described (18) into anesthetized mice by passing the sponge graft through a subcutaneous tunnel and depositing it in the interscapular region. 5-10 \times 10⁶ stimulator cells were injected into the center of the sponges with a 25-gauge needle and at various times thereafter sponges were removed and placed into 5 ml of cold RPMI 1640 containing 5 U of heparin/ml. Cells to be used as effector cells in a ⁵¹Cr-release assay were removed by squeezing the sponge with a forceps multiple times. In addition, a small fragment of the sponge was cultured to test for possible bacterial contamination of the removed implant. For the generation of cytolytic lymphocytes in vitro, tumor-bearing or tumor-free mice were first immunized by injecting subcutaneous sponges or the peritoneal cavity with mitomycin C-treated or untreated tumor cells or spleen cells. At the time mice were killed, sponges were examined for absence of tumor growth since at the dose used tumors may occasionally grow out; however, no tumors have ever been observed in mice injected intraperitoneally with viable tumor cells. Spleen cells were restimulated in vitro in a mixed lymphocyte tumor cell culture (MLTC) as previously described (14). Cytotoxicity was determined by the ability of effectors to lyse ⁵¹Cr-labeled target cells during a 6-h assay as previously described (14). The percentage specific lysis was calculated by the formula: [(experimental release - spontaneous release)/(maximum release - spontaneous release)] \times 100.

Thymocyte and DC Preparations. Thymocyte suspensions were prepared using the same methods as used for spleen cell preparations for an MLTC (13). DC preparations were prepared as described (19). Briefly, spleen cells were adhered to plastic culture dishes for 2 h and the nonadherent cells were removed by washing the plate three times with fresh medium.

The adherent macrophages and DC were incubated for an additional 22 h. DC detach during this second incubation so that the nonadherent cells recovered consist of ~50–60% DC. For convenience, the cells in this preparation that contain ~1 to 2% of all nucleated cells present in a normal spleen are referred to as DC.

Flow Cytometric Analyses Cell Preparations. The antibody CP28, specific for the K^{216} gene, has been described (20). The anti-MHC class I mAbs were gifts from Keiko Ozato (National Institutes of Health, Bethesda, MD) and their specificities have been described (21). The FACS IV (Becton Dickinson & Co., Mountain View, CA) or EPICS-753 (EPICS Coulter Corp., Hialeah, FL) was used to quantify the degree of MHC class I antigen expression of tumor cells or normal transgenic cells. Cells were incubated with 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.

Results

Generation of Transgenic Mice Expressing the K^{216} MHC Class I Antigen. Transgenic mice were generated by microinjecting fertilized C3H/HeN oocytes with the K^{216} gene. DNA isolated from tails of the offspring was analyzed by Southern blotting. Fig. 1 A shows that two of seven animals contained a 950-bp polymorphic fragment characteristic of the K^{216} gene. To establish two independent K^{216} -transgenic lines of mice, these two male founder mice were mated to normal C3H/HeN female mice. Fig. 1 B left panel shows that thymocytes from K^{216} gene-positive offspring expressed the K^{216} antigen in addition to the endogenous normal K^k and D^k MHC class I antigens. The transgenic mice also expressed the K^{216} antigen on all cell types tested including dendritic cells (Fig. 1 B, right), fibroblasts, hepatocytes, kidney cells, spleen cells, and white blood cells; the peripheral blood cells were used to identify K^{216} antigen-positive offspring by flow cytometric analysis. The level of expression in the normal transgenic tissues was in the range of 10–20-fold above the level of fluorescence of cells stained with the fluorescein-coupled goat anti-mouse Ig antibody alone, quite comparable to levels of expression of the K^{216} antigen in the parental 1591-RE tumor (20) or 1591-PRO tumor cells that have been transfected with the K^{216} gene (13).

K^{216} -Transgenic Skin Is Rejected by Normal C3H/HeN Mice. Several experiments were performed to test the immune response of normal C3H/HeN mice to K^{216} transgenic tissues. As can be seen in Table I, normal C3H/HeN mice rejected K^{216} -transgenic skin after the same time interval as they rejected fully MHC I-disparate allogeneic skin grafts. A possible trivial explanation for the highly effective rejection of the transgenic skin by normal mice might be that expression of additional antigens had been induced by insertional mutagenesis and that the rejection of the K^{216} -transgenic skin was not specific for the K^{216} antigen. However, Table I also shows that the independent lineages of K^{216} -transgenic mice derived from two different microinjected oocytes accepted skin grafts exchanged between them suggesting that no additional, artificially generated antigens contributed to the efficient rejection. In addition, polyurethane sponges placed under the skin of normal mice and injected with K^{216} -transgenic spleen cells led to the generation of sponge-infiltrating lymphocytes that lysed the K^{216} -transfected tumor cells but not untransfected pro-ressor tumor cells. Also, spleen cells from these K^{216} -immunized mice gave rise in culture to CTL that specifically killed the K^{216} -positive tumor cells when restimulated in vitro with the K^{216} -transgenic spleen cells (data not shown). Together, these

experiments suggest that normal C3H/HeN mice rejected the transgenic grafts with the same efficiency as fully allogeneic skin grafts in a K²¹⁶-specific way.

Mice Bearing a Progressor Tumor Do Not Reject a K²¹⁶-positive Tumor but Do Reject Non-malignant Grafts Expressing the Same Antigen. Table II shows that mice bearing an established 1591-PRO tumor (K²¹⁶-negative) fail to reject K²¹⁶-positive 1591 regressor tumors or 1591-PRO tumors transfected with the K²¹⁶ MHC class I gene (encoding the antigen for tumor rejection). An important finding, shown in Table II, is that even though the progressor tumor-bearing mice failed to reject K²¹⁶-positive tumors, these PRO-TBA's regularly rejected K²¹⁶-transgenic skin grafts as rapidly as they rejected allogeneic skin grafts. Both types of grafts were rejected by the PRO-TBA with only a slight delay of 1-2 d as compared with the time required for normal tumor-free mice.

It is conceivable that differences between the proliferative state of the normal and malignant tissues were responsible for the differences between the response of tumor-bearing mice to K²¹⁶-positive skin versus K²¹⁶-positive tumors, thus favoring tumor outgrowth. However, Table II shows that these TBA rejected allogeneic tumors that rapidly proliferated and formed tumors before they were rejected. Furthermore, these TBA also rejected K²¹⁶-positive fetal gut transplants from K²¹⁶-transgenic mice. These fetal gut transplants showed histologically high mitotic activity and formed tumor-like masses of 3-10 mm in diameter in syngeneic transgenic controls. Thus, differences in the proliferative state of the transplanted tissue cannot account for the different responses of TBAs to transplanted normal and malignant tissues.

Progressor Tumor-bearing Mice Generate K²¹⁶-specific CTL in Response to the Antigen on Normal But Not on Malignant Cells. Since progressor tumor-bearing mice accepted K²¹⁶-positive tumors but rejected K²¹⁶-positive skin grafts, we explored whether these differences correlated with differences in the cytolytic T cell response to the K²¹⁶ antigen on normal or malignant cells. K²¹⁶-transfected tumor cells or K²¹⁶-

TABLE I
K²¹⁶ Skin Grafts are Rejected by Normal C3H/HeN Mice but Survive Indefinitely in Independently Derived K²¹⁶-Transgenic Lines of Mice

Recipient		Donor of skin graft		Take of graft	Survival of graft
Strain	MHC I haplotype	Strain	MHC I haplotype		
C3H/HeN	K ^b D ^k	C57BL/6	K ^b D ^b	0/5	12 ± 0
		C3H/HeN transgenic line 1	K ^k D ^k K ²¹⁶	0/5	12 ± 0
		C3H/HeN	K ^k D ^k	5/5	>90†
C3H/HeN transgenic line 1	K ^k D ^k K ²¹⁶	C3H/HeN transgenic line 1	K ^k D ^k K ²¹⁶	5/5	>100†
		C3H/HeN transgenic line 2	K ^k D ^k K ²¹⁶	5/5	>100†

* Day of rejection as mean ± standard deviation.

† Represents mice killed at some time after the day indicated. All animals had normal soft grafts with new hair growth at the time they were killed.

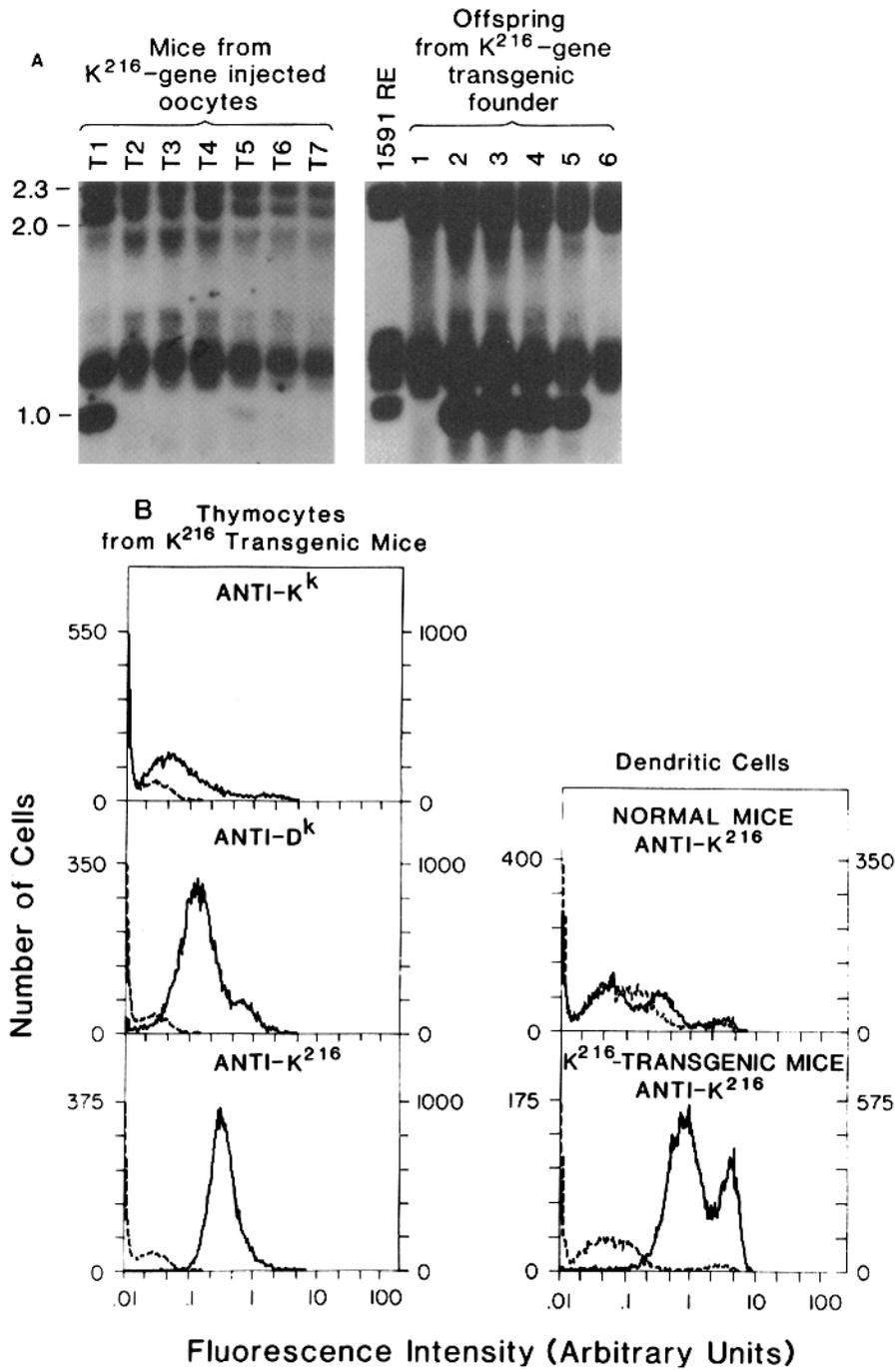


FIGURE 1. Derivation of transgenic C3H mice containing the K^{216} gene in their genome and coexpressing the K^{216} gene-encoded antigen with the endogenous D^k and K^k antigens. (A) Southern blot analysis of tail DNA from seven mice born after the fertilized oocytes had been injected with the K^{216} gene (*left*). The right panel shows a Southern blot analysis of six offspring

TABLE II
Mice Bearing K^{216} -negative Tumors Reject K^{216} -positive Grafts of
Nonmalignant Tissues but Fail to Reject K^{216} -positive Tumor Grafts

Host*		Challenge		Take of graft	Survival of graft
Strain	Tumor burden*	Type of tissue	MHC I haplotype		
					<i>d ± SD†</i>
C3H/HeN (K^kD^k)	1591-PRO tumor (K^kD^k)‡	K^{216+} transfected tumor	$K^kD^kK^{216}$	5/5	—
		1591-RE tumor	$K^kD^kK^{216}D^qL^q$	5/6	—
		5128-PRO BALB/c tumor	K^dD^d	0/6	—
		5117-RE BALB/c tumor	K^dD^d	0/7	—
		P815 DBA/2 tumor	K^dD^d	0/5	—
		K^{216+} transgenic fetal gut	$K^kD^kK^{216}$	0/8	—
		K^{216+} transgenic fetal heart	$K^kD^kK^{216}$	0/5	—
		K^{216+} transgenic skin	$K^kD^kK^{216}$	0/13	14 ± 1
		BALB/c skin	K^dD^d	0/6	14 ± 1
		C57BL/6 skin	K^bD^b	0/9	14 ± 0
	None	K^{216+} transfected tumor	$K^kD^kK^{216}$	0/6	—
		1591-RE tumor	$K^kD^kK^{216}D^qL^q$	0/6	—
		K^{216+} transgenic skin	$K^kD^kK^{216}$	0/6	13 ± 1
		BALB/c skin	K^dD^d	0/4	12 ± 2
	C57BL/6 skin	K^bD^b	0/6	12 ± 2	
C3H/HeN transgenic ($K^kD^kK^{216}$)	None	K^{216+} transgenic skin	$K^kD^kK^{216}$	5/5	
		K^{216+} transgenic gut	$K^kD^kK^{216}$	5/5	
		K^{216+} transgenic heart	$K^kD^kK^{216}$	3/3	

* C3H/HeN mice (K^kD^k) were injected with three 1-mm³ fragments of the K^{216} -negative progressor tumor 1591-PRO at one subcutaneous site (right flank). 4–5 wk later, when tumors had reached an average volume of 2–5 cm³, these tumor-bearing mice were then challenged with the type of tissue indicated.

† Day of rejection as mean ± standard deviation.

‡ This K^{216} -negative progressor tumor also designated as 1591-PRO represents the 1591 progressor tumor variant that has lost K^{216} , D^q , and L^q and is used for transfection. Here it is used as untransfected control tumor. For further details on derivation of this tumor see Materials and Methods.

|| 1591-PRO transfected with the K^{216} gene.

transgenic spleen cells were injected into the subcutaneous sponge implants of PRO-TBA. Two animals in each group were killed 7, 11, 15, and 17 d later to isolate and test the infiltrating lymphocytes. The PRO-TBA failed to mount a K^{216} -specific CTL response to the K^{216} -positive tumor cells but did mount a K^{216} -specific CTL response to K^{216} -transgenic spleen cells with a peak response observed at about day

derived from a cross between the founder animal T1 and a normal C3H mouse. The Southern blots were done with Pst I-digested genomic DNA and probed with the class I-specific probe 149.6-6 (10). The presence of a 950-bp polymorphic band indicates the presence of the K^{216} gene (10). 1591-RE DNA containing the class I genes L^q , D^q , and K^{216} was used as a control. The size markers are indicated by numbers in kilobases. (B) Flowcytometric analysis of splenic dendritic cells of normal or K^{216} -transgenic mice of the T1 lineage (line 1) (right panels) and thymocytes (left panels) from transgenic C3H mice of the T5 lineage (lane 2) derived from founder mice T5 described in A. The cells were incubated with the mAbs CP28 (K^{216} antigen), 11-4.1 (K^k antigen), and/or 15-5-5 (D^k antigen) followed by incubation with a fluorescein-labeled goat anti-mouse antibody.

15. Fig. 2 gives an example of the differences observed 15 d after injection of malignant or normal stimulator cells. Spleen cells of these TBA were then restimulated in vitro with K^{216} -transgenic spleen cells or the K^{216} -transfected mitomycin C-treated tumor cells. Fig. 3 shows that cultures of spleen cells from progressor tumor-bearing mice responded with K^{216} -specific CTL when the cells had been stimulated by K^{216} -transgenic spleen cells (A-D), but not when stimulated with the K^{216} -positive tumor cells in vivo and in vitro (E-G). K^{216} -positive tumor cells, however, effectively stimulated the in vitro generation of K^{216} -specific CTL from spleen cells of tumor-free K^{216} -immune mice (Fig. 4).

K^{216} Regressor Tumor-bearing Mice Which Reject K^{216} Skin Nevertheless Do Not Select for Antigen Loss Variants. We have shown previously that the K^{216} -negative progressor tumor grows at about an 80% incidence when transplanted into tumor-free mice (14). If the K^{216} antigen is the only antigen that causes the change from progressor to regressor phenotype, then the K^{216} -positive and the K^{216} -negative tumors should grow at about the same 80% tumor incidence in K^{216} transgenic mice, and this appears to be the case, Table III.

We have shown previously (6) that K^{216} -positive regressor tumors injected into progressor tumor-bearing mice grow progressively, even if the progressor tumor is subsequently removed (9 d or longer after challenge with the regressor).⁴ Though the tumor grows as an antigen-positive tumor in these mice (7), nevertheless the mice rejected K^{216} -positive transgenic skin (Table III), shown in the following way.

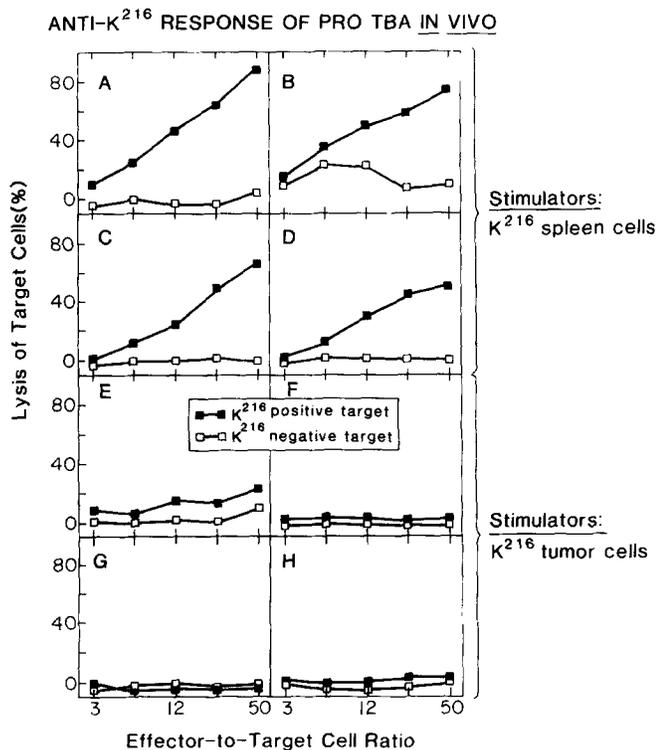


FIGURE 2. Generation of antigen-specific CTL in vivo by tumor-bearing mice in response to the antigen on normal cells (A-D) and failure to respond to the same antigen on malignant cells (E-H). Each panel represents an individual animal. All animals had the PRO tumor growing for 4 wk (average size 2-5 cm^3) when $0.5-1 \times 10^7$ transgenic spleen cells or K^{216} -transfected tumor cells, untreated (E and F) or mitomycin C-treated (G and H) were injected into 0.5-cm^3 polyurethane foam matrix sponge grafts immediately following implantation under the skin. 11-17 d later, sponge-infiltrating cells were harvested and used directly as effectors against 1591-RE and K^{216} -positive target cells and PRO tumor cells as K^{216} -negative control cells in a 6-h ^{51}Cr -release assay.

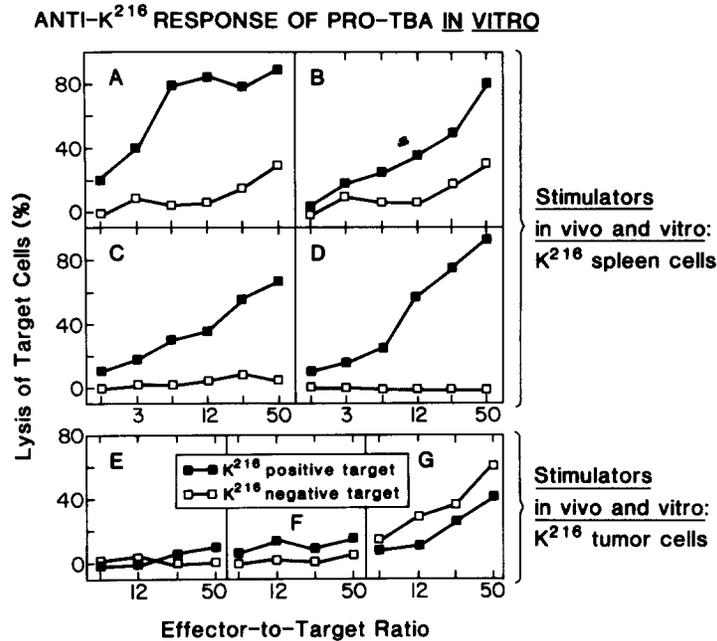


FIGURE 3. Generation of antigen-specific CTL in vitro from spleen cells of tumor-bearing mice primed in vivo and restimulated in vitro with the antigen on normal cells (A-D), and failure of tumor-bearing mice to respond in vitro when primed in vivo and restimulated in vitro with the same antigen but on malignant cells (E-G). Each panel represents an individual animal. All animals had PRO tumors growing for 4 wk (average size 2–5 cm³) when $0.5\text{--}1 \times 10^7$ K²¹⁶-positive spleen cells (A-D) or K²¹⁶-transfected tumor cells (E-G) were injected into either the intraperitoneal cavity (B, C, E, G) or subcutaneous sponge matrix grafts (A, D, F). 14–17 d later spleen cells were harvested and restimulated in vitro at the optimal responder to stimulator (R/S) ratio with the same form of antigen they received in vivo (A–D, K²¹⁶-positive spleen at a 30:1 R/S ratio; E–G, K²¹⁶-transfected tumor cells at a 300:1 R/S ratio). All cultures were analyzed after 6 d of incubation using 1591-RE tumor cells as the K²¹⁶-positive target and PRO tumor cells as the K²¹⁶-negative control target in a 6-h ⁵¹Cr-release assay.

Progressor tumor fragments were first injected into the right flank of mice. After 4–5 wk of growth, fragments of the K²¹⁶-transfected tumor were implanted subcutaneously in the contralateral left flank. 10–14 d after challenge with the K²¹⁶-positive regressor tumor, the progressor tumor was excised. 5–7 d after removal of the progressor tumor the mice received a transplant of K²¹⁶-positive skin. These mice rejected K²¹⁶ skin but more slowly than normal recipients. The delay did not seem to correlate directly with the size of the regressor tumor nor to the duration of tumor engraftment (data not shown); nor was the delay specific for the K²¹⁶ antigen since rejection of allografts was similarly delayed (Table III). Nevertheless, all of the K²¹⁶-transgenic skin grafts were rejected by the mice bearing K²¹⁶-positive tumors. Surprisingly, however, none of these mice rejected their tumors even though cytofluorometric analysis of cells isolated by biopsy of the tumor showed that the tumors were K²¹⁶-positive at the time when the skin was transplanted (data not shown). Tumors were biopsied again at the time of rejection of the K²¹⁶-positive skin graft or during the following 3 wk, and tumor cell lines were readapted to culture and analyzed

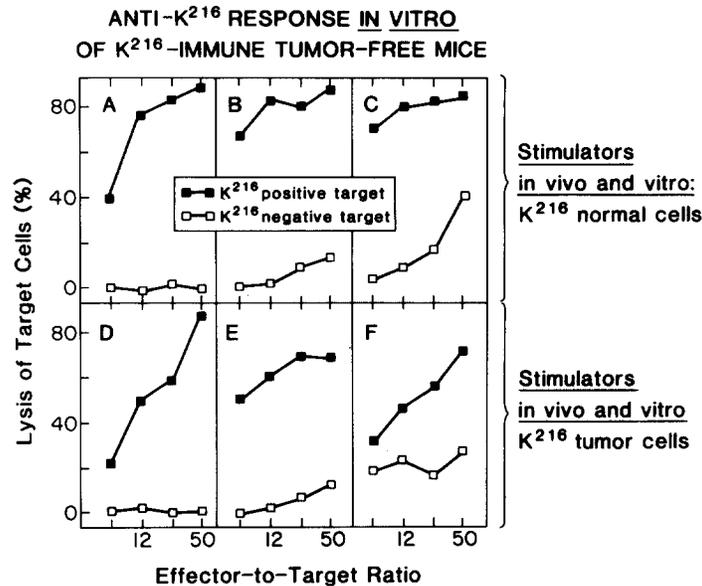


FIGURE 4. Generation of specific cytolytic T cells in vitro from spleen cells of tumor-free mice primed in vivo with the antigen on normal tissues (A, spleen; B, skin; C, HLF) or on K²¹⁶-positive tumor cells (D, 1591-RE tumor cells injected into a subcutaneous polyurethane sponge; E, 1591-RE tumor cells ip; F, K²¹⁶-transfected tumor cells i.p.). Tumor-free animals were injected with $0.5-1 \times 10^7$ K²¹⁶-tumor cells (D-F), K²¹⁶-positive normal cells (A, C), or grafted with K²¹⁶-positive skin (B) from K²¹⁶ transgenic mice. 5-14 d later spleen cells were harvested and restimulated in vitro at the optimal R/S ratio with K²¹⁶ tumor cells (D-F) at a 300:1 R/S ratio; or K²¹⁶ normal spleen cells (A-C) at an R/S ratio of 30:1. All cultures were analyzed after 6 d of incubation using the 1591-RE tumor cells as the K²¹⁶-positive target and PRO tumor cells as the K²¹⁶-negative control target in a 6-h ⁵¹Cr-release assay.

by flow cytometry for expression of the K²¹⁶ antigen (Fig. 5). In only one animal did the tumor show partial but significant K²¹⁶ antigen loss after rejection of the K²¹⁶-positive skin graft.

From previous experiments, we know that K²¹⁶ antigen loss occurs before the K²¹⁶-transfected tumors can grow in immunocompetent mice (13) and that K²¹⁶ antigen expression is not lost after prolonged growth of these tumors in T cell-deficient hosts such as nude mice or progressor TBA (8). We do not have sufficient concurrent controls to suggest that this observation of partial loss in one of the tumors was due to induction of immunity to the K²¹⁶ antigen by the skin graft. Even if this was so, it is very striking that all the other K²¹⁶ tumor-bearing mice failed to show any evidence of immunoselection in the progressively growing K²¹⁶ tumors, despite the fact that all of these tumor-bearing mice generated sufficient K²¹⁶-specific immunity to reject skin grafts expressing the same K²¹⁶ target antigen.

Discussion

We demonstrate that immune responses to a model tumor antigen, the immunogenic MHC class I antigen K²¹⁶ can be induced in hosts with long term (≥ 3 wk)

TABLE III
Mice Bearing K²¹⁶-positive Tumors Reject K²¹⁶-positive Skin Transplant but Show Delayed Skin Graft Rejection Compared with Nonimmune Tumor-free Mice

Strain	Host*		Challenge		Take of graft	Survival of graft <i>d ± SD</i> †
	Tumor burden		Type of tissue	MHC I haplotype		
C3H/HeN (K ^k D ^k)	1591-RE tumor (K ^k D ^k K ²¹⁶ D ^{qLq})	K ²¹⁶⁺	transfected tumor	K ^k D ^k K ²¹⁶	7/7	
			K ²¹⁶⁺ transgenic skin	K ^k D ^k K ²¹⁶	0/13	20 ± 4
	K ²¹⁶ tumor [§] (K ^k D ^k K ²¹⁶)	C57BL/6 skin	K ²¹⁶⁺ transgenic skin	K ^b D ^b	0/6	17 ± 1
			C57BL/6 skin	K ^k D ^k K ²¹⁶	0/8	18 ± 6
			C57BL/6 skin	K ^b D ^b	0/5	16 ± 1
None	K ²¹⁶⁺ transgenic skin	K ^k D ^k K ²¹⁶	0/7	12 ± 1		
C3H/HeN transgenic (K ^k D ^k K ²¹⁶)	None	K ²¹⁶⁺	transfected tumor	K ^k D ^k K ²¹⁶	1/20	
			transgenic skin	K ^k D ^k K ²¹⁶	9/9	>100
			1591-PRO tumor (untransfected)	K ^k D ^k K ²¹⁶	14/18	
C57BL/6 (K ^b D ^b)	None	C57BL/6 skin	K ²¹⁶⁺ transgenic skin	K ^b D ^b	13/16	
			transfected tumor	K ^b D ^b	6/6	>100

* C3H/HeN mice (K^kD^k) 3 wk after implantation and establishment of the 1591-RE or K²¹⁶ tumors were challenged with a second K²¹⁶ tumor or full-thickness skin grafts from K²¹⁶-transgenic or K^bD^b allogeneic donor mice as indicated.

† Day of rejection as mean ± standard deviation.

established tumors by presenting the tumor antigen on normal cells or tissues. However, these induced responses failed to cause rejection of the tumors though tumor-bearing animals rejected nonmalignant tissue grafts expressing the same K²¹⁶ antigen. Furthermore, rejection of the K²¹⁶-positive skin grafts was not accompanied by any reduction in tumor size, or percentage of K²¹⁶-positive cells in the tumors of these mice. The rejection of K²¹⁶-positive transgenic or allogeneic skin was delayed by 1–2 d in the regressor tumor-bearing mice, or by 4–8 d in the K²¹⁶-positive regressor tumor-bearing mice; nevertheless, both types of animals rejected the MHC class I-disparate skin grafts. There was also no evidence for an antigen-specific enhancement by tumors since mice bearing the K²¹⁶-positive tumors rejected K²¹⁶-positive grafts or K²¹⁶-negative allografts expressing different MHC class I antigens at about the same time.

Three reasons that are commonly proposed to explain the failure of the immune system to destroy established tumors: (a) lack of a strong rejection antigen, (b) release of large amounts of tumor antigen causing host immune cells to become refractory, and (c) rapid proliferation of tumor cells that outstrips the capacity of the immune system to respond effectively. Our experiments suggest that none of these three mechanisms are very important in the present model.

The target antigen clearly had the strong antigenicity of an MHC class I alloan-

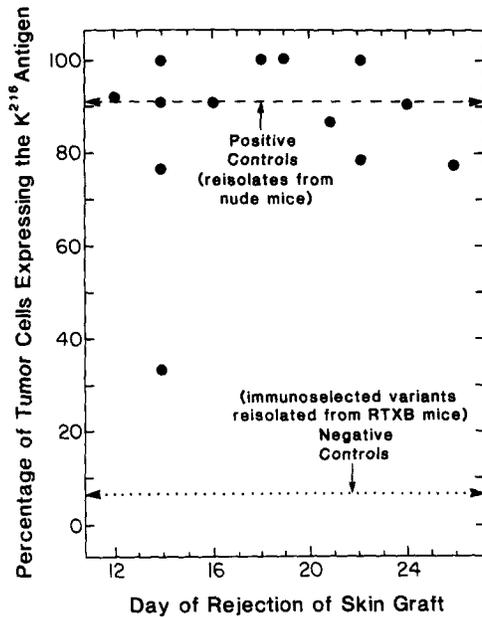


FIGURE 5. K²¹⁶ tumors growing in mice that reject K²¹⁶-positive skin grafts continue to express K²¹⁶ target antigen. K²¹⁶-RE TBA were challenged with K²¹⁶-positive transgenic skin, and 2 wk after rejection of the skin all tumors were sampled by fine needle aspiration biopsy. These tumor cells were readapted to tissue culture and stained with the K²¹⁶-specific mAb CP28 (20) and analyzed by flow cytometry. The percentage of tumor cells that express the K²¹⁶ antigen is presented on the y axis and the day of skin graft rejection is listed on the x axis. Despite the ability of the K²¹⁶-RE TBA to reject the K²¹⁶ skin graft there is only one example where this response was associated with significant selection against the K²¹⁶ antigen expression by a tumor growing in the same animal.

tigen in that it caused tumor regression in mice even when challenged with large amounts of tumor tissue without prior immunization. (And we do not consider it likely that human tumors will be more antigenic.) If large amounts of antigen due to the tumor load prevented the stimulation or effector function of anti-K²¹⁶ T cells, then these mice should not have been able to reject the K²¹⁶-positive transgenic skin grafts. Though the rejection of K²¹⁶-positive skin was delayed in the K²¹⁶ TBA, these mice showed a similar delay of rejection of antigenically unrelated allogeneic skin grafts, which is consistent with the apparent absence of antigen specificity of the suppression in the TBA (7). If the proliferative capacity of the tumor was simply greater than the killing capacity of the immune response generated, then anti-K²¹⁶ immunity caused by K²¹⁶-positive skin rejection should have caused some slowdown in K²¹⁶ tumor growth. Alternatively, at least some evidence for immune selection for K²¹⁶ loss variants should have been found after weeks of continued tumor growth in the nominally immune mice. Selection for variants readily occurs in the absence of a detectable effect on tumor size in short-term UV-irradiated mice (22) or in X-irradiated, thymectomized, spleen cell-reconstituted mice with partially compromised but still demonstrable tumor antigen-specific immunity (8). Thus, selection for antigen loss variants appears to be a particularly sensitive measure of antitumor immunity. The observed absence of selection seen here strongly suggests the absence of any antigen-specific tumor cell destruction in the tumor, and it is therefore unlikely that the tumor simply grew because it outstripped the killing capacity of the immune system. Certainly, differences in the mitotic activity of the transplanted tissues seem not to be sufficient to explain why TBA reject transgenic skin, since TBA also rejected K²¹⁶-positive fetal gut transplants that display a very high mi-

otic index. Together, our experiments fail to support the notion that low antigenicity, large antigen load, or inadequate proliferative capacity of the immune cells (as compared with the tumor) were responsible for the failure of the anti-K²¹⁶ immunity to be effective.

At present, we do not know why TBA fail to reject the K²¹⁶ tumors when these animals rejected nonmalignant tissue transplants expressing the same target antigen. Kaliss (23) noted many years ago that it was considerably easier to enhance tumor allografts than normal tissue allografts with alloantisera and he suggested that the ability of tumors to be enhanced more easily "characterizes a fundamental difference between cancerous and normal tissues." Possibly grafts of normal tissues are rejected consistently because they contain Langerhans cells, which are absent in tumors but which can powerfully stimulate allogeneic responses (24). Even though the tumor-free host can reject regressor tumors despite an absence of Langerhans cells or dendritic cells, antigen presentation by such cells may be important for the rejection by host with enhanced allografts. For example, injection of dendritic cells can abruptly terminate long-term allograft enhancement (25, 26). Although dendritic cells pulsed with lyophilized tumor antigen failed to induce tumor rejection (27) it is not known whether dendritic cells transfected to express tumor-specific antigens can lead to rejection of an established cancer.

Local factors at the tumor site may prevent immune cells from entering and rejecting tumor grafts, and the nature and specificity of tumor infiltrating lymphocytes need to be determined. Malignant cells are metabolically very active and can produce substances that may be immunosuppressive by inhibiting leukocyte attraction, antigen presentation, or effector function of T cells. Such substances produced at the site of the established tumor may act as a local barrier to infiltration by tumor-specific lymphocytes and/or prevent immune destruction. Such substances could reduce systemic immune reactivity, and the fact that TBA showed delayed allogeneic skin rejection is consistent with some degree of systemic immune suppression. In previous studies, we have shown that mice bearing UV-induced tumors have suppressor lymphocytes that can prevent the tumor rejection of regressor tumors by normal host lymphocytes upon adoptive transfer (7, 8). Such suppressor cells induced by malignant tissues might possibly lead to local intratumor suppression; however, the specificity and function of these suppressor cells that are absent from athymic tumor-bearing mice remains to be determined.

There are some interesting parallels between allograft and tumor enhancement. Pregnant individuals reject paternal skin allografts without aborting (28, 29), and rats harboring long-term enhanced renal allografts reject donor type skin without rejecting the renal transplants (30). In both instances, the survival time of the skin graft is slightly prolonged, similar to that observed for K²¹⁶-positive skin grafts in the mice carrying K²¹⁶-positive tumors. While some of the parallels between the different systems are striking, none of the systems have resolved the precise mechanisms for: (a) the enhancement of the primary graft, (b) the slight prolongation of the secondary graft, and (c) the failure of immune cells that must participate in rejecting the second graft to affect the survival of the first graft. Possibly the tumor escapes through a loophole in the immune defense that had to be left open because allogeneic fetuses must not be rejected by the pregnant mother. Thus, if we can un-

derstand why the mother fails to reject her fetus we may learn why an individual fails to respond to immunogenic tumors.

Whatever the mechanism(s) underlying our observations, it appears that responses can be induced in the TBA by presenting the "tumor" antigen on nonmalignant rather than malignant tissues. A potentially powerful approach to be used in the future may be the genetic transfer of the expression of a tumor antigen into nontumor cells, particularly into cells that have potent immunostimulatory activity, such as dendritic cells (31). Although induction of tumor-specific immune responses in the tumor-bearing host may be essential for tumor rejection, additional therapeutic manipulations may be required to cause immunological rejection of established tumors.

Summary

Breaking the state of immunological unresponsiveness of tumor-bearing individuals to cancer is a prerequisite for active or passive tumor-specific immunotherapy. To study this problem the immunogenic MHC class I antigen, K²¹⁶ was transfected into a progressor tumor. The transfected tumors were regularly rejected by normal mice but grew progressively in mice bearing nontransfected tumors. In addition, transgenic mice were derived to obtain normal cells and tissues expressing the same K²¹⁶ gene product. Normal mice rejected K²¹⁶-positive normal or malignant tissue grafts and generated K²¹⁶-specific CTL in vitro and in vivo in response to these challenges. In contrast, mice bearing nontransfected tumors, though rejecting K²¹⁶-positive nonmalignant tissue grafts, did not reject K²¹⁶-positive tumors nor generate K²¹⁶-specific CTL in response to K²¹⁶-positive tumor cells. Mice bearing K²¹⁶-positive tumors also rejected the nonmalignant K²¹⁶-positive tissue grafts, but this in vivo response failed to lead to rejection of the simultaneously present tumor graft expressing the same antigen; in fact, immunity had no measurable effect whatsoever on tumor size or incidence and caused no selection for antigen loss variants. Taken together, the present findings suggest that transfer of expression of a target antigen into nonmalignant cells provides a way for obtaining effective stimulation of antigen-specific CTL in tumor-bearing mice, but that additional manipulations will be required to cause immunological rejection of established tumors.

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