MULTIPLE CANCERS

Tumor Burden Permits the Outgrowth of Other Cancers

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The latent period between exposure to carcinogen and development of cancer in a given individual can range from a few years to several decades. Even genetically identical mice treated with the same dose of carcinogen under controlled experimental conditions develop tumors after greatly different latent periods. It is, therefore, puzzling why patients that have developed one cancer are at a much increased risk of developing a second cancer within a relatively short time (1–4). One mechanism that might lead to the successive appearance of multiple primary malignancies was suggested by our experimental studies involving the ultraviolet light (UV)[1]-induced fibrosarcoma, 1591-RE. Like most UV-induced cancers (5), this tumor consists of "potentially malignant," "premalignant," or "regressor" cells that grow in immunodeficient mice but are regularly rejected by normal hosts. In rare instances, heritable variants arise that have lost tumor antigens, and these tumors do grow progressively in normal hosts (6). In the present study, we made a striking and puzzling observation when mice were challenged with the premalignant 1591-RE cells at two anatomically distinct locations. In the rare animals that developed one progressively growing tumor, a second tumor regularly emerged at the other location a short time later. This led us to ask if the development of one tumor could facilitate the outgrowth at another location of a second cancer from premalignant cells. Our results show that indeed this occurs and that the first cancer enhances immunologically the outgrowth of other cancers from premalignant cells.

Materials and Methods

Mice. 5–10-wk-old female C3H/HeN (MTV$^-$, mammary tumor virus–negative) and female BALB/cAn mice from colonies of germfree-derived, specific pathogen–free animals were purchased from the NCI Frederick Cancer Research Facility. They were kept at the La Rabida Institute in laminar air flow hoods and were given sterilized food (Purina

This work was supported by grants RO1 CA-22677, PO1 CA-19266, and RO1 AI-22351 from the National Institutes of Health. C. Mullen is supported by grant 1-T32 HD 97009 from the National Instutute of Child Health and Human Development; J. Urban, grant 5-T32 GMO 7281-04 from the National Institute of General Medical Sciences and by Research Service Award 5-T32 from NIH; and H. Schreiber, Research Career Development Award CA-00432 from NIH and by the Brinton Memorial Fund.

[1] *Abbreviations used in this paper:* C′, complement; CMEM, complete minimum essential medium; CTL, cytotoxic T lymphocytes; MCA, methylcholanthrene; MLTC, mixed lymphocyte–tumor cell culture; MTV, mammary tumor virus; TBA, tumor-bearing animal; UV, ultraviolet light.

5010 C; Purina, Inc., St. Louis, MO) and water. 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. Unless otherwise specified, the abbreviation TBA refers to animals bearing an established progressor tumor >0.7 and <1.2 cm in average diameter (>0.5 and <1.5 cm^3 in volume).

Fibrosarcoma Lines. The fibrosarcomas 1591-RE, 1316-RE, 2240-RE, 2237-PRO (clone 42), 948-PRO, and 968-PRO were induced in C3H/HeN MTV^- mice by repeated exposure to UV light (5). The fibrosarcomas 1130-RE and 3152-PRO were induced in UV-irradiated C3H/HeN MTV^- mice by the subcutaneous injection of 3-methylcholanthrene under the ventral non–UV-exposed skin. Tumors with the postscript "-RE" were strongly immunogenic in that they regressed after an initial 1–2 wk of growth. This phenomenon of regression occurred even when large numbers of the tumor cells (e.g., multiple 1-mm^3 tumor fragments) were injected, although the same tumor inoculum grew and killed nude mice or mice immunosuppressed by x irradiation. Tumors with the postscript "-PRO" grew progressively in (and killed) normal syngeneic mice. Tumors 2237-PRO, 968-PRO, 948-PRO, and 3152-PRO are tumors that grew progressively in normal mice in the first transplant generation. The fibrosarcomas 1591-PRO, 1316-PRO, and 1130-PRO are progressively growing variants of 1591-RE, 1316-RE, and 1130-RE, respectively; these variants arose spontaneously after transplantation of the regressor tumors into normal mice. 1590-PRO has been referred to previously (6) as 1591-PRO.4 and has lost the "A" antigen while retaining the B antigen (7). 1591-ASBS is a progressor variant of 1591-RE, sequentially selected in vitro with a cloned anti-A and then anti-B T cell line; this variant lacks the A and the B antigens and has previously been referred to as 1591-V5 (7). K101-RE is a UV-induced BALB/c fibrosarcoma and P815 is a mastocytoma that spontaneously arose in DBA/2 mice. Using previously described (6) methods tumors were readapted to growth in vitro, expanded in tissue culture within 2 wk of explantation, and cryopreserved in aliquots. Cells were cultured in minimum essential medium containing 10% heat-inactivated fetal bovine serum (CMEM). Whenever tumor cells were required for experiments, frozen aliquots were thawed and used within 48 h. For tumor challenges, solid tumors grown in nude C3H mice were implanted subcutaneously as viable 1-mm^3 fragments with a 13 gauge trocar, or suspensions of cultured cells were injected subcutaneously in 0.2 ml of CMEM. 15 min before surgery mice received 0.5 mg of sodium pentobarbital intraperitoneally. Deep anesthesia was then induced by inhaled ethyl ether and tumors were removed by dissection of their margins.

Generation of Cytotoxic Lymphocytes In Vitro. Tumor-specific cytotoxic T lymphocytes (CTL) were generated from spleen cells of animals immunized with either three 1-mm^3 fragments of solid viable tumor or a single subcutaneous injection of 10^7 viable cultured tumor cells. Spleen cells were restimulated in vitro with the immunizing tumor in a mixed lymphocyte–tumor culture (MLTC) as previously described (6). Alloantigen-specific cytolytic cells were generated by culturing nonimmune C3H/HeN spleen cells for 6 d with 2,000-rad x-irradiated BALB/c spleen cells. Cytotoxicity was determined by the ability of effectors to lyse ^{51}Cr-labeled target cells during a 6 h assay as previously described (6). The percentage of specific lysis was calculated by the formula: [(experimental release − spontaneous release)/(total release − spontaneous release)] × 100.

Adoptive Transfer Assay. Lethally (800 rad) x-irradiated mice received, 3–24 h later, spleen cells, intravenously, and tumor fragments, subcutaneously. Spleens were removed from donor mice using sterile techniques, homogenized in a glass tissue grinder, filtered through nylon mesh, centrifuged, and resuspended in CMEM. Recipients received one-third organ equivalents (about 3×10^7 spleen cells) in 0.25 ml CMEM. In some experiments, the spleen cells of the tumor-bearing animals were treated with designated antibodies and complement or complement alone before transfer. Absorbed rabbit complement and monoclonal antibody at approproate concentrations were added to 10^7 spleen cells in 1 ml CMEM. The mixture was incubated for 30 min at 37°C, then centrifuged, resuspended, and treated again with the complement and antibody the same way. Monoclonal anti-Thy-1.2 (AT83A) and anti-Ly-2.1 (GS3.168) supernatants (8) (gift of Dr. Frank Fitch, University of Chicago) were used at a dilution of 1:10, which eliminates standard

control cells. Adherent cells were removed by suspending spleen cells at 10^8 cells/ml CMEM into which 200 mg of sterile carbonyl iron particles had been added. The suspension was placed in a 10 cm diam petri dish and incubated 60 min at 37°C; the iron was then moved to one side with a strong magnet and the remaining nonadherent cells were removed, centrifuged, and washed once in CMEM.

Results

Successive Development of Progressing Tumors at Anatomically Distinct Injection Sites in the Same Animal. When 100 normal immunocompetent mice were engrafted in both the right and left inguinal regions with the regressor tumor 1591-RE (fifth transplant generation in x-irradiated thymectomized mice), 95 mice rejected both tumor challenges. Curiously, in 5 mice, when progressively growing tumors arose at one of the two engraftment sites, a second progressively growing tumor also developed at the other injection site 2–3 wk later. Probability analysis[2] (9) showed that this phenomenon is incompatible with a model in which the occurrence of a second tumor is independent of the occurrence of the first, suggesting that either the recipient mice showing progressive tumor growth had less immunological resistance than the rest of the mice, or that growth of the first malignancy promoted the growth of the other later-appearing tumor. When the bilateral tumors were individually transplanted into normal mice the tumor that had appeared first always grew out at a higher incidence than the tumor that had emerged later. The comparative tumor incidences for the first and second tumors reisolated from each of the three mice tested and transplanted into normal mice were 6/12 vs. 1/12, 8/10 vs. 4/10, and 5/6 vs. 2/6, while the same tumors transplanted into nude mice as controls showed no differences (5/5 vs. 5/5, 4/4 vs. 4/4, and 2/2 vs. 2/2). In summary, an average of 68% of first-appearing tumors grew while only 25% of grafts from the later tumors grew in normal mice ($P < 0.01$), suggesting that the second-appearing tumors were less malignant and possibly more immunogenic than the first-appearing tumor.

The above experiments suggested the possibility that an established tumor may facilitate the growth of a second tumor in the same host. In the following experiments, we therefore determined whether the 1591-PRO tumor would grow faster in mice already carrying an established 1591-PRO tumor at a second site. Thus, 5×10^6 1591-PRO tissue culture cells were injected into normal tumor-free mice or into 1591-PRO TBA. In two independent experiments, the tumor inocula grew significantly faster in the TBA and achieved volumes in 1 mo that were two to six times greater than those in the controls.[3] Thus, animals already bearing a tumor on one site show an enhanced growth of a second tumor transplanted later into the same host at a different site.

Regressor Cells Grew Progressively in Animals Bearing a Progressor Tumor. The above experiments suggested that an established progressor tumor could facilitate the growth of a second progressor tumor in the same animal. We next determined the strength of this facilitation and its specificity. We analyzed first

[2] Assuming that cases of progressive tumor growth are independent of one another, the probability that the second five tumors would emerge in the animals growing the first five tumors is: $P = (5 \times 4 \times 3 \times 2 \times 1)/(100 \times 99 \times 98 \times 97 \times 96) = 1.3 \times 10^{-8}$.

[3] 0.99 ± 0.14 cm^3 for TBA compared with 0.44 ± 0.15 cm^3 for controls (7 mice per group); and 1.20 ± 0.59 cm^3 for TBA compared with 0.23 ± 0.06 cm^3 for controls (5 and 3 mice per group); $P < 0.01$ in both experiments using Student's t test.

whether the facilitation was strong enough to allow the outgrowth of even highly immunogenic 1591-RE tumor cells. Table I shows that 1591-RE regressor cells indeed developed into progressively growing tumors in most of the 1591-PRO-bearing mice but failed to grow progressively in normal mice. Since 1591-PRO was derived from 1591-RE and thus belongs to the same tumor lineage, we explored whether regressor tumor cells other than 1591-RE could also be made to grow out in mice bearing 1591-PRO. Table I shows that the 1591-PRO tumor–bearing state did indeed facilitate the outgrowth of two other syngeneic tumors that ordinarily regress; however, 1591-PRO TBA rejected the allogeneic mastocytoma P815.

These experiments showed that 1591-PRO facilitated the outgrowth of several independently derived syngeneic regressor tumors. We next determined (Table II) whether progressor tumors other than 1591-PRO could also induce such facilitation. We found that this was indeed the case for most progressor tumors whether they had been induced by either UV or MCA or whether the progressor phenotype had been found in the primary isolate or had developed as a variant during subsequent transplant generations. Viewed together, these experiments suggest (*a*) that facilitation of tumor growth is very strong since it causes the outgrowth of highly immunogenic, potentially malignant cells, and (*b*) that this facilitation is induced by most independently derived tumors. However, there were certain exceptions to this general rule: For example, 2237-PRO facilitated the outgrowth of both 1591-RE and 1130-RE, while 1130-PRO facilitated growth of 1591-RE but not 1130-RE. Such exceptions will be discussed further below. Also, Table III shows that the progressor tumors must reach a certain volume before they effectively facilitated the outgrowth of a later challenge with regressor cells. However, multiple small tumors that had grown for a short time were as effective as single large tumors that had grown for a longer time. Thus, the size of the burden was more important than the duration of the progressor tumor implant in facilitating the outgrowth of regessor tumor cells.

TABLE I

Highly Immunogenic, Potentially Malignant Cells Grow in Tumor-bearing but Not Normal Mice

Tumor challenge*	Tumor incidence in:‡		
	Nude mice	Normal mice	TBA
1591-RE	8/8 (100)	0/20 (0)	27/30 (90)§
1316-RE	9/9 (100)	0/10 (0)	17/21 (81)§
2240-RE	2/2 (100)	0/5 (0)	2/3 (67)
P815 (Allogeneic)	7/7 (100)	0/14 (0)	0/10 (0)

* Three 1-mm^3 regressor tumor fragments were injected subcutaneously into normal mice, nude mice, or mice that had borne 1591-PRO for 3–4 wk (average volume, 0.5 cm^3). All tumors were syngeneic, UV-induced regressor fibrosarcomas, with the exception of P815, which is an allogeneic ($H\text{-}2^d$) spontaneous mastocytoma.

‡ Numbers of animals with progressively growing tumors per number challenged (percent in parentheses). Tumor incidence was determined 5 wk after challenge with regressor tumor. All animals that had tumors at 5 wk eventually died because of progressive tumor growth. Data are pooled from four experiments for 1591-RE, three experiments for 1316-RE, two experiments for P815, and one experiment for 2240-RE.

§ Tumor incidence differs significantly from that in normal mice ($P < 0.001$).

TABLE II

Several Different Progressor Tumors Can Facilitate the Outgrowth of Highly Immunogenic Tumor Cells

Progressor tumor burden*		Regressor tumor challenge‡	
Name	Inducing agent	Name	Incidence
1316-PRO	UV	1591-RE	12/20 (60)§‖
		1316-RE	3/8 (38)§
None		1591-RE	1/8 (13)‖
		1316-RE	0/7 (0)
2237-PRO	UV	1591-RE	8/10 (80)§
		1130-RE	8/10 (80)§
None		1591-RE	0/5 (0)
		1130-RE	0/5 (0)
968-PRO	UV	1591-RE	5/13 (38)§‖
		1130-RE	3/8 (38)§‖
None		1591-RE	0/5 (0)‖
		1130-RE	0/5 (0)‖
948-PRO	UV	1591-RE	0/9 (0)‖
None			0/9 (0)‖
1130-PRO	MCA	1591-RE	5/10 (50)§‖
		1130-RE	0/10 (0)‖
None		1591-RE	1/10 (10)‖
		1130-RE	0/10 (0)‖
3152-PRO	MCA	1591-RE	6/10 (60)§
None			0/5 (0)
1591-ASBS	UV	1591-RE	3/5 (60)§
None			0/5 (0)

* Mice were challenged with progressor tumors that grew to at least 0.5 cm^3 before the host was challenged with regressor tumors. Progressor tumors were induced by ultraviolet light (UV) or MCA.

‡ Animals received three 1-mm^3 regressor tumor fragments subcutaneously in the inguinal region. Tumor incidence is the number of animals with growing regressor tumors 4 wk after challenge per number of animals challenged (percent in parentheses). The regressor tumors continuously grew until death of the animals.

§ Tumor incidence in the TBA significantly greater than in normal mice ($P < 0.05$).

‖ Data represents two independent experiments.

Regressor Tumors Growing Out Under the Protection of Progressor Tumors Retain their Highly Immunogenic Regressor Phenotype. We have previously shown that regressor tumors which upon rare occasions develop into progressively growing tumors in normal animals are always variants that have a heritable loss of an antigen. In contrast, regressor tumors growing out of nude athymic or x-irradiated thymectomized mice always retain their tumor-specific antigens (6). This failure of the latter mice to select for antigen loss variants seems to be a

TABLE III

Failure of the Tumor-bearing Host to Reject Regressor Tumor Cells Depends Upon the Size of the Tumor Burden

Exp.	Tumor burden				Incidence of 1591-RE*
	Name of tumor	Number of tumors	Duration of growth	Total volume	
			wk	cm^3	%
1‡	—	0	—	0	0/5 (0)
	1591-PRO	1	2	0.1	0/5 (0)
	1591-PRO	3	2	0.5	4/7 (57)§
2‖	—	0	—	0	1/29 (3)
	1591-PRO	1	2	0.1–0.2	2/19 (11)
	1591-PRO	1	2–3	0.4–1.5	11/15 (73)¶
	3152-PRO	1	1	0.1–0.2	1/7 (14)
	3152-PRO	1	2	>1.0	5/10 (50)¶

* Mice were engrafted with 1591-PRO at a single site or at three distinct sites. After the indicated duration of growth the individual tumors were measured, and then 1591-RE fragments were given to all of the animals.

‡ Number of mice growing 1591-RE per number of mice challenged (percent in parentheses). Tumor incidence assessed 1 mo after challenge.

§ Tumor incidence significantly greater than in mice bearing no progressor tumor ($P < 0.05$).

‖ Mice were given either 1591-PRO or 3152-PRO. After a period of progressor tumor growth they were challenged with three 1-mm^3 fragments of 1591-RE. Data are pooled from seven experiments.

¶ Tumor incidence significantly greater than in mice bearing no progressor tumor ($P < 0.01$).

TABLE IV

Normal Mice Reject Reisolates of 1591-RE Tumors that Grew Progressively in 1591-PRO TBA

Immunodeficient host*	Name of inoculum	Name of reisolate	Tumor incidence of reisolates in:‡	
			Normal mice	Nude mice
1591-PRO TBA	1591-RE	1591-PB.1	2/28 (7)	9/9 (100)
		1591-PB.2	0/10 (0)	4/4 (100)
		1591-PB.3	0/4 (0)	4/4 (100)
		1591-PB.4	0/4 (0)	4/4 (100)
		1591-PB.5	0/5 (0)	4/4 (100)
Nude	1591-RE	1591-RE (Nu)	0/19 (0)	6/6 (100)
	1591-PRO	1591-PRO (Nu)	30/32 (94)	4/4 (100)

* 1591 tumors which had grown for at least 4 wk in TBA or nude mice were reisolated, and injected as three 1-mm^3 fragments into normal or nude mice.

‡ Number of tumors growing per number of mice challenged (percent in parentheses) at 5 wk after challenge. Tumors grew until death of the host.

consequence of their severe immunodeficiency. We analyzed, therefore, the antigenic phenotype of the tumors growing out of progressor tumor–bearing mice in order to assess further the strength of immunoselection exerted by such hosts. Table IV shows that 1591-RE tumors reisolated from 1591-PRO tumor–bearing mice after a growth period of at least 4 wk were regularly rejected by normal mice, showing that the reisolated tumors retained the 1591-RE phenotype. To investigate whether these tumors also retained the 1591-RE tumor–

specific antigen, we tested the capacity of these tumor cells to restimulate tumor antigen–specific CTL in culture or to serve as targets for such T cells. Table V shows that all the 1591-RE tumors reisolated from TBA could restimulate 1591-RE-specific CTL in culture as effectively as the parental 1591-RE tumor cells, whereas 1591-PRO variants reisolated from normal hosts failed to stimulate. The retention of the target antigen is further demonstrated in Fig. 1, which shows that 1591-RE tumors reisolated from TBA were as effectively killed by 1591-RE-specific T cells as the 1591-RE tumor, even when reisolated from TBA bearing an unrelated progressor tumor, the methylcholanthrene (MCA)-induced fibrosarcoma 3152 (Fig. 1, *right*). Thus, like severely immunodeficient mice, mice bearing progressively growing tumors neither reject highly immunogenic tumor cells nor select for antigen loss variants.

Regressor Tumors Growing Out Under the Protection of Progressor Tumors Continue to Grow After the Complete Surgical Removal of the Progressor. Since the progressor tumor was necessary for the outgrowth of the regressor and the regressor retained the regressor phenotype, it seemed reasonable to determine whether surgical removal of the progressor tumor would result in the regression of the immunogenic tumor. Thus, 15 animals were injected with the 1591-PRO tumor and, when the tumors had grown to >0.5 cm^3, the animals were challenged at a second subcutaneous site with a 1591-RE regressor tumor. 2 wk later, the progressor tumor was completely excised surgically. To our surprise, we found in two independent experiments that 15 of 15 1591-RE tumors continued to grow (even five 1591-RE tumors <0.2 cm^3) until the demise of the mice 3–6 wk later. Tumors were removed from three moribund animals; all three retained the regressor phenotype being rejected by normal mice in vivo and remaining sensitive to the tumor-specific cytolytic cells in vitro (data not shown).

TABLE V

Generation of 1591-RE-specific Immunity by Spleen Cells of Mice Immunized with Reisolates of the "Enhanced" 1591-RE Tumors

Immunogen*	Stimulator	Lysis of target cells (percent)							
		1591-RE	1591-PB				1591-PRO	2240-RE	1316-RE
			1	2	3	5			
1591-RE	1591-RE	84 ± 5‡	81 ± 11	94 ± 8	82 ± 4	98 ± 7	9 ± 1	1 ± 2	7 ± 2
	1591-PB.2	91 ± 3	88 ± 19	96 ± 1	87 ± 3	81 ± 2	7 ± 2	3 ± 0	4 ± 1
	1591-PB.3	90 ± 14	80 ± 7	90 ± 14	85 ± 3	84 ± 9	9 ± 1	1 ± 2	0 ± 7
	1591-PRO	2 ± 2	9 ± 2	9 ± 2	9 ± 2	8 ± 4	5 ± 3	0 ± 2	6 ± 5
1591-PB.2	1591-RE	84 ± 6	89 ± 13	99 ± 4	86 ± 9	90 ± 14	8 ± 3	0 ± 2	4 ± 2
	1591-PB.2	80 ± 4	81 ± 4	98 ± 7	91 ± 0	99 ± 14	13 ± 3	3 ± 1	5 ± 2
1591-PB.3	1591-RE	81 ± 9	87 ± 16	93 ± 9	83 ± 6	82 ± 23	10 ± 2	1 ± 2	4 ± 2
	1591-PB.3	80 ± 5	85 ± 6	99 ± 6	84 ± 7	99 ± 14	12 ± 2	2 ± 1	5 ± 2
1591-PB.4	1591-PB.4	80 ± 4	85 ± 9	99 ± 7	89 ± 5	99 ± 16	12 ± 2	2 ± 1	2 ± 1
1591-PRO	1591-RE	19 ± 5	11 ± 5	17 ± 4	13 ± 3	14 ± 4	10 ± 3	12 ± 5	11 ± 4
	1591-PRO	10 ± 2	19 ± 5	16 ± 3	14 ± 1	10 ± 3	10 ± 3	10 ± 3	10 ± 3

* C3H mice were injected with 10^7 1591-RE, 1591-PB, or 1591-PRO tumor cells subcutaneously. 3 wk later, their spleen cells were restimulated with these tumor cells, as indicated, in a 6 d MLTC, and then tested against the indicated target cells in a 6 h ^{51}Cr-release assay (effector/target ratio, 5:1).

‡ Mean ± SE for four separate experiments.

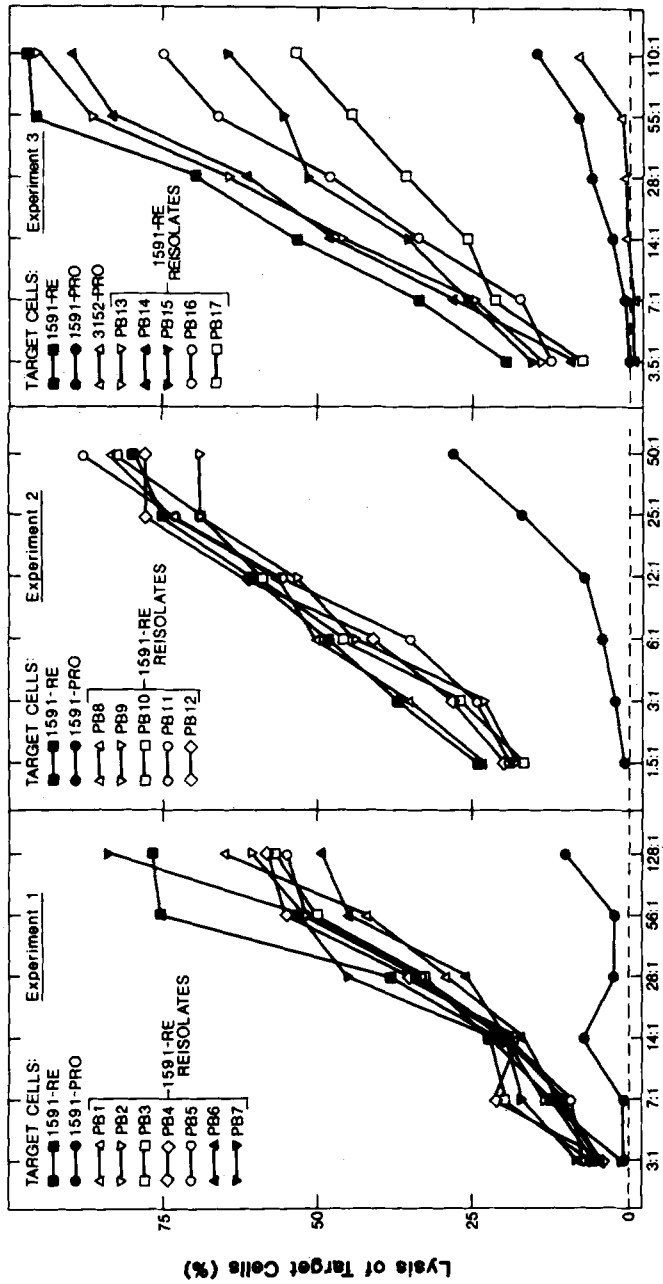

FIGURE 1. Retention of 1591-RE tumor-specific antigens by reisolates of the 1591-RE tumor that have grown progressively in 1591-PRO (*left* and *middle*) or 3152-PRO (*right*) progressor tumor–bearing animals. The enhanced 1591-RE tumor cells grew for at least 1 mo in the progressor tumor–bearing animals before they were removed, adapted to tissue culture, and used as ^{51}Cr-labeled targets for 1591-RE-specific CTL in a 6 h assay. Effector cells were generated in a standard MLTC using 1591-RE cells for in vivo immunization as well as for in vitro restimulation. Identical results were found in a fourth experiment using six additional 1591-RE reisolates (not shown).

Facilitation Is Transferable by Spleen Cells and Is Not Accompanied by Generalized Immunodeficiency. We next examined whether the tumor-susceptible state induced by the growth of the progressor tumor could be transferred by spleen cells. The model we used for these studies was based upon the previous finding (4) that lethally irradiated mice reconstituted with normal unimmunized spleen cells regularly reject a subsequent challenge of a UV-induced regressor tumor. We tested here whether cotransfer of spleen cells from TBA could suppress this T cell–mediated protection. Thus, both lymphocyte populations were injected intravenously into lethally irradiated mice that were then challenged with a progressor tumor. In five experiments (Table VI), we found that spleen cells from tumor-bearing animals could completely suppress the resistance to 1591-

TABLE VI

Spleen Cells from TBA Suppress the Tumor Resistance Conferred by Normal Spleen Cells But Not the Resistance Conferred by Immune Spleen Cells

Exp.	Sources of transferred spleen cells*	Tumor incidence of 1591-RE‡
1	Normal	0/5 (0)
	TBA + normal	5/5 (100)
2	Normal	0/5 (0)
	TBA + normal	5/5 (100)
3	Normal	0/5 (0)
	Immune	0/4 (0)
	TBA + normal	5/5 (100)
	TBA + immune	0/5 (0)
4	Normal	1/4 (25)
	TBA	3/3 (100)
	TBA + normal	2/2 (100)
5	Immune	0/2 (0)
	TBA + immune	0/2 (0)
Total	Normal	1/19 (4)§
	TBA + normal	17/17 (100)§
	Immune	0/6 (0)
	TBA + immune	0/7 (0)

* 800-rad–irradiated mice received intravenously 3×10^7 spleen cells from normal, tumor-free mice, 3×10^7 spleen cells from immune mice which had rejected 1591-RE fragments 6 wk earlier, 4×10^7 spleen cells from TBA animals having borne the tumor 1591-PRO for 1 mo, or mixtures of 3×10^7 normal or immune spleen cells and 4×10^7 TBA spleen cells.

‡ Number of mice with growing 1591-RE tumor per number of mice challenged (percent in parentheses). Three 1-mm^3 1591-RE tumors were injected subcutaneously at one site and tumor incidence was measured at 4 wk.

§ Tumor incidence significantly greater in mice receiving both normal and TBA spleen cells than in recipients of only normal spleen cells ($P < 0.001$).

RE conferred by normal spleen cells but not that conferred by immune spleen cells. Table VII summarizes further adoptive transfer experiments in which spleen cells from tumor-bearing mice were subjected to various procedures that eliminate different cell types, and then assayed for cells suppressing the tumor rejection capacity of normal spleen cells when cotransferred into irradiated recipients. We found that treatment with anti-Thy-1.2 and complement (C′) or with x irradiation (450 rad) eliminated suppression, while treatment with carbonyl iron or with anti-Ly-2.1 and C′ did not. The fact that conventional T cells were needed for inducing the suppression is also indicated by the fact that spleen cells from nude TBA fail to cause suppression upon transfer (Table VII, Exps. 8 and 9).

As would be expected from our previous results, the suppression mediated by spleen cells of 1591-PRO TBA was not 1591 tumor–specific, since such spleen cells also suppressed the rejection of another UV-induced regressor tumor (data not shown). TBA, however, did not have suppressed responses to nontumor antigens. For example, five animals bearing a 1591-PRO tumor for 1 mo (tumor volume, >1.0 cm^3) were immunized intravenously with 0.2 ml of a 5% (vol/vol) suspension of sheep red blood cells in sterile phosphate-buffered saline, and plaque-forming responses were assayed 4 d later using standard procedures (10).

TABLE VII

Phenotype of the Suppressor Cells Isolated from Tumor-bearing Mice

Spleen cells transferred*	Incidence of 1591-RE tumors in experiments‡								
	1	2	3	4	5	6	7	8	9
Normal	0/5	0/4	1/3	1/3	0/5	0/4	0/4	0/3	0/5
Normal + untreated§ TBA	—	—	3/3	3/3	—	—	4/4	3/5	3/5
Normal + C′-treated TBA	5/5	2/4	—	—	5/5	2/3	—	—	—
Normal + anti-Thy-1 + C′-treated TBA	1/5	0/4	—	—	—	—	—	—	—
Normal + carbonyl iron–treated TBA	—	—	3/3	3/3	—	—	—	—	—
Normal + anti-Ly-2 + C′-treated TBA	—	—	—	—	4/4	2/4	—	—	—
Normal + 450-rad–treated‖ TBA	—	—	—	—	—	—	1/4¶	—	—
450-rad–treated immune‖	—	—	—	—	—	—	0/4	—	—
Normal + untreated nude TBA	—	—	—	—	—	—	—	0/5	0/5

* Recipient mice received 800 rad of x radiation and were then intravenously infused with 3×10^7 spleen cells from normal, tumor-free mice or 3×10^7 normal spleen cells plus 4×10^7 (treated or untreated) spleen cells from animals bearing progressively growing tumors.

‡ Number of mice growing 1591-RE per number of mice challenged. Mice received three 1-mm^3 1591-RE fragments subcutaneously. Tumor incidence was measured 1 mo after challenge.

§ In cases in which TBA spleen cells were treated with antibody and C′, "untreated" TBA cells were incubated with C′ only.

‖ TBA spleen cells received 450 rad of x radiation in vitro immediately before their infusion. Alternatively, a group of mice was reconstituted with 3×10^7 spleen cells from 1591-RE tumor–immune mice that had received 450 rad in vivo to rule out nonselective radiolysis of spleen cells.

¶ Tumor incidence in group receiving treated TBA spleen cells was significantly less than in group receiving untreated TBA spleen cells ($P < 0.05$).

The TBA had greater responses than four age-matched, tumor-free control animals (218,273 ± 26,000 [SEM] vs. 111,045 ± 15,800). Unimmunized animals, normal or tumor bearing, produced no anti–sheep red blood cell plaques. In other experiments (Table VIII), we found that the proliferative response of spleen cells from TBA to alloantigens was as vigorous as that of normal spleen cells, and that there was no suppression of the cytolytic response to alloantigens (Fig. 2). This is consistent with our previously mentioned finding (Table I) concerning the normal transplantation resistance of tumor-bearing animals to the allogeneic P815 mastocytoma.

Although TBA are not in a state of generalized immune deficiency, the experiments do not rule out the possibility of a generalized suppression of responses to *syngeneic* tumor antigens. We therefore carefully examined the specificity of rejection of a syngeneic regressor tumor (e.g., the MCA-induced tumor, 1130-RE) which, unlike most syngeneic regressor tumors, failed to be suppressed by a given progressor tumor (i.e., 1591-PRO). Mice bearing 1591-PRO were simultaneously engrafted with three tumors: 1591-RE, 1130-RE, and a mosaic tumor reisolated from a nude mouse that had been injected with a 1:1 mixture of 1591-RE and 1130-RE cells. Also challenged were 1591-RE-immunized mice that had subsequently received 450 rad of x irradiation; such mice can still reject tumors against which they have previously been immunized but they are incapable of mounting effective rejection responses de novo against other tumors (5). The results (Table IX) show that (*a*) normal mice rejected all

TABLE VIII

Proliferative Responses of Normal and TBA Spleen Cells to Alloantigens

Exp.*	Responder‡	Average [^{3}H]thymidine uptake§ (cpm ± SEM)		Net proliferation‖
		Anti-BALB/c	Anti-C3H	
1	Normal C3H	33,509 (±1,059)	4,102 (±347)	29,403
	TBA C3H	35,728 (±1,108)	8,727 (±521)	27,001
2	Normal C3H	99,489 (±2,048)	59,229 (±2,292)	40,260
	TBA C3H	81,226 (±5,995)	35,397 (±3,346)	45,829

* Spleens were sterilely removed, homogenized with a glass grinder, and suspended in medium consisting of RPMI 1640 supplemented with 5% fetal bovine serum, 0.1 mM MEM nonessential amino acids, 2 mM L-glutamine, 1% penicillin-streptomycin, and 5×10^{-5} M 2-mercaptoethanol. Stimulator cells received 2,000 rad of x radiation. 5×10^5 responding cells and 5×10^5 stimulator cells were incubated at 37°C in 0.2 ml of medium in flat-bottomed microwells. After 4 d, 2 μCi of methyl-[^{3}H]thymidine in 25 μl of RPMI 1640 was added to each well and harvested 24 h later.

‡ Responding spleen cells came from tumor-free C3H mice or from mice that had borne 1591-PRO for at least 1 mo.

§ Proliferation of responders against irradiated BALB/c or irradiated C3H/HeN spleen cells was measured on day 4. Values represent the average uptake of [^{3}H]thymidine by 8–10 replicate cultures in counts per minute (cpm) (SEM in parentheses) pulsed on day 4 and measured on day 5 by liquid scintillography.

‖ Net proliferation: proliferation against allogeneic BALB/c cells − proliferation against syngeneic C3H cells (autologous MLR). In all cases proliferation against BALB/c spleen cells was significantly ($P < 0.001$) greater than against C3H/HeN cells. Responses of normal and TBA cells do not differ significantly ($P > 0.05$).

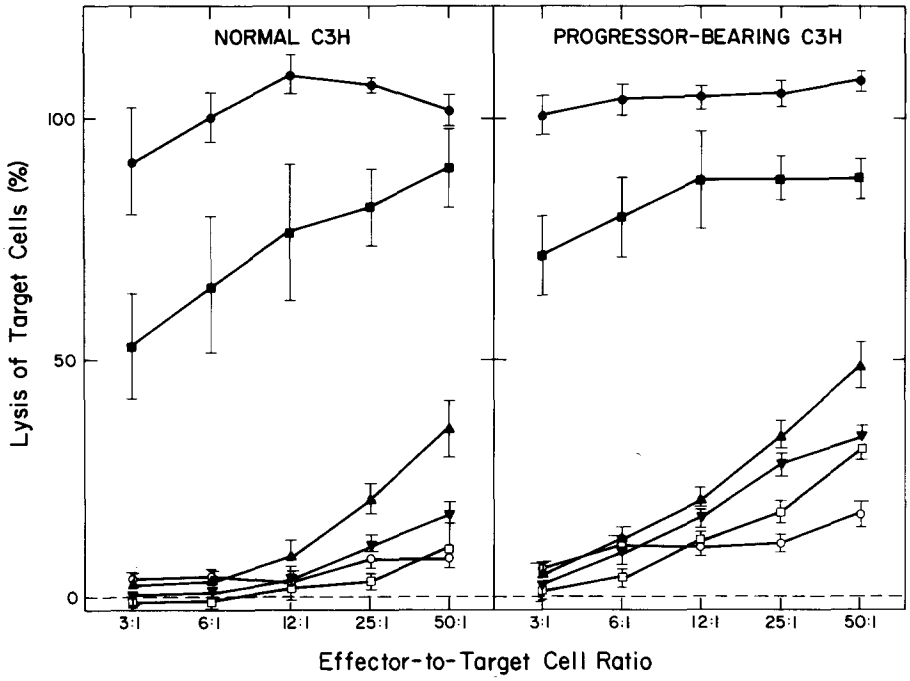

FIGURE 2. Retention of specific alloreactive CTL responses in progressor tumor-bearing animals. C3H mice were untreated (normal C3H) or challenged with two 1-mm^3 fragments of the 1591 progressor tumor 1591-PRO, 4 wk previously (progressor-bearing C3H). Spleen cells from these animals were stimulated in culture with irradiated (2,000 rad) BALB/c (H-2^d, closed symbols) or C3H (H-2^k, open symbols) spleen cells for 6 d and then tested in a 6 h ^{51}Cr-release assay against P815 (H-2^d; ●, ○), K101-RE (H-2^d; ■, □), 1591-RE (H-2^k; ▲) or EL4 (H-2^b; ▼) target cells. Data represent the mean ± SE for four separate experiments. The yield of viable cells recovered from day 6 for BALB/c stimulated cultures was 41 ± 4% of the original spleen cell input for normal C3H spleen cells and 30 ± 3% for progressor-bearing C3H spleen cells.

three grafts, (*b*) the 1591-PRO TBA grew out the 1591-RE and mosaic tumors but rejected 1130-RE, and (*c*) the 1591-RE-immunized and then irradiated mice grew out the 1130-RE and mosaic tumor but rejected 1591-RE. The mosaic tumors were reisolated and their composition analyzed. Fig. 3 shows that cells reisolated from the tumors emerging from the 1591-PRO TBA challenged with the mosaic tumor were as susceptible to lysis by 1591-RE-specific cytolytic cells as a homogeneous population of 1591-RE control cells. In contrast, cells reisolated from tumors emerging from irradiated 1591-RE-immune mice and challenged with the mosaic were as resistant to lysis as homogenous populations of 1130-RE cells, indicating that only these cells survived. Lysis of control cells reisolated from a nude mouse injected with the mosaic tumor was intermediate (Fig. 3), indicating that in these mice cells of both tumors survived. Together, the experiments show that TBA do not have a generalized immune suppression to all syngeneic tumors.[4] However, the failure of 1591-PRO TBA to suppress

[4] The resistance of the 1130-RE tumor rejection to the suppression in 1591-PRO TBA does not seem to be due to an increased immunogenicity of the 1130-RE tumor as compared with other regressor tumors that are susceptible to this suppression; at least, rejection of 1130-RE is not more resistant to immune suppression by graded doses of x rays than rejection of other regressor tumors.

TABLE IX

Growth of 1591-RE, 1130-RE, and Mosaic 1591-RE/1130-RE Tumors in Tumor-bearing and Immune Irradiated Animals

Host	Tumor incidence[‖]		
	1591-RE	1130-RE	Mosaic[¶]
Normal	0/5 (0)	0/5 (0)	0/5 (0)
Normal given 450 rad*	2/2 (100)	2/2 (100)	2/2 (100)
1591-PRO TBA[‡]	9/9 (100)	0/9 (0)	9/9 (100)
1591-RE-immune given 450 rad[§]	0/8 (0)	8/8 (100)	8/8 (100)

* Mice received 450 rad of gamma radiation 24 h before tumor challenge.

‡ Mice had borne 1591-PRO for 3 wk.

§ Mice had rejected 1591-RE fragments 1 mo earlier and received 450 rad 24 h before tumor challenge.

‖ Number of animals with growing tumors at each particular injection site 1 mo after challenge per number of animals challenged (percent in parentheses). All mice with tumors at 1 mo died from progressive tumor growth. Three 1-mm^3 fragments of each tumor were injected subcutaneously into each mouse; 1130-RE was placed on one flank, 1591-RE on the other, and the mosaic on the ventral midline.

¶ 5×10^6 1591-RE cells and 5×10^6 1130-RE cells were mixed and injected into a nude mouse. After 3 wk the tumor was removed, minced, and injected into these mice.

the rejection of the syngeneic tumor 1130-RE is one of the exceptions to the general rule that TBA usually facilitate the outgrowth of other syngeneic regressor tumors, as shown earlier in this report.

Discussion

This study shows that the presence of an established tumor can favor decisively the outgrowth of a second tumor at a different location. This enhancement appears to be a consequence of an immunological suppression caused by the established tumor, since the potentially malignant cells forming the second tumor in the TBA do not normally grow in immunocompetent mice. Furthermore, theta-bearing spleen cells from TBA, but not spleen cells from nude TBA, suppressed tumor rejection by normal spleen cells in adoptive transfer experiments. The suppression was caused by several different progressor tumors and affected the rejection of several different regressor tumors regardless of whether they had been induced by UV radiation. This broad crossreactivity of the described T cell–mediated suppression is in striking contrast to cytolytic and helper T–mediated immunity, which we found to be exceedingly tumor-specific for individual tumors despite extensive testing for crossreactivity (7, 11, 12, and unpublished results). The type of suppression described here also appears to be different from another type of tumor-induced immune suppression that affects immune rather than nonimmune T cells and which has specificity for an individual tumor (13–16). In many respects, however, the type of suppression we have observed in TBA simulates the suppression caused by UV radiation (5, 17–21). For example, UV-induced suppression also (*a*) permits the outgrowth of highly immunogenic UV-induced regressor tumor cells, (*b*) is not individually tumor-specific and affects UV-induced as well as non–UV-induced tumors, (*c*) does not affect allogeneic or other humoral responses, (*d*) is transferable by Ly-2^- T cells,

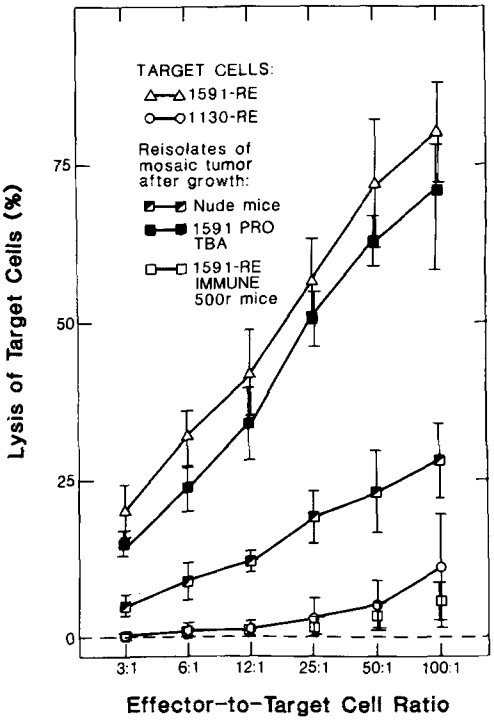

FIGURE 3. Lysis of mosaic tumors by 1591-RE-specific CTL. After adaptation of mosaic tumors to tissue culture they were used as targets for 1591-RE-specific cytolytic cells in a 6 h ^{51}Cr-release assay. 1591-PRO TBA or 1591-RE-immune x-irradiated (500 rad) mice were challenged with fragments of 1591-RE, 1130-RE, or a mosaic tumor composed of approximately equal parts of 1591-RE and 1130-RE, generated by coinjection of the two cell lines into the same site in nude mice. One month later, the progressively growing tumors were excised. Tissue culture stock 1591-RE and 1130-RE cells were used as control targets.

and (*e*) suppresses tumor rejection mounted by naive, unimmunized lymphoid cells but not the protection conferred by lymphoid cells from mice preimmunized to a particular tumor. There are even certain similarities in the exceptions to the crossreactivity of the two types of suppression, such as the failure of the MCA-induced regressor tumor 1130-RE to be enhanced by UV-induced suppression (22) or by TBA bearing certain progressor tumors. Together, these rather striking similarities suggest that progressively growing tumors and UV irradiation might bring about a similar suppression of the immune system. Indeed, in both cases, the immune system is confronted with a large number of altered cells, and it might be that UV irradiation and neoplastic transformation of cells leads to the expression of gene products that suppress the immune system in a similar way. Consistent with such a notion are experiments showing that transplantation of UV-irradiated skin can suppress tumor rejection (23). The fact that tumors other than those induced by UV radiation also induce, and are susceptible to, this type of suppression indicates its general relevance to tumor immunology.

The tumor-induced immune suppression in the TBA can be even stronger than that induced by UV irradiation, because UV-irradiated hosts still select for antigen loss variants (11) while TBA do not. This difference, however, may

simply be due to the larger numbers of altered cells to which the TBA is exposed, since the degree of suppression was clearly dependent upon the size of the tumor burden. Thus, only after the first tumor had reached a critical (>0.5 cm^3) size was the host's ability to reject highly immunogenic cells abrogated, and the cells were able to develop into a second tumor without undergoing measurable immunoselection by the host. The failure of immune selection was indicated by the fact that these second tumors were rejected regularly upon further transplantation to normal syngeneic hosts at any testable dose of tumor fragments or cells (>1 × 10^8 cells). The observed strength of suppression and the lack of immunoselection raise the possibility that second primary malignancies in man may be more antigenic than the first cancer and thus may be more susceptible to passive immunotherapy. For active immunotherapy, however, the observed strength and crossreactivity of the immune suppression may well represent a major difficulty. For example, it has been suggested that immunogenic regressor tumor variants obtained after in vitro mutagenesis could be used for active immunotherapy (24, 25). If our results are generalizable, such an approach is not likely to succeed in tumor-bearing individuals since such regressor variants might both grow out in the patient and even maintain the tumor-induced suppression. We observed that this maintainance of suppression occurred even after the first tumors were completely removed. Furthermore, we found that the second regression tumor continued to grow, even though some of them had reached sizes of only 0.2 cm^3 or less at the time of removal of the first tumor. This small size might correspond to a size that is clinically undetectable in humans. At present, we do not know how the second (regressor) tumor can maintain the suppression induced by the progressor tumor, but we have recently found that this suppression can be maintained in serial passage through animals bearing only the regressor tumor (C. A. Mullen, unpublished observation). It will be important to learn how to overcome this immune suppression in the tumor-bearing host and to accomplish rejection of these extremely immunogenic tumors after they are well established, since otherwise the outlook for active immunotherapy of other cancers, many of which may be less immunogenic, is poor. One possibility is to develop regimens by which tumor immunity can be generated de novo from the tumor-bearing host's lymphocytes in vitro, followed by adoptive transfer into the tumor-bearing host (26), since we find that T cells from immunized mice are resistant to the suppression.

Several different mechanisms, such as increased genetic susceptibility to cancer development (27) or heavy exposure to carcinogens, may also cause the development of multiple malignancies in the same host; however, our model using genetically inbred mice and cells that have previously encountered a given carcinogen allows us to describe another independent pathway which is mediated by immune suppression. Although the specificity of the described suppression was rather broad, affecting most of the different tumors tested, there was no evidence for a generalized immune suppression in the TBA. The suppression did not affect allograft rejection in vivo, proliferative and cytolytic responses to alloantigens in vitro, nor humoral responses to sheep red blood cells; furthermore, there were exceptional combinations in which a given progressor tumor failed to enhance the outgrowth of a given regressor tumor. For example, TBA

growing out 1591-RE still rejected the syngeneic tumor 1130-RE not only at an anatomically distinct site but even admixed within the same (mosaic) tumor.

At present, we can only speculate about which antigens are involved in the crossreactive suppression of tumor-bearing animals. For example, immunosuppressive molecules that are shared by many murine and human tumors such as the P15E protein could be involved (28, 29). However, we have no evidence for the existence of a single antigen shared between the progressor tumors causing suppression and the suppressed regressor tumors, and it is equally possible that the suppressor T cells do not react with tumor antigen but instead with tumor-reactive T cells (30). The fact that certain tumors are distinct exceptions to the general rule of cross-suppression will help us to separate the observed suppression from other modes of immune suppression and to identify the responsible mechanism.

Summary

We demonstrate that tumor-bearing hosts permit the outgrowth of "potentially malignant" cells that are located at a different site. These second cancers continued to grow and kill their hosts even though they retain the "premalignant" phenotype, even after removal of the original malignancy. The potentially malignant cells used in these experiments were ultraviolet light– or methylcholanthrene–induced regressor tumor cells that are rejected regularly by normal mice at any testable dose, and only form progressive tumors in immunosuppressed individuals. The immunological rejection of these highly immunogenic, potentially malignant cells was suppressed by Thy-1^+, Ly-2^-, nonadherent, radiosensitive suppressor cells in the tumor-bearing mice. These suppressor cells were absent in nude tumor-bearing mice. Unlike helper and cytolytic T cell–mediated responses, which are exquisitely tumor specific, the suppression caused by a progressively growing tumor was crossreactive among many syngeneic, independently derived tumors induced by different carcinogens. However, T cell–mediated immune responses to alloantigens, allogeneic tumors, certain syngeneic tumors, and humoral responses to xenogeneic red blood cells were normal in these mice. The immune suppression in the tumor-bearing animals closely simulated that induced by ultraviolet light irradiation, and both types of suppression might therefore share common mechanisms. Our findings may contribute to understanding the growth, development, and possible control of multicentric malignancies and add a precaution to the potential use of strongly immunogenic tumor variants for active immunotherapy in hosts bearing less immunogenic tumors.

We thank Ms. Connie Philipps for technical support, Dr. Jose Quintans for reviewing the manuscript, and Ms. Carolyn Hence for typing.

Received for publication 23 July 1985.

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