IMMUNOSELECTION OF TUMOR CELL VARIANTS BY MICE SUPPRESSED WITH ULTRAVIOLET RADIATION*

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Mice that have been exposed to subcarcinogenic doses of ultraviolet radiation (UV)[1] are generally incapable of rejecting a highly immunogenic UV-induced tumors (1–4). Thus, transplanted fragments of these tumors, which are rejected regularly by normal syngeneic mice, grow progressively in a large proportion of UV-treated animals. This lack of tumor rejection has been attributed to the fact that UV has certain suppressive effects on the immune system, such as potentiating the induction of T suppressor cells (5, 6). These suppressor cells prevent the rejection of UV-induced syngeneic tumors, but not allogeneic tumors or non-UV-induced syngeneic tumors (6, 7).

Two important questions about these UV-induced suppressor cells remain unresolved. One concerns the type of immune response that is decreased or prevented by the suppressor cells; the second relates to the specificity of the reaction that ultimately is responsible for tumor rejection. In spite of the fact that UV-treated mice have suppressor T cells that inhibit tumor rejection, there is some evidence that such UV-treated animals can still mount certain immune responses to individually specific antigens on these tumors (3, 8, 9). Thus, it is possible that UV-induced suppressor cells prevent the reaction against common (cross-reactive) antigens shared among UV-induced tumors, and that these antigens are the only antigens that are important for tumor rejection; especially because cross-protection among UV tumors has been observed (8). An alternative hypothesis is that the regulatory immune responses to UV-induced tumors by suppressor or helper T cells are predominantly directed against the common UV antigen, whereas immunologic effector reactions (e.g., T cell-mediated cytotoxicity and delayed-type hypersensitivity) are predominantly directed against individually specific tumor antigens. This latter possibility is more in keeping with our previous studies (10–12) showing that selective elimination of tumor-specific immunity by anti-idiotype suppression or loss of the tumor-specific antigen is associ-

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[1] *Abbreviations used in this paper:* CLP, cytotoxic lymphocyte precursor; E/T, effector/target cell ratio; MLTC, mixed lymphocyte-tumor cell culture; UV, ultraviolet radiation.

ated with the progressive growth of these tumors in unirradiated mice.

In the studies reported here, we have begun to address these issues by examining the antitumor immune responses that are generated in UV-treated mice under conditions that do or do not lead to progressive tumor growth of the UV-induced tumor 1591-RE. Two different approaches were used: in the first, the immune responses to the tumors were determined using tumor resistance and cytolytic T lymphocyte reactivity as measures of immunity; in the second, an analysis of antigenicity and growth behavior was performed on tumors that were recovered after growth in UV-treated mice, to determine whether immunoselection had occurred. We show here that tumors reisolated after transplantation of the 1591-RE tumor into UV-suppressed mice have regularly lost a tumor-specific determinant that is uniquely expressed on the original 1591-RE tumor. This was in striking contrast to tumors reisolated after injection of 1591-RE into nude or anti-idiotypically suppressed mice. Furthermore, a large percentage of the tumors reisolated after transplantation into UV-suppressed mice had changed to progressor tumors that were no longer rejected by normal mice. Our results emphasize the importance of tumor-specific immunity and suggest that partial immune suppression such as that caused by UV may favor the frequent appearance of immunoselected progressor tumor variants from highly immunogenic regressor tumors such as 1591-RE.

Materials and Methods

Mice. 5–10-wk-old female C3H/HeN (mammary tumor virus-negative) mice from a colony of germ-free-derived, specific pathogen-free animals were purchased from the Frederick Cancer Research Facility. They were kept at the La Rabida Institute in laminar flow hoods and were given sterilized food (Teklad L-485 for autoclaving, Harlan Sprague Dawley Inc., Madison, WI) and water. UV treatment of mice was done at the Frederick Cancer Research Facility as described earlier (6). Briefly, beginning at 8 wk of age, mice were shaved weekly on the dorsum and housed 20 cm below a bank of six Westinghouse FS40 sun lamps. They were irradiated for 1 h three times per wk for 3 mo before their use at an irradiance of ~2 $J/m^2/s$. This UV treatment has been shown repeatedly to induce susceptibility to challenge with UV-induced tumors that lasts for at least 6 mo in the absence of further treatments (2). None of the animals had developed primary tumors from the UV at the time of these experiments. Age- and sex-matched untreated mice served as controls for the UV-treated group. The original stock of nude C3H mice was in the 23rd backcross generation when it was obtained from a colony at the Biology Division of the Oak Ridge National Laboratory.

Fibrosarcoma Lines. The fibrosarcomas 1591-RE, 1316-RE, and 2240-RE were induced in C3H/HeN (mammary tumor virus-negative) mice by repeated exposure to UV (2). These fibrosarcomas have non-cross-reacting tumor-specific transplantation antigens, and they are strongly immunogenic in that they regress when transplanted into young syngeneic mice, after an initial period of growth during the first 1–2 wk (6). Variant tumors 1591-PRO.1 to 1591-PRO.4 were derived from each of four progressively growing tumors observed in 5 out of 100 unirradiated animals implanted with fragments of the fifth transplant generation of the parental 1591 regressor tumor (referred to here as 1591-RE), as described previously (12). These progressor variant tumors no longer express the 1591-RE tumor-specific antigen (12). As control tumors, we also used four 1591 tumors, 1591-IS24T1 to 1591-IS24T2, that were reisolated from anti-idiotypically suppressed mice developing progressive tumor growth after injection of 1591-RE tumor cells (experiment 2 in ref. 10). All of the fibrosarcomas used in this study grow progressively in nude mice, syngeneic UV-treated mice, or thymectomized and x-irradiated mice, and eventually kill these hosts (2). Using previously described methods (12), tumors were adapted to growth in vitro, and expanded in tissue culture within 2 wk of explanation, and cryopreserved in aliquots (13) unless otherwise indicated. Whenever tumor cells were required for experiments, selected frozen aliquots were thawed and used within 24 h. For tumor

challenge, solid tumors grown in vivo were implanted as viable 1-mm^3 fragments subcutaneously with a trocar into both inguinal regions, or cell suspensions from cultured cells were injected intraperitoneally or subcutaneously underneath the ventral skin.

Generation of Cytotoxic Lymphocytes In Vitro and In Vitro. Tumor-specific cytotoxic T lymphocytes were generated from spleen cells of animals immunized with either two 1-mm^3 fragments of solid tumor implanted subcutaneously with a trocar, or a single subcutaneous injection of 10^7 viable tumor cells. Spleen cells from tumor-immune animals were restimulated in vitro in a mixed lymphocyte-tumor cell culture (MLTC) as previously described (12). The syngeneic continuous T cell lines were a gift of Richard D. Wortzel, University of Chicago, and were derived from syngeneic MLTC cells specific for determinants uniquely expressed by tumor cells of the 1591 tumor lineage (14); a detailed characterization of these T cell lines will be published separately (R. D. Wortzel, manuscript in preparation). Cytolytic peritoneal lymphocytes were induced by a modification of the method described previously by Newcomb et al. (15). Mice were injected once intraperitoneally with 1×10^7 cultured tumor cells, and 8 d later, nonadherent peritoneal cells were recovered and used as effectors for a 6-h ^{51}Cr-release assay as described previously (12).

Limiting Dilution Analysis. MLTC were set up as microcultures in 6 × 50-mm round-bottomed glass tubes (Kimble Div., Owens-Illinois, Inc., Toledo, OH). Each well contained, in a volume of 0.4 ml MLTC medium (containing 33% secondary mixed lymphocyte culture supernatant) (16), serially diluted numbers of responder spleen cells, 10^6 x-irradiated (2,000 rad) spleen cells as fillers, and 2×10^3 mitomycin C-treated 1591-RE tumors cells. After 7 d of incubation in an upright position, the cells of each microculture were resuspended by vigorous pipetting, and the suspension was split into two 150 μl aliquots that were transferred to corresponding wells of two different microtitration plates for analysis in a ^{51}Cr-release assay. The frequency of cytotoxic lymphocyte precursors (CLP) was determined using Poisson limiting dilution formalism as described by Lefkovits and Waldmann (17). A culture was scored as positive if its ^{51}Cr-release value was more than three standard deviations above the mean of the spontaneous release value.

Cr-Release Assay. Cytotoxicity was determined by the ability of effectors to lyse ^{51}Cr-labeled target cells during a 6-h assay as described (12). The percentage of specific lysis was calculated by the formula: ([experimental release − spontaneous release]/[total release − spontaneous release]) × 100.

In some experiments, effector cells were depleted of Lyt-2^+ cells by incubation with a monoclonal antibody of the anti-Lyt-2 IgM hybridoma 2.155.2 (18), a gift of Dr. F. W. Fitch of the University of Chicago, and rabbit complement as described (12).

Results

Comparison of 1591-RE Tumor Resistance of Normal, UV-treated, and Nude Mice. We first compared the ability of normal, UV-treated, and nude mice to resist a primary subcutaneous challenge with varying numbers of 1591-RE tumor cells. Single-cell suspensions were obtained from an established tissue culture cell line of 1591-RE, and 1-mm^3 fragments were prepared from a solid 1591-RE tumor taken from a nude C3H mouse that had been injected with the same tissue culture cell line. In agreement with earlier reports (3, 4), Table I shows that UV-treated animals, in contrast to normal mice, were highly susceptible to challenges with fragments or larger doses (5×10^7) of cultured 1591-RE tumor cells. In fact, the 1591-RE fragments failed to grow in only 5 of 23 UV-treated mice. (Further analysis of the immune response of these five "exceptional" mice will be presented below in Table II.) However, UV-treated animals, like normal mice, resisted challenges of lower doses (2×10^6 or 1×10^7) of tumor cells. This relative resistance probably was not caused by insufficient UV treatment, as 3 of 10 uninjected animals from the same batch of UV-treated mice subsequently developed autochthonous tumors on their dorsal skin >6 mo later, showing that these mice had received a carcinogenic dose of UV. The lower doses of

TABLE I

Growth of Various Numbers of Transplanted 1591-RE Tumor Cells in Normal, UV-treated, and Nude Mice

Inoculum of 1591-RE*	Tumor incidence‡		
	Normal	UV-treated	Nude
2×10^6 tumor cells	0/10 (0)	0/9 (0)	4/4 (100)
1×10^7 tumor cells	0/47 (0)	0/7 (0)	7/7 (100)
5×10^7 tumor cells	0/3 (0)	3/3 (100)	1/1 (100)
Two 1-mm^3 fragments	0/37 (0)	18/23 (78)	10/10 (100)

* C3H mice were injected with 1591-RE tumor cells or viable tumor fragments, and the tumor incidence was determined 6 wk later. For challenges consisting of 2×10^6 or 1×10^7 tumor cells, about one-half of the mice were injected intraperitoneally and the other half subcutaneously. All challenges consisting of 5×10^7 tumor cells or two 1-mm^3 fragments were administered subcutaneously, and all challenges were given on the ventral aspect of the mice 7 d after the last exposure to UV. All animals that had tumors at 6 wk eventually died because of progressive tumor growth. The data are pooled from three independent experiments.

‡ Number of mice with progressively growing tumors/number challenged. Parentheses indicate percentage of mice affected.

tumor cells that failed to grow in UV-treated mice were highly tumorigenic in nude mice; this suggested that a T cell-dependent mechanism might be responsible for the tumor regression observed in UV-treated mice at the low cell inoculum. We next determined whether the UV-treated animals that rejected a small primary dose of 1591-RE tumor cells were resistant to a secondary challenge with 1591-RE tumor fragments. Thus, eight UV-treated animals were injected with 10^7 1591-RE tumor cells, and 1 mo later received implants consisting of two 1-mm^3 1591-RE tumor fragments. Table II shows that all of these UV-treated animals were now as fully capable of rejecting the 1591-RE fragment challenge as all of 27 normal unprimed animals tested. In contrast, all of 12 UV-treated control mice and 9 of 10 nude animals that had not been preimmunized were fully susceptible to progressive tumor growth. As might be expected, all of the 5 exceptional UV-treated animals that rejected a primary challenge of 1591-RE tumor fragments without preimmunization (5 out of 23 animals in Table I) were also resistant to a secondary challenge with these fragments. Table II also shows the specificity of the immune response in vitro of the UV-treated mice that had resisted a secondary challenge with fragments of the 1591-RE tumor. For this analysis, the spleen cells of these animals were restimulated with 1591-RE tumor cells in vitro in an MLTC 3 wk after the last tumor challenge. The cultures were then tested for specific cytolytic activity in a short-term ^{51}Cr-release assay. We found that those UV-treated animals that had been immunoprotected by preimmunization generated 1591-RE-specific cytolytic T cells in culture, similarly to normal immune mice. Thus, their restimulated spleen cells lysed 1591-RE tumor targets but not 2240-RE or 1316-RE control tumor targets.

Generation of Tumor-specific Immune Responses by UV-treated Mice. To further delineate the conditions under which UV-irradiated mice would respond specifically to the 1591-RE tumor, we used an in vivo system for the generation of cytotoxic T lymphocytes. Normal, UV-treated, and nude mice were challenged with a single intraperitoneal injection of 1×10^7 1591-RE tumor cells, or with two such injections 3 wk apart. These injections did not result in tumor growth in UV-treated or normal mice, but did result in progressive tumor growth in nude mice. 8 d after the last

TABLE II

Tumor Resistance and Specificity of Cytolytic Responses of UV-treated Animals Rejecting a Previous Challenge of the 1591-RE Tumor

Mice	Type of preimmunization with 1591-RE tumor cells	Outcome of subsequent challenge with 1591-RE fragments (tumor incidence)	Number of mice tested in MLTC	Percent lysis of target cells*		
				1591-RE	2240-RE	1316-RE
Normal	None	Rejected (0/27)	10	83 ± 2‡	5 ± 2	4 ± 1
	1×10^7 tumor cells	Rejected (0/5)	5	85 ± 2	3 ± 2	1 ± 2
	Two 1-mm^3 fragments	Rejected (0/6)	6	82 ± 6	12 ± 8	11 ± 4
UV-treated	None	Accepted (12/12)	8	29 ± 4	24 ± 5	29 ± 5
	1×10^7 tumor cells	Rejected (0/8)	8	83 ± 2	−1 ± 1	1 ± 4
	Two 1-mm^3 fragments	Rejected (0/5)§	5	64 ± 4	8 ± 4	7 ± 4
Nude	None	Accepted (9/10)	—	—	—	—

* C3H mice were challenged once or twice, as indicated, with 1591-RE tumor cells subcutaneously. 3 wk after the last tumor challenge, the spleen cells of the animals were restimulated for 6 d with 1591-RE stimulator cells and then tested in a ^{51}Cr-release assay against the indicated target cells (E/T ratio 5:1).

‡ Mean ± SEM for the number of animals indicated, individually analyzed in four separate experiments.

§ 5 of the 23 UV-treated mice that rejected the challenge with fragments in experiments shown in Table I.

immunization, the peritoneal exudate cells of these animals were removed, depleted of adherent cells, and tested for cytolytic activity in a ^{51}Cr-release assay. The results from three independent experiments were similar, and therefore were pooled for Fig. 1. It can be seen that both normal and UV-treated mice were capable of mounting 1591-RE-specific primary and secondary immune responses in vivo, as effector cells from these animals lysed 1591-RE tumor cells but not the unrelated UV-induced tumor 1316-RE. These specific effector cells were cytolytic Lyt-2^+ T cells, as treatment of the peritoneal cells before the ^{51}Cr-release assay with anti-Lyt-2 antiserum and complement abrogated their activity. No cross-reactive cytotoxicity by Lyt-2^+ cells was observed in any of the experiments. Not shown in Fig. 1 is that nonadherent peritoneal cells from five normal unimmunized control animals caused <10% specific lysis of 1591-RE cells even at a 100:1 effector/target cell (E/T) ratio. Nude mice failed to generate any appreciable Lyt-2^+ effector cells against the 1591-RE tumor, but did generate high levels of Lyt-2^- effector cells against control 1316-RE tumor cells. We have shown previously that such Lyt-2^- cytolytic activity can be due to natural killer cells (12).

We next analyzed the generation of cytotoxic T lymphocytes in culture after a single tumor challenge in vivo, including UV-treated mice with progressively growing tumor fragments in the analysis. Thus, normal, UV-treated, and nude mice were challenged subcutaneously with 10^7 1591-RE tumor cells, or alternatively with 1591-RE tumor fragments. 3 wk later spleen cells were removed, restimulated for 6 d with 1591-RE tumor cells in an MLTC, and then tested for specific cytolytic activity in a ^{51}Cr-release assay. In agreement with the data obtained in the above experiments in vivo, the upper panel of Fig. 2 shows that 1591-RE-specific cytolytic T cells were generated in vitro from spleens of normal and UV-treated animals injected with 10^7 cultured 1591-RE cells; this dose is nontumorigenic for UV-treated or normal mice. These effector cells lysed neither 1316-RE nor 2240-RE control tumor cells. Furthermore, they did not lyse progressor variants of 1591-RE derived in vivo, which we

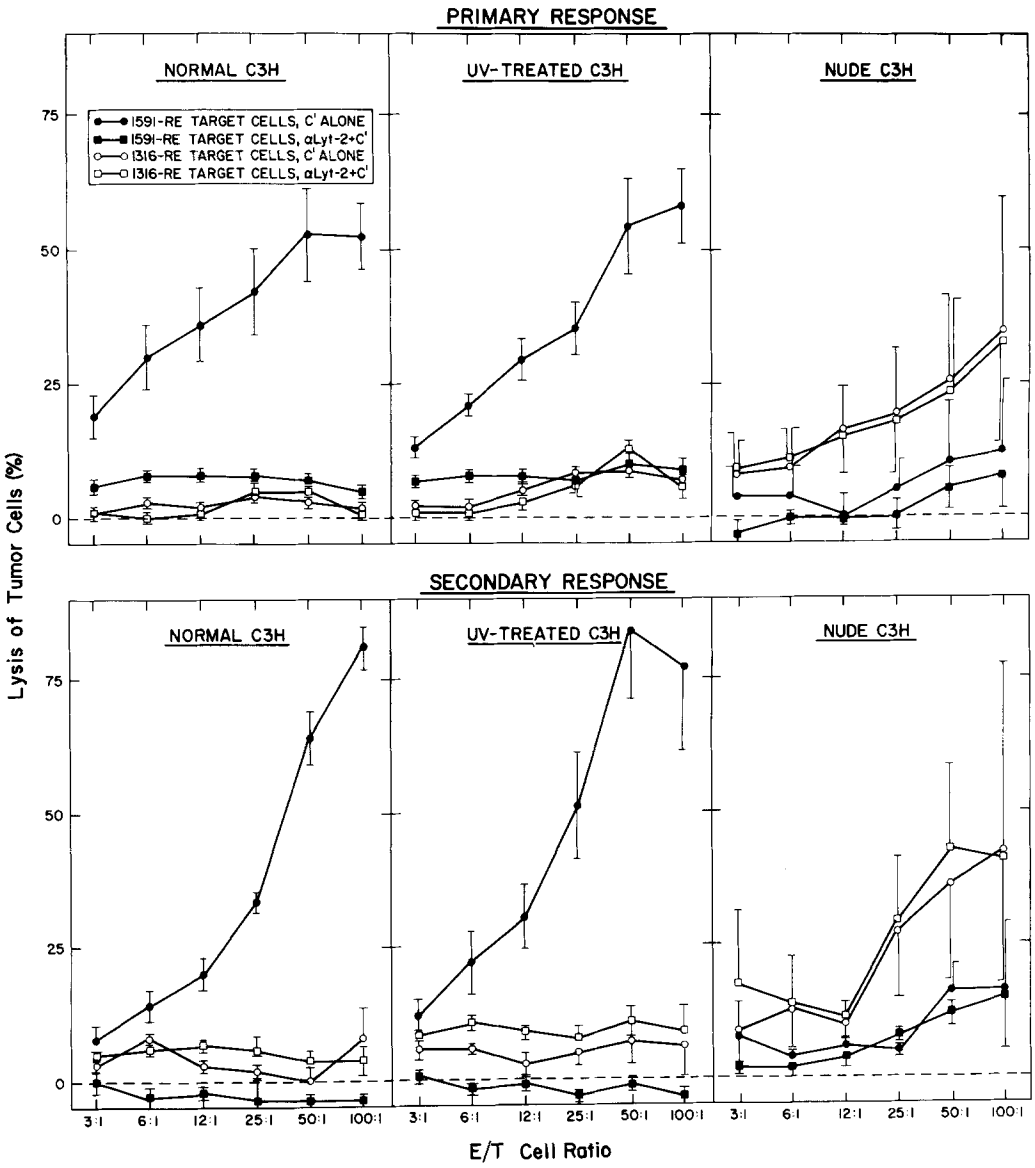

FIG. 1. Induction of primary and secondary tumor-specific immune responses in vivo in normal and UV-treated but not in nude mice. Animals received either one (primary response) or two (secondary response) intraperitoneal injections of 1×10^7 1591-RE tumor cells, 3 wk apart, and 8 d after the last injection their nonadherent peritoneal exudate cells were treated with anti-Lyt-2 antiserum and/or complement before testing in a 6-h ^{51}Cr-release assay against 1591-RE or 1316-RE tumor target cells. Vertical bars represent the SEM of the individual analysis of three to five animals in three independent experiments.

have previously shown to have lost the 1591-RE-specific determinant (12). In contrast to the immune response induced by tumor challenge of 10^7 1591-RE cells, challenge with 1591-RE tumor fragments produced profound differences between normal and UV-treated mice, shown in the lower panel of Fig. 2. Whereas normal mice that

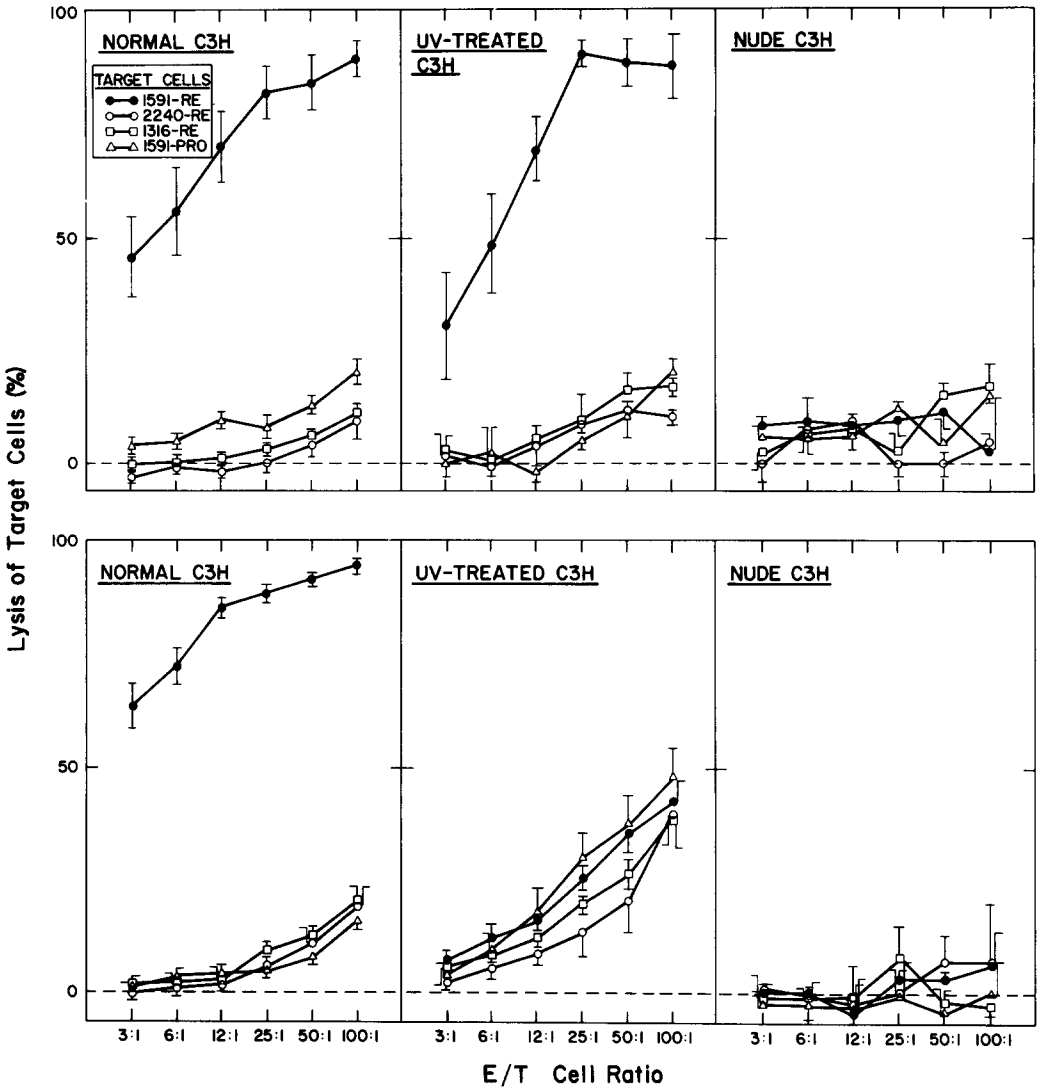

FIG. 2. Cell-mediated immune reactivity generated in vitro by normal and UV-treated but not by nude mice subsequent to in vivo challenge with 10^7 1591-RE tumor cells (upper panels), or with 1591-RE tumor fragments (lower panels). Spleen cells were removed 3 wk after tumor challenge, restimulated in culture with the parental 1591-RE tissue culture cell line in a 6-d mixed lymphocyte-tumor cell culture, and then used as effectors in a 6-h ^{51}Cr-release assay. UV-treated animals generated normal tumor-specific immunity in response to an injection of 10^7 1591-RE tumor cells, but generated a lower level of cross-reactive immunity in response to a challenge with fragments of the tumor. Normal animals responded specifically after both types of challenge. Vertical bars represent the SEM of the individual analysis of 6–12 animals in six independent experiments.

rejected the tumor fragments generated high levels of 1591-RE-specific immunity, the UV-treated mice, which showed progressive growth of the tumor fragments, generated much lower levels of cytotoxicity that also appeared to be cross-reactive. This cross-reactive response was absent in spleen cell cultures of nude mice, which had progressively growing tumor fragments.

Analysis of the Cross-Reactive Cytolytic Immunity by Limiting Dilution. The cross-reactive cytolytic activity generated in the spleens of UV-treated tumor-bearing mice could represent a response to an antigen shared on the different tumor targets, or it could represent the result of a polyclonal activation of multiple T lymphocyte clones, including those with specificity for 1591-RE. Limiting dilution analysis was used to distinguish between these two possibilities. Normal animals were challenged with two 1-mm^3 fragments of the 1591-RE tumor, and UV-treated mice were challenged with 10^7 1591 tumor cells or with 1591-RE tumor fragments. 3 wk after tumor challenge, spleen cells from these animals were serially diluted and restimulated in vitro with 1591-RE tumor cells as described in Materials and Methods. At the end of the 6-d culture period, each microculture was split into two separate wells and each well was then tested for cytolytic activity against either 1591-RE or 2240-RE tumor target cells in a ^{51}Cr-release assay. The minimal frequencies of CLP reactive with 1591-RE was determined for the three different groups of mice in two independent experiments. Semilogarithmic plots of the frequency of negative wells as a function of the dose of responding cells were linear, as seen in Fig. 3. From the slope of the regression lines, a minimal estimate can be made of the frequency of CLP (17). In this manner, the minimal CLP frequency for normal animals injected with 1591-RE tumor fragments was found to be 1 CLP/4.02 × 10^4 spleen cells. The estimated minimal CLP frequency for UV-treated animals injected with 10^7 1591-RE tumor cells was practically identical to this and was calculated to be 1 CLP/4.04 × 10^4 spleen cells. In contrast, the CLP frequency for UV-treated animals with progressively growing fragments of the 1591-RE tumor was only 1 CLP/1.74 × 10^5 spleen cells, approximately fourfold less than

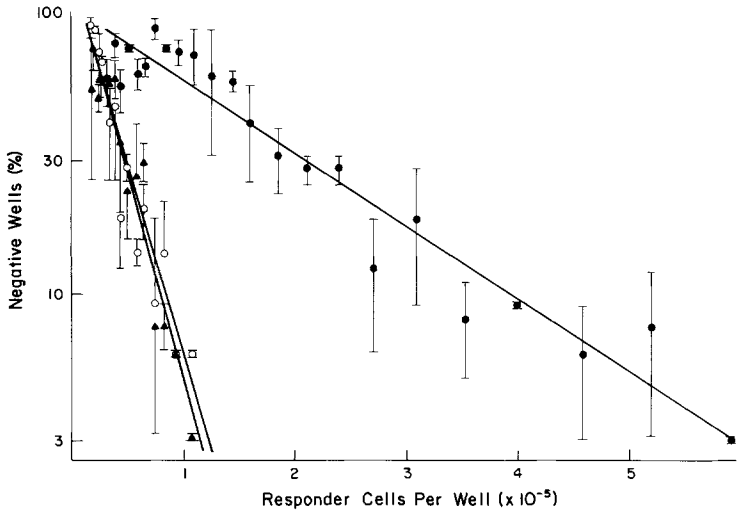

FIG. 3. Decreased frequency of CLP in UV-treated animals challenged with fragments of the 1591-RE tumor (●) as compared with UV-treated animals challenged with 10^7 1591-RE tumor cells (○) or with normal animals challenged with 1591-RE tumor fragments (▲). Spleen cells were removed 3 wk after tumor challenge, cultured in limiting numbers with 2 × 10^3 1591-RE tumor cells and 10^6 irradiated (2,000 rad) normal syngeneic spleen cells for 6 d, and then tested for cytolytic activity in a 6-h ^{51}Cr-release assay. All wells with ^{51}Cr release values <3 SD above the mean of the spontaneous release level were considered negative. CLP frequencies were determined from the slopes of the fitted regression lines. Vertical bars indicate the SEM for two separate experiments, each testing 32–160 wells/responder cell dose.

for the other two groups of animals.

We then determined the specificity of individual cytotoxic cells by analyzing the reactivity of single split microcultures against 1591-RE and 2240-RE targets. It was apparent that most of the individual cultures of cells from immunized normal mice or from UV-treated mice injected with 10^7 1591-RE cells developed cytotoxic activity with preferential specificity for 1591-RE targets, as most cultures reacted against 1591-RE, but not against 2240-RE (Fig. 4, panels A and B). In contrast, the cross-reactive cytolytic activity generated in cultures of cells from the UV-treated mice challenged with tumor fragments (panel C) segregated into an approximately equal number of CLP reactive with either 1591-RE or the 2240-RE target. The pattern of segregation that would be expected if the CLP were directed against an antigen expressed on both 1591-RE and 2240-RE (common antigen) is shown in panel D, in which the microcultures were split and each half was tested against the same 1591-RE target cells. This control experiment shows that the pattern of reactivity was not artifactually generated during the splitting of the microcultures. The results from panel C are analyzed further in Table III, which indicates that the degree of independent assortment between wells reacting to 1591-RE and wells reacting to 2240-RE target cells is >75%. This indicated that the apparent cross-reactivity observed with spleen cells from UV-treated tumor-bearing mice results from the activation of multiple specific clones, and not from activation of a single clone directed against a common antigen.

Recovery of 1591-RE Tumors from UV-treated Mice. Because UV-treated mice were capable of generating tumor-specific T cells in response to a 1591-RE tumor challenge, we wondered whether the immunity in these mice might select for 1591-RE tumor variants during the progressive growth of the tumor. To test this possibility, 11 UV-treated animals were challenged with two 1-mm^3 fragments of the solid 1591-RE

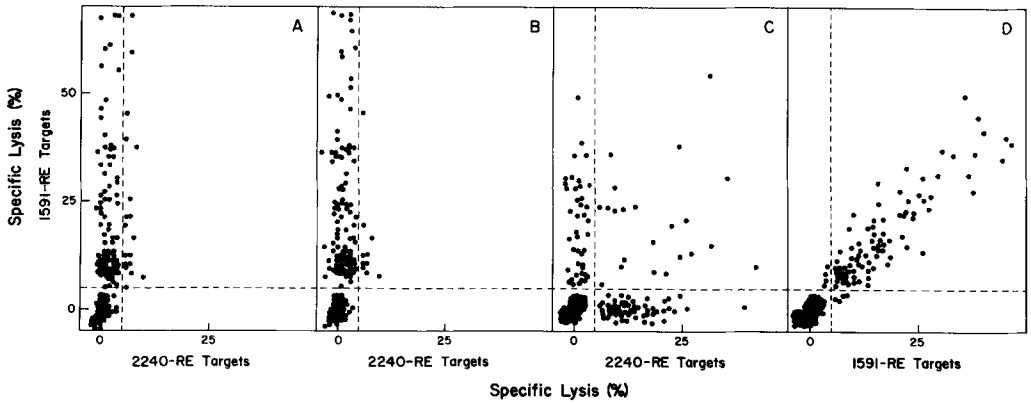

FIG. 4. Cellular basis of the immune response generated by normal animals challenged with fragments of the 1591-RE tumor (A), UV-treated animals challenged with 10^7 1591-RE tumor cells (B), or UV-treated animals challenged with fragments of the 1591-RE tumor (C, D). Individual symbols represent the analysis of single cultures, split and tested in a ^{51}Cr-release assay on different (1591-RE and 2240-RE) or the same (1591-RE and 1591-RE) tumor target cells, as indicated. Cultures contained an average of 0.5 CLP (calculated from the slopes of the corresponding fitted regression lines of Fig. 3). The dashed horizontal and vertical lines are 3 SD above and to the right of the mean of the spontaneous release values for the respective targets, our definition of positive cytotoxic activity. The data are pooled from two experiments, 160 cultures analyzed in the first experiment and 96 in the second.

TABLE III

Independent Assortment in Limiting Dilution Analysis of Tumor-reactive CPL from UV-treated Mice Bearing the 1591-RE Tumor

Experiment*	Mean CLP/well‡	Fraction of responding cultures			Number of double responding cultures			Percent independent assortment‖
					Expected			
		1591-RE	1591-RE	2240-RE	Assuming linked assortment	Assuming independent assortment§	Observed	
1	1.0	64/96	63/96	—	63	42	62	5
		58/96	—	61/96	58	37	39	90
	0.5	34/96	33/96	—	33	12	32	5
		33/96	—	28/96	28	10	7	>100
2	1.0	129/160	127/160	—	127	102	127	0
		123/160	—	97/160	97	75	79	82
	0.5	56/160	57/160	—	56	20	54	6
		31/160	—	51/160	31	10	15	76

* UV-treated animals were injected with two 1-mm^3 fragments of the 1591-RE tumor. 3 wk later, their spleen cells were cultured in microwells under limiting dilution conditions, as described in Materials and Methods. Microwells were split after 6 d of incubation, and each half was tested in a 6-h ^{51}Cr-release assay on either different (1591-RE and 2240-RE) or the same (1591-RE and 1591-RE) targets, as indicated. Dot plots of these responses appear in Fig. 4.

‡ Calculated from the linear regression plot in Fig. 3 for UV-treated mice injected with 1591-RE tumor fragments. 1.0 CLP/well was contained in 1.82×10^5 responding cells.

§ Calculated as the frequency of responders to one target times the frequency of responders to the other target multiplied by the total number of cultures per target.

‖ The percentage of independent assortment was calculated by the formula: $100 \times (1 - |(c - b)/(a - b)|)$; where a is the expected number of double responders assuming linked assortment, b is the expected number of double responders assuming independent assortment, and c is the observed number of double responders.

tumor, and 1 mo later these tumors were excised and coded as 1591-UVS.1 to 1591-UVS.11. Tissue culture cell lines were immediately and readily established for each of these tumors and expanded into large batches in <2 wk for cryopreservation. Furthermore, fragments dissected from each of these tumors were reimplanted into groups of normal mice. This procedure allowed us to test for a possible change in the growth behavior of the tumor that may have resulted from the transplantation of 1591-RE tumor fragments into UV-treated mice. Table IV shows that 8 out of the 11 tumors recovered from the UV-treated mice had indeed acquired an increased tumorigenic potential in normal mice. Fragments of seven of these tumors produced progressively growing tumors in 50% or more of the recipients, whereas none of the eight 1591-NU tumors reisolated after growth of 1591-RE fragments in nude C3H mice exhibited this behavior.

We have previously demonstrated that progressively growing tumor variants arise occasionally in normal mice injected with the 1591-RE tumor (1591-PRO tumors), and that these progressor variants always have lost a 1591-RE-specific determinant (12). We therefore tested whether this determinant also had been lost from the 1591-

TABLE IV

Effect of Prior Passage Through Nude or UV-treated Mice on the Growth Potential of the Reisolated 1591 Tumors in Normal Mice

Experiment	Injected 1591 tumor*		Tumor incidence‡	
	Donor	Designation	Normal mice	Nude mice
1	Nude mice	1591-NU.1	0/18	2/2
		1591-NU.2	2/17	2/2
2		1591-NU.3	0/10	4/4
		1591-NU.4	0/20	ND§
3		1591-NU.5	0/4	3/4
		1591-NU.6	0/4	1/1
		1591-NU.7	0/6	4/4
		1591-NU.8	0/8	4/4
Total			2/87 (2)	20/21 (95)
1	UV-treated mice	1591-UVS.1	0/6	1/1
		1591-UVS.2	0/6	1/1
		1591-UVS.3	4/6	1/1
2		1591-UVS.4	5/10	2/2
		1591-UVS.5	10/38	2/2
3		1591-UVS.6	4/4	4/4
		1591-UVS.7	4/4	1/1
		1591-UVS.8	0/4	1/1
		1591-UVS.9	4/4	1/1
		1591-UVS.10	2/4	4/4
		1591-UVS.11	4/4	3/4
Totals			37/90 (41)	21/22 (95)

* Normal C3H mice were challenged with two 1-mm^3 tumor fragments reisolated from 1591-RE-injected UV-treated or nude mice.

‡ Number of mice with progressively growing tumors 6 wk after tumor challenge/number challenged (percent). All animals that had tumors at 6 wk eventually died because of the progressive tumor growth.

§ Not done.

UVS tumors. Thus, effector T cells with this specificity were generated in vitro from the spleen cells of normal 1591-RE-immune mice in an MLTC; these effector cells were then used to test 1591-UVS target cells in a ^{51}Cr-release assay. In addition, these targets were tested using effector cells from an established T cell line (anti-A-I-4) that specifically recognizes a 1591-RE tumor-specific determinant (14). Fig. 5 shows that all of the nine 1591-UVS tumors tested were highly resistant to the cytolytic effects of both types of 1591-specific T lymphocytes, indicating that they no longer expressed this 1591-RE-specific determinant. In contrast, the tumor lines reisolated from tumors that developed in 1591-RE-injected nude mice or tumor cell lines reisolated from tumors that developed in anti-idiotypically suppressed mice fully retained the expression of this tumor-specific determinant. This resistance of 1591-UVS tumors to the 1591-specific T cells remained heritably stable over 3 mo of continuous passage in vitro or repeated passage of the tumors in nude C3H mice (data not shown). To prove that the tumors reisolated from the UV-treated mice at the site of fragment implantation were actually derived from the challenge with 1591-RE tumor cells, we used a

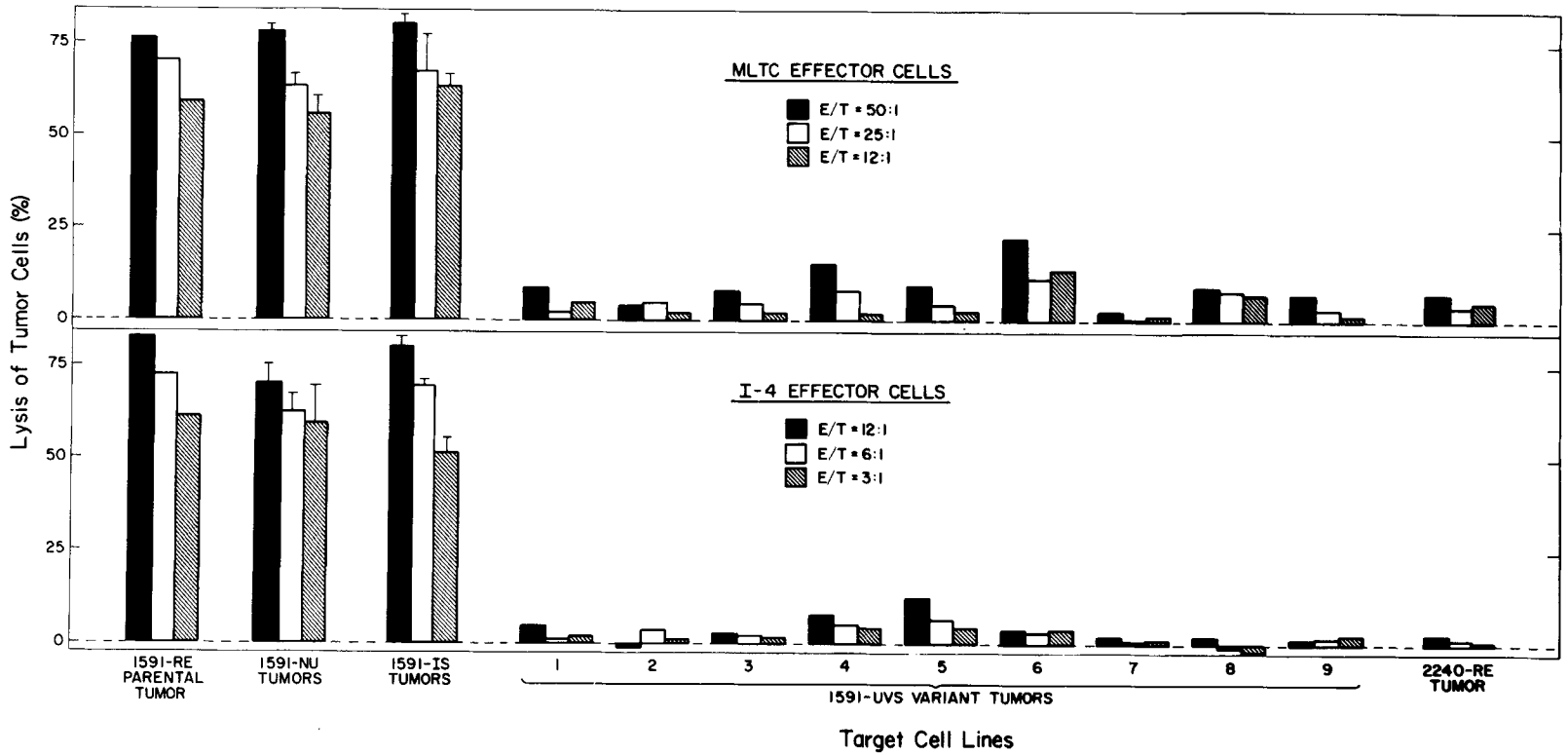

FIG. 5. Loss of a 1591-RE tumor-specific determinant from 1591-RE tumor cells passaged once through UV-treated mice (1591-UVS tumors). 1591-UVS tumors 1–5 and 7–9 had been reisolated after challenge of UV-treated mice with two 1-mm^3 fragments of the 1591-RE tumor, and 1591-UVS-6 had been reisolated from a UV-treated mouse challenged with 5×10^7 tumor cells from the established cloned 1591-RE tissue culture cell line. Control tumor cell lines were reisolated from nude mice (1591-NU tumors) or from anti-idiotypically-suppressed mice (1591-IS tumors) and then used as targets in a 6-h ^{51}Cr-release assay. Effector cells were spleen cells from a normal 1591-RE-immune mouse, restimulated for 6 d in a mixed lymphocyte-tumor cell culture (MLTC effector cells, upper panel), or lymphocytes from an established 1591-RE-specific T cell line (I-4 effector cells, lower panel).

syngeneic cytolytic T cell line (anti-B-Br1) directed against a different and independent 1591 tumor-specific epitope that we have recently found to be retained by 1591-PRO tumor variants isolated from normal mice (14). We found that all nine of the 1591-UVS tumors also retained this 1591-RE tumor-specific marker, as they remained as susceptible as the parental 1591-RE tumor cells to this cytolytic T cell probe (70% specific lysis ±3 SEM, E/T = 5:1), whereas control UV-induced fibrosarcoma lines such as 2240-RE and 1316-RE were not lysed significantly by the same probe under the same conditions (4% specific lysis ±2 SEM, E/T = 5:1).

Discussion

One of the most intriguing features of UV-induced tumors is their often extremely high immunogenicity (1, 2). Thus, most of these tumors are rejected by normal syngeneic mice but they can be transplanted into nude or x-irradiated thymectomized mice. The fact that UV-treated mice also readily accept transplants of UV-induced regressor tumors (3, 4) appears to provide a rational explanation for the question of why such highly immunogeneic tumors could ever arise in the UV-treated host. Because of this, it has been assumed that UV-treated mice do not alter the antigenic make-up of transplanted UV-induced tumors. In fact, serial passage of UV-induced tumors through UV-treated animals has been used by some investigators for the routine maintenance of the tumor cell lines in vivo. We show in this study that this assumption is unwarranted, at least for the highly immunogenic tumor 1591-RE.

Our data clearly show that there is a striking difference in the 1591-RE tumor susceptibility of nude mice and UV-treated mice. After injection of 2×10^6 or 1×10^7 1591-RE tumor cells, all C3H nude mice developed tumors, whereas all of the UV-treated mice rejected the challenge (Table I). Only at higher doses of the tumor cells did nude and UV-treated animals show a similarly high tumor incidence: ~80% of UV-treated mice injected with 5×10^7 1591-RE tumor cells or 1591-RE tumor fragments died of progressive tumors, as did all of the nude mice injected with these doses of tumor cells. The exceptional 20% of UV-treated mice that rejected a challenge with higher doses of tumor cells were also resistant to a rechallenge with the tumor fragments, and spleen cells of these mice generated tumor-specific cytolytic immunity in culture (Table II).

Previous work has already demonstrated that UV-treated mice can mount certain tumor-specific immune responses (3, 8, 9). The present study shows that UV-treated mice challenged with lower doses of 1591-RE tumor cells were as capable as normal mice of mounting tumor-specific primary and secondary cytolytic immune responses in vivo (Fig. 1) and also of generating such tumor-specific T cells after restimulation in vitro (Fig. 2). Similarly, there was no difference in the minimal estimated cytolytic precursor frequency between these UV-treated and normal mice (Fig. 3). In contrast, UV-treated mice bearing progressively growing tumor fragments showed cross-reactive cytolytic immune responses in culture (Table II and Fig. 2). Analysis of this cross-reactive reactivity at the clonal level (Fig. 4) clearly indicated that the cross-reactivity was not the result of activation of clones directed against a common UV antigen, as the experimentally observed degree of independent reactivity of individual wells fully coincided with the degree of independent reactivity that is expected statistically assuming unique reactivities of all clones (Table III). Thus, it appears that UV-treated mice with progressively growing tumor fragments show polyclonal activation

of their spleen cells in culture. Furthermore, although 1591-RE-specific T cells are then apparently responsible for the anti-1591-RE reactivity in the cross-reactive response of UV-treated progressor mice, the level of reactivity is markedly reduced as compared with the level of 1591-RE reactivity generated by UV-treated mice not bearing progressor fragments (Fig. 2). This defect is further evidenced by the much lower 1591-RE-specific cytolytic precursor frequency in the spleens of these mice (Fig. 3).

It is clear from our data (Fig. 5) that UV-treated animals regularly selected against the outgrowth of 1591-RE tumor cells that express a 1591-RE tumor-specific antigen. In contrast to all of the UV-treated recipients, there was no selection by any of the nude C3H recipients against tumor cells expressing the 1591-RE-specific determinant. As would be expected, normal mice whose 1591-RE-specific lymphocytes have been suppressed by anti-idiotypic immunity (10) also did not select against the expression of this antigen. These findings taken together suggest that the selection against a 1591-RE tumor-specific antigen in the UV-treated host is due to immune selective pressure. On the other hand, we have no evidence for a selection against a common UV antigen during the development of the 1591-UVS tumors. In fact, cross-protection among UV-induced tumors serially passed through UV-treated mice (19) and the specificity of UV suppressor cells for UV tumors (7) may suggest the retention of such common UV antigens. Thus, we would like to hypothesize that the common UV antigens may be analogous to class II cell surface antigens coded for by the major histocompatibility complex (20), which are preferentially recognized by regulatory (helper or suppressor) T cells, whereas the unique tumor-specific UV antigens may be analogous to class I antigens, recognized by effector lymphocytes mediating cytolytic responses and perhaps delayed-type hypersensitivity responses.

The regularity by which we isolated antigenic variants from UV-treated mice after transplantation with the 1591-RE tumor suggests that nonheritable changes in antigen expression might account for our findings. It has been shown, for example, that murine leukemia virus-induced thymoma cells can rapidly lose viral surface antigens to escape destruction by the host's immune system; interestingly, such escape was reportedly (21) rare in fully immunocompetent normal mice but rather frequent in partially immunosuppressed animals. So far, however, we have failed to detect any spontaneous phenotypic reversion to the parental (i.e., the 1591-RE) phenotype in the 1591-UVS tumor lines isolated from UV-treated recipients or in the 1591-PRO tumor cell lines isolated from normal recipients (12), despite prolonged passage of several of both types of these tumor cell lines in vitro or repeated passage in vivo in nude mice. Thus, it appears that the phenotypically different 1591-UVS sublines we have isolated are heritable variants. In addition to the loss of a 1591-RE-specific antigen, we found that most of the variant 1591-UVS tumors showed a progressive growth behavior in normal mice. However, 3 of the 11 1591-UVS tumors remained regressor tumors after passage through UV-treated mice, and three other 1591-UVS tumors were still rejected by >50% of the normal recipients. In contrast, all of the 1591-PRO tumors progressed in more than >80% of the normal recipients (12). We are presently exploring the reasons for the differences in progressive growth behavior between some 1591-UVS tumors and the 1591-PRO tumors.

Considering the very effective variant selection by the UV-treated mice, one has to ask how these antigenic tumors, such as 1591-RE, could ever have arisen in UV-

treated mice. The answer may lie in our previous observation that beginning at 8 mo of age normal mice increasingly fail to mount a primary tumor-specific immune response to the 1591-RE tumor in vivo and in vitro (11). (These old mice do not select for antigenic variants [J. Urban, unpubished results].) Thus, in agreement with earlier suggestions of others (25, 26) it is not unlikely that at least in some of the older mice developing tumors, both UV-treatment and advanced age jointly contribute to the development of many highly antigenic tumors after UV-treatment; especially as UV-induced tumors tend to have a long latency period (such as >6 mo in C3H mice [2]). Whereas other and especially younger UV-treated mice may have retained partial immunocompetence at the time of tumorigenesis, the escape of UV-induced tumors from the immune response in these mice might have involved not only the loss of existing antigenic determinants, but also the acquisition of new determinants. These antigenic changes may be analogous to the so-called "antigenic variation" of slow viruses during persistent infection, in which the host fails to adjust quickly enough to the constantly arising antigenic variants (27).

In conclusion, we find that UV-treated mice select for antigenically altered tumor variants, many of which exhibit an increased progressive growth potential in normal mice. This immunoselection appears to be related to the residual potential of UV-treated mice to activate tumor-specific lymphocyte clones despite the reduced tumor resistance (3, 4) and the partial immunosuppression (22, 23) in these mice. Such partial immune suppression may allow more time for the tumor to generate and expand antigenic variants that constantly arise because of the high genetic instability inherent in the malignant cell populations (24). One might expect that in humans as well, the degree of immunocompetence of an individual markedly influences the immunogenicity of the developing tumor. For example, it is possible the concomitant tumor immunity in cancer-bearing individuals leads to the selection of antigenic tumor variants. Our findings also suggest that incomplete or partially effective immunotherapeutic regimens may lead to the rapid development of lowly immunogenic tumor variants.

Summary

It has previously been shown that mice exposed to ultraviolet radiation (UV) fail to reject highly immunogenic UV-induced tumors, which are regularly rejected by normal mice. The present study shows, however, that this immunosuppresion is incomplete, as UV-treated mice can still mount certain tumor-specific immune responses and reject smaller inocula of tumor cells that regularly grow progressively in athymic nude mice. Furthermore, all tumor cell lines that were reisolated from the tumor mass resulting from one tumor passage through UV-treated recipients heritably lost a tumor-specific determinant present on the parental tumor cells used for transplantation, and a large percentage of these reisolated variant tumors had changed to progressively growing tumors, in that they were no longer rejected by normal mice. In contrast, none of the tumors reisolated from passage through athymic nude mice or anti-idiotypically suppressed mice showed this change in antigenicity and progressive growth behavior. Thus, it appears that the phenotypic change in tumors reisolated from UV-treated mice was caused by immunoselection, and that the tumor-specific immunity in these mice apparently restrained the outgrowth of the parental tumor cells despite the partial immunosuppression. Because of the regularity at which tumor

variants arose in the UV-treated mice after tumor transplantation, it appears that the partial immunosuppression caused by UV-treatment may have favored the outgrowth of antigenic variants from the parental tumor cell population, possibly by allowing more time for the generation of tumor variants. A similar immunoselection process might be part of tumor progression during tumor development and preferentially occur in cancer-bearing individuals showing concomitant tumor immunity.

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