

SELECTION OF MACROPHAGE-RESISTANT PROGRESSOR TUMOR VARIANTS BY THE NORMAL HOST

Requirement for Concomitant T Cell-mediated Immunity*

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The evolutionary progression from an initial carcinogen-exposed target cell to a cancer cell is characterized by a series of alterations in heritable phenotypic properties of the cells (1). Obviously, malignant cells that succeed in forming progressive tumors must have developed some means of subverting relevant host defenses and homeostatic control mechanisms. Thus, an analysis of how potentially malignant cells change when they acquire progressive growth behavior in normal mice should help us to understand the hierarchy and relative importance of the different naturally occurring defense mechanisms, including immunologic ones. Using this general approach, we have recently shown that the ultraviolet (UV)¹-induced 1591 regressor fibrosarcoma (1591-RE) could evade the T cell-mediated immunity of the host by the heritable loss of a 1591-RE tumor-specific transplantation antigen (2). This implied that the T cell recognition of this antigen played an important role in the rejection of the regressor tumor by normal mice. On the other hand, natural killer (NK) cells probably did not play a major role in the rejection of this tumor since progressor variants did not demonstrate a loss in sensitivity to NK cells.

Macrophages (M ϕ) can show highly selective cytotoxicity towards malignant cells *in vitro* (3) and there is some evidence suggesting that M ϕ may also destroy neoplastic cells *in vivo* (see ref. 4 for review). These data support but do not rigorously test the proposition that M ϕ exert a surveillance function against the outgrowth of potentially malignant cells (4). In the present study we have extended the type of approach referred to above to determine the role of the M ϕ in the resistance of normal mice to 1591-RE tumor cells *in vivo*. We discovered that all of the host-selected progressor variants showed a heritably reduced sensitivity to cytotoxic M ϕ . Furthermore, we found that this change could occur independently of the loss of the tumor-specific antigen *in vitro*, but that selection *in vivo* for tumor cells with decreased macrophage-sensitivity only occurred when the host also exhibited functional tumor-specific T cell

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¹ Abbreviations: E/T, effector/target cell ratio; [³H]TdR, [³H]thymidine; LK, secondary mixed lymphocyte culture supernatant containing lymphokines; LPS, purified bacterial lipopolysaccharide; M ϕ , macrophage; MLTC, mixed lymphocyte-tumor cell culture; NK, natural killer; PRO, progressor; RE, regressor; UV, ultraviolet.

immunity. This suggested that both T cells and macrophages acted together *in vivo* to suppress the outgrowth of potentially malignant cells in normal mice.

Materials and Methods

Mice. 5–10-wk-old female C3H/HeN (mammary tumor-virus negative) and BALB/c mice were from a colony of germ-free-derived, specific pathogen-free animals purchased from the National Cancer Institute Frederick Cancer Research Facility. They were kept at the La Rabida Institute in laminar flow hoods and were given sterilized food (Purina 5010C [Ralston Purina Co., Chow Div., St. Louis, MO] for autoclaving) and water. The original stock of nude C3H/HeN mice were in the 23rd backcross generation when they were obtained from a colony at the Biology Division of the Oak Ridge National Laboratory.

Effector Cells. Peritoneal M ϕ from mice injected intraperitoneally 5 d previously with 2 ml Brewer's Thioglycollate medium (0236-02; Difco Laboratories, Detroit, MI) were collected, enumerated, and purified (5). Over 95% of the purified cells belonged to the M ϕ series, as assessed by morphology, avid uptake of neutral red, and phagocytosis of heat-killed yeast, colloidal carbon, and antibody-coated sheep erythrocytes. M ϕ were activated (6) by incubating for 5 h in 200 μ l minimal essential medium (410-1100; Grand Island Biological Co., Grand Island, NY) containing 10% fetal bovine serum, 100 ng/ml purified bacterial lipopolysaccharide (LPS) (3122-25; Difco Laboratories), and 10% secondary mixed lymphocyte culture supernatant containing lymphokines (LK) prepared as described (7). Cytolytic T cells specific for the parental 1591 regressor tumor (referred to here as 1591-RE) were generated *in vitro* from spleen cells of 1591-RE-immunized animals (2).

Target Cells. The fibrosarcomas 1591-RE and 2240 were induced in C3H/HeN (mammary tumor virus negative) mice by repeated exposure to ultraviolet radiation (8). These fibrosarcomas have non-cross-reacting, tumor-specific transplantation antigens (9) and are strongly immunogenic in that they regress when transplanted into young syngeneic mice, after an initial growth during the first 1–2 wk. Eight progressively growing variants of the 1591-RE parental tumor developed in 8 out of 200 immunocompetent 1591-RE-challenged mice, and are designated as 1591-PRO tumors (2). Tumors derived by passaging the 1591-RE tumor through syngeneic athymic nude, idiotypically suppressed (10), or 1591-PRO.3-bearing animals, were designated as 1591-NU, 1591-ID, and 1591-PB tumors, respectively. All of the fibrosarcomas used in this study grow progressively in syngeneic nude mice or in mice that are immunosuppressed by either UV irradiation or adult thymectomy and x irradiation, and eventually kill these hosts (8).

Using previously described methods (2), tumors were adapted to growth *in vitro*, expanded within 2 wk of explantation, and cryopreserved in aliquots (11) unless otherwise indicated. Whenever tumor cells were required for experiments, selected frozen aliquots were thawed and used within 24 h. For tumor challenge, 1-mm³ fragments of solid tumor that had grown in a nude mouse were implanted subcutaneously in each inguinal area, or cell suspensions were injected subcutaneously underneath the ventral skin.

Normal syngeneic fibroblast lines (referred to as HLF) were derived from heart and lung tissue (2). The mastocytoma P815 and the Moloney virus-induced lymphoma YAC were maintained *in vitro* in supplemented RPMI 1640 (12). All cell lines used in these experiments were routinely examined for and found to be free of mycoplasma contamination using bacterial culture as well as Hoechst 33258 DNA staining techniques.

Cytolysis Assay. The degree of T cell-mediated or macrophage-mediated lysis of tumor cells prelabeled with chromium-51 was determined as described (2). Briefly, tumor cells were labeled with 100 μ Ci ⁵¹Cr for 1 h and then 1×10^4 of the labeled tumor cells were added to individual wells of flat-bottomed 96-well plates (76-003-05; Linbro Scientific, Hamden, CT) (0.28 cm² growth area per well) containing various numbers of washed T cells or M ϕ . Incubation continued for 6 or 16 h, respectively, unless otherwise indicated. The percentage of specific lysis was calculated by the formula: [(experimental release-spontaneous release)/(total release-spontaneous release)] \times 100.

Cytostasis Assays. M ϕ -mediated inhibition of tumor cell growth was determined by measuring [³H]thymidine ([³H]TdR) incorporation (5). Briefly, 1.0 μ Ci of [³H]TdR was added 24 h before harvest to individual wells of 96-well plates containing 2×10^3 tumor cells and varying

numbers of M ϕ . A ^{51}Cr postlabeling assay (5) was also used to measure M ϕ -mediated growth inhibition of tumor cells. Briefly, 2×10^3 tumor cells and varying numbers of M ϕ were cocultured in quadruplicate microwells for up to 4 d, washed, and labeled for 1 h with $2.0 \mu\text{Ci } ^{51}\text{Cr}$. Under standard conditions, the labeling of target cells was always 10,000–30,000 cpm/well. With both types of cytostasis assays, controls consisting of M ϕ alone were run in each experiment but isotope uptake by these cells was negligible (<200 cpm) and therefore not taken into account. The percentage target cell growth inhibition was calculated as follows: $100 \times [1 - (\text{cpm}(\text{target cells} + \text{M}\phi) / \text{cpm}(\text{target cells alone}))]$. Under the above assay conditions, M ϕ were equally effective in inhibiting tumor cell growth whether derived from normal, T cell-deficient (nude), or allogeneic animals, provided that the peritoneal cells had first been activated *in vitro* by incubating in LPS and secondary mixed lymphocyte culture supernatant containing lymphokines (LK) as described above. For example, in two separate experiments activated M ϕ from normal or nude C3H or BALB/c mice inhibited the growth of 1591-RE tumor cells 74–81% at an effector/target cell ratio (E/T) of 1:1, and 33–43% at an E/T of 1:400. The mean percent growth inhibition using nonactivated cells from these same groups of animals was only 14–19% at an E/T of 1:1, and 2–7% at an E/T of 1:400. The above data suggest that the adherent effector cell is probably not a mature T cell but most likely belongs to the M ϕ series (6, 13–15). The effects observed in this assay do not appear to reflect the depletion of essential nutrients such as arginine (16), since in repeated experiments, supernatants from 4-d primary cultures containing activated M ϕ and 1591-RE tumor cells (average 83% tumor cell growth inhibition) did not transfer significant cytotoxicity to secondary cultures containing 1591-RE tumor cells alone (average <1% tumor cell growth inhibition).

Antisera. In some experiments, effector cells were depleted of M ϕ by incubation with a 1:4 dilution of an anti-LyM-1.1 antisera (AST-101, ref. 17) for 45 min at 4°C, followed by incubation with rabbit complement (final dilution 1:2) for 45 min at 37°C. The anti-LyM-1.1 antisera was a generous gift of Dr. R. C. Burton, The University of Newcastle, Newcastle, Australia. Using the same general procedures, effector cells were depleted of Thy-1.2⁺ or Lyt-2⁺ cells using a 1:50 dilution of an anti-Thy-1.2 hybridoma antibody (AT83AB) or a 1:2 dilution of anti-Lyt-2 hybridoma antibody (2.155.2), both obtained from Dr. F. W. Fitch of this University.

Results

Selection In Vitro for 1591-RE Variants with Low M ϕ Sensitivity. First, we analyzed the differentiation markers and activation requirements of the M ϕ to be used for the selection of M ϕ -resistant variants *in vitro*. We found that adherent peritoneal exudate cells activated by exposure to both LPS and LK exhibit marked cytolytic activity towards 1591-RE fibrosarcoma cells, but not towards normal HLF fibroblasts, in an 8- or 16-h ^{51}Cr release assay (Table I). This reactivity appeared to be mediated by M ϕ and not by contaminating nonadherent T cells because it was almost totally abolished by pretreatment with anti-LyM-1.1 antiserum (17) and complement, but not by pretreatment with either anti-Thy-1.2 antiserum and complement or anti-Lyt-2 antiserum and complement (Table I). Although 1591-RE tumor cells appeared to be highly sensitive to activated M ϕ , we found in further experiments that a few of these tumor cells always survive even prolonged treatment with these effector cells (e.g., <1% survival after 120 h treatment, E/T = 20:1). We questioned whether these tumor cells represented variants with a decreased sensitivity to M ϕ -mediated killing. To test this possibility, we seeded 3×10^5 1591-RE tumor cells into each of two 150-cm² tissue culture flasks, each containing 6×10^6 activated M ϕ . After 120 h of incubation, the tumor cells were primarily localized to 3–10 clusters per flask, each cluster containing 10–30 cells. In contrast, control flasks containing 1591-RE tumor cells alone or 1591-RE tumor cells plus nonactivated macrophages displayed a near confluent monolayer of tumor cells. The tumor cells surviving M ϕ treatment were

TABLE I
*Differentiation Markers and Activation Requirements In Vitro of Adherent Peritoneal Exudate Cells
 Mediating Cytolysis of 1591-RE Tumor Cells*

Target cells	Mode of activation of effector cells*	Pretreatment of effector cells‡	Lysis of target cells§					
			50:1		10:1		2:1	
			8 h	16 h	8 h	16 h	8 h	16 h
			%		%		%	
1591-RE	LPS + LK	None	31	70	18	43	8	17
		C alone	—	78	—	42	—	17
		C + anti-Thy1.2	—	82	—	40	—	25
		C + anti-Lyt-2	—	79	—	44	—	14
		C + anti-LyM-1.1	—	17	—	5	—	3
1591-RE	LPS alone	None	6	18	2	6	2	6
HLF	LPS + LK	None	2	1	1	-3	2	0

* Adherent thioglycollate-elicited peritoneal exudate cells were cultured for 5 h in medium containing 100 ng/ml purified bacterial LPS alone or in combination with 10% LK.

‡ Activated M ϕ were treated with antisera and/or complement (C) before the addition of tumor target cells. The lytic activity of 1591-RE tumor-specific T cells generated in an MLTC was reduced from 64% to <5% by pretreatment with C and either anti-Thy-1.2 or anti-Lyt-2, but was not significantly affected by pretreatment with C and anti-LyM-1.1 (data not shown).

§ M ϕ were tested in an 8-h and 16-h ⁵¹Cr release assay against 1591-RE or HLF target cells at the indicated E/T ratios. An E/T of 50:1 corresponds to a M ϕ population density of $1.8 \times 10^4/\text{mm}^2$.

|| Not tested.

selectively dislodged from the plastic by replacing the medium with 5 ml versene for 5 min, a procedure that did not remove M ϕ in control flasks containing M ϕ alone. The tumor cells were expanded in the absence of M ϕ for cryopreservation after an additional cycle of exposure to activated M ϕ (there was no apparent inhibition of tumor cell growth during the second exposure to M ϕ). Two cell lines independently derived in the above manner were designated 1591-M ϕ .1 and 1591-M ϕ .2. To test for the stability of the altered phenotype of the tumor cells, 1591-M ϕ .1 was also passaged once through a nude C3H mouse, readapted to tissue culture, and cryopreserved. This cell line was designated 1591-M ϕ .1.NU.

To determine if these M ϕ -selected cell lines showed increased resistance to M ϕ -mediated cytolytic activity in vitro, we tested tumor cells from each of these cell lines or from the parental 1591-RE tumor for sensitivity to activated M ϕ in a 16-h ⁵¹Cr release assay. The pooled results of five such assays, analyzing the response to all three 1591-M ϕ cell lines, are presented in Table II. In agreement with the data presented in Table I, 1591-RE tumor cells were lysed by M ϕ that had been activated by lipopolysaccharide and lymphokines. In contrast, the 1591-M ϕ tumor cells were completely unaffected by the presence of these effector cells. This differential susceptibility could not be explained by differences in the spontaneous and maximum release of radiolabel because these parameters were almost identical for the two types of cell lines. Table II also shows that under these conditions, tumor cells from the relatively NK-resistant P815 tumor (14, 18) were lysed to a significant degree, whereas tumor cells from the highly NK-sensitive YAC tumor (18) were only moderately affected. This pattern of reactivity is consistent with M ϕ -mediated killing but not with NK-mediated killing (14, 18). The resistance of 1591-M ϕ to M ϕ has remained stable over 2 mo of continuous culture of the cell lines, and was retained by further

TABLE II
Resistance of the M ϕ -selected Tumor Cell Variants (1591-M ϕ) to Lysis by Activated M ϕ

Target cells	E/T*	Mode of activation	Release of ⁵¹ Cr	Lysis of target cells
			<i>cpm</i>	<i>%</i>
1591-RE	Spontaneous release	—	2672 ± 143‡	0
	Maximum release	—	9501 ± 367	100
	50:1	LPS alone	3052 ± 167	6
	25:1	LPS alone	3152 ± 199	7
	12:1	LPS alone	3076 ± 188	6
	50:1	LPS + LK	7176 ± 337	66
	25:1	LPS + LK	7190 ± 359	66
	12:1	LPS + LK	4292 ± 347	24
1591-M ϕ	Spontaneous release	—	2513 ± 119	0
	Maximum release	—	9471 ± 423	100
	50:1	LPS alone	2282 ± 254	-3
	25:1	LPS alone	2406 ± 320	-2
	12:1	LPS alone	2490 ± 294	0
	50:1	LPS + LK	2711 ± 301	3
	25:1	LPS + LK	2426 ± 231	-1
	12:1	LPS + LK	2691 ± 294	3
P815	Spontaneous release	—	998 ± 223	0
	Maximum release	—	3126 ± 65	100
	50:1	LPS alone	1075 ± 106	4
	25:1	LPS alone	1089 ± 56	4
	12:1	LPS alone	1048 ± 56	2
	50:1	LPS + LK	2376 ± 239	65
	25:1	LPS + LK	2336 ± 131	63
	12:1	LPS + LK	1453 ± 102	21
YAC	Spontaneous release	—	1840 ± 48	0
	Maximum release	—	5278 ± 93	100
	50:1	LPS alone	1716 ± 159	-4
	25:1	LPS alone	1879 ± 282	1
	12:1	LPS alone	1757 ± 123	-2
	50:1	LPS + LK	2644 ± 115	23
	25:1	LPS + LK	2471 ± 35	18
	12:1	LPS + LK	2458 ± 22	18

* 1×10^4 ⁵¹Cr-labeled tumor target cells were cultured in normal growth medium (spontaneous release), in saponin-containing medium (maximum release), or in normal growth medium containing effector M ϕ at the indicated E/T ratios, and the release of radiolabel from the target cells was determined 16 h later. An E/T of 50:1 corresponds to a M ϕ population density of 1.8×10^4 /mm².

‡ Mean ± SEM for five independent experiments, two analyzing 1591-M ϕ .1, two analyzing 1591-M ϕ .2, and one analyzing 1591-M ϕ .1.NU.

passage of these cell lines through nude mice; thus it appears that the low M ϕ -sensitivity of these M ϕ -selected cell lines is a stable heritable characteristic. In agreement with the above findings, microscopic examination of M ϕ -tumor cell cultures revealed a marked inhibition of 1591-RE, but not of 1591-M ϕ , tumor cell growth during a 4-d observation period. This cytotoxic effect was confirmed by analyzing for the incorporation of [³H]TdR by the tumor cells during this time period, and by postlabeling the residual tumor cells with ⁵¹Cr. The pooled results of five such experiments, analyzing all three of the 1591-M ϕ cell lines described above, are presented in Fig. 1. The resistance of the 1591-M ϕ tumor cell lines was not simply due

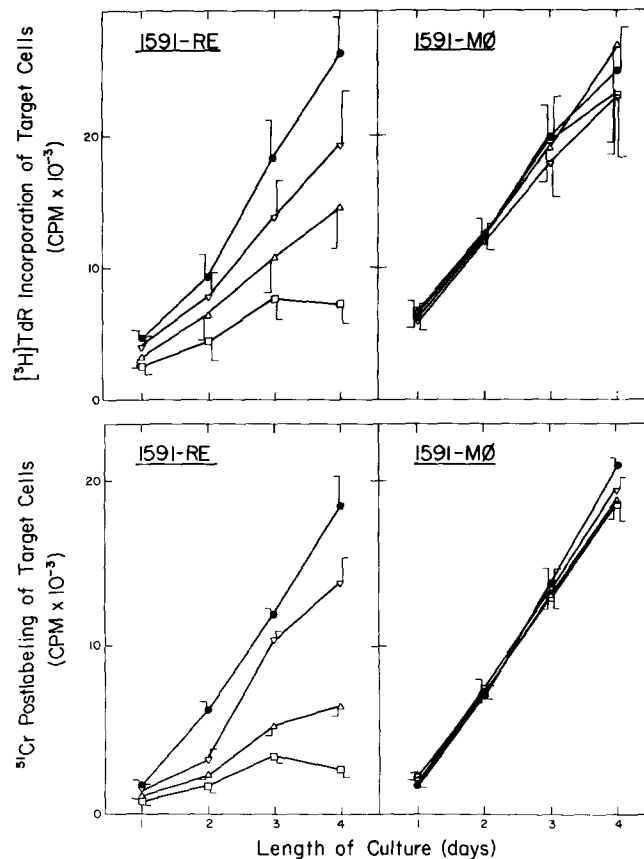


FIG. 1. Lack of Mφ-mediated growth inhibition of 1591-RE tumor variants selected in vitro for resistance to activated Mφ. Activated Mφ were added at day 0 to the parental tumor cells (1591-RE) or the variant tumor cells (1591-Mφ) at three different E/T ratios (20:1 [□], 1:1 [Δ], 1:20 [▽], no Mφ [●] for the measurement of target cell $[^3\text{H}]\text{TdR}$ incorporation (upper panels); 1:1 [□], 1:20 [Δ], 1:400 [▽], no Mφ [●] for the measurement of target cell ^{51}Cr postlabeling [lower panels]). Vertical bars represent the SEM for five separate experiments, three analyzing 1591-Mφ.1 and two analyzing 1591-Mφ.2; each experiment included 1591-RE tumor cells as concurrent controls.

to an altered growth rate of the cells, since the $[^3\text{H}]\text{TdR}$ incorporation and ^{51}Cr postlabeling at each time point was not significantly different from that of the parental 1591-RE tumor.

Analysis of Host-selected Progressor Variants for Mφ-sensitivity. The above experiments demonstrated that Mφ which have been activated by lymphokines released from T cells can select for Mφ-resistant variants in vitro; in the next series of experiments, we determined whether the intact host would select for similar Mφ-resistant variants in vivo. Our approach was based on the fact that the parental regressor 1591-RE tumor occasionally progresses upon transplantation into normal immunocompetent mice, due to the generation of progressor tumor variants (2). We have previously described the isolation of such variants (referred to as 1591-PRO tumors) and have shown that all of the variants have lost a 1591-RE-specific transplantation antigen (2). We now determined if these variant tumors had also lost sensitivity to Mφ. As controls, we isolated 1591 tumors from immunodeficient animals 1 mo after implantation of 1591-

RE tumor fragments, and adapted them to tissue culture. Two such control tumor cell lines (1591-NU.1, 1591-NU.2) were derived from congenitally athymic nude hosts, two (1591-ID.1, 1591-ID.2) from idiotypically-suppressed hosts (experiment 2, ref. 10), and one (1591-PB.3) from a host that already carried a progressively growing 1591-PRO.3 variant tumor, a condition that we recently discovered renders syngeneic mice highly susceptible to challenge with 1591-RE tumors (J. Urban, manuscript in preparation). We have previously shown that spleen cells from these three different types of hosts were unable to generate tumor-specific T lymphocytes in a mixed lymphocyte-tumor cell culture, whereas spleen cells from nonsuppressed control mice injected with the same tumor fragments generated these T cells (2, 10). We then compared the ability of activated M ϕ to inhibit the growth of each of these tumor cell lines in a ^{51}Cr release assay using the 1591-RE parental tumor as a positive control and the 1591-M ϕ variant tumors as negative controls. The pooled results of five experiments are shown in Fig. 2. The left panel indicates that 1591-NU, 1591-ID, and 1591-PB tumors obtained from the three types of immunodeficient mice described above were as sensitive to M ϕ as the parental 1591-RE tumor. In contrast, the middle panel shows that all of the rare progressor tumors (1591-PRO) isolated from normal T cell-competent mice were uniformly less sensitive to M ϕ than the parental 1591-RE tumor and almost as insensitive as the 1591-M ϕ variant tumors derived in vitro (right panel). To demonstrate that the 1591-PRO tumors are not only less sensitive to M ϕ -mediated lysis, but also to growth inhibition mediated by M ϕ , the sensitivities of the different cell lines were compared in a ^{51}Cr postlabeling assay. The pooled results of five separate experiments shown in Fig. 3, similar to the results shown in Fig. 2,

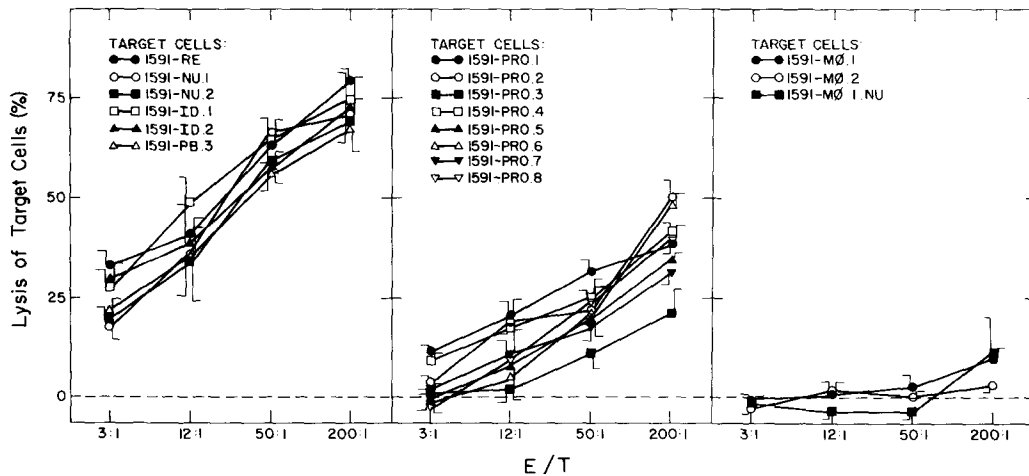


FIG. 2. Decreased sensitivity to M ϕ -mediated lysis in host-selected progressor tumor variants (1591-PRO) isolated after one passage of the parental 1591-RE tumor through mice possessing immunocompetent T cells. The 1591-RE tumor was implanted subcutaneously into mice (NU, nude; ID, idiotypically suppressed; PB, progressor-bearing; PRO, normal). 1 mo later, the progressing tumors were adapted to growth in vitro and the resulting cell lines shown in the three panels were compared for M ϕ sensitivity in a 16-h ^{51}Cr release assay as described in Materials and Methods. An E/T of 200:1 corresponds to a M ϕ population density of $7.2 \times 10^4/\text{mm}^2$. Vertical bars represent the SEM for five separate experiments, each including the parental 1591-RE tumor, at least one 1591-PRO host-selected variant, and at least one 1591-M ϕ variant derived in vitro.

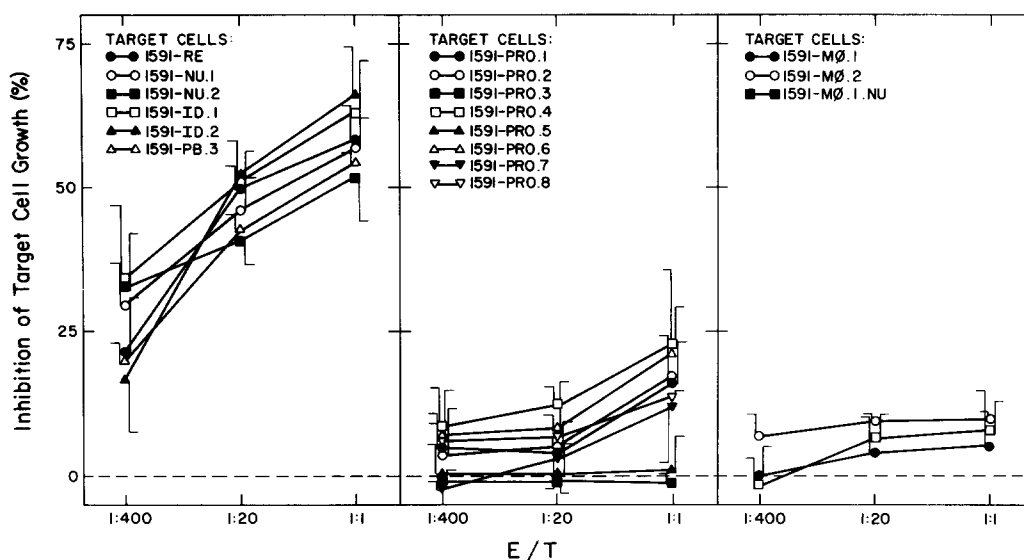


FIG. 3. Decreased sensitivity to M ϕ -mediated growth inhibition of host-selected progressor tumor variants (1591-PRO) isolated after one passage of the parental 1591-RE tumor through mice possessing immunocompetent T cells. The various tumor cell lines were tested as target cells in a ^{51}Cr postlabeling assay using activated M ϕ as effector cells (for details on target lines see legend to Fig. 2). Vertical bars represent the SEM for five separate experiments, each including the parental 1591-RE tumor, at least one 1591-PRO host-selected variant, and at least one 1591-M ϕ variant derived in vitro.

demonstrate that selection for M ϕ -resistance only occurred in mice possessing tumor-specific T cell immunity.

Independence of M ϕ -sensitivity and Expression of the 1591-RE Tumor-specific Antigen. The data above have demonstrated that progressor variants selected in vivo by normal immunocompetent mice regularly showed a reduced sensitivity to M ϕ . Because we had already shown (2) that these variants had also lost a T cell-recognized 1591-RE-specific tumor antigen, we tested whether there was an obligatory link between the loss of the immunodominant 1591-RE tumor-specific antigen and the loss of M ϕ -sensitivity. We wanted to determine whether the 1591-M ϕ tumor cell lines derived in vitro, which had lost M ϕ -sensitivity, were also less sensitive to tumor-specific T cells directed against the 1591-RE tumor-specific antigen. We generated cytolytic 1591-RE-specific T lymphocytes by culturing the spleen cells of 1591-RE-injected animals with 1591-RE stimulator cells for 6 d in a mixed lymphocyte-tumor cell culture (MLTC) and used the resulting T cells as effectors in a 6-h ^{51}Cr release assay. As shown in Fig. 4, the 1591-M ϕ variants (right panel) were fully sensitive to these T effector cells, whereas control 2240 tumor cells were not lysed. This indicated that M ϕ -sensitivity could be lost independently of the tumor-specific antigen recognized by T cells, and supported the hypothesis (19) that M ϕ do not recognize the same antigen that T cells recognize. In addition, it confirmed that the 1591-M ϕ variant tumor cells derived in vitro were of the 1591 lineage, and that the resistance of the 1591-M ϕ tumor cells to M ϕ was not solely due to a general resistance of these tumor cells to lysis. As expected, the cytolytic T cells effectively lysed the 1591-RE tumor as well as all of the other 1591 tumors reisolated after passage of the 1591-RE tumor

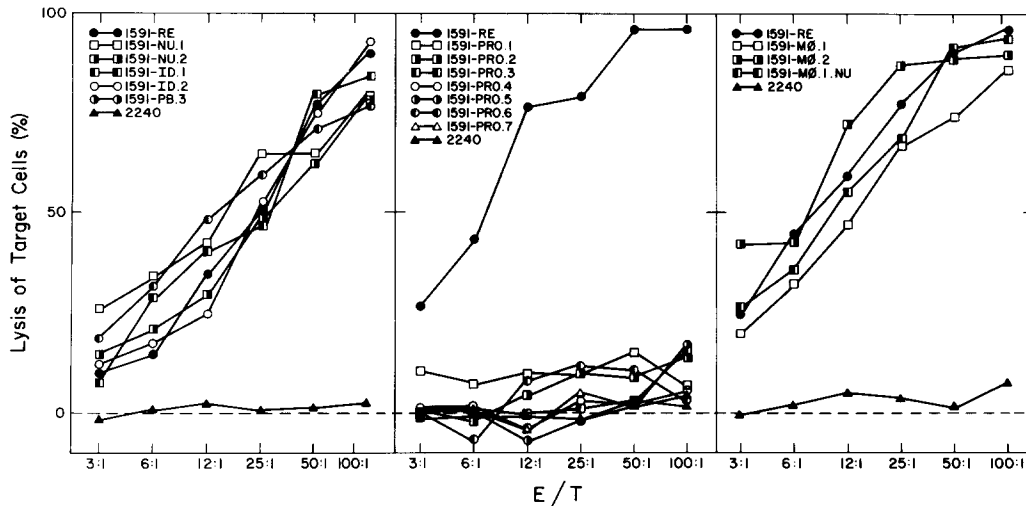


FIG. 4. Retention of the 1591-RE tumor-specific target antigen by the M ϕ -resistant 1591-M ϕ tumor cells. The indicated tumor cell lines were exposed to 1591-RE-specific T cells in a 6-h ^{51}Cr release assay. The specific T cells were generated in MLTC cultures using spleen cells from 1591-RE-immunized mice. Experiments shown in each panel were done independently of those shown in the other panels and were therefore individually controlled with 1591-RE and 2240 target cells used in each of the experiments.

through T cell-deficient mice (left panel). Confirming earlier results (10), the middle panel shows that all of the 1591-PRO tumors apparently lost the expression of the 1591-RE antigen since they were not recognized by the MLTC cells.

To further demonstrate that the 1591-M ϕ variants selected *in vitro* still possessed normal amounts of the parental 1591-RE antigen, we also tested the ability of these variant tumors to induce T cell immunity *in vivo*. Normal animals were injected with fragments of the 1591-RE or 1591-M ϕ tumors, and 3 wk later their spleen cells were removed and restimulated with tissue culture cells from the respective tumor line in an MLTC. The resulting T effector cells were tested in a 6-h ^{51}Cr release assay using 1591-RE and control 2240 tumor cells as targets. Fig. 5 shows the pooled results from four separate experiments, each analyzing the response to one of the three 1591-M ϕ variant tumors. While spleen cells of noninjected animals failed to generate significant cytolytic responses *in vitro*, injection of either the 1591-RE or 1591-M ϕ variant tumors primed spleen cells *in vivo* to produce high 1591-RE-specific cytolytic responses upon restimulation *in vitro*. Thus the 1591-RE antigen appeared to be present on the M ϕ -insensitive 1591-M ϕ variants and this antigen was recognized *in vivo* as well as *in vitro*.

Growth Potential In Vivo of M ϕ -resistant Variants. To assess the effect of decreased M ϕ -sensitivity on the tumorigenic potential of the 1591 tumor, we challenged normal or nude mice with fragments of each of the two M ϕ -resistant variant tumors that had been selected *in vitro*, and that had retained the 1591-RE specific antigen. All of the nude mice injected with 1591-RE or the 1591-M ϕ variants died of progressive tumor growth, whereas all of the normal mice injected with tumors eventually rejected the tumor, regardless of the tumor implanted. This suggested that a decrease in M ϕ -sensitivity alone was not sufficient to allow for tumor progression. Interestingly,

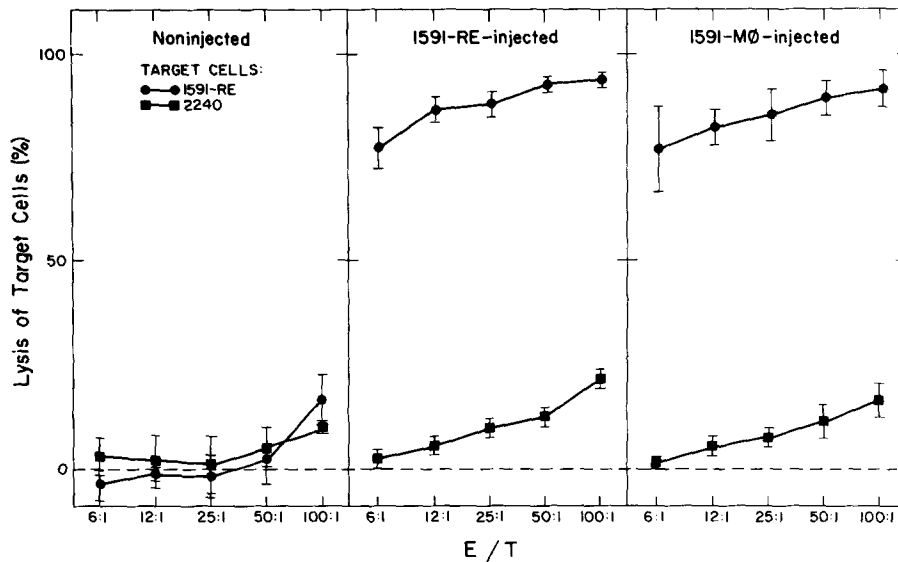


FIG. 5. Retention of the 1591-RE tumor-specific antigen by the M ϕ -resistant variant tumor cells (1591-M ϕ), as shown by their capability to elicit *in vivo* and restimulate *in vitro* 1591-RE-specific cytolytic T cells. Injected mice received two 1-mm³ fragments subcutaneously of either 1591-RE or 1591-M ϕ tumors. 3 wk later, the spleen cells of these animals were restimulated for 6 d with 1591-RE (left and middle panels) or 1591-M ϕ (right panel) tumor cells in a MLTC culture, and then tested in a 6-h ⁵¹Cr release assay against 1591-RE or 2240 control tumor cells. Vertical bars represent the SEM for four separate experiments, two analyzing 1591-M ϕ .1 and two analyzing 1591-M ϕ .2.

however, there appeared to be repeatable differences between the 1591-RE and 1591-M ϕ tumors in tumor growth during the early phase of growth in normal mice before rejection (Fig. 6, left panels). Both of the 1591-M ϕ variants grew initially to a larger volume in normal mice than the parental 1591-RE control tumor. Furthermore, the time interval required for the complete rejection of the M ϕ -resistant variants (average 34 d) was slightly longer than for the 1591-RE tumor (average 17 d). No differences in the growth curves of the 1591-M ϕ and 1591-RE tumors were observed in nude mice (Fig. 6, right panels). Similar results were found in a separate experiment (data not shown).

Discussion

We have demonstrated that tumor cell variants with greatly reduced sensitivity to M ϕ can be selected for *in vivo* or *in vitro*. In neither case was this phenotypic alteration related to the acquisition of a general resistance to lysis. The variants selected *in vitro* were still fully sensitive to 1591-RE tumor-specific T cells (Fig. 4) and even the variants selected *in vivo*, which had lost the 1591-RE-specific antigen, were still fully sensitive to T cells directed against a different tumor antigen (ref. 20, and J. L. Urban and H. Schreiber, manuscript in preparation). At present we do not know the mechanism(s) for the M ϕ -resistance of the variants selected *in vivo* and *in vitro*. M ϕ -mediated target cytolysis probably involves several independent functions including binding to a target structure on the neoplastic cells (21) and subsequent mediation of cytolysis (22). The binding of M ϕ to certain target structures present within the plasma membranes of the tumor cells is known to be an initial and

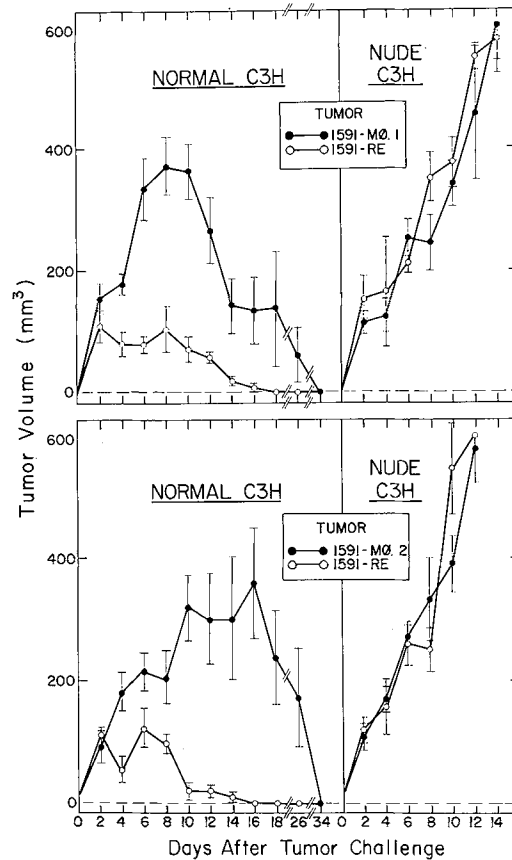


FIG. 6. Differences in growth kinetics of the parental 1591-RE tumors and the variant 1591-M ϕ tumors in normal but not in nude mice. The tumors were implanted subcutaneously in the shaved inguinal fossae of groups of 10 normal or 4 nude mice each, two 1-mm³ fragments per mouse. The mean tumor size per mouse was measured every 2 d thereafter with a caliper, and the tumor volume was computed as the average product of three perpendicular tumor diameters. Vertical bars represent the SEM for the individual analysis of the number of mice indicated above.

necessary event in the cytolysis of these targets (21). A recent study comparing a variety of other, different tumors indicated that M ϕ may still bind to a large number of target cells that are insensitive to lysis (23). Therefore the difference between our sensitive and resistant targets may not necessarily be a consequence of a specific change in the target structure on the tumor cell recognized by M ϕ , but rather may reflect differences in susceptibility to the cytolytic effects of M ϕ after binding. Thus the variants might be more resistant to the damaging effects of one or several of the lytic substances reported to be secreted by M ϕ , such as hydrogen peroxide (24), neutral protease (22), or arginase (16). For example, it has been shown that tumor cells can differ markedly in their potential to repair the oxidative injury exerted by M ϕ (24). It will be interesting to determine whether the M ϕ -resistant variants selected in vitro and in vivo subvert M ϕ -mediated cytolysis by the same or different mechanisms, to gain insight into the relative importance of these mechanisms.

The fact that we found M ϕ resistance to be regularly selected for in vivo argues

strongly for the relevance of this arm of the host immunity against tumor cells. Nevertheless, we found independent evidence in the present study that functional tumor-specific T cells were also required for the selection for M ϕ -resistance to occur *in vivo*. Thus, such selection did not occur in mice that were unable to generate tumor-specific T cells, such as athymic nude mice or mice suppressed by the induction of antiidiotypic immunity. These latter mice could only generate tumor-specific T cells against tumors other than 1591-RE (10) and, as we show here, also failed to select for M ϕ -resistant 1591-RE variants. At least indirectly, the selection for M ϕ resistance *in vitro* was also critically dependent on T cells, since the M ϕ used for the selection had to be activated with lymphokines released by alloantigen-stimulated T cells (6, 25). The influence of immunologically committed lymphocytes on M ϕ activity *in vivo* and *in vitro* has been well documented (26, 27), although little information is available on the importance of this cell-cell interaction in tumor rejection. Interestingly, however, endotoxin-induced tumor regression of an established tumor has been shown to be critically dependent upon a pre-existing state of anti-tumor T cell immunity (28), and this tumor rejection may, at least in part, be mediated by M ϕ .

M ϕ -activating lymphokines are released by T cells of the Lyt-1⁺ phenotype, and it has recently been proposed that these cells play a major role in allograft and possibly tumor rejection (for review see ref. 29). It is conceivable that a major contribution of the tumor-specific T cells or their factors lies in their ability to effectively attract M ϕ to the site of tumor growth. In this regard, it has been shown that M ϕ and T cells are distributed uniformly throughout the tumor tissue of regressing Moloney sarcomas, but only infiltrate the margins of progressively growing Moloney sarcomas (30). However, other studies analyzing UV-induced tumors have failed to reveal differences in M ϕ content between tumors which differed widely in immunogenicity and showed either regressive or progressive behavior in normal mice (31). Furthermore, it has been shown that the M ϕ content of such tumors is unaltered even in UV-irradiated animals (31, 32), which develop progressively growing tumors after implantation of UV-induced regressor tumors. It is possible that the T cells in mice bearing progressively growing UV-induced tumors were effective in attracting the M ϕ to the tumors but the host failed to activate the M ϕ sufficiently. Alternatively, the M ϕ content as well as their degree of activation may be comparable in regressing and progressing UV-induced tumors, but the progressor tumors may have acquired a decreased sensitivity to the M ϕ , as observed in the present study. In this respect it is interesting that UV-irradiated mice are only partially immunosuppressed, and we have recently shown that these mice therefore select for the outgrowth of antigenic progressor tumor variants when challenged with a UV regressor tumor (33). It will be interesting to determine whether these variants show a relative decrease in sensitivity to M ϕ , similar to the progressor variants isolated from the tumor-challenged normal mice and described in this study.

Previous attempts to isolate M ϕ -resistant variants after selection *in vivo* have apparently failed (34). One of the reasons for this may have been that the tumors that were used were probably already immunoselected for a relative resistance to M ϕ along with the loss of T cell-recognized antigens at the time of their isolation as autochthonous tumors or after repeated transplantation in normal mice. In contrast, the highly M ϕ -sensitive 1591-RE tumor line had been isolated from the autochthonous tumor of a mouse that was immunodeficient due to UV irradiation (8) and advanced

age (35). Interestingly, other malignant cell lines which have been derived in the absence of immunocompetent host defenses, such as cell lines spontaneously transformed in vitro, also show extreme sensitivity to $M\phi$ -mediated cytotoxicity (15).

In conclusion, we show that immunoselection for $M\phi$ -resistant regressor variants of the 1591-RE tumor occurs in vivo in the normal unimmunized host and that this selection requires an intact T cell-mediated tumor-specific immune response. This finding suggests that T cell-mediated activation of $M\phi$ is a relevant defense mechanism of normal hosts against 1591-RE tumor cells. Thus it appears that T cell-mediated tumor-specific immunity may protect hosts from the outgrowth of 1591-RE tumor cells by at least two different mechanisms: (a) the direct destruction of the tumor cells by cytolytic T cells, and (b) the T cell-mediated activation of $M\phi$, which then actually effect tumor destruction. Our studies suggest that the conversion of the highly immunogenic regressor tumor 1591-RE to a regressor tumor may require the loss of the strong regressor-specific tumor antigen as well as a decrease in the sensitivity to $M\phi$. Our experiments also suggest that a decrease in $M\phi$ sensitivity alone, without the loss of the strong T cell-recognized antigen, influences the rate of tumor growth but not the final tumor incidence. However, the presence of the strong T cell-recognized antigen may have overshadowed the possible contribution of decreased $M\phi$ -sensitivity in restraining tumor growth, and it will be interesting to compare tumor variants derived in vitro that have lost the strong T cell-recognized antigen and either have lost or retained sensitivity to $M\phi$. We consider it likely that the relative contribution of each of these cytolytic effector mechanisms will vary depending on the phenotype of the tumor cells, and this is currently being analyzed using $M\phi$ and/or syngeneic cytolytic tumor-specific T cell clones (20) as defined probes to reconstruct in a stepwise fashion in vitro the immunoselection for regressor variants that occurs in the intact host.

Summary

The ultraviolet radiation-induced fibrosarcoma 1591 is generally rejected by normal syngeneic mice, but occasionally the tumor succeeds in growing progressively. Analysis of these progressively growing tumors has regularly demonstrated the development of tumor variants that have acquired a heritable progressive growth potential. We have analyzed the phenotypic changes of these variants to determine which kind of selection pressure had occurred during the evolution of the variants, thus giving insight into the relative importance and hierarchy of the different immune defense mechanisms that may be operating in normal individuals as a defense against neoplastic cells. We discovered that all of the host-selected regressor variants had lost not only a strong T cell-recognized and tumor-specific antigen, but also their high sensitivity to cytotoxic macrophages. No selection for macrophage-resistance or loss of the tumor antigen was observed in 1591 tumors reisolated from idiotypically-suppressed mice or from other mice lacking tumor-specific T cell immunity. Analysis of other tumor variants selected in vitro showed that 1591 tumor cells have the potential to lose sensitivity to tumoricidal macrophages without losing the T cell-recognized tumor antigen. Thus the data suggest that T cells and macrophages act together to suppress the outgrowth of potentially malignant cells in vivo.

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