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 (Mϕ) can show highly selective cytotoxicity towards malignant cells in vitro (3) and there is some evidence suggesting that Mϕ may also destroy neoplastic cells in vivo (see ref. 4 for review). These data support but do not rigorously test the proposition that Mϕ 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 Mϕ 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 Mϕ. 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; [^{3}H]TdR, [^{3}H]thymidine; LK, secondary mixed lymphocyte culture supernatant containing lymphokines; LPS, purified bacterial lipopolysaccharide; Mϕ, macrophage; MLTC, mixed lymphocyte-tumor cell culture; NK, natural killer; PRO, progressor; RE, regressor; UV, ultraviolet.

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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 Mϕ 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 Mϕ series, as assessed by morphology, avid uptake of neutral red, and phagocytosis of heat-killed yeast, colloidal carbon, and antibody-coated sheep erythrocytes. Mϕ were activated (6) by incubating for 5 h in 200 μl 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-mm^3 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 μCi ^{51}Cr 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 cm^2 growth area per well) containing various numbers of washed T cells or Mϕ. 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)] × 100.

Cytostasis Assays. Mϕ-mediated inhibition of tumor cell growth was determined by measuring [^3H]thymidine ([^3H]TdR) incorporation (5). Briefly, 1.0 μCi 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 Mϕ. A ^{51}Cr postlabeling assay (5) was also used to measure Mϕ-mediated growth inhibition of tumor cells. Briefly, 2×10^3 tumor cells and varying numbers of Mϕ were cocultured in quadruplicate microwells for up to 4 d, washed, and labeled for 1 h with 2.0 μCi ^{51}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 Mϕ 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: $100 \times [1 - ([\text{cpm(target cells + M}\phi)]/[\text{cpm(target cells alone)}])]$. Under the above assay conditions, Mϕ 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 Mϕ 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 Mϕ 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 Mϕ 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 Mϕ 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 Mϕ Sensitivity. First, we analyzed the differentiation markers and activation requirements of the Mϕ to be used for the selection of Mϕ-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 Mϕ 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 Mϕ, 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 Mϕ-mediated killing. To test this possibility, we seeded 3×10^5 1591-RE tumor cells into each of two 150-cm^2 tissue culture flasks, each containing 6×10^6 activated Mϕ. 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 Mϕ 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 Mϕ 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).

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

‖ Not tested.

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

To determine if these Mϕ-selected cell lines showed increased resistance to Mϕ-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 Mϕ in a 16-h ^{51}Cr release assay. The pooled results of five such assays, analyzing the response to all three 1591-Mϕ cell lines, are presented in Table II. In agreement with the data presented in Table I, 1591-RE tumor cells were lysed by Mϕ that had been activated by lipopolysaccharide and lymphokines. In contrast, the 1591-Mϕ 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 Mϕ-mediated killing but not with NK-mediated killing (14, 18). The resistance of 1591-Mϕ to Mϕ has remained stable over 2 mo of continuous culture of the cell lines, and was retained by further

TABLE II

Resistance of the Mϕ-selected Tumor Cell Variants (1519-Mϕ) to Lysis by Activated Mϕ

Target cells	E/T*	Mode of activation	Release of ^{51}Cr	Lysis of target cells
			cpm	%
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-Mϕ	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 ^{51}Cr-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 Mϕ 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 Mϕ population density of $1.8 \times 10^4/mm^2$.

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

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

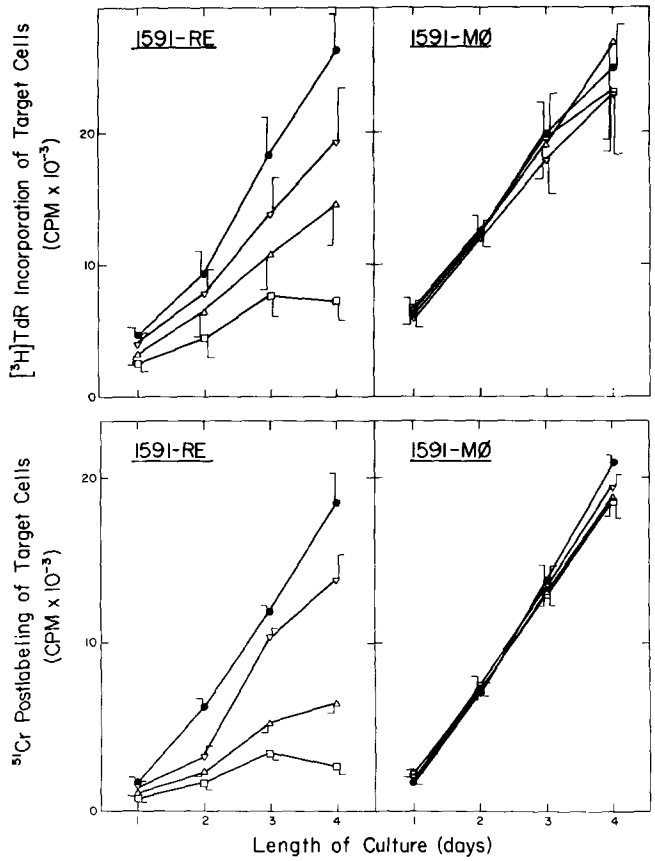

FIG. 1. Lack of Mϕ-mediated growth inhibition of 1591-RE tumor variants selected in vitro for resistance to activated Mϕ. Activated Mϕ were added at day 0 to the parental tumor cells (1591-RE) or the variant tumor cells (1591-Mϕ) at three different E/T ratios (20:1 [□], 1:1 [△], 1:20 [▽], no Mϕ [●] for the measurement of target cell [^{3}H]TdR incorporation (upper panels); 1:1 [□], 1:20 [△], 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}H]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 conditon 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 Mϕ 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-Mϕ 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 Mϕ 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 Mϕ than the parental 1591-RE tumor and almost as insensitive as the 1591-Mϕ variant tumors derived in vitro (right panel). To demonstrate that the 1591-PRO tumors are not only less sensitive to Mϕ-mediated lysis, but also to growth inhibition mediated by Mϕ, 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,

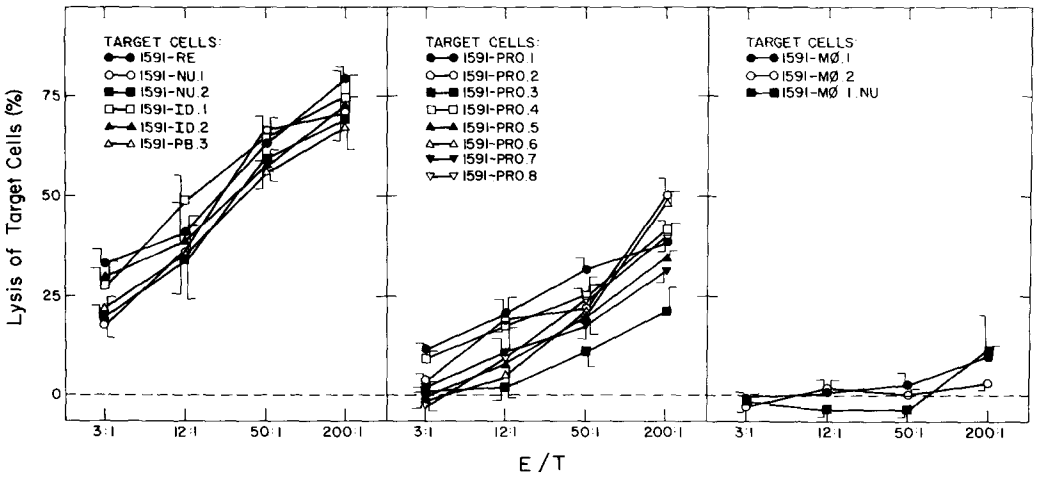

FIG. 2. Decreased sensitivity to Mϕ-mediated lysis in host-selected progressor tumor variants (1591-PRO) isolated after one passage of the parental 1591-RE tumor through mice possessing immunocompetent T cells. The 1591-RE tumor was implanted subcutaneously into mice (NU, nude; ID, idiotypically suppressed; 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 Mϕ sensitivity in a 16-h ^{51}Cr release assay as described in Materials and Methods. An E/T of 200:1 corresponds to a Mϕ population density of $7.2 \times 10^4/mm^2$. 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-Mϕ variant derived in vitro.

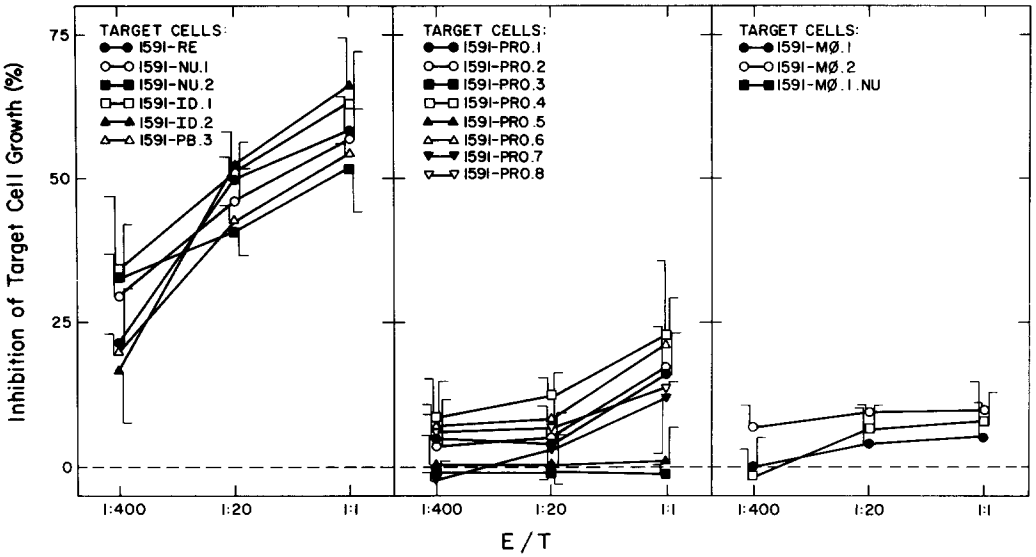

FIG. 3. Decreased sensitivity to Mϕ-mediated growth inhibition of host-selected progressor tumor variants (1591-PRO) isolated after one passage of the parental 1591-RE tumor through mice possessing immunocompetent T cells. The various tumor cell lines were tested as target cells in a ^{51}Cr postlabeling assay using activated Mϕ 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-Mϕ variant derived in vitro.

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

Independence of Mϕ-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 Mϕ. 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 Mϕ-sensitivity. We wanted to determine whether the 1591-Mϕ tumor cell lines derived in vitro, which had lost Mϕ-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-Mϕ variants (right panel) were fully sensitive to these T effector cells, whereas control 2240 tumor cells were not lysed. This indicated that Mϕ-sensitivity could be lost independently of the tumor-specific antigen recognized by T cells, and supported the hypothesis (19) that Mϕ do not recognize the same antigen that T cells recognize. In addition, it confirmed that the 1591-Mϕ variant tumor cells derived in vitro were of the 1591 lineage, and that the resistance of the 1591-Mϕ tumor cells to Mϕ 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

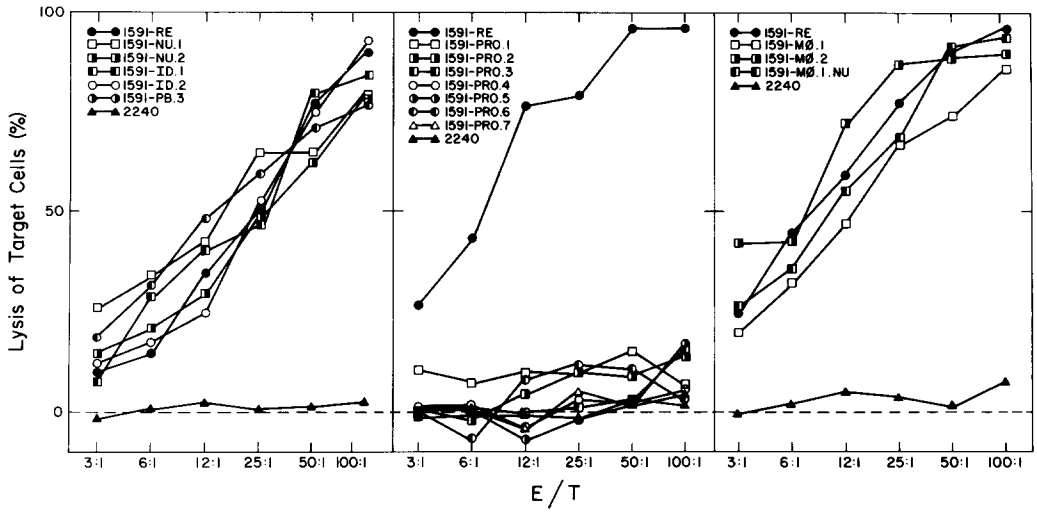

FIG. 4. Retention of the 1591-RE tumor-specific target antigen by the Mϕ-resistant 1591-Mϕ tumor cells. The indicated tumor cell lines were exposed to 1591-RE-specific T cells in a 6-h ^{51}Cr release assay. The specific T cells were generated in MLTC cultures using spleen cells from 1591-RE-immunized 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-Mϕ 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-Mϕ 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-Mϕ variant tumors. While spleen cells of noninjected animals failed to generate significant cytolytic responses in vitro, injection of either the 1591-RE or 1591-Mϕ variant tumors primed spleen cells in vivo to produce high 1591-RE-specific cytolytic responses upon restimulation in culture. Thus the 1591-RE antigen appeared to be present on the Mϕ-insensitive 1591-Mϕ variants and this antigen was recognized in vivo as well as in vitro.

Growth Potential In Vivo of Mϕ-resistant Variants. To assess the effect of decreased Mϕ-sensitivity on the tumorigenic potential of the 1591 tumor, we challenged normal or nude mice with fragments of each of the two Mϕ-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-Mϕ 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 Mϕ-sensitivity alone was not sufficient to allow for tumor progression. Interestingly,

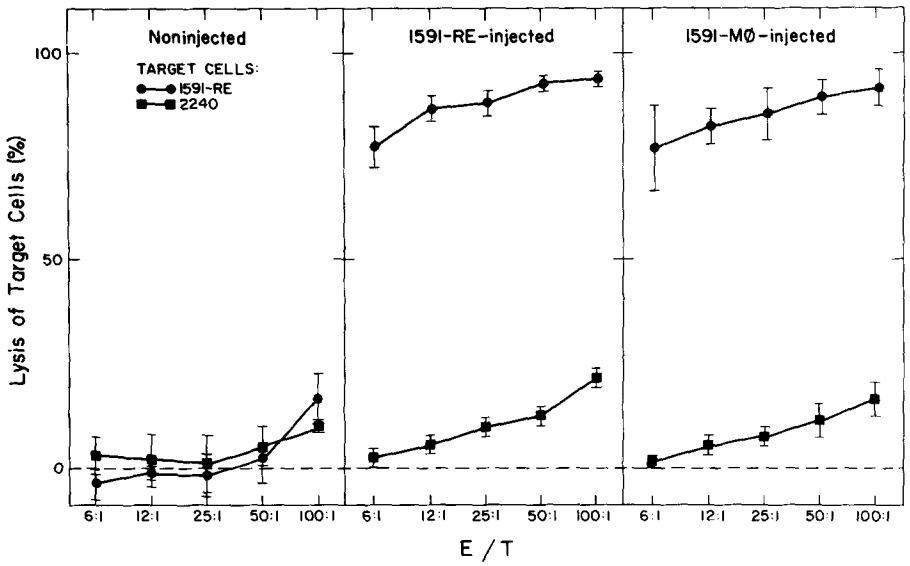

FIG. 5. Retention of the 1591-RE tumor-specific antigen by the Mϕ-resistant variant tumor cells (1591-Mϕ), as shown by their capability to elicit in vivo and restimulate in vitro 1591-RE-specific cytolytic T cells. Injected mice received two 1-mm^3 fragments subcutaneously of either 1591-RE or 1591-Mϕ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

however, there appeared to be repeatable differences between the 1591-RE and 1591-Mϕ tumors in tumor growth during the early phase of growth in normal mice before rejection (Fig. 6, left panels). Both of the 1591-Mϕ 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 Mϕ-resistant variants (average 34 d) was slighly longer than for the 1591-RE tumor (average 17 d). No differences in the growth curves of the 1591-Mϕ 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 Mϕ 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 Mϕ-resistance of the variants selected in vivo and in vitro. Mϕ-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 Mϕ to certain target structures present within the plasma membranes of the tumor cells is known to be an initial and

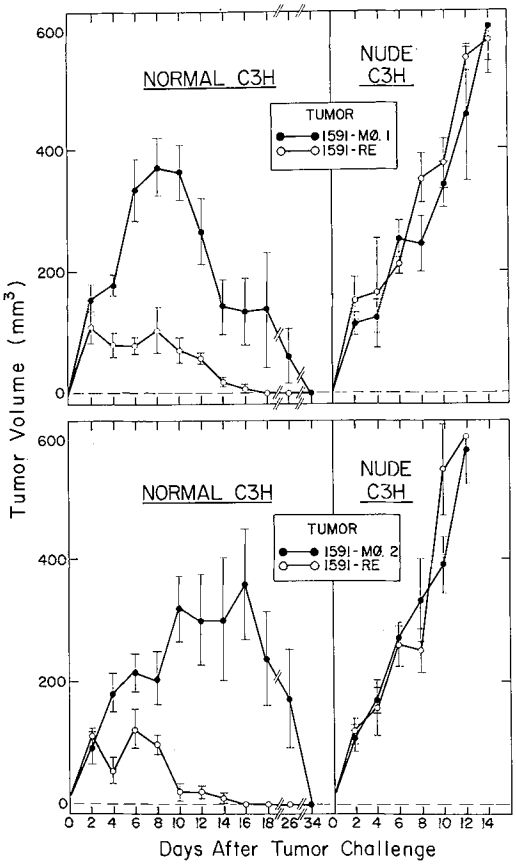

FIG. 6. Differences in growth kinetics of the parental 1591-RE tumors and the variant 1591-Mϕ tumors in normal but not in nude mice. The tumors were implanted subcutaneously in the shaved inguinal fossae of groups of 10 normal or 4 nude mice each, two 1-mm^3 fragments per mouse. The mean tumor size per 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 Mϕ 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 Mϕ, but rather may reflect differences in susceptibility to the cytolytic effects of Mϕ 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 Mϕ, 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 Mϕ (24). It will be interesting to determine whether the Mϕ-resistant variants selected in vitro and in vivo subvert Mϕ-mediated cytolysis by the same or different mechanisms, to gain insight into the relative importance of these mechanisms.

The fact that we found Mϕ 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 Mϕ-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 Mϕ-resistant 1591-RE variants. At least indirectly, the selection for Mϕ resistance in vitro was also critically dependent on T cells, since the Mϕ 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 Mϕ 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 Mϕ.

Mϕ-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 Mϕ to the site of tumor growth. In this regard, it has been shown that Mϕ 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 Mϕ 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 Mϕ 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 Mϕ to the tumors but the host failed to activate the Mϕ sufficiently. Alternatively, the Mϕ 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 Mϕ, 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 Mϕ, similar to the progressor variants isolated from the tumor-challenged normal mice and described in this study.

Previous attempts to isolate Mϕ-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 Mϕ 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 Mϕ-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 Mϕ-mediated cytotoxicity (15).

In conclusion, we show that immunoselection for Mϕ-resistant progressor 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 Mϕ 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 differnt mechanisms: (*a*) the direct destruction of the tumor cells by cytolytic T cells, and (*b*) the T cell-mediated activation of Mϕ, which then actually effect tumor destruction. Our studies suggest that the conversion of the highly immunogenic regressor tumor 1591-RE to a progressor tumor may require the loss of the strong regressor-specific tumor antigen as well as a decrease in the sensitivity to Mϕ. Our experiments also suggest that a decrease in Mϕ 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 Mϕ-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 Mϕ. 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 Mϕ and/or syngeneic cytolytic tumor-specific T cell clones (20) as defined probes to reconstruct in a stepwise fashion in vitro the immunoselection for progressor 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 progressor 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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