MECHANISMS OF SYNGENEIC TUMOR REJECTION

Susceptibility of Host-selected Progressor Variants to Various Immunological Effector Cells*

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Malignant cells can be destroyed in vitro by different cell types of the immune system (1–4). If lymphoid cells have important surveillance functions in restraining malignant growth in vivo, malignant cells must circumvent immunity before progressive growth can occur. Neoplasms frequently develop as a clone from a single transformed cell (5, 6), and during clonal evolution, phenotypic variants commonly arise, apparently as a result of the genetic instability that malignant cells acquire as a consequence of malignant transformation (6). If these phenotypic variants have a selective growth advantage and can escape immunological or other homeostatic control mechanisms of the host, they become the precursors of a newly emerging subpopulation that then becomes dominant. Therefore, these phenotypic changes indicate which kind of selection pressures have occurred (6), and a study of these "fingerprints" should give insight into the relative importance and hierarchy of the different naturally occurring immune defense mechanisms.

Fibrosarcomas that have been induced experimentally in C3H mice by ultraviolet radiation (UV)[1] seem ideal for studying the selection of progressor variants by the normal host. Most of these fibrosarcomas will not grow progressively when transplanted into normal young syngeneic C3H mice and are termed regressor fibrosarcomas (7); however, the tumor will grow progressively when transplanted into x-irradiated thymectomized, nude (7), or old animals (8, 9). Therefore, these fibrosarcomas consist of potentially malignant cells that can be used to study the relative importance of different immune surveillance mechanisms that may function in normal immunocompetent animals.

In this paper, we demonstrate that tumor variants can arise spontaneously from a regressor fibrosarcoma and can be selected for by normal nonimmunized hosts; these

* Supported by grants CA-27326, CA-22677, CA-19266, CA-20044, CA-17800, and contract CO-75380 from the National Cancer Institute.

‡ Supported by grant PHS 5 T32 GM0 7281-04 from the National Institute of General Medical Sciences.

§ Supported by research career development award CA-00432 from the U. S. Public Health Service.

‖ Supported by the John Mitchell Crouch Fellowship of the Royal Australasian College of Surgeons.

[1] *Abbreviations used in this paper:* ^{51}Cr, chromium-51; E:T, effector-to-target cell ratio; HLF, heart-lung fibroblasts; MLTC, mixed lymphocyte tumor cell culture; MTV^-, mammary tumor virus-negative; NK, natural killer; UV, ultraviolet.

variants are termed progressor fibrosarcomas. We describe several progressively growing variants of the UV-induced 1591 regressor fibrosarcoma and report studies on changes in susceptibility of the variants to natural killer cells and to tumor-specific or cross-reactive T cell-mediated immunity. These progressor variants lose their original tumor-specific transplantation antigen in a heritably stable fashion but retain their sensitivity to natural killer (NK) cells and to cross-reactive T cell-mediated killing. In fact, the variants seem to induce cross-reactive T cells more efficiently than the parent regressor tumor, and the variant tumor cells are more sensitive to lysis by NK cells even though these tumors grow progressively. This suggests that, at least in this tumor system, the ability of normal immunocompetent animals to reject potentially tumorigenic cells depends absolutely on the presence of an immune response against tumor-specific transplantation antigens and not upon the presence of NK activity and/or cross-reactive T cell-mediated immunity in vivo.

Materials and Methods

Mice. 5- to 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 Center Animal Production Area. They were kept at the La Rabida Institute in laminar flow hoods and were given sterilized food (Purina 5010C 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.

Fibrosarcoma Lines. The fibrosarcomas 1591, 1316, 1463, and 2240 were induced in C3H/HeN mammary tumor virus-negative (MTV^-) mice (7). These fibrosarcomas have noncross-reacting tumor-specific transplantation antigens (10). Tumors 1591, 1316, 1463, and 2240 are strongly immunogenic, i.e., these tumors, when transplanted into young syngeneic mice, grow during the first 10 d and then regress. These tumors can grow progressively, however, in nude mice or in syngeneic mice that are immunosuppressed by either UV irradiation or by adult thymectomy and x-irradiation (7), eventually killing these animals by infiltrative growth without macroscopic evidence of distant metastases.

Tumors were adapted to growth in vitro by mincing ~1 cm^3 of viable tumor into fragments and seeding the fine suspension into a 75-cm^2 tissue culture flask. Nonadherent particles were removed after 3 h, and the cells were immediately grown up to mass culture and frozen in aliquots for later use. The 1591, 1316, and 2240 cell lines were then cloned by diluting out the cells in Terasaki tissue culture plates (3034; Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.). 24 h later, each well was examined microscopically for cell attachment, and wells that contained a single attached cell were chosen for clonal amplification. This procedure was repeated once to ensure the clonal nature of each cell line.

Normal fibroblast lines were derived from heart and lung tissue obtained from an adult C3H/HeN (MTV^-) mouse (referred to as HLF). These fibroblast lines are not killed by activated macrophages and do not form tumors when as many as 1×10^7 cells are injected into a syngeneic nude mouse (3-mo observation period). All fibroblast cell lines were cultured in minimal essential medium, containing 10% heat-inactived fetal bovine serum and 1% penicillin-streptomycin (CMEM). YAC (Moloney virus-induced lymphoma) cells were cultured in RPMI supplemented as described by Burton and Winn (11). All cell lines used in these experiments were routinely examined for the possibility of mycoplasma contamination using culture and DNA staining techniques.

The results obtained by other investigators with virally-induced tumor cell lines have indicated that the sensitivity to lysis by NK cells may increase after about 3 wk of culturing (12, 13). We, therefore, only used the progressor cell lines in the first 2 wk after explantation from the host. Furthermore, the low NK sensitivity of the parental 1591 regressor cell line has not increased with prolonged passage in vitro (observation period >2 mo), in agreement with the results of others (14, 15) that NK sensitivity can be a stable characteristic of cell lines. Nevertheless, for better standardization of experiments, large batches of tumor cells generated

within 2 wk of explantation were cryopreserved in aliquots (16). When these cells were used as stimulator cells in a mixed lymphocyte-tumor cell culture (MLTC), as targets in a chromium-51 (^{51}Cr)-release assay, or for in vivo challenge, an aliquot was thawed and used within 24 h. Batches of various types of effector cells were also cryopreserved for use as internal controls in present and future experiments.

MLTC. Culture medium was RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 5×10^{-5} M 2-mercaptoethanol added immediately before use. Spleens were removed aseptically from animals that had received either bilateral subcutaneous implants of 1-mm^3 tumor fragments in the inguinal fossae or an intraperitoneal injection of 1×10^7 cultured tumor cells. Spleens were made into single cell suspensions using a ground glass homogenizer. The cells were then washed with complete culture medium, treated once with 0.83% (wt/vol) ammonium chloride to lyse erythrocytes, and then washed once again with culture medium. Tumor cells used as stimulators were treated with 50 μg/ml mitomycin C (Sigma Chemical Co., St. Louis, Mo.) for 45 min at 37°C and then washed three times with culture medium. MLTC for the generation of tumor-specific cytolytic T cells were made by adding 2.5×10^7 responder lymphocytes and 2.5×10^5 mitomycin C-treated stimulator cells in 20 ml medium in a 25-cm^2 tissue culture flask (3013; Falcon Labware). The flasks were incubated upright at 37°C in a fully humidified 5% CO_2 atmosphere for 6 d.

Generation of Cytotoxic Lymphocytes In Vivo. Cytolytic peritoneal lymphocytes were induced by a modification of a method described by Newcomb et al. (17). Mice were injected once intraperitoneally with 1×10^7 mitomycin C-treated or untreated cultured tumor cells in phosphate-buffered saline. At various times thereafter, the mice were killed by cervical dislocation, and their peritoneal cavities were rinsed with 5 ml of CMEM containing 5 U/ml heparin. The peritoneal cells were washed with CMEM and then incubated in 96-well tissue culture plates (Linbro Chemical Co., McLean, Va.), with 1×10^6 to 3×10^6 cells in 200 λ CMEM per well for 1 h at 37°C. Cells not adhering to the plastic were washed and used as effectors for 6-h or 16-h chromium release assays.

Chromium Release Assay. Target cells (1×10^6 to 2×10^6) were labeled with 100 μCi ^{51}Cr for 1 h at 37°C. Each batch of effector cells in a volume of 100 λ was serially diluted in V-bottomed 96-well microtiter plates (Cooke, Alexandria, Va.) and mixed with 5×10^3 to 10×10^3 ^{51}Cr-labeled cells in 100 λ CMEM. After 1, 6, or 16 h of incubation as specified, 100 λ of supernatant medium was withdrawn and assessed for radioactivity using a gamma counter. The SD of replicate samples was 5–10% of the mean. The percentage of specific lysis was calculated by the formula:

$$\text{percent specific lysis} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}} \times 100$$

Spontaneous release was <15% of the maximum release of radiolabel during the 1-h and 6-h incubation periods and <25% of the maximum release during the 16 h incubation period. The hypotonic release was 80% of the incorporated counts for all target cells.

In some experiments, effector cells were depleted of Lyt-2$^+$ cells by incubation with a monoclonal anti-Lyt-2 hybridoma antibody for 30 min at 4°C, followed by incubation with rabbit complement for 45 min at 37°C. A 1:2 dilution of culture supernatant from the anti-Lyt-2 IgM hybridoma 2.155.2 (18) was used and was a generous gift of Dr. F. W. Fitch of the University of Chicago, Chicago, Ill. Control effectors were treated with medium instead of anti-Lyt-2 supernatant followed by rabbit complement. Rabbit complement used in these experiments was serum from selected animals with low natural cytotoxicity to mouse spleen cells. This complement was subsequently absorbed with 80 mg of agar per ml of serum and used at a final concentration of 1:12. Using the same general procedures, effector cells were depleted in some experiments of NK1.2$^+$ or Thy1.2$^+$ cells using a 1:50 dilution of an anti-NK-1.2 serum (CE × NZB-F_1 anti-CBA) (11) or a 1:50 dilution of an anti-Thy-1.2 hybridoma antibody (AT83AB), obtained from Dr. F. W. Fitch of The University of Chicago.

Results

Derivation of Progressor Variants. The 1591 tumor rarely grows progressively when implanted subcutaneously as 1-mm^3 fragments into young immunocompetent female

mice; for example, after two passages of the tumor in x-irradiated thymectomized mice, two 1-mm^3 fragments of the 1591 tumor were implanted into each of 300 8- to 10-wk-old mice, and only one of the animals developed a progressively growing tumor. We anticipated that the chance of obtaining variants that can grow progressively in normal mice might be increased if the tumor was serially passed in immunosuppressed mice because the likelihood of generating a progressor variant should be a direct function of the number of generations of tumor cells (6). Therefore, we passaged the tumor serially in adult-thymectomized, sublethally-irradiated (450 rad) mice that will accept the 1591 tumor (75–85% of the recipients develop 1-cm tumors within 6 wk). After five such passages, a tumor inoculum consisting of five 1-mm^3 fragments of the tumor was implanted subcutaneously into the inguinal region of 100 3-mo-old normal mice. Progressively growing tumors were observed in five of these animals 1–4 mo after implantation. These tumors were isolated when they had grown to a size of 1.5–2.5 cm in diameter and coded as 1591 progressor-1 to 1591 progressor-5. Tissue culture cell lines were immediately established for each of these tumors and expanded into large batches in <2 wk for cryopreservation. Fragments dissected from each of these tumors, when reinjected as single 1-mm^3 fragments subcutaneously into ≥20 normal mice, produced progressively growing tumors in >80% of the animals, thus demonstrating the heritable nature of the progressive growth behavior of each of the five newly isolated tumors. Furthermore, fragments from tumors developing in nude mice receiving an injection of progressor tumor cells from the established tissue culture lines also showed a tumor incidence of ~80%. The tumor incidence in normal mice did not decrease when tumor fragments obtained after two additional passages in nude mice were used; the same was true for fragments obtained from tumors arising in nude mice injected with tumor cells from the established tissue culture cell lines.

Secondary Response of Spleen Cells from Animals in Which the Progressor Tumors Arose. We have previously demonstrated (19) that the presence of 1591-specific lymphocyte clones is necessary for animals to resist a challenge with this tumor. Therefore, we tested whether or not the five 1591-injected animals in which the progressor tumors developed possessed specific immunity to the parental 1591 tumor. Spleen cells from each of these animals were tested in individual experiments for specific cytolytic activity after a 6-d stimulation with parental 1591 cells in a mixed lymphocyte tumor cell culture. In each of these experiments, at least one control animal that had rejected the 1591 tumor was tested concurrently in the same assay. The results of the assays with lymphocytes obtained from the different tumor-bearing animals were similar; therefore, the results were pooled. Similarly, the results for the controls were similar and were pooled. Fig. 1 A shows that spleen cells from tumor-bearing animals developed significant cytolytic activity towards the parental 1591 tumor cells, although it appeared to be somewhat less than in control animals that had rejected the tumor (Fig. 1 B). The most striking difference between tumor-bearing and control animals was the high degree of cross-reactivity observed in the cultured spleen cells from the five tumor-bearers. This reactivity did not appear to be selective for malignant targets because nontumorigenic syngeneic HLF were also lysed to a significant degree.

Secondary Immune Response of Spleen Cells to Transplanted Progressor Tumors. The above results show that lymphocytes from normal animals in which the progressor tumors developed exhibited cross-reactive cytotoxic activity in culture. However, the kinetics

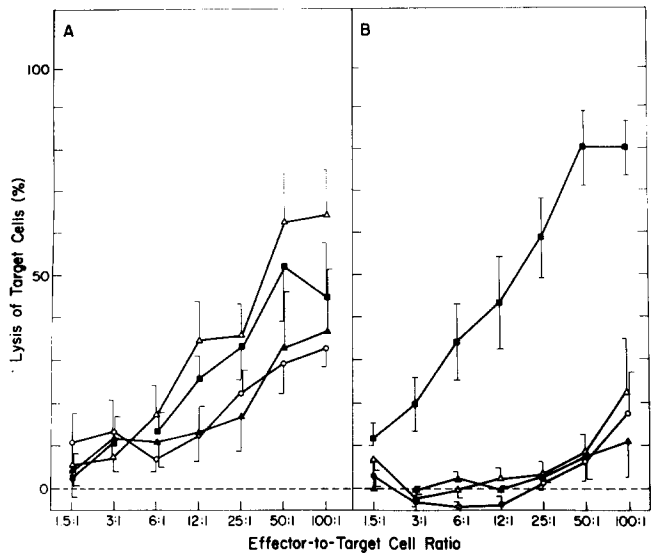

FIG. 1. Cross-reactive cytolytic immunity of animals that developed progressively growing tumors after implantation of fragments of the parental 1591 tumor (A) and specific cytolytic immunity of animals that had rejected the parental 1591 tumor (B). Spleen cells were removed 1–4 mo after tumor challenge, restimulated with the parental 1591 tissue culture cell line for 6 d in a mixed lymphocyte tumor cell culture, and then used as effectors in a 6-h ^{51}Cr release assay. Five animals that developed progressively growing tumors were individually analyzed and compared with seven concurrent control animals that had rejected the tumor fragments in five independent experiments. The results of these experiments were similar and therefore pooled. Vertical bars indicate the SE of the results for individual animals. (A) Progressor spleen cells, and (B) regressor spleen cells. Target cells are indicated as follows: ■——■, 1591 regressor (parental); Δ——Δ, 2240; O——O, 1316; ▲——▲, HLF.

of development, antigen dependence, and phenotype of the cross-reactive cells generated in vitro had not been studied. Thus, nude and normal animals were injected with either progressor or parental tumor cells. At various times thereafter, spleen cells were removed and restimulated in a 6-d MLTC and then tested for cytolytic activity in a 6-h chromium release assay. Fig. 2 shows that injection of progressor cells into normal animals induced a significant cytolytic response directed against the progressor targets, which peaked 20–30 d after tumor challenge. The lymphocyte response was totally cross-reactive with the unrelated UV-induced fibrosarcoma 2240. In contrast, normal animals injected with the regressor tumor cells exhibited a response that was specifically directed against the parental regressor tumor. This specific response peaked earlier than the cross-reactive response elicited in progressor-injected animals and was greater in magnitude (note difference in effector-to-target ratio (E:T) for regressor-injected vs. progressor-injected animals). Under the same conditions, lymphocytes from nude mice injected with progressor cells did not exhibit significant cytolytic activity. For normal animals immunized with progressor tumor cells, one animal at each time point had been injected with a nontumorigenic dose (1×10^7) of tissue culture cells, and therefore a direct comparison could be made between animals with and without progressively growing tumors. However, no difference in the magnitude of cross-reactive cell-mediated immunity could be observed, indicating that reactivity was not influenced by the presence of a tumor mass.

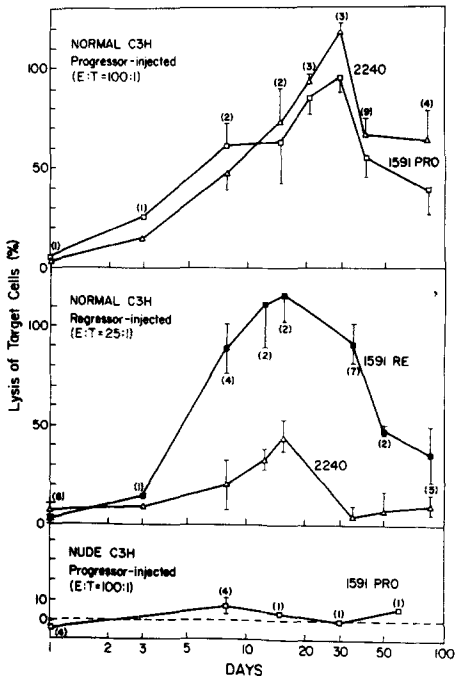

FIG. 2. Kinetics and specificity of cell-mediated immune reactivity generated by normal but not by nude animals in response to previous in vivo challenge with progressor or regressor tumor cells. Spleen cells were removed at various times after tumor challenge, restimulated in vitro with the same tumor for 6 d, and then tested for cytolytic activity against target cells 2240, 1591 progressor (1591 PRO), or 1591 regressor (1591 RE) in a 6-h ^{51}Cr release assay. The number of animals analyzed at each time point is given in parentheses, and the vertical bars indicate the SE of their individual analysis. Differences in E:T are indicated in the figure.

We analyzed further the reactivity pattern of cultured cytolytic lymphocytes obtained from normal animals injected with either progressor or regressor tumor cells and tested for reactivity against a variety of target cell lines near the time of peak response (30 d post-injection). Fig. 3 shows that animals injected with progressor cells generated tumor-reactive lymphocytes that lysed equally well two unrelated UV-induced fibrosarcomas and the nontransformed syngeneic HLF. Lymphocytes generated in animals injected with regressor cells exhibited selective cytolytic activity. Thus the pattern of cross-reactive cytolytic activity demonstrated by culture-generated spleen cells from progressor-injected animals was similar to that of spleen cells from the animals in which the progressor tumors developed. Furthermore, progressor tumor cells when used as targets were as resistant as other unrelated target cell lines to the effects of the in vitro-generated 1591-specific T cells. This suggested that the progressor variants had lost the original tumor-specific antigen, and this antigenic change remained heritably stable over 3 mo of continuous passage of the cell lines. Table I shows that the cross-reactive cytolytic activity generated in cultures of lymphocytes from progressor-injected animals was dependent on previous injection of the mice with progressor cells but did not require antigen-specific restimulation in vitro. In contrast, the generation of 1591-specific T cells depended on the presence of the specific antigen in the mixed lymphocyte-tumor cell culture.

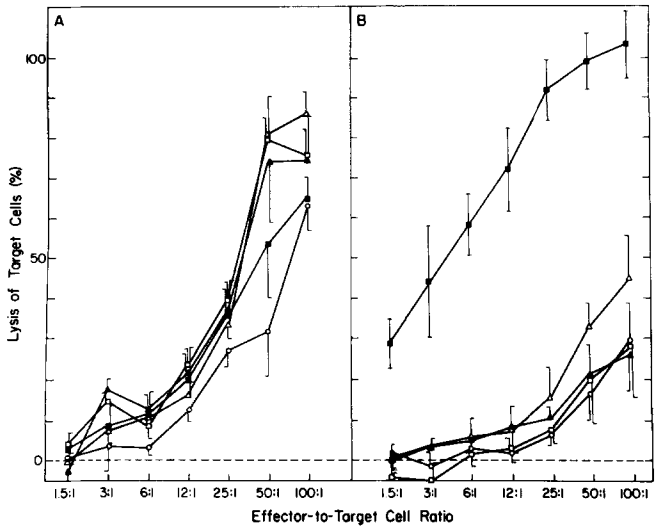

FIG. 3. Cross-reactive cytolytic immunity of animals injected with progressor tumor cells (A) and specific cytolytic immunity of animals injected with regressor tumor cells (B). Spleen cells were removed 20–40 d after tumor challenge, restimulated for 6 d in culture with the same cell line used for in vivo injection, and then used as effectors in a 6-h ^{51}Cr release assay. 13 animals injected with progressor tumor cells and 11 animals injected with regressor tumor cells were individually analyzed in nine separate experiments, and a total of five progressor cell lines were separately tested in these experiments. The results of these experiments were similar and therefore pooled. Vertical bars indicate the SE of the results for individual animals. Target cells are indicated as follows: ■——■, 1591 regressor (parental); □——□, 1591 progressors (variants); △——△, 2240; ○——○, 1316; ▲——▲, HLF.

TABLE I

Lack of Specific Immunologic Memory of Animals Injected with Progressor Cells and Failure of Progressor Cells to Restimulate a Specific Memory Response to the Parental Regressor Cells

Animal	Number of animals analyzed	Pretreatment of host*	Stimulator cells in MLTC	Percent specific lysis of target cells‡					
				100:1			25:1		
				Regressor	Progressor	2240	Regressor	Progressor	2240
Normal	8	Regressor-injected	Regressor	99 ± 6§	26 ± 9	29 ± 4	90 ± 7	9 ± 3	9 ± 4
			Progressor	20 ± 2	43 ± 7	31 ± 5	12 ± 3	9 ± 2	8 ± 2
			None	22 ± 3	27 ± 10	36 ± 7	10 ± 3	9 ± 4	7 ± 3
	18	Progressor-injected	Regressor	58 ± 8	55 ± 7	74 ± 9	25 ± 7	22 ± 5	24 ± 5
			Progressor	56 ± 5	70 ± 5	84 ± 6	18 ± 5	22 ± 4	27 ± 4
			None	42 ± 5	53 ± 7	73 ± 8	9 ± 3	17 ± 4	15 ± 5
	5	None	Regressor	6 ± 3	6 ± 4	4 ± 4	2 ± 1	4 ± 3	1 ± 3
			Progressor	25 ± 4	23 ± 11	17 ± 2	3 ± 2	3 ± 2	2 ± 2
			None	10 ± 3	18 ± 1	15 ± 7	3 ± 2	−1 ± 3	−1 ± 3
Nude	7	Progressor-injected	Regressor	11 ± 3	6 ± 5	10 ± 5	3 ± 4	3 ± 3	0 ± 3
			Progressor	2 ± 1	5 ± 7	−1 ± 3	−1 ± 3	−4 ± 2	0 ± 3
			None	0 ± 2	5 ± 8	−2 ± 4	−1 ± 1	0 ± 4	0 ± 3

* Normal or nude C3H mice were injected subcutaneously either with the parental regressor 1591 or with one of the five 1591 progressor variants. Spleen cells from these animals were removed 20–30 d later and restimulated in a 6-d MLTC with the regressor or corresponding progressor tumor cells or with the medium alone.

‡ Effector cells generated in MLTC were tested in a 6-h ^{51}Cr release assay using regressor, progressor, or 2240 tumor cells as targets at a 100:1 or 25:1 E:T. Percent specific lysis was calculated as described in Materials and Methods.

§ Values represent the mean ± SE for the individual analysis of the number of animals indicated. The data are pooled from eight separate experiments and the responses to all five progressor variants were analyzed.

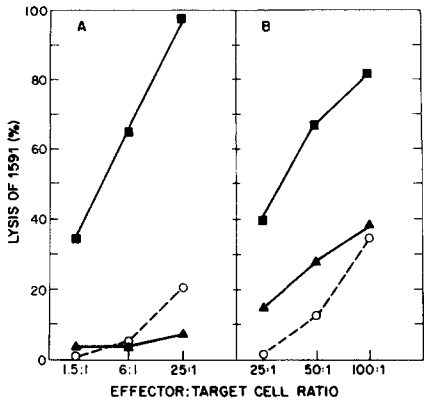

FIG. 4. Characterization of specific effector cells from animals injected with regressor tumor cells (A) and of nonspecific cells from animals injected with progressor tumor cells (B). Spleen cells were removed 35 d after challenge with the 1591 tumor, restimulated for 6 d in culture with 1591 tumor cells, and then used as effector cells in a 6-h ^{51}Cr-release assay using 1591 tumor cells as targets. Before the ^{51}Cr-release assay, effector cells were treated with anti-Lyt-2 or anti-Thy-1.2 and complement or with complement alone, as described in Materials and Methods. The target specificity of such effector cells is shown in Fig. 3. ■——■, rabbit complement alone; ▲——▲, α-Lyt-2 + rabbit complement; O- - -O, α-Thy-1.2 + rabbit complement.

The tumor-specific cytolytic activity of culture-generated lymphocytes from animals injected with the parental tumor was eliminated effectively by pretreatment with anti-Thy-1.2 or anti-Lyt-2 antibody and complement (Fig. 4). In contrast, the much lower cytolytic activity of the cross-reactive lymphoid cells from progressor-injected animals (note differences in E:T) was diminished by <60% after pretreatment with anti-Thy-1.2 and complement and only by ~40% after pretreatment with anti-Lyt-2 and complement. This was markedly less than the >90% reduction of the tumor-specific cytolytic activity after anti-Lyt-2 pretreatment of the antigen-specific lymphocytes obtained from regressor-injected animals. Pretreatment of the immune spleen cells from progressor-injected mice with anti-NK antiserum and complement at the beginning of the 6-d culture period did not affect the degree of cross-reactivity generated by these cells, whereas pretreatment with anti-Thy-1.2 and complement virtually abolished the generation of this reactivity (data not shown), indicating that cross-reactivity could not be attributed to NK cells.

Primary Immune Response of Peritoneal Lymphocytes to Transplanted Progressor Tumors. The results of the above in vitro experiments suggested that animals bearing progressively growing tumors may be incapable of generating tumor-specific lymphocytes and that all of the five different progressor tumor cell lines were resistant to the effects of in vitro generated tumor-specific cytolytic T cells. To confirm the significance of these results in vivo, we challenged normal animals intraperitoneally with either parental 1591 or progressor 1591 tumor cells. 8 d thereafter, nonadherent peritoneal cells were studied for their cytolytic activity towards parental or progressor 1591 tumor cells and towards control tumor cells derived from the unrelated UV-induced fibrosarcoma 1316. Figure 5A shows that only animals challenged with the parental tumor generated cytolytic activity that was tumor-specific; furthermore, cytotoxicity was totally eliminated by pretreatment of the effector cells with anti-Lyt-2 and complement. Interestingly, animals challenged with progressor tumor cells also generated a

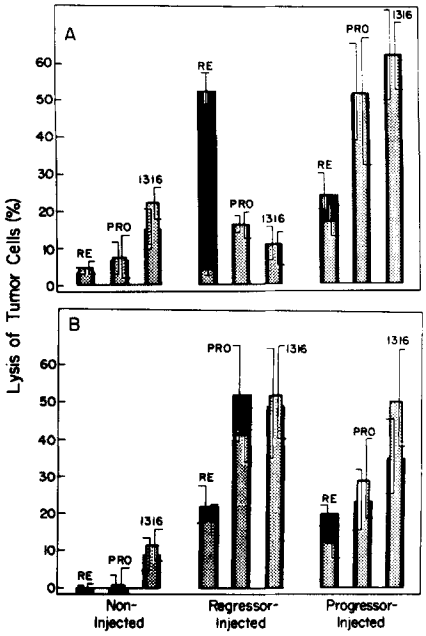

FIG. 5. (A) Induction of cross-reactive cytolytic immunity by intraperitoneal injection of progressor tumor cells from the five different progressor tumors 8 d previously and induction of tumor-specific cytolytic immunity by a similar injection of regressor tumor cells into normal C3H mice. The vertical bars indicate the percent lysis of target cells (RE, regressor 1591 parental tumor cells; PRO, progressor 1591 variant tumor cells; 1316 tumor cells) in a 6-h ^{51}Cr release assay after treatment of the effector cells with anti-Lyt-2 and complement (□) or after treatment with complement alone (■). Progressor targets are totally insensitive to Lyt-2$^+$ regressor-specific T cells, and regressor targets are less sensitive than progressor targets to Lyt-2$^-$ lymphocytes. Vertical lines show the SE of values obtained from individual analysis of animals injected with regressor tumor cells (11 animals), progressor tumor cells (eight animals), or noninjected (five animals), obtained in four independent experiments. E:T, 200:1. (B) Induction of cross-reactive cytolytic immunity by injection of either progressor or regressor tumor cells into nude C3H mice, individually tested in three independent experiments (one animal per injection per experiment). For other details see part A.

significant level of cytolytic activity. These lymphocytes, however, were completely insensitive to treatment with anti-Lyt-2 and complement and lacked the exquisite specificity observed with lymphocytes from animals immunized with the parental 1591 tumor. Finally, in agreement with our earlier in vitro findings, it can be seen from Fig. 5 A that the progressor tumor cells were not sensitive to lymphocytes specific for the parent 1591 tumor, indicating again that the progressor tumor cells lost the original 1591 tumor-specific antigen. Fig. 5 B shows that nude mice were also capable of generating significant cytolytic activity; however, this activity was relatively insensitive to anti-Lyt-2 and complement treatment regardless of whether the nude mouse had been injected with regressor or progressor cells. Thus, this cytolytic activity resembled that of normal mice injected with progressor cells.

To test whether the progressor-reactive cells generated by nude and normal mice were related to NK cells, we tested their ability to kill YAC target cells and their expression of the NK1.2 surface marker. In a chromium release assay, these progressor-induced, progressor-reactive lymphocytes lysed not only the progressor target cells, but also YAC target cells (Table II). This activity was almost totally removed by

TABLE II

Differentiation Markers on NK Lymphocytes and Increase of NK Activity after Injection of the Host with Progressor Tumor Cells

Pretreatment of host*	Source of effector cells	Pretreatment of effector cells‡	Percent specific lysis of target cells§			
			Progressor cells		YAC cells	
			6 h	*16 h*	*6 h*	*16 h*
Noninjected	Peritoneal cavity	C′ alone	10	17	38	53
		C′ + anti-Lyt-2	10	19	30	62
		C′ + anti-NK-1.2	2	11	<0	<0
	Spleen	C′ alone	10	15	66	74
		C′ + anti-Lyt-2	11	16	66	81
		C′ + anti-NK-1.2	7	2	6	7
Progressor-injected	Peritoneal cavity	C′ alone	45	83	85	94
		C′ + anti-Lyt-2	37	82	81	90
		C′ + anti-NK-1.2	7	20	28	17
	Spleen	C′ alone	20	56	93	100
		C′ + anti-Lyt-2	19	51	87	86
		C′ + anti-NK-1.2	6	16	15	21

* C3H mice were injected with 10^7 live progressor 1591 tumor cells intraperitoneally. Spleen or peritoneal exudate cells were removed and tested 8 d after injection.

‡ Effector cells were depleted of adherent cells and then treated with antisera and/or complement before testing in a ^{51}Cr-release assay as described in Materials and Methods.

§ Effector cells were tested in a 6-h and 16-h ^{51}Cr-release assay using as target cells progressor 1591 or YAC tumor cells at a 200:1 E:T. Percent lysis was calculated as described in Materials and Methods.

pretreatment with anti-NK-1.2 antibody and complement. In the same experiment, this reduction in activity was demonstrated to be equivalent to a >95% reduction in the number of effector cells (data not shown). As shown above, the effector cells were insensitive to anti-Lyt-2 and complement treatment. The results were similar when splenic rather than peritoneal lymphocytes were used as effectors.

The results we have presented show that, in normal mice, progressor variants can induce natural killer activity much more effectively than the parental regressor tumor (Fig. 5 A), whereas in the nude mouse, the parental tumor could induce natural killer activity equally effectively (Fig. 5 B). Furthermore, the parental tumor cells were consistently less sensitive than the progressor variants to the NK cells. This relatively lower NK sensitivity of the parental tumor cells has not increased even with prolonged passage in vitro for many months. Furthermore, the higher sensitivity of progressor variants and their increased capacity to induce NK activity in normal mice was apparent even in primary progressor cell cultures and has not changed thereafter (data not shown).

Growth Kinetics of Progressor Tumors in Normal and Nude Mice. Because in our experience C3H nude mice demonstrate the same NK activity as normal C3H mice, we expected that the progressor variants would grow in nude and normal animals at the same rate. To test this hypothesis, nude and normal animals were injected with 1-mm^3 fragments of the progressor tumors and then monitored for differences in the rate of tumor appearance. Interestingly, several differences in the growth behavior of the progressor tumors were found between normal and nude hosts.

First, as shown in Fig. 6, tumors appeared earlier in nude mice, although >80% of

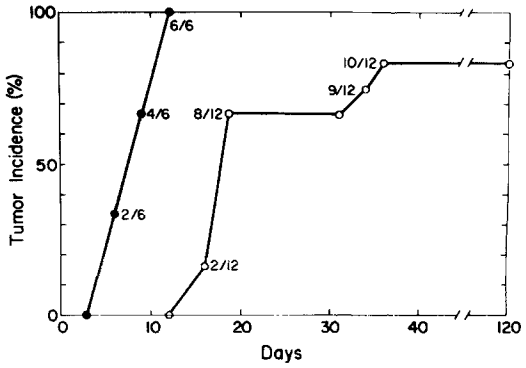

FIG. 6. Rate of tumor appearance in nude (●——●) and normal (○——○) C3H mice injected with the progressor variants (two 1-mm^3 fragments per mouse). The results of three independent experiments, each of which tested a different PRO line in two and four normal animals, were similar and therefore pooled. Animals were scored as positive for tumor growth when tumors reached 0.4-cm Diam and continued to progress to eventually kill the animals.

normal as well as nude animals eventually developed progressively growing tumors. At day 12 post-injection, all nude mice had developed tumors, whereas no tumors were yet observed in normal animals. The average time interval after which the progressor tumors became macroscopically visible in nude mice was 9.0 d (± 2.7, SD), whereas the average time interval for tumor appearance in normal mice was 21.0 d (± 7.4 d, SD; $P < 0.01$).

Second, tumors in nude mice reached an average diameter of 1-cm as early as 12 d after implantation, whereas tumors transplanted in normal mice did not reach this average size until 36 d after transplantation. A third characteristic difference between the progressor tumors growing in normal and nude mice was the conspicuous absence of macroscopic central necrosis in even 3-cm diam tumors growing in nude mice as compared to the regular presence of marked central necrosis in tumors exceeding even 1-cm Diam in normal mice.

Lineage Relationship of the Progressor Tumors. The lineage relationship of the progressor variants was tested by determining whether the progressively growing tumors exhibited a surface marker that is restricted to a small percentage of UV-induced fibrosarcomas, including tumor 1591. This marker was defined by reactivity with a hybridoma antibody (P67) obtained from a fusion of mouse P3 myeloma cells with spleen cells from a 1591 tumor cell-immunized rat. Of 10 syngeneic fibroblast lines tested, only the 1591 and the 1463 regressor fibrosarcomas selectively express this marker, either measured by susceptibility of the fibrosarcoma cells to lysis by the hybridoma antibody and complement or measured by cytofluorometry analysis. When the expression of the P67 marker was tested on the five 1591 progressor tumor lines, four of the five expressed the marker, and this expression was stably heritable as determined by three repeated analyses several weeks apart. The retention of at least some level of expression of the P67 marker in four of five progressor tumors suggested a lineage relationship with the parental 1591 tumor. Furthermore, there were differences in the level of P67 expression that suggested that the different progressor tumors were not identical. The results of these studies will be published separately.[2]

[2] Phillips, C., J. L. Urban, R. D. Wortzel, M. Loken, P. M. Flood, and H. Schreiber. Analysis of antigenic cell surface changes in progressor variants using quantitative cytofluorography. Manuscript in preparation.

Discussion

Our studies show that during the growth of the 1591 tumor, heritably stable variants arose that could neither be killed by 1591-specific T cells nor stimulate the generation of such 1591-specific effector cells in vivo or in vitro. The generation of heritably stable tumor cell variants is thought to be a direct function of the number of generations of tumor cells (6). Consistent with this notion is the fact that several repeated passages in immunosuppressed hosts were required to allow for the development of a significant number of tumor variants before they could be selected for in normal animals. Presumably, the 1591 parental tumor was of unicellular (clonal) origin. This is suggested by the fact that UV-induced cancers originate as single foci in the relatively large area of UV-exposed skin. Nevertheless, more than one cell may have been simultaneously affected (transformed) by the carcinogen, and multicellular transformation appears to be a frequent occurrence for fibrosarcomas induced by subcutaneous injection of methylcholanthrene (20). In any case, passage of the 1591 tumor in immunosuppressed and then normal mice led to the emergence of tumor cells that were phenotypically different from the previously dominant phenotype of the parental tumor cell population.

Whenever phenotypic changes are found to occur in tumor populations, the possibility of laboratory contamination needs to be excluded. Several lines of evidence strongly argue against such a possibility. First, the P67 marker, whose expression is restricted to few UV fibrosarcomas, was retained by four of the five isolated progressor tumors. Second, there were significant differences among the progressors in cell morphology, growth behavior in vitro, and expression of the P67 marker. These findings make it highly unlikely that all the variant tumors could have derived from a single laboratory contamination. Thirdly, we have recently discovered (unpublished results) that the twice-cloned parental 1591 fibrosarcoma cell line is capable of spontaneously generating similar heritable variants in vitro, including those that differ in expression of the tumor-associated P67 marker and in expression of the tumor-specific transplantation antigen. Thus, at least in culture, a single clone of 1591 cells is capable of generating variants differing in expression of the tumor-associated marker, and a multicellular origin of the tumor is not necessary to explain the differences observed in the 1591 progressor tumors isolated from the animals.

The argument can be raised that the loss of the tumor-specific antigen was incidental and not related to selection by a tumor-specific immunologic host defense mechanism. Several lines of evidence make this highly unlikely. First, all of the progressor tumors we independently isolated showed a complete loss of the tumor-specific transplantation antigen. Second, we have previously shown (19) that the 1591 antigen is never lost from 1591 tumors growing in hosts whose 1591-specific immunity had been selectively suppressed with anti-idiotypic immunity. These animals retained their normal resistance to other UV-induced fibrosarcomas. Third, in vitro exposure of the parental 1591 tumor to a 1591-specific cytolytic T cell line is sufficient for the selection of variant tumor cell lines that have lost the expression of the original 1591-specific antigen (Richard Wortzel et al., manuscript in preparation). Thus, we propose from the above (*a*) that the outgrowth of tumorigenic variant cells, all of which lost the original 1591-specific antigen, was due to a resistance to the selection pressures of the host and (*b*) that the absolute resistance of normal animals to the potentially

malignant parental 1591 cells depended upon the expression of the 1591-specific antigen.

Collins et al. (15) report that the tumorigenicity of fibroblast cell lines chemically transformed in vitro correlated inversely with sensitivity to NK cells. However, no selection for progressor variants from the nontumorigenic NK-sensitive cells was performed. Such studies would determine whether sensitivity to NK cells was lost by progressor variants and would indicate whether a causative link between progressive growth and the loss of sensitivity to NK effector cells exists. In contrast, we have used cells transformed in vivo to study how NK-sensitivity changes when progressor variants arise in normal animals. Interestingly, we found that no selection against NK activity occurred during the evolution of progressor variants from the parental 1591 tumor. In fact, tumor cells of the different progressor cell variants induced natural killer activity more effectively than the parental tumor cells. These natural killer cells exhibited the classical reactivity and surface differentiation markers previously described to be present on natural killer cells (11).

At present we do not know whether the increased capacity of our progressor variants to induce and to be killed by natural killer cells is an obligatory event occurring simultaneously with the loss of a strong tumor-specific antigen. This is presently being investigated using other highly antigenic UV-induced fibrosarcomas. Interestingly, it has recently been demonstrated (21) that the sensitivity to NK cells may depend on the given stage of differentiation of the tumor cells and that only tumor cells expressing a more immature phenotype may be effectively killed by natural killer cells. Tumor progression is associated with a loss of differentiated cell function and an increased malignant growth behavior. Therefore, an increase in sensitivity to natural killer cells may commonly develop in tumor cell populations undergoing tumor progression.

Several other studies (22–24) have demonstrated the development of immunoresistant variants with decreased expression of the tumor-specific antigen after immunoselection in preimmunized animals. However, these investigators did not test nonimmune animals, nor did they test for possible simultaneous changes in susceptibility to NK or other immune cells. If, after the loss of a strong tumor antigen, tumor cells commonly show an increased ability to induce and be killed by natural killer cells, then natural killer cells may commonly play a protective role against local or metastatic growth of tumors that escape tumor-specific immunity. For example, NK-1.2$^+$Thy-1.2$^-$ cells have been shown (25) to adoptively transfer resistance to artificial blood-borne metastases of the UV-induced 2237 progressor tumor. Nevertheless, we found that the progressor variants we have isolated grew faster in nude mice, which have significant levels of natural killer activity, than in normal animals. Therefore, the protective significance of NK cells may be restricted at least in this system to lower numbers of tumor cells or to tumor cells that have entered the vascular circulation.

The differences in growth behavior of the progressor tumors in nude vs. normal animals suggest the participation of a T cell-mediated host defense mechanism in normal animals. The fact that progressor tumors isolated from normal but not nude animals show marked central necrosis strongly suggests that the normal host is at least partially capable of destroying progressor tumor cells. We do not yet know which effector cells are attracted to the site of tumor growth, but it is conceivable that the nonspecific T lymphocyte reactivity induced by the injection of progressor tumor cells

participates in the partial destruction of the tumor. In fact, other investigators (26) have shown that hyperimmunization can generate cross-protection against antigenically distinct tumors, implying the existence of cross-reactive tumor transplantation antigens. It is questionable, however, whether cross-protection among different tumors can always be taken as evidence for the presence of shared tumor antigens. In elegant experiments, it has, for example, been shown (27, 28) that endotoxin can also induce complete tumor regression that requires T cell-mediated immunity. This does not necessarily indicate that a common antigen is present on both the endotoxin and the tumors.

In any case, the nonspecific tumor cytotoxicity we describe here does not appear to be directed solely against such a putative cross-reacting tumor antigen because nontumorigenic adult syngeneic fibroblasts were also killed. It has been suggested by Shustik and co-workers (29) that this type of promiscuous T cell-mediated cytotoxicity might represent a polyclonal differentiation of effector lymphocytes. In support of this conclusion, we have recently found that the cross-reactive cytolytic immunity generated in vitro after in vivo priming with progressor cells segregates to a significant degree into noncross-reactive clones when analyzed under limiting dilution conditions (J. L. Urban, manuscript in preparation). Thus, injection of normal animals with progressor cells might induce a nonspecific increase in the immune responsiveness to various antigens in vivo and an increased susceptibility of the T cells to polyclonal activation in vitro.

Several groups of investigators (30–32) have used cultured cell lines to isolate in vitro clones of tumor cells that were either tumorigenic or nontumorigenic when injected as "pure" clones into a host. Interestingly, it was shown recently (32) that such progressor clones derived in vitro may not be selected for in vivo. This was demonstrated by the fact that injection of low numbers of cells from the regressor clone, when mixed with even a 10-fold higher number of cells from the progressor clone, still resulted in the regressor (parental) phenotype. Thus, the biologic relevance of progressor clones derived in vitro in the emergence of variant tumor cell populations in the host during normal tumor progression remains uncertain. It is possible that the in vitro derived progressor clones did not have a selective growth advantage in the host because they were found not to be antigenically different from the regressor clones. In contrast, the variant progressor subpopulations selected for by the normal host and described in our study all lacked the original tumor-specific transplantation antigen.

In conclusion, we studied the relative importance and hierarchy of different immune defense mechanisms that may enable a host to reject a syngeneic tumor without prior immunization. This was done by studying the fingerprints that the selective pressure of the host left on the progressively growing variant tumor cell population that emerged from the parental regressor cell population. Apparently, selective pressure was exerted by tumor-specific cytotoxic T cells or by other lymphocyte subclasses with the same specificity. On the other hand, there was no selection by NK cells or nonspecific T cells, and sensitivity to these cells was not sufficient to allow the unimmunized host to reject the tumor. Thus, our study shows that the expression of the tumor-specific antigen is essential for the resistance of the unimmunized host to the 1591 regressor tumor. The present results are complementary to our earlier studies using anti-idiotypic immune regulation that showed the critical importance of the

presence of tumor-specific lymphocytes for this tumor resistance (9, 19). Nevertheless, these effector cells are probably not the only requirements for tumor resistance, and it is quite conceivable that the tumor must evade other effector mechanisms to grow progressively. For example, macrophages are known to kill tumor cells in vitro (2), and they are known to accumulate at the site of secondary tumor challenge in animals exhibiting concomitant tumor immunity (33). At present we do not know why the 1591 tumor, when it changes to a progressor variant and loses the 1591-specific tumor antigen, becomes capable of inducing natural killer cells and cross-reacting cytolytic T lymphocytes. This alteration may either represent a detrimental dysregulation of the host's defense mechanism against the tumor or an induction of a valuable secondary defense mechanism which, if boosted, could lead to tumor rejection. Obviously, the therapeutic approach for inducing resistance to progressor tumors would critically depend on which of these two mechanisms is operative. If, in fact, the progressor tumors cause a dysregulation of the immune system, as shown in another tumor system (34), repair of such dysregulation may enable the host to reject the tumor by mounting a response against tumor antigens that remain or against new ones that appear after the loss of the original tumor-specific antigen.

Summary

The ultraviolet radiation-induced fibrosarcoma 1591 generally is rejected by normal syngeneic mice and only rarely exhibits progressive growth. We isolated five of these rare progressor tumors from normal animals to determine the selective pressures that had been exerted upon the parental tumor by normal immunocompetent hosts. We found that the variant tumor cell lines could neither induce nor be killed by tumor-specific lymphocytes, suggesting that selection had been exerted against tumor cells expressing the tumor-specific antigen. In contrast, no selection against natural killer cell activity or against nonspecific T cell-mediated immunity seems to have occurred because progressor tumor cells were highly sensitive to these types of effector cells and in fact induced these effector cells more effectively than did the parental tumor.

Nude mice were found to be as capable as normal mice in generating natural killer activity in response to a challenge with progressor tumor cells, but they were unable to mount a nonspecific T lymphocyte response. This may account for the fact that the progressor tumors grew at a significantly faster rate in nude animals than in normal mice. Thus, our study shows that in this tumor system nonspecific T cell-mediated immunity may play a role in retarding tumor growth, but the absolute resistance of normal animals to progressive tumor growth critically depends upon the presence of T cell-mediated tumor-specific immunity. Furthermore, neither NK cells nor nonspecific cytotoxic T lymphocytes appear to play a role in immunoselection against this tumor in normal immunocompetent hosts.

We thank Dr. D. A. Rowley for critically reviewing the manuscript. We also thank Ms. Connie Philipps and Ms. Bano Bohri for their excellent technical assistance.

Received for publication 24 September 1981.

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