LOSS OF TUMOR-SPECIFIC AND IDIOTYPE-SPECIFIC IMMUNITY WITH AGE*

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Most tumors induced in experimental animals are immunologically rejected only if the host has been previously immunized against the tumor (1). Recently, however, tumors have been induced by ultraviolet (UV) light that are also rejected when transplanted into normal syngeneic mice that have not been preimmunized (2). These tumors appear to be ideal for the study of naturally occurring host immunological mechanisms that protect against the growth of malignant cells.

Previously we reported that resistance to UV-induced tumors depends on the generation of tumor-specific lymphocytes (3). Mice immunized against the tumor-specific lymphocytes developed anti-idiotype lymphocytes that selectively lysed tumor-specific lymphocytes and such mice selectively lost the ability to reject a primary tumor challenge. Furthermore, this loss of resistance correlated with the loss of the capability of lymphocytes to respond to the tumor cells in vitro. Together, these results demonstrated the importance of tumor-specific immunity in protecting against malignant growth, and suggested that such immunity can be regulated by autogenous anti-idiotype immunity.

In the present study, we show that the capability of mice to reject a transplanted UV-induced fibrosarcoma is lost progressively with age. One possible explanation for this loss (4-7) is that older animals might develop an elevated level of anti-idiotype immunity (8), but this does not appear to be the case. Rather, aging mice appear to lose the capacity to mount both idiotype and anti-idiotype responses involved in the regulation of specific immunity to the tumor. This loss in turn is correlated with an increasing degree of “nonspecific” or “cross-reactive” immunity that is ineffective in controlling tumor growth.

Materials and Methods

Mice. Mammary tumor virus-negative (MTV⁻) C3H/HeN mice were obtained from a colony of germ-free-derived, specific pathogen-free animals at the Frederick Cancer Research...
Center. The mice were 1.5–3 mo old when shipped and were used by age 2–6 mo unless otherwise stated. The mice were kept at the La Rabida Institute in laminar flow hoods using sterilized cages, food and water; under these conditions, the 50% survival for female animals was >22 mo.

**Fibrosarcoma Lines.** The two fibrosarcomas, 1591 and 1316, were induced in C3H/HeN (MTV⁻) mice by repeated exposure to UV light (9). The 1591 and 1316 fibrosarcoma lines have non-cross-reacting tumor antigens (10). Both of these lines were adapted to culture from the first transplant generation and both are strongly immunogenic in that they regularly regress when transplanted into young syngeneic mice as tumor cell suspensions or tumor fragments, after an initial growth during the first 10 d. These tumors regularly grow progressively, however, in nude mice or in syngeneic C3H mice that were immunosuppressed by either UV irradiation or adult thymectomy and x-irradiation (9, 10). Both of these tumors kill the animals by infiltrative growth and direct extension of the tumor into vital organs without macroscopic evidence of distant metastases. So far, we have observed the development of a progressively growing variant tumor in only one of >300 young normal animals injected with cloned cultured cells or fragments from early transplant generations. All fibrosarcoma lines were cultured in minimal essential medium (410-1100; Grand Island Biological Co., Grand Island, N. Y.) containing 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin (600-5140; Grand Island Biological Co.).

**Mixed Lymphocyte-Tumor Cell Cultures (MLTC).** Culture medium was Dulbecco’s modified Eagle’s medium (H-21; Grand Island Biological Co.) supplemented with 5% heat-inactivated fetal bovine serum, 470 μg l-glutamine/ml, 580 μg l-arginine/ml, 180 μg l-asparagine/ml, 60 μg folic acid/ml, 550 μg sodium pyruvate/ml, 1% penicillin-streptomycin, and 5 × 10⁻⁵ M 2-mercaptoethanol (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.) added immediately before use. Spleens were aseptically removed, pressed through a wire screen, and further dispersed with a Pasteur pipette. The cells were then washed three times with culture medium, treated once with 0.83% (wt:vol) ammonium chloride to lyse erythrocytes, and then washed once again with culture medium. Tumor cells were treated with 50 μg/ml mitomycin C (Sigma Chemical Co., St. Louis, Mo.) for 45 min at 37°C and then washed four times with culture medium. MLTC for the generation of tumor-specific cytolytic T cells were made by adding 7 × 10⁶ responder lymphocytes and 2 × 10⁴ mitomycin C-treated stimulator cells in 2.0 ml medium per culture (24-well tissue culture plate 3524; Costar, Data Packaging, Cambridge, Mass.). MLTC for the measurement of DNA synthesis of responding cells were made by adding 10⁶ responder lymphocytes and 2.9 × 10³ mitomycin C-treated stimulator cells in 200 μl of medium per culture (96-well tissue culture plate 76-004-05; Linbro Chemical Co., Hamden, Conn.). 1.0 μCi [³¹P]TdR was added 12 h before harvest. Cells were harvested by absorption onto glass fiber filter paper with the use of a cell harvester (Otto Hiller Co., Madison, Wis.).

**Generation and Purification of Tumor-Specific Lymphoblasts and Induction of Anti-Idiotype Immunity.** The induction of anti-idiotype immunity by immunization with syngeneic alloantigen-specific T lymphoblasts has recently been described (11–14). We have slightly modified this scheme for the induction of anti-idiotype immunity to tumor-specific lymphocytes, and we have previously described the general scheme for the generation, purification, and subsequent immunization with tumor-specific lymphoblasts or nonresponding lymphocytes (3). Briefly, mice were immunized with viable 1-mm³ fragments of the 1591 tumor implanted subcutaneously with a trocar into both inguinal regions. Spleen cells from tumor-immune animals were removed aseptically 30 d after the primary immunization. Lymphocytes were restimulated in an MLTC by adding 7.5 × 10⁶ mitomycin C-treated 1591 tumor cells to 2.6 × 10⁶ lymphocytes in a 75-cm² tissue culture flask (3705; Costar, Data Packaging) containing 75 ml of MLTC medium. After 5 d in culture, tumor-reactive lymphoblasts were separated from nonresponding lymphocytes (NRL) by equilibrium density centrifugation (15). Cells harvested from 5-d cultures were washed three times with medium, and resuspended in a solution of bovine serum albumin (fraction V; Armour Pharmaceutical Co., Chicago, Ill.), (ρ = 1.082), which was overlaid with a less dense solution of bovine serum albumin (ρ = 1.060). Suspensions were spun to equilibrium at 10,000 g for 20 min. The small dense NRL formed a pellet at the bottom of the tube, whereas the less dense lymphoblasts floated at the interface of the two solutions. The
purity of the separated fractions was routinely determined by three different criteria: size, specific cytolytic activity, and \[^{3}H\]Tdr uptake. Gradient-purified anti-1591 and anti-1316 lymphocytes showed at least 60-80% specific lysis with <5% lysis of the control cell line using a 50:1 effector:target cell ratio. A suspension of such purified tumor-specific T lymphoblasts or NRL was mixed with equal parts Freund's complete adjuvant; 0.2 ml of the mixture containing \(10^7\) cells was injected intraperitoneally. Two booster injections of cells in Freund's incomplete adjuvant were given 3 and 6 wk later. 10 d after the final immunization, the immune reactivity of spleen cells from immunized mice was tested in vitro, or the spleen cells were used as a source of anti-idiotypic cytolytic T cells.

**Generation of Cytotoxic Lymphocytes In Vivo.** Cytolytic peritoneal exudate cytotoxic lymphocytes (PECL) were induced by a modification of a method previously described (16). Mice were injected once intraperitoneally with \(1 \times 10^7 - 2 \times 10^7\) 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 5–10 ml of CMEM containing 5 U/ml heparin to obtain PECL. The cells were washed with CMEM, and then incubated in 96-well tissue culture plates (Linbro Chemical Co.), \(1 \times 10^6 - 3 \times 10^6\) cells in 200 \(\mu\)l CMEM per well for 1 h at 37°C. Cells not adhering to plastic were washed and used as effectors for chromium release assays.

**Chromium Release Assay.** Cytotoxicity assays for cell-mediated cytolysis of tumor cells, tumor-specific lymphoblasts, or nonresponding lymphocytes were identical. \(5 \times 10^6 - 10 \times 10^6\) target cells were labeled with 100 \(\mu\)Ci \[^{51}Cr\] for 1 h at 37°C. 100 \(\mu\)l of effector cells were placed in V-bottomed 96-well microtiter plates (Cooke Engineering Co., Alexandria, Va.), and mixed with \(10^4 \ ^{51}Cr\)-labeled cells in 100 \(\mu\)l CMEM. Unless stated otherwise, the effector:target cell ratio was always 50:1. Plates were spun at 800 g for 5 min. After 3-6 h of incubation, 100 \(\mu\)l of supernate was withdrawn. Spontaneous release was 10–15% of the maximum release of radiolabel during the incubation period for the fibrosarcoma lines and <25% of the maximum release for the purified lymphocytes. The percentage of specific lysis was calculated by the formula:

\[
\text{specific lysis} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}} \times 100.
\]

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 an incubation with rabbit complement for 45 min at 37°C. A 1:2 dilution of culture supernate from the anti-Lyt-2 IgM hybridoma 2.155.2 (17) was used and was a generous gift from Dr. F. W. Fitch of The University of Chicago. Control effectors were treated with medium instead of the anti-Lyt-2 supernate, followed by rabbit complement treatment. Rabbit complement used in these experiments was serum from selected animals with low natural cytotoxicity to mouse myeloma cells. This complement was subsequently absorbed with 80 mg of agar per ml of serum and used at a final concentration of 1:8.

**Results**

**Definition of the “Anti-Idiotypic Probe” and the “Common Anti-1591 Idiotype”.** We have previously shown (3) that anti-1591 lymphoblast-immunized animals developed cytolytic T cells that lysed lymphoblasts generated in culture from spleen cells of mice immunized to 1591. These cytolytic lymphocytes were called “anti-idiotypic” because they selectively killed 1591-specific lymphocytes and recognized neither mitogen-induced lymphoblasts nor lymphoblasts that were specific for other UV-induced tumors having different antigens. Furthermore, these effector cells did not recognize the lymphocytes obtained from animals that broke idiotype suppression after blast-immunization.

In the present study, we test whether different animals immunized with 1591 regularly have lymphocytes that express an idiotype that is recognized by the anti-
idiotypic effector cells. Therefore, spleen cell suspensions from a large number of tumor-immunized animals were individually cultured with stimulator tumor cells. The lymphoblasts responding to the tumor cells in these cultures were used as $^{51}$Cr-labeled target cells. Effector cells were spleen cells from animals that had been immunized repeatedly with 1591-specific lymphoblasts as described in Materials and Methods. Table I shows that 1591-specific lymphoblasts from different normal young 1591-immune animals were regularly sensitive to effector spleen cells from blast-immune animals (average of 54% specific lysis), whereas control target cells consisting of 1316-specific lymphoblasts were not affected. This indicated the regular presence of shared antigenic determinants on 1591-specific lymphocytes of young immune animals. Thus, the putative anti-idiotypic effector cells (anti-idiotypic probe) recognize a common idiotype on 1591-specific T cells. Table I also shows that anti-idiotypic cells generated on five different occasions showed comparable cytolytic activity. Thus the variability in the amount of specific lysis of the 1591-specific lymphoblasts from cultures of the different animals was small regardless of whether a single batch

| Table I |
|---|
| Lymphocytes from 15 Mice Immunized with 1591 Share an Idiotype Recognized by Anti-Idiotypic Effector T Cells |

| Experiment | Mouse providing anti-idiotypic lymphocytes$^*$ | Mouse providing 1591 tumor-specific lymphocytes$^\dagger$ | Percent specific lysis of 1591 tumor-specific lymphocytes$^\ddagger$ |
|---|---|---|---|
| 1 | A | 1 | 43 |
| | A | 2 | 50 |
| | A | 3 | 56 |
| | A | 4 | 54 |
| | A | 5 | 44 |
| | A | 6 | 43 |
| 2 | B | 7 | 47 |
| | C | 8 | 76 |
| | D | 9 | 53 |
| | E | 10 | 44 |
| 3 | F | 11 | 56 |
| | G | 12 | 58 |
| 4 | H | 13 | 68 |
| 5 | J | 15 | 55 |

$^*$ Effector cells were spleen cells from C3H mice immunized three times with 1591 tumor-specific lymphoblasts in adjuvant and tested 10 d to 3 mo after the final lymphoblast immunization.

$^\dagger$ 1591-specific lymphoblasts were generated in a secondary MLTC from individual animals immunized with 1591 fragments 1–3 mo earlier. Animals 1–6 were all tested in the same experiment. Animals 7–15 were tested on different days.

$^\ddagger$ Cells were tested in a 4-h $^{51}$Cr release assay using as target cells purified 1591 tumor-specific lymphoblasts from experimental animals or 1316 tumor-specific lymphoblasts from 1316-immunized control animals at a 250:1 effector:target cell ratio. Percent lysis was calculated as described in Materials and Methods. The killing potential of the anti-idiotypic effector cells was completely eliminated by treatment with anti-Thy-1.2 and complement. Furthermore, the percent lysis of 1316 specific control target lymphocytes by the anti-idiotypic effector cells was $<2$ in all experiments (data not shown).
(experiment 1, 48% ± SD 6) or different batches of anti-idiotypic killer cells were used (experiments 2–5, 57% ± SD 10). This indicated that we can repeatedly generate an anti-idiotypic probe that recognized a common anti-1591 idiotype.

Parameters for Induction of Anti-Idiotype Immunity. Normal 10-wk-old C3H mice were immunized three times with 1591 tumor-specific lymphoblasts in adjuvant. Control animals were immunized with NRL that had been isolated from the same MLTC culture as the 1591-specific lymphoblasts. 10 d after the last immunization, spleen cells from test and control mice were stimulated with 1591 or 1316 tumor cells. The proliferative response was measured by [3H]Tdr incorporation. Fig. 1 shows that spleen cells from 1591 lymphoblast-immunized animals were unresponsive to 1591 tumor cells, whereas spleen cells from NRL-immunized animals responded normally to stimulation with 1591 cells. Also, spleen cells from anti-1591 lymphoblast-immunized animals were unable to generate cytolytic T cells specific for 1591 tumor cells, even though they were able to generate a normal cytolytic T cell response to 1316 tumor cells when stimulated with these cells in a primary MLTC (Fig. 2). Table II shows that this specific unresponsiveness and cytolytic anti-idiotype activity persisted for at least 6 mo after the blast immunization.

We then investigated in repeated experiments the number of blasts required for the generation of cytolytic anti-idiotype activity and the induction of unresponsiveness.
Fig. 2. Lack of cytolytic response of spleen cells from lymphoblast-immunized animals to 1591 tumor cells. Spleen cells from lymphoblast-immunized, NRL-immunized, or normal animals were incubated with 1591 or 1316 tumor cells and after 5 d of culture tested at a 100:1 effector to target cell ratio. □, stimulated with 1591, tested on 1591 sarcoma; ◊, stimulated with 1591, tested on 1316 sarcoma; ▼, stimulated with 1316, tested on 1591 sarcoma; and □, stimulated with 1316, tested on 1316 sarcoma.

Table II
Persistence of Specific Anti-Idiotype Immunity and Concurrent Lack of Specific Immunity to 1591 Tumor Cells

| Cells from mice immunized with* | Time after immunization† | Age of mouse at analysis | Percent specific lysis of§ | Percent specific lysis of‖ |
|---------------------------------|---------------------------|--------------------------|---------------------------|---------------------------|
|                                 | mo | mo | Anti-1591 lymphoblasts | Anti-1316 lymphoblasts | 1591 tumor cells | 1316 tumor cells |
| Anti-1591 blasts                | 1  | 5  | 55 ± 1                  | 1 ± 1                   | 1 ± 0                     | 3 ± 2                     |
| NRL                             | 1  | 5  | 1 ± 0                   | 1 ± 1                   | 48 ± 16                   | 1 ± 1                     |
| Anti-1591 blasts                | 3  | 7  | 61 ± 4                  | 0 ± 0                   | 1 ± 1                     | 4 ± 3                     |
| NRL                             | 3  | 7  | NT†                     | NT                      | 29 ± 7                    | 3 ± 2                     |
| Anti-1591 blasts                | 6  | 10 | 64 ± 8                  | 2 ± 2                   | 0 ± 0                     | 1 ± 1                     |
| NRL                             | 6  | 10 | NT                       | NT                      | 27 ± 7                    | 3 ± 2                     |

* Mice were immunized with 10⁷ purified lymphoblasts or 10⁷ NRL injected intraperitoneally in Freund's complete adjuvant. Animals were boosted twice at 3-wk intervals with the same number of lymphocytes in incomplete adjuvant.

† At different times after the immunizations, spleen cells from blast-immunized and control animals were tested (a) for their capability to specifically lyse 1591 tumor-specific lymphoblasts of the dominant clones and (b) for their capability to generate 1591 tumor-specific T cells in a primary 5-d MLTC culture. Three animals per group were killed at 1 and 3 mo and two animals per group were killed at 6 mo.

§ Spleen cells were tested in a 4-h ⁵¹Cr release assay using as target-purified 1591-specific or 1316-specific lymphoblasts at a 250:1 effector:target cell ratio. These lymphoblasts were obtained after standard in vitro restimulation of 1591- or 1316-immune spleen cells and purified by density gradient centrifugation. The percentage of specific lysis was calculated as described in Materials and Methods.

‖ Effector cells generated in a 5-d MLTC by spleen cells from blast-immunized or control animals were tested in a 3-h ⁵¹Cr release assay using as target 1591 or 1316 tumor cells at a 50:1 effector:target cell ratio. Percent specific lysis was calculated as described in Materials and Methods.

† Not tested.
Table III shows that a dose of $10^7$ lymphoblasts per injection generated significant anti-idiotypic activity. Furthermore, this high dose was capable of totally abrogating the response of the animals to 1591 tumor cells; however, lymphocytes from animals receiving half as many lymphoblasts responded specifically and almost as well as 1591 tumor cells in vitro, as did lymphocytes from control animals.

**Effect of Age on the Development of Anti-Idiotypic Immunity.** Animals of different age groups were immunized repeatedly with $10^7$ anti-1591 lymphoblasts or $10^7$ NRL in adjuvant. Table IV shows that as the animals increased in age, anti-idiotypic immunity was less readily induced. Animals that had been immunized at the age of 3 mo showed the highest anti-idiotypic activity, and spleen cells from these mice were unresponsive to 1591 tumor cells in vitro. Animals immunized at the age of 6 mo showed a lower but nevertheless significant level of cytolytic anti-idiotypic activity. Interestingly, cells from these animals respond specifically to 1591 tumor cells in vitro. However, the anti-1591 lymphoblasts of these blast-immunized animals were insensitive to the anti-idiotypic probe, indicating that idiotypically different 1591-specific lymphocyte clones were involved in the response of the 11-13-mo-old animals.

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**Table III**

*A Threshold Number of Lymphoblasts is Required to Induce Anti-Idiotypic Immunity and Concurrent Unresponsiveness to 1591 Tumor Cells*

| Experiment | Dose | Cells from mice immunized with* | Anti-idiotypic cytolytic activity (percent specific lysis)$^\dagger$ | Tumor-specific immune response (percent specific lysis)$^\ddagger$ |
|------------|------|-------------------------------|-------------------------------------------------|-------------------------------------------------|
|            |      |                               | Anti-1591 lymphoblasts                          | Anti-1316 lymphoblasts                          |
|            |      |                               | 1591 tumor cells                                | 1316 tumor cells                                |
| 1          | $1 \times 10^7$ | 1591-specific blasts | 72                                               | 3                                               |
|            | $5 \times 10^6$ | 1591-specific blasts | 8                                                | <0                                              |
|            | $1 \times 10^6$ | 1591-specific blasts | 2                                                | <0                                              |
|            | $5 \times 10^5$ | 1591-specific blasts | <0                                               | <0                                              |
|            | $1 \times 10^7$ | NRL                         | 7                                                | <0                                              |
| 2          | $1 \times 10^7$ | 1591-specific blasts | 76                                               | <0                                              |
|            | $5 \times 10^6$ | 1591-specific blasts | <0                                               | <0                                              |
|            | $1 \times 10^6$ | 1591-specific blasts | <0                                               | <0                                              |
|            | $5 \times 10^5$ | 1591-specific blasts | <0                                               | <0                                              |
|            | $1 \times 10^7$ | NRL                         | <0                                               | <0                                              |
| 3          | $1 \times 10^7$ | 1591-specific blasts | 44                                               | 2                                               |
|            | $5 \times 10^6$ | 1591-specific blasts | 2                                                | <0                                              |
|            | $1 \times 10^6$ | 1591-specific blasts | 2                                                | 1                                               |
|            | $5 \times 10^5$ | 1591-specific blasts | 1                                                | 1                                               |
|            | $1 \times 10^7$ | NRL                         | 1                                                | 3                                               |

$^\ast$ Mice were immunized with different doses of 1591-specific lymphoblasts or NRL intraperitoneally in Freund's complete adjuvant. Animals were boosted twice at 3-wk intervals with the same number of lymphocytes in incomplete adjuvant and tested 10 d to 3 mo thereafter.

$^\dagger$ Spleen cells from blast-immunized and control animals were tested for their capability to specifically lyse 1591 tumor-specific lymphoblasts of the dominant clonotype in a 4-h $^{51}$Cr release assay using as targets purified 1591-specific or 1316-specific lymphoblasts at a 250:1 effector:target cell ratio. These lymphoblasts were obtained after standard in vitro restimulation of 1591- or 1316-immune spleen cells and purified by density gradient centrifugation. The percentage of specific lysis was calculated as described in Materials and Methods.

$^\ddagger$ See footnote \| to Table II.
**TABLE IV**

*Effect of Age on the Induction of Cytolytic Anti-Idiotype Activity and on the Induction of Unresponsiveness to 1591 Tumor Cells*

| Experiment | Age at start of immunization | Age at analysis | Immunogen* | Anti-idiotypic cytolytic activity (percent specific lysis)‡ | Tumor-specific immune response (percent specific lysis)§ | Sensitivity of tumor-reactive lymphoblasts to lysis by anti-idiotypic probe||
|---|---|---|---|---|---|---|
| 1 | 9 mo | 12 mo | Anti-1591 blasts | 53 | 7 | <0 |
| 6 | 9 mo | 12 mo | Anti-1591 blasts | 28 | 16 | 3 |
| 9 | 9 mo | 12 mo | Anti-1591 blasts | 2 | 25 | 5 |
| 12 | 9 mo | 12 mo | Anti-1591 blasts | <0 | 32 | 44 |
| 3 | 13 | 16 | NRL | <0 | 19 | 1 |
| 6 | 13 | 16 | NRL | <0 | 24 | 4 |
| 9 | 13 | 16 | NRL | <0 | 31 | 17 |
| 12 | 13 | 16 | NRL | <0 | 38 | 45 |

* Mice of different ages were immunized with 10⁷ purified lymphoblasts or 10⁷ NRL intraperitoneally in Freund's complete adjuvant. Animals were boosted twice at 3-wk intervals with the same number of lymphocytes in incomplete adjuvant.
‡ See footnote ‡ to Table III.
§ See footnote § to Table II.
|| The tumor-reactive lymphocytes from blast-immunized and control animals were gradient-purified and used as ²⁵Cr-labeled target cells using anti-idiotypic killer cells obtained from a separate group of young blast-immunized animals as effector cells at a 250:1 effector:target cell ratio in a 4-h assay. The percentage of specific lysis was calculated as described in Materials and Methods.

Animals that had been immunized at the age of 9 or 12 mo did not develop any detectable anti-idiotypic activity. Furthermore, the responses of these blast-immunized animals to the 1591 tumor cells in vitro showed high cross-reactivity to 1316 target cells. Tumor-reactive lymphocytes from spleens of older NRL-immunized controls also demonstrated a high degree of cross-reactivity; however, there were marked differences in the sensitivity of the tumor-reactive lymphocytes to the anti-idiotypic probe for this group as compared with the blast-immunized group. Spleen cells from blast-immunized animals of any age failed to develop tumor-reactive lymphocytes expressing the common 1591-specific idiotype, whereas young NRL-immunized control animals developed tumor-reactive lymphocytes expressing the common anti-1591 idiotype. It can be seen in Table IV, however, that the tumor-reactive lymphocytes
generated by the NRL-immunized control animals demonstrated a progressive decrease in sensitivity to the anti-idiotypic probe with increasing age. This change was paralleled by the increase in cross-reactivity of these tumor-reactive lymphocytes to 1316 tumor cells described above.

Effect of Age on the Generation of Tumor-specific Responses In Vitro. The above experiments showed that aging animals did not generate significant anti-idiotypic immune responses after blast immunization. Furthermore, older blast- and NRL-immunized animals generated nonspecific 1591-reactive lymphocytes in culture that lacked the exquisite specificity of cytolytic lymphocytes from young animals. We then wished to determine whether such age-dependent change in the tumor reactivity pattern would also be observed in normal untreated animals because it was possible that this age-dependent pattern in the blast- and NRL-immunized animals was related to the chronic nonspecific stimulation by intraperitoneal adjuvant present in these animals. Therefore, we compared in three independent experiments young, middle-aged, and older nonimmunized animals in terms of their capability to generate tumor-specific lymphocytes in vitro. Spleen cells from animals of different ages were stimulated in a primary MLTC with 1591 tumor cells. After 5 d the effector cells were tested on either 1591 or 1316 tumor cell targets in a ⁵¹Cr release assay. The results from these studies were similar and therefore pooled. Fig. 3 shows that as the age of the animals increased, their reactivity to 1591 tumor cells remained constant. The cross-reactivity of the effector cells, however, increased considerably between 10 and 13 mo of age. All the cytolytic activity could be totally eliminated by pretreatment of the effector cells with anti-Lyt-2 and complement (data not shown). The tumor-reactive lymphocytes

![Graph](image-url)  
**Fig. 3.** Age-related loss of the specificity of tumor-reactive lymphocytes generated in a primary MLTC of spleen cells from normal animals of different ages. Spleen cells of normal mice of different ages were stimulated with 1591 tumor cells in a 5-d MLTC. The culture-generated lymphocytes were then tested in a 4-h ⁵¹Cr release assay using 1591 (•) or 1316 (○) tumor cells as targets. The percent lysis was calculated as described in Materials and Methods. Data from two independent experiments are pooled. Vertical bars indicate ± 1 SEM, two or three individual animals. The effector to target cell ratio is 50:1.
generated by spleen cells from the same animals as those used in Fig. 3 were also gradient purified and used as $^{51}$Cr-labeled target cells for cytolytic anti-idiotypic effector cells or control effector cells. Fig. 4 shows that the participation of the common 1591 tumor-specific lymphocyte clone in an in vitro immune response to 1591 tumor cells markedly declined with increasing age beginning at about 8 mo of age.

Effect of Age on Development of Tumor-specific Immunity. We had previously demonstrated that the presence of tumor-specific lymphocyte clones was necessary for young animals to resist the 1591 tumor (3). Therefore, normal animals might be expected to lose their natural resistance to the 1591 tumor cells during middle age. To test this hypothesis we implanted 1-mm$^3$ 1591 tumor fragments subcutaneously into mice of different ages and monitored the mice for progressive tumor growth during the following 8 wk. Table V shows that, as anticipated, the tumor resistance of animals challenged with 1591 decreased with increasing age.

We also studied the immune response of PECL of mice of different ages to 1591 tumor cells. Animals were injected intraperitoneally with 1591 tumor cells and the responding PECL were removed 10 d later. Fig. 5 shows that cells from mice 2-6 mo old showed strong cytolytic reactivity to 1591 tumor cells. PECL from middle-aged animals (9-15 mo) showed considerably less reactivity to 1591 tumor cells and those from 16-22-mo-old animals were completely nonresponsive.

Interestingly, the increased cross-reactivity to 1316 tumor cells with increasing age,
TABLE V
Effect of Age on the Resistance of Normal Mice to Challenge with the 1591 Tumor

| Age of mice at time of challenge* (mo) | Number of mice challenged | Sex  | Number of mice with progressively growing tumors$ |
|--------------------------------------|---------------------------|------|--------------------------------------------------|
| 2                                    | 20                        | Female | 0                                                  |
| 2                                    | 20                        | Male  | 0                                                  |
| 6                                    | 20                        | Female | 1                                                  |
| 6                                    | 20                        | Male  | 2                                                  |
| 10                                   | 20                        | Female | 3                                                  |
| 10                                   | 20                        | Male  | 7                                                  |

* Normal C3H mice of different age groups were implanted with two 1-mm³ fragments of the 1591 fibrosarcoma in both inguinal regions.

$ Number of mice that showed progressively growing tumors 8 wk after tumor challenge.

Fig. 5. Effect of age on the generation of PECL specific for 1591 tumor cells. Female mice of different ages were injected intraperitoneally with 10⁷ 1591 tumor cells that had either been mitomycin C-treated or left untreated. PECL were removed 8 d later and tested at an effector:target cell ratio of 100:1. Percent lysis was calculated as described in text. (*), amount of specific lysis by PECL from an individual 1591-immunized animal; (O), amount of specific lysis by PECL from nonimmunized controls. The results of two independent experiments were similar and therefore pooled in this figure. Lyt-2 and complement treatment regularly reduced the reactivity of effector cells to 1591 cells to <10% lysis and untreated effector cells caused <10% lysis of 1316 cells (data not shown).

which we had observed in culture-generated tumor-reactive lymphocytes, was not found for lymphocytes recovered from the peritoneal cavity of these animals. The lysis of 1316 tumor cells by PECL was <5% at an effector:target cell ratio of 100:1. All the reactivity to 1591 tumor cells could be eliminated by anti-Lyt-2 antibody and complement.
Discussion

In recent studies, we have shown that the expression of the 1591 tumor-specific antigen on 1591 tumor cells (18) and the immune response of tumor-specific T cells to this antigen (3) are critically important for resistance of normal animals to the 1591 tumor. In the present study, we found that progressing age caused qualitative as well as quantitative changes in the immune response of the animals to the 1591 tumor-specific antigen. Apparently as a consequence of aging, animals became progressively more susceptible to challenge with the 1591 tumor.

We have found that young mice regularly recognize the 1591 tumor antigen with lymphocytes that share idiotypic determinants. This suggests the common expression of a selected group of clonotypes or idiotypes on lymphocytes responding in the young animal to the 1591 tumor. By 8-9 mo of age, however, the participation of these lymphocytes expressing the common idiotype is reduced, although there are considerable variations among individual animals in the extent of this reduction (Fig. 4). Nevertheless, the tumor-reactive lymphocytes at this age still show the same high specificity for 1591 tumor cells as had been found in the younger animals (Fig. 3). Thus, aging animals begin to lose clonal dominance at this age in that they respond with idiotypically different lymphocyte clones that are still specific for the 1591 tumor. The age period during which animals respond with these idiotypically different but tumor-specific clonotypes is apparently short because only slightly older animals (10-13 mo) respond to tumor challenge with different tumor-reactive lymphocytes that show marked cross-reactivity with 1316 tumor cells (Fig. 3 and Table IV).

Because this and the previous studies (3) indicate the host-protective value of the dominant 1591-specific lymphocyte clones for the resistance of the host to the tumor, it is important to determine how the dominance of these T cell clones is controlled. Several independent studies in other systems have suggested that the relative participation of B cell idiotypes in an immune response depends on naturally present anti-idiotypic helper or suppressor T cells (19-22). In some instances, the activity of naturally present anti-idiotypic helper T cells seems to control the dominance of a certain clonotype in an antibody response (19, 20). In other instances, normal individuals seem to have anti-idiotypic suppressor T cells that are responsible for the lack of participation of certain B cell clonotypes in an immune response (21, 22). Unfortunately, little is known about the various factors that may control dominance of T cell idiotypes in an immune response. We have previously shown that autogenous anti-idiotypic immunity can regulate 1591 tumor-specific lymphocyte clones (3). Thus, age-dependent changes in the anti-idiotypic compartment may be at least partially responsible for the age-dependent changes of clonal dominance in the 1591 system.

Anti-idiotypic helper cells may, for example, participate in the control of the dominant 1591-specific clonotypes, and aging may cause a loss in the response of anti-idiotypic helper T cells. This hypothesis is consistent with our finding that the ability of animals to develop anti-idiotypic immunity declines with age. Our findings also suggest that a spontaneous generation of suppressive anti-idiotypic T cells is probably not responsible for the loss of clonal dominance. Certainly, we have not detected a spontaneous development of anti-idiotypic killer cells in aging animals. Thus, the mechanism for the changes we have observed in clonal dominance in aging mice
appears to be different from the one mediating the immune suppression in young mice caused by immunization with 1591-specific lymphocytes of the common idioype.

Immunization of young mice to produce anti-idiotypic immunity (which permits tumor growth) may switch the young host from the production of anti-idiotypic helper cells to the production of the observed anti-idiotypic killer cells. Such a phenomenon may in fact be the reason for the threshold effects of the dose of lymphocytes needed to induce effective idioype-suppression as observed in this study (Table III) and in a previous study in the alloantigen system (11).

We know of no published studies using carefully defined, idiootypically restricted systems that report changes in T cell clonal dominance with increasing age. Recent studies at this institution, however, indicate that the loss of B cell clonal dominance and the development of minor, previously absent or suppressed B cell clones specific for phosphorylcholine antigens occur not only after idioype suppression (23) but also as a result of aging of the animals (D. Kaplan and J. Quintans, unpublished results). Thus, aging may have a parallel effect on B and T cell clonal dominance.

In contrast to the qualitative age-dependent changes mentioned above, there was no change in the absolute quantitative level of cytolyis of 1591 tumor cells by cultured lymphocytes from unprimed animals of different ages (Fig. 3). This result is in agreement with earlier investigations that also found no age-dependent change in the cytotoxicity of short-term-cultured lymphocytes to syngeneic fibrosarcomas of three different mouse strains, even when mice beyond their 50% survival mode were used (24). Although T cells generated from spleens of older animals can lyse 1591 tumor cells effectively in vitro, the value of these lymphocytes, if generated in vivo, in protecting the host against a challenge of 1591 tumor cells must be relatively small, because individuals lose their natural resistance to the 1591 tumor cells in spite of generating such T cells.

We have no evidence to support the possibility that cytotoxic lymphocytes generated in short-term culture are specific for a putative cross-reacting tumor antigen on UV tumors, because such lymphocyte suspensions also kill non-UV-induced, nontransformed, nonmalignant syngeneic fibroblast cell lines (unpublished results). Possibly the spleen cells of older animals have an increased tendency to generate self-reactive rather than tumor-specific T cells in culture when stimulated with immunogenic tumor cells, because it has recently been suggested that alloreactive T lymphocytes from aged mice are cross-reactive and express increased lysis of autologous and third-party target cells (25). It is possible that the age-dependent increase in the generation of such cross-reactive cells occurs preferentially in vitro, because we find it difficult to retrieve such highly cross-reactive Lyt-2+ cells in vivo from the peritoneal cavities of 1591-immunized mice. Such cells may be generated in large numbers in vivo but we may not be able to retrieve the majority of them from the peritoneal cavity due to trapping by normal tissues of the host. In any case, because the total immune response of an individual to the tumor antigen is not found to increase with increasing age, an increased participation of nonspecific T cell clones must mean a decreased contribution by tumor-specific clones and therefore a decreased protection against tumor growth.

The capability of normal mice or their cells in culture to mount a tumor-specific immune response declines during middle age, i.e., between 9 and 14 mo. At this time, individual mice begin to become susceptible to the tumor challenge. The high degree
of individual variation in the capability of different animals to mount a tumor-specific immune response may explain why only some animals become susceptible to the tumor challenge at this age. The decline in tumor-specific immune responses may also reduce immune selection pressures against tumor variants that occur. In fact, the unusually high immunogenicity of UV or methylcholanthrene-induced tumors induced in UV-exposed mice has been attributed to the lack of immunoselection in such mice because their immune system had been suppressed by the UV light (10, 26–28). Because most of these tumors developed in 8–15-mo-old animals (9, 28), we feel that the decreased immune potential of the animals at this age may have contributed to the lack of immunoselection observed in these animals. In any event, our results show that older animals, even in the absence of carcinogen-induced immunosuppression, permit the growth of tumors that are regularly rejected by the intact immune system of the young. If tumors developing in older individuals have indeed retained tumor-specific antigens, we may be able to recover the response of the tumor-specific lymphocytes in aging individuals, and thus induce strong resistance to their developing tumors.

Summary

The ultraviolet light-induced fibrosarcoma 1591 undergoes “first-set rejection” when transplanted into normal syngeneic mice. We found, however, that the primary resistance of normal mice decreases with age, beginning at 9–12 mo, equivalent to middle age for mice. Mice lose with age the capacity to mount both idiotypic and anti-idiotypic responses responsible for controlling the growth of the tumor. This loss was correlated with quantitative as well as qualitative changes in the response, such as changes in specificity and clonotype. Normal young mice regularly expressed a dominant common anti-1591 “idiotype” as defined by an anti-idiotypic probe. The capability of normal mice to respond with lymphocytes of this dominant common idiotype began to decline at about 8 mo of age. At this time, animals still generated tumor-specific lymphocytes, but these lymphocytes appear to be idiotypically different lymphocyte clones. With further increase in age, animals responded with tumor-reactive lymphocytes that showed a marked cross-reactivity to other tumor target cell lines. Both in vivo and in vitro, the capability of normal mice to mount an immune response that was specific for the 1591 tumor cells decreased between 9 and 14 mo, which was the age individual mice became increasingly susceptible to a challenge with 1591 tumor cells. Thus, our data suggest that clones of tumor-specific T cells provide primary and early protection of young animals against challenge with malignant 1591 cells. However, the dominance of these tumor-specific T cell clones in a primary immune response is lost in middle-age.

Because the ability of animals to mount anti-idiotypic immune responses also declined in middle-aged animals, it is possible that the observed loss of clonal dominance of tumor-specific clones with increasing age is at least partially related to age-dependent changes in the anti-idiotypic compartment.

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