HIGHLY MALIGNANT TUMOR VARIANTS RETAIN TUMOR-SPECIFIC ANTIGENS RECOGNIZED BY T HELPER CELLS

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Cancers are usually advanced when diagnosed. An important problem in developing immunotherapy to cancer is to determine whether such advanced cancers express antigens that can serve as specific targets for immune destruction. Murine skin tumors induced by UV are especially suited for study of changes in the expression of tumor-specific antigens during development of cancer (reviewed in reference 1). The "parental" UV-induced tumors are usually highly immunogenic and regress (RE)[1] when transplanted into normal immunocompetent mice, but will usually grow progressively in T cell–deficient or nude mice. Rarely, however, do these tumors give rise to variants that grow progressively in normal mice (2). The progressor (PRO) variants lose antigens recognized by CTL (2), so that "tumor progression" (3, 4) leading to an advanced cancer apparently results from Darwinian selection by immunologic and possibly other homeostatic defense mechanisms (5, 6).

We have previously shown (2) that PRO variants derived from the UV-induced tumor 1591-RE have lost a tumor-specific antigen recognized by CTL, and this antigen was designated the "A" antigen. We suspected that PRO tumor variants retained additional antigens, since a relatively larger tumor inoculum ($>10^7$) of these variant tumor cells was required for growth in normal mice as compared with the number of tumor cells ($\leq 10^6$) needed for outgrowth in T cell–deficient nude mice, and because the tumor take in normal mice was only ~80% of that in nude mice (2). Indeed, we found that 1591-PRO variants could be rejected and did induce specific CTL if mice were first immunized with RE or a subtumorigenic number of PRO tumor cells (7); we therefore considered the 1591-PRO variants to still express other antigens, designated "B" (8).

In this paper, we now describe a highly malignant (HM) variant of 1591 that metastasizes, grows even in immune hosts, and that has lost all known CTL-

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[1] *Abbreviations used in this paper:* DTH, delayed-type hypersensitivity; HM, highly malignant; MLTC, mixed lymphocyte–tumor cell culture; MTV, mammary tumor virus; PRO, progressor; RE, regressor; R/S, responder/stimulator ratio.

defined antigens. This HM variant, however, as well as all other tumors of the 1591 lineage, retain antigen(s) recognized by Th cells. Such Th-recognized tumor-specific antigens may, therefore, represent potential targets for immunotherapy.

Materials and Methods

Mice. 4–10-wk-old germ-free–derived, specific pathogen-free female C3H/HeN mammary tumor virus (MTV)-negative mice were purchased from the National Cancer Institute Frederick Cancer Research Facility, Bethesda, MD. The mice were housed at the La Rabida Institute in laminar flow hoods. Original stock of nude C3H/HeN mice used to breed a nude C3H colony were in the twenty-third backcross generation when they were obtained from a colony at the Biology Division of the Oak Ridge National Laboratory, Oak Ridge, TN. To prepare immunodeficient mice, 4-wk-old C3H mice were thymectomized, irradiated (900 rad, ^{137}Cs source), and then bone marrow reconstituted a wk later (9).

Monoclonal Antibodies. The hybridomas secreting anti-Thy-1.2 (AT83A), anti-Lyt-2 (3.155), and anti-L3T4 (GK 1.5) were a kind gift of Dr. Frank W. Fitch of the University of Chicago. The anti-H-2D^k (15.5.5) hybridoma was a gift of Dr. David Sachs, National Cancer Institute, Bethesda, MD. Anti-I-A^k (10-2.16) and anti-H-2K^k (11-4.1) hybridomas were obtained from the Salk Cell Distribution Center (La Jolla, CA) and anti-I-E^k (17.3.3S) was from the American Type Culture Collection, Rockville, MD.

Tumor Lines and Variants. The RE fibrosarcomas 1591-RE, 1316-RE, and 2240-RE were induced in C3H/HeN(MTV$^-$) mice by repeated exposure to UV light (10) and the RE fibrosarcoma 1130-RE was induced in a UV-irradiated C3H/HeN(MTV$^-$) mouse by subcutaneous injection of 3-methylcholanthrene under the unshaved ventral skin. The immunogenic RE tumors grow initially for 1–2 wk in young syngeneic mice but then regress. Regression occurs even when large doses of the tumor are injected, e.g., multiple 1-mm^3 tumor fragments, while much smaller tumor grafts grow progressively and kill immunodeficient or nude mice (11). Noncrossreacting, tumor-specific antigens have previously been shown on these tumors using transplantation assays (11), and through the generation of CTL (2, 10, 12) and mAb probes (13).

PRO tumors 1591-PRO.4 (2) and 1316-PRO.1 (referred to in this paper as 1591-PRO and 1316-PRO) were reisolates from rare tumors (<5%) that grew progressively in normal mice challenged with the parental 1591-RE and 1316-RE. These variants grow progressively upon subsequent transplantation in ~80% of young immunocompetent mice (2), and they kill the host through local invasion, without macroscopic evidence of distant metastases. As a result of immunoselection by the normal host, the 1591-PRO variants have lost a 1591-tumor-specific antigen (designated A) that is only expressed on 1591-RE tumors (7, 8). The 1591-PRO tumors retain another 1591-specific tumor antigen designated B, which is recognized by CTL from 1591-PRO-immunized mice (7, 8). 1591-AS6 (A$^-$B$^+$) and 1591-BS1 (A$^+$B$^-$) (referred to here as 1591-AS and 1591-BS, respectively) were selected in vitro by CTL specific for the A and B determinants, respectively (8). The induction of MHC class II antigens has been reported for some tumors after manipulation of tumor cells in vitro and in vivo (14), but such antigens were not inducible in the 1591 tumors by culture with recombinant IFN-γ (Genentech, Inc., San Francisco, CA) or secondary mixed lymphocyte culture supernatants (8), although these factors induced an increased expression of class I antigens. Furthermore 1591-RE did not express detectable levels of class II antigen when tissue sections of tumors isolated during rejection were stained by immunoperoxidase using I-A$^+$ tumor-associated macrophages as positive controls (C. Van Waes, unpublished results).

All tumors have been adapted to tissue culture, expanded for 2 wk, and cryopreserved in aliquots. Aliquots were thawed and used within 1–3 d for all experiments. All cell lines used were routinely examined for and found to be free of mycoplasma by Hoechst 33258 (Flow Laboratories, Inc, McLean, VA) DNA staining. Tissue culture lines were injected into C3H nude or immunodeficient mice to obtain solid tumor fragments for immuniza-

tion or challenge in most experiments. The solid tumors were finely minced in cold MEM and three 1-mm^3 fragments were injected subcutaneously in the inguinal region of mice by a trocar. This precaution was taken to prevent priming Th cells to heterologous serum components of tissue culture medium.

Delayed-Type Hypersensitivity (DTH) Assay. A DTH response is defined as an antigen-specific, T cell–dependent, cutaneous inflammatory reaction with a peak intensity at 24–48 h after antigen challenge of a preimmunized animal (15). DTH reactions can be sensitively measured by an ear swelling assay (16, 17) that we have adapted for testing DTH responses to live tumor cells. In the DTH assay, responses of unsensitized mice or mice sensitized with three 1-mm^3 tumor fragments 8 d previously were elicited with 2×10^5 tumor cells suspended in 10 μl culture medium. The injection for elicitation was given subcutaneously on the outer dorsal surface of both ears using a precision syringe (710LT; Hamilton Co., Reno, NV) and a 30 g needle. The increment in ear thickness represents the average difference between three prechallenge and three 24-h postchallenge measurements using a precision spring-loaded dial caliper (Mitutoyo No. 7326; Precision Gage Co., Chicago, IL). The measurements were carried out independently by two investigators who did not know the identity of the experimental groups. Controls in all experiments included comparison of immune and nonimmune mice, and immune mice challenged with the related and unrelated tumor cells in opposite ears, to show that the DTH responses required priming and were antigen specific. Pilot experiments (not shown) confirmed that the ear swelling had the characteristic peak at 24–48 h followed by decay of swelling, which excluded the possibility that swelling was simply due to growth of tumor. For adoptive transfer assay of DTH, the hind footpads of nonimmune recipients were each injected with 3×10^6 nonimmune or immune spleen cells and 2×10^5 tumor cells suspended in 50 μl of hybridoma culture supernatant from the monoclonal anti-L3T4 hybridoma or from the nonsecreting fusion partner cell line. Pre- and postchallenge measurements were carried out with the leg fully extended using a precision spring-loaded dial caliper (Mitutoyo No. 7300; Precision Gage Co.).

Assay for Th In Vitro. Tumor-specific Th cells were detected in vitro by coculture with cloned IL-2-dependent CTL that were tested for cytotoxicity against tumor targets at the end of a 7-d culture period (18). The number of spleen cells containing optimal numbers of Th as predetermined by limiting dilution analysis were added to microcultures containing 2×10^3 mitomycin C–treated tumor cells as antigen, 3×10^5 irradiated (2,000 rad) T cell–depleted filler spleen cells, with cloned CTL (10^3 anti-1591-A or 200 anti-2240) for detection of IL-2. Cultures were set up in 6 × 50 mm round-bottomed glass tubes (Kimble Div., Owens-Illinois, Inc., Toledo, OH) containing a volume of 0.4 ml mixed lymphocyte–tumor culture (MLTC) medium. After 7 d of incubation, we resuspended cultures by pipetting, and we split two 150-μl aliquots to corresponding wells of two microtitration plates to which we added the specific or an unrelated ^{51}Cr-labeled tumor target in 50 μl medium. In some experiments, the phenotype of responder cells was assayed by depletion of Thy-1.2$^+$ or Lyt-2$^+$ cells before culture using the AT83A or 3.155 mAb and rabbit complement, as described (2). In other experiments, the ability of anti-I-A^k, anti-L3T4, or anti-thy-1.2 antibody to functionally block precursor Th activity by addition to microcultures in the absence of complement was assayed with or without the addition of supernatant containing added IL-2. For the Poisson analysis of the limiting dilution assay, cultures containing responder cells were scored "negative" for Th activity when the specific release of ^{51}Cr by the CTL in these cultures <3 SD above the mean ^{51}Cr release caused by CTL without responders (control cultures; 18). Th precursor frequencies were calculated using the maximum likelihood method of Finney (19) as modified by Porter and Berry (20), and the minimum χ^2 method presented by Taswell (21). The "relative cytolytic activity" for cultures of different responder cell numbers was also analyzed as described by Coppleson and Michie (22).

Cytolytic T Cells and Lines. To generate CTL, spleen cells from tumor-immunized animals were restimulated in vitro in a 7-d MLTC (2). Continuous anti-1591 (anti-A) and anti-2240 CTL lines were derived by cloning the CTL generated in MLTC with mitomycin C–treated tumor cells as stimulator cells, irradiated (2,000 rad) spleen cells as fillers

cells, and a supernatant from secondary mixed lymphocyte cultures as a source of IL-2 (8). The resultant lines were L3T4$^-$, Lyt-2$^+$, tumor-specific, and IL-2-dependent (8) for growth.

Chromium Release Assay. Target cells (1–2 × 10^6) were labeled with 100 μCi ^{51}Cr (Amersham Corp., Arlington Heights, IL) for 1–2 h at 37°C in a volume of 200 μl. Cells were washed twice with 1 ml of medium before addition of 2–5 × 10^3 targets per well containing effector cells from helper assay cocultures or MLTC. CTL effector cells generated in MLTC were serially diluted in V-bottomed 96-well microtiter plates just before the addition of labeled target cells. 0.1 ml of supernatant from the 0.2 ml cultures was removed after 4–6 h of incubation at 37°C, and the released radioactivity was counted in a gamma counter. The percentage of lysis was calculated by the formula: [(experimental release-spontaneous release)/(total release-spontaneous release)] × 100.

Cytofluorometric Analysis. Methods for quantifying levels of normal MHC class I surface antigen expression on the surface of tumor cells have been described (23). Briefly, 10^6 cells were incubated with a saturating concentration of the first antibody for 20 min at 4°C. The cells were then washed twice and resuspended in PBS containing 0.1 sodium azide and fluorescein-coupled goat anti–mouse Ig (Hyclone 5 1081-A; HyClone Laboratories, Logan, UT). After 20 min, cells were washed twice again and 10^4 cells were analyzed on a FACS IV (Becton Dickinson Immunocytometry Systems, Mountain View, CA.). The number of cells was plotted as a function of the fluorescence intensity in arbitrary units obtained using a log amplifier.

Results

Loss of CTL-defined, Tumor-specific Antigens during Tumor Progression. We previously found (7, 24) that selection for locally invasive 1591-PRO variants of the 1591-RE tumor required loss of the A antigen recognized by 1591-RE-specific CTL. The 1591-PRO variant, in turn, retained a less immunogenic B antigen defined by CTL (7, 24). In the present study, we sought to derive more malignant variants of the UV-induced tumor 1591 by further selection of 1591-PRO in vivo, and determine if further antigen loss was associated with these changes in malignancy. Thus, two 1-mm^2 tumor fragments of 1591-PRO and 10^8 spleen cells from 1591-PRO-immune mice were coinjected into nude C3H mice in a Winn-type assay (25). The growth of tumors in mice coinjected with 1591-PRO immune cells was inhibited when compared with that of tumors coinjected with normal cells or medium only as controls (Fig. 1). Tumors grew rapidly in all animals that received normal (nonimmune) or no spleen cells, and these mice died 22–30 d after challenge. In contrast, all mice that received the mixture of immune spleen cells and tumor cells were initially protected during this period, and only later did progressive tumor growth overcome these mice. Such a progressively growing tumor was reisolated from one of these mice at day 50, and it was transplanted into normal mice. The transplanted reisolate was found to grow and invade rapidly and to develop distant metastases. Based on these characteristics, the variant was designated 1591-HM, because it was a highly malignant variant of the 1591 tumor. The increase in malignancy after selection was seen by comparison of the survival of normal mice challenged with the 1591-HM variant with that of mice challenged with the 1591-PRO variant (Fig. 2). The 1591-HM variant was more malignant than the 1591-PRO variant from which it was derived, since it killed all mice within 35 d, whereas the original 1591-PRO tumor required 120 d before it killed 50% of the mice by local growth. In addition to the 1591-HM variant, we reisolated an additional

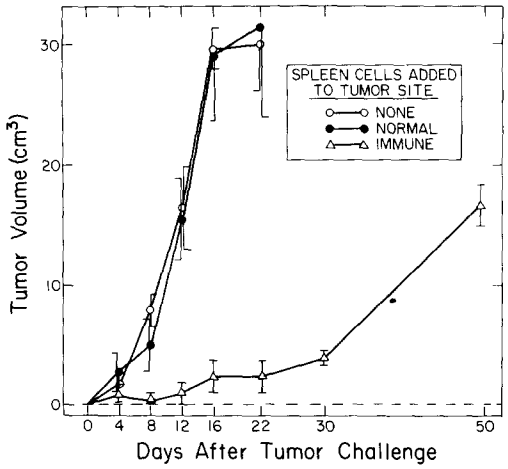

FIGURE 1. Temporary inhibition of the outgrowth of 1591-PRO tumor cells by 1591-PRO immune cells leading to immune selection for 1591-HM tumor variants. 1591-PRO tumor cells either alone or mixed with either normal or immune spleen cells were injected subcutaneously into nude mice in a Winn-type assay (25). Immune spleen cells were from animals injected twice with a sublethal dose of 10^7 1591-PRO tumor cells 4 wk apart. 10^8 spleen cells from either normal or immunized mice were injected with two 1-mm^3 fragments of the 1591-PRO tumor. Tumor volumes were determined by caliper measurements in three dimensions. Vertical bars represent the SEM for three animals tested in two separate experiments. Animals not receiving immune spleen cells died between 22 and 30 d after challenge. The animals receiving immune cells were all alive 50 d after implantation; at this time the mice were killed for readaptation of the progressively growing tumors to tissue culture. These lines were designated 1591-HM.

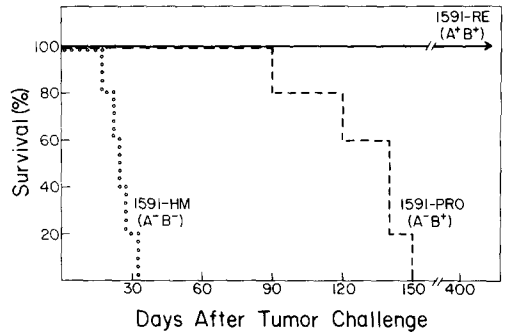

FIGURE 2. Highly malignant behavior of 1591-HM reisolated after immune selection from 1591-PRO tumor cells. Five normal C3H mice per group were each injected subcutaneously with two 1-mm^2 fragments of variants 1591-PRO or 1591-HM or the parental tumor 1591-RE. The rapid death of mice injected with 1591-HM reflected more rapid growth of this tumor when compared with the 1591-PRO variant from which it was selected. For definition of the CTL-defined antigenic phenotypes A and B, consult Fig. 3.

tumor cell line from an axillary metastasis of 1591-HM, which also regularly metastasized on transplantation, and have called this metastatic tumor cell line 1591-MET. Thus, both 1591-HM and 1591-MET represented HM variants of the 1591 tumor.

We next asked if complete loss of the 1591-specific A and B antigens resulted from the step-wise in vivo selection resulting in the 1591-HM variant. Anti-A

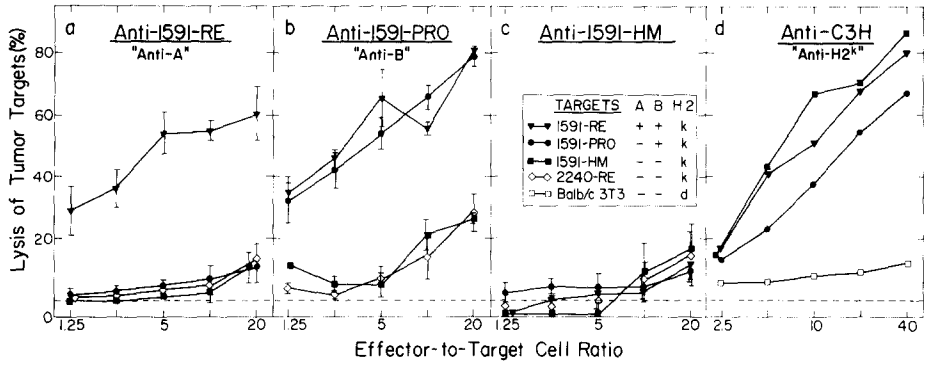

FIGURE 3. Loss of A and B antigens recognized by CTL during variant selection. To raise 1591 tumor-specific CTL, mice were immunized twice with 1591-RE (anti-A), 1591-PRO (anti-B), or 1591-HM tumor cells inactivated by 10,000 rad to prevent outgrowth of the 1591-PRO and 1591-HM variants. Spleen cells from these mice were restimulated in vitro with appropriate tumor cells inactivated with mitomycin C (2). BALB/c anti-C3H allospecific CTL were stimulated in a primary mixed lymphocyte culture to test the sensitivity of the tumor variants to CTL specific for a nontumor antigen (H-2^k alloantigen). The cytotoxicity of effector cells recovered after 7 d of culture was tested in a 5-h ^{51}Cr release assay against the various ^{51}Cr-labeled target cells (see legend).

and anti–B CTL were generated from 1591-RE and 1591-PRO immune spleen cells in MLTC and the susceptibility of the sequentially selected variants of the 1591 lineage to lysis was tested in a ^{51}Cr-release assay. As shown in Fig. 3, *a* and *b*, the 1591-HM variant was lysed by neither 1591-RE (anti-A)- nor 1591-PRO (anti-B)-specific CTL. The insensitivity of the 1591-HM tumor to 1591-specific CTL could be due to (*a*) loss of the target antigens, (*b*) resistance to the lethal hit delivered by CTL, or (*c*) loss of MHC class I, K, and D antigens which restrict CTL responses (26). Antigen loss is the most likely basis of insensitivity to lysis since 1591-HM was not only resistant to lysis by anti–A and anti–B CTL, but also failed to induce anti–A or anti–B CTL that could lyse targets 1591-RE and 1591-PRO in the same experiment (Fig. 3, *a–c*). Further, we have excluded the second alternative explanation by showing that 1591-HM tumor cells remained susceptible to the lethal hit by CTL that reacted with C3H H-2^k alloantigen (Fig. 3*d*). Finally, the failure of the 1591-HM variant to be lysed by anti–A or anti–B CTL could not be accounted for by loss of MHC class I, K, and D antigens, because the 1591-HM variant had not lost MHC class I antigens recognized by class I alloreactive CTL (Fig. 3*d*), or class I–specific mAbs (Fig. 4). Therefore, the phenotypes of the tumors have been designated 1591-RE [A^+B^+], 1591-PRO [A^-B^+], and 1591-HM [A^-B^-], according to sensitivity to 1591-specific CTL, as in Fig. 3. Together, the above experiments are consistent with the hypothesis that multiple tumor-specific antigens that are recognized by CTL were lost with tumor progression.

Retention of Th-defined, Tumor-specific Antigens by Malignant Variants. The selection of an HM variant that has lost CTL-defined antigens should make it possible to determine whether an independent antigen recognized by Th cells exists and is retained despite tumor progression. We tested whether the 1591 variants expressed antigens recognized by tumor-specific Th cells in an ear swelling assay that measures DTH responses (16, 17). Responses of unsensitized

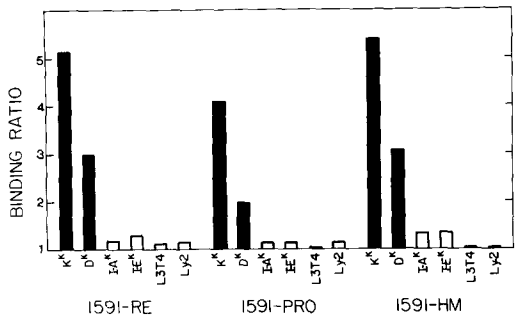

FIGURE 4. Retention of MHC class I antigens by 1591-PRO and 1591-HM. Tumor cells were incubated with class I–reactive mAb 11-4.1 (K^k) or 15.5.5 (D^k) (*solid bars*), class II–reactive mAb 10.2.16 ($I\text{-}A^k$) or 17.3.3 ($I\text{-}E^k$), or T lymphocyte–reactive mAb GK1.5 (L3T4) and 3.155.3 (Lyt-2) (*open bars*) as the first antibody as indicated. All samples were then washed and incubated with fluorescein-coupled goat anti-mouse antibody as a second step. The amount of fluorescence for 10^4 cells was quantified using a FACS IV. Binding ratio represents the ratio of the mean fluorescence of staining with both first and second antibodies divided by the mean fluorescence of staining with the second antibody alone (background fluorescence). The use of the binding ratio allows a comparison between different cell lines that may differ in the amount of background fluorescence. Mean binding ratios ar calculated from two independent experiments that were similar and therefore pooled. 1591-PRO and 1591-HM tumor cells do not express MHC class II antigens (A^k,E^k), nor could class II antigens be induced in vitro by treatment with IFN-γ (not shown).

mice and mice sensitized by injection of 1591-RE or 1316-RE tumor fragments were elicited after 8 d with the parental1591-RE and 1316-RE tumors and their variants. Fig. 5 shows that the 24-h ear swelling responses elicited by 1591 or 1316 cells are tumor-specific, in that they require specific sensitization and elicitation with the same tumor. In the same experiments, we found that tumor variants that had lost the 1591 CTL-defined tumor antigens also elicited the 1591-specific DTH responses (Fig. 5, *left*). To our surprise, the DTH responses elicited by challenge with the variants 1591-HM and 1591-MET were not reduced despite the loss of the CTL-defined A and B antigens. Although Fig. 5 only shows tumor-specific DTH immunity elicited by the 1591 tumor variants, such A^-B^- 1591 variant cells could also sensitize for 1591-specific DTH responses. Thus, we saw that the DTH swelling responses ($\times 10^{-3}$ cm) elicited by 1591-RE tumor cells in 1591-RE- (5.5 ± 0.7), 1591-PRO- (6.4 ± 2.0), and 1591-HM- (5.9 ± 0.7) sensitized mice were similar and significantly greater ($p < 0.01$) than responses elicited by the 1316 tumor (-1.0 ± 0.6, 0.2 ± 0.5, and 0.4 ± 0.4, respectively). These results indicate that an antigen found on all tumors of 1591 lineage, but not on any other UV-induced tumor tested (i.e., 1316-RE, Fig. 5; 2240-RE or 1463-RE, ref. 27) can sensitize as well as elicit 1591 tumor-specific DTH independent of expression of antigens recognized by tumor-specific CTL. The finding of a retained tumor lineage–specific antigen on PRO variants has been extended to another tumor, since Fig. 5 (*right*) shows that 1316-PRO variant cells also retain a 1316 tumor lineage–specific DTH-inducing antigen. To confirm that the DTH responses to 1591 were mediated by the $L3T4^+$ Th subset, we tested if the response could be blocked by L3T4-specific mAb in a local adoptive transfer footpad swelling assay (see Materials and Methods). 1591-RE-sensitized spleen cells, but not unsensitized spleen cells, were found to

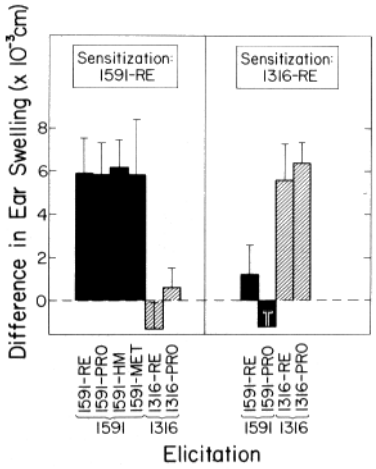

FIGURE 5. Retention of a tumor-specific, DTH-inducing antigen during tumor progression. The DTH responses are "tumor lineage–specific," i. e., all tumor cells and variants derived from the same parental tumor (but not from another independently derived tumor) express the same antigen. Mice were sensitized with three 1-mm^3 tumor fragments of 1591-RE or 1316-RE, or remained unsensitized. 1591-RE, 1316-RE, 1591-PRO, 1316-PRO, 1591-HM, or 1591-MET tumor cells were used for elicitation 8 d later. The difference in ear swelling responses between sensitized and unsensitized mice (± SE of the difference) are shown at 24 h after elicitation with the RE and the variant tumor cells. The bars represent measurements from two to three independent experiments, each with five mice per group that were similar and therefore pooled. Responses elicited by tumor cells in immune mice were greater than those of nonimmune controls as long as sensitization and elicitation were carried out using tumor cells of the same lineage ($p < 0.05$). The responses to 1591-PRO and 1591-MET (but not to 1316-RE) were significantly greater than in nonimmune control mice ($p < 0.01$), indicating the retention of a 1591 tumor-specific, DTH-inducing antigen during tumor progression of the 1591 tumor.

produce a local DTH response 24 h after coinjection with 1591 tumor cells (6.9×10^{-2} cm increase in thickness above the swelling of 1.0×10^{-2} cm transferred by unsensitized spleen cells; $p < 0.05$; five mice per group). The addition of anti-L3T4 antibody (but not of nonsecreting parent hybridoma supernatant used as control) blocked the response (0.2×10^{-2} cm swelling above nonimmune control). These results indicated that the DTH response to the 1591 tumor was mediated by an L3T4$^+$ Th cell, which is distinct from the Lyt-2$^+$ phenotype of 1591-specific CTL (2).

The findings above indicated the existence of a 1591 lineage-specific antigen that is retained on the HM tumor variants 1591-HM and 1591-MET. We next investigated whether the Th response to this tumor lineage–specific antigen could be defined in vitro. We first determined if Th cells were required for the generation of 1591-specific cytolysis in vitro and if Th could be detected reliably and characterized under limiting dilution conditions in a 7-d MLTC microculture assay (for details see Materials and Methods). Dilutions of spleen cells from 1591-immune mice were cultured with excess numbers of tumor cells as antigen and APCs to increase the likelihood that titrated immune lymphocytes would be the limiting population. The number of cultures that lysed 1591-RE in a ^{51}Cr-release assay was scored and the percentage of nonresponding cultures was plotted as a function of number of titrated 1591 immune cells (Fig. 6*a*, *filled circles*); the

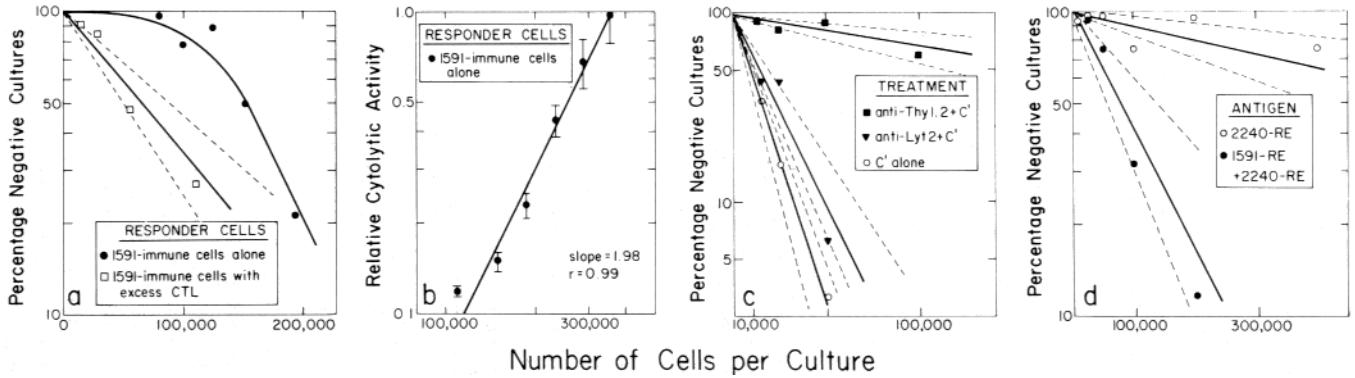

FIGURE 6. Demonstration of 1591 tumor-specific Th by coculture with IL-2-dependent CTL in limiting dilution assays. Spleen cells from 1591-immune mice were diluted and cultured with tumor cells, T-depleted irradiated filler cells, and without or with added CTL for readout of IL-2 activity as described in Materials and Methods. (*a*) Poisson analysis (18) of responses of responder spleen cells from 1591-immune mice cultured in limiting dilution without or with excess IL-2-dependent CTL. The generation of cytolysis from cultures with immune cells alone (*closed symbol*) required multiple interacting cell types since responsiveness followed a multi-hit kinetics (curved line in the semi-log plot, Poisson hypothesis rejected). After addition of IL-2-dependent 1591-specific CTL (*open symbol*), a single population became limiting as CTL were provided in nonlimiting quantity (linear line in a semi-log plot, Poisson hypothesis accepted, $p < 0.05$). The *dashed lines* represent the 95% confidence intervals. Each point represents 48 microcultures per dilution. The minimum χ^2 estimate of the minimum helper cell precursor frequency was 9×10^{-4}. (*b*) Log–log plot after Coppleson and Michie (22), correlating cytolytic activity to the dose of responder spleen cells from 1591-immune mice (without excess IL2-dependent CTL, same experiment as in a; *closed symbols*). There is a slope of 2 (1.98), indicating that two cell types were required for generation of cytolysis from cultures without added CTL. (*c*) Demonstration that the 1591-immune helper cell is a Thy-1$^+$, Lyt-2$^-$ T cell. 1591-immune spleen cells were treated with rabbit C′ (1:10) and anti-Thy-1 (AT83A, 1:10) or anti-Lyt-2 (3.155, 1:5), or C′ alone for 45 min at 37°C and washed before coculture with excess IL-2-dependent CTL used as readout for helper activity (same method as in *a*). Optimal conditions for elimination of subsets by mAb and C′ were predetermined by elimination of function in adoptive transfer, in vitro proliferative and CTL assays, and by FACS analysis (not shown). 32 cultures were assayed per dilution and limiting dilution data were analyzed as described in Materials and Methods. Help for the cloned CTL was diminished by pretreatment of the immune cells with anti-Thy-1, but not anti-Lyt-2 + C′ or C′ alone. (*d*) 1591 antigen specificity of Th from 1591-immunized mice. Coculture of 1591-immune cells with an unrelated tumor antigen such as 2240-RE (using an excess of 2240-specific cloned CTL as readout cell line) fails to stimulate anti-1591 Th, while addition of the relevant antigen, i. e., 1591-RE tumor cells, stimulate help for the anti-2240 CTL line. 32 cultures were assayed per dilution.

curved plot that resulted indicated that cytolysis was generated by a multihit kinetics and, therefore, was dependent on more than one limiting cell per culture. The slope of a plot of the mean cytolytic response as a function of number of titrated immune cells using the analysis of Coppleson and Michie (Fig. 6*b*, reference 22) provided an estimate of approximately two interacting cell types. Thus, on the hypothesis that a Th cell would be the remaining limiting cell if IL-2-dependent, tumor-specific CTL were present in excess, we added cloned 1591-specific CTL, which depend upon Th-derived growth factor (IL-2) for proliferation (8). As shown in Fig. 6*a*, addition of limiting but increasing numbers of immune cells to an excess of cloned CTL (*open squares*) produces a linear increase in the numbers of positive cultures, verifying that a single cell type was limiting under conditions of the assay. The limiting cell type was found to be a T cell belonging to the Lyt-2^- Th subset, since treatment of the immune spleen cells with complement and anti-Thy-1, but not anti-Lyt-2, eliminated the help for the response of the cocultured CTL line (Fig.6 *c*). To test whether these Th cells were specific for 1591 antigen, 1591 immune cells were cocultured with CTL against an unrelated antigen, 2240-RE. Fig. 6*d* shows that in the absence of 1591-RE tumor cells, 1591 immune cells failed to provide help, while upon addition of 1591-RE antigen we saw an increase in response of 2240-specific CTL. Together, the experiments in Fig. 6 show that the response of CTL in vitro depends upon the presence of tumor-specific Th cells. Furthermore, the experiments document the quantitative response of indicator CTL as a function of dose of Th since, in the range of 10^4–2×10^5 immune cells per culture, the cytolytic activity of the cloned CTL increased proportionally with the increase in numbers of immune cells.

We next investigated whether 1591 tumor variants expressed a 1591 tumor lineage–specific antigen that was recognized by Th in vitro. Thus, we cultured different tumor cells as a source of antigen, together with a single concentration of Th, CTL, and APCs, as determined to be optimal in the limiting dilution experiments above. We first tested if 1591 variants expressing different combinations of the A and B antigens (8) shared an additional antigen that was not defined by CTL. Exp. 1 of Table I shows that sharing of the CTL-defined, 1591-specific antigens between the sensitizing and eliciting tumor cells did not seem to be required for generation of 1591-specific help. Thus, Th cells sensitized by A^-B^+ tumor cells were restimulated by an A^+B^- tumor variant in coculture. This indicates that the 1591 tumors express a lineage-specific, Th-recognized antigen independently of the CTL-defined antigens. Exp. 1 also confirms that the elicitation of help for the CTL in vitro was antigen-specific since it required sensitization in vivo with tumors of the 1591 lineage (i.e., spleen cells of mice immunized with another UV- or a methylcholanthrene-induced tumor did not provide help). In addition, the 1591 lineage-specific Th cells were blocked by anti-L3T4 and anti-I-A^k but not anti-Thy-1 antibodies in the absence of C′ (Exp. 2), suggesting that the 1591 antigen-specific cells providing help for CTL in vitro had a classical Th cell phenotype. Similar results were obtained using parental 1591-RE tumor cells as antigen in Exps. 3 and 4 of Table I, which also show that the blocking effect of anti-L3T4 could be abrogated by the addition of IL-2.

TABLE I

Sensitization and Elicitation of Th Requires Sharing of the Tumor Lineage–specific Antigen that Is Independent of the CTL-defined Tumor-specific Antigens

Exp.	Tumor antigen*		Blocking mAb specificity‡	Lysis by helper factor–dependent anti-1591 CTL§
	Sensitization in vivo	Elicitation in vitro		
1	—	1591-[A^+B^-]	—	11 ± 3 (control)
	2240-RE‖		—	12 ± 4
	1130-RE		—	15 ± 3
	1591-[A^+B^+]		—	37 ± 6¶
	1591-[A^+B^-]		—	40 ± 6¶
	1591-[A^-B^+]		—	30 ± 5¶
2	1591-[A^-B^+]	—	—	7 ± 2 (control)
		1591-[A^+B^-]	—	40 ± 4¶
			Thy-1.2	37 ± 4¶
			L3T4	4 ± 1
			I-A^k	8 ± 4
			D^k	49 ± 4¶
3	1591-[A^+B^+]	—	—	12 ± 4 (control)
		1591-[A^+B^+]	—	62 ± 4¶
			L3T4	6 ± 1
			I-A^k	19 ± 2
			D^k	51 ± 5¶
4	1591-[A^+B^+]	—	—	6 ± 1 (control)
		1591-[A^+B^+]	—	59 ± 2¶
			L3T4	13 ± 3
			L3T4 + IL-2	41 ± 3¶

* Nonimmune, 2240-RE, 1130-RE, 1591-RE [A^+B^+], 1591-BS [A^+B^-], 1591-AS [A^-B^+] tumor-immune spleen cells were cultured with a 1591-specific IL-2-dependent cytolytic T cell line (which is used as a readout for Il-2 production by Th cells), mitomycin c–treated stimulating tumor cells and T cell–depleted filler spleen cells in a limiting dilution assay (for details see Materials and Methods). The 1591-specific, CTL-defined antigenic phenotypes are shown in brackets.

‡ Anti-Thy-1.2, anti-L3T4, anti-I-A^k, or anti-D^k mAb or secondary mixed lymphocyte culture supernatant as a source of IL-2 were added at a 1:10 dilution at initiation of culture.

§ Numbers represent the mean percent lysis ± SEM for 8 replicate cultures in Exps. 1 and 2, and 16 replicates in Exps. 3 and 4 in a 6-h ^{51}Cr-release assay after 7 d of culture. Lysis of the unrelated target 2240-RE in duplicate assay was 0% in Exp. 1, ≤3% in Exp. 2, ≤1% in Exp. 3, and ≤8% in Exp. 4 for all culture groups.

‖ The failure to elicit help from spleen cells of mice injected with the tumors unrelated to 1591 in Exp. 1 was apparently not due to less efficient priming by these tumors. This is suggested by the fact that 2240-RE immune spleen cells from the same group of mice provided help when the relevant tumor antigen to which they had been immunized (2240-RE cells) was added (not shown).

¶ Mean response is significantly greater than that of controls, as determined by Student's t test ($p < 0.01$).

We next tested whether 1591-specific Th cells could be restimulated with the 1591-HM variant that had lost all known 1591-specific, CTL-defined antigens. Since these variant cells no longer have the CTL-defined antigens required for stimulation of 1591-specific CTL used for readout, we used another IL-2-dependent CTL line stimulated by an unrelated tumor as indicator. Thus, 2240-RE tumor-specific CTL and 2240 tumor cells were added to cultures to provide a readout for 1591-specific Th activity in the experiments of Table II. Exp. 1 shows that 1591-specific Th activity was detectable when 1591 antigen was added, despite relatively high anti-2240 background lytic activity using the 2240

TABLE II

Retention of the 1591 Lineage–specific Antigen by Highly Malignant 1591 Variant Tumor Cells

Exp.	Tumor antigen*		Lysis by helper factor–dependent anti-2240 CTL‡ at responder/stimulator ratio of:				
	Sensitization in vivo	Elicitation in vitro	200	100	50	25	12
1	1591-RE	2240-RE	20 ± 3	18 ± 2	20 ± 4	15 ± 2	11 ± 2 (control)
		1591-RE + 2240-RE	41 ± 2§	59 ± 4§	34 ± 8	25 ± 4	10 ± 2
2	None	2240-RE	—	37 ± 3	—	—	—
	1591-RE	2240-RE	—	35 ± 3 (control)	—	—	—
		1591-RE + 2240-RE	—	63 ± 4§	—	—	—
		1591-PRO + 2240-RE	—	62 ± 5§	—	—	—
		1591-HM + 2240-RE	—	57 ± 3§	—	—	—

* 1591-RE immune spleen cells were cocultured with a 2240-RE-specific IL-2-dependent CTL line (which is used as a readout for IL-2 production). Antigen specificity of the help provided by the 1591-RE spleen cells was measured by the presence or absence of tumor cells of the 1591 lineage. 2240-RE cells were always added to provide antigen for the antigen-dependent anti-2240 CTL line used as readout. T cell–depleted filler cells were added to all cultures.

‡ The cloned 2240-specific CTL line was added at 200 cells per culture. After 7 d of coculture, 16 replicate cultures were tested in a 6-h ^{51}Cr-release assay against 2240-RE. The mean percentages of lysis ± SEM are reported. Lysis of 2240-RE by 1591 immune cells or anti-2240.3 CTL cultured alone with appropriate antigen and fillers was 13 and 4%, respectively.

§ All responses significantly greater than control cultures ($p < 0.0001$).

readout system. The relatively high anti-2240 background activity was apparently due to a primary Th response to 2240, since the 2240 antigen elicited a similar response from nonsensitized, as well as 1591-sensitized, spleen cells (Exp. 2). Analysis of different responder/stimulator (R/S) ratios in Exp. 1 indicated that the maximum specific activity was detected at 2×10^5 responders per culture (R/S, 100:1), and thus spleen cells at this concentration were cultured with 2240-RE, anti-2240-RE CTL, and the different 1591 variants as antigen in Exp. 2. When compared with the parental 1591-RE cells, 1591-HM fully retained the ability to elicit the increment due to 1591 tumor-specific Th responses ($p < 0.0001$; Table II, Exp. 2). Thus, the results of Table II are consistent with the experiments in Fig. 5 and Table I, which indicated the existence and retention of a 1591 lineage-specific, Th-recognized antigen expressed independently of CTL-recognized antigens.

Tumor Variants Lacking CTL-Recognized Antigens Can Induce Tumor Rejection. We next wished to determine the significance of the response to the antigen recognized by Th in tumor rejection. Therefore, we determined which of the antigens recognized by CTL or Th cells was needed to induce protective 1591-specific immunity to challenge in transplantation assay. Tumor-specific immunity to transplanted UV-induced tumors can be detected after immunization with the same tumor and sublethal irradiation, which eliminates detection of protective immunity generated by primary or crossreactive immune responses (11). Thus, mice were immunized with variants of the 1591 tumor and control tumors, and 1 mo later, were challenged with the A^-B^+ 1591-PRO tumor after sublethal (600 rad) irradiation. Experiments comparing tumor incidence in irradiated and unirradiated recipients confirm that radioresistant tumor-specific immunity can only be induced by preimmunization with tumors of the 1591 lineage (Table III). The table shows that an A^+B^- 1591 variant was as effective as the A^+B^+ 1591-RE tumor in protecting against a challenge with the A^-B^+

TABLE III

1591 Lineage-specific Immune Protection Persists After Irradiation

Preimmunization*	Tumor incidence (%) of 1591-PRO[A^-B^+]‡	
	Nonirradiated recipient	Irradiated recipient
2240-RE	1/7 (14)§	7/7 (100)
1316-RE	4/11 (36)	8/8 (100)
1130-RE	12/18 (67)	5/5 (100)
1591-[A^+B^+]	1/33 (3)§	1/8 (13)
1591-[A^+B^-]¶	1/8 (13)	1/7 (14)
1591-[A^-B^+]¶	0/8 (0)	1/8 (13)
1591-[A^-B^+]‖	0/58 (0)	0/5 (0)
Normal fibroblasts	5/5 (100)	ND
None	50/59 (85)	6/8 (75)

* C3H mice received either an intraperitoneal injection of 10^7 cultured cells or a subcutaneous implantation of three 1-mm^3 fragments of tumor grown in C3H nude or adult-thymectomized, γ-irradiated bone marrow–reconstituted C3H mice. 4 wk later, mice received 600 rad or no irradiation, as indicated, and 24 h later all mice were challenged with three 1-mm^3 fragments of the 1591-PRO tumor.

‡ Number of mice with progressively growing tumors at 6 wk after challenge per number of mice challenged. The percentage is given in parentheses. Similar results were obtained in two independent experiments and, therefore, pooled; each experiment included the four tumors of the 1591 lineage and two or three non-1591 tumors. All animals that had tumors at 6 wk eventually died or were killed when moribund. Tumors regularly showed an initial period of growth even when later rejected. Mice that had rejected their tumor by 6 wk remained tumor free (≥3 mo observation period).

§ Not included in this table are mice that were challenged with 1591-PRO 5 mo rather than 1 mo after preimmunization; six mice each were preimmunized with 2240-RE or 1591-RE in the same experiment. Four of six of 2240-RE immunized mice developed tumors, while zero of six 1591-RE immunized animals succumbed to progressive tumor growth when challenged at this later time.

‖ This 1591-[A^-B^+] variant is 1591-PRO, derived by selection in vivo (2).

¶ 1591-[A^+B^-] is the variant 1591-BS, and 1591-[A^-B^+] is the variant 1591-AS, both derived by in vitro selection with 1591-specific CTL (8).

tumor 1591-PRO. This indicated that there was a 1591 lineage-specific antigen that was independent of the CTL-defined A or B antigens, since transplant immunity to the A^-B^+ tumor was obtained without prior immunization with the B antigen. Further experiments showed that the A^-B^- variant 1591-HM also induced radioresistant 1591-specific immunity, despite the absence of any detectable CTL-defined antigens on the tumor used for immunization (Fig. 7*b*). While immunization with the A^-B^- 1591-HM variant provided protection to 1591-PRO tumor challenge (the B^+ variant from which 1591-HM had been derived), none of the tumors of the 1591 lineage could induce protective immunity against challenge with the rapidly growing 1591-HM tumor itself (Fig.7*a*), or to an unrelated progressor tumor, 1316-PRO (Fig. 7*c*). Thus, these data indicate the importance of a 1591 lineage-specific antigen that does not seem to be the target of protective immunity, but nevertheless induces immunity

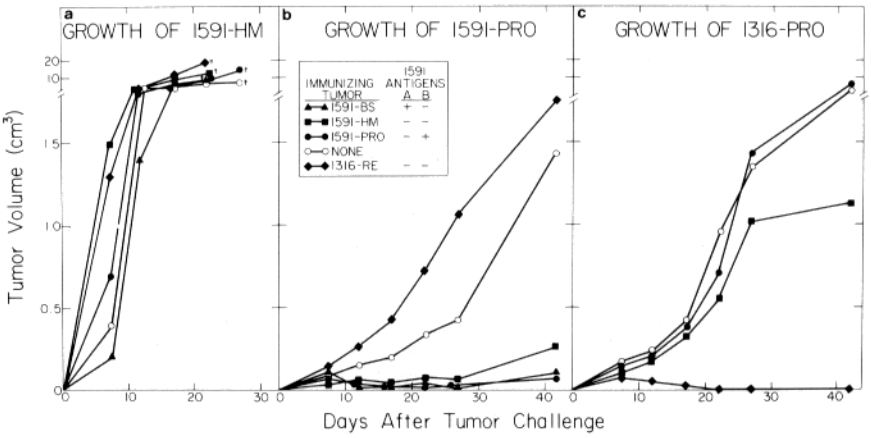

FIGURE 7. 1591 tumor lineage–specific protection conferred by immunization with variants not sharing 1591-specific, CTL-recognized antigens. Mice were immunized subcutaneously with three 1-mm³ γ-irradiated (10,000R) tumor fragments of 1591-BS, 1591-HM or 1591-PRO variants, or the unrelated UV-induced 1316-RE tumor. One mo later, mice were sublethally irradiated (600R), and after 24 h groups of 5 mice were challenged with two 1-mm³ fragments of 1591-HM (*a*), 1591-PRO (*b*) or 1316-PRO (*c*). Tumor volumes were calculated from caliper measurements in three dimensions. Death of 1591-HM-challenged mice for all immunized groups is indicated by crosses.

to challenge with a tumor of the same lineage that retains a CTL-defined target structure.

Discussion

This study shows that tumor-specific antigens can be retained by cancer cells isolated during progression from a rather "benign" RE tumor to an HM cancer. We dissected the components of T cell–mediated immunity to a tumor-specific antigen, and found that the antigens recognized by CTL were lost (Fig. 3), while those recognized by Th cells were retained (Fig. 5, Table II). Such Th cell–recognized antigens were even retained by cancer cells isolated from a distant metastasis (Fig. 5). It seems unlikely for several reasons that the Th-recognized antigens merely represent retained or quantitatively reduced levels of CTL-recognized antigens. First, we excluded the possibility that the variants had retained the CTL-defined antigens by becoming resistant to lysis (Fig. 3), or through loss of MHC class I antigens that restrict Lyt-2$^+$ CTL responses (Fig. 4). Second, the antigenic differences among different 1591 tumor variants were not due to differences in quantitative levels of a single antigen, since we have shown that the CTL-defined antigens A and B can be lost independently in any combination as well as altogether by selection with A and B antigen-specific CTL in vitro (12); further, we now know that loss of the A antigen results from complete loss of the gene encoding this antigen (28). Third, it is likely that the Th antigen is completely independent of the A and B antigens since we have shown that Th responses and tumor rejection do not require sharing or the presence of A and B antigens during sensitization and elicitation (Tables I–III; Figs. 5 and 7). Thus, it appears that the Th cell–recognized antigens represent an independently expressed tumor-specific antigen or complex of antigens. These

Th cell–recognized antigens are retained during tumor selection and are specific for all tumor variants derived from the same original tumor, i. e., these antigens are "lineage-specific."

The demonstrated persistence of certain tumor-specific antigens and the loss of others as a result of host-selective pressures may provide important clues regarding the mechanisms of tumor recognition and destruction in vivo. The results suggest that tumor-specific CTL directly recognize and destroy tumor cells expressing the appropriate target structures in vivo, and hence, exert direct selective pressure upon the tumor for the generation of antigen-loss variants. In contrast, the lineage-specific antigens recognized by Th cells are not a direct target of immune destruction in vivo since they persist during tumor progression, and since such antigens provide no protection to 1591-HM in 1591-immune mice (Fig. 7). However, we found that the antigens retained by 1591-HM play a significant role in tumor rejection, since immunization with 1591-HM may induce protective immunity to parental RE or variant PRO cells that retain target antigens recognized by CTL (Fig. 7). Thus, the Th-recognized antigens may also be distinguished by an ability to induce, but not serve as the target of tumor rejection. The presence of these antigens would therefore have been missed by classical tumor transplantation assays in which the same HM tumor was used for immunization and challenge. Thus, similar Th-defined, unique tumor-specific antigens may not have been detected on other HM tumors that have previously been considered to be nonantigenic.

To account for the retention of Th-recognized antigens, it may be important that, unlike the CTL-recognized antigens, the Th cell–recognized antigen(s) are not recognized directly on the tumor cell surface. The observed blocking of the tumor-specific Th responses by anti-Ia antibody suggests that Th cells recognize this antigen in the context of I-A class II MHC determinants, and indeed both the cells required for the DTH and Th responses were found to belong to the $L3T4^+$ subset; i.e., cells that recognize antigen in the context of MHC class II molecules (29). Since the 1591 tumor is itself Ia^- (Fig. 4), it is likely that the Th-recognized antigens are only recognized on Ia^+ APCs and not directly on tumor cells.

Possibly the PRO and HM tumors have retained Th cell–recognized antigens by selectively losing susceptibility to lymphotoxin, a factor that mediates killing by Th cells (30–32).At present, we know that these tumors are resistant to macrophage tumor necrosis factor and to B cell lymphotoxin (33). B cell lymphotoxin seems to use a similar effector pathway as tumor necrosis factor (33), and it appears to be closely related to T cell lymphotoxin, as shown by DNA sequence homology (N. Ruddle, personal communication). This question may be critically addressed when purified recombinant murine lymphotoxin of the type produced by Th cells becomes available.

Tumor-specific Th cells, though not directly tumoricidal, may play an important role in tumor rejection through the release of lymphokines that activate other effector cells (9, 34), and we have shown such cooperation between Th cells and CTL (Fig.6). Lake and Mitchison (35) predicted some time ago that Th cells and CTL could cooperate by "associative recognition" of antigens expressed independently, and our findings are consistent with this hypothesis.

In agreement with others (36), we found that unrelated UV-induced fibrosarcomas provided varying levels of crossprotective immunity against a PRO tumor in unirradiated mice. However, the mechanism and importance of crossprotection is unclear. As noted by Brent and Medawar (37), immune responses acquire radioresistance once lymphocytes are exposed to antigen. Unlike immunity that is specific for individual tumors, crossprotection is sensitive to low doses of ionizing radiation (Table III) and fades with time (see footnote of Table III). Thus at present, there is no evidence for an antigen-specific response to a crossprotective antigen that would be commonly expressed by independently induced tumors, since radioresistant immunity (11, this study), CTL (2, 12, 38), and Th cell responses (this study) show specificity for antigens that are individually specific for each independently induced tumor (see reference 1 for review). Therefore, we have concentrated in the present study on developing an understanding of the tumor-specific protection that is radioresistant, long-lived, and antigen specific.

Th cell–recognized antigens could represent target structures for immunotherapy, but at present, we do not know if tumoricidal effector cells could be induced to such antigens. Tumor lineage–specific Th cell–recognized-antigens might have a distinct advantage over artifically introduced helper cell–recognized antigens, such as those provided by direct injection of TNP (39) or adjuvants into a tumor mass. Only the lineage-specific antigens would be present on MET or deeply invasive tumor cells. If specific antibodies could be generated, such lineage-specific antigens might also be useful as diagnostic markers to identify cancer cells wherever they may spread during the course of disease, and to distinguish them from a second primary malignancy. Since these Th-recognized antigens appear to be individually specific for each independently derived tumor, it would be interesting to determine if different Th-recognized antigens belong to a family of related molecules. We do not know if the differences in recognition of Th cell and CTL-recognized antigens are due to distinct molecular properties that determine their association with different MHC antigens or T cell receptors. As an alternative hypothesis, we propose that Th cell–recognized antigens may represent intracellular determinants, which can be recognized only after antigen presentation. Presumably, mutations resulting in changes in intracellular molecules are as likely as those affecting cell surface determinants. This hypothesis can be tested, since methods are available for screening mAbs to intracellular as well as cell surface antigens. Since Th-recognized antigens are retained during tumor progression they might well reflect an alteration essential to the expression of the malignant phenotype.

Summary

We have studied the components of a complex of tumor-specific antigens to determine if all of the components of the complex were lost during progression from a rather benign regressor tumor to a highly malignant (HM) cancer. We find that the HM tumor cells have lost antigens recognized by CTL but retained antigens recognized by Th cells. Immunization with variants expressing Th-defined antigens induced tumor-specific immunity to challenge with a parental variant that expressed a CTL-recognized target antigen, but did not induce

immunity to challenge with the variant that expressed the Th-defined antigen alone. Together, these findings suggested that Th cells fail to exert direct selective pressure upon the tumor, resulting in retention of "lineage-specific," Th-recognized antigens by highly immunoselected variants. Possible advantage could be taken of this fact for the development of specific immunotherapy.

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