Loss of a Unique Tumor Antigen by Cytotoxic T Lymphocyte Immunoselection from a 3-Methylcholanthrene–induced Mouse Sarcoma Reveals Secondary Unique and Shared Antigens

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Summary

Most chemically induced tumors of mice express unique antigens that can be recognized by cytotoxic T lymphocytes (CTL) and thereby mediate tumor rejection. The number of different antigens expressed by a single tumor and their interplay during immunization and rejection are largely unexplored. We used CTL clones specific to individual tumor antigens to examine the number and distribution of CTL antigens expressed by cell lines derived from 3-methylcholanthrene–induced sarcomas of (C57BL/6J × SPRET/Ei)F_1 mice. Each tumor cell line expressed one or more antigens that were unique, that is, not detected on cell lines from independent sarcomas. Immunoselection against an immunodominant antigen produced both major histocompatibility complex class I antigen and unique tumor antigen loss variants. Immunization of mice with antigen-negative immunoselected variants resulted in CTL that recognized additional antigens that were also expressed by the progenitor tumor. Some CTL recognized additional unique tumor antigen(s); other CTL recognized a shared antigen expressed not only by the immunizing cell line, but also by independent sarcoma cell lines and untransformed myoblastoid cell lines. CTL that recognized the shared antigen were also recovered from mice immunized in vivo with an untransformed myoblastoid cell line. These findings support a model of immunodominance among chemically induced tumor antigens in which shared antigens are masked by unique immunodominant antigens.

Chemically induced, transplantable tumors of mice have been used extensively to investigate immunologically mediated tumor rejection (1), and understanding the cellular mechanisms and molecular antigens responsible for tumor rejection in such mouse models may enable the development of more effective immunotherapies. Historically, studies have used in vivo tumor rejection assays to indirectly investigate antigens expressed by 3-methylcholanthrene (MCA)[1]-induced tumors. These studies demonstrated that immunization with a chemically induced tumor usually protects against in vivo challenge by the same tumor, but only rarely or sporadically protects against challenge by independent syngeneic tumors (2–4). These observations led to the suggestion that the antigens responsible for mediating antitumor immunity to MCA-induced tumors are unique to individual tumors.

More recently, $CD8^+$ CTL have been shown to be necessary for immunity to transplantable sarcomas (5, 6), and the antigens recognized by tumor-specific CTL have come under investigation. Short-term CTL lines from immunized mice have been used to demonstrate that the CTL antigens of MCA-induced sarcomas consist of MHC class I–bound peptides on the cell surface (7). Each peptide antigen is expressed only by a single sarcoma, correlating with the pattern of in vivo protection. The unique CTL antigens expressed by MCA-induced mouse tumors contrast with the CTL antigens of human melanomas, which are lineage-specific (8, 9) or "activation" (10) antigens expressed by multiple tumors.

The contribution of single antigens to tumor rejection has been investigated in mouse models using in vitro immunoselection to generate CTL-resistant tumor variants that lack expression of the selected antigen. For some highly immunogenic tumors, including spontaneously regressing, UV-induced sarcomas and SV-40 large T antigen–induced sarcomas, multiple epitopes independently mediate rejection (11–14). For other UV-induced and some chemically induced tumors, selected loss of an individual antigen produces variants that display a more malignant phenotype than their antigen-expressing progenitor tumors (15–17), indicating that the selected antigen is the principal target of tumor re-

[1] *Abbreviations used in this paper:* CML, cell-mediated lympholysis; MCA, 3-methylcholanthrene; MLTC, mixed lymphocyte tumor culture.

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jection. These studies emphasize the need to assess the spectrum of antigens that are available as targets, and immunoselection provides a powerful tool for exposing secondary epitopes to evaluation.

The use of monoclonal CTL to study antigenic diversity of MCA-induced tumors has been limited. In this report, we use CTL lines and clones to investigate the antigens expressed by cell lines derived from MCA-induced sarcomas of highly heterozygous, (C57BL/6J × SPRET/Ei)F_1, termed (B6 × SPE)F_1 mice. The antigenic complexity of one tumor cell line was investigated by selecting CTL-resistant variants in vitro and characterizing the immune response to the variants. CTL with new specificities were derived, identifying previously undetected antigens. Some variant-reactive CTL lines defined one or more uniquely expressed antigen(s); other CTL lines defined a shared antigen whose expression could not be inferred from the CTL response against primary cell lines.

Materials and Methods

Tumor Cell Lines. The tumors and their derivative cell lines examined in this study have been previously described in detail (18). Briefly, cell lines were derived from MCA-induced sarcomas generated in male and female mice from an F_1 cross between C57BL/6J (B6) and SPRET/Ei (SPE). All tumor lines were diagnosed histologically as poorly differentiated sarcomas or rhabdomyosarcomas that grew progressively and could be transplanted into syngeneic hosts. Cell lines derived from tumors were grown in 100-mm tissue culture–treated petri plates (Corning Glass Works, Corning, NY) in DMEM-based Vc medium (18) supplemented with 5% FCS (Hyclone Laboratories, Inc., Logan, UT). Cell lines were passaged weekly by preparation of a single-cell suspension using trypsin-EDTA (Sigma Chemical Co., St. Louis, MO) and vigorous pipetting and reseeding fresh plates. For immunization and T cell–mediated lympholysis (CML) assays, cells were harvested from petri plates and washed twice in PBS before use. The male-derived tumor cell lines used in this study were bs2 and its clonal derivative, bs2.1; bs4 and its clone, bs4.1; and bs15 and its clone, bs15.1; and the female-derived tumor cell lines were bs9 and its clone, bs9.1. The NK-sensitive Yac-1 cell line was passaged weekly in Vc5 medium.

Untransformed Myoblastoid Cell Lines. Untransformed myoblasts were derived from (B6 × SPE)F_1 neonates. Muscle tissue was carefully dissected from skin, bone, and fat, minced into fine pieces, and rocked for 1 h at 37°C in 10 ml of HBSS (GIBCO BRL, Gaithersburg, MD) containing 1 mg/ml collagenase and 2.5 U/ml hyaluronidase (Sigma). Large pieces of tissue were allowed to settle out, then suspended cells were washed twice in PBS (GIBCO BRL) and plated in Vc10 medium in a 100-mm tissue culture–treated cell culture dish (Corning). Myoblastoid cells were passed weekly into fresh Vc10 (same formulation as Vc5, except with 10% FCS) at a 1–10 dilution.

Mouse Immunizations. (B6 × SPE)F_1 mice (same sex as immunizing tumor) were immunized by intraperitoneal injection of 3–5 × 10^6 irradiated (1,000 Gy) tumor cells admixed with 150 μg heat-killed *Corynebacterium parvum* (19; culture kindly provided by Dr. C. Cummins, Virginia Polytechnical Institute and State University, Blacksburg, VA). Immunized mice were boosted at weekly intervals two or three times with 3 × 10^6 irradiated tumor cells without *C. parvum*. 10 d to 2 wk after the final boost, animals were euthanized by carbon dioxide asphyxiation, spleens were removed, and mononuclear splenocyte preparations were obtained. Mice for these experiments were bred and housed at The Jackson Laboratory Research Animal Facility following protocols approved by the institutional Animal Care and Use Committee and conforming to the American Association for Accreditation of Laboratory Animal Care (AAALAC) standards.

Mixed Lymphocyte/Tumor Cultures (MLTC) and CTL Lines. Primary MLTC were generated by in vitro culture of 2 × 10^7 mononuclear splenocytes, 2 × 10^6 irradiated (1,500 Gy) tumor cells, 6 ml Vc10 media, and 25 U/ml IL-2 (a generous gift of M. Widmer, Immunex Corp., Seattle, WA). After 3 d of in vitro culture, MLTC were expanded 1:3 into Vc10 with 25 U/ml IL-2. MLTC were passaged weekly thereafter in 16-mm tissue culture wells (Costar Corp., Cambridge, MA) by restimulation of 1–3 × 10^5 MLTC cells, 5 × 10^6 irradiated (200 Gy) syngeneic splenocytes, 1–3 × 10^5 irradiated (1,000 Gy) tumor cells, and 25 U/ml IL-2 in Vc medium supplemented with 5–10% fetal bovine serum. Some MLTC were supplemented with 1:20 volume anti-CD4 ascites fluid (clone GK1.5) during the third and fourth passages to block antigen-specific $CD4^+$ cell proliferation. After five or six passages in vitro, MLTC usually exhibited consistent growth characteristics from week to week, and were considered to be stable CTL lines. CTL clones were derived from established CTL lines by micromanipulation of single cells as previously described (20). For restimulation before CML assays, MLTC and CTL were passaged as described above, but with 30–40 U/ml IL-2; and cell supernatants were replaced 24 h before assay with fresh Vc5 supplemented with 5 U/ml IL-2.

The (B6 × SPE)F_1 CTL lines used in this study (and their cognate tumor lines, used for immunization and restimulation) were BxS/2 (bs2), BxS/4 (bs4) BxS/9 (bs9), BxS/15 (bs15), BxS/V (15V.1), and BxS/Δ (15Δ1). The (B6 × SPE)F_1 CTL clones used (and their cognate tumor lines) were BxS/15.4 (bs15.1); and BxS/Δ.11 (15Δ1). The SPE CTL line S/4 ($H2K^b$ restricted) and the B6 CTL clone B/4.4 ($H2^{SPE}$ restricted) were restimulated using tumor lines bs4 or bs4.1

Immunoselection. Variant tumors lines were obtained by two different immunoselection procedures that involved slight variations on previously described protocols (21, 22). The variant 15V.1 cell line was cloned by micromanipulation from a bulk population of variant cells derived from the bs15 tumor cell line that survived multiple rounds of selection with the CTL clone BxS/15.4. The variant 15Δ1 cell line was obtained as 1 of 13 growth positive wells out of 384 total wells that were seeded with bs15.1 tumor cells and subjected to multiple rounds of selection with the CTL clone BxS/15.4.

Flow Cytometry. FACS® (Becton Dickinson & Co., Mountain View, CA) was performed at The Jackson Laboratory Flow Cytometry Facility with a FACScan® fluorocytometer (Becton Dickinson). Dead cells were excluded by propidium iodine gating, and relative fluorescence (expressed in arbitrary units) of fluorescein- or rhodamine-conjugated mAbs was determined. The mAbs that were used included 28-13-3 (K^b) and 28-14-8 (D^b), neither of which stains $H2^{SPE}$ MHC alleles (not shown); YTS169 (CD8); GK1.5 (CD4); and H57-597 (α/β TCR).

CML Assay. 4–6-h CML assays were performed as previously described (23). ^{51}Cr released into the supernatant was determined, and specific lysis was calculated using the following ratio:

$$\textit{specific lysis} = (\textit{experimental} - \textit{spontaneous})/(\textit{maximum} - \textit{spontaneous})$$

Data are reported as the mean of three wells. SDs (generally <5%) are omitted for clarity of presentation. The ability of vari-

ous antibodies to inhibit target lysis was determined by the addition of 1/20 vol antibody ascites to microplate wells. In some experiments, tumor target cells were grown in 5 U/ml IFN-γ (a generous gift from Dr. van der Meide, Biomedical Primate Research Center, Rijswijk, The Netherlands) for 48 h before assay to augment surface expression of MHC class I molecules. T cell lymphoblast target cells were cultured from splenocytes in Vc5 supplemented with 25U/ml IL-2 and 2 μg/ml Con A (Sigma). These cells were used as CML targets 2–5 d later.

Results

(B6 × SPE)F_1 Tumors Express Unique Tumor Antigens. Tumor cells lines were used to immunize (B6 × SPE)F_1 mice, and CTL lines were derived from immunized splenocytes by in vitro restimulation. Fig. 1 shows CML assays using CTL lines established from four mice, each immunized with a different tumor cell line. Each CTL demonstrated specific lysis of its cognate tumor cell line, and failed to show significant lysis of independent tumor cell lines, untransformed syngeneic cell lines, or Yac-1 cells. CTL clones exhibiting identical specificity as the bulk cultures have been obtained for each CTL line. These CML results are representative of multiple experiments for each tumor, and they indicate that CTL derived by these techniques from mice immunized with (B6 × SPE)F_1 sarcomas recognize exclusively unique antigens.

Antigen expression in the cloned, bs15.1 tumor cell line was characterized further. A $CD8^+$, α/β TCR^+ CTL clone, BxS/15.4, which specifically lysed a clonal derivative of its cognate tumor cell line, bs15.1, was isolated (Fig. 1). Lysis was inhibited by anti-CD8 MAb and anti-$H2K^b$ mAb, but not by anti-CD4 mAb or anti-$H2D^b$ mAb (not shown). These data indicate that tumor cell line bs15.1 expresses a tumor-specific, K^b-restricted CTL antigen.

CTL-resistant Tumor Variants Express Different Phenotypes. To further investigate antigen expression by bs15.1 tumor cells, CTL-resistant variants were isolated by immunoselection with CTL clone BxS/15.4. Variant 15V.1 was isolated by micromanipulation of a single cell from a population of CTL-resistant cells. Variant cell line 15Δ1 was isolated in an independent, microwell-based immunoselection, and based on the Poisson distribution is highly likely to be clonally derived. Variants 15V.1 and 15Δ1 were examined by flow cytometry for MHC antigen expression, and by CML assay for sensitivity to various CTL (Fig. 2). Tumor variant line 15V.1 expressed $H2D^b$ (not shown), but not $H2K^b$, when analyzed by flow cytometry. Cell line 15V.1 retained sensitivity to the $H2^{SPE}$-restricted CTL, B/4.4, indicating that 15V.1 was capable of presenting endogenous antigens; however, 15V.1 was resistant to K^b-restricted CTL S/4, as well as the CTL BxS/15.4. These results indicate that variant 15V.1 acquired resistance to the selecting CTL (BxS/15.4) because of lack of $H2K^b$ cell-surface expression. In contrast, variant 15Δ1 cells expressed both $H2K^b$ and $H2D^b$ (not shown) by FACS® analysis, and they retained sensitivity to both CTL B/4.4 and CTL S/4. These results indicate that variant 15Δ1 acquired resistance to the selecting CTL through loss of the tumor-specific antigen recognized by CTL BxS/15.4.

CTL Generated to Antigen-loss Variants of bs15.1 Define Additional bs15.1 Antigens. To examine the antigenic complexity of tumor cell line bs15.1, CTL lines with additional antigenic specificities were generated (Fig. 3). Splenocytes from a mouse immunized with variant line 15V.1 (lacking $H2K^b$) were restimulated with irradiated 15V.1 cells to generate a $CD8^+$, α/β TCR^+ CTL line BxS/V. The (uncloned) CTL BxS/V lysed all tumor cell lines derived from tumor bs15, including the immunizing tumor 15V.1, its progenitor tumor bs15.1, and the unique antigen loss variant 15Δ1. However, CTL BxS/V did not lyse Yac-1 cells, independently derived sarcoma cell lines including bs4.1, or untransformed syngeneic myoblastoid cells. Thus, the antigen recognized by BxS/V was unique to the bs15 tumor, but not restricted by $H2K^b$, and not identical to the bs15.1 epitope recognized by CTL BxS/15.4.

In contrast, the $CD8^+$, α/β TCR^+ CTL BxS/Δ, derived from splenocytes of a mouse immunized with variant cell line 15Δ1, detected an antigen with an unexpectedly

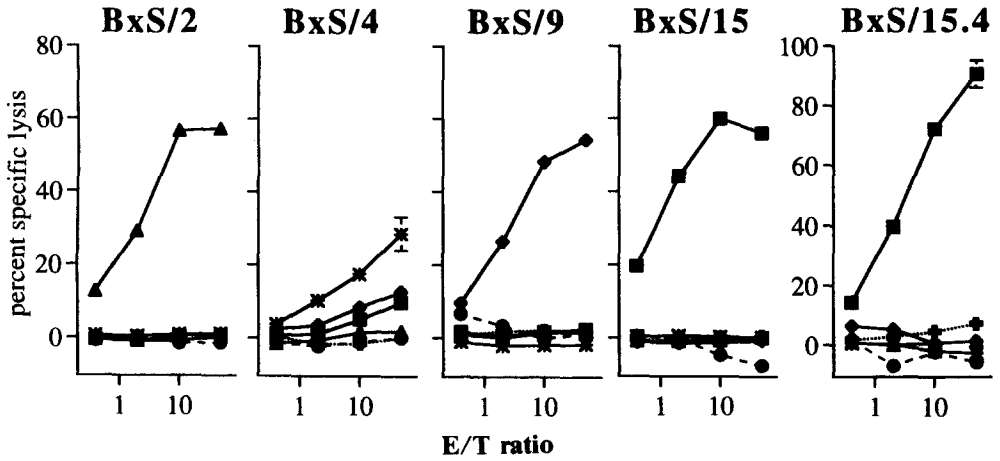

Figure 1. Long-term antitumor CTL lines lyse their cognate tumor. Each graph represents the results of a CML assay using a single, independently derived CTL versus its cognate tumor and five other targets. The CTL line is indicated above each graph, and the number in the CTL name corresponds to the number of the cognate, uncloned tumor against which the CTL was derived. BxS/15.4 is a cloned CTL line derived from BxS/15. Targets for each assay included independently derived sarcoma cell lines bs2 (-▲-), bs4 (-✕-), bs9 (-◆-), bs15 (-■-), untransformed syngeneic myoblasts (-●-), and Yac-1 cells (+). Each symbol represents the mean of three replicate wells. The SD (cognate tumor only) is indicated by a vertical bar or is smaller than the plot symbol.

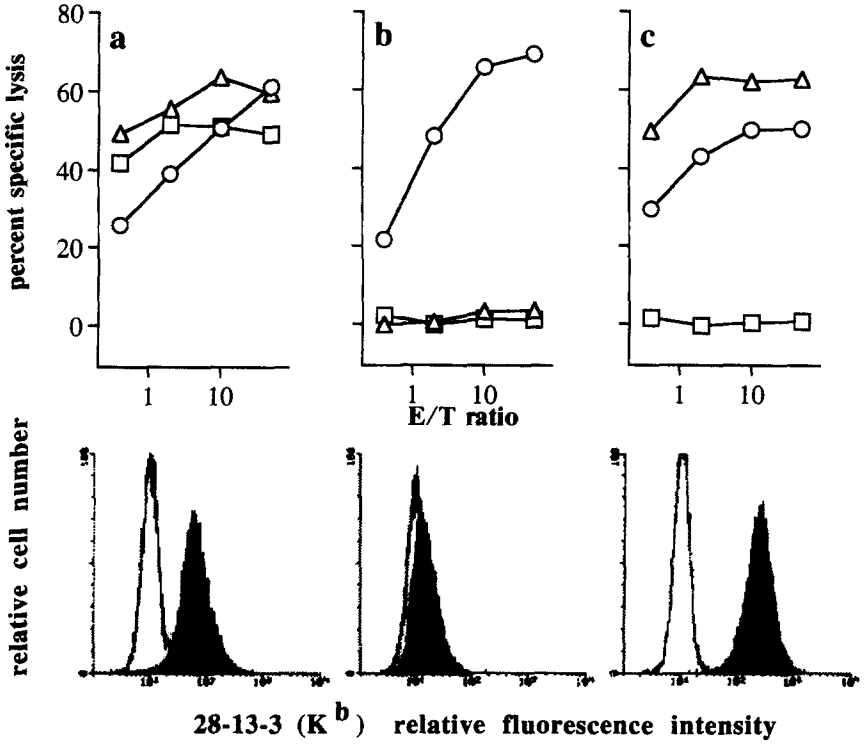

Figure 2. CML assays (*top graphs*) and FACS® analysis (*bottom graphs*) reveal that immunoselected variants express different phenotypes. (*a*) Progenitor tumor clone bs15.1 is lysed by the selecting CTL BxS/15.4 (–□–), allo-H2^{SPE}–specific CTL clone B/4.4 (–○–), and allo-H2K^b–specific CTL line, S/4 (–△–). Flow cytometric analysis shows expression of H2K^b by bs15.1. (*b*) Immunoselected variant 15V.1 is lysed by B/4.4, but not by BxS/15.4 or S/4 (legend key same as in *a*). Flow cytometric analysis demonstrates lack of expression of H2K^b by 15V.1 cells. (*c*) Immunoselected variant 15Δ1 is not lysed by BxS/15.4, but is lysed by B/4.4 and S/4 (legend key same as in *a*). Flow cytometric analysis demonstrates expression by 15Δ1 of H2K^b.

broad expression pattern. This CTL line lysed cell lines bs15.1, 15V.1, and 15Δ1, but not Yac-1 cells or untransformed Con A–stimulated T cell blasts. Additionally, CTL BxS/Δ lysed independently derived tumor clones bs2.1, bs4.1, and bs9.1, as well as untransformed syngeneic myoblasts, indicating that BxS/Δ recognized an antigen expressed in common among multiple independent sarcoma cell lines. A CTL clone, BxS/Δ.11, was derived from the BxS/Δ CTL line and demonstrated identical target specificity (Fig. 3). The failure of BxS/Δ to lyse Con A–stimulated splenic T cell blasts, LPS-stimulated splenic B cell blasts (not shown), or Yac-1 cells indicated that the antigen is not expressed as an artifact of in vitro cell growth, and is not expressed by cells of the lymphoid lineage. Moreover, trypsin-EDTA treatment of splenic T cell blasts did not sensitize them to lysis by BxS/Δ.11, while cell cultures freshly prepared ex vivo from progressing bs15.1 tumors retained sensitivity (not shown), indicating that cell preparation or culture conditions are unlikely to account for the observed cross-reactive antigen.

Shared Antigens Are Weak Elicitors of CTL. To test the relative efficacy of different antigens to elicit CTL, additional MLTC were derived and tested for lytic activity. Six unimmunized (B6 × SPE)F_1 mice were examined. MLTC derived from all unimmunized mice did not proliferate well upon restimulation, and they failed to demonstrate lytic activity against any target tested (e.g., 8/Δ, Fig. 4). The inability to derive tumor-reactive MLTC from naive (unimmunized) mice indicates the requirement for in vivo priming

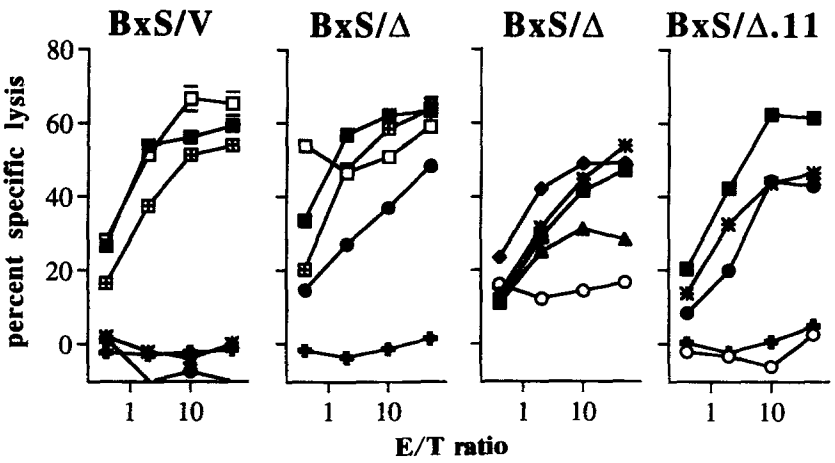

Figure 3. CTL lines derived against immunoselected variants recognize additional antigens. Each graph represents the results of a CML assay with a single CTL and multiple targets. CTL lines are indicated above each graph. Target cell lines are represented thus: bs15.1 (–■–) and its variant lines 15V.1 (–□–) and 15Δ1 (–⊞–); independently derived syngeneic MCA tumor cell lines bs2.1 (–▲–), bs4.1 (–✕–), and bs9.1 (–◆–); untransformed syngeneic myoblasts (–●–) and syngeneic splenic blasts (–○–); and Yac-1 cells (+).

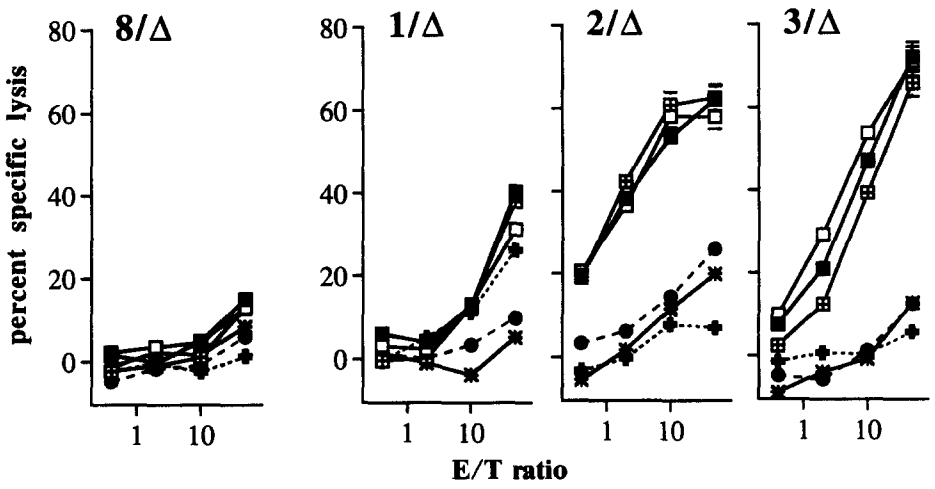

Figure 4. After four passages in vitro, some MLTC lyse bs15.1-derived tumors when tested in a CML assay. Mice 1–3 were immunized with tumor cell line 15Δ1; mouse 8 was not immunized. Splenocytes from each mouse were restimulated with tumor cell line 15Δ1. MLTC line 1/Δ from mouse 1 and 8/Δ from mouse 8 display no sarcoma-specific lysis; MLTC 2/Δ from mouse 2 and MLTC line 3/Δ from mouse 3 express lytic activity against secondary unique antigens. Targets: tumor bs15.1 (—■—), variant 15V.1 (—□—), variant 15Δ1 (—⊞—), independently derived sarcoma bs4.1 (—✕—), untransformed syngeneic myoblasts (—●—), and Yac-1 cells (+).

to generate CTL with the antigen specificities exhibited by lines BxS/15.4, BxS/V, and BxS/Δ.11.

Four additional mice were immunized, and their splenocytes were restimulated in vitro using tumor cell line bs15.1. All four MLTC specifically lysed the cognate tumor, bs15.1, but failed to lyse the tumor antigen-specific variant, 15Δ1 (not shown), indicating that the CTL populations in these additional MLTCs recognize the same unique tumor antigen as CTL BxS/15.4. The $H2K^b$-restricted bs15.1 antigen recognized by all these CTLs is therefore immunodominant, in that it is highly effective at eliciting CTL under the culture conditions used.

Three additional MLTC were established from splenocytes of mice immunized with tumor cell line 15Δ1 and restimulated with 15Δ1 cells (Fig. 4). MLTC 2/Δ and 3/Δ demonstrated potent specific lysis of bs15.1 cells and variants 15V.1 and 15Δ1, but not of tumor cell line bs4.1 or untransformed myoblasts. This pattern of reactivity is identical to that exhibited by the CTL line BxS/V, and indicates recognition of a secondary tumor-specific antigen(s). MLTC 1/Δ demonstrated relatively high lysis of Yac-1 targets, indicating NK-like or LAK-like nonspecific cytotoxicity, but little additional sarcoma-specific lytic activity. MLTC 2/Δ and 3/Δ also initially demonstrated low levels of lysis of sarcoma bs4.1 and untransformed myoblastoid lines (replicate experiments, not shown). This lytic activity could indicate a minor population of CTL that recognized a shared antigen with the same specificity as the BxS/Δ line.

Immunization with Untransformed Myoblasts Primes against a Shared Tumor Antigen. Four mice were immunized with untransformed myoblasts. Splenocytes from these mice were split, and one half was restimulated using untransformed myoblasts while the other half was restimulated using tumor cell line 15Δ1. All MLTC derived by restimulating in vitro using myoblasts failed to lyse any target tested. However, three of the four MLTC from myoblast-immunized splenocytes that were restimulated with tumor cell line 15Δ1 demonstrated target cell lysis. All three MLTC exhibited lysis of bs15.1, bs4.1, and untransformed myoblasts, but not of Yac-1 cells or splenic T cell blasts. One example of a pair of MLTCs derived from splenocytes of a single mouse, but restimulated using two different antigen sources, is shown in Fig. 5. The antigen specificity exhibited by the MLTC from myoblast-immunized, tumor-restimulated MLTC recapitulates the target specificity exhibited by CTL BxS/Δ.

Discussion

Despite the widespread use of MCA tumors for investigating immunological tumor rejection (1, 17), the use of monoclonal CTL lines to probe tumor antigen expression has been limited. The results presented here demonstrate that CTL lines and clones derived from (B6 × SPE)F_1 mice

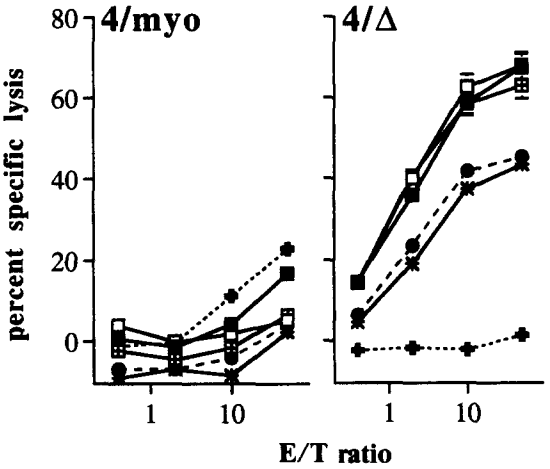

Figure 5. Development of shared antigen-specific lytic activity from immunized splenocytes in MLTC depends on the source of antigen used for in vitro restimulation. Splenocytes from representative, myoblast-immunized mouse 4 were divided into two aliquots; one was restimulated with myoblasts (*4/myo*), while the other was restimulated with tumor cell line 15Δ1 (*4/Δ*). MLTC 4/myo fails to generate lytic activity against any target tested, while MLTC 4/Δ lyses the same array of targets as the BxS/Δ CTL line. Legend key is the same as the one in Fig. 4.

immunized and restimulated with syngeneic MCA-induced sarcoma cell lines exhibit specific lysis of their cognate (immunizing) tumor cell lines. Lysis of independent tumor cell lines was not observed, indicating that the primary CTL antigens expressed by MCA-induced sarcomas are uniquely expressed by individual tumors. These results recapitulate and extend the extensively replicated observation that chemically induced and UV-induced sarcomas are immunogenic, but fail to elicit cross-protective immunity (7, 24).

The genetic mechanisms by which tumor cells escape CTL lysis can be studied by applying an in vitro CTL immunoselection approach. We observed that selective pressure on the highly heterozygous tumor cell line bs15.1 resulted in both MHC antigen variants and unique tumor antigen variants. It is interesting that these variant phenotypes reflect mutational alterations also observed in human and mouse tumors in vivo (25–27), suggesting that CTL selective pressure can shape tumor progression in patients. Because (B6 × SPE)F_1 tumor cell lines comprise abundant polymorphism between B6 and SPE alleles throughout the genome, they should expedite genetic analysis of mutations accompanying antigen loss after immunoselection and provide a powerful tool for understanding genetic mechanisms of tumor progression.

To investigate the antigenic complexity of tumor cell line bs15.1, variants lacking expression of the immunodominant antigen were exploited for detecting additional CTL antigens. Two qualitatively different, secondary antigens of tumor bs15.1 elicit CTL. A secondary tumor-specific antigen is recognized by CTL BxS/V. Expression of multiple tumor-specific antigens by a single tumor has been previously reported, including UV-induced sarcomas with highly immunogenic "regressor" phenotypes (11, 17). Expression of multiple tumor-specific antigens by MCA-induced tumors is therefore not unexpected, although it has not been extensively reported. More surprising is the observation that a shared antigen recognized by CTL BxS/Δ.11 is expressed by independent MCA-induced sarcomas as well as untransformed myoblastoid cell lines. Shared antigens were unexpected under these experimental circumstances because no cross-reactive lytic activity was generated in bulk MLTCs against the progenitor bs15.1 cell line, and shared antigens of UV-induced and MCA-induced tumors are not routinely observed using in vitro approaches or in vivo cross-protection assays.

The three CTL antigens expressed by tumor cell line bs15.1 exhibit a gradation of immunodominance under the experimental conditions used to generate MLTC. The primary tumor-specific antigen monopolized the CTL response when it was expressed by the immunizing/restimulating tumor. Most MLTC generated by using primary antigen-loss variants 15Δ1 and 15V.1 recognized the tumor-specific secondary antigen, although one MLTC recognized the shared antigen. Finally, although the shared antigen appears to be the weakest of the three CTL-defined antigens, under appropriate conditions, the shared antigen-specific CTL activity was reproducibly generated. The failure to observe persistent lytic activity aimed at more than a single antigen suggests that these activities may be mutually exclusive. Thus, these results support the hypothesis that immunodominant, tumor-specific antigens of MCA-induced tumors not only provoke a strong, tumor-specific CTL response, but they may also suppress or mask responses against weaker antigens, including both secondary tumor-specific antigens and shared antigens.

The coexpression of immunodominant tumor-specific antigens and secondary, shared antigens may explain sporadic reports of in vivo cross-protection in immunized animals. While most studies investigating in vivo rejection indicate that cross-protection between MCA sarcomas is rare (3, 4, 28), sporadic cases of cross-protection have been reported: Basombrio (4) demonstrated replicable cross-protection with one combination out of 14 MCA-induced tumors, and Prehn and Main (2) showed significant cross-protection in two of the four tumor combinations they tested. This rare in vivo cross-protection may reflect sporadic priming against a shared sarcoma antigen in the context of frequent priming against unique antigens. Further studies are needed to characterize the nature of the shared antigen detected on (B6 × SPE)F_1 sarcomas and to evaluate the efficacy of shared-antigen specific CTL for tumor rejection in vivo. The use of untransformed myoblastoid cell lines to prime a CTL response against shared antigens offers a new tool to dissect the potential role of shared antigens in tumor rejection.

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References

1. Boon, T., J.C. Cerottinin, B. Van den Eynde, P. van der Bruggen, and A. Van Pel. 1994. Tumor antigens recognized by T lymphocytes. *Annu. Rev. Immunol.* 12:337–365.
2. Prehn, R.T., and J.M. Main. 1957. Immunity to methylcholanthrene-induced sarcomas. *J. Natl. Cancer Inst.* 18: 769–778.
3. Klein, G., H.O. Sjogren, E. Klein, and K.E. Hellstrom. 1960. Demonstration of resistance against methylcholanthrene-induced sarcomas in the primary autochthonous host. *Cancer Res.* 20:1561–1572.
4. Basombrio, M.A. 1970. Search for common antigenicity among twenty-five sarcomas induced by methylcholanthrene. *Cancer Res.* 30:2458–2462.
5. Shu, S., T. Chou, and S.A. Rosenberg. 1987. Generation from tumor-bearing mice of lymphocytes with in vivo therapeutic efficacy. *J. Immunol.* 139:295–304.
6. Chou, T., and S. Shu, 1987. Cellular interactions and the role of interleukin 2 in the expression and induction of immunity against a syngeneic murine sarcoma. *J. Immunol.* 139:2103–2109.
7. Kono, K., M. Petersson, A.-M.T. Ciupitu, T. Wen, G. Klein, and R. Kiessling. 1995. Methylcholanthrene-induced mouse sarcomas express individually distinct major histocompatibility complex class I-associated peptides recognized by specific $CD8^+$ T-cell lines. *Cancer Res.* 55:5648–5655.
8. Robbins, P.F., M. el Gamil, Y. Kawakami, E. Stevens, J.R. Yannelli, and S.A. Rosenberg. 1994. Recognition of tyrosinase by tumor-infiltrating lymphocytes from a patient responding to immunotherapy [published erratum appears in *Cancer Res.* 1994. 54:3952]. *Cancer Res.* 54:3124–3126.
9. Kawakami, Y., S. Eliyahu, C.H. Delgado, P.F. Robbins, K. Sakaguchi, E. Appella, J.R. Yannelli, G.J. Adema, T. Miki, and S.A. Rosenberg. 1994. Indentification of a human melanoma antigen recognized by tumor-infiltrating lymphocytes associated with in vivo tumor rejection. *Proc. Natl. Acad. Sci. USA.* 91:6458–6462.
10. Van Der Bruggen, P., C. Traversari, P. Chomez, C. Lurquin, E. De Plaen, B. Van Den Eynde, A. Knuth, and T. Boon. 1991. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science (Wash. DC).* 254:1643–1647.
11. Kitajima, T., M. Iwashiro, K. Kuribayashi, and S. Imamura. 1994. Immunological characterization of tumor-rejection antigens on ultraviolet-light-induced tumors originating in the CB6F1 mouse. *Cancer Immunol. Immunother.* 38:372–378.
12. Urban, J.L., M.L. Kripke, and H. Schreiber. 1986. Stepwise immunologic selection of antigenic variants during tumor growth. *J. Immunol.* 137:3036–3041.
13. Wortzel, R.D., C. Philipps, and H. Schreiber. 1983. Multiple tumour-specific antigens expressed on a single tumour cell. *Nature (Lond.).* 304:165–167.
14. Lill, N.L., M.J. Tevethia, W.G. Hendrickson, and S.S. Tevethia. 1992. Cytotoxic T lymphocytes (CTL) against a transforming gene product select for point mutations within sequences encoding CTL recognition epitopes. *J. Exp. Med.* 176:449–457.
15. Frassanito, M.A., D. Loftus, R.M. De Leo, L.W. Law, E. Appella, and A.B. De Leo. 1994. Characterization of cloned class I MHC-restricted, $CD8^+$ anti-Meth A cytotoxic T-lymphocytes: recognition of an epitope derived from the Meth A gp110 tumor rejection antigen. *Cancer Res.* 54:4424–4429.
16. Maryanski, J., and T. Boon. 1982. Immunogenic variants obtained by mutagenesis of mouse mastocytoma P815. IV. Analysis of variant-specific antigens by selection of antigen-loss variants with cytolytic T cell clones. *Eur. J. Immunol.* 12: 406–412.
17. Urban, J.L., and H. Schreiber. 1992. Tumor Antigens. *Annu. Rev. Immunol.* 10:617–644.
18. Dudley, M.E., J.P. Sundberg, and D.C. Roopenian. 1995. Motif-primed polymerase chain reaction-based allelotype of sarcomas induced by 3-methlycholanthrene in interspecific mice. *Oncogene.* 11:517–524.
19. Dye, E.S., R.J. North, and C.D. Mills. 1981. Mechanisms of anti-tumor action of *Corynebacterium parvum:* potentiated tumor-specific immunity and its therapeutic limitations. *J. Exp. Med.* 154:609–620.
20. Roopenian, D.C., C.G. Orosz, and F.H.J.I. Bach. 1984. Responses against single minor histocompatibility antigens. II. Analysis of cloned helper T cells. *J. Immunol.* 132:1080–1084.
21. King, T.R., G.J. Christianson, M.J. Mitchell, C.E. Bishop, D. Scott, I. Ehrmann, E. Simpson, E.M. Eicher, and D.C. Roopenian. 1994. Deletion mapping by immunoselection against the H-Y histocompatibility antigen further resolves the Sxra region of the mouse Y chromosome and reveals complexity of the Hya locus. *Genomics.* 24:159–168.
22. Zuberi, A.R., M.E. Dudley, G.J. Christianson, and D.C. Roopenian. 1994. Gene mapping in a murine cell line by immunoselection with cytotoxic T lymphocytes. *Genomics.* 19: 273–279.
23. Wunderlich, J., and G. Shearer. 1992. In vitro assays for mouse lymphocyte function. *In* Current Protocols in Immunology. J.E. Coligan, A.M. Kruisbeek, D.H. Marguiles, E.M. Shevach, and W. Strober, editors. John Wiley & Sons, New York. 3.11.1–3.11.15.
24. Barth, R.J., Jr., S.N. Bock, J.J. Mule, and S.A. Rosenberg. 1990. Unique murine tumor-associated antigens identified by tumor infiltrating lymphocytes. *J. Immunol.* 144:1531–1537.
25. Restifo, N.P., Y. Kawakami, F. Marincola, P. Shamamian, A. Taggarse, F. Esquivel, and S.A. Rosenberg. 1993. Molecular mechanisms used by tumors to escape immune recognition: immunogenetherapy and the cell biology of major histocompatibility complex class I. *J. Immunother.* 14:182–190.
26. Lehmann, F., M. Marchand, P. Hainaut, P. Pouillart, X. Sastre, H. Ikeda, T. Boon, and P.G. Coulie. 1995. Differences in the antigens recognized by cytolytic T cells on two successive metastases of a melanoma patient are consistent with immune selection. *Eur. J. Immunol.* 25:340–347.
27. Seung, S., J.L. Urban, and H. Schreiber. 1993. A tumor escape variant that has lost one major histocompatibility complex class I restriction element induces specific $CD8^+$ T cells to an antigen that no longer serves as a target. *J. Exp. Med.* 178:933–940.
28. Ramarathinam, L., S. Sarma, M. Maric, M. Zhao, G. Yang, L. Chen, and Y. Liu. 1995. Multiple lineages of tumors express a common tumor antigen, P1A, but they are not cross-protected. *J. Immunol.* 155:5323–5329.