Comparison of Tumor-associated Transplantation Antigens of Sublines of Methylcholanthrene-induced Murine Tumors Passaged Separately in Vivo for over a Decade

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ABSTRACT

Two sublines of the methylcholanthrene-induced P815 mastocytoma, which had been passaged in vivo in separate institutions for over a decade, were compared for the expression of tumor-associated antigens in common. In cytotoxicity assays in vitro, the sublines were found to differ in expression of tumor-associated antigen(s) that were recognized by cytotoxic lymphocytes. However, in assays of tumor rejection in vivo, the sublines were found to express tumor-associated transplantation antigens in common. Both sublines were capable of inducing reciprocal cross-protection against the growth of a challenge implant of tumor cells of either subline, and both sublines were capable of inducing immunity that upon transfer of spleen cells from immunized mice to appropriate immunodeficient recipients would result in the complete regression of already established tumors of either subline. Similarly, two sublines of the methylcholanthrene-induced Meth-A fibrosarcoma that had been passaged separately in vivo for over a decade were also found to induce reciprocal cross-protection against a subsequent implant of cells of either subline.

These results indicate that the expression of tumor-associated transplantation antigens by two methylcholanthrene-induced immunogenic tumors is quite stable and suggest that the generation of tumor-associated transplantation antigen variant cell occurs infrequently. Therefore, an explanation for the progressive growth of immunogenic tumors based on the emergence of nonimmunogenic variants is unlikely, and the probability that the emergence of antigenic variants will lead to the failure of specific adoptive immunotherapy is low. Furthermore, the results indicate that the specificity of the immune response of mice to tumors as defined by transplantation immunity in vivo and lymphocyte cytotoxicity in vitro may be quite different. Therefore, using antigenic differences defined by in vitro cytotoxicity assays to explain the behavior of immunogenic tumors in vivo should be done with caution.

INTRODUCTION

The immunogenicity of chemical carcinogen-induced murine tumors has been demonstrated by showing that syngeneic mice can be immunized to reject a subsequent challenge implant of the same tumor. This immunity to syngeneic tumors is mediated by T-cells and is directed against TATAs (1). The TATAs of tumors induced in rodents by MCA have been studied extensively (2), and most have been found to be antigenically distinct for each tumor. Indeed, even within individual MCA-induced tumors, separate clones may express unique non-cross-reacting TATAs (3, 4). The molecular basis for the large number of unique TATAs expressed by MCA-induced tumors is unknown. However, despite the large number of unique TATAs expressed by MCA-induced tumors, the expression of any TATA by a particular tumor has generally been perceived to be quite stable (5, 6).

The progressive growth of an immunogenic MCA-induced tumor in a syngeneic host has been shown to evoke the generation of a concomitant immune response that is of sufficient magnitude to prevent the growth of a second implant of the same tumor, despite the fact that the original tumor will not be rejected but will grow progressively and kill the host (1, 7). The knowledge that immunogenic tumors can evoke an immune response during their growth has led to a number of theories to explain how such tumors escape rejection. These theories include the suggestions: (a) that the immunity generated is too weak and occurs too late to reject an already rapidly growing tumor mass (8); (b) that the generation of antigen-specific suppressor T-lymphocytes down-regulates the generation of the effector T-cells that mediate concomitant immunity (1); (c) that tumor cells emerge that evade destruction because they are not recognized by antigen-specific immune effector mechanisms (9–11); and (d) that tumors may avoid confrontation with host effector cells by secreting anti-inflammatory factors that prevent host cells from migrating into the tumor (12). It should be realized that these theories are not mutually exclusive, and that all or some of these factors may function in concert to permit the growth of any particular tumor.

Uyttenhove et al. (9) and H. Schreiber and colleagues (11) have recently described the isolation of cells from immunogenic tumors growing in syngeneic mice that are antigen-loss variants, in that they do not express tumor antigens present on the cells originally implanted. These antigen-loss variants have been characterized primarily based on killing by cytotoxic T-lymphocytes in vitro. Despite the fact that the antigen-loss variants differ in expression of tumor antigens recognized by cytotoxic T-cells, they apparently express other tumor antigens and are immunogenic. Besides indicating that individual cells may express multiple tumor antigens, these results, along with others (13–17), have been interpreted as suggesting that alterations in expression of tumor antigens may be frequently involved in the progressive growth of immunogenic tumors, and that emergence of antigen-loss variants may present a significant obstacle to treatment of tumors by specific immunotherapy in a manner analogous to the way that emergence of drug-resistant variants frequently leads to the failure of chemotherapy (18, 19).

It was reasoned that if generation and selection of stable clones of tumor cells that are antigen-loss variants are routinely involved in the progressive growth of an immunogenic tumor (in the face of an antitumor immune response), then, after many passages in vivo in immunocompetent hosts, sublines of a tumor would be expected to express different tumor-associated antigens. This possibility was investigated for two MCA-induced tumors by comparing antigen expression between sublines that had been passaged in vivo in separate institutions for over 10 years. The purpose of this report is to show that, for both the P815 and Meth-A tumors, TATA expression, analyzed by in vivo assays of tumor rejection, is very stable in that the apparent frequency of cell emergence failing to express TATAs in common with other cells within the tumor is very low. It will
also be shown that sublines of the P815 mastocytoma that express TATAs in common may differ in the expression of tumor antigens recognized by cytotoxic lymphocytes.

**MATERIALS AND METHODS**

Mice. B6D2F1 (C57BL/6 × DBA/2), CB6F1 (BALB/c × C57BL/6), AB6F1 (A × C57BL/6), and BALB/c female mice were used between 8 and 12 weeks of age. They were supplied by the Trudeau Institute Animal Breeding Facility and were derived from stocks originally obtained from The Jackson Laboratory, Bar Harbor, ME. The mice were free of known viral pathogens as evidenced by the results of routine screening performed by the Diagnostic Testing Service of Microbiological Associates, Bethesda, MD.

Mice were made TXB at 4 weeks of age by thymectomy, followed 7 days later by 1000 rads of whole-body gamma-irradiation delivered from a 137Cs irradiator at a dose of 29.5 rads/min. After irradiation the mice were infused i.v. with 2 × 10^7 syngeneic bone marrow cells and were used in experiments after an additional 5 weeks.

Tumors. The MCA-induced P815 mastocytoma, syngeneic in DBA/2 mice, was originally obtained from Dr. Virginia Evans (National Cancer Institute, Bethesda, MD) in 1970. This subline of the P815 tumor, P815-TRU, has been routinely passaged at the Trudeau Institute as an ascites in B6D2F1, mice. In 1974 the P815-TRU tumor was obtained by Dr. Eugenia Hawrylko, and was passed continuously by her as an ascites in DBA/2 mice (obtained from The Jackson Laboratory) at the Memorial Sloan-Kettering Cancer Center, New York, NY, until 1982, and then from 1982 to 1984 at the Long Island College Hospital, Brooklyn, NY. In 1984 the P815 tumor passed by Dr. Hawrylko, subsequently referred to as P815-HAW, was returned to the Trudeau Institute and passaged once as an ascites in B6D2F1, mice. The P815-HAW cells were then cryopreserved over liquid nitrogen in RPMI1640 medium (GIBCO, Grand Island, NY) containing 10% fetal calf serum (GIBCO), 5% dimethyl sulfoxide, and 5% glycerol.

The MCA-induced Meth-A fibrosarcoma was originally obtained from Dr. Lloyd Old (Memorial Sloan-Kettering Cancer Center) in 1970. The Meth-A fibrosarcoma, syngeneic in BALB/c mice, has been routinely passaged at the Trudeau Institute as an ascites in CB6F1, mice at the Trudeau Institute, and is referred to as Meth-A-TRU. In 1974 Dr. Hawrylko obtained the Meth-A-TRU tumor and routinely passaged it in BALB/c mice (from The Jackson Laboratory) at the Memorial Sloan-Kettering Cancer Center from 1974 to 1982, and then at the Long Island College Hospital, Brooklyn, NY, from 1982 to 1984. In 1984, the Meth-A tumor passed by Dr. Hawrylko, now referred to as Meth-A-HAW, was passaged once as an ascites in CB6F1, mice at the Trudeau Institute, and then the cells were cryopreserved over liquid nitrogen in Dulbecco’s modified Eagle’s medium (GIBCO) containing 10% calf serum (GIBCO), 5% dimethyl sulfoxide, and 5% glycerol.

The SA-1 spindle-cell sarcoma, syngeneic in A/J mice, was originally obtained from The Jackson Laboratory. All of the tumor lines were free of known viral pathogens as evidenced by the results of serological screening performed by the Animal Diagnostic Testing Service of Microbiological Associates.

Before use in experiments, a vial of cryopreserved tumor cells was thawed, and 10^6 cells were implanted in the peritoneal cavity of semisyngeneic mice that had previously received 700 rads of gamma-irradiation. Tumor cells were harvested 6 days later in PBS, pH 7.2, containing heparin, 10 units/ml, and resuspended to the desired concentration for implantation.

**Immunizations.** Mice were immunized for tumor-challenge tests by receiving 2 i.d. injections, on the abdomen, of 10^6 irradiated (5000 rads of gamma-irradiation) tumors cells. The 2 immunizing injections were given 2 weeks apart. The immunized mice were challenged, 1 week after the second immunization, with an i.d. implant of tumor cells on the opposite side of the abdomen.

B6D2F1 mice were immunized, for use as donors of spleen cells in passive transfer studies, by i.d. injection, on the midline of the abdomen, of 2 × 10^7 P815-TRU or P815-HAW tumor cells admixed with 50 or 100 μg of heat-killed Corynebacterium parvum. Injection of this admixture i.d. results in an initial 8- to 10-day period of tumor growth, followed, in most animals, by complete tumor regression in about 2 weeks (20).

Effector cells for use in cytotoxicity assays were obtained from B6D2F1 mice that were given i.d. injections of 2 × 10^7 P815-TRU or P815-HAW cells admixed with C. parvum (anti-P815 specificity), or from AB6F1, or BALB/c mice that were given 2 × 10^6 tumor cells alone (anti-DBA/2 specificity). The effector cells were obtained between 7 and 12 days after tumor implantation from lymph nodes draining the site of tumor rejection on the lateral thorax.

**Passive Transfer of Immunity.** Spleens were removed from donor mice 10 days after i.d. immunization with tumor cells admixed with C. parvum as described above. A single-cell suspension of spleen cells was prepared by dicing the spleens into small pieces and pushing the pieces through a 60-mesh stainless steel screen into PBS containing 1% fetal calf serum. The cell suspension was repeatedly pipetted with a Pasteur pipet, and then filtered through sterile surgical gauze to remove debris. The cells were then washed, resuspended in PBS, and infused via a lateral tail vein into TXB mice that had received 10^6 tumor cells i.d. 4 days before. All mice received 1.5 × 10^6 spleen cells in a volume of 1.0 ml. Tumor growth was followed by measuring 2 perpendicular diameters of the tumor at regular intervals and plotting the mean diameter against time.

**Cell-mediated Cytotoxicity and Cold-target Inhibition Assays.** Cell-mediated cytotoxicity was measured using a modification of the ^51Cr release assay described by Brunner et al. (21). Tumor cells for use as targets were obtained either from tissue culture during log-phase growth in RPMI1640 medium containing 10% fetal calf serum and gentamicin, or from peritoneal ascites. The tumor target cells were labeled for 1 h at 37°C with 100 μCi of sodium chromate (^51Cr) (CJ5;11; Amersham Corp., Arlington Heights, IL) per 10^6 cells in RPMI 1640 medium containing 10% fetal calf serum.

Lymph node cells for use as effector cells were obtained 7–12 days after immunization of B6D2F1 mice (anti-P815 effector cells) and 7–9 days after immunization of AB6F1, or BALB/c mice (anti-DBA/2 effector cells). Effector cells were obtained by pressing finely diced pieces of lymph nodes through a stainless steel screen into cold RPMI 1640 medium containing 10% fetal calf serum, 10 mM morpholinopanesulfonic acid (Sigma Chemical Co., St. Louis, MO), and gentamicin sulfate, 50 μg/ml (GIBCO). The cells were then centrifuged and resuspended to 10^7/ml. The assay was performed in quadruplicate in plates containing 96 round-bottomed wells (Costar Corp., Cambridge, MA). Each well contained 10^4 effector cells and 10^5 ^51Cr-labeled target cells (100:1, effector:target) in a total volume of 0.2 ml. The assay was initiated by centrifugation of the microtiter plate at 300 × g for 5 min. After 6 and/or 18 h of incubation at 37°C in an atmosphere of 5% CO2 in air, 0.05 ml of medium was removed from each well and counted in a 1282 Compugamma gamma counter (LKB Instruments, Inc., Gaithersburg, MD). Controls for the assay included labeled target cells incubated alone (spontaneous release) and treatment of labeled target cells with 0.5% Triton X-100 (maximum release). The maximum release was approximately 90% of the incorporated label. The spontaneous release was between 5 and 9% of the maximum release after 6 h of incubation, or between 15 and 30% after 18 h of incubation. The percentage of specific ^51Cr release was calculated as [(experimental cpm – spontaneous cpm)/(maximum cpm – spontaneous cpm)] × 100.

The ability of unlabeled tumor cells to inhibit cell-mediated cytotoxicity, i.e., the cold-target inhibition assay, was measured using a modification of the cell-mediated cytotoxicity assay described above. Unlabeled tumor cells (10^6, 4 × 10^5, or 1.6 × 10^5) were added to each well of a microtiter plate that already contained 10^4 effector cells and 10^5 ^51Cr-labeled target cells to give unlabeled tumor cells to labeled target cell ratios of 1:1, 4:1, and 16:1, respectively. The assays were then performed as described above for the cell-mediated cytotoxicity assay. Controls for specific and nonspecific inhibition were run simultaneously. The specific inhibition control consisted of adding unlabeled tumor cells to wells that contained ^51Cr-labeled target cells of the same type. Unlabeled SA1 spindle cell sarcoma cells, syngeneic in A/J (H-2^d) mice, were used as a control for nonspecific inhibition due to crowding.
RESULTS

Subline Variation in Expression of Tumor Antigen(s) Recognized by Cytotoxic Lymphocytes. The ability of the P815-TRU and P815-HAW sublines to induce and to be killed by cytotoxic lymphocytes was compared. Previous publications from this institute have shown that rejection of an i.d. P815-TRU tumor was associated with the detection of Lyt-2+ cytotoxic T-lymphotyes in the lymphatic drainage of the site of the i.d. injection (22, 23). This observation was confirmed. Lymph node cells, from B6D2F1 mice, injected 8–10 days previously with an admixture of P815-TRU cells and C. parvum, lysed P815-TRU cells in a 6 h 51Cr release assay (Table 1). However, lymph node cells from B6D2F1 mice that lysed P815-TRU cells were not cytotoxic to P815-HAW cells (Table 1) even if the assay time was increased from 6 to 18 h (data not shown). In addition, attempts to generate cytotoxic cells by inoculation of B6D2F1 mice with P815-HAW cells admixed with C. parvum, using the same protocol that consistently generated cytotoxic lymph node cells using the P815-TRU subline, failed to generate detectable cytotoxic activity against either P815-HAW or P815-TRU target cells.

It should be pointed out that the failure to detect cytotoxicity to P815-HAW cells was not due to a general resistance of P815-HAW cells to lysis by cytotoxic lymphocytes. Both P815-TRU and P815-HAW cells were equally susceptible to lysis by lymph node cells from allogeneic AB6F1 mice (H-2a) immunized with P815-TRU cells (Table 1) and to lymph node cells from P815-TRU immunized H-2a-compatible BALB/c mice that differ from P815 at multiple minor histocompatibility loci (data not shown). Furthermore, P815-HAW cells were as efficient as P815-TRU cells in eliciting cytotoxic anti-DBA/2 effector cells in the draining lymph nodes of allogeneic AB6F1 mice given injections of tumor cells. These experiments indicate that, as expected, the P815-TRU and P815-HAW cells express DBA/2 histocompatibility antigens in common.

The inability of lymph node cells of B6D2F1 mice, immunized against the P815-TRU tumor line to lyse P815-HAW cells suggested that P815-HAW cells, originally derived from the P815-TRU subline, may not express a tumor-associated antigen(s) recognized by the anti-P815-TRU cytotoxic cells. This possibility was examined further by comparing the relative ability of unlabeled tumor cells of both sublines to inhibit lysis of 51Cr-labeled P815-TRU cells. The data in Fig. 1 indicate that unlabeled P815-TRU and P815-HAW cells were equally effective at inhibiting the lysis of 51Cr-labeled P815-TRU target cells. Further evidence that the P815-TRU and P815-HAW tumors differ in expression of a tumor-associated antigen that induces the generation of cytotoxic cells in the lymph nodes of B6D2F1 mice immunized with the P815-TRU tumor. Unlabeled P815-TRU (•) and P815-HAW (A) cells were equally effective at inhibiting the lysis of 51Cr-labeled P815-TRU cells by AB6F1 anti-DBA/2 effector lymph node cells (left panel). However, P815-HAW cells were inefficient, compared with P815-TRU cells, at inhibition of lysis of 51Cr-labeled P815-TRU cells by anti-P815-TRU effector cells from the lymph nodes of immunized, semisyngeneic B6D2F1 mice (right panel). The unrelated SA-1 sarcoma (H-27) (©) was used in both assays as a control for nonspecific inhibition due to crowding. The SA-1 cells were very efficient at nonspecific inhibition of the anti-P815-TRU effector cells because they are twice the diameter (8 times the volume) of the P815-HAW or P815-TRU cells. Similar results were obtained in 3 separate experiments.

Table 2 Cross-protection with sublines of the P815 tumor

<table>
<thead>
<tr>
<th>Immunizing tumor</th>
<th>Challenge inoculum</th>
<th>Tumor incidence</th>
<th>Latent period</th>
</tr>
</thead>
<tbody>
<tr>
<td>None P815-TRU</td>
<td>5/5</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>None P815-HAW</td>
<td>5/5</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>P815-TRU</td>
<td>5/5</td>
<td>9.5 ± 2.6</td>
<td></td>
</tr>
<tr>
<td>P815-HAW</td>
<td>0/5</td>
<td>7.9 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>P815-TRU</td>
<td>105 P815-TRU</td>
<td>1/15</td>
<td>20.7 ± 6.4</td>
</tr>
<tr>
<td>P815-HAW</td>
<td>0/5</td>
<td>17.6 ± 6.1</td>
<td></td>
</tr>
<tr>
<td>P815-TRU</td>
<td>105 P815-HAW</td>
<td>11/15</td>
<td>19.8 ± 13.6</td>
</tr>
<tr>
<td>P815-HAW</td>
<td>105 P815-HAW</td>
<td>4/15</td>
<td>19.3 ± 11.0</td>
</tr>
</tbody>
</table>

* B6D2F1 mice were immunized by 2 i.d. injections, 2 weeks apart, with 106 irradiated tumor cells (5000 rads gamma-radiation).
* Immunized mice were challenged i.d. 1 week after the second immunization.
* Number of mice developing tumors over the number of mice challenged. Data are pooled for 3 separate experiments at a 106 challenge inoculum.
* Sample mean latent period (days) ± SD. The latent period was defined as the day when the mean diameter of the challenge tumor was 3.0 mm or greater.
* ND, not determined.
* Statistically significant (P < 0.01, Student's t test) compared to the latent period in unimmunized mice.
* Statistically significant (P < 0.05, x2 = 4.16) compared to the incidence of P815-TRU tumors occurring in P815-TRU immunized mice challenged with 105 cells.

Table 1 Lysis of P815 cells by lymph node cells from mice immunized with sublines of the P815 tumor

<table>
<thead>
<tr>
<th>Immunizing tumor</th>
<th>Mouse strain immunized</th>
<th>Target cells</th>
<th>No. of independent experiments</th>
<th>Median specific 51Cr release (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P815-TRU</td>
<td>B6D2F1</td>
<td>P815-TRU</td>
<td>12</td>
<td>30.9 (11.6–61.0)</td>
</tr>
<tr>
<td>P815-TRU</td>
<td>B6D2F1</td>
<td>P815-HAW</td>
<td>12</td>
<td>2.0 (–0.8–28.8)</td>
</tr>
<tr>
<td>P815-HAW</td>
<td>B6D2F1</td>
<td>P815-TRU</td>
<td>8</td>
<td>2.9 (–0.5–7.2)</td>
</tr>
<tr>
<td>P815-TRU</td>
<td>AB6F1</td>
<td>P815-TRU</td>
<td>7</td>
<td>1.8 (0.7–6.4)</td>
</tr>
<tr>
<td>P815-HAW</td>
<td>AB6F1</td>
<td>P815-TRU</td>
<td>7</td>
<td>42.6 (11.6–66.3)</td>
</tr>
<tr>
<td>P815-TRU</td>
<td>AB6F1</td>
<td>P815-HAW</td>
<td>7</td>
<td>28.8 (7.3–77.5)</td>
</tr>
</tbody>
</table>

- Semisyngeneic B6D2F1 mice were given i.d. injections of 2 × 106 tumor cells admixed with C. parvum allogeneic AB6F1 mice, were given injections of 2 × 106 tumor cells alone. In each experiment lymph node cells were tested for cytotoxicity in a 6-h 51Cr release assay at an effector to target ratio of 100:1 on days 7–10 after immunization. The data present the median (and the range) of the highest activity observed within each experiment.
- Statistically significant by Wilcoxon matched-pairs signed-ratio test (P < 0.005) compared to the percent specific 51Cr release for P815-TRU targets tested with the same effector cells.
- Not statistically significant compared to the percentage of specific 51Cr release for the P815-TRU targets tested with the same effector cells.
was used, mice immunized with the P815-HAW subline were equally protected against challenge by either subline, but mice immunized with the P815-TRU subline were less resistant to challenge by the P815-HAW subline than to challenge by the P815-TRU subline. In all groups of immunized mice, the mean latent period preceding the emergence of a measurable (>3.0 mm) challenge tumor was significantly longer than the mean latent period for the development of tumor in nonimmunized mice challenged with 10^5 cells. However, once tumor size exceeded 3.0 mm, the growth rate of tumor in nonimmunized and immunized mice was the same. These results have been interpreted as indicating that the P815 sublines still express TATAs in common even after separate passage in vivo for over a decade.

It had previously been shown that spleen cells from mice immunized against the P815-TRU tumor were capable, upon transfer to TXB recipients, of mediating the specific rejection of an established P815-TRU tumor, but not the rejection of antigenically unrelated P388 or L5178Y tumors (24). Furthermore, it had also been shown that the onset of tumor regression was associated with the occurrence of anti-P815-TRU cytotoxic T-cells in the lymph nodes of the TXB recipients (23). Therefore, to determine if the immune response to either the P815-TRU or P815-HAW subline could cause the rejection of established tumors of the other subline, 1.5 x 10^5 spleen cells (approximately one spleen cell equivalent) from B6D2F1 mice immunized to either subline were transferred to TXB recipients with a growing P815-TRU or a P815-HAW tumor that had been established 4 days earlier by i.d. injection of 10^6 cells. The immune spleen cells were from donor mice that had received an i.d. injection of tumor cells admixed with C. parvum 10 days earlier. This was the same immunization protocol used in the cytotoxicity experiments shown in Table 1. The results in Fig. 2 show that transfer of spleen cells from immunized to either subline caused the complete regression of an i.d. tumor of either subline. All mice that received immune spleen cells were free of detectable tumor, primary or metastatic, at 60 days after the transfer. The transfer of 1.5 x 10^5 spleen cells from normal, age-matched B6D2F1 mice had no effect on the growth of either tumor subline. The same results were obtained in three separate experiments. These results indicate that the antigenic difference between the P815-TRU and the P815-HAW sublines detected in the in vitro cytotoxicity assays was not sufficient in an adoptive immunotherapy protocol to permit either tumor to escape rejection in vivo. Thus, these results are consistent with the results of the immunization and challenge tests (Table 2) in that both in vivo assays of tumor rejection indicate the P815-TRU and P815-HAW sublines retain expression of TATAs in common.

Tumor-associated Transplantation Antigen Expression in Two Sublines of the Meth-A Fibrosarcoma Passaged Separately for Over a Decade. The data in Table 3 show that semisyngeneic CB6F1 mice immunized with either the TRU or the HAW subline of the Meth-A fibrosarcoma were protected against subsequent challenge with either the Meth-A-TRU or the Meth-A-HAW subline. These results have been interpreted as indicating that these two Meth-A sublines still express at least one strong TATA in common even after 10 years of separate passage. The susceptibility of the Meth-A-TRU and Meth-A-HAW sublines to cytotoxic lymphocytes could not be compared. Neither the Meth-A-TRU nor the Meth-A-HAW subline was susceptible to killing in vitro by cytotoxic lymphocytes, including lymph node cells from allogeneic AB6F1 (H-2^d), HAW tumor (A), to cause regression, upon transfer to TXB B6D2F1 hosts, of an established tumor (A), to cause regression, upon transfer to TXB B6D2F1 hosts, of an established P815-TRU (H-2^d) cells. These results have been interpreted as indicating that these two Meth-A sublines still express at least one strong TATA in common even after 10 years of separate passage. The susceptibility of the Meth-A-TRU and Meth-A-HAW sublines to cytotoxic lymphocytes could not be compared. Neither the Meth-A-TRU nor the Meth-A-HAW subline was susceptible to killing in vitro by cytotoxic lymphocytes, including lymph node cells from allogeneic AB6F1 (H-2^d), that were demonstrated in concurrent tests to be highly cytotoxic to P815-TRU (H-2^d) cells.

**DISCUSSION**

This paper demonstrates that TATA(s) expression is a stable property of 2 MCA-induced tumors. Two sublines of the P815 mastocytoma, passaged separately in vivo for over 10 years, were compared for the expression of tumor-associated antigens in common. Experiments designed to test the expression of TATA(s) in common between the P815-TRU and P815-HAW sublines indicated that immunization of semisyngeneic mice with either subline provided protection against subsequent challenge by either subline. This result, combined with the demonstration that TXB mice infused with spleen cells from mice immunized against either subline rejected established tumors of either subline, indicated that the P815-TRU and P815-HAW sublines still shared TATA(s) in common. Furthermore, immunization of semisyngeneic mice with either of 2 sublines of the Meth-A fibrosarcoma also protected against challenge by either tumor subline, indicating that the sublines shared TATA(s) in common. The expression of TATA(s) in common between sublines passaged separately in vivo for over a decade suggests that the frequency of generation and selection of TATA-variant cells is low. This implies that the probability of TATA-variant cell generation playing a significant role in the progressive growth of these immunogenic tumors is quite low. However, these results indicate only that the two sublines still have at least one TATA in common, and they do not exclude the possibility of alterations in expression of other TATA(s).

<table>
<thead>
<tr>
<th>Immunizing tumor*</th>
<th>Challenge tumor*</th>
<th>Tumor incidence Exp. 1</th>
<th>Exp. 2</th>
<th>Total % with tumors</th>
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<tr>
<td>Meth-A-TRU</td>
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<td>0/5</td>
<td>0</td>
</tr>
<tr>
<td>Meth-A-TRU</td>
<td>Meth-A-HAW</td>
<td>0/5</td>
<td>0/5</td>
<td>0</td>
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<tr>
<td>Meth-A-HAW</td>
<td>Meth-A-TRU</td>
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<td>1/5</td>
<td>10</td>
</tr>
<tr>
<td>Meth-A-HAW</td>
<td>Meth-A-HAW</td>
<td>0/5</td>
<td>0/5</td>
<td>0</td>
</tr>
<tr>
<td>None</td>
<td>Meth-A-TRU</td>
<td>5/5</td>
<td>5/5</td>
<td>100</td>
</tr>
<tr>
<td>None</td>
<td>Meth-A-HAW</td>
<td>5/5</td>
<td>3/3</td>
<td>100</td>
</tr>
</tbody>
</table>

* CB6F1 mice were immunized by 2 i.d. injections, 2 weeks apart, with 10^6 irradiated tumor cells (5000 rads of gamma-irradiation).

* Immunized mice were challenged i.d. with 4 x 10^6 cells 1 week after the second immunization.

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Fig. 2. Ability of 1.5 x 10^5 transferred spleen cells from normal B6D2F1 mice (○), or B6D2F1 mice immunized against the P815-TRU tumor (△), or the P815-HAW tumor (▲), to cause regression, upon transfer to TXB B6D2F1 hosts, of an established P815-TRU tumor (left panel) or P815-HAW tumor (right panel). The immune spleen cells were from B6D2F1 mice 10 days after immunization by the i.d. implantation of 2 x 10^6 tumor cells admixed with C. parvum and were infused i.v. into tumor-bearing mice that had received 10^6 tumor cells i.d. 4 days previously. Means of 5 mice per group. Similar results were obtained in 3 of 3 experiments.
Although they share common TATA(s), the P815-TRU and P815-HAW sublines do, however, appear to differ in the expression of some tumor-associated antigens, as indicated by the following observations: (a) The P815-TRU subline does not immunize as effectively against challenge by the P815-HAW subline as it does against itself; (b) immunization of semisynthetic mice with the P815-TRU subline, but not the P815-HAW subline, admixed with C. parvum as an adjuvant, results in a potent CTL response detectable in the draining lymph nodes; and (c) the CTL in the draining lymph nodes of mice immunized against P815-TRU do not recognize or lyse P815-HAW cells. Collectively, these results are consistent with the hypothesis that the P815-HAW subline does not express a tumor-associated antigen(s) recognized by CTL which lyse P815-TRU cells. Whether the prolonged passage of the P815-HAW cells in syngeneic mice (DBA/2), as compared to passage of the P815-TRU cells in semisynthetic (DBA/2 × C57BL/6 F₁) mice, contributed to this difference is unknown.

The results presented here do not indicate whether the P815-TRU tumor antigens that are recognized by CTL function as TATA(s). Although a previous report has indicated that mice adoptively immunized against an established P815-TRU tumor generate CTL in the recipient (23), evidence of active involvement of CTL in P815-TRU tumor rejection in vivo remains indirect. On the other hand, it has been observed that the P815-HAW tumor is not recognized by CTL (which recognize the P815-TRU tumor) and spleen cells from animals immunized to the P815-TRU tumor can cause the rejection of the P815-HAW tumor in vivo, subsequent to transfer to appropriate recipients. This does not formally eliminate the possibility that CTL may participate in tumor rejection. Rather, these results only indicate that CTL that recognize P815-HAW cells in vitro are not detectable in the lymph nodes of immunized animals. Further experiments using antibody and complement depletion of lymphocyte subsets prior to transfer of immune populations may help characterize the mechanism(s) of in vivo immunity generated by immunization with each subline. But, regardless of the mechanism(s) of immunity, the immunity generated by immunization with either subline is cross-protective.

P815-TRU and P815-HAW cells differ in expression of antigen(s) recognized by tumor-specific CTL and continue to express tumor-specific TATA(s) in common. This suggests that P815-TRU cells probably express a minimum of two distinct tumor-associated antigens although a purely quantitative difference in the expression of a single antigen cannot be ruled out. Uyttenhove et al. (9), using CTL clones, have detected multiple tumor-associated antigens on cells of the P815-X2 subline. The relationship between the antigenic specificities defined by the cloned CTL used by Uyttenhove et al. and the antigens recognized by the anti-P815-TRU CTL are unknown. Partial immune rejection of the P815-TRU tumor growing as an ascites in normal mice, as described by Uyttenhove et al. for the P815-X2 subline, has not been seen with the P815-TRU subline. However, it has been observed that P815-TRU cells recovered very late during the i.p. growth of the tumor have a reduced sensitivity to lysis by anti-P815-TRU specific CTL. This observation is in agreement with the observations of Biddison and Palmer (13) with the P815-Y subline. The reduced sensitivity to CTL lysis is seen, however, when the P815-TRU tumor is grown in either normal or immunodefpressed mice, and is rapidly reversible upon culture of the tumor cells in vitro;

therefore, it is probably not due to immunoselection of stable antigen-loss variants.⁴

The occurrence of multiple tumor-associated antigens is not unique to the P815 mastocytoma. Evidence for multiple tumor antigens, which may function as TATA(s), has been obtained for UV light-induced tumors (25, 26), for other chemical carcinogen-induced tumors, including the Meth-A fibrosarcoma (27), and with murine retrovirus-induced tumors (28, 29).

In recent years, investigation into the multifaceted and dynamic nature of cancer has centered on the theme of “tumor cell heterogeneity” (30). Nowell (31), in his theory of “clonal evolution of tumor cell populations,” postulated that onset of neoplasia results in an acquired genetic instability that ultimately leads to an increase in the production of mutations. Thus genetic mechanisms would play a major role in generating tumor cell diversity. Evidence supporting this concept has been obtained particularly from studies of metastasis (32) and the development of drug resistance (33). Furthermore, the significance of the generation of tumor cell heterogeneity has been dramatically illustrated by the observations that, for both clinical and experimental tumors, a major cause of failure of chemotherapy is the selection for drug-resistant mutants (34). However, the generation of TATA-loss variants need not occur at similar frequencies as seen for the generation of metastatic and drug-resistant variants. Indeed, the results reported here indicate that TATA(s) were found in common between sublines of MCA-induced tumors even after extensive passage in normal immunocompetent mice, an environment that would appear to favor selection for TATA-loss variants; this suggests that the frequency of loss of TATA(s) may be quite low. Clearly, specific immunotherapy would be expected to be most efficacious if it were directed toward stably expressed tumor antigens.

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Comparison of Tumor-associated Transplantation Antigens of Sublines of Methylcholanthrene-induced Murine Tumors Passaged Separately in Vivo for over a Decade

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