In Situ Augmentation of Class I Major Histocompatibility Antigen Expression on Immunogenic Variants of a Spontaneous Murine Mammary Carcinoma

B. E. Elliott, D. A. Carlow, L. Ivimey, M. Arnold, N. Hampton, and P. Bosman

ABSTRACT

The relationship between expression of cell surface glycoproteins encoded by the major histocompatibility complex (MHC) and immunogenicity of a recently obtained spontaneous murine mammary adenocarcinoma (designated CBA.SP1) was examined. Immunogenic and nonimmunogenic variant clones were isolated from a subclone of the parent tumor after treatment with the mutagen N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) or the DNA hypomethylating agent and "gene activator," 5-aza-2'-deoxycytidine (5-aza-dCyd). All clones from the untreated tumor population were tumorigenic in normal syngeneic recipients. In contrast, immunogenic variant clones, isolated at high frequencies after drug treatment (ranging from 5% (5-aza-dCyd treated) to greater than 90% (MNNG treated)), were rejected in normal syngeneic mice but grew progressively in T-cell deficient nude mice. Consistent with our previous report (J. Natl. Cancer Inst., 75: 291, 1985), all 5-aza-dCyd induced immunogenic clones expressed elevated levels of class I (particularly D^P^) MHC antigens. However some (three out of nine) nonimmunogenic clones also showed enhanced class I MHC expression, implying that not all high MHC expressors were immunogenic. In contrast to 5-aza-dCyd induced variants, only 50% of MNNG induced immunogenic variants showed elevated levels of D^P^ or D^E^ antigens in vitro. Strong augmentation of class I MHC antigens in situ was observed on all immunogenic, but not nonimmunogenic, clones following transplant into syngeneic mice; no increase in MHC expression on variants during progressive growth in athymic nude mice occurred. Although no class II (A^a^ or E^e^) antigens were detected on the parent line or any of the immunogenic variants, a strong infiltration of host I-A bearing cells occurred during immune rejection of SP1 variants. These results are consistent with the hypothesis that induction of class I MHC antigen expression on certain low MHC expressing tumors, although not the sole requirement for immunogenicity, can facilitate immune rejection of the SP1 tumor and, conversely, that the reduced level of MHC observed in certain clinical cancers may significantly affect the immunological aspects of the tumor-host relationship.

INTRODUCTION

There is a growing body of evidence supporting the view that, at least in some tumor systems, tumorigenicity and cell surface expression of glycoproteins encoded by the MHC are inversely related (1). First, reduced or absent MHC expression has been correlated with a significant proportion of human and rodent malignancies [especially carcinomas (1-3)]. Second, adenovirus 12 (4, 5) or Moloney murine leukemia virus (6) mediated repression of class I MHC expression was found to specifically correlate with tumorigenicity. Third, transfection of foreign H-2K genes into an H-2K^d^ negative murine (AKR) leukemia (7), and an H-2K^k^ negative methylcholanthrene induced fibrosarcoma (8), produced H-2K positive transfectants which grew poorly in vivo and (in the latter case) metastasized less well compared to the parent tumor. In some of the above studies (7, 8), regression of high MHC expressing nontumorigenic transfectants appeared to be T-cell dependent in that they grew progressively in T-cell deficient nude or irradiated mice. One plausible explanation for these observations is that increased MHC expression triggers MHC restricted T-cell mediated recognition and subsequent rejection of gene transfected tumor lines (9). However there is evidence that a complementary "parimmune" defense system detects deleted or reduced expression of self-MHC, as suggested by Karre et al. (10) who showed that certain murine lymphoma cells selected for loss of H-2 expression are less malignant, and more sensitive to natural killer cells, than wild type tumor cells. Thus depending on antigenic status and tumor type, changes in MHC expression could have diverse effects on recognition by T-cells and natural killer cells.

Although most experimentally induced animal tumors are immunogenic, many human malignancies (11, 12) and the majority of spontaneously arising animal tumors (i.e., that arise in low cancer incidence strains without apparent or intended carcinogen exposure) (13-15) fail to stimulate any detectable host-immune response. About 60% of human carcinomas of different origin have been shown to express heterogeneous, or low, levels of MHC antigens (2). Such tumors would evade host T-cell recognition regardless of their antigenic status. It is therefore possible, as suggested by MHC gene transfection approaches using experimentally induced tumor systems (3, 6, 7), that quantitative changes in surface MHC antigen expression could significantly affect host-immune surveillance against this category of low MHC expressing tumors.

Our laboratory has developed a spontaneous murine carcinoma model with which to examine the contribution of cell surface MHC expression on tumor immunogenicity and tumor progression (16). Our previous results have shown that three out of four immunogenic variants derived from the parent line exhibited 4- to 12-fold elevated levels of class I D^P^ MHC, compared to nonimmunogenic variant clones and the parent tumor; consistent with an inverse correlation between MHC expression and tumorigenicity. In the present report we further examine the proposed relationship, using a wide spectrum of immunogenic and nonimmunogenic variants, independently isolated following treatment with drugs known to affect DNA metabolism: i.e., the DNA alkylating agent MNNG (17) and the DNA hypomethylating agent 5-aza-dCyd (18). All immunogenic variants derived from the parent line following 5-aza-dCyd treatment, and a large proportion of MNNG induced immunogenic variants exhibited in vitro elevated levels of class I, but not class II, MHC antigens. Most, but not all, nonimmunogenic variants maintained a low level of class I MHC expression. The level of class I MHC antigens on all immunogenic, but not on nonimmunogenic, clones greatly increased (up to 50-fold) with time after transplantation into normal...
syngeneic mice; correlating with the immune rejection. A strong host I-A bearing cell infiltrate was also observed at the site of tumor rejection. The hypothesis is presented that augmentation of class I MHC antigen expression and infiltration of host I-A bearing cells contribute to the immune rejection of immunogenic variants.

MATERIALS AND METHODS

Mice

Female CBA/J, and C3H mice between 8 and 30 weeks of age, were obtained from The Jackson Laboratories at Bar Harbor, ME. BALB/c nude mice were bred in the mouse colony at Queen's University.

Media

The media for all manipulations was RPMI 1640 (GIBCO, No. 430-1800) supplemented with 5-7% fetal calf serum (Flow Laboratories), l-glutamine (290 mg/liter; GIBCO No. 320-5030), asparaginase (36 mg/liter), I-arginine HCl (116 mg/liter), folic acid (10 mg/liter), and sodium pyruvate (100 mg/liter).

Tumors

CBA.SP1 (abbreviated, SP1) is a mammary adenocarcinoma which was isolated on January 25, 1982. The tumor arose spontaneously in an 18-month-old CBA/J female retired breeder obtained from The Jackson Laboratories and housed in the mouse colony at Queen's University. The histological and ultrastructural morphology of the original tumor was consistent with that of an infiltrating mammary ductal carcinoma. The tumor was adapted to culture, and could be passaged in vivo. When passaged in vivo by s.c. injection, the cultured line produced a well defined, solid, encapsulated tumor without metastasis. The growth characteristics of the SP1 tumor have been described previously (16). All tumors were tested periodically for mycoplasma by assessing for growth in pleuropneumonia-like organism agar plates and frozen normal and malignant tissue were air dried and acetone fixed for 5 min at 4°C on precleaned gelatinized (2%) glass slides. Sections (4-μm thick) of normal and malignant tissue were air dried and acetone fixed for 5 min at 4°C on precleaned gelatinized (2%) glass slides. Sections were overlaid overnight at 4°C with an appropriate dilution of monoclonal antibodies specific for a monomorphic class I MHC determinant (M1/42) or an unrelated polymorphic class I MHC determinant (H-2.4 (34-2-12S)). Positive controls included sections of normal CBA and BALB/c lymph node. For detection of rat monoclonal antibody, sections were incubated with fluorescein isothiocyanate labeled avidin (Biocan, Mississauga, Ontario). For detection of mouse antibody, fluorescein isothiocyanate (FITC) labeled antibody protein was added to yield approximately 40,000-80,000 MFI (irrelevant control MoAb) minus MFI (experimental MoAb). For detection of rat monoclonal antibody, sections were incubated with fluorescein isothiocyanate labeled avidin (Biocan, Mississauga, Ontario). For detection of mouse antibody, fluorescein isothiocyanate labeled antibody protein was added to yield approximately 40,000-80,000 MFI (irrelevant control MoAb) minus MFI (experimental MoAb) was used at 750 mamps with a photomultiplier voltage of 600. Histograms were collected from gated scatter analysis in which the number of cells was plotted as a function of linear mean fluorescence intensity.

Quantitation of Antibody Binding

Analysis by Immunofluorescence. Tumor cells were harvested with 0.02 M EDTA/PBS (no serum or trypsin) and washed in medium containing 50-75% fetal calf serum over Ficoll-Urovison (density, 1.09 g/cc) (16). Normal spleen cells were used as controls. A two step staining procedure was used, as previously described (16). Samples, 10,000 cells each, were then analyzed on the fluorescence activated cell sorter (FACS IV; Becton Dickinson), outfitted with a 4-decade log amplifier (Nazi Associates, Palo Alto, CA). The 488-nm laser line was used at 750 mamps with a photomultiplier voltage of 600. Histograms were collected from gated scatter analysis in which the number of cells was plotted as a function of linear mean fluorescence intensity. Antibody binding was expressed in terms of delta (Δ) MFI [MFI (experimental MoAb) minus MFI (irrelevant control MoAb)].

Analysis by Radioimmunoassay. A two step RIA was used to detect antibody binding to viable cells immobilized on polyvinylchloride microtiter plates (Falcon Plastics, No. 3912). Plates were coated with 1.0 μg/ml poly-D-lysine (Sigma) in PBS (100 μl/well) overnight at 4°C. After three times washing with PBS, tumor cells or normal spleen cells (100 μl) at 5×10¹⁰/well were added and pelleted (2000 rpm, 5 min). The supernate was removed and free sites were blocked with PBS + 1% bovine lacto transfer technique optimizer ([BLOTTO, Carnation skim milk powder (2%)] + 0.02% sodium azide). After 1 h at room temperature, wells were washed with PBS and 50 μl antibody at a predetermined dilution were added. The first antibody incubation was for 30 min at 4°C; wells were then washed four times with PBS. 125I labeled sheep anti-mouse IgG (Amersham; specific activity, 10-50 μC/μg antibody protein) was added to yield approximately 40,000-80,000 cpm/well. After a 2-h incubation, wells were washed four times in PBS and each well was paraffin sealed, cut out, and counted in a Beckman γ 4000 counter. Results were expressed as the mean cpm per well (of triplicate) + SD.

Immunohistochemical Staining. Cryostat sections (4-μm thick) of frozen normal and malignant tissue were air dried and acetone fixed for 5 min at 4°C on precoat gelatinized (2%) glass slides. Sections were overlaid overnight at 4°C with an appropriate dilution of monoclonal antibodies specific for a monomorphic class I MHC determinant (M1/42) or an unrelated polymorphic class I MHC determinant (H-2.4 (34-2-12S)). Positive controls included sections of normal CBA and BALB/c lymph node. For detection of rat monoclonal antibody, sections were incubated for 60 min with biotin-labeled mouse anti-rat IgG antibody (Jackson Immunoresearch, PA), followed by a 30-min incubation with fluorescein isothiocyanate labeled avidin (Biocan, Mississauga, Ontario). For detection of mouse antibody, fluorescein isothio-
cyanate labeled goat anti-mouse IgG (Fc-specific) was used. After each step the sections were washed three times in PBS. After the final wash, sections were air dried, mounted in Fluormount (Sigma Chemical Company, St. Louis, MO), and viewed with a Leitz Dialux microscope with epillumination. Photography was performed using Kodak Ektachrome P800/1600 film (push processed American Standard Association 3200); constant exposure times (30–60 s) for each group were used.

RESULTS

Derivation and Properties of Immunogenic Clones. In order to independently derive a wide variety of immunogenic clones, we treated 10.1, a subclone of a spontaneous murine mammary adenocarcinoma (CBA.SP1), with the DNA alkylating agent MNNG or the DNA hypomethylating agent and "gene activator" 5-aza-dCyd (18). The frequency of immunogenic variant clones (i.e., exhibiting attenuated growth in normal syngeneic mice compared to immunodeficient nude mice) before and after drug treatment was determined by isolating clones, clonal expansion, and in vivo challenge experiments. The results in Fig. 1 summarize our experience to date with the SP1 tumor. All clones from the untreated line were tumorigenic in syngeneic mice compared to athymic nu/nu mice (Table 2). All (seven of seven) immunogenic variants derived following 5-aza-dCyd treatment showed significantly elevated levels of class I MHC expression compared to the parent line 10.1 (See Table 3 and Fig. 3). Weak but detectable binding of anti-Kk/Dk monoclonal antibodies to the parent line 10.1 was observed, confirming our previous demonstration by quantitative absorption that trace levels of class I antigens are expressed (e.g., A.22 (Fig. 1), A.2.5, A.2.29, and M10.1.15 (Table 2)). Analysis of in vivo growth properties of the 16 variants generated following one or two cycles of 5-aza-dCyd treatment revealed seven clones which were consistently immunogenic (designated +, i.e., exhibited reduced frequency of tumor takes), and one clone (A.2.29) which was mildly immunogenic (designated ±, i.e., exhibited growth, but with reduced rate). Clones A.2.38 and A.2.43 though initially immunogenic became non-immunogenic in subsequent testing (designated −, i.e., 100% tumor takes with no reduced rate). In contrast, all MNNG induced immunogenic clones examined consistently exhibited strongly immunogenic properties (i.e., no growth, or attenuated growth in normal mice and progressive growth in athymic nude mice).

It should be noted that some immunogenic variants grew more slowly than the parent tumor in nude mice, [e.g., M10.1.21 (Table 2) or exhibited reduced clonogenic frequencies in vitro [e.g., A.2.29 and M10.1.21 (Table 3)]. Thus, non-immunological factors may also be important in determining the attenuated growth properties of certain variant clones.

Analysis of Class I and Class II MHC Levels on Immunogenic and Nonimmunogenic Variants in Vitro. We have previously reported that elevated expression of class I MHC (D^4) antigens was frequently associated with immunogenic variants (three of four) derived from the SP1 tumor (16), an observation consistent with the idea that enhancement of MHC expression might influence the T-cell dependent rejection process. In order to establish the generality of this phenomenon we analyzed by FACS and RIA the levels of class I MHC expression on the 5-aza-dCyd and MNNG-induced variants described above (compared to the parent line 10.1). The results of the FACS analysis are summarized in Table 2. Consistent with our previous results, all (seven of seven) immunogenic variants derived following 5-aza-dCyd treatment showed significantly elevated levels of class I K^a and/or D^4 antigen expression. However, some (three of nine) nonimmunogenic variants also showed enhanced class I MHC expression, implying that not all high MHC expressing clones were immunogenic. In contrast to 5-aza-dCyd induced variants, no all (i.e., three of six) immunogenic variants generated following MNNG treatment showed enhanced class I MHC levels (see Tables 2 and 3). Class I and II MHC levels on four 5-aza-dCyd and four MNNG-induced variants were further quantitated by RIA and compared to the parent tumor 10.1 (See Table 3 and Fig. 3). Weak but detectable binding of anti-K^a/D^4 monoclonal antibodies to the parent line (10.1) was observed, confirming our previous demonstration by quantitative absorption that trace levels of class I antigens are
The nonimmunogenic variant A.22.43 showed no significant change in class I MHC level. Of four MNNG-induced immunogenic variants examined, two (M10.1.2 and M10.1.21) exhibited 3- to 5-fold enhanced expression of D^a antigen, one (M10.1.21) of which also showed an increased level of K^k antigen. No class II (A^k or E^k) antigens were detected on the parent line or any of the variants. Thus all 5-aza-dCyd induced, but only some (approximately 50%) MNNG-induced, immunogenic variants exhibited increased class I MHC antigen levels compared to the untreated parent line 10.1.

Class I MHC Antigen Expression on Immunogenic Clones in Vivo. Since increased expression of class I MHC in vivo has been shown to correlate with the rejection of low MHC expressing tumors in allogeneic resistant hosts (29, 30), we examined the level of MHC antigens on immunogenic variants following in vivo passage in normal syngeneic and athymic nude mice. Immunogenic variants which formed solid tumors in vivo were resected at various times during the attenuated growth phase and cells were analyzed by indirect immunofluorescence FACS analysis of immunogenic variant A.22 following in vivo passage in normal syngeneic mice revealed uptake to 50-fold increased levels of class I D^a antigens. One of these variants (A.22.29) also showed a significant (11-fold) increase in K^k antigen expression.

Fig. 2. Growth characteristics of immunogenic variant A.22 in nude and normal syngeneic mice. Immunogenic variant A.22 (isolated following a single treatment of the 10.1 parent tumor with 5-aza-dCyd) was injected s.c. at a cell dose of 10^7 (A) or 10^8 (B) cells per mouse into nude (•) or normal syngeneic (A) recipients. The average tumor size (mm^2) is plotted versus the number of days following injection. The growth of 10^7 10.1 tumor cells (O) in normal mice is shown in A. The proportion of mice with tumor takes is shown for each group.

Class I MHC AND IMMUNOGENICITY OF A MURINE CARCINOMA

Table 2 Tumor growth properties and class I MHC expression of immunogenic and nonimmunogenic variants isolated from the SP1 carcinoma cell line after in vitro treatment with 5-aza-dCyd and MNNG

<table>
<thead>
<tr>
<th>Tumor clone</th>
<th>Precloning treatment</th>
<th>Growth in CBA mice</th>
<th>Day of 10-mm tumor diameter</th>
<th>Growth in nu/nu mice</th>
<th>Relative class I MHC level in vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exp. 1</td>
<td>Exp. 2</td>
<td>Exp. 1</td>
<td>Exp. 2</td>
<td>MHC status</td>
</tr>
<tr>
<td>10.1 (sub- clones)</td>
<td>None</td>
<td>5/5</td>
<td>5/5</td>
<td>14.3 ± 6.1</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>A1</td>
<td>5-aza-dCyd</td>
<td>1/1</td>
<td>4/4</td>
<td>9.0 ± 6.8</td>
<td>1/2</td>
</tr>
<tr>
<td>A2</td>
<td>(one cycle)</td>
<td>1/1</td>
<td>4/4</td>
<td>11.5 ± 5.2</td>
<td>2/2</td>
</tr>
<tr>
<td>A6</td>
<td>(one cycle)</td>
<td>1/1</td>
<td>4/4</td>
<td>10.2 ± 3.2</td>
<td>2/2</td>
</tr>
<tr>
<td>A21</td>
<td>(one cycle)</td>
<td>1/1</td>
<td>4/4</td>
<td>19.8 ± 9.0</td>
<td>2/2</td>
</tr>
<tr>
<td>A22</td>
<td>(one cycle)</td>
<td>1/4</td>
<td>3/4</td>
<td>17.6 ± 1.7</td>
<td>2/2</td>
</tr>
<tr>
<td>A24</td>
<td>(one cycle)</td>
<td>1/4</td>
<td>2/4</td>
<td>18.8 ± 3.8</td>
<td>2/2</td>
</tr>
<tr>
<td>A2.5</td>
<td>5-aza-dCyd</td>
<td>0/1</td>
<td>1/4</td>
<td>24.7 ± 3.8</td>
<td>2/2</td>
</tr>
<tr>
<td>A2.15</td>
<td>(two cycles)</td>
<td>1/5</td>
<td>3/4</td>
<td>NT</td>
<td>6/6</td>
</tr>
<tr>
<td>A2.20</td>
<td>(two cycles)</td>
<td>1/1</td>
<td>0/4</td>
<td>1.4 ± 1.9</td>
<td>2/2</td>
</tr>
<tr>
<td>A2.29</td>
<td>(two cycles)</td>
<td>3/5</td>
<td>8/11</td>
<td>26.7 ± 3.8</td>
<td>2/2</td>
</tr>
<tr>
<td>A2.38</td>
<td>(two cycles)</td>
<td>6/9</td>
<td>5/5</td>
<td>15.7 ± 4.8</td>
<td>2/2</td>
</tr>
<tr>
<td>A2.43</td>
<td>(two cycles)</td>
<td>6/9</td>
<td>4/4</td>
<td>NT</td>
<td>4/4</td>
</tr>
<tr>
<td>A2.21</td>
<td>(two cycles)</td>
<td>1/0</td>
<td>0/4</td>
<td>6.7 ± 1.6</td>
<td>2/2</td>
</tr>
<tr>
<td>A2.22</td>
<td>(two cycles)</td>
<td>1/1</td>
<td>6/6</td>
<td>20.3 ± 2.2</td>
<td>2/2</td>
</tr>
<tr>
<td>A2.23</td>
<td>(one cycle)</td>
<td>1/1</td>
<td>4/4</td>
<td>17.7 ± 2.6</td>
<td>2/2</td>
</tr>
<tr>
<td>A2.24</td>
<td>(one cycle)</td>
<td>1/1</td>
<td>4/4</td>
<td>13.7 ± 3.3</td>
<td>2/2</td>
</tr>
</tbody>
</table>

a Clones were derived following 5-aza-dCyd (4 μM) treatment or MNNG (3 μg/ml) treatment as indicated.

b Experiments are listed in sequence of testing variant clones from continuous cultures. All clones were injected at 10^5 cells per mouse, except MNNG induced clones which were injected at 10^6 cells per mouse. Representative data for one of six subclones of the parent tumor 10.1 are shown.

c Calculated using linear regression analysis of tumor growth in individual mice (28). Values, mean ± SD.

d All variants grew progressively in nude mice (mean number of days to reach 10-mm tumor diameter, 17.9 ± 5.6); except M10.1.21, which exhibited slow growth in nude mice (data not shown).

e Values, Δ mean fluorescence intensity (MFI experimental MoAb - MFI irrelevant MoAb). The mean and standard deviation of six subclones of the parent line 10.1 is shown.

f Immunogenicity is assessed by attenuated growth in normal compared to nude mice. +, reduced tumor takes; ±, tumor takes but with slower growth rate; -, 100% tumor takes with no attenuation of growth.

g Tumors grew more slowly in normal compared to nude mice.

h Positive MFI values. Significance level is arbitrarily defined as four SD units above the mean as follows: K^k = 2.1; D^a = 1.6.

i Significant increase in number of days to reach 10-mm tumor diameter (P ≤ 0.05 in a Mann-Whitney U test (nonparametric analysis).

j NT, not tested.

k See RIA data in Table 3.
Table 3: Relative level of surface class I and class II MHC expression on immunogenic variant clones of the CBA.SPI carcinoma

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Clone</th>
<th>Treatment*</th>
<th>Immuno-</th>
<th>Relative MHC level in vitro*</th>
<th>Percentage of cloning efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10.1</td>
<td>-</td>
<td>-</td>
<td>7.9 ± 2.1 (1)</td>
<td>1.8 ± 0.31</td>
</tr>
<tr>
<td></td>
<td>A.2.15</td>
<td>5-aza-dCyd</td>
<td>+</td>
<td>22.7 (3)</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>A.2.29</td>
<td>(two cycles)</td>
<td>+/-</td>
<td>84.3* (11)</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>A.2.38</td>
<td>(two cycles)</td>
<td>-</td>
<td>18.4 (2)</td>
<td>2.1*</td>
</tr>
<tr>
<td></td>
<td>A.2.43</td>
<td>(two cycles)</td>
<td>-</td>
<td>11.0 (1)</td>
<td>2.9*</td>
</tr>
<tr>
<td>M10.1.2</td>
<td>MNNG</td>
<td>+</td>
<td>15.5 (2)</td>
<td>0.7</td>
<td>1.6</td>
</tr>
<tr>
<td>M10.1.15</td>
<td>MNNG</td>
<td>+</td>
<td>3.1 (-1)</td>
<td>-2.4*</td>
<td>26.2</td>
</tr>
<tr>
<td>M10.1.12</td>
<td>MNNG</td>
<td>+</td>
<td>42.4* (5)</td>
<td>3.8</td>
<td>82.1*</td>
</tr>
<tr>
<td>M10.1.33</td>
<td>MNNG</td>
<td>+</td>
<td>11.0 (1)</td>
<td>-0.4*</td>
<td>20.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Clone</th>
<th>Treatment*</th>
<th>Immuno-</th>
<th>Relative MHC level in vitro*</th>
<th>Percentage of cloning efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>10.1</td>
<td>-</td>
<td>-</td>
<td>3.4 ± 0.7 (1)</td>
<td>0.1 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>EL-4</td>
<td>-</td>
<td>-</td>
<td>0.4 ± 0.1</td>
<td>-1.2 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>A.22</td>
<td>5-aza-dCyd passed</td>
<td>+</td>
<td>10.6 (3)</td>
<td>0.4</td>
</tr>
<tr>
<td>A.2.29</td>
<td>(in vivo)</td>
<td>+/-</td>
<td>86.4* (25)</td>
<td>0.1</td>
<td>3.0</td>
</tr>
<tr>
<td>A.22E</td>
<td>spleen</td>
<td>NONE</td>
<td>NA</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>A.22E</td>
<td>collagenase digested</td>
<td>+</td>
<td>252*</td>
<td>-1.6*</td>
<td>3.7*</td>
</tr>
</tbody>
</table>

* See Table 2 for clone designations and details of treatments.

Table 2 footnote f.

* Data correspond to RIA experiments in Figs. 3 and 5. Values, relative cpm per 5 x 10^5 cells (minus background with unrelated antibody 34-2-12S) compared to CBA spleen (normalized to 100%). Numbers in parentheses, relative class I MHC levels compared to the parent line 10.1.

* Values are > 3 times the corresponding values for 10.1 in each experiment. Antibody hybridoma clones used: Kk (clone 11-4-1); Aa (116/32R5); E (13/18); D (15-5-5S).

* A mixture of anti-I-A^a and anti-I-E^d antibodies was used.

* NT, not tested; NA, not applicable.

* A.22E is a subline isolated from A.22 following transplantation into a normal CBA mouse. The tumor was resected after 50 days and adapted to tissue culture.

* A.22E cells were passaged in (CBA x B6)F1 recipients, resected 14 days later, and dispersed by collagenase treatment. Tumor cells were separated from host cells by discontinuous Percoll gradient centrifugation (31).

* Values represent mean fluorescence intensity calculated as in Table 2. K^a/D^a, clone 20-8-4S; A^a, clone B21-2.

---

### Figure 3

Expression of class II MHC antigens on variant clones following MNNG or 5-aza-dCyd (5-aza-CR) treatment. The parent tumor and derivative variant clones were assessed for relative expression of class I (Kk, Dk) and class II (V, I-A) antigens using a radioimmunoassay. Monoclonal antibodies used were: 11-4.1 (Kk), 15-5-5S (Dk), 116/32R5 (Aa), and 13/18 (Ea). An unrelated antibody, 31-3-4S (Kk), was used as a negative control. Cell lines tested were MNNG induced immunogenic variants M10.1.2.1 (X), M10.1.33 (A), M10.1.15 (D), and M10.1.2 (O), 5-aza-dCyd induced immunogenic variants A.2.15 (*), A.2.29 (•) and nonimmunogenic variants A.2.13 (O) and A.2.43 (O). Controls were the untreated parent tumor line 10.1 (O) and normal CBA spleen cells (O). RIA analysis was performed as described in the text and [125I]IgG bound (cpm) per 5 x 10^6 cells (mean of three replicates) was plotted versus reciprocal of antibody titer. Bars, SD values.

### Figure 4

Class I MHC antigen expression on immunogenic variant (A.22) and parent tumors after in vivo passage. Immunogenic variant A.22 or the parent tumor (10.1) was injected into normal syngeneic mice (A) or BALB/c nude mice (B). Regressive tumors (in normal mice) or progressive tumors (in nude mice) were resected at various times, adapted to tissue culture, and assessed for class I MHC expression by IIF using the class I MHC specific monoclonal antibody M/142. A control consisted of an unrelated antibody (K^a/D^a, 20-8-4S). A mean fluorescence intensity (see Table 2) is plotted versus number of days of in vivo growth.
contrast, the level of MHC expressed on the same variant during progressive growth in athymic nude mice was variable and generally decreased with time, consistent with in vitro passage of A.22 cells as reported previously (16). No class II MHC antigens were detected by RIA on either tumor variants or the parent tumor following explantation (Fig. 5).

The second approach involved immunohistochemical staining of frozen sections of solid tumors following s.c. injection of immunogenic or nonimmunogenic variants into CBA or (CBA × B6)F1 mice. Sections were stained with either M1/42 or an antibody of irrelevant specificitiy (anti-Dd, clone 34-2-12S). The parent tumor line (10.1) exhibited no significant staining with class I specific monoclonal antibody M1/42 (Fig. 6C), although significant staining was observed during rejection of 10.1 in allogeneic C3H mice (Table 4). In contrast, the EMS induced immunogenic variant 800.11 exhibited a uniformly strong class I specific membrane staining pattern during rejection in syngeneic mice (Fig. 6C). As expected, no staining of the irrelevant anti-Dd antibody was observed (Fig. 6D), indicating that non-specific binding had not occurred. A similar pattern of in situ staining occurred with all immunogenic variants examined (Table 4), including those variants [e.g., M10.1.15 (Table 3) and 800.11 (16)] which showed no alteration in MHC expression in vitro compared to the parent tumor. No significant augmentation of class I MHC expression was observed during progressive growth in nude mice (Fig. 6E and Table 4). Very slight class I MHC specific staining of some cells (5–10%) of non-immunogenic variants (A.20, A.2.38, and 800.7) was observed during growth in immunocompetent recipients. Thus, enhancement of class I MHC expression in situ correlated with immune rejection in normal syngeneic mice for all variants examined.

Infiltration of Class II Bearing Host Cells during Rejection of Immunogenic Variants. Although class II MHC antigens were not detected on the parent tumor line or any immunogenic variants in vitro, there is the possibility that class II molecules were expressed transiently in situ during tumor rejection in response to la-inducing factors as proposed by Callahan (32) and Walker et al. (33). Staining of tissue sections with class II specific MoAbs showed a high frequency of class II (Aa and Eb) positive cells at the site of rejection of immunogenic variants (Fig. 6F, Table 4). In (CBA × B6)F1 recipients, I-A molecules of both parental haplotypes were present, implicating a significant infiltration of host I-Ak/b bearing cells. To distinguish unequivocally between host and tumor derived class II molecules, 10.1 tumor cells (Ak genotype) were injected in B6 (Ab genotype) mice. Staining with Aa versus Ab specific MoAbs revealed that class II molecules in the tumor site were of host (Aa) and not of tumor (Ab) origin (Fig. 6, G and H). No I-Aa bearing host cell infiltrate was detected during growth of non-immunogenic variants, or during growth of immunogenic var-

Table 4. Comparison of class I and class II MHC expression in situ on immunogenic and nonimmunogenic variants of SP1

<table>
<thead>
<tr>
<th>Clone</th>
<th>Pretreatment</th>
<th>Immunogenicity</th>
<th>Recipient</th>
<th>Class I (K + D)</th>
<th>Class II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Aa</td>
<td>Ea</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.1</td>
<td>MNNG</td>
<td>–</td>
<td>CBA</td>
<td>++</td>
<td>–</td>
</tr>
<tr>
<td>10.1</td>
<td>MNNG</td>
<td>+</td>
<td>CBA</td>
<td>++</td>
<td>–</td>
</tr>
<tr>
<td>10.1</td>
<td>A.20, A.2.29</td>
<td>A.2.38, A.2.15</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* See Table 2 for clone designation and immunogenicity.
* MHC levels were assessed on frozen sections by immunohistochemical staining using monoclonal antibodies specific for a monomorphic determinant on class I (K/D) molecules (clone M1/42); Aa (clone 116/32R5), Ea (clone 13/18), and Aa (clone B21-2) class II molecules. No staining with an unrelated Dd specific antibody (34-2-12S) was observed (data not shown). + +, all cells brightly stained; – (+), overall negative with a few positive cells; –, all cells negative.
* A palpable tumor mass appeared transiently 8 to 12 days post injection and was resected.

4920
Fig. 6. Immunohistochemical staining of class I MHC antigens on the parent tumor 10.1 and immunogenic variant 800.11. Paraffin embedded sections of the parent tumor 10.1 (A) and immunogenic variant 800.11 (B) were stained with hematoxylin phloxine and saffron. Immunohistochemical staining of frozen sections was carried out as follows: C, the parent tumor 10.1 was stained with the class I specific antibody M1/42 following transplantation into a CBA mouse (positive areas most likely represent capillary endothelium); D, immunogenic variant 800.11 during rejection in a CBA mouse was stained with antibody M1/42; E, immunogenic variant 800.11 was stained with antibody M1/42 following transplantation into a nude mouse; F, tissue in D was stained with an unrelated antibody 31-2-12S (D11); G, parent tumor 10.1 (H-2b) was injected into B6 (H-2^b) mice, and stained with anti-A^b antibody (116/32R5); H, tissue in G was stained with anti-A^b antibody (B21-2). All tumors were resected 15 days postinjection. Concentrations of first antibodies were as follows: B21-2, M1/42, 1/500; 116/32R5, 31-2-12S, 1/50. Magnification: A and B, x 352; C-H, x 432.

variants in nude mice (Table 4). Thus, infiltration of host I-A^b bearing cells correlated with immune rejection of immunogenic variants.

DISCUSSION

In this report we continue to examine the generality of the contention previously raised by us (16) and others (1) that the level of class I MHC antigen expression can affect the immunogenicity and in vivo growth properties of certain tumors. The spontaneous murine mammary adenocarcinoma CBA.SP1, which arose in our own mouse colony was well suited for this study in that: (a) artifacts, due to long term culture conditions (e.g., 10 years) and genetic drift of inbred mouse strains are minimized (34) and (b) that SP1 is poorly immunogenic and expresses very low amounts of cell surface MHC (16), representative of a significant proportion of human carcinomas.

The results reported here, together with our earlier study (16), support a positive correlation between cell surface class I MHC expression and immunogenicity of SP1; at least in the analysis of 5-aza-dCyd induced variants. First, all 5-aza-dCyd induced immunogenic variants (nine of nine) tested in both studies exhibited elevated K^k and/or D^k MHC antigens. Second, most (seven out of 10) nonimmunogenic variants studied so far were found to be low MHC expressors. Third, we have previously shown that some immunogenic variants reverted back to the nonimmunogenic low MHC expressing parental phenotype after 6 to 8 weeks in culture (16). Low MHC expressing, nonimmunogenic, revertant subclones of immunogenic variant A.22 have also been isolated following in vivo passage. Despite the positive relationship between class I MHC expression and immunogenicity of 5-aza-dCyd induced SP1 variants, a significant proportion (30%) of nonimmunogenic variants were high MHC expressors.

In an independent study it was found that high class I MHC
expressing variants directly selected by cell sorting from 5-aza-dCyd treated SP1 cells exhibited virtually no attenuation of growth in normal mice compared to the parent line.4 Taken together, these results are consistent with the view that class I MHC may be a significant, but not the only, determinant of immunogenicity leading to rejection of SP1 variants.

Contrary to 5-aza-dCyd induced variants, a significant proportion (approximately 50%) of immunogenic clones generated following treatment with DNA alkylating agents (i.e., EMS or MNNG) exhibited no change in class I antigen expression. These results indicate that changes in non-MHC determinants can also contribute to the immunogenic properties of at least some variants. If tumor cells directly stimulate host T-cells, one would predict that immune responsiveness is dependent on both antigen and MHC expression as defined in T-cell recognition systems (35). Thus the relative contribution of MHC versus non-MHC determinants to the immunogenic phenotype would be different for each variant and dependent on the nature of the mutagenizing agent and the tumor cell type. For example, Boon et al. (15) using several tumor models [e.g., teratocarcinoma, a mastocytoma (P815), and two spontaneously arising leukemias] have shown that “unique” clone specific antigens are expressed on immunogenic variants isolated following treatment with MNNG, under conditions in which no detectable change in MHC expression is observed. Frost et al. (17) have obtained similar findings with an undifferentiated tumor, MDAY-D2, and a spontaneous adenocarcinoma, TA3 (36). In these systems, immunogenic variants were able to induce CTL response specific for unique tumor associated antigens on the variant clones as well as CTL which recognize common antigenic determinant(s) shared between the variant and parent lines. Functional studies are currently in progress to determine whether unique or common antigens are present on 5-aza-dCyd versus MNNG induced immunogenic variants generated in the SP1 tumor system.

Although some EMS or MNNG induced immunogenic variants retained the low MHC expressing parental phenotype in vitro, all immunogenic variants exhibited a marked augmentation of class I MHC when examined in situ, during rejection in normal syngeneic recipients. A similar augmentation of class I MHC on the parent tumor 10.1 during rejection in C3H mice was also detected. No increase in class I MHC expression occurred during progressive growth in T-cell deficient nude mice, implying that the observed increased in MHC antigens is dependent on infiltrating host T-cells which secrete interleukins or interferon at the site of rejection in normal mice (37). In an independent study, we have shown that the enhanced MHC levels on variants isolated from SP1 are immunologically relevant, in that high class I MHC expressing variants were much more effective than the parent line in stimulating an allogeneic H-2 specific CTL response in vitro.7 Class I restricted T-cells can mediate strong delayed type hypersensitivity and CTL reactions against viral or chemically induced immunogenic tumors (38-40). Thus, the observed increase in class I MHC antigen expression on immunogenic variants in situ could be an important step in the maintenance of syngeneic T-cell responses against the tumor, and in rendering tumor cell susceptible to host effector T-cells (1).

In contrast to the observed augmentation of class I MHC expression, no tumor derived class II molecules were detected on any of the variants in vitro or in situ. Thus induction of class II molecules by Ia-inducing factors, as shown to occur during allograft and graft versus host reactions (37, 41, 42) and immune rejection of certain carcinomas and sarcomas (32), did not occur during rejection of SP1 variants. Nevertheless, a strong infiltration of host I-A bearing cells correlated with rejection of immunogenic variants in normal mice. The histological identity and origin of the host I-A bearing cell infiltrate is currently being determined. These results raise the possibility that activation of class II restricted T-cells by tumor associated antigens presented by host I-A bearing cells, is an important step in the immune rejection of SP1 derived immunogenic variants as demonstrated in allograft reactions (42) and in the presentation of soluble antigens to class II restricted T-cells (43). Experiments are now in progress to determine, using MHC transfection strategies, the relative role of class I and class II self-MHC molecules in the immune rejection of SP1, and to assess the genetic/epigenetic factors involved in regulating the observed patterns of class I and class II MHC expression.

ACKNOWLEDGMENTS

The authors wish to thank Dr. R. S. Kerbel and Dr. Bill Mackillop for critical discussion of the data, and to acknowledge W. Longhurst for his competent expertise in FACS analysis, Dr. Andrew Wade for carrying out statistical analyses, and Beverley Fluhrer and Sandra Tirelli for secretarial assistance.

REFERENCES


In Situ Augmentation of Class I Major Histocompatibility Antigen Expression on Immunogenic Variants of a Spontaneous Murine Mammary Carcinoma


Updated version  Access the most recent version of this article at:  http://cancerres.aacrjournals.org/content/47/18/4915

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.