ABSTRACT

Four wheat germ agglutinin-resistant (WGA\textsuperscript{r}) variants of the highly malignant murine tumor MDAY-D2 were examined for membrane alterations which might correlate with their decrease in tumorigenicity described previously. Injection s.c. of as many as $5 \times 10^6$ cells of the WGA\textsuperscript{r} variants MDW1 or MDW3 was rejected by the normal syngeneic DBA/2 host, but they grew rapidly and progressively in athymic nude mice, suggesting that a vigorous T-cell-mediated immune response was responsible for rejection of the variants in the immunocompetent host. In contrast, an inoculum of as few as $10^2$ MDW4, MDW5, or MDAY-D2 cells gave rise to progressively growing metastatic tumors in normal DBA/2 mice. Hence, the MDW1 and MDW3 variants appeared more immunogenic than did the MDW4, MDW5, or MDAY-D2 parent tumor.

Two lines of evidence suggested that all of the WGA\textsuperscript{r} variants and parental tumor in fact shared a common tumor-associated antigen; (a) rejection of MDW1 tumor cells by DBA/2 mice immunized them against a subsequent challenge of either MDAY-D2 or MDW4 cells; (b) antitumor cytotoxic T-lymphocyte (CTL) activity was detected in a 4-hr \textsuperscript{51}Cr release assay in spleens taken from tumor-bearing mice and restimulated \emph{in vitro} with mitomycin C-treated tumor cells. MDAY-D2 and WGA\textsuperscript{r} variants were cross-reactive, both as immunogens at the restimulation stage and as CTL targets. Compared to the tumorigenic lines, MDW1 and MDW3 were more effective stimulators of tumor-specific CTL and were superior targets in the CTL assay. Similarly, stimulation of syngeneic DBA/2 spleen cells with trinitrophenol-modified tumor cells showed MDW1 and MDW3 were more effective stimulators of the trinitrophenyl-specific response and were more readily lysed by trinitrophenyl-directed CTL than were the other WGA\textsuperscript{r} variants or MDAY-D2.

Separation of microsomal membrane proteins on sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed an elevated level of two glycoproteins with apparent molecular weights of 43,000 and 47,000, present in the microsomes of the poorly tumorigenic variants MDW1 and MDW3. The glycoproteins were identified as H-2D and H-2K, respectively. Absorption of alloantiserum by whole tumor cells indicated that the WGA\textsuperscript{r} variants and MDAY-D2 had similar amounts of H-2 on their cell surface. In contrast, absorption tests, using tumor cell endoplasmic reticulum and plasma membrane fractions, as well as analysis of the membrane fractions by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, showed an increased level of H-2 in the endoplasmic reticulum of the poorly tumorigenic MDW1 and MDW3 variants.

The results demonstrate an inverse correlation between the tumorigenicity and both the immunogenicity and cellular H-2 levels of the variant tumor cell lines.

INTRODUCTION

The cells of most chemically or virally induced tumors have been found to bear TATA\textsuperscript{r} which are capable of evoking an immunologically mediated rejection-type response in the syngeneic host (12, 13). The intrinsic immunogenicity of tumor cells and, in turn, the intensity of the cell-mediated immune response, produced by the host, appear to depend on the nature of the TATA itself [e.g., virally induced tumors contain viral protein antigens which are often more immunogenic than the antigens associated with chemically induced or spontaneously arising tumors (13)] as well as the physical relation of the antigens to other normal cellular structures, such as the murine major histocompatibility complex gene produces H-2D and H-2K in both the immunogenicity of the murine tumor cells and their susceptibility to CTL-mediated lysis (12, 35).

It has been shown that CTL and target cells, bearing the sensitizing antigen, must share the same serologically detectable H-2 gene products in order to give maximal cytotoxicity (8, 31). By coating target and effector cells with anti-H-2 antibodies, it has been found that the presence of antibody-free syngeneic H-2 antigens on the target cells was necessary for lysis, whereas H-2 antigens on the effector cells were not involved (11, 30). In particular, the H-2D antigen on the tumor target appeared to be necessary for CTL-mediated lysis (30).

In addition to the apparent contribution of H-2 gene products in CTL tumor cell interactions, these antigens also influence the immunogenic potential of tumor cells injected into animals. Virally induced tumors of different genetic backgrounds, but expressing the same viral TATA, have been shown to evoke immune responses in the syngeneic host of varying intensities, a difference which appears to be H-2K or H-2D dependent (12, 28). Both levels of H-2D and/or H-2K and their relation to the tumor antigen appear to affect the intensity of the cell-mediated immune response of the host against the tumor. For example, variants of the YAC lymphoma, selected for a reduction in cell

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surface H-2, have been shown to be less immunogenic by immunization-challenge experiments and in their ability to elicit a CTL response in the syngeneic host (4). Similarly, mouse strain variation in resistance to radiation-leukemia virus-induced leukemia has been found to be associated with increased H-2 expression on host thymocytes; the onset of leukemia was associated with mouse strains showing low thymocyte H-2 expression (21). Resistance was shown to be associated with the ability of virus-infected high-H-2-expressing thymocytes to elicit a strong CTL response in contrast to virus-infected low-H-2-expressing cells, which induced a poor CTL response (22). The thymomas produced by radiation-leukemia virus were low-H-2-expressing cells, and, as in the case of the low-level H-2 virus-infected thymocytes, were suggested to be poorly immunogenic (22). In contrast, tumor cell variants have been found (e.g., myelomas) which express unaltered amounts of cell surface H-2 but which nonetheless have a reduced ability to elicit an allogeneic or syngeneic CTL response; the defect appeared to be related to an altered orientation of H-2 in the tumor cell membrane (27).

In the present study, 4 WGAl variants of the highly malignant tumor MDAY-D2 were examined for changes in membrane glycoprotein content which may correlate with their tumorigenicity in the syngeneic DBA/2 host. Inoculations of the poorly tumorigenic variants MDW1 or MDW3 s.c. were rejected from the syngeneic DBA/2 host after a week of transitory growth, while the same tumors grew progressively in immunodeficient nude mice (5, 14), suggesting a cell-mediated immune response involving T-cells was involved in host rejection of the tumors. Previous studies in our laboratory have shown that MDW1 and MDW3 elicited a much stronger antitumor CTL response in DBA/2 mice than did the tumorigenic variants MDW4, MDW5, and the parental MDAY-D2 tumor (5).

In addition, MDW1 and MDW3 showed a reduced rate of cell surface shedding and were poorly agglutinated by WGA compared to the tumorigenic lines. The results reported in this paper indicate that the contrasting immunogenic and tumorigenic properties of the variants also correlated with the levels of cellular H-2 glycoproteins.

**MATERIALS AND METHODS**

**Mice.** All DBA/2 mice used in the present study were purchased from The Jackson Laboratory, Bar Harbor, Maine. The mice were used at 8 to 12 weeks of age.

**Tumor Cells.** The origin of the highly malignant DBA/2 tumor MDAY-D2 and the selection of the WGA variants of MDAY-D2 have been described in detail elsewhere (14-16). The cloned WGA variants MDW1, MDW3, and MDW4 have been shown to be resistant to 10, 10, and 50 µg of WGA per ml, respectively (14). MDW5 is an uncloned population of WGA variants resistant to 100 µg of WGA per ml. In contrast, the parental tumor MDAY-D2 showed the same level of resistance to 1 µg WGA per ml. Esb is a metastatic variant of the DBA/2 strain L5178Y (Ebu) lymphoma (29), and EL4 is a C57BL lymphoma.

**H-2 Antiserum and Absorption Tests.** The congeneric anti-H-2b antiserum was made by repeated immunization of B10.Br mice with B10.D2 spleen cells in our laboratory, and the anti-D2 serum (B10.AKM × A.SW)anti-ATTH) was obtained from the NIH with the help of Dr. John G. Ray. The expression of H-2 on the tumor cells was determined by measuring the ability of the cells to absorb the anti-H-2 antibody. The antiserum was used at the highest dilution at which 95% cytotoxicity (in the presence of rabbit complement) could be obtained. Tumor cells were incubated with 0.1 ml of the diluted antiserum at 37°C for 30 min and pelleted, and aliquots of the absorbed serum were tested in a standard rabbit complement cytotoxicity assay (14). Sonicated plasma membrane and endoplasmic reticulum preparations were also used in place of whole tumor cells for antisera absorption tests.

**CTL Assay.** The assay was performed as described by Schirrmacher et al. (29). DBA/2 mice received s.c. injections of either MDW1 or MDW3 (107 cells/mouse) or MDW4, MDW5, or MDAY-D2 (106 cells/mouse), which gave rise to 0.5- to 1-cm tumors in 2 weeks. If tumor-bearing animals were left for longer time periods, MDW1 and MDW3 regressed completely, while MDW4, MDW5, and MDAY-D2 grew and killed the hosts in 4 to 6 weeks. The spleens from animals that had received injections 2 weeks previously were removed, and the washed spleen cells were cocultured with mitomycin C-treated tumor cells in Roswell Park Memorial Institute Tissue Culture Medium 1640 containing 10% heat-inactivated fetal calf serum, 7 mM glutamine, 50 µM 2-mercaptoethanol, 50 units of penicillin per ml, and 50 µg of streptomycin per ml at a spleen cell:tumor cell ratio of 20:1 for 4 days. Tumor targets (2 × 105) were labeled with 100 µCi 51Cr (New England Nuclear, Lachine, Quebec, Canada) for 1 hr at 37°C, washed, and incubated with effector cells in microtiter plates for 4 hr at 37°C. Maximum 51Cr release was determined by adding 1% Nonidet P-40 to target cells, and the spontaneous release, by incubating target cells in medium alone. The percentage of 51Cr specifically released was calculated to be

\[
\frac{(O - S) \times 100}{M - S}
\]

where O is the observed experimental 51Cr release, S is the spontaneous release, and M is the maximum release. Spontaneous release was 10 to 20% of the maximal release.

**TNP-directed CTL was performed as described by Finberg et al. (10). Cells were TNP modified by adding an equal volume of 10 µM TNBS (Sigma Chemical Co., St. Louis, Mo.) in PBS to cells at 107/ml PBS for 10 min at 37°C followed by 3 washes to remove free TNBS. Stimulator TNP-modified cells were pretreated with mitomycin C and cocultured with DBA/2 spleen cells as described above. TNP-modified target cells were first 51Cr labeled, followed by TNBS treatment as described above.

**Preparation of Microsomes and Separation of Endoplasmic Reticulum and Plasma Membrane Functions.** Cells were disrupted by the nitrogen cavitation method described previously by Ferber et al. (9). (Cells (106) were washed in serum-free medium, taken up in 4 ml of 20 mM HEPES (pH 7.4):0.5 mM MgCl2:0.13 M NaCl, and stirred on a magnetic stirrer box while 4 ml of ice-cold 0.5 M sucrose were slowly added. After adding 100 µl Tranylol (Sigma), the cells were placed in an Artisan "bomb" (Artisan Metal Products, Waltham, Mass.) and equilibrated with 600 psi of N2 at 4°C for 10 min and with constant gentle stirring. Cell disruption occurred after dropwise release of the suspension from the bomb. The cell lysate was made 1 mM EDTA and centrifuged at 1,000 × g for 10 min, and the resulting supernatant again was centrifuged at 20,000 × g for 15 min to pellet cell organelles. The supernatant was spun at 120,000 × g, using a 40 rotor, in a Beckman ultracentrifuge for 60 min, to pellet the microsomal membranes.

**Endoplasmic reticulum and plasma membrane were separated on a one-step Ficoll gradient. The microsomal pellet was resuspended in 0.5 ml of 1 mM HEPES (pH 8.2):1 mM MgCl2, layered on top of 2.5 ml of Ficoll (1.09 g/ml), and dissolved in the same buffer. The tubes were centrifuged for 5 hr at 50,000 rpm in SW50.1 rotor. The top cm of the gradient was removed, and the resulting supernatant was loaded at 20,000 × g for 30 min in a Beckman ultracentrifuge for 60 min, to pellet the microsomal membranes.**
SDS-PAGE and 125I-labeled Con A Staining of Glycoproteins. Samples of membrane fractions were solubilized in sample buffer [10% glycerol:5% 2-mercaptoethanol:3% SDS:62.5 mM Tris-HCl (pH 6.8)] and a trace of bromophenol blue and boiled for 5 min. Aliquots of 40 μl containing 80 μg of protein, were applied to slab gels with a 5% polyacrylamide stacking and a 12.5% polyacrylamide resolving gel (20) and electrophoresed at 25 mA for 3 hr. Gels were then stained with Coomassie brilliant blue, destained, and photographed.

Glycoproteins were stained by incubating the protein-stained gels with 125I-labeled Con A (2). The gel was equilibrated in 0.02 M PBS, pH 6.8, followed by overnight incubation with 5 mg of 125I-labeled Con A in 100 μl of PBS. The gel was then washed exhaustively in PBS, dried, and exposed to X-ray film for 7 to 14 days. Con A was iodinated using chloroglycoluronil (Pierce Chemical Co., Rockford, IL) as described previously (21).

[35S]Methionine Labeling of Tumor Cells and Immunoprecipitation of H-2. Tumor cells were suspended in methionine-free Roswell Park Memorial Institute Tissue Culture Medium 1640 at 2 x 10^6 cells/ml, and 100 μCi of [35S]methionine (New England Nuclear) were added to 2 ml of cells for 4 hr. The cells were washed and solubilized in 0.5 ml of 0.5% Nonidet P-40:10 mM Tris-HCl (pH 7.5):0.15 M NaCl:2 mM EDTA:50 μl Tranylcol. Cell lysates containing 2 μCi were then incubated with 6 μl of nonimmune serum and 50 μl of a 1:1 slurry of protein A: Sepharose 4B [Pharmacia (Canada) Ltd., Dorval, Quebec, Canada] to remove nonspecifically bound material. The lysate was then incubated with 6 μl of immune serum for 2 hr on ice, and then 50 μl of 1:1 slurry of protein A-Sepharose 4B were added and left for 30 min. The beads were then washed 4 times in the lysate buffer and finally in H2O (7). The immune complexes were eluted from the beads in SDS sample buffer at 100° for 5 min, followed by application to SDS-PAGE.

RESULTS

Increased Expression of H-2 Glycoproteins in the WGA Variants. For comparison of the membrane glycoproteins of the tumor variants and the parent tumor, microsomes were isolated from cell lysates, and the protein was separated on SDS-PAGE. Proteins were stained with Coomassie brilliant blue (Fig. 1a), followed by staining with 125I-labeled Con A and autoradiography to identify the Con A binding (i.e., mannose-containing) glycoproteins (Fig. 1b). Two microsomal proteins with molecular weights of 43,000 and 47,000 were present in high levels in MDW1 and MDW3, moderate levels in MDW4 and MDW5, and very low levels in MDAY-D2, as can be seen in the gel stained with Coomassie brilliant blue (Fig. 1a).

The proteins with molecular weights of 43,000 and 47,000 were distinguished from actin, a species with a molecular weight of 44,000 which comigrated with rabbit skeletal muscle actin, and affinity-purified actin from the tumor cell microsomes (Fig. 1a). Unlike the proteins with molecular weights of 43,000 and 47,000, actin appeared to be present in similar amounts in the microsomes prepared for the WGA variants and MDAY-D2. Actin is not a glycoprotein, and, as shown in Fig. 1b, the protein species with a molecular weight of 44,000 was not stained with 125I-labeled Con A.

The species with molecular weights of 43,000 and 47,000 were identified as glycoproteins by their ability to bind 125I-labeled Con A (Fig. 1b). The glycoproteins from the 5 tumor lines showed a similar relative staining intensity with both the Coomassie brilliant blue and the 125I-labeled Con A (Fig. 1, a and b), suggesting that the species with molecular weights of 43,000 and 47,000 were glycosylated (i.e., contained core mannose residues), to a similar degree, in each of the WGA lines.

In common with the species with molecular weights of 43,000 and 47,000 observed on the SDS-PAGE (Fig. 1), H-2D and H-2K gene products have been found to be glycoproteins with molecular weights of 43,000 and 47,000, respectively (7). Furthermore, immunoprecipitation of H-2 gene products from [35S]methionine-labeled tumor cells and separation of the material on SDS-PAGE showed a band which migrated with a molecular weight of 43,000 and was present in much larger amounts in MDW1 cells, compared with MDAY-D2 cells (Fig. 2). The band (M.W. 43,000) was immunoprecipitated using both a congeneric B10. Br anti-B10.D2 serum, as well as anti-H-2Dd serum (NIH), identifying the glycoprotein as H-2D. Our efforts to immunoprecipitate H-2K were less successful, and therefore, the species with a molecular weight of 47,000 was only tentatively identified as H-2K. It is possible that H-2 glycoprotein and, in particular, H-2K are more labile proteins which are subject to proteases during the immunoprecipitation procedure, since a low-molecular-weight band was also specifically associated with the immunoprecipitate. A protein with a molecular weight of 69,000 appeared to be specifically immunoprecipitated by the anti-H-2Dd serum and was present in similar amounts in MDW1 and MDAY-D2. Samples of H-2 immunoprecipitated from tumor cells have been shown previously to contain a contaminant of the same size (3).

H-2 in the Plasma Membrane and Endoplasmic Reticulum of the WGA Variants. Since the majority of the cellular H-2 antigens have been shown to be present in the plasma membrane fraction (23), it seemed likely that the WGA variants would have an increased cell surface H-2 content. However, absorption of small aliquots of diluted alloantisera (i.e., B10. Br anti-B10.D2) with increasing numbers of tumor cells and subsequent testing of the absorbed serum by a microcytotoxicity assay on normal DBA/2 spleen cells revealed that the WGA variants appeared to have similar levels of cell surface H-2 (Chart 1). The elevated levels of H-2, present in
the microsomes of the WGAR variants and observed on the SDS-PAGE (Fig. 1), appear to be associated with a cellular fraction other than the plasma membrane.

H-2D and H-2K have been shown to be synthesized and glycosylated in the rough endoplasmic reticulum and are largely restricted to a membrane environment (7). Therefore, the increased H-2 of MDW1 and MDW3 may be present in the endoplasmic reticulum of the cells. To examine this possibility, plasma membrane and endoplasmic reticulum were separated from microsomal preparations by centrifugation on a one-step Ficoll gradient. The plasma membrane marker enzyme 5'-nucleotidase and plasma membrane-associated actin were used as indicators of plasma membrane purity. The plasma membrane fraction constituted approximately 5% of the microsomal preparation and showed a 3- to 5-fold increase in the specific activity of 5'-nucleotidase (data not shown). Microfilaments which are polymers of actin have been shown to be anchored to plasma membrane (32, 36) rather than endoplasmic reticulum, and, as can be seen in Fig. 3, actin was present in the plasma membrane fraction and largely absent from the endoplasmic reticulum. In contrast, the majority of the species with molecular weight of 43,000 (i.e., H-2D) was found in the endoplasmic reticulum fraction of the WGAR variants. The glycoprotein with a molecular weight of 47,000 (i.e., H-2K) was present in both fractions with a larger representation in the endoplasmic reticulum (Fig. 3).

Preparations of endoplasmic reticulum and the plasma membrane of MDW1 and MDAY-D2 were also tested for H-2 content in an alloantiserum absorption assay. The results in Chart 2 indicate that MDW1 endoplasmic reticulum contained much greater levels of H-2 than did the same membrane preparation from MDAY-D2. Calculation of the total H-2 absorptive capacity of each membrane fraction indicated that MDW1 cells contained 16 times more H-2 antigen compared to MDAY-D2, 95% of which was found in the endoplasmic reticulum fraction. The cellular H-2 of MDAY-D2 had a distribution similar to that reported previously for other tumor lines (23), with approximately 75% of the microsomal H-2 present in the plasma membrane fraction.

Antitumor CTL Induction by the WGAR Variants and MDAY-D2. Rejection of the poorly tumorigenic WGAR variants from the syngeneic DBA/2 host and their ability to grow in an immunosuppressed host suggested that a graft-type rejection response was induced by MDW1 and MDW3 in normal DBA/2 host (5, 14). However, a primary CTL response by spleen cells from tumor-bearing mice, as assayed by 51Cr-labeled WGAR variant targets, was not detected. Only on restimulation of the spleen cells in vitro using mitomycin C-treated tumor lines, and CTL was tested against 51Cr-labeled MDW1 (O) and MDAY-D2 (X), endoplasmic reticulum from MDW1 (□) and MDAY-D2 (△).

The results in Chart 3 suggest that the poorly tumorigenic variants, MDW1 and MDW3, were more immunogenic than were the highly malignant lines, MDW4, MDW5, and MDAY-D2. Since the tumor lines are cross-reactive as CTL targets and therefore appear to share the same tumor antigen, the difference in tumor immunogenicity should be demonstrable at the level of the secondary in vitro stimulation. Spleen cells from MDW3 tumor-bearing mice were cocultured with each of the mitomycin C-treated tumor lines, and CTL was tested against MDW3 targets (Chart 4). The most effective inducers of CTL were MDW1 and MDW3; MDW5 was intermediate, and MDW4 and MDAY-D2 produced the lowest CTL response. A further demonstration of the immunogenicity of MDW3 at the secondary level was shown by the enhanced CTL produced by incubating mitomycin C-treated MDW3 cells with spleen cells from MDAY-D2 tumor-bearing mice (Chart 8).

In some experimental models, tumor growth can induce suppressor cell activity which can dampen the specific CTL response against the tumor (37). However, suppressor cell

\[ \text{CTL activity induced by various combinations of in vivo inocula and in vitro spleen cell-tumor cell coculturing.} \]

MDW3-induced CTL's have been shown previously to kill MDAY-D2 tumor cell targets and each of the WGAR variants, suggesting that the tumor lines share a common tumor antigen (5). However, MDW1 and MDW3 appear to be better targets, since they were more efficiently lysed by an MDW3-induced CTL, compared to the MDW4, MDW5, and MDAY-D2 targets (Chart 3C). Further evidence of the cross-reactivity of the tumor antigens on the parental strain and the WGAR variants was produced by immunization-challenge experiments in vivo. Rejection of a s.c. inoculum of MDW1 cells protected the DBA/2 host against a subsequent challenge of MDAY-D2 or MDW4 cells (Table 1). Previous attempts to demonstrate immune protection against MDAY-D2 by immunization with lethally X-irradiated MDAY-D2 cells were unsuccessful.4

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4 R. S. Kerbel, unpublished observation.
Chart 3. Stimulation of tumor-specific CTL by MDAY-D2 and the WGA<sup>a</sup> variants. DBA/2 mice were given an s.c. injection of tumor cells, and after 2 weeks, spleen cells from the mice were cocultured for 4 days with mitomycin C-treated tumor cells of the same lineage. In A, CTL activity was tested against 5<sup>1Cr</sup>-labeled tumor targets of the same lineage as those used for the primary immunization and secondary stimulation: MDW1 (x); MDW3 (•); MDW4 (O); MDW5 (A); MDAY-D2 (•); and lysis of MDW3 by normal spleen cells (O). In B, the CTL effector groups in A were each tested for their ability to lyse 5<sup>1Cr</sup>-labeled MDW3 cells. Symbols for the CTL effector groups are the same as those in A. In C, cross-reactivity of the tumor targets was examined by comparing the lytic activity of CTL produced by an inoculum of MDW3 cells followed by coculture of the immune spleen cells with mitomycin C-treated MDW3 cells. Targets were MDW1 (x), MDW3 (•), MDW4 (O), MDW5 (A), and MDAY-D2 (•). Points, mean of triplicate determinations; bars, S.E.

Table 1

<table>
<thead>
<tr>
<th>Immunization&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Challenge&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Tumor takes at 20 days</th>
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<tr>
<td>MDW1</td>
<td>MDW-D2</td>
<td>0/5</td>
</tr>
<tr>
<td>MDW1</td>
<td>MDW4</td>
<td>0/5</td>
</tr>
<tr>
<td>Nil</td>
<td>MDW-D2</td>
<td>5/5</td>
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<tr>
<td>Nil</td>
<td>MDW4</td>
<td>4/5</td>
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<sup>a</sup> Normal DBA/2 mice received a s.c. injection of 10<sup>6</sup> MDW1 cells which were completely rejected within 3 weeks.

<sup>b</sup> A s.c. injection of 10<sup>6</sup> MDAY-D2 or 10<sup>6</sup> MDW4 tumor cells was administered 5 weeks after the first injection; 20 days later, tumor takes were scored. Tumor growth in the immunized animals was not observed even 60 days after the challenge.

Stimulation of TNP-specific CTL by TNP-modified Tumor Variants. The tumor lines showed an inverse correlation between tumorigenicity in vivo and both immunogenicity (as determined by CTL stimulation) and cellular H-2 content. It is possible that the poor tumorigenicity of MDW1 and MDW3 was related to an alteration in the tumor antigen, resulting in an increased immunogenicity. Alternatively, as the results thus far indicate, changes in a normal cellular structure, such as increased H-2 levels, may be responsible for the elevated immunogenicity of MDW1 and MDW3. To distinguish between these possibilities, the ability of TNP-modified tumor variants to stimulate TNP-specific CTL, rather than a tumor antigen-specific response, was examined. TNP-modified spleen cells, as well as TNP-modified tumor lines, were used as stimulators of unprimed DBA/2 spleen cells in a 5-day culture. The best TNP-modified stimulators of the TNP-specific CTL response were MDW1 and MDW3, then DBA/2 spleen cells, followed by MDAY-D2, MDW4, and MDW5 (Chart 6A). The CTL activity was TNP specific, since unmodified MDW3 targets were poorly lysed. TNP-modified EL4 tumor cells (H-2<sup>b</sup>) did not produce CTL activity against TNP-modified MDW3 targets, suggesting stimulation of the TNP-specific CTL was "H-2 restricted" (§).

The TNP-modified tumor variants also showed differences in
their ability to be lysed by TNP-specific CTL (Chart 6B). CTL stimulated by syngeneic TNP-modified spleen cells showed the following preference for TNP-modified targets: MDW3 > MDW1 > MDW4, MDW5, MDAY-D2 > EL4.

The increased immunogenicity of the poorly tumorigenic variants was evident in both the tumor-specific CTL response and in the TNP-directed CTL response, suggesting that immunogenicity of MDW1 and MDW3 was not dependent on the tumor antigen alone but was dependent on changes in a normal cellular component, such as the elevated cellular H-2.

**DISCUSSION**

It has been demonstrated previously that in vitro selection of lectin-resistant tumor variants may produce tumor lines with altered tumorigenic or metastatic capacities in vivo (6, 26, 33). The derivation of such variants provides a useful means of examining features of the tumor cell which may contribute to its relative malignant capacity. In the present study, certain WGA\textsuperscript{R} variants of the highly malignant murine tumor MDAY-D2 were examined for membrane alterations which might correlate with their decrease in tumorigenicity described previously (5, 14).

A preliminary examination of microsomal membrane proteins, separated on SDS:PAGE, showed an increase in the levels of several proteins in the poorly tumorigenic variants, MDW1 and MDW3. Two of these proteins having molecular weights identical to those reported previously for H-2D and H-2K (7), 43,000 and 47,000, respectively, were shown to be glycoproteins. The species with a molecular weight of 43,000 comigrated on SDS:PAGE with immunoprecipitated H-2D, and a greater amount of the glycoprotein was immunoprecipitated from MDW1 than from MDAY-D2 cells, confirming the identity of the species with a molecular weight of 43,000 as H-2D. The glycoprotein with a molecular weight of 47,000 was tentatively identified as H-2K on the basis of its molecular weight. The increase in H-2 in the microsomes of the WGA\textsuperscript{R} variants was similar for both H-2D and H-2K, suggesting that synthesis and turnover of the 2 proteins may be regulated by the same mechanism.

The higher levels of H-2 in MDW1 and MDW3 cells were accounted for by an increased H-2 content in the endoplasmic reticulum, rather than in the plasma membrane fraction or on the cell surface. The variants and parental strain appeared to have similar levels of cell surface H-2, as determined by an alloantiserum absorption test using whole cells. In contrast, alloantiserum absorption, using endoplasmic reticulum and plasma membrane cell fractions, showed a much higher level of H-2 in the endoplasmic reticulum of MDW1, compared to that of MDAY-D2. SDS:PAGE analysis also showed a higher concentration of the species with molecular weights of 43,000 and 47,000 (i.e., H-2D and H-2K) in the endoplasmic reticulum of MDW1 and MDW3 cells, compared to that of the tumorigenic lines.

The results demonstrate an inverse correlation between the levels of cellular H-2 and the tumorigenicity of the 5 tumor cell lines in the syngeneic, immunologically normal DBA host. The relative H-2 content of the 5 tumor lines, as observed on the SDS gels, was MDW1, MDW3, >MDW4, MDW5, and >MDAY-D2. The relative tumorigenicity of the tumor lines, determined by measuring the time after an s.c. injection of 10\textsuperscript{5} tumor cells, necessary for a 50% mortality rate was: MDW1 and MDW3, no tumor takes; MDW4, 63 days; MDW5, 50 days; and MDAY-D2, 25 days. Since H-2D and/or H-2K is thought to be closely associated with tumor antigens (12, 13) and has been shown to contribute to tumor immunogenicity, as well as susceptibility of target cells to lysis mediated by T-cells (12, 35), we considered the possibility that the immunogenicity of the tumor variants might be incorporated into the above correlation. This...
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appeared to be the case, since the tumor cell variants, showing high H-2 levels, were also more immunogenic and more susceptible to cell-mediated lysis, a situation presumably contributing to their poor tumorigenicity in the normal DBA/2 host.

Five experimental approaches were used to confirm the inherently higher immunogenicity of the MDW1 and MDW3 variants in the syngeneic DBA/2 host. (a) It was noted that, unlike the tumorigenic variants MDW4 and MDW5 and the parental tumor MDAY-D2, s.c. injections of MDW1 and MDW3 cells were consistently rejected by the DBA/2 host after 1 to 2 weeks of transitory growth but grew as rapidly as the tumorigenic lines in immunosuppressed mice (5, 14). This suggests a T-cell-mediated immune response was involved in rejection of MDW1 and MDW3 tumors. Rapid immune rejection of a tumor variant by its syngeneic host could be explained by the acquisition of strong tumor rejection antigens by the variants (34). The results suggested that MDW1, MDW3, and the parental strain shared a common TATA, since they were found to be cross-reactive as immunogens in vivo, or in vitro when used to stimulate CTL, as well as being cross-reactive when used as CTL targets. (b) MDW1 was shown to be immunogenic in vivo as determined by an immunization-challenge experiment; host rejection of MDW1 cells resulted in protection against a subsequent injection of MDAY-D2 or MDW4 cells, while attempts to immunize mice with X-irradiated MDAY-D2 cells were unsuccessful. (c) Restimulation of the spleen cells of tumor-bearing mice with the same line of mitomycin C-treated tumor cells resulted in much higher frequency of tumor-directed CTL when MDW1 or MDW3 cells were used as stimulators, compared to the 3 tumorigenic lines. The differences in lytic activity were apparent whether CTL activity was tested against one target (i.e., MDW3) or against the same target used for the CTL stimulation. (d) Regardless of which tumor lines were used for the primary in vivo stimulation, the secondary in vitro stimulation indicated that MDW1 and MDW3 were superior inducers of CTL activity during the 4-day coculture period. (e) The possibility that an increased amount or altered orientation of the tumor antigen in MDW1 and MDW3 cells (i.e., the same cross-reactive tumor antigen on each of the 5 tumor lines) may be responsible for the immunogenicity of MDW1 and MDW3 was tested by substituting TNP, in a functional sense, for the tumor antigen. TNP-modified tumor variants cocultured with unprimed DBA/2 spleen cells stimulated a TNP-specific and H-2-restricted CTL response. DBA/2 spleen cells cocultured with TNP-modified EL4 (H-2k) lymphoma cells did not lyse TNP-modified MDW3 cells, and DBA/2 (H-2b) spleen cells cocultured with syngeneic TNP-modified spleen cells did not lyse TNP-modified EL4 targets, demonstrating H-2 restriction at both stimulator and target levels. The ability of the TNP-modified tumor variants to both stimulate the TNP-specific CTL and serve as CTL targets showed the same pattern as that observed for the tumor-specific CTL. Thus, TNP-modified MDW1 and MDW3 cells were superior stimulators of CTL activity and were more readily lysed by TNP-directed CTL.

Since the relative ability of the 5 tumor lines to stimulate a cell-mediated immune response against 2 unrelated antigens (i.e., the tumor antigen or TNP) was similar, it appears that another structural feature of MDW1 and MDW3 cells other than the tumor antigen itself may be responsible for the variable immunogenicity of the tumor variants. In this regard, it should be noted that a number of experimental models have been used to demonstrate the critical contribution of self-H-2 in both CTL stimulator and CTL lysis of mouse target cells (35). Preliminary results in our laboratory using radiation chimeras have shown that MDW3 will grow progressively in lethally irradiated CBA mice which had been reconstituted with DBA/2 bone marrow cells but that the tumor was rejected from lethally irradiated DBA/2 mice which had been reconstituted with DBA/2 bone marrow cells. The results show that recognition of self-H-2, as well as the TATA, is necessary for CTL-mediated tumor rejection. Furthermore, the growth of MDW3 in the CBA chimeras would suggest that the tumor antigen was not an altered or "foreign" H-2 which would be expected to induce an allogeneic-like graft rejection-type of response, even in the CBA chimeras.

The high content of H-2 glycoproteins in MDW1 and MDW3 cells was accounted for inside the cell, on the endoplasmic reticulum, rather than on the cell surface, thus supposedly unavailable for stimulation of CTL. However, it is possible that the endoplasmic reticulum, containing tumor antigens as well as H-2, may stimulate CTL when the tumor cells die and release their contents as a result of necrosis and autolysis in vivo (or in vitro as a result of the mitomycin C treatment). Similarly, it is unclear how high levels of H-2 in the endoplasmic reticulum may influence the susceptibility of cells to CTL lysis. However, Russel et al. (27) have shown recently that a BALB/c myeloma variant expressing unaltered levels of cell surface H-2 (intracellular H-2 content was not examined) had a reduced ability to stimulate or serve as a target in an allogeneic or syngeneic CTL response; they have suggested that a change in the membrane or cytoskeletal structure of the variant may be responsible for the observed changes in CTL susceptibility.

A small portion of the cell surface H-2 molecule has been shown to extend into the cell and appears to be anchored to the cytoskeletal structure (25). Control of the physical relation of H-2 to other membrane structures, including tumor antigens, by cytoskeletal elements may be necessary for optimal reactivity with CTL. A similar explanation may be veiled in the present experimental model. In this connection, previous studies in our laboratory have shown that MDW1 and MDW3 cells are also poorly agglutinated by WGA and shed cell surface proteins at a reduced rate compared to the tumorigenic lines (5); these changes were thought to be due to an increased involvement of microtubules in the anchorage of cell surface proteins (for example, H-2). The dual recognition of antigen and self (i.e., H-2) by effector T-cells (8, 31) may require an optimal physical relationship between the tumor antigen and H-2 on the tumor cells, which in turn is controlled by their relation to other membrane-associated structures.

In conclusion, structures other than the tumor antigen, in this case the levels or disposition of cellular H-2, may to a large degree determine the relative immunogenicity, and consequently the relative tumorigenicity, of the tumor in the syngeneic immunologically intact host. The development of rapid procedures for the production of these types of highly immunogenic, but poorly tumorigenic, variants, from a highly malignant (and poorly immunogenic) parent tumor, such as described here, may be a very useful tool in tumor immunotherapy, especially for inherently poorly immunogenic tumors as

8 B. E. Elliott and J. W. Dennis, unpublished observations.
suggested by Klein and Klein (17). Systemic infusion of CTL reduce their metastatic burden.

REFERENCES


Fig. 1. a, SDS-PAGE separation of 80 μg of microsomal membrane protein from MDAY-D2 (B), MDW1 (C), MDW3 (D), MDW4 (E), and MDW5 (F). Actin was affinity purified from MDW1 microsomal membranes using a DNase I-Sepharose 4B column, as described previously (19), and applied to SDS-PAGE (G). Lanes A and H contain bovine serum albumin with a molecular weight of 68,000, ovalbumin with a molecular weight of 43,000, and cytochrome C with a molecular weight of 12,000. Rabbit skeletal muscle actin was applied to Lane I. The gels were stained with Coomassie brilliant blue. Arrows, positions of the actin (M.W. 47,000) and the proteins (M.W. 43,000), top to bottom, respectively. b, detection of microsomal membrane glycoproteins in Lanes A to F of Fig. 1a by incubating the gel with 125I-labeled Con A, followed by exhaustive washing and autoradiography. The region of the gels with a molecular weight of 30,000 to 100,000 is shown. Arrows, position of the glycoproteins with molecular weights of 43,000 and 47,000. The samples are in the same order as those of Fig. 1a. Ovalbumin (M.W. 43,000) is a glycoprotein and was stained by 125I-labeled Con A; bovine serum albumin (M.W. 68,000) was not stained (A).
Fig. 2. SDS-PAGE of H-2D immunoprecipitated from Nonidet P-40 extracts of [35S]methionine-labeled MDW1 and MDAY-D2 cells. Aliquots of extract containing $2 \times 10^6$ trichloroacetic acid-insoluble cpm were immunoprecipitated and applied to SDS-PAGE, and proteins were detected by autoradiography. Lanes A and B are extracts of MDW1 and MDAY-D2 immunoprecipitated with normal mouse serum; Lanes C and D are extracts of MDW1 and MDAY-D2 immunoprecipitated with anti-D$^b$ serum, respectively. 43K, molecular weight of 43,000.

Fig. 3. SDS-PAGE profiles of plasma membrane (PM) and endoplasmic reticulum (ER) proteins from MDAY-D2 cells and the WGA variants. Plasma membrane and endoplasmic reticulum fractions were separated from microsomal membrane preparations by centrifugation, and 60 μg of protein from each fraction were applied to the gel. Molecular weight markers were applied to lanes designated M. The gel was stained with Coomassie brilliant blue. Arrows, positions of the actin (M.W. 47,000) and proteins (M.W. 43,000).
Demonstration of a Correlation between Tumor Cell H-2 Antigen Content, Immunogenicity, and Tumorigenicity Using Lectin-resistant Tumor Variants

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