Differential Extraction of Tumor-specific Antigens from Two Ultraviolet Light-induced Murine Fibrosarcomas with the Use of 1-Butanol

William J. Simčík, Margaret L. Kripke, Tasi-Ling Sheu, and Stephen J. LeGrue

ABSTRACT

The purpose of this study was to investigate the immunobiological characteristics of the tumor-specific cell surface antigen expressed by the UV-induced murine fibrosarcoma, UV-2240. UV-2240 is classified as a regressor UV tumor because it is immunologically rejected by normal syngeneic mice but grows in immunocompromised or UV-irradiated hosts. The strong tumor-specific rejection antigen expressed by UV-2240 was found on the plasma membrane, and unlike the previously characterized antigen of UV-1591, the UV-2240 antigen was removed by using the noncytolytic butanol extraction technique. The tumor antigen activity in butanol extracts was resistant to digestion by endoglycosidase F and α-mannosidase, but was destroyed by pronase. In addition, the immunoprotective activity in extracts of UV-2240 was thermostable. These data demonstrate that the UV-2240-specific tumor antigen possesses physicochemical properties distinct from those of its well-characterized counterpart UV-1591.

INTRODUCTION

The tumor-specific antigens of many murine tumors are defined by their ability to immunize syngeneic hosts against subsequent challenge with the same, but not other, tumors (1-3). One interesting feature to emerge from recent studies of MCA-induced murine tumors is the similar physicochemical characteristics of some tumor-specific antigens generated in a particular mouse strain, when compared to tumor-specific antigens between strains (4-7). Thus, specific tumor immunity can be engendered with structurally and serologically related, predominantly cytosolic phosphoproteins with molecular weights of 75,000 to 82,000 from the Meth-A and CI-4 tumors of BALB/c mice (4, 5). Meth-A also expresses a second set of cytoplasmic Mr 84,000 and 86,000 tumor antigens related to murine heat shock proteins, which are also found in the SV-40-induced tumor mKSA (6, 7), and perhaps the L1210 leukemia (8). Finally, Meth-A and other MCA-induced BALB/c sarcomas express Mr 96,000 membrane glycoproteins that are structurally and serologically related, but which bear tumor-specific epitopes (9, 10). Conversely, the tumor-specific antigens expressed by MCA-induced sarcomas of C3H mice such as MCA-F appear to be carbohydrate-defined epitopes carried on a common framework polypeptide that may be derived from or related to the murine mammary tumor virus envelope glycoprotein with a molecular weight of 36,000 (11, 12).
1-Butanol Extraction. Single cell suspensions of tumor cells were extracted with 2% 1-butanol, as previously described (11, 25). Plasma membranes were prepared from cultured cells by using the two-phase polymer system (31). Extracts were digested with proteases and glycosidases with methods previously described (11). Briefly, aliquots of CBE (70 to 400 µg/ml) were incubated for 3 h at 37°C with PBS; or buffer containing 50 units of papain (EC 3.4.22.2) or pronase (nonspecific protease from Streptomyces griseus), 0.5 unit of endoglucosidase F (EC 3.2.1.96) or 2 units of α-mannosidase (3.2.1.24). Pronase was from Calbiochem (La Jolla, CA), while all other enzymes were obtained from Boehringer (Indianapolis, IN). To examine thermostability, CBE-2240 in PBS was heated to 100°C for 10 min by using a heating block. Mice were immediately immunized with the mixtures following incubation. Controls received 0.2 ml of 50 units/ml papain in PBS, 10⁴ viable UV-2240 tumor cells, or PBS alone.

Modified Immunoprotection Assay. The standard immunoprotection assay (2, 32) was modified to allow the assessment of tumor antigen activity in a regressor tumor model (15). Mice are immunized once s.c. on the flank with cells or extracts in 0.1 to 1.0 ml of PBS. Eight days later the animals were sublethally irradiated with 450 R from a 60Co source to eliminate primary immune responses without affecting secondary or memory responses (15). Two days later (10 days after immunization), the mice were challenged s.c. on the contralateral flank with 10 times the minimum tumorigenic dose (2, 33) of tumor cells. For UV-2240 and UV-1591, the challenge dose was 2 x 10⁴ cells. Tumor incidence and tumor diameter were monitored by twice weekly inspections and caliper measurements. Statistical evaluation of tumor incidence was by Fisher's exact test, whereas tumor diameters were analyzed by using Student-Newman-Keuls multiple comparison test that uses both an analysis of variance and a pooled error rate (11).

Flow Cytfluorography. Tissue culture propagated UV-1591 and UV-2240 cells were analyzed for the expression of the immunodominant antigen of UV-1591 before and after butanol extraction by using the syngeneic monoclonal antibody CP28 (23). One aliquot of UV-1591 cells was extracted with 2% 1-butanol in PBS for 5 min at room temperature, while another aliquot was incubated in PBS alone. The cells were washed twice and then preincubated in 10% serum to block nonspecific sites on the extracted cells (30). After additional washes, the cells were subdivided into two groups. One group was treated for 60 min on ice with a 1:800 dilution of CP28, followed by a 1:50 dilution of fluorescein-conjugated rat anti-mouse second antibody. The control groups was treated with second antibody alone. The cells were then washed in PBS supplemented with 1% bovine serum albumin and resuspended in 1 ml of 1% paraformaldehyde. Green fluorescence was analyzed by using a Coulter EPICS V flow cytometer (Coulter Electronics, Hialeah, FL).

**RESULTS**

UV-2240 Tumor Antigen on Plasma Membrane. The strong tumor rejection antigen expressed by the UV-2240 tumor was localized to the plasma membrane by immunization of normal syngeneic mice with isolated membrane preparations (Table 1). A modified immunoprophylaxis assay was used to allow the transient growth of regressor tumors in immunized animals (15). Immunization of mice with 10⁶ viable UV-2240 cells afforded strong, tumor-specific protection against subsequent challenge with UV-2240, but not the antigenically distinct tumor, UV-1591. Similarly, pretreatment of hosts with 30 µg of plasma membrane protein also engendered strong, tumor-specific protection (Table 1).

UV-2240 Antigen Extracted with 1-Butanol. Cell surface, tumor-specific antigens can be assigned into two general categories: transmembrane and peripheral. The immunodominant tumor-rejection antigen of UV-1591 is a recombinant class I MHC antigen of the transmembrane type (23, 24). On the other hand, the immunoprotective antigens of a number of other murine tumors are peripheral components that can be removed by using single-phase butanol extraction (29). To determine whether the UV-2240 antigen resembled the peripheral antigens, the cells were extracted with 1-butanol.

We observed significant immunoprotective activity with the UV-2240 butanol extracts. Immunization with 10 or 30 µg of CBE-2240 significantly reduced both the incidence and size of the UV-2240 challenge tumors (Table 2). Although two to three mice in these groups developed tumors, in other experiments 30 µg CBE-2240 completely protected against a challenge with 2 x 10⁶ UV-2240 cells (data not shown). Immunization with 100 µg CBE-2240 did not significantly reduce the incidence of UV-2240, although the size of the developing tumors was significantly less than in mice receiving PBS (Table 2). Immunization with CBE-2240 did not influence the growth of the UV-1591 tumor (Table 2), demonstrating the specificity of the immunoprotective response engendered by CBE-2240. The dose-response curve obtained by immunizing with 10, 30, or 100 µg of CBE-2240 is consistent with the results from other tumor antigen systems, in which a high-dose unresponsive state is observed following treatment with 100 to 500 µg of soluble antigen (15, 22, 27, 28, 31).
We have also attempted to solubilize the tumor-specific immunoprotective antigen from isolated UV-2240 plasma membranes by using the nonionic detergents Nonidet P-40 and octyl-\(\beta\)-d-glucoside. We have been unable to demonstrate any immunoprotective activity in detergent extracts of UV-2240 membranes (data not shown), despite the fact that octyl-\(\beta\)-d-glucoside has proved to be effective for the release of other butanol-extractable, tumor-specific antigens (31).

UV-1591 Antigen Not Extractable with 1-Butanol. Next we investigated whether extracts of UV-1591 contained immunoprotective activity. Immunization of mice with 10\(^6\) UV-1591 tumor cells significantly reduced the incidence of UV-1591 tumor growth, without affecting the incidence of UV-2240 (Table 3). However, immunization with butanol extracts prepared from the UV-1591 tumor had no effect on the growth of either UV-1591 or UV-2240 tumor cells (Table 3). These results are consistent with the MHC class I derivation of the immunodominant antigen expressed by UV-1591 (23), and demonstrate that the protective response engendered by CBE-2240 is dependent on the extraction of a UV-2240-specific molecule.

To confirm that butanol extraction did not release the immunodominant, MHC-related antigen on UV-1591 cells, we analyzed cell surface antigen expression before and after extraction by using the UV-1591-specific syngeneic monoclonal antibody, CP28 (23). Flow cytofluorographic analysis of unextracted UV-1591 cells revealed a 46\% positive reactivity with CP28, with a binding ratio (23) of 38, when compared to cells stained with second antibody alone (Fig. 1). Butanol-extracted UV-1591 cells displayed a 34\% positive reactivity with CP28 and a binding ratio of 8.6. The lower binding ratio of the butanol-extracted UV-1591 cells was attributable to their higher nonspecific second antibody binding, relative to unextracted controls (4.0% versus 1.2%, respectively). UV-2240 cells did not bind monoclonal antibody CP28, either before or after extraction (data not shown).

Biochemical Characterization of Tumor Antigen in CBE-2240. To determine whether the immunoprotective activity observed with CBE-2240 was attributable to a protein moiety, we incubated extracts with either papain (11). Controls consisted of animals immunized with (a) viable UV-2240 cells; (b) CBE-2240 incubated without enzyme; or (c) enzyme alone (Table 4, Experiment 1). Mice immunized with 50 units (0.43 mg) of papain in PBS all developed tumors, while mice receiving 10\(^6\) viable UV-2240 cells were all protected from tumor growth. Mice immunized with 30 \(\mu\)g CBE-2240 displayed a significantly reduced tumor incidence and mean tumor diameter, as compared to the PBS/papain control group, which was not affected by digestion with papain (Table 4, Experiment 1). As expected, CBE-2240 had no effect on the growth of UV-1591 tumors, regardless of the enzymatic pretreatment. In a second experiment, CBE-2240 was resistant to digestion with endoglycosidase F and \(\alpha\)-mannosidase, but was destroyed by treatment with the nonspecific protease pronase (Table 4, Experiment 2). In addition, the immunoprotective activity in CBE-2240 was completely resistant to denaturation by heat. Thus, the protective activity in CBE-2240 is attributable to a thermostable epitope of a protein or glycoprotein.

**DISCUSSION**

In this study, we used noncytolytic butanol extraction to release an immunoprotective, tumor-specific antigen from the surface of the UV-induced regressor fibrosarcoma, UV-2240. Conversely, the immunodominant protective antigen from a related UV-induced regressor tumor, UV-1591, was not extracted with butanol. The differential extractabilities of the immunogenic activities from these two UV-induced tumors demonstrates that their biochemical characteristics are distinct, although these two tumors were induced in the same group of experimental mice and have been maintained under similar conditions (14). The inability of butanol to extract the UV-1591 antigen is consistent with the reported MHC derivation for the UV-1591 immunodominant antigen (22, 23), because the noncytolytic conditions used in this study do not release transmembrane proteins (26, 29, 30). The sensitivity of the UV-2240 antigen to extraction by butanol suggests that this moiety is a peripheral membrane component and not a transmembrane or MHC-related glycoprotein (29, 30).

The immunoprotective antigen of UV-2240 was clearly associated with the plasma membrane fraction, similar to the UV-1591 antigen, and the \(M_f\) 36,000 and 96,000 antigens expressed by MCA-induced tumors of C3H and BALB/c mice, respectively (9–11). The lack of immunoprotective activity in cytosolic preparations of UV-2240 (data not shown) distinguishes it from the \(M_f\) 82,000 and \(M_f\) 84,000 and 86,000 antigens of Meth-A and other tumors (4, 6, 7). Despite plasma membrane localization of the immunoprotective antigen from UV-2240, detergent

---

**Table 3** 1-Butanol extraction of UV-1591

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Dose</th>
<th>Challenge</th>
<th>Tumor</th>
<th>Tumor incidence*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td></td>
<td>UV-1591</td>
<td>8/10</td>
<td></td>
</tr>
<tr>
<td>UV-1591 cells</td>
<td>10^6</td>
<td>UV-1591</td>
<td>1/6^a</td>
<td></td>
</tr>
<tr>
<td>CBE-1591</td>
<td>10 (\mu)g</td>
<td>UV-1591</td>
<td>6/6</td>
<td></td>
</tr>
<tr>
<td>CBE-1591</td>
<td>30 (\mu)g</td>
<td>UV-1591</td>
<td>6/6</td>
<td></td>
</tr>
<tr>
<td>CBE-1591</td>
<td>100 (\mu)g</td>
<td>UV-1591</td>
<td>6/6</td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td></td>
<td>UV-2240</td>
<td>9/10</td>
<td></td>
</tr>
<tr>
<td>UV-1591 cells</td>
<td>10^6</td>
<td>UV-2240</td>
<td>6/6</td>
<td></td>
</tr>
<tr>
<td>CBE-1591</td>
<td>10 (\mu)g</td>
<td>UV-2240</td>
<td>6/6</td>
<td></td>
</tr>
<tr>
<td>CBE-1591</td>
<td>30 (\mu)g</td>
<td>UV-2240</td>
<td>6/6</td>
<td></td>
</tr>
<tr>
<td>CBE-1591</td>
<td>100 (\mu)g</td>
<td>UV-2240</td>
<td>6/6</td>
<td></td>
</tr>
</tbody>
</table>

* Number of animals with tumor/number challenged, at 16 days after challenge.

Statistically significant from PBS control, \(P < 0.01\) by using Fisher's exact test.

---

**Fig. 1.** Flow cytofluorographic analysis of CP28 binding to UV-1591. The binding of monoclonal CP28 antibody, specific for the immunodominant antigen of UV-1591 cells, was examined before and after butanol extraction. A, unextracted UV-1591 cells treated with fluorescein-labeled second antibody only; B, unextracted cells treated with CP28; C, second antibody control for butanol-extracted UV-1591 cells; D, reactivity of CP28 with butanol-extracted cells.
extracts of isolated plasma membranes were not active in immunoprotection assays. A greatly diminished immunogenicity of antigens from UV-induced tumors was also reported when 3 M KCl was used to extract soluble antigens (18, 19). One explanation for our observations is that the immunogenic epitope was destroyed by detergent extraction, possibly by protein denaturation or disruption of combinatorial associations. Alternatively, detergent-extracted antigens may be processed differently by accessory cells, leading to a weaker or absent cellular response. The very strong protection engendered by CBE-2240 may result from the slightly hydrophobic character of butanol-extracted materials (29, 34), leading to spontaneous interaction with tissue macrophages or other antigen-presenting cells. However, the protective activities of both CBE and isolated membranes were not as strong as that engendered by intact UV-2240 cells, consistent with the weaker immunogenic potential of solubilized and subcellular materials (3, 5, 11). The activity removed from UV-2240 with butanol was tumor specific, since CBE-2240 did not protect against challenge with UV-1591, nor did CBE-1591 protect against challenge with either UV-2240 or UV-1591. The narrow dose-response profile observed with CBE-2240, together with the reduced protection observed at higher antigen concentrations is consistent with our previous results with butanol-extracted tumor antigens (11, 29). We have yet to determine whether the reduced activity of large immunizing doses is attributable to reduced immunogenicity or to active suppression.

Preliminary biochemical characterization of the protective antigen in CBE-2240 was undertaken by using degradative enzymes. The antigen was resistant to papain digestion, but was completely destroyed by pronase. Thus, the immunoprotective epitope is probably carried on a protein. The immunogen appeared to be resistant to trypsin, since the use of this protease to harvest tumor cells from both in vitro and in vivo sources did not ablate activity. The resistance of the antigen to papain suggests that a peptide or glycopeptide is sufficient to engender immunity, although the sensitivity of the antigen to pronase demonstrates that if oligosaccharides are involved in the immunogenic epitope, they must be carried on a peptide of minimum size.

The antigenic activity was not destroyed by the two glycosidases tested here, endoglycosidase F and α-mannosidase. The failure of endoglycosidase F to digest the antigen indicates that high mannose, hybrid or complex N-linked glycoconjugates do not bear the immunogenic epitope, but does not rule out the participation of O-linked oligosaccharides in the structure of the antigen. However, if O-linked oligosaccharides do contain the UV-2240-specific antigens, effective processing or immune recognition requires some associated polypeptide structures since pronase-digested extracts were not immunogenic. The resistance of the UV-2240-specific antigen to digestion by both papain and α-mannosidase differentiates it from the butanol-extracted tumor-specific antigens of MCA-induced C3H tumors, which were destroyed by both enzymes (11).

Finally, the antigen in CBE-2240 was thermostable, as are the MCA-induced antigens of C3H, but not BALB/c mice (9, 11). While resistance to heat denaturation is consistent with a carbohydrate-defined epitope, it is important to remember that (a) thermostability of proteins is correlated with renaturation of secondary structure (35), and (b) immune recognition is not dependent upon the integrity of the whole molecule. Thus, while our results cannot definitively assign the UV-2240-specific epitopes to either protein or carbohydrate structures, they do suggest that the most likely candidate is a thermostable polypeptide.

The distinct characteristics of the immunogenic antigens from UV-1591 and UV-2240 suggest that UV carcinogenesis in C3H/HeN mice does not result in the expression of unique neoantigenic epitopes on a common framework sequence. This observation differs from that with chemically induced sarcomas of C3H mice, in which unique epitopes seem to be generated on common framework sequences (11). The ability of UV-B

---

**Table 4 Enzyme digestion of CBE-2240**

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Dose</th>
<th>Treatment</th>
<th>Challenge</th>
<th>Tumor incidencea</th>
<th>Tumor diameter (mm ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td></td>
<td></td>
<td>UV-2240</td>
<td>4/4</td>
<td>4.4 ± 0.9</td>
</tr>
<tr>
<td>UV-2240</td>
<td>10⁶ cells</td>
<td>Papain®</td>
<td>UV-2240</td>
<td>0/5</td>
<td>0.2 ± 0.2*</td>
</tr>
<tr>
<td>CBE-2240</td>
<td>30 μg</td>
<td>PBS</td>
<td>UV-2240</td>
<td>1/5*</td>
<td>0.7 ± 0.7*</td>
</tr>
<tr>
<td>CBE-2240</td>
<td>30 μg</td>
<td>Pronase</td>
<td>UV-2240</td>
<td>1/5*</td>
<td>0.7 ± 0.7*</td>
</tr>
<tr>
<td>PBS</td>
<td></td>
<td></td>
<td>UV-1591</td>
<td>7/9</td>
<td>5.3 ± 1.8</td>
</tr>
<tr>
<td>UV-2240</td>
<td>10⁶ cells</td>
<td>None</td>
<td>UV-1591</td>
<td>4/5</td>
<td>6.9 ± 1.8</td>
</tr>
<tr>
<td>CBE-2240</td>
<td>30 μg</td>
<td>PBS</td>
<td>UV-1591</td>
<td>5/5</td>
<td>3.6 ± 1.1</td>
</tr>
<tr>
<td>CBE-2240</td>
<td>30 μg</td>
<td>Pronase</td>
<td>UV-1591</td>
<td>4/5</td>
<td>5.2 ± 1.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Experiment 2</th>
<th>PBS</th>
<th>UV-2240</th>
<th>None</th>
<th>UV-2240</th>
<th>9.9 ± 0.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBE-2240</td>
<td>10⁶ cells</td>
<td>PBS</td>
<td>UV-2240</td>
<td>0/5*</td>
<td>0.7 ± 0.7*</td>
</tr>
<tr>
<td>CBE-2240</td>
<td>100 μg</td>
<td>PBS</td>
<td>UV-2240</td>
<td>1/5*</td>
<td>0.7 ± 0.7*</td>
</tr>
<tr>
<td>CBE-2240</td>
<td>100 μg</td>
<td>100°C 10 min</td>
<td>UV-2240</td>
<td>0/5*</td>
<td>0.7 ± 0.7*</td>
</tr>
<tr>
<td>CBE-2240</td>
<td>100 μg</td>
<td>Endoglycosidase F®</td>
<td>UV-2240</td>
<td>1/5*</td>
<td>0.4 ± 0.4*</td>
</tr>
<tr>
<td>CBE-2240</td>
<td>100 μg</td>
<td>α-Mannosidase®</td>
<td>UV-2240</td>
<td>1/5*</td>
<td>0.4 ± 0.4*</td>
</tr>
<tr>
<td>CBE-2240</td>
<td>100 μg</td>
<td>Pronase</td>
<td>UV-2240</td>
<td>5/5</td>
<td>7.6 ± 2.0</td>
</tr>
</tbody>
</table>

---

*a Number of animals with tumor/number of animals challenged; incidence assessed 24 days after challenge.
® Fifty units of papain/ml of CBE (70 μg).
* Statistically significant difference from PBS control, P ≤ 0.01 by using Fisher's exact test.
® Statistically significant difference from PBS control, P ≤ 0.05 by using Fisher's exact test.
* Statistically significant difference from PBS control, P ≤ 0.005 by using the Student-Newman-Keuls t test.
* Statistically significant difference from PBS control, P ≤ 0.001 by using the Student-Newman-Keuls t test.
* Endoglycosidase F (0.5 units)/ml of CBE (400 μg).
* Two units of α-mannosidase/ml of CBE.
® Fifty units of pronase/ml of CBE.
irradiation to induce strong tumor-specific neoantigens in vitro (36, 37) may mean that several genes can serve as potential targets for mutation or activation by this physical carcinogen. Furthermore, the data presented here suggest that strong neoantigens expressed by UV-induced tumors need not be altered MHC antigens in order to mediate the regressor phenotype.

ACKNOWLEDGMENTS

The authors thank Dr. R. Goodenow for providing the CP28 antibodies. The secretarial assistance of Alice Burnett is gratefully acknowledged.

REFERENCES

Differential Extraction of Tumor-specific Antigens from Two Ultraviolet Light-induced Murine Fibrosarcomas with the Use of 1-Butanol

William J. Simick, Margaret L. Kripke, Tasi-Ling Sheu, et al.