ABSTRACT

In vitro exposure of nonimmunogenic murine tumor cells to UV radiation (UVR) generates highly antigenic variants that are immunologically rejected by normal, syngeneic mice. The purpose of this study was to determine whether these antigenic variants cross-react immunologically with the parental tumor and whether the UVR-associated antigen unique to UVR-induced tumors is also present on the variants. Antigenic (regressor) variants and nonimmunogenic (progressor) clones derived from UV-irradiated cultures of the C3H K1735 melanoma and SF19 spontaneous fibrosarcoma cell lines were used to address these questions. In an in vivo immunization and challenge assay, the antigenic variants did not induce cross-protection among themselves, but each induced immunity against the immunizing variant, the parent tumor cells, and nonimmunogenic clones derived from UV-irradiated parent cultures. Therefore, the variants can be used to induce in mice a protective immunity that prevents the growth of the parent tumor and nonimmunogenic clones, but not other antigenic variants. In contrast, immunization with cells of the parental tumor or the nonimmunogenic clones induced no protective immunity against challenge with any of the cell lines. Utilizing the K1735 melanoma-derived cell lines in vitro, T-helper (Th) cells isolated from tumor-immunized mice were tested for cross-reactivity by their ability to collaborate with trinitrophenyl-primed B-cells in the presence of trinitrophenyl-conjugated tumor cells. Also, the cross-reactivity of cytotoxic T-lymphocytes from tumor-immunized mice was assessed by a 4-h 3Cr-release assay. Antigenic variants induced cytotoxic T-lymphocytes and Th activity that was higher than that induced by the parent tumor and nonimmunogenic clones from the UV-exposed parent tumor and cross-reacted with the parental tumor cells and nonimmunogenic clones, but not with other antigenic variants. Furthermore, upon transplantation, the UVR-induced antigenic variants grew in UV-irradiated and immunosuppressed mice, but not in untreated mice indicating that the variants expressed the determinant recognized by suppressor T-cells present in UV-irradiated mice. These results demonstrate that highly antigenic cells generated by the in vitro exposure of two different murine tumors to UV radiation express a determinant shared with the parental tumor cells and nonimmunogenic clones, a unique variant-specific determinant and the suppressor cell-defined determinant present on UVR-induced tumors. Based on these results, two models are proposed to explain the make-up of the antigenic determinants present on the UVR-induced antigenic variants.

INTRODUCTION

Chronic exposure of mice to (280–320 nm) UVR results in the production of highly antigenic skin cancers that fail to grow when transplanted into normal, syngeneic recipients (1). The rejection is associated with an immune response specific for the immunizing tumor, indicating the presence of antigens unique to each tumor (2, 3). In contrast, every UVR-induced tumor is recognized by Ts present in the lymphoid organs of mice chronically irradiated with UVR (2, 4, 5). Even though these same tumors express individually specific transplantation antigens, the Ts appear to inhibit the immune response to all cancers induced by UVR but not the response to other allogeneic or syngeneic tumors (6). Recognition of UVR-induced tumors as a group by UVR-induced Ts cells suggests that they express a common regulatory antigen, in addition to a unique determinant. Roberts demonstrated that a cloned suppressor cell line derived from spleen cells of UV-irradiated mice recognizes multiple tumors, providing direct evidence that a common determinant expressed on all UVR-induced tumors is the target for the Ts cells (7).

Antigens on UVR-induced tumors have also been demonstrated in vitro by utilizing CTL assays and an indirect assay for Th activity. Individually specific antigens have been demonstrated on UVR-induced tumors by assessing the specificity of CTL (8, 9) and Th cells (10) generated after immunization of mice with UVR-induced tumors. In addition, Schreiber and colleagues (11, 12) have isolated and cloned CTL lines from mice immunized with UVR-induced tumors. These cloned CTL recognize transplantation antigens specific for the immunizing tumor and show no evidence of cross-reactivity with other tumors whether or not they are UVR-induced.

Recently we reported that in vitro exposure of cells from two weakly immunogenic murine tumors, a melanoma and a fibrosarcoma, to UVR generated a high frequency of antigenic (regressor) variants. Approximately 50% of the clones isolated from UV-irradiated cultures of the melanoma and fibrosarcoma were immunologically rejected in normal, syngeneic mice but grew progressively in immunosuppressed mice (13, 14). Antigenic variants derived from the fibrosarcoma cell lines were like UVR-induced tumors in that the variants were not cross-protective in vivo, but were recognized by UVR-induced Ts (13). In this study, we investigated whether antigenic variants derived from the UV-irradiated melanoma cells also possess the antigenic characteristics of UVR-induced tumors or whether the induction of UVR-associated antigens was a unique property of the fibrosarcoma-derived variants. Second, we wished to determine whether the antigenic variants share antigens with the parental cells from which they are derived that can be detected by in vivo immunization and challenge. In addition, we compared the specificity of the variant-induced immune response observed in vivo with that of Th and CTL obtained from variant-immunized mice and assayed in vitro.

MATERIALS AND METHODS

Mice. Specific pathogen-free C3H/HeN (MTV-) mice were supplied by the Animal Production Area of the Frederick Cancer Research Center. Mice were used at 8–12 weeks of age for all experiments, and within a single experiment, all mice were age- and sex-matched. The animals were housed in a pathogen-free, barrier facility where ambient light was automatically controlled to produce 12-h light, 12-h dark cycles; they were maintained in a facility accredited by the American Association for Cancer Research.
Association for Accreditation of Laboratory Animal Care according to the NIH guide for Laboratory Animal Care.

Tumors. K1735 is a melanoma cell line derived from a tumor induced in a C3H/HeN (MTV\textsuperscript{+}) mouse by UVR and croton oil treatment (15). SF19 fibrosarcoma is a spontaneous tumor that arose in a C3H/HeN (MTV\textsuperscript{+}) mouse (16). Both of these cell lines grow progressively when transplanted into syngeneic mice. Tumor cell lines K1735-OR.5, K1735-LD.1, SF19-OR.26, and SF19-OR.58 are clones derived from UV-irradiated K1735 or SF19 cultures following in vitro exposure to UVR as described previously (13, 14). These cell lines are highly antigenic in that they are rejected following s.c. injection into normal syngeneic mice. The K1735-OR.3 and SF19-LD.3 cell lines are non-immunogenic (progressor) clones derived from UV-irradiated K1735 and SF19 cultures, respectively (13, 14). UV 2240 was induced in C3H/HeN (MTV\textsuperscript{+}) mice by chronic exposure to UVR. It grows in UV-irradiated and immunosuppressed mice, but not untreated syngeneic hosts (17). The MCA-FMI cell line, produced by Dr. Philip Frost (Department of Cell Biology, University of Texas M. D. Anderson Cancer Center, Houston, TX), is an antigenic (regressor) variant derived from the 3-methylcholanthrene-induced MCA-\textsuperscript{+} fibrosarcoma of C3H/He strain origin after in vitro exposure to the mutagen N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). This cell line is immunologically rejected upon transplantation into normal syngeneic mice, and was obtained from Dr. Stephen LeGrue (Department of Immunology, University of Texas M. D. Anderson Cancer Center, Houston, TX).

All tumors were maintained in tissue culture using Eagle's minimal essential medium (GIBCO, Grand Rapids, MI) supplemented with 10% FBS, 2% vitamin solution, 10% sodium pyruvate, 1% nonessential amino acids, and 1% L-glutamine, without antibiotics (complete minimal essential media). All cell lines were tested routinely to assure the absence of Mycoplasma, and all lines were free of pathogenic murine viruses (Microbiological Associates, Rockville, MD). Subconfluent monolayers of the tumor cells were harvested by a 30-s trypsinization (0.25% trypsin-verseone). The single-cell suspensions were washed and resuspended in HBSS (GIBCO). Cell number was determined using a hemocytometer, and viability was measured by the exclusion of trypan blue.

**In Vitro Cross-Protection Experiment.** Normal C3H mice were immunized by s.c. injection of 2 \times 10\textsuperscript{5} tumor cells on Day 0. Because both progressor and regressor tumors were used for immunization, all cell lines were exposed to 10,000-rad X-irradiation before injection to make the immunizing inocula comparable. Control, unimmunized mice were injected with an equal volume of culture medium. On Day 14, after regression of the immunizing tumors, the mice were exposed to 450 rads of X-irradiation. This procedure reduces the primary response against challenge implants while the secondary response in immunized mice is left intact (18). On Day 16, a challenge dose of 2 \times 10\textsuperscript{5} cells of the K1735 cell lines or 2 \times 10\textsuperscript{5} cells of the SF19 cell lines was given s.c. on the opposite side. These challenge doses produce tumors in 100% of the mice challenged. Mice were checked weekly for tumor growth for 6-10 weeks.

**In Vitro Th Cell Assay.** To generate tumor-immune T-helper cells, C3H mice were immunized with three i.p. injections of 2 \times 10\textsuperscript{5} X-irradiated (10,000-rad) tumor cells at 1-week intervals. Spleens from these mice were used as a source of tumor-immune Th cells 2 weeks after the third immunization.

Concurrently, other groups of mice were immunized with TNP-conjugated SRBC to generate TNP-SRBC-primed B-cells. SRBC in Alsever's solution were obtained from the Science Park Veterinary Division of the University of Texas M. D. Anderson Cancer Center. TNP-conjugated erythrocytes were freshly prepared for each experiment. Red cells were washed four times in PBS, pH 7.4, before conjugation with TNP. This was accomplished by mixing, 1-mll packed volume of washed SRBC and 7 ml of 20 mM TNP in PBS, pH 8.0. After 30 min at room temperature, the cells were washed four times with PBS, pH 7.4. Glycylglycine (10 mg/ml) was added to the first wash. To prime mice against TNP-SRBC, they were given two i.p. injections of 0.1 ml of 2% (v/v) TNP-SRBC, 2 weeks apart. These mice were used as a source of TNP-primed B-cells 2 weeks after the second immunization.

Spleens were removed from tumor- and TNP-SRBC-immune mice, and single-cell suspensions were prepared. The cells were washed, then resuspended in RPMI 1640, and the number of viable cells was determined by trypan blue exclusion.

B-cells were purified from mice immunized against TNP-SRBC. Spleen cells were suspended at 4 \times 10\textsuperscript{7}/ml in RPMI containing 2% FBS, and an equal volume of RPMI containing anti-Thy-1.2 (Becton-Dickinson, Oxnard, CA) was added. The final dilution of the Thy-1.2 monoclonal antibody was 1/166. After 30 min on ice, the cells were washed twice and resuspended in RPMI with 2% FBS and a 1/16 dilution of rabbit H2 complement (Pel-Freez, Brown Deer, WI). The cells were incubated for 1 h at 37°C and washed twice before the number of viable spleen cells was counted as above.

Spleen cell preparations from tumor-immunized mice were enriched for T-cells by incubation on NW columns by the method of Julius et al. (19). The NW-nonadherent cells were washed and used as Th in the mixed lymphocyte-tumor cell cultures.

Cultures were set up in RPMI 1640 containing 10% FBS, 2 mM L-glutamine, 5 \times 10\textsuperscript{-5} M 2-mercaptoethanol, 100 U/ml penicillin, 100 \mu g/ml streptomycin, 10% sodium pyruvate, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, and 1 x vitamins and nonessential amino acids (GIBCO). To test for Th activity, we used the method of Howie and McBride (20). Cultures consisting of 10\textsuperscript{7} T-depleted spleen cells from TNP-SRBC-primed mice, 5 \times 10\textsuperscript{5} TNP-conjugated, X-irradiated (10,000-rad) tumor cells, and 10\textsuperscript{6} NW-nonadherent spleen cells from tumor-immunized mice were grown in 3 ml of medium in Falcon six-well tissue culture plates (Becton-Dickinson, Oxnard, CA).

To determine the background PFC response for each experiment, B-cells were incubated alone and with TNP tumor cells.

After a 5-day incubation, the cultures were harvested and washed in HBSS. Anti-TNP PFC were assayed by using the Mishell and Dutton (21) modification of the Jerne and Nordin (22) technique. Each sample was assayed in the presence of 8% TNP-HRBC, guinea pig complement (Pel Freez), and rabbit anti-mouse IgG serum (Cappel, Cochranville, PA). Th cell activity was assayed by standard methods (19) for TNP-PFC and measured as total (direct plus indirect) plaques per culture. Cultures were also tested for PFC against SRBC and HRBC to demonstrate the specificity of the antibody response for TNP.

**In Vitro CTL Assay.** C3H mice were immunized with one i.p. injection of 2 \times 10\textsuperscript{5} X-irradiated (10,000-rad) tumor cells to generate tumor-immune CTL. 2 weeks later, spleens were removed from mice, and single-cell suspensions were prepared. The cells were washed, then resuspended in RPMI 1640, and the number of viable cells was determined by trypan blue exclusion.

As a source of T-enriched CTL, spleen cell preparations from tumor-immunized mice were incubated on nylon wool columns using the method of Julius et al. (19). The NW-nonadherent cells were washed and used as CTL in the mixed lymphocyte-tumor cell cultures.

Cultures were set up in the same media as described above for the Th cell assays. Five \times 10\textsuperscript{5} NW-purified splenocytes and 10\textsuperscript{5} X-irradiated (10,000-rad) tumor cells were incubated in 2 ml of medium in Falcon 24-well tissue culture plates for 5 days (Becton-Dickinson, Oxnard, CA).

To assay for CTL activity, we adapted the method described by Thorn (9). Tumor targets were labeled with Na\textsuperscript{51}CrO\textsubscript{4}, immediately after trypsinization, and 100\mu l aliquots of CTL and \textsuperscript{51}Cr-labeled tumor target cells were added to each well of a V-bottom microtest plate (Falcon). The plates were centrifuged for 4–5 min at 400 RPM and incubated for 4 h at 37°C. Cytotoxicity was determined by sampling 100 \mu l directly from each well. The percentage of cytotoxicity was calculated as [(E-S)/(T-S)] \times 100, where E is the counts released in the presence of CTL cells, S is the counts released by targets incubated only with medium, and T is the counts released by target cells in the presence of 1% Triton X-100. The total release (T) was at least 75% of the incorporated counts for all target cells. The spontaneous release (S) was consistently <15% of releasable counts.

Chronic UVR Irradiation of Mice. Mice were exposed to UVR according to the procedure of Kincade and Cochranville, PA. The light source was a bank of six FS40 sunlamps (Westinghouse). Dorsal hair of the mice was removed with electric clippers once a week, and mice were exposed to UVR for...
1 h, three times a week, for at least 12 weeks. The incident dose rate was approximately 4 W/m² over the wavelength range from 280 to 320 nm. None of the animals had yet developed primary tumors from the UV irradiation at the time of these experiments.

RESULTS

In Vivo Cross-Reactivity between the Antigenic Variants and Parental Cell Lines. An in vivo cross-protection experiment was performed to determine whether the immune responses generated by immunization with the antigenic variants were protective against challenge with the parent tumor. Nonimmunogenic clones of UV-irradiated parental cultures and other antigenic variants were also tested for cross-reactivity. Tables 1 (SF19 cell lines) and 2 (K1735 cell lines) show that mice immunized with one antigenic variant cell line generated an immune response that resulted in the rejection of the immunizing antigenic variant as expected. In addition, the variant-immune mice were able to reject a challenge with the nonimmunogenic parental cells, and the nonimmunogenic clones, SF19-LD.3 (Table 1) and K1735-OR.3 (Table 2), derived from UV-irradiated SF19 and K1735 cells, respectively. However, animals immune to the antigenic variant did not reject a challenge with a different antigenic variant or an unrelated control tumor. Mice immunized with cells of the parent tumors or nonimmunogenic clones generated no detectable resistance to tumor challenge. Control, X-irradiated, unimmunized mice were unable to reject a challenge with any of the cell lines.

The lack of cross-protection among antigenic variants indicates that the variants exhibit individually specific, non-cross-reacting antigens. This individual specificity of the variant cell lines resembles that of skin cancers induced in mice in vivo by repeated exposure to UVR (2). The presence of cross-reactivity between an antigenic variant and parental cells and cells of a nonimmunogenic clone indicates that the antigenic variants, parental cells, and nonimmunogenic clones share at least one tumor rejection antigen that by itself is not sufficient to induce a protective immune response.

Specificity of Th Cells and CTL from Tumor-immunized Mice. Immune responses to tumor antigens, similar to responses against other antigens, involve a complex immune network that includes B-cells, various subsets of T-cells, and macrophages. This network includes antigen recognition (afferent) and antitumor effector (efferent) phases. Therefore, when evaluating the specificity of the immune rejection response to any tumor, it is important to define specificity in terms of the various afferent and effector cell types. In this study, the specificity of antigenic variant-induced Th cells (afferent) and CTL (effector) was determined in vitro and compared with the specificity of the antitumor rejection response observed in vivo.

In order to determine whether immunization with the antigenic variants induces Th activity, an indirect in vitro assay was performed. In addition, the specificity of the Th cells was assessed by determining their ability to recognize unique and/or shared antigens present on the parental cells and the other clonal cell lines derived from the UV-irradiated parent cell line. Four tumor lines (K1735, K1735-OR.5, K1735-LD.1, and K1735-OR.3) were injected into different groups of mice to generate Th. C3H mice were injected i.p. three times weekly with X-irradiated (10,000-rad) tumor cells. Splenic NW-nonadherent cells from the tumor-immunized mice were cocultivated for 5 days in vitro with TNP-primed B-cells and TNP-conjugated tumor cells of the appropriate cell line. The results shown in Table 3 demonstrate that the amount of Th activity induced by the antigenic variants was much higher than that measured after immunization with the cells of the parental tumor or nonimmunogenic clone. The antigenic variant-induced Th cells recognized the immunizing variant, the parent tumor, and the nonimmunogenic clone, but did not cross-react with a different antigenic variant or unrelated, control tumor cells. These results suggest that Th cells generated by immunization of mice with antigenic variant cells detect variant-specific antigens as well as antigens present on the parental and nonimmunogenic clone cells. This experiment was repeated and yielded the same results.

Splenic T cells from mice immunized with tumor cells were tested for reactivity against a battery of target cells in a 51Cr-release CTL assay. Table 4 shows that CTL from mice immunized with antigenic variant cells recognized the immunizing variant cells, the parent cells, and the nonimmunogenic clone cells, but not the other antigenic variant cells. The amount of cytotoxicity against the parent tumor and nonimmunogenic clone was less than that against the immunizing variant. These results suggest that there are CTL-defined antigens on the variant cells specific for the variant, as well as antigens shared with the parental and nonimmunogenic clone cells. Immunization with cells of the parent tumor or nonimmunogenic clone generated a small amount of CTL activity, but this was not greater than that generated against an unrelated tumor. Because this activity is nonspecific, it may represent a low level of natural killer cell activity. This experiment was performed twice with the same results.

Growth of an Antigenic Variant in UV-irradiated Mice. To determine whether antigenic variants derived from K1735 mel-
anoma express the common, UVR-associated antigenic determinant recognized by UVR-induced Ts cells, K1735-LD.1 was injected into chronically UV-irradiated mice. As a positive control, the UV-2240 fibrosarcoma was also injected into mice. This tumor cell line grows in UV-irradiated and immunosuppressed mice but not in untreated mice. In addition, mice were challenged with the MCA-FM1 fibrosarcoma, an antigenic variant derived from a tumor induced in a C3H mouse by injection of the chemical carcinogen 3-methylcholanthrene and treated with the mutagen MNNG in vitro. MCA-FM1 cells grow in immunosuppressed but not in normal mice. The results, shown in Table 5, indicate that the K1735 antigenic variant LD.1, like the UV-2240 cells, grew in UV-irradiated and immunosuppressed mice, but not in untreated mice. These results are the same as those of previous experiments utilizing antigenic variants derived from UV-irradiated SF19 fibrosarcoma cells (13). In contrast, MCA-FM1 cells grew only in the immunosuppressed mice, but not in normal or UV-irradiated mice. The parental K1735 cell line produced tumors in all groups of mice. Therefore, the K1735-LD.1 cells exhibited a pattern of growth unique to tumors bearing the UVR-associated antigen recognized by UVR-induced Ts cells. The rejection of the MNNG-induced antigenic variant MCA-FM1 in UV-irradiated mice suggests that the expression of the UVR-associated common antigen may be restricted to antigenic variants induced by UVR.

**DISCUSSION**

In these studies, we investigated whether the antigenic variants generated from UV-irradiated, nonimmunogenic murine tumor cells exhibit the UVR-associated antigen unique to UVR-induced tumors and whether the variants share antigens with the parental cells and other clones derived from UV-irradiated cultures.

Our study demonstrates that in vitro UV irradiation of a weakly antigenic melanoma cell line generates antigenic clones that exhibit the UVR-associated common antigen determinant recognized by the Ts cells induced in chronically UV-irradiated mice. In our experience, this antigen is found only on skin cancers induced in vivo or in vitro by UVR (4, 6, 23-25). Previously, we reported the presence of the UVR-associated antigen on antigenic variants derived from a spontaneous C3H strain fibrosarcoma (13). The induction of the UVR-associated antigen on a weakly antigenic tumor cell by in vitro exposure to UVR implies that at least some UVR-associated antigens arise as a consequence of exposing cells to UVR and that they may occur independently of the initial neoplastic transformation event.

The results of our *in vivo* and *in vitro* studies indicate that in addition to the UVR-associated antigen, the antigenic variants exhibit both unique, variant-specific antigens and parent-tumor-derived antigens. The antigenic variants produce a high level of protective immunity in vivo, which enables the murine host to reject the immunizing variant, the parental cells, and cells of the nonimmunogenic clone. These results indicate that the antigenic variants express a determinant that is also expressed by the parental and nonimmunogenic clone cells. This determinant seems to be nonimmunogenic by itself, but very immu-

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**Table 3** Specificity of helper T-lymphocytes for individual K1735 tumor lines

<table>
<thead>
<tr>
<th>T-helper cells immunized to*</th>
<th>TNP-K1735</th>
<th>TNP-OR.5</th>
<th>TNP-LD.1</th>
<th>TNP-OR.3</th>
<th>TNP-MCA113</th>
</tr>
</thead>
<tbody>
<tr>
<td>K1735</td>
<td>131 ± 11†</td>
<td>113 ± 15</td>
<td>169 ± 27</td>
<td>192 ± 12</td>
<td>19 ± 1</td>
</tr>
<tr>
<td>K1735-OR.5</td>
<td>1419 ± 79</td>
<td>1735 ± 64</td>
<td>131 ± 13</td>
<td>1181 ± 171</td>
<td>46 ± 6</td>
</tr>
<tr>
<td>K1735-LD.1</td>
<td>1328 ± 137</td>
<td>2076 ± 84</td>
<td>206 ± 37</td>
<td>148 ± 9</td>
<td>10 ± 9</td>
</tr>
<tr>
<td>K1735-OR.3</td>
<td>171 ± 7</td>
<td>210 ± 136</td>
<td>27 ± 4</td>
<td>18 ± 7</td>
<td>24 ± 8</td>
</tr>
<tr>
<td>No immunization</td>
<td>21 ± 9</td>
<td>30 ± 5</td>
<td>27 ± 4</td>
<td>18 ± 7</td>
<td>24 ± 8</td>
</tr>
</tbody>
</table>

*Normal C3H mice were immunized i.p. three times at weekly intervals with 2 × 10⁸ cells of the appropriate tumor. 1 week after the third immunization, NW-nonadherent spleen cells were isolated from tumor-immunized and nonimmunized mice and test for Th activity in vitro. The NW-purified Th cells were cocultivated in vitro for 5 days with TNP-SRBC-primed B-cells and TNP-conjugated tumor cells.

**Table 4** Specificity of CTL for individual K1735 tumor lines

<table>
<thead>
<tr>
<th>CTL from mice immunized with*</th>
<th>K1735</th>
<th>K1735-OR.5</th>
<th>K1735-LD.1</th>
<th>K1735-OR.3</th>
<th>MCA113</th>
</tr>
</thead>
<tbody>
<tr>
<td>K1735</td>
<td>12.5 ± 4.1†</td>
<td>12.9 ± 6.2</td>
<td>17.3 ± 5.7</td>
<td>14.0 ± 2.5</td>
<td>12.0 ± 4.9</td>
</tr>
<tr>
<td>K1735-OR.5</td>
<td>42.3 ± 5.4</td>
<td>70.7 ± 9.9</td>
<td>19.9 ± 5.4</td>
<td>36.4 ± 3.1</td>
<td>12.8 ± 4.2</td>
</tr>
<tr>
<td>K1735-LD.1</td>
<td>53.6 ± 9.0</td>
<td>15.0 ± 5.2</td>
<td>67.5 ± 13.3</td>
<td>41.6 ± 18.2</td>
<td>10.1 ± 5.6</td>
</tr>
<tr>
<td>K1735-OR.3</td>
<td>16.3 ± 5.8</td>
<td>13.7 ± 3.9</td>
<td>17.9 ± 6.6</td>
<td>13.9 ± 5.5</td>
<td>13.0 ± 6.1</td>
</tr>
<tr>
<td>No immunization</td>
<td>0.0 ± 0.0</td>
<td>2.0 ± 3.2</td>
<td>1.5 ± 4.3</td>
<td>0.8 ± 0.9</td>
<td>4.2 ± 5.6</td>
</tr>
</tbody>
</table>

*Nylon wool-purified spleen cells from mice immunized with 2 × 10⁸ cells of the appropriate tumor cell line 2 weeks prior to *in vitro* culturing. A 4-h, ⁵¹Cr-release assay was done as described in "Materials and Methods." Effector-to-target ratio was 100:1.

**Table 5** Growth of K1735 melanoma-derived antigenic variant cells in normal, UV-irradiated, and immunosuppressed mice

<table>
<thead>
<tr>
<th>Tumor cell line*</th>
<th>Normal mice</th>
<th>UV-irradiated mice</th>
<th>Immunosuppressed mice</th>
<th>P &lt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>K1735 (parental)</td>
<td>5/5</td>
<td>5/5</td>
<td>4/4</td>
<td>NS*</td>
</tr>
<tr>
<td>K1735-LD.1</td>
<td>0/5</td>
<td>5/5</td>
<td>4/4</td>
<td>0.025</td>
</tr>
<tr>
<td>UV-2240</td>
<td>0/5</td>
<td>5/5</td>
<td>4/4</td>
<td>0.004</td>
</tr>
<tr>
<td>MCA-FM1</td>
<td>0/5</td>
<td>0/5</td>
<td>4/4</td>
<td>NS</td>
</tr>
</tbody>
</table>

*C3H mice were injected with 2 × 10⁸ K1735, K1735-LD.1, or MCA-FM1 cells and 2 × 10⁶ UV-2240 cells s.c., according to their minimal tumorigenic dose. The mice were checked weekly for tumor growth for 6-8 weeks.

*Thymectomy at 4 weeks of age, followed by whole-body exposure to 450-rad X-irradiation.

*Fisher’s exact test utilized to compare growth of tumor cells in normal mice versus UV-irradiated mice; NS, nonsignificant.
nonsensitive. Despite the apparent inability of the parental or nonimmunogenic cells to induce a detectable immune response, they are nevertheless rejected by an immune response induced by the antigenic variants. The specificities of Th and CTL in the in vitro assays are identical to the specificity of the immune response observed in vivo, suggesting that these cells may be important in the immune-mediated resistance to tumor challenge.

The ability of the variants to cross-react with the parental cells is consistent with the results of other investigators using different mutagenic agents (26–33). In most of these studies, the variant lines induced a weak immune protection against other antigenic variant lines (26–32), and Peppoloni et al. (33) reported that UVC radiation (254 nm)-induced antigenic variants exhibited complete interclonal cross-reactivity. In our studies in which UVB radiation was used, mice immunized with one variant were not able to reject a challenge with cells of another variant. Therefore, no evidence of cross-reactivity between antigenic variants in vivo was found (Tables 1 and 2). This lack of cross-reactivity is not attributable to our use of X-irradiated cells for immunization because identical results were obtained in studies using non-X-irradiated cells (13). The reason for this difference between antigenic variants induced with UVC and UVB radiation is not clear, but it may be due to individual properties of the cell lines used, to shared viral antigens, or to the fact that various wavelengths of UVR have different biological effects. The individual specificity of variant cell lines produced by UVB radiation resembles that of skin cancers induced in mice in vivo by repeated exposure to UVR, as tested by both in vivo (1, 3) and in vitro (8, 9, 11, 12, 34) assays.

The lack of detectable in vivo and in vitro cross-reactivity between the antigenic variants in this study is interesting in light of the fact that in the same assays, the variants share antigens with all other cell lines derived from the parent, i.e., parent cells themselves and the nonimmunogenic clone cells. From these observations, it appears that the presence of a unique, variant-specific antigen on an antigenic variant permits the induction of a tumor rejection response against all tumors derived from the parental cells unless a different, dominant, variant-specific antigen is exhibited by the cells. In support of this theory, Wortzel et al. have identified multiple antigens on a tumor induced in mice by UVR, which are expressed in a hierarchical manner (11, 12). Thus, at least for one UVR-induced tumor, the immune system responds to different combinations of antigens present on tumor cells in a specific and unique way.

The structural arrangement of the antigens induced in the parent tumor cells by exposure in vitro to UVR is not known. However, based on the results of these experiments, two models can be proposed. Evidence from studies in other systems has suggested that the immune response against a weakly immunogenic determinant can be elicited only if another (helper) determinant is corecognized on the same cell (35, 36). Likewise, UVR may induce a new determinant on native, nonimmunogenic, cell surface molecules thereby producing an "altered self" molecule that is now recognizable by the immune system. These determinants may occur on the same (Fig. 1A) or different (Fig. 1B) cell surface molecules exhibited on a single cell. In the first model (Fig. 1A), exposure to UVR of a parental cell exhibiting a nonimmunogenic cell surface molecule results in a new, unique epitope(s) on this molecule. Each antigenic variant expresses its own neoepitope, as well as the nonimmunogenic (parent) molecule, whereas the nonimmunogenic clones express no new epitopes on the common molecule. In addition, the

UVR-associated common antigen that is recognized by UVR-induced suppressor cells is expressed by the UVR-exposed parent cells. Immunization with an antigenic variant results in immune effector cells that recognize the neoepitope alone, the neoepitope/parent molecule in combination, and the parental molecule by itself. Cells that recognize the neoepitope mediate rejection of the immunizing antigenic variant and are therefore variant-specific immune cells. The cells that recognize the neoepitope/parental combination or the parent molecule by itself mediate the cross-reactive immunity that results in the rejection of the parental and nonimmunogenic clones. These same effector cells specific for the parental antigen do not recognize the

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**Fig. 1.** Models for the arrangement of the antigenic determinants present on UVR-induced antigenic variants. According to these models, UVR radiation induces new epitopes on native, nonimmunogenic cell surface molecules. The new epitopes induced by UV radiation may be exhibited on (4) the same or (B) different molecules present on the surface of the tumor cells exposed to UV radiation. In addition, UV radiation induces the expression of the UVR-associated common determinant on the cells.
parental molecule on other antigenic variants because the parent molecule has been altered by the neoepitope unique to other antigenic variants. The presence of a different neoantigen may change the conformation of the parental molecule or sterically hinder its recognition such that parent antigen-specific effector cells no longer recognize the parent molecule.

This model is based on the assumption that the mutagenic effect of UVR resulting in increased antigenicity selectively alters one particular gene encoding a specific cell surface protein. In support of a selective mutation by UVR, bacterial mutagenesis studies have shown that UVR produces lesions in only a few specific places in bacterial DNA (37), and studies by Bohr et al. (38) suggest that repair of UVR-induced lesions may be selective for certain genes and not others. The major problem with this model is, however, that UVR-induced skin cancers do not seem to exhibit their unique antigens on the same cell surface molecule. The unique antigen on the UVR-induced parent 1591 is an altered histocompatibility antigen (39), whereas a different UVR-induced tumor (UV-2240) possesses unique antigens that are biochemically extractable with 1-butanol, a solvent that does not extract major histocompatibility complex antigens (40). In addition, the expected frequency of multiple mutations in the same gene would be exceedingly low, resulting in a very low frequency of antigenic variants. However, a high frequency of antigenic variants is produced by in vitro UVR irradiation.

Alternatively, UVR may induce the expression of unique antigenic determinants on different cell surface molecules as shown in Fig. 1B. In this model, following UV irradiation of the parental tumor cultures, the epitope unique for one antigenic variant is present on a cell surface molecule that is different from the molecule bearing the unique epitope of a second variant. UVR also induces the expression of the UVR-associated common antigen on the tumor cells. As in the first model, immunization with one antigenic variant would produce effector cells that recognize the unique epitope alone, the neoepitope in combination with a particular parent molecule, and the parental molecule by itself. Effector cells generated by immunization with a different antigenic variant would be specific for both a different unique epitope and a different parent molecule. The main problem with this model is that if effector cells can be generated that recognize the original parental molecules, why then do they not recognize and react against the same parent molecule present on another antigenic variant cell? One possibility is that the parent molecule exhibiting the neoantigen blocks the binding of the effector cells specific for other parent molecules by sterically hindering or allosterically changing the conformation of these molecules on the cell surface. It may even be that the expression of the molecule bearing the neoantigen somehow leads to a decrease in the expression of other parent molecules on the cell surface. In light of the high frequency of antigenic variants produced in vitro by UVR, this second model may be more likely than the first, because exposure of a cell to UVR probably produces mutations in a variety of genes rather than multiple mutations in a single, exclusive gene. In this model, UVR may still produce lesions in selective regions of the genome as described for the first model, but no conclusive evidence exists that only one gene within this selected subset of genes is targeted for mutation. Finally, it is not yet clear whether the UVR-associated antigen and the tumor-specific antigen are induced in the same or separate cell surface molecules.

These models of course do not take into account other possible tumor-associated antigens, such as suppressogenic determinants and determinants recognized by other effector cells (macrophages, natural killer cells, and lymphokine-activated killer cells). All of these may be involved in the complex reaction that results in selective recognition and rejection of the tumor cells. Additional analysis of the immunogenic determinants present on the antigenic variants at a molecular level, as well as analysis of the specificity of cloned effector/effector cells, is necessary to distinguish between the two models proposed above.

Our studies demonstrate that in vitro exposure of nonantigenic tumors to UVB radiation induces antigenic variants that exhibit antigenic characteristics similar to that expressed by UVR-induced tumors and are capable of immunizing against a challenge with the parental tumor from which the variants were derived. The ability to produce in vitro a highly antigenic variant that is recognized and rejected by the immune system and that also cross-reacts with the parental nonantigenic tumor is of considerable importance for the immunotherapy of cancer. As a result of the antigenic cross-reactivity between UVR-induced tumor variants and the parent tumor cells, tumors that elicit a poor immune response because of low antigenicity could be removed surgically, established in short term culture, exposed to UVR in vitro, and used for immunization against recurrence of the primary tumor or metastases.

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REFERENCES


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Specificity of Antigens on UV Radiation-induced Antigenic Tumor Cell Variants Measured \textit{in Vitro} and \textit{in Vivo}

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