Immunogenic Variants of a Murine Fibrosarcoma Induced by Mutagenesis: Requirement of Viable Cells for Antigen-specific Cross-Protection

Stephen J. LeGrue, William J. Simcik, and Philip Frost

Department of Immunology [S. J. L., W. J. S./] and Cell Biology [P. F.], The University of Texas System, M. D. Anderson Hospital and Tumor Institute at Houston, Houston, Texas 77030

ABSTRACT

The purpose of the study was to investigate the immunological and biological consequences of neoantigen expression by immunogenic tumor variants (Imm*) following in vitro treatment with the mutagen 1-methyl-3-nitro-1-nitrosoguanidine. The weakly immunogenic murine fibrosarcoma MCA-F was used because we have previously characterized the tumor-specific transplantation antigen expressed by this tumor. Immunogenic variant clones were obtained at high frequency following four treatments with 1-methyl-3-nitro-1-nitrosoguanidine. The immunogenicity of the Imm* clones was confirmed by their progressive growth in immunosuppressed C3H/HeN mice and their lack of growth in normal syngeneic C3H/HeN mice. The immune response engendered in immunocompetent mice after a single immunization with viable Imm* cells was tumor specific, completely protecting hosts against challenge with 10,000-fold the minimum tumorigenic dose of parental MCA-F cells, but not against 10 minimum tumorigenic doses of the non-cross-reactive tumor MCA-D. The strong cross-protection elicited by Imm* neoantigens against the parental tumor-specific transplantation antigen was not observed when soluble extracts or isolated plasma membranes of Imm* cells were used for immunization. Immunogenic variant cells inactivated using either mitomycin C or γ-irradiation also demonstrated a significantly diminished immunoprotective activity against challenge with the parent tumor. However, inactivated Imm* cells and their isolated plasma membranes still expressed sufficient neoantigen to completely protect mice against homotypic Imm* but not parental challenge. These results suggest that (a) the MCA-F Imm* variants express neoantigens capable of engendering a strong specific as well as cross-protective immunity against challenge with either the parent or the variant and (b) the associative recognition of neoantigen and TSTA that results in strong cross-protection against challenge with the parent tumor requires immunization with viable Imm* cells for full expression of the immunogenic phenotype.

INTRODUCTION

One of the major impediments to the use of tumor-derived products for active specific immunotherapy has been the lack of strongly immunogenic tumor rejection antigens on many human and chemically induced or spontaneous murine neoplasms (1–3). One strategy to circumvent this problem has been the induction of highly immunogenic tumor rejection antigens on weakly immunogenic tumors by treatment with mutagens such as the alkylating compounds MNNG and ethyl methanesulfonate or by the hypomethylating agent 5-aza-2'-deoxycytidine (4–9). The diversity of physical and chemical agents that produce Imm* variants at high frequency suggests that epigenetic mechanisms may be involved (6, 8).

We have used MNNG to induce highly immunogenic antigen expression on the weakly immunogenic, 3-methylcholanthrene-induced murine fibrosarcoma MCA-F. This model was chosen because the TSTA of MCA-F that serves as the focus of the host antitumor immune response has been characterized (10). Thus, we hoped to distinguish between altered or enhanced expression of existing tumor antigens and the induction of neoantigens.

One feature of the Imm* system that has attracted the attention of human tumor immunologists is the strong cross-protective immunity induced against antigens expressed on the weakly immunogenic parental cells (4–9). This stimulation of immunogenic potential following the expression of an additional antigen or epitope has been termed associative recognition (11).

Useful application of this model to cancer therapy would require a similar associative recognition be induced using nonviable tumor cells or subcellular vaccines. We report here that isolated Imm* plasma membranes and inactivated Imm* cells bear both the parental TSTA and the mutagen-induced neoantigen but lack the ability to mediate the associative recognition that results in strong cross-protection between the two antigens.

MATERIALS AND METHODS

Tumors and Mice. The MCA-F and MCA-D fibrosarcomas were induced in female C3H/HeJ mice using 3-methylcholanthrene, as described (12, 13). Tumors were stored in liquid N₂ and were used between the fourth and eighth transplant generations. Tumor cell lines were routinely screened and shown to be free of Mycoplasma infection and pathogenic viruses. Specific-pathogen-free C3H/HeN mice were purchased at 4 to 6 weeks of age from the Animal Production Facility of the Frederick Cancer Research Center (Frederick, MD) and maintained according to NIH guidelines for the care and use of laboratory animals. Syngeneic C3H/HeN mice were rendered immunoincompetent by aspiration thymectomy at 4 to 5 weeks of age, followed 2 or 3 weeks later by 450 R X-irradiation.

Treatment with MNNG. MCA-F parent cells (3 × 10⁶) in 5 ml medium containing 10% fetal bovine serum were incubated for 1 h at 37°C with 3 µg/ml MNNG (Aldrich Chemical, Milwaukee, WI). The cells were washed 3 times and returned to culture. This regimen was repeated every 7 days for a total of 4 treatments to stabilize immunogenic phenotype (6, 7). Following the fourth treatment, the cells were propagated in vitro for 3 days, at which time they were cloned by limiting dilution at a multiplicity of 1 cell/well. Wells containing a single cell were expanded in vitro, and 15 clonal isolates were analyzed for their capacity to grow in syngeneic mice to identify the immunogenic clones (7). Four clones were isolated that failed to grow in normal syngeneic hosts but grew in adult thymectomized, 450-R-X-irradiated hosts. The frequency of variant generation (4 of 15) is in agreement with previous reports (6–9). The immunogenic clones were denoted MCA-F-4C, MCA-F-4D, MCA-F-4H, and MCA-F-4J.

Immunoprotection Assays. Mice were immunized by s.c. injection of 5 × 10⁴ tumor cells or various doses of subcellular vaccines 10 days before challenge on the contralateral flank with a supralethal number (10⁴ to 10⁵) of in vitro propagated MCA-F parental tumor cells. To demonstrate tumor specificity, some mice were challenged with the non-cross-reactive tumor MCA-D (10, 13).

Partially purified plasma membranes and crude butanol extracts were prepared as described previously (14–16). Nonviable tumor cell vaccines were prepared by incubating 10⁴ MCA-F-4C variant tumor cells in 1 ml PBS containing 50 µg mitomycin C for 30 min at 37°C or by

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To whom requests for reprints should be addressed, at Department of Immunology, M.D. Anderson Hospital and Tumor Institute, 6723 Bertner Avenue, Houston, TX 77030.

The abbreviations used are: MNNG, 1-methyl-3-nitro-1-nitrosoguanidine; TSTA, tumor-specific transplantation antigen; MCA, 3-methylcholanthrene; PM, plasma membrane; PBS, phosphate-buffered saline.
treatment with 12,000 R from a $^{137}$Cs source. Cells were washed twice prior to injection.

To evaluate the immunoprotective activity of variant cells against homotypic challenge, a modified procedure adapted for regressor tumors was used (17). Briefly, normal mice immunized as described above were irradiated with 450 R 8 days after immunization to dampen primary immune responses, while sparing memory responses (17). Two days after irradiation, mice were challenged, and tumor growth was monitored.

Statistical Analyses. Differences in tumor incidence were determined using the Fisher exact probability test. The extent of tumor growth was analyzed by the Student-Newman-Kuels multiple comparison test, which uses both an analysis of variance and a pooled error rate (10).

RESULTS

Immunogenicity of the Variant Clones. The immunogenicity of the 4 variant clones isolated following MNNG treatment of the MCA-F parent fibrosarcoma was demonstrated using normal and immunosuppressed syngeneic hosts. Challenge of C3H/HeN mice with 10$^5$ cells from any of the variant clones failed to produce tumors, while challenge with the parental MCA-F line invariably resulted in tumor outgrowth (Table 1). Challenge of adult thymectomized 450-R-X-irradiated mice with either parent or variant cells produced progressive tumor growth in all instances. Thus, the immune status of the host was a critical factor in determining the growth of immunogenic variants.

The specificity of the immune response engendered by 2 of the variant clones was investigated using the parental MCA-F cell line and an immunologically distinct tumor, MCA-D. Mice immunized with MCA-F-4J or MCA-F-4C cells vigorously resisted the growth of MCA-F (Table 2). Growth of the non-cross-reactive MCA-D tumor was unaffected by immunization with the variant clones, demonstrating that the Imm+ cells engender a strong, antigen-specific cross-protection against parental antigen.

Subcellular Vaccines. The strong immunoprotective response elicited by immunization with intact variant cells suggested that the strong rejection antigen might be localized at the plasma membrane. As reported previously (16, 18, 19), membranes from the parental MCA-F cell line protected mice at doses of 30 and 100 $\mu$g of membrane protein, yielding a specific activity of 33 units/mg (Table 3). Plasma membrane preparations from the variant cell lines MCA-F-4C and MCA-F-4J were only marginally more active than the materials derived from the parental cell line, with specific activities of about 100 units/mg.

The inability to demonstrate the strong immunoprotective antigen activity using Imm+ plasma membrane might have been attributable to a dosage effect. To test this hypothesis, mice were immunized every 7 days for 3 weeks using 30 $\mu$g of either parent or variant plasma membranes (Fig. 1). Mice immunized once with either PBS or viable MCA-F-4C cells served as negative and positive controls, respectively. Ten days after the last immunization, mice were challenged with MCA-F. Although multiple immunizations with MCA-F-4C membranes did engender a stronger immunoprotective response than did parental membranes ($P < 0.02$), the effect was not as profound as a single immunization with viable MCA-F-4C cells ($P < 0.05$). The growth rates of tumors from membrane-immunized hosts were intermediate between the positive (MCA-F-4C) and negative controls.
IMMUNOGENIC TUMOR VARIANTS INDUCED BY MUTAGENESIS

In a manner analogous to our results with isolated Imm* PM, inactivated MCA-F-4C variant cells unable to induce protection against the parental MCA-F cell line did protect mice against challenge with Imm* cells (Table 6). Again using the sublethal irradiation experimental paradigm (17), we observed that MCA-F-4C cells inactivated with mitomycin C completely protected mice against challenge with Imm* cells, but not with the parental cells (Table 6). Interestingly, immunization with a mixture of viable MCA-F-4C cells and MCA-D cells did not stimulate a strong anti-MCA-D immune response (data not shown), confirming that the observed associative recognition between neoantigen and TSTA requires coexpression on the same cell (11).

DISCUSSION

A number of physical and chemical agents have been shown to induce the expression of strong rejection antigens by weakly or nonimmunogenic tumors (4-8, 20-23). The protective immune response is cell mediated (6, 22) and specific for the tumor of origin (6, 7, 9, 22). The relative ease and high frequency of immunogenic variant generation has suggested that such cell lines might prove useful in the active specific immunotherapy of human neoplastic diseases.

The molecular nature of the mutagen-induced tumor rejection antigens remains obscure. Increased expression of class I or class II major histocompatibility antigens has been suggested as one possible explanation for the generation of immunogenic variants (24). Conversely, enhanced immunogenicity of existing tumor antigens or associative recognition of existing antigens in the context of immunogenic neoantigens are also attractive models. In an attempt to examine these possibilities, we chose to generate immunogenic variants of the chemically induced murine fibrosarcoma MCA-F. This tumor was used for three reasons: (a) the line expresses both K and D class I major histocompatibility antigens, without detectable class II expression (14); (b) immunization with the weak TSTA expressed by materials derived from the parent tumor. Inactivation of the cells using irradiation or mitomycin C also resulted in the loss of the strong immunogenic activity. Similar results were reported by Koyama and Ishii (21) following freezing and thawing of Imm* tumor challenged s.c. with either MCA-F or MCA-F-4C cells (Table 4). In this model, Imm* cells completely protected mice against challenge with either parent or Imm* cells. However, Imm* PM were effective only in immunizing hosts against Imm* challenge. Thus, Imm* PM express the strong neoantigen but do not mediate associative recognition of parental antigen.

The absence of the strong cross-protective tumor rejection antigen activity by membranes from Imm* lines led us to investigate the immunoprotective capacity of whole cells rendered nonviable by treatment with mitomycin C (Table 5). Surprisingly, only viable MCA-F-4C cells could engender a completely protective response against challenge with parental MCA-F cells. Similar results were obtained when variant cells were inactivated by γ-irradiation (data not shown). Thus, only intact viable immunogenic variant cells can elicit a strong protective immune response against subsequent challenge with parent tumor.

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Table 4 Expression of immunogenic neoantigen by variant plasma membranes

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Tumor</th>
<th>Incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBA</td>
<td>MCA-F</td>
<td>5/5</td>
</tr>
<tr>
<td>MCA-F-4C</td>
<td>10° cells</td>
<td>0/5*</td>
</tr>
<tr>
<td>4C-PM</td>
<td>10 µg</td>
<td>MCA-F</td>
</tr>
<tr>
<td>4C-PM</td>
<td>30 µg</td>
<td>MCA-F</td>
</tr>
<tr>
<td>4C-PM</td>
<td>100 µg</td>
<td>MCA-F</td>
</tr>
<tr>
<td>PBS</td>
<td>MCA-F-4C</td>
<td>5/5</td>
</tr>
<tr>
<td>MCA-F-4C</td>
<td>10° cells</td>
<td>0/5*</td>
</tr>
<tr>
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</tr>
<tr>
<td>4C-PM</td>
<td>100 µg</td>
<td>MCA-F-4C</td>
</tr>
</tbody>
</table>

* Statistically significant difference from PBS controls at P < 0.01, using the Fisher exact test.

Table 5 Immunoprotective activity of untreated and mitomycin C-treated MCA-F-4C cells

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Tumor</th>
<th>Incidence</th>
<th>Mean tumor diameter (mm ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>MCA-F</td>
<td>5/5</td>
<td>9.6 ± 1.2</td>
</tr>
<tr>
<td>MCA-F-4C</td>
<td>MCA-F</td>
<td>1/5*</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td>MCA-F-4C (mitomycin C)</td>
<td>MCA-F</td>
<td>5/5</td>
<td>8.6 ± 0.9</td>
</tr>
</tbody>
</table>

* Statistically significant at P < 0.01, using the Fisher exact test.

* Statistically significant at P < 0.001, using the Student-Newman-Keuls multiple comparison test.

* Statistically significant at P < 0.05.

Table 6 Effect of immunization with mitomycin C-treated MCA-F-4C cells on homotypic challenge with variant cells

<table>
<thead>
<tr>
<th>Immunizing cell</th>
<th>Challenge cell</th>
<th>Incidence</th>
<th>Mean tumor diameter (mm ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>MCA-F</td>
<td>5/5</td>
<td>9.6 ± 1.2</td>
</tr>
<tr>
<td>MCA-F-4C</td>
<td>MCA-F</td>
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* Statistically significant at P < 0.01, using the Fisher exact test.

* Statistically significant at P < 0.001, using the Student-Newman-Keuls multiple comparison test.

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lines and by Georlette and Boon (23) using irradiated teratocarcinoma variants. In other studies, irradiated Imm* variants did confer protection (4).

Many transplantable murine neoplasms engender the strongest antitumor immune response following immunization with metabolically active tumor cells (13, 16, 19, 25, 26). Progressive tumor growth from the immunizing cells is usually avoided by treating the cells with genotoxic agents such as radiation or mitomycin C just prior to use (2, 25). Conversely, subcellular vaccines and soluble extracts usually display a lower specific immunogenic activity, when compared to immunization with an equivalent number of cells or amount of cellular protein (1, 10, 13, 14, 27). Thus, one possible explanation for the observed absence of the strong immunogenic activity from subcellular vaccines of the immunogenic variants might be that the viable cells provided a larger antigen dose over a more prolonged period than did a single bolus of soluble antigen or isolated plasma membrane. Although this model is supported by the observation that repeated immunizations with plasma membranes from MCA-F-4C yielded an enhanced resistance to challenge with MCA-F, our results also demonstrate that a single immunization with inactivated Imm* cells or PM yields a strong protective primary response to the neoantigen.

Tumor cells treated with irradiation or mitomycin C may remain metabolically active but do not grow. Thus, differential antigen expression during cell cycle progression might account for the highly immunogenic phenotype of these Imm* cells. The similar results obtained with the two different methods of cell inactivation should rule out unanticipated “side effects” of either treatment alone. Conversely, neoantigen gene expression may be more sensitive to radiation or mitomycin C. This seems unlikely, however, because other immunogenic variant systems exhibit a similar phenomenon (21, 23), and sufficient neoantigen was expressed to induce protection against Imm* challenge.

The most striking observation of the present study was the ability of inactivated Imm* cells to completely protect against challenge with variant but not parental cells. These data demonstrate that the defect in immunogenic potential of inactivated variant cells is not attributable to altered neoantigen expression, but rather to a loss of associative recognition (11) with the parental TSTA. The simple coexpression of neoantigen and TSTA on the surface of the Imm+ cells is required but is not sufficient for the strong immunoprotective response against the parent tumor. Thus, inactivated variant cells express TSTA activity (Table 5) and neoantigen activity (Table 6) without the strong cross-protection afforded by immunization with viable cells. However, data presented here cannot exclude the possibility that the neoantigen-mediated associative recognition is mediated by a parental antigen other than the TSTA.

Possible mechanisms to account for the apparent requirement of cellular viability for the expression of the immunogenic phenotype and the resulting associative recognition of neoantigen and parental TSTA include: (a) interaction of surface components on viable cells may provide a combinatorial epitope that is lost upon inactivation or isolation; (b) changes in membrane fluidity or architecture may destroy one or more cross-protective epitopes; or (c) diminished local production of monokines and lymphokines may alter lymphocyte recruitment or activation. Whatever the mechanism of immunogenicity engendered by mutagen treatment, evidence presented here demonstrates that Imm* phenotype results from expression of a neoantigen and that associative recognition of both neoantigen and TSTA requires viable cells. Furthermore, these studies suggest that immunogenic variants may be useful in human tumor immunotherapy only when intact, viable cells are used.

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