Immune Response to Progressor Variants Derived from Transfection of an Ultraviolet Radiation-induced C3H Mouse Regressor Tumor Cell Line with Activated Harvey-ras Oncogene

David S. Kaba, William E. Pierceall, Janet E. Price, and Honnavara N. Ananthaswamy

Departments of Immunology (D. S. K., W. E. P., H. N. A.) and Cell Biology (J. E. P.), The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

ABSTRACT

Skin cancers induced in mice by UV radiation often exhibit a regressor phenotype. In order to determine how tumors escape the immune defenses of the normal immunocompetent host, we sought to isolate progressor variants from a UV radiation-induced C3H mouse regressor fibrosarcoma cell line, UV-2240, by transfection with an activated Ha-ras oncogene. A cotransfection protocol using pSV2-neo DNA, which confers resistance to the antibiotic G418, was used to select transfected cells. Injection of Ha-ras-transfected UV-2240 cells s.c. into immunocompetent C3H mice produced tumors in four of 36 animals. In contrast, UV-2240 cells transfected with pSV2-neo DNA alone or mock transfected with CaPO4 did not produce tumors in normal C3H mice. DNAs from cell lines established from Ha-ras-induced tumors contained unique Ha-ras sequences in addition to those sequences endogenous to UV-2240 cells. However, the Ha-ras-induced progressor variants did not overexpress the M, 21,000 protein. The Ha-ras-induced progressor variants produced experimental lung metastasis in both normal C3H and nude mice, although they induced more lung nodules in nude mice than in normal C3H mice. In addition, all four Ha-ras-induced progressor variants produced significantly more experimental lung metastases in nude mice than did the parent UV-2240 cell line. However, both the parental UV-2240 cell line and the Ha-ras-induced progressor variants expressed similar levels of H-2K and H-2D antigens and were immunologically cross-reactive, as determined by in vitro cytotoxic T-lymphocyte and in vivo immunization-challenge assays. These results indicate that the progressor phenotype of the Ha-ras-induced tumor variants is not due to loss of tumor-specific transplantation or Class I major histocompatibility complex antigens. This implies that some tumor cells can escape the immune defenses of the normal immunocompetent host by mechanisms other than loss of tumor-specific transplantation and Class I major histocompatibility antigens.

INTRODUCTION

Most skin cancers induced in C3H mice by UV radiation exhibit a regressor phenotype; i.e., they are rejected when transplanted into syngeneic immunocompetent hosts (1, 2). However, these tumors grow progressively and kill the host when transplanted into immunosuppressed mice. The ability of these tumors to grow in immunosuppressed hosts but not in normal syngeneic recipient mice suggests that the regression seen in normal hosts is immunologically mediated. In fact, it has been shown by in vivo (1) and in vitro (3–6) methods that tumor regression is accompanied by the development of immunity to the transplanted tumor; the resulting transplantation immunity is specific for the immunizing tumor and does not cross-react with other UV-induced tumors (7). These studies demonstrated that UV-induced murine regressor tumor variants express highly immunogenic TSTAs. Although the antigenic properties of UV-induced murine skin cancers resemble those of chemical carcinogen-induced tumors in rats and mice, the UV-induced tumors exhibit a much higher degree of antigenicity as a group than do chemically induced tumors.

In rare instances, variant cells that can grow progressively in normal syngeneic hosts arise from a population of regressor tumor cells. Such progressor variants from chemical (8) and physical (9–11) carcinogen-induced murine regressor tumors have been isolated. Schmitt and Daynes (9) isolated progressor variants from a UV-induced regressor tumor, RD-1024, by cloning in soft agar without any selection pressure and showed that the progressor clones still expressed the same major rejection antigens of the parent tumor. More recently, Lin et al. (12) isolated two types of transformed cells from normal BALB/c mouse cells by in vitro chemical carcinogenesis and found that one type of transformed cell produced tumors only in immunosuppressed mice, whereas the other type produced tumors in both normal and immunosuppressed mice. Interestingly, both types of transformed cells expressed cross-reactive antigens. These data suggest that both immune cells and nonimmune natural cytotoxic cells play a role in protecting mice from transplanted tumors. Taken together, these results point to the possibility that tumor cells escape the immune defenses of the normal host by more than one mechanism.

The cellular and molecular events that result in cancer are poorly understood. The specific biological transitions that take place during tumor initiation and progression may be consequences of the activation of cellular protooncogenes. Recent studies have shown that activation of ras oncogenes may play a role in tumor progression (13–22). Tainsky et al. (22) and Vousden and Marshall (13) have shown that the appearance of an activated ras gene in late passages of a human teratocarcinoma and a mouse T-lymphoma cell line correlates with a more aggressive growth behavior. Thus, changes in ras expression or function may play a crucial role in tumor progression. Therefore, we transfected a cloned activated Ha-ras oncogene from a human bladder carcinoma (23, 24) into a regressor UV-2240 cell line in an attempt to isolate progressor variants. In this report, we describe the isolation and characterization of Ha-ras-induced progressor variants from the regressor UV-2240 tumor.

MATERIALS AND METHODS

Animals. Six- to 8-wk-old female C3H/HeNCr (mammary tumor virus negative) and athymic (nu/nu) nude mice were purchased from the Frederick Cancer Research Animal Production Area, Frederick, MD. The mice were housed in a specific-pathogen-free animal facility.

Received 7/25/88; revised 1/26/90.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported by NIH Grant CA 40454.

2 Supported by predoctoral fellowships from the R. E. "Bob" Smith Foundation.

3 To whom requests for reprints should be addressed, at Department of Immunology, Box 178, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030.

* The abbreviations used are: TSTAs, tumor-specific transplantation antigens; MHC, major histocompatibility complex; MEM, minimal essential medium; BCS, bovine calf serum; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodeyl sulfate-polyacrylamide gel electrophoresis; FITC, fluorescein isothiocyanate; GAM, goat antimitomouse; CTLs, cytotoxic T-lymphocytes; MLTC, mixed lymphocyte tumor culture.
Tumor Cell Lines. The regressor UV-2240 fibrosarcoma was induced in a C3H/HeN (mammary tumor virus negative) mouse by repeated exposure to UV radiation from a bank of six FS40 sunlamps (250 to 400 nm) (1, 2). The UV-2237 tumor was also induced in a C3H/HeN mouse by chronic UV radiation. However, this tumor grows progressively in normal C3H mice. The 10T-1 cell line arose by spontaneous transformation of C3H mouse embryo fibroblast 10T½ cells. A highly tumorigenic clonal cell line of 10T-1 was established by serial transplantation in normal C3H mice. Tumor cells were grown in Eagle’s MEM (Grand Island Biological Co., Grand Island, NY) supplemented with 10% FCS (Hyclone Laboratories, Inc., Logan, UT), 2% vitamin solution, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 2 mM glutamine, 100 IU/ml of penicillin, and 100 µg/ml of streptomycin. All the cell lines have been tested for and found free of Mycoplasma and other pathogenic viruses.

Plasmids. The pEJ plasmid contains the 6.6-kilobase BamHI fragment of the human EJ bladder carcinoma c-Ha-ras oncogene (23, 24). This plasmid (a gift from Dr. Mein Chi Hung, Department of Tumor Biology, M. D. Anderson Cancer Center) was introduced into Escherichia coli HB101 and selected for ampicillin resistance in our laboratory. The pSV2-neoEJ plasmid (25) is a recombinant of pSV2-neo into which the 6.6-kilobase BamHI fragment of EJ was inserted (kindly provided by Dr. Ruth Sager, Dana Farber Cancer Institute, Boston, MA). The pEJ and pSV2-neoEJ plasmids are biologically active; i.e., they transform NIH 3T3 and other normal cells (23-26). The pSV2-neo plasmid was obtained from Bethesda Research Laboratories, Inc. (Gaithersburg, MD). Plasmid DNAs were isolated according to the procedure described by Maniatis et al. (27). Supernatized plasmid DNAs were purified on cesium chloride gradients.

DNA Transfection. Recipient UV-2240 cells were plated at a density of 4 x 10⁶ cells/60-mm dish in MEM. Twenty-four h later, the cells were cotransfected by the calcium phosphate precipitation technique of Graham and Van der Eb (28) and Southern and Berg (29). Five µg of pEJ DNA, 1 µg of pSV2-neo DNA (which confers resistance to the antibiotic G418), and 20 µg of carrier DNA (C3H mouse skin) were applied to each dish. In experiments involving transfection with pSV2-neoEJ, 5 µg of pSV2-neoEJ DNA and 20 µg of C3H mouse skin DNA were added to each dish. As controls, UV-2240 cells were transfected with pSV2-neo plus carrier DNAs or mock transfected with CaPO₄.

About 24 h after transfection, cells from each dish were trypsinized and transferred to one T-150 flask. After another 16 to 20 h, G418 was added to the medium at a concentration of 200 µg/ml. The cells were exposed to G418 for 10 to 12 wk, when the tumors reached a diameter of about 10 mm, and were examined regularly, and weekly measurements of the tumors were taken using calipers. When the tumors reached a diameter of about 10 mm, the tumors were excised, and the skin incisions were closed with wound clips. The mice were killed 90 days after injection of cells, or sooner if they were moribund, and examined for the presence of lung metastases.

Spontaneous Metastasis Assay. Cells were harvested by trypsinization and washed with serum-free RPMI 1640 medium, and 2 x 10⁶ viable cells in 0.2 ml of RPMI 1640 medium were injected s.c. into groups of normal C3H or nude mice. Tumor growth was monitored weekly for up to 12 wk.

Expression of MHC Class I Antigens. Cell surface expression of H-2K² and H-2D² antigens was analyzed by indirect immunofluorescence using the monoclonal antibodies 16-1-11N and 15-5-5s, respectively (33). Single cell suspensions derived from monolayer cultures by treatment with trypsin and EDTA were washed with PBS, and 1 x 10⁶ cells were incubated with 100 µl of 16-1-11N or 15-5-5s antibodies (hybridoma culture supernatants) on ice for 60 min. After two washes with PBS containing 1% goat serum and 0.02% sodium azide, the cells were incubated on ice for 30 min with 50 µl of appropriately diluted FITC-conjugated GAM antibody (Cappel Laboratories, Cochranville, PA). After two additional washes, the cells were suspended in 1 ml of PBS containing 1% paraformaldehyde and were analyzed on a fluorescence-activated cell sorter (Ortho Cytocounter II; Ortho Diagnostics, Inc., Westwood, MA). Each cell line was stained with the second antibody alone to determine the background fluorescence.

Cytotoxic T-Lymphocyte Assay. CTLs were generated in MLTC from the spleen cells of syngeneic tumor-immune mice, as described by Kaba et al. (34). In brief, C3H mice were immunized by i.p. injection of 5 x 10⁶ UV-2240, 2240-EJ1, or 2240-EJ2 tumor cells that had been irradiated with 10,000 R of γ-radiation. Fourteen days later, their spleen cells were cocultured with 10,000 R of γ-irradiated UV-2240, 2240-EJ1, or 2240-EJ2 cells in RPMI 1640 medium supplemented with 10% BCS, 100 IU/ml of penicillin, 100 µg/ml of streptomycin, and 5 x 10⁻⁵ m 2-mercaptoethanol. The MLTC cultures set up in 24-well plates (5 x 10⁶ spleen cells plus 1 x 10⁶ γ-irradiated tumor cells) were incubated at 37°C for 5 days and then assayed for cytolytic activity against various targets. Target cells (5 x 10¹⁰) were labeled with 300 µCi of Na¹¹¹I for 45 min at 37°C. The washed target cells were mixed with various ratios of effector cells (in U-bottomed microtest plates) and incubated at 37°C for 4 h. The plates were centrifuged, and an aliquot of the supernatant from each well was counted for radioactivity.

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To locate the position of the bands complementary to the radioactive Ha-ras probe.

p21 Assay. Logarithmic phase cells were lysed with lysis buffer (0.5% Nonidet P-40-125 mM KC1-3.6 mM CaCl₂-5.7 mM MgCl₂-0.5 mM EDTA-30 mM Tris-HCl, pH 7.5), homogenized with a Dounce homogenizer, and centrifuged at 15,000 x g for 15 min. Aliquots of the supernatant (~100 µg of protein) were run on 12.5% SDS-PAGE (31), and the proteins were transferred to nitrocellulose filters by Western blotting technique (32). The blots were incubated overnight at 4°C with 3% bovine serum albumin and then with a mouse monoclonal antibody (ras;1; New England Nuclear) against the ras p21 overnight at 4°C. The blots were washed and incubated at room temperature for 2 h with 125I-labeled goat antiserum secondary antibody (IgG) (New England Nuclear). The blots were washed thoroughly, dried, and autoradiographed using Kodak XAR-5 film.

Tumor Growth Rate. Nearly confluent cells were harvested by trypsinization and washed once with serum-free RPMI 1640 medium, and 2 x 10⁴ viable cells in 0.2 ml of RPMI 1640 medium were injected s.c. into groups of normal C3H or nude mice. Tumor growth was monitored weekly for up to 12 wk.

Experimental Metastasis Assay. Cells were harvested by trypsinization and washed with serum-free RPMI 1640 medium, and 1 x 10⁶ cells (for nude mice) or 2 x 10⁵ cells (for normal C3H mice) in 0.2 ml of serum-free RPMI 1640 medium were injected i.v. into the lateral tail vein. The mice were killed 60 days after injection, or earlier if they were moribund, and examined for metastases as described above.

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The percentage of cytotoxicity was calculated as follows:

\[
\% \text{ of cytotoxicity} = \frac{\text{cpm}_{\text{test}} - \text{cpm}_{\text{max}}}{\text{cpm}_{\text{max}} - \text{cpm}_{\text{max}}} \times 100
\]

where \(\text{cpm}_{\text{test}}\) is counts obtained in the presence of effector cells, \(\text{cpm}_{\text{max}}\) is counts obtained when target cells were incubated with medium alone, and \(\text{cpm}_{\text{max}}\) is total counts obtained by treatment of the target cells with Triton X-100.

Immunization and Challenge Assay. 2240-EJ1 and 2240-EJ2 cells were tested for cross-reactivity with UV-2240 in an in vivo immunization challenge assay. When used as the immunizing cell lines, 2240-EJ1 and 2240-EJ2 were exposed to 10,000 R of \(\gamma\)-radiation to prevent progressive growth of these tumor cells. When UV-2240 was used as an immunogen, it was also \(\gamma\)-irradiated, even though UV-2240 does not grow in normal C3H mice. Cells were harvested by trypsinization and washed with serum-free RPMI 1640 medium, and 0.2 ml of suspension (2 \(\times 10^6\) cells) were injected s.c. into groups of normal C3H mice. As a control, groups of mice were given injections s.c. with 0.2 ml of RPMI 1640 medium alone. Two wk later, the mice were exposed to 450 R of X-irradiation to prevent a primary response to tumor challenge but leave a secondary response intact (35). Forty-eight h later, 2 \(\times 10^6\) 2240-EJ1, 2240-EJ2, UV-2240, or syngeneic, non-cross-reactive tumor cells such as UV-2237 or 10T-1 were injected s.c. on the opposite side. Tumor growth was monitored for 4 to 6 wk after challenge.

RESULTS

Efficiency of Transfection. UV-2240 cells were cotransfected with pEJ plus pSV2-neo plus C3H mouse skin DNAs (Table 1, Experiments 1 and 2) or with pSV2-neoEJ plus C3H mouse skin DNAs (Table 1, Experiment 3). Following transfection and selection in G418 medium, G418-resistant colonies were obtained at a frequency of 250 to 300 per 4 \(\times 10^5\) transfected cells. Approximately the same number of G418-resistant colonies was obtained when UV-2240 cells were cotransfected with pSV2-neo plus carrier DNAs. However, the number of G418-resistant colonies was reduced 10-fold when UV-2240 cells were transfected with pSV2-neo DNA alone in the absence of any carrier DNA. This suggests that the carrier DNA enhances the ability of UV-2240 cells to incorporate the cotransfected plasmid DNAs.

Tumorigenicity of Ha-ras-transfected UV-2240 in Normal C3H Mice. The tumorigenicity of UV-2240 transfecants was assessed by injecting 5 \(\times 10^6\) cells s.c. into normal C3H mice. Only a small percentage of immunocompetent mice developed progressively growing tumors (Table 1). In three separate experiments, 4 of 36 mice given injections of Ha-ras-transfected UV-2240 cells produced progressively growing tumors. In contrast, UV-2240 cells cotransfected with pSV2-neo and carrier DNA or mock transfected with CaPOj did not produce tumors in normal C3H mice. As expected, the regressor UV-2240 cell line did not produce tumors in normal C3H mice. In fact, the UV-2240 cell line was so highly antigenic that it did not induce tumors in any of 300 normal C3H mice given injections of >1 \(\times 10^7\) cells/mouse (data not shown). When the Ha-ras-induced tumors reached a mean diameter of 15 mm, they were excised and established in culture. The resultant cell lines were designated 2240-EJ1, 2240-EJ2, 2240-EJ3, and 2240-EJ4.

Presence of Ha-ras Sequences and p21 Expression in 2240 Progressor Variants. The Ha-ras-induced progressor variants were analyzed for the presence of the transfected Ha-ras gene. The proofs for molecular biology and monoclonal antibodies that ras protein was not highly amplified in progressor variants (Fig. 2). The slightly higher levels of p21 in 2240-EJ cell lines as compared with the UV-2240 cell line could be due to the expression of an additional copy of the transfected Ha-ras oncogene.

Growth Characteristics of Ha-ras-induced Progressor Variants. To ascertain whether the Ha-ras-induced progressor variants had acquired some growth advantage over the parental UV-2240 tumor cell line, we assessed the in vitro and in vivo growth characteristics of Ha-ras-induced progressor variants. In vitro, both the regressor UV-2240 and the Ha-ras-induced progressor variants exhibited more or less identical growth rates in medium containing either 0.5% or 5% BCS (data not shown).

The in vivo growth kinetics of Ha-ras-induced progressor variants and of the parent UV-2240 cell line was assessed by injecting 2 \(\times 10^6\) cells s.c. into normal C3H and nude mice. The results shown in Figs. 3 and 4, respectively, reveal that all four Ha-ras-induced progressor variants produced tumors in both the normal immunocompetent C3H and the immunodeficient nude mice. In contrast, the parent UV-2240 cell line induced tumors only in nude mice. The growth rates of UV-2240 and the Ha-ras-induced progressor variants in immuno-
Ha-ras-INDUCED TUMOR PROGRESSION

Fig. 1. Presence of unique Ha-ras sequence in 2240-EJ cell lines. Ten μg of DNA from each cell line were digested with BamHI and SstI restriction enzymes and analyzed by Southern blot hybridization to a 32P-labeled Ha-ras probe, as described in "Materials and Methods." Lane 1, UV-2240; Lane 2, 2240-EJ1; Lane 3, 2240-EJ2; Lane 4, 2240-EJ3; Lane 5, 2240-EJ4. Numbers on the ordinate are HindIII-digested λ-phage DNA molecular weight markers (in kilobases). The arrow indicates the unique 2.9-kilobase band present in 2240-EJ cell lines but absent in the UV-2240 cell line.

Fig. 2. Expression of p21 in Ha-ras-induced progressor variants. Cell lysates (~100 μg of protein/lane) were run on 12.5% SDS-PAGE, Western blotted onto nitrocellulose filters, and analyzed for p21 expression using ras-specific mouse monoclonal primary antibody and 125I-labeled goat anti-mouse secondary antibody, as described in "Materials and Methods." (A). Duplicate blots without treatment with the ras-specific primary antibody, but treated with secondary 125I-labeled goat antimouse antibody, did not reveal the p21 band (B). Lane 1, UV-2240; Lane 2, 2240-EJ1; Lane 3, 2240-EJ2; Lane 4, 2240-EJ3; Lane 5, 2240-EJ4. Numbers on the ordinate are molecular weight markers (in thousands).

Fig. 3. Growth kinetics of Ha-ras-induced progressor variants in normal C3H mice. Cells were harvested by trypsinization and washed with serum-free RPMI 1640 medium, and 2 × 10^6 cells in 0.2 ml of serum-free RPMI 1640 medium were injected s.c. into groups of normal C3H mice (5 mice/cell line). Tumor growth was monitored weekly. □, UV-2240; ●, 2240-EJ1; ●, 2240-EJ2; △, 2240-EJ3; ◊, 2240-EJ4.

Fig. 4. Growth kinetics of Ha-ras-induced progressor variants in athymic nude mice. Cells were prepared and injected s.c. into nude mice (5 mice/cell line), as described in the legend to Fig. 3. □, UV-2240; ●, 2240-EJ1; ●, 2240-EJ2; △, 2240-EJ3; ◊, 2240-EJ4.

deficient nude mice were similar (Fig. 4). However, there appeared to be some difference in the growth rate of the Ha-ras-induced progressor variants in normal C3H versus nude mice. The Ha-ras-induced progressor variants produced tumors in nude mice, with an average diameter of 20 to 25 mm, as early as 3 wk after injection (Fig. 4), whereas it took about 6 wk for them to produce tumors of comparable diameter in normal C3H mice (Fig. 3).

Metastatic Ability of Ha-ras-induced Progressor Variants.

Since Ha-ras gene transfection is known to enhance the metastatic potential of tumor cells (13–21), it was of interest to determine whether the Ha-ras-induced progressor variants of UV-2240 tumor had also acquired the metastatic phenotype. To study this, we used two types of assays. One involved injection of cells s.c. (spontaneous metastasis) and the other, injection of cells i.v. into the tail vein (experimental metastasis) of mice. Normal C3H and nude mice were used for these experiments.

When the Ha-ras-induced progressor variants were injected s.c. into normal C3H and nude mice, none of them produced visible colonies in the lungs or other internal organs in either normal C3H or nude mice (data not shown). As expected, the UV-2240 cell line produced s.c. tumors in nude mice but not
in normal C3H mice, whereas the four Ha-ras-induced progressor variants produced s.c. tumors in both normal C3H and nude mice (data not shown).

To determine whether the Ha-ras-induced progressor variants were capable of inducing experimental lung metastasis, we injected cells i.v. directly into the blood stream. All four Ha-ras-induced progressor variants produced lung metastasis in both normal C3H (Table 2) and nude mice (Table 3) following i.v. injection, whereas the parent UV-2240 cell line and UV-2240 transfected with pSV2-neo alone produced lung colonies only in nude mice (Table 3). Furthermore, the Ha-ras-induced progressor variants produced significantly more lung nodules in nude mice than did UV-2240 cells or UV-2240 cells transfected with pSV2-neo (2240-neo) (Table 3). The mean survival time (i.e., when mice became moribund or died) of nude mice given injections of the Ha-ras-induced progressor variants was shorter (15 to 17 days) than that of nude mice given injections of UV-2240 or 2240-neo cell lines (60 days). Although the Ha-ras-induced progressor variants produced experimental lung metastases in both normal and nude mice, there was a significant difference between normal and nude mice in the number of lung colonies produced. All four Ha-ras-induced progressor variants produced 4- to 20-fold more lung colonies in nude mice than in normal C3H mice.

Expression of H-2K\(^k\) and H-2D\(^d\) Antigens. To determine whether loss or decreased expression of MHC Class I antigens was associated with the progressor phenotype in Ha-ras-induced progressor variants, we analyzed cell surface expression of H-2K\(^k\) and H-2D\(^d\) antigens by indirect immunofluorescence. The results indicated that the Ha-ras-induced progressor variants expressed at least as much H-2K\(^k\) (Fig. 5) and H2-D\(^d\) (data not shown) antigens on their cell surfaces as did the parent UV-2240 cells. In fact, the percentage of antigen-positive cells was higher in 2240-EJ1 (83% for H2-K\(^k\) and 42.4% for H2-D\(^d\)) compared with the regressor UV-2240 (50.2% for H2-K\(^k\) and 22.1% for H2-D\(^d\)). This suggests that the progressor phenotype of Ha-ras-induced variants of UV-2240 tumor cells does not arise from loss or decreased expression of MHC Class I antigens.

Expression of TSTAs. To determine whether loss or decreased expression of TSTAs is the cause for the growth of Ha-ras-induced progressor variants in normal, immunocompetent hosts, we performed in vitro and in vivo cross-reactivity assays. If similar or the same TSTAs are expressed on two different tumors, an immune response induced by one tumor can also recognize and react with the second tumor. We thus explored differences between the regressor UV-2240 cells and the two representative Ha-ras-induced progressor variants, 2240-EJ1 and 2240-EJ1.

In vitro cross-reactivity was determined by a CTL assay. As shown in Fig. 6, CTLs generated against UV-2240 cells were quite capable of killing both 2240-EJ1 and 2240-EJ2 cells. In contrast, UV-2240-immune CTLs were unable to kill the unrelated UV-induced tumor cell line, UV-2237. As expected, CTLs generated by immunization with UV-2240 lysed \(^{51}\)Cr-labeled UV-2240 targets effectively.

In order to determine whether the Ha-ras-induced progressor variants were capable of generating CTLs, we performed a reciprocal experiment in which the MLTC derived from the spleen cells of mice immunized with \(\gamma\)-irradiated 2240-EJ1 progressor cells was tested for its ability to kill various targets. The results shown in Fig. 7 reveal that CTLs generated against 2240-EJ1 were able to kill not only the immunizing tumor target, 2240-EJ1, but also the second Ha-ras-induced progressor variant, 2240-EJ2, and the parent regressor UV-2240. As before, there was no significant killing of UV-2237 tumor targets by 2240-EJ1-specific CTLs.

Cross-reactivity between the regressor UV-2240 and the Ha-ras-induced progressor variants was also investigated by in vivo

### Table 2 Production of experimental lung metastasis in normal C3H mice by 2240-EJ cell lines

<table>
<thead>
<tr>
<th>Cell line injected</th>
<th>Time of autopsy (days)</th>
<th>Incidence</th>
<th>Median no. of lung tumor colonies/mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV-2240</td>
<td>60</td>
<td>0/5</td>
<td>0 (0)</td>
</tr>
<tr>
<td>2240-neo</td>
<td>60</td>
<td>0/5</td>
<td>0 (0)</td>
</tr>
<tr>
<td>2240-EJ1</td>
<td>25-60</td>
<td>5/5</td>
<td>95 (10-150)</td>
</tr>
<tr>
<td>2240-EJ2</td>
<td>25-60</td>
<td>5/5</td>
<td>75 (5-200)</td>
</tr>
<tr>
<td>2240-EJ3</td>
<td>35-60</td>
<td>3/5</td>
<td>12 (0-75)</td>
</tr>
<tr>
<td>2240-EJ4</td>
<td>60</td>
<td>3/5</td>
<td>15 (0-26)</td>
</tr>
</tbody>
</table>

* Tumor cells (1 x 10⁷/mouse) were injected i.v. into the lateral tail vein of normal C3H mice.
* The mice were killed and autopsied 60 days after injection or when they became moribund.
* Number of mice with lung tumor nodules per number of mice given injections.
* Numbers in parentheses, range.

### Table 3 Production of experimental lung metastasis in nude mice by 2240-EJ cell lines

<table>
<thead>
<tr>
<th>Cell line injected</th>
<th>Time of autopsy (days)</th>
<th>Incidence</th>
<th>Median no. of lung tumor colonies/mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV-2240</td>
<td>60</td>
<td>5/5</td>
<td>16 (8-31)</td>
</tr>
<tr>
<td>2240-neo</td>
<td>60</td>
<td>5/5</td>
<td>26 (11-63)</td>
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<tr>
<td>2240-EJ1</td>
<td>17</td>
<td>5/5</td>
<td>&gt;300 (290-&gt;300)</td>
</tr>
<tr>
<td>2240-EJ2</td>
<td>17</td>
<td>5/5</td>
<td>260 (94-269)</td>
</tr>
<tr>
<td>2240-EJ3</td>
<td>15</td>
<td>5/5</td>
<td>&gt;300 (280-&gt;300)</td>
</tr>
<tr>
<td>2240-EJ4</td>
<td>15-17</td>
<td>5/5</td>
<td>&gt;300 (187-&gt;300)</td>
</tr>
</tbody>
</table>

* Tumor cells (5 x 10⁷/mouse) were injected i.v. into the lateral tail vein of nude mice.
* The mice were killed and autopsied 60 days after injection or when they became moribund.
* Number of mice with lung tumor nodules per number of mice given injections.
* Numbers in parentheses, range.
or 2 X 10^5 cells of UV-2240. On Day 14, the mice were exposed to 450 R of X-
2237.

days. The lytic activity of these effector cells was tested against various target
cells in a 4-h Cr release assay. D, UV-2240; O, 2240-EJ1; •, 2240-EJ2; A, UV-
C3H mice were immunized by i.p. injections of 5 x 10^6 UV-2240 cells. Two wk
later, their spleen cells were cocultured with γ-irradiated UV-2240 cells for 5
days. The lytic activity of these effector cells was tested against various target
cells in a 4-h Cr release assay. D, UV-2240; O, 2240-EJ1; •, 2240-EJ2; A, UV-

Immunogen* Challenge tumor Tumor incidence † (no. of mice with tumor/no. of mice challenged)

RPMI 1640
UV-2240 10/10
2240-EJ1 10/10
2240-EJ2 10/10
UV-2237 10/10
10T-1 10/10

UV-2240
UV-2240 0/10
2240-EJ1 0/10
2240-EJ2 0/10
UV-2237 9/10
10T-1 10/10

* C3H mice were immunized with a single s.c. injection of RPMI 1640 medium or 2 x 10^5 cells of UV-2240. On Day 14, the mice were exposed to 450 R of X-
irradiation. Two days later, the mice were challenged on the opposite side with 2 x 10^5 cells of the indicated tumor cell lines. The results shown are pooled data from two separate experiments.

† Data are for 4 wk after challenge.

immunization and challenge assays. As shown in Table 4, immunization with the regresor UV-2240 tumor generated protective immunity against the immunizing UV-2240 tumor. In addition, there was complete cross-protection against challenge with both 2240-EJ1 and 2240-EJ2, but not against chal-

lenge with other syngeneic, non-cross-reactive progressor tumors, UV-2237 or 10T-1. As expected, all the mice immunized with RPMI 1640 medium and challenged with various tumor cell lines developed tumors at the challenge site.

In a reciprocal experiment, immunization with γ-irradiated 2240-EJ1 or 2240-EJ2 induced protective immunity not only against challenge with the immunizing tumor but also against challenge with the other Ha-ras-induced progressor variant and the parent UV-2240 tumor (Table 5). Once again, immunization with either 2240-EJ1 or 2240-EJ2 did not afford protection against challenge with unrelated syngeneic progressor tumors, UV-2237 or 10T-1. Taken together, these results suggest that the antigenic molecules expressed on Ha-ras-induced progressor variants are functionally cross-reactive with the parental UV-2240 cells.

**DISCUSSION**

In this study, we showed that transfection of a highly antigenic C3H mouse regressor tumor cell line with an activated Ha-ras oncogene produces progressor variants capable of inducing tumors in normal syngeneic hosts. Injection s.c. of UV-2240 cells cotransfected with pEJ and pSV2-neo or transfected with pSV2-neoEJ DNAs produced progressively growing tumors in 4 of 36 normal C3H mice in three separate experiments (Table 1). In contrast, neither the parent, untransfected UV-2240 cell line nor UV-2240 cells transfected with pSV2-neo DNA alone nor mock-transfected with CaPO4 produced tumors in normal C3H mice (0 of 72 mice). This contrast suggests that mere integration of any foreign DNA into the genome of UV-2240 cells is not sufficient to produce variants capable of growing in normal syngeneic hosts. In addition, these results indicate that the progressor variants were induced by Ha-ras oncogene transfection and not spontaneously. This conclusion is further supported by the fact that our attempts to isolate spontaneous progressor variants from the regressor UV-2240 cell line were unsuccessful. Injection of over 300 normal C3H mice with high cell numbers (1 x 10^7 cells/mouse) of UV-2240 did not give rise to a single progressor tumor (data not shown). The reason for this unusual behavior of UV-2240 cell line is unknown. In any case, based on these observations, we can conclude that the progressor variants were induced by Ha-ras oncogene transfection and not spontaneously.

DNAs from all four Ha-ras-induced progressor variants contained both unique and endogenous Ha-ras sequences (Fig. 1). However, none of the Ha-ras-induced progressor variants overexpressed the p21 product (Fig. 2), which suggests that the

immunization and challenge were performed as described in the legend to Table 4, except that 2240-EJ1 and 2240-EJ2 cells were exposed to 10,000 R of γ-irradiation before immunization. The results shown are pooled data from two separate experiments.

Data are for 4 wk after challenge.
progressor phenotype of the Ha-ras-induced tumor variants is not due to overexpression of p21.

Many tumor cells secrete growth factors and thus acquire partial or complete autonomy that permits proliferation in the absence or presence of very low concentrations of serum. Since ras and other oncoproteins have been shown to stimulate the production of growth factors (36, 37), we measured the growth rates of the UV-2240 parental cell line and its Ha-ras-induced progressor variants in liquid medium containing either 5% serum or 0.5% serum. However, growth rates did not significantly differ (data not shown). These results suggest that the progressor phenotype of 2240-EJ cell lines is not due to an serum or 0.5% serum. However, growth rates did not significantly differ (data not shown). These results suggest that the progressor phenotype of 2240-EJ cell lines is not due to an increased growth rate in vitro. In agreement with this observation, Vousden et al. (18) reported that mouse carcinoma cells transfected with an activated Ha-ras oncogene did not show an increased growth rate in vitro, even though they produced significantly more metastasis. However, Price et al. (38) found that cloned K-1735 melanoma cells transfected with an activated Ha-ras oncogene proliferated faster in medium containing 0.5% serum than did untransfected cells. In addition, transfection of some normal and tumorigenic cell lines with an activated Ha-ras oncogene has been shown to accelerate the growth of primary tumors (17, 18, 38, 39). However, in our study, the in vitro growth rates in nude mice of Ha-ras-induced progressor variants and the parental UV-2240 cell line did not significantly differ (Fig. 4). Thus, these contradictory results point to the possibility that any correlation between various biological parameters may depend in part on the intrinsic properties of the recipient cell used in transfection.

Even though both UV-2240 and 2240-EJ cell lines produced tumors in nude mice, only the 2240-EJ cell lines were tumorigenic in normal immunocompetent mice. In addition, the 2240-EJ cell lines also produced experimental lung metastasis in normal C3H and nude mice, although they induced more lung colonies in nude mice than in normal C3H mice (Tables 2 and 3). In contrast, the parental UV-2240 cell line and UV-2240 cells transfected with pSV2-neo DNA alone produced experimental lung metastasis only in nude mice (Table 3). However, the Ha-ras-induced progressor variants showed a marked increase in their metastatic potential in nude mice compared with the parent UV-2240 cell line or UV-2240 cells transfected with pSV2-neo DNA alone. These results are similar to those reported by other investigators (13–21, 38).

The progressor variants, even though they grew more slowly in normal mice than in nude mice, escaped attack from the normal host immune system. Previous studies have shown that loss or decreased expression of TSTAs (10, 11, 40) and MHC Class I antigens (41–46) on the surface of tumor cells can confer a selective growth advantage in immunocompetent hosts. In order to ascertain whether loss or decreased expression of MHC Class I antigens was involved in the escape of the Ha-ras-induced progressor variants from the defenses of the normal immunocompetent host, we analyzed the progressor variants for cell surface expression of MHC Class I antigens using monoclonal antibodies against H-2K^d (16-11-11N) and H-2D^d (15-5-5s) antigens. The results indicated that the Ha-ras-induced progressor variants expressed H-2K^d (Fig. 5) and H-2D^d antigens equal to or greater than the UV-2240 parent. In addition, CTL results also showed that MHC Class I antigens were functional, not just present in the progressor variants.

We next analyzed the progressor variants for loss of TSTAs by in vitro CTL and in vivo immunization and challenge assays. The results indicated that CTLs generated against UV-2240 were able to kill the Ha-ras-induced progressor variants effectively (Fig. 6). In addition, γ-irradiated progressor variants were quite capable of generating CTLs that killed not only the immunizing tumor cells but also the parent UV-2240 and the other Ha-ras-induced progressor variant (Fig. 7). Similarly, in vivo immunization and challenge experiments revealed that prior immunization with the regressor UV-2240 tumor protected mice against subsequent challenge with the Ha-ras-induced progressor variants (Table 4). In addition, immunization with the Ha-ras-induced progressor variants cross-protected mice against challenge with the regressor UV-2240 (Table 5). Taken together, these results suggest that the Ha-ras-induced progressor variants express similar or even the same MHC Class I antigens and TSTAs as the parent UV-2240 tumor. In addition, these data indicate that nondividing (γ-irradiated) Ha-ras-induced progressor tumor cells are perfectly capable of inducing an immune response in normal mice, as measured by in vitro CTL and in vivo immunization and challenge assays. In spite of this, viable Ha-ras-induced progressor variants are able to grow progressively in normal immunocompetent hosts. Previously, expression of TSTAs and MHC Class I antigens on the surface of neoplastic cells was thought to be the major requirement for the rejection of transplanted tumors in secondary hosts. Our studies suggest that expression of TSTAs and MHC Class I antigens is not the only criterion for growth or rejection of a tumor. It is likely that some tumor cells, despite the absence of strong TSTAs and MHC Class I antigens, evade immune surveillance by other mechanisms.

These results are similar to those of Schmitt and Daynes (9), who found that both the regressor UV-induced tumor RD1024 and its progressor variants expressed similar or the same TSTAs. Analogous to these results are those of Lin et al. (12), who established murine cell lineages that represent a phenotypic progression of cells from nontransformed (N-lines) to transformed and susceptible to immune surveillance (T-lines) to transformed and resistant to immune surveillance (C-lines). Both their I- and C-lines expressed cross-reactive transformation-associated antigens. Still, viable C-lines were not capable of inducing an immune response in normal mice, although X-irradiated cells did. These observations lend support to our conclusion that the Ha-ras-induced progressor variants may escape the immune defense of the normal host by mechanisms other than loss of TSTAs or MHC Class I antigens. For example, progressor variants may induce a suppressor cell pathway or acquire resistance to killing by natural killer cells or activated macrophages. In fact, Urban and Schreiber (47) have isolated UV-1591 progressor variants that exhibit resistance to killing by activated macrophages. Alternatively, progressor variants may also display an ability to protect themselves from immune attack by producing immunosuppressive factors. Recent studies have shown that some tumor cells produce large amounts of immunosuppressive factors, such as transforming growth factor β (48, 49), that inhibit mitogen-induced proliferation of T- and B-lymphocytes (50–52). In addition to its inhibitory effects on T- and B-cells, transforming growth factor β also inhibits natural killer cell activity (53). Whether our Ha-ras-induced progressor variants escape the immune surveillance by any of these mechanisms is being investigated.

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