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Immunology

Reduced tumorigenicity of a spontaneous mouse lung carcinoma following *H-2* gene transfection

(major histocompatibility antigen/class I molecule/tumor growth/DNA-mediated gene transfer)

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ABSTRACT **Cultured cells of the murine lung carcinoma called line 1 express very low levels of H-2 class I antigens and are resistant to lysis mediated by alloreactive T cells. In order to investigate how the expression of class I antigens affects the *in vivo* growth of this spontaneous tumor, *H-2D^p* genes were transferred into line 1 cells. Cloned transfectants that displayed H-2D^p surface antigens were identified using flow cytometry. The transfected H-2D^p antigens appeared normal by two-dimensional gel electrophoresis and could also function as excellent targets for T-cell-mediated lysis *in vitro*. Marked differences in tumorigenicity (defined as tumor growth in immunologically competent hosts) were observed between the *D^p* transfected cells and untransfected or control transfected line 1 cells in syngeneic mice only if the animals had previously received injections of irradiated *D^p* transfectants. Expression of D^p antigens did not appreciably affect the growth of line 1 tumors in immunologically naive syngeneic mice or necessarily cause rejection in allogeneic mice. Our *in vivo* results show that increased expression of class I antigens can reduce the growth of tumors like line 1 that lack all class I antigens. Our results also suggest that increasing class I antigens alone on some spontaneous tumors deficient in expression will not by itself be sufficient for tumor rejection.**

Class I major histocompatibility complex (MHC) antigens (classically H-2K, H-2D, and H-2L in the mouse) function as major transplantation antigens during graft rejection and as restriction elements for T lymphocytes during the recognition of other antigens (1). Specifically, recognition of non-MHC-encoded molecules by cytotoxic T lymphocytes (CTL) depends on the surface expression of both the appropriate target-specific and MHC antigens. Limiting quantities of either type of antigen can prevent effective cell lysis (2).

There are a number of studies in which the ability to form tumors, or the aggressiveness of tumors, appears to be inversely correlated to the expression of class I antigens (3–12, for review see ref. 10). However, in many of these studies it is difficult to assign the altered tumorigenicity of these cells solely to the expression of class I molecules. More recently, gene transfection has been used to study the effect of class I expression in the methylcholanthrene-induced T10 tumor system (7) and in the AKR leukemia (9) and adenovirus systems (8).

Intriguingly, some studies have also shown that class I antigen expression positively correlates with tumor growth. In one study, mouse lymphoma cells selected for loss of H-2 antigen expression were less capable of forming tumors than were the parental H-2 class I-expressing cells (12). Even more interesting was the observation that a gene, later shown to be encoded in the *H-2D* gene region (13, 14), was dramatically elevated in a variety of tumor cells (3). Similarly, in the T10-sarcoma system it was suggested that the expression of the *D^k* gene product actually enhanced the metastatic ability of these cells (11). Thus, it is of particular interest to examine the role of *H-2D* region genes in tumor growth.

In this report, we use transfection of an *H-2D^p* gene to evaluate how expression of class I antigens affects the tumor growth of a spontaneous BALB/c lung carcinoma called line 1 (15). The *D^p* gene is one of a variety of genomic clones that have been isolated and encode fully functional class I MHC molecules upon transfection into mouse L cells (16, 17). Line 1 cells provide an excellent system to study the role of class I expression in tumor growth because they are markedly deficient in expression of all class I MHC antigens (H-2K, H-2D, H-2L, and TL) by flow cytometry (ref. 18 and unpublished data), and are not normally susceptible to CTL-mediated lysis *in vitro* (19). In addition, line 1 cells resemble human small cell lung carcinoma cells (20) and embryonal carcinoma cells in MHC expression (21) and thus may be a good model for these tumors.

MATERIALS AND METHODS

Cell Lines. The BALB/c line 1 lung carcinoma was maintained in tissue culture as described (22). The cloned lines described in this report were negative for all 12 viruses analyzed (Sendai, mouse hepatitis virus, pneumonia virus of mice, reovirus type 3, Theiler encephalomyelitis, K-virus, Ectromelia, minute virus of mice, polyoma, lactic dehydrogenase virus, mouse adenovirus, and lymphocytic choriomeningitis virus) as tested by Microbiological Associates.

Mice. The BALB/cByJ and P/J mice were purchased from The Jackson Laboratory. The (BALB/c × P/J)F_1 mice were produced within our animal facility. Primed mice received two intraperitoneal injections of 5×10^6 irradiated (5000 rads) C1.6 cells as described in the text. Tumors were induced in mice (8–10 weeks of age) by intramuscular thigh injection. Viability of these cells was routinely checked by trypan blue exclusion and plating efficiency at the time of injection. Tumor size is expressed as the average of two perpendicular diameters, which were measured using vernier calipers at regular intervals. Mice were sacrificed when their tumors attained average diameters near 1.5 cm.

Gene Transfer. The genomic *D^p* clone F12 was obtained from J. A. Frelinger (University of North Carolina, Chapel Hill) (23). The neomycin resistance gene plasmid pko-neo was constructed by Doug Hanahan and coworkers (24). Our transfection procedure is similar to that of Wigler *et al.* (25). Briefly, 2×10^6 line 1 cells, plated out 1 day before in fresh medium, received 1.5 ml of a $CaPO_4$–DNA precipitate that contained 5 μg of F12, 5 μg of pko-neo, and 50 μg of high-molecular-weight line 1 DNA. After 30 min of incuba-

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Abbreviations: CTL, cytotoxic T lymphocyte; *neo*, neomycin resistance gene; MHC, major histocompatibility complex.

tion, 10 ml of culture medium were added to the cells, and 8 hr later this was replaced by 15 ml of fresh culture medium. Selection of transfectants with 400 μg of Geneticin (G418, GIBCO) per ml was initiated following 36 hr of additional culture (26).

Flow Cytometric, Immunological, and Biochemical Analyses. The D^p-specific monoclonal antibody 7-16.10 (27) was obtained from J. A. Frelinger. The D^d-specific monoclonal antibody AF4-62.4 and the K^k-specific monoclonal antibody AF3-12.1 have been described (18). Cells were stained for H-2 antigens using a two-step immunofluorescence technique (18), and average densities of fluorescently labeled antigens were calculated as described (18). The H-2^p-reactive CTL were generated by culturing 2.5×10^7 BALB/c spleen cells (H-2^d) with 1.25×10^7 irradiated P/J spleen cells (H-2^p) using the above media (19). The chromium release assays (19) and the two-dimensional gel electrophoresis (28) were done as previously described and are described in the figure legends.

RESULTS

Line 1 Transfectants Express D^p Molecules. The F12 plasmid containing an *H-2D^p* genomic clone was cotransfected into line 1 cells along with pko-neo, a plasmid that carries the neomycin resistance gene (*neo*). Approximately one per 10^4 starting cells containing functional *neo* copies were obtained by selection in media containing G418. Four of the fifty G418-resistant colonies that were screened by flow cytometry contained cells reacting with the D^p antibody 7-16.10, and clones that expressed D^p were subsequently obtained by limiting dilution. Fig. 1 shows typical fluorescence histograms for one positive clone, C1.2, after staining with anti-K^k (control) and anti-D^p antibodies. All of the transfectants stably expressed the *D^p* gene product, although at a density significantly lower than normal spleen cells. Two of the three cloned transfectants used in this report, C1.2 and C1.6, were chosen because they expressed the highest constitutive levels of D^p and the other, C13.10, because it expressed a lower but detectable level of the D^p antigen.

Transfected *H-2* Genes Produce Normal D^p Proteins. Because a major goal of this report was to evaluate recognition of the transfectants in syngeneic hosts, it was necessary to establish that the transfected *H-2* genes produced normal D^p antigens. To this end, biosynthetically [^{35}S]methionine-labeled D^p molecules were immunoprecipitated from Nonidet P-40 extracts of transfectants or normal P/J spleen cells and analyzed by two-dimensional gel electrophoresis followed by fluorography (28). The D^p molecules isolated from the transfected line 1 cells are identical to those isolated from P/J spleen cells, as shown by the representative autoradiographs in Fig. 2. Thus, by the technique of two-dimensional gel analysis no abnormality was observed in the transfected *D^p* gene product.

Alloreactive T Cells Specifically Lyse Line 1 *D^p* Transfectants *in Vitro*. In order to demonstrate that the D^p on the transfectants was functional, we compared the lysis of *D^p* transfectants and control cells by cytotoxic T lymphocytes with specificity for H-2^p antigens using a standard *in vitro* ^{51}Cr release assay (Fig. 3). Lysis of the *D^p* transfectants by the D^p-specific cytotoxic T-cell population was comparable in magnitude to lysis of P/J spleen blast cells that express high levels of H-2^p class I antigens. In contrast, lysis of the *D^p*-negative target cells was much lower. Irrelevant BALB/c anti-C3H effectors did not lyse the transfected or untransfected line 1 cells (data not shown). Although these experi-

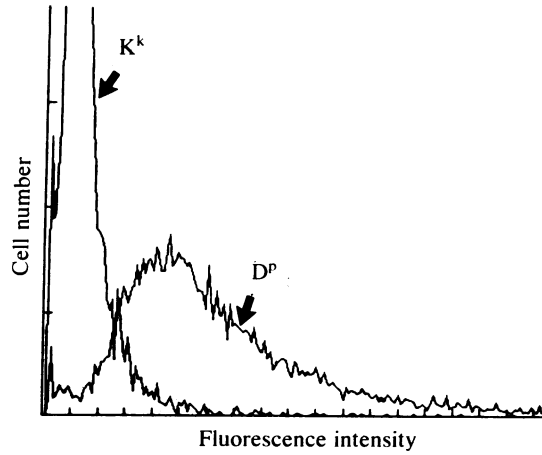

FIG. 1. Surface expression of D^p antigens on transfected line 1 cells. Cells from cloned transfectant C1.2 were incubated with the indicated hybridoma culture supernatants, followed by incubation with fluoresceinated goat anti-mouse F(ab')$_2$ serum. Fluorescence was quantitated for 10,000 cells using an Epics V (Coulter) flow cytometer. Functions on both axes are linear, and the units are arbitrary. The relative surface density of the D^p antigen calculated from flow histograms is as follows: normal (BALB/c × P/J) spleen cells, 100; clone C1.2, 20; clone C1.6, 22; and clone C13.10, 7.

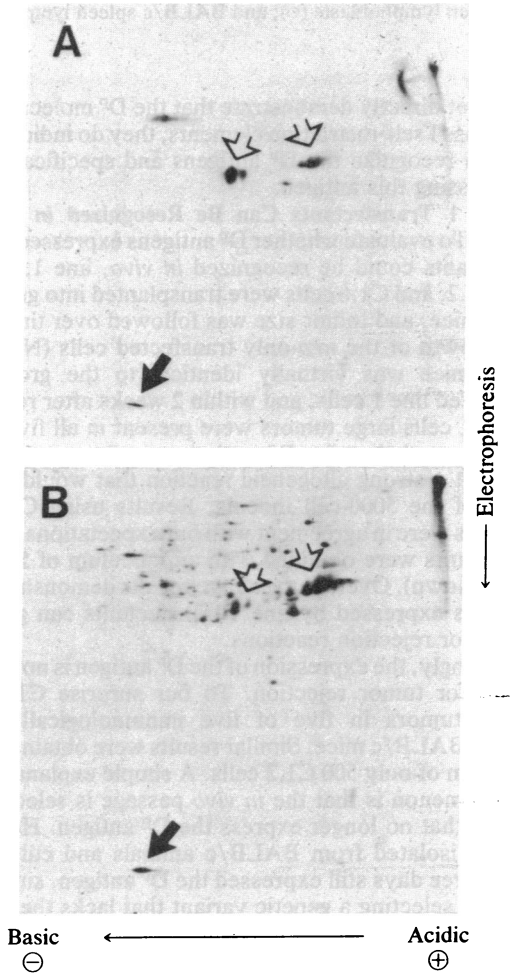

FIG. 2. Two-dimensional gel electrophoresis of D^p antigens. Normal P/J spleen cells (*A*) and line 1 cells transfected with a *D^p* gene, clone C1.2, (*B*) were incubated with [^{35}S]methionine and then solubilized with Triton X-100. Extracts representing equal numbers of cells were precipitated with a D^p-specific monoclonal antibody and *Staphylococcus aureus* bacteria. Precipitates were eluted into lysis buffer and separated in the first dimension (horizontal axis) by isoelectric focusing and in the second dimension (vertical axis) by NaDodSO$_4$ gel electrophoresis. Open arrows, D^p molecules; solid arrows, β_2-microglobulin.

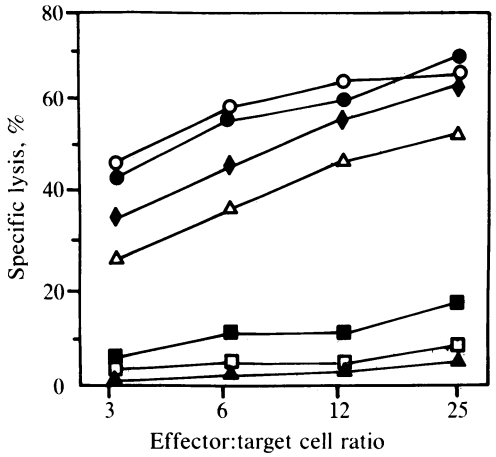

FIG. 3. Lysis of transfected line 1 cells by cytotoxic T lymphocytes. Target cells were labeled with ^{51}Cr and incubated for 4 hr with graded numbers of BALB/c anti-P/J CTL. Triplicate determinations of released ^{51}Cr were made at each point. Spontaneous release was never more than 10% of maximal release induced with 1 M HCl. Target cells were as follows: cloned D^p transfectants C1.2 (♦), C1.6 (●), C13.10 (△); *neo*-only transfected line 1 (□); untransfected line 1 (▲); P/J spleen lymphoblasts (○); and BALB/c spleen lymphoblasts (■).

ments do not directly demonstrate that the D^p molecules can serve as class I self-restriction elements, they do indicate that T cells can recognize the D^p antigens and specifically lyse cells expressing this antigen.

***D^p* Line 1 Transfectants Can Be Recognized *in Vivo* as Allografts.** To evaluate whether D^p antigens expressed by line 1 transfectants could be recognized *in vivo*, line 1, Neo-1, C13.10, C1.2, and C1.6 cells were transplanted into groups of BALB/c mice, and tumor size was followed over time (Fig. 4). The growth of the *neo*-only transfected cells (Neo-1) in BALB/c mice was virtually identical to the growth of untransfected line 1 cells, and within 2 weeks after receiving 5000 Neo-1 cells large tumors were present in all five mice. It was expected that the D^p antigens on the transfectants would elicit a strong allogeneic reaction that would lead to rejection of the 5000-cell inocula. Results using C1.6 and C13.10 cells were in agreement with our expectations (Fig. 4). Similar results were obtained with an inoculum of 500 cells (data not shown). Overall, the above results demonstrate that D^p antigens expressed by line 1 transfectants can generate strong tumor rejection reactions.

Interestingly, the expression of the D^p antigen is not always sufficient for tumor rejection. To our surprise C1.2 cells produced tumors in five of five immunologically naive allogeneic BALB/c mice. Similar results were obtained using an inoculum of only 500 C1.2 cells. A simple explanation for this phenomenon is that the *in vivo* passage is selecting for C1.2 cells that no longer express the D^p antigen. However, C1.2 cells isolated from BALB/c animals and cultured *in vitro* for three days still expressed the D^p antigen, suggesting we are not selecting a genetic variant that lacks the expression of D^p. Also C1.2 tumor cells isolated by centrifugal elutriation (29) from BALB/c mice also still expressed the D^p class 1 antigen suggesting we are not seeing antigenic modulation. Furthermore, C1.2 cells are capable of being rejected, as BALB/c mice primed with P/J spleen cells 4 and 2 weeks before challenge can reject an inoculum of at least 5000 C1.2 cells—suggesting the D^p molecule is still present (Fig. 4).

***D^p* Line 1 Transfectants Are Rejected Only in Primed Syngeneic Mice.** To determine whether expression of H-2 antigens affected line 1 tumorigenicity in syngeneic hosts, groups of primed and unprimed (BALB/c × P/J)F_1 mice

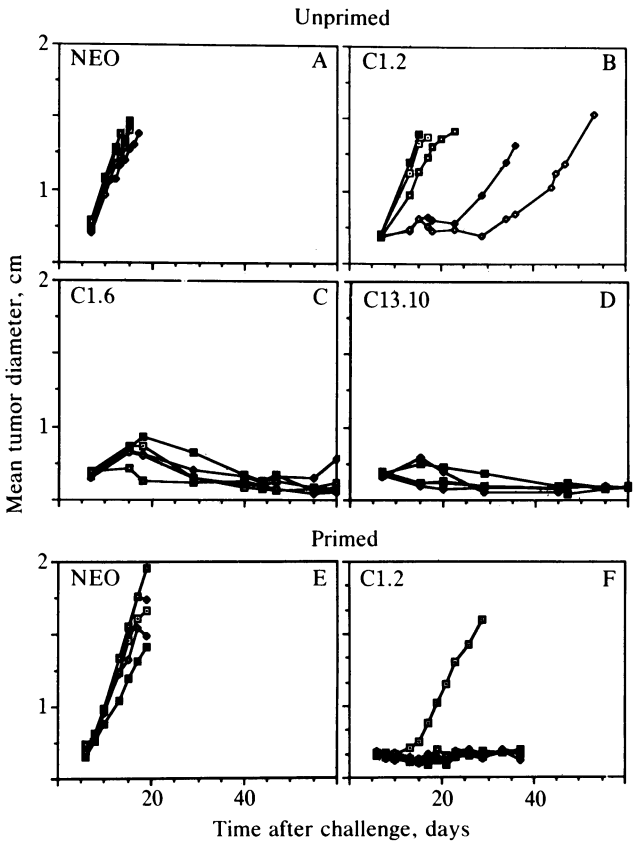

FIG. 4. Growth of transfected line 1 cells in BALB/c mice. Five thousand cells from various cloned transfectants were injected intramuscularly into naive (*A–D*) and primed (*E, F*) BALB/c mice. Individual curves represent the tumor size of one mouse. Transfectants used were as follows: Neo-1 (*A* and *E*); and the D^p-transfected C1.2 (*B* and *F*), C1.6 (*C*), and C13.10 (*D*).

were challenged with line 1, Neo-1, C1.2, C1.6, or C13.10 cells, and tumor size was followed over time (Fig. 5). The primed mice received irradiated C1.6 cells prior to tumor challenge as described above. With an inoculum of 5000 cells, all D^p transfectants and controls (line 1 and Neo-1) produced tumors in unprimed mice, although perhaps at slightly different rates. When primed mice were used, the D^p-transfected line 1 cells were significantly less tumorigenic than control cells. With one exception, none of the primed mice developed tumors when challenged by any of the transfectants. Similar results were obtained with inocula of 500 and 50,000 cells, the only difference being the time required for tumors to reach a specific size (data not shown). These data strongly suggest that the expression of class I antigens can dramatically lower the growth of line 1 tumor cells in syngeneic hosts.

DISCUSSION

We have shown that transfection of *H-2D^p* genes into line 1 cells dramatically decreases the ability of this tumor to grow in (BALB/c × P/J)F_1 mice. The simplest interpretation of these results is that the decreased tumorigenicity of the D^p transfectants results from increased expression of H-2 class 1 antigens and their recognition by T cells. Previous studies had suggested that the expression of the *D* region gene actually enhanced the ability of transformed cells to grow as tumors. In the T10 system, the ability to grow as metastatic tumors correlated with the expression of the D^k antigen (11). Transfection of the K^k gene into these cells reduced their ability to grow as tumors and metastasize, but transfection of

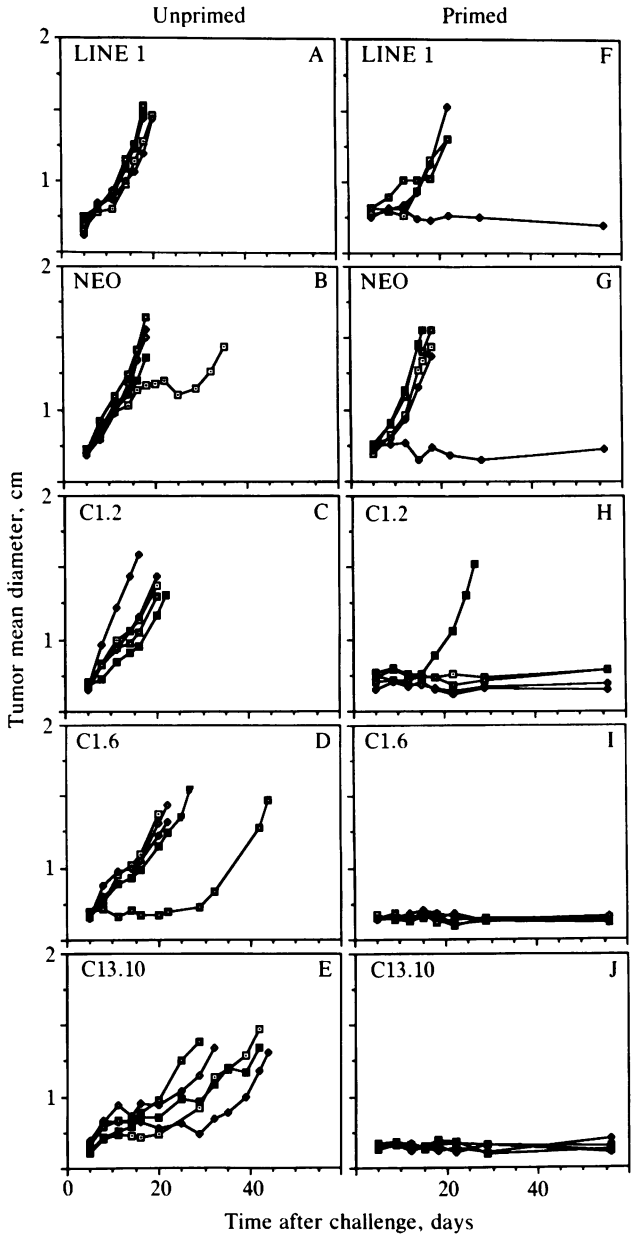

FIG. 5. Growth of transfected line 1 cells in syngeneic (BALB/c × P/J)F_1 mice. Five thousand cells from various lines were injected intramuscularly into naive (*A–D*) and primed (*F–J*) F_1 mice. Individual curves represent the tumor size of one mouse. Cell lines used were as follows: Line 1 (*A* and *F*), Neo-1 (*B* and *G*), C1.2 (*C* and *H*), C1.6 (*D* and *I*), and C13.10 (*E* and *J*); the latter three lines were D^p-transfected.

the D^k gene was not reported (7). Even more striking was the report that the expression of a *D* region gene was a general feature of oncogenesis in the mouse (3, 13). In light of these reports, it is significant that the expression of a *D* region gene in line 1 cells can lead to reduced tumor growth.

In contrast to other reports describing the malignancy of tumor cells transfected with class I genes (7–9), alterations of tumorigenicity were not observed in this study using immunologically naive mice. One explanation for this observation is that naive mice do, in fact, mount immune responses against the D^p transfectants, but these responses are not strong enough to significantly affect the rapid growth of this tumor. Priming may serve to augment this weak response, which may be related to the poor intrinsic antigenicity reported for untransfected line 1 cells (30). This priming can be very effective, as our results with C1.2 indicate, because primed syngeneic mice can generate an effective immune response to a tumor that can otherwise grow across a major transplantation barrier. It has been appreciated for some time that spontaneous tumors, such as line 1, are poorly immunogeneic and may be better able to evade host tumor defense mechanisms than can induced tumors (31–33). This point is significant because other reports of tumorigenicity following the transfection of a *K* or an *L* class I gene utilized tumors that had been induced with either viruses or chemicals and therefore likely had strong tumor-associated antigens. Our results indicate that both manipulating the immune system and increasing class I expression may be required to reduce the growth of some tumors.

Class I-deficient tumors have been found originating from a wide variety of types of cells (4–6, 34, 35). There is experimental evidence for several mechanisms by which such cells might arise. Viruses may directly interfere with the expression of class I molecules as in the adenovirus system (8, 36). Induced expression of class I on these cells (37), or preimmunization with induced cells (38), leads to reduced tumor growth. CTL may also select variants that have lost class I antigens that served as restriction elements. This appears to explain why *in vitro* simian virus 40-transformed fibroblasts lost the expression of the K^k antigen when adapted to *in vivo* growth (39). Such a mechanism may also be operating in both the methylcholanthrene-induced T10 sarcoma system and the AKR K36 leukemia system (9, 11), which lack one or more class I antigens. Consistent with this mechanism is that the transfection and expression of K^k class I gene can cause the reduced growth of these tumors (9, 11). Finally, tumors may also represent the clonal expansion of cells that normally lack class I antigens. Such cells seem to be normally present in a number of tissues (4, 40, 41) and may be represented by tumors such as embryonal carcinoma cells, human small lung cell carcinoma, and line 1 cells.

Even the expression of a strong transplantation class I antigen by itself is not always sufficient for tumor rejection. Our unexpected results with the growth of the D^p transfectant C1.2 in BALB/c mice were interesting in that they showed that surface expression of foreign class I molecules does not necessarily lead to an effective allograft rejection response. This could not be explained by a low level of D^p antigens because clone C1.6, which expresses the same density of D^p as does C1.2, was easily rejected. A similar result was obtained in the Sal tumor line in which allogeneic MHC class I antigen expression was insufficient for tumor rejection (S. Ostrand-Rosenberg, personal communication). Perhaps the growth of C1.2 is due to a suppressive effect as seen in the ultraviolet-induced fibrosarcoma system (42), the activation of oncogenes, or because of a yet unknown mechanism.

Using recombinant DNA techniques we showed that the deficient expression of class I molecules can contribute to the tumorigenicity of a lung carcinoma. This is particularly interesting because the most common human malignancy associated with deficient class I antigen expression is small cell lung carcinoma (20). Human small cell carcinoma cells, like line 1 cells, express little or no class I antigens due to the low levels of β_2-microglobulin and class I mRNA (ref. 20 and unpublished data). Further, the expression of these class I antigens on both the human tumor and line 1 cells can be induced with γ-interferon treatment. Because of these similarities, line 1 may serve as an animal model for this class of human tumors.

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