Dissimilar Immunogenicities of Human Papillomavirus E7 and Adenovirus E1A Proteins
Influence Primary Tumor Development

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Although human papillomaviruses (HPV) and adenoviruses (Ad) both transform cells by expressing functionally related oncoproteins (Ad-E1A/E1B; HPV-E7/E6), only HPV are oncogenic in humans. Prior studies have shown that HPV-transformed cells are resistant to NK cell lysis and E7- and E6-specific CTL are inefficiently generated in women with HPV-induced cervical cancer. Therefore, we postulated that the dissimilar oncogenicities of Ad and HPV may be caused by a protective NK and T cell response that is triggered by transformed cells expressing E1A, but not by E7. To test this hypothesis, mice that were either immunologically intact, lacked T cells, or lacked both NK and T cells were challenged with Ad serotype 5 (Ad5)-E1A- or HPV16-E7-transfected tumor cells. E7-expressing tumor cells were resistant to NK cell lysis in vitro and failed to elicit a measurable anti-tumor NK or T cell response in vivo. The concomitant expression of E6 did not change this phenotype. In contrast, E1A-expressing tumor cells were sensitive to NK lysis in vitro and triggered a protective NK and T cell immune response in vivo. These data suggest differences in the capacities of E1A or E7 oncoproteins to trigger protective anti-tumor immune responses may contribute to the dissimilar oncogenicities of Ad and HPV in humans. © 2000 Academic Press

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INTRODUCTION
Human papillomaviruses (HPV) and adenoviruses (Ad) are common human pathogens with a proclivity for causing persistent infections. In the United States, 95% of adults are seropositive for one of the group C Ad, and over 12 million adults are infected with genital tract HPV (Koutsky et al., 1988; Straus, 1984). Although both viruses are competent to transform human cells, only HPV are oncogenic in humans (Bosch et al., 1995; Green et al., 1979).

In HPV-induced human malignancies or Ad-transformed cells, there is viral gene integration into the host genome, and expression of two viral genes (HPV, E6 and E7; Ad, E1A and E1B) is found consistently (Graham et al., 1977; Schwarz et al., 1985; Smotkin and Wettstein, 1986). E1A and E7 are the primary immortalizing genes of Ad and HPV, respectively. Ad-E1B and HPV-E6 serve as "helper" genes that increase the efficiencies of E1A- and E7-induced transformation (Halbert et al., 1991; Houwel-
viruses indicate that there are factors other than cellular transformation efficiency that distinguish tumorigenic from nontumorigenic viruses. For example, although non-oncogenic group C Ad (Ad serotype 2, Ad2, Ad5) and highly oncogenic group A Ad (Ad 12) transform rodent cells in vitro with similar efficiencies, only cells transformed by highly oncogenic serotypes form tumors in immunocompetent animals. Conversely, cells transformed in vitro by both types of viruses are tumorigenic in immunodeficient animals (Lewis and Cook, 1984). These and other data suggest that the outcomes of interactions between oncogene-expressing, virally transformed cells and the host cellular immune response are pivotal in determining whether primary tumors will or will not form in vivo.

In previous studies we showed that the oncogenicities of HPV- or Ad-transformed human cells correlated with their resistance to lysis by human NK cells (Routes and Ryan, 1995). Oncogenic, HPV transformed human cells were resistant to NK lysis, whereas Ad-transformed human cells were NK sensitive. The Ad-E1A and HPV-E7 oncoproteins regulated this difference in susceptibility to NK cell lysis. Additionally, several laboratories have reported that HPV-tumor-specific CTL responses are weak in women with cervical cancer (Borysiewicz et al., 1996; Evans et al., 1996; Ressing et al., 1996). In contrast, studies in rodents show that robust CTL responses are induced following injection of Ad5-transformed cells (Bellgrau et al., 1988). These data suggested to us that differences in the capacities of E7 and E1A proteins to target virally transformed cells for destruction by the cellular immune response might contribute to the dissimilar oncogenicities of HPV and Ad in humans. However, there previously had been no way to directly compare the immunogenicities of E1A- and E7-expressing tumor cells to determine whether oncoprotein immunogenicity could influence primary tumor development in vivo. Therefore, it was uncertain whether these in vitro findings were relevant to primary tumor development in vivo. (For these considerations, tumor cell immunogenicity is defined as the ability of the tumor cell to elicit an effective antitumor response in vivo that includes both innate (NK cell) and acquired (T cell) immune defenses).

To test the hypothesis that dissimilar NK and T cell responses directed against cells expressing E1A or E7 affect primary tumor formation, a murine model was developed using the C57/BL6-derived tumor cell line MCA-102. MCA-102 cells were chosen for several reasons. First, MCA-102 cells are highly oncogenic in immunocompetent animals. Second, MCA-102 cells are nonimmunogenic, even when transduced by the costimulatory molecules B7.1 or B7.2 (Chen et al., 1994; Mule et al., 1987; Yang et al., 1995). Therefore, by transfecting the E1A and E7 oncogenes into MCA-102 cells, the immunogenicities of the E1A and E7 proteins could be directly compared and the relevance of oncoprotein immunogenicity on primary tumor formation could be assessed. Thus, if E1A were more immunogenic than E7, then immunocompetent mice should more efficiently reject MCA-102-E1A cells than MCA-102-E7 cells. Furthermore, if NK and T cells were important in the preferential rejection of E1A-expressing cells, the NK cell-related and T-cell-related components to the tumor rejection response could be quantitated through the use of mice that lack T cells (nude mice) or T cells and NK cells (CD3-e transgenic mice). Finally, because nontransfected MCA-102 cells were inherently resistant to killing by NK cells, it was also possible to test the effect of E1A and E7 oncogene expression on NK cell susceptibility in vivo and to correlate these results with in vivo tumor development data.

These results showed that the E1A- or E7-expressing mouse tumor cells exhibited phenotypes similar to those observed for virally transformed human tumor cells. E7-expressing cells were resistant to rejection by animals with competent NK cell and T cell responses and were resistant to NK cell lysis in vitro. Conversely, E1A-expressing cells were highly NK susceptible and over 1000 times less tumorigenic in immunocompetent mice than either E7-expressing cells or nontransfected MCA-102 tumor cells. The decreased tumorigenicity of E1A-expressing MCA-102 cells was dependent on an intact T cell and NK cell antitumor immune response. These studies provide the first animal model in which the immunogenicities of these viral oncoproteins have been directly compared and shown to influence primary tumor development. The possible biological implications of these findings are discussed.

RESULTS

Establishment of MCA-102 transfectants expressing high levels of HPV-E7 or Ad-E1A oncoproteins

MCA-102 cells were transfected with either pLSXN16E7 or pE1A-neo. Geneticin-resistant colonies were screened for HPV-16-E7 or Ad5-E1A expression by Western analysis. Levels of E7 oncoprotein expression in MCA-102-E7-CL1 and MCA-102-E7-CL2 were compared to those in SiHa and H4-E7. SiHa is an HPV16-transformed human cervical cancer line that expresses low levels of HPV16-E7, whereas H4-E7 is an HPV16-E7-transfected human fibrosarcoma cell line that expresses high levels of E7 (Routes and Ryan, 1995). As shown in Fig. 1, both MCA-102-E7-CL1 and MCA-102-E7-CL2 expressed considerably higher levels of the HPV16-E7 oncoprotein than SiHa, but slightly lower levels than H4-E7. Similarly, high levels of the Ad5-E1A oncogenes were expressed in both MCA-102-E1A-CL1 and MCA-102-E1A-CL2 cells. The cell morphologies and in vitro doubling times of these E7- and E1A-expressing MCA-102 lines were indistinguishable from those of nontransfected, MCA-102 cells (data not shown).
Failure of E7-expressing MCA-102 cells to elicit a protective cellular immune response to primary tumor development

The effects of E7 oncogene expression on primary tumor development by MCA-102 cells was tested in mice with varying abilities to mount an antineoplastic cellular immune response (Fig. 2). If E7 expression were effective in increasing the immunogenicity of MCA-102 cells, it would be predicted that expression of this oncoprotein would reduce tumorigenicity in immunocompetent mice compared with that in T-cell-deficient, nude mice. This was not the observation. Only a few thousand parental MCA-102 cells were required to induce subcutaneous tumors in immunocompetent mice (TPD$_{50}$ mean ± SEM = 3.3 ± 0.4 cells) (Fig. 2 and Table 1). Expression of high levels of E7 in two different transfected clones did not alter this level of tumorigenicity (TPD$_{50}$ = 3.4 ± 0.3 and 2.8 ± 0.2). This failure of E7 expression to alter tumorigenicity was also observed with cells co-expressing HPV E6 (TPD$_{50}$ = 3.0 ± 0.3). There was also no differential effect of E7 expression on MCA-102 tumor development in T-cell-deficient nude mice (Fig. 3). Both parental MCA-102 cells and E7-expressing cells were slightly more tumorigenic in nude than in immunocompetent mice, as indicated by the slightly lower TPD$_{50}$ values of each cell type in nude mice (Fig. 3 vs Fig. 2). There were, however, no significant differences in the tumor-inducing capacities of E7-expressing cells compared with parental cells when compared in either nude (Fig. 3) or normal mice (Fig. 2). These data suggested that E7 expression does not induce an effective T-cell-dependent defense against primary tumor formation by MCA-102 cells.

MCA-102 cells are inherently nonimmunogenic in syngeneic C57/BL6 mice (Chen et al., 1994; Mule et al., 1987; Yang et al., 1995). Therefore, it was possible that these cells possessed some trait that limited the immunogenic effects of any transfected viral oncoproteins. This possibility was tested in two ways. MCA-102 cells expressing E1A, rather than E7, were tested in the same tumorigenicity assays in immunocompetent mice (Fig. 2). In contrast to E7 expression, E1A expression caused a three- to four-log decrease in tumorigenicity of transfected MCA-102 cells, as indicated by the respective increases in the numbers of cells required to induce tumors in 50% of animals (TPD$_{50}$ of E1A-positive cells, E1A-CL1 = 6.8 ± 0.4, E1A-CL12 = 7.3 ± 0.3 cells vs TPD$_{50}$ of parental cells = 3.3 ± 0.4 cells). This comparison of different MCA-102 clones transfected with different viral oncoproteins could not exclude the possibility that clonal variation in the different transfectants contributed to the differences in tumorigenicity. Therefore, in a second type of experiment, an E7-transfected clone of MCA-102 cells was supertransfected with E1A and retested for sensi-

![FIG. 1](image1.png)

**FIG. 1.** (A) Expression of HPV16-E7 oncoprotein MCA-102-E7-CL1, MCA-102-E7-CL2, SiHa, and H4-E7 cell lines measured by Western analysis. (B) Expression of Ad5-E1A protein in MCA-102-E1A-CL1 and -CL2 cells measured by Western analysis.

![FIG. 2](image2.png)

**FIG. 2.** Tumor induction studies in normal, nude, and CD3-e-transgenic mice. Immunologically normal (A), nude (B), and CD3-e-transgenic mice (C) were injected subcutaneously in the flank with log dilutions of MCA-102, MCA-102-E1A, and MCA-102-E7 cells and observed weekly for 12 weeks. Animals were sacrificed when tumors reached a mean diameter of 20 mm or at the end of a 12-week observation period. The calculated TPD$_{50}$ value represents the log$_{10}$ of the number of tumor cells required to produce tumors in 50% of the mice.
tivity to rejection by immunocompetent mice (Table 1). These E7/E1A coexpressing cells exhibited the same three-log reduction in tumorigenicity in immunocompetent mice that was observed with cells expressing E1A alone. This eliminated clonal selection as an explanation for the observed differences in tumorigenic phenotypes of the cells and further excluded the possibility that there was some trait of E7-expressing cells that blocked expression of a rejection-susceptible phenotype. These data suggested that, in contrast to E1A expression, E7 expression was weakly immunogenic (or nonimmunogenic) in MCA-102 cells and failed to elicit a protective host defense against primary tumor formation.

E1A-expressing MCA-102 cells elicit both NK cell- and T-cell-specific responses to primary tumor challenge

The results of two types of animal experiments suggested that, in addition to a T-cell-dependent rejection response, the reduced tumorigenicity of E1A-expressing MCA-102 cells was also dependent on NK cell-mediated rejection. First, depletion of NK cells in vivo by pretreatment of normal mice with the anti-NK antibody PK136 (Koo et al., 1986) increased the tumorigenicity of E1A-expressing cells, as evidenced by a 1.7-log reduction in the number of MCA-102-E1A cells required to produce tumors (TPD50 in NK cell-depleted mice = 4.8; TPD50 in untreated mice = 6.5). Second, E1A-expressing MCA-102 cells continued to be significantly less tumorigenic in T-cell-deficient nude mice than either parental MCA-102 or E7-expressing MCA-102 cells (TPD50 values, MCA-102-E1A = 4.5 vs MCA-102 = 1.8 and MCA-102-E7 = 2.3). Nude mice have normal or increased NK cell activity. These results indicated that E1A but not E7 expression sensitized MCA-102 cells to an NK cell-dependent rejection response that is discernible from CTL-dependent rejection.

It was also possible that T-cell-independent defenses other than NK cells contributed to the enhanced rejection of E1A-, but not E7-, expressing cells in nude mice (Cook et al., 1982). To test this, tumor induction studies were done in CD3- α- transgenic mice, which lack both NK cells and T cells (Fig. 2C). There were no significant differences in the tumor-forming capacities of MCA-102-E1A cells in these mice, compared with either parental MCA-102 cells or MCA-102-E7 cells. Therefore, the incremental deletion of the NK cell response in addition to the T cell response that resulted from using CD3- α-transgenic mice, rather than nude mice, resulted in complete elimination of the host’s ability to reject E1A-expressing cells. These results also showed that the reduced tumorigenicity of E1A-expressing MCA-102 cells in nude mice was NK cell-dependent and was not explained by some other, lymphocyte-independent rejection mechanism.

Inverse correlation between NK susceptibility of oncogene-expressing tumor cells tested in vitro and tumorigenicity in NK-competent animals

Next, we tested the susceptibilities of several MCA-102 lines expressing either E7 or E1A to NK cell killing to determine if patterns of lysis in vitro correlated with cell line susceptibility to protective NK and T cell responses in vivo (Fig. 3). Expression of E1A as a single oncogene induced MCA-102 cells to become highly susceptible to NK cell killing. In contrast, E7 expression resulted in no increase in NK susceptibility compared with nontransfected, MCA-102 cells. To exclude the possibility that these NK susceptibility patterns were specific to the cell clone, rather than the oncogene expressed, an E7-expressing clone of MCA-102 cells that had been supertransfected with E1A and a second clone that had been established by co-transfection with E7 and E6 were tested in NK cell assays. E1A expression converted the

<table>
<thead>
<tr>
<th>Cell line</th>
<th>TPD50 Normal mice</th>
<th>Class I MHC antigen surface expression* (%)</th>
<th>Sensitivity to NK cell lysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCA-102</td>
<td>3.3 ± 0.4</td>
<td>100</td>
<td>No</td>
</tr>
<tr>
<td>-E1A-CL1</td>
<td>6.8 ± 0.4</td>
<td>120 ± 5</td>
<td>Yes</td>
</tr>
<tr>
<td>-E1A-CL2</td>
<td>7.3 ± 0.3</td>
<td>400 ± 7</td>
<td>Yes</td>
</tr>
<tr>
<td>-E7-CL1</td>
<td>3.4 ± 0.3</td>
<td>200 ± 6</td>
<td>No</td>
</tr>
<tr>
<td>-E7-CL2</td>
<td>2.8 ± 0.2</td>
<td>200 ± 4</td>
<td>No</td>
</tr>
<tr>
<td>-E7/E6</td>
<td>3 ± 0.3</td>
<td>220 ± 3</td>
<td>No</td>
</tr>
<tr>
<td>-E1A/E7</td>
<td>6.7 ± 0.2</td>
<td>290 ± 5</td>
<td>Yes</td>
</tr>
</tbody>
</table>

* Surface class I MHC antigen levels were normalized to the levels expressed on MCA-102 cells.
E7-expressing clone from an NK-resistant to an NK-susceptible cell. This result indicated that E7 expression did not select for an inherently NK-resistant clone. The failure of the E7/E6 coexpressing MCA-102 clone to exhibit NK susceptibility further indicated that the E7-related resistant phenotype observed with the single oncogene E7 transfectant was not a result of clonal selection.

**NK resistance and tumorigenicity of oncogene-expressing MCA-102 cells do not correlate with the level of class I MHC antigen expression**

NK cells express inhibitory receptors that recognize class I MHC antigens expressed on target cells (Lanier, 1998). In some experimental systems, cells that express high levels of class I antigens are resistant to NK cell killing, whereas cells that express low levels of class I are NK sensitive (Lanier, 1998). If this were the explanation for the oncogene-related NK and tumorigenic phenotypes observed in these studies, it would be predicted that cells in which E1A expression had induced high-level NK susceptibility would express very low levels of class I MHC antigens, relative to NK-resistant parental cells and E7-expressing cells. In contrast, it has also been proposed that the escape of Ad- or HPV-transformed cells from the T-cell-mediated cellular immune response occurs as a result of downregulation of cell surface expression of class I MHC antigens (Bernards et al., 1983; Cromme et al., 1994; Schrier et al., 1983). If this were the case with the MCA-102 cells tested here, it would be predicted that the nonimmunogenic, E7-expressing MCA-102 cells tested here would express very high levels of class I antigen compared with the E1A-expressing cells. It was also possible that there was no correlation between class I MHC antigen expression and susceptibility to NK killing or to T-cell-mediated rejection, as had been reported from some studies of DNA virus-transformed rodent and human cells (Haddada et al., 1986, 1988; Routes and Cook, 1995).

To examine these relationships using the oncogene-expressing MCA-102 cells studied here, surface class I MHC antigen expression was measured by FACS analysis (Table 1). The results showed that all oncogene-transfected clones of MCA-102 cells expressed higher levels of class I MHC antigen than did nontransfected, parental MCA-102 cells. More important for the purpose of the above considerations, there was no correlation between class I antigen expression and either tumorigenicity or NK susceptibility of the clones. Levels of K<sup>+</sup> and D<sup>+</sup> surface expression on all MCA-102 lines were also measured by flow cytometry and directly correlated with total class I antigen levels. Therefore, levels of surface class I MHC antigen expression did not explain either the NK phenotypes or the relative tumorigenicities of these oncogene-expressing cells.

**DISCUSSION**

Viral oncogene-induced cellular immortalization is a prerequisite for establishment of stable, neoplastic cell clones, but this immortalization step is only part of the explanation for tumorigenicity. Comparison studies of different Ad serotypes and other papovaviruses have shown that another important factor contributing to the differences in the tumorigenicities of cells immortalized by different viral oncogenes is the variable outcomes of interactions between oncogene-immortalized cells and components of the host cellular immune response (Lewis and Cook, 1984, 1985). For example, group A and C adenoviruses are equally competent to transform mammalian cells, but only group A Ad are oncogenic in immunocompetent rodents (Gallimore and Paraskeva, 1980). Studies using Ad5 (nontumorigenic)–Ad12 (highly tumorigenic) chimeric viruses showed that the differences in the oncogenicities of these viruses are regulated primarily by the functions of their E1A oncoproteins (Bernards et al., 1982; Jelinek et al., 1994; Telling and Williams, 1994). These E1A differences also determine whether Ad2/5- or Ad12-transformed cells elicit protective NK and T cell responses. Expression of Ad2/5, but not Ad12, E1A gene products sensitizes cells to lysis by NK cells (Cook and Lewis, 1984; Cook et al., 1986). Similarly, Ad2/5- but not Ad-12-, transformed cells elicit robust CTL responses (Bellgrau et al., 1988; Pereira et al., 1995). We speculate that a weak NK response to Ad12-transformed cells may contribute to the subsequent failure to generate a strong Ad12-E1A-CTL response in vivo (see below). Alternative explanations for a blunted CTL response include the capacity of Ad12-E1A gene products to transrepress the expression of class I MHC antigens and the TAP1 and TAP2 transporter genes (Bernards et al., 1983; Schrier et al., 1983), an activity not shared by Ad5-E1A gene products.

These observations suggest that, in addition to E1A-induced cellular immortalization, E1A-induced cellular traits that modulate the cellular immune response to oncogene-expressing cells are an important determinant of tumor progression or rejection. These studies may be relevant for discerning the reasons for the dissimilar oncogenicities of HPV and Ad in humans. In a manner analogous to that of differences between highly oncogenic Ad12 and nononcogenic Ad2/5-E1A genes, the dissimilarities in oncogene function between HPV16-E7 and Ad5-E1A may influence the tumorigenicities of Ad- and HPV-transformed cells in humans.

The studies presented here are consistent with this hypothesis and suggest that one reason for the tumorigenicity of E7 oncogene-expressing tumor cells is the failure of these cells to initiate an effective antineoplastic cellular immune response. For example, E1A expression caused a marked reduction of the tumorigenicity of MCA-102 cells in immunocompetent mice and a significant
reduction of MCA-102 tumorigenicity in T-cell-deficient, NK-competent nude mice. The observation that E1A had no effect on tumor development in CD3-e transgenic mice that lacked both NK cell and T cell defenses indicates that this E1A-induced reduction in tumorigenicity was caused by the combined effects of T cell and NK cell defenses (Figs. 2 and 3 and Table 1). In contrast, E7 expression failed to induce any detectable tumor rejection response in NK- and T-cell-competent mice. The lack of a protective T-cell-dependent response against E7-expressing MCA-102 cells (Fig. 2) cannot be explained by the lack of antigenicity in the E7 protein itself in the H-2^b background of the C57/B16 mouse used in these experiments. There are several reports showing that E7 is highly antigenic in these mice. For example, E7 peptide immunization of C57/B16 mice induces E7-specific CTL activity (Feltkamp et al., 1993; Sadovnikova et al., 1993). Furthermore, such CTL-inducing vaccination strategies protect mice from tumor development by E7-expressing tumor cells (Feltkamp et al., 1995; Ossevoort et al., 1995). This same pattern of viral oncogene-induced protection following preimmunization but absence of protection during primary tumor development has been reported for several other oncogenic DNA tumor viruses, including SV40 expressed in hamster cells and Ad12 expressed in hamster and mouse cells (Levine et al., 1984; Lewis and Cook, 1984, 1985). It is clear, therefore, that the antigenicity of an oncoprotein does not guarantee its ability to induce tumor cell rejection during primary tumor formation. In fact, this is usually not the case.

This failure of E7 expression to induce primary tumor rejection is common to both the human host faced with HPV-transformed cervical carcinoma cells and the C57/B16 mouse challenged with E7-expressing MCA-102 cells. One possible explanation for this phenomenon that would be consistent with the data presented in this report and with our previous studies of HPV-transformed human cells is that there is a requirement for an "initiating interaction" between oncogene-expressing tumor cells and NK cells that is required for effective generation of an oncoprotein-specific CTL response. There is precedent for such an accessory role for NK cells in the generation of a CTL response (Kos and Engleman, 1996). For example, it has been reported that depletion of NK cells reduces generation of syngeneic tumor-specific, virus-specific, and allospecific CTL (Kos and Engleman, 1996; Kurosawa et al., 1995; Suzuki et al., 1985). Our data and those in previous reports also show that certain viral oncogenes fail to sensitize the cells they immortalize to NK killing and that these NK-insensitive oncogene-expressing cells do not induce protective, oncoprotein-specific anti-tumor responses. There are several examples of this correlation between the ability of a DNA viral oncogene to induce cellular sensitization to NK killing and the tumor-inducing capacity of the oncogene-expressing cell in the immunocompetent animal. This correlation has been reported for hamster and mouse cells expressing Ad2/5-E1A (NK susceptible and nontumorigenic), hamster and mouse cells expressing Ad12-E1A (NK resistant and tumorigenic), SV40 T antigen-expressing hamster cells (NK resistant and tumorigenic), SV40 T antigen-expressing mouse and rat cells (NK sensitive and nontumorigenic), polyoma T antigen-expressing hamster cells (NK resistant and tumorigenic), and human cells expressing HPV16- or HPV18-E7 and -E6 (NK resistant and tumorigenic) (Cook et al., 1980, 1982; Cook and Lewis, 1984; Fresa et al., 1987; Raska and Gallimore, 1982; Routes and Ryan, 1995; Sawada et al., 1985). The data reported here extend this correlation to E7-expressing MCA-102 cells (NK resistant and tumorigenic) and suggest that this same pattern of resistance for HPV16- or HPV-E7-expressing human tumor cells may be relevant in vivo. Furthermore, neither the NK sensitivity nor tumorigenicities of the different MCA-102 lines were related to class I MHC antigen expression. These data are in agreement with other studies relating NK sensitivity and either tumorigenicity or class I expression on Ad-transformed or E1A-transfected rodent and human cell lines (Haddada et al., 1986, 1988; Routes and Cook, 1995). Studies are ongoing to determine if the expression of nonclassical class I antigens influences the NK sensitivities of E1A- and E7-expressing murine and human tumor cell lines.

The inability of E7-expressing MCA-102 cells to induce protective T-cell-dependent responses in immunocompetent mice is also consistent with observations of HPV-transformed cells in humans and other types of E7-transfected tumor cells in mice. For example, E7- or E6-specific CTL are inefficiently generated in women with HPV-induced cervical cancer (Borysiewicz et al., 1996; Evans et al., 1996; Ressing et al., 1996). Similarly, injection of HPV16-E7-transfected tumor cells that are not co-transfected with the co-stimulatory molecule B7.1 fail to induce E7-specific CTL in mice (Chen et al., 1992). Thus, our findings are consistent with the hypothesis that the E7 and E6 oncoproteins are ignored by the immune system despite their antigenicity, a state referred to as immunological ignorance (Melero et al., 1997).

HPV infect only human keratinocytes with complete viral replication linked to the differentiation of the infected cell. HPV gene products that help circumvent the host immune response to HPV infection may exist. In addition, the urogenital location of HPV-induced malignancies as well as differences in the replicative cycle and the unique cell tropism of HPV may contribute to the dissimilar oncogenicities of HPV and Ad. However, we believe that these are not the only factors leading to the dissimilar oncogenicities of Ad and HPV. Ad are ubiquitous human pathogens that cause persistent infections with asymptomatic fecal excretion for months to years following the initial infection (Fox et al., 1969). Ad infect
epithelial (including keratinocytes), fibroblastic, and lymphoid cells. Ad can cause both asymptomatic and symptomatic urogenital tract infections (Mufson et al., 1973). Like HPV-E7/E6, Ad-E1A/E1B are competent to completely transform both epithelial and fibroblastic cells in vitro. Furthermore, Ad-transformed human cells are tumorigenic in immunodeficient animals (Chang et al., 1990). Therefore, the fact that Ad can infect and potentially transform cells at virtually any anatomic location, including locations in which HPV replicate, precludes the possibility that the cell tropism and anatomic site of HPV-induced malignancy is the only explanation for the dissimilar oncogenicities of Ad and HPV. Finally, although there may be HPV proteins produced that circumvent cellular immunity to HPV infection, these proteins likely would not affect the cellular immune response following viral transformation. HPV-transformed cells are virion free and no further viral replication is possible (Galloway and Mc Dougall, 1989). Only two viral proteins are consistently expressed in HPV-transformed cells, the E7 and E6 oncoproteins.

In summary, the data reported here show that the dissimilar immunogenicities of the E1A and E7 oncoproteins can influence primary tumor development. We postulate that differences similar to those observed in this mouse model might also exist in the cellular immune responses to human cells expressing Ad5-E1A or HPV16-E7. This hypothesis predicts that, following Ad5 transformation of human cells, E1A expression would elicit a robust NK cell and T cell response that results in destruction of these cells in vivo. In contrast, following HPV16 transformation, E7 expression would fail to elicit such an immune response and would, therefore, persist to allow cellular transformation, subsequent cellular mutations, and tumor progression. These observations provide a basis for future studies contrasting E7 and E1A to test cellular mechanisms that explain the failure of E7 to sensitize tumor cells to NK killing and to induce T-cell-dependent tumor rejection by immunocompetent animals.

MATERIALS AND METHODS

Cell lines

The methylcholanthrene-induced sarcoma cell line MCA-102 was provided by Dr. Nicholas Restifo (National Institutes of Health, Bethesda, MD) (Mule et al., 1987). H4-E7 cells are HT1080-derived, human fibrosarcoma cells expressing high levels of the HPV16-E7 oncoprotein (Routes and Ryan, 1995). MCA-102 cells expressing Ad5-E1A or HPV16-E7 were derived from clones selected in G418 following transfection with pLSXN16E7 or pE1A-neo, which code for G418 resistance and HPV16-E7 or Ad5-E1A, respectively. G418-resistant clones were expanded and screened for the expression of Ad5-E1A or HPV16-E7 by Western analysis. pLSXN16E7 was provided by Denise Galloway (Fred Hutchinson Cancer Research Center, Seattle, WA) (Halbert et al., 1991). pE1A-neo was provided by Elizabeth Moran (Temple University, Philadelphia, PA) (Ruley et al., 1985). Two independently derived clones of MCA-102 cells expressing high levels of either Ad5-E1A (MCA-102-E1A-CL1, MCA-102-E1A-CL2) or HPV16-E7 (MCA-102-E7-CL1, MCA-102-E7-CL2) were used for all studies. The MCA-102 cell lines were maintained in DMEM supplemented with antibiotics, 15 mM glucose, and 5% FCS. Cell lines were periodically tested for contamination with mycoplasma using the Mycotec assay (Bethesda Research Labs, Bethesda, MD) and were negative.

NK cell cytolysis assays

NK cytolysis assays were performed as described using spleen cells from athymic nude C57/B6 mice as the source of NK cells and target cells labeled with [51Cr] (100 mCi/ml for 1 h; 1 Ci = 37 gBq) (Routes and Cook, 1995). The results shown represent the means ± SEM of at least four separate experiments. The mean percentage spontaneous release from all types of target cells was less than 20%.

Western analysis

For quantitation of E1A proteins, 60-mm plates of MCA-102-E1A-CL1 or MCA-102-E1A-CL2 cells were lysed in RIPA buffer (1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 7.4, 150 mM NaCl), and protein concentrations of RIPA supernatants were determined by the BCA protein assay (Pierce, Rockford, IL). An equal amount of protein from each cell lysate was separated on 10% SDS–PAGE polyacrylamide gels and electrophoretically transferred to PVDF membrane (Bio-Rad, Hercules, CA). The membrane was blocked in 5% nonfat milk solution and incubated with the anti-E1A monoclonal antibody, M73, supplied by E. Harlow (Massachusetts General Hospital, Charleston, MA) for 1 h (Harlow et al., 1985). Following several washes with PBST (PBS with 0.05% Triton X-100), the membranes were incubated for 1 h with rabbit anti-mouse antibody (Cappel, Durham, NC) and washed extensively with PBST. The E1A protein was then visualized per the manufacturer's instructions using the Renaissance Chemiluminescence Kit (DuPont–NEN, Boston, MA).

For quantitation of E7 proteins, 100-mm plates of H4-E7, SiHa, or MCA-102-E7-CL1 and CL2 cells were lysed with the E7 lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 0.5% SDS, 0.5% NP-40, 0.5% deoxycholate, 1 mM EDTA, 1% aprotinin, and 1 µM PMSF). Protein concentrations from each lysate were determined and equal amounts of lysate were immunoprecipitated using Protein A Sepharose CL-4B beads (Amersham Pharmacia Biotech, Uppsala, Sweden) and the E7 monoclonal antibody ED17 (Santa Cruz Biotechnology, Santa Cruz, CA). Immunopre-
cipitated proteins were resolved on 16% SDS–PAGE and transferred to PVDF membrane. With the exception of the use of the monoclonal E7 antibody, the remainder of the Western analysis was identical to the procedure used for the detection of the E1A proteins.

**Tumor induction studies**

CD3-ε-transgenic mice and congenitally athymic nude and normal C57/BL6 mice were obtained from Jackson Laboratories. CD3-ε-transgenic mice do not express either T cells or NK cells (Wang et al., 1994). Quantitative tumor induction studies were performed as previously described (Walker et al., 1991). Briefly, mice (three animals per cell dose for normal and athymic nude mice, two animals per dilution for CD3-ε-transgenic mice) were injected subcutaneously with serial log concentrations of cells and observed weekly for tumor development for 12 weeks. Animals were sacrificed when tumors reached a mean diameter of 20 mm or at the end of the 12-week observation period. Tumor cells from animals injected with either MCA-102-E1A or MCA-102-E7 cells were tested for E1A or E7 expression. TPD_{50} (log_{10} of the number of tumor cells required to produce tumors in 50% of the mice) were calculated by the method of Karber (1931).

**Measurement of class I MHC antigen levels**

M1/42.3.98, a monoclonal antibody that is panreactive to all murine class I antigens, was obtained from the American Tissue Culture Collection. The MCA-102 lines were stained with M1/42.3.98, and 5000 cells were analyzed on an Epics C flow cytometer as described (Routes and Cook, 1990). The levels of surface class I antigens on the E1A- or E7-transfected cell lines were normalized to parental MCA-102 cells using linearized values of log mean fluorescence. The results in the text represent the means ± SEM of at least three experiments. Levels of K\* and D\* surface expression on all MCA-102 lines were also measured by flow cytometry and directly correlated with total class I antigen levels.

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