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Adenovirus E1A Gene Induction of Susceptibility to Lysis by Natural Killer Cells and Activated Macrophages in Infected Rodent Cells

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Rodent cells immortalized by the E1A gene of nononcogenic adenoviruses are susceptible to lysis by natural killer (NK) cells and activated macrophages. This cytolysis-susceptible phenotype may contribute to the rejection of adenovirus-transformed cells by immunocompetent animals. Such increased cytolytic susceptibility has also been observed with infected rodent cells. This infection model provided a means to study the role of E1A gene products in induction of cytolytic susceptibility without cell selection during transformation. Deletion mutations outside of the E1A gene had no effect on adenovirus type 2 (Ad2) or Ad5 induction of cytolytic susceptibility in infected hamster cells, while E1A-minus mutant viruses could not induce this phenotype. E1A mutant viruses that induced expression of either E1A 12S or 13S mRNA in infected cells were competent to induce cytolytic susceptibility. Furthermore, there was a correlation between the accumulation of E1A gene products in Ad5-infected cells and the level of susceptibility of such target cells to lysis by NK cells. The results of coinfection studies indicated that the E1A gene products of highly oncogenic Ad12 could not complement the lack of induction of cytolytic susceptibility by E1A-minus Ad5 virus in infected cells and also could not block induction of this infected-cell phenotype by Ad5. These data suggest that expression of the E1A gene of nononcogenic adenoviruses may cause the elimination of infected cells by the immunologically nonspecific host inflammatory cell response prior to cellular transformation. The lack of induction of this cytolysis-susceptible phenotype by Ad12 E1A may result in an increased persistence of Ad12-infected cells in vivo and may lead to an increased Ad12-transformed cell burden for the host.

Resistance of experimental animals and humans to virus infection involves a diverse array of factors, including host humoral and cellular immune responses. While virus-specific antibodies are the major immunological defense against the extracellular spread of virus infection in vivo, host cellular immune responses are the major mechanism by which virus-infected cells are eliminated (reviewed in reference 24). These antiviral cellular immune defenses can be divided into early-appearing nonspecific, and late-appearing specific responses. The major effectors of the nonspecific component of the host antiviral cellular immune response appear to be naturally cytotoxic lymphocytes (termed natural killer [NK] cells) and activated macrophages. The specific arm of the host cellular immune response to virus infection is composed of cytotoxic thymus-derived lymphocytes and a variety of host mononuclear cells that collaborate with virus-specific antibody in what is termed antibody-dependent cell-mediated cytotoxicity. Mechanisms by which specific cellular immune responses recognize and eliminate virus-infected host cells have been defined (34). However, the reasons for the selective recognition and destruction of virus-infected cells by NK cells and activated macrophages are less well understood. Specifically, there are few models in which the viral gene(s) has been defined whose expression results in susceptibility of infected cells to lysis by NK cells and activated macrophages.

Hamster embryo fibroblasts infected with nononcogenic human adenovirus type 2 (Ad2) are susceptible to lysis by NK cells and activated macrophages (10). This Ad2-induced increased cytolytic susceptibility of infected cells is due to the function of early Ad2 genes expressed prior to viral DNA replication. Conversely, hamster cells infected with highly oncogenic Ad12 are resistant to lysis by these two host killer cell populations (10). We reported recently that the increased susceptibility to destruction by NK cells and activated macrophages of cells transformed by Ad2 or Ad5 is associated with a function of the E1A gene (11). This conclusion is compatible with the results of studies by Sawada et al. of the effects of rat NK cells on transformed rat cells (28). The data presented in this report are the results of a series of experiments performed to examine the viral genetic regulation of induction of susceptibility of Ad2- and Ad5-infected cells to the lytic effects of NK cells and activated macrophages and to test the hypothesis that E1A gene expression is sufficient for induction of this infected-cell phenotype.

MATERIALS AND METHODS

Cells and cell lines. Ad2HE1 is a clonally derived, Ad2-transformed LSH Syrian hamster embryo cell line that is highly susceptible to lysis by hamster NK cells and activated macrophages (8). A2T2C4 is an Ad2-transformed Hooded Lister rat embryo cell line that is highly susceptible to lysis by rat NK cells (11). These two cell lines were used as susceptible controls in hamster and rat NK cell and macrophage cytolysis assays. Hamster embryo cells (HEC) and rat embryo cells (REC) were prepared from near-term embryos as described (9) and were used in cytolysis assays at the first

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TABLE 1. Mutant viruses

Mutant virus[a]	Mutation coordinates[b]	Effect of mutation on early gene expression
E1A (1.4–4.5 m.u.)		
H5*hr*1	1 bp at 1,055	E1A 13S mRNA product truncated; E1A 12S mRNA product unchanged
H5*dl*312	Δ bp 448–1349	E1A 12S and 13S mRNA absent
H5*dl*520	Δ bp 1107–1117	E1A 13S mRNA absent due to removal of donor splice site; E1A 12S mRNA unchanged
H5*dl*521	Δ bp 920–1139	E1A 12S and 13S mRNA absent
H2*dl*1500	Δ bp 1110–1118	Same as H5*dl*520, except in Ad2 rather than Ad5
H2/5*pm*975	1-bp change at 975 (T→G)	E1A 12S mRNA absent due to alteration in donor splice site; E1A 13S mRNA product unchanged due to degeneracy of code
E1A + E1B (H5*dl*313)	Δ bp 1334–3639	Deletion of sequences encoding carboxyl-terminal 70 amino acids of E1A polypeptides; E1B 19- and 58-kDa polypeptides absent
E1B (4.7–11.2 m.u.)		
H5*hr*6	1 bp deleted at 2,347	E1B 58-kDa protein truncated
	1 bp change at 2,947 (G→T)	E1B 19-kDa protein not altered but synthesized at a reduced rate
H5*dl*337	Δ bp 1770–1916	E1B 19-kDa protein absent
H5*dl*338	Δ bp 2804–3329	E1B 58-kDa protein absent
E3 (76.8–85.9 m.u.)		
H2*dl*801	m.u. 78.5–83.5	E3 glycoproteins absent
E4 (91.3–99.1 m.u.)		
H2*dl*808	m.u. 92.0–97.1	E4 polypeptides absent

[a] Mutant names indicate the human (H) adenovirus serotype (Ad2 or Ad5) from which the mutants were derived and whether the mutant has been characterized as a host range (*hr*), deletion (*dl*), or point (*pm*) mutation.

[b] Mutation coordinates are given, where possible, in base pairs deleted (Δ) or changed. Where mutations have not been identified by sequencing, the mutation coordinates are given as map units (m.u.) (5, 35, 38).

or second tissue culture passage. Transformed cell lines were maintained in Dulbecco modified Eagle medium containing antibiotics and supplemented with 5% calf serum; embryo cells were maintained in the same medium supplemented with 10% calf serum. Cell lines were tested for contamination with *Mycoplasma* by the Mycotect assay (Bethesda Research Laboratories) and were negative.

Viruses. The prototype strain of human Ad2 (strain adenoid 6) and the Ad-75 strain of human Ad5 were grown in either A549 or 293 cells. The wild-type human Ad5 strains H5*wt*300 and H5*dl*309 were provided by T. Shenk and were grown in 293 cells. Ad12 (Huie strain) was grown in human embryonic kidney cells. The Ad5 deletion (*dl*) mutants H5*dl*312, H5*dl*313, H5*dl*337, and H5*dl*338 were provided by T. Shenk. The Ad5 deletion mutant *dl*1500 and the point mutant *pm*975 were provided by A. Berk. The Ad5 host range (*hr*) mutants H5*hr*1 and H5*hr*6 were provided by F. Graham. All stocks of Ad5 mutant viruses were grown in 293 cells. The Ad2 deletion mutant H2*dl*801 was provided by G. Ketner and was grown in A549 cells. A titered stock of the Ad2 mutant H2*dl*808 was provided by Dr. Ketner. Titers of viral stocks were determined by plaquing in the cells in which they were grown. The genetic changes in these mutant viruses compared with wild-type adenoviruses are described in Table 1 and Fig. 1. The cell lines in which these virus stocks were grown were screened for contamination with *Mycoplasma* by the Mycotect assay and were negative. Ad5-specific neutralizing antibody was used in certain experiments to treat the viral inoculum prior to infection of target cells. Neutralizing antibody was induced in a rabbit by repeated intravenous immunization with 10^{11} particles (4×10^9 PFU) of cesium chloride gradient-purified Ad5 (strain Ad-75). A 1:100 dilution of this rabbit antiserum prevented plaque formation by 10^5 PFU of Ad5 on A549 cells for 2 weeks. Ad5 inocula were neutralized with a 1:10 dilution of this rabbit antiserum for 30 min at 20°C.

Cytolysis assays. Adult (2- to 5-month-old) random-bred golden Syrian hamsters and Sprague-Dawley rats of either sex were used as spleen cell donors for NK cell cytolysis assays. Assays of NK cell and activated macrophage killing of control or infected target cells were performed in Dulbecco modified Eagle medium containing HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (20 mM) and antibiotics and supplemented with glucose (final concentration, 15 mM) and 10% (<1 ng/ml) endotoxin-free fetal bovine serum (Sterile Systems, Logan, Utah) as described (8) with minor modifications. Briefly, target cells were labeled overnight with [^{3}H]thymidine (0.5 μCi/ml), washed to remove unincorporated label, and cocultured (3×10^4 cells per well) with donor spleen cells for 48 h at optimal 100:1 (hamster) or 200:1 (rat) spleen cell-to-target cell ratios in 48-well cluster plates (Costar 3548). Monolayers of BCG-activated macrophages were prepared as described (8). Briefly, peritoneal macrophages were elicited with proteose peptone from hamsters infected intraperitoneally 2 weeks previously with 10^7 CFU of BCG. Confluent macrophage monolayers in 48-well cluster plates were obtained by adding 2×10^6 peritoneal exudate cells and removing nonadherent cells by repeated washing, resulting in effector-to-target cell ratios of approximately 33:1.

Virus-infected target cells were incubated for 24 h (HEC) or 48 h (REC) with the indicated viral inoculum and simultaneously radiolabeled. In certain experiments, the effects on NK cell killing of assay medium conditioned by virus-infected target cells were tested. For these studies, HEC were infected with 100 PFU of H5*dl*520 or H5*dl*309 per cell for 24 h and then washed and used at the same infected cell-to-volume ratio used in the cytolysis assays to condition assay medium for 48 h. This infected-cell-conditioned medium was then used without storage for cytolysis assays as described. The mean ± standard error of the mean (SEM) percent spontaneous release of [^{3}H]thymidine from control target cells during these 48-h cytolysis assays was as follows: Ad2HE1, 24.5 ± 2.0%; A2T2C4, 10.1 ± 0.9%; HEC, 14.1 ± 1.2%; REC, 11.7 ± 0.6%. The mean ± SEM percent spontaneous release of radiolabel from virus-infected em-

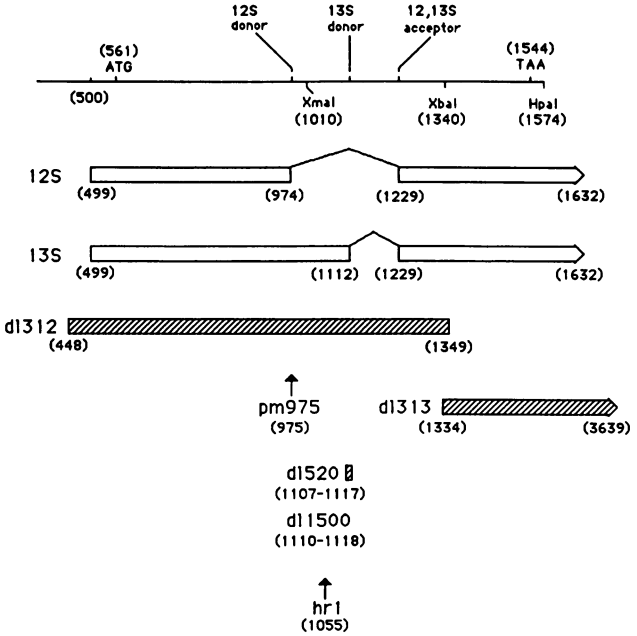

FIG. 1. Genetic map locations of lesions in E1A viral mutants. The left-hand 5% of the Ad5 genome map is represented by the line at the top of the figure. The locations of the Ad5 E1A translational initiation (561) and termination (1,544) codons and the locations of the E1A 12S donor (974), 13S donor (1,112), and 12S and 13S acceptor (1,229) splice site are indicated on the map along with selected restriction endonuclease cleavage sites for reference. The open-bar arrows below the map represent the locations of the sequences (exons) from which the E1A 12S and 13S mRNAs are transcribed; interruptions in these arrows indicated by carets joining the bars represent intervening sequences (introns) removed during RNA processing. Shaded bars and vertical arrows represent the sequences deleted from the Ad5 and Ad2 E1A mutants described in Table 1.

bryo cells was as follows. Infected HEC (100 PFU/cell for 24 h prior to assay): Ad2, 18.9 ± 2.8%; Ad5, 12.0 ± 1.2%; *dl*309, 15.0 ± 0.8%; *dl*801, 10.5 ± 1.4%; *dl*338, 30.2 ± 0.6%; *hr*6, 13.2 ± 1.0%; *dl*808, 30.9 ± 1.3%; *dl*312, 17.8 ± 0.9%; *dl*520, 44.3 ± 3.3%; *pm*975, 25.0 ± 1.6%; *dl*520 + *pm*975, 22.5 ± 3.4%; *dl*1500, 46.6 ± 2.8%; Ad12, 32.1 ± 2.4%; *dl*309 + Ad12, 25.1 ± 2.2%; *dl*312 + Ad12, 23.9 ± 1.2%. Infected REC (200 PFU/cell for 48 h prior to assay): Ad2, 11.8 ± 1.2%; Ad12, 22.7 ± 1.6%; *dl*520, 21.6 ± 1.5%. The slightly increased spontaneous release of radiolabel from HEC infected with viruses that can express only the 12S mRNA of E1A (*dl*520 and *dl*1500) may reflect the E1A 12S mRNA product-induced increase in cellular DNA degradation after infection of certain cell types with adenoviruses that do not express the E1B 19 kilodalton (kDa) protein (35). This conclusion is supported by the reduction in spontaneous radiolabel release in HEC coinfected with *dl*520 and *pm*975 to levels seen with other mutants and wild-type virus. The fact that this *dl*520 effect on spontaneous release of radiolabel was much less prominent in *dl*520-infected REC than in infected HEC suggests that REC may be less susceptible to this E1A-induced degradation of cellular DNA. Since the results of cytolysis assays with either *dl*520-infected HEC or *dl*520-infected REC were the same (see below), it is unlikely that this viral effect on the target cells is a determinant in the results of these experiments. Data are expressed as the percentage of total radiolabel released from target cells due to NK cell-induced lysis, calculated as described (8).

Protein blotting studies. Cells (2×10^7) were lysed and digested in a volume of approximately 200 μl by the micrococcal nuclease-RNase A-DNase I method of Gaynor et al. (13). Extracts were then boiled in Laemmli gel sample buffer (17a) and electrophoresed on 12% polyacrylamide gels. Proteins were electrophoretically transferred to 0.2-μm-pore-size nitrocellulose filters (Fisher) overnight at 50 mA (4). The filter was incubated with 5% nonfat dry milk to block nonspecific protein binding. The filter was then incubated for 2 h with a 1:1,000 dilution of a polyclonal rabbit anti-E1A antiserum provided by B. Ferguson (17). After washing, the filter was incubated for 1 h at 37°C with 1 μCi of ^{125}I-labeled protein A (New England Nuclear Corp.). Immune complexes were detected by autoradiography.

RESULTS

Nononcogenic Ad2 and Ad5 induce susceptibility of infected HEC and REC to NK cell-mediated killing. To justify using both Ad2 and Ad5 mutant viruses in studies of viral genetic regulation of induction of cytolytic susceptibility in infected rodent cells, we first compared the effects of infection with wild-type Ad2 and Ad5 on the susceptibility of HEC to hamster NK cell-mediated lysis under optimal conditions for target cell killing with a 100:1 spleen cell/target cell ratio (Fig. 2, left panel). These results and those from subsequent experiments show that infection with Ad5 is at least as effective as infection with Ad2 in the induction of increased susceptibility of embryo fibroblasts to lysis by NK cells and activated macrophages (data not shown). Furthermore, when NK cell experiments similar to those performed in the

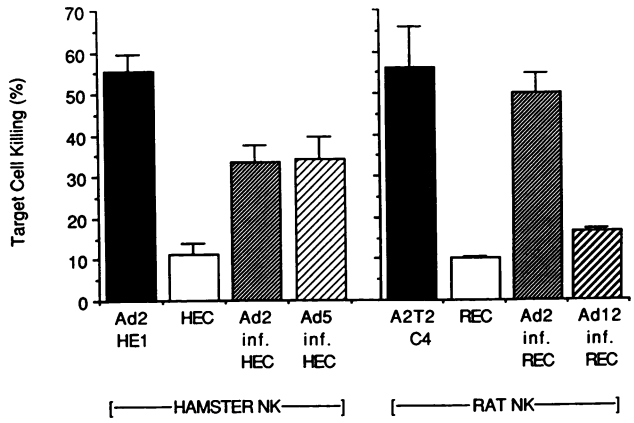

FIG. 2. Susceptibility of adenovirus-infected HEC and REC to lysis by NK cells. Bars represent the results (mean ± SEM) of four hamster or two rat NK cell cytolysis assays. Ad2HE1 and A2T2C4 are Ad2-transformed cell lines from these species, respectively, that were used as cytolysis-susceptible control target cells in these experiments. The NK cell-mediated killing of Ad2-, Ad5-, or Ad12-infected HEC or REC was compared with that of mock-infected cells at optimal 100:1 (hamster) or 200:1 (rat) spleen cell-to-target cell ratios. HEC were infected for 18 to 24 h prior to the assay with the indicated serotype at a multiplicity of infection of 100 PFU/cell. REC were infected with 200 PFU/cell of the indicated serotype for 48 h prior to the assay. HEC infected with Ad2 or Ad5 and REC infected with Ad2 were significantly more susceptible to NK cell-mediated lysis than the respective mock-infected embryo cells ($P < 0.003$ to 0.01). Furthermore, Ad12 was significantly less effective in inducing susceptibility to rat NK cell-mediated lysis in infected REC than was Ad2 ($P < 0.013$).

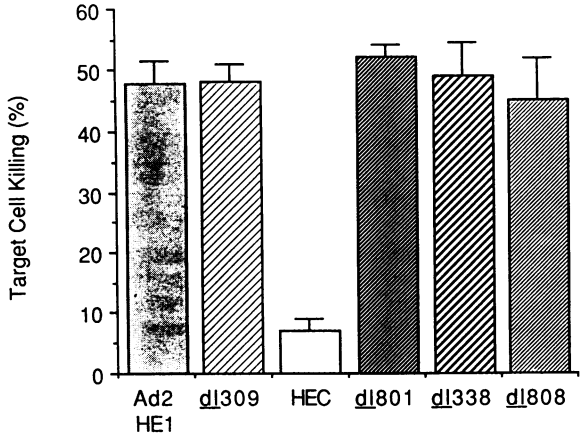

FIG. 3. Lack of effect of deletions in adenovirus early regions E3, E4, and E1B on induction of cytolytic susceptibility in HEC infected with mutant adenoviruses. Bars represent the results (mean ± SEM) of four hamster NK cell cytolysis assays. Ad2HE1 cells (Ad2-transformed hamster cell line) and *dl*309-infected HEC were used as cytolysis-susceptible control target cells in these assays. Mock-infected HEC target cells were compared with HEC target cells infected with the indicated Ad2 (*dl*801, *dl*808) or Ad5 (*dl*338) deletion mutant virus at 100 PFU/cell (*dl*801 and *dl*338) or with 1 ml of virus stock (*dl*808) for 18 to 24 h prior to the assay. HEC infected with wild-type *dl*309 and with all of the mutant viruses were significantly more susceptible to lysis by hamster NK cells than mock-infected cells ($P < 0.003$).

spleen cell-target cell hamster system were repeated with infected REC as target cells in rat NK cell assays at an optimal 200:1 ratio, an identical increase in the cytolytic susceptibility of Ad2-infected cells was observed (Fig. 2, right panel). Since we had reported that Ad12 infection did not induce increased cytolytic susceptibility in infected hamster cells (10), we repeated the comparison between target cells infected with nononcogenic Ad2 or highly oncogenic Ad12 in the REC-rat NK cell assay system (Fig. 2, right panel). A similar lack of induction of cytolytic susceptibility by Ad12- compared with Ad2-infected cells was observed in the rat system. These data indicate that it is appropriate to compare the results of assays with virus-infected hamster and rat cells when evaluating the effects of viral mutations on induction of cytolytic susceptibility. These results also extend to adenovirus-infected target cells the correlations observed between NK cell susceptibility of weakly oncogenic Ad2-transformed cells and NK cell resistance of highly oncogenic Ad12-transformed cells.

Mutations involving the E1B, E3, or E4 gene do not affect virus induction of cytolytic susceptibility in infected cells. To begin mapping the region(s) important for induction of the cytolysis-susceptible phenotype in infected cells, a series of deletion mutants lacking specific early viral gene functions were tested (Table 1). Many of the Ad5 deletion mutants used in these studies were derived from the parental wild-type virus, H5*dl*309 (hereafter referred to as *dl*309), which was originally selected for three small deletions located at *Xba*I sites at 26, 79, and 85 map units on the Ad5 genetic map (16). Preliminary studies of the ability of *dl*309 to induce cytolytic susceptibility in infected rodent cells were performed. As shown in Fig. 3, this virus is highly effective in inducing susceptibility to NK cell-mediated lysis in infected hamster cells. In other studies, we observed no difference in the induction of cytolytic susceptibility in infected hamster cells when comparing *dl*309 with the nonmutant wild-type virus H5*wt*300 from which it was derived (not shown). In subsequent experiments, *dl*309 was used as the wild-type control virus with which Ad5 deletion mutant viruses were compared.

Due to the selection process through which *dl*309 was derived, one of the small deletion mutations in this virus involves a 2% deletion in the Ad5 E3 coding region (16). Induction of cytolytic susceptibility by *dl*309 suggests that expression of E3 may not be required for induction of this infected-cell phenotype. To study further the role of the E3 gene in this process, the mutant H2*dl*801 was tested. The deletion in this Ad2 mutant spans most of the E3 region (Table 1) (5). As shown in Fig. 3, this mutant is highly effective in inducing susceptibility of infected HEC to killing by hamster NK cells. This result is of interest since the E3 encoded 19-kDa glycoprotein is one of the few adenovirus early proteins that has been shown to be integrated into cell membranes and expressed on the cell surface (23). Such a viral gene product might be an ideal candidate for a target for NK cells or activated macrophages. The results obtained with the *dl*801 mutant suggest, however, that expression of this viral glycoprotein on the cell surface is not required for recognition and destruction of adenovirus-infected cells by hamster NK cells. These data are compatible with most of the reported data on the NK cell susceptibility of virus-infected cells which do not support the conclusion that virus-specific proteins are the cell surface molecules recognized by NK cells on target cells (24).

It has been observed in a murine NK cell-tumor target cell system that inhibition of target cell protein synthesis renders formerly resistant target cells susceptible to NK cell-mediated lysis (7). Infection of mammalian cells with Ad2 and Ad5 results in inhibition of host cell macromolecular synthesis within hours after infection (reviewed in reference 12). Therefore, an adenovirus-induced inhibition of protein synthesis-dependent repair mechanisms in NK cell-injured target cells provides one possible mechanism by which adenovirus infection might render infected cells susceptible to NK cell adenovirus killing. The early adenovirus proteins responsible for inhibition of host cell protein synthesis are the E1B-encoded 58-kDa protein and the E4-encoded 34-kDa protein. These two viral proteins have been shown to exist as a complex in infected cells.

To test the hypothesis that adenovirus-induced interference with host cell protein synthesis in the cause of increased cytolytic susceptibility, HEC infected with two types of deletion mutants were tested as target cells in hamster NK cell assays (Fig. 3). H5*dl*338 is an Ad5 mutant derived from *dl*309 that has a large deletion in the coding region for the E1B 58-kDa protein (Table 1). As a result of this deletion, cells infected with *dl*338 do not contain detectable amounts of this early viral protein. HEC infected with this E1B 58-kDa-minus mutant exhibit the same level of susceptibility to killing by hamster NK cells observed with the wild-type parental virus *dl*309. This indicates that synthesis of E1B 58-kDa protein in infected cells is not required for expression of the cytolysis-susceptible phenotype. The results of studies with the Ad5 host range mutant H5*hr*6 (Table 1), which has a point mutation in the E1B 58-kDa protein coding region and does not induce the synthesis of E1B 58-kDa protein in infected cells, support this conclusion. HEC infected with either low or high multiplicities of this host range mutant were highly susceptible to lysis by hamster NK cell populations. In three hamster NK cell assays, the mean ± SEM percent target cell killing was as follows: uninfected HEC, 11.8 ± 1.5%; HEC infected for 24

h with H5*hr*6 at 10 PFU/cell, 47.0 ± 4.4% ($P < 0.005$); HEC infected for 24 h with H5*hr*6 at 100 PFU/cell, 52.6 ± 6.8% ($P < 0.02$). To evaluate the importance in induction of the cytolysis-susceptible infected-cell phenotype of the other early-region protein, E4 34 kDa, which inhibits host cell protein synthesis, an Ad2 E4 deletion mutant, H2*dl*808 (Table 1) (5), was tested. This E4-minus mutant also induced susceptibility to NK cell killing that was similar to that observed with wild-type Ad2 (Fig. 3). These results suggest that adenovirus-induced inhibition of host cell protein synthesis mediated by the E1B 58- kDa protein, the E4 34-kDa protein, or a complex of these two early viral proteins is not the explanation for the increased susceptibility of infected rodent cells to the lytic effects of NK cells.

The E1B 19-kDa protein is encoded from a reading frame in the E1B gene that is different from that which encodes the E1B 58-kDa protein (3). Since this E1B 19-kDa protein is present in the plasma membranes of infected cells (23), its possible role in altering the susceptibility of adenovirus infected cells was also considered. H5*hr*6 induces synthesis of a reduced amount of this protein in infected cells with delayed kinetics compared with infection with wild-type virus (26). The fact that H5*hr*6 induced high levels of cytolytic susceptibility in infected hamster cells even at a low multiplicity of infection (see above) suggests that wild-type levels of expression of E1B 19-kDa protein may not be required for expression of this phenotype. The best test of this possibility would use target cells infected with deletion mutants lacking the ability to express the E1B 19-kDa protein. Unfortunately, such mutants cause the rapid induction of degradation of host cellular DNA (35). In the experiments we attempted, the high spontaneous release of incorporated [^{3}H]thymidine from cells infected with such mutants made it impossible to interpret the role of NK cells or macrophages in the lysis of the target cells. The results of the studies described below with target cells infected with E1A mutant viruses reduce the importance of this experimental limitation and suggest that E1B 19-kDa proteins are, in fact, not required for expression of the cytolysis-susceptible phenotype.

Expression of Ad5 E1A proteins in infected cells is sufficient for induction of susceptibility to lysis by NK cells and activated macrophages. We recently reported that immortalization of rodent cells with the Ad2 or Ad5 E1A gene alone is sufficient to induce high levels of transformed target cell cytolytic susceptibility in assays of NK cell and activated macrophage killing (11). This observation, in addition to the previously described data, stimulated studies of the cytolytic susceptibility of rodent cells infected with E1A mutants. The first of these E1A mutants tested was H5*dl*312 (Table 1, Fig. 1), an Ad5 mutant that contains a large deletion spanning most of the E1A gene and that does not induce detectable E1A proteins in infected rodent cells even at high multiplicities of infection (data not shown). In numerous NK cell and activated macrophage cytolysis assays with both infected HEC and infected REC as target cells, *dl*312 failed to induce a significant increase in susceptibility to killing over that observed with uninfected cells (Fig. 4, 5, and 6; REC data not shown). An identical lack of induction of cytolytic susceptibility in infected HEC was observed with another E1A-minus Ad5 mutant, H5*dl*521 (14) (data not shown).

A trivial explanation for the failure of *dl*312 to induce increased cytolytic susceptibility in HEC and REC is that *dl*312 cannot infect these cells. The fact that infection of HEC with *dl*312 partially rescued the cells from the DNA degradation effects of the E1B 19-kDa-minus Ad5 mutant H5*dl*313 (Table 1, Fig. 1) indicates that this explanation is incorrect. The mean ± SEM percent spontaneous release of incorporated [^{3}H]thymidine (as a measure of DNA degradation) in five coinfection experiments was as follows: HEC infected with *dl*312 alone, 5.8 ± 0.4%; HEC infected with *dl*313 alone, 59.5 ± 9.0%; HEC coinfected with *dl*312 and *dl*313, 29.6 ± 3.6% ($P = 0.01$). A similar *dl*312-induced rescue of infected cells from DNA degradation was observed when HEC were coinfected with *dl*312 and the E1B 19-kDa-minus mutant, H5*dl*337 (data not shown). Supporting the conclusion that *dl*312 can infect hamster cells is the recent observation that this virus can infect an Ad2-transformed HEC line, Ad2HE3 (A. M. Lewis, Jr., unpublished data). Successful *dl*312 infection of these cells was evidenced by the ability of the E1A products expressed in Ad2HE3 to complement the replicative defect of *dl*312 after low-multiplicity infection. The results of these NK cell and activated macrophage cytolysis assays with *dl*312-infected target cells indicate that Ad5 E1A must be expressed in infected rodent cells for the cytolysis-susceptible phenotype to be observed. The next question addressed was whether expression of only the Ad2 or Ad5 E1A gene in such infected rodent cells was sufficient to induce this phenotype.

The Ad5 E1A mutant H5*dl*520 contains an 11-base-pair (bp) deletion that removes the E1A 13S mRNA donor splice junction (Table 1, Fig. 1) (14). The RNA sequences involved in this mutation are removed from the E1A 12S mRNA during splicing. Therefore, cells infected with this virus synthesize 12S mRNA and its products but do not synthesize 13S mRNA. E1A 13S mRNA products are the major activators of transcription of the other early adenovirus genes (E1B, E2, E3, and E4); 12S mRNA products are inefficient in this process of transcriptional activation under the conditions of virus infection (20, 21, 37). It is not surprising, then, that cells infected with *dl*520 have been observed to express little or no detectable virus-specific proteins from other early adenovirus regions or that this mutant has a host range phenotype, growing efficiently only in 293 cells, which contain and express the Ad5 E1 region. Another Ad5 E1A mutant, H5*pm*975, has an E1A phenotype different from that of *dl*520 (Table 1, Fig. 1) (21). H5*pm*975 has a point mutation at bp 975 that is within the consensus sequence of the donor spice junction of E1A 12S mRNA, preventing splicing of this mRNA in infected cells. Due to genetic-code degeneracy, this mutation does not affect the transcriptional activation function of 13S mRNA products. Therefore, cells infected with *pm*975 express other early genes similarly to cells infected with wild-type virus, and this mutant does not have a host range phenotype.

HEC infected with each of these two E1A mutants at low (10) and high (100) multiplicities or coinfected with both mutants at low multiplicity were used as target cells in hamster NK cell assays (Fig. 4). Both *dl*520 and *pm*975 induced significant levels of cytolytic susceptibility at high multiplicities. In neither case was the level of susceptibility of the infected cells equal to that observed with the wild-type parental virus, *dl*309. Only *pm*975 induced a detectable level of cytolytic susceptibility in NK cell assays at low multiplicity. In identical studies in which the host range mutant H5*hr*1 (a point mutant with a phenotype identical to that of *dl*520; Table 1, Fig. 1) was used to infect HEC, results identical to those shown for *dl*520 in Fig. 4 were obtained (data not shown). When HEC were coinfected with low multiplicities of *dl*520 and *pm*975, a significant increase in cytolytic susceptibility was always observed over that seen when either virus was used alone to infect HEC target cells. In

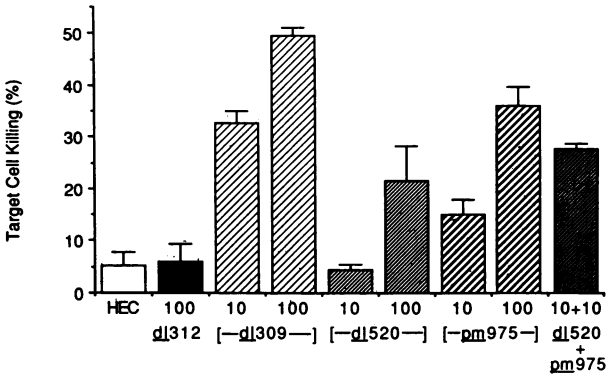

FIG. 4. Induction of susceptibility to hamster NK cell-mediated lysis in infected hamster embryo cells by Ad5 E1A 12S only (*dl*520) and E1A 13S only (*pm*975) mutant viruses but not by the E1A-minus Ad5 mutant virus *dl*312. Bars represent the results (mean ± SEM) of three hamster NK cell cytolysis assays. Mock-infected HEC target cells were compared with HEC target cells infected with 10 or 100 PFU of the indicated virus per cell or doubly infected with 10 PFU of both *dl*520 and *pm*975 per cell for 18 to 24 h prior to the assay. HEC infected with *dl*309, *dl*520, or *pm*975 were significantly more susceptible to lysis by hamster NK cells than mock-infected cells ($P < 0.001$ to 0.05). There were no significant differences in the cytolytic susceptibilities of HEC infected with *dl*312 or infected with *dl*520 at low multiplicity compared with mock-infected HEC. HEC doubly infected with *dl*520 and *pm*975 at low multiplicities were significantly more susceptible to NK cell-mediated lysis than HEC infected with either virus alone at low multiplicity ($P < 0.003$). Such *dl*520 plus *pm*975-infected HEC were equally susceptible to NK cell-mediated lysis compared with HEC target cells infected with *dl*309 at low multiplicity ($P > 0.10$).

fact, there is no significant difference in the NK cell susceptibility of HEC coinfected with low multiplicities of *dl*520 plus *pm*975 compared with HEC infected with a low multiplicity of *dl*309 alone. These results indicate that expression of the products of either of the E1A mRNAs is sufficient to induce this infected-cell phenotype. The results of the coinfection studies suggest two possible conclusions, neither of which can be excluded by these data. Either the functions of the products of the 12S and 13S mRNAs in the induction of this phenotype are the same and therefore additive on coinfection, or the functions of these two sets of E1A products are complementary.

Studies were also performed to evaluate the possibility that effects of the viral inoculum on HEC cells that were unrelated to E1A gene expression were a factor in increased infected target cell cytolytic susceptibility. To determine whether induction of cytolytic susceptibility is related to a toxic effect of viral structural proteins on infected target cells (33), the experiments were repeated with very high multiplicities of infection of the E1A-minus virus *dl*312 (Fig. 5). Even at a multiplicity of infection of 400 PFU of *dl*312 per cell, there were no detectable increases in the susceptibility of infected HEC to lysis by hamster NK cells. Conversely, both *dl*520 and *dl*1500, the Ad2 equivalent of *dl*520 (Table 1, Fig. 1) (20), induced a multiplicity-dependent increase in NK cell cytolytic susceptibility of infected HEC. The conclusions that simply cocultivating viral particles with HEC in the absence of infection is not sufficient for induction of cytolytic susceptibility and that viral gene expression in infected cells is required are also supported by the observation that induction of cytolytic susceptibility was blocked when the viral inoculum was neutralized with Ad5-specific rabbit antiserum. In two cytolysis assays, the mean ± SEM percent killing of *dl*309-infected HEC by hamster NK cell populations was reduced from 56.5 ± 6.3% to 12.3 ± 0.6% by pretreating the *dl*309 inoculum with neutralizing antibody. Another indirect effect of viral infection that was considered was the possibility that interferon produced in infected-cell cultures could increase the cytolytic activity of the NK cell populations, resulting in an apparent increase in the cytolytic susceptibility of otherwise resistant HEC (24). To test this possibility, the effect on hamster NK cell activity of medium conditioned by *dl*520-infected HEC was compared with that of control medium. In two comparison experiments, there were no significant differences in the cytolytic activity of hamster spleen NK cells against cytolysis-susceptible Ad2-transformed cells or cytolysis-resistant HEC. The mean ± SEM percent killing of these target cells was as follows. Ad2HE1: control medium, 44.5 ± 7.0% versus conditioned medium, 47.9 ± 3.6%; HEC: control medium, 5.9 ± 0.1% versus conditioned medium, 5.6 ± 1.4% ($P > 0.10$). A similar lack of effect on NK cell activity was observed with medium conditioned by HEC infected with wild-type virus (data not shown). These data support the conclusions that increased cytolytic susceptibility of infected rodent cells is not due to the indirect effects of the viral inoculum but rather that a direct effect of Ad5 and Ad2 E1A gene-encoded proteins is required for expression of this infected-cell phenotype.

When cytolysis assays were repeated with the same battery of virus-infected target cells and activated macrophage monolayers as the host killer cells instead of spleen NK cells, the same pattern of induction of cytolytic susceptibility was observed (Fig. 6). Both "12S-only viruses," *dl*520 and *dl*1500, and the "13S-only virus" *pm*975 induced high levels of susceptibility to macrophage-mediated target cell lysis, while the E1A-minus virus *dl*312 did not. In contrast to the relatively lower levels of killing of *dl*520-infected HEC compared with wild-type *dl*309-infected cells observed in NK cell assays (Fig. 4 and 5), the susceptiblity of *dl*520-infected HEC to killing by activated macrophages was not

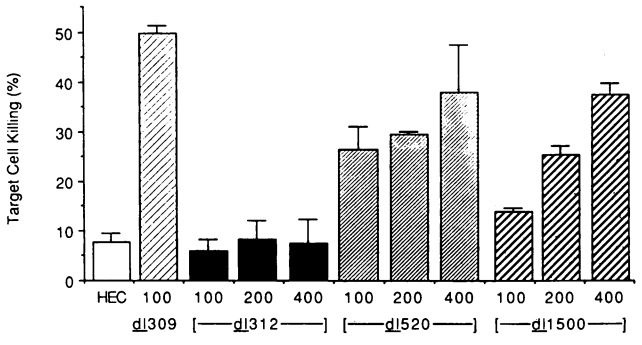

FIG. 5. Multiplicity-dependent induction of increasing levels of susceptibility to hamster NK cell-mediated lysis in HEC infected with Ad5 (*dl*520) or Ad2 12S-only (*dl*1500) virus. Bars represent the results (mean ± SEM) of three hamster NK cytolysis assays. HEC infected with wild-type Ad5 (*dl*309) were used as cytolysis-susceptible control target cells in these studies. Mock-infected HEC target cells were compared with HEC target cells infected with increasing amounts (100, 200, or 400 PFU/cell) of the indicated virus for 18 or 24 h prior to the assay. HEC infected with *dl*312 were not significantly more susceptible to lysis by hamster NK cells than mock-infected HEC. HEC infected with increasing amounts of *dl*520 ($P < 0.001$ to 0.01) or of *dl*1500 ($p < 0.001$ to 0.03) were significantly more suceptible to NK cell-mediated lysis than mock-infected HEC or *dl*312-infected HEC.

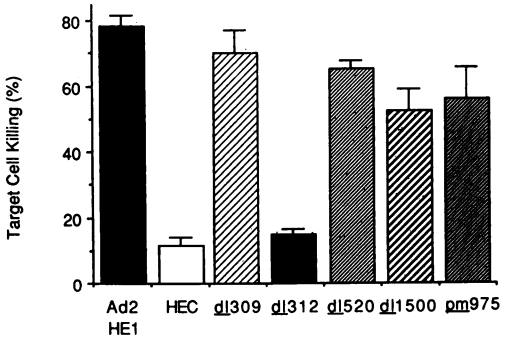

FIG. 6. Induction of susceptibility to activated hamster macrophage-mediated lysis in infected HEC by E1A 12S-only (*dl*520) or E1A 13S-only (*pm*975) mutant viruses but not by the E1A-minus Ad5 mutant virus *dl*312. Bars represent the results (mean ± SEM) of four hamster macrophage cytolysis assays. Ad2HE1 cells (Ad2-transformed hamster cell line) and *dl*309-infected HEC were used as cytolysis-susceptible control target cells in these assays. Mock-infected HEC target cells were compared with HEC target cells infected with 100 PFU of the indicated virus per cell for 18 to 24 h prior to the assay. HEC infected with wild-type Ad5 (*dl*309), *dl*520, *dl*1500, and *pm*975 were significantly more susceptible to lysis by activated macrophages than mock-infected HEC ($P < 0.001$ to 0.003). There was, however, no significant increase in the cytolytic susceptibility of *dl*312-infected HEC.

significantly different from that of *dl*309-infected HEC. This result may be due to the previously observed increased cytolytic potential of BCG-activated macrophages compared with NK cells against a given cytolysis-susceptible target cell type (8).

At high multiplicities of infection, cells infected with E1A-minus mutants may still contain transcripts and proteins encoded by other early adenovirus gene regions (30)—so-called E1A mutant leakiness. The greater cytolytic activity of BCG-activated hamster macrophages allowed an evaluation of the possibility that induction of infected-cell cytolytic susceptibility might require expression of other early adenovirus gene regions (e.g., the E1B 19-kDa protein region) due to multiplicity-dependent leakiness of the 12S-only virus *dl*520. To test this possibility, primary HEC were infected with low multiplicities (10, 20, and 40 PFU/cell) of *dl*520 for 18 h prior to use as targets in activated macrophage cytolysis assays (Fig. 7). In contrast to the results of similar hamster NK cell cytolysis assays, significant increases in infected HEC cytolytic susceptibility were seen when activated hamster macrophages were used as the killer cell populations. These results suggest that the increased susceptibility of HEC infected with high multiplicities of *dl*520 is more likely a reflection of the killer cell type used than an indication of a requirement for expression of an early region outside of E1A for detection of this infected-cell phenotype.

To further test the correlation between the expression of E1A proteins and the development of cytolytic susceptibility in infected rodent cells, a dose-response and time-response study was performed (Fig. 8). In these experiments, REC were used as the host cells for *dl*520 infection. REC are more resistant to the cytopathic effects of virus infection over prolonged periods of culture than are HEC. This resistance results in lower spontaneous release of incorporated radiolabel from infected REC in cytolysis assays (see Materials and Methods section). As had been observed in hamster target cell-NK cell experiments (Fig. 4 and 5), there was a multiplicity-dependent increase in the susceptibility of *dl*520-infected REC target cells to rat NK cell-mediated lysis (Fig. 8A). The NK cell susceptibility of *dl*520-infected REC also increased with time after infection (Fig. 8A). Infected REC lysates obtained under the same conditions of infection were examined for the presence of E1A proteins by protein-blotting studies (Fig. 8B). E1A proteins accumulated to detectable levels in *dl*520-infected REC at the same times and at the same multiplicities of infection at which infected target cells were found to show significantly increased susceptibility to rat NK cell-mediated lysis. Such protein-blotting assays may be relatively insensitive indicators of the presence of newly synthesized E1A proteins. In spite of this limitation of detection, these results suggest that there is a correlation between the levels of E1A proteins accumulating in primary embryo cells after infection with a 12S virus and the susceptibility of such infected cells to the lytic mechanisms of NK cells.

The above data suggest that the products of either the E1A 12S mRNA or the E1A 13S mRNA may mediate the increased susceptibility of infected rodent cells to lysis by NK cells or activated macrophages. It does not appear that the sequences unique to the E1A 13S mRNA coding region are essential for induction of this infected-cell phenotype. The results of NK cell assays with HEC target cells coinfected with the E1A-minus mutant *dl*312 and the E1B-minus mutant *dl*313, whose genetic deletion extends into the carboxyl terminus of the E1A gene (Fig. 1), also provide information relevant to the question of mapping the E1A gene region(s) required for induction of the cytolysis-susceptible infected-cell phenotype. As was mentioned previously, coinfection of HEC with *dl*312 and *dl*313 prevented induction of host cell DNA degradation and the accompanying high spontaneous release of incorporated [^{3}H]thymidine in labeled target cells by the E1B 19-kDa-minus *dl*313 mutant. This increased survival and associated decreased spontaneous release of radiolabel from infected cells made studies of such coinfected HEC target cells in NK cell cytolysis assays possible. The results (mean ± SEM percent target cell killing) of five such hamster NK cell cytolysis assays are as follows: uninfected HEC controls, 9.5 ± 1.8%; HEC in-

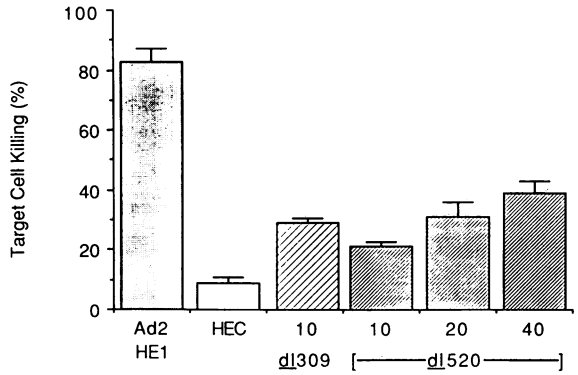

FIG. 7. Induction of susceptibility to lysis by activated hamster macrophages in HEC infected at low multiplicity with the E1A 12S-only virus *dl*520. Bars represent the results (mean ± SEM) of four hamster macrophage cytolysis assays. Ad2HE1 cells (Ad2-transformed hamster cell line) and *dl*309-infected HEC were used as cytolysis-susceptible control target cells in these assays. Mock-infected HEC target cells were compared with HEC infected with 100 PFU of the indicated virus per cell for 18 h prior to the assay. HEC infected with *dl*309 ($P < 0.001$) or with *dl*520 at low multiplicity ($P < 0.003$ to 0.01) were significantly more susceptible to lysis by activated hamster macrophages than mock-infected cells.

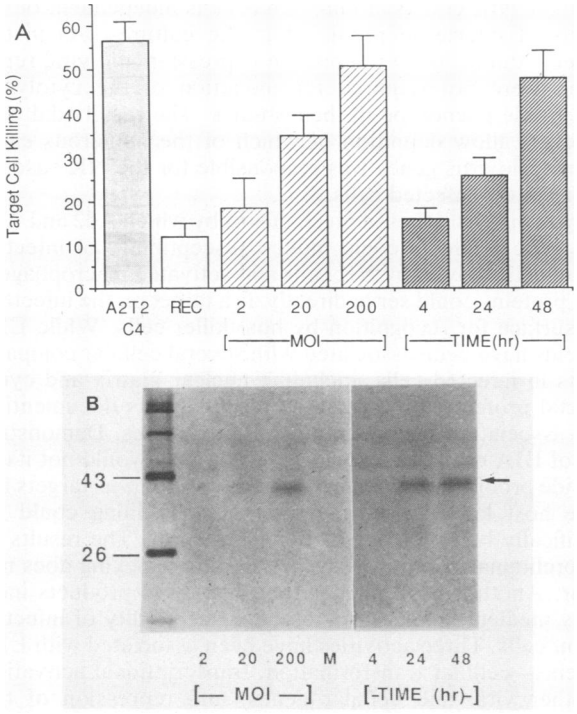

FIG. 8. Comparison of NK cell susceptibility of *dl*520-infected REC and accumulation of detectable E1A 12S mRNA products at different multiplicities of infection and at different times after infection. (A) Bars represent the results (mean ± SEM) of four rat NK cell cytolysis assays. A2T2C4 cells (Ad2-transformed rat cell line) were used as cytolysis-susceptible control target cells in these assays. Mock-infected REC target cells were compared with REC target cells infected with *dl*520 at increasing amounts (2, 20, or 200 PFU/cell) for 48 h prior to the assay (light crosshatched bars) and with REC infected with *dl*520 at 200 PFU/cell for increasing times (4, 24, or 48 h) prior to the assay (dark crosshatched bars). REC infected with *dl*520 at 20 PFU/cell for 48 h or at 200 PFU/cell for 24 or 48 h were significantly more susceptible to rat NK cell-mediated lysis than mock-infected REC ($P < 0.025$ to 0.003). REC infected with *dl*520 at 2 PFU/cell for 48 h or at 200 PFU/cell for 4 h were not significantly more susceptible to lysis by NK cells than mock-infected REC. (B) Lysates were prepared from mock-infected REC (M) or REC infected with *dl*520 under the same conditions of increasing multiplicity (2, 20, or 200 PFU/cell) for 48 h and of increasing time (4, 24, or 48 h) at a fixed multiplicity (200 PFU/cell) and were analyzed for the presence of detectable E1A 12S mRNA proteins by an immunoblotting study with E1A-specific rabbit antiserum. Immune complexes were detected on filters with ^{125}I-labeled protein A and autoradiography. E1A proteins (arrow) were detected at a multiplicity of infection (MOI) of 20 and 200 at 48 h after infection and at an MOI of 200 at 24 and 48 h after infection. No E1A proteins were detected at 2 PFU/cell after 48 h of infection or at 200 PFU/cell after 4 h of infection. Molecular size marker protein locations (in kilodaltons) are shown at the left.

fected for 24 h with *dl*312 at 100 PFU/cell, 10.7 ± 1.7%; HEC coinfected for 24 h with *dl*312 + *dl*313 at 100 PFU/cell of each virus, 46.2 ± 4.5% ($P < 0.001$). These data indicate that the E1A gene region deleted from *dl*313 (the carboxyl 70% of the E1A second exon) is not essential for induction of increased cytolytic susceptibility in infected HEC. A more precise definition of the E1A region(s) required by infectious adenoviruses to induce cytolytic susceptibility in rodent cells will require testing additional E1A mutants in these assays.

E1A gene of highly oncogenic Ad12 is ineffective in the induction of cytolytic susceptibility in infected cells. We have previously reported that, in contrast to nononcogenic Ad2, highly oncogenic Ad12 is ineffective in the induction of infected-cell susceptibility to lysis by NK cells or activated macrophages (10). Such data can be viewed from two different mechanistic perspectives. It is possible that Ad12 E1A products are simply not as efficient in the induction of infected-cell cytolytic susceptibility as are the E1A products of nononcogenic Ad2 and Ad5. Conversely, Ad12 E1A products might actively induce resistance of infected cells to the lytic mechanisms of these host effector cells. To test these possibilities, we performed studies of the effects of coinfection of HEC with Ad12 and Ad5 mutants on HEC susceptibility to NK cell-mediated lysis (Fig. 9). As had been observed previously, infection of HEC with Ad12 alone induced little increase in the susceptibility of target cells over the uninfected-cell background, while the wild-type Ad5 mutant *dl*309 induced a marked increase in HEC susceptibility to NK cell-mediated killing. When HEC were preinfected with Ad12 for 24 h and then infected with *dl*309 (Fig. 9) or coinfected with these two viruses (data not shown), there was no reduction in the NK cell susceptibility of the infected cells compared with cells infected with *dl*309 alone. This result suggests that Ad12 E1A either has no inhibitory or resistance-inducing effect on the cytolytic phenotype of infected HEC or that the active induction of cytolytic susceptibility by Ad5 (*dl*309) is dominant over the cytolysis resistance-inducing effect of Ad12 E1A. Another approach to evaluating the effect of Ad12 E1A on the cytolytic phenotype of infected cells is to coinfect HEC with Ad12 and the E1A-minus Ad5 mutant *dl*312. The expression

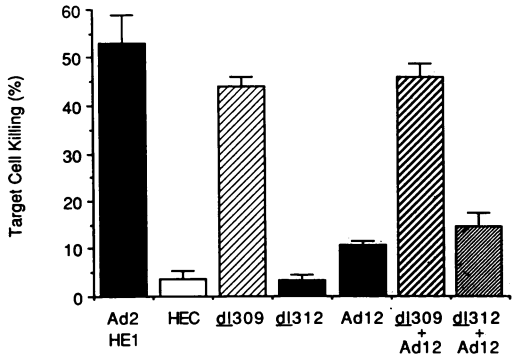

FIG. 9. Inability of Ad12 to block induction of cytolytic susceptibility by Ad5 or to complement the lack of induction of cytolytic susceptibility by the E1A-minus virus *dl*312. Bars represent the results (mean ± SEM) of three hamster NK cell cytolysis assays. Ad2HE1 cells (Ad2-transformed hamster cell line) were used as cytolysis-susceptible control target cells in these assays. The effect of Ad12 on induction of HEC susceptibility to lysis by hamster NK cells were tested by comparing the cytolytic susceptibilities of mock-infected HEC target cells and HEC target cells infected with the indicated single viruses (100 PFU/cell for 18 to 24 h) with the cytolytic susceptibilities of doubly infected HEC. To evaluate the possible blocking effects of Ad12 infection on the induction of NK cell susceptibility by wild-type Ad5 (*dl*309), HEC target cells were preinfected for 24 h with Ad12 (100 PFU/cell) and then were infected for 18 h with *dl*309 (100 PFU/cell) prior to use in cytolysis assays. For studies of the possible complementing effect of Ad12 infection on the induction of cytolytic susceptibility by *dl*312, HEC were simultaneously infected with both viruses at 100 PFU/cell for 24 h prior to the assay. Ad12 infection of HEC caused no significant reduction in the cytolytic susceptibilities of *dl*309-infected HEC and no significant increase in the cytolytic susceptibilities of HEC infected with *dl*312 compared with HEC infected with only *dl*309 or *dl*312, respectively.

of Ad12 E1A complements the virus replication and transformation defects of *dl*312 in human and hamster cells (1, 27, 31; A. M. Lewis, Jr., unpublished data). The question asked in these experiments is whether the E1A of Ad12 can complement this mutant's inability to induce increased susceptibility to NK cell killing in infected cells. The results of these studies show that coinfection with Ad12 and *dl*312 did not result in a significant increase in the susceptibility of HEC to NK cell killing over the low levels of cytolytic susceptibility observed with Ad12 infection alone. These results indicate that Ad12 E1A function cannot replace that of Ad5 E1A in the induction of this infected-cell phenotype.

DISCUSSION

When different human adenoviruses are inoculated into rodents or used to infect rodent cells in vitro, several serotype-dependent differences are observed (33). Ad2 and Ad5 are nononcogenic when inoculated into newborn hamsters or rats, while Ad12 is highly oncogenic in such animals, inducing tumors in the majority of animals within 2 months. In addition, Ad12 can induce tumors when inoculated into immunologically maturing weanling hamsters, although at a reduced incidence compared with that observed in immunodeficient newborn animals (38). In spite of their lack of oncogenicity in vivo, Ad2 and Ad5 can neoplastically transform rodent cells in vitro. There is no correlation between the efficiencies with which different serotypes transform rodent cells in vitro and their oncogenicity in vivo (reviewed in reference 18). For example, nononcogenic Ad2 and highly oncogenic Ad12 transform hamster and rat cells with approximately equal efficiency. These data imply that the factors that determine the tumor-inducing capacities of these viruses involve interactions between virus-infected, and eventually transformed, cells and the rodent host. The results of previous studies indicate that the nonspecific host cellular immune response in the form of NK cells and activated macrophages selectively destroys hamster and rat cells infected with or transformed by nononcogenic Ad2 while sparing Ad12-infected and Ad12-transformed cells (8, 10, 19, 25, 28). Recent studies show that expression of the E1A gene of nononcogenic adenoviruses in transformed hamster and rat cells is sufficient to induce susceptibility of the transformed cells to destruction by host NK cells and activated macrophages (11, 28). The results of the present study extend those observations to the adenovirus-infected rodent target cell system. These data indicate that the same early viral gene, E1A, expressed shortly after infection renders Ad2- or Ad5-infected cells susceptible to lysis by the same two types of host killer cells, while the E1A gene of highly oncogenic Ad12 is inefficient in inducing this cytolysis-susceptible phenotype in infected rodent cells.

There have been few other studies of the requirement for specific viral gene expression for induction of susceptibility of infected target cells to lysis by NK cells or activated macrophages. Macrophages have been reported to have the ability to limit virus replication in infected target cells (22, 32). However, prior to the present report, specific viral genes that induce susceptibility to macrophage-mediated effects on virus-infected target cells had not been identified. In a report by Borysiewicz et al. on the effects of human cytomegalovirus infection on target cell susceptibility to lysis by human NK cells, findings similar to those in the current study were presented (2). They observed that expression of early cytomegalovirus antigens in infected human fibroblasts was associated with increased susceptibility to the lytic effects of human NK cells independent of the effects of interferon produced in the cultures. As in the present studies, late viral protein expression and viral replication were not required for induction of the cytolysis-susceptible phenotype in their studies. The results did not, however, allow definition of which of the numerous early cytomegalovirus genes was responsible for the NK susceptibility in the infected cells.

There are two types of mechanisms by which Ad2 and Ad5 E1A gene products could induce susceptibility of infected rodent cells to lysis by NK cells and activated macrophages. E1A proteins could serve directly as a target on the infected-cell surface for recognition by host killer cells. While E1A proteins have been associated with several cellular compartments in infected cells, including nuclear matrix and cytoskeletal proteins (6, 29), there are no reports documenting E1A association with infected-cell membranes. Demonstration of E1A on the surface of infected cells would not itself provide proof of the direct role of E1A proteins as targets for these host killer cells unless target cell killing could be specifically blocked by anti-E1A antibodies. The results of our preliminary studies suggest that such blocking does not occur. Another possibility is that E1A gene products indirectly mediate increased cytolytic susceptibility of infected rodent cells. Three activities have been associated with E1A proteins—cellular transformation, transcriptional activation of other viral and cellular genes, and repression of the activity of enhancers of other viral and cellular gene promoters (reviewed in reference 15). Since the current studies and those previously reported demonstrate that E1A gene expression can induce cytolytic susceptibility in cells in the absence of neoplastic transformation, the transforming function of E1A can be excluded as a requirement for induction of this cellular phenotype. Both the transcription-activating and enhancer-repressing activities of E1A can affect cellular genes. These remain possible pathways through which the cytolysis-susceptible phenotype of adenovirus-infected cells could be mediated.

The results presented here may provide a more complete explanation for the observed differences in the oncogenicity of Ad2 and Ad5 (nononcogenic) compared with Ad12 (highly oncogenic). We speculate that the following sequence of events may occur upon infection of a rodent host with a given adenovirus serotype. The population of infected cells undergoes a certain amount of attrition due to virus-induced cytopathology or chromosomal damage. Those infected cells that survive these virus-induced insults then exhibit a serotype-dependent, E1A-induced level of susceptibility to destruction by NK cells and activated macrophages. Ad2- or Ad5-infected cells would be highly susceptible to NK cell- and activated macrophage-mediated lysis, while Ad12-infected cells would be relatively resistant to elimination by such host inflammatory cell responses. This cytolysis-susceptible or cytolysis-resistant phenotype of infected cells would be expressed in advance of any signs of virus-induced cellular transformation. The selective host effector cell-mediated destruction of Ad2- or Ad5-infected cells compared with Ad12-infected cells would result in a greater number of surviving Ad12-infected cells that could ultimately exhibit the fully transformed phenotype. Once transformation has occurred, the Ad12-transformed cells would also be resistant to destruction by the same immunologically nonspecific host cellular immune defenses, as has been reported for both Ad12-transformed hamster and rat cells (8, 25).

The reasons for the differences in the susceptibility of cells infected with nononcogenic Ad2 and Ad5 compared with

oncogenic Ad12 remain to be defined. The data presented here suggest that Ad12 E1A is simply inefficient in induction of the cytolysis-susceptible phenotype, rather than there being an active induction of cytolytic resistance by Ad12 E1A; however, the latter possibility is not excluded by these results. Definition of the mechanisms by which Ad2 and Ad5 E1A gene expression induces increased cytolytic susceptibility of infected cells will provide a basis for further comparative evaluation of the functions of the E1A genes of nononcogenic and oncogenic adenovirus serotypes. Furthermore, this model of virus-specific early gene regulation of infected-cell phenotypes may provide information of general importance for understanding the reasons for selective recognition and destruction of virus-infected cells by NK cells and activated macrophages.

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LITERATURE CITED

1. **Bernards, R., A. Houweling, P. I. Schrier, J. L. Bos, and A. J. Van der Eb.** 1982. Characterization of cells transformed by Ad5/Ad12 hybrid early region plasmids. Virology **120**:422–432.
2. **Borysiewicz, L. K., B. Rodgers, S. Morris, S. Graham, and J. G. P. Sissons.** 1985. Lysis of human cytomegalovirus infected fibroblasts by natural killer cells: demonstration of an interferon-independent component requiring expression of early viral proteins and characterization of effector cells. J. Immunol. **134**:2695–2701.
3. **Bos, J. L., L. J. Polder, R. Bernards, P. I. Schrier, A. J. Van den Elsen, and H. Van Ormondt.** 1981. The 2.2 Kb E1B mRNA of human Ad12 and Ad5 codes for two tumor antigens starting at different AUG triplets. Cell **27**:121–131.
4. **Burnette, W. N.** 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radiodinated protein A. Anal. Biochem. **112**:195–203.
5. **Challberg, S. S., and G. Ketner.** 1981. Deletion mutants of adenovirus 2: isolation and initial characterization of virus carrying mutations near the right end of the viral genome. Virology **114**:196–209.
6. **Chatterjee, P. K., and S. J. Flint.** 1986. Partition of E1A proteins between soluble and structural fractions of adenovirus-infected and -transformed cells. J. Virol. **60**:1018–1026.
7. **Collins, J. L., P. Q. Patek, and M. Cohn.** 1981. Tumorigenicity and lysis by natural killers. J. Exp. Med. **153**:89–106.
8. **Cook, J. L., J. B. Hibbs, Jr., and A. M. Lewis, Jr.** 1982. DNA virus-transformed hamster cell-host effector cell interactions: level of resistance to cytolysis correlated with tumorigenicity. Int. J. Cancer **30**:795–803.
9. **Cook, J. L., and A. M. Lewis, Jr.** 1979. Host response to adenovirus 2-transformed hamster embryo cells. Cancer Res. **39**:1455–1461.
10. **Cook, J. L., and A. M. Lewis, Jr.** 1984. Differential NK cell and macrophage killing of hamster cells infected with nononcogenic or oncogenic adenovirus. Science **224**:612–615.
11. **Cook, J. L., T. A. Walker, A. M. Lewis, Jr., H. E. Ruley, F. L. Graham, and S. H. Pilder.** 1986. Expression of the adenovirus E1A oncogene during cell transformation is sufficient to induce susceptibility to lysis by host inflammatory cells. Proc. Natl. Acad. Sci. USA **83**:6965–6969.
12. **Flint, J. S.** 1986. Alteration in cellular function in adenovirus-infected and transformed cells, p. 311–341. *In* W. Doerfler (ed.), Adenovirus DNA: the viral genome and its expression. Martinus Nijhoff Publishing, Boston.
13. **Gaynor, R. B., A. Tsukamoto, C. Montell, and A. J. Berk.** 1982. Enhanced expression of adenovirus transforming proteins. J. Virol. **44**:276–285.
14. **Haley, K. P., J. Overhauser, L. E. Babiss, H. S. Ginsberg, and N. C. Jones.** 1984. Transformation properties of type 5 adenovirus mutants that differentially express the E1A gene products. Proc. Natl. Acad. Sci. USA **81**:5734–5738.
15. **Imperiale, M. J., and J. R. Nevins.** 1986. Transcriptional and post-transcriptional regulation of adenovirus gene expression, p. 129–160. *In* W. Doerfler (ed.), Adenovirus DNA: the viral genome and its expression. Martinus Nijhoff Publishing, Boston.
16. **Jones, N., and T. Shenk.** 1979. Isolation of adenovirus type 5 host range deletion mutants defective for transformation of rat embryo cells. Cell **17**:683–689.
17. **Krippl, B., B. Ferguson, M. Rosenberg, and H. Westphal.** 1981. Functions of purified E1A protein microinjected into mammalian cells. Proc. Natl. Acad. Sci. USA **81**:6988–6992.

17a. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.

18. **Lewis, A. M., Jr., and J. L. Cook.** 1984. The interface between adenovirus-transformed cells and cellular immune response in the challenged host. Curr. Top. Microbiol. Immunol. **110**:1–22.
19. **Lewis, A. M., Jr., and J. L. Cook.** 1985. A new role for DNA virus early proteins in viral carcinogenesis. Science **227**:15–20.
20. **Montell, C., G. Courtois, C. Eng, and A. Berk.** 1984. Complete transformation by adenovirus 2 requires both E1A proteins. Cell **36**:951–961.
21. **Montell, C., E. F. Fisher, M. H. Caruthers, and A. J. Berk.** 1982. Resolving the functions of overlapping genes by site-specific mutagenesis at a mRNA splice site. Nature (London) **295**: 380–384.
22. **Morahan, P. S., S. S. Morse, and M. B. McGeorge.** 1980. Macrophage extrinsic antiviral activity during herpes simplex virus infection. J. Gen. Virol. **46**:291–300.
23. **Persson, H., M. G. Katze, and L. Philipson.** 1982. Purification of a native membrane-associated adenovirus tumor antigen. J. Virol. **42**:905–917.
24. **Rager-Zisman, B., and B. R. Bloom.** 1982. Natural killer cells in resistance to virus-infected cells. Springer Semin. Immunopathol. **4**:397–414.
25. **Raska, K., and P. H. Gallimore.** 1982. An inverse relation of the oncogenic potential of adenovirus-transformed cells and their sensitivity to killing by syngeneic natural killer cells. Virology **123**:8–18.
26. **Rowe, D. T., P. E. Branton, and F. L. Graham.** 1984. The kinetics of synthesis of early viral proteins in KB cells infected with wild-type and transformation-defective host-range mutants of human adenovirus type 5. J. Gen. Virol. **65**:585–597.
27. **Rowe, D. T., and F. L. Graham.** 1981. Complementation of adenovirus type 5 host range mutants by adenovirus type 12 in coinfected HeLa and BHK-21 cells. J. Virol. **38**:191–197.
28. **Sawada, Y., B. Föhring, T. E. Shenk, and K. Raska.** 1985. Tumorigenicity of adenovirus-transformed cells: region E1a of adenovirus 12 confers resistance to natural killer cells. Virology **147**:413–421.
29. **Schmitt, R. C., M. L. Fahnestock, and J. B. Lewis.** 1987. Differential nuclear localization of the major adenovirus type 2 E1A proteins. J. Virol. **61**:247–255.
30. **Shenk, T., N. Jones, W. Colby, and D. Fowlkes.** 1979. Functional analysis of adenovirus-5 host-range deletion mutants defective for transformation of rat embryo cells. Cold Spring Harbor Symp. Quant. Biol. **44**:367–375.
31. **Shiroki, K., I. Saito, K. Maruyama, and H. Shimojo.** 1983. Isolation of a nondefective recombinant between adenovirus type 5 and early region 1A of adenovirus type 12. J. Virol. **46**:632–637.
32. **Stohlman, S. A., J. G. Woodward, and J. A. Frelinger.** 1982. Macrophage antiviral activity: extrinsic versus intrinsic activity. Infect. Immun. **36**:672–677.
33. **Tooze, J. (ed.).** 1973. The molecular biology of tumor viruses, p. 420–469. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
34. **Townsend, A. R. M., and A. J. McMichael.** 1985. HLA class-I

restriction of T cell mediated cytotoxicity. Specificity of cytotoxic T lymphocytes stimulated with influenza virus. Prog. Allergy **36**:10–43.

35. **White, E., and B. Stillman.** 1987. Expression of adenovirus E1B mutant phenotypes is dependent on the host cell and on synthesis of E1A proteins. J. Virol. **61**:426–435.
36. **Williams, J.** 1986. Adenovirus genetics, p. 247–309. *In* W. Doerfler (ed.), Adenovirus DNA: the viral genome and its expression. Martinus Nijhoff Publishing, Boston.
37. **Winberg, G., and T. Shenk.** 1984. Dissection of overlapping functions within the adenovirus type 5 E1A gene. EMBO J. **3**:1907–1912.
38. **Yabe, Y., J. J. Trentin, and G. Taylor.** 1962. Cancer induction in hamsters by human type 12 adenovirus. Effect of age and of virus dose. Proc. Soc. Exp. Biol. Med. **111**:343–344.
39. **Young, C. S. H., T. Shenk, and H. S. Ginsberg.** 1984. The genetic system, p. 125–172. *In* H. S. Ginsberg (ed.), The adenoviruses. Plenum Publishing Corp., New York.