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The E1B 19,000-Molecular-Weight Protein of Group C Adenoviruses Prevents Tumor Necrosis Factor Cytolysis of Human Cells but Not of Mouse Cells

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Tumor necrosis factor (TNF) is a multifunctional immunoregulatory protein that is secreted by activated macrophages and is believed to have antiviral activities. We reported earlier that when mouse C3HA fibroblasts are infected with human adenoviruses, the 289R and 243R proteins encoded by region E1A render the cells susceptible to lysis by TNF, and a 14,700-molecular-weight protein (14.7K protein) encoded by region E3 protects the cells against lysis by TNF. We now report that the 19,000-molecular-weight (19K) (176R) protein encoded by the E1B transcription unit can protect human HEL-299 fibroblasts and human ME-180 cervical carcinoma cells against lysis by TNF. This was determined by infecting cells with adenovirus double mutants that lack region E3 and do or do not express the E1B-19K protein and by measuring cytolysis by using a short-term (18-h) ^{51}Cr-release assay. Under these assay conditions, the ^{51}Cr release was specific to TNF and was not a consequence of the *cyt* phenotype associated with E1B-19K protein-negative mutants. Also, by using virus double mutants that lack E3 in combination with other early regions, we found that E1A, the E1B-55K protein-encoding gene, E3, and E4 are not required to protect HEL-299 cells against TNF cytolysis. Three additional human cancer cell lines (HeLa, HCT8, and RC29) and a simian virus 40-transformed WI38 cell line (VA-13) also required E1B for protection against TNF cytolysis, indicating that the E1B-19K protein is required to protect many if not all human cell types against lysis by TNF when infected by adenovirus. The E1B-19K protein was not able to protect six different adenovirus-infected mouse cell lines against TNF lysis, even though the protein was shown to be efficiently expressed in one of the cell lines. HEL-299 or ME-180 cells infected by a mutant that lacks the E1B-19K protein but retains region E3 were not lysed by TNF, indicating that one or more of the E3 proteins can protect these cells against TNF lysis in the absence of the E1B-19K protein. Thus, the E3-14.7K but not the E1B-19K protein can protect adenovirus-infected mouse cells against TNF cytolysis, whereas the E1B-19K protein as well as one or more of the E3 proteins can protect adenovirus-infected human cells against TNF cytolysis.

Tumor necrosis factor (TNF) is a multifunctional cytokine which is secreted by activated macrophages and which regulates a wide variety of aspects of the immune system and inflammatory response (reviewed in reference 7). A notable feature of TNF is that it is cytotoxic or cytostatic to certain tumor cells. TNF also lyses cells infected with certain viruses (13, 16, 20, 28, 39, 46, 89, 90), inhibits the replication of a number of RNA and DNA viruses (2, 17, 34, 41, 42, 50, 75, 89, 90), and inhibits the activation of B cells by Epstein-Barr virus (30). TNF is released during infections by respiratory viruses such as influenza virus (72). These observations support the hypothesis that TNF inhibits virus replication in vivo.

Adenovirus (Ad) encodes several proteins that have remarkable effects on the cellular response to TNF. Expression of the region E1A 289-amino-acid (289R) or 243R proteins, either in Ad-infected mouse cells (15, 16) or in permanent transfected mouse cell lines (1, 10, 12, 16) but not in all cell types (76), renders the cells susceptible to cytolysis by TNF. The mechanism by which this occurs and the significance to the biology of Ad are not known. Since E1A-induced TNF-mediated cytolysis would be expected to be deleterious to Ad replication in vivo, it would not be surprising if Ad had evolved genes to prevent TNF cytolysis. Consistent with this, we have shown that the 14,700-molecular-weight protein (14.7K protein) encoded within the E3 transcription unit (48, 71, 77) can protect Ad-infected C3HA or NIH 3T3 mouse cells against TNF cytolysis (20, 21, 28). The 14.7K protein was the only Ad protein that was found to provide protection against TNF in these cells. Recently we have shown (21) that the 14.7K protein prevents TNF cytolysis not only when cells are sensitized to TNF by expression of E1A but also when they are sensitized by inhibition of protein synthesis by cycloheximide (CHI) or by treatment with cytochalasin E, an agent that disrupts microfilaments and sensitizes cells to TNF (51). Also, the 14.7K protein has been found to prevent TNF lysis in nearly all mouse cell lines tested so far, including "normal" mouse fibroblasts, transformed fibroblasts and epithelial cells, and two of three cell lines that are spontaneously susceptible to TNF cytolysis (21). Thus, the 14.7K protein appears to be a general inhibitor of TNF lysis. We have also observed that permanent transfected cells that express the 14.7K protein are not killed by TNF, indicating that this protein can function in the absence of other Ad proteins (27a).

In our earlier results (15, 16, 20, 21, 28), the TNF lysis experiments were performed in the C3HA mouse fibroblast

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cell line. These cells were used because extensive TNF cytolysis was reproducibly observed after infection with 14.7K protein-negative mutants. Although we occasionally observed TNF lysis of human 293 and human embryo lung (HEL) cells after infection with Ad E3 deletion mutants, the results were not consistent and the amount of cytolysis was low. These latter results were puzzling, because we were working with human Ads. We now report that the 19K protein (176R) encoded by the E1B transcription unit can protect human HEL-299 and ME-180 cells from TNF lysis when infected with Ad mutants that lack region E3. This explains our previous inability to observe consistent lysis of human cells, because all our mutants expressed the E1B-19K protein. Our observations add to the growing list of properties that have been associated with the E1B-19K protein, and they add new perspective to the physiological role of this interesting molecule. Our studies also provide insight into the mechanisms that exist in Ad to allow it to escape immune surveillance.

MATERIALS AND METHODS

Cell lines. Human cell lines are as follows. HEL-299 fibroblasts, ME-180 epidermoid carcinoma of the cervix, HeLa epitheliod carcinoma of the cervix, HCT8 ileocecal adenocarcinoma, and VA13 simian virus 40 (SV40)-transformed WI38 lung fibroblasts were obtained from the American Type Culture Collection (ATCC). RC29 renal cell carinoma cells were obtained from S. Graham (Emory University, Atlanta, Ga.). Mouse cell lines are as follows. NIH 3T3 contact-inhibited fibroblasts were obtained from ATCC. C3HA is a 3T3-like line derived from C3H mouse embryo fibroblasts (19). Mel B16 is a cultured explant of the C57B1/6 B16 melanoma obtained from the Jackson Laboratory. 10T1/2 cells, contact-inhibited C3H fibroblasts, and SV40-transformed 10T1/2 cells (SV-10T1/2) were obtained from J. Pipas (University of Pittsburgh, Pittsburgh, Pa.). MCA cells were derived from a methylcholanthrene-induced fibrosarcoma in C57B1/6 mice and were obtained from D. Adams (Duke University, Durham, N.C.). All cells were grown in Dulbecco modified Eagle medium (GIBCO, Grand Island, N.Y.) supplemented with 10% fetal calf serum (Hyclone, Logan, Utah).

Viruses. Schematics have been presented elsewhere for the Ad2 genome (16), E1A and E1B (e.g., see references 62 and 82), E3 (87), and E4 (25). *dl*708 (14), *dl*758 (20), and *dl*799 (see below) were derived from *rec*700 (88). *rec*700 is an Ad5/Ad2/Ad5 recombinant which consists of the Ad5 *Eco*RI-A (map position 0 to 76), Ad2 *Eco*RI-D (76 to 83), and Ad5 *Eco*RI-B (83 to 100) fragments. Nucleotides 1654 to 2207 in the E3 transcription unit are deleted in *dl*708; this deletes genes encoding the E3 10.4K, putative 7.5K, and 11.6K proteins, as well as a portion of the gene encoding the gp19K protein. *dl*758 (Δ3003-3251 in region E3) deletes the gene encoding the E3-14.7K protein. *dl*799 (Δ2229-2803 in region E3) was prepared by building the E3 deletion of *dl*753 (8) into the genome of *dl*764 (70).

Mutants *dl*309, *dl*312, *dl*313 (32), *sub*304 (31), *dl*366 (25), and *dl*327 were obtained from T. Shenk (Princeton University, Princeton, N.J.). *dl*327 is identical to *dl*324 (68); it lacks the Ad5 *Xba*I-D fragment so it lacks genes encoding the E3 6.7K, gp19K, 11.6K, 10.4K, 7.5K, 14.5K, and 14.7K proteins, and it retains the remaining genes encoding the putative E3 12.5K and 3.6K proteins. *dl*366 has the same E3 deletion as in *dl*327 and it also lacks all the predicted E4 genes. *dl*309 lacks the genes encoding the E3 10.4K, 7.5K, 14.5K, and 14.7K proteins and it has a mutation in the gene encoding the E3 6.7K protein (see reference 9). *dl*312 has the same E3 deletion as in *dl*309 and it also deletes most of E1A. *pm*975 (43) and *dl*1520 (4) were obtained from A. Berk (University of California, Los Angeles, Calif.), and *dl*110, *dl*111, and *dl*118 (3) were obtained from H. Ginsberg (Columbia University, New York, N.Y.); these mutants are all in a *dl*309 background. *pm*975 synthesizes the E1A-289R but not the E1A-243R protein, *dl*110 and *dl*1520 synthesize the E1B-19K but not the E1B-55K protein, *dl*111 synthesizes the E1B-55K but not the E1B-19K protein, and *dl*118 does not synthesize either E1B protein. All the above mutants are Ad5. *dl*237 (63) and *dl*250 (62) were obtained from G. Chinnadurai (St. Louis University Medical Center, St. Louis, Mo.). *dl*237 has the Ad2 cDNA copy of the 12S mRNA in place of wild-type E1A, placed into a *dl*309 background; it synthesizes the E1A-243R but not the E1A-289R protein. *dl*250 is an Ad2 mutant that deletes the gene encoding the E1B-19K protein but retains region E3.

Mutants *dl*708, *dl*758, *dl*799, *rec*700, *dl*309, and *dl*327 were grown in suspension cultures of KB cells and banded in CsCl as described (24). *dl*366 was grown in monolayers of W162 cells, a monkey cell line that expresses region E4 and complements the E4 defect in *dl*366 (78). The remaining mutants were prepared in monolayers of 293 cells, a human cell line that expresses regions E1A and E1B and complements the defects in the mutants (22). The procedure used to prepare virus stocks (not banded in CsCl) from monolayer cultures has been described (16).

Immunoprecipitation. Procedures for protein labeling, immunoprecipitation, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were as previously described (71). Monoclonal antibody to adenovirus E1B-p21 (19K) was purchased from Oncogene Sciences, Inc.

Assay of TNF cytotoxicity (^{51}Cr release). The ^{51}Cr-release assay for TNF cytotoxicity was performed essentially as previously described (20). Briefly, cells were infected with virus and then incubated 18 h prior to the addition of $Na_2{}^{51}CrO_4$ (200 μCi per 60-mm culture dish; Dupont, NEN Research Products, Boston, Mass.). Cells were harvested for the ^{51}Cr-release assay after a further 6 h of incubation at 37°C (total of 24 h postinfection [p.i.]). Assay of ^{51}Cr released from infected and control cells was performed by incubation with human recombinant TNF (hr TNF) protein (13×10^6 U/mg) (the generous gift of Cetus Corporation, Emeryville, Calif.) for 18 h at 37°C. Exceptions to this general protocol were as follows. Assays of TNF lysis in the presence of CHI were incubated for 16 h instead of 18 h. TNF lysis of ME-180 cells was determined in a 12-h assay. Cells infected with *dl*312 were assayed at 48 h p.i. to ensure complete expression of viral proteins in the absence of E1A (16). Cells infected with *dl*110, *dl*118, and *dl*250, all of which lack the E1B-19K protein, were assayed at 6 h p.i. Further incubation prior to assay resulted in high spontaneous release values. In contrast, *dl*313-infected cells could be assayed at 24 h p.i. without increases in spontaneous release, apparently because the deletion in E1A slows infection somewhat.

Results are expressed as the percentage of specific lysis which equals 100 times the counts per minute of ^{51}Cr released from cells in the presence of TNF minus the counts per minute of ^{51}Cr released in the presence of medium alone (spontaneous release) divided by the counts per minute of ^{51}Cr released in 1 N HCl (maximum release) minus the counts per minute of ^{51}Cr released in medium alone. Results shown are calculated from triplicate samples ± the standard

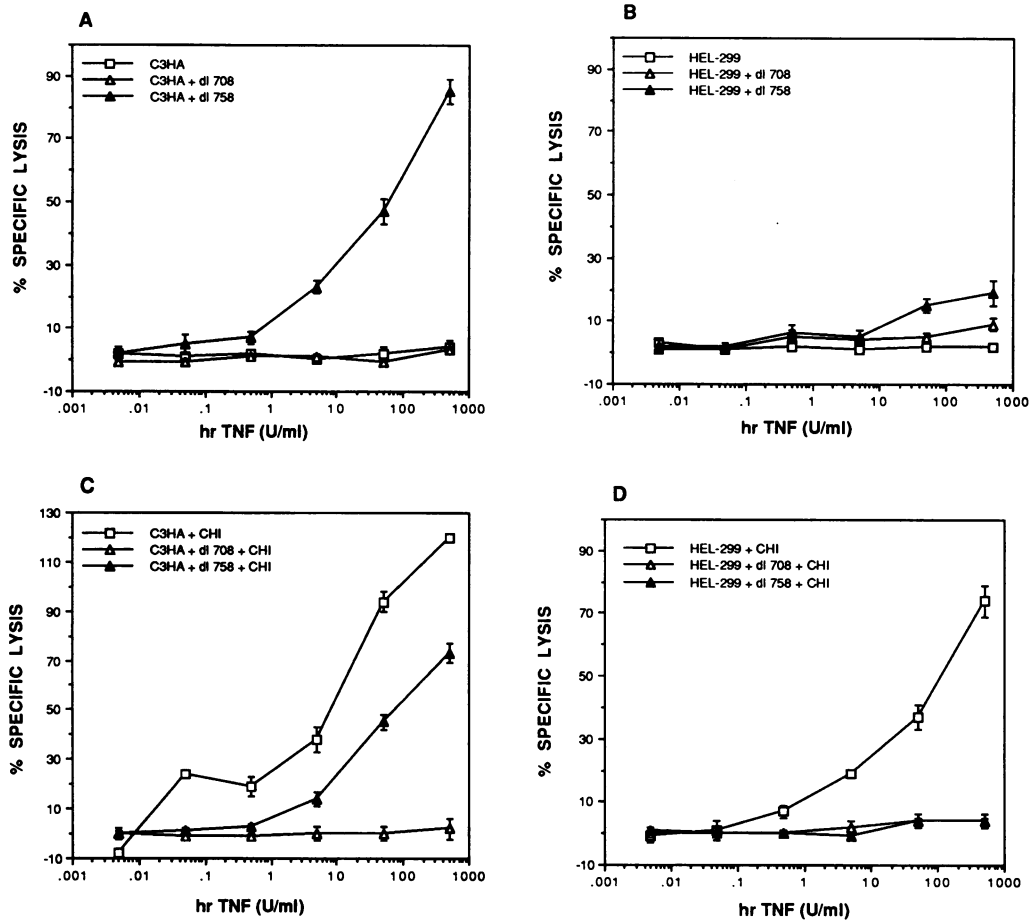

FIG. 1. ^{51}Cr-release assay for TNF-induced cytolysis of mouse (C3HA) or human (HEL-299) fibroblasts infected with *dl*708 or *dl*758 and either untreated or treated with CHI. In all cases, TNF was added at 24 h p.i. CHI treatment renders the cells susceptible to TNF lysis. *dl*708 expresses the E3-14.7K protein, whereas *dl*758 does not. The figure shows that C3HA cells, either untreated (A) or treated with CHI (C), are lysed by TNF when infected with a mutant (*dl*758) that lacks the E3-14.7K protein but not when infected with a mutant (*dl*708) that expresses the 14.7K protein. HEL-299 cells are not lysed by TNF when infected with a 14.7K protein-negative mutant (B); however, an Ad protein other than the E3-14.7K protein or the genes deleted in *dl*708 can prevent lysis of HEL-299 cells in the presence of TNF plus CHI (D).

error of the mean (typically 0 to 2%) in representative experiments. Protocols were designed to maintain constant spontaneous release levels for all target cells and conditions of virus infection. Average spontaneous release values for the experiments shown in the figures, expressed as a percentage of maximum release, were as follows. Mouse cell lines: uninfected, 38 ± 3; uninfected treated with CHI, 44 ± 1; infected with Ads lacking the E1B-19K protein, 43 ± 2; infected with all other Ads, 36 ± 3; Ad infected and treated with CHI, 41 ± 3. Human cell lines (except ME-180): uninfected, 39 ± 2; uninfected plus CHI, 45 ± 2; infected with *dl*313 (assayed at 24 h p.i.), 47 ± 1; infected with other mutants lacking the E1B-19K protein (assayed at 6 h p.i.), 44 ± 2; infected with all other Ads, 41 ± 1; Ad infected and treated with CHI, 45 ± 2. ME-180: uninfected, 16 ± 1; infected with mutants lacking the E1B-19K protein, 35 ± 3; infected with all other Ads, 15 ± 1. Each experiment was repeated a minimum of three times and usually many more than three times. In all experiments, infected cells were plated onto glass slides, fixed, and stained by using anti-Ad5 serum (ATCC) to confirm the percentage of cells infected in the cell population. Cells were 95% infected in experiments reported here.

RESULTS

The E1B-19K protein protects human HEL-299 and ME-180 cells against lysis by TNF. We have reported previously that TNF does not lyse mouse C3HA or NIH 3T3 fibroblasts when they are either uninfected or infected with Ads that express the E3-14.7K protein (16, 20, 28). This is illustrated in Fig. 1A: uninfected C3HA cells were not lysed by TNF, nor were cells infected with *dl*708, an E3 mutant that deletes the C-terminal eight amino acids of the gp19K protein, does not express the 11.6K, 10.4K, or putative 7.5K proteins, and overproduces the 14.7K protein. On the other hand, C3HA cells were killed by TNF when infected with *dl*758, a mutant that deletes the gene encoding the 14.7K protein. We have also reported that inhibition of protein synthesis in C3HA cells by treatment with CHI (or actinomycin D) renders these cells susceptible to TNF lysis and that the 14.7K protein, when synthesized in the cells by a 14.7K protein-expressing mutant prior to treatment with CHI, can protect the cells against TNF lysis (21, 28); this observation is illustrated in Fig. 1C.

Human (HEL-299) cells also were not lysed by TNF when uninfected or infected with *dl*708 (Fig. 1B). However, in

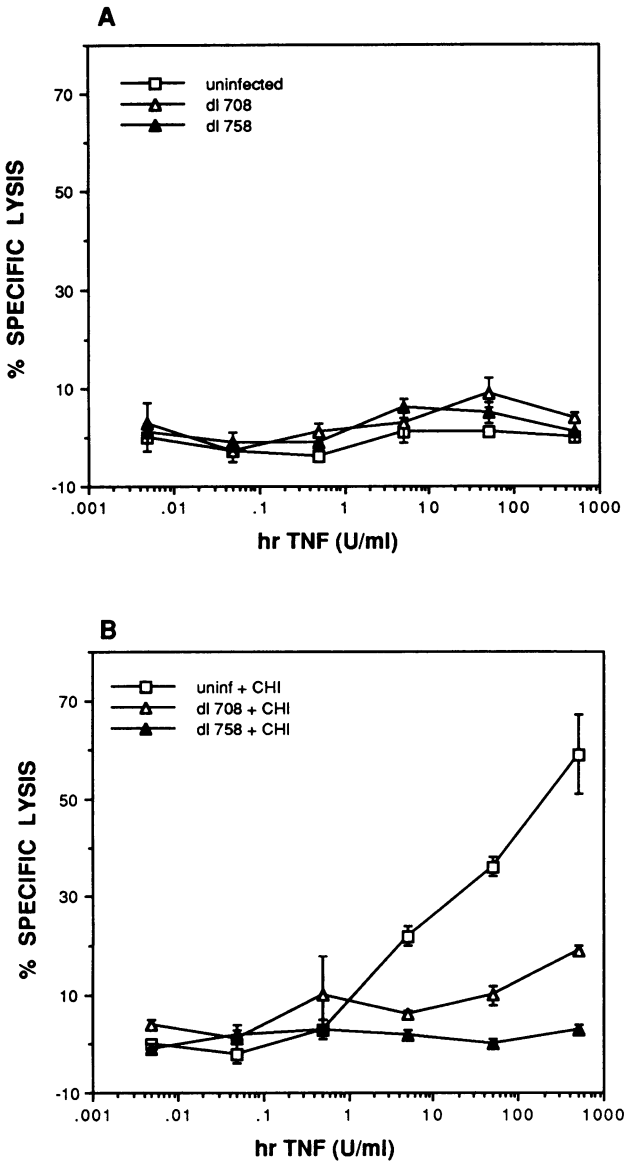

FIG. 2. An adenovirus early protein protects HEL-299 cells against lysis by TNF (A) or TNF plus CHI (B). TNF was added at 24 h p.i. Hydroxyurea (10 mM) was added at 2 h p.i. and maintained at at least that concentration throughout the assay of TNF cytolysis.

contrast to C3HA cells, *dl*758-infected HEL-299 cells were not lysed by TNF. HEL-299 cells were sensitized to TNF when treated with CHI, but again, in contrast to C3HA cells, *dl*758-infected HEL-299 cells were not lysed by TNF in the presence of CHI (Fig. 1D). This latter result indicates that an Ad protein other than (or in addition to) the 14.7K protein (as well as the proteins lacking in *dl*708) can protect CHI-treated cells against TNF lysis.

To determine whether the Ad protein that blocks TNF cytolysis in Ad-infected HEL-299 cells is an "early" protein, TNF lysis experiments were carried out in the presence of hydroxyurea. Hydroxyurea inhibits viral DNA synthesis and accordingly maintains the infection in the early phase. Whereas CHI-treated uninfected HEL-299 cells were lysed by TNF, *dl*758- and *dl*708-infected cells were not lysed (Fig. 2B). Hydroxyurea alone did not affect TNF lysis (Fig. 2A). Similar results were observed when DNA replication was inhibited by 1-β-D-arabinofuranosylcytosine (data not shown). We conclude that an Ad early protein(s) can inhibit TNF lysis of Ad-infected HEL-299 cells.

In the next series of experiments, we examined Ad double mutants with deletions in region E3 in combination with the various early transcription units in order to identify which Ad early protein(s) counteracts TNF lysis of HEL-299 cells. *dl*327 deletes all the region E3 genes except those for the putative 12.5K and 3.6K proteins. *dl*366 has the same deletion as *dl*327 but it also lacks region E4. HEL-299 cells were not killed by TNF when infected with either *dl*327 or *dl*366, and both *dl*327 and *dl*366 blocked TNF cytolysis of CHI-treated cells (data not shown). This indicates that none of the deleted E3 or E4 genes is required to prevent TNF cytolysis.

*dl*309 deletes the genes encoding the E3 10.4K, putative 7.5K, 14.5K, and 14.7K proteins, and it has a mutation in the gene encoding the 6.7K protein. *pm*975, *dl*237, and *dl*312 have the same E3 deletion as in *dl*309 but they also have mutations in region E1A. *pm*975 makes 289R but not 243R protein, *dl*237 makes 243R but not 289R protein, and *dl*312 makes neither E1A protein. HEL-299 cells infected with these mutants were not lysed by TNF (Fig. 3A). This is perhaps not surprising for *dl*312, because E1A is required to induce sensitivity to TNF (at least in mouse C3HA cells). For *dl*309, *pm*975, and *dl*237, these mutants presumably induce sensitivity to TNF lysis but some other Ad protein protects against TNF lysis. TNF also did not lyse CHI-treated cells infected with *dl*309, *pm*975, *dl*312, or *dl*237 (Fig. 3B). Thus, the E1A proteins are not required to inhibit TNF lysis when cells are sensitized to TNF by inhibition of protein synthesis.

We next examined whether region E1B proteins can protect human cells against TNF lysis. The first mutant tested was *dl*313, which lacks the E1B-19K and E1B-55K proteins and produces truncated E1A proteins on a *dl*309 background (53). HEL-299 cells infected with *dl*313 were sensitive to TNF cytolysis (Fig. 4A). Furthermore, cells infected with *dl*313 became extremely sensitive to lysis by TNF in the presence of CHI (Fig. 4B), indicating that one or both of the E1B proteins were responsible for the suppression of TNF lysis seen in human cells.

Additional mutants, all in a *dl*309 background, were analyzed to determine which E1B proteins were responsible for protection from TNF and to establish what role, if any, was played by the removal of a portion of the C terminus of E1A such as occurs in *dl*313. It was found that all mutants lacking the E1B-19K protein but containing wild-type E1A caused the release of ^{51}Cr in the absence of TNF under our standard assay conditions. This is presumably because of the *cyt* phenotype of E1B-19K protein deletion mutants leading to premature death. This phenotype is also expressed by *dl*313 but is delayed in its onset by 24 h, probably because of the E1A deletion (data not shown). Thus, for E1B-19K protein deletions other than *dl*313, a modified assay procedure was adopted in which cells were infected with virus, immediately exposed to ^{51}Cr, and assayed at 6 h p.i. instead of the usual 24 h p.i. *dl*110 and *dl*1520 synthesize the E1B-19K but not the E1B-55K protein. HEL-299 cells infected with *dl*110 or *dl*1520 were not killed by TNF (Fig. 5A), indicating that the E1B-55K protein is not required for protection against TNF. *dl*111 makes the E1B-55K but not the E1B-19K protein, and *dl*118 makes neither E1B protein. HEL-299 cells infected with either *dl*111 or *dl*118 were lysed by TNF (Fig. 5B).

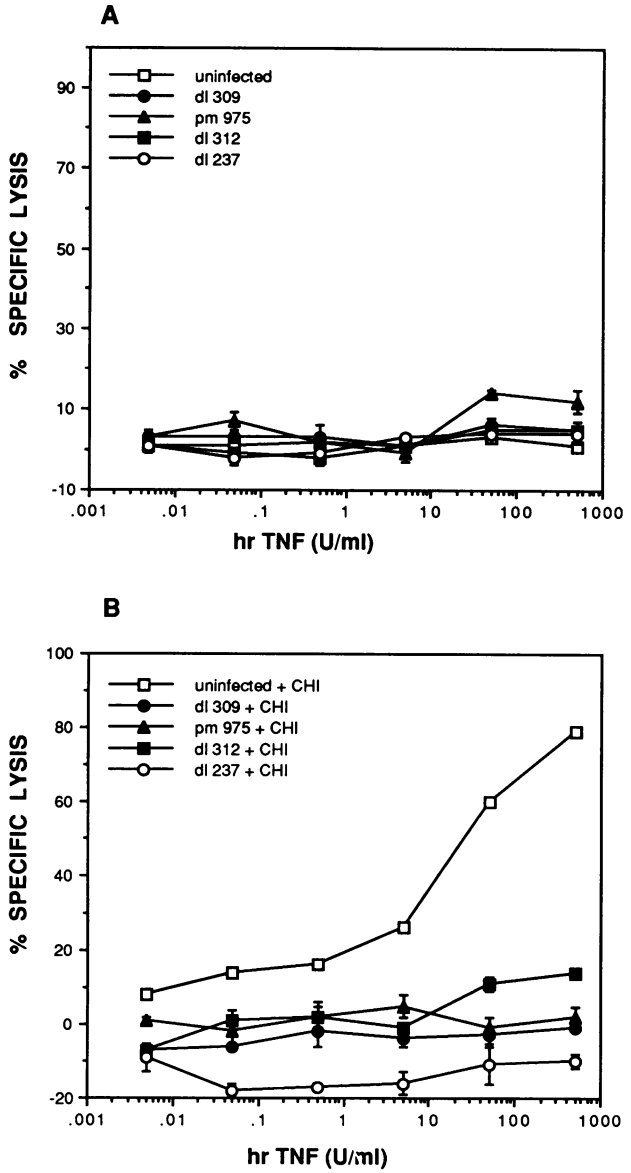

FIG. 3. Region E1A proteins are not required for the protection of HEL-299 cells against lysis by TNF (A) or TNF plus CHI (B). In panel B, TNF plus CHI was added at 24 h p.i. (except with *dl*312, in which case it was added at 48 h p.i.). *dl*309 lacks E3, and the other mutants are in a *dl*309 background. *pm*975 makes the E1A-289R but not the E1A-243R protein, *dl*237 makes the E1A-243R but not the E1A-289R protein, and *dl*312 makes neither E3 protein.

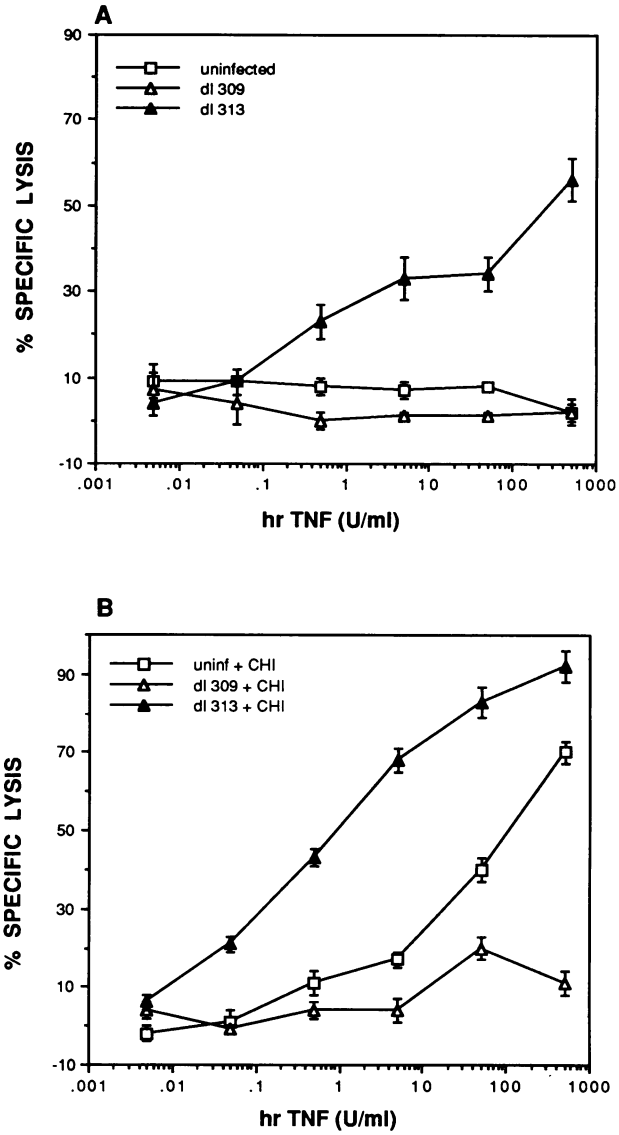

FIG. 4. HEL-299 cells infected with *dl*313 are lysed by TNF (A), and infection with *dl*313 does not prevent lysis by TNF plus CHI (B). *dl*313 lacks region E1B and a portion of the C terminus of the E1A proteins. TNF was added at 24 h p.i.

Thus, the E1B-19K protein prevents TNF cytolysis of HEL-299 cells under these conditions.

In the absence of the E1B-19K protein, region E3 proteins can protect human HEL-299 and ME-180 cells against lysis by TNF. The data so far show that in the absence of the E1B-19K protein and the E3 proteins that *dl*309 lacks, no other early or late Ad protein can provide protection against TNF cytolysis. *dl*309 synthesizes the E3 11.6K, gp19K, and 6.7K proteins (unpublished results) and is expected to synthesize the putative 12.5K and 3.6K proteins. (Although the 6.7K protein is synthesized, there may be a mutation in the protein, because *dl*309 lacks the *Xba*I site at map position 78.5 in the gene encoding the 6.7K protein.) Thus, we can conclude that these E3 proteins are not sufficient to prevent TNF cytolysis. However, until now, we have not addressed whether the E3 10.4K, 14.5K, 14.7K, and putative 7.5K proteins can protect human cells against TNF because these proteins as well as the E1B-19K protein are not present in *dl*313, *dl*118, and *dl*111 (all in a *dl*309 background). Accordingly, *dl*250 was analyzed to examine the role of these E3 proteins. *dl*250 is an Ad2 mutant that lacks the E1B-19K protein but retains the E1B-55K protein as well as region E3. As shown in Fig. 6A, HEL-299 cells infected with *dl*250 were lysed to a much lesser degree than those infected with *dl*111 or *dl*118. Thus, one or more of these E3 proteins can protect HEL-299 cells against lysis in the absence of the E1B-19K protein. We also examined a second human cell line, ME-180, a cervical cancer cell. ME-180 cells infected with Ad5, *dl*327, or *sub*304 were not killed by TNF, whereas cells infected with *dl*118 were killed (Fig. 6B). Thus, the E1B-19K

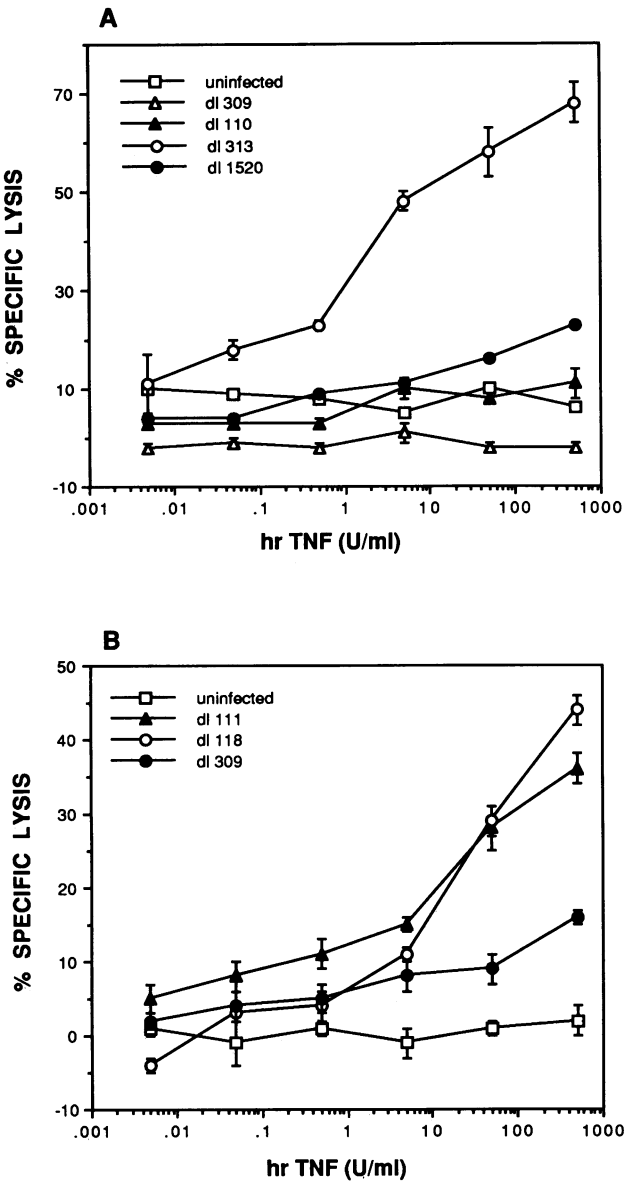

FIG. 5. The E1B-19K protein (A) but not the E1B-55K protein (B) prevents lysis of HEL-299 cells by TNF. All the mutants have the same E3 deletion as in *dl*309. *dl*118 and *dl*313 make neither the E1B-19K nor the E1B-55K protein, *dl*111 makes the E1B-55K but not the E1B-19K protein, and *dl*110 and *dl*1520 make the E1B-19K but not the E1B-55K protein. TNF was added at 24 h (A) and 6 h (B) p.i.

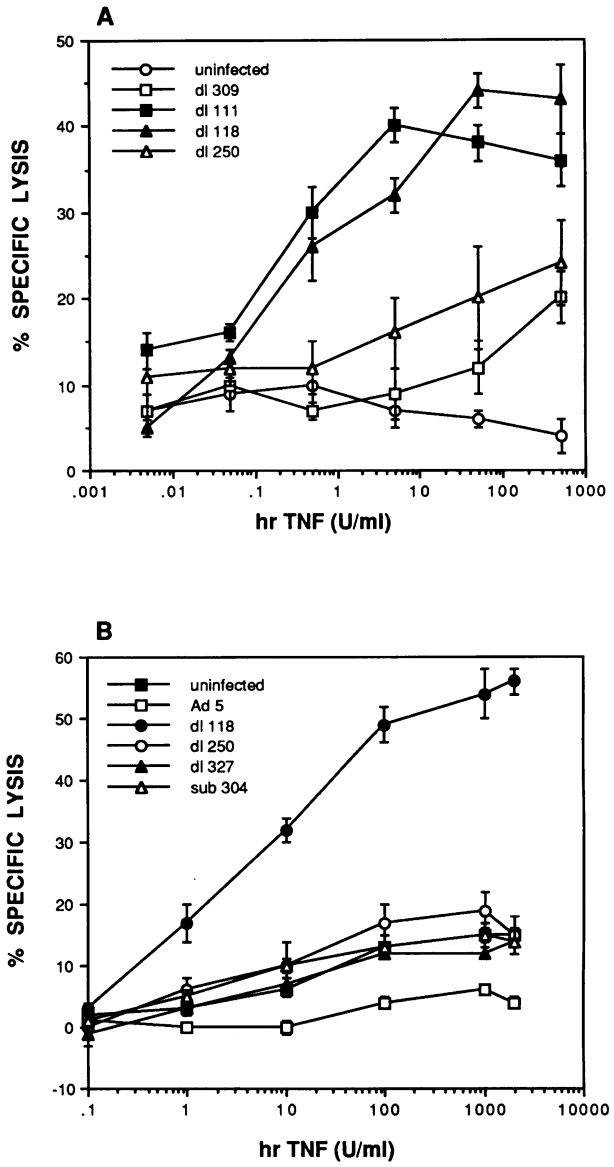

FIG. 6. Region E3 proteins protect human HEL-299 cells (A) and ME-180 cells (B) from lysis by TNF in the absence of the E1B-19K protein. *dl*250 lacks the E1B-19K protein but retains E3. TNF was added at 6 h p.i.

protein apparently is required to protect these cells against TNF. (A role for the E1B-55K protein cannot be excluded, because both E1B genes are deleted in *dl*118.) ME-180 cells infected with *dl*250 were not lysed by TNF (Fig. 6B), indicating that E3 protein(s) also can protect these cells against TNF in the absence of the E1B-19K protein.

The E1B-19K protein does not protect mouse cells against TNF lysis. Considering that the E1B-19K protein blocks TNF lysis of HEL-299 and ME-180 cells, we reexamined whether it played any role in TNF lysis of mouse C3HA fibroblasts. The C3HA cells were infected with various E1B mutants, all in a *dl*309 background and therefore lacking the E3-14.7K protein, and TNF lysis was assayed. As was done with the human cells, a 6-h infection followed by an 18-h ^{51}Cr-release assay was used; under these conditions, TNF-independent release of ^{51}Cr was comparable among uninfected cells and cells infected with all mutants examined (data not shown). As shown in Fig. 7, C3HA cells infected with *dl*309, *dl*118, *dl*110, or *dl*111 were lysed to approximately the same extent by TNF. Therefore, the presence or absence of the E1B-19K (or the E1B-55K) protein has no observable effect on TNF cytolysis of C3HA cells infected with E3-14.7K protein-negative mutants.

One possible explanation for the lack of E1B-19K protein function in C3HA cells is that the protein is not efficiently synthesized. To address this, C3HA cells were infected with *dl*309, the proteins were labeled with [^{35}S]Met, and the E1B-19K protein was analyzed by immunoprecipitation and

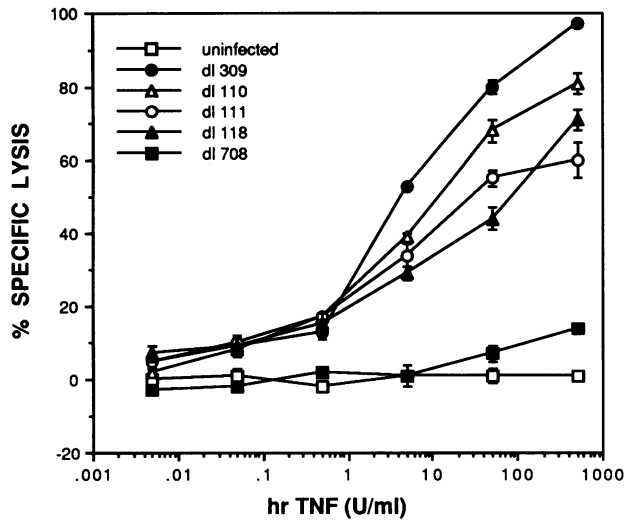

FIG. 7. The E1B-19K protein does not protect mouse C3HA cells from lysis by TNF. TNF was added at 6 h p.i.

SDS-PAGE. In a parallel experiment, HEL-299 cells were mock infected or infected with *dl*309 or *dl*118. Roughly comparable amounts of the E1B-19K protein were obtained from *dl*309-infected C3HA cells (Fig. 8A, α19K) and from *dl*309-infected HEL-299 cells (Fig. 8B, α19K). As expected, the E1B-19K protein was not immunoprecipitated from mock-infected (Fig. 8A, α19K) or *dl*118-infected (Fig. 8B, α19K) HEL-299 cells. We conclude that the E1B-19K protein is efficiently synthesized and is reasonably stable in Ad-infected C3HA cells.

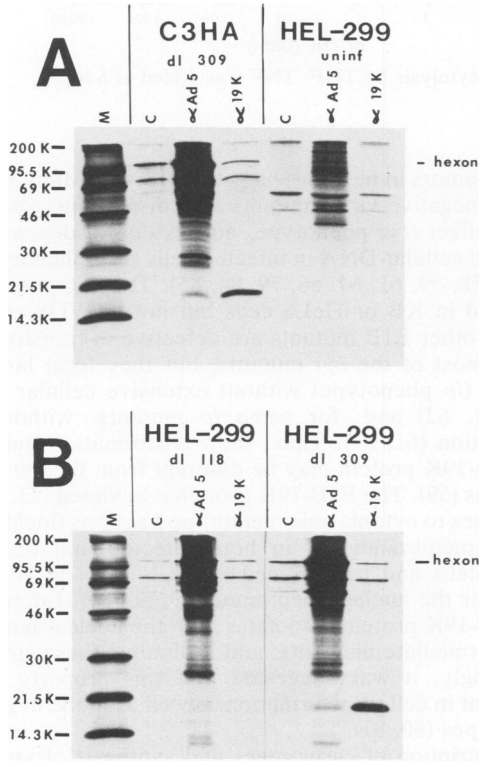

FIG. 8. Immunoprecipitation of the [^{35}S]Met-labeled E1B-19K protein from infected human (HEL-299) or mouse (C3HA) cells.

The E1B-19K protein protects from TNF lysis all human cells tested but has no effect on lysis of any mouse cells. The results described above indicate that in two human cell lines, HEL-299 and ME-180, the E1B-19K protein functions to prevent TNF cytolysis. Earlier studies with two mouse cell lines, C3HA and NIH 3T3, failed to reveal protection from TNF outside the E3 region (20). To determine the extent to which the relative function of the E1B-19K protein in human and mouse cells could be generalized, a panel of human and mouse cell lines was examined for their degrees of sensitivity to TNF when infected with Ads. Four human cell lines were selected for testing. Their selection was based solely on low (less than 35%) spontaneous release of ^{51}Cr when infected with Ads and assayed by using the 6-h p.i. protocol described above. Of the four lines tested, only VA-13, an SV40-transformed human diploid fibroblast, was weakly sensitive to TNF without Ad infection (Fig. 9A). Furthermore, none of the lines became sensitive when infected with Ads lacking only E3, i.e., *dl*327 or *dl*309 (Fig. 9). However, infection with viruses lacking both E3 and the E1B-19K protein caused significant TNF-mediated cytolysis in all four human cell lines, indicating that the E1B-19K protein functions in many, if not all, human cells to suppress sensitivity to TNF lysis. In contrast, of the four mouse cell lines selected by the same criteria, all four became sensitive to TNF when infected with Ads lacking region E3 but retaining the E1B-19K protein (Fig. 10). In addition, as was the case with C3HA, no further lysis of mouse SV-10T1/2 cells was seen when cells were infected with a virus lacking both E3 and E1B (*dl*313) (Fig. 10B).

DISCUSSION

We have shown that HEL-299 and ME-180 human cells are lysed by TNF when infected with mutants that fail to synthesize the 19K protein coded by the E1B transcription unit. In contrast to the E1B-19K protein mutants, HEL-299 cells were not killed by TNF when infected with mutants that express the E1B-19K protein but have mutations in various other Ad early genes, namely E1A, E1B-55K, all the E3 genes except those for the putative 12.5K and 3.6K proteins, and all of E4. We conclude that the E1B-19K protein is necessary to prevent TNF cytolysis of HEL-299 or ME-180 cells under these conditions. We suggest that this property of E1B-19K protein-negative mutants be referred to as the *lyt* phenotype (*l*ysis by *T*NF). We do not yet know if the E1B-19K protein is sufficient to prevent TNF lysis of HEL-299 or ME-180 cells; this will require the analysis of cells that express the E1B-19K protein in the absence of other Ad proteins.

We have also shown that HEL-299 or ME-180 cells are lysed much less efficiently when infected with *dl*250 (which lacks the E1B-19K protein but retains E3) than when infected with mutants that lack the E1B-19K protein and have the *dl*309 E3 deletion. Thus, one or more of the E3 proteins that are deleted in *dl*309 can protect HEL-299 or ME-180 cells against TNF lysis in the absence of the E1B-19K protein. The E3 proteins deleted in *dl*309 are the 10.4K, putative 7.5K, 14.5K, and 14.7K proteins, and there is a mutation in the gene encoding the 6.7K protein which destroys the *Xba*I site. The 14.7K protein is the most likely candidate, although some of the other E3 proteins could also be involved. The 10.4K (70) and 14.5K (69) proteins are also potential candidates, because both proteins have recently

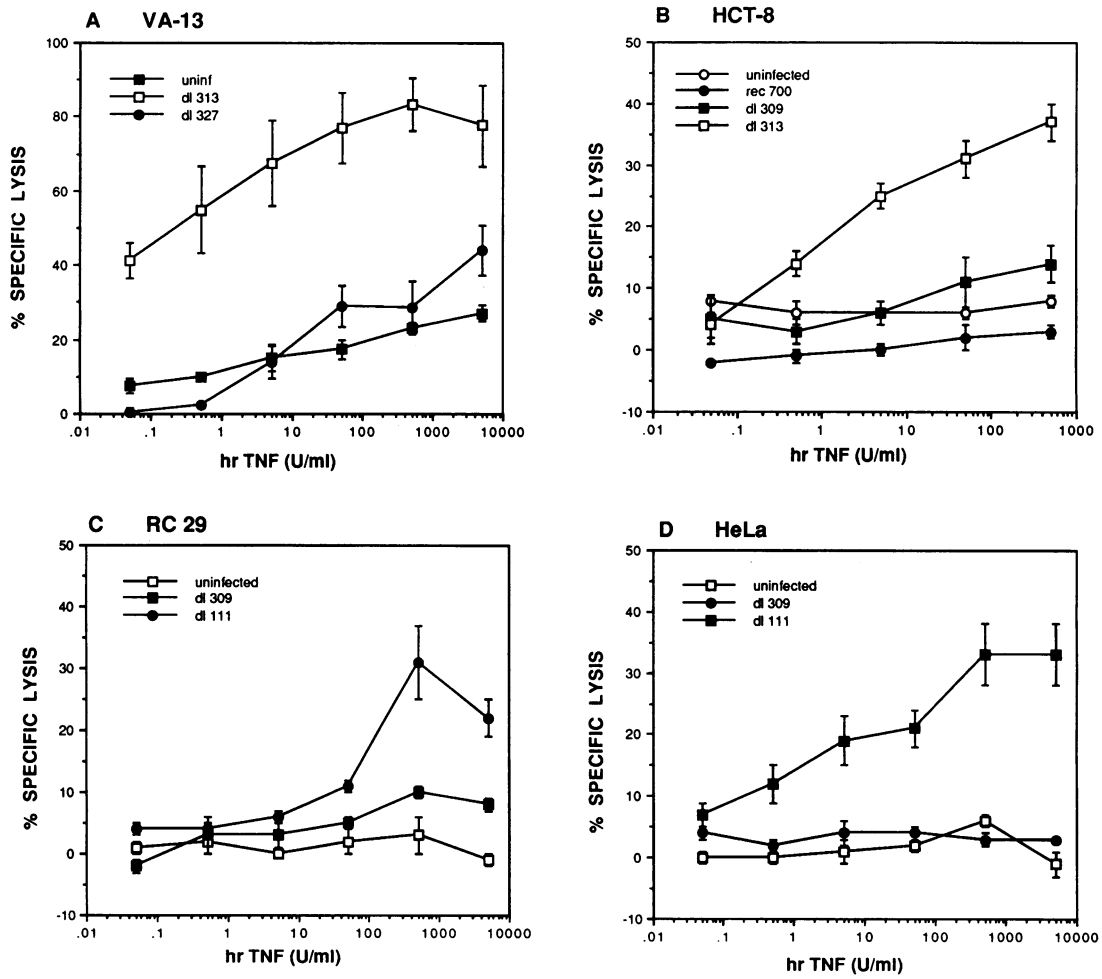

FIG. 9. The E1B-19K protein protects all human cells tested from cytolysis by TNF. TNF was added at 6 h p.i.

been shown to be required to protect many mouse cell lines against TNF lysis in the absence of the 14.7K protein (20a). Protection against TNF apparently cannot be provided by the E3 11.6K, gp19K, 6.7K, and putative 12.5K and 3.6K proteins because these are expressed or are expected to be expressed in the E1B-19K protein-negative mutants in a *dl*309 background.

Studies with virus mutants and plasmids have ascribed a number of properties and functions to the E1B-19K protein. Viruses with mutations in the gene encoding the 19K protein have a host range (*hr*) phenotype and usually grow well in HEK cells (5, 18, 65), usually show reduced growth (10- to 1000-fold) in KB cells but grow nearly as well as the wild type in HeLa cells (4, 5, 49, 62, 65, 82, 84, 85), and show increased growth in human WI38 cells, apparently because of higher early gene expression (83). Viruses with mutations in the gene encoding the 19K protein are transformation defective (*tra* phenotype) (3, 4, 5, 11, 18, 32, 38, 49, 62, 65, 66). In plasmid transfection experiments, the gene encoding the E1B-19K protein confers what appears to resemble a fully transformed phenotype in cooperation with E1A (81). Also, transformed cells that lack the E1B-19K protein do not grow in semisolid medium (18, 49, 62, 81) or in Ca^{2+}-deficient medium (60), and they are nontumorigenic (6). E1B-19K protein mutants of Ad12 have lost their ability to induce tumors in newborn hamsters (18, 66). Many E1B-19K protein-negative virus mutants also display increased cytopathic effect (*cyt* phenotype) and extensive degradation of viral and cellular DNA in infected cells (*deg* phenotype) (35, 36, 49, 58, 59, 61, 64, 66, 79, 84, 85). The *deg* phenotype is observed in KB or HeLa cells but not in WI38 cells (85). Certain other E1B mutants are defective in transformation, as are most of the *cyt* mutants, but they form large clear plaques (*lp* phenotype) without extensive cellular destruction (11, 62) and, for some *lp* mutants, without DNA degradation (61, 84). Thus, the transformation function of the E1B-19K protein may be different from the *cyt* and *deg* functions (59). The E1B-19K protein is acylated (23, 40), and it localizes to cytoplasmic membrane fractions (including the plasma membrane; 56) in both infected and transfected human cells and transformed rat cells (47, 79, 80, 81), in particular the nuclear membrane (79, 80, 81). Interestingly, the E1B-19K protein associates with the nuclear lamina (79) and intermediate filaments, and it disrupts these structures; accordingly, it was suggested that this property may be important in cell transformation as well as in the *deg* and *cyt* phenotypes (80, 81).

Transcription of early genes and synthesis of viral DNA were reported to be much higher in WI38 (84) and HeLa (82) cells infected with E1B-negative mutants, and it was sug-

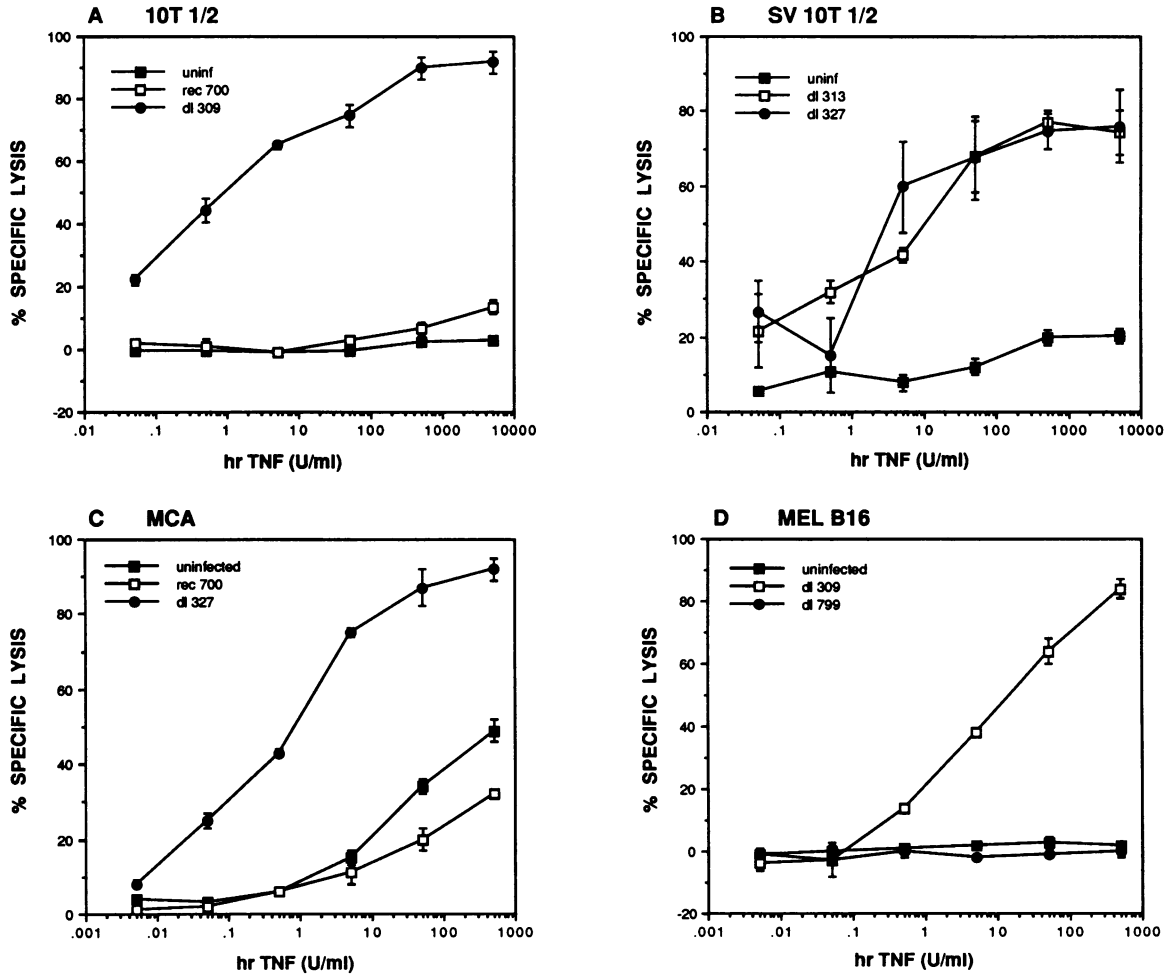

FIG. 10. The E1B-19K protein does not protect infected mouse cells from cytolysis by TNF. TNF was added at 6 h p.i.

gested that the 19K protein may act directly or indirectly to repress E1A-dependent early gene transcription. However, in plasmid transfection experiments, it was concluded that the E1B-19K protein stimulates expression from Ad and certain cellular promoters (26, 45, 54, 73, 91). The activation may be due, at least in part, to a relief from E1A-mediated enhancer repression (91). Increased gene expression by the E1B-19K protein has also been detected in E1A-negative Ads (26, 73, 85). However, a later report suggested that the increased transcription by the E1B-19K protein of transfected plasmids was indirect and resulted from stabilization by the E1B-19K protein of the transfected plasmid DNA (27), although this was not observed with the Ad12 E1B-19K protein (54). Therefore, the role of the E1B-19K protein in gene regulation is unclear at present.

How do these properties of the E1B-19K protein relate to its ability to protect HEL-299 cells against TNF cytolysis? The answer is unknown, but it seems likely to involve the close relationship between the E1B-19K protein and the E1A proteins (82, 85). First, the E1A and E1B-19K proteins are related in terms of cell transformation, i.e., the E1A proteins alone can immortalize cells with low efficiency, but together they generate what appears to be fully transformed cells (see reference 81 and references therein). Second, in virus-infected WI38 and HeLa cells, the E1B-19K protein may be a negative regulator of the E1A transactivation activity (82, 85). Third, either the 289R or the 243R E1A protein can induce in cells a state that leads to DNA degradation and enhanced cytopathic effect (85), and the E1B-19K protein prevents DNA degradation and enhanced cytopathic effect. The E1A proteins have a modular structure, with specific domains in the proteins being associated with specific functions (44). Experiments with virus double mutants that lack the E1B-19K protein and that also have deletions in E1A have determined that the deletion of residues 22 to 85 in E1A abrogates the ability of E1A to induce DNA degradation (78a). E1A renders cells susceptible to cytolysis by TNF, at least in mouse cells (1, 10, 12, 16, 76), and as shown here, the E1B-19K protein protects HEL-299 and ME-180 cells against lysis by TNF. Mapping studies using double mutants that lack E3 and have fairly large deletions in E1A have indicated that deletion of residues 30 to 85 in E1A abrogates the ability of E1A to induce susceptibility to TNF cytolysis in mouse C3HA cells (15). Thus, similar E1A sequences are required to induce DNA degradation and TNF susceptibility, and the E1B-19K protein can prevent DNA degradation, enhanced cytopathic effect, and TNF cytolysis. Interestingly, DNA fragmentation can also accompany TNF-induced cell death (51a), suggesting that a potential route whereby E1A "sensitizes" cells to TNF occurs via its ability

to induce DNA degradation. However, TNF lysis clearly occurs in the absence of gross damage to DNA in some cell lines, including Ad-infected C3HA (36a) and HEL-299 (unpublished data). Hence, the precise relationship between E1A's ability to sensitize cells for TNF lysis and to induce DNA fragmentation is currently unknown. Large deletions in this same region of E1A also generally abrogate the ability of E1A to immortalize cells, to induce DNA synthesis in quiescent cells, to transform cells in cooperation with E1B or *ras*, to repress certain cellular genes, to activate certain cellular genes, and to bind to the cellular p300 and p105 (RB) proteins (29, 33, 37, 52, 55, 57, 74, 75, 86).

Given the points discussed above, it will clearly be important to determine whether the TNF protection function (lyt^+) of the E1B-19K protein is related to the lp^+, cyt^+, deg^+, and tra^+ functions. This should be possible through the analysis of different mutations throughout the gene encoding the E1B-19K protein to determine which functions are linked in the mutants and which can be separated. This approach should be feasible because it has been shown that certain *lp* mutants are not *cyt* or *deg* but are *tra* (59, 62, 61, 84). With *cyt tra* mutants, the E1B-19K protein does not localize to the nuclear membrane, whereas with *lp3*, which is deg^+, the E1B-19K protein is similar to the wild-type protein in localizing to the nuclear membrane. In addition, some Ad12 *cyt* mutants are tra^+ (38, 67).

As reported previously (16, 20, 21, 28) and as shown in Fig. 1, the E3-14.7K protein protects mouse C3HA fibroblasts against lysis by TNF. The E1B-19K protein was unable to protect Ad-infected mouse C3HA fibroblasts against lysis by TNF when such cells were infected with mutants that lack region E3. Similar results were obtained with a variety of mouse cell lines (Fig. 10; data not shown). The lack of protection against TNF in C3HA cells was not due to lack of synthesis of the E1B-19K protein; rather, it most likely was due to an inability of the E1B-19K protein to function properly. Either the E1B-19K protein cannot function at all in mouse cells or, perhaps, it functions poorly so that much higher levels of the E1B-19K protein are required in mouse cells than in human cells to prevent TNF cytolysis. An understanding of why the E1B-19K protein functions to protect human cells but not mouse cells against TNF may provide insights into the mechanism of TNF cytolysis.

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