JOURNAL OF VIROLOGY, Apr. 1996, p. 2431–2439 Vol. 70, No. 4
0022-538X/96/$04.00+0

The Role of Human Adenovirus Early Region 3 Proteins (gp19K, 10.4K, 14.5K, and 14.7K) in a Murine Pneumonia Model

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Received 30 October 1995/Accepted 12 December 1995

Products of human adenovirus (Ad) early region 3 (E3) inhibit both specific (cytotoxic T lymphocytes [CTLs]) and innate (tumor necrosis factor alpha [TNF-α]) immune responses in vitro. The E3 gp19K protein prevents CTL recognition of Ad-infected fibroblasts by sequestering major histocompatibility complex class I proteins in the endoplasmic reticulum. E3 proteins 10.4K, 14.5K, and 14.7K function to protect infected cells from TNF-α cytolysis. To address the in vivo functions of these proteins, Ad mutants that lack the E3 genes encoding these proteins were inoculated intranasally into C57BL/10SnJ (*H-2^b*) mice. Mutants that lack the gp19K gene failed to alter CTL generation or to affect Ad-induced pulmonary infiltrates. Since gamma interferon (IFN-γ) is capable of overcoming gp19K suppression of CTL lysis in vitro, mice were depleted of IFN-γ and inoculated with gp19K mutants. Even when IFN-γ was depleted, gp19K was incapable of altering pulmonary lesions. These results are not in accord with the function of gp19K in vitro and suggest that gp19K does not affect immune recognition in vivo during an acute virus infection, yet they do not exclude the possibility that gp19K blocks immune recognition of Ad during a persistent infection. In contrast, when mice were inoculated with Ad mutants that lack the TNF resistance genes (14.7K and either 10.4K or 14.5K), there was a marked increase in alveolar infiltration and no change in the amounts of perivascular/peribronchiolar infiltration compared with wild-type-Ad-induced pathology. These findings demonstrate the importance of TNF susceptibility and TNF by-products for recruiting inflammatory cells into the lungs during Ad infections.

An emerging theme in viral immunology is the discovery that many of the "nonessential" genes in the viral genome are actually immune modulators (reviewed in references 25 and 57). Practically all aspects of the immune response are circumvented by a variety of viruses. For example, both the innate (e.g., complement [33] and alpha/beta interferon [IFN-α/β] [5]) and the specific (e.g., cytotoxic T lymphocytes [CTLs]) (18, 36, 74) arms of the immune response are countered by the herpesviruses. Human adenoviruses (Ads) have evolved a cassette of genes, early region 3 (E3), which counteract both arms of the immune response (reviewed in references 26 and 72).

The specific arm of the immune response includes CTLs that are responsible for viral clearance (15, 37, 38). The Ad E3 protein gp19K prevents CTL lysis in vitro by binding and retaining major histocompatibility complex (MHC) class I molecules in the endoplasmic reticulum (2, 9, 52). The role of gp19K in vivo was initially addressed in an Ad pneumonia model in the cotton rat (*Sigmodon hispidus*) (47, 49). Unlike mouse cells, cotton rat cells are permissive for replication of human Ads; therefore, they provide a realistic model for Ad-induced pathogenesis. Using gp19K deletion mutants, Ginsberg et al. (22) found that viruses lacking gp19K induced an increased perivascular/peribronchiolar cellular infiltration following intranasal (i.n.) inoculation. Unfortunately, immunological tools are not available to assess the binding of gp19K to cotton rat MHC class I molecules.

To further evaluate gp19K's role in vivo, Grunhaus et al. (32) inoculated mice with a recombinant vaccinia virus expressing gp19K. In this model, expression of gp19K had no effect on disease severity, $CD4^+/CD8^+$ ratios, or vaccinia virus clearance. Also using a vaccinia virus-gp19K construct, Cox et al. (12) confirmed the findings of Grunhaus et al. and demonstrated that the expression of gp19K did not affect natural killer (NK) cell or CTL responses. The difference between these findings and those of Ginsberg et al. (22) could be due to the overwhelming immune response to the vaccinia virus background, which could mask the role of gp19K. In studies reported here, the mouse model of Ad-induced pneumonia was used to reevaluate the function of gp19K in vivo. Even though Ad does not replicate in mouse cells, the murine Ad pneumonia model was validated when Ginsberg et al. (20, 21) demonstrated that viral replication is not necessary for pathogenesis and that the pulmonary pathology in the mouse mimicked that in the cotton rat.

The innate response initiates downstream events in the immune response through cytokines which activate and attract cells of the specific and innate immune responses. In addition, innate cytokines, such as tumor necrosis factor alpha (TNF-α), have direct antiviral effects. TNF-α prevents Ad replication (44, 45, 73) and induces lysis of some Ad-infected cells in vitro (28, 30). To counteract this assault, Ad produces the E3 proteins 10.4K (64), 14.5K (63), and 14.7K (66), which prevent cytolysis of virally infected cells by TNF-α (28, 29). The 10.4K

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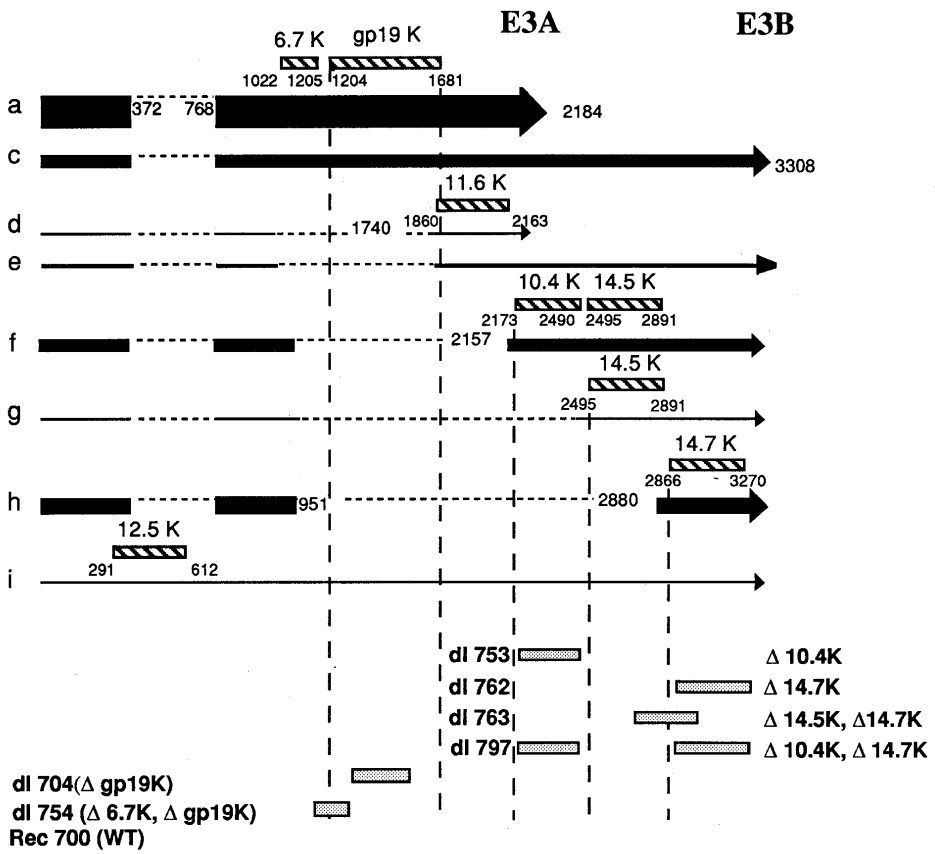

FIG. 1. Viral deletion mutants used in this study. Shown is a schematic illustration of region E3 of *rec*700. Arrows indicate the splice structures of the mRNAs; solid parts are exons, dashed parts are introns, and the thickness implies the relative abundance (4, 6, 7). The bars above the arrows indicate proteins. The stippled bars at the bottom indicate the deletions in the viral mutants. WT, wild type.

and 14.5K proteins form a complex (29, 65) that localizes to the plasma and cytoplasmic membranes (42, 43, 59), while the 14.7K protein is found in the nuclear and cytoplasmic fractions (62). 14.7K and 10.4K/14.5K function independently and redundantly in preventing TNF-α lysis in vitro (28). In order for TNF-α to lyse most types of virally infected mouse cells, both 14.7K and one member of the 10.4K/14.5K complex must be eliminated (29).

The in vivo role of the TNF-α resistance gene encoding 14.7K is more clearly defined than that of the gene encoding gp19K. Insertion of the E3 14.7K and TNF-α genes into vaccinia virus revealed that when 14.7K is present there is an increase in vaccinia virus virulence, and this increase is not due to T or B cells (67, 68). In this case, 14.7K presumably increases virulence by negating TNF's inhibitory effect on viral replication and spread. Experiments reported here explore the functions of 10.4K, 14.5K, and 14.7K in vivo using the murine Ad pneumonia model developed by Ginsberg et al. (21).

MATERIALS AND METHODS

Cells and viruses. Simian virus 40-transformed fibroblasts (SVB6KHA and SV2R) (23, 24) were grown in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, N.Y.) supplemented with 10% fetal calf serum (HyClone, Logan, Utah) and 1% L-glutamine (GIBCO). Viruses were grown on human KB cells (American Type Culture Collection, Rockville, Md.), CsCl banded, and titered on human A549 cells as previously described (31). All mutants were derived from *rec*700, an Ad5-Ad2-Ad5 recombinant whose genome consists of the Ad5 *Eco*RI-A fragment (map position 0 to 76), Ad2 *Eco*RI-D (76 to 83), and Ad5 *Eco*RI-B (83 to 100) (71). The E3 transcription unit and the E3 deletion mutants used in this study are shown in Fig. 1. Descriptions of these mutants are as follows: *dl*704, Δ(1289–1441) (4); *dl*753, Δ(2229–2436) (7); *dl*754, Δ(1200–1244) (28); *dl*762, Δ(2904–3251) (6); *dl*763, Δ(2804–3002) (6); and *dl*797, Δ(2229–2436) and Δ(2804–3002) (29).

Mouse infections. C57BL/10SnJ ($H\text{-}2^b$) mice (6- to 8-week-old males) were purchased from Jackson Laboratory (Bar Harbor, Maine). For CTL generation, mice were inoculated intraperitoneally (i.p.) with 2×10^7 PFU of either *rec*700 or *dl*754 at least 10 days prior to assay or i.n. with 25 μl of CsCl-banded virus (10^{11} PFU/ml) or CsCl control (0.51 g/ml) after anesthetization with Avertin (2,2,2-tribromoethanol) (Aldrich Chemical Co., Milwaukee, Wis.).

Histopathological scoring. At 9 days postinoculation (p.i.), mice were anesthetized and exsanguinated, and all lobes of the lungs were removed, inflated with acetone, and fixed for 24 h in acetone at −20°C. The lungs were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Pulmonary lesions on all lobes were scored in a semiquantitative, double-blind manner. Lung sections were scored by assigning a value from 0 to 4 (4 being the most severe lesion) to each of four different anatomical sites: the bronchiolar epithelium, alveoli, alveolar septa, and perivascular/peribronchiolar space. The total damage for the four areas was then expressed as a percentage of the maximum damage (i.e., 16); therefore, maximum damage is 16/16 (1.00). The histopathological scores from several experiments were pooled and averaged (± the standard error of the mean). Statistical analysis of these data was performed with a one-tailed Student *t* test assuming unequal variances ($\alpha = 0.01$).

CTL assays. The spleens or mediastinal lymph nodes from i.n. or i.p. inoculated mice were removed, stimulated in vitro for 6 days with syngeneic, Ad-infected stimulators, and assayed against ^{51}Cr-labeled targets as previously described (52). Briefly, primed lymphocytes were stimulated in vitro with gamma-irradiated stimulators (SVB6KHA [K^b D^b] infected with *dl*754 at 100 PFU per cell) for 6 days. Target cells (unless otherwise noted) were SV2R (K^k D^b) cells infected with *rec*700 or *dl*754 (100 PFU per cell) and labeled with 200 μCi of $Na_2{}^{51}CrO_4$ (New England Nuclear, Boston, Mass.) the day prior to assay. CTL activity was measured by a 5-h ^{51}Cr release assay. Results were expressed as means (± the standard errors of the means) of triplicate samples and were calculated as follows: $(E-C)/(M-C) \times 100\%$ specific lysis, where C is counts per minute released by nonstimulated lymphocytes, E is counts per minute released by sensitized lymphocytes, and M is maximum releasable counts per minute, determined by the addition by 0.05 ml of 1 N HCl to 0.05 ml of target cells.

IFN-γ treatment of targets. SVB6KHA target cells were either mock infected or infected with 100 PFU of *rec*700 or *dl*754 per cell. At various times p.i., the cells were treated with 50 U of mouse recombinant IFN-γ (Genzyme, Cambridge, Mass.). At 24 h p.i., the cells were collected and assayed with Ad-specific CTLs.

In vivo depletion of IFN-γ. C57BL/10SnJ mice were inoculated i.n. with 25 μl of *rec*700 or *dl*704 (10^{11} PFU/ml), and 12 h p.i. the mice were injected i.p. with 200 μg of purified anti-IFN-γ antibody R46A2 (58) or phosphate-buffered saline control. Clearance of *Listeria monocytogenes* was used as a measure of IFN-γ depletion (8). Briefly, C57BL/10SnJ mice were injected i.p. with 1.3×10^5 CFU of *L. monocytogenes* per mouse and depleted of IFN-γ as described above. At 7 days p.i., the surviving mice were sacrificed and colony counts were performed on the livers and spleens.

UV inactivation of virus. *dl*797 (0.5 ml, 10^{11} PFU/ml) was placed in a 35-mm-diameter petri dish (Falcon, Lincoln Park, N.J.) and exposed to 875 μW of short-wavelength UV (254 nm) per cm^2, provided by a handheld UV lamp (model UVGL-25; UVP, Inc., San Gabriel, Calif.) (19), for 30 min. Inactivation of the virus was confirmed by a plaque assay.

RESULTS

gp19K does not prevent CTL generation. Rawle et al. (52) had previously shown that gp19K does not alter the generation of Ad-specific CTLs following i.p. injection with Ad. Since the amount of gp19K MHC class I suppression varies from cell to cell (55, 56), the effect of gp19K expression on CTL generation was tested in a more relevant model system: Ad infection of the bronchiolar epithelium. C57BL/10SnJ mice were inoculated i.n. with 2.5×10^9 PFU of *rec*700 (wild type) or *dl*704 (Δ19K). At 7 days p.i., spleens and mediastinal lymph nodes were removed, stimulated in vitro with syngeneic Ad-infected cells, and assayed for CTL activity. With spleen responder cells, CTLs were generated equally as well in the presence or absence of gp19K expression in vivo (Fig. 2). The data for the mediastinal lymph nodes were identical to those for the splenocytes; i.e., there was no measurable diminution in the bulk CTL response (data not shown). Whether or not gp19K alters CTL precursor frequencies remains to be examined. These data indicate that gp19K does not function on the afferent arm (priming stage) of the immune response even when mice are infected via the bronchiolar epithelium to mimic natural infection.

gp19K does not alter Ad pneumonia. Although gp19K is unable to prevent CTL generation, it may function in vivo to prevent lysis of virally infected bronchiolar cells. Thus, its true role would be to act on the efferent arm (effector stage) of the immune response, decreasing Ad pathogenesis. Mice either were inoculated i.n. with *rec*700 or *dl*704 or were mock infected (CsCl); then at 9 days p.i., lungs were fixed, stained, and scored semiquantitatively. When vehicle alone was used as the inoculum, bronchioles, alveoli, and septa all appeared normal (Fig. 3A). When mice were infected with either *rec*700 (Fig. 3B and C) or *dl*704 (Fig. 3D and E), a minimal cellular infiltrate consisting predominately of lymphocytes was seen in the perivascular/peribronchiolar regions, with a few cells infiltrating the alveolar septa. The bronchiolar epithelium and the alveoli were normal. In sum, the absence of gp19K did not significantly alter Ad-induced pulmonary lesions (*rec*700 versus *dl*704, $P \leq 0.16$) (Fig. 3F). These data indicate that the specific arm of the immune response is unaffected by gp19K in vivo.

IFN-γ does not mask the role of gp19K in vivo. One possible explanation for the failure of gp19K to alter Ad-induced lesions in vivo may be that IFN-γ overcomes the gp19K effect. IFN-γ may upregulate MHC class I expression such that there is insufficient gp19K to retain all the MHC class I molecules in the endoplasmic reticulum, thus exposing the Ad-infected cells to CTL lysis. To address this possibility in vitro, target cells were infected 24 h prior to incubation with Ad-specific CTLs. IFN-γ was added either immediately, 8 h p.i., or 12 h p.i. Only when Ad-infected targets were exposed to IFN-γ for 24 h were the gp19K effect overcome and CTL recognition restored (Fig. 4). These data indicate that exposure of virally infected cells to IFN-γ for a long period of time in vitro overcomes gp19K blockage of CTL target lysis.

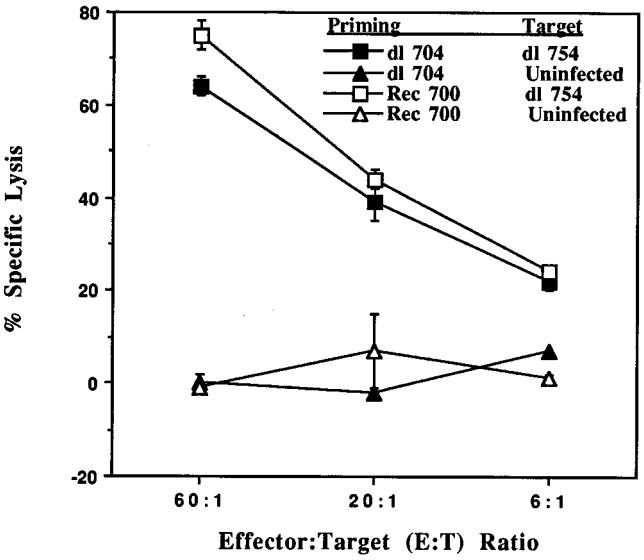

FIG. 2. CTL priming following i.n. inoculation with Ad. Splenocytes were harvested 9 days following i.n. inoculation with 2.5×10^9 PFU of either *rec*700 (wild type) or *dl*704 (Δ19K). After secondary in vitro restimulation, CTLs were assayed with SVB6KHA cells that were either mock infected or infected with *dl*754 (Δ19K).

In vivo, IFN-γ, produced by either T cells or NK cells (70), could function as it had in vitro and overcome gp19K's retention of MHC class I molecules. The possibility that IFN-γ may overcome the effect of gp19K in vivo was tested by depleting IFN-γ with an anti-IFN-γ monoclonal antibody. Mice were inoculated i.n. with *rec*700, *dl*704, or CsCl, and 12 h p.i. they were depleted of IFN-γ with an anti-IFN-γ antibody. At 7 days p.i., the lungs were scored for cellular infiltration. Once again, gp19K did not affect pulmonary pathology, even when IFN-γ was depleted (Fig. 5). To control for IFN-γ depletion in this experiment, colony counts were performed on the spleens and livers of mice infected with *L. monocytogenes* and depleted of IFN-γ as described above. Since IFN-γ is vital for the resolution of *L. monocytogenes* infection in vivo (8), depleting IFN-γ should increase the number of *Listeria* cells isolated from infected livers and spleens. A total of $\approx 10^3$ more colonies (1.3×10^3 CFU per liver for nondepleted mice versus 6×10^5 CFU per liver for IFN-γ-depleted mice) were found in the depleted mice than in the mock-depleted mice, indicating that IFN-γ had been depleted (data not shown). We conclude that an increase in the expression of MHC class I in vivo due to elevated IFN-γ does not explain why the presence of gp19K has no effect on Ad-induced pneumonia in the mouse lung.

Viruses lacking the TNF-α resistance genes induce a more severe pulmonary disease. The role of the other E3 proteins, 10.4K, 14.5K, and 14.7K, in the pathogenesis of Ad-induced pneumonia was investigated by inoculating C57BL/10SnJ mice i.n. with mutants that lack one or more of the genes for these proteins and semiquantitatively scoring the pulmonary lesions. The lung lesions associated with viruses that lack only one of the TNF-α resistance genes closely resembled the wild-type lesions (Fig. 3B and C and Fig. 6A and B). A comparison of the

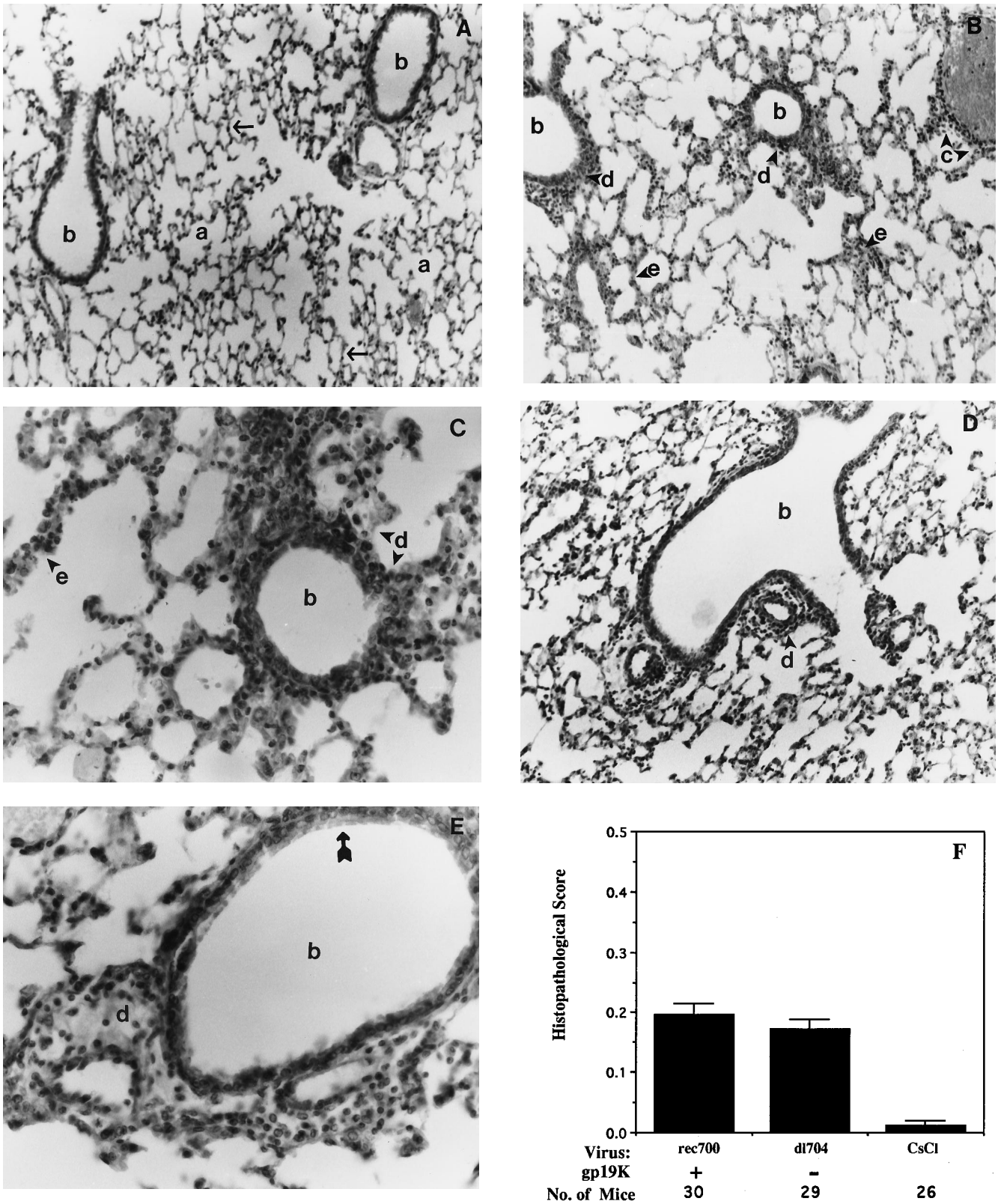

FIG. 3. Histopathology following i.n. inoculation with viruses that lack gp19K. Mice were infected with 2.5×10^9 PFU of *rec*700 (wild type), *dl*704 (Δ19K), or CsCl (diluent), and 9 days p.i., lungs were evaluated for pathological changes. Shown are representative hematoxylin-and-eosin-stained sections from mice inoculated with various agents. (A) CsCl; magnification, ×66; (B) *rec*700; magnification, ×66; (C) *rec*700; magnification, ×165; (D) *dl*704 (Δ19K); magnification, ×66; (E) *dl*704 (Δ19K); magnification, ×165. Shown are normal alveoli (a), bronchiole (b), perivascular infiltration (c), peribronchiolar infiltration (d), and swelling of the alveolar septa (e). The thick arrow indicates normal bronchiolar epithelium. The thin arrow indicates normal alveolar septa. (F) Histopathological scores (means ± standard errors) from several experiments.

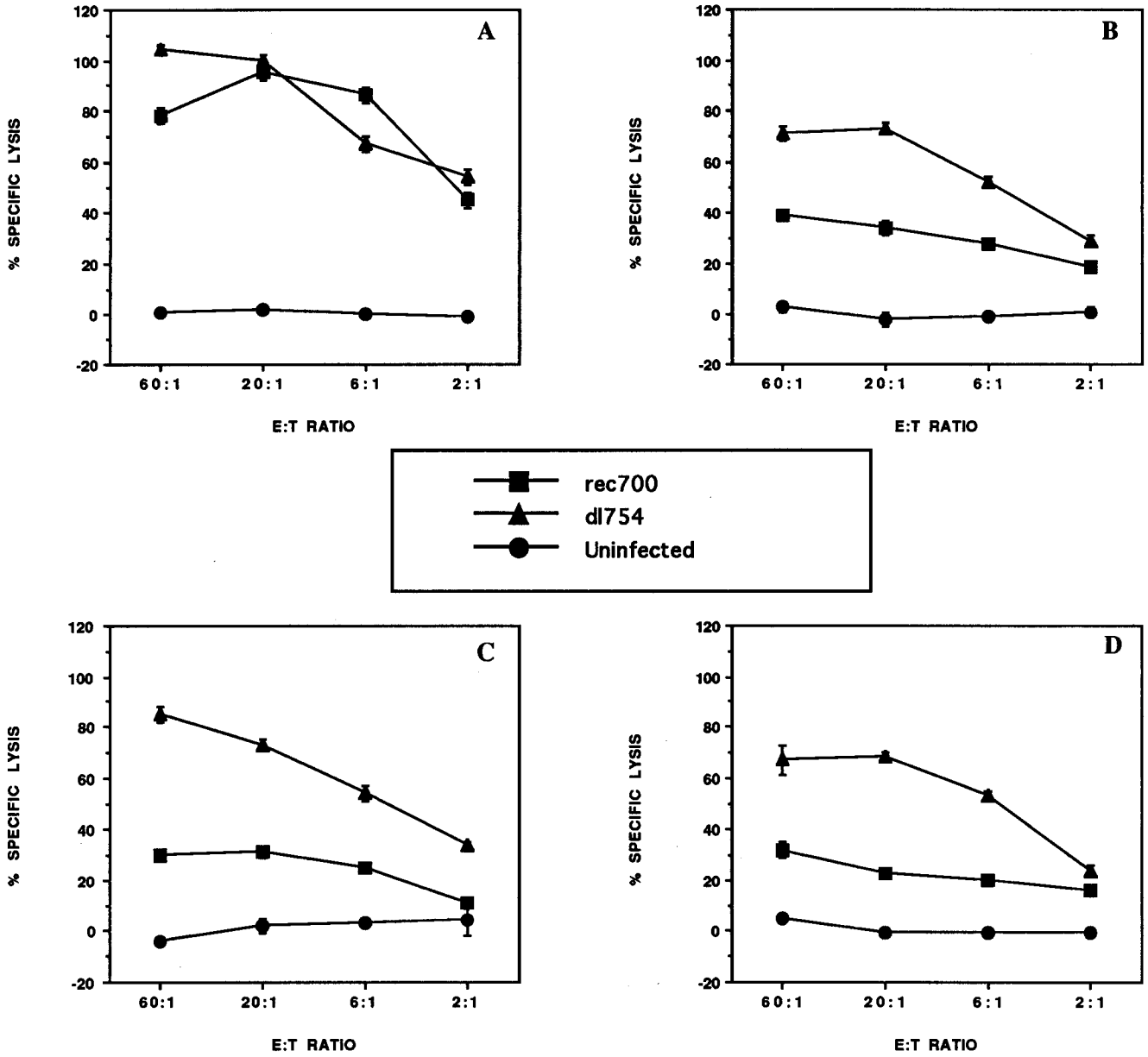

FIG. 4. IFN-γ treatment of CTL targets in vitro. Virally infected targets were treated with 50 U of mouse recombinant IFN-γ 24 h (A), 16 h (B), or 12 h (C) prior to assay with Ad-specific CTLs or were mock treated (D). E, effector; T, target.

mean histopathological scores illustrates that wild-type virus and mutant viruses lacking either 10.4K or 14.5K generate the same degree of pulmonary pathology (*rec*700 versus *dl*753, $P \leq 0.175$; *rec*700 versus *dl*762, $P \leq 0.701$) (Fig. 7). Only when one member of the 10.4K/14.5K complex and 14.7K were eliminated did the severity of the lesions change. The perivascular/peribronchiolar spaces, alveolar septa, and alveoli were diffusely infiltrated by many lymphocytes and only a few macrophages; the bronchiolar epithelium remained normal (Fig. 6C and D). The histopathological scores increased two- to threefold over wild-type-virus scores (*rec*700 versus *dl*763, $P \leq 2.27 \times 10^{-3}$; *rec*700 versus *dl*797, $P \leq 7.7 \times 10^{-15}$; *dl*797 versus *dl*763, $P \leq 0.117$) (Fig. 7). The increased histopathological score was due to a marked increase in alveolar infiltration, while the perivascular/peribronchiolar infiltration was equal to that of *rec*700 (wild type). The increase in disease severity with the viral mutants correlates with the in vitro susceptibility of virally infected cells to TNF-α. These data indicate that the E3 proteins which inhibit TNF cytolysis in vitro play an important role in viral pathogenesis in vivo.

The differences in Ad-induced pneumonia are not due to differences in particle counts or fiber content. With the induction of lung lesions one presupposes that disease requires expression of viral genes and is not simply an inflammatory response to the presence of viral particles. If the latter were true, then the difference in pulmonary lesions induced by mutant and wild-type Ad could conceivably be due to differences in the ratios of defective particles to infective particles. Particle counts were performed, and no significant differences among the mutants and the wild-type virus were found (data not shown). Furthermore, to confirm that the *dl*797-induced pneumonia was not due to fiber or other Ad coat proteins, mice

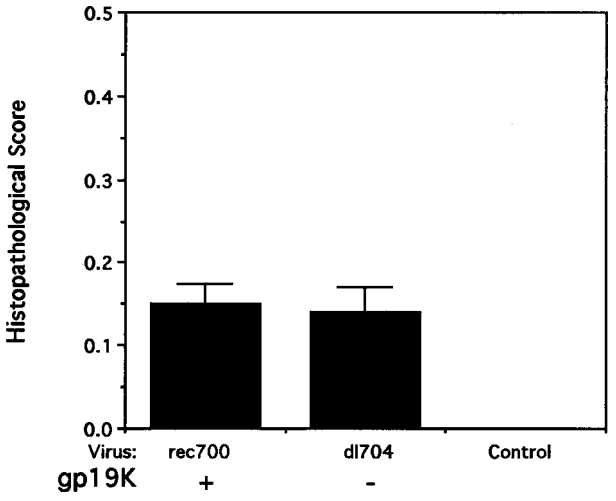

FIG. 5. Depletion of IFN-γ in vivo. Mice were inoculated i.n. with *rec*700 or *dl*704, and 12 h p.i. they were depleted of IFN-γ with 200 μg of anti-IFN-γ (R46A2) antibody. At 7 days p.i., lungs were scored for pulmonary lesions; the mean scores of five mice (± standard errors) are shown.

were infected with UV-inactivated *dl*797 and their lung lesions were scored. When *dl*797 was inactivated, the histopathological score resembled that of noninfected mice (Fig. 8); therefore, the observed pneumonia is not due to the toxic effects of input virion proteins. Instead, the induction of pulmonary pathology requires the expression of viral genes subsequent to infection.

DISCUSSION

These data illustrate an apparent dichotomy in the immune regulatory role of Ad E3 proteins. Our data verify the role of the TNF resistance genes in suppressing the inflammatory response but also indicate that the E3 protein gp19K alters neither afferent nor efferent immune responses. There are several possible explanations for gp19K's lack of immunomodulation. First, gp19K is not expected to alter CTL priming in vivo because in vivo priming is not restricted to the MHC of the immunizing cell (3, 27). Most cells can present only endogenous proteins to CTLs, but Rock et al. (53, 54) have shown that a subset of macrophages can present exogenous antigen in the context of MHC class I molecules. Recently, Suto and Srivastava (61) have elucidated the molecular basis for this phenomenon. They have shown that a subset of macrophages can load exogenous peptides onto MHC class I molecules of the macrophage via the heat shock protein gp96. In light of these data, gp19K could retain MHC class I molecules in the endoplasmic reticula of infected cells but macrophages at the site of infection could process and present Ad proteins by utilizing gp96. Since the macrophage has not been infected with Ad, the Ad peptides could be presented to CTLs without interference from gp19K.

A second possibility that cannot be completely eliminated is that in the mouse lung, gp19K could be underexpressed and/or MHC class I molecules could be overexpressed. The degree of suppression of MHC class I molecules depends on the levels of gp19K and the levels of class I molecule expression on Ad-infected cells (40, 51). If the Ad-infected bronchiolar epithelial cells are capable of reducing gp19K expression or the Ad immune response causes an increase in MHC class I molecule expression, then gp19K would not function in vivo as it does in vitro. Whether or not gp19K is produced at reduced levels in vivo remains to be addressed.

Grunhaus et al. (32) detected gp19K in vivo when it was overexpressed from a vaccinia virus promoter in a replicating vaccinia virus. In the experimental model used here, human Ad does not replicate, making the detection of gp19K in vivo difficult. Our laboratory has been unable to detect gp19K in the tissues of Ad-infected mice by in situ staining, Western blotting (immunoblotting), immunoprecipitation, or membrane-enhanced enzyme-linked immunosorbent assay. Even though gp19K production is below detectable levels, it seems likely that gp19K is produced, since it is regulated from the same promoter as the other E3 proteins (i.e., 10.4K/14.5K and 14.7K). The lack of a discernible function for gp19K in vivo may be due to a combination of any of these possibilities.

Even though gp19K does not appear to function in vivo as it does in vitro, this does not eliminate the possibility that gp19K functions in a different manner in vivo. Perhaps the true role of gp19K in vivo is to prevent immune recognition during a persistent infection. Routes et al. (56) have suggested that gp19K functions to allow persistence in a minor population of E1-transformed human cells. This study and the studies of Cox et al. (12) and Grunhaus et al. (32) evaluated gp19K's function during an acute infection; gp19K's role in persistent infections remains unexplained. Ad persists in humans and is shed asymptomatically for years in the face of a vigorous anti-Ad immune response (17). In order to survive immune recognition, Ad must have evolved ways to subvert the immune response. gp19K proteins sequenced from 17 clinical isolates of Ad7 were nearly identical (35). The conservation of gp19K in persistent Ad infections strongly suggests that gp19K is essential for viral persistence in the human population.

The results presented here reveal that the other E3 proteins (10.4K/14.5K and 14.7K) function in reducing the severity of the pulmonary lesions. These data directly correlate with the ability of these proteins to protect cells from lysis by TNF-α in vitro. That is, Ad-infected NIH 3T3 fibroblasts that express either 14.7K or 10.4K/14.5K are resistant to cytolysis by TNF-α (28). Although the in vitro susceptibility to TNF-α cytolysis correlates with pulmonary pathology, no visible cytolysis of the bronchiolar epithelium was observed. This is not unusual since apoptotic cells and cellular debris are rapidly cleared in vivo (10). Even though there was no evidence of TNF-α cytolysis, TNF-α is induced during Ad infections (21) and may be responsible for the inflammation observed when cells lack the TNF protection proteins.

The ability of the E3 proteins to reduce pathogenesis may be due to their ability to inhibit TNF-α cytolysis or to prevent the generation of by-products of the TNF-α cytolytic pathway. An emerging player in the TNF-α cytolytic pathway is cytoplasmic arachidonate-selective phospholipase A_2 ($cPLA_2$) (75). Pharmacologic inhibitors of $cPLA_2$ prevent TNF cytolysis in many cell lines (39, 41, 60). Also, Hayakawa et al. (34) showed that cells selected for resistance to TNF-α lacked $cPLA_2$ and became sensitive to TNF cytolysis when transfected with the cDNA for $cPLA_2$.

In turn, $cPLA_2$ metabolizes membrane phospholipids to generate arachidonic acid, which is further metabolized into a vast array of inflammatory mediators (50). These mediators include leukotrienes that act as chemoattractants and aggregate neutrophils (16) and prostaglandins that increase vascular permeability (48). Although the final effector for TNF-α cytolysis is still unknown, reactive oxygen intermediates from the lipoxygenase pathway probably contribute to TNF-α cytolysis since inhibitors of the 5-lipoxygenase pathway reportedly protect against TNF-mediated killing (11). Not only do the 10.4K/

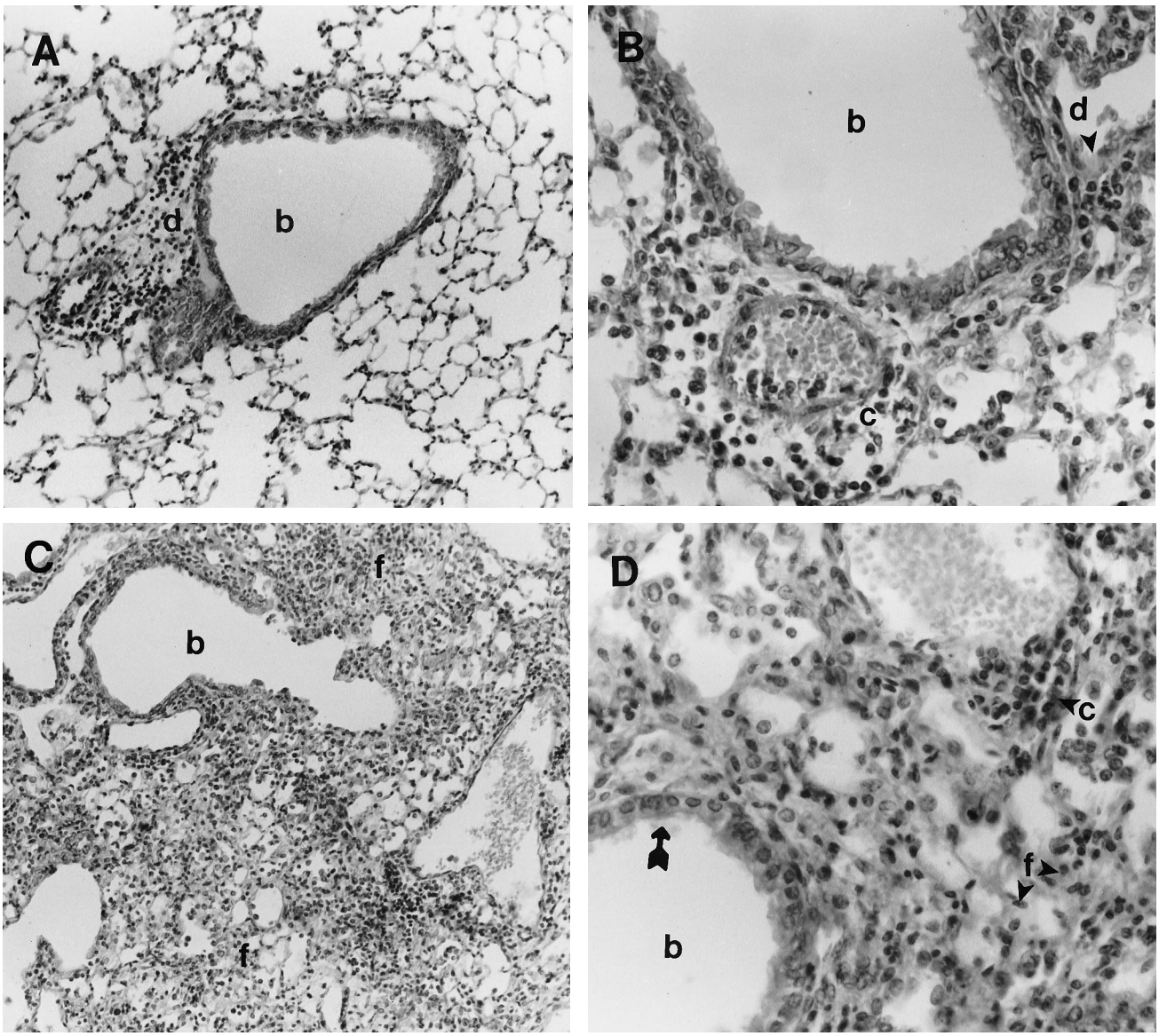

FIG. 6. Histopathology of lungs from mice infected with Ad that lack the TNF-α resistance genes. Mice were infected with *dl*762 (Δ14.7K) or *dl*797 (Δ10.4K, Δ14.7K) and evaluated for pulmonary lesions as described in the text. Shown are representative hematoxylin-and-eosin-stained sections for mice infected with the following: *dl*762; magnifications, ×80 (A) and ×200 (B); *dl*797; magnifications, ×80 (C) and ×200 (D). See legend to Fig. 3 for an explanation of letters and symbols used. (f) Intra-alveolar infiltration.

14.5K and 14.7K proteins prevent TNF-α cytolysis, but they also prevent the generation of arachidonic acid and the subsequent inflammatory mediators (41).

The inhibition of inflammatory mediators by the E3 proteins may be crucial for dampening cellular infiltration. In the lungs of mice infected with mutants that lack 10.4K/14.5K and 14.7K, soluble or membrane-associated TNF from activated resident macrophages (14) would liberate the inflammatory mediators from Ad-infected bronchiolar epithelial cells. These mediators would increase the vascular permeability and recruit cells into the lungs, which would lead to the observed increase in cellular infiltration. Therefore, the generation of inflammatory metabolites and the subsequent recruitment of inflammatory cells into the lungs is prevented by 10.4K/14.5K and 14.7K.

The inflammatory response observed in infections with both the wild type and mutants lacking the TNF resistance genes is also typical of responses to other respiratory viruses, such as influenza virus (1), Sendai virus (37), and respiratory syncytial virus (46). During a Sendai virus infection, 35% of the cells from bronchoalveolar lavage are macrophages and the nonadherent population is 85% $Thy1^+$. The nonadherent population has a $CD4^+/CD8^+$ ratio of 1:1 to 1:2 depending on the number of days p.i., and the polymorphonuclear leukocyte population is minuscule. Similarly, the Ad-induced pulmonary infiltrate consists mostly of mononuclear cells. Flow cytometric analysis of bronchoalveolar lavage cells showed that ≈50% were macrophages, with 70 to 80% of the nonadherent cells being $CD3^+$ lymphocytes (data not shown). Although there were two to three times as many cells isolated from mice inoculated with *dl*763 (Δ14.5K, Δ14.7K) as from *rec*700 (wild-type)-inoculated

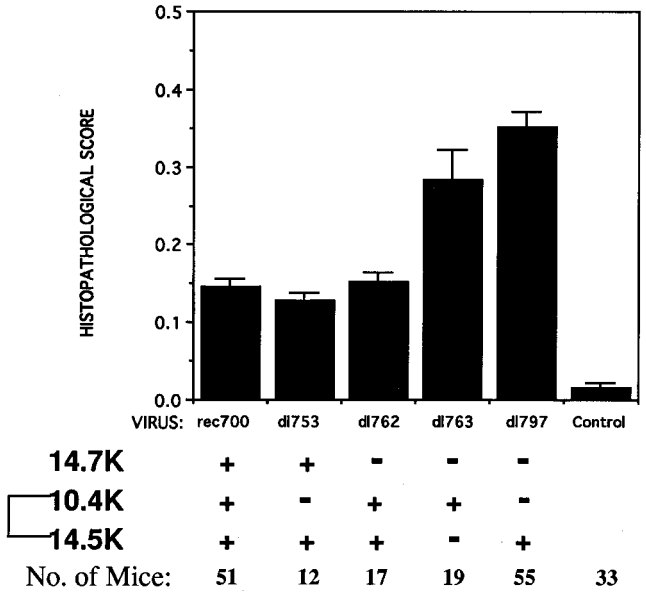

FIG. 7. Summary of histological scores for mice infected with viruses that lack the TNF-α resistance genes. Mice were infected with *rec*700 (wild-type), *dl*753 (Δ10.4K), *dl*762 (Δ14.7K), *dl*763 (Δ14.5K, Δ14.7K), or *dl*797 (Δ10.4K, Δ14.7K) and evaluated for pulmonary lesions. Mean histopathological scores from several experiments are shown.

mice, the $CD4^+/CD8^+$ ratios were the same (≈1:1.4) (data not shown). These data indicate that although there is quantitatively more cellular infiltration with *dl*763, qualitatively the response is the same as that seen with wild-type virus, thus illustrating that TNF magnifies the inflammatory response to Ad in a generalized fashion.

The role of the Ad E3 proteins has become increasingly important because Ad is a leading gene therapy vector for the treatment of cystic fibrosis (69). When Ad vectors that delete E3 were administered to cystic fibrosis patients, a major inflammatory response ensued (13). Results reported here suggest that maintaining expression of E3 in the vector, especially expression of 10.4/14.5K or 14.7K, may lessen the severity of the inflammatory response.

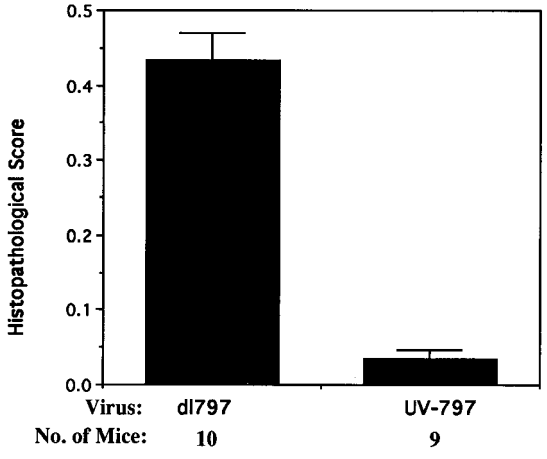

FIG. 8. Effect of UV inactivation on Ad-induced pneumonia. Mice were infected i.n. with *dl*797 that was UV inactivated (UV-797) or untreated (*dl*797). At 9 days p.i., pulmonary lesions were scored and the data were averaged. Error bars represent standard errors.

ACKNOWLEDGMENTS

We thank Jennifer Hull and Sonji K. Webb for technical assistance and Marianne Skeen for helpful discussions. We also thank Skip Melson for the particle counts.

This work was funded by NIH grants CA58736 (to L.R.G.) and CA24710 and CA58538 (to W.S.M.W.) and by a grant from the Genzyme Corp.

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