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NOTES

Role of Viral Antigens in Destructive Cellular Immune Responses to Adenovirus Vector-Transduced Cells in Mouse Lungs

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Adenoviruses missing E1 have been used as gene delivery vectors to the lungs for the treatment of cystic fibrosis. Transient expression of the recombinant gene and the development of inflammation have been two major limitations to the application of first-generation recombinant adenoviruses for gene therapy. Studies with mouse models of liver- and lung-directed gene therapy suggested that $CD8^+$ cytotoxic T lymphocytes (CTLs) are effectors that contribute to extinction of transgene expression. The precise antigens responsible for activation of CTLs have not been identified. In this study, we examine the relative contributions of viral proteins versus the transgene product to the activation of CTLs which eliminate transgene-containing cells in mouse lungs. Instillation of a *lacZ*-expressing virus into the lungs of C57BL/6 mice elicited CTL responses to both viral proteins and the transgene product, β-galactosidase, which collectively contribute to loss of transgene expression in mouse airways. Similar results were obtained in two experimental models in which the animals should be tolerant to the transgene, i.e., *lacZ* virus delivered to an animal transgenic for *lacZ* and a virus expressing the liver-specific enzyme ornithine transcarbamylase administered to the lungs of various strains of immune-competent mice. These data confirm the hypothesis that CTLs specific for viral antigens contribute to the problem of transgene instability in mouse lungs and indicate that CTLs specific for transgene product alone cannot account for the observed problem.

Adenoviruses have been used as vectors in many applications of somatic gene transfer. Gene transfer directed to airway epithelia has been evaluated in preclinical and clinical models of cystic fibrosis (5, 14). In virtually all models, expression of the transgene is transient, lasting less than 3 to 4 weeks, and is associated with the development of inflammation characterized by infiltration of polymorphonuclear leukocytes and lymphocytes. Our previous studies with mouse liver and lung suggested that adenoviral vectors missing E1 activate $CD8^+$ T cells to form cytotoxic T lymphocytes (CTLs), leading to loss of transgene expression (10, 11). One mechanism by which this occurs appears to be elimination of the infected cell; however, other antigen- and non-antigen-specific mechanisms likely contribute. In vitro studies indicated that viral proteins expressed from adenoviral vectors missing E1 are targets for CTLs in most strains of mice (references 10 and 11 and unpublished data). The presence of CTLs specific for the transgene product was not formally excluded from these initial experiments. In fact, a recent study by Tripathy et al. indicated that immune responses to transgene products are responsible for the loss of transgene expression in mouse skeletal muscle (7). Humoral immune responses to proteins of the input recombinant adenovirus underlie an unrelated but equally important problem of neutralizing antibodies that prevent gene transfer upon a second administration of virus (2, 3, 6, 11, 13).

Models of lung-directed gene therapy have been used in the current study to define the relative contributions of viral antigens versus transgene product as neoantigens that activate cellular immunity, which leads to the loss of transgene expression in airway epithelial cells.

The experimental paradigm used in this study has been described previously (11). Briefly, adenoviral vectors missing E1 (10^9 PFU/50 μl of phosphate-buffered saline [PBS]) were instilled into the tracheas of 6- to 8-week-old female mice who were evaluated for T-cell responses to viral and transgene antigens, as well as for efficiency and stability of gene expression with histochemical stains specific for the reporter gene.

Instillation of an adenovirus missing E1 expressing nucleus-targeted β-galactosidase into C57BL/6 mouse lungs led to high-level *lacZ* expression in >80% of the airways at day 3 (Table 1), which declined to undetectable levels by day 28 (Table 1) and which was associated with the development of substantial peribronchial and perivascular lymphocytic infiltrates (data not shown). This is consistent with our previous studies utilizing vectors expressing other reporter and therapeutic genes. Lymphocytes harvested from mediastinal lymph nodes (MLNs) were analyzed for the presence of CTLs specific for viral proteins and β-galactosidase with a chromium release assay (Fig. 1). Substantial cytolysis was observed with target cells infected with the *lacZ* adenovirus. A reduced level of specific lysis was observed with target cells infected with alkaline phosphatase-expressing adenovirus (Fig. 1). Specific lysis was also detected with target cells that were stably expressing *lacZ* via a retrovirus (i.e., PLJ-*lacZ*), whereas no cytolysis was observed with target cells expressing retrovirus-encoded alkaline phosphatase (i.e., PLJ-ALP). These results demonstrate activation of CTLs specific for both viral proteins and the transgene target β-galactosidase after the delivery of adenovirus missing E1 to the respiratory epithelia of C57BL/6 mice. It is impossible on the basis of in vitro studies to predict the

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TABLE 1. Morphometric analysis of mouse lungs for efficiency of transgene expression[a]

Animal, vector	% of transgene-containing respiratory epithelial cells:		
	Per airway	In total airways	
		Day 3	Day 28
C57BL/6, H5.CMVNT*lacZ*	0	6	100
	1–25	20	0
	>25	74	0
ROSA26, H5.CMV*lacZ*	0	8	100
	1–25	19	0
	>25	73	0
C57BL/6, H5.CMVmOTC	0	12	100
	1–25	28	0
	>25	60	0
C3H, H5.CMVmOTC	0	14	100
	1–25	24	0
	>25	62	0
BALB/c, H5.CMVmOTC	0	18	100
	1–25	26	0
	>25	56	0

[a] Data were analyzed by examination of a total of 50 airways from three mice for the presence of transgene-containing respiratory epithelial cells.

relative contribution of CTL subsets to in vivo activity, as defined by target cell elimination.

To test the relative role of immune responses to transgene versus viral antigens in the extinction of vector from mouse lung, a similar study was performed with an animal that is transgenic for a gene encoding cytoplasmic β-galactosidase (i.e., the ROSA-26 mouse). The animal should be tolerant to the vector-derived reporter gene, which would allow a direct analysis of the importance of immune responses to virus-derived proteins. The germ line-encoded *lacZ* transgene is expressed in many tissues, including airway epithelial cells. Vector-derived β-galactosidase was distinguished from the germ line-transmitted *lacZ* product because it was targeted to the nucleus. Similar to what is observed in C57BL/6 mice, instillation of virus into the lungs of ROSA-26 mice resulted in high levels of nucleus-localizing β-galactosidase in the majority of airways at day 3 (Table 1), which were reduced to undetectable levels by day 28 (Table 1), associated with the development of inflammation (data not shown). Lymphocytes from the MLNs of vector-treated ROSA-26 mice demonstrated no cytolysis to PLJ-*lacZ*-infected target cells (Fig. 1); however, substantial lytic activity was observed on target cells infected with adenovirus missing E1 (Fig. 1).

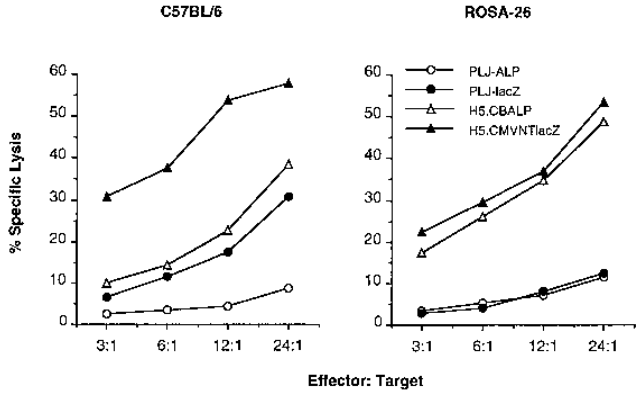

FIG. 1. CTL responses to viral proteins versus those to β-galactosidase in C57BL/6 and ROSA-26 mice. Lymphocytes of MLNs from C57BL/6 and ROSA-26 mice 10 days after administration of H5.CMVNT*lacZ* were restimulated in vitro for 5 days and tested for specific lysis on PLJ-ALP-, PLJ-*lacZ*-, H5.CBALP-, and H5.CMVNT*lacZ*-infected H-2-compatible target cells (C57SV) in a 6-h ^{51}Cr release assay. The methods are described in reference 11. The percentage of specific lysis is expressed as a function of different effector/target cell ratios (3:1, 6:1, 12:1, and 24:1). The viruses used in this study have been described in references 11 and 12.

These experiments used a *lacZ* transgenic animal in an attempt to eliminate immune responses to the reporter gene of the vector. It is theoretically possible that a CTL response was generated specific for the NH_2 terminus of the reporter gene product, where it differs from the germ line-transmitted *lacZ* gene product having incorporated a nuclear targeting sequence from simian virus 40 T antigen. To further clarify the relative contributions of cellular immune responses to transgene versus viral antigens in the elimination of the vector from airways, ornithine transcarbamylase (OTC), an endogenous hepatic enzyme involved in the urea cycle, was used as a novel reporter gene in mouse lungs (12). The enzyme is expressed only in the liver and bowel; a convenient histochemical stain exists that is sensitive and specific (i.e., there is no background staining in the lungs). Instillation of a recombinant virus expressing murine OTC into the lungs of BALB/c (Fig. 2 and Table 1), C3H (Table 1), or C57BL/6 (Fig. 2 and Table 1) mice resulted in high-level OTC expression in airway epithelial cells on the basis of histochemical or immunohistochemical staining at day 3. However, expression of OTC protein and enzyme was again reduced to undetectable levels at day 28 in all three mouse strains. For a summary of the results of morphometric analysis of these experiments, see Table 1.

Lymphocytes harvested from MLNs were analyzed in vitro for cytolytic activity against virus-infected target cells with the chromium release assay (Fig. 3). MLN-derived lymphocytes from infected C57BL/6 mice demonstrated significant cytolysis of class I-restricted, H-2-compatible targets infected with the OTC vector. The specificity of this activity for viral proteins was demonstrated by the lack of cytolysis of mock-infected targets. MLN-harvested lymphocytes from C3H or BALB/c mice demonstrated levels of cytolysis to H2-compatible targets infected with OTC vector similar to what was observed with C57BL/6 mice. Taken together, these data suggest that CTLs specific for viral antigens are sufficient to eliminate virus-transduced epithelial cells in mouse lungs.

Mice were evaluated for the development of intrapulmonary pathology by criteria that Ginsberg and colleagues (1, 4) have used to evaluate adenovirus pneumonia in cotton rats and mice. A quantitative summary of the findings is presented in Fig. 4. Primary administration of recombinant adenovirus in C57BL/6 mice led to inflammation that peaked at day 10 and that declined to certain levels at day 28. This pathology was characterized by perivascular and peribronchial mononuclear infiltrates with some alveolar infiltration. Similar results were observed in C3H and BALB/c mice (Fig. 4).

The purpose of this study was to determine if cellular immune responses to viral proteins are sufficient to diminish transgene stability in mouse lungs. This is difficult to assess when the reporter gene encodes a foreign protein such as β-galactosidase, chloramphenicol acetyltransferase, luciferase, green fluorescent protein, or alkaline phosphatase. A humoral immune response to β-galactosidase has been demonstrated in mice after intratracheal instillation of *lacZ* adenovirus (8). The strategy was to perform experiments in a setting in which the transgene product cannot be viewed as a neoantigen. The use of *lacZ*-expressing adenoviruses in a *lacZ* transgenic mouse and a vector expressing the liver-specific enzyme OTC deliv-

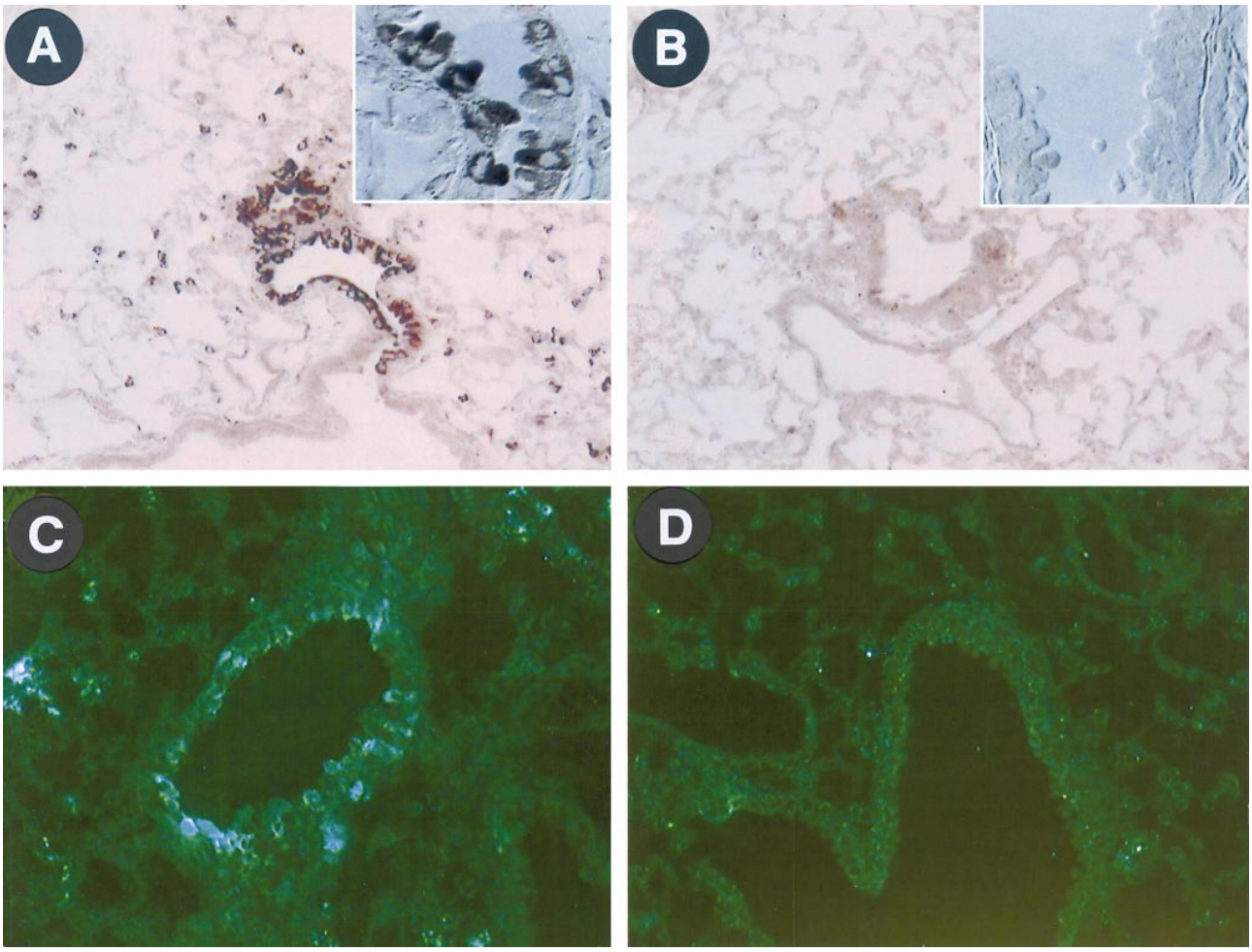

FIG. 2. Localization of adenovirus-mediated OTC expression in mouse lungs. Suspensions of virus (3×10^9 PFU) were instilled into the lungs of BALB/c (A and B) and C57BL/6 (C and D) mice. The mice were sacrificed, and lung tissues were evaluated for OTC expression by histochemical staining at days 3 (A) and 28 (B) and by immunohistochemical staining at days 3 (C) and 28 (D). Magnification, ×125; inset magnification, ×400. For histochemical staining, sections (6 μm thick) were first treated with 50 mM triethanolamine (pH 7.2) for 10 min and then bathed in OTC incubation buffer for 10 min. The incubation buffer (10 ml) was prepared by dissolving 0.8 g of sucrose, 3 mg of carbamylphosphate (dilithium salt), and 5 mg of L-ornithine in 50 mM triethanolamine. Lead nitrate (1 ml of 1%) was subsequently added with stirring, and the buffer was readjusted to pH 7.2, filtered, and used immediately. The sections were washed twice with water and stained with 1% ammonium sulfide for 1 min, followed by washes in water and mounting on coverslips. For OTC immunohistochemical staining, frozen sections (6 μm) were fixed with methanol for 10 min as described above. After being blocked with 10% goat serum in PBS, sections were incubated with a polyclonal antibody specific for rat OTC (1:500 dilution; a gift from Frank Kalousek) for 60 min, followed by incubation with 5 μg of donkey anti-rabbit fluorescein isothiocyanate-conjugated immunoglobulin G per ml for 30 min. Sections were washed and mounted with the antifadent Citifluor (Citifluor, Canterbury, United Kingdom).

ered to the lung accomplished this objective. Elimination of the transgene as an immune target did not diminish the problems of transient expression or inflammation. We believe that for cystic fibrosis gene therapy, it will be prudent to further engineer the vector to minimize, if not eliminate, viral protein expression.

Several factors could influence the contribution of transgene-specific CTLs to the destruction of recombinant virus-transduced cells. (i) One factor is the immunogenicity of the transgene product. We have used similar vectors to express rabies glycoprotein, which in mice elicits potent CTLs specific for this viral antigen that effectively protect against lethal infection with a virulent strain of rabies virus (9). (ii) Another factor is the organ to which adenoviral vectors are targeted. A recent study by Tripathy et al. indicated that immune responses to transgene product are responsible for the loss of transgene expression in mouse skeletal muscle (7). (iii) The genotype of the gene therapy recipient may have an impact on

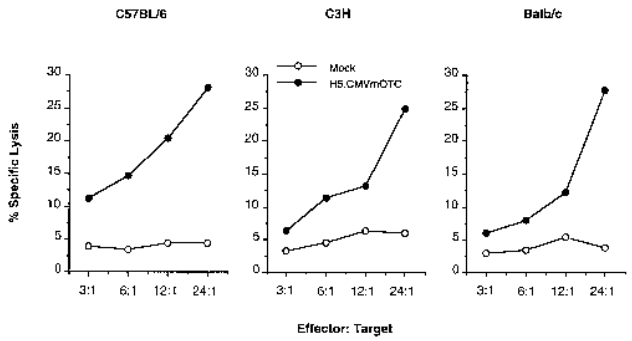

FIG. 3. CTL responses in mice infected with H5.CMVmOTC. Lymphocytes of MLNs harvested from C57BL/6, C3H, and BALB/c mice 10 days after administration of H5.CMVmOTC were restimulated in vitro for 5 days and tested for specific lysis on mock-infected or H5.CMVmOTC-infected H-2-compatible target cells (C57SV, L929, and P815 for C57BL/6, C3H, and BALB/c mice, respectively) in a 6-h ^{51}Cr release assay. The percentage of specific lysis is expressed as a function of different effector/target cell ratios (3:1, 6:1, 12:1, and 24:1).

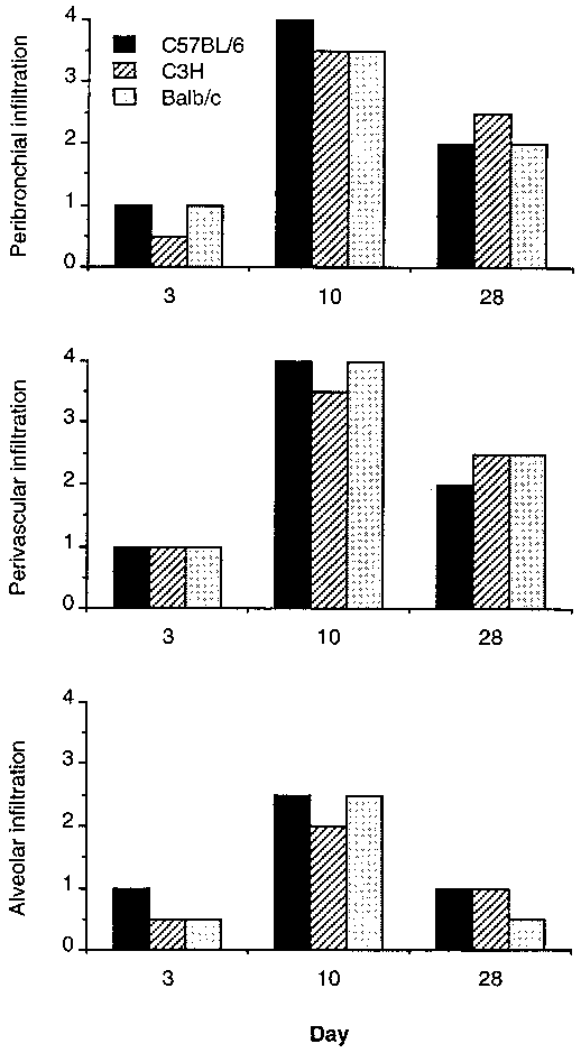

FIG. 4. Pathological responses of the recipient mouse lungs to recombinant adenoviruses. Lungs were harvested after instillation of H5.CMVmOTC and evaluated for evidence of histopathology by light microscopic inspection of paraffin sections stained with hematoxylin and eosin. Samples were characterized with respect to peribronchial infiltration, perivascular infiltration, and alveolar infiltration. The extent of pathology was scored from 0 (no pathology) to 4 (severe pathology [see reference 11]). This figure summarizes the extent of pathology observed in C57BL/6, C3H, and BALB/c mice as a function of time after instillation of virus (days 3, 10, and 28).

the magnitude of the CTL response to the therapeutic protein, especially in the treatment of patients homozygous for a deficiency state. (iv) Finally, allelic variation in major histocompatibility complex loci may have an impact directly on the presentation of transgene epitopes.

Another interesting point that arose from this study is the use of the gene coding for OTC as a novel reporter gene for adenovirus-mediated gene transfer to mouse lungs. There are several advantages to this new method. (i) As an endogenous protein, OTC (a hepatocyte-specific enzyme) is not expressed in the lungs; therefore, it can be more effectively used to evaluate the performance of new generations of adenoviral vectors. (ii) In addition, OTC is easily detected by both histochemical and immunohistochemical stainings.

In summary, this study attempts to define the role of the CTL response to the transgene product versus that to viral antigens in the loss of transgene expression. Our basic findings are that CTLs are activated in response to both viral antigens and transgene product; in the absence of transgene-specific CTLs, virus-specific CTLs are sufficient to explain the problem of transient expression of the transgene in mouse lung. This study does not rule out that CTLs specific for β-galactosidase contribute to the loss of transgene expression in this model. The data for mouse lungs underscore the complexity of antigen-specific immune responses to adenoviral gene therapy, which are likely influenced by the species and strain of recipient, major histocompatibility complex haplotype, target organ, route of vector administration, and vector structure. In some applications, it may be necessary to develop new generations of recombinant adenovirus vectors that are void of viral protein expression.

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