Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 4407–4411, May 1994
Medical Sciences

Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy

(liver/cytotoxic T lymphocytes/genetic diseases)

YIPING YANG*†, FREDERICK A. NUNES*†, KLARA BERENCSI‡, EMMA E. FURTH§, EVA GÖNCZÖL‡, AND JAMES M. WILSON*†‡¶

*Institute for Human Gene Therapy and Departments of †Molecular and Cellular Engineering, and §Pathology and Laboratory Medicine, University of Pennsylvania Medical Center, and ‡The Wistar Institute of Anatomy and Biology, Philadelphia, PA 19104

Communicated by Francis S. Collins, January 4, 1994 (received for review November 3, 1993)

ABSTRACT **An important limitation that has emerged in the use of adenoviruses for gene therapy has been loss of recombinant gene expression that occurs concurrent with the development of pathology in the organ expressing the transgene. We have used liver-directed approaches to gene therapy in mice to study mechanisms that underlie the problems with transient expression and pathology that have characterized *in vivo* applications of first-generation recombinant adenoviruses (i.e., those deleted of E1a and E1b). Our data are consistent with the following hypothesis. Cells harboring the recombinant viral genome express the transgene as desired; however, low-level expression of viral genes also occurs. A virus-specific cellular immune response is stimulated that leads to destruction of the genetically modified hepatocytes, massive hepatitis, and repopulation of the liver with nontransgene-containing hepatocytes. These findings suggest approaches for improving recombinant adenoviruses that are based on further crippling the virus to limit expression of nondeleted viral genes.**

Human adenoviruses have emerged as a promising technology for *in vivo* gene therapy. The 36-kb double-stranded DNA genome of these viruses contains 25 a series of early genes encoding regulatory products and late genes encoding structural products. An important step in the design of recombinant adenoviruses for gene therapy was the isolation of a packaging cell line that provides in trans the essential regulatory genes *E1a* and *E1b* (1). By using this cell line, it is possible to produce recombinants containing a transgene in which the virus was rendered replication-defective by deleting *E1* sequences.

Recombinant adenoviruses have many of the requisite features for a useful *in vivo* gene delivery vehicle in that they can be purified and grown in large quantities, and they very efficiently transduce genes into a wide spectrum of nondividing cells *in vivo* (2–8). Application of adenoviral technology for the treatment of chronic disorders such as genetic diseases will require very prolonged, if not stable, expression of the recombinant gene or repeated administration of the vehicle without the development of destructive or inhibiting responses.

Critical evaluation of this technology for *in vivo* gene therapy has revealed several important limitations. In the best characterized systems (i.e., gene transfer to lung and liver), expression of the transgene has been transient and associated with the development of pathology in the target organ (9–13). In addition, it appears that repeated administration of the recombinant virus is associated with confounding immune responses. In this report, we have used mouse models to evaluate the role of the immune system in the pathology and transient recombinant gene expression that has characterized *E1*-deleted viruses for *in vivo* gene therapy.

MATERIALS AND METHODS

***In Vivo* Delivery of Recombinant Adenoviruses to Mouse Liver.** Ad.CB*lacZ*, an *E1*-deleted replication-deficient recombinant adenovirus carrying the *lacZ* minigene driven by a cytomegalovirus-enhanced β-actin promoter, was constructed (14). It is based on human adenovirus type 5 (Ad5) with a small deletion of the *E3* region. None of the viral stocks used in the experiments contained detectable replication-competent virus. Ad5dlE3, a replication-competent Ad5 with deletion of E3 region, was described (15). Virus (1×10^{11} plaque-forming units/ml) in 0.1 ml of phosphate-buffered saline (PBS, pH 7.4) was infused retrograde into the biliary tract of female CBA and *nu/nu* mice as described (16). All animals that received recombinant virus survived to necropsy.

DNA and RNA Analyses. Total cellular DNA and RNA were prepared from liver tissues and analyzed as described (17, 18). Probes included a 2.0-kb *Escherichia coli lacZ* DNA fragment (*Cla* I–*Eco*RI) for DNA hybridization and a 1.6-kb Ad5 hexon DNA fragment for RNA hybridization.

Morphological Analyses. *5-Bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) histochemistry.* Frozen sections (6 μm) were fixed in 0.5% glutaraldehyde and stained for β-galactosidase activity as described (16).

Histopathology. Paraffin sections (5 μm) were stained with hematoxylin/eosin. Random sections were examined for histopathology in a blinded fashion.

Immunohistochemical detection of viral proteins. Frozen sections (6 μm) were fixed in 2% (wt/vol) paraformaldehyde and permeabilized as described (19). Indirect immunofluorescence was performed with a hybridoma supernatant to Ad5 DNA binding protein (an *E2a* gene product) (20), and Ad5 hexon protein was detected with fluorescein isothiocyanate-labeled polyclonal antibody (Ab1056F, Chemicon).

BrdUrd labeling and detection of mitotically active cells. Animals were labeled for 4 h with BrdUrd at 100 mg/kg before sacrifice at each time point. Frozen sections were fixed in methanol and analyzed by immunohistochemical method using a monoclonal antibody to BrdUrd as described (21). The percentage of BrdUrd labeling was calculated by counting 2000 cells from 10 random fields for each animal.

Cytotoxic T-Lymphocyte (CTL) Assay. CBA mice infused with Ad.CB*lacZ* were sacrificed at day 14 and the spleens were aseptically removed for preparation of cell suspensions. Cells were suspended at 2.5×10^6 viable cells per ml and restimulated *in vitro* using Ad5dlE3 with multiplicity of

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: Ad5, adenovirus type 5; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside; CTL, cytotoxic T lymphocyte; MOI, multiplicity of infection; MHC, major histocompatibility complex.

¶To whom reprint requests should be addressed.

infection (MOI) of 20 for 5 days in 24-well plates. Cytolytic activity of nonadherent spleen cells (effectors) was tested in a chromium release assay.

RESULTS AND DISCUSSION

The most extensive evaluation of adenoviral technology for *in vivo* gene therapy has been performed in the lung and liver (9–13). In these organs, expression of the recombinant virus has been efficient but transient and, when carefully examined, associated with inflammation. Transient expression of the transgene has also been associated with adenoviral-mediated gene transfer to other organs (5, 8). Exceptions to this include studies that evaluated adenovirus-mediated gene transfer in (*i*) newborn animals (2, 22), (*ii*) immunoprivileged organs (i.e., retina) (7), and (*iii*) human xenografts grown in immunodeficient animals (23). In each case, transgene ex-

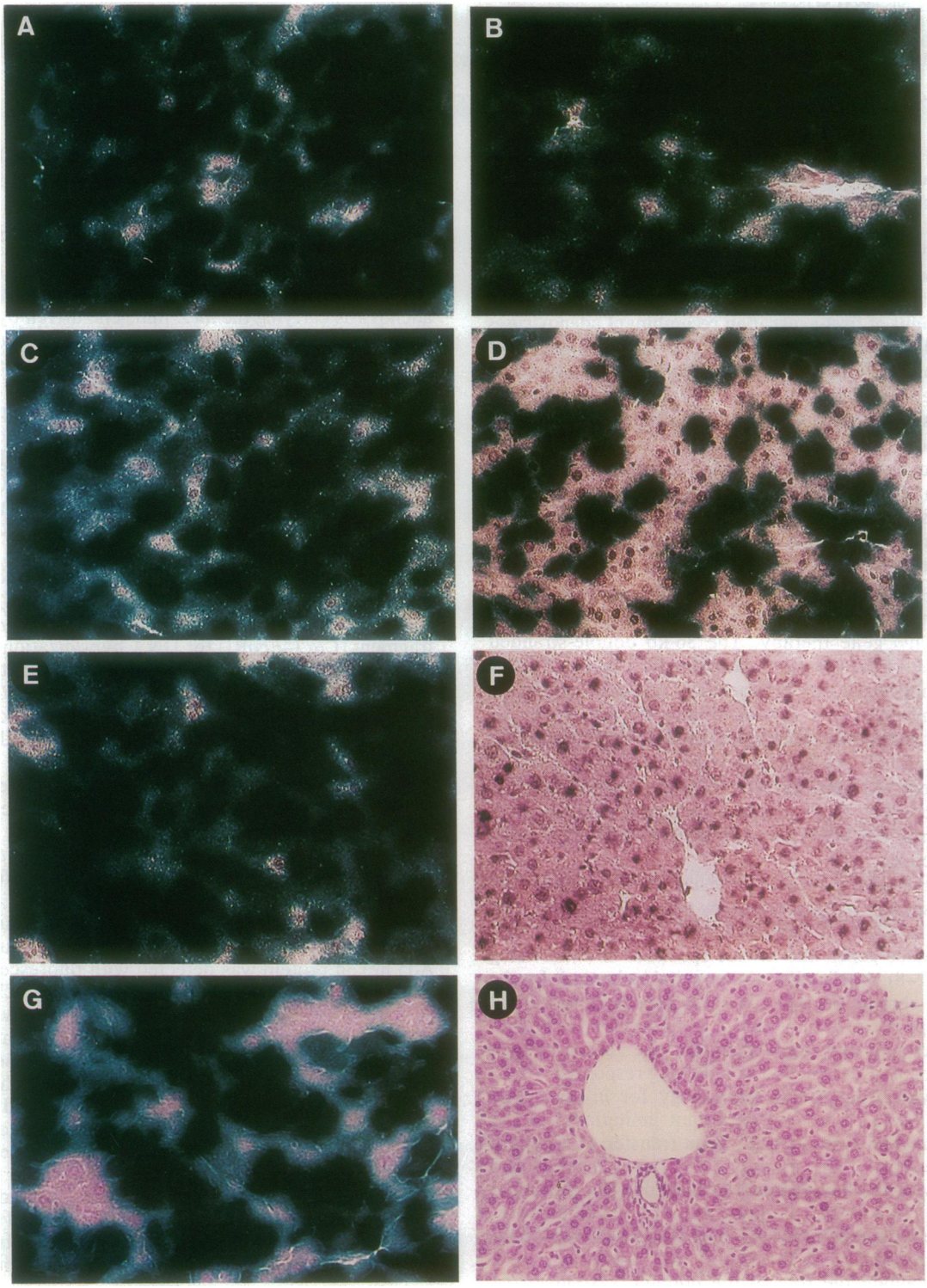

FIG. 1. X-Gal histochemical analysis to detect *lacZ* expression. Mice were infused with Ad.CB*lacZ* (1×10^{10} plaque-forming units in 0.1 ml of PBS) and euthanized, and liver tissues were evaluated for *lacZ* expression using the X-Gal histochemical staining. Ad.CB*lacZ*-infected *nu/nu* mice at 3 days (*A*), 7 days (*C*), 21 days (*E*), and 60 days (*G*) and Ad.CB*lacZ*-infected CBA mice at 3 days (*B*), 7 days (*D*), 21 days (*F*), and 35 days (*H*) are shown. (×160.)

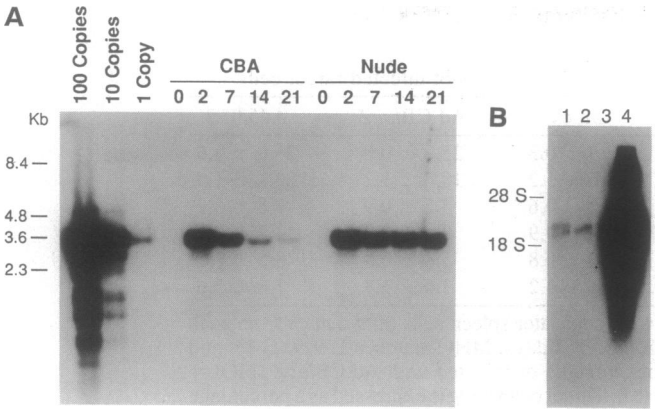

FIG. 2. Analysis of liver for viral DNA and RNA. (*A*) Total cellular DNA (10 μg) was prepared from liver tissues of CBA (lanes CBA) and *nu/nu* (lanes Nude) mice, digested with *Bam*HI, and analyzed on a Southern blot using an *E. coli lacZ* probe. Numbers indicate the day after gene transfer on which tissue was harvested. DNA (10 μg) from uninfected mice plus 7.5 pg (lane 1 copy), 75 pg (lane 10 copies), and 750 pg (lane 100 copies) of pAd.CB*lacZ* plasmid DNA was used as copy number controls. Molecular size standards are indicated to the left. (*B*) Total cellular RNA (10 μg) from uninfected (lane 3), Ad.CB*lacZ*-infected (lanes 1 and 2), and Ad5dlE3-infected (lane 4) liver tissues of CBA mice were analyzed. The intensity of the ribosomal markers was identical in each lane, indicating equivalent quantities of electrophoresed RNA.

pression was stable for prolonged periods of time without significant pathology. These informative exceptions suggest that the immune system may play a role in mediating the major limitations of adenovirus technology.

We propose a hypothesis to explain the mechanisms that underlie the development of pathology and loss of gene expression that have limited the utility of *E1*-deleted adenoviruses for *in vivo* gene therapy. We suggest that cells harboring the recombinant genome express adenoviral proteins despite the absence of *E1a* and *E1b*. This could occur through transactivation by cellular E1-like factors or basal expression of adenoviral promoters (24, 25). Presentation of the newly synthesized viral proteins in the context of histocompatibility antigens would lead to the generation of specific cellular immune responses to, and destruction of, the genetically modified cells. The end result is a pathological inflammatory response and loss of gene expression as the tissue is replaced with cells that do not contain the transgene.

Adenovirus-mediated gene transfer to mouse liver *in vivo* was used as a model to evaluate this hypothesis. The mouse is an attractive species for studying immunological mechanisms because of the availability of congenic strains and extensive panels of reagents for *in vitro* immunologic assays. In addition, previous studies have shown that wild-type human Ad5 is capable of lytic infection and replication in mouse hepatocytes that, when administered *in vivo*, leads to dose-dependent hepatitis (26).

The role of the immune system in the stability of recombinant adenoviral transgene expression was demonstrated in experiments in which livers from both immunocompetent (CBA) and genetically athymic (*nu/nu*) strains of mice were exposed to Ad.CB*lacZ* by instillation of virus retrograde into the biliary tract. *lacZ* expression was demonstrated by X-Gal

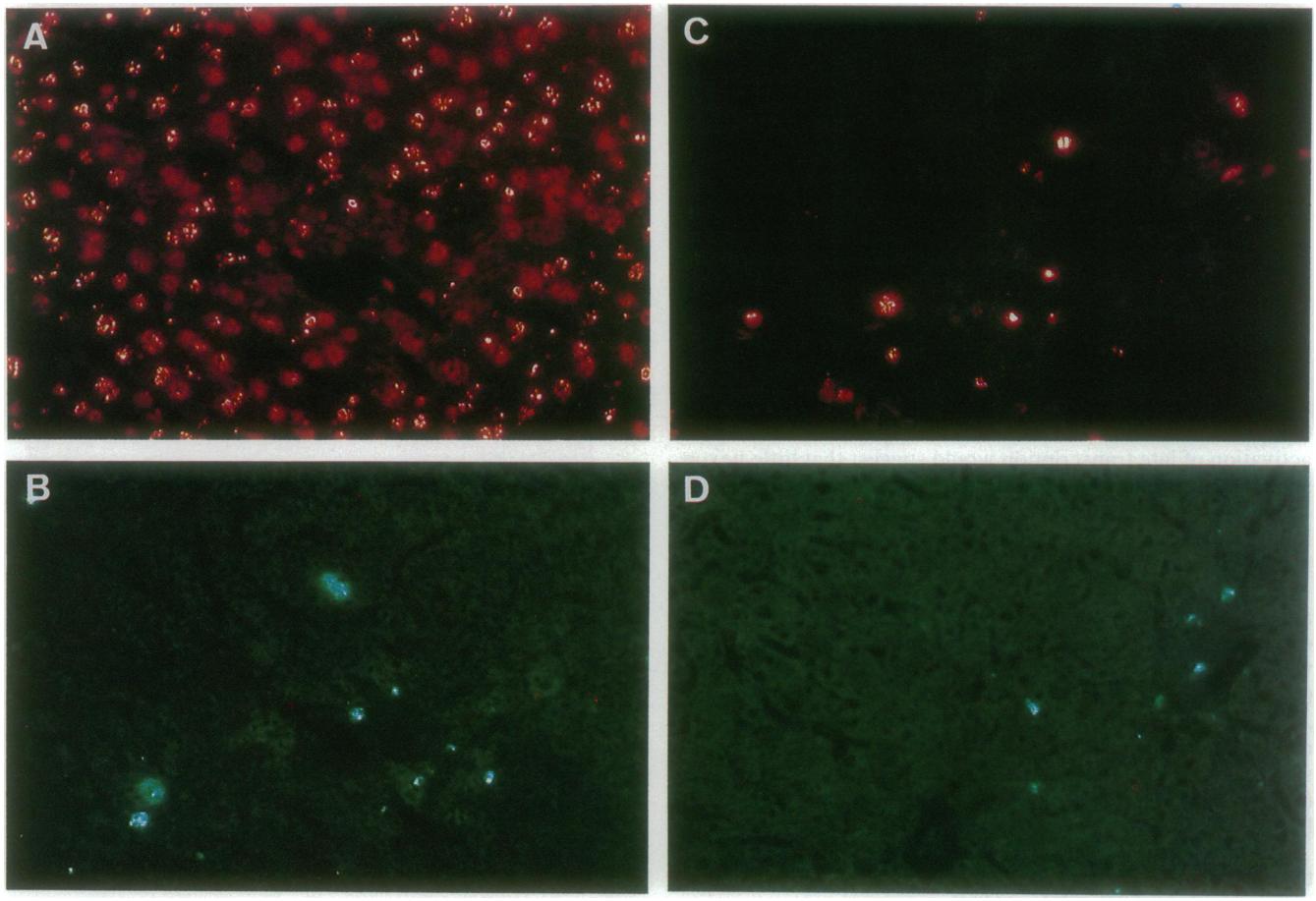

FIG. 3. Immunocytochemical analysis to detect viral proteins. Liver sections of CBA mice infused with Ad5dlE3 (*A* and *B*) and Ad.CB*lacZ* (*C* and *D*) were analyzed for viral protein expression by double immunofluorescence using antibodies to Ad5 DNA binding protein (*A* and *C*) and hexon protein (*B* and *D*). (×150.)

Table 1. Adenovirus-specific MHC class I-restricted lysis of target cells by spleen cells from Ad.CB*lacZ*-infected CBA mice

Mice	Target cells	E/T ratio	Specific lysis of infected target cells		
			Mock	Ad.CB*lacZ*	Ad5dlE3
Ad.CB*lacZ*-infected	L-929	100:1	12.9 ± 0.3	25.0 ± 0.8	26.8 ± 0.6
		50:1	9.6 ± 1.2	18.8 ± 1.2	20.1 ± 1.5
	MC57	50:1	4.7 ± 0.6	5.2 ± 0.9	7.8 ± 3.2
Uninfected	L-929	100:1	10.3 ± 0.9	8.8 ± 0.7	11.7 ± 0.2
		50:1	3.0 ± 0.8	5.6 ± 0.8	8.5 ± 2.1
	MC57	50:1	3.1 ± 0.2	3.0 ± 1.3	7.6 ± 5.6

CBA(H-2^k) mice were infected with Ad.CB*lacZ*. Two weeks later spleen cells of two mice from each group were pooled and restimulated *in vitro* with Ad5dlE3 for 5 days. MHC-matched L-929 (H-2^k) and MHC-mismatched MC57 (H-2^b) target cells were mock-infected or infected with Ad.CB*lacZ* (MOI = 100–200) for 72 h or with Ad5dlE3 (MOI = 50–100) for 48 h. Target cell killing is expressed as a percentage (mean ± SD) of CTL-induced release of incorporated ^{51}Cr from target cells in a 4-h cytolysis assay.

histochemistry in >80% of hepatocytes of both strains of mice when analyzed 2 days after gene transfer (Fig. 1 *A* and *B*). In CBA mice, expression diminished to undetectable levels by day 21 (Fig. 1*F*), whereas no diminution in expression was detected in *nu/nu* mice during the longest interval studied, which was 60 days in the initial studies (Fig. 1*G*). Southern blot analysis of total cellular DNA from liver of recipient animals was performed to further delineate the mechanism for the selective loss of gene expression in immunocompetent animals (Fig. 2*A*). Viral DNA remained at high levels (≈5 copies per cell) for the duration of the experiment in *nu/nu* mice, whereas the viral DNA progressively diminished from ≈5 copies per cell to <0.2 copy per cell over 21 days in CBA mice. This indicates that loss of the viral genome, rather than extinction of expression, is the primary mechanism responsible for loss of *lacZ* activity.

CBA mice were exposed to either recombinant adenovirus and liver tissue was analyzed for presence of viral proteins by immunocytochemistry and viral RNAs by RNA hybridization. Expression of the early gene *E2a*, which encodes a DNA binding protein essential for DNA replication, was detected in the nuclei of the majority of hepatocytes in animals treated with Ad5dlE3 (Fig. 3*A*) and in a subpopulation of hepatocytes in animals treated with Ad.CB*lacZ* (Fig. 3*C*). Expression of this gene in the absence of *E1a* was not surprising since it can be activated with the inducible cellular factor E2F (27). Expression of the prototypic late gene product hexon was demonstrated in a smaller population of periportal hepatocytes in Ad.CB*lacZ*-infected animals (Fig. 3*D*). Hexon-containing transcripts were also detected in these tissues, at levels ≈200-fold lower than that achieved with Ad5dlE3 (Fig. 2*B*, compare lanes 1 and 2 to lane 4). The requirement of E1a for activation of late transcription would suggest that little hexon should be present in Ad.CB*lacZ*-infected animals. The finding of small but detectable hexon RNA and protein suggests low-level transactivation of the major late promoter by E1a-independent mechanisms.

Animals were analyzed for the development of CTLs to adenoviral proteins in splenocytes harvested 2 weeks after exposure to Ad.CB*lacZ* (Table 1). Significant lysis ($P < 0.01$) was demonstrated to major histocompatibility complex (MHC)-matched target cells (L-929) infected with either Ad5dlE3 or Ad.CB*lacZ*, compared to mock-infected L-929 cells. Specificity of the assay was confirmed by absence of significant lysis with infected MHC-mismatched target cells (MC57). A CTL response was not detected in splenocytes from naive animals.

Analysis of liver tissue 10 days after gene transfer revealed the development of substantial hepatic pathology in CBA mice infected with Ad.CB*lacZ* (Fig. 4*B*) that was not present in naive CBA mice (Fig. 4*A*) or *nu/nu* mice exposed to similar doses of this virus (Fig. 4*C*). By day 2, liver from CBA mice showed dramatic ballooning degeneration of hepatocytes with the beginning of apoptosis, which by day 7 is associated with a light lymphocytic infiltrate and mitotically active hepatocytes. The lymphocytic infiltrate and regenerative response increased dramatically in intensity over the next 7 days, having resolved in all sections of liver except the portal tracts by day 21. The

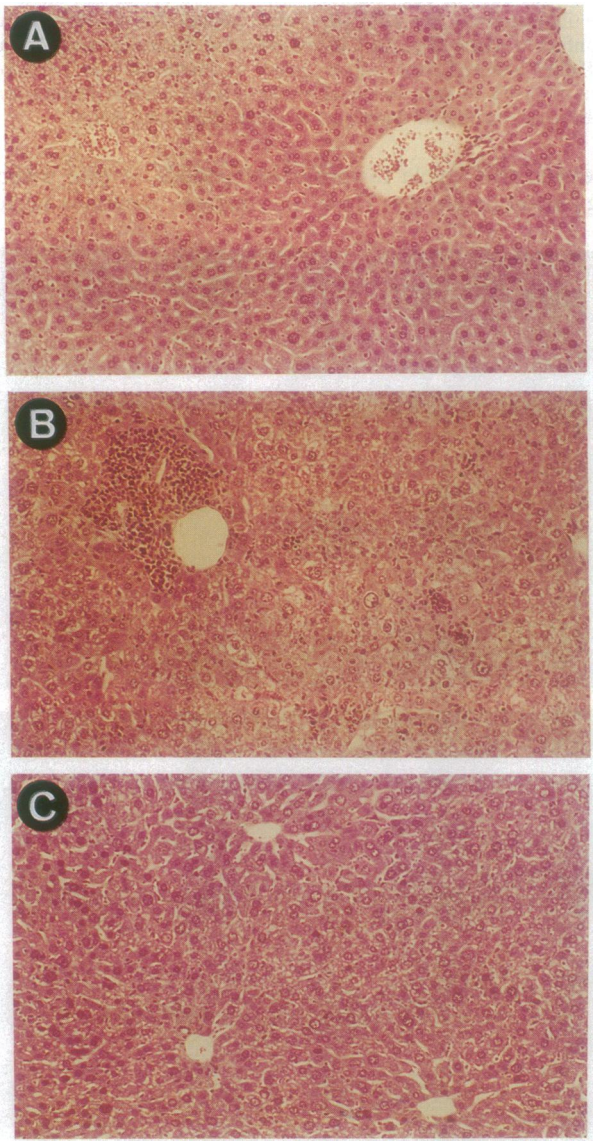

FIG. 4. Histopathology in liver of recipient animals. (*A*) Uninfected CBA mouse. (*B*) CBA mouse infected with Ad.CB*lacZ* and analyzed at day 10. (*C*) *nu/nu* mouse infected with Ad.CB*lacZ* and analyzed at day 10. (×130.)

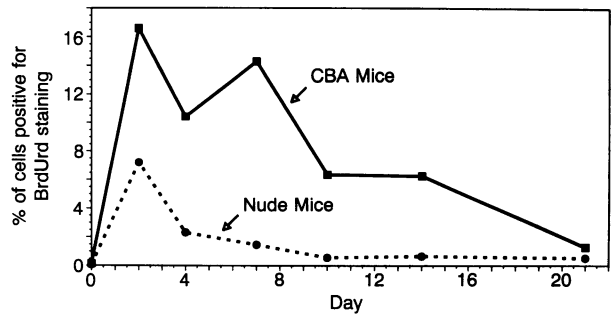

FIG. 5. Labeling of hepatocytes with BrdUrd after exposure to virus. Animals were labeled with BrdUrd (100 mg/kg) and analyzed for its incorporation into DNA by immunofluorescent staining using a monoclonal antibody to BrdUrd. The percentage of cells positive for BrdUrd labeling over time with the solid line and the dash line indicating CBA and *nu/nu* mice, respectively.

extensive pathology noted in liver of Ad.CB*lacZ*-infected animals is not due to direct toxicity of the virus because it was not present when UV-inactivated virus was introduced into these animals (data not shown). The *nu/nu* mice developed similar abnormalities in hepatocyte morphology 2 days after exposure to virus but they did not progress to develop lymphocytic infiltrates and hepatocyte regeneration. The level of hepatocyte regeneration was quantitated by labeling hepatocytes with the nucleoside analog BrdUrd whose incorporation into DNA can be detected by immunocytochemistry. On selected days animals were infused with a bolus of BrdUrd and sacrificed 4 h later for analysis. Less than 0.01% of nuclei were labeled in uninfected control animals, whereas at its peak (day 2 after instillation of virus), 7% of hepatocytes were labeled in *nu/nu* animals and 15–20% of hepatocytes were labeled from the CBA mice (Fig. 5). Nuclei labeling was primarily periportal and it quickly diminished to baseline levels in *nu/nu* animals, whereas, in CBA mice, the labeling was distributed throughout the hepatic lobule and it persisted at very high levels for several weeks (data not shown). The massive regeneration observed in CBA mice is consistent with that which would be required to repopulate this organ with nontransgene-containing cells.

Our data are consistent with at least two mechanisms of injury to the liver in the context of gene transfer with *E1*-deleted recombinant adenoviruses. One mechanism illustrated in the *nu/nu* mouse is characterized by low level and transient hepatocyte degeneration with compensatory regeneration in the areas of the liver in which expression of *lacZ* was the highest (i.e., periportal). These are areas of liver exposed to the highest MOI of virus consistent with direct toxicity of the virus as possibly mediating this effect; the development of pathology and regeneration within days after infusion of virus is consistent with this hypothesis. The major mechanism that underlies loss of recombinant gene expression with the development of pathology is a cellular immune response to *de novo*-synthesized viral antigens. This effect is independent of MOI (data not shown) and occurs several days later. Our findings are consistent with results of Ginsberg and coworkers (28, 29) who studied the mechanisms of human adenovirus pneumonia in cotton rat and mouse models. They describe a biphasic inflammatory response the latter of which is mediated by CTLs to viral antigens and is absent in *nu/nu* mice.

The hypothesis that emerges from this study suggests mechanisms to explain the major limitations of first generation adenoviruses. The data presented in this report are from mouse liver; however, similar results have been obtained in mouse and cotton rat lung, suggesting this may be a general paradigm (Y.Y., J.M.W., and J. Engelhardt, unpublished results). These studies provide a basis for rationally improving this technology. One approach would be to manipulate the immune system of the recipient to abrogate the cellular immune response. This could be accomplished by initially tolerizing the recipient to viral antigens or subjecting the recipient to chronic immunosuppression. A more attractive approach would be to further cripple the virus to diminish or ablate viral protein expression.

We thank Kim Alston for technical support and the Cell Morphology Core and Vector Core of the Institute for Human Gene Therapy. This work was supported by the Cystic Fibrosis Foundation and National Institutes of Health Grants P30 DK 47757 and RO1 HL 49040.

1. Graham, F. L., Smiley, J., Russell, W. L. & Nairn, R. (1977) *J. Gen. Virol.* **36**, 59–72.
2. Stratford-Perricaudet, L. D., Levrero, M., Chasse, J. F., Perricaudet, M. & Briand, P. (1990) *Hum. Gene Ther.* **1**, 241–256.
3. Rosenfeld, M. A., Siegfried, W., Yoshimura, K., Yoneyama, K., Fukayama, M., Stein, L. E., Pääkö, P. K., Gilardi, P., Stratford-Perricaudet, L. D., Perricaudet, M., Jalla, S., Pavirani, A., Lecocq, J.-P. & Crystal, R. G. (1991) *Science* **252**, 431–434.
4. Quantin, B., Perricaudet, L. D., Tajbakhsh, S. & Mandel, J.-L. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 2581–2584.
5. Lemarchand, P., Ari Jaffe, H., Danel, C., Cid, M. C., Kleinman, H. K., Stratford-Perricaudet, L. D., Perricaudet, M., Pavirani, A., Lecocq, J.-P. & Crystal, R. G. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 6482–6486.
6. Le Gal La Salle, G., Robert, J. J., Berrard, S., Ridoux, V., Stratford-Perricaudet, L. D., Perricaudet, M. & Mallet, J. (1993) *Science* **259**, 988–990.
7. Bennett, J., Wilson, J., Sun, D., Forbes, B. & Maguire, A. (1993) *Invest. Ophthalmol. Visual Sci.*, in press.
8. Roessler, B. J., Allen, E. D., Wilson, J. M., Hartman, J. W. & Davidson, B. L. (1993) *J. Clin. Invest.* **92**, 1085–1092.
9. Jaffe, H. A., Danel, C., Longenecker, G., Metzger, M., Setoguchi, Y., Rosenfeld, M. A., Gant, T. W., Thorgeirsson, S. S., Stratford-Perricaudet, L. D., Perricaudet, M., Pavirani, A., Lecocq, J.-P. & Crystal, R. G. (1992) *Nat. Genet.* **1**, 372–378.
10. Li, Q., Kay, M. A., Finegold, M., Stratford-Perricaudet, L. D. & Woo, S. L. C. (1993) *Hum. Gene Ther.* **4**, 403–409.
11. Ishibashi, S., Brown, M. S., Goldstein, J. L., Gerard, R. D., Hammer, R. E. & Herz, J. (1993) *J. Clin. Invest.* **92**, 883–893.
12. Engelhardt, J. F., Simon, R., Yang, Y., Zepeda, M., Weber-Pendelton, S., Doranz, B., Grossman, M. & Wilson, J. M., (1993) *Hum. Gene Ther.* **4**, 759–769.
13. Simon, R., Engelhardt, J. F., Yang, Y., Zepeda, M., Weber-Pendelton, S., Grossman, M. & Wilson, J. M. (1993) *Hum. Gene Ther.* **4**, 771–780.
14. Kozarsky, K., Grossman, M. & Wilson, J. M. (1993) *Somatic Cell Mol. Genet.* **19**, 449–458.
15. Berencsi, K., Rando, R. F., deTaisne, C., Paoletti, E., Plotkin, S. A. & Gonczol, E. (1994) *J. Gen. Virol.* **74**, in press.
16. Yang, Y., Raper, S. E., Cohn, J. A., Engelhardt, J. F. & Wilson, J. M. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 4601–4605.
17. Wilson, J. M., Johnston, D. E., Jefferson, D. M. & Mulligan, R. C. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 4421–4425.
18. Chomczynski, P. & Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159.
19. Yang, Y., Devor, D. C., Engelhardt, J. F., Ernst, S. A., Strong, T. V., Collins, F. S., Cohn, J. A., Frizzell, R. A. & Wilson, J. M. (1993) *Hum. Mol. Genet.* **2**, 1253–1261.
20. Reich, N. C., Sarnow, P., Durpey, E. & Levine, A. (1983) *Virology* **128**, 480–484.
21. Engelhardt, J. F., Yankaskas, J. R. & Wilson, J. M. (1992) *J. Clin. Invest.* **90**, 2598–2607.
22. Ragot, T., Vincent, N., Chafey, P., Vigne, E., Gilgenkrantz, H., Couton, D., Cartaud, J., Briand, P., Kaplan, J. C., Perricaudet, M. & Kahn, A. (1993) *Nature (London)* **361**, 647–650.
23. Engelhardt, J. F., Yang, Y., Stratford-Perricaudet, L. D., Allen, E. D., Kozarsky, K., Perricaudet, M., Yankaskas, J. R. & Wilson, J. M. (1993) *Nat. Genet.* **4**, 27–34.
24. Spergel, J. M., Hsu, W., Akira, S., Thimmappaya, B., Kishimoto, T. & Chen-Kiang, S. (1992) *J. Virol.* **66**, 1021–1030.
25. Imperiale, M. J., Kao, H.-T., Feldman, L. T., Nevins, J. R. & Strickland, S. (1984) *Mol. Cell. Biol.* **4**, 867–874.
26. Duncan, S. J., Gordon, F. C. A., Gregory, D. W., McPhie, J. L., Postlethwaite, R., White, R. & Willcox, H. N. A. (1978) *J. Gen. Virol.* **40**, 45–61.
27. Reichel, R., Kovesdi, I. & Nevins, J. R. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 387–390.
28. Ginsberg, H. S., Moldawer, L. L., Sehgal, P. B., Redington, M., Kilian, P. L., Chanock, R. M. & Prince, G. A. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 11651–11655.
29. Prince, G. A., Porter, D. D., Jenson, A. B., Horswood, R. L., Chanock, R. M. & Ginsberg, H. S. (1993) *J. Virol.* **67**, 101–111.