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Ablation of *E2A* in recombinant adenoviruses improves transgene persistence and decreases inflammatory response in mouse liver

(temperature sensitive)

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ABSTRACT **First-generation recombinant adenoviruses that lack *E1* sequences have shown tremendous promise in animal and human models of gene therapy. Important limitations of these vectors are that recombinant gene expression is transient and inflammation occurs at the site of gene transfer. Our hypothesis for generating vectors with increased persistence is that present recombinant adenoviruses express viral proteins that stimulate cellular immune responses leading to destruction of the infected cells and repopulation of the organ with non-transgene-containing cells. This model predicts that further crippling of the virus will improve persistence and diminish pathology. We describe in this report second-generation recombinant adenoviruses harboring a β-galactosidase-expressing transgene in which a temperature-sensitive mutation has been introduced into the *E2A* gene of an *E1*-deleted recombinant. At nonpermissive temperature, this virus fails to express late gene products, even when *E1* is expressed in trans. The biology of this recombinant was studied *in vivo* in the context of mouse liver, a setting that is permissive for adenovirus type 5 replication. Animals that received the second-generation virus expressed the transgene for at least 70 days, whereas expression of the first-generation virus was no longer than 14 days. In addition, the inflammatory response, as measured by infiltration of $CD8^+$ T cells, was blunted and delayed in livers infected with second-generation virus. These studies illustrate that modifications that disrupt structural protein expression in recombinant adenoviruses may be useful in enhancing their utility for gene therapy.**

Recombinant adenoviruses hold tremendous promise for gene therapy (1). First-generation recombinant viruses have been rendered replication defective by virtue of the deletion of *E1A* and *E1B* genes, which are thought to control virus replication and the expression of other early and late genes. Experiments with *E1*-deleted adenoviruses in animal models have demonstrated extremely high levels of recombinant gene expression in a variety of cellular targets (1). In most systems, however, expression is transient (i.e., <3 weeks), and the recipient develops inflammation in the organ expressing the transgene (2–5). These problems do not arise when the virus is administered to newborn animals or to immunoprivileged organs, suggesting that the immune response plays an important role (6, 7).

Recent studies in genetically defined strains of mice have begun to elucidate the mechanisms by which the immune system leads to limited persistence of transgene expression and pathology from current *E1*-deleted recombinant adenoviral vectors (4). From these studies in mouse liver, a hypothesis has emerged whereby viral proteins expressed from the *E1*-deleted adenoviral genome, when presented as foreign antigens, lead to the generation of cytotoxic T lymphocytes (CTL) that destroy the genetically modified cells. Replication of *E1*-deleted viruses and expression of viral proteins have been noted in several *in vitro* systems, consistent with the hypothesis that the first-generation recombinant adenoviruses are not completely replication incompetent (8, 9). These observations suggest potential alterations in vector design that might increase the stability of transgene expression by further crippling the ability of the virus to express viral proteins and hence decrease the extent of CTL-mediated rejection of virally infected cells.

This report describes the introduction of a temperature-sensitive (ts) mutation within the *E2A*-encoded DNA-binding protein (DBP) of current *E1*-deleted recombinant adenoviral vectors. We have evaluated the effect of this alteration on the stability of transgene expression and extent of elicited inflammatory response. Such alterations, which may increase the safety and efficacy of current adenoviral vectors, will have important implications for the use of these vectors for gene therapy.

MATERIALS AND METHODS

Construction and Propagation of ts Recombinant *lacZ* Vector. The *E1*-deleted recombinant adenoviral vector containing the *Escherichia coli lacZ* gene encoding β-galactosidase (designated Ad.CBlacZ) was generated as described (10). The *lacZ* transgene cassette, for which expression is driven by the cytomegalovirus (CMV) enhancer and β-actin gene promoter, has been inserted within the *E1*-deleted region. In addition, sequences from *E3B* (150 bp within the 14.6 kDa protein) have been deleted. The *E1*-deleted recombinant *lacZ* vector harboring a ts mutation in the *E2A* region (G-to-A conversion at base 1064 of the DPB cDNA, generating a proline-to-serine amino acid change) was generated in two steps. First, the previously isolated ts mutant strain Ad5.Hst125 (11) was cloned within the *E3*-deleted background of Ad5sub360 by ligation of the 5′ genomic viral *Eco*RI fragment of Ad5.Hst125 to the 3′ genomic viral *Eco*RI fragment from Ad5.sub360, followed by transfection into 293 cells. Viral isolates were purified by two rounds of plaque purification and designated Ad5.Hst125sub360. Recombinant ts *lacZ* virus was generated by homologous recombination in transfected 293 cells with *Cla* I-digested Ad5.Hst125sub360 genomic DNA and *Nhe* I-digested pE1CBlacZ plasmid DNA, which contains 360 bp of adenoviral sequence 5′ to the CMV enhancer/β-actin promoter/*lacZ* transgene cassette. The resultant adenoviral mutants Ad5.CBlacZHst125sub360 (referred to as Ad.ts125CBlacZ) were screened for their ability to express the *lacZ* transgene and cause a cytopathic effect (CPE) on 293 cells at 32°C but

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Abbreviations: DBP, DNA-binding protein; ts, temperature sensitive; CTL, cytotoxic T lymphocytes; pfu, plaque-forming units; CPE, cytopathic effect(s); moi, multiplicity of infection.

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not 39°C. Plaques that produced β-galactosidase and showed no evidence of growth on 293 cells at 39°C were purified through a second round and amplified for studies of gene transfer efficiency and stability.

Analysis of ts Growth *in Vitro* on 293 Cells. Ad.CBlacZ and Ad.ts125CBlacZ were analyzed for growth at 32°C, 35°C, 37°C, and 39°C on 293 cells. Subconfluent monolayers of 293 cells were infected with either Ad.CBlacZ or Ad.ts125CBlacZ at a multiplicity of infection (moi) of 0.5 plaque-forming unit (pfu) per cell for 4 hr in Dulbecco's modified Eagle's medium (DMEM) at the appropriate temperature. A moi of 0.5 pfu per cell was used because higher levels of infection (i.e., moi = >5 pfu per cell) caused CPE at 39°C in the absence of viral replication. After infection, the plates were washed three times with DMEM, refed with DMEM containing 10% (vol/vol) fetal calf serum, and allowed to incubate for an additional 4 days. Cells were harvested by scraping into incubation medium. The resultant cell suspension was frozen and thawed three times in a dry-ice/ethanol bath and centrifuged at 3000 × *g* for 20 min. This cell lysate was titered by limiting dilution for *lacZ* adenoviral particles by infecting plates of 293 cells and staining for β-galactosidase at 21 hr. The resultant titers are expressed as *lacZ*-forming units per ml.

Immunocytochemical Detection of Adenoviral Proteins and CD8 Antigen. The 293 cells were plated onto polylysine-coated glass slides, infected with either Ad.CBlacZ or Ad.ts125CBlacZ, and grown at 32°C and 39°C for 48 and 16 hr, respectively. Variation in the incubation time was necessary because of differences in the growth rates of adenovirus at 32°C and 39°C. Slides were harvested and fixed in methanol (−20°C) for 10 min, followed by air-drying. Cells were stained by indirect triple immunofluorescence for *lacZ*, DBP, and fiber as described (9). $CD8^+$ lymphocytic infiltrate was analyzed in 6-μm cryosections postfixed in −20°C methanol for 10 min followed by air-drying. Sections were blocked in phosphate-buffered saline (PBS) containing 20% (vol/vol) goat serum for 30 min, followed by incubation in a 1:10 dilution of rat anti-mouse CD8–fluorescein isothiocyanate (FITC) conjugate (BioSource International, Camarillo, CA) in PBS containing 1.5% goat serum for 90 min. $CD8^+$ lymphocytes were quantitated by counting the number of $CD8^+$ cells per unit area. Ten random fields (×20 magnification) from at least four sections were counted from each liver time point. Each time point represents the average ± SE from three independent animals for each experimental condition.

Histochemical Detection and Quantification of β-Galactosidase-Encoding Transgene Activity in Liver. Frozen sections (6 μm) were postfixed in glutaraldehyde and stained for 2 hr as described (4). Quantification of the percent $lacZ^+$ area was performed on a Lica DM100 morphometric analysis system by analyzing 10 fields from two to four sections from each liver. Each time point represents the average ± SE from three independent animals for each experimental condition. This analysis was not designed to differentiate the intensity of staining but rather the percentage of the liver that expressed transgene. Hence, the thresholds were set for the weakest staining intensity and held constant throughout the entire quantification analysis.

RESULTS

ts Mutation in *E2A* Causes a Block in Late Gene Expression at 39°C. A second-generation *lacZ* recombinant adenoviral vector was generated by the introduction of a single base-pair mutation in the *E2A* gene of a virus lacking *E1* and *E3*. This mutation renders the protein product of the *E2A* gene (DBP) sensitive to temperature. The new recombinant is called Ad.ts125CBlacZ, while the corresponding first-generation virus without the *ts* mutation is called Ad.CBlacZ. Initial studies characterized these matched *lacZ* recombinant adenoviruses for the effect of temperature on growth *in vitro*. These studies were performed in 293 cells, where high levels of *E1* are expressed in trans, in an attempt to simulate the *in vivo* situation where transcomplemented *E1* function may occur through cellular factors or other viruses. The mutation in *E2A* completely disabled the ability of the virus to replicate at 39°C and diminished detectable growth at intermediate temperatures of 35°C and 37°C (≈15% of growth achieved with Ad.CBlacZ) (Fig. 1). At the permissive temperature of 32°C, Ad.ts125CBlacZ growth was ≈50% of that of Ad.CBlacZ and allowed for production of sufficient quantities for *in vivo* experiments. These results predict that in the setting of extremely high levels of *E1* expression, the Ad.ts125CBlacZ would remain partially replicative at an *in vivo* temperature of 37°C.

In an attempt to define the mechanism by which Ad.ts125CBlacZ failed to replicate at 39°C, we sought to determine the relationship between expression of DBP and transition into the late phase of adenoviral replication. Experiments were performed that compared the level of expression of the transgene (i.e., *lacZ*), DBP, and a late gene product (fiber protein) by triple immunofluorescence within infected 293 cells. At 32°C equivalent levels of DBP and fiber were expressed in cells infected with either Ad.ts125CBlacZ or Ad.CBlacZ; however, at nonpermissive temperatures (39°C) the Ad.ts125CBlacZ vector failed to express late gene product, presumably because the defective DBP was incapable of activating the major late promoter (Fig. 2). With the demonstration of a functional defect in the transition from early to late gene expression within Ad.ts125CBlacZ, we sought to determine whether the introduction of the ts mutation would affect the persistence of transgene expression and the development of pathology *in vivo*.

ts Mutation in *E2A* Leads to Increased Persistence of Transgene Expression in Mouse Liver. We compared *lacZ* recombinant adenoviral vectors harboring wild-type and ts sequences in the *E2A* DBP for their efficiency of infection and persistence of transgene expression in mouse liver. Recombinant Ad.ts125CBlacZ or AdCBlacZ was injected into the tail veins of mice at a total dose of 1×10^9 pfu, and livers were harvested at 3, 7, 14, 21, and 28 days after infection for histologic analysis of transgene expression. Histochemical staining for β-galactosidase activity revealed approximately equivalent levels of expression at early time points of 3 and 7 days (Fig. 3). However, as expression rapidly diminished from 26 ± 3.5% at day 14 to undetectable levels by day 21 in the Ad.CBlacZ-infected animals, livers infected with Ad.ts125CBlacZ demonstrated persistence of β-galactosidase (10 ± 4%) to 28 days (Fig. 3). Transgene expression was quantitated by morpho-

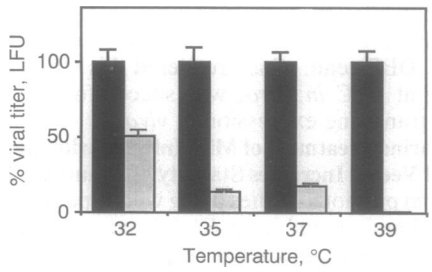

FIG. 1. Comparison of Ad.ts125CBlacZ and Ad.CBlacZ replication on 293 cells. Subconfluent monolayers of 293 cells were infected at a moi of 0.5 pfu per cell, incubated at the indicated temperature, and harvested by scraping at day 4. Cell lysates were titered for the number of *lacZ*-forming units (LFU) by infection on confluent monolayers of 293 cells and histochemically staining for *lacZ* product 21 hr after infection. Experiments were performed in triplicate, and the average (± SE) percent was normalized to Ad.CBlacZ as 100%. ■, Ad.CBlacZ; ▨, Ad.ts125CBlacZ.

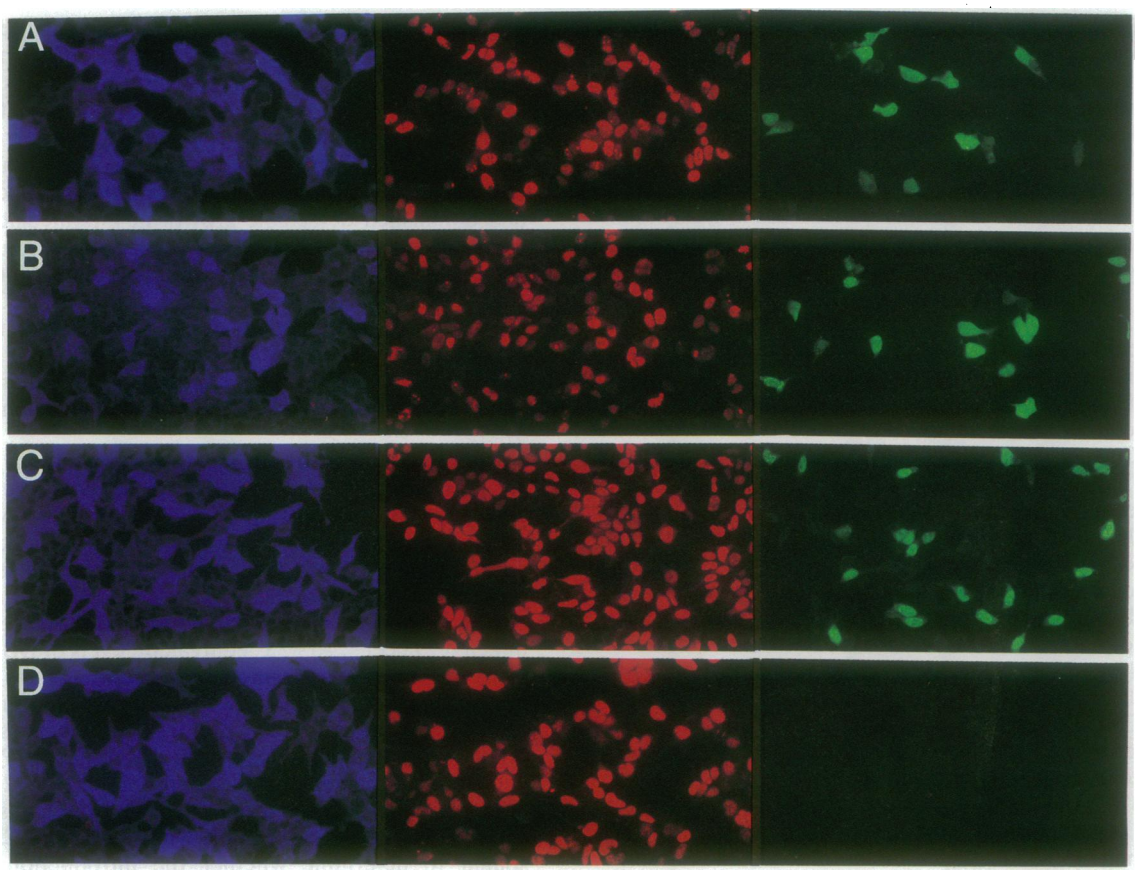

FIG. 2. Evaluation of DBP and fiber expression from recombinant Ad.ts125CBlacZ and Ad.CBlacZ vectors at 32°C and 39°C. Monolayers of 293 cells were infected with Ad.ts125CBlacZ and Ad.CBlacZ at a moi of 0.5 pfu per cell and incubated for 24 hr at 39°C and 48 hr at 32°C. Triple immunofluorescence shows immunoreactive β-galactosidase [AMCA (7-amino-4-methylcoumarin-3-acetic acid) in blue] (*Left*), DBP (Texas red) (*Center*), and fiber (fluorescein isothiocyanate in green) (*Right*). (*A*) Ad.CBlacZ at 32°C. (*B*) Ad.ts125CBlacZ at 32°C. (*C*) Ad.CBlacZ at 39°C. (*D*) Ad.ts125CBlacZ at 39°C.

metric analysis as described in *Materials and Methods* and is expressed as a percentage of $lacZ^+$ cellular area. Morphometric analysis of histochemically stained sections for the percent $lacZ^+$ area demonstrated a statistically significant ($P < 0.001$) higher level of transgene expression in livers infected with Ad.ts125CBlacZ as compared with Ad.CBlacZ at time points beyond 7 days. No transgene expression was ever seen in Ad.CBlacZ-infected livers beyond 14 days. Animals infected long-term with Ad.ts125CBlacZ showed stable transgene expression (≈10% of liver area) between 28 and 70 days (see Fig. 3 *E*, *K*, and *L*). By 100 days the level of expression from this vector was significantly reduced; of the five animals analyzed at 100 days, some animals showed no transgene expression, while others showed levels ranging between 0.1% and 1%. These results show that a single base-pair substitution in the *E2A* DBP gene, which rendered this virus incapable of replicating at 39°C *in vitro*, was successful in substantially extending transgene expression *in vivo*.

Cyclosporine Treatment of Mice Infected with the Nonmutant *E1*-Deleted Vector Increases Stability of Transgene Expression. Results from previous studies using wild-type adenovirus type 5 (Ad5) (12) and recombinant *E1*-deleted adenovirus (4) have suggested that CTL-mediated responses play an important role in the clearance of adenoviral-infected cells from the lung and liver. In these studies, pathology was prevented, and the viral genome persisted with little reduction when experiments were performed in athymic mice. These observations were extended in the current study by evaluating the effect of pharmacological inhibition of T-lymphocyte function with cyclosporine on the persistence of adenoviral transgene expression. Animals treated with daily injections of cyclosporine (15 mg/kg) were compared to untreated animals for the persistence of Ad.CBlacZ-mediated transgene expression at 7, 14, 21, and 28 days. Histochemically stained sections from these experiments (Fig. 4) showed that cyclosporine treatment was capable of extending *lacZ* transgene expression to 21 days as compared with 14 days in untreated animals. Morphometric analysis of the percent $lacZ^+$ area showed a statistically significant ($P < 0.001$) higher level of *lacZ* expression at 14 and 21 days in animals treated with cyclosporine as compared with untreated controls. The differences in expression of littermates infected with Ad.CBlacZ and Ad.CBlacZ (+ cyclosporine) were most notable at the 21-day time point; transgene expression was only retained in cyclosporine-treated animals at a level of 22 ± 4%. Only infrequent $lacZ^+$ cells (accounting for <0.1% of the total cellular area) were noted in 28-day animals treated with cyclosporine. While these studies confirm the importance of T-cell populations in mediating the diminution of transgene expression from first-generation viruses, they differ quantitatively from the results obtained with *nu/nu* mice, where transgene expression persists for at least 6 months. This may be due to incomplete ablation of CTL function with cyclosporine under the conditions of this experiment or the absence of other relevant immunologic functions in the athymic mouse model that are not inhibited by cyclosporine.

Both Cyclosporine Addition and ts *E2A* Mutation Act by Similar Mechanisms to Increase the Stability of Transgene Expression. Previous studies in immunocompetent mice with first-generation adenoviruses described the development of a lymphocytic infiltrative hepatitis at the time transgene expression is diminishing (4). Our hypothesis would predict that this inflammatory response is blunted and/or delayed in animals infected with the second-generation virus because the stimulus

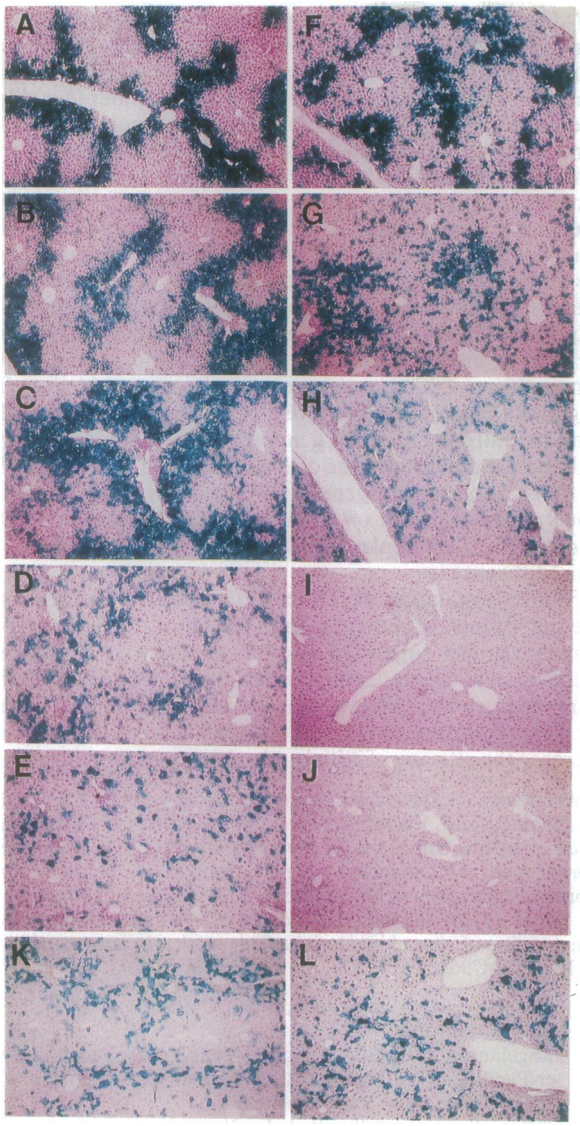

FIG. 3. Evaluation of *lacZ* transgene expression in sections from livers infected with Ad.ts125CBlacZ (*A–E*, *K*, *L*) and Ad.CBlacZ (*F–J*). Mice were injected through the tail vein with 1×10^9 pfu of each Ad.ts125CBlacZ and Ad.CBlacZ in 100 μl of PBS. Livers were harvested on day 3 (*A* and *F*), 7 (*B* and *G*), 14 (*C* and *H*), 21 (*D* and *I*), 28 (*E* and *J*), 36 (*K*), and 70 (*L*). Frozen sections (6 μm) were histochemically stained in 5-bromo-4-chloro-3-indolyl β-D-galactoside and counterstained briefly in hematoxylin.

for CTL response (i.e., viral protein expression) is diminished. In fact, immunocytochemical detection of the late adenoviral protein hexon in livers infected with Ad.ts125CBlacZ demonstrated a lower frequency of positive cells as compared with AdCBlacZ infection (data not shown). To evaluate this hypothesis, the kinetics, magnitude, and nature of the inflammatory infiltrate were characterized by immunocytochemical analysis of liver for CD8-reactive T cells.

Livers from animals infected with Ad.CBlacZ, Ad.CBlacZ (+ cyclosporine), and Ad.ts125CBlacZ were evaluated for $CD8^+$ inflammatory infiltrate by immunocytochemical detection; data are summarized in Fig. 5. A representative field showing the lack of CD8 expression 3 days after infection (Fig. 5*A*) is contrasted to that seen in an average field 7 days after infection with Ad.CBlacZ (Fig. 5*B*) and Ad.ts125CBlacZ (Fig. 5*C*). Morphometric analysis of $CD8^+$ cells demonstrated a >200-fold increase in the number of $CD8^+$ cells between 3 and 7 days in livers infected with Ad.CBlacZ virus. This response significantly decreased between 14 and 21 days in agreement

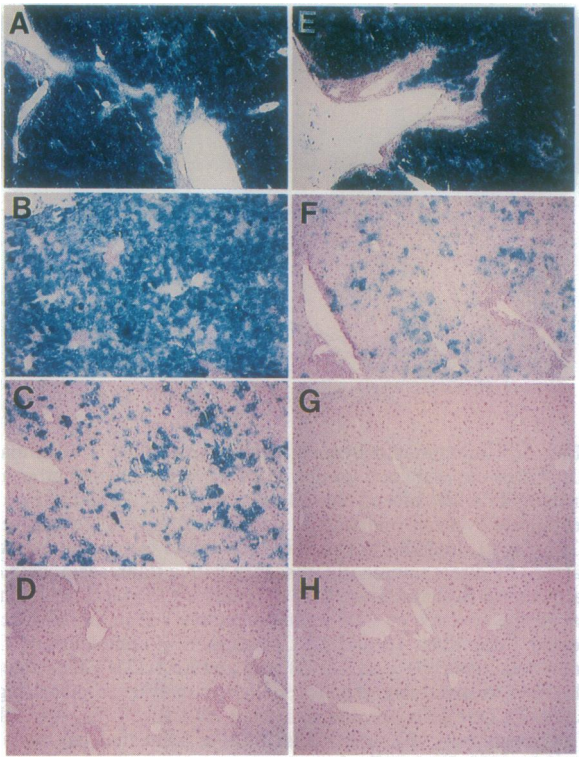

FIG. 4. Effect of cyclosporine on the persistence of Ad.CBlacZ transgene expression in mouse liver. Mice were injected through the tail vein with 1×10^9 pfu of Ad.CBlacZ in 100 μl of PBS. One set of mice was treated with daily injections of cyclosporine. Livers were harvested on days 7 (*A* and *E*), 14 (*B* and *F*), 21 (*C* and *G*), and 28 (*D* and *H*). Frozen sections (6 μm) were histochemically stained in 5-bromo-4-chloro-3-indoyl β-D-galactoside and counterstained briefly in hematoxylin. Sections from livers infected with Ad.CBlacZ (+ cyclosporine) (*A–D*) and Ad.CBlacZ alone (*E–H*) are shown.

with the diminution in transgene expression. Analysis of the $CD8^+$ cellular infiltrate in livers infected with Ad.ts125CBlacZ and Ad.CBlacZ (+ cyclosporine) showed a significantly lower response (reduction by a factor as high as 7; $P < 0.001$) at 7 and 14 days as compared with that seen with Ad.CBlacZ (compare Fig. 5 *B* and *C*). By day 28 Ad.ts125CBlacZ and Ad.CBlacZ (+ cyclosporine) infected livers were indistinguishable from those infected with Ad.CBlacZ as to the level of $CD8^+$ infiltrate. The time course of $CD8^+$ cellular infiltration was also significantly different within Ad.CBlacZ-infected livers, peaking on day 7 as compared with day 21 in Ad.CBlacZ (+ cyclosporin)- and Ad.ts125CBlacZ-infected livers (Fig. 5*D*). The time course of $CD8^+$ T-cell response after infection of mouse liver with Ad.CBlacZ was similar to that previously described for wild-type Ad5 in cotton rat lung (12). These results show that introduction of the ts mutation in Ad.ts125CBlacZ both blunts and reduces the host $CD8^+$ T-cell response in a similar manner to that seen by a known inhibitor of CTL ($CD8^+$) replication. Furthermore, this inflammatory response occurs at the time that transgene expression is diminishing.

DISCUSSION

Current *E1*-deleted recombinant adenoviruses have shown tremendous promise for the treatment of inherited and acquired diseases by gene therapy. However, the application of these vectors has been limited by their lack of persistence and associated host inflammatory response. Importantly, studies targeting *E1*-deleted recombinant adenoviruses to the liver of immunocompetent and athymic mice have demonstrated that a cellular immune response to virally infected cells may contribute to problems of instability of transgene expression

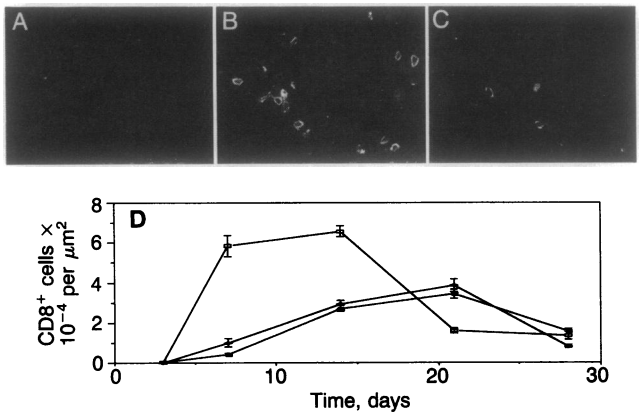

FIG. 5. Immunocytochemical evaluation of CD8+ inflammatory cells. Three experimental groups of mice were evaluated after injection with 1×10^9 pfu of Ad.CBlacZ, Ad.CBlacZ treated by daily injections of cyclosporine, or Ad.ts125CBlacZ. Livers were harvested at 3, 7, 14, 21, and 28 days after infection, and frozen sections were analyzed by direct immunofluorescence with a fluorescein isothiocyanate-labeled rat anti-mouse CD8 antibody. (*A*) Typical field demonstrating the absence of CD8 immunoreactive cell in an Ad.CBlacZ-infected 3-day liver. (*B* and *C*) Comparison of abundance of CD8-immunoreactive cells in an Ad.CBlacZ- and Ad.ts125CBlacZ-infected 7-day liver, respectively. (*D*) Morphometric analyses summarizing the results of CD8+ cells per unit area from Ad.CBlacZ (□), Ad.CBlacZ (+ cyclosporine)- (◆), and Ad.ts125CBlacZ-infected (■) livers are shown in graph format. Each time point represents the average ± SE of three independent mice.

and inflammation (4). These results confirm that current *E1*-deleted vectors are only partially defective in their ability to express viral genes. Such studies have laid the ground work for the development of second-generation adenoviral vectors that have reduced capacity to express viral genes.

Based on the current hypothesis that the CTL inflammatory response was in part responsible for the rejection of virally infected cells, we propose two mechanisms by which viral protein expression induced by host cell factors (13, 14) could lead to the recognition and rejection of virally infected cells through major histocompatibility complex presentation: (*i*) viral replication and generation of intact viral particles or (*ii*) viral protein expression from early and/or late genes in the absence of viral replication. Hence, we constructed *E1*-deleted recombinant adenoviral vectors that harbored a ts mutation within the *E2A* DBP gene in an attempt to decrease both the extent of viral replication and the level of late viral gene expression. We reasoned that a mutation that decreases the virus' ability to activate late gene expression might blunt the CTL response and lead to increased persistence of transgene expression. The generation of two identical LacZ recombinant vectors that differed only by a single base-pair substitution in the *E2A* DBP gene has allowed us to generate a recombinant vector that incorporates all of these criteria, including (*i*) an inability to replicate at 39°C in 293 cells because of a block in late gene expression, (*ii*) an increased stability of transgene expression in mouse liver, and (*iii*) a decreased CD8+ (most likely CTL) inflammatory response in mouse liver.

The successful construction of recombinant adenoviral vectors harboring the ts mutation within the DBP *E2A* gene has allowed us to evaluate methods of increasing stability of transgene expression by reducing the ability of recombinant adenoviral vectors to promote a CTL response that leads to cellular-mediated clearance of infected cells. Results of our studies in mouse liver are as predicted; the ts mutation in the *E1*-deleted virus leads to prolonged gene expression and diminished CD8+ lymphocyte infiltration. This effect represented only a partial improvement in that approximately one-fifth of the $lacZ^+$ cells at day 3 were rendered stable by the introduction of the ts mutation into recombinant virus. One explanation for these results includes the possibility of a second mechanism, such as adenoviral genome instability, which could also effect transgene expression independent of the immune response. Results in athymic mice, in which transgene expression from first-generation viruses has persisted 6 months with little diminution, argue against this alternative mechanism of genome instability. A more likely explanation is that the block in late gene expression conferred by the ts mutation in DBP is incomplete at the *in vivo* temperature of 37°C, an observation borne out *in vitro*. This suggests that a more complete block due to a deletion of *E2A* may be more useful. The generation of such a recombinant will require new packaging cells that provide both *E1* and *E2A* in trans. Such second-generation vectors will have a tremendous positive impact on the use of recombinant adenoviral vectors for gene replacement therapy of inherited and acquired diseases.

Studies evaluating the effect of cyclosporine treatment on the persistence of recombinant adenoviral transgene expression have also suggested additional potential strategies to increase persistence through direct manipulation of the host immune response. A better understanding of the mechanisms by which tolerance may be induced to adenoviral vectors may increase the efficacy of initial and subsequent repetitive doses.

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1. Kozarsky, K. F. & Wilson, J. M. (1993) *Curr. Opin. Genet. Dev.* **3**, 499–503.
2. Engelhardt, J. F., Simon, R. H., Yang, Y., Zepeda, M., Weber-Pendleton, S., Doranz, B., Grossman, M. & Wilson, J. M. (1994) *Hum. Gene Ther.*, in press.
3. Simon, R. H., Engelhardt, J. F., Yang, Y., Zepeda, M., Weber-Pendleton, S., Grossman, M. & Wilson, J. M. (1994) *Hum. Gene. Ther.*, in press.
4. Yang, Y., Nunes, F. A., Berencsi, K., Furth, E. E., Gonczol, E. & Wilson, J. M. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 4407–4411.
5. Herz, J. & Gerard, R. D. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 2812–2816.
6. Bennett, J., Wilson, J., Sun, D., Forbes, B. & Maquire, A. (1994) *Invest. Ophthalmol. Visual Sci.*, in press.
7. Davidson, B. L., Allen, E. D., Kozarsky, K. F., Wilson, J. M. & Roessler, B. J. (1992) *Nature Genet.* **3**, 219–223.
8. Rich, D. P., Couture, L. A., Cardoza, L. M., Guiggio, V. M., Armentano, D., Espino, P. C., Hehir, K., Welsh, M. J., Smith, A. E. & Gregory, R. J. (1993) *Hum. Gene Ther.* **4**, 461–476.
9. Engelhardt, J. F., Yang, Y., Stratford-Perricaudet, L. D., Allen, E. D., Kozarsky, K., Perricaudet, M., Yankaskas, J. R. & Wilson, J. M. (1993) *Nature Genet.* **4**, 27–34.
10. Kozarsky, K., Grossman, M. & Wilson, J. M. (1993) *Somatic Cell Mol. Genet.* **19**, 449–458.
11. Ensinger, M. J. & Ginsberg, H. S. (1972) *J. Virol.* **10**, 328–339.
12. 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**, 1651–1655.
13. Spergel, J. M., Hsu, W., Akira, S., Thimmappaya, B., Kishimoto, T. & Chen-Kiang, S. (1992) *J. Virol.* **66**, 1021–1030.
14. Imperiale, M. J., Ikao, H.-T., Feldman, L. T., Nevins, J. R. & Strickland, S. (1984) *Mol. Cell. Biol.* **4**, 867–874.