JOURNAL OF VIROLOGY, July 1997, p. 5189–5196 Vol. 71, No. 7
0022-538X/97/$04.00+0

Adenovirus Vector-Infected Cells Can Escape Adenovirus Antigen-Specific Cytotoxic T-Lymphocyte Killing In Vivo

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Received 20 December 1996/Accepted 24 March 1997

The recent findings that prolonged expression of certain adenovirus (Ad) vector-encoded proteins, including human α_1-antitrypsin (huAAT), mouse erythropoietin (EPO), and human factor IX, can be achieved in animals that do not mount an immune response to the reporter protein were obtained with mouse strains which have been shown to be capable of mounting a cellular immune response against Ad vector antigens. This suggests either that Ad vectors expressing nonimmunogenic transgenes fail to elicit a cellular immune response or that an Ad-specific cellular immune response does develop but is ineffective against cells expressing nonimmunogenic transgenes. Here we demonstrate that an Ad vector expressing huAAT administered by intravenous injection does stimulate an Ad-specific cellular immune response but that this response fails to abolish vector-directed gene expression in vivo. Moreover, expression of huAAT remained stable in animals stimulated by concurrent and multiple administrations of different Ad vectors or viruses. We also demonstrate prolonged expression of huAAT in CD1 mice transgenic for the huAAT gene, indicating that long-term expression is not restricted to C57BL/6 mice. These results demonstrate that under some circumstances, an Ad vector can direct prolonged expression of a nonimmunogenic transgene despite the presence of a robust Ad-specific cellular immune response.

Recombinant adenovirus (Ad) vectors have many advantages for human gene therapy, but immune responses may present a barrier to their efficacy. Early studies used vectors in which the E1 region was replaced by the transgene expression cassette, the E3 region was in some cases fully or partially deleted, and the remainder of the vector genome was wild type. In vivo administration of such vectors, generally referred to as first generation, resulted in gene transfer that was robust initially but declined rapidly to near background levels (8, 12, 15, 18). Ad-specific cytotoxic T lymphocytes (CTL) developed in parallel with declining expression (22). These results led to the hypothesis that low levels of Ad-encoded proteins expressed in vector-infected cells induced Ad-specific CTL which were responsible, at least in part, for lysis of cells expressing vector-encoded reporter proteins and that this response would limit intrinsically the utility of Ad vectors for gene therapy (23, 24). Interpretation of the results of these studies was complicated, however, by the use of Ad vectors encoding reporter gene products such as *Escherichia coli* β-galactosidase that were immunogenic and capable of stimulating a vigorous CTL response. Thus, the relative contribution of Ad antigens to the stimulation of a CTL response and to subsequent CTL killing was unclear.

More recent studies have shown that long-term Ad vector-directed expression of alternative transgenes such as human α_1-antitrypsin (huAAT) (2) and murine erythropoietin (EPO) (20) can be achieved in certain mouse strains. Such prolonged expression appears to be correlated with the absence of antibody responses to the transgene product (14, 20). These results are not consistent with the postulate that the longevity of Ad vector-mediated gene expression is inherently limited by Ad-specific CTL responses.

A key question that has not been addressed in any of the previous studies is whether first-generation Ad vectors encoding weakly immunogenic or nonimmunogenic transgene proteins can persist because they are ineffective in stimulating Ad-specific CTL and/or because they are poor targets for Ad-specific CTL in vivo. To investigate this question, we examined the Ad-specific CTL response to a first-generation vector expressing huAAT as the reporter gene product. We found that intravenous (i.v.) administration of an Ad vector encoding huAAT can direct transgene expression for long periods in C57BL/6 mice despite the presence of circulating CTL specific for Ad antigens. Even when Ad-specific CTL levels were boosted by various means, there was no detectable impact on the persistence of AdhuAAT expression. Thus, expression of Ad vector antigens appears to be insufficient, at least in some circumstances, to target infected cells for CTL lysis.

MATERIALS AND METHODS

Animals and cell lines. C57BL/6 mice were obtained from Taconic (Germantown, N.Y.) and were used at 8 to 12 weeks of age. Simian virus 40-transformed fibroblasts derived from C57BL/6 mice have been described elsewhere (6, 7, 16). Vector was administered to mice intranasally in a 50- to 100-μl volume or by tail vein injection in a volume of approximately 150 μl. Mice transgenic for huAAT were derived from the CD1 mice, an outbred mouse strain obtained from Charles River Laboratories. In these animals, the huAAT cDNA is controlled by the rabbit whey acidic promoter (5, 19, 20a).

Ad vectors. The Ad vectors used in this study are based on the Ad5 serotype. In these vectors, the E1 region of Ad5 has been replaced with a gene expression cassette encoding either huAAT (AdhuAAT) or *E. coli* β-galactosidase (Adβgal) (25). The E3 and E4 regions of these vectors are wild type (WT). All vectors were propagated in 293 cells and purified by CsCl centrifugation as previously described (17). The Ad2Δ2.9 and Ad5Δ2.9 viruses used to infect fibroblasts consist of the full-length Ad2 or Ad5 genomes from which the E3 coding sequences have been removed.

Quantitation of transgene expression. For quantitation of βgal expression, lung samples from individual animals were homogenized and βgal activity in the homogenate was assessed with a commercially available assay kit as specified by the manufacturer (Galactolight Kit; Tropix, Bedford, Mass.). The protein concentration of lung homogenates was determined with the DC reagent (Bio-Rad, Hercules, Calif.), and the results are expressed as relative light units (RLU) per microgram of protein.

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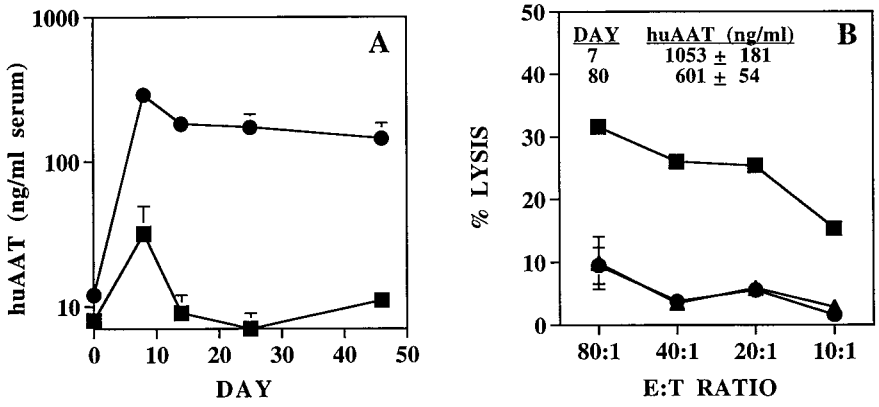

FIG. 1. Expression of huAAT and CTL response following administration of AdhuAAT. (A) CD1 mice transgenic for huAAT (●) and the nontransgenic parental CD1 strain (■) were given an i.v. injection of AdhuAAT vector (2×10^{10} IU). Serial serum samples were collected, and levels of huAAT were measured by an enzyme-linked immunosorbent assay as described in Materials and Methods. (B) C57BL/6 mice were given an i.v. injection (2×10^{10} IU) of AdhuAAT vector. (Inset) Levels of huAAT in serum were measured on days 7 and 80. On day 80, the animals were sacrificed and their spleen cells were restimulated in vitro with syngeneic fibroblasts infected with Ad2Δ2.9 to expand virus-specific CTLs. The E3$^-$ Ad2Δ2.9 virus was used to avoid interference of E3 gp19K with antigen presentation through MHC class I. Cultured effector cells were then tested for cytolytic activity against noninfected target fibroblasts (●) or target fibroblasts infected with either AdhuAAT (▲) or Ad2Δ2.9 (■). Results shown are the mean percent lysis and standard error of the mean (SEM) obtained from triplicate wells at the indicated effector/target (E:T) ratios.

The amount of huAAT present in mouse serum was evaluated by an enzyme-linked immunosorbent assay. The wells of a 96-well plate were coated with goat anti-huAAT (INCSTAR/Atlantic Antibodies, Stillwater, Minn.) to capture huAAT present in mouse serum samples. Antibody-bound huAAT was then detected by the addition of horseradish peroxidase (HRP)-conjugated goat anti-huAAT (Cappel, Durham, N.C.) with 3,3′,5,5′-tetramethyl benzidine dihydrochloride (TMBD) as the substrate for the HRP enzyme. For quantitation, a standard curve was constructed by adding known amounts of huAAT (INCSTAR/Atlantic Antibodies) to the goat anti-huAAT-coated plates. The optical densities at 490 nm obtained following the addition of HRP-conjugated goat anti-huAAT were plotted against the amounts of huAAT standard added to the wells. The concentrations of huAAT present in mouse serum samples were then derived from the standard curve by linear regression analysis. The limit of detection of the assay was approximately 10 ng of huAAT/ml of serum.

CTL assay. To evaluate CTL activity, spleen cells from animals in the same group (three or four mice/group) were pooled and stimulated in vitro with mitomycin-inactivated, infected syngeneic fibroblasts. The cells were cultured in 24-well plates containing 5×10^6 spleen cells and 6×10^4 stimulator fibroblasts per well in a 2-ml volume. The culture medium consisted of RPMI 1640 medium (Gibco BRL, Grand Island, N.Y.) supplemented with 100 U of penicillin per ml, 100 μg of streptomycin per ml, 2 mM glutamine, 5×10^{-5} M 2-mercaptoethanol, 20 mM HEPES buffer, and 10% heat-inactivated fetal calf serum (HyClone Laboratories, Inc., Logan, Utah). Cytolytic activity was assayed after 6 days of culture. Target fibroblasts were infected at a multiplicity of infection of 100 for 48 h and were treated with 100 U of recombinant mouse gamma interferon (Genzyme, Cambridge, Mass.) per ml for the last 24 h to enhance major histocompatibility complex (MHC) class I expression and antigen presentation to effector CTLs. The fibroblasts were labeled with ^{51}Cr (New England Nuclear, Boston, Mass.) overnight (50 μCi/10^5 cells) and added to the wells of a round-bottom 96-well plate in a 100-μl volume (5×10^3 fibroblasts/well). Effector cells were added in a 100-μl volume at various effector/target cell ratios in triplicate. After 5 h of incubation at 37°C under 5% CO_2, 100 μl of cell-free supernatant was collected from each well and counted in a Microbeta Trilux scintillation counter (Wallac Inc., Gaithersburg, Md.). The amount of ^{51}Cr spontaneously released was obtained by incubating target fibroblasts in medium alone, and the total amount of ^{51}Cr incorporated was determined by adding 1% Triton X-100 in distilled water. The percent lysis was calculated as follows: percent lysis = [(sample cpm − spontaneous cpm)/(total cpm − spontaneous cpm)] × 100.

RESULTS

Expression of AdhuAAT persists despite the presence of Ad-specific CTL. Undiminished levels of huAAT expression from a first-generation Ad vector in the C57BL/6 mouse strain have been reported by Barr et al. (2), although expression of huAAT from the same vector in C3H and BALB/c mice declined rapidly. The vector used by Barr et al. (2) was an Ad5-based E1 replacement vector, contained the Rous sarcoma virus promoter to control expression of huAAT, and had a small deletion within E3b. The E2 and E4 regions were WT. huAAT was chosen as the reporter gene product in these studies (2) because it generally does not produce an immune response in C57BL/6 mice (14), although a small proportion of animals do produce huAAT-specific antibodies (data not shown).

To test whether long-term expression of huAAT from a first-generation Ad vector was a peculiarity limited to the C57BL/6 mouse strain, we used an outbred mouse strain, CD1, that was transgenic for huAAT with the transgene under the direction of the whey acidic protein promoter (5, 19). The vector used in this experiment, AdhuAAT, and in the remainder of the present study is based on Ad5, uses the PGK promoter to control the expression of huAAT and is otherwise WT (25). AdhuAAT (2×10^{10} IU) was administered i.v. to CD1 mice or to CD1-huAAT transgenic mice, and huAAT expression in the serum was measured over time (Fig. 1A). Expression of huAAT peaked in both groups of animals on day 7, declined slightly in the huAAT transgenic animals, but then was maintained at steady levels throughout the remainder of the experiment. As expected, no serum antibodies reactive with huAAT were observed in the transgenic, and therefore tolerant, animals. In contrast, huAAT expression declined rapidly to background levels in the control CD1 mice and was accompanied by a sharp increase in the number of serum antibodies reactive with huAAT (antibody titer, >1/50,000). Thus, long-term expression of huAAT from a first-generation Ad vector is not limited to the C57BL/6 mouse strain or to the H2b haplotype.

A cellular immune response to Ad vector antigens in the C57BL/6 mouse strain has been documented in several studies with a spectrum of different vectors (9, 23). Such a cellular immune response might be predicted to destroy Ad vector-expressing cells in the animal irrespective of an immune response to the transgene. To investigate this apparent paradox, AdhuAAT (2×10^{10} IU) was administered to C57BL/6 mice i.v. and the Ad-specific CTL response and huAAT expression were measured. Consistent with previous studies in which similar vectors were used, huAAT was detected in the serum of these animals 80 days postadministration at approximately 60% of the day 7 levels (Fig. 1B, inset). Because the half-life of

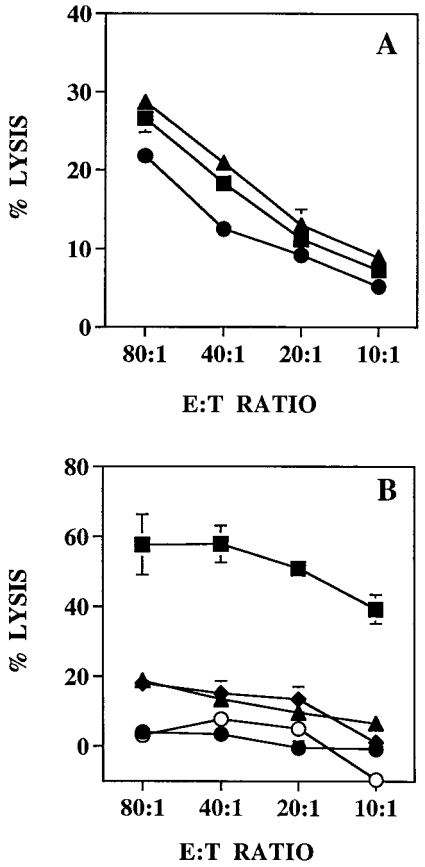

FIG. 2. (A) ConA-redirected lysis. To assess susceptibility to lysis independent of antigen recognition, Ad-specific effector cells were incubated with target fibroblasts in the presence of 5 μg of ConA per ml. The targets consisted of syngeneic fibroblasts either uninfected (●) or infected with Ad5huAAT (■) or Ad5βgal (▲). Results shown are the mean percent lysis and SEM from triplicate wells. (B) Protective effect of $E3^+$ vectors against lysis by Ad-specific CTL. To investigate the basis of the observed resistance of AdhuAAT-infected targets against CTL lysis, Ad-specific effector CTL were tested against syngeneic fibroblasts that were either uninfected (●), infected with Ad2Δ2.9 (■) or AdhuAAT (▲), or coinfected with both Ad2Δ2.9 and either AdhuAAT (○) or Ad5βgal (◆). The AdhuAAT and Ad5βgal vectors are both $E3^+$.

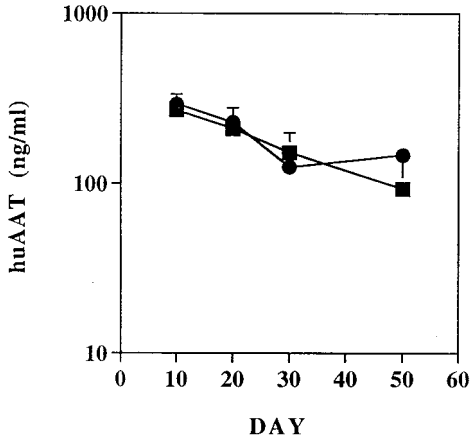

FIG. 3. Effect of concurrent administration of Adβgal on AdhuAAT expression. C57BL/6 mice were given AdhuAAT vector alone (2×10^{10} IU) by tail vein injection (●) or were given AdhuAAT vector (2×10^{10} IU) by tail vein injection and concurrently Adβgal (3×10^9 IU) by the i.n. route (■). Levels of huAAT were measured in serum on the indicated days.

AAT in the bloodstream has been reported to be approximately 4 to 5 days (3), we conclude that the continued presence of huAAT within the serum reflects continued expression of huAAT from the Ad vector.

At the time of sacrifice on day 80, spleen cells were harvested, restimulated in vitro with Ad-infected syngeneic fibroblasts, and tested for lytic activity against Ad-infected target cells (Fig. 1B). These effector cells were capable of lysing Ad-infected target cells, confirming that Ad vector administration to C57BL/6 mice does stimulate an Ad-specific CTL response. However, the same population of Ad-specific CTL did not have detectable lytic activity against syngeneic target cells infected with AdhuAAT (Fig. 1B).

The poor lysis of AdhuAAT-infected targets by Ad-specific CTL raised the concern that the antiprotease activity of huAAT expressed in target cells might inhibit CTL-derived proteases and thus artifactually mask the lytic activity of Ad-specific CTL. To test this possibility, a concanavalin A (ConA)-mediated redirected lysis experiment was carried out with Ad-specific CTL. In this method, specific antigen recognition is bypassed and adhesion between CTL and target cells is mediated by the lectin ConA rather than by T-cell receptor–MHC interactions. Under these conditions, similar levels of lysis were observed with uninfected target cells and with target cells infected with AdhuAAT or Adβgal (Fig. 2A) indicating that huAAT expression within the target cell in itself did not offer protection against CTL killing.

Since the AdhuAAT vector has an intact E3 region, we tested whether resistance of AdhuAAT-infected cells to CTL killing in vitro could be attributed at least in part to gp19K expression. It has been established that expression of the Ad E3 gene product, gp19K, can inhibit CTL killing in vitro by trapping nascent MHC class I molecules of certain haplotypes within the endoplasmic reticulum and inhibiting antigen presentation (21). Ad-specific CTL were tested for lytic activity against syngeneic target cells infected with E3-deleted Ad alone (Ad2Δ2.9) or coinfected with either AdhuAAT or Adβgal, which are both WT for the E3 region. Cotransfection of cells with Ad2Δ2.9 and either of the $E3^+$ vectors imparted resistance to Ad-specific CTL lysis (Fig. 2B), suggesting that the E3 region from these vectors has a strong suppressive effect on CTL killing, at least in vitro.

Concurrent administration of AdβGal vector in *trans* does not limit AdhuAAT expression. The above results demonstrated that Ad-specific CTL were present in the animals but that they failed to interfere with the expression of huAAT. It was possible that a single administration of AdhuAAT vector did not stimulate sufficient levels of Ad-specific CTL to eliminate vector-expressing cells in vivo. One method to increase the levels of Ad-specific CTL is adoptive transfer of spleen cells from animals immunized with the Ad vector. This procedure has been used to demonstrate successfully that Ad-specific CTL raised in an animal expressing a highly immunogenic transgene can lyse Ad vector-infected cells in the lungs and liver (9, 23). However, a potential complication of adoptive transfer protocols is that they may not reflect accurately either the conditions that develop over time in an intact animal during the process of antigen stimulation or the number of antigen-specific CTL that would be present in vector-treated animals.

To address more directly the in vivo effects of Ad-specific CTL, concurrent administration of Adβgal and AdhuAAT vectors to C57BL/6 mice was used as a means to increase the

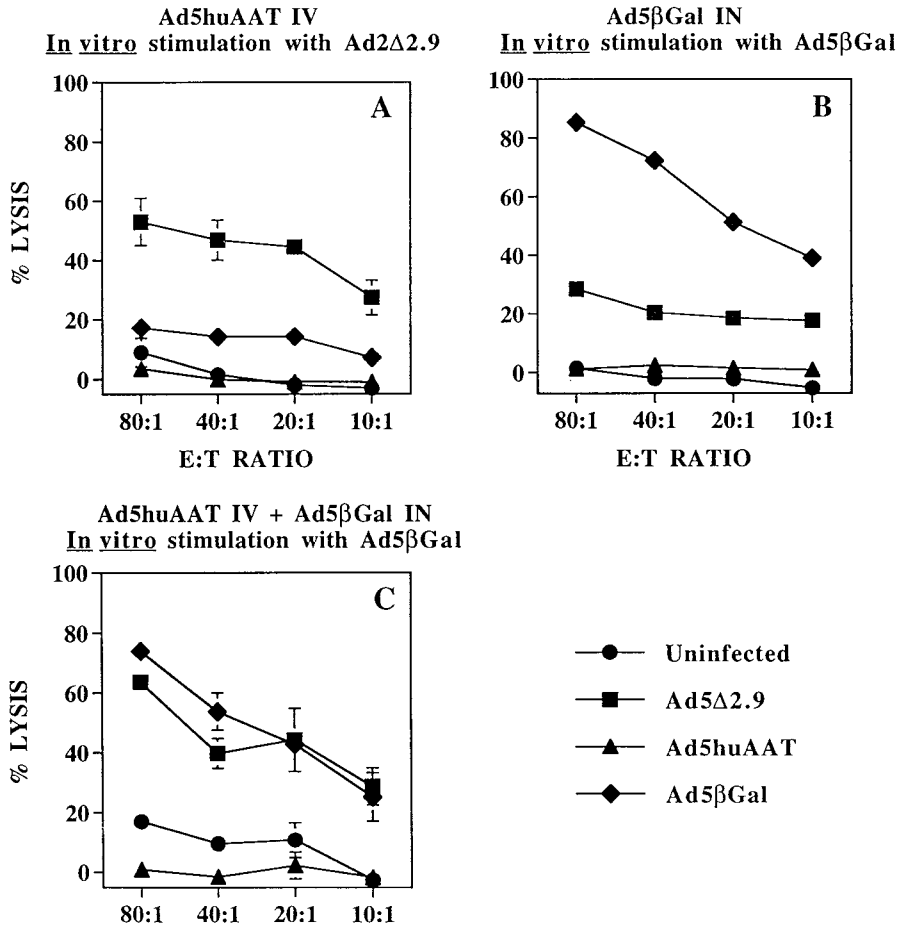

FIG. 4. CTL response in C57BL/6 mice following i.v. administration of AdhuAAT alone or concurrent administration of AdhuAAT (i.v.) and Adβgal (i.n.). Animals from the experimental groups in Fig. 3 were sacrificed on day 50, and their spleen cells were restimulated in vitro with syngeneic fibroblasts infected with Ad2Δ2.9 or with Adβgal to expand respectively virus-specific CTL or CTL specific for Adβgal. Cultured effector cells were then tested for cytolytic activity against noninfected target fibroblasts or target fibroblasts infected with either AdhuAAT, Adβgal, or Ad2Δ2.9. Results shown are the mean percent lysis and SEM obtained from triplicate wells at the indicated effector/target (E:T) ratios.

antigenic load and to boost the Ad-specific CTL response. To assess the impact of Ad-specific CTL independent of βgal-specific CTL and to avoid the complication of doubly infected cells, it was necessary to administer the AdhuAAT and Adβgal vectors by different routes. AdhuAAT (2×10^{10} IU) was administered i.v. either alone or in parallel with Adβgal (3×10^{9} IU) by the intranasal (i.n.) route. i.n. administration of this type of Adβgal vector is known to result in a strong CTL response to Ad antigens and to the βgal protein (9). As shown in Fig. 3, however, huAAT levels in the serum of animals treated with Adβgal in parallel with AdhuAAT were indistinguishable from those in animals treated with AdhuAAT alone, implying that huAAT vector-transduced cells were maintained.

Spleen cells harvested from these animals on day 51 were restimulated in vitro with syngeneic fibroblasts infected with different Ads and tested for CTL activity against a panel of Ad-infected target cells. CTL from animals treated with AdhuAAT i.v. alone and restimulated in vitro with Ad-infected fibroblasts had lytic activity against Ad-infected target cells, indicating that this vector is fully capable of stimulating a CTL response against Ad antigens (Fig. 4A). CTL from animals treated with Adβgal i.n. alone and restimulated in vitro with Adβgal-infected cells had lytic activity for Ad-infected and Adβgal-infected target cells consistent with the development of a CTL response against Ad antigens as well as the βgal gene product (Fig. 4B). Similarly, CTL from animals treated with both Adβgal and AdhuAAT and stimulated in vitro with Adβgal-infected cells had high lytic activity against Ad-infected and Adβgal-infected cells (Fig. 4C). As in the previous experiment, however, lysis of AdhuAAT-infected targets was undetectable in all cases (Fig. 4).

These results indicate that Ad-specific CTL precursors were present in all three groups but did not eliminate AdhuATT-infected cells in vivo. Evidence of the in vivo activity of vector-induced CTL was provided by measurement of the βgal activity within the lungs of animals treated with Adβgal alone or in combination with AdhuAAT (Fig. 5). At the time of sacrifice (day 51 postadministration), levels of βgal activity were 1.2 and 5% of the day 3 levels in animals that had been treated with Adβgal alone and with Adβgal in combination with AdhuAAT, respectively. The loss of βgal expression within the lung presumably was due to CTL lysis by Ad-specific and/or βgal-specific CTL.

Repeat administration of Adβgal vector or WT Ad does not limit AdhuAAT expression. The above results indicated that Ad-specific and βgal-specific CTL were present within animals continuing to express huAAT and that at least those CTL specific for Adβgal-infected cells had lytic activity in vivo. To

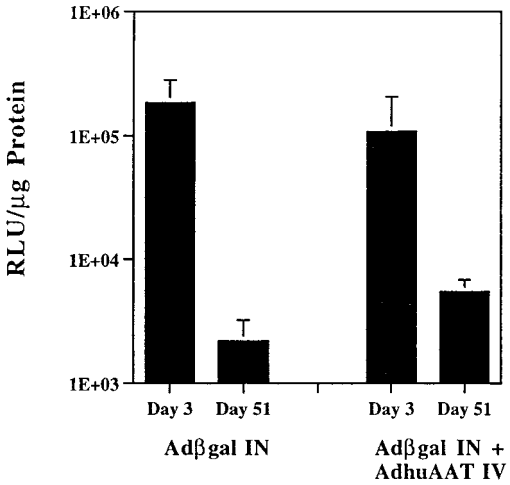

FIG. 5. Loss of expression of *E. coli* β galactosidase encoded by Adβgal vector over time in animals infected with Adβgal alone or concurrently with Adβgal and AdhuAAT. Animals from the experimental groups in Fig. 3 were sacrificed on day 3 or 51. The right caudal lobe from each animal was homogenized, and the level of *E. coli* βgal activity was measured as described in Materials and Methods. Results shown are the mean RLU per microgram of protein and SEM obtained from three individual animals.

increase further the levels of CTL specific for Ad antigens, AdhuAAT (2×10^{10} IU) was administered i.v. alone or in parallel with Adβgal (3×10^9 IU) or WT Ad5 (10^9 IU) by the i.n. route. At 20 days after the first administration, half of the animals that had received Adβgal or WT Ad5 i.n. were given a second dose of the same virus by the same route. A second i.n. administration of Adβgal is expected to boost the levels of CTL specific for Ad antigens expressed from the vector backbone and common to the Adβgal and AdhuAAT vectors, while a second administration of WT Ad should boost levels of CTL for all Ad antigens expressed in the mouse. A potential block to administration of the booster viruses was the presence of neutralizing antibodies. To ensure that neutralization would not occur, Ad-specific antibody levels in serum were measured in animals at the time of readministration and were found to be at a titer of 1/12,800 or less. From numerous previous experiments, we have determined that at this antibody level, readministration of Ad vector by the i.n. route is highly efficient (data not shown).

No significant differences in the expression levels of huAAT were observed among the different treatment groups regardless of whether the AdhuAAT vector was administered alone or in parallel with the Adβgal vector or with WT Ad5 or whether the Adβgal vector or WT Ad5 administration was repeated on day 20 (Fig. 6). These results indicated that boosting the immune response to viral antigens by these methods was not effective in limiting AdhuAAT expression.

To test the possibility that the route of vector administration influenced the site of action of CTL, we attempted to increase Ad antigen-specific CTL levels by the i.v. administration route. The groups that had previously received the AdhuAAT vector i.v. in parallel with WT Ad5 i.n. were given an i.v. administration (10^9 IU) of an E1-deleted Ad2 vector lacking a transgene (Ad2EV). The dose was selected to minimize reinfection of AdhuAAT-infected cells and to avoid damage to the liver. An Ad2-based vector was used for this challenge to circumvent neutralization by Ad5 type-specific serum antibodies, taking advantage of the fact that CTL against Ad2 and Ad5 are cross-reactive (Fig. 4). This third dose of Ad antigen given by the same route as the AdhuAAT vector still failed to cause a significant decrease in the expression of AdhuAAT (Fig. 6), even though Ad-specific CTL precursors were clearly present within these animals at the time of sacrifice, 50 days after administration of Ad2EV and 99 days after administration of AdhuAAT (Fig. 7A).

The final strategy for increasing Ad-specific CTL levels in animals expressing AdhuAAT was to administer WT Ad2 by the i.v. route. Again, Ad2 was used to avoid neutralization by Ad5-specific antibodies, and a dose of 10^9 IU of virus was used to avoid direct damage to the liver. The animals that had been given AdhuAAT vector i.v. and Adβgal vector i.n. on day 0 of the experiment were challenged with an i.v. dose of WT Ad2 on day 90. The huAAT expression levels in serum were monitored for an additional 30 days and did not change significantly (Fig. 6) despite the high levels of Ad-specific CTL activity detected within these animals at the time of sacrifice, 30 days after administration of WT Ad2 and 120 days after administration of AdhuAAT (Fig. 7B).

DISCUSSION

It has been suggested that administration of Ad-based gene therapy vectors triggers a cellular immune response against viral antigens expressed from the vector backbone and that this represents an inherent limitation on the longevity of gene expression from Ad vectors (23, 24). However, this broad conclusion is potentially complicated since many studies have used vectors that express very high levels of transgene products, such as *E. coli* βgal, that are themselves immunogenic and therefore capable of provoking immune system-mediated rejection. More recent studies have demonstrated that long-term expression of transgenes can occur from first-generation Ad vectors (2, 20) under circumstances where the immune response to the transgene product is minimal. The primary purpose of the present study was to monitor Ad-specific CTL levels in the context of persistent expression of a transgene protein that did not provoke an immune response in the test animal, the goal being to reconcile these apparently disparate findings.

We have shown that administration of an Ad vector encoding a transgene that is minimally immunogenic for the experimental animal, huAAT in the C57BL/6 mouse in this study, stimulates the production of CTL. Furthermore these CTL have lytic activity for Ad antigens when assayed in vitro but have no detectable activity on AdhuAAT-expressing cells in vivo. AdhuAAT treatment does not cause a general suppression of immune function, because titers of anti-Ad antibodies in serum rose following administration and Adβgal-expressing cells in animals simultaneously treated with AdhuAAT were eliminated. Furthermore, we have subjected AdhuAAT-expressing animals to various Ad vector treatment regimens designed to boost the immune response to vector-expressing cells. In all cases, stimulation of Ad-specific CTL by repeat dosing regardless of the route of administration failed to provoke the elimination of AdhuAAT expressing cells. Since the presence of Ad-specific CTL precursors in vivo following each of the various treatment schemes was documented, these results suggest that prolonged survival of AdhuAAT-expressing cells is not due to a lack of Ad-specific CTL within treated animals but, rather, that the Ad-specific CTL present appear to be ineffective in the elimination of AdhuAAT-infected cells. These results are consistent with a recent report that the activity of CTL in vitro may not accurately reflect their activity in vivo (1).

Although the majority of studies have been conducted in the

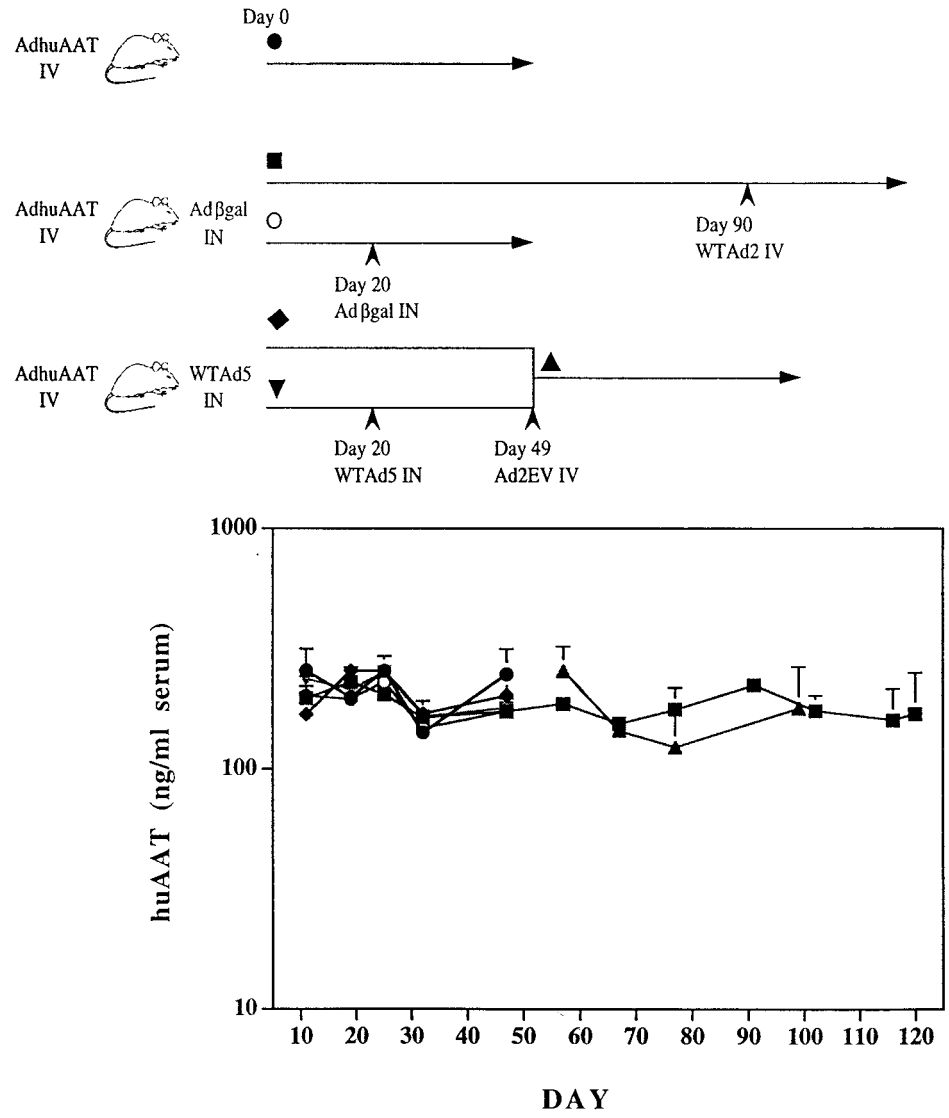

FIG. 6. (Top) Schematic diagram of treatment protocol. Twenty C57BL/6 mice were given AdhuAAT (2×10^{10} IU) by tail vein injection (i.v.), and the animals were then divided into three groups. Levels of huAAT in serum were measured over time in four of the AdhuAAT-treated animals (group 1) without further treatment until they were sacrificed on day 49. Each of the eight AdhuAAT-treated animals in group 2 was given Adβgal concurrently (3×10^9 IU) by the i.n. route. Four of the animals within group 2 were also given WT Ad2 i.v. (10^9 IU) on day 90. Levels of huAAT in serum were measured in these animals until they were sacrificed on day 120. The remaining four animals from group 2 were given a second i.n. dose (3×10^9 IU) of Adβgal on day 20, and the levels of huAAT in serum were measured in these animals until they were sacrificed on day 49. Each of the eight AdhuAAT-treated animals in group 3 was given WT Ad5 i.n. concurrently (10^9 IU). Four of the animals in group 3 were also given a second i.n. dose (10^9 IU) of WT Ad5 on day 20. On day 49, all animals in group 3 were given an i.v. dose (10^9 IU) of an E1-deleted Ad2 vector that is lacking a transgene (Ad2EV). Levels of huAAT in serum in animals in group 3 that received a single dose of WT Ad5 are plotted separately from the levels in animals that received a second dose of WT Ad5. After day 49, the huAAT expression levels from all animals in group 3 were combined. (Bottom) huAAT levels in serum of animals in treatment groups 1 through 3.

C57BL/6 mouse strain, prolonged expression of minimally antigenic reporter proteins is not unique to the huAAT-C57BL/6 system. The huAAT CD1 transgenic mice used in the present study represent a direct model to assess the impact of immunogenicity of the transgene on the longevity of vector expression in vivo. The transgenic animals differ from the parental CD1 strain only by the presence of the huAAT transgene under the control of the whey acidic protein promoter, and this results in expression in the serum at baseline levels. Prolonged and elevated levels of AdhuAAT vector-directed huAAT expression was seen in the transgenic animals, while in animals of the parental strain the same vector directed transient huAAT expression, which was accompanied by an antibody response to the huAAT protein. Similarly, other studies have demonstrated prolonged expression of mouse but not human EPO in a variety of mouse strains following intramuscular injection of first-generation Ad vectors. Persistent expression was characterized by the lack of an antibody response to mouse EPO and retention of vector genomes at the site of injection (20).

Our results are consistent with several recent published reports of studies in which alternate transgenes were used in the C57BL/6 strain. Kozarsky et al. (10) observed prolonged expression of human very-low-density lipoprotein (VLDL) receptor following i.v. administration of a first-generation vector but, in contrast, transient expression of human low-density lipoprotein (LDL) receptor from an identical vector backbone.

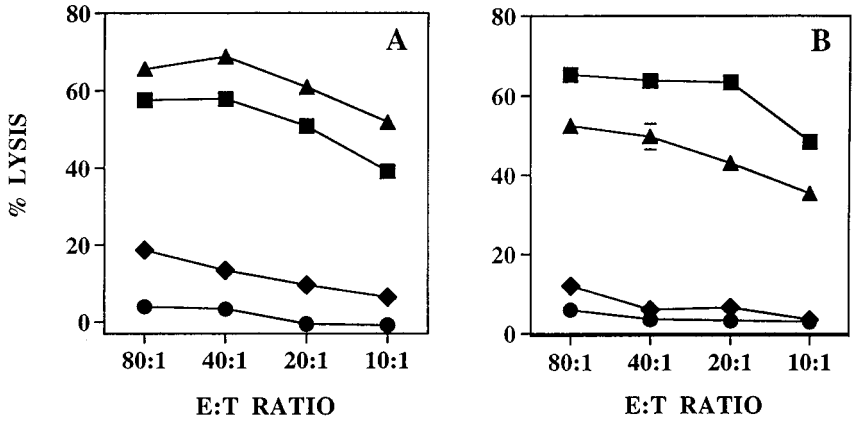

FIG. 7. (A) Levels of CTL activity in mice treated with AdhuAAT i.v. (day 0) plus WT Ad5 i.n. (days 0 and 20) followed by an Ad2 vector lacking a transgene i.v. on day 49. (B) Levels of CTL activity in mice treated with AdhuAAT i.v. plus Adβgal i.n. on day 0 followed by WT Ad2 i.v. on day 90. Spleen cells collected from the animals at the time of sacrifice on day 99 (A) or day 120 (B) were pooled and stimulated with Ad5Δ2.9-infected syngeneic fibroblasts to expand virus-specific CTL. The CTL activity of the cultured cells was tested against target fibroblasts that were either uninfected (●) or infected with Ad5Δ2.9 (■), Ad2Δ2.9 (▲), or AdhuAAT (◆). Results shown are the mean percent lysis and SEM from triplicate wells.

Humoral and cellular immune responses to the human LDL receptor were documented and could account for rejection of vector-infected cells. Neither humoral nor cellular immune responses to the human VLDL receptor were observed, although Ad-specific CTL were reported to be present in that study. Connelly et al. (4) have shown that long-term expression of human factor VIII from a first-generation Ad vector can be achieved following i.v. administration in C57BL/6 mice if the vector dose is adjusted to avoid hepatotoxicity. Poller et al. (13) reported long-term expression from a first-generation vector encoding human factor IX administered i.v. in the C57BL/6 mouse but showed that an equivalent vector lacking the E3 region exhibited transient expression. Persistence of huAAT expression in the experiments reported here could likewise be dependent on the presence of the E3 region, a conclusion supported by the in vitro lysis experiments shown in Fig. 2B. Lee et al. (11) reported a reduced CTL response as assayed in vitro to an Adβgal vector constitutively expressing the E3gp19K protein. Further research on the in vivo effects of the E3 region would appear to be warranted.

Taken together, the results of the present study and those of published studies suggest that many factors contribute to the duration of Ad vector-mediated gene expression. These include immune responses to the transgene, shutoff of the transgene promoter, the presence or absence of the E3 region, other changes to the vector backbone, and vector-induced tissue damage. The literature and the results reported here imply that neglect of these issues may well result in transient expression. When these variables are controlled, however, the cellular immune response to Ad vector antigens may be less of a limitation than previously suggested, at least in mice, and prolonged expression can result. We caution against overinterpretation of these conclusions, but if they can be extended to larger animals and to humans, first-generation Ad vectors might be effective for gene therapy applications provided that the therapeutic gene product is weakly immunogenic or nonimmunogenic, the transgene expression cassette has prolonged activity, and single or infrequent administrations would be required.

ACKNOWLEDGMENTS

We acknowledge K. Hehir, D. Pratt, M. Perricone, M. Nichols, C. Sacks, W. Smith, L. DeFalco, K. Couture, and N. Morral for technical support; Y. Echelard for making available the mice transgenic for human AAT; and Judith St. George, Arthur Beaudet, and Richard J. Gregory for helpful discussions.

Work at Baylor College of Medicine was supported by a grant from the North American Cystic Fibrosis Foundation (CFF 984).

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