

Prolonged Metabolic Correction in Adult Ornithine Transcarbamylase-deficient Mice with Adenoviral Vectors*

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A murine model of ornithine transcarbamylase (OTC) deficiency was used in this study to evaluate the efficacy of recombinant adenoviruses for correcting the metabolic defect in liver. Recombinant adenoviruses deleted in E1 and containing a human OTC cDNA expressed little functional OTC enzyme *in vivo* and had no observable impact on the underlying metabolic abnormalities of the OTC-deficient mouse (i.e. elevated urinary orotate and serum glutamine). E1-deleted vectors were improved through the use of the strong constitutive promoter from cytomegalovirus driving the normal murine homolog of OTC cDNA and the ablation of E2a with a temperature-sensitive mutation. Infusion of this improved vector into the mouse model was associated with a complete normalization of liver OTC enzyme activity that persisted for at least 2 months with complete but transient correction in serum glutamine and urine orotic acid. These studies illustrate the utility of improved adenoviral vectors in the treatment of liver metabolic disease.

A deficiency of ornithine transcarbamylase (OTC)¹ is associated with derangements in nitrogen metabolism leading to hyperammonemic encephalopathy in humans. This X-linked recessive disorder is the most common inborn error of urea synthesis, with an estimated prevalence of 1:40,000 to 1:80,000 births (1). Approximately one-half of affected males develop marked elevations of ammonia leading to coma in the 1st week of life. These episodes are associated with 50% mortality (2). Survivors of the neonatal crisis often experience recurrent episodes of potentially life-threatening hyperammonemia that are precipitated by excessive protein intake or catabolic stress (3). Since the urea cycle is principally localized to the liver, gene therapy directed to hepatocytes has the potential to correct the underlying metabolic derangements. The success of orthotopic liver transplantation in this disease indicates that

hepatocyte-directed gene transfer should be sufficient for metabolic correction (4–6).

Murine models of OTC deficiency are available for the development and evaluation of *in vivo* liver-directed gene therapies. The best characterized model is the sparse fur (*spf*) mouse, in which a missense mutation in codon 117 of the OTC gene leads to a functionally defective enzyme with hepatic OTC activity reduced to approximately 5–20% of wild-type levels at physiologic pH (7, 8). The other mutant is the *spf^{ash}* (abnormal skin and hair) mouse, in which a point mutation in the final base of exon 4 of the OTC gene leads to aberrant splicing with markedly reduced levels of OTC mRNA and only 5% of normal OTC activity (9, 10). In these two mouse strains, hemizygous male and homozygous female pups are runted and have wrinkled skin with little to no fur early in development. On a normal diet, adult *spf* or *spf^{ash}* hemizygotes develop symptomatic hyperammonemia, glutaminemia and severe orotic aciduria (8, 9), essentially identical with the findings in affected humans. They also have a markedly shortened lifespan and behavioral and learning abnormalities (11).

Recombinant adenoviruses have been evaluated as vectors for liver-directed gene therapy in a variety of metabolic disorders including OTC deficiency (12–15). Adenovirus is rendered defective for use as a vector by deleting the immediate early genes E1a and E1b and incorporating a minigene expressing the therapeutic protein. Adenovirus is efficiently targeted to hepatocytes *in vivo* following intravenous infusion; high level transgene expression can be achieved in virtually 100% of hepatocytes, most of which are fully differentiated and not dividing. The first use of E1-deleted viruses for gene therapy was in newborn *spf^{ash}* mice (14). Infusion of a vector containing a rat OTC cDNA into the newborn animals led to an increase in hepatic OTC activity in 4/15 mice which persisted for 1–2 months and was associated with decreased urinary orotic acid excretion.

Experiments of adenovirus-mediated gene transfer to liver have not been as encouraging when performed in other species or in adult mice. Gene transfer to liver is similarly efficient in these experimental models; however, transgene expression is transient, often lasting less than 14–21 days, and associated with substantial hepatitis (13, 16–18). We believe this is due, in part, to destructive cellular immune responses to the genetically corrected hepatocytes of the adult animal. This does not occur in the newborn mouse, who is exposed to gene therapy prior to immunologic maturity when tolerance can be induced. Deletion of E1a and E1b is insufficient to prevent either expression of other viral genes or actual replication at high multiplicities of infection (18, 19). A concerted immune response to exogenous and endogenously produced viral protein (or trans-

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¹ The abbreviations used are: OTC, ornithine transcarbamylase; CMV, cytomegalovirus; PBS, phosphate-buffered saline; CTL, cytotoxic T lymphocytes.

gene product) ensues. This results in activation of both CD4 (T helper) and CD8 (cytotoxic T) lymphocytes against the virus-infected cell and extinction of transgene expression by either destruction of the cell or through other indirect mechanisms (18, 20). This is particularly problematic for treatment of humans when the window for inducing tolerance is in the prenatal period of 14–18 weeks gestation (21).

We describe in this report the use of adenoviral vectors for treatment of OTC deficiency in the previously described murine models of the human disease. Use of a sufficiently strong promoter with a species homologous OTC cDNA was important in achieving curative therapeutic gene expression. An E1-deleted adenoviral vector, made temperature-sensitive in the E2a gene, normalized hepatic activity of OTC in the *spf/y* mouse and completely corrected metabolic abnormalities in urinary orotate and serum glutamine.

EXPERIMENTAL PROCEDURES

Animals—The mouse strains *spf*, *spf^{ash}*, and C3HeB/J were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in the Wistar animal facility. Female heterozygous *spf/x* or *spf^{ash}/x* mice were bred with normal male (x/y) C3HeB/J mice to generate experimental *spf/y* or *spf^{ash}/y* mice. All animals used in the experiment were between 6 and 10 weeks of age.

Construction and Propagation of the First and the Second Generation Recombinant Adenoviruses—A summary of recombinant adenoviruses used in this study is provided in Fig. 1. Nomenclature used for naming the vectors was described in Ref. 32. E1-deleted first generation recombinant adenovirus, designated H5.010CBhOTC, was derived from a retroviral vector containing a human OTC minigene. The coding sequence for human OTC was removed from plasmid pHO-731 on a 1.0-kilobase *HinfI* fragment (22), blunted with Klenow, ligated with *BclI* linkers, and cloned in direct orientation into the *BamHI* site of the retroviral vector pgagBA (23). A *XhoI* to *NheI* restriction fragment from this vector containing the chicken β -actin promoter, human OTC cDNA, 3'-untranslated sequences including 130 base pairs of retroviral sequence was cloned in place of *lacZ* in the adenoviral vector pAd.CMV-*lacZ* (13). This new plasmid, called pAd.CBhOTC, expresses human OTC cDNA from a CMV-enhanced β -actin promoter and is deleted of adenoviral sequences spanning 1 to 9.6 map units. In preparation for production of virus, pAd.CBhOTC was linearized with *NheI* and transfected into 293 cells with *Clal/XbaI*-restricted sub360 genomic DNA, which contains a substitution of sequence in E3b (24). The resulting recombinants (H5.010CBhOTC) were grown and purified through three rounds of plaque isolations. H5.110CBhOTC differs from H5.010CBhOTC only by a single base pair substitution in the E2a gene which generates a temperature-sensitive viral DNA-binding protein capable of growth at 32 °C but not 39 °C. The mutation contained within this recombinant adenovirus was generated from the wild-type Ad5 mutant strain H5.ts125 (25). Mouse OTC cDNA was generated by reverse transcription-polymerase chain reaction, cloned into pGEM-T vector (Promega), and restricted with *SpeI* and *SacII*. A 1.5-kilobase fragment containing mouse cDNA was isolated, blunted, and cloned into the *EcoRV* site of an adenoviral vector pAd.CMV-link (A CMV promoter-polylinker cassette was cloned into a plasmid containing the adenoviral sequences 0 to 16 map units deleted of E1a and E1b as described in the other adenovirus vectors). The new plasmid, designated pAd.CMVmOTC, was linearized with *EcoRI* and cotransfected into 293 cells with *Clal/XbaI*-restricted sub360 or sub360/ts125 DNA. The resulting recombinant virus (H5.010CMVmOTC or H5.110CMVmOTC) was purified through three rounds of plaque isolation. The integrity of the murine OTC cDNA was confirmed by DNA sequence analysis.

OTC Lysate Assay—Cells were harvested by scraping into mitochondria lysis buffer (0.5% Triton, 10 mM Hepes, pH 7.4, 2 mM dithiothreitol), and total protein was extracted by three freeze-thaw cycles. Liver tissue was homogenized in mitochondria lysis buffer with a Polytron homogenizer. The homogenate was centrifuged in a Microfuge at the maximum speed for 5 min, the supernatant was transferred to a new tube, and OTC enzyme activity was measured as described by Lee and Nussbaum (26) with modifications. Briefly, 2–10 μ g of total cellular protein was added to 700 μ l of reaction mixture (5 mM ornithine, 15 mM carbamyl phosphate, and 270 mM triethanolamine, pH 7.7) which was incubated at 37 °C for 30 min. Reactions were stopped by adding 250 μ l of 3:1 phosphoric acid/sulfuric acid (by volume). Citrulline production was then determined by adding 50 μ l of 3% 2,3-butanedione monoxime,

incubating at 95–100 °C in the dark for 15 min, and measuring absorbance at 490 nm.

OTC Histochemistry—Slides of liver tissue, less than 3 mm thick, were fixed in 4% paraformaldehyde in PBS for 4–6 h at room temperature and subsequently washed with PBS containing 10% sucrose for 2 h, PBS containing 20% sucrose for 2 h, and finally PBS containing 30% sucrose overnight. The liver slides were then embedded in OCT and sectioned for histochemical staining as described by Mizutani (27). Briefly, a reaction medium was prepared first, which contained 12 mg of carbamyl phosphate, lithium salt; 20 mg of L-ornithine dihydrochloride, 3.2 g of sucrose; 20 ml of 0.05 M triethanolamine buffer, pH 7.2; 16 ml of distilled water, and 4 ml of 1% lead nitrate. The lead nitrate solution was added dropwise with continuous stirring, and the solution was readjusted to pH 7.2 with 1 N NaOH. The slightly turbid substrate mixture was filtered and used immediately. Sections were incubated for 30 min in reaction medium at room temperature, washed with distilled water three times, immersed in 0.37% ammonium sulfide for 1 min, rinsed with distilled water again, and mounted for light microscopic observations. The dark brown deposits of lead sulfide indicated the sites of OTC activity.

RNA Hybridization Analyses—Total cellular RNA was isolated, fractionated on formaldehyde gel, and transferred onto Hybond-N nylon filters (Amersham). DNA fragments used as probes in RNA hybridizations were gel-purified and labeled with [α - 32 P]dCTP by random priming.

In Vivo Delivery of Recombinant Adenoviruses to Mouse Liver—*spf/y*, *spf^{ash}/y*, and male C3HeB/J mice at 6–10 weeks of age were used in this study. Blood samples were collected by retro-orbital bleeding the day before the experiment (day -1). Urine samples were collected at day -3 and day -1. On day 0, virus suspended in 0.1 ml of phosphate-buffered saline (PBS) was administered to animals via the tail vein. Urine and plasma samples were collected at weekly intervals after viral infusion. The animals were sacrificed at day 4, 7, 14, or 28, depending on the experimental protocol. Liver tissues were prepared for histochemical, biochemical, and molecular biological analysis.

Immunocytochemical Analysis—Immunofluorescence staining of adenoviral late gene products was performed as described by Kozarsky *et al.* (13). The primary antibody was a polyclonal rabbit antibody specific to Ad5 late gene products (produced in Dr. Wilson's laboratory). The secondary antibody was a fluorescein isothiocyanate-labeled goat anti-rabbit IgG (Chemicon, Temecula, CA).

Determination of Urinary Orotate—Mouse urine was collected at day -3, day -1, and at different time points after virus infusion by leaving the animals in a metabolic cage overnight. Urinary orotic acid levels were measured in duplicate for each sample according to Brusilow *et al.* (28).

Determination of Plasma Amino Acids—Plasma amino acids were analyzed by precolumn derivatization with *o*-phthalaldehyde as described previously by Robinson *et al.* (29). After centrifugation of heparinized blood, an aliquot of plasma was immediately precipitated with an equal volume of 0.8 N perchloric acid which contained the internal standards L- α -amino adipate and L- α -amino-n-butyric acid. After centrifugation, an aliquot of the supernatants was neutralized with 2 M KHCO₃. These samples were derivatized using an Autosampler. External standards were injected after every fifth specimen.

Evaluation of Liver Pathology—Histopathology was evaluated using the criteria developed by Knodell *et al.* (30). The three criteria scored were: I, periportal and bridging necrosis; II, intralobular degeneration and focal necrosis; and III, portal inflammation. Analyses were performed on three to four animals per time point.

RESULTS AND DISCUSSION

Ineffective Genetic Reconstitution in *spf* Mice Using Adenoviral Vectors Containing Human OTC—The *spf* and *spf^{ash}* mice are well described animal models for human OTC deficiency we have used to develop and evaluate adenoviral vectors for liver-directed gene therapy (7–10). In the two available models, point mutations lead to dysfunctional or reduced OTC enzyme protein resulting in metabolic and clinical abnormalities consistent with a partial deficiency in humans. The model used primarily in this study, the *spf* mouse, has an 85% reduction in liver OTC activity which leads to an activation of *de novo* pyrimidine synthesis as evidenced by a 13-fold increase in excretion of orotic acid in the urine (11). Nitrogen accumulates in these animals, as evidenced by a 60% increase in serum glutamine. Conversion of $^{15}\text{NH}_3$ to urea is decreased to 50% of

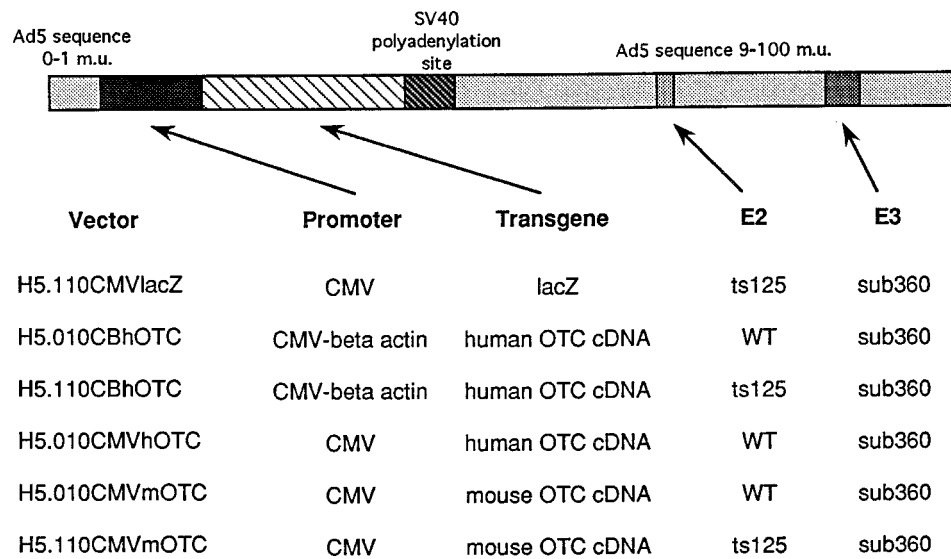


FIG. 1. **Recombinant adenoviral vectors.** Diagrammatic vector map was not drawn to scale. Nomenclature of adenoviral vectors is described in Ref. 32.

control, and the level of serum citrulline, a urea cycle intermediate, is decreased to 25% of control (11).

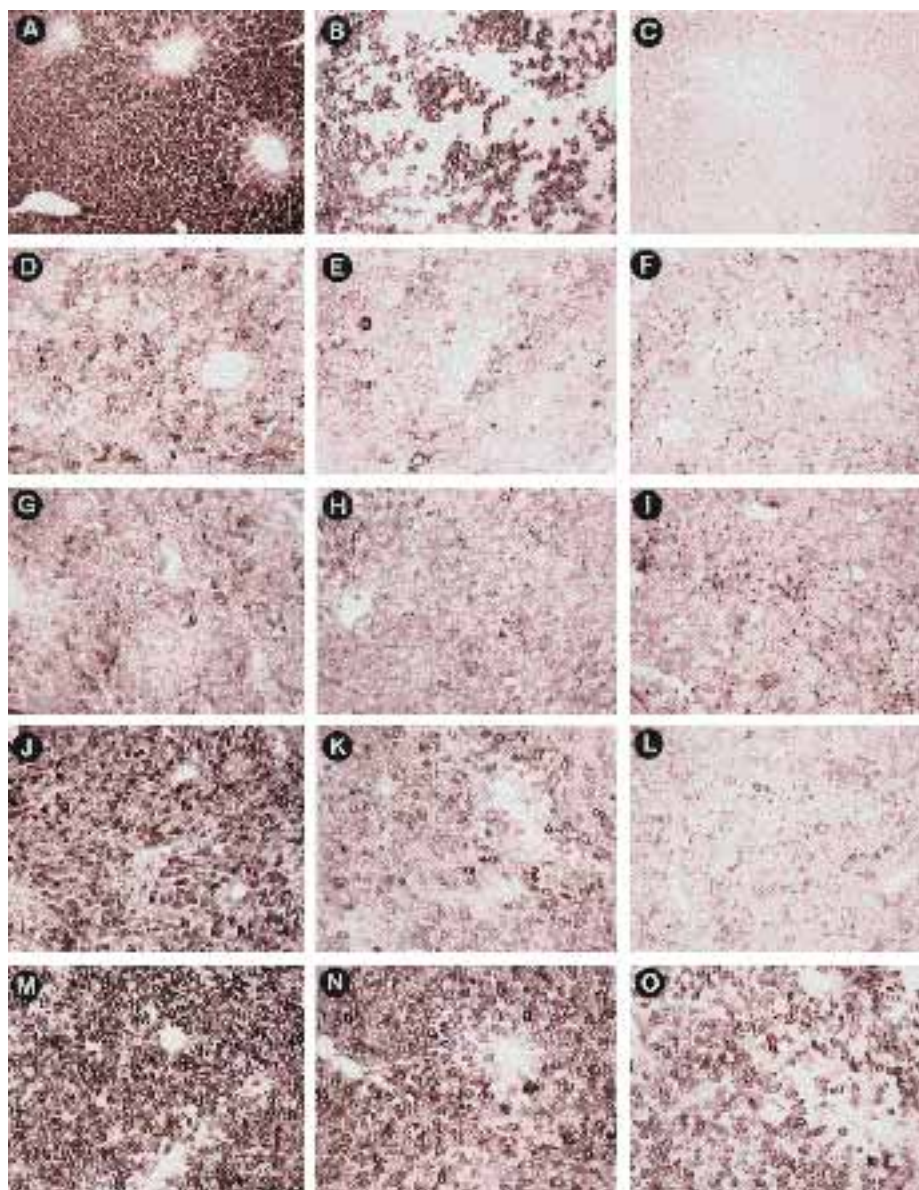
An E1-deleted adenoviral vector was constructed that contains human OTC cDNA (referred to as a first generation virus) expressed from a CMV-enhanced β -actin promoter. Pilot experiments were performed to determine the dose of virus necessary to increase OTC enzyme activity significantly in liver when vector was infused into *spf* mice. We found that the maximally tolerated dose of a first generation virus containing human OTC (*i.e.* 5×10^{11} particles) resulted in only a modest increase in OTC activity over baseline. This represents 5-fold more virus than what is necessary to transduce >80% of hepatocytes based on experiments with similar vectors expressing a variety of reporter genes (13, 15, 31). A histochemical stain for OTC activity was used to better characterize the distribution and level of OTC expression (Fig. 2). The specificity of this assay was demonstrated in analyses of C3H animals (Fig. 2A), which show a dark brown reaction product in 100% of cells that was absent in *spf* hemizygotes (Fig. 2C); heterozygotes show two populations of OTC-expressing cells consistent with lyonization of the x-chromosome (Fig. 2B). Histochemical analysis of *spf* liver removed 4 days after infusion of 5×10^{11} particles of first generation human OTC vector was underwhelming with expression detected at low levels in most cells (Fig. 2D), diminishing to baseline by days 7–14 (Fig. 2, E and F), concurrent with the development of substantial but self-limited hepatitis (Fig. 3A). Not surprising, there was no significant change in either urinary orotate (Fig. 4A) or serum glutamine (data not shown) when compared to animals that received identical doses of *lacZ* virus; urinary orotate nonspecifically decreased to approximately 50% of pretreatment levels with both viruses, possibly due to the associated hepatitis.

The relatively poor performance of the E1-deleted vector was felt to be, in part, due to the inherent immunogenicity of first generation constructs. We described in other systems that E1-deleted viruses express viral genes whose proteins are targets for destructive cellular immune responses (13, 18, 20, 31). Our first attempt to improve E1-deleted adenoviral vectors, by inactivating the essential gene product of E2a with a temperature-sensitive mutation, has shown promise in mouse liver, and mouse, rat, and primate lung, using *lacZ*-containing constructs (19, 31–33). In each case, expression of the transgene is prolonged for a variable period of time and associated with diminished inflammation. A second generation vector was con-

structed that was deleted in E1, defective in E2a due to the ts125 mutation, and contained a human OTC cDNA minigene. As expected, the levels of viral late gene RNA (Fig. 5A, lanes 1 and 2) and protein (Fig. 6, A and B) are diminished over that observed with the E1-deleted virus; the associated hepatitis is also decreased (Fig. 3A). Expression of the human OTC cDNA is still low; however, it appears slightly more stable (Fig. 2, G–I) from what is observed with the E1-deleted virus (Fig. 2, D–F). A nonspecific decrease (~50% of pretreatment levels) in urine orotate (Fig. 4B) and nonsignificant increase in serum glutamine (Fig. 4C) were again found in animals treated with either the *lacZ* or OTC virus. We concluded from these experiments that E1-deleted adenoviral vectors containing human OTC cDNA driven by the CMV-enhanced β -actin promoter were inadequate for gene therapy in the *spf* mouse, and the benefit of incorporating the ts125 mutation was minimal.

Efficient Expression of OTC from Adenoviral Vectors with Strong Constitutive Promoters and Species-homologous cDNAs—The most striking aspect of the studies presented above is the paucity of OTC expression achieved with the human OTC-based vectors and the lack of enhanced performance in vectors containing the ts125 mutation. This suggested problems with the human OTC cDNA functioning in the mouse model. Direct sequence analysis of viral DNA confirmed the structure of the human OTC minigene ruling out the trivial explanation of mutation or rearrangement (data not shown). Analysis of RNA from liver of treated animals revealed recombinant derived human OTC mRNA in excess of the level of endogenous OTC transcript found in normal human liver suggesting possible post-transcriptional inefficiencies (data not shown). We hypothesized that greater expression of the therapeutic protein could be achieved in a vector that contains a stronger promoter and uses isogenic OTC cDNA. Differences in amino acid sequence between the OTC murine and human homologs may affect the ability of the human enzyme to fold, oligomerize, and/or traffic to the mitochondria where it can function. In fact, 10 of the 26 amino acid differences present between mouse and human OTC are located in the leader sequence. In addition, the human protein may be immunogenic in the mouse. To evaluate these hypotheses, first and second generation viruses were constructed in which human OTC cDNA was replaced with mouse OTC cDNA, and the CMV enhancer/chicken β -actin promoter (CB) was replaced with the strong constitutive viral enhancer/promoter from CMV.

FIG. 2. Cytochemical demonstration of OTC activity in liver tissues from *spf* mice infused with recombinant adenoviruses. *spf/y* mice infused with 5×10^{11} particles of first generation H5.010CBhOTC (D, E, and F) or second generation H5.110CBhOTC (G, H, and I) human OTC-based recombinant adenovirus were sacrificed at day 4 (D and G), 7 (E and H), and 14 (F and I) postinfusion. *spf/y* mice infused with 2×10^{11} particles of first generation H5.010CMVmOTC (J, K, and L) or second generation H5.110CMVmOTC (M, N, and O) mouse OTC-based recombinant adenovirus were sacrificed at day 7 (J and M), 14 (K and N), and 28 (L and O). Liver tissues were analyzed for OTC activity by histochemical staining. Liver sections from uninfected C3HeB/J (A), heterozygous *spf*⁺ (B), and hemizygous *spf/y* (C) mice were also stained as controls. Representative photomicrographs are presented. Magnification $\times 100$.



First generation vectors that differed by the promoter, the OTC cDNA, or both were constructed and infused into *spf* mice. Liver tissue was harvested 3 days later and analyzed for OTC activity by lysate enzyme analysis (Fig. 7). The original vector expressing human OTC from the CMV/ β -actin promoter produced little enzyme activity above background in *spf* liver. A 3-fold increase in activity was achieved when the CMV promoter/enhancer was used to express the human OTC cDNA. An additional 2- to 3-fold increase was realized when the human OTC cDNA was replaced with the murine homolog in the CMV-based vector.

Metabolic Correction Is Complete and Prolonged in *spf* Mice Treated with E2a-defective Adenoviral Vectors Containing Mouse OTC cDNA—Gene therapy experiments were repeated with first and second generation versions of the vector expressing mouse OTC cDNA from the CMV promoter. The first generation mouse OTC cDNA vector was infused into *spf* mice, and lysates of liver harvested 3 days after gene transfer were analyzed for expression of OTC activity. OTC activity was normalized with a dose of mouse OTC virus that was 2–5-fold less than the maximally tolerated dose of human OTC virus that only partially corrected OTC deficiency. Histochemical analysis demonstrated OTC activity in the majority of hepatocytes of *spf*

animals treated with mouse OTC virus (Fig. 2J). Transgene expression diminished with time, although OTC activity was detected in at least one-third of hepatocytes at day 28 (Fig. 2L). Urinary orotate decreased to 10% of pretreatment levels by day 7 and gradually returned to baseline by day 42 (Fig. 8A). Correction of urinary orotate was significantly greater than the nonspecific reduction seen with the *lacZ* virus, although it was not complete. A 30% decline in serum glutamine was measured with the most substantial improvement, realized 20 days after gene transfer, approaching normal levels, thereafter returning to baseline within 42 days of gene transfer (Fig. 8B).

Experiments were repeated with the second generation virus in which the ts125 mutation in E2a was introduced into the E1-deleted vector containing the mouse OTC minigene. This vector performed substantially better in all categories in comparison to the corresponding first generation construct. Expression of OTC enzyme as measured by the histochemical stain was higher (Fig. 2M) and prolonged (Fig. 2, N and O). Inflammation was reduced (Fig. 3B), as was expression of late viral genes at the level of RNA (Fig. 5A, lanes 3 and 4) and protein (Fig. 6, C and D). Abnormalities in urine orotate and glutamine were completely normalized within 2–3 weeks of gene transfer; both metabolic parameters gradually returned to

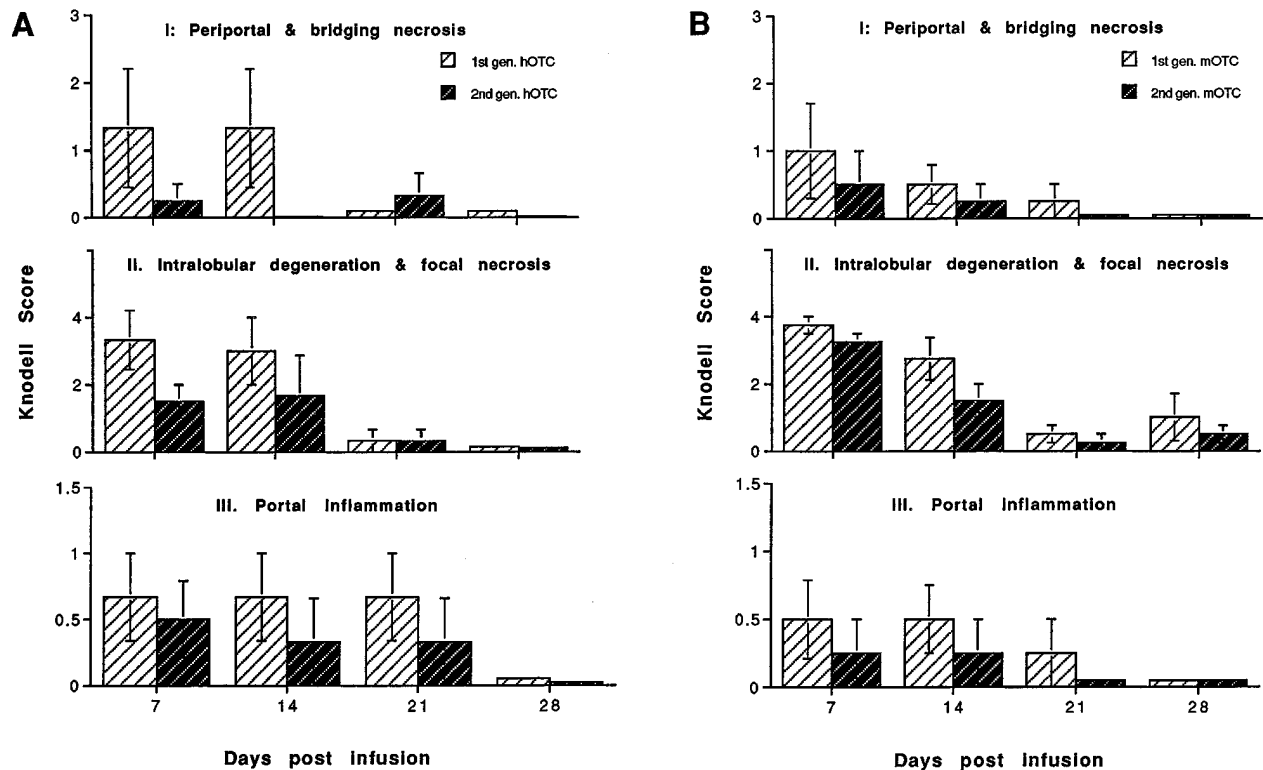


FIG. 3. Evaluation of pathological responses of the recipient mouse liver to recombinant adenovirus. Pathological response in mice receiving human OTC viruses (A) or mouse OTC viruses (B). Liver tissues were harvested at indicated time points following infusion of first generation (H5.010CBhOTC or H5.010CMVmOTC, light hatched boxes) or second generation (H5.110CBhOTC or H5.110CMVmOTC, heavy hatched boxes) recombinant adenovirus (5×10^{10} particle/mouse in A and 1×10^{11} particle/mouse in B) and evaluated for evidence of histopathology by light microscopic inspection of paraffin sections stained with hematoxylin and eosin. The pathological responses were characterized in three categories: I, periportal and bridging necrosis; II, intralobular degeneration and focal necrosis; and III, portal inflammation. The severity in each category was quantified by the Knodell score system. The histogram shown is the average of at least three independent observations with S.E. shown as error bars.

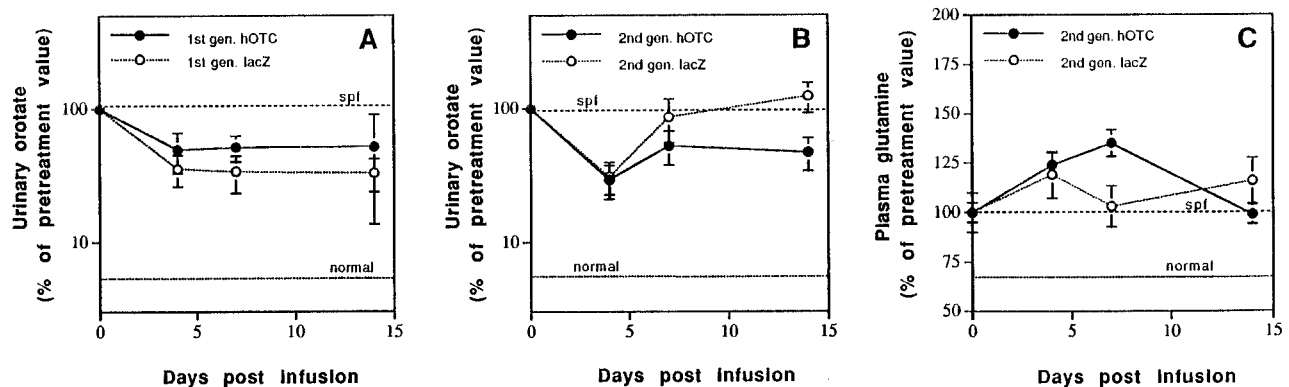


FIG. 4. Urinary orotate excretion and plasma glutamine levels in *spf* mice infused with recombinant adenoviruses carrying human OTC cDNA. A and B, urine orotate in mice infused with first and second generation recombinant virus. C, plasma glutamine in mice infused with the second generation virus. *spf* mice at 6–8 weeks of age were infused with 5×10^{11} particles of first generation viruses (H5.010CBhOTC or H5.010CMVlacZ) or second generation viruses (H5.110CBhOTC or H5.110CMVlacZ) through tail vein. Urine and plasma samples were collected the day before the virus infusion, and at day 4, 7, and 14 postinfusion. Urinary orotic acid levels were measured in duplicate for each sample. Urinary orotate/mg of creatinine are presented as a percent of pretreatment levels and are the mean \pm S.E. of at least 6 determinations. Plasma glutamine levels were determined as described previously (29). The levels are presented as a percent of pretreatment levels and are the mean \pm S.E. of between 4 and 10 determinations.

baseline levels but remained significantly improved as compared to animals infused with *lacZ* virus for 2–3 months (Fig. 8A and B). A similar correction of urine orotate and serum glutamine was achieved in the other murine model of OTC deficiency, the *spf*^{ash} mouse, following infusion of second generation vector containing mouse OTC cDNA (Fig. 8, C and D).

Relationship between Genetic Complementation and Metabolic Correction—Previous studies in mice and humans deficient in OTC suggested reconstitution of OTC in liver should be

sufficient for some level of metabolic correction. Several lines of transgenic mice have been established that express normal OTC cDNA in the murine models genetically deficient in OTC. Introduction of a minigene expressing rat OTC cDNA from an SV40 promoter into the germ line of *spf*^{ash} mice resulted in phenotype conversion as evidenced by normal hair growth and diminished excretion of orotic acid (34). OTC activity was increased to 80–90% of control in both liver and intestine. A similar approach was taken by Jones *et al.* (35) in *spf* mice with

a construct carrying human OTC cDNA under control of the mouse OTC promoter. Two transgenic hemizygous males demonstrated normal fur and orotic acid excretion. Interestingly, these animals expressed high levels of the transgene in intestine but low levels in liver. Both studies implicate an important role of OTC expression in bowel and/or liver although neither measured the impact of gene transfer on serum amino acids

which should represent more directly urea cycle function.

Our study demonstrates complete correction of orotic acid overproduction following hepatic reconstitution of OTC in both the *spf* and *spf^{ash}* mouse. We also measured the impact of gene therapy on serum amino acids and showed a normalization of serum glutamine reflecting a decrease in nitrogen stores. It was interesting that in the case of the first generation, murine OTC vector, correction of serum glutamine lagged behind biochemical correction of enzyme activity and the peak reduction in urine orotate. The reason for this is unclear; however, we speculate it may be due to the combined effects of increased OTC enzyme expression with the superimposed but transient decline in liver function that occurs because of adenovirus-induced hepatitis. The relative impact of gene therapy on various metabolic parameters will be determined by the balance of gene correction *versus* liver injury.

Despite normalizing serum glutamine and urine orotic acid, adenovirus-mediated gene transfer did not significantly impact on depleted serum citrulline (data not shown). This is consistent with the experience of orthotopic liver transplantation in carbamyl-phosphate synthetase and OTC deficiencies that all serum amino acids are corrected except citrulline which remains low (6, 36). One explanation is that serum citrulline is primarily contributed to by extrahepatic nitrogen ureagenesis which is not corrected using liver-directed approaches. Through the action of a partial urea cycle in gut, including OTC, glutamine nitrogen is converted to citrulline which is transported via the blood to kidney, where it is converted to arginine. The role of this extrahepatic pathway in humans appears minor as suggested by the observation that transplant recipients realize substantially improved tolerance to nitrogen challenges despite maintaining low serum citrulline levels (36).

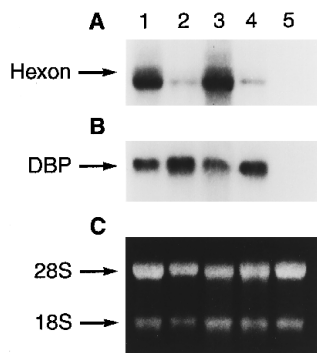


FIG. 5. RNA blot analysis of liver tissues from *spf* mice infused with recombinant adenoviruses. *spf* mice were infused with 2×10^{11} particles of the first generation (H5.010CBhOTC or H5.010CMVmOTC) or second generation (H5.110CBhOTC or H5.110CMVmOTC) recombinant adenovirus. At day 4 postinfusion, total liver RNA (10 μ g) was isolated, fractionated in denaturing formaldehyde-agarose gels, transferred to nylon filter, and hybridized with probes specific to the later viral gene product hexon (A) or DNA-binding protein (B). Lanes 1 and 2, liver RNA from *spf* mouse received first and second generation human OTC virus. Lanes 3 and 4, liver RNA from *spf* mouse received first and second generation mouse OTC virus. Lane 5, RNA from untreated *spf* liver. The intensity of ribosomal RNA (18S and 28S) was similar in each lane (C), indicating equivalent quantities of electrophoresed RNA.

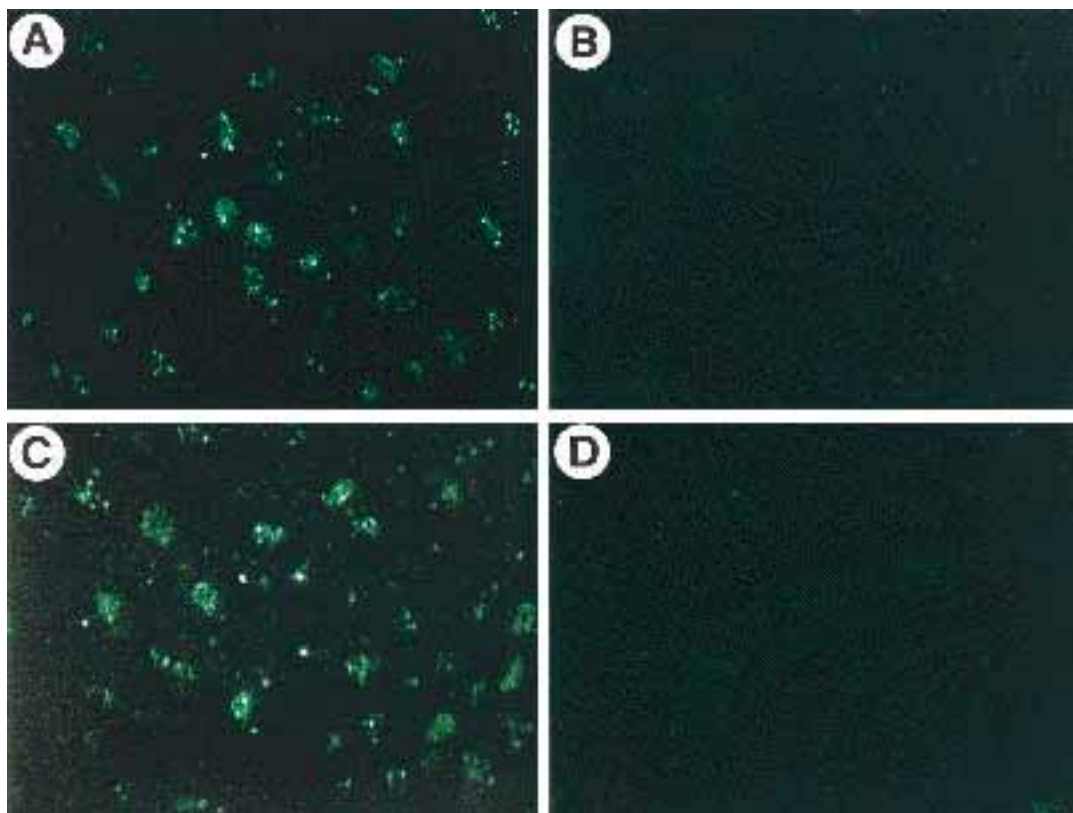


FIG. 6. Evaluation of viral late gene expression in liver tissue from *spf* mice infused with recombinant adenoviruses. Liver tissues of *spf* mice infused with 2×10^{11} particles of recombinant virus were harvested 4 days later. Fresh frozen sections (6 μ m) were fixed in 100% methanol for 10 min and analyzed for viral late gene expression by immunofluorescence using an antibody specific to viral late gene products. Representative sections are presented. CMV/ β -actin-driven human OTC cDNA constructs, first generation (A) and second generation (B). CMV-driven mouse OTC cDNA constructs, first generation (C) and second generation (D). Magnification $\times 200$.

Implications for Gene Therapy of Liver Metabolic Diseases— This study systematically evaluated the impact of both the transgene and vector on the safety and efficiency of adenovirus-mediated gene transfer to liver. Vectors containing human

OTC cDNA did not function as well in mouse liver as did the corresponding murine OTC constructs. One explanation is that the human OTC protein is not properly processed in a mouse cell to form catalytically active, mitochondrial-localized enzyme. Another explanation for the apparent dysfunction of the human OTC cDNA is that its protein is viewed as a neoantigen in the *spf* mouse eliciting confounding and destructive immune responses. Analysis of mice infected with human OTC adenovirus detected cytotoxic T cells but not antibodies to human OTC protein (data not shown). While primary antigen-specific cellular immune responses may decrease stability of transgene expression at day 7 and beyond, they cannot limit efficiency at early time points such as day 3.

Previous studies have clearly implicated cellular immunity in the loss of transgene expression and associated inflammation that has characterized E1-deleted adenoviruses (18–20, 37). Both T helper cells of the T_H1 subset and CTLs are necessary for the observed destabilization of transgene expression. T_H1 cells are activated to the input viral capsid proteins in a class II-dependent manner, while CTLs are activated to newly synthesized peptides presented by class I major histocompatibility complex. Viral proteins expressed from intact open reading frames in the recombinant virus as well as the transgene product itself are potential targets of CTL. It has been difficult quantifying the relative contribution of viral *versus* transgene

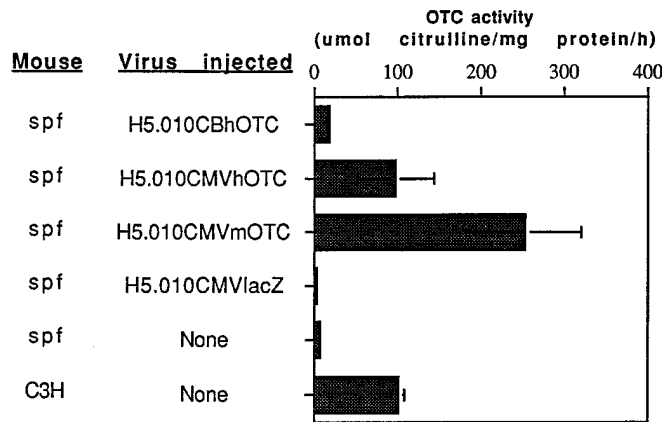


FIG. 7. Liver OTC activity in *spf* mice infused with recombinant adenovirus. *spf* mice were infused with 2×10^{11} particles of recombinant adenovirus and sacrificed at day 3 postinfection. Data are presented as OTC activity (μmol of citrulline/mg of protein/h) in mice (*spf* or C3H) infused with a variety of vectors. OTC activity in liver tissue was determined as described under "Experimental Procedures."

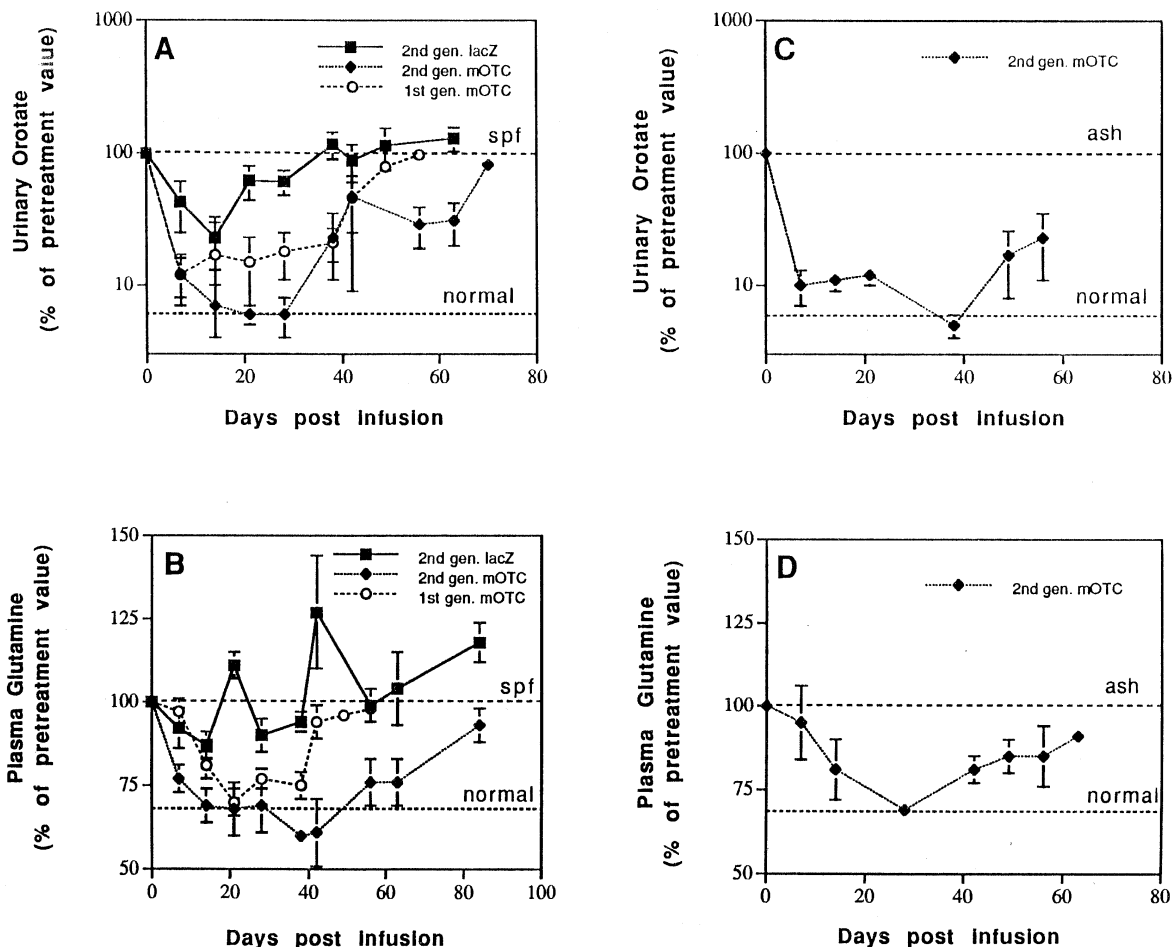


FIG. 8. Urinary orotate excretion and plasma glutamine levels in *spf* and *spf*^{ash} mice infused with recombinant adenoviruses carrying mouse OTC cDNA. *spf* mice (A and B) at 6–8 weeks of age were infused with 2×10^{11} particles of first generation (H5.010CMVmOTC) or second generation virus (H5.110CMVmOTC or H5.110CMVlacZ) through tail vein. *spf*^{ash} mice (C and D) of similar age were infused with 2×10^{11} particles of the second generation viruses (H5.110CMVmOTC). Urine and plasma samples were collected at day –3, day –1, and at weekly intervals after virus infusion. Urinary orotate levels were measured in duplicate for each sample. Urinary orotate/mg of creatinine are presented as a percent of pretreatment levels and are the mean \pm S.E. of at least 4 determinations. Plasma glutamine levels were determined as described previously by Robinson *et al.* (29). The levels are presented as a percent of pretreatment levels and are the mean \pm S.E. of between 4 and 10 determinations.

protein to activation of destructive cellular immune responses. Most experiments have used transgenes whose protein products are easily distinguished from endogenous protein but which run the risk of being neoantigens (e.g. *lacZ*, luciferase, chloramphenicol acetyltransferase, etc.) Previous experiments with E1-deleted viruses containing *lacZ* demonstrated CTLs to both viral proteins and *Escherichia coli* β -galactosidase; however, adaptive transfer experiments indicated immune responses to viral antigens from β 1-deleted viruses are sufficient to ablate transgene expression (38).

Characterization of the performance of adenoviral vectors containing normal mouse OTC cDNA provides an opportunity to evaluate directly the relative contribution of viral protein *versus* transgene product in eliciting destabilizing cellular immunity. This is the first example in which adenovirus-mediated gene transfer has been performed with a transgene whose product should not be viewed as a neoantigen; transgene-derived OTC differs by only one amino acid from the *spf* protein and is identical with the product of the *spf*^{ash} allele. Transgene expression with the mouse OTC cDNA vectors persisted longer than what we have consistently observed with vectors expressing non-self transgenes such as β -galactosidase. Incorporating the ts125 mutation into this vector diminished late viral gene expression and further prolonged transgene expression. However, even under optimal conditions of isogenic OTC cDNA in a second generation virus, the expression of transgene eventually diminished to undetectable levels within 3–4 months of gene transfer. This is less stable than what has been observed when E1-deleted *lacZ* viruses are infused into athymic or RAG2 knockout mice (data not shown and Refs. 18 and 20). Eliminating antigenicity of the transgene product will not be sufficient to prevent all destructive cellular immune responses.

Our data in the authentic animal models of OTC deficiency support the utility of recombinant adenoviruses for treating liver metabolic diseases. Complete and prolonged correction of the metabolic defect has been demonstrated following a single infusion of purified virus. Current limitations of the technology are the potential of CTL responses to residual expressed viral protein and possibly to OTC in patients with null mutations. In addition, previous studies have described the development of neutralizing antibodies to input viral proteins that block gene transfer upon second administration (13). Improved vectors may diminish CTL to viral protein but will have no effect on neutralizing antibody to capsid protein or CTL to OTC. Activation of CD4 T helper cells to input viral proteins is necessary for both B cell and CTL activation (37). Transient blockade of the initial CD4 T cell activation at the time of virus instillation has been shown to both prolong transgene expression and prevent formation of neutralizing antibody (39). This suggests a strategy for treating OTC deficiency based on coadministration of an immune modulator together with an improved recombinant adenovirus.

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REFERENCES

- Nagata, N., Matsuda, I., and Oyanagi, K. (1991) *Am. J. Med. Genet.* **39**, 223–229
- Batshaw, M. L., Brusilow, S., Waber, L., Blom, W., Brubakk, A. M., Burton, B. K., Cann, H. M., Kerr, D., Mamunes, P., Matalon, R., Myerberg, D., and Schafer, I. A. (1982) *N. Engl. J. Med.* **306**, 1387–1392
- Brusilow, S. W., and Horwich, A. L. (1995) in *The Metabolic and Molecular Bases of Inherited Disease* (Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., eds) Vol. I, pp. 1187–1232, McGraw-Hill, Inc., New York
- Broelsch, C. E., Emond, J. C., Whittington, P. F., Thistlethwaite, J. R., Baker, A. L., and Lichtor, J. L. (1990) *Ann. Surg.* **212**, 368–377
- Todo, S., Starzl, T. E., Tzakis, A., Benkov, K. J., Kalousek, F., Saheki, T., Tanikawa, K., and Fenton, W. A. (1992) *Hepatology (Baltimore)* **15**, 419–422
- Largilliere, C., Houssin, D., Gottrand, F., Mathey, C., Checoury, A., Alagille, D., and Farriaux, J. (1989) *J. Pediatr.* **115**, 415–417
- Veres, G., Gibbs, R. A., Scherer, S. E., and Caskey, C. T. (1987) *Science* **237**, 415–417
- Qureshi, I. A., Letarte, J., and Ouellet, R. (1979) *Pediatr. Res.* **13**, 807–811
- Doolittle, D. P., Hulbert, L. L., and Cordy, C. (1974) *J. Hered.* **65**, 194–195
- Hodges, P. E., and Rosenberg, L. E. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 4142–4146
- Batshaw, M. L., Yudkoff, M., McLaughlin, B., Gorry, E., Anegawa, N. J., Smith, I. A., Hyman, S. L., and Robinson, M. B. (1995) *Gene Ther.* **2**, 743–749
- Herz, J., and Gerard, R. D. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 2812–2816
- Kozarsky, K., McKinley, D., Austin, L., Raper, S., Stratford-Perricaudet, L., and Wilson, J. (1994) *J. Biol. Chem.* **269**, 13695–13702
- Stratford-Perricaudet, L. D., Levvero, M., Chasse, J. F., Perricaudet, M., and Briand, P. (1990) *Human Gene Ther.* **1**, 241–256
- Morsy, M., Alford, E., Bett, A., Graham, F., and Caskey, C. (1993) *J. Clin. Invest.* **92**, 1580–1586
- Ishibashi, S., Brown, M., Goldstein, J., Gerard, R., Hammer, R., and Herz, J. (1993) *J. Clin. Invest.* **92**, 883–893
- Li, Q., Kay, M., Finegold, M., Stratford-Perricaudet, L., and Woo, S. (1993) *Human Gene Ther.* **4**, 403–409
- Yang, Y., Nunes, F., Berencsi, K., Furth, E., Gönczöl, E., and Wilson, J. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 4407–4411
- Yang, Y., Nunes, F., Berencsi, K., Gönczöl, E., Engelhardt, J., and Wilson, J. (1994) *Nature Genet.* **7**, 362–369
- Yang, Y., Ertl, H., and Wilson, J. (1994) *Immunity* **1**, 433–442
- Crombleholme, T. M., and Bianchi, D. W. (1994) *Semin. Perinatol.* **18**, 376–384
- Horwich, A. L., Fenton, W. A., Williams, K. R., Kalousek, F., Kraus, J. P., Doolittle, R. F., Konigsberg, W., and Rosenberg, L. E. (1984) *Science* **224**, 1068–1074
- Grossman, M., Raper, S., and Wilson, J. (1992) *Human Gene Ther.* **3**, 501–510
- Logan, J., and Shenk, T. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81**, 3655–3659
- Ensinger, M. J., and Ginsberg, H. S. (1972) *J. Virol.* **10**, 328–339
- Lee, J. T., and Nussbaum, R. L. (1989) *J. Clin. Invest.* **84**, 1762–1766
- Mizutani, A. (1968) *J. Histochem. Cytochem.* **16**, 172–180
- Brusilow, S. W., and Hauser, E. (1989) *J. Chromatog.* **493**, 388–391
- Robinson, M. B., Djali, S., and Buchhalter, J. R. (1993) *J. Neurochem.* **61**, 2099–2103
- Knodell, R., Ishak, K., Black, W., Chen, T., Craig, R., Kaplowitz, N., Kiernan, T., and Wollman, J. (1981) *Hepatology (Baltimore)* **1**, 431–435
- Engelhardt, J., Ye, X., Doranz, B., and Wilson, J. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 6196–6200
- Engelhardt, J., Litzky, L., and Wilson, J. (1994) *Human Gene Ther.* **5**, 1217–1229
- Goldman, M. J., Litzky, L., Engelhardt, J. F., and Wilson, J. M. (1995) *Human Gene Ther.* **6**, 839–851
- Cavard, C., Grimber, G., Dubois, N., Chasse, J., Bennoun, M., Minet-Thuriaux, M., Kamoun, P., and Briand, P. (1988) *Nucleic Acids Res.* **16**, 2099–2110
- Jones, S. N., Grompe, M., Munir, M. I., Veres, G., Craigen, W. J., and Caskey, C. T. (1990) *J. Biol. Chem.* **265**, 14684–14690
- Tuchman, M. (1989) *N. Engl. J. Med.* **320**, 1498–1499
- Yang, Y., Xiang, Z., Ertl, H. C. J., and Wilson, J. M. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 7257–7261
- Yang, Y., Jooss, K. U., Su, Q., Ertl, H. C. J., and Wilson, J. M. (1996) *Gene Ther.*, in press
- Yang, Y., Trinchieri, G., and Wilson, J. M. (1995) *Nature Med.* **1**, 890–893