Long Term Correction of Bilirubin-UDP-glucuronosyltransferase Deficiency in Gunn Rats by Administration of a Recombinant Adenovirus during the Neonatal Period*

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Injection of a recombinant adenovirus expressing human bilirubin-UGT, (Ad-hBUGT,) (3 × 10⁹ plaque-forming units (pfu) intravenously) in adult bilirubin-UDP-glucuronosyltransferase-1 (BUGT)-deficient Gunn rats resulted in biliary excretion of bilirubin glucuronides and a 70% reduction of serum bilirubin levels. However, the effect was transient, and host humoral and cellular immune response prevented transgene expression after subsequent injections. To determine whether injection during the neonatal period would tolerize the host to the recombinant virus, we injected 1 × 10⁹ pfu of Ad-hBUGT, or Ad-LaCZ (a recombinant adenovirus expressing Escherichia coli β-galactosidase) into 1–3-day-old Gunn rats. Two subsequent injections (3 × 10⁹ pfu) were given 56 and 112 days after the initial injection. Injection of Ad-hBUGT, but not Ad-LaCZ, reduced serum bilirubin by 70–76% of the levels in untreated pups (9 ± 1.3 mg/dl), followed by a gradual increase to 3.25 ± 0.3 mg/dl in 56 days; similar or greater reductions occurred after the second and third injection. Serum neutralizing antibody titer and cytotoxic lymphocyte activity against adenovirus-infected hepatocytes were low or undetectable. Thus, tolerization by injection of the virus during the neonatal period permits long term gene therapy by repeated injection of the virus.

Hepatic glucuronidation is required for efficient excretion of bilirubin. Bilirubin glucuronidation is catalyzed predominantly by a single isoform of the uridine diphosphoglucuronate-glucuronosyltransferase family, termed BUGT,¹ (1), the inherited genetic lesions of which leads to marked accumulation of bilirubin in the plasma, causing Crigler-Najjar syndrome type I, which is characterized by neurotoxicity and death (2). Mutant homozygous Gunn rats have an inherited deficiency of hepatic glucuronidation. Like patients with Crigler-Najjar syndrome type I, Gunn rats do not glucuronidate bilirubin, exhibit life-long hyperbilirubinemia, and develop spontaneous bilirubin encephalopathy (3). The human (4) and the rat (5) BUGT, are products of the gene, UGT1. Deletion of a single guanosine residue in this gene in Gunn rats results in a premature translational stop codon, producing a truncated and inactive enzyme (6). At present, liver transplantation is the only definitive treatment for Crigler-Najjar syndrome type I. Because the liver is structurally normal, substitution of the mutant gene to correct the metabolic abnormality would be an attractive alternative treatment. We have been exploring various avenues for developing an effective long term method for gene therapy for inherited bilirubin-UGT, deficiency, using the homozygous Gunn rat as an animal model.

Recombinant retroviruses and adenoviruses are being used for somatic gene therapy (7). Retroviruses integrate into the host genome, providing long term gene expression, but retroviral gene transfer requires cell division, which is infrequent in hepatocytes in vivo (7). In contrast, recombinant adenoviruses are highly efficient in transferring foreign genes to quiescent cells, can be generated at high titers (8), and are potentially useful for gene therapy (8–11). Furthermore, we and others have shown that after systemic administration, recombinant adenovirus 5 (Ad5) localizes preferentially to the liver, and can express the transgene in over 90% of the hepatocytes (8, 12).

The early transcription region of the adenoviral genome (E1) controls viral gene expression and replication (9). First generation replication-defective recombinant adenoviruses are generated by disrupting the E1 region. These vectors achieve a high level of gene transfer, but integration into the cell genome is rare (9, 10) and the transgene expression is of short duration. Studies in immune-deficient mice indicate that the duration of gene expression may be limited by host immune response (13, 14). Moreover, neutralizing antibodies and cytotoxic lymphocytes (CTL) that develop in the host after the initial injection of the virus prevents transgene expression upon subsequent administrations (13). In an attempt to prevent any possible viral gene expression in vivo, “second generation” vectors were produced using an adenoviral strain with a mutated E2a gene that expresses a temperature sensitive DNA-binding protein (15). These viruses caused less liver and lung inflammation, but long term gene expression was difficult to achieve (15). It is possible that antigens present in the input virus may be sufficient to evoke the immune response.

Administration of antigens during the neonatal period induces tolerance by several mechanisms, including clonal deletion of antigen-specific T cells, T cell-mediated suppression of
regulatory cells (e.g. helper T cells), and secretion of factors that suppress the generation of antigen-specific effector cells (16, 17). In this study, we show that injection of recombinant adenoviruses into neonatal Gunn rats prevents the development of neutralizing antibodies and cytotoxic lymphocytes, thereby permitting long term gene therapy by repeated injection of the vector.

**MATERIALS AND METHODS**

**Animals**

Homozygous Gunn rats were bred in our colony at the Albert Einstein College of Medicine, maintained on standard laboratory chow, and kept in 12-h light/dark cycles. Newborn and adult rats of both genders were used for these studies.

**Plasmids**

pJM17, containing the sequences of adenovirus Type 5, was kindly provided by Dr. F. L. Graham, McMaster University, Hamilton, Canada.

**Generation of E1-disrupted Recombinant Adenovirus 5**

pBC/CMV (Invitrogen) was partially digested with PvuII, and BglII linkers (Boehringer Mannheim, IN) were inserted at nucleotide position 1290. The NotI-SpeI fragment of pSVK3-hBUGT, was subcloned into pRe-CMV-BglII. The resulting expression cassette including the promoter and enhancer sequence for the immediate early gene of cytomegalovirus, the structural region of human BUGT, and the polyadenylation signal from bovine growth hormone was cloned into a shuttle vector, pAD-BglII, generating pAD-BglII-hBUGT. The recombinant adenovirus Ad-hBUGT, was generated by cotransfection of pAD-BglII-hBUGT, and pJM17 into 293 cells as described (18). Briefly, 293 cells were seeded at 70–80% confluence in 24-well plates and transfected for 20 h with 250 μl of transfection mixture containing 2 μg of pAD-BglII-hBUGT, 5 μg of pJM17 mixed with 1 ml of OptiMEM containing 20 μl of Lipofectin® (Life Technologies, Inc.)/well. After transfection, 293 cells were maintained in RPMI 1640 medium with 2% fetal calf serum until 72 h after infection and resuspended in 10 mM Tris-HCl (pH 8.0).

**Assessment of Transgene Expression**

β-galactosidase and human BUGT expression were determined as described below.

**Large Scale Virus Preparation**

Recombinant adenoviruses were grown on 293 cells, a human embryonic kidney cell line that supplies the product of the E1 region in trans, allowing the recombinant vectors to propagate (18). The 293 cells were grown in suspension culture at a cell density of 5 × 10^6/ml using calcium-free essential medium (Life Technologies, Inc.) supplemented with 10% calf serum. The cells (1.6 × 10^8) were then infected by suspending them in 3 liters of supernatant from adenovirus-infected 293 cells that had been allowed to lyse completely. Cells were harvested 72 h after infection and resuspended in 10 ml Tris-HCl (pH 8.0). Recombinant adenovirus was purified from the cell lysate by two consecutive CsCl density gradient ultracentrifugations on preformed gradients, and stored in 30% glycerol at −20 °C. Virus was dialyzed overnight at 4 °C against an isotonic solution containing 135 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 10 mM Tris-HCl, pH 7.4, and 10% glycerol, and sterilized by filtration through 0.2-μm filters before use.

**Injection of Newborn Rats with Ad-hBUGT and Ad-LacZ**

Six groups of 10 rats each were studied (Table I). Group A were Gunn rats that received three intravenous injections Ad-hBUGT at the ages of 1–3, 56, and 112 days, respectively. Group B Gunn rats received Ad-hBUGT injection intravenously as newborns, and the second and third injections, at ages 56 and 112 days, respectively, into the portal vein. Group C were newborn Gunn rats injected with Ad-LacZ intravenously. Group D were adult Gunn rats injected intravenously with Ad-hBUGT. Group E were newborn normal RHA rats injected intravenously with Ad-hBUGT, for the first injection, followed by Ad-LacZ intravenously for the second injection. Newborn rats were infected with 1 × 10^8 pfu of the recombinant adenovirus into the right jugular vein. All other injections (3 × 10^9 pfu) were given into tail veins or intraorally where indicated (Table I).

**Distribution of Human BUGT DNA after Administration of Ad-hBUGT**

Four adult Gunn rats injected with Ad-hBUGT, were killed 4–48 h after virus administration. Various tissue samples were snap-frozen in acetone/dry ice baths. To determine the distribution of the transgene, Southern blot analysis was performed as follows. The tissue was homogenized in 1.2 ml/100 mg tissue of 10 mM Tris-HCl, 100 mM NaCl, 25 mM EDTA, 0.5% SDS, pH 8.0, for preparation of DNA. The lysate was incubated overnight with 200 μg/ml proteinase K at 37 °C. After three extractions of phenol-Chloroform-isomyl alcohol, RNA was removed from the lysate by RNase treatment, followed by phenol-Chloroform-isomyl alcohol extraction. DNA was precipitated with ethanol. DNA samples (10 μg) were digested with HindIII, separated by electrophoresis on 0.8% agarose gel, and transferred to a nylon membrane (GeneScreen R, DuPont NEN) by capillary blotting. The filter was hybridized with a 32P-labeled 2.1-kilobase pair NotI to SpeI fragment of pSVK3-BUGT, or a 5881-base pair EcoRI fragment of adenovirus type 5. The filter was reprobed with rat albumin cDNA to correct for small variations in DNA loading. The blot was washed under high stringency conditions according to standard protocols and exposed to x-ray film.

**Table I**

| Group | Rat strain and age at first injection | Virus | Route of injection |
|-------|-------------------------------------|-------|--------------------|
| A     | Newborn Gunn                        | Ad-hBUGT₁ | Intravenous        |
| B     | Newborn Gunn                        | Ad-hBUGT₁ | Intravenous for the first injection; intraportal injection for the second and third injections |
| C     | Newborn Gunn                        | Ad-LacZ  | Intravenous        |
| D     | Adult Gunn                          | Ad-hBUGT₁ | Intravenous        |
| E     | Newborn RHA                         | Ad-hBUGT₁ | Intravenous        |
| F     | Newborn Gunn                        | Ad-hBUGT₁, followed by Ad-LacZ | Intravenous        |

of 1–3, 56, and 112 days, respectively. Group B Gunn rats received Ad-hBUGT injection intravenously as newborns, and the second and third injections, at ages 56 and 112 days, respectively, into the portal vein. Group C were newborn Gunn rats injected with Ad-LacZ intravenously. Group D were adult Gunn rats injected intravenously with Ad-hBUGT. Group E were newborn normal RHA rats injected intravenously with Ad-hBUGT, for the first injection, followed by Ad-LacZ intravenously for the second injection. Newborn rats were infected 1 × 10^8 pfu of the recombinant adenovirus into the right jugular vein. All other injections (3 × 10^9 pfu) were given into tail veins or intraorally where indicated (Table I).
mRNA Expression—Surgical wedge biopsies were performed as described for β-galactosidase assay and stored at −80 °C until use. RNA was extracted, and a segment of the 5′ region of human BUGT1 mRNA was amplified by reverse transcription-primed polymerase chain reaction (RT-PCR) as described previously using the following primers: sense, 5′-AAGGAAGGCTGGCTGACCA-3′ (nucleotides 141–180 from the translational start codon), and antisense: 5′-CCACGACGTCGAGCAGCAGAGG-3′ (nucleotides 441–462 from the translational start codon) to amplify a segment of the unique amino-terminal domain of human BUGT1 mRNA. RT-PCR was performed as described previously (1) (denaturation: 94 °C for 30 s, annealing 58 °C for 30 s, extension 72 °C for 90 s, 30 cycles).

Immunoblot Analysis of Human BUGT1, Expression—A fragment of the biopsy specimens was homogenized in 0.25% sucrose, 10 mm Tris-HCl, pH 7.4, using a glass homogenizer fitted with a motorized Teflon pestle. Proteins (100 μg/lane) were resolved by 7.5% SDS-polyacrylamide gel electrophoresis and electroblotted to nitrocellulose membranes. Human BUGT1 bands were visualized using a monoclonal antibody WP1 directed at the common carboxyl-terminal domain of UGT isoforms expressed by human ugt1 gene (4), and a goat anti-mouse IgG Fab′ fragment second antibody coupled to horseradish peroxidase (Sigma Diagnostics).

UGT Activity toward Bilirubin—Tissue homogenates were prepared from liver biopsy specimens from two different lobes of the liver and UGT activity toward bilirubin was determined by a sensitive HPLC-based method as described previously (1, 20).

Serum Bilirubin Levels—Approximately 0.3 ml blood samples were collected from tail veins every 6–10 days in all groups, and serum bilirubin concentrations were determined (21).

Bile Pigment Analysis—For definitive evaluation of bilirubin glucuronidation, selected rats were provided with a polyethylene bile duct stent to allow bile duct cannulation and bile was removed using HPLC using a μBondapak C-18 column (Millipore-Waters, Milford, MA) as described (20).

Evaluation of Immune Tolerance

Humoral and cell-mediated immune response to the initial and subsequent injections of the recombinant adeno viruses were determined as described below. In addition, biochemical and histological evidence for liver damage were evaluated. Evaluation of Liver Damage—Liver biopsy specimens collected at 3–4-week intervals were fixed in 10% formaldehyde. Paraffin sections were stained with hematoxylin-eosin by standard procedures and examined by light microscopy. The sections were graded for hepatic inflammation as follows: Grade 0, normal; Grade 1, mild periportal or focal lobular lymphocytic infiltration; Grade 2, extension of lymphocytic infiltration into the lobules and “piecemeal necrosis”; and Grade 3, disruption of the lobular architecture by “bridging necrosis” and extensive lymphocytic infiltrates from portal to central, portal to portal, and central to central zones.

Serum Alanine Aminotransferase (ALT) Levels—As a measure of hepatic inflammation, ALT levels were quantified using a commercially available kit (Sigma).

Neutralizing Antibodies against the Recombinant Adenoviruses—Rat anti-adenovirus-neutralizing serum antibodies were measured every 3–4 weeks. 293 cells were seeded at 3 × 10⁴/well in 96-well plates, and cultured until 90% confluence. Ad-LacZ was diluted in cell culture medium to 3 × 10⁴ pfu/10 μl. Serum samples were treated with heat-inactivated at 55 °C for 30 min and diluted in medium in two steps. 0.1 ml of each serum dilution was mixed with 5 × 10⁴ of the recombinant virus, incubated at 37 °C for 90 min, and applied to the confluent 293 cells from 10–14 h. The medium, containing serum and virus, was then replaced by RPMI with 10% fetal calf serum. After 18 h, the cells were fixed and stained for β-galactosidase expression. In the absence of neutralizing antibodies, all of the cells stained blue. The neutralizing antibody titer for each serum sample was reported as the highest dilution at which less than 25% of the cells stained blue.

Cytotoxic T Lymphocyte Assay—Adeno-293-transfected primary hepatocytes, obtained as described above, were plated on collagen-coated dishes with Chex’s medium (22) (2 × 10⁵ cells/well in six-well plates) and were used as target cells for the effector lymphocytes. Two rats from each group were studied for CTL activity, on days 28, 56, 112, and 140 after the first injection. Inguinal and paraaortic lymph nodes were removed and gently disrupted using a rubber policeman. Red blood cells were removed using lysis buffer containing 8.3 g/liter NH₄Cl, 1 g/liter KHCO₃, 100 μl of EDTA at pH 7.4 (1 ml/spleen or 5–10 lymph nodes) for 2 min. Lymphocytes were pelleted by centrifugation and plated at 5 × 10⁵/ml in RPMI with 10% fetal calf serum. Cells were restimulated with Ad-hBUGT1, with at a multiplicity of infection of 10, for 4–5 days. The stimulated effector cells were harvested, counted and added to the primary hepatocyte cultures at a ratio of 50–100:1, and incubated at 37 °C for 5 h. Hepatic cell lysis was estimated by measuring ALT levels using a commercial kit (Sigma Diagnostics) according to the manufacturer’s instructions, except that the reagent and the test samples were mixed at equal volumes and the incubation was for 90 s. Product formation was proportional to the duration of incubation. ALT levels were expressed in international units. ALT levels in the medium of culture dishes containing adenovirally infected hepatocytes with the addition of CTLs from naive animals ranged from 110 to 200 IU (150 ± 41, mean ± S.D.; n = 20) and were considered background. CTL activity was expressed in IU of ALT averaged from six wells after subtraction of background. Maximum possible ALT release from the cells was estimated by incubating the cells with 5 mm D-(-)-galactosamine overnight, which resulted in ALT levels of 3500–4000 IU.

Infection of Tolerized Rats with Wild-type Adenovirus 5—Two tolerized rats from each group were injected with wild-type adenovirus 5 (1 × 10¹⁰ pfu), 132 and 142 days after the third injection. All rats were reexamined for serum bilirubin levels, the appearance of neutralizing antibodies and CTL response 4 days after each injection of the wild-type virus.

RESULTS

Distribution of the Recombinant Adenoviruses in Various Tissues

Four hours and 24 h after the injection of Ad-hBUGT1, adenoviral DNA in various tissues was examined by Southern blot analysis using a probe for the hBUGT1. The Ad-hBUGT1 DNA was detectable predominantly in the liver, with only traces being present in other tissues (Fig. 1). Based on the assumption of a body weight:liver weight ratio of 25:1, 90–95% of the injected viral dose was estimated to be recovered in the liver.

Expression of β-Galactosidase Activity

Liver specimens of two rats in Groups C and F were examined 10 days after each Ad-LacZ injection. Nearly all hepatocytes showed positive nuclear staining for β-galactosidase activity following each injection (Fig. 2). In addition, liver biopsy was performed in two rats immediately before the third injection (day 108); only 1–2% of the cells in these specimens showed positive staining.
Fig. 2. β-Galactosidase expression in liver after the injection of Ad-LacZ. Three-day-old Gunn rats were injected 1 × 10^11 pfu of Ad-LacZ, and two subsequent injections of 3 × 10^11 pfu of AdLacZ were given at age 56 days and 112 days. Cryostat sections were prepared, from serial liver biopsy specimens from rats in Group C and stained for β-galactosidase activity as described in the text. Specimens from a single rat from a representative experiment are shown. Panel A, 10 days after the second Ad-LacZ injection (age 66 days); panel B, 4 days before the third injection (age 108 days); panel C, 10 days after the third injection (age 122 days). The data shown are from a single rat, representative of a group of 10 rats in Group C.

Expression of h-BUGT1 after Injection of Ad-hBUGT1 in Gunn Rats

Time Required for Expression of BUGT Activity after Ad-hBUGT1 Injection—After glucuronidation, bilirubin is excreted in bile almost immediately, which permitted the determination of the time required for the expression of functional bilirubin-UGT activity in Gunn rat livers after the injection of Ad-hBUGT1. HPLC analysis of bile samples showed that significant amounts of bilirubin monoglucuronide and diglucuronide began to be excreted in bile 3.5 h after the virus injections. After 6 h, the pigment composition of bile resembled that in normal rats.

Persistence of Ad-hBUGT1 DNA in Liver after Injection into Newborn Gunn Rats—By Southern blot analysis, positive signals for hBUGT1 were identified in Group A after each of the three injections, while positive signals were found only after the first injection in Group D (data not shown). Biopsy specimens collected before the third injection (day 108) showed that the DNA was barely detectable by Southern blot.

Expression of hBUGT1 mRNA after Injection of Ad-hBUGT1—RT-PCR of RNA extracted from liver biopsy specimens (Fig. 3) showed the expected 321-base pair amplicon of the human BUGT1 mRNA after each injection of Ad-hBUGT1, in Groups A and B, but only after the first injection in Group D.

Expression of Human BUGT1 Protein—Liver biopsy specimens from two rats in each group 14 days after each injection showed immunoreactive 52-kDa bands, corresponding to human bilirubin-UGT1 in the treated Gunn rats in Groups A and B (Fig. 4). In Group D, the protein was expressed only after the first injection.

BUGT Activity—BUGT activity was not detectable in the liver of untreated Gunn rats or Gunn rats that received Ad-LacZ (Group C). Two days and 56 days after the first injection of Ad-hBUGT1 in newborn Gunn rats (Groups A and B), hepatic bilirubin-UGT activities were 80 ± 28 and 27 ± 5.8 nmol/mg liver wet weight-min (mean ± S.E., n = 6), respectively. Ten days after the third injection in Groups A and B, hepatic BUGT activity again increased to 90 ± 25 and 108 ± 45 nmol/mg liver wet weight-min (mean ± S.E., n = 6), respectively. In Group D (rats receiving the first dose as adults), comparable levels of hepatic BUGT activities were detected in the liver 2 and 56 days after the first injection (70 ± 22 and 6.8 ± 0.77 nmol/mg liver wet weight-min (mean ± S.E., n = 6), respectively), but no BUGT activity was detectable after the third injection. Hepatic bilirubin-UGT activities achieved in Gunn rat livers after Ad-hBUGT1 injections were comparable to those in normal Wistar rats (75 ± 20 nmol/mg liver weight-min; mean ± S.E., n = 6).

Pigments Excreted in Bile—HPLC profiles of pigments (Fig. 5) in the bile from rats in Groups A and B 3–4 weeks after each Ad-hBUGT1 injection resembled that in congenic normal Wistar RHA rats. Bilirubin monoglucuronide and diglucuronide constituted 53% and 46% of the pigments, respectively, only 1–1.4% being unconjugated bilirubin. Ad-LacZ-injected rats did not excrete detectable amounts of bilirubin glucuronides in bile, and more than 98% of the pigments was unconjugated bilirubin. In Group D, after the first injection, the bile pigment profile resembled that in Groups A and B, but only minimal amounts of bilirubin glucuronides were excreted after subsequent injections.

Serum Bilirubin Levels—In initial experiments, four newborn Gunn rats were injected with Ad LacZ and four were injected with Ad-hBUGT1 24 h after birth. Four additional newborns were injected with normal saline (control). All groups were killed by exsanguination 48 h after the injection. Serum bilirubin levels, 48 h after the injection, in the control group and the Ad LacZ-injected pups were 9 ± 1.3 and 9.1 ± 1.1 mg/dl (mean ± S.D.), respectively. In the Ad-hBUGT1-injected pups, serum bilirubin concentrations were reduced to 2.0 ± 0.12 mg/dl (Fig. 6).

In Groups A, B, and C (newborn Gunn rat recipients), bilirubin levels were studied every 7–14 days starting on day 21 in the newborn rats. In Group D (adult Gunn rat recipients), serum bilirubin levels were determined from the beginning of the study.
the experimental protocol. Newborn Gunn rats have distinctly yellow skin color within 24 h of birth and are easily distinguishable from normal or heterozygous pups. Twenty-four hours after the injection of Ad-hBUGT1, the skin color of treated Gunn rat pups resembled that of normal pups. The skin color did not change in pups that received Ad-LacZ.

Marked decrease in serum bilirubin levels occurred after each Ad-hBUGT1 injection to Gunn rats, with mean levels reaching as low as 2.02, 1.67, and 1.45 mg/dl after first, second, and third injections respectively (Group A, Fig. 6). Intraportal injections (Group B) reduced serum bilirubin to a similar extent (1.67 and 1.02 mg/dl after second and third injections). Bilirubin levels remained low for 2 months and then increased gradually. In contrast, rats receiving Ad-LacZ (Group C), serum bilirubin levels remained unchanged. Adult Gunn rats (Group D) had a shorter duration of hypobilirubinemic response after the first Ad-hBUGT1 injection, and subsequent injections had no effect on serum bilirubin levels. Ad-hBUGT1 injections did not affect serum bilirubin concentrations in normal Wistar-RHA rats.

Evaluation of Immune Tolerance

Antibody Levels—In adult rats injected with Ad-hBUGT1 (Group D), all recipients developed antibodies during the first month after injection (Fig. 7). Titer of the neutralizing antibodies exceeded 1:4096 in all cases. In contrast, of the newborn recipients, only 25–33% had detectable antibodies, and the antibody titers did not exceed 1:16. The magnitude of the decrease in bilirubin levels after subsequent injections of Ad-hBUGT1 did not differ between the rats that developed low level antibodies and those that did not.

CTL Activity—Cytotoxic T cells were tested against rat hepatocytes infected with adenoviruses four times during the study in Groups A–D. CTL response, as determined by release of ALT into the medium from the responder hepatocytes, remained below 40 IU in all rats that received the initial injection during the newborn period. In contrast, a strong CTL response in the non-tolerized rats receiving the first injection as an adult was shown by ALT concentrations of over 500 IU in the medium (Table II).

Serum ALT Levels—Serum ALT levels increased minimally
the wild-type virus did not increase the serum bilirubin levels, indicating that the transgene was not lost despite the immune response (Table II).

**DISCUSSION**

The Gunn rat is both a genetic and metabolic model of the human Crigler-Najjar syndrome type I (2, 3). A major advantage of the Gunn rat as a model for gene therapy is that the biliary excretion of bilirubin glucuronides are virtually absent, allowing direct and precise quantitation of the metabolic effect of gene transfer of the therapeutic gene. Although a moderate fluctuation of serum bilirubin levels in Gunn rats can occur as a result of a variety of metabolic factors, direct demonstration of bilirubin glucuronide excretion in bile in our experiments constitutes an unequivocal evidence for glucuronidation of bilirubin. After formation in the liver, conjugated bilirubin is normally excreted in bile within minutes. This enabled us to demonstrate that detectable UGT activity toward bilirubin is expressed in Gunn rat livers *in vivo* within 3.5 h of the injection of Ad-hBUGT1.

Clinically useful gene therapy methods will require methods that allow a therapeutic level of gene expression on a long term basis or the ability to achieve prolonged therapeutic effect by repeated administration of the vector. As shown in this study and elsewhere (8–10, 12), recombinant adenoviruses preferentially localized in the liver after systemic administration and transfer genes to quiescent hepatocytes with high efficiency. However, adenoviruses are episomal and persist in the cell for a limited duration. In our experiments, Southern analysis indicated that the reduction of human bilirubin-UGT1 expression paralleled the changes in the transgene content of the liver, suggesting that the lack of gene persistence, rather than “sileencing” of gene expression is involved in the loss of transgene expression. Currently the major obstacles to the application of adenoviral gene therapy are that the development of neutralizing antibodies precludes transgene expression after subsequent injections (7, 13) and CTL response against adenovirally infected cells causes hepatic inflammation after repeated administration of the virus (13). Studies in transgenic or naturally mutant mice with humoral, cellular, or combined deficiency of the immune system indicate both antibodies and CTL are responsible for the loss of transgene expression (22).

Injection of antigens in the neonatal period is known to induce specific tolerance (23). Neonatal allograft tolerance was previously shown to involve several mechanisms, including clonal deletion of antigen-specific T cells, T cell-mediated suppression of T helper cells, and secretion of factors that suppress the generation of antigen-specific effector cells (16, 17, 23–26). Injection of a recombinant adenovirus expressing the coagulation factor IX in a newborn mouse was reported to allow transgene expression after a second injection (14). However, in this study the cell-mediated immunity of the host was not examined, and long term gene therapy by multiple injections of the adenovirus was not attempted. Abrogation of CTL response is particularly important in preventing the hepatic inflammation that occurs after repeated injection of the virus. In the present study, we demonstrate, for the first time, that the initial injection of a recombinant adenovirus in neonatal rats abrogates both humoral and CTL responses against the recombinant adenovirus. This permitted transgene expression after repeated injection of Ad-hBUGT1 in Gunn rats, resulting in prolonged and marked amelioration of jaundice.

Infection with wild-type adenovirus 5 is common, and the consequences of inducing long term tolerance to this virus must be considered. Therefore, we wanted to determine whether rats that are tolerized to the E1-disrupted recombinant adenoviruses are capable of mounting an immune response to wild-
type adenoviruses, which have the E1 region intact. Our results demonstrate that the tolerized rats retain the capability to mount a cell-mediated immune response against the wild-type virus. Although the mechanism of this finding is not established, the results suggest that the anti-E1 immune response may protect tolerized animals when infected naturally with the wild-type virus. Interestingly, this immune response was not associated with a loss of the transgene expression.

Many inherited metabolic disorders are now being diagnosed in utero. Recently, we have identified a Crigler-Najjar type I mutation prenatally by genetic analysis of amniotic cells (27). Therefore, neonatal tolerization to therapeutic recombinant adenoviruses may be a potential clinical benefit. Other methods of inducing tolerance to the recombinant viral vector are also being explored.

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