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Stable delivery of physiologic levels of recombinant erythropoietin to the systemic circulation by intramuscular injection of replication-defective adenovirus

SANDEEP K. TRIPATHY*, EUGENE GOLDWASSER†, MIN-MIN LU‡, ELIAV BARR‡, AND JEFFREY M. LEIDEN*‡

Departments of *Pathology, †Biochemistry and Molecular Biology, and ‡Medicine, University of Chicago, Chicago, IL 60637

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ABSTRACT **A number of inherited and acquired serum protein deficiencies including hemophilias A and B, diabetes mellitus, and the erythropoietin-responsive anemias are currently treated with repeated subcutaneous or intravenous infusions of purified or recombinant proteins. The development of an *in vivo* gene-transfer approach to deliver physiologic levels of recombinant proteins to the systemic circulation would represent a significant advance in the treatment of these disorders. Here we describe the construction of a replication-defective adenovirus (AdEF1hEpo) containing the human erythropoietin (hEpo) cDNA under the transcriptional control of the cellular elongation factor 1α (EF1α) promoter and the 4F2 heavy chain (4F2HC) enhancer. Neonatal CD-1 and adult SCID mice injected once intramuscularly (i.m.) with 10^7 to 10^9 plaque-forming units (pfu) of this virus displayed significant dose-dependent elevations of serum hEpo levels and increased hematocrits, which were stable over the 4-month time course of these experiments. Adenovirus injected i.m. remained localized at the site of injection and there was no evidence of either systemic infection or a localized inflammatory response. These results suggest that i.m. injection of recombinant replication-defective adenovirus vectors may serve as a paradigm for the treatment of human serum protein deficiencies.**

Erythropoietin (Epo) is a 30-kDa glycoprotein synthesized in adult renal cells that functions as the major regulator of mammalian erythropoiesis (1). Serum human Epo (hEpo) levels are controlled transcriptionally in response to tissue hypoxia or anemia (2, 3). The secreted protein increases erythrocyte production by stimulating the proliferation and preventing the apoptosis of erythroid precursors including erythroid colony-forming units and erythroid blast-forming units (4). Most patients with end-stage renal disease treated with hemodialysis (5) and many patients with human immunodeficiency virus treated with 3′-azido-3′-deoxythymidine (6) suffer from severe anemias resulting from inappropriately low levels of serum hEpo. These patients are currently treated with repeated (two or three times per week) subcutaneous or intravenous infusions of recombinant hEpo (5, 6). The Epo-responsive anemias represent an excellent model system for studies designed to develop an *in vivo* gene transfer approach for the treatment of serum protein deficiencies. The hEpo gene (7) and cDNA (8) have been cloned and hEpo functions in multiple mammalian species including mice (9). There is a sensitive ELISA assay that can be used to distinguish hEpo from murine Epo (10). Most importantly, the hematocrit is an easily measured physiologic assay of serum Epo levels (9).

Previous studies have demonstrated that skeletal myoblasts, genetically engineered to produce secreted proteins *in vitro*, can be used to stably deliver physiologic levels of recombinant proteins to the systemic circulation after intramuscular implantation (11–15). Although effective, such an *ex vivo* gene therapy approach is both labor intensive and expensive because it requires the isolation, growth, and transfection of primary human myoblasts from each patient to be treated. Moreover, recent clinical trials in patients with Duchenne muscular dystrophy have suggested that myoblast implantation may be less efficient in human muscle than in murine muscle (16–18). The development of an *in vivo* gene transfer approach to stably deliver recombinant proteins to the systemic circulation would be an important advance in our ability to treat human serum protein deficiencies.

Replication-defective adenoviruses represent an efficient and safe method of *in vivo* gene transfer. These vectors can be prepared at high titer [up to 10^{11} plaque-forming units (pfu)/ml] and infect many replicating and nonreplicating cell types *in vivo* (17–24). Adenoviruses are common and relatively benign human pathogens that have not been associated with persistent infections or neoplasias in humans (19). Wild-type adenoviruses have been used previously for human vaccination (25). In the studies described in this report, we demonstrate that a single i.m. injection of a replication-defective adenovirus encoding hEpo can be used to produce dose-dependent elevations in serum Epo levels and hematocrits that were stable over the 120-day time course of these experiments. The injected adenovirus remains localized at the site of administration and does not cause muscle pathology. Thus, these results suggest that i.m. injection of replication-defective adenoviruses will be useful for the treatment of a number of acquired and inherited human serum protein deficiencies.

MATERIALS AND METHODS

Adenovirus Vectors. The polyadenylylation site from the bovine growth hormone gene (26) was cloned into the *Bgl* II site of pAdBglII (20). The multiple cloning site from Bluescript II KS (Stratagene) was then cloned into the regenerated *Bgl* II site followed by insertion of the elongation factor 1α (EF1α) promoter (bp −203 to +986) into the *Cla* I site of the polylinker. The 480-bp enhancer from the first intron of the human 4F2 heavy chain gene (bp 535–1015) (27) was then cloned into the *Not* I site of the polylinker. Finally, the 816-bp *Bst*EII–*Bgl* II fragment containing the human Epo cDNA from pHU13 (28) (generously provided by Amgen Biologicals) was cloned into the *Eco*RV and *Bam*HI sites of the polylinker. The resulting plasmid (pAdEF1hEpo) was cotransfected into 293 cells (20) with *Xba* I/*Cla* I-digested Ad5sub360 wild-type adenovirus DNA by using calcium phosphate (20) to generate the AdEF1hEpo adenovirus. The virus was plaque-purified three times and subjected to struc-

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Abbreviations: pfu, plaque-forming unit; hEpo, human erythropoietin; mU, milliunit(s); EF1α, elongation factor 1α; SCID, severe combined immunodeficiency.

tural analysis using the PCR and restriction enzyme digestion. The AdβAc.lacZ virus has been described (29).

Isolation and Infection of Primary Human Myoblasts. Primary human myoblasts isolated from fetal muscle (30) were plated at a density of 10^6 cells per 75-cm^2 plate, allowed to fuse into myotubes for 3 days, and infected for 2 h at 37°C with AdEF1hEpo or the AdβAc.lacZ control virus at 5 pfu per cell. After infection, the cultures were overlaid with 10 ml of serum-free medium (OPTI-MEM I; GIBCO).

Western Blot Analysis. Culture supernatants from primary human myotubes infected with AdEF1hEpo (5 pfu per cell) were concentrated 20-fold using a Centricon-10 filter (Amicon). An aliquot of the concentrated supernatant was denatured and subjected to digestion with 0.5 unit of N-Glycanase (Genzyme) for 18 h at 37°C according to the manufacturer's suggested protocol. Untreated and N-Glycanase-treated myotube culture supernatants were fractionated by SDS/PAGE, transferred to nitrocellulose, and subjected to Western blot analysis using a commercially available polyclonal anti-hEpo antibody (R & D Systems).

Injections i.m. of Replication-Defective Adenovirus. High titer (2–5 × 10^{10} pfu/ml) stocks of AdEF1hEpo and AdβAc.lacZ were produced by infection of 293 cells followed by discontinuous CsCl density gradient centrifugation as described (20). Neonatal (day 2–4) CD-1 or adult severe combined immunodeficiency (SCID) mice were injected i.m. once with 10^6 to 10^9 pfu of virus in 50 μl of Hepes-buffered saline. All animal experimentation was performed in accordance with National Institutes of Health guidelines in the A. J. Carlson Animal Research Facility of the University of Chicago.

Histological Analyses. *LacZ* expression was detected by staining of glutaraldehyde-fixed tissues with 5-bromo-4-chloro-3-indolyl β-D-galactoside as described (20).

Epo Assays. hEpo levels were measured in serum, tissue culture supernatants, and tissue lysates using a commercially available assay (R & D Systems). hEpo bioassays were performed using the hEpo-dependent cell line UT-7/Epo, as described (31).

PCR Assay. One microgram of genomic DNA prepared as described (20) was subjected to 35 cycles of the PCR using primers complementary to sequences within the EF1α promoter (sense primer, GTCACCCACACAAAGGAAAAGGGCC) and the hEpo cDNA (antisense primer, GCTGCAGTGTTCAGCACAGCCCG) (20). PCR products were fractionated by electrophoresis in 1.5% agarose gels, transferred to nitrocellulose, and analyzed by Southern blot hybridization using a ^{32}P-labeled 350-bp *Sac* I–*Cla* I fragment from pAdEF1hEpo (20).

RESULTS

To test the feasibility of using i.m. injection of replication-defective adenoviruses to deliver physiologic levels of recombinant proteins to the systemic circulation, we constructed a replication-defective adenovirus (AdEF1hEpo) containing the hEpo cDNA under the transcriptional control of the ubiquitously active cellular EF1α promoter and the 4F2HC enhancer (Fig. 1) (27, 32). To assess the ability of this virus to program hEpo synthesis and secretion after infection of terminally differentiated muscle cells *in vitro*, we infected cultured primary human myotubes with AdEF1hEpo at a multiplicity of infection of 5 pfu per cell. Control cultures of myotubes were injected with AdβAc.lacZ (5 pfu per cell) (29), which contains the bacterial *LacZ* gene under the control of the chicken β-actin promoter and the cytomegalovirus enhancer. Culture supernatants from the infected myotubes were assayed for hEpo by ELISA. Myotubes infected with AdEF1hEpo produced and secreted 980 ± 96 milliunits (mU) of hEpo per 24 h (Fig. 2*A*). In contrast, only

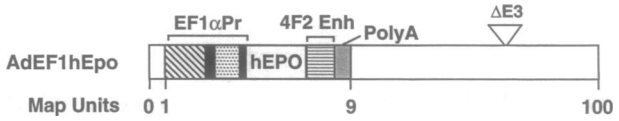
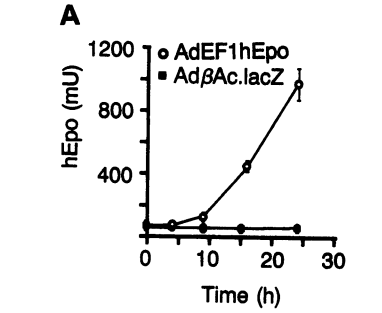
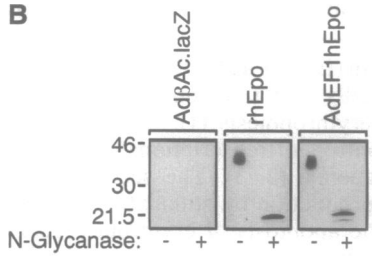


FIG. 1. Schematic illustration of the replication-defective AdEF1hEpo adenovirus. This virus was derived from adenovirus serotype 5 and lacks both the E1 (map units 1–9) and E3 (map units 78.5–84.7) (ΔE3) regions of the viral genome. The E1 region has been replaced by the 5′ end of the EF1α gene (EF1α Pr) (32), which contains the promoter (hatched box), exon 1 (solid box), intron 1 (stippled box), and 10 bp of exon 2 (solid box); the hEpo cDNA (8); the 4F2 heavy chain first intron enhancer (4F2 Enh) (27); and the bovine growth hormone polyadenylylation site (poly A) (26). Adenovirus map units are shown below the map.

background levels of hEpo were detected in culture supernatants from the AdβAc.lacZ-infected myotubes.

Mature hEpo contains three N-linked and one O-linked oligosaccharide chains (33, 34). N-linked glycosylation is important for both the solubility and stability of the protein *in vivo* (35). To determine whether hEpo synthesized after infection of primary human myotubes is appropriately glycosylated, culture supernatants from AdEF1hEpo-infected myoblasts were assayed by Western blot analysis before and





C

Assay	Activity (mU/ml)
ELISA	1110 ± 50
Bioassay	1150 ± 120

FIG. 2. hEpo production by AdEF1hEpo-infected primary human myotubes. (*A*) Time course of hEpo secretion after AdEF1hEpo infection of cultured primary human myotubes. The results are expressed as the total hEpo production per 75-cm^2 plate of cells at each time point. Each point represents the mean ± SEM of two duplicate plates that were each assayed in duplicate at each time point. (*B*) Western blot analysis of hEpo secreted by AdEF1hEpo-infected primary human myotubes. Untreated and N-Glycanase-treated culture supernatants from primary human myoblasts infected with either AdEF1hEpo or AdβAc.lacZ, along with 5 units of commercially available recombinant hEpo (rhEpo), were subjected to Western blot analysis using a commercially available polyclonal anti-hEpo antibody. (*C*) Bioassay of hEpo produced by AdEF1-hEpo-infected primary human myotubes. Primary human myotubes were infected with AdEF1hEpo at 5 pfu per cell as described in *A*. Twenty-four hours after infection, culture supernatants were assayed for hEpo activity by using a commercially available ELISA or a bioassay using the hEpo-dependent cell line UT-7/Epo (31).

after treatment with N-Glycanase to remove N-linked oligosaccharide chains (Fig. 2*B*). Prior to N-Glycanase treatment, hEpo from AdEF1hEpo-infected myotube culture supernatants displayed an electrophoretic mobility identical to that of fully glycosylated commercially available recombinant hEpo (Amgen Biologicals) (≈34 kDa). After treatment with N-Glycanase, both the commercially available recombinant hEpo and the hEpo from myotube supernatants displayed reductions in molecular mass to ≈19.5 kDa (Fig. 2*B*). These results suggested that primary human myotubes are capable of producing hEpo containing appropriate N-linked oligosaccharide chains. To assess the bioactivity of the hEpo secreted by AdEF1hEpo-infected primary human myotubes directly, we compared the specific activity of the hEpo in muscle supernatants as determined by ELISA with that determined by bioassay on the hEpo-dependent cell line UT-7/Epo (31) (Fig. 2*C*). Both assays demonstrated Epo levels of ≈1100 mU/ml, confirming that the hEpo produced by the adenovirus-infected muscle cells was fully bioactive.

To determine whether i.m. injection of AdEF1hEpo could be used to produce physiologically significant levels of serum hEpo *in vivo*, neonatal CD-1 mice were injected once i.m. with 10^6 to 10^8 pfu of AdEF1hEpo. Control mice were injected i.m. with 10^8 pfu of AdβAc.lacZ. Hematocrits were measured immediately prior to and 30–120 days after injection (Fig. 3*A*). Mice injected with AdEF1hEpo showed dose-dependent increases in hematocrits from baseline values of 35% to peak values of 71%. These elevations in hematocrits were both significantly different from preinjection hematocrits ($P < 0.0001$) and significantly elevated as compared to those observed in the control animals injected with AdβAc.lacZ ($P < 0.0001$). Most importantly, the observed elevations in hematocrits were stable over the 120-day duration of the study.

To test the feasibility of using i.m. injection of adenovirus to deliver hEpo to the systemic circulation of adult animals, 8-week-old SCID mice were injected once i.m. with 10^7 to 10^9 pfu of AdEF1hEpo. Control mice received a single injection of 10^9 pfu of AdβAc.lacZ. SCID mice were used in these experiments to avoid potential murine immune responses to either the hEpo or adenoviral proteins. Similar to the results obtained with neonatally injected animals (Fig. 3*A*), i.m. injections of AdEF1hEpo produced statistically significant dose-dependent increases in hematocrits to peak values of ≈85% in the adult SCID mice (Fig. 3*B*). These increases were also stable over the 120-day time course of the experiment.

To confirm that the changes in hematocrits observed in the AdEF1hEpo-injected animals reflected increases in serum hEpo levels, we measured hEpo levels in the serum of AdEF1hEpo- and AdβAc.lacZ-injected mice 90 days after injection (Fig. 3*C*). Mice injected with 10^7 pfu of AdEF1hEpo had serum hEpo levels of 15 ± 3 mU/ml while mice injected with 10^8 pfu of AdEF1hEpo had serum hEpo levels of 630 ± 260 mU/ml. In contrast, the AdβAc.lacZ-injected control animals had serum hEpo levels of 2 ± 0.1 mU/ml as measured using the same ELISA. Thus, the dose-dependent elevations in hematocrits seen in the AdEF1hEpo-injected animals correlated with dose-dependent increases in serum hEpo levels.

Previous studies using intravascular injections of replication-defective adenoviruses have demonstrated infection of multiple organs including liver, heart, lung, and brain (20, 21). To determine whether the i.m.-injected AdEF1hEpo remained localized to the site of injection and to exclude the possibility of productive infection of the neonatally injected mice, we analyzed tissue extracts from the AdEF1hEpo-injected mice for the presence of adenovirus DNA by using a PCR-based assay (Fig. 4). Mixing experiments demonstrated that this assay could detect one copy of the adenovirus genome in 10^5 cells (Fig. 4*A*). AdEF1hEpo DNA was detected by PCR in the injected muscle but was not seen in brain, lung, heart, liver, spleen, kidney, gonads, or uninjected muscle from these animals 90 days after injection. In additional experiments (data not shown), we assayed hEpo levels in tissue homogenates from mice 90 days after a single i.m. injection of AdEF1hEpo. Both serum and injected muscle from the AdEF1hEpo-injected animals showed significant elevations in hEpo compared to control values. In contrast, hEpo levels in uninjected muscle from the contralateral limb, lung, heart, liver, spleen, and kidney were identical in treated and control animals. Thus, we concluded that the elevations in serum hEpo levels observed in these mice reflected recombinant gene expression that was limited to the site of i.m. injection.

To determine directly which cell types were responsible for recombinant gene expression after i.m. injection of replication-defective adenovirus, neonatal mice were injected once i.m. with the AdβAc.lacZ virus and recombinant *LacZ* gene expression was assayed histologically 7, 30, and 90 days after injection (Fig. 4 *B–G*). Stable *LacZ* gene expression, manifested as blue staining, was observed in myotubes at each time point assayed. Of note, there was no evidence of an inflammatory response in any of the sections during the

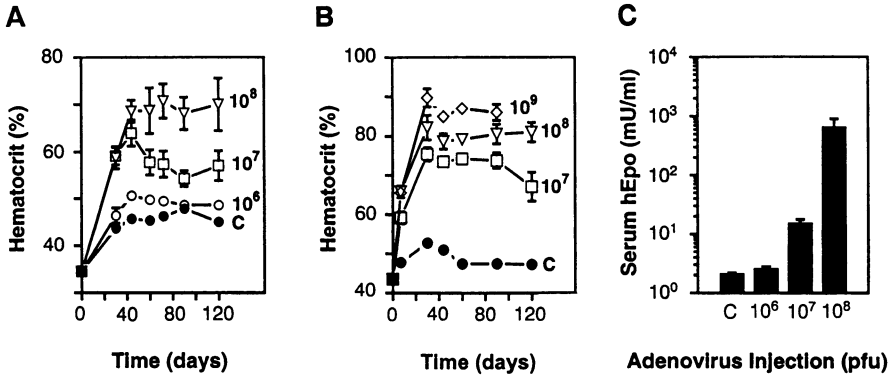


FIG. 3. Hematocrits and serum hEpo levels after i.m. injection of neonatal CD-1 and adult SCID mice with AdEF1hEpo. (*A* and *C*) Four-day-old CD-1 mice were injected once i.m. into hind limb muscle with 10^6 ($n = 10$), 10^7 ($n = 9$), or 10^8 ($n = 9$) pfu of AdEF1hEpo (open symbols) or 10^8 pfu of the AdβAc.lacZ control virus (solid circles) ($n = 10$). Hematocrits (*A*) were measured by centrifugation of blood obtained from tail veins at the times indicated. Serum hEpo levels (*C*) were measured 90 days after injection by using a commercially available ELISA (R & D Systems) ($n = 4$ to 6 for each experimental group). The results are presented as mean ± SEM. (*B*) Eight-week-old SCID mice ($n = 3$ to 8) were injected once i.m. with 10^7 to 10^9 pfu of AdEF1hEpo (open symbols) or 10^9 pfu of AdβAc.lacZ control virus (solid circles). Hematocrits were measured by centrifugation of blood obtained from tail veins at the times indicated.

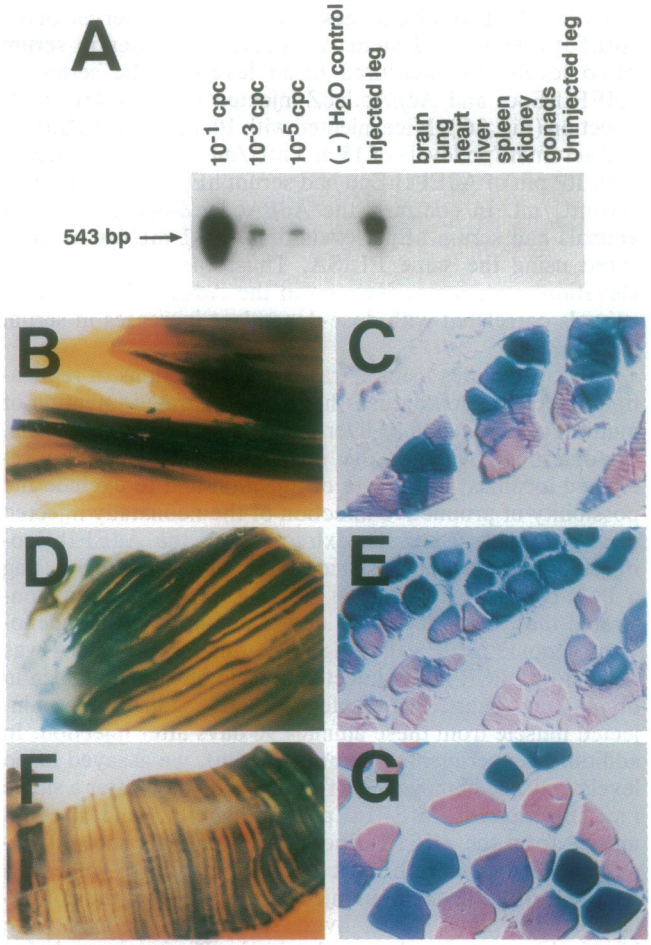


FIG. 4. Localization of adenovirus after i.m. injection of neonatal mice. (*A*) Distribution of AdEF1hEpo DNA in mice after i.m. injection with AdEF1hEpo. Four-day-old CD-1 mice were injected once i.m. in hind limb muscle with 10^8 pfu of AdEF1hEpo. Ninety days after injection, mice were sacrificed and organ lysates were assayed for AdEF1hEpo DNA using the PCR. In control experiments, purified AdEF1hEpo plasmid DNA corresponding to 0.1, 0.001, and 0.00001 copy per cell (cpc) was added to uninfected cell DNA and amplified in parallel. The size of the expected PCR product is shown by the arrow. (*B–G*) Histologic analysis of recombinant gene expression after i.m. injection of AdβAc.lacZ. One- to 4-day-old CD-1 mice were injected once i.m. into hind limb muscle with 10^8 pfu of AdβAc.lacZ. Mice were sacrificed at 7 days (*B* and *C*), 30 days (*D* and *E*), and 90 days (*F* and *G*) after injection and the injected muscle was assayed for *lacZ* expression as described (20). Photographs of gross muscle samples (*B*, *D*, and *F*) were taken on a Wild M3Z dissecting microscope (×18–30). Photomicrographs of muscle sections (*C*, *E*, and *G*) were taken on a Leitz Fluovert FU microscope using diffraction interference contrast optics. (*C*, ×292; *E*, ×146; *G*, ×146.)

90-day time course of the experiment. These results confirmed the hypothesis that i.m. injection of neonatal mice with replication-defective adenoviruses results in the stable expression of recombinant gene products in myotubes *in vivo*.

DISCUSSION

In the studies described in this report, we have demonstrated that a single i.m. injection of relatively small amounts of a replication-defective adenovirus encoding a secreted protein under the transcriptional control of a potent cellular promoter and enhancer can be used to stably deliver physiological levels of this recombinant protein to the systemic circulation of mice. Of equal importance, we have shown that the replication-defective adenovirus remains localized at the site of i.m. injection and does not cause systemic infection or localized inflammation. Our results demonstrate a clear dose–response relationship between the amount of adenovirus injected i.m. and both the serum hEpo levels and the elevations in hematocrits observed over the 120-day time course of these experiments.

Several previous reports have described *ex vivo* and *in vivo* gene transfer approaches for delivering secreted proteins to the systemic circulation. These include the i.m. implantation of genetically modified myoblasts (11–15) and the intravascular administration of replication-defective retroviruses (36, 37) and adenoviruses (38, 39). The i.m. injection of replication-defective adenoviruses, as described in this report, displays several advantages as compared to these previously described approaches. First, i.m. injections of adenovirus are less labor intensive and significantly more efficient than the implantation of genetically modified myoblasts. It should be emphasized that the experiments described in this report employed a single i.m. injection equivalent to 0.1–10.0 μl of high-titer adenovirus to produce physiologically significant increases in hematocrits of mice. By extrapolation, we estimate that the successful treatment of the Epo-responsive anemia of renal failure in humans would require a single i.m. injection of 3.5×10^9 pfu of AdEF1hEpo or 0.1–0.2 ml of high-titer virus. Moreover, the observed dose–response relationship between virus dose and hematocrit suggests that it would be possible to program predictable increases in human hematocrits by i.m. injection of graded doses of AdEF1hEpo. The i.m. injection of adenoviruses would also appear to be superior to intravascular administration of these vectors in that i.m. injection avoids systemic and hepatic infection. Moreover, after i.m. injection, the adenovirus-infected muscle cells could be removed by simple surgical excision. This would be difficult, if not impossible, after intravascular administration. Finally, as opposed to replication-defective retroviruses, adenoviruses can be prepared in much higher titer, infect nonreplicating cell types such as skeletal myotubes *in vivo*, and do not integrate into the host genome (19), thereby reducing the risk of malignant transformation of the infected cells.

The studies described in this report demonstrate long-term recombinant gene expression after i.m. injection of replication-defective adenoviruses into adult immunocompromised and normal neonatal mice. Previous studies have suggested that recombinant gene expression programmed by similar E1-deleted replication-defective adenoviruses may be transient in adult immunocompetent hosts, presumably as a result of an immune response directed against adenoviral or recombinant proteins (19–24, 38–40). In agreement with these results, our preliminary studies have demonstrated transient elevations in hematocrits in adult CD-1 mice after i.m. injection with AdEF1hEpo. However, ongoing studies in adult animals using other adenovirus vectors and immunosuppressive strategies may circumvent this host immune response and thereby allow long-term recombinant gene expression in adult animals similar to that observed by us and others (21, 39) in neonates. Nevertheless, our results demonstrate that i.m.-injected adenovirus can be used to produce stable and physiological levels of recombinant proteins in the circulation of adult animals. Thus, they suggest that it may ultimately be possible to use i.m. injection of adenovirus vectors to treat inherited human serum protein deficiencies.

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