HUMAN GENE THERAPY 15:405–413 (April 2004)

Brief Report

Comparison of Adenoviral and Adeno-Associated Viral Vectors for Pancreatic Gene Delivery *In Vivo*

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ABSTRACT

Although effective gene therapy vectors have been developed for organ systems such as the liver, an effective delivery vector to the pancreas *in vivo* has remained elusive. Of the currently available viral vectors, adenovirus and adeno-associated virus (AAV) are two of the most efficient at transducing nondividing cells. We have constructed recombinant adenovirus (AdVLacZ), adeno-associated virus serotype 2 (AAV2LacZ), and pseudotyped adeno-associated virus serotype 5 and 8 (AAV5LacZ, AAV8LacZ) carrying the *LacZ* reporter, and compared the transduction efficiency of these four vectors in the pancreas of mice *in vivo*. We showed that adenovirus, AAV2, and AAV8 are capable of transducing the pancreas *in vivo*, but with different expression kinetics, efficiencies of transduction, and persistence. AdVLacZ-transduced pancreas exhibited maximum LacZ expression at 1 week postdelivery, with greater than 90% of expression lost at 4 weeks. AAV2LacZ-transduced pancreas displayed peak LacZ levels at 4 weeks postdelivery, with no significant decrease in expression for up to 8 weeks. AAV8LacZ was at least 10-fold more efficient than AAV2LacZ in transducing the pancreas *in vivo*, with significant levels of expression detectable at 1 week, whereas AAV5LacZ did not result in any detectable transgene expression at all tested time points. All three vectors primarily transduced pancreatic acinar cell types, with limited transduction of pancreatic endocrine cells. AdVLacZ elicited a significant leukocyte infiltration early after delivery into the pancreas, whereas none of the AAV vectors elicited a significant leukocyte response. None of the tested vectors caused significant changes in serum amylase or blood glucose levels, suggesting that they do not significantly alter pancreatic function. These vectors will be useful for studying novel gene delivery based treatments in animal models for diabetes and other pancreatic disorders.

INTRODUCTION

THE PANCREAS is a complex organ that serves many critical endocrine and exocrine functions crucial for survival (Edlund, 2002). Common maladies of the pancreas include diabetes mellitus, pancreatitis, and pancreatic cancer. No effective long-term therapies are available for any of these. Viral-mediated gene delivery is currently the most efficient gene transfer method, and of these viral vectors, recombinant adenovirus (AdV) is probably the most efficient, and has been shown to transduce a variety of organ systems, including the liver, skeletal muscle, heart, brain, and lung (Breyer *et al.*, 2001). In mouse and rat pancreas, adenovirus has been shown to efficiently transduce pancreatic cells *in vivo*, but expression of the transgene is transient, and the viral proteins elicit a potent immune response (Raper and Dematteo, 1996; McClane *et al.*, 1997a). In addition, administration of AdV to the mouse pancreas *in vivo* results in significantly decreased intracellular amylase levels and associated histologic damage, suggesting that the vector is quite toxic to the organ (McClane *et al.*, 1997b).

Adeno-associated virus (AAV) is a single-stranded DNA virus, which unlike adenoviral vectors, does not elicit a significant inflammatory response, making it a particularly promising gene delivery vector (Snyder, 1999; Monahan and Samul-

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ski, 2000; Zaiss *et al.*, 2002; Stilwell and Samulski, 2003). AAV has been shown to efficiently transduce a variety of cell types *in vivo*, including liver, muscle, and lung, resulting in long-term gene expression from these tissues (Carter and Samulski, 2000). *Ex vivo*, AAV has been shown to transduce pancreatic endocrine cells (Flotte *et al.*, 2001), but successful transduction of the pancreas by AAV has not been demonstrated *in vivo*. It has been demonstrated that *ex vivo* viral transduction efficiencies do not always correlate with *in vivo* efficiencies (Kay *et al.*, 1992; Snyder *et al.*, 1997; Park *et al.*, 2000), so we wanted to determine whether AAV could also efficiently and safely transduce pancreatic cells *in vivo*. Several novel serotypes of AAV have been identified, each which exhibit different structural and functional characteristics (Rutledge *et al.*, 1998; Gao *et al.*, 2002; Grimm and Kay, 2003a). Recent studies have shown that the different serotypes of AAV have different cellular specificities, and in particular, AAV serotype 5 was shown to transduce human pancreatic islets *ex vivo* with a much higher efficiency than AAV serotype 2 (Flotte *et al.*, 2001). We also wanted to determine if different serotypes of AAV could result in greater efficiency transduction of the pancreas *in vivo*.

MATERIALS AND METHODS

Recombinant viruses

The LacZ expression cassette (consisting of a Rous Sarcoma virus [RSV] promoter driving the expression of an *Escherichia coli* β-galactosidase gene with a nuclear localization signal, flanked by a bovine growth hormone polyadenylation site) was excised from plasmid pRSVnlsLacZ using *Bgl*II, and cloned into the *Bgl*II site of the pΔE1 adenovirus shuttle plasmid, or blunt-ended and cloned into the *Not*I site of the pAAV shuttle plasmid. AdVLacZ was amplified in 293 cells and subsequently purified in a cesium chloride (CsCl) density gradient as previously described (Kay *et al.*, 1994). AAV2LacZ (AAV serotype 2) was produced by the triple-transfection method in 293 cells, and purified using a high-performance liquid chromatography (HPLC) method as described earlier (Nakai *et al.*, 2000). Pseudotyped AAV5LacZ and AAV8 LacZ were produced by cross-packaging the AAV2 vector genome (containing the ITRs/LacZ expression cassette) into AAV5 or AAV8 capsids by cotransfection of the pAAV plasmid with DF5 (containing AAV2 Rep, AAV5 capsid, and adenovirus helper genes) or triple transfection of pAAV with R2C8 (containing AAV2 Rep and AAV8 capsid genes) and plAd5 (containing adenovirus helper genes) plasmids (Gao *et al.*, 2002; Grimm *et al.*, 2003b). The viruses were purified using CsCl density centrifugation. AdVLacZ was titered by a plaque-forming assay, whereas AAV2LacZ, AAV5LacZ, and AAV8LacZ were titered by a quantitative densitometric dot-blot assay. To confirm the expression of the LacZ transgene from the two recombinant viruses, 293 cells were transduced with 1×10^8 AdVLacZ and 1×10^8 AAV2LacZ, and stained with X-gal 2 days posttransduction. Positive LacZ expression was observed in AdVLacZ-, AAV2LacZ-, AAV5-LacZ-, and AAV8LacZ-transduced 293 cells.

Surgical procedures

All animal procedures were done according to the guidelines for animal care at Stanford University (Stanford, CA). Eight- to 12-week-old male C57Bl/6 mice were anesthetized using a single intraperitoneal injection of a mixture of ketamine, acepromazine, and xylazine suspended in phosphate-buffered saline (PBS). Mice were subjected to laparatomy, and injected with 100 μl of purified virus solution suspended in PBS using a 26-gauge needle into a single location in the splenic lobe of the pancreas. Laparotomies were subsequently closed with a two-layer suture. Mice were then sacrificed at various days postinjection, and pancreatic tissue was harvested for X-gal staining and immunohistochemistry.

Tissue embedding and X-gal staining

Pancreatic tissue was washed in PBS, and placed in optimal cutting temperature (OCT) compound and immediately frozen on dry ice. Ten-micrometer sections were then cut on a cryostat and placed on VWR Superfrost plus microscope slides (Erie Scientific Company, Portsmouth, NH). Tissue sections were fixed in 1.25% glutaraldehyde in PBS for 10 min and rinsed 3 times in PBS prior to staining. Slides were then placed in X-Gal solution (1 mg/ml X-gal, 5 m*M* $K_3Fe(CN)_6$, 5 m*M* $K_4Fe(CN)_6$, and 1 m*M* $MgCl_2$ in PBS) and incubated overnight at 37°C. Slides were then washed three times in PBS, and then counterstained with hematoxylin.

Immunohistochemistry

Rat anti-mouse CD45 antibodies were obtained from BD Pharmingen (San Diego, CA). Slides were processed using the Vectastain ABC kit (Vector Labs, Burlingame, CA). Pancreas sections were blocked with 10% normal goat serum and avidin for 30 min, and then incubated with 1:250 dilution of primary rat anti-mouse CD45 antibody in PBS/1% bovine serum albumin (BSA)/10% goat serum and biotin for 2 hr. Subsequently, the slides were then washed 3 times in PBS, and biotinylated mouse anti-IgG2 secondary antibody diluted 1:500 in PBS/1% BSA/10% goat serum was applied for 30 min. Slides were washed 3 times in PBS, and then incubated with the ABC reagent for 30 min. The sections were thoroughly washed, and incubated with 0.06% 3,3′-diaminobenzidine and 0.01% H_2O_2 for 5 min, and counterstained with hematoxylin.

Monitoring changes in pancreatic function

Whole blood and sera from mice injected with saline, AdVLacZ, AAV2LacZ, AAV5LacZ, and AAV8LacZ were collected at various days postinjection by retro-orbital eye bleeds. Glucose levels were measured using a Glucometer Elite glucometer (Bayer Healthcare, Pittsburgh, PA) and Ascensia Elite blood glucose test strips (Bayer Healthcare). Amylase levels were measured from the collected sera samples by the Stanford University Department of Comparative Medicine Diagnostic Laboratory.

RESULTS AND DISCUSSION

Transduction of mouse pancreas by AdV and AAV

To compare the transduction efficiencies of AdV and AAV in the pancreas, we delivered 1×10^8 plaque-forming units (pfu) AdVLacZ and 1×10^{11} particles AAV2LacZ, AAV5LacZ, or AAV8LacZ by direct injection into the splenic lobe of the

pancreas of 8- to 12-week-old C57Bl/6 mice. Successful administration was confirmed by a uniform swelling of the injected area. The majority of the mice survived this operation with no observed complications, and histologic examination of mouse pancreas injected in this manner showed no significant inflammatory or mechanical lesions. Mice were sacrificed at 1, 2, 4, and 8 weeks postinjection ($n = 3$ or 4 per group), and their pancreas harvested for X-gal staining and immunohistochemistry.

Significant LacZ expression was detected in the pancreas of mice injected with AdVLacZ starting at 1 week postinjection, with expression decreasing over 99% by 4 weeks (Figs. 1 and 2). In contrast, immunodeficient C57Bl/6 SCID mice injected intrapancreatically with 1×10^8 pfu of AdVLacZ displayed increased persistence of gene expression, with significant LacZ expression detectable for at least 4 weeks (Fig. 2), in agreement with previously published results (McClane *et al.*, 1997a). In

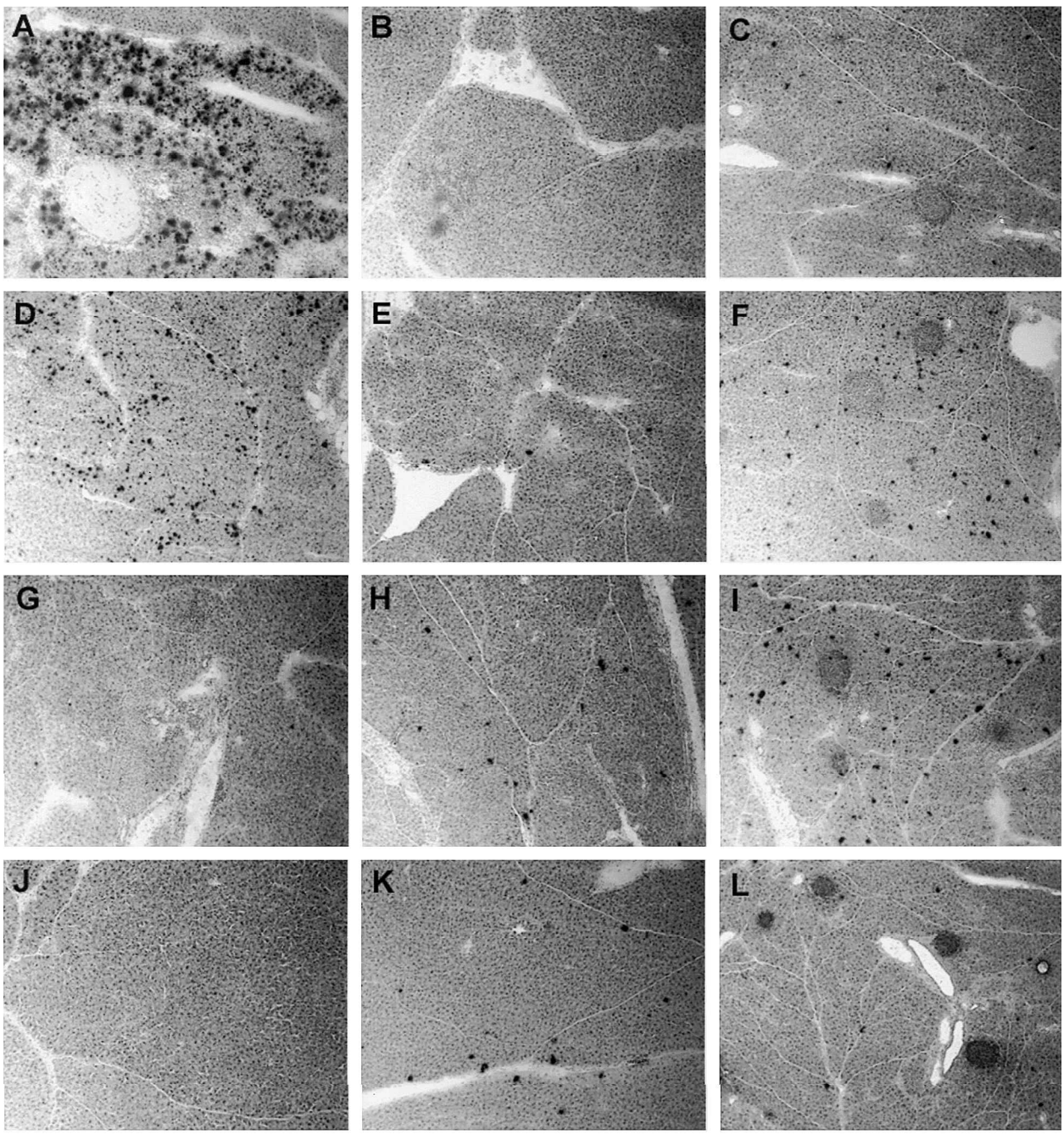

FIG. 1. Pancreas sections from C57Bl/6 mice injected with 1×10^8 plaque-forming units (pfu) AdVLacZ, 1×10^{11} particles AAV2LacZ, or 1×10^{11} particles AAV8LacZ stained with X-gal at various days postinjection. AdVLacZ-injected mouse pancreas at 7 days (**A**), 14 days (**D**), 28 days (**G**), or 56 days (**J**) postinjection. AAV2LacZ-injected mouse pancreas at 7 days (**B**), 14 days (**E**), 28 days (**H**), or 56 days (**K**) postinjection. AAV8LacZ-injected mouse pancreas at 7 days (**C**), 14 days (**F**), 28 days (**I**), or 56 days (**L**) postinjection. Magnification ×10.

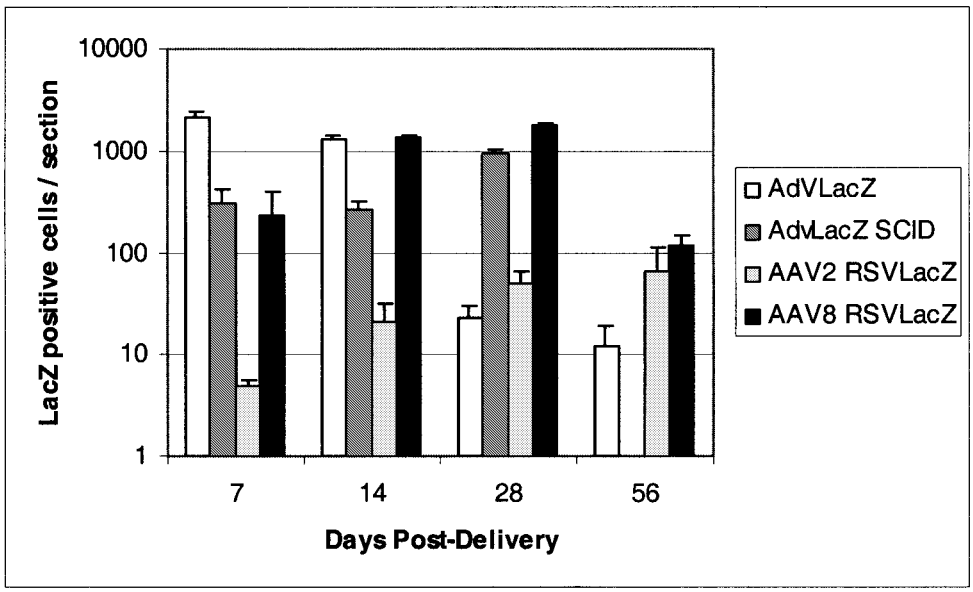

FIG. 2. Quantitation of the number of LacZ-positive cells/slide obtained by AdVLacZ, AAV2LacZ, or AAV8LacZ transduction of the pancreas in C57Bl/6 mice or by AdVLacZ in C57Bl/6 SCID mice. The number of LacZ-positive cells were determined by counting the total number of LacZ expressing cells per section (n = 4/mouse) and averaging the number of cells counted between the different mice (n = 3 or 4 mice per time point).

addition, LacZ expression was primarily concentrated in a small area near the area of injection, with much of the pancreas left untransduced. Transduction of other tissues (liver, spleen) was not observed.

LacZ expression was detectable in the pancreas at 7 days postdelivery in mice injected with AAV2LacZ, but expression did not peak until 4 weeks postdelivery. The total number of cells transduced by AdVLacZ was at least 10-fold higher than the number of cells transduced by AAV2LacZ at the time points of maximum expression. AAV8LacZ transduced pancreas exhibited at least 10-fold higher levels of expression compared to AAV2LacZ, and significant levels of expression were detectable beginning at 1 week postinjection. The more rapid onset of expression from AAV8 is likely caused by the more rapid

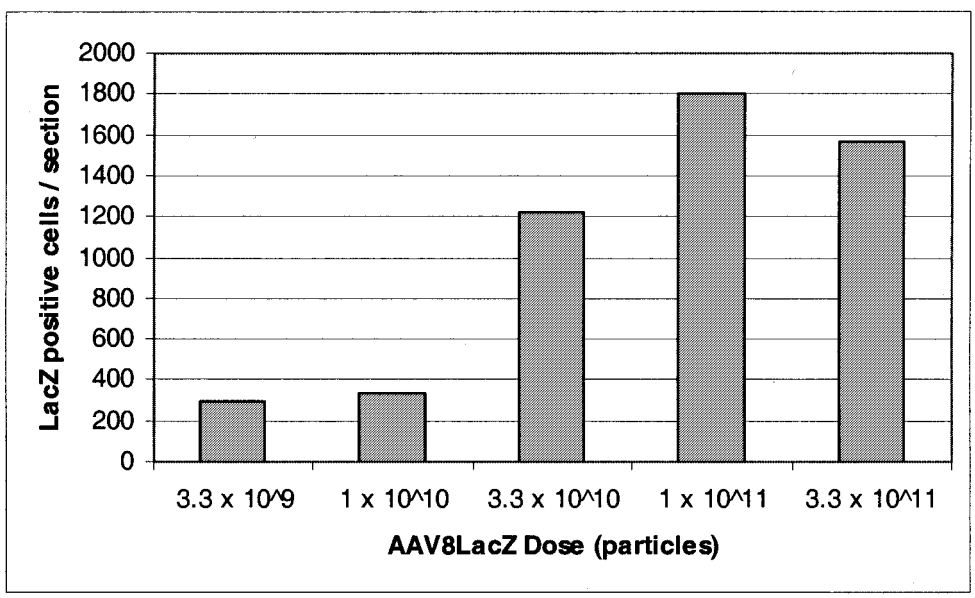

FIG. 3. Dose response of 5 different titers of AAV8LacZ (3.3×10^9, 1×10^{10}, 3.3×10^{10}, 1×10^{11}, 3.3×10^{11} particles) administered intrapancreatically to C57Bl/6 mice at 28 days postinjection.

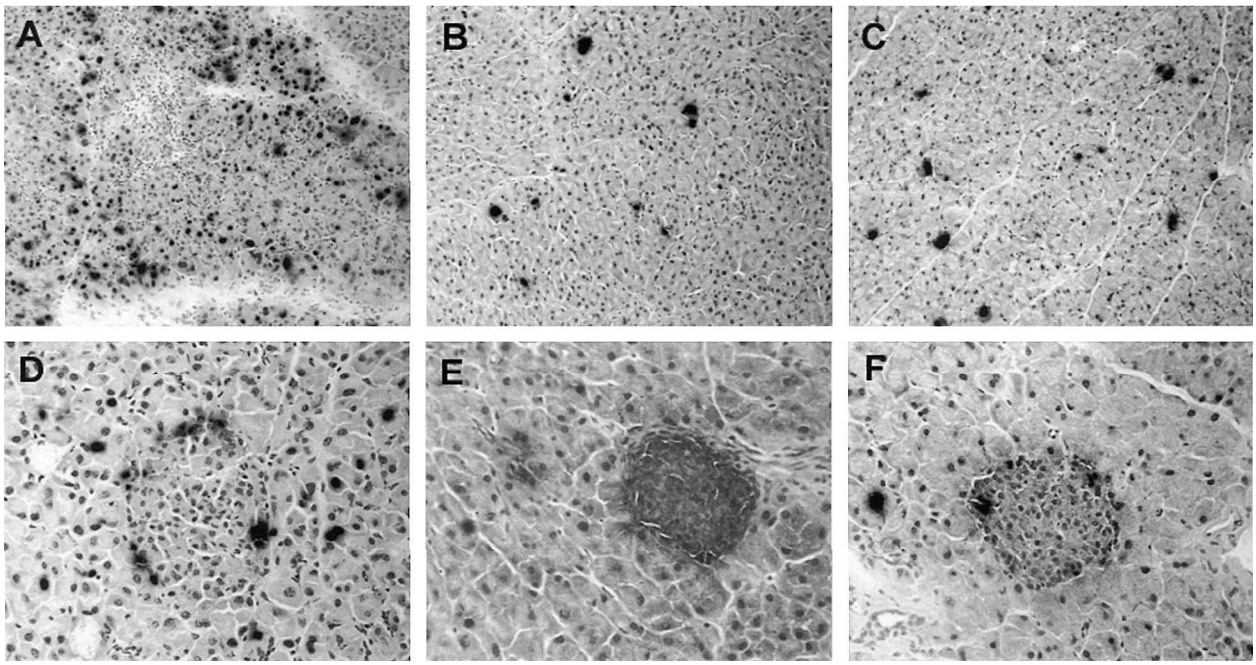

FIG. 4. Sections from C57Bl/6 mouse pancreas injected with AdVLacZ, AAV2LacZ, or AAV8LacZ at 7 days (AdVLacZ) or 4 weeks (AAV2LacZ, AAV8LacZ) postinjection showing the different transduced cell types. Pancreatic acinar cells transduced by AdVLacZ (**A**), AAV2LacZ (**B**), or AAV8LacZ (**C**) 20× magnification. Pancreatic islets of Langerhans transduced by AdVLacZ (**D**), AAV2LacZ (**E**), or AAV8LacZ (**F**) and stained with anti-insulin antibody. Magnification ×40.

uncoating of the AAV8 capsid in the cell compared to AAV2 (Thomas *et al*., 2004). Expression from AAV8LacZ-transduced pancreas peaked at 4 weeks postdelivery, but interestingly, expression declined dramatically (~90%) by 8 weeks. This decrease in gene expression may be the result of an immune response elicited to the LacZ transgene, or a result of pancreatic cell turnover. The half-lives of murine pancreatic acinar cells and islet cells are approximately 70 days and 47 days, respectively (Magami *et al*., 2002), so it is not unlikely that many of the transduced cells were lost because of cell cycling. In contrast, mice injected intrapancreatically with AAV5LacZ did not result in any detectable LacZ expression in the pancreas *in vivo* at any of the tested time points (data not shown). Expression of the LacZ transgene from both AAV2LacZ and AAV8LacZ was present through the entire pancreas, and transduction of other organs was not observed. The ability of the adeno-associated viral vectors to transduce a larger area of the pancreas was likely caused by the smaller particle size of AAV com-

TABLE 1. QUANTITATION OF THE CELL TYPES TRANSDUCED BY AdvLacZ, AAV2LacZ, AAV5LacZ, AND AAV8LacZ

	1 week	*2 weeks*	*4 weeks*	*8 weeks*
AdvLacZ				
Acinar cells	29.2	17.7	0.8	0.8
Beta cells	2.7	<0.1	<0.1	<0.1
AAV2LacZ				
Acinar cells	0.2	0.7	0.5	1.2
Beta cells	<0.1	<0.1	<0.1	<0.1
AAV5LacZ				
Acinar cells	<0.1	<0.1	<0.1	<0.1
Beta cells	<0.1	<0.1	<0.1	<0.1
AAV8LacZ				
Acinar cells	1	3.5	4.1	0.6
Beta cells	0.4	1.2	4.9	<0.1

Numbers represent the percentage of the total cells in a 10× field of view. The maximum number of LacZ-positive cells in a 10× field of view for each cell type was determined, and averaged between the different mouse pancreas samples ($n = 3$ or 4 per group).

pared to adenovirus, allowing it to diffuse more readily through the injected tissue.

Dose response of AAV8

In order to test the maximum levels of transduction achievable with AAV8LacZ, we performed a dose-response experiment to determine if higher doses of AAV8LacZ would result in greater transduction levels. Five doses of AAV8LacZ (3.3×10^9, 1×10^{10}, 3.3×10^{10}, 1×10^{11}, 3.3×10^{11} particles) were delivered intrapancreatically to 8- to 12-week-old C57Bl/6 mice, and the mice were sacrificed at 4 weeks and their pancreas harvested for X-gal staining. Increasing doses of AAV8LacZ resulted in increased of levels of LacZ expression in the pancreas of mice, with comparable levels of expression achieved by the highest two doses (Fig. 3). Higher doses of AAV8LacZ could not be tested because of the relatively low titer of our AAV8LacZ viral preparation (2.9×10^{12} particles

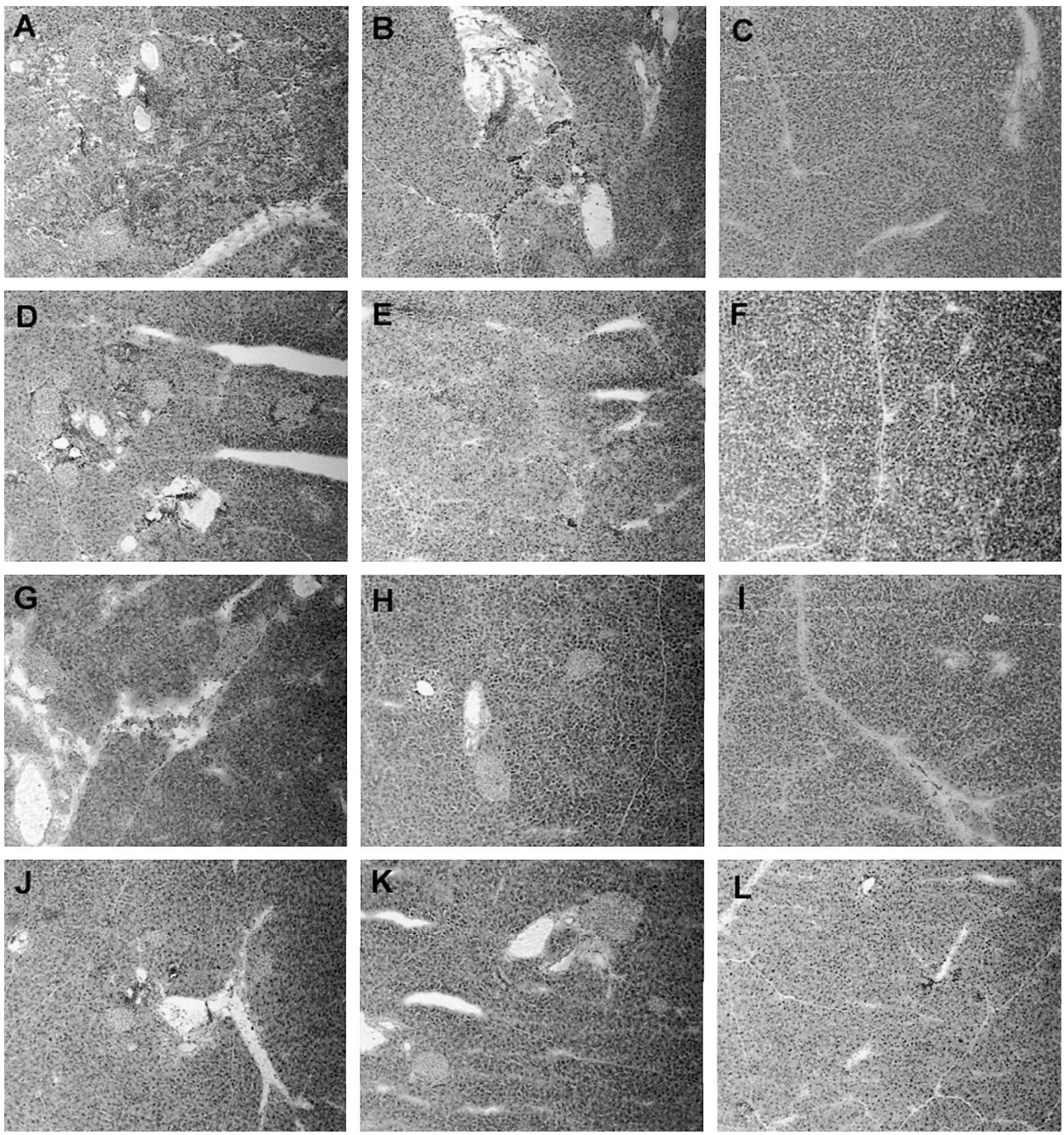

FIG. 5. Sections of AdVLacZ-, AAV2LacZ-, and AAV8LacZ-transduced C57Bl/6 mice pancreata at various days postinjection stained with rat anti-mouse CD45 antibody. AdVLacZ-injected mouse pancreas at 7 days (**A**), 14 days (**D**), 28 days (**G**), or 56 days (**J**) post-injection. AAV2LacZ injected mouse pancreas at 7 days (**B**), 14 days (**E**), 28 days (**H**), or 56 days (**K**) postinjection. AAV8LacZ-injected mouse pancreas at 7 days (**C**), 14 days (**F**), 28 days (**I**), or 56 days (**L**) postinjection. Magnification ×10.

per milliliter) and the risk of inducing pancreas damage by using larger injection volumes. It is likely that higher doses of AAV8 (>3.3×10^{11} particles) may result in even greater levels of transduction of the pancreas *in vivo*.

Cell types transduced by AdV and AAV

AdVLacZ-, AAV2LacZ-, and AAV8LacZ-injected pancreatic tissue stained with X-gal and an antibody for insulin revealed similar cellular distributions for the three viruses. Pancreas transduced with AdVLacZ showed expression primarily in the pancreatic acinar cells, with some pancreatic endocrine cells expressing LacZ at the periphery of the insulin-positive beta cell clusters (Fig. 4). The location of the transduced cells were primarily clustered around the site of injection. Pancreas transduced by AAV2LacZ and AAV8LacZ showed positive LacZ expression primarily in the acinar cells, with punctate LacZ positive staining throughout the entire pancreas. Few insulin-positive cells were transduced by AAV2LacZ, whereas AAV8LacZ clearly resulted in transduction of several insulin-positive cells.

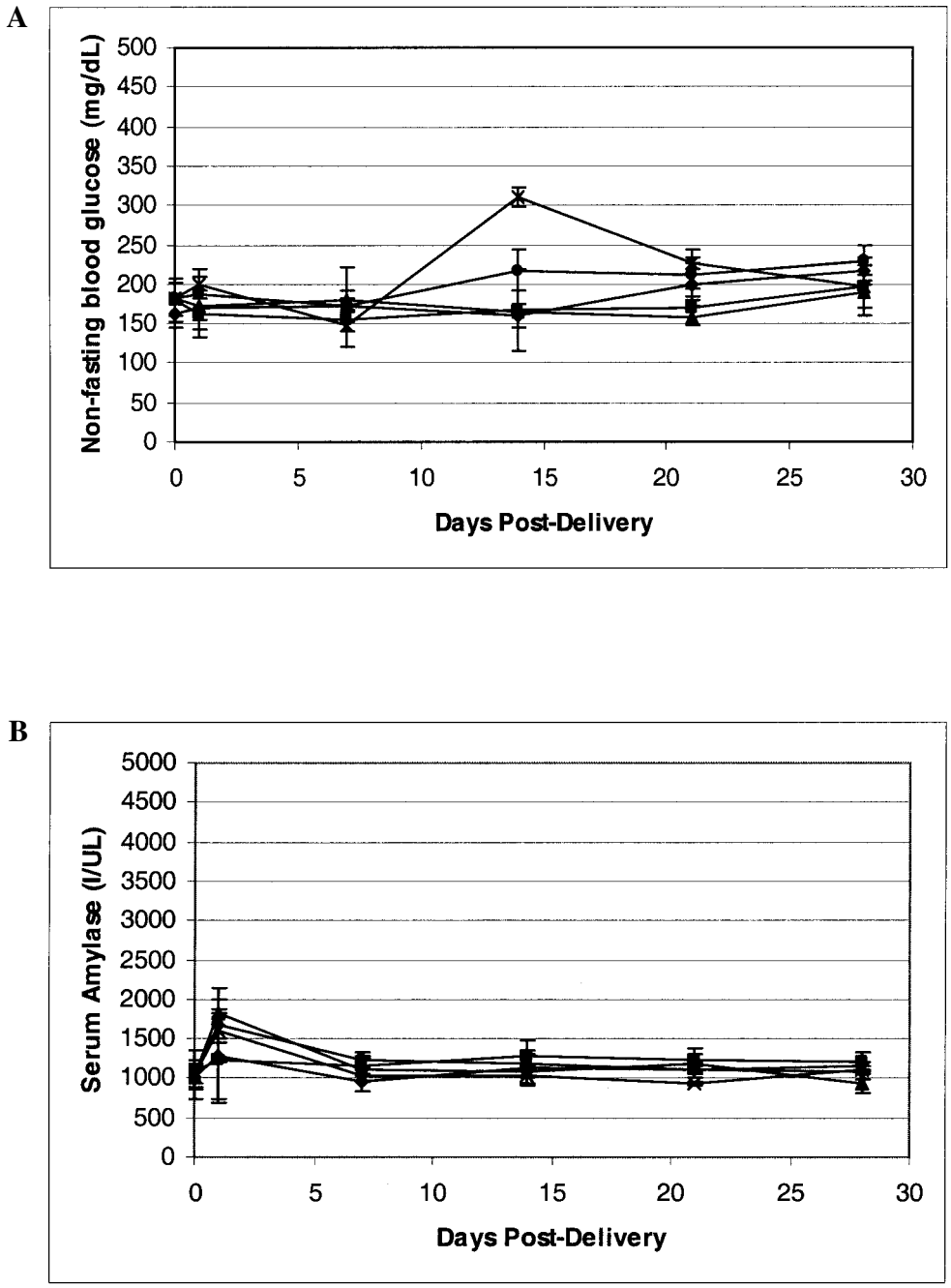

FIG. 6. Nonfasting blood glucose (**A**) and serum amylase (**B**) measurements of C57Bl/6mice injected intrapancreatically with saline (◆), 1×10^8 plaque-forming units (pfu) AdVLacZ (■), 1×10^{11} particles AAV2LacZ (▲), 1×10^{11} particles AAV5LacZ (×), and 1×10^{11} particles AAV8LacZ (●) at various days postinjection (n = 3–5 mice per group).

Ex vivo, the pancreatic islets of Langerhans have been successfully transduced by AAV (Prasad *et al.*, 2000), but there has been no demonstration thus far of successful islet transduction by AAV *in vivo*. AdVLacZ was able to transduce both pancreatic acinar and endocrine cell types, but transduction of the endocrine islet cells was primarily limited to the periphery of the cell clusters. This may be because of the limited access to the islets, which do not feed into the vast network of pancreatic ducts that connect the acinar cells. AAV2LacZ was shown primarily to transduce pancreatic acinar cells, with limited transduction of pancreatic endocrine cells. This may be because of the lower efficiency at which AAV2 transduces pancreatic cells. AAV8LacZ transduced both acinar and endocrine cells with similar efficiency (Table 1). Delivery of immunoprotective molecules to the islets *in vivo* using AAV8 may be a promising method of inhibiting the onset of type 1 diabetes (Prudhomme, 2000; Kapturczak *et al.*, 2001; Bottino *et al.*, 2003).

Histopathology in treated animals

To begin to unravel the cause of the loss of transgene expression from the AdV transduced cells, we stained the transduced pancreas sections for CD45 (leukocyte common antigen), a transmembrane glycoprotein that is found on all cells of hematopoetic origin except erythrocytes (Ledbetter and Herzenberg, 1979). As expected, significant leukocytic infiltration was observed in AdVLacZ injected pancreas starting at day 7 (Fig. 5), decreasing to negligible levels by day 28, which corresponds to the decrease in LacZ expression levels. AAV2LacZ and AAV8LacZ injected pancreata had significantly less leukocytic infiltration at day 7, and little if any CD45 positive cells were observed at day 28, even though there was no decrease in LacZ expression with these vectors.

Pancreatic function parameters in treated animals

In order to determine if the two delivery vectors induced any changes in pancreatic function *in vivo*, we monitored serum amylase levels, a key pancreatic enzyme useful for the diagnosis of pancreatitis (Moridani *et al.*, 2003). In addition, we monitored the nonfasting blood glucose levels of these mice to determine if there were any changes in glucose homeostasis in response to AdV or AAV delivery. Intrapancreatic delivery of the AdV and AAV vectors to the pancreas of mice resulted in a slight (<2-fold) elevation of serum amylase levels compared to saline at 1-day postinjection, but amylase levels returned to normal at 7 days postinjection, and remained normal for the remainder of the measurement period (Fig. 6B). This suggests that neither AdV nor AAV delivery to the pancreas induces pancreatitis in mice *in vivo*. Intrapancreatic delivery of AdV and AAV also resulted in no significant changes in nonfasting blood glucose levels, with the mice in all groups remaining normoglycemic (<250 mg/dl) at all measured time points (Fig. 6A). These data suggest that AdV and AAV do not cause any significant alteration in insulin secretion or islet function in the pancreas of mice *in vivo*.

Our studies suggest that AAV8 is a highly useful vector for safe, efficient, and long-term gene delivery to the pancreas. Although our direct injection delivery method would not be suitable for human applications because of the high risk of pancreatitis, safer methods for vector delivery such as endoscopic retrograde cholangiopancreatography (ERCP) exist and are currently in wide use in humans and could be adapted for delivery of gene transfer vectors. This in combination with the improved safety of helper dependent adenoviral vectors (Ehrhardt and Kay, 2002; Yant *et al.*, 2002) and the relative safety record of AAV suggest that these approaches may be useful for developing new therapies for diabetes.

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Received for publication March 8, 2003; accepted after revision February 23, 2004.

Published online: March 17, 2004.