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Ultrarapid, highly efficient viral gene transfer to the heart

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ABSTRACT **Gene therapy for common myocardial diseases will require effective and homogeneous gene delivery throughout the intact heart. We created two experimental models to identify and optimize parameters important for adenovirus-mediated cardiac gene transfer. In cultured rabbit ventricular myocytes, the percentage of infected cells increased with higher absolute numbers of virus particles, longer durations of virus exposure, physiological temperatures, and specific culture media compositions. Simulating the *in vitro* conditions, we delivered adenovirus to intact rabbit hearts by intracoronary perfusion. The percentage of infected cells increased with higher coronary flow rates, longer virus exposure times, and higher virus concentrations. Under optimal conditions, nearly 100% of myocytes expressed the reporter gene β-galactosidase after *ex vivo* infection. This novel delivery method, the first to demonstrate virtually complete transduction of any intact organ, could be adapted to achieve widespread gene transfer *in vivo*.**

Diseases such as congestive heart failure, familial hypertrophic cardiomyopathy, and the long QT syndrome result from alterations of myocardial function on a cellular or subcellular level, and effecting a cure will necessitate modification of a majority of the diseased cells. Adenovirus-mediated gene transfer has been used to introduce recombinant genes to cardiac myocytes, offering the potential to treat both rare and common cardiac disorders. Previous attempts utilized intramyocardial injection of viral vectors and achieved locally intense gene delivery, limited to an area within 1–2 mm of the needle track (1, 2). More diffuse but less effective gene transfer was demonstrated with coronary arterial delivery, either by percutaneous *in vivo* delivery or in explanted hearts prior to cardiac transplantation (3–5). Despite all of these attempts, no strategy yet devised has achieved high levels of homogeneous gene expression throughout the intact heart. These observations motivated us to evaluate infection conditions in a more controlled environment to identify parameters that would increase infection efficiency.

Our work identifies several key variables that influence recombinant adenoviral gene transfer in cultured cardiac myocytes and intact hearts. We first evaluated infection conditions in primary cultures of adult rabbit ventricular myocytes using recombinant adenoviruses encoding the reporter gene for either β-galactosidase (β-gal) (Adβgal) or luciferase (AdLuc). Infection of cultured cells is most effective at 37°C in crystalloid solutions with elevated virus concentrations and virus-to-cell ratios. Using such conditions in intact hearts, we achieve reporter gene expression in 96% of cardiac myocytes infected by coronary perfusion. If appropriately modified for *in vivo* applications, this delivery strategy would be a viable method for gene therapy in the heart and other solid organs.

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MATERIALS AND METHODS

Adenovirus Vectors. Adβgal contained the *Escherichia coli lac Z* gene driven by the human cytomegalovirus immediate early promoter. AdLuc was created by homologous recombination in HEK 293 cells between an E1 shuttle vector and the plasmid pJM17 containing the full adenoviral genome (6); the shuttle vector contained the firefly luciferase gene driven by the Rous sarcoma virus long terminal repeat. Virus stocks were expanded as previously described (7), aliquoted in small volumes, and stored in PBS with 10% glycerol at −80°C. Viral titers were determined by the average of two plaque assays performed using a traditional method (6). Virus concentrations were reported in plaque-forming units (pfu) per ml rather than absolute virus particles per ml, by standard convention (8).

Rabbit Cardiac Myocyte Isolation. Rabbit ventricular myocytes were isolated as previously described (9). Adult New Zealand White rabbits received heparin anticoagulation (1,000 units i.v.) prior to pentobarbital (50 mg/kg i.v.). The heart was extracted and rinsed twice in ice-cold, modified Krebs buffer containing 138.2 mM Na^+, 5.4 mM K^+, 1.2 mM Mg^{2+}, 1.0 mM Ca^{2+}, 144.4 mM Cl^-, 1.2 mM SO_4^{2-}, 1.2 mM $H_2PO_4^-$, 20 mM Hepes, 15 mM glucose, saturated with O_2 at pH 7.4. Next, the aorta was cannulated, and the heart was suspended in an insulated chamber at 35–37°C. Langendorff perfusion occurred by retrograde flow from the cannula in the ascending aorta to the coronary arteries (10). The heart was perfused with Krebs buffer for 2.5 min at 30–40 ml/min and then with nominally calcium-free Krebs for 10 min at 10 ml/min, before perfusion with an enzyme solution consisting of Krebs buffer with 0.025 mM Ca^{2+}, 1 mg/ml collagenase B (Boehringer Mannheim), 0.1 mg/ml protease (fraction XIV, Sigma), 60 mM taurine, 8.0 mM glutamic acid, and 2.0 mM D,L-carnitine. After digestion, the ventricles were excised, minced, and agitated by repeated suction through a Pasteur pipette. The resulting cell suspension was strained through a 200-μm nylon mesh filter. In general, the ventricles were uniformly digested, and the residue remaining on the filter was from the valvular and tendon structures, with only minimal myocardium. Calcium was gradually repleted in four steps before the cells were added to medium 199 (M199, GIBCO). The cells were supplemented with 5.0 mM creatine, 5.0 mM D,L-carnitine, 5.0 mM taurine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin, and were placed in laminin-coated dishes in a 37°C incubator.

***In Vitro* Infection.** The number of myocytes in each culture dish was quantified by counting the cells in 10 high-power fields and averaging over the surface area of the culture dish. Each series of experiments was performed using aliquots from the same dilution of virus. Baseline conditions for infection included media volume of 1.0 ml at 37°C and M199. After the predetermined infection interval, the media was removed,

Abbreviations: β-gal, β-galactosidase; Adβgal, adenovirus with β-gal reporter gene construct; AdLuc, adenovirus with luciferase construct; M199, tissue culture media 199; pfu, plaque-forming units.

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then cells were washed twice with cold Krebs buffer and placed back in M199 at 37°C.

Langendorff Infection. Rabbits hearts were excised and mounted on a Langendorff-perfusion apparatus at 37°C in the manner detailed above. The hearts were perfused with normal Krebs buffer for 2.5 min followed by 50 ml of oxygenated Krebs buffer containing 1 mg/ml albumin and 5.0×10^9 pfu of Adβgal. Experiments evaluating high virus concentration were performed with 3.2×10^{10} pfu of Adβgal in 20 ml of Krebs/albumin. During infection, the perfusate was collected and recirculated, and the flow rate was controlled by a peristaltic pump (Masterflex, Cole–Palmer). Perfusion pressure did not change significantly for flow rates between 20–40 ml/min. At 10 ml/min, the perfusion pressure was 10–30 mm Hg, and flows between 20–40 ml/min had perfusion pressures of 40–70 mm Hg. At the end of the infection interval, the hearts were perfused with nonrecirculating, virus-free Krebs buffer at 20–30 ml/min to maintain a total Langendorff-perfusion time of 180 min before cell isolation and culture to allow time for transgene expression. The cells were cultured in M199 on laminin-coated plates. To demonstrate that any unattached virus was removed prior to cell culture, three plates from the high virus concentration experiments were initially cultured in M199 mixed with rabbit plasma known to contain anti-adenoviral antibodies. The percentage of infected cells in these experiments was identical to the cells cultured without antibody. The myocardial digestion and number of viable cells with each isolation, quantified by counting morphologically normal myocytes as detailed above, were not affected by any of the experimental manipulations.

Reporter Gene Assays and Data Analysis. Forty-eight hours after exposure to Adβgal, the myocytes were fixed in 0.05% glutaraldehyde for 5 min at room temperature. The cells were then washed twice in PBS and stained overnight at 37°C in PBS containing 1.0 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal), 15 mM potassium ferricyanide, 15 mM potassium ferrocyanide, and 1 mM $MgCl_2$. For the *in vitro* experiments, 250 cells were counted in each culture dish to quantify the percentage of blue cells. If the percentage of blue cells was <10%, 500 cells were counted, and if the percentage was <5%, 1,000 cells were counted in each dish. For the Langendorff-infected myocytes, 1,000 cells were counted for each experiment.

Cells infected with AdLuc were processed 48 hr after infection using a luciferase assay system (Promega), and the luciferase activity was quantified using a luminometer. The results were subtracted from background counts, compared with a standardization curve to determine the amount of luciferase present, and normalized to the amount of protein in each sample.

Statistical Analysis. Unless otherwise stated, all experiments were performed in triplicate and the data are presented as mean ± SD. Statistical significance was determined at the 5% level using paired Student's *t* tests.

RESULTS AND DISCUSSION

Determinants of Adenoviral Infection Efficiency in Isolated Cardiac Myocytes. Alterations in virus concentration affected both the percentage of cardiac myocytes expressing β-gal and the intensity of luciferase production. Exposure to increasing concentrations of Adβgal induced a sigmoidal increase in the percentage of infected cells (Fig. 1*A*). Infection occurred in 50% of myocytes at an Adβgal concentration of 5.5×10^4 pfu/ml and saturated at 100% for Adβgal concentrations $>5.0 \times 10^5$ pfu/ml. This dose-response relationship is similar to one previously described (11) in rat cardiac myocytes, although the half-maximal virus concentration in this study is an order of magnitude lower. In contrast, the dose-response curve for AdLuc showed a progressive increase in luciferase production without saturation up to virus concentrations of 10^8 pfu/ml (Fig. 1*B*), indicating that virally induced protein production continues to rise, far beyond the point where 100% of the target cells are infected.

To distinguish between dependence of the final infection percentage on virus concentration or total virus amount, we exposed myocytes to either 10^4 or 10^5 pfu of Adβgal for 2 hr or 48 hr (Fig. 1*C*). At 2 hr, the percentage of infected cells seemed to depend on virus concentration alone, in agreement with previous reports (12). After 48 hr of virus exposure, however, it became apparent that the final percentage of infected cells was really a function of the total amount of virus, independent of concentration. This indicated that sufficient time for virus diffusion was necessary to evaluate infection in more than minimal volume.

The infection rate also varied with virus exposure time. Different concentrations of Adβgal or AdLuc were added to myocytes for 10 sec to 2 hr before vigorous washing with cold Krebs solution. Exposure to 10^7 pfu/ml of Adβgal resulted in extremely rapid infection, >50% after just 10 sec and 100% by 10 min (Fig. 2*A*). The infection rate slowed with decreasing virus concentration. Infection with AdLuc showed a similar increase with time (Fig. 2*B*). Exposure to 10^7 pfu/ml AdLuc resulted in a luciferase concentration of 5.8×10^{-6} mg/mg protein after 10 sec, which increased to 8.8×10^{-5} mg/mg protein at 2 hr.

The role of temperature was explored by incubating cells with Adβgal at 37°, 24°, and 4°C for up to 8 hr (Fig. 3*A*). Afterwards, the cells were washed twice with cold Krebs solution to remove any unattached virus and incubated at 37°C. The time to half-maximal infection at 37°C was 19.0 ± 2.3 min, and infection peaked at 75.5 ± 2.6%. At 24°C, the time to half-maximal infection was longer, but the final infection percentage was not significantly different than at 37°C. At 4°C, both the rate and final percentage of infected cells were reduced. These data demonstrating adenovirus infection dependence on virus concentration and amount, exposure time, and temperature suggest that adenovirus attachment to myocytes can be treated like a typical receptor-ligand interaction. The reaction rate in these systems is determined by the collision probability between particles with an energy greater than the reaction activation energy, and thus depends on temperature, ligand, and receptor concentrations; the final product is related to the total amount of each starting reactant and the ratio between the reactants (13).

We also examined different culture media to evaluate infection conditions relevant to the *in vivo* environment (Fig. 3*B*). The infection percentages in M199 and Krebs solution were not significantly different. Switching to heparinized rabbit blood caused a 60% decrease in the percentage of infected cells. To investigate this effect, we separated blood into individual components. The infection rate with red blood cells reconstituted in Krebs solution was similar to that in whole blood, while the rate in plasma was nearer that in the crystalloid solutions. This indicated that the red cells were the major component of blood responsible for reducing infection efficiency, consistent with the known ability of adenovirus to hemagglutinate red blood cells (14). In M199 plus 20% iohexol, an intravascular contrast agent used for coronary angiography (e.g., prior to catheter-mediated intracoronary delivery), the infection percentage decreased by 30% compared with M199 alone.

Infection via Coronary Perfusion in the Intact Heart. Our isolated myocyte studies therefore demonstrated that adenovirus-mediated gene transfer to ventricular myocytes is most effective at 37°C in crystalloid solutions with high virus concentrations and virus-to-cell ratios. These became the starting conditions for our second experimental model, in which Adβgal was delivered to intact hearts via coronary perfusion. Using this system, we determined the effects of

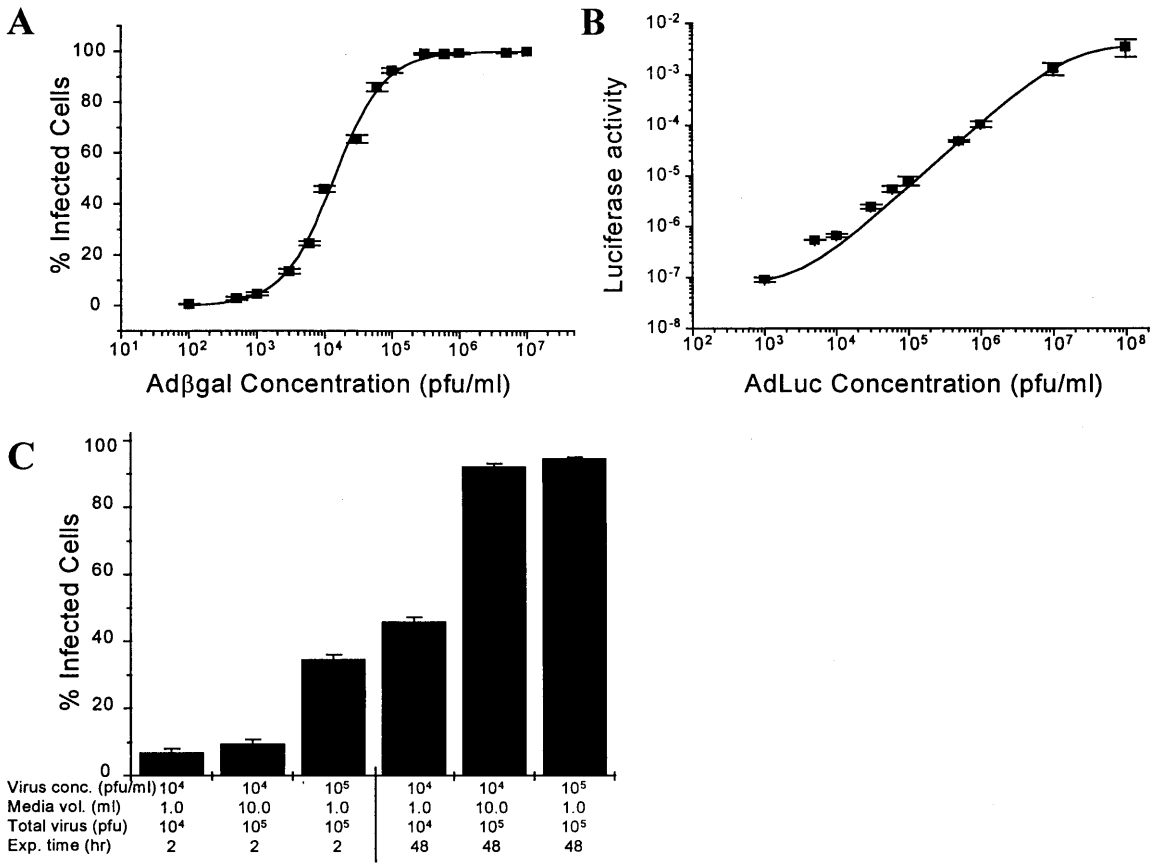

FIG. 1. Influence of virus concentration and exposure time on recombinant adenovirus infection in primary cultures of adult rabbit ventricular myocytes at 37°C. (*A*) Percentage of cells positive for β-gal after 48 hr exposure to Adβgal (1.0×10^5 cells per culture dish). Control, uninfected cells did not stain positive for β-gal activity. (*B*) Normalized luciferase activity (mg/mg protein) after 48 hr exposure to varying concentrations of AdLuc (2.0×10^5 cells per culture dish). Luciferase activity in control, uninfected cells was 1.2×10^{-9} mg/mg protein. Solid lines are logistical regression curves drawn through data. (*C*) Percentage of Adβgal-infected cells after 2 hr or 48 hr virus exposure (1.0×10^5 cells per culture dish). Columns 1–2 and 4–5 are the same virus concentration, and columns 2–3 and 5–6 are the same total virus amount. ($n = 3$.)

arterial flow rate, virus exposure time, and virus concentration on Adβgal infection.

To evaluate the dependence on coronary flow rate, the hearts were perfused with virus-containing solution for 120 min at 10–40 ml/min. Infection increased from 10.6 ± 2.5% at a flow rate of 10 ml/min to 40.3 ± 3.2% for flow rates of 30 ml/min or greater (Fig. 4*A*). Although the decreased efficiency of gene transfer at 10 ml/min may have been related to reduced perfusion pressure, the improvement at higher flow rates was independent of perfusion pressure, which did not vary significantly for coronary flow rates between 20–40 ml/min. A more likely cause of the flow dependence is the presence of precapillary sphincters that open in response to increasing flow rates (15). We surmise that flow above a critical rate opens all of the myocardial capillaries, maximizing the surface area for virus delivery and shortening diffusion distances to myocytes.

Adβgal infection of intact hearts also varied significantly with the duration of virus exposure (Fig. 4*B*). For these experiments, the infection and virus-free rinse phases were

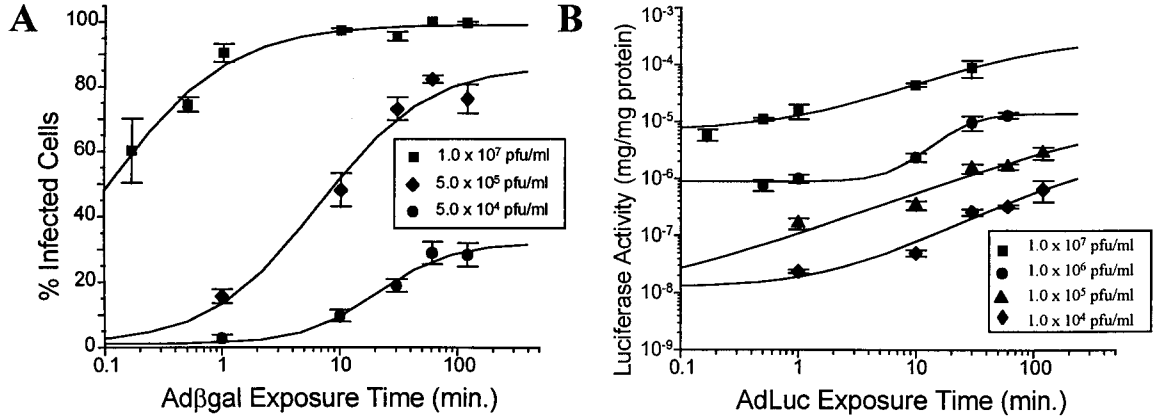

FIG. 2. Virus exposure time dependence of *in vitro* infection. Virus exposure time ranging from 10 sec to 2.0 hr at varying concentrations of Adβgal in 8.8×10^4–1.0×10^5 cells (*A*) or AdLuc in 6.4–8.7×10^4 cells (*B*). Solid lines are logistical regression curves drawn through data. ($n = 3$.)

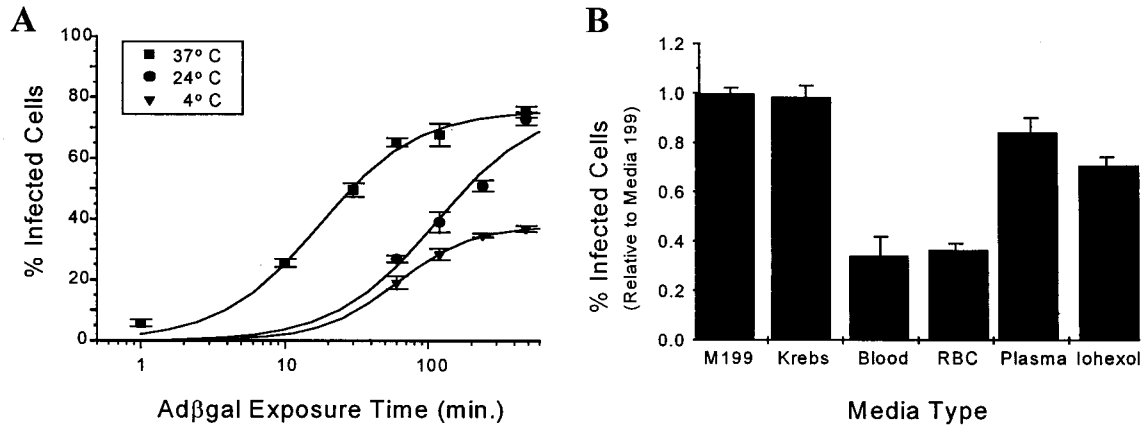

FIG. 3. Temperature and media effects on virus infection in isolated rabbit ventricular myocytes. (*A*) Percentage of cells positive for β-gal after 1 min to 8 hr exposure to 1.0×10^5 pfu of Adβgal in 1.0 ml Krebs solution at three different temperatures (8.9×10^4 cells per culture dish). Solid lines are logistical regression curves drawn through data. (*B*) Relative number of cells positive for β-gal, compared with infection in M199. Myocytes were exposed to 1.0×10^5 pfu of Adβgal in different media for 2.0 hr at 37°C (4.4×10^4 cells per culture dish). Whole rabbit blood had a hematocrit of 33%, and isolated red blood cells (RBC) were diluted in Krebs solution to a hematocrit of 33%. Iohexol was diluted in M199 to 20% by volume. ($n = 3$.)

adjusted to maintain a total perfusion time of 180 min, and the coronary flow rate was held constant at 30 ml/min. The percentage of infected cells was 0.8 ± 0.2% after a single pass of the 50 ml perfusate over 1.67 min. Infection increased with recirculation of the virus-containing solution, peaking at 41.5 ± 3.1% for virus exposures of 60 min or longer.

Exposure to a higher initial virus concentration played a critical role in the infection efficiency (Fig. 4*C*). As we have shown, perfusion of 10^8 pfu/ml Adβgal for 60 min at 30 ml/min infected 41.5% of the cardiac myocytes. Remarkably, increasing the concentration to 1.6×10^9 pfu/ml caused infection in 96.1 ± 0.2% of the myocytes throughout the intact heart. Repeating the high concentration experiments with anti-adenovirus antibody in the culture media did not change the percentage of infected cells, demonstrating that infection occurred only during coronary perfusion and was not augmented by incomplete washout of virus allowing infection during cell culture. This result significantly exceeds the most

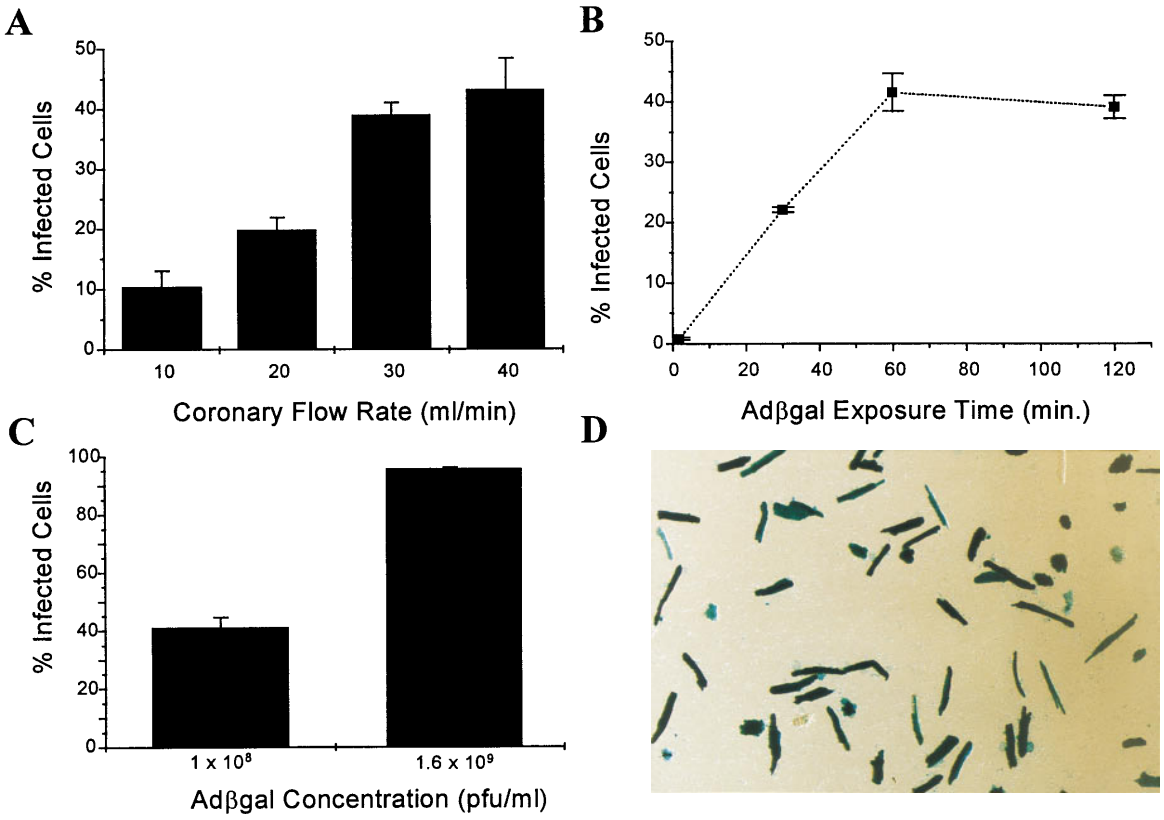

FIG. 4. Adβgal delivery to intact, Langendorff-perfused rabbit hearts at 37°C. (*A*) Percentage of myocytes positive for β-gal following 2.0 hr infections with 1.0×10^8 pfu/ml Adβgal at coronary flow rates ranging from 10 to 40 ml/min. (*B*) Effect of virus perfusion time on infection with 1.0×10^8 pfu/ml Adβgal at 30 ml/min. The exposure time varied from 1.67 min to 2.0 hr with the virus-free rinse duration adjusted to maintain a total Langendorff perfusion time of 180 min. (*C*) Effect of virus concentration on infection rates with 60 min Adβgal perfusion at 30 ml/min. ($n = 3$.) (*D*) 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal)-stained myocytes from a heart infected with 1.6×10^9 pfu/ml of Adβgal during Langendorff perfusion.

effective delivery method previously described (3), in which 10^{10} pfu of Adβgal delivered by an intracoronary catheter infected less than one-third of the myocytes in the distribution of a single target artery. In those experiments, as in our short exposure time protocols, a single pass of perfusate did not allow sufficient time for virus to overcome diffusional barriers presented by capillary endothelium, basement membrane, and extracellular matrix. Increasing virus concentration and lengthening exposure time produced highly efficient and widespread gene transfer to intact hearts.

Implications for Gene Therapy. The results of these basic studies in cultured myocytes and intact hearts are particularly relevant to *in vivo* gene transfer. The lack of success of previous attempts using a cardiac transplantation model (4) can be readily explained by our data. Cooling cardiac myocytes to 4°C, as was done in those reports, would significantly reduce infection efficiency. Similarly, catheter-mediated delivery in the presence of blood and intravascular contrast agents would result in lower levels of infection.

Contemporary cardiac surgical techniques enable similar conditions to be applied to the heart during coronary bypass procedures or at the time of cardiac transplantation. Modern coronary catheterization systems could also be modified to deliver virus in a manner analogous to the one we describe. Further animal studies will be necessary to establish the feasibility of this approach *in vivo*, but the general strategy described here opens up new prospects for gene therapy to treat a variety of common myocardial disorders, including heart failure and ventricular arrhythmias (7, 16). This delivery system need not be limited to the heart. Similar approaches may be used to achieve widespread gene transfer with other viral vectors in a wide variety of tissues and organs.

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