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#### **Research Article**

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# Established Immunity Precludes Adenovirus-mediated Gene Transfer in Rat Carotid Arteries

## Potential for Immunosuppression and Vector Engineering to Overcome Barriers of Immunity

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### Abstract

Preclinical arterial gene transfer studies with adenoviral vectors are typically performed in laboratory animals that lack immunity to adenovirus. However, human patients are likely to have prior exposures to adenovirus that might affect: (a) the success of arterial gene transfer; (b) the duration of recombinant gene expression; and (c) the likelihood of a destructive immune response to transduced cells. We confirmed a high prevalence (57%) in adult humans of neutralizing antibodies to adenovirus type 5. We then used a rat model to establish a central role for the immune system in determining the success as well as the duration of recombinant gene expression after adenovirus-mediated gene transfer into isolated arterial segments. Vector-mediated recombinant gene expression, which was successful in naive rats and prolonged by immunosuppression, was unsuccessful in the presence of established immunity to adenovirus. 4 d of immunosuppressive therapy permitted arterial gene transfer and expression in immune rats, but at decreased levels. Ultraviolet-irradiated adenoviral vectors, which mimic advanced-generation vectors (reduced viral gene expression and relatively preserved capsid function), were less immunogenic than were nonirradiated vectors. A primary exposure to ultraviolet-irradiated (but not nonirradiated) vectors permitted expression of a recombinant gene after redelivery of the same vector. In conclusion, arterial gene transfer with current type 5 adenoviral vectors is unlikely to result in significant levels of gene expression in the majority of humans. Both immunosuppression and further engineering of the vector genome to decrease expression of viral genes show promise in circumventing barriers to adenovirus-mediated arterial gene transfer. (*J. Clin. Invest.* 1997. 99:209–219.) Key words: adenovirus • arteries • gene transfer • immunity • rats

### Introduction

In vivo gene transfer into the arterial wall is used both to elucidate the role of individual genes in local arterial biology (1, 2) and to explore the potential of recombinant proteins to serve a therapeutic purpose (3–5). Vectors based on recombinant adenovirus are currently the agents of choice for in vivo arterial gene transfer (3–8). Adenoviral vectors derive their utility from a demonstrated ability to achieve high local levels of potentially therapeutic proteins while avoiding the toxicity that might result from systemic administration of the same agents (2, 3, 5). Several investigators have suggested that adenoviral vectors have particular clinical promise for the treatment of restenosis after angioplasty (3–5).

The difficulties experienced by numerous laboratories with adenoviral vectors in hepatic and pulmonary gene transfer experiments contrast with the optimism for these vectors that is expressed within the cardiovascular field. Extensive investigation of adenoviral vectors in animal models of hepatic and pulmonary gene transfer has revealed both acute and delayed toxicity after vector infusion. This toxicity includes in many cases an early neutrophil-dominated infiltrate (9, 10) followed by a lymphocyte-predominant response that includes upregulation of MHC class I in transduced cells followed by elimination of these cells by antigen-specific MHC class I-restricted cytotoxic T cells (11–13). Moreover, exposure to adenovirus in naive laboratory animals (in which antibody to human adenovirus is absent) invariably results in the generation of a humoral immune response (14). This humoral response completely blocks repeat hepatic gene transfer and significantly diminishes the efficiency of repeat pulmonary gene transfer (13, 15). In agreement with the potential problems suggested by these animal data, human clinical protocols of lung-directed adenoviral gene therapy for cystic fibrosis performed by three independent groups have found low gene transfer efficiency (16–18) with evidence of clinical toxicity at higher vector doses (17).

In previous vascular gene transfer studies performed in our laboratory with adenoviral vectors delivered to the arteries of rats and rabbits, we described both an early dose-dependent neutrophil-predominant infiltrate, as well as a delayed lymphocyte-predominant infiltrate accompanied by smooth muscle cell activation and intimal hyperplasia (19, 20). The possibilities that these untoward findings are species specific or that the proinflammatory effects of adenoviral vectors will be minimized or eliminated by lowering the vector dose await further experimentation. Also unaddressed in these previous studies, all of which were performed with naive laboratory animals, was the relationship between existing immunity to adenovirus and the success and duration of recombinant gene expression

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in the artery wall. Adenovirus is a ubiquitous human pathogen (21), and it is likely that significant numbers of angioplasty patients will have circulating antibodies [as well as memory T cells (22)] to adenovirus at the time of any clinical arterial gene transfer protocol.

In this study, performed in rats, we determined the relationships between humoral immunity to adenovirus, local cellular inflammation at the site of gene delivery, and the success and duration of adenovirus-mediated arterial gene transfer. We developed protocols with which arterial gene transfer and expression could be achieved despite the presence of established immunity, and we explored the possibility that advanced-generation adenoviral vectors, in which the adenoviral genome is rendered nonfunctional, might eventually be used to circumvent the destructive immune response that occurs subsequent to exposure to first generation adenoviral vectors.

## Methods

**Viruses, plasmid, cell lines.** AvlLacZ4, a recombinant replication-defective adenovirus, encodes a nuclear-targeted *Escherichia coli*  $\beta$ -galactosidase ( $\beta$ -gal)<sup>1</sup> gene driven by the Rous sarcoma virus long terminal repeat promoter. The construction, purification, propagation, storage, and use of this vector (initially provided by Dr. Bruce Trapnell, Genetic Therapy Inc., Gaithersburg, MD) have been described in detail (23). Titers of virus stocks used in the present experiments ( $1-2 \times 10^{11}$  pfu/ml) were determined by plaque titration on 293 cells. To ensure standardization between experiments, all left carotid infusions were performed with virus from the same preparation. The plasmid pCMVLuc (24), containing the human cytomegalovirus immediate early promoter driving expression of a firefly luciferase gene, was used in transfection studies.

Cos-7, a simian virus 40-transformed African green monkey kidney cell line, and the human embryonic kidney cell line 293 were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in improved minimal essential medium (IMEM) supplemented with 10% FBS, 1 mM glutamine, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 0.25  $\mu$ g/ml amphotericin (all from Biofluids, Rockville, MD).

**Animal studies.** A total of 83 adult male Sprague-Dawley rats (Taconic Farms, Germantown, NY) weighing 300–400 grams was used in these studies. Animal care and operative procedures, all approved by the Animal Care and Use Committee of the National Heart, Lung, and Blood Institute, were as described (25). In vivo endothelium-specific gene transfer was performed in rat left common carotid arteries (25). In certain rats, local virus infusion was also performed in the right common carotid artery. Animals were killed by pentobarbital overdose either 3, 7, 14, or 30 d after gene transfer. Arteries were fixed by in vivo perfusion at 100 mmHg, followed by excision and ex vivo incubation in fixative (25). At the time of death, none of the animals had evidence of wound infection, and all common carotid arteries were patent.

**Histochemical staining and detection of recombinant gene expression.** To confirm the identification of luminal cells as endothelial cells (EC), histological sections were stained using an antibody to von Willebrand's factor (25). Expression of  $\beta$ -gal was detected by staining of excised, fixed arteries with 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-Gal) (23). Stained arteries were examined with the aid of a dissecting microscope, providing an initial qualitative assess-

ment of the presence or absence of recombinant gene expression. The arteries were then divided transversely into segments of  $\sim 4$  mm in length (two per vessel), which were then embedded side by side in paraffin blocks. 5- $\mu$ m-thick sections were cut from the blocks and counterstained with nuclear fast red. Both total and transduced (blue-nucleated) EC per histologic section were counted in at least two sections per vessel. The sections were always spaced a minimum of 50  $\mu$ m apart to avoid counting the same cells twice. Based on data obtained from individual cross sections, a mean number of transduced EC per section was calculated for each artery. In the in vitro experiments, in which cultured cells were the objects of gene transfer, recombinant  $\beta$ -gal and luciferase activity were measured in cell extracts and normalized to protein content in the extracts, as described (25).

**Evaluation of local vascular inflammation after gene delivery.** The degree of local vascular inflammation was determined by evaluation of hematoxylin and eosin (H & E)-stained slides and by specific immunostaining for lymphocyte antigens. The degree of inflammation on H & E-stained sections was determined by two independent observers, each blinded to the identity of the sections. Inflammation on each section was scored according to a semiquantitative scale: 0, no or minimal cellular infiltrates; 1, focal, mild cellular infiltrates in the adventitia; 2, moderate cellular infiltrates in the adventitia with no or only very focal involvement of the media and/or intima; 3, moderate to severe cellular infiltrates in the adventitia with moderate to severe focal involvement of the media and/or intima; and 4, severe cellular infiltrates in the adventitia with moderate to severe, diffuse infiltrates in the media and/or intima. The two observers independently assigned a score to each of four sections taken from each artery. The mean of these eight scores (four from each of the two observers) was calculated to generate a score for the entire artery. To test whether arteries within one group had significantly more or less inflammation than arteries in another group, the inflammation scores in both groups were ranked.

We also confirmed that the inflammatory infiltrates identified on H & E-stained slides comprised lymphocytes by staining serially cut sections with antibodies to CD3 and CD45RC (both from Pharmingen, San Diego, CA). CD3 is a pan-T cell antigen (26). CD45RC is present on B cells, CD8<sup>+</sup> T cells, a subset of CD4<sup>+</sup> cells, and NK cells (27). Isotype-matched primary antibodies (IgG1 from Dako, Carpinteria, CA; IgG3k from Sigma Chemical Co., St. Louis, MO) were used as controls.

**Immunosuppression and immunization.** Rats received immunosuppression, immune stimulation, or no treatment before transduction of the left common carotid artery. The day of adenoviral infusion was designated day 0. Immunosuppressed rats received either: (a) intraperitoneal cyclosporine A (CyA; Sandoz Pharmaceuticals Corp., East Hanover, NJ) 15 mg/kg/d starting on day -1 and continuing daily until vessel harvest; (b) intraperitoneal antilymphocyte globulin (ALG; Accurate Chemical and Scientific Corp., Westbury, NY) 1 ml given on days -7 and 0; or (c) intraperitoneal antithymocyte globulin (ATG; Accurate Chemical and Scientific Corp.) 1 ml/d starting on day -1 and continuing daily until vessel harvest. Rats were immunized to human adenovirus by either intravenous (right external jugular vein) or intraarterial (right common carotid artery) injection of either AvlLacZ4 or ultraviolet (UV)-inactivated AvlLacZ4 (UV-AvlLacZ4). The specific dosages and schedules of adenoviral immunization used in each experiment are indicated below.

**UV inactivation of AvlLacZ4.** Aliquots of AvlLacZ4 were placed in the wells of a sterile 96-well plate and exposed at a distance of  $\sim 1$  cm to a UV lamp (254 nm; model UV G-54; UVP, Inc., San Gabriel, CA). Controlled UV irradiation is reported to disrupt the function of adenoviral DNA with less profound effects on the virus capsid proteins (28). We did not use psoralen-aided UV inactivation because addition and subsequent removal of unreacted psoralen would have diluted the virus stocks (29). Because our in vivo gene transfer protocol is effective only with relatively high vector concentrations (25), dilution of virus stocks would likely have interfered with our experimental protocol.

1. *Abbreviations used in this paper:* ALG, antilymphocyte globulin; ATG, antithymocyte globulin;  $\beta$ -gal,  $\beta$ -galactosidase; CyA, cyclosporine A; EC, endothelial cells; H & E, hematoxylin and eosin; IMEM, improved minimal essential medium; X-Gal, 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside.

To determine the effect of our protocol of UV irradiation on the function of adenoviral capsid proteins, we tested the ability of UV-AvLacZ4 to enhance the transfer and expression of plasmid DNA in Cos-7 cells (30). Cells were plated in 24-well tissue culture plates at  $1 \times 10^5$  cells/well. The next day, the cells were washed once with Opti-MEM1 medium (Gibco Laboratories, Grand Island, NY), then further incubated in 225  $\mu$ l of Opti-MEM1 for 2 h. pCMVLuc (5.0  $\mu$ g diluted in 20  $\mu$ l Opti-MEM1) was added to each well, followed immediately by the addition of either AvLacZ4 or UV-AvLacZ4 (both  $2.5 \times 10^7$  pfu in 25  $\mu$ l in Opti-MEM1). After 2 h at 37°C, an additional 240  $\mu$ l of Opti-MEM1 was added to each well. After 22 h, the medium was changed to IMEM containing 10% calf serum and 1 mM glutamine. After an additional 24-h incubation, the cells were washed twice with PBS, lysed, and assayed for luciferase activity.

The effect of UV irradiation on the function of the AvLacZ4 genome was evaluated *in vitro* by assessing the ability of AvLacZ4 to mediate transfer and expression of  $\beta$ -gal in Cos-7 cells and to direct the synthesis of adenoviral hexon protein in 293 cells. Because E1-deleted adenoviral vectors such as AvLacZ4 can replicate in 293 cells, assays for adenoviral genomic function performed in this cell type should be particularly sensitive. Cos-7 cells ( $1 \times 10^5$  cells/well in 24-well plates) were infected with  $1 \times 10^6$  or  $2.5 \times 10^7$  pfu/ml of either AvLacZ4 or UV-AvLacZ4. After 48 h, the cells were lysed and  $\beta$ -gal activity was measured. 293 cells ( $2 \times 10^5$  cells/well in 35-mm wells) were infected for 2 h at 37°C with  $2 \times 10^6$  pfu/well of either AvLacZ4 or UV-AvLacZ4. Immediately after infection, labeling of newly synthesized proteins was initiated by addition of [ $^{35}$ S]methionine (10  $\mu$ Ci/ml; specific activity > 1,000  $\mu$ Ci/mmol; Amersham, Arlington Heights, IL) for 12 h in methionine-free IMEM (Biofluids) (28). At the end of the incubation, the cells were washed with PBS, harvested, centrifuged (13,000 g, 5 min), and lysed by three cycles of freeze-thaw. Lysates were separated by SDS-PAGE and evaluated by autoradiography.

**Antiadenoviral antibody assay.** The immune response to adenoviral vectors is complex and includes both the humoral and cellular branches of the immune system (12, 13). In this study, we primarily measured the humoral component. Assays for humoral immunity are far more likely to be clinically applicable than those for cellular immunity because they are simple, highly reproducible, and amenable to automation. Moreover, as would be expected in immune competent animals, we found (see below) that assays of humoral immunity were often predictive of the activity of the cellular immune system. Finally, assays for humoral immunity lack an *in vitro* restimulation step, which is present in assays for cellular immunity to adenovirus (11). The absence of this restimulation step renders the humoral assays less susceptible to potential *in vitro* artifacts whose relevance to the *in vivo* behavior of the immune system may be questioned.

Neutralizing antibodies to adenovirus type 5 were assayed essentially as described (31). Briefly, rat or human serum samples were heat inactivated at 55°C for 40 min, then diluted serially with IMEM/2% FBS. Sera were assayed at dilutions of 1:2 and above. Aliquots of diluted serum (100  $\mu$ l) were mixed with AvLacZ4 ( $3 \times 10^5$  pfu in 10  $\mu$ l of IMEM/2% FBS), incubated for 1 h at 37°C, and then applied to nearly confluent 293 cells present in 96-well plates ( $3 \times 10^4$  cells/well). After 90 min at 37°C, the virus-containing solution was aspirated and replaced with 200  $\mu$ l of IMEM/10% FBS. The next day, cells were fixed with 2% formaldehyde, 0.2% glutaraldehyde in PBS, then stained with X-Gal. The antiadenoviral antibody titer was reported as the highest dilution of serum at which < 25% of the 293 cell nuclei stained blue.

Sera that had been obtained from a group of bank employees as part of a lipid study (32) were screened for the presence of neutralizing antibodies to adenovirus. The sera (kindly supplied by Dr. Thomas Bersot, Gladstone Institute of Cardiovascular Disease, San Francisco, CA) had been stored at -80°C before use in this study. Of 5,160 samples in the initial lipid study, 53 (~ 1%) were chosen randomly for analysis in the present study.

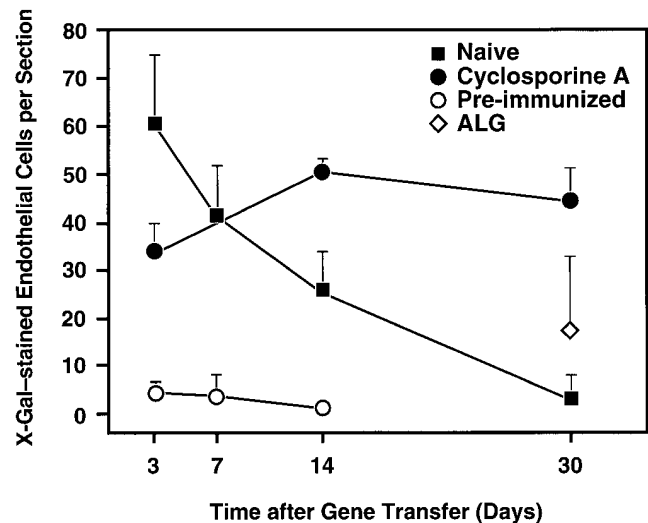
**Statistics.** Results are reported as mean  $\pm$  SD or median and

range for data not anticipated to be normally distributed. Means of most experimental groups were compared using an unpaired *t* test. Ranked inflammation scores were compared with the Mann-Whitney U test. Results of antibody titers were compared between groups by Kruskal-Wallis one-way ANOVA, and regression analysis of antibody titer data was by Spearman rank correlation (33). Differences were considered to be significant if  $P < 0.05$ .

## Results

*The level and duration of transgene expression is decreased by preimmunization and increased by immunosuppression.* 47 rats underwent left carotid artery gene transfer in a series of experiments examining the effects of systemic immunosuppression or preimmunization on the level and duration of transgene expression (Fig. 1). In addition to carotid infusion of AvLacZ4 at  $3 \times 10^{10}$  pfu/ml, each animal received either (a) no treatment (naive); (b) chronic (daily) immunosuppression with CyA starting on day -1; (c) transient (two doses) immunosuppression with ALG on days -7 and 0; or (d) immunization with intravenous AvLacZ4 ( $1 \times 10^9$  pfu) on days -35 and -14.

Vessels were harvested 3, 7, 14, and 30 d after left carotid infusion, and the number of transduced EC per histologic section was determined. In naive rats, vessels harvested 3 d after left carotid infusion ( $n = 3$ ) showed  $60 \pm 14$  transduced EC per cross section ( $43 \pm 4\%$  of total luminal EC; all data are subsequently expressed as total transduced EC per section rather than percentage of transduced EC). At 7 d ( $n = 3$ ),  $41 \pm 10$  transduced EC per cross section were present; at 14 d ( $n = 4$ ),  $17 \pm 10$  stained EC were present; at 30 d ( $n = 7$ ), only  $2 \pm 5$  transduced EC were found per cross section. Vessels harvested



**Figure 1.** Time course of recombinant gene expression after adenovirus-mediated arterial gene transfer: influence of immunity and immunosuppression. Rat left common carotid arteries were infused on day 0 with an adenoviral vector expressing  $\beta$ -gal. Arteries were harvested on days 3, 7, 14, and 30, stained with X-Gal, and the number of cells per transverse section expressing the recombinant gene was determined by counting cells in microscopic sections. Rats were either untreated (naive), treated with one of two immunosuppressive regimens (CyA or ALG), or were preimmunized by systemic injections of the adenoviral vector on days -35 and -14. Data are presented as mean  $\pm$  SD.

3 d after left carotid infusion from rats immunosuppressed with CyA ( $n = 2$ ) showed  $33 \pm 6$  transduced EC per cross section; at 14 d ( $n = 2$ ),  $50 \pm 3$  transduced EC per cross section were present; at 30 d ( $n = 5$ ),  $43 \pm 7$  transduced EC per cross section were present. Transduced carotid arteries ( $n = 10$ ) were harvested from ALG-treated animals only at 30 d after left carotid infusion. These vessels contained  $16 \pm 16$  transduced EC per cross section. Thus, at 30 d both CyA and ALG treatments resulted in a significant increase in recombinant gene expression over that present in untreated rats ( $P < 0.001$  and  $P < 0.05$ , for CyA and ALG treatments, respectively). Vessels were harvested from preimmunized rats at 3 ( $n = 4$ ), 7 ( $n = 3$ ), and 14 d ( $n = 4$ ) after virus infusion. These arteries contained  $4 \pm 3$ ,  $3 \pm 4$ , and  $0 \pm 0$  transduced EC per cross section, respectively. At all three time points, the differences in gene expression between immunized rats and both naive and CyA-treated rats were highly significant ( $P < 0.005$ ).

The titer of antiadenoviral neutralizing antibody is negatively correlated with the persistence of transgene expression. In the 22 rats from which arteries were harvested 30 d after left carotid infusion, serum samples were drawn both immediately before carotid infusion and at the time of vessel harvest. Serologic evidence of antiadenoviral immunity was absent in 100% of animals before carotid infusion. By 30 d (Fig. 2), naive animals ( $n = 7$ ) had, in association with their low levels of recombinant gene expression, all developed neutralizing antibody to adenovirus (median titer = 1:32, range 1:8 to 1:32). In contrast, at 30 d, rats treated with daily CyA ( $n = 5$ ) had no detectable antibody to adenovirus (median titer  $< 1:2$ ). The absence of

neutralizing antibody in CyA-treated rats was associated with high levels of recombinant gene expression. Rats given transient (two dose) immunosuppression with ALG ( $n = 10$ ) exhibited variable levels of humoral immunity to adenovirus (median titer = 1:8, range 1:4 to 1:32). These variable levels of antibody were associated with a broad range of recombinant gene expression in individual animals (0–42 transduced EC per cross section). When the data from all three groups were combined (Fig. 2), a significant inverse correlation between transgene expression and the titer of antiadenoviral antibodies at 30 d was apparent ( $r^2 = 0.75$ ;  $P < 0.001$ ). Thus, the development of neutralizing antibodies and the extinction of recombinant gene expression (by elimination of transduced cells by antigen-specific cytotoxic T cells [11] or possibly by antibody-dependent cellular cytotoxicity) were highly correlated.

The presence of neutralizing antibody to adenovirus is associated with immunity to adenoviral vector-mediated gene transfer and expression. 19 rats were used to investigate whether an established humoral immune response to adenovirus predicted failure of adenovirus-mediated gene transfer and expression even when gene transfer was attempted in an isolated arterial segment. 16 rats were immunized with intravenous AvlLacZ4 ( $1-2 \times 10^9$  pfu/dose) according to one of two different protocols. In 12 rats, we attempted to generate a primary immune response of variable intensity by injection of a single dose of AvlLacZ4 either 30 ( $n = 6$ ), 21 ( $n = 2$ ), 14 ( $n = 2$ ), or 7 d ( $n = 2$ ) before left carotid infusion. In four rats, a secondary immune response was generated by injection of one dose of AvlLacZ4 5 wk before left carotid infusion followed by a sec-

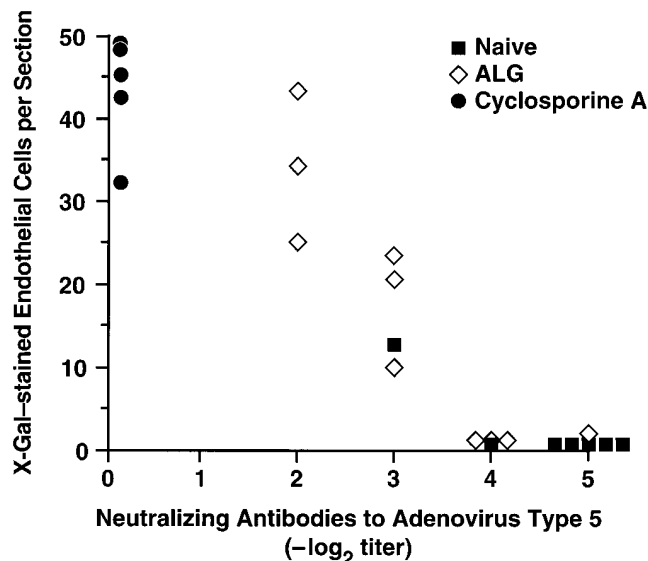


Figure 2. Humoral immunity to adenovirus 30 d after gene transfer correlates inversely with recombinant gene expression. Rat left common carotid arteries were infused with an adenoviral vector expressing  $\beta$ -gal, harvested 30 d later, and stained with X-Gal. At the time of harvest, serum was drawn and was assayed for the presence of neutralizing immunity to adenovirus. Rats were either untreated (naive) or were treated with one of two immunosuppressive regimens (CyA or ALG). The number of cells per transverse section expressing the recombinant gene was determined by counting cells in microscopic sections. Each data point represents a result from a single rat. Antibody titers of  $< 1:2$  are plotted adjacent to the ordinate.

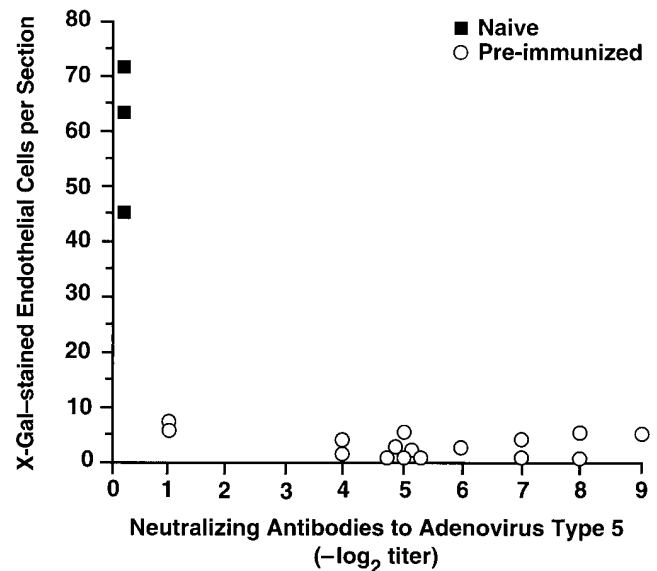


Figure 3. Humoral immunity to adenovirus at the time of virus infusion precludes successful gene transfer. An adenoviral vector expressing  $\beta$ -gal was infused into the left carotid arteries of both naive rats and rats that had been preimmunized with intravenous injections of adenovirus. At the time of vector infusion, blood was drawn and assayed for the presence of neutralizing immunity to adenovirus. 3 d later the arteries were removed, stained with X-Gal, and the number of cells per transverse section expressing the recombinant gene was determined by counting cells in microscopic sections. Each data point represents a result from a single rat. Antibody titers of  $< 1:2$  are plotted adjacent to the ordinate.

ond dose 2 wk before infusion. Three rats were not immunized with adenovirus before gene transfer and served as concurrent naive controls (gene expression data from these latter seven rats were also used in Fig. 1). Arteries were harvested 3 d after left carotid artery infusion, fixed, X-Gal stained, sectioned, and evaluated for the presence of transduced EC.

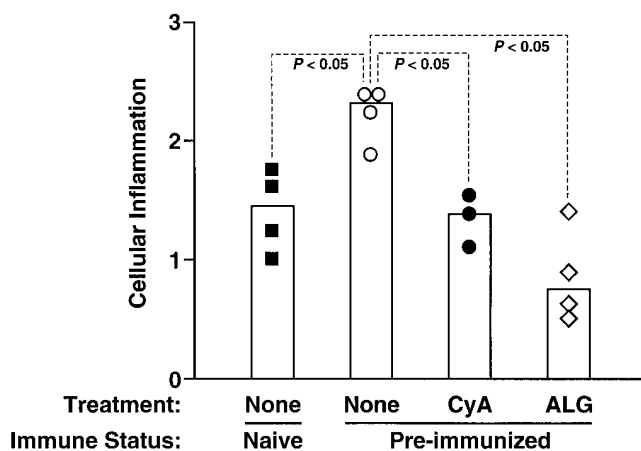
The naive control animals had no neutralizing antibodies to adenovirus at the time of left carotid infusion. 3 d later, large numbers of transduced EC ( $60 \pm 14$  per section) were detected by X-Gal stain. In contrast, at the time of left carotid infusion the immunized rats all had serum antibodies to adenovirus, ranging in titer from 1:2 to 1:512 (Fig. 3). Rats immunized only once had lower titers (1:2 to 1:32) than rats immunized twice (1:128 to 1:512). Despite this broad range of neutralizing antibody concentrations, recombinant gene expression was at a uniformly low to nonexistent level in all of the immunized rats ( $2 \pm 2$  transduced EC per section;  $P < 0.001$  vs. the naive animals). Thus, arterial gene transfer and expression were equally ineffective in the presence of either low or high levels of anti-adenoviral antibody. The data in Fig. 3, when compared with those in Fig. 2, reveal a fundamental difference between the immune response to cells transduced by a first exposure to adenovirus and the immune response to a second exposure to adenovirus. In the former case, intermediate levels of immunity and recombinant gene expression can temporarily coexist; in the latter case any detectable level of immunity essentially precludes the achievement of recombinant gene expression.

*Failure to detect recombinant gene expression in arteries of immune rats is associated with increased vascular inflammation.* The data presented in Fig. 3 establish that humoral immune status is highly predictive of the success or failure of gene transfer. However, the association of humoral immunity with failure of recombinant gene expression does not establish causality. Previous studies, including experiments performed

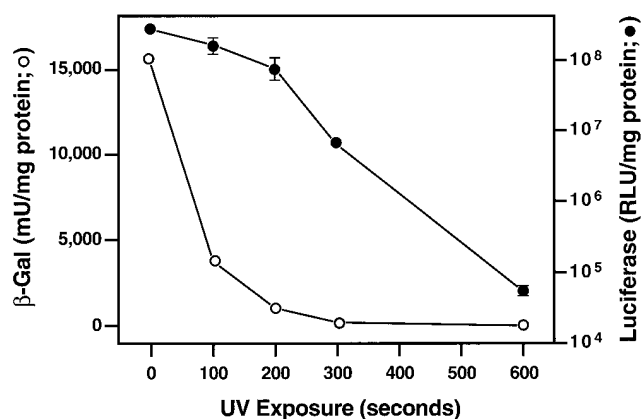
in mice with adoptive transfer of either the humoral or cellular immune components, have emphasized the ability of the cellular immune system (specifically CD8<sup>+</sup> lymphocytes) to eliminate transduced cells rapidly (12, 13). Thus, we considered that an accelerated local cellular immune response might contribute to the near absence of recombinant gene expression 3 d after gene delivery to immune rats. To investigate this possibility, we assessed the activity of the cellular immune system in arteries removed 3 d after Av1LacZ4 infusion into the carotid arteries of either naive rats ( $n = 4$ ) or immune rats ( $n = 4$ ). Cellular inflammation was significantly increased in the arteries of the immune rats ( $P = 0.014$ ; Fig. 4). Inflammatory cells were present primarily in the adventitia and along the intimal surface. Specific immunostains confirmed that many of the inflammatory cells expressed the lymphocyte markers CD3 and CD45RC (see below).

*UV-inactivated Av1LacZ4 maintains capsid functions preferentially over genomic functions.* The above results suggested that the development of immunity to adenoviral capsid proteins was associated with the disappearance of adenovirus-transduced cells. There are two sources from which rats subjected to vector infusion could have been exposed to immunogenic adenoviral proteins: the capsid proteins present in the initial vector inoculum or transgene and adenoviral proteins produced within transduced cells [the latter from intact virus open reading frames present within the vector DNA (11)]. Whereas the former exposure is obligatory in any adenovirus-based vector system, the latter could conceivably be prevented by use of species-homologous proteins (34) and by engineering further deletions into the adenoviral genome (35–37). We sought to create an investigational tool to discriminate the relative contributions of virus capsid and virus protein expression in the generation of antiadenoviral immunity. To do so, we treated aliquots of Av1LacZ4 with UV light. We anticipated that this protocol would damage adenoviral nucleic acid (decreasing or eliminating expression of adenoviral genes) while preserving the capability of virus capsid proteins to mediate internalization and endosome disruption. However, since prolonged UV exposure may also result in protein (i.e., capsid) damage, it was necessary to examine the effect of UV irradiation on both genomic and capsid functions.

Aliquots of Av1LacZ4 were irradiated for periods of time ranging from 0 to 600 s. The ability of Av1LacZ4 to express  $\beta$ -gal in Cos-7 cells and to direct hexon protein synthesis in 293 cells was used as indices of adenoviral genomic function. The abilities to enhance delivery of plasmid DNA to Cos-7 cells were used as an index of capsid function. UV irradiation decreased the ability of Av1LacZ4 to effect  $\beta$ -gal expression in Cos-7 cells in a dose-dependent manner (Fig. 5). After 600 s of irradiation,  $\beta$ -gal activity dropped by over three orders of magnitude, to near background levels (i.e., the level of endogenous  $\beta$ -gal activity in Cos-7 cells). UV irradiation of Av1LacZ4 also resulted in a time-dependent loss of the ability of Av1LacZ4 to enhance plasmid delivery to Cos-7 cells. After 600 s of irradiation, luciferase expression from cotransduced plasmid DNA also declined by over three orders of magnitude to near background levels (i.e., luciferase levels obtainable by transfection of naked plasmid DNA). With shorter periods of UV irradiation, however, the genomic functions of Av1LacZ4 were impaired proportionately more than the capsid functions. After 200 s,  $\beta$ -gal activity (a measure of genomic function) had decreased by nearly 1.5 orders of magnitude (7% of initial activ-

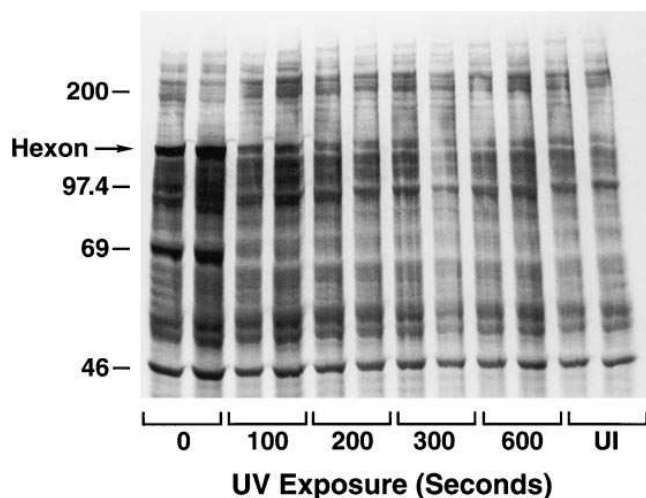


**Figure 4.** Cellular inflammation is increased in arteries of preimmunized rats, but is decreased by treatment with CyA or ALG. An adenoviral vector expressing  $\beta$ -gal was infused into the left carotid arteries of rats that were either naive (no previous exposure to adenovirus) or were preimmunized with systemic injection of adenovirus 30 d before carotid infusion. Preimmunized animals received either no treatment or were treated with 4 d of CyA or ALG. Four sections taken from each artery were stained with H & E and graded for the degree of cellular inflammation (see Methods for description of grading scale). Data points represent the scores for individual arteries; bar heights are group medians.



**Figure 5.** UV irradiation of adenoviral vectors: at early time points gene expression is lost before capsid function. Aliquots of the Av1LacZ4 vector were irradiated with UV light (254 nm) for time periods ranging from 0 to 600 s. The vectors were then assayed both for their ability to transfer a functional  $\beta$ -gal gene to Cos-7 cells and for their ability to enhance transfection into Cos-7 cells of a plasmid expressing luciferase (a function of the capsid proteins).  $\beta$ -gal and luciferase activities were measured in Cos-7 cell extracts. Data are presented as mean  $\pm$  SD and are from one of two experiments that gave similar results. The right ordinate is logarithmic to enable depiction of the full range of luciferase values.

ity retained), whereas luciferase activity (a measure of capsid function) decreased by only 0.5 orders of magnitude (32% of initial activity retained). In addition, hexon protein production (another measure of adenoviral genomic function) was still detectable after 100 s of UV irradiation, but was decreased to undetectable levels after 200 s (Fig. 6). These data, obtained in cultured cells, support the use of Av1LacZ4 irradiated for 200 s

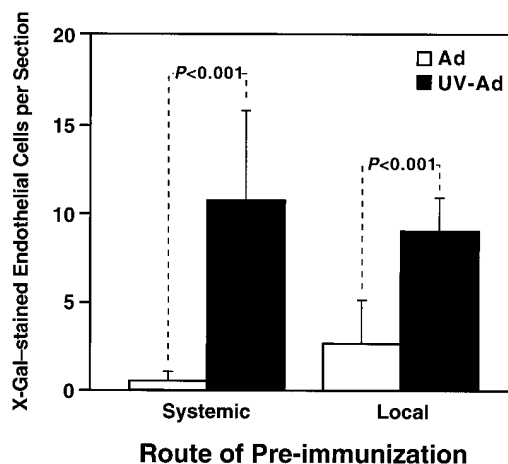


**Figure 6.** Effect of UV irradiation of adenoviral vectors on production of hexon protein after 293 cell infection. Aliquots of the Av1LacZ4 vector were irradiated with UV light (254 nm) for time periods ranging from 0 to 600 s and were then used to infect 293 cells at a multiplicity of infection of 10. The right two lanes (UI) are from uninfected 293 cells. Newly synthesized cellular proteins were then labeled by addition of [<sup>35</sup>S]methionine for 12 h. Cell lysates (from duplicate wells for each data point) were analyzed by SDS-PAGE and autoradiography. Left margin indicates  $M_r \times 10^{-3}$ . The position of migration of hexon protein is indicated.

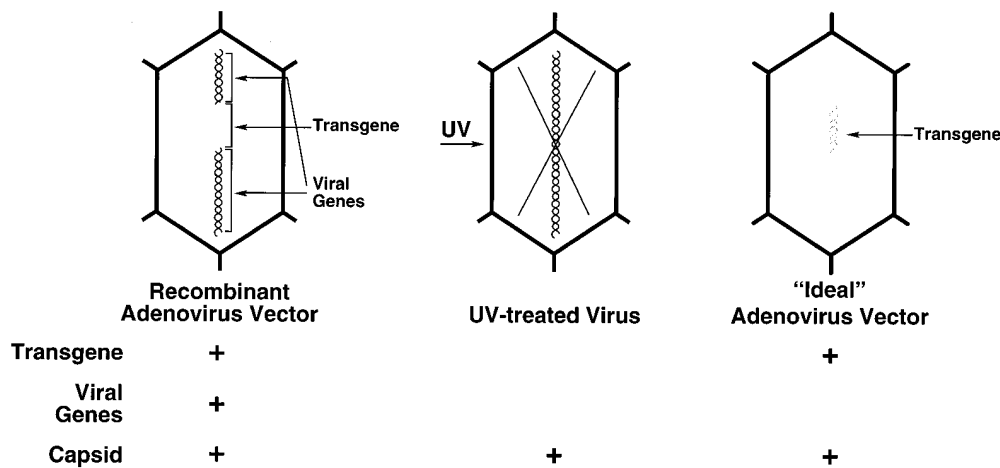
as a model of a virion that has preserved capsid protein functions but a relative impairment of the ability to express adenoviral genes.

*UV irradiation alters the immune response to Av1LacZ4.* We used Av1LacZ4 and UV-Av1LacZ4 to investigate the relative contributions of the input adenoviral capsid and genome to the induction of a neutralizing humoral immune response to adenovirus. Experiments were performed in 23 rats using either intentional systemic exposure or intended local exposure to the two types of vector (data from six of the rats are also included in Fig. 3). Systemic exposure was achieved by intravenous infusion of  $2 \times 10^9$  pfu. Limitation to local exposure was attempted by infusion of  $2 \times 10^9$  pfu into isolated right carotid arteries for 20 min followed by backflushing of infused virions into the infusion cannula from both the proximal common carotid and distal internal carotid arteries. 30 d after this initial exposure to Av1LacZ4 or UV-Av1LacZ4, rats were reanesthetized, serum samples were collected for antiadenoviral antibody assay, and gene transfer was attempted by infusion of Av1LacZ4 ( $3 \times 10^{10}$  pfu/ml) into isolated left carotid arteries. The left carotids were harvested 3 d after infusion, fixed, X-Gal-stained, sectioned, and examined for the presence of transduced EC.

Baseline antiadenoviral antibodies (i.e., before any intentional vector exposure) were undetectable in all rats. 30 d after the initial vector injection, all rats had measurable titers of neutralizing antibody to adenovirus, regardless of whether the vector was UV inactivated and regardless of whether the infusion was systemic or local. The antiadenoviral antibody titers after systemic or local infusion were [median (range)]: systemic UV-Ad, 1:64 (1:32–1:64); systemic non-UV-Ad, 1:32 (1:32–1:128); local UV-Ad, 1:32 (1:16–1:32); local non-UV-Ad,



**Figure 7.** Effect of UV irradiation and route of administration on the ability of a primary exposure to adenovirus to prevent recombinant gene expression after a repeat exposure. Rats were preimmunized to adenovirus by injection of  $2 \times 10^9$  pfu either intravenously [systemic:  $n = 6$ , adenovirus (Ad);  $n = 6$ , UV-irradiated adenovirus (UV-Ad)] or into an isolated right carotid artery segment followed by attempted aspiration of the virus inoculum [local:  $n = 5$  (Ad);  $n = 6$  (UV-Ad)]. 30 d later, local gene transfer into the isolated left carotid artery was attempted by infusion of the Av1LacZ4 vector. 3 d later the left arteries were harvested and stained with X-Gal. The number of cells per transverse section expressing the recombinant gene was determined by counting cells in microscopic sections. Data are presented as mean  $\pm$  SD.



**Figure 8.** Model of an "ideal" adenoviral vector, using UV-irradiated adenovirus as a prototype. Current recombinant adenoviral vectors consist of a capsid containing viral genes as well as an inserted transgene. UV-irradiated adenovirus has relatively impaired genomic function but relatively preserved capsid functions. An ideal adenoviral vector would have only a transgene inside an intact capsid.

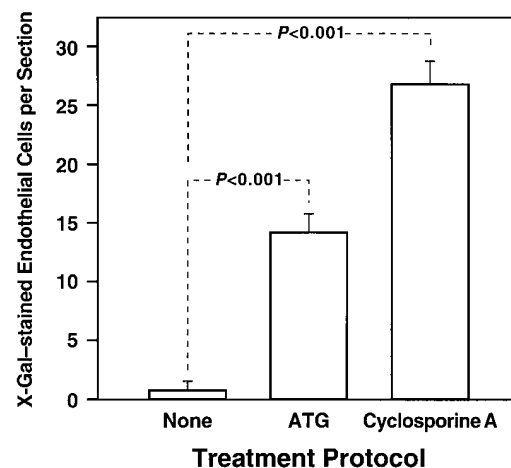
1:32 (1:16–1:32). The magnitude of the antibody response to adenovirus did not differ significantly between the four groups ( $P = 0.12$ ; Kruskal-Wallis one-way ANOVA). However, despite the absence of a quantitative difference in antibody titers, substantial levels of vector-mediated gene expression were achieved in rats preimmunized with UV-Av1LacZ4 but not in rats infused with Av1LacZ4 (Fig. 7). In both the systemically and locally infused groups, there was a statistically significant ( $P < 0.001$ ) increase in  $\beta$ -gal expression in the rats preimmunized with UV-Av1LacZ4. The relatively low level of success in redelivery after an initial exposure to UV-Av1LacZ4 ( $\sim 10$  transduced EC per section vs. 30–60 in naive animals; Fig. 1) may be due to residual expression of viral genes in adenovirus irradiated for only 200 s (Fig. 5). Nevertheless, these data suggest that the immune response to UV-irradiated adenovirus is qualitatively different from the immune response to a fully transcriptionally active virion. These results also raise the possibility that an ideal adenoviral vector (Fig. 8), in which adenoviral genomic DNA was completely eliminated and capsid functions left intact, might be delivered without the generation of a destructive immune response that both eliminates transduced cells and prevents gene expression after repeat gene transfer.

*Adenovirus-mediated gene transfer and expression can be achieved in immune rats by transient immunosuppression.* Human immunity to adenovirus (which is highly prevalent in the American population; see below) results from exposure to intact, not UV-irradiated, adenovirus. Therefore, it is critical to determine whether adenoviral vectors can successfully transfer genes to vascular tissue in the setting of immunity developed in response to intact virions. In seven rats exposed to systemic Av1LacZ4 30 d before attempted gene transfer, we tested whether acute immunosuppression could circumvent the observed block to vascular gene transfer (Fig. 7). Rats were treated with CyA (15 mg/kg/d) or ATG (1 ml/d). Both regimens were begun 1 d before left carotid artery gene transfer and were continued until vessel harvest (3 d after gene transfer).

At the time of gene transfer all rats had detectable neutralizing antibodies to adenovirus. Antibody titers were [median (range)]: naive rats, 1:32 (1:32–1:128); CyA, 1:32 (1:32–1:64); ATG, 1:32 (1:32–1:64). These titers did not differ between groups ( $P = 0.92$  by Kruskal-Wallis one-way ANOVA). Rats treated with CyA ( $n = 3$ ) had  $27 \pm 2$  transduced EC per sec-

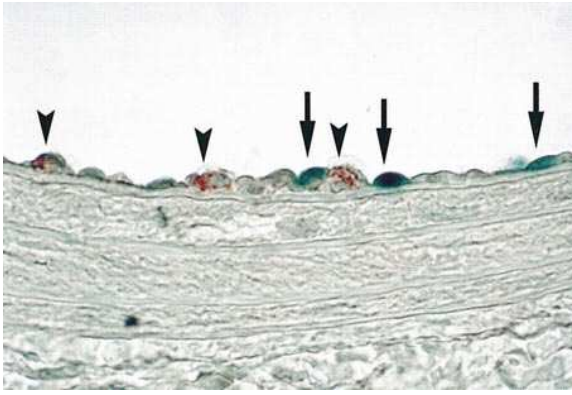
tion; rats treated with ATG ( $n = 4$ ) had  $14 \pm 2$  transduced EC per section. Both of these groups (Fig. 9) showed significantly more transduced cells than did rats ( $n = 6$  from a concurrent experiment; Fig. 7) that were exposed to systemic Av1LacZ4 but were not immunosuppressed ( $0.4 \pm 0.5$  transduced EC;  $P < 0.001$  vs. either group of treated rats). Thus, acute immunosuppression permits vascular gene transfer and expression in the presence of established immunity to adenovirus. However, the extent of recombinant gene expression remains below that achieved by use of the same gene transfer protocol performed in naive, nonimmune animals (compare Fig. 1).

*The ability of immunosuppression to permit recombinant gene expression in immune rats is associated with a decrease in local vascular inflammation.* The half-life of plasma IgG is 3



**Figure 9.** Acute immunosuppression permits gene transfer and expression in rats that have been immunized to adenovirus. Rats were immunized with the Av1LacZ4 vector by intravenous injection of  $2 \times 10^9$  pfu. 29 d later, subgroups of the rats were begun on daily doses of ATG ( $n = 4$ ) or CyA ( $n = 3$ ). On the next day, the Av1LacZ4 vector was infused into isolated left carotid arteries. Rats were continued on ATG or CyA until vessel harvest (4 d total). 3 d after local intraarterial Av1LacZ4 infusion, the arteries were harvested and stained with X-Gal. The number of cells per transverse section expressing the recombinant gene was determined by counting cells in microscopic sections. Data are presented as mean  $\pm$  SD.





**Figure 10.** Luminal T cells are present adjacent to transduced EC. Av1LacZ4 vector was infused into the carotid artery of a rat that had been preimmunized and treated with CyA. 3 d later, the artery was harvested, stained with X-Gal, paraffin-embedded, sectioned, and stained with an antibody to rat CD3. As described elsewhere (Fig. 4), recombinant gene expression is detectable in preimmunized rats only if immunosuppression is also given. Arrowheads indicate the T cells, which are identified by the presence of brown peroxidase reaction product. Transduced EC expressing  $\beta$ -gal are stained blue (arrows). Original magnification  $\times 200$ .

wk (38). It is unlikely that 4 d of treatment with either ATG or CyA would have a significant effect on established humoral immunity to adenovirus. It is more likely that both agents permit recombinant gene expression by suppressing the activity of the cellular immune system. We investigated whether ATG and CyA treatment affected the intensity of inflammation present in the arterial wall 3 d after infusion of adenovirus into immune rats. A low level of inflammation was present in all arteries examined (Figs. 4 and 10). A semiquantitative assessment of inflammation in the arteries of the CyA-treated ( $n = 3$ ) and ATG-treated rats ( $n = 4$ ) revealed significantly less inflammation than in preimmunized rats that received no immunosuppression ( $P = 0.029$  and  $0.014$ , respectively). These data support a role for the cellular immune system in preventing recombinant gene expression after adenovirus infusion in immune rats.

*The prevalence of neutralizing antibodies to adenovirus type 5 in humans is high.* Sera from 53 human subjects were tested for the presence of neutralizing antiadenoviral antibody. The population had a mean age of 39 yr (range 27–62) and included 12 males (23%). On a questionnaire, 68% of the population reported no significant health problems; nine reported hypertension; six reported hypercholesterolemia; and five reported the presence of lung disease. Neutralizing antibody was found in 30 of 53 samples (57%). Among those with neutralizing antibody, titers ranged from 1:2 ( $n = 1$ ) to 1:512 ( $n = 2$ ) with a median titer of 1:8.

## Discussion

We used a model of endothelium-specific gene delivery in the rat common carotid artery to study interactions between adenovirus-mediated arterial gene transfer and the immune system. Our major findings were as follows. First, in a population of Sprague-Dawley rats with no baseline humoral immunity to

human adenovirus type 5, recombinant gene expression can be achieved from a high ( $\sim 40\%$ ) percentage of EC. Recombinant gene expression is unstable over time but can be prolonged significantly by immunosuppression. Second, if the rats are immunized to human adenovirus by prior exposure to the vector, essentially no evidence of recombinant gene expression is found 3 d after attempted gene transfer. This observation is reproduced even in the presence of only low (i.e., 1:2) titers of neutralizing antibody to adenovirus. Third, the efficacy of two immunosuppressive regimens in permitting persistent recombinant gene expression correlates with their ability to suppress the development of a humoral immune response to adenovirus. Fourth, UV-irradiated adenoviral vectors are less effective immunogens than are nonirradiated vectors, as measured by the ability to achieve recombinant gene expression from the endothelium of immunized rats. Fifth, acute immunosuppression permits adenoviral vector-mediated recombinant gene expression within the endothelium of rats with preexisting immunity to adenoviral vectors. Finally, neutralizing antibodies that react with adenovirus type 5 are highly prevalent in the adult American population.

Our finding that recombinant gene expression was essentially unattainable in animals with humoral immunity to adenovirus was unanticipated. The primary barrier to adenovirus-mediated gene transfer in immune animals is thought to be neutralization of recombinant virions by the humoral immune system (13, 39). The rat common carotid artery has no side branches and is thoroughly rinsed with medium before instillation of virus. Thus, in our model of local gene delivery in immune rats, there is essentially no plasma (and therefore few if any neutralizing antibodies) present at the site of gene delivery. The absence of significant recombinant gene expression under these conditions may be interpreted either as evidence that neutralizing antibodies are not completely excluded from the isolated artery or, alternatively, that transduced cells are promptly eliminated by the immune system such that, by day 3 after gene delivery, no transduced cells remain. Quantitation of lymphocytic infiltrates (Fig. 4) as well as subsequent experiments, performed in immune rats treated with CyA or ATG (Fig. 9), support the latter hypothesis. These two immunosuppressive agents would not be expected to affect existing humoral immunity but would strongly affect established cellular immunity. Our data do not exclude a role for preformed antibody along with antibody-dependent cellular cytotoxicity in the elimination of transduced cells; however, the inability to achieve recombinant gene expression in carotid arteries of immune rats most likely results from the effects of established cellular immunity.

Our inability to achieve more than low level recombinant gene expression in the carotid arteries of immune rats (Figs. 3 and 6) appears to contrast with the report of Ueno et al. (40) who attempted redelivery of adenovirus using a double balloon catheter in the femoral arteries of dogs. These authors found that reinfusion of an adenoviral vector expressing a cytoplasmic lacZ gene at a site previously exposed to the same vector “reinduced”  $\beta$ -gal expression locally, even in the presence of neutralizing antibodies. While it is possible that species-specific differences permit redelivery of adenovirus to the dog but not the rat, it must also be noted that Ueno et al. (40) did not exclude the possibility that the measured reinduction of  $\beta$ -gal activity resulted from either enhanced expression from the primary delivery or enhanced endogenous  $\beta$ -gal ac-

tivity. Experiments performed using redelivery of a vector expressing a different transgene (such as luciferase) or accomplishing delivery to a site that had not yet been transduced (such as in this study) would constitute a more definitive proof of the success of redelivery. Finally, use of a cytoplasmic  $\beta$ -gal reporter gene is confounded by background endogenous  $\beta$ -gal activity in vascular tissue (41–44) and is best avoided.

Our results also shed light on the potential for development of adenoviral vectors that might circumvent aspects of immune surveillance. The humoral and cellular immune responses to recombinant adenoviral vectors, as described in several animal models, result in extinction of transgene expression, severe local inflammation, and production of neutralizing antibodies that prevent readministration (11–13, 19, 35, 39). Production of neutralizing antibodies appears to result from exposure to adenoviral capsid proteins present in the initial vector inoculum (13, 39), and therefore will be difficult to circumvent in any adenoviral vector system. The development of adenovirus-specific cellular immunity and the accompanying local inflammatory response leading to the elimination of transduced cells appear to result from persistent low level expression of adenoviral genes that remain within the vector backbone (9, 35, 45). Two strategies have shown promise in resolving this problem: further deletions and modifications of the adenoviral genome (35, 45, 46) and immunosuppression of the recipient organism (35, 39, 47). Although results with immunosuppression have been more encouraging than those obtained with vector engineering in animal models, immunosuppression is not an attractive strategy for general use in human gene therapy. To test the potential that vector engineering will ever be a useful approach, we performed experiments with UV-irradiated adenoviral vectors, which represent an informative model of a transduction-competent particle that lacks viral gene expression (Figs. 5 and 6).

We detected a qualitative difference between the host response to UV-irradiated and nonirradiated adenoviral vectors. After either systemic or local exposure to a UV-irradiated adenoviral vector, readministration of the vector led to significant (though relatively low) levels of recombinant gene expression (Fig. 7). The principal difference between non-irradiated and irradiated adenovirus is the relative lack of gene expression from the latter; therefore, we conclude that the destructive immune response to adenovirus-transduced cells might be decreased or eliminated by using a viral vector that is completely devoid of viral gene expression. Efforts to create such a “guttled” virus by performing extensive deletions of the adenoviral genome have thus far met with modest, but incomplete success (36, 37, 46). The data presented herein suggest that these efforts should be continued and that they may eventually lead to persistent gene expression, lack of local inflammation, and ability to redeliver adenoviral vectors.

It is important to consider the implications of our findings for arterial gene transfer protocols that might be performed in humans. Consideration of these implications presumes that data obtained in rats might be extrapolated to humans; thus, the extrapolations may not be borne out with time. Nevertheless, extrapolation to humans appears worthwhile, particularly given recent statements of enthusiasm for the use of adenoviral vectors for the prevention of restenosis after angioplasty (3–5). First, it is anticipated that arterial gene transfer with adenovirus type 5–based vectors in humans will not result in significant recombinant gene expression in a majority of recipi-

ents due to preexisting immunity. A recent report suggests that the presence of humoral immunity may seriously underestimate the prevalence of established cell-mediated immunity to adenovirus in humans (22). Thus, our determination of a 57% point prevalence of immunity to adenovirus is likely to be an underestimation. Second, it appears unlikely that use of a catheter-based system to perform gene delivery within a closed intravascular space will permit circumvention of preexisting immunity to adenovirus. The rat common carotid artery, which has no branches and is approached surgically in this study, represents an ideal paradigm of vascular isolation. No percutaneous intervention in a human artery (e.g., a coronary artery with numerous side branches) can approach this degree of exclusion of blood from the site of gene delivery. If contact with circulating blood has a detrimental effect on *in vivo* gene transfer (as would be expected consequent to contact between virus and neutralizing antibodies), then anticipated results in humans will only be inferior to those obtained in this study. Third, it does not appear possible to prevent generation of an immune response to adenovirus by attempting to confine vector infusion to a closed intravascular space. Even when extreme care is taken to avoid systemic exposure (see Methods and Fig. 7), it is apparent that a systemic and potentially destructive immune response to the vector is elicited. Fourth, methods by which an existing immune response to adenovirus may be at least partially circumvented (Fig. 9) consist of fairly severe immunosuppressive regimens that are not particularly palatable for generalized human use and may actually worsen underlying atherosclerotic disease (48). Any efforts to extend into clinical practice those results obtained recently with adenoviral vectors in animal models of arterial disease (2–5) should take these observations into account.

Formidable difficulties, mostly involving the immune system and not all appearing to be imminently resolvable, appear to preclude the current clinical application of adenoviral vector-mediated arterial gene transfer. Given these difficulties, are there compelling reasons to continue to investigate the clinical potential of adenovirus? We believe the answer is yes for the following reasons. First, intractable diseases such as restenosis after angioplasty merit a continued search for novel therapies; investigators interested in cures rather than palliation must explore all promising avenues. Second, adenoviral vectors appear to be uniquely capable of achieving the high *in vivo* arterial gene transfer efficiencies [ $\geq 35\%$  (5, 23, 25)] that are likely required for therapeutic efficacy. Third, production of gutted adenoviral vectors appears feasible (36, 37, 46). Fourth, more specific and potentially less harmful methods of immunosuppression than CyA or ATG have shown promise in combating the immune response to adenoviral vectors (47). Fifth, under certain circumstances, use of “self” genes (to which tolerance exists) rather than foreign transgenes may result in a diminished immune response to adenoviral vector-transduced cells (34). Extensive studies in animal models will likely be required to translate these promising observations into clinical protocols that are both safe and likely to be efficacious.

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