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TRANSGENE EXPRESSION AFTER ADENOVIRUS-MEDIATED RETRANSFECTION OF RAT LUNGS IS INCREASED AND PROLONGED BY TRANSPLANT IMMUNOSUPPRESSION

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Objectives: **Adenovirus-mediated gene therapy has been proposed as a potential treatment modality in lung transplantation. However, to date its utility has been limited by an inflammatory host immune response that not only limits the amount and duration of transgene expression but also obviates successful retransfection. Having previously shown that by administering triple-immunosuppression, as is routine in lung transplantation, we could increase and prolong transgene expression after initial transfection, we hypothesized that transgene expression after retransfection could also be increased and prolonged.** ***Methods:*** **Lewis rats underwent intratracheal adenovirus-mediated transfection with the β-galactosidase gene and were randomized to either the immunosuppression group, receiving daily cyclosporine (INN: ciclosporin), azathioprine, and methylprednisolone, or the control group (no immunosuppression). Five weeks later, rats were similarly retransfected and transgene expression and post-transfection inflammation were evaluated 1, 7, and 14 days after retransfection.** ***Results:*** **After retransfection, immunosuppressed rats had significantly higher levels of transgene**

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expression ($P < .001$), whereas control rats had virtually no detectable levels. On histologic sections of the lungs, immunosuppressed rats had overall lesser grades of post-transfection inflammation. ***Conclusions:*** **Transplant immunosuppression attenuates the severe immune response to gene transfer and permits increased, prolonged, and repeated transfection. Retransfection is now achievable in the immunosuppressed lung transplant setting to allow for chronic, repeated administration of gene therapy. (J Thorac Cardiovasc Surg 1999;117:1-7)**

With overall survivorships of only 70% and 60% at 12 and 24 months, respectively, significant improvements to the lung transplantation process can and must be developed.[1] Together, early graft dysfunction resulting from ischemia-reperfusion injury and chronic lung dysfunction, as manifested by progressive obliterative bronchiolitis, account for the major part of mortality and significant morbidity after lung transplantation.[1,2] In particular, obliterative bronchiolitis occurs in almost 50% of lung transplant recipients surviving at least 3 months after transplantation and carries a 4-fold increase in relative risk of mortality.[3] There remains, however, no known effective prevention or treatment for this post-transplantation syndrome.

Adenovirus-mediated gene therapy, with its ability to modify transfected somatic cells, has been suggested as a treatment option for a number of diseases and injuries including reperfusion injury and obliterative bronchiolitis.[4,4a] Gene therapy presents the possibility of modifying the transplanted lung in a number of potentially beneficial ways. This could be achieved, for example, either through up-regulation of protective endogenous antioxidants at the time of transplantation to counteract ischemia-reperfusion injury or by the delivery of an anti-inflammatory cytokine gene, such as interleukin-10 to forestall or prevent the onset of obliterative bronchiolitis.[4]

The potential of gene therapy has been severely limited, however, by a significant inflammatory host immune response. Transfected cells are directly targeted and destroyed by cytotoxic T lymphocytes, with virtually no transgene expression detectable by 14 to 21 days after transfection (unpublished data).[5-9] This immune response has been shown to be even stronger after repeated administration of the vector-gene construct. After adenovirus-mediated retransfection, an almost immediate anamnestic response is seen, causing transgene expression levels to be virtually nonexistent.[6,9-11] These immunologic obstacles to effective gene transfer have heretofore seriously limited any practical clinical application of gene therapy.

Inasmuch as transplant recipients are necessarily immunosuppressed after surgery to prevent organ rejection, we hypothesized that immunosuppression, as used in the transplant setting, could attenuate the deleterious immune reaction after transfection. In a previous study, we demonstrated this concept in a rat model after a 1-time adenovirus-mediated transfection. Not only was transgene expression increased significantly, but these elevated levels were prolonged to 5 weeks after transfection.[4a]

For gene therapy to be made clinically feasible, especially for such chronic and long-term injuries as obliterative bronchiolitis, it is essential to further demonstrate the ability to effectively *retransfect* the transplant recipient. In this study, we therefore investigated the ability of transplant immunosuppression to attenuate the severe immune response to retransfection and achieve increased and prolonged transgene expression levels.

Material and methods

Gene delivery and immunosuppression. Male Lewis rats (275-300 g; Harlan Sprague Dawley, Inc, Indianapolis, Ind) were anesthetized by intraperitoneal injection of ketamine (Rogar; London, Ontario, Canada) and acepromazine (Ayerst; Montreal, Quebec, Canada) and intubated with a 16-gauge Teflon angiocatheter (Becton-Dickinson, Sandy, Utah). They were then transfected intratracheally with 10^9 pfu of E1-deleted type 5 adenovirus containing the *lacZ* gene with a cytomegalovirus promoter (Ad5CMV*lacZ*; University of Iowa Gene Transfer Vector Core, Iowa City, Iowa) in 200 μL of 0.3% sucrose in phosphate-buffered saline (PBS) solution. After transfection, rats underwent simple randomization to either immunosuppressed or nonimmunosuppressed (control) groups (n = l2 and 17 per group, respectively). Starting on the day of transfection, immunosuppressed rats received daily intraperitoneal injections of cyclosporine (INN: ciclosporin) (15 mg/kg per day; Sandoz, Dorval, Quebec, Canada), azathioprine (6 mg/kg per day; Burroughs Wellcome, Kirkland, Quebec, Canada), and methylprednisolone (2.5 mg/kg per day; Upjohn, Don Mills, Ontario, Canada). Five weeks after initial transfection, rats from both groups were administered a second dose of the gene-vector construct.

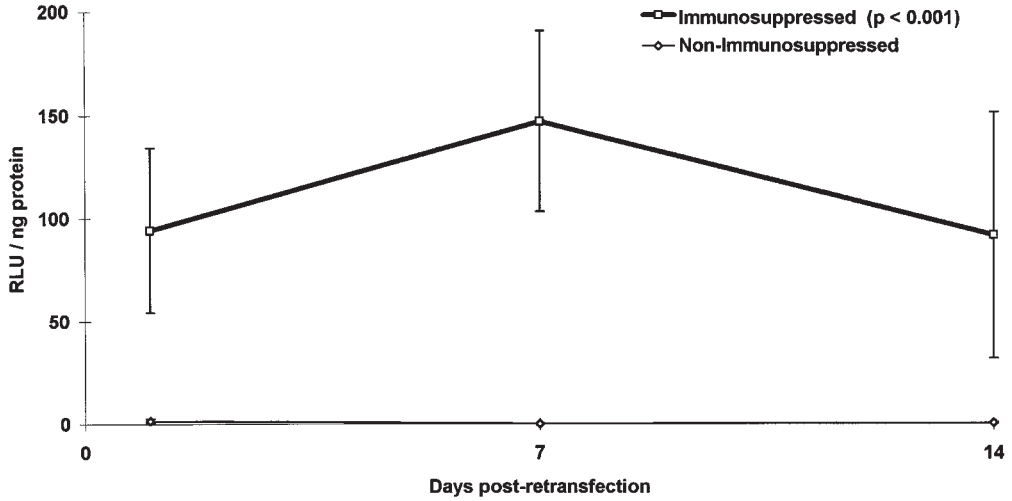

Fig 1. β-Galactosidase transgene expression is increased and prolonged in the lungs of immunosuppressed rats. As compared with control rats, which show virtually no detectable transgene expression, rats receiving transplant immunosuppression have a significantly higher level and persistence of elevated transgene expression as far as 2 weeks after retransfection. Overall difference between the 2 groups, as assessed by 1-way analysis of variance, is significant with a *P* value less than .001. *RLU,* Relative light units; *ng,* nanograms; *error bars* indicate standard deviation.

Lung excision. On days 1, 7, and 14 after retransfection, rats from both immunosuppressed (n = 3, 4, and 5, respectively) and control (n = 5, 6, and 6, respectively) groups were randomly chosen for sacrifice and evaluation of transgene expression. Rats were anesthetized with pentobarbital (MTC Pharmaceuticals, Cambridge, Ontario, Canada) and ventilated via a tracheostomy (Harvard rodent ventilator model 683, Harvard Apparatus Co, South Natick, Mass). Lungs were flushed via the pulmonary artery with 20 mL of 0.9% saline solution at 20 cm H_2O pressure before en bloc excision. Samples from each of the 4 lobes of the right lung were then sectioned and snap frozen in liquid nitrogen for subsequent determination of β-galactosidase transgene expression by cellular chemiluminescence. The left lung was processed for chromogenic staining and histologic sectioning.

Cellular chemiluminescence. β-Galactosidase transgene expression in the lung tissue was measured by the Galacto-light cellular chemiluminescence assay (Tropix, Bedford, Mass).[12] Lung sections were individually homogenized and sonicated in 5 mL of PBS and 200 μL lysis solution. After centrifugation, the supernatant samples were incubated with reaction buffer for 1 hour before the addition of the accelerator solution. Samples were measured in relative light units by means of a standard chemiluminometer (Berthold, Bad Wildbad, Germany) and standardized to each sample's protein content as measured by the Bradford assay technique.[13]

Chromogenic staining. After excision, left lungs were perfused via the trachea with standard fixative: 4% formaldehyde, 0.2% glutaraldehyde, and a 2 mmol/L concentration of $MgCl_2$ in PBS. One hour later, the fixative was washed out with PBS. The chromogenic substrate for β-galactosidase, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; Sigma Chemical Co, St Louis, Mo), in a staining solution (5 mmol/L $K_3Fe(CN)_6$, 5 mmol/L $K_4Fe(CN)_6$, 2 mmol/L $MgCl_2$, 0.05% Triton X-100, in PBS), was then instilled into the lungs and allowed to incubate overnight at 37°C.[4a] Lungs were then sectioned and embedded in paraffin blocks.

Histologic grading. Mid-sagittal slices of the left lung, stained with hematoxylin and eosin, were used to permit blinded histologic assessment and histopathologic grading of the inflammatory response to adenoviral transfection. A modification of the grading method developed by Ginsberg and colleagues[14] to characterize mouse and cotton rat models of adenoviral pneumonia was used. For each specimen, the following 4 criteria were assessed: airway epithelial injury and peribronchial, perivascular, and alveolar space inflammation. These criteria were graded on a scale ranging from normal appearance to minimal (<25%), mild (25%-50%), moderate (50%-75%), and severe (>75%) abnormalities and scored from 0 to 4, respectively.

Animal care. The investigational protocol was reviewed and approved by the Toronto Hospital Research Institute Animal Care and Biohazard committees. All animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research, the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Science and published by the National Institutes of Health (NIH publication No. 85-23, revised 1985), and the "Guide to the Care and Use of Experimental Animals" formulated by the Canadian Council on Animal Care.

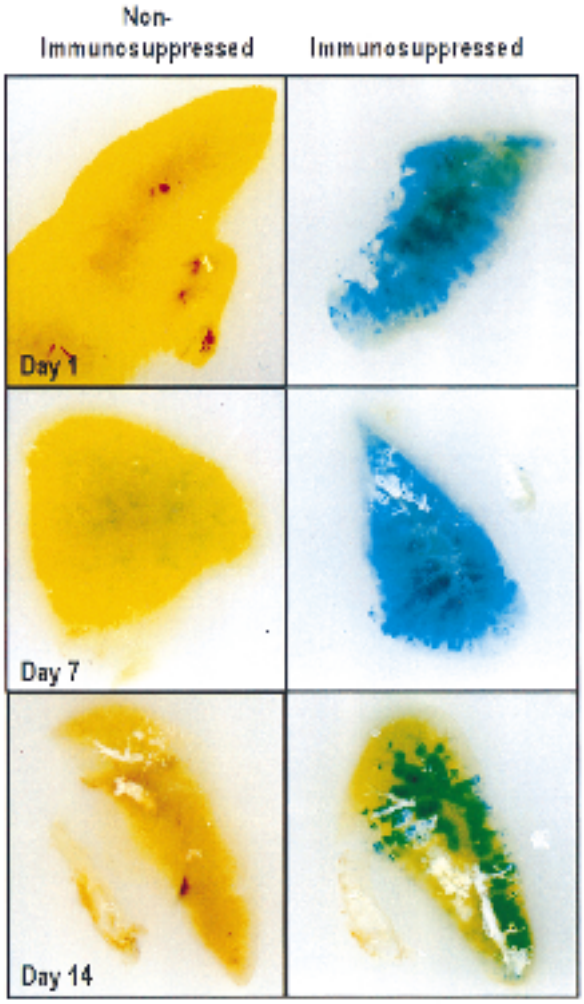

Fig 2. Chromogenic staining of rat lung blocks demonstrates positive and prolonged parenchymal expression of β-galactosidase transgene in immunosuppressed rats after retransfection. Whereas lung tissue from the nonimmunosuppressed group fails to show positive X-Gal staining after retransfection, lungs from rats receiving transplant immunosuppression demonstrate positive β-galactosidase staining at the 1-, 7-, and 14-day time points.

Statistical analysis. Differences between immunosuppressed and nonimmunosuppressed β-galactosidase transgene expression by chemiluminescence were analyzed by means of 2-tailed, unpaired Student *t* tests adjusted for unequal variances and for multiple comparisons by means of the Bonferroni correction. One-way analysis of variance was used to test for overall difference between the immunosuppressed and control groups. Kruskal-Wallis tests were performed to assess statistical differences between immunosuppressed and control groups for the nonparametric histologic grading scores. Individual time points were assessed for statistically significant differences using Dunn's post-hoc analysis. Data were analyzed with the SigmaStat version 1.0 statistical software package (Jandel Scientific, San Rafael, Calif).

Results

Quantitative transgene expression. After repeat transfection, immunosuppressed rats had significantly elevated transgene expression at all 3 evaluated time points: days 1, 7, and 14 after retransfection ($P < .001$; Fig 1). In contrast, transgene expression was virtually undetectable at all time points in control rats. As with initial transfection, seen in our earlier studies,[4a] peak levels of transgene expression were noted at day 7.

Chromogenic tissue staining. Rat lung blocks, stained with X-Gal to demonstrate the presence of β-galactosidase transgene, showed positive and diffuse staining in lungs from rats in the transplant immunosuppression group at the 1-, 7-, and 14-day time points. Corresponding lung blocks from the control (nonimmunosuppressed) group failed to show any detectable positive staining at any of the tested time points after retransfection (Fig 2).

Post-transfection inflammation. Grading of lung tissue sections for post-transfection inflammation revealed several notable results. Alveolar inflammation in the control group was significantly more severe than in the immunosuppressed group and was present from day 1 after retransfection (Fig 3, *A;* $P = .004$). Infiltrating cells were found within the alveolar air space and interstitium and continued to be observable at days 7 and 14 in the control group. Peribronchial inflammation was also more severe in the control group ($P = .002$) and was observable as early as day 1 after retransfection (Fig 3, *B*). Inflammation involving the perivascular space was also significantly higher in the control group ($P = .03$) and was readily apparent at the day 1 time point (Fig 3, *C*). Airway epithelial cell injury was apparent only in the immunosuppressed group on day 1 after retransfection and only to a mild degree (Fig 3, *D*). By day 7, evidence of airway epithelial regeneration was observable and no further injury was noted. Control animals showed no airway epithelial injury throughout the time course of this study.

Discussion

While conventional forms of therapy aim to alter function through transient exposure to external agents, such as medications, the ultimate goal of gene therapy is to effect sustained modifications in structure or function of cells and organs by the transformation of the host's genetic composition. A fundamental step in the process of applying gene therapy, in both the experimental and clinical settings, is the successful transfer of

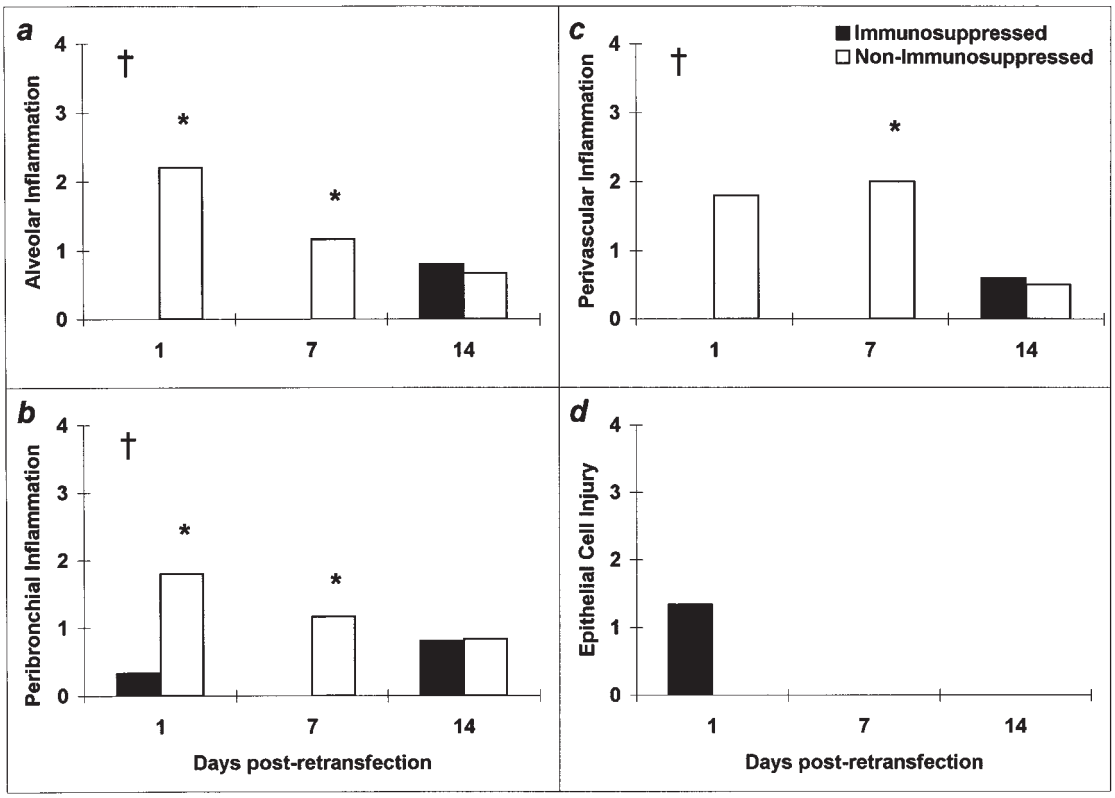

Fig 3. Pathologic grading of post-retransfection inflammation in histologic sections of rat lungs shows overall decreased inflammation in the immunosuppressed group. Lung sections from each of the tested time points were stained with hematoxylin and eosin, and inflammation was graded in the following 4 categories: **A,** Alveolar inflammation; **B,** peribronchial inflammation; **C,** perivascular inflammation; **D,** epithelial cell damage. †$P < .05$ between immunosuppressed and control groups overall; *$P < .05$ between immunosuppressed and control groups at specified time points.

new genetic material to the target cells. Previous studies have emphasized the immune host reaction to transfection as a major obstacle to successful gene transfer after both initial transfection and repeated transfection.[4-11]

In particular, retransfection, as a means to provide repeated long-term gene therapy, has been significantly limited by this immune response. The inability to effectively retransfect is believed to be due to neutralizing antibodies, produced by B cells in response to class II major histocompatibility complex presentation of input viral proteins.[5,9-11] Further studies have demonstrated that, in addition to this humoral component, there is an associated cell-mediated effect from CD4+ T cells that plays a role in the neutralizing immune activity.[10,15]

In keeping with the results of these previous studies of repeated adenovirus-mediated transfection, our results in nonimmunosuppressed (control) rats show the same type of anamnestic immune response with virtually no detectable transgene expression at any of the tested time points after retransfection. This was tested both by quantitative chemiluminescence assays and by chromogenic tissue staining. Our findings demonstrate, however, that the inability, to date, to successfully retransfect lung tissue can be overcome by the administration of triple immunosuppression of the type used in transplantation. Levels of transgene expression in the immunosuppressed lungs, in our study, were not only significantly increased over those of the control group, but they were also prolonged out to 2 weeks after retransfection.

The peak of transgene expression, noted at day 7 in the immunosuppressed group, is consistent with the peak seen after initial transfection by the intratracheal route.[4a] The levels of transgene expression in the immunosuppressed group after retransfection in this study did not reach the peak levels seen in our previous study of 1-time transfection with similar transplant immunosuppression. However, they did surpass and in

fact showed more than a 2-fold increase over the decreasing levels seen in immunosuppressed rats at 5 weeks after initial transfection. It should be further noted that transplant immunosuppression may show even higher transgene expression levels when transfecting with vectors expressing less immunogenic transgene products than the highly immunogenic, foreign β-galactosidase used in this study.[15]

Levels of inflammation, as assessed by histologic grading, were notably higher in the nonimmunosuppressed animals. These animals had patterns of inflammation consistent with previous studies of adenovirus-mediated retransfection.[9] In contrast to the control group, lungs from the immunosuppressed group show significantly attenuated levels of inflammatory infiltrate. When compared with the amount and type of inflammation seen after 1-time transfection,[4a] the inflammation seen in nonimmunosuppressed lungs after retransfection was both earlier in onset and of a more intense nature. This is in keeping with the more rapid and profound immune reaction attributed to the anamnestic response seen in previously sensitized hosts. Of particular note is the early appearance of alveolar inflammation in the control group after retransfection. The onset of this type of inflammation coincides temporally with the loss of transgene expression after both initial transfection (days 7-14[4a]) and retransfection (day 1) and may serve as a signal that transgene expression has been arrested.

Clearly, attenuation of the immune response to adenovirus-mediated transfection continues to be a major focus for those interested in the clinical application of gene therapy. However, the administration of immunosuppression combined with gene therapy in the treatment of such chronic lung diseases as cystic fibrosis and emphysema is limited by the very nature of these diseases, which predispose patients to frequent and often life-threatening infections. Fortunately, the setting of lung transplantation, where patients necessarily receive immunosuppression to prevent rejection of the transplanted organ, provides an excellent opportunity for the successful application of gene therapy. As we progress closer to clinical trials of gene therapy in the transplant setting, it will be important to pay particular attention to the clinical grade of viral vector with special emphasis on the issue of viral replication deficiency. Clearly, what is currently termed *replication deficient* has retained the potential for viral replication, at least to some degree. The impact of this issue, especially in the context of immunosuppressed patients, will have to be determined.

Enhanced and prolonged adenovirus-mediated gene therapy using transplant immunosuppression has been demonstrated, with transgene levels lasting more than 1 month after transfection.[4a] Indeed, the lungs present a unique opportunity among transplantable organs in that they provide an easily accessible and relatively noninvasive route via the trachea for local readministration of adenoviral gene therapy. The ability of transplant immunosuppression to attenuate the immune response to retransfection and produce effective and prolonged transgene expression after repeated vector administration, as shown in this study, further increases the clinical practicality of gene therapy in lung transplantation, particularly for the prevention or attenuation of such complications as obliterative bronchiolitis.

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Discussion

Dr Andrew S. Wechsler *(Philadelphia, Pa).* It is easy to understand why you would have enhanced expression of your transgene in the group that was subjected to retransfection. However, I always thought a fairly rapid transfection would occur on first exposure to an adenovirus. Could you postulate about how the immunosuppression facilitated increasing the expression of the adenovirus on the first exposure?

Dr Cassivi. That question is best illustrated if I return to the initial transfection results.

The initial rise of transgene expression in both the immunosuppressed and nonimmunosuppressed groups is virtually the same. It is only at 7 days, once the transgene expression starts to tail off in the control group, that a significant improvement in the transgene expression in the immunosuppressed group can be seen. I believe that is the effect of the immunosuppression in attenuating the immune response. With the adenoviral transfection occurring epichromosomally and the immune response attacking transfected cells, those cells that have escaped attack by the protective immunosuppression are able to continue to express the transgene.

Dr Todd Rosengart *(New York, NY).* I certainly agree with you. There are a number of studies that now show that adenovirus is influenced by immunosuppression. In fact, there are alternative strategies as well using different serotypes to circumvent the immune-mediated down-regulation of adenovirus expression.

One important point, as these data become more widespread, is that inflammation appears to be related to the amount of replication-competent particles. When we used clinical-grade vector, where there are essentially no replication-competent particles, we found little inflammation. On the other hand, when using preparations with higher replication-competent adenovirus levels, we found much more inflammation. What was the quality of your vector preparation?

My second point is that other strategies will be developed, besides immunosuppression, for prolonging adenovirus expression or gene therapy expression, such as the use of adeno-associated virus. Have you looked at vectors other than adenovirus in your studies?

Dr Cassivi. We used the first-generation adenovirus, an E1-deleted replication-deficient or supposedly replication-deficient viral vector. With continued research we found that the nomenclature of replication deficiency may not be absolutely correct. Replication deficiency of these E1-deleted vectors depends on the cell type being transfected. In an already dividing cell, those functions deleted from the E1 region may already be provided, allowing the virus to escape this control and be replication-competent.

We decided to use the most primitive form of adenoviral vector to prove the principle that immunosuppression in the transplant setting was advantageous for adenoviral transfection. In the future, with advances that are continuing to be made in the construction and development of vectors, such as the adeno-associated vector, we may be able to achieve even better results and make these findings much more widely applicable.

Dr Alain F. Carpentier *(Paris, France).* I have 2 brief questions. First, how long should you prolong your immunosuppression? Does the transgene expression persist when you stop the immunosuppression? Second, have you tried any of the immunosuppressive agents separately so as to eventually have a less aggressive therapy?

Dr Cassivi. We tried to mimic the clinical setting in lung transplantation using the triple immunosuppression of cyclosporine, azathioprine, and methylprednisolone. We did not halt the immunosuppression, because our transplant patients necessarily receive life-long immunosuppression to prevent graft rejection. That was the hypothesis and design of our studies.

As an answer to your second question, we did not do separate studies. It would be useful to investigate the individual effects on transgene expression of the 3 different immunosuppressants used currently and maybe the newer immunosuppressants that are now being used in transplantation.

Dr Robert C. Robbins *(Stanford Calif).* Is this an isograft model or an allograft model?

Dr Cassivi. These experiments were done in nontransplanted rats. They received transplant immunosuppression at the time of transfection. We are now starting to examine therapeutic gene administration in a rat single-lung transplant model.

Dr Robbins. Have you used surfactant to increase your transfection efficiency? When we have tried this in rat lungs, we have not been able to transfect them very well, but with surfactant we were able to improve the transfection efficiency.

Dr Cassivi. We did not use any surfactant to improve the distribution. If you refer to the photographs of the lung blocks stained with X-Gal from the retransfection group, you can see that there is a fairly diffuse and generalized staining of the lung, demonstrating the diffuse distribution of transfection. Furthermore, we did do studies in the right lung, which has 4 lobes in the rat, and quantitatively there was no significant difference between the transgene expression levels in any of the 4 right lung lobes.