General Thoracic Surgery

Gene transfer of tumor necrosis factor inhibitor improves the function of lung allografts

Tsutomu Tagawa, MD,[a,b] Benjamin D. Kozower, MD,[a] Samer A. Kanaan, MD,[a] Niccolò Daddi, MD,[a] Masashi Muraoka, MD,[b] Tadayuki Oka, MD,[b] Jon H. Ritter, MD,[a] and G. Alexander Patterson, MD, FRCS(C)[a]

Dr Tagawa

From the Department of Surgery, Division of Cardiothoracic Surgery, Washington University School of Medicine, Barnes-Jewish Hospital, St Louis, Mo,[a] and First Department of Surgery, Nagasaki University School of Medicine, Nagasaki, Japan.[b]

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Address for reprints: G. Alexander Patterson, MD, FRCS(C), Division of Cardiothoracic Surgery, Washington University School of Medicine, One Barnes-Jewish Hospital Plaza, 3108 Queeny Tower, St Louis, MO 63110-1013 (E-mail: pattersona@msnotes.wustl.edu).

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Background: Tumor necrosis factor is an important mediator of lung transplant acute rejection. Soluble type I tumor necrosis factor receptor binds to tumor necrosis factor-α and -β and inhibits their function. The objectives of this study were to demonstrate efficient in vivo gene transfer of a soluble type I tumor necrosis factor receptor fusion protein (sTNF-RI-Ig) and determine its effects on lung allograft acute rejection.

Methods: Three groups of Fischer rats (n = 6 per group) underwent recipient intramuscular transfection 24 hours before transplantation with saline, 1×10^{10} plaque-forming units of control adenovirus encoding β-galactosidase, or 1×10^{10} plaque-forming units of adenovirus encoding human sTNF-RI-Ig (Ad.sTNF-RI-Ig). One group (n = 6) received recipient intramuscular transfection with 1×10^{10} Ad.sTNF-RI-Ig at the time of transplantation. Brown Norway donor lung grafts were stored for 5 hours before orthotopic lung transplantation. Graft function and rejection scores were assessed 5 days after transplantation. Time-dependent transgene expression in muscle, serum, and lung grafts were evaluated by using enzyme-linked immunosorbent assay of human soluble type I tumor necrosis factor receptor.

Results: Recipient intramuscular transfection with 1×10^{10} plaque-forming units of Ad.sTNF-RI-Ig significantly improved arterial oxygenation when delivered 24 hours before transplantation compared with saline, β-galactosidase, and Ad.sTNF-RI-Ig transfection at the time of transplantation (435.8 $\pm$ 106.6 mm Hg vs 142.3 $\pm$ 146.3 mm Hg, 177.4 $\pm$ 153.7 mm Hg, and 237.3 $\pm$ 185.2 mm Hg; $P = .002$, .005, and .046, respectively). Transgene expression was time dependent, and there was a trend toward lower vascular rejection scores ($P = .066$) in the Ad.sTNF-RI-Ig group transfected 24 hours before transplantation.

Conclusions: Recipient intramuscular Ad.sTNF-RI-Ig gene transfer improves allograft function in a well-established model of acute rejection. Maximum benefit was observed when transfection occurred 24 hours before transplantation.

Lung transplantation is an established treatment for a number of end-stage pulmonary disorders.[1] Unfortunately, ischemia-reperfusion injury and rejection continue to affect clinical lung transplantation.[2] Our laboratory has demonstrated that gene transfer of transforming growth factor-β1 and interleukin (IL)-10 reduces acute rejection in an experimental model of lung transplantation.[3-5] These studies illustrate that gene transfer of immunosuppressive cytokines may be useful in reducing acute rejection in clinical lung transplantation.

Tumor necrosis factor (TNF) is a proinflammatory cytokine that mediates a variety of pathologic conditions.[6] It is primarily secreted by macrophages and influences the immune response after organ transplantation.[7,8] Soluble type I TNF receptor (sTNF-RI) is a receptor for TNF-α and -β. It has been identified in serum and urine.[9] Soluble TNF-RI and the cell-surface TNF receptor competitively bind to TNF-α and -β. After sTNF-RI binds to TNF-α and -β, it neutralizes their function.[10] Therefore, gene transfer of sTNF-RI-IgG fusion protein (sTNF-RI-Ig) has the potential to reduce the immune response meditated by TNF and ameliorate acute lung allograft rejection.

In vivo transgene expression with liposomal vectors to introduce the chloramphenicol acetyl transferase reporter gene into lung grafts has recently been examined. Chloramphenicol acetyl transferase expression was detected as early as 2 hours after in vivo transfection, and it increased in a time-dependent fashion.[11] The delay between gene transfer and gene expression is crucial for the success of cytokine gene therapy in lung transplantation. In short, to reduce acute rejection of lung allografts, gene expression needs to occur before or during the acute lung injury. Experiments to determine the influence of the timing of gene transfection on the functional outcome of lung transplantation have not been performed.

The aims of this study were to demonstrate efficient adenovirus-mediated recipient sTNF-RI-Ig gene transfer, determine its effect on lung allograft acute rejection, and define the influence of the timing of gene transfection.

Materials and Methods

Animals

Fischer 344 rats and Brown Norway rats (Harlan Sprague Dawley Inc, Indianapolis, Ind) weighing 250 to 280 g were used in all experiments. The Animal Studies Committee at Washington University approved all animal procedures. Animals received humane care in compliance with the *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Sciences and published by the National Institutes of Health (National Institutes of Health publication 85-23, revised 1985).

Adenoviral Vectors

Ad.sTNF-RI-Ig originates from a replication-deficient recombinant type 5 adenovirus lacking the *E1* and *E3* loci.[12] Soluble TNF-RI-Ig complementary DNA was inserted in place of the *E1* region, and expression was driven by the cytomegalovirus promoter. Ad.sTNF-RI-Ig encodes a fusion protein that consists of the extracellular domain of the human 55-kd TNF-α receptor and the C_H2 through C_H3 domains of a mouse immunoglobulin G1 heavy chain.[13] The adenovirus used in this study was provided as a gift from Dr Paul D. Robbins, Departments of Molecular Genetics and Biochemistry, University of Pittsburgh (Pittsburgh, Pa).[14]

The first-generation replication-deficient adenovirus serotype 5 carries the *Escherichia coli* LacZ gene. It is driven by the constitutive cytomegalovirus promoter and encodes the enzyme β-galactosidase. β-Galactosidase is a nonfunctional reporter gene that is used as an adenovirus control (provided by gene vector core, University of North Carolina, Chapel Hill, NC).

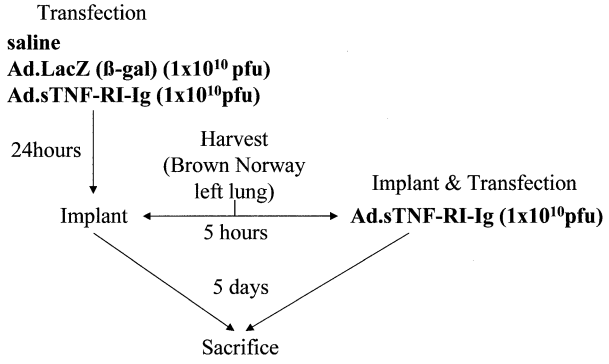

Figure 1. Experimental protocol. The experimental protocol was separated by the vector and the timing of Ad.sTNF-RI-Ig transfection.

Purified viral aliquots were stored at −70°C in 10% glycerol buffered with 10 mmol/L Tris, 140 mmol/L NaCl, and 1 mmol/L $MgCl_2$. These stocks were thawed and diluted in sterile normal saline solution immediately before use.

Experimental Design

Two experiments were performed in this study. Experiment 1 determined the time-dependent transgene expression of sTNF-RI-Ig. Experiment 2 evaluated the effects of sTNF-RI-Ig gene transfection on lung allograft acute rejection.

Experiment 1: Time-Dependent sTNF-RI-Ig Expression in Muscle and Serum

Fischer 344 rats received transfection into the right gastrocnemius muscle with 1×10^{10} plaque-forming units (pfu) of Ad.sTNF-RI-Ig. Rats were killed 6, 12, 18, and 24 hours after transfection (n = 3 each) to determine sTNF-RI-Ig transgene expression in the right gastrocnemius muscle and serum by using enzyme-linked immunosorbent assay (ELISA).

Experiment 2: The Effects of sTNF-RI-Ig Gene Transfection on Lung Allograft Acute Rejection

Fischer 344 rats were divided into 4 groups. Three groups (n = 6 each) underwent recipient intramuscular transfection 24 hours before transplantation with saline, 1×10^{10} pfu of control adenovirus encoding β-galactosidase, or 1×10^{10} pfu of Ad.sTNF-RI-Ig. One group (n = 6) underwent recipient intramuscular transfection with 1×10^{10} pfu of Ad.sTNF-RI-Ig at the time of transplantation. Brown Norway donor lung grafts were stored for 5 hours before orthotopic lung allotransplantation. This combination of rats was chosen because of the strong major and minor histocompatibility locus mismatch that results in complete lung graft rejection within the fifth postoperative day in control recipients without immunosuppression (Figure 1).

Recipient intramuscular gene transfer. Fischer 344 rats were anesthetized with halothane. Adenovirus vector was dissolved in 1 mL of saline and injected into the right gastrocnemius muscle.

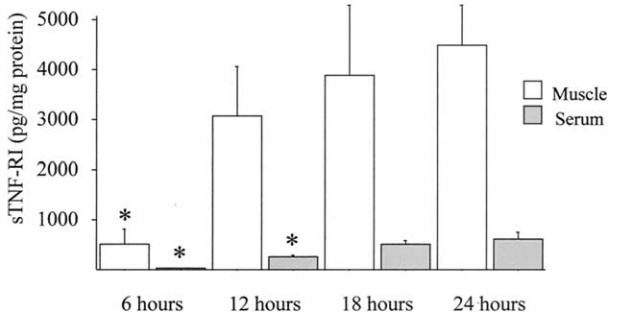

Figure 2. Time-dependent expression of sTNF-RI-Ig after intramuscular transfection. The expression of sTNF-RI-Ig after transfection with 1×10^{10} pfu of Ad.sTNF-RI-Ig into the right gastrocnemius muscle increased in a time-dependent manner in both muscle and serum. *Values in these columns were significantly lower than at the other times.

Donor lung procurement. Brown Norway rats were anesthetized with intraperitoneal pentobarbital (65 mg/kg), mechanically ventilated, and heparinized (300 U), and a median laparosternotomy was performed. Lungs were flushed through the main pulmonary artery with 20 mL of cold (4°C) low-potassium dextran glucose solution at 20 cm H_2O pressure. The heart-lung block was removed with the lungs inflated at end-inspiratory volume. The left lung was isolated and stored at 4°C for 5 hours before implantation. The 5 hours of cold ischemia is consistent with cold ischemia times in clinical transplantation.

Recipient animals were anesthetized and intubated with a 14-gauge catheter, and a left thoracotomy was performed. The pulmonary vessels and bronchus were dissected, and the lung graft was anastomosed by using the modified cuff technique.[15]

Five days after transplantation, recipient animals were reanesthetized with pentobarbital. Animals were mechanically ventilated via a tracheostomy with 100% oxygen. The right main bronchus and pulmonary artery were clamped to isolate the left lung graft. Animals were ventilated for 5 minutes at a tidal volume of 1.5 mL, a respiratory rate of 100 breaths/min, and positive end-expiratory pressure of 1.0 cm H_2O. An arterial blood gas analysis was performed, and the lungs were flushed with 20 mL of 4°C saline. Left lung grafts were divided into 2 sections. The upper half was used for histologic assessment of the rejection score. The lower half was used for ELISA of human sTNF-RI, rat TNF-α, IL-1β, IL-2, and interferon (IFN)-γ. Serum and transfected right gastrocnemius muscles from all rats were used for ELISA of human sTNF-RI.

Histologic assessment of allograft rejection. Slides stained with hematoxylin and eosin were reviewed by a blinded pathologist (J.H.R.) with extensive experience reviewing rat and human lung allograft rejection.[3-5] Histologic rejection was graded on the basis of the 1996 modification of the working formulation for the classification of pulmonary allograft rejection.[16] Vascular and airway rejection were scored independently. A grade of 0 corresponded to the absence of rejection, 1 to minimal rejection, 2 to mild rejection, 3 to moderate rejection, and 4 to severe rejection with complete allograft destruction. Vascular rejection was graded as A0 to A4 and airway rejection as B0 to B4.

ELISA of sTNF-RI. Lung and muscle samples were homogenized with a lysis solution consisting of 100 mmol/L of potassium phosphate dibasic, pH 7.8, including 0.2% Triton X-100 (Fisher Scientific, Fair Lawn, NJ), 5 μg/mL pepstatin A (Boehringer-Mannheim, Indianapolis, Ind), and the protease inhibitor Complete Mini (Roche, Inc, Nutley, NJ). After 15 minutes at room temperature, the extraction was centrifuged at 11,000*g* for 10 minutes at 4°C. Serum samples were obtained by centrifugation of arterial blood samples at 1000*g* for 10 minutes at 4°C. A human sTNF-RI ELISA kit was used (R&D Systems Inc, Minneapolis, Minn). There was no cross-reactivity between the human and rat sTNF-RI proteins. Optical density was determined with an Ultra Microplate Reader (EL808; Bio-Tek Instruments, Inc, Winooski, Vt) set to 450 nm. Total soluble protein (picograms per milligram of total protein) was determined by the method of Pierce Laboratories (Rockford, Ill).[17]

ELISA of rat TNF-α, IL-1β, IL-2, and IFN-γ. Reperfused lung grafts from all 4 groups were used for ELISA of endogenous rat TNFα, IL-1β, IL-2, and IFN-γ, as described previously for sTNF-RI. The rat TNFα, IL-1β, IL-2, and IFN-γ ELISA kits were purchased from R&D Systems.

Statistical Analysis

All values are presented as mean $\pm$ SD. Data not normally distributed were analyzed after logarithmic correction. One-way analysis of variance with pairwise comparison by Fisher's protected least significant difference was used to compare the differences among multiple groups.

Results

Experiment 1

Significant transgene expression of sTNF-RI-Ig was detected in muscle and serum, and it increased in a time-dependent fashion, with the maximum expression 24 hours after transfection. The expression in muscle and serum 12, 18, and 24 hours after transfection was significantly higher than the expression in both 6 hours after transfection ($P <$ .014, .015, and .002, respectively). The expression in serum 18 and 24 hours after transfection were also significantly higher than 12 hours after transfection ($P <$ .011; Figure 2).

Experiment 2: Isolated Lung Allograft Gas Exchange

Intramuscular transfection of Ad.sTNF-RI-Ig 24 hours before transplantation significantly improved arterial oxygen levels compared with saline, β-galactosidase, and transfection of Ad.sTNF-RI-Ig at the time of transplantation ($P =$ .002, .005, and .046, respectively; Figure 3). There were no significant differences in the mean carbon dioxide levels among the 4 groups ($P >$ .166).

Allograft Rejection Score

The vascular rejection scores for the saline, β-galactosidase, and Ad.sTNF-RI-Ig transfection 24 hours before transplantation and Ad.sTNF-RI-Ig transfection at the time of transplantation groups were 2.6 ± 0.4, 2.8 ± 0.5, 2.3 ± 0.3, and 2.7 ± 0.3, respectively. The vascular rejection score for the

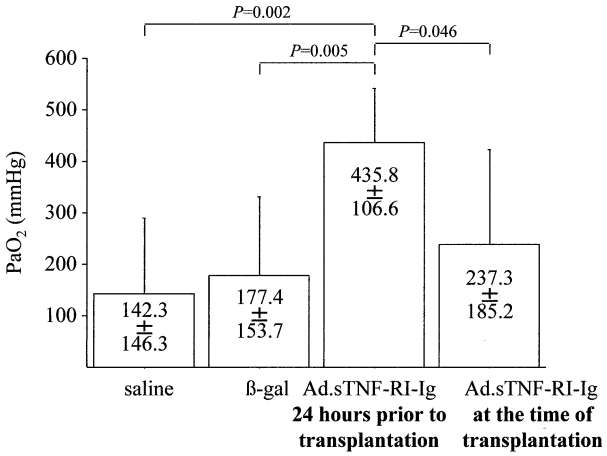

Figure 3. Isolated lung allograft oxygenation 5 days after transplantation. Five days after transplantation, the right hilum was clamped, and the left lung was ventilated with 100% oxygen for 5 minutes. The allografts transfected with 1×10^{10} pfu of Ad.sTNF-RI-Ig intramuscularly 24 hours before transplantation had significantly superior arterial oxygenation (Pao_2) compared with the allografts transfected with saline, β-galactosidase, and 1×10^{10} pfu of Ad.sTNF-RI-Ig at the time of transplantation.

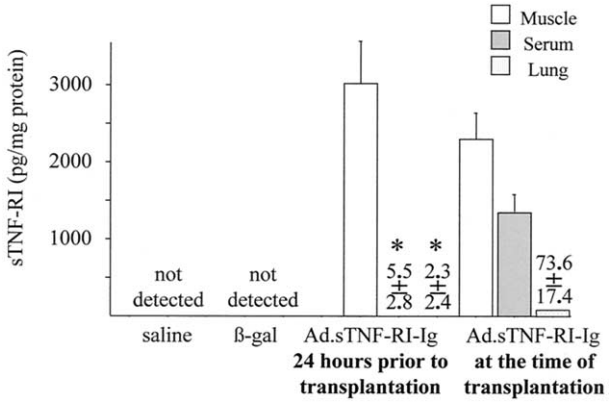

Figure 4. Expression of sTNF-RI-Ig in muscle, lung, and serum 5 days after transplantation. In recipient rats transfected 24 hours before transplantation, significant sTNF-RI-Ig transgene expression was observed in muscle, but less was observed in serum and lung samples (6 days after transfection). In recipient rats transfected at the time of transplantation with 1×10^{10} pfu of Ad.sTNF-RI-Ig, higher transgene expression was observed in muscle, serum, and lung samples (5 days after transfection). *Values in these columns were significantly lower than those in recipient rats transfected at the time of transplantation.

Ad.sTNF-RI-Ig group transfected 24 hours before transplantation was lower compared with the other 3 groups, but this did not reach statistical significance ($P = .066$). There were no significant differences in airway rejection scores among the 4 groups ($P > .836$).

Soluble TNF-RI-Ig Expression 5 Days After Transplantation

The extracts derived from muscle, lung, and serum 5 days after transplantation were used for the measurement of sTNF-RI ELISA. No transgene expression of sTNF-RI-Ig was detected in the saline or β-galactosidase control groups. In both groups transfected with Ad.sTNF-RI-Ig, recipient rats maintained strong expression of sTNF-RI-Ig in muscle. When the sTNF-RI expression in serum and lung was compared between recipient rats transfected 24 hours before transplantation and those transfected at the time of transplantation, the gene expression of sTNF-RI-Ig diminished significantly from day 5 to day 6 after gene transfection ($P < .0001$ for both; Figure 4).

Endogenous Rat TNFα, IL-1β, IL-2, and IFN-γ in Allografts

The endogenous rat TNFα, IL-1β, IL-2, and IFN-γ levels in transplanted lung allografts were measured to determine the effect of sTNF-RI-Ig transfection on the production of these cytokines. No significant differences were observed among the 4 groups ($P > .05$).

Discussion

Acute rejection is one of the most important predictors of postoperative morbidity and mortality after lung transplantation.[1,2] Conventional rescue therapy for acute rejection uses anti-lymphocyte therapy and steroid pulse therapy, but no definitive therapy has been established. The pathophysiology of acute rejection is complex, and numerous cytokines affect the multifactorial rejection process.[7]

TNF is an important proinflammatory cytokine secreted by mononuclear phagocytes and macrophages. It has been broadly recognized for its pleiotropic effects on numerous inflammatory and immunologic responses.[18,19] It is important to note that TNF plays a crucial role in inducing lung transplantation acute rejection.[7,8] TNF-α and -β promote graft destruction by increasing the cytotoxicity of target cells.[7,20] Two kinds of TNF receptors, type I (p55) and type II (p75), specifically bind TNF with equal affinity.[21] These soluble receptors are capable of neutralizing and modulating the biological activity of TNF.[9] This study evaluated the ability of adenovirus-mediated gene transfer of sTNF-RI-Ig, a TNF inhibitor, to reduce acute lung rejection.

We have recently demonstrated that adenovirus-mediated TNF-inhibitor gene therapy ameliorates ischemia-reperfusion injury in rat lung isografts.[22] However, the potential for TNF-inhibitor gene transfer to reduce acute rejection had not been examined. In this study, in vivo recipient intramuscular transfection with 1×10^{10} pfu of Ad.sTNF-RI-Ig 24 hours before transplantation improved isolated lung allograft oxygenation and showed a trend

toward decreased vascular rejection scores. Our ELISA data demonstrate that adenovirus-mediated gene transfer of sTNF-RI-Ig produces sTNF-RI protein in the muscle and that it is released into the systemic circulation. Soluble TNF-RI reaches the allograft, where it can bind endogenous rat TNF and inhibit its cytotoxic effects.

The experimental group that received Ad.sTNF-RI-Ig at the time of transplantation showed a trend toward improved graft function but did not experience as dramatic an effect as the group transfected 24 hours before transplantation. Our ELISA data demonstrate that gene production after transfection occurs in a time-dependent fashion and that the values range in the order of muscle, serum, and lung. The amount of sTNF-RI that reaches the lung allograft within the first 12 hours in the group transfected at the time of transplantation was not sufficient to reduce acute allograft rejection in this experimental model. This may be partially explained by an increased degree of ischemia-reperfusion injury in the group transfected at the time of transplantation because the sTNF-RI protein is not available for protection at the time of transplantation.[22] An alternative explanation is that the sTNF-RI protein is not available at the time of transplantation and thus does not ameliorate the early immunologic rejection process. Future work is required to more fully explain the mechanism for this effect.

In localized transfection to the grafts, the organs in which the gene product is being produced and in which the gene product functions are the same. In the case of recipient intramuscular transfection, they are different. For effect, in vivo recipient intramuscular transfection requires gene product being produced in muscle and released into the circulation for an effect on the remote lung allograft.[3,4,23] There are a number of possible advantages to intramuscular transfection. The vector is easily administered. There also may be an advantage in not introducing an inflammatory adenoviral vector directly into the lung graft. In addition, it would be relatively easy to treat the transplant recipient as soon as a donor organ is identified.[24-26] Furthermore, repetitive administration for sustained effect would be feasible in immunosuppressed recipients.

In this study, serum and lung sTNF-RI ELISA data 5 and 6 days after transfection are difficult to explain. Despite high expression in muscle at 6 days, serum and lung levels of TNF-RI were low. The sTNF-RI used in this study was of human—not rat—origin. It is possible that rat anti-human sTNF-RI antibody bound the human sTNF-RI and facilitated its elimination from the circulation. In the absence of a rat sTNF-RI gene, this speculation cannot be addressed.

Although adenovirus sTNF-RI-Ig gene transfer dramatically improved allograft function, it did not reduce the airway rejection score or the endogenous inflammatory cytokine milieu. There are a number of possible explanations for this observation. The model used in this study is a major mismatch combination that creates severe rejection and graft loss within 5 days in untreated animals. The recipients in this study received no immunosuppression. Also, inhibition of TNF action may help to improve lung graft function without completely blocking the rejection cascade.[17] Previous studies have demonstrated that the results of single anticytokine therapy have been limited because of the overlapping activities of different cytokines.[27] Combination therapy directed toward the inhibition of several cytokines, such as TNF and IFN-γ, may be more useful in improving rejection scores and graft survival. Recent work by our laboratory demonstrated that multiple genes can be co-transfected.[28] The co-transfection of Ad.sTNF-RI-Ig with other anticytokine genes is an exciting area that requires future investigation.

In conclusion, recipient intramuscular sTNF-RI-Ig gene transfer improves allograft oxygenation and demonstrates a trend toward a decreased vascular rejection score. The maximum benefit was observed when transfection occurred before transplantation. Recipient intramuscular delivery of Ad.sTNF-RI-Ig is feasible and warrants further investigation to improve the outcome of clinical lung transplantation.

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GTS

Discussion

Dr Robert J. Korst *(New York, NY).* What was the rationale for the intramuscular route as opposed to another route, such as intravenous or something like that? With an intravenous route, you probably could have saved a lot in terms of the quantity of vector that you needed to use, or is there some downside of having it all go to the liver: is there some downside of expressing that in the liver, for example? What is the thinking behind the intramuscular route?

Dr Alec Patterson *(St Louis, Mo).* It's a good question. This viral vector produces a certain inflammatory response by its application, and we wanted to avoid any confounding effect that might have on the subsequent rejection process. In previous work, we demonstrated pretty good expression using the intramuscular route, and that also has maybe the potential advantage of being perhaps amenable to repetitive treatment. That is the rationale for selection of the intramuscular route.

Dr Mark S. Allen *(Rochester, Minn).* Alec, did you look how fast the gene gets incorporated? Because the one that you gave right at the time of transplantation there didn't seem to affect it, but it did when you gave it some time before the transplant worked. Does it take a couple hours or more than 12 hours to work? How long before the transplantation do you have to give the gene before it is effective?

Dr Patterson. I don't know exactly what the ideal time is. I know that it generally takes 12 to 24 hours. We reasoned that since the inflammatory response of rejection was not likely going to be maximal for a couple of days, transfection at the time of transplantation might actually work. It wasn't quite as good, but I think maybe if it had been administered a few hours before transplantation we might have seen some additional effects.

Dr Douglas E. Wood *(Seattle, Wash).* What do you think is the best time to look at this? You chose 5 days in terms of looking at the outcomes. Obviously, it is an evolving recipient. What do you think is an advantage of 5 days versus looking at it maybe at 1 or 2 days or maybe looking at it even later and seeing what the more chronic consequences are?

Dr Tagawa. In this major mismatched model, fulminant rejection is established at 5 days in control animals. This is the reason we chose to kill animals at the 5-day point. It is quite possible that there might have been a significant difference in results had the analysis been made at 3 or 4 days, when the inflammatory response is not so maximal. We have not conducted these experiments.