TRANSFORMING GROWTH FACTOR- β 1 GENE TRANSFER AMELIORATES ACUTE LUNG ALLOGRAFT REJECTION

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Background: The aim of the current work was to study the feasibility of functional gene transfer using the gene encoding for transforming growth factor- β 1, a known immunosuppressive cytokine, on rat lung allograft function in the setting of acute rejection.

Methods: The rat left lung transplant technique was used in all experiments, with Brown Norway donor rats and Fischer recipient rats. After harvest, left lungs were transfected ex vivo with either sense or antisense transforming growth factor- β 1 constructs complexed to cationic lipids, then implanted into recipients. On postoperative days 2, 5, and 7, animals were put to death, arterial oxygenation measured, and acute rejection graded histologically.

Results: On postoperative day 2, there were no differences in acute rejection or lung function between animals treated with transforming growth factor- β 1 and control animals. On postoperative day 5, oxygenation was significantly improved in grafts transfected with the transforming growth factor- β 1 sense construct compared with antisense controls (arterial oxygen tension = 411 ± 198 vs 103 ± 85 mm Hg, respectively; $P = .002$). Acute rejection scores from lung allografts were also significantly improved, corresponding to decreases in both vascular and airway rejection (vascular rejection scores: 2.0 ± 0.5 vs 2.8 ± 0.6 ; $P = .04$; airway rejection scores: 1.3 ± 0.7 vs 2.3 ± 0.8 , respectively; $P = .02$). The amelioration of acute rejection was temporary and decreased by postoperative day 7.

Conclusions: The feasibility of using gene transfer techniques to introduce novel functional genes in the setting of lung transplantation is demonstrated. In this model of rat lung allograft rejection, gene transfer of transforming growth factor- β 1 resulted in temporary but significant improvements in lung allograft function and acute rejection pathology. (*J Thorac Cardiovasc Surg* 2000;119:913-20)

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We have previously reported on the use of liposomal vectors to introduce the chloramphenicol acetyl transferase reporter gene into transplanted lung isografts. This was shown to be feasible by means of both the in vivo¹ and ex vivo² routes of administration. Transgene expression was noted as early as 4 hours after ex vivo transfection, under conditions used routinely in clinical lung transplantation. Although adenoviral transfection was also possible in the setting of experimental lung transplantation,³ we have focused on the use of cationic lipid vectors due to its resultant lower toxicity and inflammatory responses in the host and the possibility of repeated transfection.

An exciting possibility in organ transplantation is the transfection of immunosuppressive agents to decrease or prevent acute allograft rejection. Although the ideal transfection agent has not been found, several recent reviews have discussed the limitations and potential

advantages of such an approach.^{4,5} Several candidate genes have been studied in animal models. These have included the genes encoding for inducible nitric oxide synthase,⁶ adenovirus early region 3,⁷ antisense intercellular adhesion molecule-1 oligodeoxynucleotides,⁸ viral interleukin-10,⁹ and transforming growth factor- β 1 (TGF β -1).¹⁰ These reports have established the feasibility of whole-organ gene transfection in the setting of acute allograft rejection, resulting in decreases in acute allograft rejection in recipient animals.

One particularly attractive gene with wide-ranging immunosuppressive effects is the gene encoding for TGF β -1. It is a 25-kd homodimeric peptide whose amino acid sequence is highly conserved among mammalian species. It plays several important roles in growth, development, inflammation, tissue repair, and host immunity. There are 3 mammalian isoforms, secreted as latent precursors, of which TGF β -1 is the most widely studied. It has several immunosuppressive effects *in vitro* such as the inhibition of thymocyte proliferation, T- and B-cell proliferation, cytokine production, natural killer cell activity, cytotoxic T lymphocyte development, lymphokine-activated killer cell activity, helper T-cell type 2 cellular apoptosis, monocyte function, antibody production, and cell switching.¹⁰

In this report, we study the effects and time-course of gene transfer of the murine TGF β -1 gene into lung allografts. A model of acute lung allograft rejection was developed in the rat in the absence of systemic immunosuppression. This model was used to study the effects of TGF β -1 transfection on acute lung allograft rejection in the setting of a major histocompatibility mismatch. The *ex vivo* technique was chosen since it was more clinically relevant for organ transplantation and resulted in higher organ specificity than *in vivo* transfection, as we have recently reported.¹¹

Material and methods

Animals. Inbred male Brown Norway donor rats and Fischer/F344 recipient rats, weighing 270 to 300 g, were used in all experiments (Charles River Laboratories, Wilmington, Mass). All animal protocols were approved by the Animal Studies Committee at Washington University. Animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press, revised 1996.

Experimental groups. All experiments involved left lung transplantation with Brown Norway rats used as donors and Fischer rats as recipients. Animals were transfected with either the sense or antisense TGF β -1 construct and then put to

death at 3 different time points after the operation: on post-operative days (PODs) 2, 5, and 7. The sense TGF β -1 group consisted of 3, 8, and 5 animals put to death on PODs 2, 5, and 7, respectively. The antisense TGF β -1 group consisted of 4 and 7 animals put to death on PODs 2 and 5, respectively. Animals put to death on POD 7 included only sense-transfected animals, as previous extensive experience with this model in our laboratory had invariably resulted in complete transplant allograft destruction by POD 7 in the absence of immunosuppression. This was manifested by atelectatic collapse of the transplanted lung, an inability to measure arterial oxygenation from the lung allograft because of the lack of ventilation of the allograft, and histologic evidence of complete lung destruction. Therefore, no antisense-TGF β -1 transfected animals were killed beyond POD 5, because neither lung function nor acute rejection, the end points in this study, could be measured accurately in the presence of such extensive allograft necrosis and destruction.

TGF β -1 plasmid-liposome complexes. The naked plasmid into which the TGF β -1 gene was inserted, pMP6A, consisted of an adeno-associated virus left terminal repeat, followed downstream by the cytomegalovirus promoter, an intron sequence, a multiple cloning site, the SV40 polyadenylation tail, then the adeno-associated virus right terminal repeat. The gene encoding for murine TGF β -1 is a 1579 base-pair sequence that bears a high degree of homology to the native rat TGF β -1 gene. It was cloned into the multiple cloning site of the carrier plasmid pMP6A by means of standard molecular biology techniques. Two plasmids were produced. One carried the gene in the standard orientation, producing functional TGF β -1 protein and corresponding to the sense TGF β -1 plasmid. The other plasmid was identical except for the TGF β -1 gene, which was inserted in an inverse orientation, producing no functional TGF β -1 protein, and corresponding to the antisense TGF β -1 plasmid. Both sense and antisense TGF β -1 plasmids were amplified and purified as previously described.¹² The liposomal vector used in these experiments consisted of GL-67, an amphiphile lipid, mixed in a 1:2 molar ratio with dioleoylphosphatidylethanolamine. It was supplied by Genzyme Corporation, Framingham, Massachusetts,¹² and prepared as previously described.¹

Rat lung transplantation. An orthotopic left lung rat transplant model (Brown Norway donors to Fischer/F344 recipients) was performed by a modification of the "cuff technique" as described in detail elsewhere.² In brief, after harvest, an aliquot of 660 μ g of plasmid DNA was diluted to 5 mL with normal saline solution and passively flushed into the left lung graft over 3 to 5 minutes at a pressure of 20 cm H₂O with a silicone catheter inserted into the left pulmonary vein. The pulmonary venous route of delivery was used for two reasons. First, we had used this same route of delivery in previously published experiments in which we used reporter genes in rat lung grafts.^{1,2} Second, it was technically easier to introduce a catheter into the left pulmonary vein rather than the left pulmonary artery. After storage for 3 hours in normal saline solution at 4°C, allografts were implanted. In recipients put to death on PODs 5 and 7, the bronchial anastomo-

sis was performed with a running 8-0 nylon suture, whereas the cuff technique was used in animals killed on POD 2. Our preference in the presence of acute rejection is for the sutured bronchial anastomosis technique, which was done for the POD 5 and 7 groups, correlating with the time course of acute allograft rejection in this model. On POD 2, however, there was no discernible rejection, which allowed us to use a cuffed bronchial anastomosis, a superior and easier anastomosis to perform. The cuffed anastomosis was used for both the sense and antisense groups on POD 2. When the animals were put to death and assessed, a median sternotomy was performed after induction of anesthesia and endotracheal intubation, as previously described.¹¹ The right hilum was dissected, and the right pulmonary artery and main stem bronchus were clamped for 5 minutes, resulting in one-lung ventilation of the transplanted left lung. This allowed for the measurement of the overall function of the transplanted left lung with an inspired oxygen fraction of 100%, a tidal volume of 1.5 mL, a respiratory rate of 100 breaths/min, and a positive end-expiratory pressure of 1 cm H₂O. Recipients were then put to death and native right and transplanted left lungs were harvested. The inferior portion of the lungs was flash-frozen in liquid nitrogen and stored at -70°C. The superior portion of the lungs was flushed with formalin and then prepared for standard hematoxylin-and-eosin histologic staining.

Histologic assessment of allograft rejection. Slides stained with hematoxylin and eosin were reviewed by a single pathologist (J.M.R.), who was blinded with respect to the animals being examined. He has had extensive experience reviewing rat and human lung allograft rejection.^{13,14} Histologic rejection was graded on the basis of the 1996 modification of the working formulation for the classification of pulmonary allograft rejection.¹⁵ Vascular and airway rejection was 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. Specifically, the following grading schema was developed and used to assign pathologic rejection scores: For vascular rejection (A0-A4), a grade of A1 reflected small perivascular cuffs approximately 2 to 3 cells thick, A2 reflected prominent perivascular cuffs approximately 3 to 5 cell layers thick or more but without involvement of adjacent alveoli, A3 reflected the extension of perivascular cuffing to adjacent alveoli, and A4 represented confluent inflammation, necrosis, hemorrhage, and diffuse alveolar damage. For airway rejection (B0-B4), a grade of B1 represented permeative submucosal airway infiltrates, B2 represented single-cell epithelial apoptosis with more extensive submucosal inflammatory infiltrates, B3 represented additional band-like submucosal infiltrates with more extensive epithelial cell apoptosis and lymphocytic infiltrates, and B4 represented ulceration, epithelial denudation, extensive inflammation, and in some cases airway destruction. The absence of immunosuppression in this study resulted in more fulminant rejection than is seen in the clinical setting. As a result, a large number of the lungs examined had mild to moderate rejection and, as such, were scored as 2 to 3. For statistical analysis, these were given a grade of 2.5.

Measurement of TGF β -1 levels. Homogenized lung samples flash frozen in liquid nitrogen were used to determine tissue TGF β -1 levels by means of the plasminogen activator inhibitor-1 luciferase assay, as previously described.¹⁶ Three separate measurements of luminosity were obtained per sample, averaged, and then divided by the sample's weight to obtain a value of luminosity per gram. This was performed on the first 5 sense-transfected and first 6 antisense-transfected lung allografts, in addition to 5 normal lung specimens that served as a second control for endogenous TGF β -1 levels. Given the absence of a difference among these 3 groups, the remaining lung allografts were not subjected to this assay.

Statistical analysis. All numbers were expressed as mean $\pm$ standard deviation. Data were analyzed by means of Systat version 7 for Windows (Systat, Evanston, Ill). To deal with unequal variances, the continuous data were log-transformed and separate variance *t* tests were done where appropriate. Analysis of variance was performed for 3-group comparisons. Nonparametric tests were used for the rejection score data (Mann-Whitney *U* test). Before completion of data acquisition, data from the first 4 animals were analyzed statistically, and additional animals were added to the TGF β -1 sense and antisense groups to achieve greater statistical significance. To deal with this multiple test problem, the *P* values obtained were then multiplied by 2 (Bonferroni correction).

Results

This study investigated the feasibility of whole-lung transfection with a functional gene. There was only 1 death, 24 hours after the operation, which occurred in the sense TGF β -1 group that was to have been put to death on POD 2.

Arterial oxygenation, as sampled from the transplanted lung allograft when the animal was put to death, reflected the functional status of the graft during the assessment period since the contralateral right hilum was clamped. When measured on POD 2, arterial oxygenation was similar in both sense and antisense TGF β -1 transfected animals: 507 $\pm$ 117 versus 449 $\pm$ 224 mm Hg for sense and antisense TGF β -1 transfected animals, respectively (*P* = .99). On POD 5, arterial oxygenation was significantly higher in animals transfected with sense TGF β -1 constructs than in antisense controls: 411 $\pm$ 198 versus 103 $\pm$ 85 mm Hg, respectively (*P* = .002). On POD 7, arterial oxygenation levels from sense TGF β -1 transfected animals averaged 64 $\pm$ 47, with a range of 0 to 131. One of the 5 lung allografts in this group could not support the animal for the requisite 5-minute period of ventilation before obtaining the arterial blood gas. This is contrasted to our previous observations using this model, when on POD 7, in the absence of immunosuppression, the rejecting transplanted lung allografts

Fig 1. Histologic cross sections after ex vivo transfection with TGF β -1. Representative hematoxylin-eosin-stained cross sections of lung allografts treated with TGF β -1 sense (**A**, **C**, and **E**) or antisense (**B** and **D**) plasmid-liposome complexes then transplanted and put to death on PODs 2 (**A** and **B**), 5 (**C** and **D**), or 7 (**E**). **A** and **B** (POD 2): Minimal perivascular lymphocytic inflammation is present without significant interstitial airway inflammation. In **A**, a typical vessel is shown at 3 o'clock, and two adjacent bronchioles are shown at the 7 o'clock position. In reviewing the entire slide, a rejection score of A2B0 was given for section **A** and a score of A2B1 for section **B**. **C** and **D** (POD 5): Increased density of perivascular lymphoid infiltrates and extensive peribronchiolar inflammation. Airway inflammation is now involving all epithelial layers. After review of the entire slide, a rejection score of A2-3B2 was given for both sections **C** and **D**. **E**: Extensive acute rejection with massive cuffing of vessels and airways, with extensive interstitial chronic inflammation, corresponding to a rejection score of A4B3. **F**: Representative section from the contralateral normal right lung. Note the absence of inflammation in either airways (at 1-3 o'clock) or vessels (at 5 and 8 o'clock).

could not support the animal for the 5-minute assessment period, resulting in the absence of a measurable arterial Po_2 level.

No noticeable histologic findings were observed on POD 2 between sense and antisense TGF β -1 groups, as evidenced by vascular and airway rejection scores: The vascular rejection score was 1.5 ± 0.5 versus 1.6 ± 0.3 for the sense and antisense TGF β -1 groups, respectively ($P = .99$). The airway rejection score was 0.7 ± 0.6 versus 0.3 ± 0.5 for the sense and antisense TGF β -1 groups, respectively ($P = .6$).

On POD 5, gene transfection with sense TGF β -1 constructs resulted in a significant reduction in the histologic grading of rejection, which was true for both the vascular and airway rejection scores: The vascular rejection scores were 2.0 ± 0.5 versus 2.8 ± 0.6 for sense and antisense TGF β -1 transfected recipients, respectively ($P = .04$). The airway rejection scores were 1.3 ± 0.7 versus 2.3 ± 0.8 for sense and antisense TGF β -1 transfected animals, respectively ($P = .02$).

On POD 7, the protective effects of TGF β -1 gene therapy in this model of acute lung allograft rejection decreased considerably. This was true for both vascular and airway rejection, with scores for sense TGF β -1 transfected animals of 3.7 ± 0.5 , and 2.6 ± 0.4 , respectively. Although no antisense TGF β -1 transfected control animals were put to death on POD 7, our previous experience with this model had shown extensive destruction, necrosis, and diffuse alveolar damage by PODs 6 and 7. Grossly, this was manifested by complete lung destruction, with an inability to inflate or ventilate the lung allograft. A representative sample of histologic findings is shown in Fig 1.

Tissue TGF β -1 levels were measured from lung homogenates transfected with either sense or antisense TGF β -1 constructs by means of the plasminogen activator inhibitor-1 luciferase assay. This assay yielded a value of luminosity per gram of tissue, corresponding to total TGF β -1 activity: 419 ± 58 , 378 ± 118 , and 454 ± 71 luminosity units per gram for sense TGF β -1, antisense TGF β -1, and normal rat lungs, respectively ($P = .6$).

Comment

This report describes the introduction of a novel functional gene into lung allografts before transplantation, followed by an observable functional effect after implantation. The feasibility of whole-lung transfection with a functional gene is demonstrated, with subsequent amelioration of acute rejection in a model of fulminant allograft rejection.

The administration of TGF β -1, or the gene encoding for it, to organ allografts at the time of transplantation has been shown to result in beneficial results in several experimental settings. Initial experiments focused on the administration of the recombinant TGF β -1 protein, which resulted in the prolongation of graft survival in the mouse heart,¹⁷ rat heart,¹⁸ mouse pancreatic islet allografts,¹⁹ and rat islet xenografts in mice.²⁰ The direct injection of naked TGF β -1 DNA into rat cardiac allografts significantly prolonged graft survival from 12.6 ± 1.1 days to 26.3 ± 2.5 days in the absence of systemic immunosuppression.^{10,21} The immunosuppressive effects of TGF β -1 were shown to be partially mediated via the suppression of local T-cell immunity. Specifically, TGF β -1 gene transfection reduced the precursor frequency of donor-specific cytotoxic T lymphocytes, and both activated and total interleukin-2-producing helper T lymphocytes in graft-infiltrating cells.²² More recently, the intracoronary administration of TGF β -1 adenoviral vectors in rabbits resulted in the prolongation of cardiac allograft survival from 6.9 days to 11.1 days, with significant decreases in rejection scores in treated animals.²³ The introduction of an adenoviral vector encoding TGF β -1 into liver cells before transplantation has been shown to result in decreased production of tumor necrosis factor α and γ interferon.²⁴ In another study, a retroviral vector was used to introduce the TGF β -1 gene into myoblasts before transplantation, which resulted in a 20% decrease in myoblast mortality after 3 days, correlating with lower neutrophil and macrophage cell counts in transfected muscles compared with those of control animals.²⁵ Recent evidence from human kidney transplant recipients suggests that cyclosporine (INN: ciclosporin) stimulates TGF β -1 expression, both at the messenger RNA and protein levels.²⁶ Further, we have recently reported that the ex vivo administration of TGF β -1 was superior to the in vivo approach, resulting in improved allograft function.¹¹ We have also successfully introduced the TGF β -1 constructs intratracheally, resulting in an amelioration of acute rejection, although not to the same extent as intravenous administration.²⁷ These data collectively suggest that TGF β -1 gene transfer is possible in a transplant setting and results in local, albeit transient, immunosuppressive effects.

This report further supports the immunosuppressive role of TGF β -1 in acute rejection. Significant improvements in arterial oxygenation and acute rejection were observed after transfection with sense TGF β -1 constructs. On POD 2, arterial oxygenation and acute rejection scores showed no significant differences

between the 2 groups. This was not unexpected, as acute rejection had not yet developed at this time period, corresponding to normal arterial blood gas measurements and histologic findings from transplanted lung allografts. In this model, acute rejection was at its peak on POD 5, with resultant decreases in arterial PO_2 measurements in the control (antisense TGF β -1) group compared with the experimental (sense TGF β -1) group. This correlated with the peak protective effects of TGF β -1 gene therapy in this model. The absolute improvement of arterial oxygenation, such as comparing the function of the transplanted left lung with that of the native right lung, was not investigated in this report. It is technically difficult to obtain two arterial blood gas specimens from the same animal without exsanguinating the animal. However, on the basis of our previously published results in isogeneic animals,² in which acute rejection was not a factor, an arterial PO_2 of 546 ± 18 mm Hg had been obtained from transplanted left lung isografts by means of techniques similar to the ones used in this study. The results obtained in the sense TGF β -1 group were still lower than those obtained in isogeneic controls. The histologic features of the contralateral native right lungs of recipient animals were normal compared with findings in the rejecting left lung allografts. Thus, although TGF β -1 resulted in an improvement in arterial oxygenation and histologic rejection scores, it was still not completely protective against lung dysfunction.

The immunosuppressive effects of TGF β -1 gene therapy were transient in our model. By POD 7, the multifactorial rejection process was able to overcome the local immunosuppressive effects of TGF β -1, resulting in worsening graft function and pathologic rejection scores. The model used in these series of experiments corresponded to a major histocompatibility type I mismatch, which resulted in profound allograft necrosis by POD 7. Any prolongation of graft function is therefore significant, despite transient gene expression. Another reason for the transient effects of TGF β -1 gene therapy includes the small number of transfected cells typically detected by means of gene therapy techniques, on the order of 5% to 10% of all cells.

TGF β -1 gene therapy appeared to be more immunosuppressive for airway rejection than for vascular rejection. The exact reason for this gradation in immunosuppression is not clear. It should be noted that although TGF β -1 plasmid-liposome complexes were infused via the vascular route, their effect on airway inflammation was greater than their effect on vascular rejection.

Finally, when total tissue TGF β -1 levels were measured, no significant differences were encountered

between sense and antisense transfected animals. This is likely due to the measurement of *total* TGF β -1 levels using this assay, whereas the functional effect exerted by TGF β -1 is derived from the *active* form of the peptide. The majority of TGF β -1 is stored in a *latent* form, which is also measured by the luciferase assay. Further, the assay measures endogenous TGF β -1 levels present in the rat lung. Additional transfection with murine TGF β -1 would not be differentiated from endogenous rat TGF β -1. These data support our observations that tissue TGF β -1 levels were not different when measured by another technique, the enzyme-linked immunosorbent assay, as we have recently reported.¹⁴ It should be noted, however, that by using reverse transcriptase polymerase chain reaction (RT-PCR), we have proof of gene transfection with TGF β -1 with significant differences found between sense-transfected animals compared with antisense controls.²⁷

In summary, gene transfer of TGF β -1 into rat lung allografts before transplantation resulted in statistically significant improvements in graft function and acute rejection. The feasibility of gene transfer with a functional gene in the lung transplant setting is demonstrated. This may make it possible one day to alter the lung allograft before or after allograft implantation and observe beneficial downstream effects such as decreases in ischemia-reperfusion injury or acute and chronic allograft rejection or even the induction of tolerance.

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Discussion

Dr Verdi DiSesa (*Chicago, Ill*). To say that this is functional gene transfer, you have to show that you are actually expressing the gene that you transferred. Have you looked for expression of the gene and do you hypothesize that the reason rejection occurs is that you lose expression of the gene?

Second, therapy that delays the onset of rejection usually will enhance the survival of the allograft, but your allografts were pretty much gone by POD 7 no matter what you did. Do you know the reason for that?

Dr Mora. Thank you, Dr DiSesa. We did not include data on TGF β -1 gene expression in this presentation because of time constraints. We have recently used RT-PCR to document TGF β -1 gene expression by the production of the TGF β -1 messenger RNA in transfected allografts. The RT-PCR experiments were carried out with the use of the same TGF β -1 plasmid described in this report, except that the route of plasmid administration was via the airways as opposed to the left pulmonary vein, as in this report. We also attempted to measure TGF β -1 protein levels using both a luciferase assay and an enzyme-linked immunosorbent assay but did not find significant differences between the sense and antisense groups. One reason for this could be that the protein assays currently available measure total TGF β -1. The active form of TGF β -1 that results in a functional effect, and that was presumably transferred by the plasmid vectors, represents a small percentage of this TGF β -1 protein pool, where the majority of TGF β -1 is stored in a latent, nonfunctional form. Thus a small increase in active TGF β -1 would result in an even smaller effect on total TGF β -1 levels, presumably below the levels of detection by the assays.

With regard to your second question concerning graft loss despite TGF β -1 transfection, we think this is due to the multifactorial process involved in acute rejection. Thus, although one pathway of the rejection cascade may be suppressed by TGF β -1 gene transfer, other pathways remain intact and result in the continuation of rejection.

Dr Beat H. Walpoth (*Bern, Switzerland*). Do you think repetition of TGF β -1 gene transfer would help in postponing allograft rejection? How are the kinetics of TGF β -1 gene transfer?

Second, is aerosol administration feasible? Perhaps that will be the best method to use in lung transplantation.

Dr Mora. Thank you. With regard to your first question, we have not attempted to readminister plasmid-liposome complexes in these animals. Repeated transfection with plasmids could potentially be easily done since liposomal vectors do not incite a host inflammatory response, as opposed to

adenoviral vectors. One can theoretically administer the plasmid-liposome complexes at various time points in the rejection process, although we have not looked at this using TGF β -1. When we used the chloramphenicol acetyl transferase reporter gene and did repeated injections in our rat lung transplant model, we did notice prolonged gene expression of the chloramphenicol acetyl transferase protein up to 77 days after initial administration. With repeated administration, there was continued expression of the gene.

With regard to your second question regarding aerosol administration, one of our fellows in the laboratory is actually performing that study; he is administering the TGF β -1 plasmid DNA, with or without the liposome, intratracheally at the time of organ procurement. He has observed an amelioration of allograft rejection and an improvement in arterial oxygenation. Interestingly, the amount of allograft rejection was slightly worse with an intratracheal route of administration compared with the intravascular route as described in the current study.

Dr Harvey I. Pass (*Detroit, Mich.*). I appreciate that you measured the TGF β levels. Is the antisense the proper control here? Is there the theoretic possibility that your antisense is actually interfering with native TGF β -1 levels in your control situation so that your effect in your controls is actually worse than it really is in the actual situation of a negative cassette control?

Dr Mora. The antisense TGF β -1 group consisted of the same TGF β -1 gene as in the sense group except that it was inserted in an inverse sequence in the plasmid vector. This controls for nonspecific effects of the plasmid or the liposome, while at the same time producing a nonfunctional protein. We have performed other experiments in the laboratory using normal saline controls in the same strain combination used here, and we have uniformly measured a Pao₂ on POD 5 between 50 and 80 mm Hg. This is certainly similar to what we have observed in the antisense TGF β -1 controls used in this study.