IMMUNOSUPPRESSANT-FREE ALLOTRANSPLANTATION OF THE TRACHEA: THE ANTIGENICITY OF TRACHEAL GRAFTS CAN BE REDUCED BY REMOVING THE EPITHELIUM AND MIXED GLANDS FROM THE GRAFT BY DETERGENT TREATMENT

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Objective: To develop a method for eliminating the epithelium and mixed glands from tracheal grafts by detergent treatment and evaluate these grafts for immunosuppressant-free allotransplantation in dogs.

Methods: Fresh canine tracheal grafts were treated with a detergent (1% Triton X-100 t-octylphenoxypolyethoxyethanol; T-9284; Sigma Chemical Co, St Louis, Mo) at 4°C for 48 hours. The grafts were then used for intrathoracic 5-ring tracheal replacement in other dogs without immunosuppressant treatment (n = 6, detergent treatment group). In the control group (n = 6) fresh untreated canine tracheal segments were implanted as allografts. All the implanted grafts were covered with an omental pedicle.

Results: In the detergent treatment group the chondrocytes in the graft had a similar appearance to those in the fresh trachea, indicating that the chondrocytes remained viable after the detergent treatment. In 5 of the 6 grafts, the epithelium and mixed glands had been removed completely. After transplantation, these 5 grafts were incorporated by the host trachea without stenosis. In the remaining treated tracheal graft, in which removal of the epithelium was incomplete, moderate stenosis was observed at the fourth week after implantation, although this was not progressive. In the control group, granulation tissue of the graft and significant stenosis were observed after transplantation.

Conclusion: The antigenicity of tracheal grafts can be greatly reduced by removing the epithelium and mixed glands by the use of detergent treatment. The epithelium and mixed glands of the graft appear to be the determining elements involved in rejection after tracheal allotransplantation. (J Thorac Cardiovasc Surg 2000;120:108-14)

It is widely known that recipients of allografts tend to reject them after implantation. The rejection of allografts is elicited by foreign histocompatibility antigens on the grafted cells. Many antigens can act as histocompatibility antigens. The strongest transplantation

antigens are those expressed by a single chromosomal region known as the major histocompatibility complex (MHC), which in human subjects is located on chromosome 6. MHC has been found to play an important role in graft rejection.^{1,2}

It has previously been reported that MHC-II and MHC-I antigens are expressed in the epithelium and mixed glands of the trachea but not in tracheal cartilage.³⁻⁵ This suggests that the mucosa, and not the cartilage, is the major antigenic structure of the trachea and thus responsible for transplant rejection. If the antigenicity of tracheal grafts could be eliminated, then immunosuppressant-free tracheal allotransplantation could possibly be performed. We have therefore developed a detergent treatment technique that removes the antigenic structures from tracheal grafts while maintaining cartilage viability and used the grafts treated in

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Received for publication Sept 3, 1999; revisions requested Oct 14, 1999; revisions received Jan 26, 2000; accepted for publication Feb 21, 2000.

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0022-5223/2000 \$12.00 + 0 12/1/106655

doi:10.1067/mtc.2000.106655

Fig 1. Light microscopic and scanning electron microscopic examination revealed that the epithelium and mixed glands of the grafts had been completely removed from grafts 1 to 5 in the detergent treatment group. (A, Hematoxylin and eosin staining, original magnification 25 $\times$; B, original magnification 2500 $\times$.) The chondrocytes in the graft appeared similar to those in the fresh trachea.

this way for tracheal allotransplantation without immunosuppressants.

Material and methods

Preparation of tracheal allografts. Fresh tracheal grafts, including 7 to 8 cartilage rings, were harvested from beagle dogs weighing 9 to 16 kg.

In the detergent treatment group the inner surface of the graft was scraped with a plastic blade and then rinsed with running water at room temperature. After immersion in 3.0% saline solution for 10 minutes at 4°C, the inner surface of the graft was scraped again. It was then immersed and stirred in 3 L of 1.0% *t*-octylphenoxypolyethoxyethanol solution (Triton X-100; T-9284; Sigma Chemical Co, St Louis, Mo) at 4°C for 12 hours, and the inner surface was scraped again. The above procedure was repeated 4 times, requiring a total of about 48 hours. Finally, the graft was thoroughly rinsed with physiologic saline solution to remove the detergent, and it was stored in physiologic saline solution at 4°C until use (storage time, 2-39 hours). A total of 6 grafts were prepared in this way.

Histologic examination and demonstration of MHC-II antigen. Before transplantation, one ring was resected from each end of every graft to check the preoperative condition of the graft by means of light microscopy and scanning electron microscopy.

Immunohistochemical staining for MHC-II antigen was performed by the labeled streptavidin biotin method on frozen sections (5 μ m). The primary antibody used was a rat anti-canine MHC-II antibody (MCA 1044; Serotec, Oxford, United Kingdom) diluted 1:50, and the secondary antibody was a biotin-conjugated rabbit anti-rat antibody (STAR52, Serotec) diluted 1:300. The sections were incubated with the primary antibody at room temperature for 1 hour and then with the secondary antibody at room temperature for 1 hour.

Fig 2. The epithelium and mixed glands are no longer seen in grafts 1 to 5 in the detergent treatment group. No MHC-II antigen is identified. (Original magnification 25 $\times$.)

The horseradish peroxidase conjugated with streptavidin was detected by means of the diaminobenzidine reaction.

Tracheal allotransplantation. After endotracheal intubation of the recipient beagle dog (weight, 7-16 kg), anesthesia was maintained with 50% oxygen, 50% nitrous oxide, and 1% halothane. An omental pedicle graft was prepared and drawn into the right hemithorax through the diaphragm, and the right side of the chest was opened at the fourth intercostal space. A 5-ring segment of the intrathoracic trachea was then removed, and the defect was reconstructed with the treated graft ($n = 6$, detergent treatment group). Telescopic anastomosis was performed by interrupted suturing with 4-0 Prolene suture (Ethicon, Inc, Somerville, NJ). In the control group, fresh untreated tracheal segments were transplanted as allografts immediately after harvest ($n = 6$). The anastomotic site and the graft were wrapped with the omental pedicle in both groups.

Postoperative care and observation. No immunosuppressants were given at any time during the course of the experi-

Table I. Results in the detergent treatment group

No.	Epithelium and mixed glands on the graft	Status of graft	Survival (d)	Cause of death
1	Removed completely	Incorporated	321	Alive
2	Removed completely	Incorporated	183	Killed
3	Removed completely	Incorporated	189	Killed
4	Removed completely	Incorporated	191	Killed
5	Removed completely	Incorporated	108	Killed
6	Epithelium remaining	Moderate stenosis	91	Killed

Table II. Results in the control group

No.	Epithelium and mixed glands on the graft	Status of graft	Survival (d)	Cause of death
1	Intact	Severe stenosis	42	Killed
2	Intact	Granulation	63	Killed
3	Intact	Granulation	56	Killed
4	Intact	Severe stenosis	70	Killed
5	Intact	Severe stenosis	35	Killed
6	Intact	Granulation	63	Killed

ment. An antibiotic (ampicillin sodium–cloxacillin sodium for injection, 500 mg/d) was administered intramuscularly on the day of the operation and continued daily for 1 week. The dogs were then given ampicillin–cloxacillin tablets orally (500 mg/d) from the second to the fourth week. Bronchoscopic examination was performed at the second and fourth weeks after the operation and monthly thereafter in the detergent treatment group and once a week in the control group. When the animals were killed, the implanted grafts were removed en bloc for histologic examination.

All the animals used in this study received humane care in compliance with the “Guide for the Care and Use of Laboratory Animals” prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (National Institutes of Health publication No. 86-23, revised 1985).

Results

In the detergent treatment group (Table I), complete removal of the epithelium and the mixed glands was confirmed by both light and scanning electron microscopy in 5 of the 6 grafts (Fig 1). The appearance of the cartilage cells was similar to that in fresh untreated trachea, indicating that the cartilage cells remained viable. MHC-II was not identified in these grafts (Fig 2). In the remaining tracheal graft, some residual epithelium was detected. Deformation of the remaining epithelium was severe, and our immunohistochemical staining system did not demonstrate expression of MHC-II antigen. All animals ($n = 6$) in the detergent treatment group survived uneventfully for more than 3 months (Table I). The observation period for 4 dogs

was more than 6 months, with the longest being 321 days. Two dogs were killed at 3 months and 3 dogs were killed at 6 months after transplantation. Bronchoscopic examination showed no stenosis or granulation tissue in the grafts in dogs 1 to 5 (Fig 3). The inner surface of the grafts was shiny, and the grafts had been incorporated by the host trachea in all 5 dogs. Macroscopically, no necrosis or atrophy was observed in any of the grafts. Complete epithelial regeneration was confirmed in all the implanted grafts by both light and scanning electron microscopy. In dog 6, moderate stenosis was detected at the fourth week after surgery, but it did not progress any further, and the dog was asymptomatic.

In the control group (Table II), the fresh tracheal grafts had intact epithelium, subepithelial mixed glands, and viable chondrocytes (Fig 4). MHC-II antigen was identified in both the epithelium and the mixed glands but not in the cartilage (Fig 5). During these immunohistochemical staining procedures, both the positive and negative controls clearly stained as expected, and no background or other nonspecific staining was observed. Graft necrosis and dissolution were observed from 2 to 3 weeks after the operation in the control group (Fig 6). Granulation tissue gradually began to obstruct the airway, and fatal stenosis occurred in 3 dogs during the observation period. Histologic examination of the grafts showed that absorption of the tracheal cartilage was accompanied by severe lymphocyte and monocyte infiltration into the subepithelium.

Fig 3. Bronchoscopic (A, 2 weeks after transplantation; B, 6 months after transplantation) and macroscopic (C, 6 months after transplantation) findings in dog 2 in the detergent treatment group. The graft has been incorporated by the host trachea without stenosis. Complete regeneration of the epithelium is confirmed. (D, Hematoxylin and eosin staining, original magnification 100 $\times$.)

Fig 4. Pseudostratified columnar ciliary epithelium and subepithelial mixed glands are observed in the fresh trachea. (A, Hematoxylin and eosin staining, original magnification 25 $\times$.) Scanning electron microscopic examination reveals dense cilia covering the fresh tracheal surface. (B, Original magnification 2500 $\times$.)

Discussion

Direct end-to-end anastomosis is possible when less than 6.4 cm of the trachea has been resected.⁶ When longer segments are removed, another method of reconstruction is necessary. Tracheal transplantation has been studied for several decades. The reasons for the lack of

success are mainly due to graft ischemia and rejection. Anatomically, it is difficult to anastomose tracheal vessels.⁷ Neville and colleagues⁸ reported that dogs could not survive longer than 1 month with a 5-ring free tracheal graft, even in the case of an autograft. The cause of death was stenosis of the airway induced by collapse of

Fig 5. MHC-II antigen is identified in the epithelium and mixed glands of the fresh trachea (arrows; original magnification 25 $\times$). The chondrocytes are negative.

the graft. For graft viability to be maintained, promotion of graft revascularization appears to be necessary. Messineo,⁹ Nakanishi,¹⁰ and their colleagues reported that omentopexy was an effective method for facilitating neovascularization in tracheal autografts. The trachea was long believed to have weak antigenicity.¹¹ However, despite omentopexy, all the animals died of airway obstruction between 14 and 78 days after fresh tracheal allotransplantation.¹²⁻¹⁴ Histologic examination revealed epithelial defects, granulation tissue with fibrosis, mononuclear cell infiltration into the subepithelium, and absorption of the tracheal cartilage. Beigel and Muller-Ruchholtz^{15,16} tried both orthotopic and heterotopic transplantation in rats and found that the trachea was subject to the same immunologic laws as all other tissues when used for transplantation. In our present control group, similar serious airway stenosis was observed after transplantation of fresh untreated tracheal segments. This suggested that graft rejection occurred after fresh tracheal allotransplantation and, accordingly, the use of immunosuppressants was necessary to control the immune reaction.^{17,18} However, use of immunosuppressants has certain disadvantages, especially in patients with malignant disease. Therefore, there have been several attempts at immunosuppressant-free tracheal transplantation.

Yokomise and colleagues^{12,19} succeeded in performing immunosuppressant-free tracheal transplantation with high-dose irradiated grafts and long-term cryopreserved grafts. Mukaida,¹³ Tojo,¹⁴ and their colleagues reported that immunosuppressant-free tracheal transplantation could be achieved by using cryopreserved grafts. However, the mechanism of reduction of graft antigenicity after cryopreservation is still unclear, and

the question of how long a tracheal graft can be preserved is unanswered. With a variety of clinical needs in mind, we considered that it is necessary to develop a more practical method of preparing grafts.

It has long been known that cartilage can be transferred successfully between individuals of different genetic backgrounds without any need for immunosuppression therapy.²⁰ This immunologic privilege is attributable to the absence of a blood supply in adult articular cartilage. The absence of direct vascularization and the presence of a dense proteoglycan-collagen matrix will insulate chondrocytes from the host antigens.²¹

We thought that it would be difficult to eliminate the antigenicity of tracheal allografts completely, while maintaining their viability. However, if it were possible to completely remove the major antigenic structures (ie, the epithelium and mixed glands) from the graft while maintaining the viability of the tracheal cartilage, we thought that this graft might be successful in immunosuppressant-free tracheal allotransplantation.

Because MHC antigen expression is observed mainly in the tracheal mucosa,³⁻⁵ in a preliminary experiment we directly scraped the inner surface of the trachea in an attempt to remove the mucosa. However, we abandoned this method because removal of the mucosa was inadequate and the cartilage was severely damaged.

The detergent Triton X-100 is a nonionic surfactant used to extract membrane proteins and inactivate viruses in human plasma.²² It converts lipids into micelles and can destroy cell membranes, which consist of a lipid bilayer. In this way surfactants can remove antigens expressed on cell membranes, lipids, and glycosaminoglycans.^{23,24} Yamamoto and colleagues²⁵ reported that the use of the detergent was effective in preparing an acellular matrix membrane from human amnion. Sondell and colleagues²⁶ reported the removal of myelin and Schwann cells from nerve allografts with Triton X-100 and application of these grafts for sciatic nerve replacement in rats.

In the present study a 1.0% detergent solution (hypotonic solution), 3.0% saline solution (hypertonic solution), and scraping were used alternately and repeatedly to enhance removal of the epithelium. In grafts 1 to 5 of the detergent treatment group, not only the epithelium but also the subepithelial mixed glands were eliminated, and the viability of the chondrocytes seemed to be maintained. Neither stenosis nor granulation tissue occurred in the dogs that received these well-treated grafts. Dog 6, which received a graft from which the epithelium had not been sufficiently removed, had mild stenosis within 4 weeks after the operation. However,

Fig 6. Bronchoscopic (**A**, 2 weeks after transplantation; **B**, 5 weeks after transplantation) and macroscopic (**C**, 5 weeks after transplantation) findings reveal serious stenosis of the graft in the control group. Lymphocyte-monocyte infiltration and absorption of the tracheal cartilage are evident. (**D**, Hematoxylin and eosin staining, original magnification 100 $\times$.)

this stenosis did not advance thereafter. This may have been because most of the antigenic structures had already been removed by the detergent treatment, and therefore the rejection was too mild to cause progressive stenosis. Accordingly, we think it is important to determine whether any residual epithelium is present before implantation.

In this experiment the grafts containing living host chondrocytes maintained a normal luminal shape. The acellular matrix on the graft surface provided a site for regeneration of the epithelium, and the regenerated epithelium covered the inner surface of the graft.

With regard to the origin of the regenerated epithelium after tracheal transplantation, Mukaida and colleagues,²⁷ using polymerase chain reaction–restriction fragment length polymorphism, concluded that the regenerated epithelium was of recipient origin in a cryopreserved canine tracheal allotransplantation model. The recipient epithelium migrated gradually from the anastomosis site to the transplanted graft and covered the allograft within about 50 days after transplantation. Tojo and colleagues²⁸ investigated the origin of the epithelium in transplanted cryopreserved tracheal allografts in rats by using immunohistochemical staining and found that the epithelium of the trans-

planted segment originated from the recipient epithelium, whereas the cartilage retained the structure of the donor trachea 2 months after transplantation. Beigel and colleagues²⁹ also confirmed that the tracheal allograft epithelium would be replaced by recipient epithelium after transplantation.

The bulk of experimental and clinical evidence suggests that cartilaginous allografts have a greater tendency for late deterioration and absorption than autografts.²¹ The long-term changes in the cartilage of allotransplanted tracheal tissue are still unclear. Therefore, an additional period of observation will be required to confirm whether malacia of the graft will occur after transplantation.

Conclusion

We have found that the epithelium and subepithelial mixed glands of tracheal grafts are the determining factors involved in rejection after tracheal allotransplantation. We developed a new practical method for eliminating the epithelium and subepithelial mixed glands from tracheal allografts by detergent treatment while maintaining the viability of the chondrocytes and used the treated grafts for successful immunosuppressant-free tracheal transplantation.

REFERENCES

1. Bach FH, Sachs DH. Transplantation immunology. *N Engl J Med* 1987;317:489-92.
2. Batchelor JR, Welsh KI, Maynard A, Burgos H. Failure of long surviving, passively enhanced kidney allografts to provoke T-dependent alloimmunity. I. Retransplantation of (AS X AUG) F1 kidneys into secondary AS recipients. *J Exp Med* 1979;150:455-64.
3. Daar AS, Fuggle SV, Fabre JW, Ting A, Morris PJ. The detailed distribution of MHC class II antigens in normal human organs. *Transplantation* 1984;38:293-8.
4. Daar AS, Fuggle SV, Fabre JW, Ting A, Morris PJ. The detailed distribution of HLA-A, B, C antigens in normal human organs. *Transplantation* 1984;38:287-92.
5. Bujia J, Wilmes E, Hammer C, Kastenbauer E. Tracheal transplantation: demonstration of HLA class II subregion gene products on human trachea. *Acta Otolaryngol (Stockh)* 1990;110:149-54.
6. Grillo HC, Dignan EF, Miura T. Extensive resection and reconstruction of mediastinal trachea without prosthesis or graft: an anatomical study in man. *J Thorac Cardiovasc Surg* 1964;48:741-9.
7. Salassa JR, Pearson BW, Payne WS. Gross and microscopical blood supply of the tracheal. *Ann Thorac Surg* 1977;24:100-7.
8. Neville WE, Bolanowski PJP, Soltanzadeh H. Homograft replacement of the trachea using immunosuppression. *J Thorac Cardiovasc Surg* 1976;72:596-601.
9. Messineo A, Filler RM, Bahoric B, Smith C, Bahoric A. Successful tracheal autotransplantation with a vascularized omental flap. *J Pediatr Surg* 1991;26:1296-300.
10. Nakanishi R, Shirakusa T, Takachi T. Omentopexy for tracheal autografts. *Ann Thorac Surg* 1994;57:841-5.
11. Rose KG, Sesterhenn K, Wustrow F. Tracheal allotransplantation in man. *Lancet* 1979;1:433.
12. Yokomise H, Inui K, Wada H, Goh T, Yagi K, Hitomi S, et al. High-dose irradiation prevents rejection of canine tracheal allografts. *J Thorac Cardiovasc Surg* 1994;107:1391-7.
13. Mukaida T, Shimizu N, Aoe M, Andou A, Date H, Okabe K, et al. Experimental study of tracheal allotransplantation with cryopreserved grafts. *J Thorac Cardiovasc Surg* 1998;116:262-6.
14. Tojo T, Niwaya K, Sawabata N, Kushibe K, Nezu K, Taniguchi S, et al. Tracheal replacement with cryopreserved tracheal allograft: experiment in dogs. *Ann Thorac Surg* 1998;66:209-13.
15. Beigel A, Muller-Ruchholtz W. Tracheal transplantation. I. The immunogenic effect of rat tracheal transplants. *Arch Otorhinolaryngol* 1984;240:185-92.
16. Beigel A, Muller-Ruchholtz W. Tracheal transplantation. II. Influence of genetic difference and degree of sensitization on reactions to the tracheal transplant. *Arch Otorhinolaryngol* 1984;240:217-25.
17. Davreux CJ, Chu NH, Waddell TK, Mayer E, Patterson GA. Improved tracheal allograft viability in immunosuppressed rats. *Ann Thorac Surg* 1993;55:131-4.
18. Delaere PR, Liu Z, Sciort R, Welvaart W. The role of immunosuppression in the long-term survival of tracheal allografts. *Arch Otolaryngol Head Neck Surg* 1996;122:1201-8.
19. Yokomise H, Inui K, Wada H, Ueda M, Hitomi S. Long-term cryopreservation can prevent rejection of canine tracheal allografts with preservation of graft viability. *J Thorac Cardiovasc Surg* 1996;111:930-4.
20. Gibson T, Davis WB, Curran RC. The long-term survival of cartilage homografts in man. *Br J Plast Surg* 1959;11:177-87.
21. Simmons RL, Sutherland DER, Lower RR, Najarian JS. Transplantation. In: Schwartz SI, Shires GT, Spencer FC, Storer EH, editors. *Principles of surgery*. 4th ed. New York: McGraw-Hill; 1984. p. 396-7.
22. Strancar A, Raspor P, Schwinn H, Schutz R, Josic D. Extraction of Triton X-100 and its determination in virus-inactivated human plasma by the solvent-detergent method. *J Chromatogr A* 1994;658:475-81.
23. Wilson GJ, Courtman DW, Klement P, Lee JM, Yeager H. Acellular matrix: a biomaterials approach for coronary artery bypass and heart valve replacement. *Ann Thorac Surg* 1995;60:S353-8.
24. Courtman DW, Pereira CA, Kashef V, McComb D, Lee JM, Wilson GJ. Development of a pericardial acellular matrix biomaterial: biochemical and mechanical effects of cell extraction. *J Biomed Mater Res* 1994;28:655-66.
25. Yamamoto Y, Ueda H, Matsumoto K, Sekine T, Inoue H, Suzuki K, et al. Production of acellular matrix membranes from human amnion for medical application. *Jpn J Artif Organs* 1997;26:219-24.
26. Sondell M, Lundborg G, Kanje M. Regeneration of the rat sciatic nerve into allografts made acellular through chemical extraction. *Brain Res* 1998;795:44-54.
27. Mukaida T, Shimizu N, Aoe M, Andou A, Date H, Moriyama S. Origin of regenerated epithelium in cryopreserved tracheal allotransplantation. *Ann Thorac Surg* 1998;66:205-8.
28. Tojo T, Kitamura S, Gojo S, Kushibe K, Nezu K, Taniguchi S. Epithelial regeneration and preservation of tracheal cartilage after tracheal replacement with cryopreserved allograft in the rat. *J Thorac Cardiovasc Surg* 1998;116:624-7.
29. Beigel A, Steffens-Knutzen R, Muller B, Schumacher U, Stein H. Tracheal transplantation. III. Demonstration of transplantation antigens on the tracheal mucosa of inbred rat strains. *Arch Otorhinolaryngol* 1984;241:1-8.

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