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# Reproduction of the Obliterative Bronchiolitis Lesion after Heterotopic Transplantation of Mouse Airways

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***Obliterative bronchiolitis, characterized histopathologically by airway inflammation and occlusion of small airways by vascularized fibrous tissue, constitutes an important threat to the long-term survival of lung and heart-lung transplant recipients. The pathogenesis of obliterative bronchiolitis is poorly understood, and successful preventative or treatment strategies are not available. We sought to develop a preclinical model system of obliterative bronchiolitis by transplanting murine airway grafts, consisting of tracheas and main bronchi, into the subcutaneous tissue of allogeneically mismatched recipient animals. By 10 days after transplantation, allografts demonstrated subepithelial and/or peritracheal inflammation, epithelial necrosis, and early fibroproliferation. Grafts harvested 21 days after transplantation demonstrated fibroproliferation in the airway wall or lumen in nine of 10 allografts versus 0 of 10 isografts (P = 0.0001). In addition, abnormal epithelium (ie, nonciliated cuboidal, squamous, or absent) was seen in all allografts, while nine of nine isografts demonstrated normal respiratory epithelium (P = 0.0003). Although differences exist between this model and the chronic rejection process in human lung transplant recipients, these findings reproduce the characteristic features of obliterative bronchiolitis and demonstrate that this lesion can result from allograft rejection. This model will be useful for studying the pathogenesis, prevention, and treatment of obliterative bronchiolitis after lung transplantation. (Am J Pathol 1993, 142:1945–1951)***

Lung and heart-lung transplantation, introduced into clinical medicine in 1981, have now been performed in over 2000 recipients for whom effective alternative medical therapy was not available. Initial results of these procedures have been impressive, and current 2-year survival rates are approximately 70%.[1] Despite these remarkable successes, problems remain. In the first 3 months after transplantation acute lung rejection and infections are the major clinical problems encountered. During the later posttransplant period, approximately one-third of recipients develop progressive deterioration of graft function due to obliterative bronchiolitis, characterized clinically by dyspnea and progressive airflow obstruction.[2] This devastating complication leads to irreversible losses of lung function and is frequently fatal. Histologically, the characteristic lesion of obliterative bronchiolitis is defined by small-airway inflammation and lumenal occlusion by vascularized fibrous tissue.[3–5]

The pathogenesis of obliterative bronchiolitis is poorly understood, and effective treatment is lacking. Clinical investigation of the human disease is hampered by the limited quantities of biological material that can be obtained from each patient and the small number of affected individuals available for study. These factors make the development of preclinical models a highly desirable goal. Such models would allow sequential studies of the fibroinflammatory lesions and would afford the opportunity to test promising therapeutic strategies. The most satisfactory model to date, a whole-lung rat allograft model,[6] reproduces many characteristic changes of obliterative bronchiolitis but suffers from two major limitations: first, orthotopic lung transplantation in small animals requires the use of specialized microsurgical equipment and techniques, limiting its applicability; and second, the coexistence of severe vascular and pa-

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renchymal damage complicates the study of the airway lesion. In this report, we describe a new model of heterotopic allogeneic transplantation of mouse trachea and bronchi that is technically simple, allows the specific study of transplanted airways, and replicates the histopathological features of obliterative bronchiolitis independently of other pulmonary lesions.

## Materials and Methods

### Donor and Recipient Surgery

The trachea and main bronchi or peripheral pulmonary tissue from donor mice were transplanted heterotopically into the subcutaneous tissue of recipient mice. Six- to 8-week-old male inbred BALB/c and C3H mice were obtained from Harlan Sprague-Dawley (Indianapolis, IN). Isogeneic (BALB/c donor into BALB/c recipient) and allogeneic (BALB/c donor into C3H recipient) transplants were performed. All experiments were carried out in accordance with the guidelines of the University of Minnesota Animal Care Committee.

Donor mice were anesthetized with intraperitoneal sodium pentothal (Nembutal, Abbott Laboratories, North Chicago, IL) (150 mg/kg) containing heparin sulfate (150 U/ml). Animals were exsanguinated, and the trachea, heart, and lungs were exposed via an anterior midline incision. The lungs were flushed with 3 ml of ice-cold Euro-Collins solution (Transplant Technologies, Dallas, TX) containing penicillin G (100 U/ml), streptomycin sulfate (100 μg/ml), and amphotericin B (0.25 μg/ml) (Antibiotic/Antimycotic, Gibco, Grand Island, NY), injected into the right ventricle of the beating heart. The thymus was removed, and the trachea, lungs, and heart were excised *en bloc*, and care was taken to divide the trachea distal to the thyroid cartilage. To prepare the airway graft, the bronchi were divided at the pulmonary hilum, and the tracheobronchial preparation was trimmed of excess surrounding tissue and submerged in ice-cold Euro-Collins solution until implantation. Following removal of the trachea and main bronchi, the pulmonary parenchymal grafts were prepared by removing the pulmonary hilum and sectioning the remaining lung into 1–2-mm-thick slices, which were submerged in ice-cold Euro-Collins solution until implantation.

Recipient mice were anesthetized with intraperitoneal sodium pentothal (25 mg/kg). A small area on the back was shaved and prepared with betadyne solution. An airway graft was implanted into a subcutaneous pocket via a small skin incision, which was closed with nylon sutures. In 30 of 38 recipient animals, a pulmonary parenchymal graft was implanted into a separate subcutaneous pocket, as previously described.[7] The time interval between graft harvest and implantation was less than 15 minutes.

### Histopathological Evaluation

Grafts were harvested at 10 and 21 days after transplantation, fixed in formalin, and prepared for histopathological assessment in routine fashion. All sections were analyzed by a single pathologist who was blind to the identity of the individual specimens. Each specimen was scored by the following criteria: the airway was scored for the intensity (mild, moderate, severe), distribution (intraepithelial, subepithelial, transmural, peritracheal), and predominant cell type of the inflammatory infiltrate. Fibroproliferation in the airway wall and lumen was scored by distribution (eccentric or circumferential), and the percentage of airway occlusion was estimated. The respiratory epithelium was scored for the percentage of luminal circumference it covered and for necrosis (focal, patchy, diffuse). The epithelium was classified as normal or abnormal, and the types of abnormalities, including metaplastic changes, were described. Submucosal glands were categorized as normal, absent, or atrophic and were scored for inflammation (none, mild, moderate, severe).

### Statistical Analysis

Associations between categorical variables and graft type were assessed using a $\chi^2$ test or a Fisher's exact test if the comparison resulted in a $2 \times 2$ table. Differences between proportion of airway surface lined by epithelium and percentage reduction of the lumen caliber were assessed using the Mann-Whitney U-test. A nonparametric test was chosen because of small sample sizes and inability to determine if the samples were normally distributed. All analyses were performed using Statistical Package for the Social Sciences.

## Results

Nineteen isogeneic transplants (BALB/c into BALB/c) and 19 allogeneic transplants (BALB/c into C3H) were performed. Of the 38 recipient animals, histologically evaluable tissue was recovered from

33. Reasons for graft loss included anesthetic-related recipient death (one isograft, one allograft), unexplained posttransplant recipient death (one isograft), and absence of recognizable graft tissue at harvest (two isografts). No evidence of infection was observed in any animal.

## *Heterotopic Pulmonary Parenchymal Grafts*

Pulmonary parenchymal grafts harvested 10 and 21 days after transplantation showed extensive organization of the central portion of the graft, which involved all but a small rim of peripheral lung and completely effaced the normal pulmonary architecture. Many grafts demonstrated ductlike structures lined by respiratory epithelium or less frequently by squamous epithelium. However, these changes were not consistent, and in many cases no evidence of regenerating epithelium was observed. Therefore, our attention focused on the examination of transplanted airway tissue.

## *Heterotopic Airway Grafts*

Airway isografts harvested 10 days after transplantation ($n$ = 6) were histologically normal or nearly normal. In marked contrast, allografts harvested 10 days after transplantation ($n$ = 8) demonstrated inflammation, epithelial abnormalities, and early fibroproliferative changes (Figure 1). Inflammation in the allografts was primarily subepithelial but also involved the peritracheal tissue in several specimens. In most instances, lymphocytes and neutrophils were the predominant cells, although plasma cells and histiocytes were also observed. Epithelial necrosis was observed in seven of eight allografts versus 0 of six isografts ($P$ = 0.007). Normal respiratory epithelium was observed in five of six isografts, while epithelial abnormalities (ie, squamous epithelium or a denuded basement membrane) were present in all allografts ($P$ = 0.008). Early fibroproliferative changes were observed in five of eight allografts but did not result in significant compromise of the airway lumen at this time point.

Histological abnormalities typical of the obliterative bronchiolitis lesion observed after orthotopic lung transplantation were clearly present in allografts by 21 days after transplantation, while isografts continued to demonstrate normal or near-normal histology (Figure 2A, 2B, Table 1). Airway inflammation was observed in each of the 10 allografts, was graded moderate or severe in eight

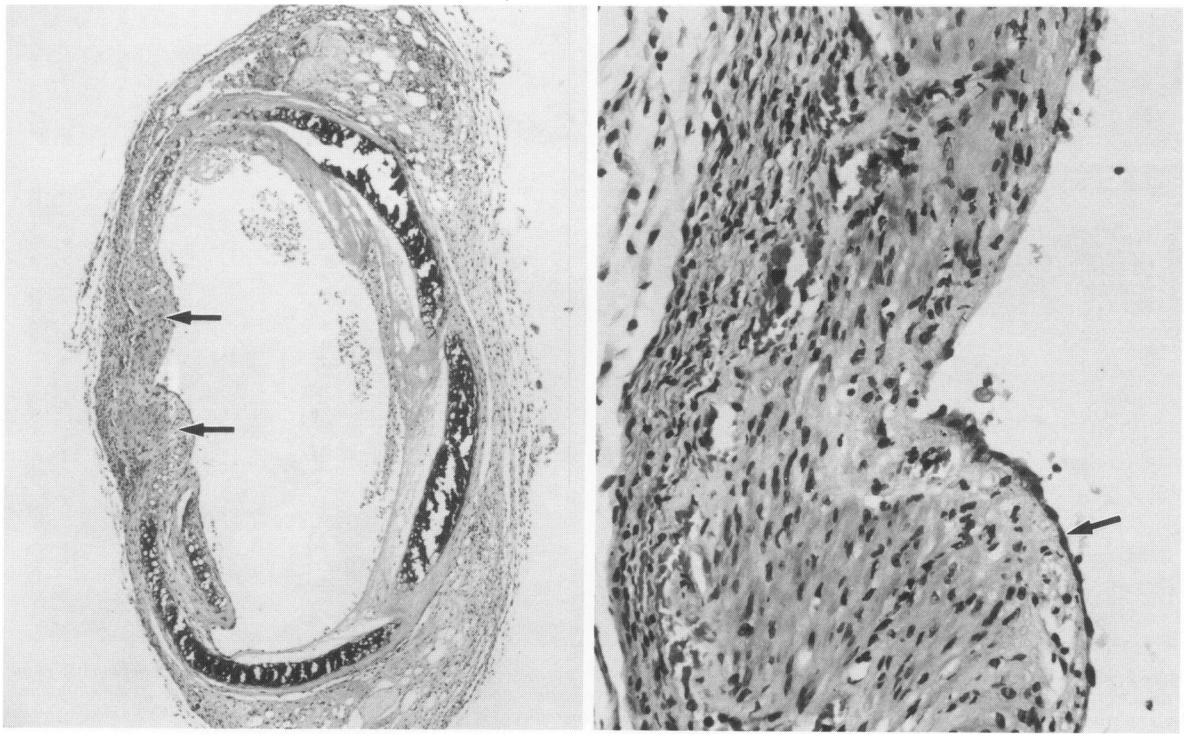

Figure 1. *Allograft harvested 10 days after transplantation. In* A *the graft is devoid of epithelium on most of its inner surface as a result of epithelial necrosis. Note sloughed epithelium and inflammatory cells in lumen. Focal areas of fibroproliferation are present (arrows).* B, *higher-power view of early fibroproliferative change composed of typical granulation tissue. Note focal area of residual flattened epithelium (arrow).* A, *H&E, ×62;* B, *H&E, ×300.*

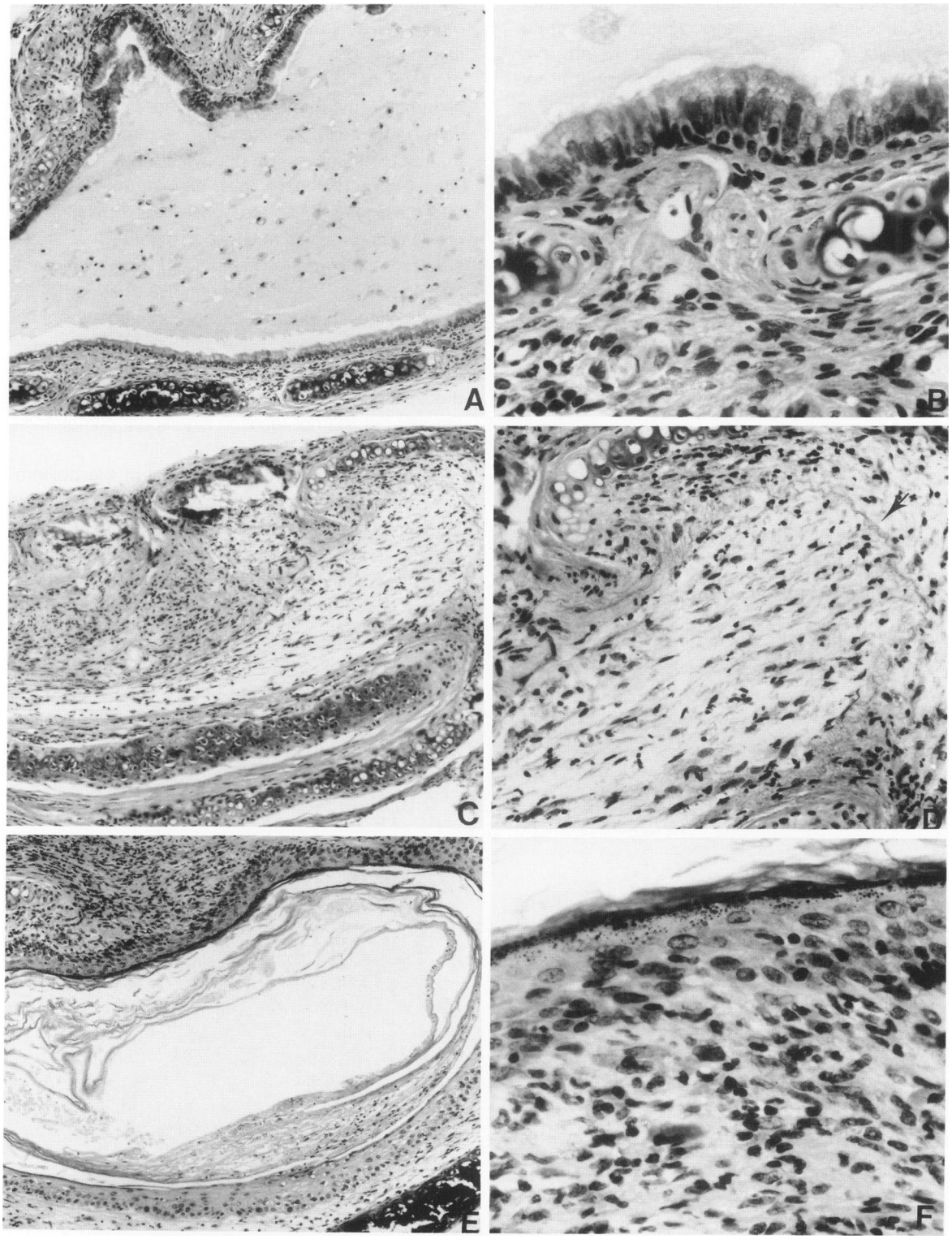


Figure 2. *Grafts harvested 21 days after transplantation. All of the isografts were patent* (A) *and lined by normal respiratory epithelium* (B). *Some of the allografts show total luminal occlusion by granulation tissue* (C). *Note residual basement membrane (arrow), the absence of epithelium, and the proliferation of fibroblasts and intermixed inflammatory cells* (D). *In other allografts, the lumen was occluded by keratin* (E) *formed by metaplastic squamous epithelium* (F). A,C,E, *H&E,* ×*35;* B,D,F, *H&E,* ×*300.*

**Table 1.** *Comparison of Isografts and Allografts Harvested 21 Days after Transplant*

| Histological abnormality | Isografts ($n$ = 9) | Allografts ($n$ = 10) | $P$ |
|---|---|---|---|
| Airway inflammation | 2 | 10 | 0.0007* |
| Fibroproliferation in airway wall or lumen | 0 | 9 | 0.0001* |
| Abnormal or absent epithelium | 0 | 10 | 0.0001* |
| Type of epithelium | | | |
| Respiratory | 9 | 0 | |
| Cuboidal | 0 | 2 | 0.0003† |
| Squamous | 0 | 3 | |
| None | 0 | 5 | |
| Epithelial necrosis | 0 | 3 | NS*‡ |
| Proportion of airway surface lined by epithelium | | | |
| Range | 95–100% | 0–100% | |
| Median | 100% | 20% | 0.006§ |

* Fisher's exact test of association.
† $x^2$.
‡ Not significant.
§ Mann-Whitney U-test.

specimens, and was found in a patchy or diffuse transmural distribution in nine. In contrast, only two isografts demonstrated mild or moderate inflammation that was focal in distribution. Allografts also demonstrated marked fibroproliferation involving the airway wall and lumen (Figure 2C, 2D). Circumferential or eccentric granulation tissue and fibrosis that reduced the lumen caliber to a variable degree were observed in nine of 10 allografts. The granulation tissue was generally quite cellular and comprised typical fibroblastlike cells and many small capillaries. The graft was lined by normal respiratory epithelium in all isografts. In marked contrast, the epithelium in the allografts was absent in five and abnormal (cuboidal or squamous) in five. In some cases, complete squamous metaplasia with keratin formation was evident (Figure 2E, 2F). Submucosal glands were identified in four isografts but were absent in all allografts.

## Discussion

The model described in this report replicates the histopathological lesions seen in obliterative bronchiolitis following clinical lung transplantation and has several attractive features. The donor and recipient operations are technically simple and can be accomplished without specialized equipment and expertise. In addition, the use of a murine model across defined major histocompatibility complex (MHC) barriers will facilitate studies of the immunopathogenesis of the obliterative bronchiolitis lesion. Finally, tracheas are readily maintained in explant culture for prolonged periods, which will allow *ex vivo* pharmacological and immunological manipulation of this system following disease induction. These characteristics define a model system which will allow preclinical studies of strategies designed to prevent or reverse obliterative bronchiolitis.

The histopathological features of obliterative bronchiolitis suggest that inflammation and injury of bronchiolar structures are followed by a fibroproliferative process that results in airway obstruction. Although the cause of these lesions is incompletely understood, most investigators favor an immune-mediated attack directed against airway targets, ie, a form of allograft rejection. Evidence supporting this hypothesis includes *ex vivo* studies of bronchoalveolar lavage-derived lymphocytes demonstrating donor-specific primed lymphocyte test reactivity[8,9] as well as clinical studies identifying severe and persistent episodes of acute rejection as a risk factor for the subsequent development of obliterative bronchiolitis.[10,11] The development of obliterative bronchiolitis lesion in allografts and not in isografts in the present study is in agreement with observations in rat lung transplants[12–14] and clearly supports an immune pathogenesis. However, our findings do not exclude the possibility that the disease process may be initiated or modified by factors other than alloimmunity, such as ischemia, infections, or other environmental factors.

Following airway injury, a fibroproliferative response ensues, characterized by new capillary formation (ie, angiogenesis) and migration and replication of mesenchymal cells within the airways,

leading to progressive airflow obstruction and graft failure. Previous work in our laboratory has demonstrated increased platelet-derived growth factor concentrations in bronchoalveolar lavage fluid of patients with obliterative bronchiolitis after lung transplantation.[15] This model will allow further investigation of a possible pathogenetic role of platelet-derived growth factor and other growth factors, as well as studies designed to interdict their action.

In the present model, epithelial necrosis was observed in the day 10 allografts, followed by universal epithelial abnormalities at day 21 after transplantation. Tazelaar et al have shown that fully allogeneic single lung transplants in nonimmunosuppressed rats develop bronchiolar injury and intraluminal granulation tissue following acute rejection.[14] Often, however, these allografts demonstrated complete necrosis of the airways, which the authors attributed to vascular insufficiency due to concomitant destruction of the remainder of the lung. More recently, epithelial abnormalities and fibroproliferative changes have been described in the large airways of lung allografts in cyclosporine A-treated rats.[13] Thus, epithelial abnormalities are consistently observed along with fibroproliferation in rodent models of obliterative bronchiolitis. One explanation for this is that the airway epithelium is a primary target of the alloimmune response, perhaps facilitated by the incresased expression of class II MHC antigens on allograft epithelial cells.[12] Alternatively, the observed epithelial abnormalities may result from prolonged ischemic injury due to an immune-mediated impairment of revascularization of allografts. Future studies will attempt to clarify the role of epithelial injury in the pathogenesis of obliterative bronchiolitis and test strategies designed to prevent its occurrence by promoting epithelial protection from and regrowth after injury.

While the histopathological changes described in this report recapitulate many features of the human obliterative bronchiolitis lesion, differences exist. Most notable is the fact that in our system the pathologic lesion involves the trachea and major bronchi, while the obliterative bronchiolitis lesion in humans has been thought to occur primarily in terminal bronchioles. Several studies, however, have identified associated pathologic abnormalities, including submucosal inflammation and fibrosis, loss of submucosal glands, squamous metaplasia, and bronchiectasis,[3,16,17] in the large airways of affected patients. These findings suggest that while similar pathologic events occur throughout the airways, only small airways display obliterative changes, perhaps rendered vulnerable by virtue of their small caliber. A second difference between our model and human obliterative bronchiolitis is that the heterotopic airway grafts are not primarily vascularized, which may alter early events in immune recognition.[18] Finally, the animals were not immunosuppressed, which is likely to be responsible for the rapid development of the obliterative process and its occurrence in 100% of the fully MHC-mismatched recipients. The effects of immunosuppression and of specific MHC mismatches on the development of obliterative changes in our model system are currently under investigation.

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