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Obliterative airway disease in a porcine heterotopic bronchial allograft model

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Abstract Representative animal models are needed for the study of posttransplant obliterative bronchiolitis (OB). Because human OB originates in terminal bronchi, the validity of tracheal models can be questioned. Using our hetrotopic model, we implanted pieces of a lobar bronchus subcutaneously into domestic pigs. Five groups were included: autograft implants, allograft implants, allograft implants with 2 regimens of cyclosporine (CsA)-azathioprine (AZA)-methylprednisolone (MP), and allograft implants with CsA-SDZ RAD-MP. Samples were harvested at 2 weeks and at 1, 2, and 3 months. The histological findings were graded from 0 to 3. Following initial ischemic epithelial damage, autograft implants recovered, but untreated allografts and those treated with CsA-AZA-MP were totally and permanently damaged within one month. In the group treated with CsA-SDZ RAD-MP, a maximal grade 1.5 ± 0.5 epithelial injury was seen at one month. Epithelial damage preceded and correlated with luminal obliteration. The obliterative lesions histologically resembled human OB. Differences from our previous findings with terminal bronchioles were minor. This study supports the use of larger-size airways in the study of OB.

Key words Experimental · Obliterative airway disease · Lung transplantation · Immunosuppression

Abbreviations *AZA* Azathioprine · *CsA* Cyclosporin A · *MP* Methylprednisolone · *SDZ RAD* 40–0-(2-hydroxyethyl)-rapamycin, the new macrolide immunosuppressant · *OAD* Obliterative airway disease

Introduction

Obliterative bronchiolitis (OB) is the most common and life-threatening long-term complication following human lung transplantation [3]. To elucidate its pathogenesis and prevention, representative experimental models are needed. Obliterative airway disease (OAD) has been studied in rodent models with respect to heterotopic tracheal implants [2, 4, 9, 10, 12, 14, 15]. These models may, however, have drawbacks; because human OB originates in terminal bronchioles [22], the pathogenesis of the tracheal lesions may differ.

To study experimental OB, our group previously developed a heterotopic model using terminal bronchi with or without pulmonary tissue [7]. In this model, autografts stay patent with functioning mucous glands, and untreated allografts are obliterated within 21 days [6, 7]. In this model we have been able to delay and even prevent experimental OB [16, 17]. These findings suggest that our model is valid for studying the mechanisms of experimental OB and for comparing the effects of varying immunosuppressive regimens.

This study focuses on models of intermediate-size bronchi. We studied auto- and allografts, as well as allo-

grafts from animals either on the standard triple immunosuppression [i.e., a combination of cyclosporin A (CsA), azathioprine (AZA) and methylprednisolone (MP)], or on triple immusuppression with CsA, MP and the new macrolide immunosuppressant, SDZ RAD [i.e., 40–0-(2-hydroxyethyl)-rapamycin] [19, 20]. The aim was to discover the histologic findings and their time-table to test the use of larger-size airways in heterotopic models.

Materials and methods

Bronchial autografts and allografts were transplanted subcutaneously into random-bred domestic piglets weighing ca 20 kg (see below). The animals received humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society of Medical Research and the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 86–23, revised 1985). The study protocol was accepted by the institutional committee for animal research and by the Uusimaa Provincial Government in Finland.

Anesthesia

The animals were anesthetized for the surgical procedure with ketamine sulphate (10–15 mg/kg), azaperone (10–15 mg/kg), sodium pentobarbital (6–12 mg/kg), pancuronic bromide (2–4 mg), and atropine sulphate (0.05 mg/kg). They were ventilated after intubation with 40% oxygen and enflurane at a rate of 18 to 20 breaths/min. Postoperatively, diclophenac acid (37.5–50 mg) was given intramusculary for pain control. Ranitidine was given for ulcer prophylaxis, 50 mg intravenously in induction of anesthesia and 150 mg orally for 1–3 weeks. Ceftriaxone 500 mg was given intramuscularly for 3 days for infection control. Atropine sulphate (0.05 mg/kg), ketamine sulphate (6–10 mg/kg), and azaperone (6–10 mg/kg) were used for anesthesia during harvesting of the samples. At the end of the follow-up, the animals were killed with a high-dose sodium pentobarbital infusion.

Surgical procedures

Left thoracotomy was performed, and the caudal lobe removed for preparation of 0.5–0.8 cm long implants of the lobar bronchus. In the autograft group, 6 autograft implants were transplanted subcutaneously into the ventral side of each animal. In the other groups, where each animal was used both as a donor and as a recipient, animals received 4–6 allograft implants from a non-related donor. The implants were harvested at 2 weeks and 1, 2, and 3 months following the procedure. In addition, two of the groups were followed up for 6 months.

Experimental groups

The minimum follow-up for all groups was 3 months.

Group 1

Five pigs. Autograft implants. No immunosuppression.

Group 2

Five pigs. Allograft implants. No immunosuppression.

Group 3

Five pigs. Allograft implants. Surgical treatment: intravenous doses of CsA (100 mg), AZA (50 mg), and MP (125 mg). Postoperative treatment: daily oral CsA 10 mg/kg, AZA 2 mg/kg, and MP 3 mg/kg for 1 week, 2 mg/kg the second week, and 20 mg at 2 months, thereafter reduced to 4 mg/week until 3 months.

Group 4

Six pigs. Allograft implants. Preoperative treatment: CsA 10 mg/kg for 5 days. Surgical treatment: intravenous doses of CsA (100 mg), AZA (50 mg), and MP (250 mg). Postoperative treatment: daily oral CsA 10 mg/kg, AZA 2 mg/kg, and MP 3 mg/kg for 3 days, 2 mg/kg for 4 days, then 20 mg.

Group 5

Five pigs. Allograft implants. Preoperative treatment: SDZ RAD 1.5 mg/kg for 4 days. Surgical treatment: SDZ RAD just before anesthesia 1.5 mg/kg p.o., intravenous doses of CsA (100 mg) and MP (250 mg). Postoperative treatment: daily oral SDZ RAD 1.5 mg/kg, CsA 10 mg/kg, MP 3 mg/kg for 3 days, 2 mg/kg for 4 days, thereafter 20 mg.

Monitoring of immunosuppressive agents

Venous blood was drawn for drug-level monitoring when appropriate prior to implant harvestings. Whole blood CsA and SDZ RAD concentrations were measured with specific radioimmunoassay and ELISA respectively.

Long-term follow-up

Two of the groups, one with autografts and one with allografts and immunosuppression (group 3), were followed up for 6 months. In group 3 all immunosuppression was, stopped at 3 months.

Histological analysis

The tissue samples were fixed in 4% buffered formalin and embedded in paraffin. Slides 4 μm thick were cut and stained with hematoxylin and eosin, van Gieson and diastase periodic acid Schiff method for histological assessment. Damage to the bronchial epithelium, epithelial atypia, destruction and proliferation of the bronchial cartilage, pericartilagenous inflammation, and fibrosis were analyzed in the histological specimens, as well as mural inflammation, granulation tissue, necrosis, edema, neovascularization, and fibrosis. Our studies with heterotopic terminal bronchioles have shown that the most prominent accumulation of mononuclear cells appears in the pericartilagenous area; therefore mural and pericatilagenous inflammation were graded separately [7]. Likewise, luminal obliteration was separately analyzed. All alterations were graded semiquantitatively on a scale of 0–3 (0 = no alteration, 1 = mild alteration including a minor portion of the ob-

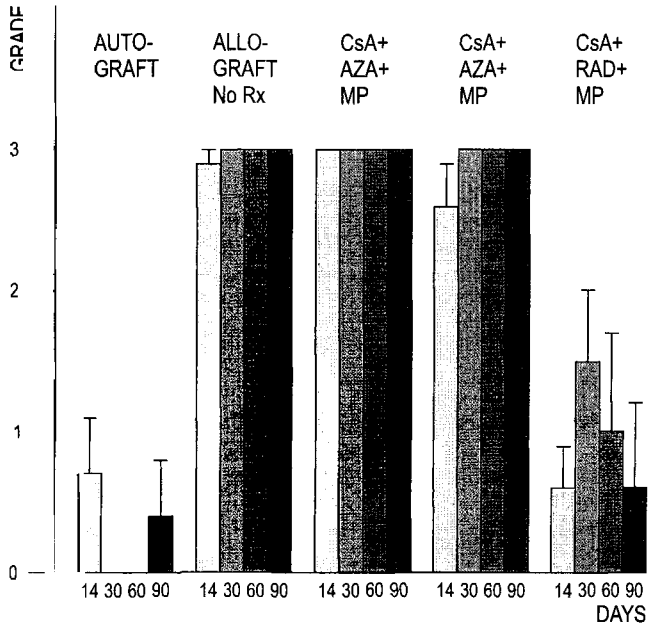

Fig. 1 Epithelial destruction (mean + SEM) during follow-up. *0* None, *3* severe alteration. *Rx* Treatment, *CsA* cyclosporine, *AZA* azathioprine, *RAD* SDZ RAD, *MP* methylprednisolone

served area, 2 = moderate alteration in which pathologic alterations were equal to the normal tissue, 3 = severe alteration in which pathologic changes were the predominant component). Luminal obliteration was quantified as 0–1 in incipient obliteration. It was 1, when approximately one-third of the lumen was occluded, it was 2, when two-thirds were occluded and 3 in total occlusion.

Statistical analysis

All data are expressed as mean ± SEM. The variation between nonimmunosuppressed and immunosuppressed auto- and allografts was analyzed with the nonparametric Kruskal-Wallis one-way analysis by ranks (Statistica v. 5, StatSoft Inc., Tulsa, OK). The rank sums were then used for Dunn's test at a significance level of 5% (Medstat, Astra Group A/S, Copenhagen, Denmark). Values of $p < 0.05$ were considered statistically significant.

Results

Toxicity and drug-level monitoring

Except for a tendency to less weight-gain in the immunosuppressed animals, no major toxicities were evident. The mean CsA whole blood levels were 555 ± 157 ng/ml, 583 ± 221 ng/ml, and 462 ± 207 ng/ml in groups 3, 4, and 5, respectively. The mean whole blood SDZ-RAD concentration was 18 ± 11 ng/ml in group 5.

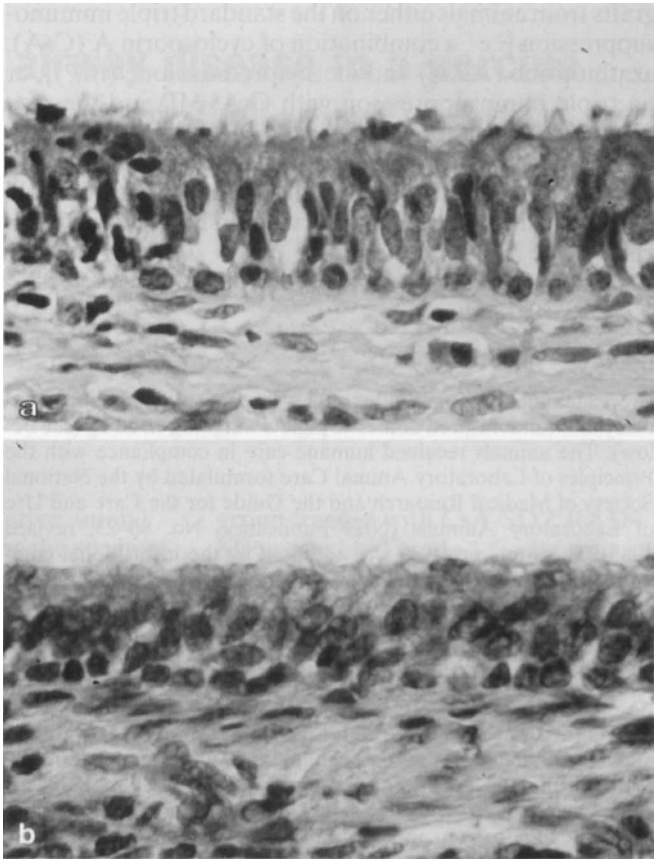

Fig. 2 Recovered epithelium **a** in an autograft at 60 days and **b** in an allograft treated with cyclosporine, SDZ RAD, and methylprednisolone at 90 days. H&E staining, × 100

Histological alterations

Bronchial epithelium

After some initial epithelial damage, the autografts regained normal epithelium, which was thereafter essentially retained throughout the follow-up (Fig. 1). In the autografts, the lumen was filled with mucus as a sign of normal epithelial secretory function. (Fig. 2). Epithelial atypia was not apparent to any significant extent. By one month, the bronchial epithelium was totally and permanently damaged in all allografts without immunosuppression as well as in all those treated with the standard triple therapy (CsA, AZA, MP) (Fig. 1). The difference between the autografts and these allografts reached statistical significance. In the group in which SDZ RAD (Fig. 3) was combined with CsA and MP, bronchial epithelial damage reached a grade of 1.5 ± 0.5 at 1 month, thereafter decreasing to partial damage at 3 months (Fig. 1). Mild atypia (mainly of squamous metaplasia) was seen in the preserved epithelium after 1 month in the group on triple immunosup-

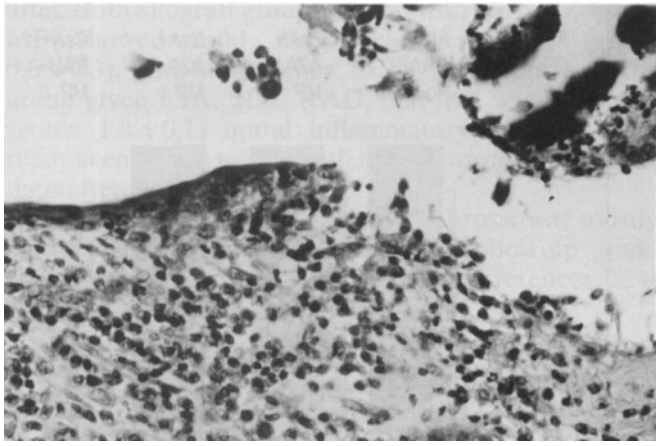

Fig. 3 Partial epithelial destruction and luminal obliteration in an allograft treated with cyclosporine, SDZ RAD, and methylprednisolone at 30 days. H&E staining, × 40

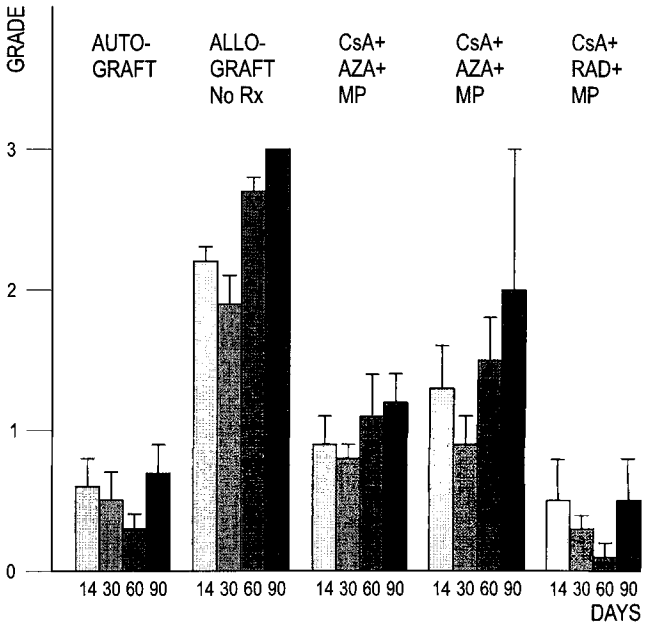

Fig. 4 Luminal obliteration (mean + SEM) during follow-up. *0* None, *3* severe alteration. *Rx* Treatment, *CsA* cyclosporine, *AZA* azathioprine, *RAD* SDZ RAD, *MP* methylprednisolone

pression with SDZ RAD. In the other allograft groups after two weeks no epithelium was left.

Luminal obliteration

In the autografts some initial obliteration was seen, but thereafter the bronchi remained patent (Fig. 4). In the allografts without immunosuppression and those treated with the standard therapy the bronchi were totally and permanently occluded by one month (Fig. 4). The obliterative plug first consisted of fibroblasts (granulation tissue with capillaries) and inflammatory cells (Fig. 5). About 2 months later, the plug evolved from cellular fibrosis to collagen-rich tight, scarlike fibrosis. At this stage inflammation disappeared. The plug was well vascularized at all assessment points. When AZA was swithched to SDZ RAD in the combination with CsA and MP, luminal obliteration was almost totally prevented. In this group the maximum grade of 0.6 ± 0.6 obliteration was seen at 3 months.

Fig. 5 Partial luminal obliteration in an allograft treated with cyclosporine, azathioprine, and methylprednisolone at 14 days. H&E staining, × 4

Cartilagenous alterations

Partial destruction of the bronchial cartilage was seen in the autografts throughout the study (Fig. 6). On the other hand, moderate to severe cartilagenous damage was present in the allografts with no immunosuppression from the beginning of the follow-up (Fig. 6), and by 3 months this destruction was total. The difference between the autografts and non-immunosuppressed allografts was statistically significant at every assessment point. In the allografts treated with the standard therapy, cartilage damage was moderate throughout the study. When AZA was switched to SDZ RAD in the drug combination, cartilage destruction was partial and almost identical to that of the autografts (Fig. 6). In the CsA-MP-SDZ RAD group, the difference from non-immunosuppressed allografts was statistically significant at all time-points.

In the autografts, chondrocytic proliferation was moderate (mean total follow-up grade 1.3 ± 0.3) and showed a statistically significant difference throughout the study from the non-immunosuppressed allografts which had no proliferation. In the group treated with

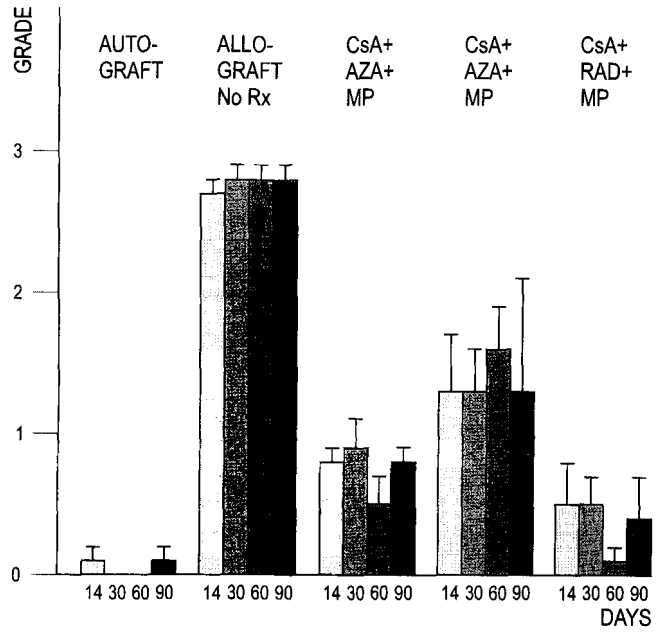

Fig. 6 Cartilageneous destruction (mean + SEM) during the follow-up. *0* None, *3* severe alteration. *Rx* treatment, *CsA* cyclosporine, *AZA* azathioprine, *RAD* SDZ RAD, *MP* methylprednisolone

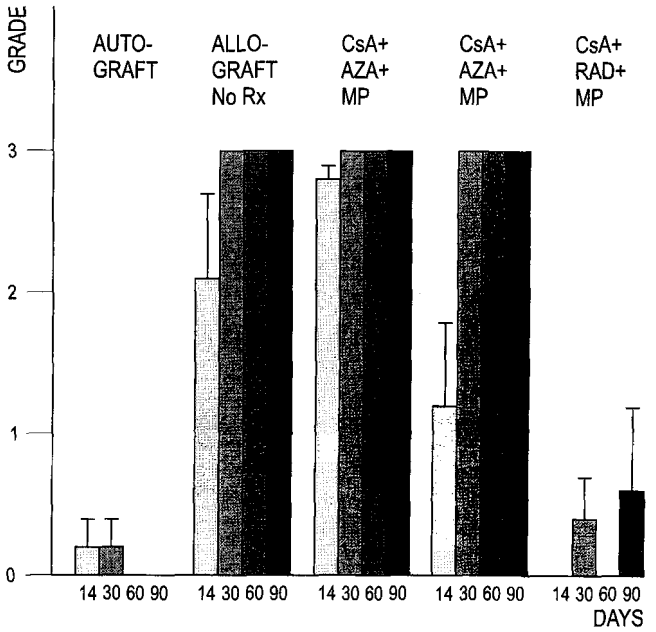

Fig. 7 Cartilagenous inflammation (mean + SEM) during the follow-up. *0* None, *3* severe alteration. *Rx* Treatment, *CsA* cyclosporine, *AZA* azathioprine, *RAD* SDZ RAD, *MP* methylprednisolone

CsA, MP, and SDZ RAD, mainly mild cartilage cell proliferation was seen throughout the study with a tendency for this to be more prominent than in the groups treated with the standard therapy (data not shown).

Virtually no pericartilagenous mononuclear inflammatory cells were present in the autografts. In the allografts with no immunosuppression this inflammation was almost maximal (Fig. 7), with a statistically significant difference from the autografts during the whole study period. In the allografts treated with the standard therapy, accumulation of inflammatory cells was mainly mild with a tendency for less inflammation in group 3 (Fig. 7). The allografts treated with CsA, SDZ RAD, and MP resembled the autografts and showed only some mononuclear cell accumulation on the cartilage surface. The difference from untreated allografts as well was statistically significant throughout the follow-up.

In the autografts, at 2 weeks and 1 month, pericartilagenous fibrosis was moderate (grade 1.3 ± 0.3) with a decline at 3 months to almost zero. The allografts with no immunosuppression showed the reverse patttern. In the allografts on any immunosuppressive regimen, pericartilagenous fibrosis was moderate (mean total follow-up grades from 0.8 ± 0.3 to 1.4 ± 0.4) with no consistent changes or differences.

Mural alterations

During the follow-up except for the autografts, mild mural edema (mean total follow-up grades from 0.3 ± 0.3 to 0.7 ± 0.3) was a constant finding.

Necrotic foci of the bronchial wall was evident in all groups at two weeks, being only partial in the autografts. In the untreated allografts or those treated with the standard therapy, necrosis went on to full destruction of the bronchial wall, so that mucous glands or lamina propria could not be identified at one month or thereafter. Mural necrosis was resolved totally by one month in the autografts and by two months in the allografts treated with the combination of CsA, MP, and SDZ RAD.

No granulation tissue with fibroblastic and vascular proliferation was visible in the autografts, but was apparent in all allografts at 2 weeks, disappearing by 1 month. At 2 weeks, mild to moderate mural neovascularization (grades from 0.9 ± 0.4 to 1.7 ± 0.3) was visible in all study groups. The situation during the follow-up was essentially unchanged.

The inflammation reaction of the bronchial wall consisted mainly of mononuclear cells, with a majority of lymphocytes as a marker of rejection. Very mild (grade 0.5 ± 0.3) mural inflammation was present in the autografts at 2 weeks, with a tendency to decline thereafter. In the allografts without immunosuppression, mononuclear infiltration was already mild to moderate (grade 1.2 ± 0.1) at 2 weeks, with only minor fluctuation there-

after. Both allograft groups treated with CsA, AZA, and MP showed mild mononuclear reaction (grade 0.6 ± 0.3), with a tendency to decrease. Only in the group given CsA, SDZ RAD, and MP, was moderate (grade 1.8 ± 0.1) mural inflammatory infiltration already seen at two weeks, with a trend toward a decrease thereafter.

During the study period, mural fibrosis was mostly moderate in all groups (mean total follow-up grade 1.3 ± 0.4), with no clear-cut changes or differences.

Long-term follow-up

In the autografts, no changes were evident when the findings at 3 months were compared to the situation up to 6 months (data not shown). In the allografts (group 3), after all immunosuppression was stopped at 3 months, the remaining cartilagenous structures were destroyed, and the samples were partially replaced by fat tissue.

Discussion

OB is currently thought to be a manifestation of immunologically mediated chronic rejection [13]. Models with OB following conventional lung transplantation have been reported in rodents [4], mini-pigs [1], and pigs [5]. However, in all these porcine models a demanding surgical procedure is needed, and a long follow-up after withdrawal of immunosuppression is a perquisite before OB lesions develop [1, 5]. With the heterotopic models, the operative procedure is far less demanding, and lesions histologically resembling human OB develop more rapidly [7, 10, 12].

In this study as well as in our previous studies of terminal bronchioles [7, 18], we observed the same two extremes: recovery of autografts following initial ischemic damage, and total obliteration of untreated allografts by one month. Although with this sampling frequency, the standard immunosuppression with CsA-MP-AZA was not shown to delay epithelial damage consistently, this regimen still preserved the bronchial allografts to some extent, since the implants were rapidly and almost totally replaced by fat tissue once this immunosuppression was stopped. The finding that standard immunosuppression does not prevent OB is specific for our model, which probably represents accelerated OB.

When SDZ RAD was used instead of AZA in the triple combination, a significant reduction in epithelial damage and subsequent luminal obliteration was observed. In this respect, no differences were seen in comparison to our previous study [16], although epithelial damage was at the first assessment point more severe in these larger-size bronchial allografts treated with CsA-SDZ RAD-MP than has to date been reported for terminal bronchioles [16].

The bronchial epithelium has been proposed as a potential allogenic target for chronic rejection following lung transplantation [11]. Furthermore, obliterative airway disease has been suggested to rely on a host T-cell response that includes $CD8^+$ cells, which are directed against allo-class I-bearing donor cells within the graft in heterotopically transplanted murine tracheas [8]. In accordance with these data, here too, luminal obliteration followed epithelial injury. Autografts stayed patent till the end of the study, and the allografts treated with CsA-SDZ RAD-MP showed only minor obliteration. In all the other groups, obliteration was as total as the preceding epithelial damage had been.

The cartilagenous destruction and pericartilagenous accumulation of mononuclear inflammatory cells seen here were essentially as previously described in terminal bronchial implants [7, 18]. Cartilage destruction was seen to some extent in all groups, but was very mild in autografts and in the allografts treated with CsA-SDZ RAD-MP. In the untreated allografts, cartilage destruction reached a maximal level at 3 months, but immunosuppression with CsA-AZA-MP limited this destruction to mainly moderate levels. Mural fibrosis was, as well, a constant finding in this study.

The current implants were taken more proximally and were larger than previously in our studies [7, 18], a fact which might increase the likehood of ischemic or lymphatic damage. We also previously found that the bronchial lesions were more intense in allografts which had surrounding lung tissue, possibly as a result of greater ischemia [7]. Initial, probably ischemic, changes – mural edema, necrosis, and epithelial injury – recovered rapidly, however. Neoangiogenesis was seen in all groups at all assesssment points. Thus, the use of a lobar instead of a terminal bronchus or the use of a somewhat larger sample size in this heterotopic model did not alter the amount of vascular injury.

The present findings are consistent with those of the rat heterotopic tracheal allograft model [10, 12] and with those in heterotopic transplantation of mouse airways [2, 4, 9]. Likewise, in tracheal allografts, respiratory epithelium was replaced by cuboidal or squamous cell epithelium early after transplantation [4, 10]. Later, total epithelial necrosis developed, and intense proliferation of granulation tissue occluded the airway lumen [4, 10]. Such long-term changes were not evident in heterotopic isografts [4, 10, 12]. The time-table and histologic findings in tracheal heterotopic models thus closely resemble our findings with both terminal and lobar bronchi.

Today, the pathogenesis of OB is largely unknown [3]. In the present study, as in our previous studies of terminal bronchioles [7], and in the studies with rodent heterotopic models [4, 10], histological findings resemble

those of human OB [21], suggesting the usefulness of these models in studying OB.

In contrast to our previous findings with terminal bronchioles some differences were noticed in bronchial implants from the lobar bronchus. These differences were, however, insignificant, and would indicate that the size of the airway plays a minor role in heterotopic models of experimental OB.

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