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ORIGINAL ARTICLE

Immune cells in a heterotopic lamb-to-pig bronchial xenograft model*

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Summary

We developed our porcine model to elucidate the cellular rejection mechanisms of xenografts. Bronchial segments from a donor lamb were implanted into domestic pigs. The immunosuppressive regimens consisted of no immusuppression, or of daily oral cyclosporine A (CsA) 15 mg/kg, or of everolimus, 1.5 mg/kg, or of both. Implants were serially harvested during 17 days. Epithelial damage and obliteration were graded histologically, followed by a count of CD4+, CD8+, MHC class II-expressing cells, and macrophages. Furthermore, we studied the pharmocokinetics of everolismus. Epithelial damage preceded luminal obliteration, which was eventually total, except when both drugs had been given. In xenografts, an influx of cells with CD8+ cells dominating peaked on day 9, thereafter declining, except in the combination drug group. There, the immunological reaction was delayed and blunted, with CD4+ cells dominating. More macrophages appeared in xenografts than in allografts except with the combination CsA and everolimus. A dose of 1.5 mg/kg everolimus yields adequate blood concentrations for porcine studies. In this xenograft model, chronic rejection appears to be caused by an immune response to the graft, but it is more short-lived than the response in allografts. The combination of CsA and everolimus was able to blunt the response and delay the subsequent obliteration.

Introduction

In recent years, the treatment of choice for patients with otherwise untreatable pulmonary disease has become lung allotransplantation [1]. A manifestation of chronic rejection in the lung transplant is believed to be obliterative bronchiolitis (OB) [2]. With its prevalence in lung transplant patients of more than 34% at 5 years, OB is the major factor increasing mortality and limiting long-term outcome [1]. Furthermore, clinical transplantation suffers from the shortage of available and suitable donors [1]. That the lung is also more susceptible than other organs to *antemortem* deterioration means even fewer lungs available from the already limited number of human donors [1].

Because mechanical lung-replacement devices have had no significant clinical impact, one potential source of organs considered is animal donors [3]. Along with ethical problems and the undetermined potential risk of transferring infectious organisms from animal organs into human recipients, the problem of xenograft rejection comes [3]. Four major immunological barriers to solid organ xenotransplantation exist: hyperacute rejection, acute vascular rejection, acute cellular rejection, and chronic rejection [3]. With current techniques, hyperacute rejection can be prevented and acute vascular rejection delayed, but data on prevention of chronic rejection are thus far either inconclusive or very limited [3].

Prolongation of lung or tracheal xenograft survival has been achieved with immunosuppression in rodents [4–6]. Such concordant hamster-to-rat models have shown that OB occurs also after xenotransplantation and that subepithelial infiltration of neutrophils and the appearance

of plasma cells and eosinophils in the peritracheal infiltrates distinguishes the histology of the rejected xenografts from that of allografts [6]. The deposition of rat immunoglobulin in the peritracheal area during the inflammation process in immunofluorescence studies, together with the appearance of plasma cells in histology, provides evidence of a humoral component in the rejection process [6]. To study lung xenografts, a simplified en block double lung hamster-to-rat model has been developed [7], but a useful, easily reproducible large animal model has been lacking.

To study OB, our group has developed a porcine heterotopic bronchial model [8]. Autografts are seen to normalize once they have recovered from a brief postoperative ischemia [8]. Nonimmunosuppressed allografts are rapidly obliterated, but this process can be delayed with a combination of cyclosporine A (CsA), azathioprine (AZA) and methylprednisolone, and prevented when AZA is replaced with everolimus [40–0-(2-hydroxyehtyl)-rapamycin)] [9]. In immunosuppressed allografts, decrease in number of immune cells alone does not prevent OB in this model and supports the view of OBs being T-cell-mediated [10].

To study xenografts, we further developed the pig model [9]. To achieve discordance [11], a lamb was the selected donor. In xenografts, the inevitable obliteration of the nonimmunosuppressed bronchi was markedly delayed with immunosuppression comprising CsA and everolimus [12]. The present study focuses on the components of cell-mediated rejection of bronchial xenografts and the effect of immunosuppression on inflammatory cell accumulation in the grafts. Furthermore, the pharmocokinetics of oral everolimus was studied to verify the appropriate dose.

Materials and methods

All 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). All study protocols were accepted by the institutional committee for animal research and by the South Finland Provincial Government. At the end of the studies, the animals were euthanized with a high-dose sodium pentobarbital infusion.

Everolimus pharmacokinetic study

Two groups each comprised three domestic, random-bred piglets weighing 20 kg, Group 1 received a single oral dose of 1.5 mg/kg everolimus and Group 2 a daily oral dose of 1.5 mg/kg for 14 days. Blood was drawn prior to the application and 30 min, 1 h, and 2, 4, 8, 24, 48, 72, and 96 h after the end of dosing. Group 2 was studied after the 14th dose.

For blood sampling, the overnight-fasted piglets were anesthetized with atropine sulfate (0.05 mg/kg), ketamine sulfate (6 mg/kg), and azaperone (6 mg/kg), given intramuscularly. Up to the 8th hour, the blood samples were obtained from a cannula placed in the auricular artery and kept patent with heparin. After 24 h, the samples were taken by subclavian vein puncture. At each time-point, 2 ml of whole blood was collected into EDTA tubes, frozen, and stored at −70 °C. Whole blood everolimus concentration was measured with specific ELISA.

Transplantation studies

Ten random-bred domestic pigs weighing about 20 kg served as xenograft and allograft recipients, and a lamb weighing about 40 kg was used as the xenograft donor. Each allograft control pig served both as donor and recipient.

Anesthesia and medication

The donor lamb was anesthetized with xylazin hydrochloride (2 mg/kg) intramuscularly and heparinized. Just prior to cutting the bronchi and pulmonary vessels, the animal was euthanized.

The xenograft recipients were anesthetized with ketamine sulfate (8–12 mg/kg), azaperone (8–12 mg/kg), sodium pentobarbital (4–8 mg/kg), diazepam (0.25 mg/kg) and atropine sulfate (0.05 mg/kg). Allograft controls were anesthetized as described previously [9].

Postoperative medication consisted of diclophenic acid 37.5 mg given intramuscularly for pain control; intramuscular cephtriaxone 500 mg was continued orally. Oral ranitidine was given postoperatively for ulcer prophylaxis.

For the graft harvesting; all animals were anesthetized with a subcutaneous injection of ketamine sulfate (6–10 mg/kg), azaperone (6–10 mg/kg), and atropine sulfate (0.05 mg/kg).

Surgical procedures

The lungs of the donor lamb were removed through bilateral thoracotomy. The donor lungs were flushed with physiological saline and segments of intrapulmonary third order bronchi including terminal bronchioli about 1.5 cm long were dissected out. Fifty grafts were transplanted into subcutaneous pockets on the ventral side of each recipient pig, lateral to the nipples. Five to six implants in all groups were serially harvested at each time-point through small cutaneous incisions at 2, 4, 7, 9, 11, 14, and 17 days following the operation. After harvesting, the

samples were cut into two pieces; two-thirds were fixed in 4% buffered formalin and embedded in paraffin, and one-third snap-frozen in liquid nitrogen and stored at −70 °C.

Allograft controls underwent surgery as described previously [9].

Experimental animals

Allograft recipients received no immunosuppression.

Xenograft recipients received no immunosuppression, or were treated with daily oral CsA 15 mg/kg [13] starting 4 days preoperatively or with daily oral everolimus 1.5 mg/kg/starting 4 days preoperatively, or with both.

Histological analysis

The frozen sections were cut 4 μm and stained with hematoxylin & eosin. Epithelial destruction, obliteration of the lumen, mural fibrosis, and inflammation were each graded semiquantitatively on a scale from 0 to 3 (0 = no alteration, 1 = mild alteration including a minor portion of the observed area, 2 = moderate alteration in which pathologic alterations were equal to normal tissue, 3 = severe alteration in which the pathological alterations were the predominant component).

Immunohistological analysis

Frozen cross-sections 4 μm thick were cut. They were air-dried onto silane-coated slides, fixed in acetone at −20 °C for 10 min, and stored at −20 °C until used.

The method used involved three-layer indirect immunoperoxidase plus monoclonal mouse antibodies. To avoid unspecific staining due to endogenous peroxidase, the slides were refixed at room temperature chloroform for 30 min before staining, and then air-dried. Monoclonal antibodies detecting swine CD4 and CD8 (VMRD Inc., Pullman, WA, USA) were used, and an antibody against mouse anti-human macrophages (MCA 874G) (Serotec, Oxford, UK). In addition, MHC class II antigens were demonstrated by a monoclonal, anti-porcine, MHC class II-DQ antibody (MCA 1335) (Serotec). Mouse anti-human CD62E/CD62P (MCA 883) (Serotec) demonstrated induction of the early marker of the leukocyte adhesion endothelial-leukocyte adhesion molecule (ELAM)-1. The slides were first incubated with a primary antibody, then with peroxidase-conjugated rabbit anti-mouse antibody (Dako A/S, Glostrup, Denmark), and thereafter with a peroxidase-conjugated goat anti-rabbit antibody (Zymed, San Francisco, CA, USA). The reaction was revealed by an AEC (3-amino-9-ethylcarbazole) solution containing hydrogen peroxidase. The incubation periods at room temperature were 30 min for the first three phases and 20 min for the last one. Counterstaining was performed with Mayer's hemalum solution.

The number of inflammatory cells positive for the cellular markers per high-power visual field was counted. The endothelial intensity of staining with ELAM-1 and epithelial as well as endothelial MHC class II expression was graded from 0 to 3 (0 = negative, 1 = positive cells showing weak intensity, 2 = more intense and/or irregular staining, 3 = staining intense and even).

Three separate bronchi, when present, were analyzed from each of the five samples harvested at the assessment points. We always evaluated three microscopic visual fields at ×40 objective magnification. All stainings (and assessments) were performed from the same site.

Statistical analysis

All histological and immunohistochemical data are expressed as mean + SEM. Variation between the various specimens was analyzed with the nonparametric Kruskal–Wallis one-way analysis by ranks (Statistica™ v. 5, StatSoft Inc., Tulsa, OK, USA). The rank sums were then used for Dunn's test at a significance level of 5% (Medstat, Astra Group A/S, Copenhagen, Denmark). Spearman's rank correlation served for correlation analysis (Statistica™). Values of $P \leq 0.05$ were considered statistically significant.

Results

Everolimus pharmacokinetics

No toxicity occurred in any of the animals. After single oral administration, individual pharmacokinetic profiles of everolimus displayed variability (Fig. 1). After once-daily multiple dosing for 14 days, the mean 24-hour everolimus trough concentration was 16.3 ± 6.6 ng/ml.

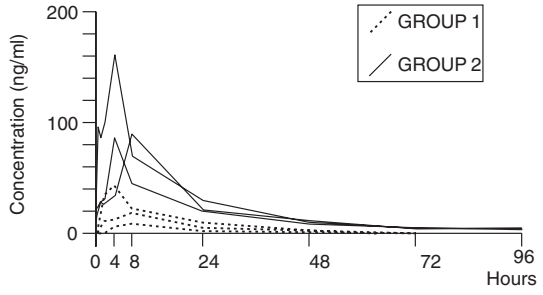


Figure 1 Whole blood everolimus concentration-time profiles in individual pigs after a single oral dose of 1.5 mg/kg (Group 1) and following once-daily doses of 1.5 mg/kg for 14 days (Group 2).

Once-daily multiple dosing for 14 days in Group 2 indicated some likely accumulation of everolimus blood concentrations when compared with that with the single administration in Group 1 (Fig. 1).

Histologic findings

A total of 196 samples were scored blindly in regard to group, medication, and assessment point. Epithelial damage was practically total by day 9 in all samples, except with the combination of CsA and everolimus (Fig. 2). In these xenograft samples, epithelial damage was significantly ($P < 0.05$) delayed on days 7–11. Epithelial damage preceded luminal obliteration, which was total by day 17 in all allograft and xenograft samples except the ones with everolimus alone or in combination with CsA. (Fig. 2). From day 7 onwards, the grade of obliteration in the xenograft samples treated with the combination was significantly less in comparison with the other groups ($P < 0.05$).

Likewise, luminal inflammation and fibrosis were less severe following the combination, this difference reaching significance at most assessment points from day 7 onwards. Both these variables were severe by the end of follow-up, reaching, on average, grade 2 (data not shown).

Immunohistochemical findings

Based on our earlier experience, immunohistochemical findings were assessed from the bronchial wall because the epithelium in most samples is too rapidly destroyed [10].

In nonimmunosuppressed control allografts, an influx of CD4+ and CD8+ lymphocytes, of macrophages, and of MHC class II-expressing cells was apparent early on, persisting throughout follow-up (Figs 3–6). CD8+ lymphocytes were most numerous, followed by CD4+ lymphocytes and class II cells, with fewer macrophages.

In nonimmunosuppressed xenografts, the immunological response started after day 4, peaking on day 9 and then declining. CD8+ lymphocytes were most numerous also in these xenograft samples, but the numbers of CD4+ lymphocytes, macrophages, and MHC class II-expressing cells were equal during the early phase; only from day 11 on did the number of CD4+ cells remain higher, with the other two cell types sharply declining.

In CsA-treated xenografts, the numbers of CD4+ and CD8+ lymphocytes remained at the peak level of day 9 till the end of follow-up (Figs 3–6). Numbers of macrophages and MHC class II-expressing cells declined similarly to those in nonimmunosuppressed xenografts.

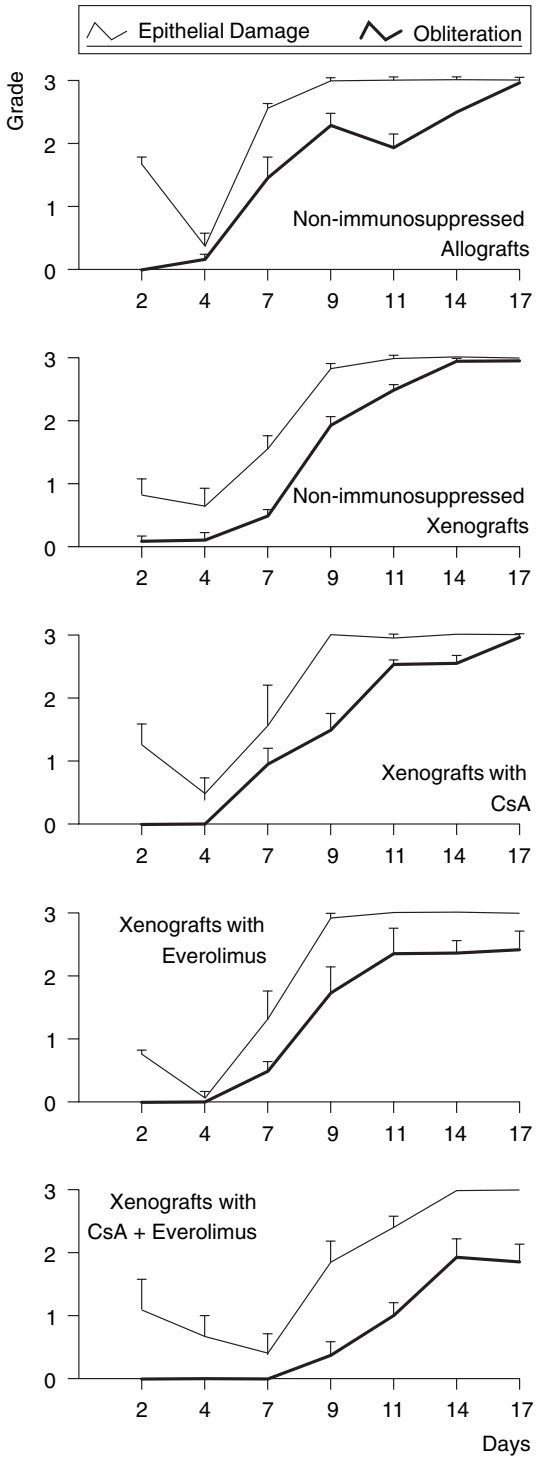


Figure 2 Grade of epithelial damage and luminal obliteration (mean + SEM) in groups during follow-up. CsA = cyclosporine A.

Likewise, in everolimus-treated xenografts, the immunological response was blunted but neither delayed nor prevented. In comparison to nonimmunosuppressed

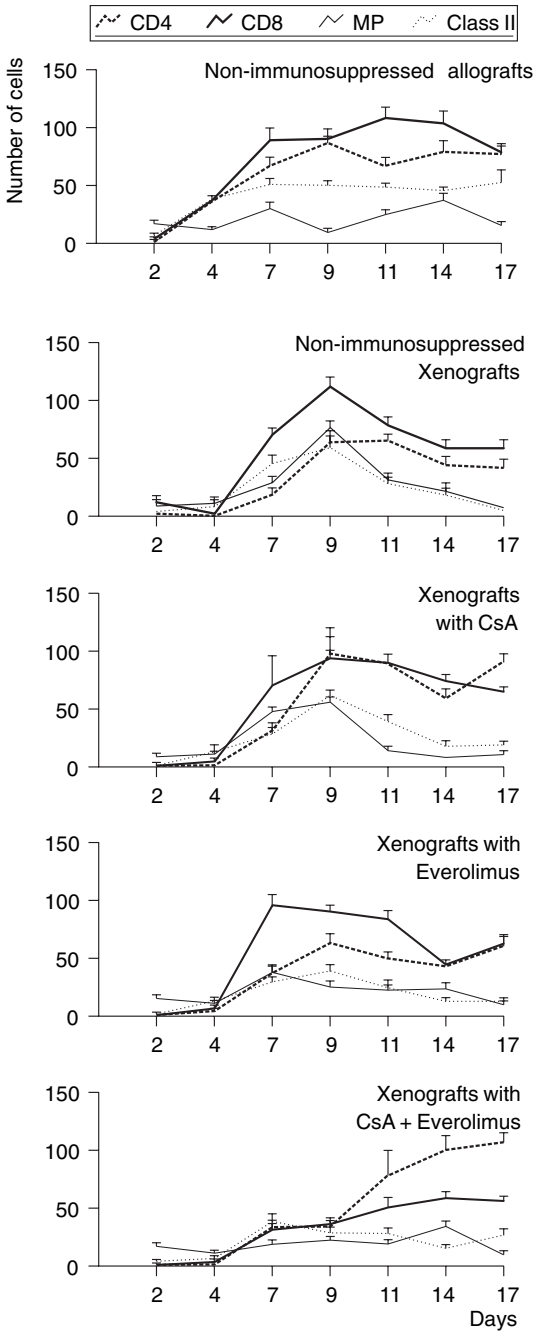


Figure 3 Numbers (mean + SEM) of CD4+ and CD8+ lymphocytes (CD4, CD8), macrophages (MP) and class II-expressing cells (Class II) in bronchial wall in the groups during follow-up. CsA = cyclosporine A.

xenografts, everolimus reduced the numbers of all cell types, but most markedly CD4+ lymphocytes (Figs 3–6).

Combination of CsA and everolimus led to a clear delay in initiation of immunological response (Figs 3–6). Numbers of macrophages and MHC class II-expressing cells remained low throughout follow-up, but from day 11 onwards, both CD4+ and CD8+ lymphocytes were on the increase, with CD4+ cells dominating in contrast to all other study groups.

Throughout the study, the number of CD8+ lymphocytes was significantly ($P < 0.001$) smaller when the combination of CsA and everolimus was given to xenografts in comparison with all the other groups at most assessment points before day 17. On day 17, the difference was significant ($P < 0.05$) only in comparison with allografts. As regards CD4+ cells in xenografts, with the combination, on days 4–9, numbers were significantly smaller ($P < 0.05$), but on days 14–17 significantly higher than in most other groups, in which the xenogeneic reaction was already subsiding. Numbers of macrophages were similar in allografts and in xenografts treated with the combination throughout the study, both these numbers being smaller ($P < 0.05$) than in the other groups at most assessment points. More ($P < 0.05$) class II-expressing cells were evident in allografts on day 4 and at the two last assessment points than in any of the xenografts.

In xenografts, numbers of both CD4+ and CD8+ cells on day 9 correlated significantly with obliteration at the end of follow-up on day 17 ($R = 0.48$–0.49, $P < 0.05$) implying that these cell numbers could be used as predictive markers of obliteration.

Intensity of expression of ELAM-1 reached grade 3 in the xenografts on day 14, but grade 2 in allografts. Intensity of expression of MHC class II in endothelium at the end of the study averaged grade 2 in the xenografts and the maximal grade 3 in the allografts. Epithelial MHC class II expression up to grade 2 was evident at the beginning of follow-up, assuming that any epithelium was left. (Data not shown.)

Discussion

Despite interindividual variation in blood concentrations, a daily oral dose of 1.5 mg/kg everolimus is expected to achieve therapeutic blood concentrations in pigs based on reports on such rapamycin-class compounds in other species [14, 15].

In experimental allotransplantation, airway epithelial loss correlates with progression of luminal obliteration and this finding supports the hypothesis that the epithelium plays a significant role in the pathogenesis of OB [16]. Likewise, our xenograft study showed initial epithelial injury followed by airway obliteration. That immunological response and the subsequent airway obliteration apparent in nonimmunosuppressed xenografts were more short-lived than in the control allografts indicating the greater susceptibility of xenografts to chronic rejection, as reported previously [7, 12]. Furthermore, our findings are in accordance with findings that in xenografts chronic

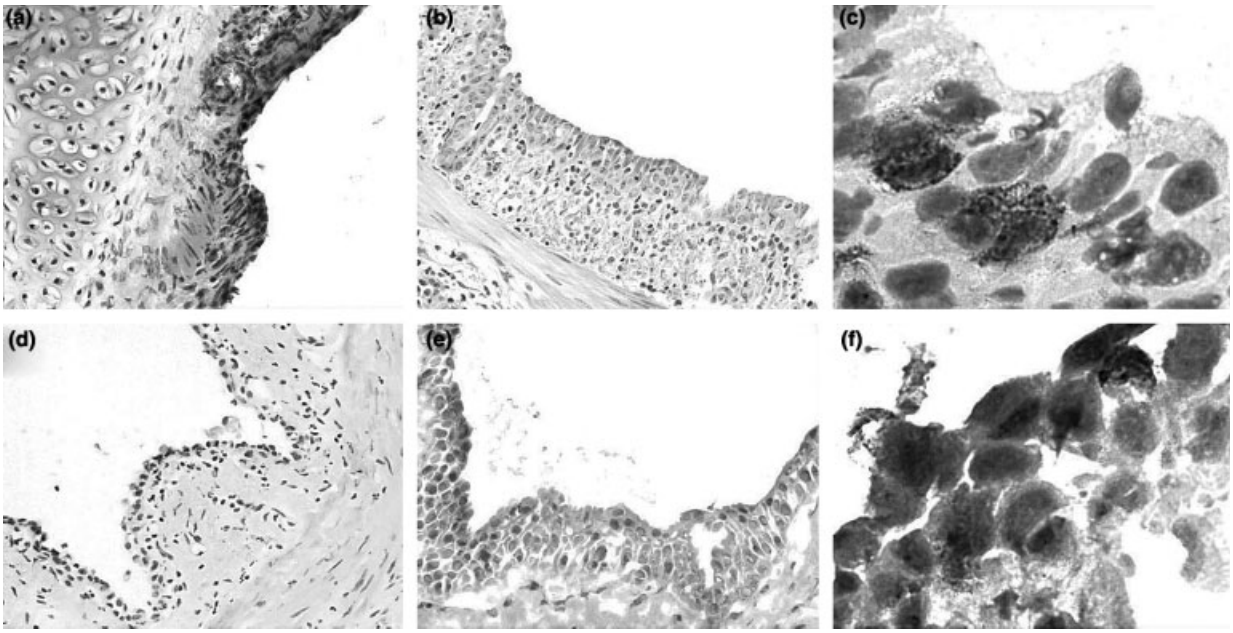


Figure 4 Initial ischemic injury in the respiratory epithelium was evident on day 2 both in bronchial allografts (a) and in nonimmunosuppressed xenografts (d). Recurrence toward normal epithelium, here with normal epithelial cells and metaplastic atypia on day 4, occurred as well in allografts (b) as in xenografts (e) (H&E staining; original magnification ×20 objective). In allografts (c), class II-expressing cells were evident on day 4 prior to epithelial loss. In xenografts (f), expression was also observed, here in metaplastic epithelial cells (immunocytochemistry against MHC class II, hematoxylin counterstaining, original magnification ×100 objective).

rejection is caused by an immune response to the graft, not by qualities of the graft itself [17].

The mechanisms of cell-mediated xenograft rejection have been less intensively studied than have those of humoral rejection. The importance of type of vascularization in determining the nature of immune responses is nowhere more important than in xenotransplantation [18]. Ability to sustain angiogenesis determines whether tissues successfully engraft and are thereafter subject to cellular rejection [19]. After neovascularization, epithelial repair first occurs after the ischemic damage, but is followed by progressive destruction and subsequent obliteration. The fact that the immune response in our model started faster in the allografts than in the xenografts is probably due to xenografts' slower neovascularization [8, 12]. Furthermore, our model, in addition to, being initially nonvascularized is also permanently nonaerated. These factors limit the direct applicability of the data to the clinic. At best, this model can be useful to study the mechanisms of OB.

Most studies in large animal species have indicated that mechanisms of cell-mediated immunity to discordant xenografts are fundamentally similar to those involved in allograft rejection but stronger [18]. Both CD4+ and CD8+ T cell-mediated human anti-pig immune responses have been documented [20]. In the present study, an influx of CD4+, CD8+ cells, macrophages and class II-expressing cells was evident in nontreated xenografts, and the peak numbers of CD4+ and CD8+ cells even predicted grade of obliteration at the end of follow-up. In allografts, we have earlier shown that cell-mediated rejection in nontreated samples was still active when total obliteration had been reached, supporting the fact of OADs being a continuation of the initial acute reaction in this model [10]. In xenografts with single therapy, and especially in those without immunosuppression, the cell-mediated reaction already seemed to be subsiding at the end, indicating a faster process. Furthermore, both ELAM-1 and MHC class II-expression in the endothelium, as well as epithelial class II expression, was evident in xenografts and allografts, supporting the findings of an immune-mediated reaction.

Neither CsA nor everolimus alone was able to significantly alter the immunological response seen in our non-immunosuppressed xenografts. On the other hand, when both CsA and everolimus were used with xenografts, the immunological response was delayed and blunted. In these samples, CD4+ lymphocytes still appeared in increasing numbers at the end of follow-up, indicating the continuation of the process. Studies in rodents have consistently indicated that CD4+ T cells are necessary for the rejection of xenogeneic skin and that in the absence

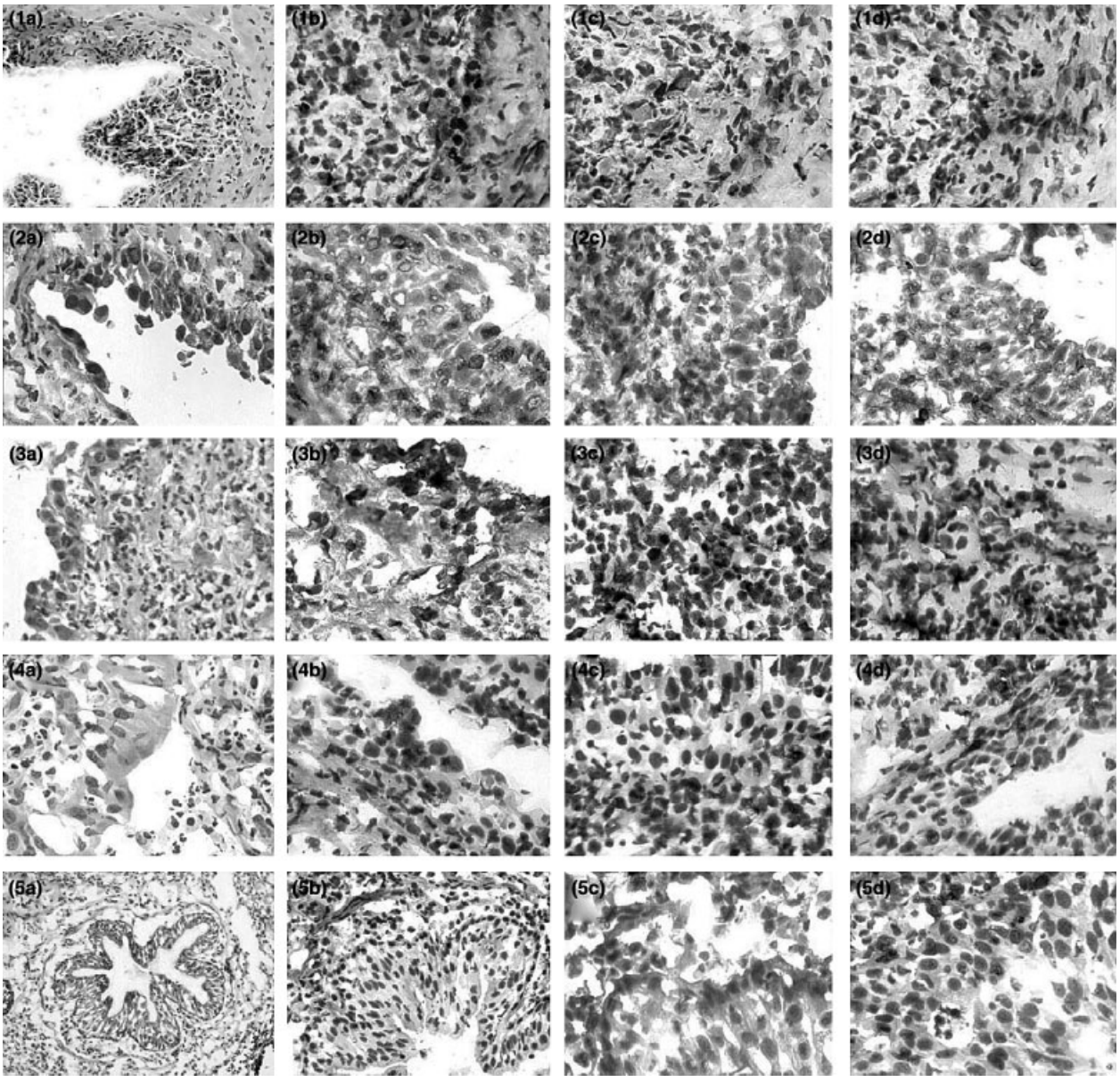
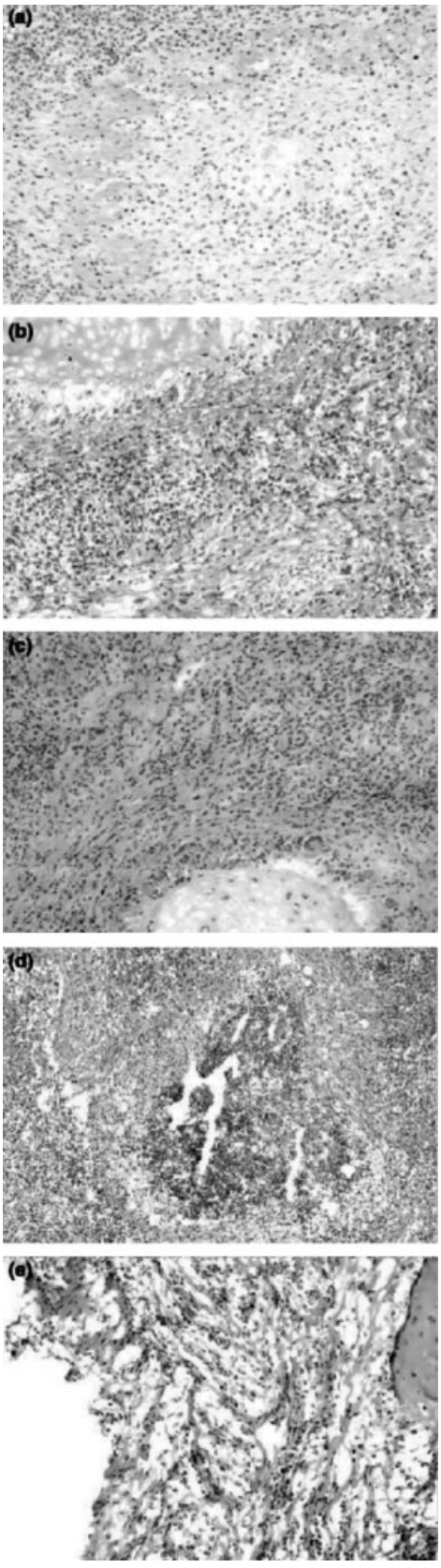


Figure 5 Histology and immunocytochemistry on day 7 in bronchial allografts and xenografts. H&E staining; original magnification ×20 in A, ×40 in E, I, and M, ×10 in Q. Immunocytochemistry against MHC class II (1b, 2b, 3b, 4b, 5b), CD4 (1c, 2c, 3c, 4c, 5c) and CD8 (1d, 2d, 3d, 4d, 5d), hematoxylin counterstaining, original magnification ×40 in all except ×20 in 1a, and 5b. CsA = cyclosporine A. On the first line control allografts. Epithelial destruction followed by gradual obliteration. The early obliterative plug (1a) consists of fibroblasts and inflammatory cells and is vascularized. Fibroblasts and inflammatory cells express class II (1c), and CD4+ (1c), and CD8+ (1d) lymphocytes both in the obliterative plug, and in the bronchial wall. On the second line nonimmunosuppressed xenografts. Epithelial destruction with some preserved epithelium (2a), and class II-expressing cells in the bronchial wall and in the atypical epithelium (2b). CD4+ (2c), and CD8+ (2d) lymphocytes in the bronchial wall. On the third line xenografts given CsA. Metaplastic atypia of the airway epithelium and inflammatory cells intramurally (3a). Intense class II-expression in the destructing epithelium, in endothelial, and in inflammatory cells, as well as, in fibroblasts (3b). CD4+ (3c), and CD8+ (3d) lymphocytes in the bronchial wall. On the fourth line xenografts given everolimus. Partly normal respiratory epithelium, inflammatory cells in the bronchial wall, and early obliterative plug formation (4a). Class II-expressing fibroblasts, and inflammatory cells in addition to, epithelial cells comprising the basal cell layer (4b). CD4+ (4c), and CD8+ (4d) lymphocytes in the bronchial wall, preserved epithelium with patent bronchial lumen. On the fifth line xenografts given CsA and everolimus. Normal epithelium in a patent bronchiolus (5a), some inflammatory cells in the bronchiolar wall. Class II-expression in inflammatory, and endothelial cells, as well as, in the muscular ring (5b). Some CD4+ (5c) and CD8+ (5d) lymphocytes in the bronchiolar wall.



of CD8+, T cells, NK cells, and B cells islet transplants and CD4+ T cells can cause rejection [21, 22]. CD4+ T cells are thus especially important in xenograft rejection, as was seen also in our surviving xenograft samples.

Another special feature of cell-mediated xenograft rejection is that xenografts appear to be more susceptible to nonspecific inflammatory processes of graft destruction than are allografts [18]. Macrophages are considered the predominant cell in the area of xenogeneic tissue destruction in some models [23, 24], although there is evidence that recipient T cells are required to initiate these reactions [25]. In general, more macrophages were visible also in our xenografts in the absence of everolimus than was the case for allografts, but these were not the predominant cell type. The reason may, however, be that we assessed cells in the bronchial wall.

Cyclosporine A, despite being the drug most commonly used in clinical transplantation, has shown no great effect after concordant xenotransplantation in rodents [6]. Although it influences the extent of the cellular immune response, it is unable to affect the humoral component of the rejection process or the development of OB [6]. On the other hand, rapamycin significantly decreases severity of luminal obliteration, but fails to prevent loss of respiratory epithelium [6]. In our model, a combination of CsA and everolimus was able to prevent epithelial damage early on and to reduce severity of the subsequent airway obliteration, but to a lesser extent than in allotransplants [12]. Furthermore, based on number of macrophages, everolimus had an effect on the nonspecific inflammatory processes of graft destruction here as earlier [26], which may indicate a potential role for everolimus also in xenotransplantation.

In conclusion, in this xenograft model, chronic rejection appeared to be caused by an immune response to the graft, with peak numbers of CD4+ and CD8+ cells predicting grade of obliteration. In contrast to allografts, CD4+ cells and macrophages seemed to have a greater influence in this xenograft model. The combination of

Figure 6 Bronchial remodeling at the end of the follow-up (day 17). A totally obliterated bronchial allograft (a) with numerous lymphocytes in the bronchial wall but less intense inflammation in the fibrotic obliterative plug. Severe inflammation in the wall of a nonimmunosuppressed xenograft (b), between necrotic cartilage and the obliterative plug. Dense fibrosis, lymphocytic inflammation, and necrotic cartilage in a xenograft given CsA (c). Severe bronchial wall inflammation, and a obliterative plug in addition to, cellular debris in a xenograft given everolimus (d). An almost totally patent bronchial lumen to the left with a tiny early plug formation in a xenograft given CsA and everolimus (e). Bronchial remodeling is less severe than in the other xenografts, or in the allograft controls (H&E staining; original magnification ×10 objective in all, but ×5 in d). CsA = cyclosporine A.

CsA and everolimus was able to blunt the xenogeneic immunological response and delay the subsequent obliteration. Human everolimus-dosing regimens do not apply in porcine studies.

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