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# The Development of Obliterative Airway Disease under the Implication of Epithelium Damage and Proliferation:

## *An Experimental Study Using Heterotopic Tracheal Transplantation in Rats*

*Ina Schade, Sylke Roth-Eichhorn, Katrin Ploetze, Michael Kasper, and Stephan Schueler*

Cyclosporin A (CsA) and Rolipram, an inhibitor of phosphodiesterase 4 (PDE4) and established as an antiinflammatory drug, were compared examining their immunosuppressive effect. Using an acknowledged heterotopic trachea graft model, 4 groups were investigated: CsA-, Rolipram-, and CsA+Rolipram-treated, and untreated animals. At different time points, the grafts were removed for histology and immunohistochemistry. In untreated and Rolipram-treated animals, the epithelium was completely destroyed by day 28, whereas in CsA-treated rats it was conserved until day 60 (55% ± 39%). A similar protective effect was also seen in the group treated with CsA+Rolipram (50% ± 35%). Rolipram treatment led to decreased proliferation (day 28: 330 ± 130, 370 ± 30, 160 ± 20, 70 ± 20 cells/mm<sup>2</sup>; untreated, CsA-, Rolipram-, CsA+Rolipram-treated, respectively). Rolipram significantly inhibited the infiltration of monocytes/macrophages but was less effective than CsA (day 5: 4860 ± 420, 2370 ± 210, 3720 ± 280, 2320 ± 30 cells/mm<sup>2</sup>; untreated, CsA-, Rolipram-, CsA+Rolipram-treated, respectively). This resulted in the luminal obliteration of 100% ± 1%, 28% ± 2%, 82% ± 6%, 15% ± 6% in control, CsA-, Rolipram-, and CsA+Rolipram-treated animals, respectively (day 60). In isogenic grafts, no changes were observed. The authors' results suggest that PDE4-inhibitor is not suitable for mono-immunosuppressive therapy after lung transplantation. In the acute phase, PDE4-inhibitor is less effective than CsA and does not prevent obliteration. The strong antiproliferating effect of Rolipram, however, is beneficial for the prevention of chronic changes. The combination of immunosuppressive and antiproliferating drugs could be a new strategy for pharmacological intervention of airway obliteration.

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### Introduction

Despite the development of potent immunosuppressive strategies, the half-life of patients after lung transplantation has not changed. Chronic rejection processes, including obliterative bronchiolitis (OB), limit the long-term survival. OB is clinically

manifested in a progressive decline of lung function.<sup>1-3</sup> Clinical and experimental evidence suggests multiple causes and risk factors for the development of OB,<sup>4</sup> which is characterized by the infiltration of inflammatory cells, and the accumulation of fibrous tissue in the airway lumen.<sup>5</sup>

Currently, no data are available on the use of phosphodiesterase 4 (PDE4) inhibitors to prevent acute and chronic rejection. Because of the inhibition of proliferation and cytokine release by inflammatory cells (monocytes, alveolar macrophages, lymphocytes), PDE4-inhibitors have been established as drugs particularly for pulmonary disease.<sup>6,7</sup> In this report, we describe the effects of the PDE4-inhibitor Rolipram on the parameters of graft rejection including epithelial integrity, leukocyte infiltration, proliferation, and obliteration.

## Material and Methods

### *Transplantation Model*

Following anesthesia by xylacin (2%, Sanofi-Ceva, Germany) and ketamin (Velonarcon 5%, Asta-Medica, Germany) v (2:8 (v/v), donor 1.6 and recipient 1.1 ml/kg body weight IM), the trachea was implanted in the greater omentum (ischemia time 1-5 min, allogenic Brown-Norway to Lewis and isogenic Lewis to Lewis, body weight circa 250 g).

Four treated groups were studied: cyclosporin A CsA (10 mg/d/kg), Rolipram (5 mg/d/kg), CsA+ Rolipram, and untreated (4-8 animals per time point). The drugs were from AWD, Asta Medica, Dresden, Germany, dissolved in olive oil and given postoperatively, one time daily. The tracheal grafts were harvested on days 1, 3, 5, 7, 14, 21, 28, and 60.

### *Histology and Immunohistochemistry*

Cross-sections were stained with Mayer's hematoxylin-eosin solution (H&E) and analyzed quantitatively according to the procedure described<sup>8</sup> for

- the type of epithelium (respiratory, attenuated, none);
- the percentage of airway surface lined by epithelium;
- the percentage of luminal obliteration.

The epithelial integrity, and the number of proliferating and inflammatory cells, were analyzed using the following antibodies: ASML (Dr U. Günthert, Basel, Switzerland) against epithelial CD44v6, MNF116 (Dako, Glostrup, Denmark) against epithelial cytokeratin, ED1 (Augst, BMA, Switzerland) against monocytes/macrophages, and MIB5

(Dianova, Hamburg, Germany) against proliferating cells. The immunostaining was performed using Vectastain ELITE ABC-Kit (Vector/Alexis, Grunberg, Germany), according to the manufacturer's instructions. Cell density was calculated by digital morphometry. The groups were analyzed using Kruskal-Wallis and Mann-Whitney tests ( $P < 0.05$ ).

## Results

### *Epithelium*

In untreated controls, the epithelium disappeared starting on day 5 (acute rejection). CsA treatment showed a temporary protective effect. Treatment with Rolipram caused an aggravation of epithelium destruction observed in control (day 5). The combination of CsA and Rolipram, the CsA-mediated protected the epithelium from destruction (Fig 1).

### *Proliferation*

Rolipram significantly inhibited cell proliferation in the acute phase (day 5) but was less effective than CsA. From day 28 onward, the inhibiting effect of CsA weakened. In contrast, Rolipram showed significant inhibition even at late time points. The strongest inhibition was found in the group treated by the combination of CsA and Rolipram (Fig 2).

### *Monocytes/Macrophages*

Rolipram significantly inhibited the infiltration of monocytes/macrophages on day 5, but less effectively than CsA. Whereas CsA treatment led to a significant increase in the number of infiltrating cells at later time points, Rolipram had no effect on infiltration. On day 28 onward, CsA+Rolipram-treated group had the same density of monocytes/macrophages as the group treated with CsA alone (Fig 3).

### *Obliteration*

Treatment with CsA led to a strong inhibition of obliteration as compared with untreated animals. A single dose of Rolipram showed significant inhibition, but not to the extent shown by CsA. The combination of CsA and Rolipram resulted in the complete inhibition of obliteration (Fig 4).

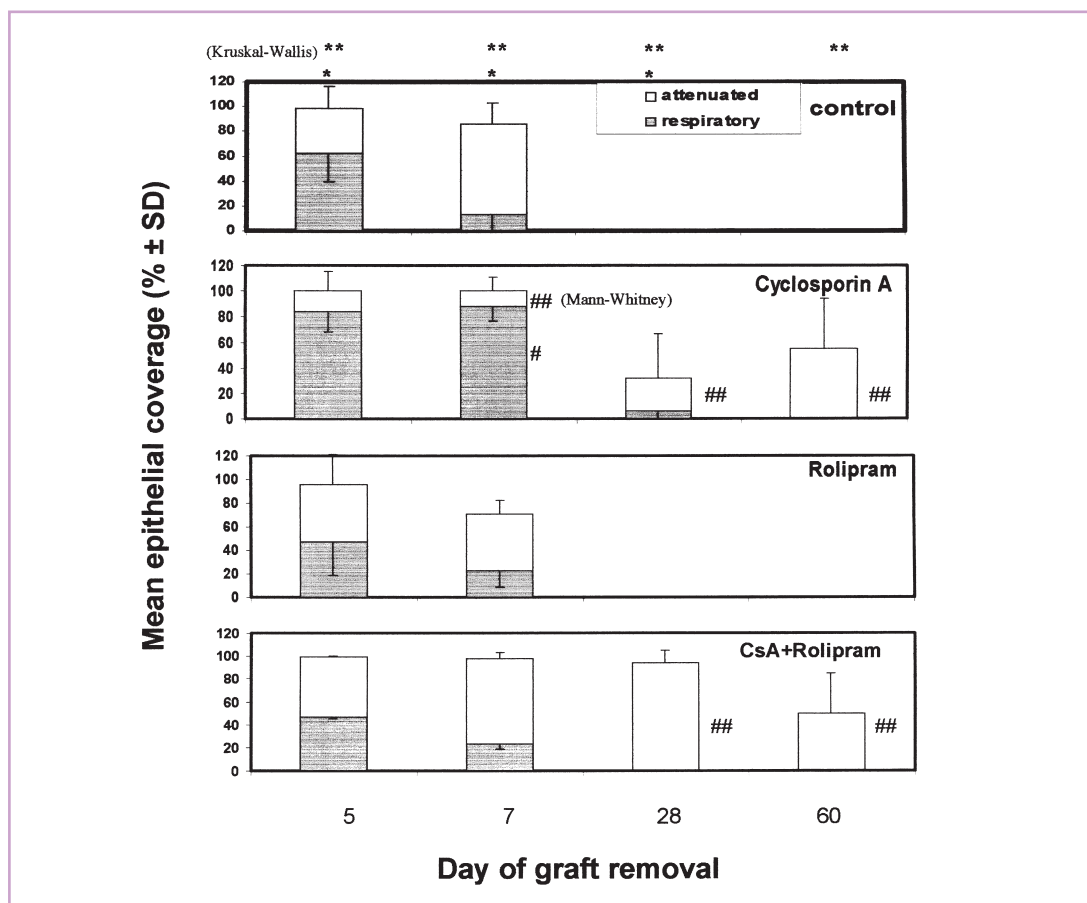


Figure 1. Morphometric analysis of epithelial coverage of transplanted tracheae after different treatment (Control, cyclosporin A (CsA), Rolipram, CsA+Rolipram). Significant differences between all groups were tested using the Kruskal-Wallis test (KW; \*\*attenuated, \*respiratory epithelium;  $P < 0.05$ ). At time points showing significant differences, the untreated control was additionally compared with treated groups by pairs using the Mann-Whitney test (MW; ##attenuated, #respiratory epithelium;  $P < 0.05$ ).

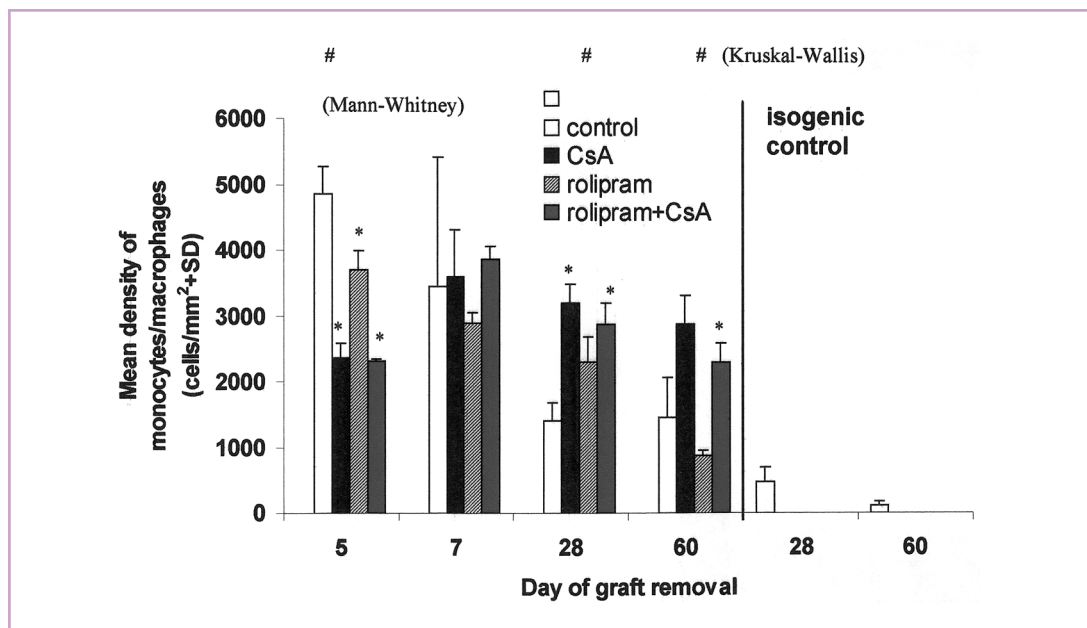


Figure 2. Mean density of proliferating cells (Ki-67 positive) in allografts after different treatment. CsA = cyclosporin A. Statistical analysis was performed by pairs using the Mann-Whitney test (MW; \*,  $P < 0.05$ ) and Kruskal-Wallis test (KW; #,  $P < 0.05$ ).

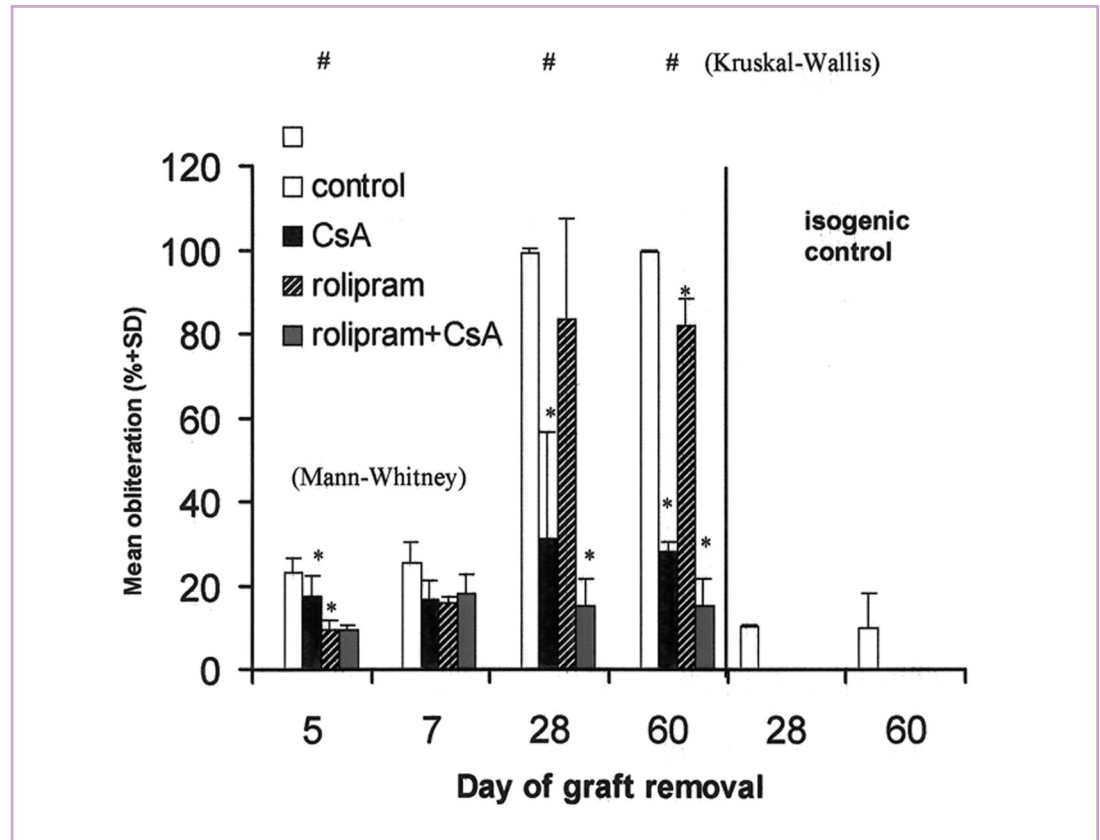


Figure 3. Mean density of monocytes/macrophages in allografts after different treatment. CsA = cyclosporin A. Statistical analysis was performed by pairs using the Mann-Whitney test (MW; \*,  $P < 0.05$ ) and Kruskal-Wallis test (KW; #;  $P < 0.05$ ).

### Discussion

The rationale of this study was to compare the effects of CsA and the PDE4 inhibitor Rolipram on epithelium preservation and proliferation in transplantation. Rolipram demonstrates antiinflammatory properties in inflammatory pulmonary diseases, such as asthma,<sup>9</sup> and may prove to be effective in inhibition of graft rejection.

In untreated allografts, the typical time course of acute rejection was characterized by maximal peritracheal infiltration of monocytes/macrophages on day 5, followed by gradual loss of epithelial coverage. These findings are in agreement with earlier studies.<sup>10-12</sup> CsA treatment has only a temporary protective effect on the epithelium at early time points. Rolipram seems to be less effective than CsA. We even found a negative effect of Rolipram on the respiratory epithelium, probably caused by

the inhibition of epithelial regrowth after ischemia. This action of Rolipram during acute rejection may contribute to the obliteration.

In the chronic phase (day 28 and day 60), significant inhibition of proliferation by Rolipram seems to contribute to prevention of obliteration. On day 60, no proliferating cells were observed. In contrast, CsA at these time points caused a significant increase in proliferating cells, partially myofibroblast-like. A variety of clinical studies including lung-transplanted patients treated long-term with CsA demonstrated that immunosuppression with CsA alone is not potent enough for prevention of OB.<sup>13</sup>

Our study shows that Rolipram is not suitable for mono-immunosuppressive therapy after lung transplantation. As shown by our treatment with CsA+Rolipram, this combination that protects the epithelium and minimizes proliferation could be

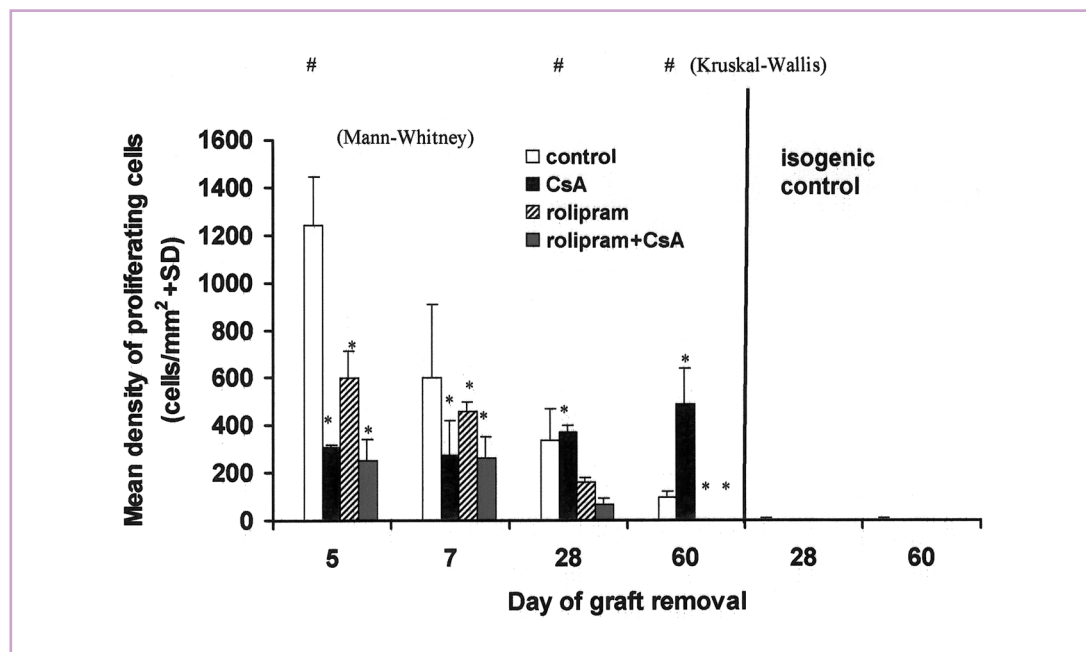


Figure 4. Morphometric analysis of obliteration. CsA = cyclosporin A. Statistical analysis was performed by pairs using the Mann-Whitney test (MW; \*;  $P < 0.05$ ) and Kruskal-Wallis test (KW; #;  $P < 0.05$ ).

efficient for pharmacological intervention of airway obliteration.

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