Experimental rhinovirus 16 infection increases intercellular adhesion molecule-1 expression in bronchial epithelium of asthmatics regardless of inhaled steroid treatment


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Summary

Background  Rhinovirus infections in airway epithelial cells in vitro have been shown to upregulate intercellular adhesion molecule-1 (ICAM-1) expression. Epithelial ICAM-1, in its dual role as the major rhinovirus receptor and as adhesion molecule for inflammatory cells may be involved in the pathogenesis of rhinovirus-induced exacerbations of asthma.

Objective  We aimed to investigate the effect of experimental rhinovirus 16 (RV16) infection on ICAM-1 expression in bronchial mucosal biopsies in asthma. In addition, the effect of 2 weeks pretreatment with inhaled budesonide (800 μg b.d.) on RV16-associated changes in ICAM-1 expression was studied.

Methods  The study had a parallel, placebo-controlled design in 25 steroid-naive nonsmoking atopic asthmatic subjects. After 2 weeks budesonide (BUD) or placebo (PLAC) pretreatment bronchoscopy was performed 2 days before (day −2) and 6 days after (day 6) RV16 inoculation (on days 0 and 1). Immunohistochemical staining for ICAM-1 was performed on snap-frozen bronchial biopsies. ICAM-1 staining intensity on the basal epithelial cells was scored semiquantitatively from 1 (weak) to 3 (intense). Similarly, epithelial intactness was noted (1 = basal cells only, 2 = basal and parabasal cells, 3 = intact epithelium).

Results  ICAM-1 scores were not significantly different between the groups at day −2 (P ≥ 0.08). Subsequent RV16 infection was associated with a trend towards an increase in ICAM-1 expression in the BUD-group (P = 0.07), whereas the increase was significant in the PLAC-group (P = 0.03). However, the increase was not significantly different between the groups (P = 0.74). Epithelial intactness score was not different between the groups before RV16 infection (P ≥ 0.07), and no significant changes were observed in either group (P ≥ 0.59). Moreover, ICAM-1 score did not correlate significantly with epithelium score in either group, at any time-point (P ≥ 0.27).

Conclusion  We conclude that an RV16 common cold in atopic asthmatic subjects is associated with increased ICAM-1 expression in the bronchial epithelium, which is not related to epithelial intactness. Glucocorticoid treatment does not appear to prevent the RV16-associated increased ICAM-1 expression. This suggests that other treatment modalities are required to protect against the spreading of infection during rhinovirus-induced exacerbations in asthma.

Keywords: airways inflammation, asthma, bronchial biopsy, bronchial epithelium, budesonide, common cold, exacerbation, glucocorticoids, intercellular adhesion molecule-1, respiratory virus infection, rhinovirus
Introduction

Asthma is a chronic airways disease that is characterized by episodic worsening of symptoms such as chest tightness, dyspnea and cough, and is associated with variable airways obstruction and airway hyperresponsiveness [1]. This is associated with airway mucosal shedding [2], cellular infiltration and increased expression of pro-inflammatory cytokines and adhesion molecules [3,4]. The long recognized temporal relationship between asthma exacerbations and respiratory viral infections has been reconfirmed in recent studies, showing that a respiratory virus can be found in up to 83% of all asthma exacerbations in children, about half of these viruses being identified as rhinovirus [5]. In experimental studies it has been shown that rhinovirus 16 (RV16) colds increase airway hyperresponsiveness to inhaled histamine in patients with atopic rhinitis [6] or asthma [7]. In addition, elevated numbers of submucosal lymphocytes and epithelial eosinophils have been reported in bronchial mucosal biopsies after experimental RV16 infection in a sample of both normal subjects and asthmatic subjects [8].

Adhesion molecules are involved in leucocyte infiltration in asthma [4,9]. Intercellular adhesion molecule-1 (ICAM-1), a member of the immunoglobulin supergene family, is the major ligand for the \( \beta_2 \) integrins lymphocyte function-associated antigen (LFA-1, CD11a/CD18), which is expressed on most leucocytes, and macrophage-1 antigen (Mac-1, CD11b/CD18), which is expressed on neutrophils and mononuclear cells [10]. Interestingly, 90% of rhinoviruses use ICAM-1 as their cellular receptor [11]. There is evidence to suggest that respiratory viruses, including rhinoviruses can upregulate ICAM-1 transcription and expression in pulmonary epithelial cells in vitro [12–16], which is associated with enhanced adherence of polymorph mononuclear cells [12] and migration of T cells [17]. Such upregulated expression may in part be mediated by cytokines such as IL-1 [9,13,16], which is an inducer of ICAM-1 expression [16]. Thus, one could envisage an interactive mechanism, in which a increased ICAM-1 promotes both rhinoviral spreading [16] and influx of inflammatory cells [8], thereby potentially contributing to the development of an exacerbation of asthma.

Patients with asthma have been reported to demonstrate elevated ICAM-1 expression in their bronchial epithelium as compared to normal controls [4,18]. It its presently unclear as to whether such expression is dependent on the clinical control of the disease. Glucocorticoids, a commonly used class of drug in asthma, appear to down-regulate both basal- and cytokine-induced epithelial ICAM-1 expression in vitro [19]. However, in steady state asthma regular treatment with glucocorticoids did not seem to have an effect on epithelial ICAM-1 expression [3]. The influence of an asthma exacerbation on epithelial ICAM-1 expression has not been studied.

In the present study we hypothesized that a rhinovirus-induced cold in patients with asthma is accompanied by elevated ICAM-1 expression in the bronchial epithelium. In addition, we hypothesized that glucocorticoids are able to prevent such elevated ICAM-1 expression. To that end, we investigated first, the effect of experimental RV16 infection on the expression of ICAM-1 in bronchial mucosal biopsies. Secondly, we examined the effect of 2 weeks pretreatment with inhaled budesonide (a glucocorticoid) on RV16-associated changes in ICAM-1 expression in atopic, mildly asthmatic subjects.

Materials and methods

Design

The study had a randomized double-blind, parallel, placebo-controlled design, including two groups of asthmatics receiving RV16 inoculation during treatment with either budesonide (BUD/RV16 group) or placebo (PLAC/RV16 group). Budesonide by dry powder inhaler (Turbohaler®, 800 \( \mu \)g, b.i.d.) or placebo was given for a period of 4 weeks, starting 16 days before RV16 inoculation (day –16). After two weeks of treatment (day –2), bronchoscopy was performed and bronchial biopsies were taken in all patients. Two days later this was followed by experimental RV16 infection on two consecutive days (day 0 and day 1). The bronchoscopy was repeated 6 days after infection (day 6).

To ascertain that the bronchoscopy procedure itself would not interfere with ICAM-1 expression, bronchial biopsy specimens were obtained at an 8-days interval without intervention or treatment in seven asthmatic subjects, thereby serving as controls. This third group of patients (PLAC/PLAC group) had the same inclusion criteria as the other subjects.

Subjects

Twenty-five nonsmoking or ex-smoking (>12 months, <5 pack years) adult asthmatics (13 female, 12 male; age 19–25 years.) were recruited. The subjects’ characteristics are shown in Table 1. The subjects had low titres of
circulating antibodies specific to rhinovirus 16 using a RV16 serum neutralization assay (≤ 1:4 serum dilution against 20–25 × 50% tissue culture infective dose TCID₅₀), and were atopic, as reflected by one or more weal (> 3 mm) and flare response to skin prick tests to 10 common aero-allergen extracts (Soluprick, ALK, Benelux). In the 3 months preceding the study the subjects had not used oral or inhaled glucocorticoids or any other medication for their asthma or allergies except for inhaled short-acting β₂ agonists on demand. All subjects were healthy, apart from their asthma during the 6 weeks preceding the study. The characteristics of the seven control patients (PLAC/PLAC group) (three female, four male; age 19–34 years.) are presented in Table 2. The study was approved by the Medical Ethics Committee of the Leiden University Medical Center, and the subjects gave their written informed consent before entering the study.

**Rhinovirus 16 inoculation**

The RV16 inoculum was obtained from the same strain and stock as used in previous experiments in humans *in vivo* [6,7]. The virus was cultured according to standards of good laboratory practice and the inoculum was tested to be safe for human *in vivo* usage [20]. A total dose of 0.6–2.1 × 10⁴ TCID₅₀ RV16 was administered to each subject according to a previously described procedure [7]. Briefly, the total RV16 dose, suspended in Hanks’ Balanced Salt Solution (HBSS) containing 0.5% (w/v) gelatine, in a volume of 3 mL, was divided over 2 days. On each day, 0.5 mL of the

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**Table 1. Characteristics of rhinovirus-inoculated patients**

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Sex (M/F)</th>
<th>Age (years)</th>
<th>Baseline FEV₁ (% predicted)</th>
<th>Baseline PC₂₀ histamine (mg/mL)</th>
<th>titre pre/post inoculation (1: . . .)*</th>
<th>culture nasal lavage†</th>
</tr>
</thead>
<tbody>
<tr>
<td>BUD/RV16</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>M</td>
<td>24</td>
<td>73.5</td>
<td>0.14</td>
<td>1/1</td>
<td>pos/pos</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>21</td>
<td>86.4</td>
<td>0.17</td>
<td>1/8</td>
<td>neg/neg</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>23</td>
<td>82.5</td>
<td>0.29</td>
<td>1/16</td>
<td>pos/pos</td>
</tr>
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<td>4</td>
<td>F</td>
<td>24</td>
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<td>0.30</td>
<td>1/1</td>
<td>pos/neg</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>23</td>
<td>95.2</td>
<td>0.54</td>
<td>1/8</td>
<td>pos/pos</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>24</td>
<td>76.4</td>
<td>0.54</td>
<td>1/32</td>
<td>pos/neg</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>23</td>
<td>81.7</td>
<td>0.65</td>
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</tr>
<tr>
<td>8</td>
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<td>23</td>
<td>91.0</td>
<td>1.28</td>
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<td>neg/neg</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
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<td>76.8</td>
<td>1.34</td>
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</tr>
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<td>10</td>
<td>M</td>
<td>25</td>
<td>82.6</td>
<td>2.13</td>
<td>1/8</td>
<td>pos/pos</td>
</tr>
<tr>
<td>11</td>
<td>F</td>
<td>24</td>
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<td>3.32</td>
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<td>neg/neg</td>
</tr>
<tr>
<td>12</td>
<td>F</td>
<td>25</td>
<td>103</td>
<td>5.92</td>
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<td>pos/pos</td>
</tr>
<tr>
<td>mean ± SEM</td>
<td></td>
<td></td>
<td>84.6 ± 2.4</td>
<td>0.74 ± 0.49 ‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLAC/RV16</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>M</td>
<td>22</td>
<td>80.9</td>
<td>0.06</td>
<td>1/16</td>
<td>pos/neg</td>
</tr>
<tr>
<td>14</td>
<td>F</td>
<td>19</td>
<td>80.5</td>
<td>0.14</td>
<td>1/4</td>
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</tr>
<tr>
<td>15</td>
<td>F</td>
<td>19</td>
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<td>0.30</td>
<td>1/1</td>
<td>pos/pos</td>
</tr>
<tr>
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<td>0.31</td>
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</tr>
<tr>
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<td>M</td>
<td>19</td>
<td>101</td>
<td>0.46</td>
<td>1/1</td>
<td>neg/neg</td>
</tr>
<tr>
<td>18</td>
<td>M</td>
<td>23</td>
<td>74.6</td>
<td>0.52</td>
<td>1/32</td>
<td>pos/neg</td>
</tr>
<tr>
<td>19</td>
<td>F</td>
<td>20</td>
<td>83.9</td>
<td>0.53</td>
<td>1/1</td>
<td>pos/neg</td>
</tr>
<tr>
<td>20</td>
<td>F</td>
<td>23</td>
<td>84.0</td>
<td>0.65</td>
<td>1/32</td>
<td>pos/pos</td>
</tr>
<tr>
<td>21</td>
<td>F</td>
<td>24</td>
<td>94.2</td>
<td>1.44</td>
<td>1/16</td>
<td>neg/neg</td>
</tr>
<tr>
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<td>F</td>
<td>20</td>
<td>96.6</td>
<td>1.81</td>
<td>1/1</td>
<td>pos/pos</td>
</tr>
<tr>
<td>23</td>
<td>M</td>
<td>23</td>
<td>103</td>
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<td>neg/neg</td>
</tr>
<tr>
<td>24</td>
<td>F</td>
<td>20</td>
<td>95.9</td>
<td>2.40</td>
<td>1/8</td>
<td>pos/pos</td>
</tr>
<tr>
<td>25</td>
<td>M</td>
<td>22</td>
<td>84.1</td>
<td>4.34</td>
<td>1/256</td>
<td>pos/pos</td>
</tr>
<tr>
<td>mean ± SEM</td>
<td></td>
<td></td>
<td>89.2 ± 2.5</td>
<td>0.65 ± 0.49 ‡</td>
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</table>

*Serum RV16 neutralizing antibody titre obtained at days −2 and 28; † nasal lavage obtained at days 3 and 6; ‡ geometric mean ± SEM (doubling dose).
inoculum was administered by nasal inhalation (DeVilbiss 646 nebulizer, connected to a face mask). Then, 0.5 mL was administered by spraying equal portions into each nostril (DeVilbiss 286 atomiser, powered by a compressor). Finally, 0.5 mL of the inoculum was instilled in equal portions into each nostril by pipette.

The confirmation of RV16 infection was established by a fourfold or greater increase in virus-specific neutralizing antibody titre in serum and/or by recovery of the virus from the nasal washes [7]. The nasal washes were inoculated onto Human Embryonic Lung fibroblast (HEL) cultures and incubated at 32°C for 14 days. The identity of rhinovirus in a positive culture was confirmed by neutralization assay, using RV16-specific guinea pig immune serum (1126AS/GP-VR; American Type Culture Collection, Rockville, MD, USA). In order to exclude any intercurrent respiratory infection, all nasal washes were inoculated into rhesus monkey kidney cells (LCC-MK2), Hep-2 and HEL cell cultures and cultured at 37°C [7].

Bronchoscopy

Fiberoptic bronchoscopy was carried out by experienced investigators (JJB, JV, LW, EB), using a standardized protocol based on international guidelines, as has been used in previous studies in our department [21]. After 6 h of fasting, premedication consisting of atropine 0.5 mg subcutaneously, codeine 20 mg orally and salbutamol 400 µg by metered dose inhaler was administered to the subjects. Local anaesthesia was performed with 10% (w/v) lignocaine aerosol in the oropharynx, and with 2% (w/v) lignocaine solution in the lower airways. If necessary, additional lignocaine 2% was administered through the bronchoscope during the procedure. Fiberoptic bronchoscopy was performed using a Pentax bronchoscope (outer diameter 6 mm; Pentax Optical Co., Japan). The bronchoscope was introduced through the mouth, with the patient in supine position. Six bronchial biopsies were taken at (sub)segmental level from either the right lung (right lower and middle lobe) or the left lung (lingula and left lower lobe), using a pair of cup forceps (Olympus FB-20C, Tokyo, Japan). Alternate biopsy sites (right or left lung) were randomized over the two bronchoscopy visits.

Throughout the procedure oxygen was delivered through the nasal canal at a rate of 4 L/min while oxyhaemoglobin saturation was monitored continuously in all subjects using a transcutaneous oximeter (N-180, Nellcor Inc., Hayward, CA, USA) with a finger probe placed on a finger. Afterwards, the subjects were observed for 1 h for any adverse events.

Processing of the bronchial biopsies

The biopsy samples were immediately embedded in OCT medium (Miles Inc. Diagnostics Division, Elkhart, USA), and snap-frozen in isopentane cooled by iced CO2. Thereafter, the samples were stored in airtight containers at −70°C, pending further processing [21].

Immunohistochemistry

Four micrometer thick cryostat sections of frozen biopsies were air dried for 1 h, and fixed in acetone for 10 min. Immunohistochemical staining was performed using monoclonal antibodies against the markers of interest, and the avidin-biotin complex (ABC) as visualizing method. In short, the sections were incubated with an optimal dilution of monoclonal antibodies in 1% BSA/PBS at room temperature for 60 min. After washing with PBS (3 × 5 min), the slides were incubated with mouse primary antibodies in appropriate dilution for 30 min (1:500 for ICAM-1, CD54-clone MEM-112, MONOSAN, Uden, The Netherlands; 1:100 for platelet-endothelial cell adhesion molecule-1 or PECAM-1, NCI-C131, NOVOCASTRA Laboratories, 2000 Blackwell Science Ltd, Clinical and Experimental Allergy, 30, 1015–1023

### Table 2. Characteristics of control patients

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Sex (M/F)</th>
<th>Age (year)</th>
<th>Baseline FEV₁ (% predicted)</th>
<th>Baseline PC₂₀ methacholine* (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLAC/PLAC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>M</td>
<td>20</td>
<td>111</td>
<td>0.31</td>
</tr>
<tr>
<td>27</td>
<td>M</td>
<td>23</td>
<td>115</td>
<td>0.68</td>
</tr>
<tr>
<td>28</td>
<td>M</td>
<td>34</td>
<td>94</td>
<td>1.41</td>
</tr>
<tr>
<td>29</td>
<td>F</td>
<td>22</td>
<td>99</td>
<td>1.45</td>
</tr>
<tr>
<td>30</td>
<td>F</td>
<td>19</td>
<td>115</td>
<td>1.95</td>
</tr>
<tr>
<td>31</td>
<td>F</td>
<td>23</td>
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<td>mean ± SEM</td>
<td></td>
<td></td>
<td>107.0 ± 3.4</td>
<td>1.57 ± 0.56†</td>
</tr>
</tbody>
</table>

* methacholine chloride; † geometric mean ± SEM (doubling dose).
Newcastle upon Tyne, UK). The slides were washed with PBS (3×5 min) and incubated with biotinylated rabbit antimouse antibody (dilution 1:200) for 30 min. Subsequently, the slides were again washed with PBS (3×5 min) incubated for another 30 min with avidin-biotin complex. Then, the slides were washed with PBS, rinsed in sodium acetate buffer 0.1 M (pH 5.0) for 5 min and the reaction was revealed by putting the slides in 5% AEC (3-amino-9-ethylcarbazol) for 7 min. The reaction was stopped in demineralized water and the sections were counterstained with Mayer’s Haematoxylin. For negative controls, the primary antibody was omitted from this procedure.

Analysis of stained sections

All coded biopsy specimens examined at a 250× magnification by one observer (KG), who was blinded to the patient’s characteristics, treatment, and the study day on which the biopsy was taken. Biopsy sections were analysed semiquantitatively, with regard to intensity and distribution pattern of the staining of ICAM-1 positive basal epithelial cells on a three-point scale from 1 = weak, 2 = medium, focal 3 = intense, continuous staining of basal cell layer) [18,21]. Areas with nonadjacent epithelial cells, or layers of epithelial cells not connected to the basement membrane were not taken into account. Since ICAM-1 staining was most intense on basal cells, less intense on parabasal cells, and not present on ciliated and goblet cells, we focused on ICAM-1 staining in basal epithelial cells only, in order to avoid bias by epithelial damage. The association between epithelial damage and ICAM-1 staining was further investigated by scoring epithelial intactness in a semiquantitative way, based on a qualitative description of the bronchial epithelium in asthma by Jeffery et al. [2]: 1 = predominantly (>50%) adjacent basal cells, 2 = predominantly basal and parabasal cells, 3 = predominantly intact epithelium. Each biopsy specimen was scored twice and the mean value of the two scores was used in the analysis. Following this procedure, the intra-observer repeatability of ICAM-1 score and epithelium score of the same slides over a 2-day interval was satisfactory (κ = 0.69). Platelet endothelial cell adhesion molecule-1 (PECAM-1), which has been described to be constitutively expressed on the endothelium of all vessel types [22], was used as a technical internal staining control.

Non-parametric statistical analysis was applied in order to examine the effect of RV16 or bronchoscopy on ICAM-1 immunostaining within the groups (Wilcoxon rank test), and the effect of inhaled steroids in the BUD/RV16 group as compared to the PLAC/RV16 group (Mann–Whitney U-test). Spearman rank test was used to test the relationship between ICAM-1 score and epithelium score. P-values less than 0.05 were considered to be statistically significant.

Results

Thirty-two subjects completed the study. One subject (patient 3) did not undergo the bronchoscopies due to strong subjective discomfort. All the other subjects underwent bronchoscopy twice. The quality and size of the biopsies obtained during the first bronchoscopy of subjects (1, 19, 26, 27 and 32), and during the second bronchoscopy of subjects (1, 10 and 20) were inadequate for staining or analysis. RV16 infection was confirmed in all RV16 inoculated subjects, except subjects 2 and 17, who were only included in the between-group statistical analysis at day 1. All RV16-treated subjects had an anti-RV16 titre serum ≥ 1:1 before entering the study. However, reassessment just before inoculation of RV16 revealed slightly elevated titres in subjects 7, 8 and 23, coinciding with symptoms of a common cold in subjects 8 and 23, which was confirmed in subject 8 by rhinovirus-positive (RV16-negative) culture of the nasal lavage. Subjects 8 and 23 were therefore excluded from the analysis. Since low levels of neutralizing antibodies per se have been shown not to preclude a symptomatic common cold [7], subject 7 was not excluded from the analysis. Hence, seven-paired samples were available for analysis in the BUD/RV16 group, nine-paired samples were available in the PLAC/RV16 group, and four-paired samples were available in the PLAC/PLAC group.

PECAM-1 and ICAM-1 staining

Examples of ICAM-1 staining in biopsy specimens before and after rhinovirus inoculation are shown in Fig. 1(a,b), respectively. Epithelial ICAM-1 staining was most intense on basal cells, less intense on parabasal cells, whereas ciliated cells and goblet cells did not stain in any of the biopsy sections. Positive staining varied from focal staining of light to medium intensity, to continuous, intense staining of the basal cell layer. The control staining of PECAM-1 showed endothelial staining of similar intensity throughout all biopsy specimens (data not shown).

Before RV16 infection, the ICAM-1 scores were not significantly different between the groups (MWU test: P = 0.08). The ICAM-1 score increased significantly after RV16 infection within the PLAC/RV16 group (P = 0.03), whilst there was a similar trend within the BUD/RV16 group (P = 0.07). These increases in ICAM-1 staining were not significantly different between the two groups (P = 0.74) (Fig. 2). Consequently, at the time of the second bronchoscopy, ICAM-1 intensity levels were no longer significantly different between the BUD/RV16 group and the PLAC/RV16 group (P = 0.53). Repeated bronchoscopy alone did not affect the intensity of ICAM-1 staining (PLAC/PLAC: P = 0.32) (Fig. 2).

At both time-points, the epithelial intactness score was not different between the BUD/RV16 group and the
However, the epithelial intactness score tended to be higher in the BUD/RV16 group as compared to the PLAC/PLAC group (P ≈ 0.07). There was no significant effect of RV16 infection or bronchoscopy on epithelial quality either group (P ≈ 0.32). There was no significant correlation between epithelium score and ICAM-1 score within the treatment groups for each study visits (P ≈ 0.27). Likewise, there was no significant correlation between the change in epithelium score and the change in ICAM-1 score within the treatment groups (P ≈ 0.79). Hence, we did not find evidence that epithelial intactness affected the ICAM-1 scores in our study.

**Discussion**

This study has demonstrated an increased expression of ICAM-1 in the epithelium of bronchial biopsy specimens after experimental infection with RV16 in patients with atopic asthma. Regular treatment with inhaled budesonide did not prevent the RV16-induced upregulation of ICAM-1. These data indicate that RV16 infection triggers elevated expression of its cellular receptor within the intrapulmonary airways in asthmatics *in vivo*, irrespective of inhaled glucocorticoid treatment. This suggests that by upregulating its own receptor, rhinovirus may potentially facilitate its own bronchial epithelial infection and leucocyte infiltration, which might contribute to the development of rhinovirus-induced exacerbations of asthma.

This is the first study examining the effect of a common cold on the expression of ICAM-1 within the lower airways in asthmatic subjects *in vivo*. Such rhinovirus-associated upregulation of ICAM-1 expression fits in with reports of increased ICAM-1 expression *in vivo* after several other
pro-inflammatory stimuli such as hyperoxia [23], ozone exposure [24] and allergen inhalation challenges [4,9]. In addition, evidence of in vitro studies suggests that respiratory viruses, including rhinoviruses can upregulate ICAM-1 transcription and subsequent expression in epithelial cells [12–16]. Hence, our results in asthmatics in vivo confirm previous observations in vitro, and extend these by providing evidence of a potential mechanism for the development of rhinovirus-induced exacerbations of asthma.

The present findings have been obtained after careful patient selection and study design, and by applying validated methods for RV16 experimental infection. RV16 inoculation was performed as previously described [7], by using a combination of three methods of virus administration, including nasal inhalation, thus mimicking the natural ways of transmission [25]. By choosing the dosage of inhaled glucocorticoids (800 μg b.i.d.) which was higher than the recommended dose for mild persistent asthma [1], we intended to affect inflammatory mechanisms within the airways [26]. We purposely added a third group of seven patients who did not receive an experimental RV16-infection, nor any other intervention. This control group did not show a significant change in ICAM-1 expression, indicating that there was no confounding effect of a bronchoscopy or any of the other procedures on ICAM-1 expression. Although these asthmatic subjects were selected according to the same inclusion criteria, baseline FEV1 was slightly higher as compared to the other 25 asthmatics, indicating that their asthma may have been slightly milder. However, since this group was added to investigate the effect of the bronchoscopy procedure on ICAM-1 expression only, we feel that such a minor difference is not relevant to the outcome of this study. The present results may be negatively affected by the loss of statistical power caused by missing data. However, data in large samples on the repeatability of assessment of ICAM-1 expression in bronchial biopsies over time are presently not available. Since the reasons for loss of data points were various, and the success rate of RV16 infection was equally distributed over the two RV16-treated groups, one can argue that the trend towards an increase in ICAM-1 expression in the BUD/RV16 group is comparable to the significant increase in the PLAC/RV16 group.

The present data critically depend on the immunohistochemical analysis. We used the CD54-clone MEM112 antibody because of its ability to detect ICAM-1 [27]. We focused on the expression of ICAM-1 in the bronchial epithelium because the available evidence indicates that the epithelium is the primary target of rhinovirus infection in the nose [28]. Furthermore, primary tracheal epithelial cell cultures as well as a bronchial epithelial cell line appear to be susceptible to rhinovirus infection [14,16]. The stained sections were analysed by semiquantitative analysis, as has been previously described by others [4], and by ourselves [21] for measuring the expression of molecular markers in bronchial biopsies. A potential bias by epithelial damage in the ICAM-1 score was avoided by focusing on staining in the basal cell layer. The lack of effect of budesonide treatment and the RV16 intervention on epithelial intactness and the lack of a significant correlation between ICAM-1 staining and epithelial intactness support the absence of such a bias. Finally, the validity of the semiquantitative analysis of both ICAM-1 staining and epithelial quality is supported by a satisfactory intra-observer repeatability (κ>0.69). As a technical control for staining of the biopsies we chosePECAM-1, because it is equally and constitutively expressed in the endothelium and it has not been reported to be affected by steroids [22].

How can the present results be interpreted? A possible pathway of the rhinovirus-induced ICAM-1 upregulation is mediation by cytokines such as IL-1β [16]. In addition, one could speculate that ICAM-1 cross-linking by rhinovirus particles, which contain multiple ICAM-1 binding sites, could induce AP-1-mediated IL-1β transcription and expression [29]. Indeed, elevated IL-1β levels have been demonstrated in nasal lavage after rhinovirus infection [30]. Alternatively, the rhinovirus infection itself could lead to enhanced transcription of the ICAM-1 gene [14] and other genes [31]. This may occur through activation of transcription factors such as NF-κB, subsequent increased DNA binding [31,32], and activation of the ICAM-1 promoter region [14]. The latter two mechanisms require the presence of rhinovirus particles within the intrapulmonary airways, which seems likely [33], although conclusive evidence is presently lacking. We did not observe a difference in epithelial ICAM-1 expression in the budesonide-pretreated group as compared to the placebo group. The present study was not designed to examine the effect of inhaled steroids on ICAM-1 expression per se. Nevertheless, the budesonide pretreatment did not appear to influence the RV16-associated increase in ICAM-1 expression. Few studies are available on the effect of glucocorticoids on pulmonary epithelial ICAM-1 expression. In vitro, glucocorticoids appear to decrease basal and IFNγ-induced ICAM-1 expression [19]. In asthmatic patients in vivo, 6 weeks pretreatment with inhaled glucocorticoids had no effect on bronchial epithelial ICAM-1 staining [3], fitting in with our data, whereas it was recently demonstrated that such a treatment did decrease the ICAM-1 expression on BAL cells (mainly macrophages) [34]. The lack of protection against RV-associated enhanced expression of ICAM-1 may not be surprising, since a combination of systemic and nasal glucocorticoid treatment of experimental rhinovirus infection in normal volunteers did not affect the virus-induced increase in IL-1β in nasal lavage [30]. However, it can be speculated that the pretreatment period might have been too short and/or the steroid
dose too low for some anti-inflammatory effects to take place.

What are the clinical implications of this study? The observed rhinovirus-associated increase in its own cellular receptor in the bronchial epithelium might facilitate the spreading of the infection within the intrapulmonary airways in asthmatics [16], thereby potentially increasing the patients susceptibility to a severe immune response and its consequences, such as an asthma exacerbation. It is remarkable that the widely used inhaled steroids in a clinically relevant dose do not protect against this apparent positive feedback mechanism. It can be speculated that other treatment modalities such as cromolyns [35] or soluble ICAM-1 [36] will appear to be more useful in breaking this vicious circle, thus potentially providing effective protection against rhinovirus-induced asthma exacerbations.

In conclusion, experimental RV16 infection upregulates ICAM-1 expression in the bronchial epithelium of asthmatics in vivo. Inhaled steroids do not affect baseline ICAM-1 expression, and do not appear to have a protective effect against the RV16-associated upregulation. This suggests that other treatment modalities are required to treat or prevent rhinovirus-induced exacerbations in patients with asthma.

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