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Rhinovirus infection in nonasthmatic subjects: effects on intrapulmonary airways

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Rhinovirus infection in nonasthmatic subjects: effects on intrapulmonary airways. J. de Kluijver, K. Grünberg, J.K. Sont, M. Hoogeveen, W.A.A.M. van Schadewijk, E.P.A. de Klerk, C.R. Dick, J.H.J.M. van Krieken, P.J. Sterk. ©ERS Journals Ltd 2002.

ABSTRACT: The common cold is a highly prevalent, uncomplicated upper airway disease. However, rhinovirus (RV) infection can lead to exacerbation of asthma, with worsening in airway hyperresponsiveness and bronchial inflammation. The current authors questioned whether such involvement of the intrapulmonary airways is disease specific.

Twelve nonatopic, healthy subjects (forced expiratory volume in one second (FEV_1) >80% predicted, provocation concentration causing a 20% fall in FEV_1 (PC_{20}) >8 mg·mL^{-1}) were experimentally infected with RV16. Next to PC_{20} and the maximal response to methacholine ($MFEV_1$ and $M\dot{V}'_{40p}$), the numbers of mucosal inflammatory cells and epithelial intercellular adhesion molecule (ICAM)-1 expression in bronchial biopsies were assessed before and 6 days after RV16 inoculation.

RV16 infection induced a small but consistent increase in maximal airway narrowing, without a change in PC_{20}. There was a significant increase in bronchial epithelial ICAM-1 expression after RV16, whereas inflammatory cell counts did not change. Nevertheless, the change in the number of submucosal CD3+ cells was correlated with the change in $M\dot{V}'_{40p}$.

In conclusion, rhinovirus infection in normal subjects induces a limited, but significant increase in maximal airway narrowing, which is associated with changes in bronchial T-cell numbers. Together with the upregulation of bronchial epithelial intercellular adhesion molecule-1, these findings indicate that, even in healthy subjects, rhinovirus infection affects the intrapulmonary airways.

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Acute upper respiratory tract infection is the most prevalent illness in humans [1]. It not only causes school and work absence, but also results in a high expenditure for treatment each year [1]. The viruses most frequently involved are adenoviruses, influenza viruses, parainfluenza viruses, respiratory syncytial viruses, coronaviruses, and rhinoviruses [2, 3]. The latter are the major cause of the common cold and have >100 serotypes [1–3]. Fortunately, in healthy humans the common cold is usually a self-limiting upper airway disease with a short duration.

However, common colds in individuals with pre-existing airway diseases, such as asthma, are often associated with transient worsening of the disease, sometimes even leading to life-threatening exacerbations [4]. Such clinical and epidemiological data are supported by human studies using experimental rhinovirus (RV) infection. Asthmatic subjects who were experimentally infected with RV16 showed worsening of symptoms, variable airway obstruction, and airway hyperresponsiveness, associated with both increased sensitivity [5] and augmented maximal airway narrowing to bronchoconstrictor agents [6]. These physiological findings strongly suggest lower (intrapulmonary) airway involvement. Indeed, an increase in bronchial mucosal lymphocytes and eosinophils was found during an experimental RV16 cold in a study using a combined group of asthmatic and nonasthmatic subjects [7]. However, it remains to be established whether such lower airway inflammation is disease specific or whether it equally occurs in nonatopic nonasthmatic subjects.

In this study, the null-hypothesis, that RV infection in healthy subjects does not result in worsening of airway responsiveness or demonstrable lower airway inflammation, was tested. Therefore, the dose/response curve to inhaled methacholine and the degree of mucosal inflammation in bronchial biopsy specimens before and after experimental RV16 infection in a carefully selected group of nonatopic nonasthmatic subjects was investigated.

Materials and methods

Subjects

Twelve nonsmoking, nonatopic, nonasthmatic healthy subjects were recruited (table 1). They had a

Table 1. – Characteristics of subjects

Patient no.	Sex	Age yrs	FEV_1[#] % pred	$PC_{20}FEV_1$[¶] $mg \cdot mL^{-1}$	Cold score[+]	Antibody titre[§] pre/post	Culture[f] days -2/3/6
1	F	21	99.3	>16	3	<1/32	neg/neg/neg
2	M	20	110.3	>16	9	1/128	neg/RV16/RV16
3	F	20	108.5	>16	2	2/128	neg/RV16/RV16
4	M	24	104.5	>16	6	<1/1	neg/RV16/RV16
5	F	21	105.8	>16	4	1/8	neg/RV16/RV16
6	M	22	97.9	>16	5	1/4	neg/RV16/RV16
7	M	20	100.1	13	1	<1/8	neg/neg/neg
8	M	23	107.5	>16	1	<1/8	neg/RV16/RV16
9	M	19	107.1	>16	3	1/8	neg/neg/neg
10	M	21	87.1	>16	2	2/2	neg/neg/neg
11	M	20	99.9	>16	2	1/4	neg/RV16/RV16
12	M	27	97.9	>16	5	<1/8	neg/RV16/RV16

FEV_1: forced expiratory volume in one second; PC_{20} FEV_1: provocation concentration causing a 20% fall in FEV_1; M: male; F: female. [#]: FEV_1 % from predicted; [¶]: airway responsiveness to methacholine measured at baseline; [+]: total cold score at the time point with the highest score (between days 0–5); [§]: titre (circulating antibodies against RV16) before and 4 weeks after inoculation (*e.g.* pre/post=1:1/1:4=1/4); [f]: rhinovirus culture of nasal washing fluid.

baseline lung function (spirometry) within the normal range (forced expiratory volume in one second (FEV_1) >80% predicted), and normal responsiveness to inhaled methacholine (provocative concentration causing 20% fall in FEV_1 (PC_{20}) >8 $mg \cdot mL^{-1}$) [8]. Their nonatopic status was determined by negative skin-prick tests to 16 common aero-allergen extracts (ALK Abelló, Nieuwegein, the Netherlands). The subjects had low titres of circulating antibodies specific to RV16 using a RV16 serum neutralisation assay ($\leqslant$1:2 serum dilution against 20–25×50% tissue culture infective dose ($TCID_{50}$)). The study was approved by the Medical Ethics Committee of the Leiden University Medical Center, Leiden, the Netherlands, and the subjects gave their written, informed consent.

Design

Experimental RV infection was performed by RV16 inoculation on 2 consecutive days (days 0 and 1). Methacholine challenges were performed on days -3, 5 and 14, to obtain the degree of airway sensitivity and maximal airway narrowing. Blood samples were drawn on days -2, 3, 6 and 27, and nasal washes were carried out on days -2, 3 and 6. Bronchial biopsies were taken by fibreoptic bronchoscopy on days -2 and 6.

Rhinovirus-16 inoculation

The RV16 inoculum was obtained from the same strain and stock as used in previous experiments in humans *in vivo* [5, 6]. A total dose of $0.6–24 \times 10^4$ $TCID_{50}$ diluted in 3 mL Hanks′ balanced salt solution (HBSS) with 0.5% (weight/volume (w/v)) gelatin was administered to each subject according to a previously described procedure [5, 6]. Confirmation of RV16 infection was established by an at least four-fold increase in virus-specific neutralising antibody titre in serum and/or by recovery of the virus from the nasal washes. Possible intercurrent respiratory infections were excluded [5, 6]. In addition, the subjects scored their cold symptoms three-times daily on a four-point scale [5]. The highest cold score after infection is presented in table 1.

Leucocyte counts in peripheral blood

Before and on days 3 and 6 after virus inoculation, differential leucocyte counts were assessed by automated blood count analysis (Technicon H1; Technicon, Tarrytown, NY, USA).

Airway responsiveness

Standardised methacholine challenge tests were performed according to the 2-min tidal breathing method [8]. The response was measured by FEV_1 and the more sensitive partial flow/volume curves (flow when 40% of the forced vital capacity remains to be expired (V'_{40p})) [5, 8]. Airway sensitivity was determined by the provocative concentration of methacholine causing a 10% fall in FEV_1 ($PC_{10}FEV_1$) and 40% fall in V'_{40p} ($PC_{40}V'_{40p}$). Maximal responses ($MFEV_1$ and MV'_{40p}) were calculated by averaging the consecutive points on the dose-response plateau or, in absence of a plateau, by taking the highest data point [6].

Bronchoscopy and biopsy processing

Fibreoptic bronchoscopy was performed according to a standardised and validated protocol [9]. Six biopsy specimens were taken at (sub)segmental level. Three biopsies were immediately fixed in phosphate buffered saline (PBS) buffered formalin 10% (v/v) and three were embedded in ornithyl carbamyltransferase (OCT) medium (Miles Inc. Diagnostics Division, Elkhart, USA) and snap-frozen in isopentane cooled by iced carbon dioxide [9]. The formaline-fixed biopsies were embedded in paraffin

and stored until further processing. The snap-frozen samples were stored in airtight containers at -70°C.

Immunohistochemical staining of bronchial biopsy specimens

Cell markers. Immunohistochemical staining on paraffin embedded tissue was performed on 3 μm thick biopsy sections. Cell-type specific antibodies against CD3, CD4, CD8, CD68, tryptase (AA1) and neutrophil elastase were obtained from DAKO (Glostrup, Denmark), and the antibody against eosinophils (EG2) was purchased from Pharmacia (Uppsala, Sweden). Antigen expression was demonstrated with appropriate dilutions of the primary antibodies, followed by a secondary biotinylated antibody and a tertiary complex of streptavidin-biotin conjugated to horseradish peroxidase (SABC/HRP). 3-amino-9-ethyl-carbazole (AEC) was used as a chromogen. The horseradish peroxidase conjugated antimouse EnVision system (DAKO) was used for the detection of CD4, with NovaRED (Vector, Burlingame, CA, USA) as the chromogen. The sections were counterstained with Mayer's haematoxyline (Klinipath, Duiven, the Netherlands). For negative controls, the primary antibody was omitted from this procedure [9].

Intercellular adhesion molecule-1. Since studies in asthma have demonstrated that bronchial mucosal ICAM-1 is upregulated by RV infection [10], staining for ICAM-1 was performed on 4 μm sections of the snap-frozen tissue using monoclonal anti-CD54 as primary antibody (clone MEM-111; Monosan, Uden, the Netherlands) [10].

Analysis of bronchial biopsies

Cell markers. Digital images from the stained sections were obtained using a three-chip colour camera (1.732×10^6 pixels; 1320×992 μm^2; 3×256 grey values) (Zeiss Vision KS-400 system; Kontron/Zeiss, Weesp, the Netherlands). Fully-automated or point-interactive (CD3+ and CD4+ cells in epithelium) cell counts were performed in the epithelium and in the lamina propria by a validated method [9]. Positively stained cells were expressed as the number of cells·0.1 mm^{-2}.

Intercellular adhesion molecule-1. Staining of ICAM-1 in the epithelium was analysed semiquantitatively, using a four-point scale for intensity of the staining (0=absent, 1=weak, 2=medium, 3=intense) [10]. Variability of the intra-observer scoring of the semiquantitative ICAM-1 staining, expressed as weighted kappa (κw), was satisfactory (κw>0.7).

Statistical analysis

$PC_{10}FEV_1$, $PC_{40}V'_{40p}$, and cell counts were log-transformed before statistical analysis. Paired t-tests were applied for comparing cell counts and functional data before and after infection. The Wilcoxon signed-ranks test was applied to test for differences in ICAM-1 staining. Relationships between outcome parameters were investigated using Spearman correlation tests. A p-value of $\leqslant0.05$ was considered statistically significant.

Results

RV16 infection was confirmed in all subjects, except patient 10 who was excluded from analysis. Patient 1 dropped out on day 6 after the virus inoculation, because of lignocaine-associated adverse effects during the second bronchoscopy. No other respiratory viruses were detected in any of the nasal washings (table 1). The common cold symptom score increased significantly after infection compared with before (mean±SEM) (day -3: 0.91±0.34; postinfection: 3.73±0.73; p=0.003). The differential leucocyte counts in peripheral blood showed a significant decrease in lymphocyte numbers on day 3, as compared with day -2 (day -2: 35.5±1.4; day 3: 29.9±1.3%; p=0.006), with a subsequent significant increase back to baseline value on day 6 (35.9±1.6%; p=0.0008). The neutrophil counts in peripheral blood, however, did not change significantly during RV16 infection (days -2, 3 and 6: 51.6±1.7, 55.3±1.6, 51.3±1.5%; p>0.08).

Airway responsiveness

During the study, there were no significant changes in baseline FEV_1 as compared to the values at entry of the study (p>0.1). After infection, there were no significant changes in $PC_{10}FEV_1$ and $PC_{40}V'_{40p}$ (p>0.1). However, the maximal response to methacholine, as measured by both $MFEV_1$ and MV'_{40p}, increased significantly 5 days after virus infection as compared with before. At day 14, both $MFEV_1$ and MV'_{40p} were not significantly different from baseline values (p>0.1) (fig. 1).

Bronchial biopsies

Cell markers. There were no significant changes in the numbers of EG2-, elastase-, AA1-, CD68-, CD3-, CD4-, and CD8-positive cells after RV infection as compared to before, both in epithelium (p>0.4) and in the lamina propria (p>0.2) (tables 2 and 3). However, there was a significant and positive correlation between the change in CD3+ cell numbers in the lamina propria and the change in maximal airway narrowing as measured by MV'_{40p} (between days -3 and 5: r_s=0.65, p=0.03) (fig. 2).

Intercellular adhesion molecule-1. RV16 infection was associated with a significant increase in ICAM-1 expression of the bronchial epithelium (scores 0, 1, 2 and 3: preRV16: 0, 37.5, 62.5 and 0%; postRV16: 0, 0, 62.5 and 37.5%; p=0.048).

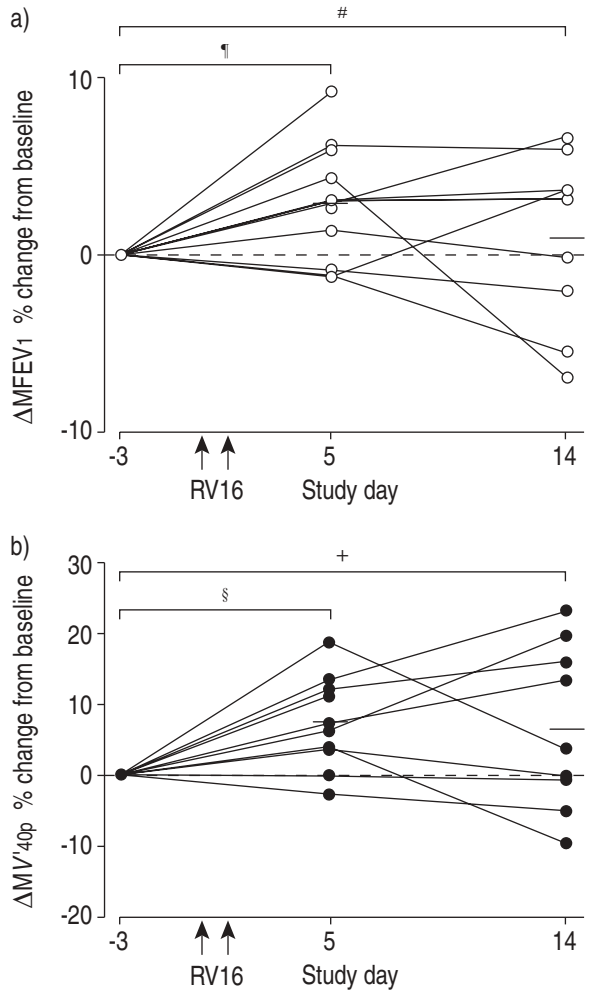

Fig. 1. – Change in maximal airway narrowing to methacholine for each individual subject as measured by a) maximal forced expiratory volume in one second ($MFEV_1$) and b) maximal flow when 40% of the forced expiratory volume remains to be expired (MV'_{40p}), on days 5 and 14 postinfection (% change from baseline day -3). RV: rhinovirus; Bar: mean value. #: p=0.6; ¶: p=0.014; +: p=0.1; §: p=0.004.

Discussion

The results of this study show a small, but consistent, effect of experimental RV16 infection on maximal airway narrowing to methacholine in nonatopic, nonasthmatic volunteers. There was a significant increase in ICAM-1 expression in the bronchial epithelium. Even though there were no significant changes in bronchial inflammatory cell counts as such, it appeared that the changes in CD3+ cells in the bronchial lamina propria occurring after RV16 infection were positively correlated with the changes in maximal airway narrowing. These data indicate that a common cold leads to physiological and cellular changes in the intrapulmonary airways, even in normal subjects.

There are very few comparative data on the relationship between the physiological and inflammatory outcomes of RV infection in nonasthmatic healthy controls. This is of fundamental importance in order to assess the disease-specific mechanisms of virus-induced asthma exacerbations [11]. Firstly, when considering the physiological changes in normal subjects, the observation of an increase in maximal airway narrowing extends previous findings as obtained by experimental RV infection in asthmatics [6]. Secondly, systemic effects, such as the fall in peripheral lymphocytes, do occur in RV-infected normal subjects, as has been observed in asthmatics [6, 7]. However, at the level of histopathology in

Table 2. – Cell markers in bronchial lamina propria

	Lamina propria		
	PreRV16	PostRV16	p-value#
EG2	0.61 (3.7)	0.49 (0.4)	0.3
Elastase	7.46 (4.5)	7.93 (6.5)	0.4
AA1	7.95 (27.3)	6.64 (19.2)	0.8
CD68	11.68 (13.1)	6.54 (10.8)	0.6
CD3	49.28 (35.4)	45.33 (34.8)	0.2
CD4	23.34 (25.6)	18.66 (19.7)	0.3
CD8	21.46 (31.3)	22.08 (21.7)	0.8

Data are presented as number of cells·0.1 mm^{-2} and are shown as median (interquartile range). RV: rhinovirus. #: p-values refer to paired t-tests on log-transformed data.

Table 3. – Cell markers in bronchial epithelium

	Epithelium		
	preRV16	postRV16	p-value#
EG2	0 (0)	0 (0.5)	0.5
Elastase	0 (8.0)	1.06 (2.3)	0.6
AA1	0 (1.7)	0 (1.7)	0.5
CD68	1.25 (2.0)	0 (5.6)	0.9
CD3	31.94 (16.5)	25.93 (9.7)	0.6
CD4	2.74 (4.9)	5.61 (5.3)	0.4
CD8	55.22 (28.1)	48.62 (40.7)	0.9

Data are presented as number of cells·0.1 mm^{-2} and are shown as median (interquartile range). RV: rhinovirus. #: p-values refer to paired t-tests on log-transformed data.

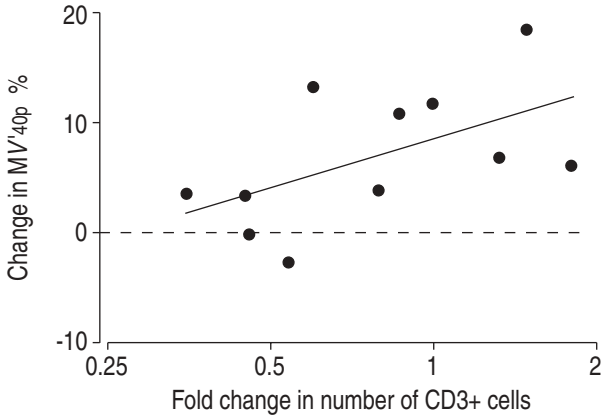

Fig. 2. – Relationship between the change in maximal airway narrowing measured by % fall in the maximal flow when 40% of the forced expiratory volume remains to be expired (MV'_{40p}) (between days -3 and 5) and the change in CD3+ cells in the lamina propria. r_s=0.65, p=0.03.

the intrapulmonary airways, normal subjects seem to behave differently from asthmatics [7, 12], since significant increases in airway infiltrative cells were not observed.

All the study procedures were carried out according to validated protocols. The sample size of 12 subjects was carefully chosen, especially concerning the analysis of intrapulmonary cell counts. With the current analysis of 11 subjects, the statistical power to detect a two-fold change in, for example, mucosal eosinophils or PC_{20}, was 0.77 and 0.85, respectively [8, 9]. Objective confirmation of infection with RV16 was established by a rise in antibody titre and/or positive virus culture. In this study, in nonatopic normal subjects, the cold scores were relatively low, especially when compared with the studies by GRÜNBERG and coworkers [10, 12], in which the same virus batch was used. Apparently, nonatopic healthy subjects have less severe cold symptoms than asthmatics, even though this is still a controversial issue [13, 14].

How can these data be interpreted in normal subjects? In this study, both the physiological and cellular findings are indicative of intrapulmonary effects of RV infection in nonasthmatic subjects. The increase in maximal airway narrowing *per se* is likely to reflect small airway involvement [15]. This might be associated with effects of RV on microvascular leakage leading to airway wall swelling [16], or direct effects on airway smooth muscle [17, 18]. It has been suggested that infection of the bronchial epithelial cells in the lower airways by RV [19, 20] may be enhanced by the virus-induced upregulation of ICAM-1 [21]. Indeed, the present findings confirm such increased epithelial ICAM-1 expression during RV infection in healthy subjects similar to the observations in asthmatic subjects [10]. Release of various mediators by infected epithelial and other cells [22] can attract inflammatory cells, such as eosinophils, neutrophils and T-lymphocytes, thereby enhancing intrapulmonary inflammation. An increase in CD3+ T-cells in the epithelium or the lamina propria in normal subjects, as has been observed in asthma [7, 12], was not observed. However, the correlation between the changes in maximal response and those in T-cell counts supports a possible inflammatory origin of physiological changes. Bronchial eosinophilic infiltration after RV has been controversial in asthma [7, 12]. The absence of an increase in eosinophil counts in normal subjects is in agreement with the current authors' recent observations in asthma [12]. However, a virus-induced increase in the activity of eosinophils in normal subjects, as has been demonstrated in asthmatics by Grünberg *et al.* [23], who showed elevated sputum ECP levels after RV infection in asthmatics, cannot be excluded.

In conclusion, rhinovirus infection leads to systemic and intrapulmonary effects even in nonatopic, nonasthmatic subjects. However, changes in airway responsiveness and bronchial biopsies are certainly limited. The authors speculate that any differences in response to a common cold between normal and asthmatic subjects might be explained by host factors, such as pre-existing allergic inflammation and/or airway hyperresponsiveness [24], thereby potentially facilitating the infection, intrapulmonary inflammation, and clinical worsening in pre-existing disease.

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