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Allergen challenge-induced acute exudation of IL-8, ECP and α_2-macroglobulin in human rhinovirus-induced common colds

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ABSTRACT: Rhinovirus infections cause exacerbations of eosinophilic airway disease. The acute effects of allergen-challenge on nasal interleukin-8 (IL-8), eosinophil cationic protein (ECP), and α_2-macroglobulin were examined in atopic subjects with common cold symptoms.

Twenty-three patients with seasonal allergic rhinitis were inoculated with human rhinovirus 16 outside the pollen season. Diluent and allergen challenges, followed by nasal lavages, were carried out about 3 months before and 4 days after virus inoculation.

Seventeen patients developed significant common cold symptoms with increased nasal lavage fluid levels of α_2-macroglobulin, IL-8, and ECP at baseline ($p<0.001$–0.05 *versus* before inoculation), and were further increased by allergen challenge ($p<0.001$–0.05); IL-8 and ECP levels were correlated ($r=0.63$, $p<0.001$). Before inoculation, the six patients who later did not develop common cold symptoms had high levels of IL-8 and myeloperoxidase (MPO), and exhibited strong allergen-induced plasma exudation responses (α_2-macroglobulin). After inoculation, IL-8 and ECP did not increase in these symptomless subjects.

In conclusion, high nasal interleukin-8 and myeloperoxidase levels and exudative hyperresponsiveness may protect against infection. The association between nasal interleukin-8 and eosinophil cationic protein in common cold, particularly that observed in nasal lavage fluids after allergen-induced acute exudation of plasma, suggests the involvement of interleukin-8 in exacerbation of airway mucosal eosinophil activity.
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Upper respiratory tract infections induced by rhinoviruses and coronaviruses are recognized as a cause of exacerbations of eosinophilic airway disease. The interest is particularly focused on the role of common cold in episodes of asthma in children and adults [1, 2]. Common colds cause nasal mucosal output of a different spectrum of cytokines to that seen in allergic conditions [3]. Rhinovirus infection-induced increased levels of interleukin (IL)-8, with reputed neutrophil chemotactic and activating properties [4], has recently been associated with virus-provoked asthma exacerbations [5–7].

It has become increasingly evident that virus-provoked airway disease may also involve eosinophilic activity. GAROFALO *et al.* [8] and others [9, 10] have thus observed high nasopharyngeal levels of eosinophil cationic protein (ECP) during acute respiratory syncytial virus (RSV) infection in children with bronchiolitis and common cold-induced bronchial hyperresponsiveness has been demonstrated in atopic subjects [11]. CALHOUN *et al.* [12] observed an increase in eosinophils in bronchoalveolar lavage (BAL) fluids obtained 48 h after segmental bronchial allergen challenge that was greater in rhinovirus-infected atopic subjects than in either uninfected atopic subjects or infected nonatopic subjects. THOMAS *et al.* [13] further observed a trend towards increased mucosal eosinophils in asthmatic subjects at the height of rhinovirus inoculation-induced nasal infection. However, it was also reported that rhinovirus infection in atopic individuals caused no significant change in the number of eosinophils in nasal mucosal biopsies [14].

There appears to be an association between local IL-8 levels and airway eosinophil activity. Although their data were statistically insignificant, DOUGLASS *et al.* [15] reported a "marked eosinophil infiltrate" in response to topical nasal IL-8 challenge in three atopic donors of nasal secretions. Further, IL-8 was increased in BAL fluids obtained from asthmatic subjects [16] and nasal allergen challenge in patients with allergic rhinitis caused a late phase increase in nasal surface IL-8 levels and eosinophil numbers [17]. GRÜNBERG *et al.* [18], who examined samples of induced sputum in atopic asthma, reported that rhinovirus inoculation-evoked infection approximately doubled the sputum levels of IL-8. Simultaneously, there was a 1.6-fold increase in mean sputum ECP. However, no changes in sputum eosinophil numbers were detected, and a small increase in mean ECP levels was also recorded in control subjects "inoculated" with diluent [18]. *In vitro* observations have added the possibilities that IL-8 might induce eosinophil chemotaxis [19, 20] and that eosinophils might produce and release IL-8 [16].

ZHU *et al*. [7] noted in passing that subjects who did not develop infection at rhinovirus inoculation happened to exhibit abnormally high nasal lavage fluid levels of IL-8 prior to the inoculation. It may further be deduced from the data reported by GRÜNBERG *et al*. [5] that subjects developing severe rhinovirus inoculation-induced infection had lower mean nasal surface levels of IL-8 than those who only showed mild symptoms of infection. If these data are confirmed, it may be hypothesized that airway mucosal output of IL-8 somehow reflects a capacity to resist common cold infections.

This study explores the possibility of an association between IL-8 and eosinophil activation in rhinovirus-inoculation-associated common cold symptoms in atopic subjects *in vivo*. Owing to the controlled methodology that can be applied, the present focus is on effects involving the nasal airway mucosa. The approach has been to carry out allergen challenges before and after human rhinovirus 16 inoculation in atopic subjects without active eosinophilic allergic disease (subjects with seasonal allergic rhinitis outside the season) and to determine nasal lavage fluid levels of IL-8, ECP and α_2-macroglobulin both at baseline and in the acute plasma exudation phase after topical allergen challenge. The inducement and determination of luminal entry of the plasma protein α_2-macroglobulin is of interest because this macromolecule is known to bind cytokines and other inflammatory factors [21, 22] and may thus bring tissue IL-8 and ECP, if present in the extracellular matrix of the mucosa [23], to the airway surface. The induced levels of α_2-macroglobulin also reflect the exudative responsiveness of the airway mucosa, which may be increased in allergic and infectious inflammatory disease conditions [24, 25]. This study also aimed to determine whether subjects who are high nasal producers of IL-8 exhibit a reduced tendency to show common cold symptoms after rhinovirus inoculation.

Materials and methods

Study design

Nasal inoculation with human rhinovirus 16 was performed out of season in 23 patients with seasonal allergic rhinitis. Nasal diluent and allergen challenges (see below) were carried out before and on day 4 after inoculation and were followed by nasal lavages. Nasal lavage fluid levels of ECP, IL-8 and α_2-macroglobulin were measured.

Patients

Twenty-four subjects of both sexes and aged between 18 and 50 yrs were recruited for the study. Inclusion criteria were a history of seasonal allergic rhinitis (verified by skin prick test for birch and/or timothy pollen allergen), no history of chronic airway disease, no infectious nasal disease during the 2 months preceding each allergen challenge series, a negative skin prick test for perennial allergen and no history of smoking. Exclusion criteria were nasal polyps, septal deviation of clinical significance, known impairment of liver and kidney function, pregnancy or nursing, drug treatment and ongoing desensitization therapy.

Nasal rhinovirus inoculation

Human rhinovirus 16 was obtained and cultured according to the guidelines described previously [26]. Human rhinovirus 16 (10^5–10^6 tissue culture infective dose 50 ($TCID_{50}$)·mL^{-1}) was diluted 1:20 in phosphate-buffered saline at 4C to a concentration of 5×10^3–5×10^4 $TCID_{50}$·mL^{-1}. With the subjects in a supine and extended neck position, 0.25 mL of this solution was administered to each nasal cavity (study day 0). A further 100 μL of the solution was administered to each nasal cavity using a spray device.

Determination of infection

In previous virus inoculation studies, this group [24] and others [5] have noticed that seropositive, infected subjects may be free from symptoms. The present study focused on common colds with clear symptoms. Hence, subjects with and without common cold were distinguished exclusively by symptoms, which were scored and entered in diaries by the subjects before and once daily for 5 days after inoculation: nasal blockage, sneezes, rhinorrhea, muscle ache, fever, headache and sore throat were each scored on a four-point scale, *i.e*. 0 for no symptoms, 1 for mild symptoms, 2 for moderate symptoms and 3 for severe symptoms. The criteria for the development of common cold were a total diary score of >12 points and a convincing progress of the diary score from the second to fifth day after inoculation, *i.e.* a 4-point increase for at least two consecutive days. By focusing only on common cold symptoms, but with a maintained demand regarding increment and progression of these symptoms, the score is a modification of that suggested by BEARE and REED [27]. Furthermore, as an adjunct, the subjects were asked about their own opinion of whether or not they had developed a common cold.

Nasal allergen challenges and lavages

Approximately 3 months before and at day 4 after inoculation, nasal challenges with diluent and allergen (10^3 and 10^4 standard quantity units (SQ-U)) were carried out as single actuations with 20-min intervals using a nasal spray device delivering 100 μL per actuation. Nasal lavages were carried out 10 min after each diluent and allergen challenge using a nasal pool device containing 15 mL of isotonic saline [28]. The lavage fluids were kept in the nasal cavity for 10 min. Nasal lavage fluid levels of IL-8, ECP and α_2-macroglobulin were determined. In addition, levels of myeloperoxidase (MPO) were determined in nasal lavages obtained after diluent challenge before inoculation. The recovered lavage fluid was centrifuged ($105 \times g$, 10 min, 4°C) end aliquots were prepared from the supernatants and frozen (-30°C) for later analysis. (In addition, two 30-s isotonic saline lavages were carried out before the above challenge and lavage series in order to remove solutes that might have accumulated on the mucosa for an unknown period.)

Analysis

The nasal lavage fluid levels of α_2-macroglobulin were measured using a radioimmunoassay (RIA) sensitive to 8 ng·mL^{-1}. Rabbit anti-human α_2-macroglobulin (Dakopatts, Copenhagen, Denmark) was used as antiserum and human serum (Behringwerke Diagnostica, Marburg, Germany) as standard. Human α_2-macroglobulin (Cappel-Organon Teknika, Turnhout, Belgium) was iodinated using the lactoperoxidase method. Tracer and standard or sample was mixed with antiserum before adding goat anti-rabbit antiserum (Astra Draco, Lund, Sweden). The bound fraction was measured using a gamma counter. The intra- and inter-assay coefficients of variation were between 3.8–6.0% and 3.1–7.2%, respectively.

The nasal lavage fluid levels of IL-8 were measured using a commercially available enzyme-linked immunosorbent assay (ELISA; R&D Systems Europe, London, UK). The nasal lavage fluid levels of ECP were measured using a commercially available FEIA (Pharmacia-Upjohn, Uppsala, Sweden) and those of MPO were measured using a commercially available RIA (Pharmacia-Upjohn).

Statistics

Differences in nasal indices between observations before and after nasal virus inoculation were examined using the Wilcoxon signed rank test. Differences between subjects who remained asymptomatic and those who developed common cold symptoms were examined using the Mann–Whitney U-test. A p-value of <0.05 was considered significant. Data are presented as means±SEM.

Table 1. – Diary symptom scores, subjective impression of whether a common cold had developed during the study period and overall grouping

Subject No.	Diary symptom score*						Subjective cold	Grouping
	Before	1	2	3	4	Total		
4	1	12	13	12	8	45	Yes	Common cold
14	1	10	12	13	9	44	Yes	Common cold
22	4	13	17	8	2	40	Yes	Common cold
20	3	7	9	8	7	31	Yes	Common cold
5	1	4	8	10	4	26	Yes	Common cold
8	2	6	7	7	6	26	Yes	Common cold
1	3	8	8	5	4	25	Yes	Common cold
2	3	4	9	8	3	24	Yes	Common cold
3	0	7	9	7	1	24	Yes	Common cold
19	0	2	7	8	5	22	Yes	Common cold
10	1	5	11	3	2	21	Yes	Common cold
16	0	6	8	3	3	20	Yes	Common cold
11	1	3	3	6	6	18	Yes	Common cold
23	1	6	5	2	3	16	Yes	Common cold
7	0	4	3	3	4	14	No	Healthy
18	0	2	6	4	2	14	Yes	Common cold
9	0	1	4	4	4	13	Yes	Common cold
13	0	4	6	2	1	13	Yes	Common cold
17	2	2	4	3	3	12	No	Healthy
12	4	3	2	1	1	7	No	Healthy
15	1	4	1	1	1	7	No	Healthy
21	0	1	1	0	0	2	No	Healthy
6	0	0	0	0	0	0	No	Healthy

Data have been ranked by order of the sum of diary symptom score entered after inoculation. Subject number 7 scored more than 12 points but did not meet the criteria of a progression of the symptom score, *i.e.* a 4-point increase on 2 consecutive days, after inoculation. *: Score before and day 1–4 after inoculation and total score day 1–4.

Results

Twenty-four patients were recruited for the study. One patient failed to attend at the time of inoculation and, therefore, 23 patients were inoculated. Seventeen patients developed common cold symptoms, whereas six patients remained asymptomatic (table 1). The cumulative levels of IL-8 in lavage fluids obtained after diluent challenge were greater in patients who later were to remain asymptomatic after nasal virus inoculation than in those who later were to develop common cold symptoms ($p<0.01$, Mann–Whitney U-test) (figs. 1 and 2). The levels of IL-8 in these baseline lavages correlated significantly with the levels of MPO (r_s=

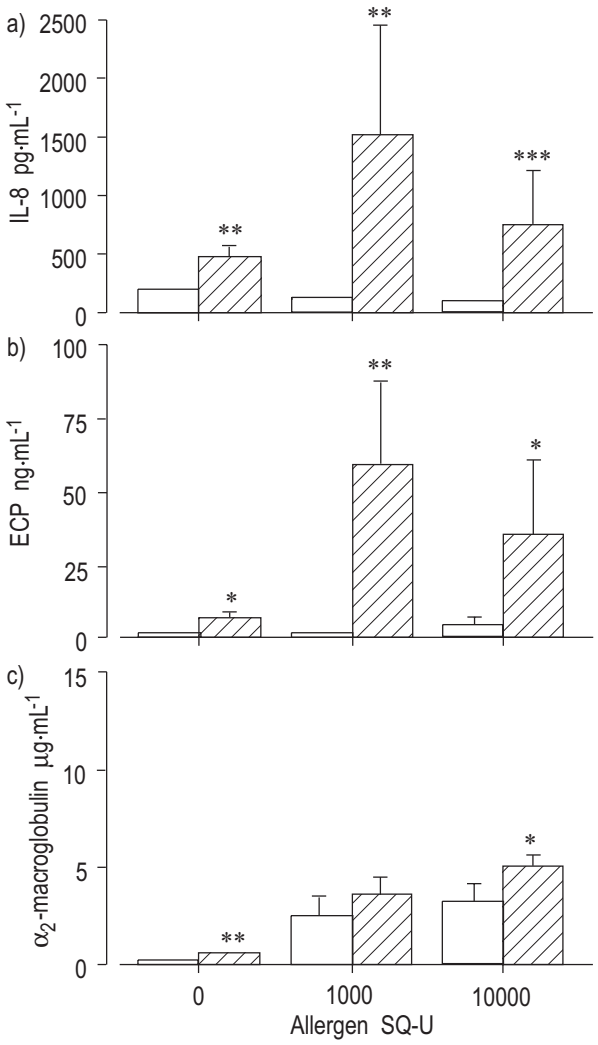

Fig. 1. – Levels of a) interleukin-8 (IL-8), b) eosinophil cationic protein (ECP) and c) α_2-macroglobulin in nasal lavage fluids obtained after challenge with diluent and allergen well before (□) and on day 4 after (▨) human rhinovirus 16 inoculation in the 17 subjects who developed common cold symptoms after inoculation. The development of common cold symptoms was associated with elevated mucosal output of IL-8, ECP and α_2-macroglobulin at baseline (see diluent challenge data before and after inoculation). These levels were further increased on exudative allergen challenge (a, b) in these subjects with common cold symptoms. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ (Wilcoxon signed rank test *versus* before inoculation).

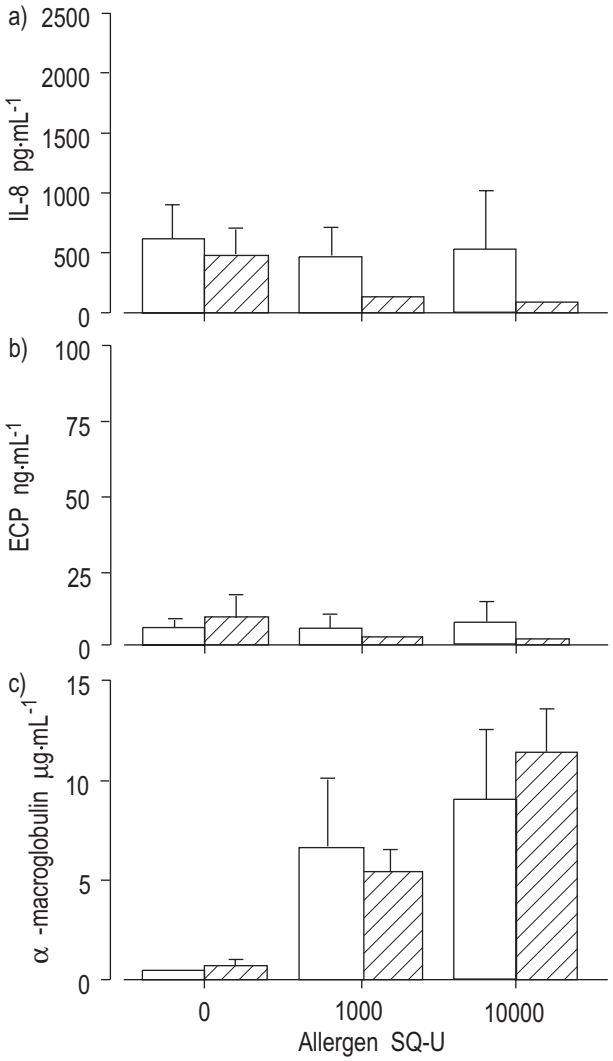

Fig. 2. – Levels of a) interleukin-8 (IL-8), b) eosinophil cationic protein (ECP) and c) α_2-macroglobulin in nasal lavage fluids obtained after challenge with diluent and allergen well before (□) and on day 4 after (▨) human rhinovirus 16 inoculation in the six subjects who remained asymptomatic after inoculation. There were no changes in the mucosal output of IL-8, ECP or α_2-macroglobulin in response to diluent and allergen challenges before and after inoculation in these asymptomatic subjects (Wilcoxon signed rank test *versus* before inoculation).

0.514. $p<0.05$). Furthermore, the initial allergen-challenge (10^3 SQ-U) resulted in greater luminal entry of IL-8 in these patients than in those who later were to develop common cold ($p<0.05$, Mann–Whitney U-test).

The nasal lavage fluids obtained by the 10-min saline lavage performed after diluent challenge demonstrated increased levels of IL-8 ($p<0.01$), ECP ($p<0.05$) and α_2-macroglobulin ($p<0.01$) in patients who had developed common cold symptoms after rhinovirus inoculation (Wilcoxon signed rank test *versus* before inoculation) (fig. 1). These changes were not seen in patients who remained symptomless (fig. 2).

Allergen challenge produced dose-dependent nasal symptoms and caused acute nasal mucosal exudation of bulk plasma (α_2-macroglobulin) before as well as after rhinovirus inoculation (figs. 1c and 2c). Before inoculation, the six subjects that did not develop common cold symptoms had greater exudative responses to allergen challenge than those who later developed common cold symptoms after rhinovirus inoculation ($p<0.05$, Mann–Whitney U-test) (figs. 1c and 2c). In patients who developed common cold symptoms, the plasma exudation response to allergen was greater than the response before inoculation ($p<0.05$, Wilcoxon signed rank test) (fig. 1c). In subjects who remained asymptomatic, the exudative response to allergen did not change (fig. 2c).

Table 2. – Individual levels of eosinophil cationic protein (ECP) and interleukin-8 (IL-8) in lavages obtained after the initial allergen challenge (10^3 SQ-U) which was carried out before as well as after nasal rhinovirus inoculation in the 17 patients who developed common cold symptoms

Subject No.	ECP µg·mL^{-1}		IL-8 pg·mL^{-1}	
	Before	After	Before	After
1	3.1	255.0	50.1	1451.7
2	0	26.7	99.4	383.8
3	3.1	7.7	197.4	191.2
4	0	8.4	56.9	707.4
5	0	0	101.5	212.9
6	0	6.9	67.7	506.2
7	0	4.1	278.0	139.1
8	4.1	460.0	319.7	16390.5
9	0	21.9	91.0	278.2
10	0	0	42.2	375.7
11	0	2.2	206.2	365.0
12	4.0	9.3	152.9	720.3
13	0	0	74.3	94.2
14	0	0	129.4	281.6
15	2.1	0	254.8	88.3
16	0	19.4	57.5	2334.3
17	0	156.0	59.6	1092.4

The exudative allergen challenge caused acute luminal entry of IL-8 ($p<0.01$) as well as ECP ($p<0.01$) after inoculation when these subjects experienced common cold symptoms. SQ-U: standard quantity units.

In subjects who developed common cold symptoms, the exudative allergen challenge caused acute luminal entry of IL-8 ($p<0.01$–0.001) (fig. 1a, table 2) as well as ECP ($p<0.05$–0.01) (fig. 1b, table 2) (Wilcoxon signed rank test *versus* before inoculation). This response was not seen before virus inoculation (fig. 1a and 1b). There was also a significant positive correlation between levels of IL-8 and ECP in lavage fluids obtained from subjects with common cold symptoms after allergen challenge ($r=0.63$, $p<0.001$) (fig. 3). In subjects who remained symptom free, exudative allergen challenges failed to produce mucosal output of IL-8 and ECP either before or after nasal virus inoculation (fig. 2a and b).

Discussion

The present study of experimental rhinovirus inoculation in atopic individuals without active allergic disease has demonstrated the coappearance of IL-8 and ECP in the nasal mucosal lavage fluids in subjects exhibiting common cold symptoms. This association was particularly evident in acutely induced exudation conditions, under which bulk plasma would be moved through the extracellular matrix of the lamina propria and paracellularly across the epithelium into the airway lumen. The present observations are of interest in view of the role of rhinovirus infections in

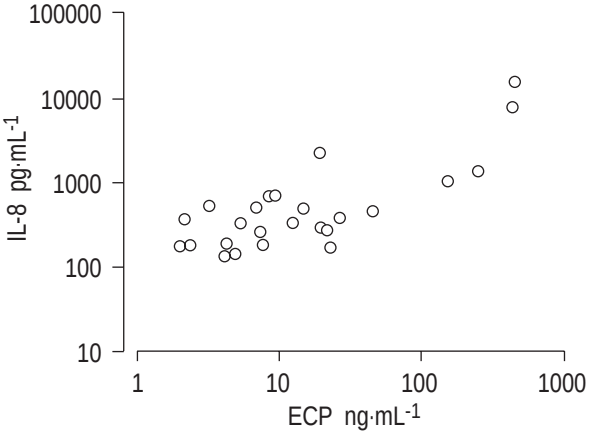

Fig. 3. – Correlation of nasal lavage fluid levels of interleukin-8 (IL-8) and eosinophil cationic protein (ECP) in lavages obtained after allergen challenge in subjects who developed common cold symptoms. The correlation between these two cell products was significant ($r=0.63$, $p< 0.001$). (Nine out of a total of 34 paired observations are not shown as they comprise levels of IL-8 or ECP that are lower than can be depicted in this logarithmic graph.)

provocations of eosinophilic airway disease. However, high IL-8 levels and strong exudative responses were seen before inoculation in subjects who remained asymptomatic after rhinovirus inoculation. These data may reflect dual inflammatory and defence roles of IL-8 and plasma exudation. Further, a potentially important association between mucosal output of IL-8 and development of eosinophilic airway inflammation is being resolved.

Plasma exudation is a general feature of inflammatory airway diseases, occurring irrespective of the mechanisms driving the inflammation. The elevated levels of α_2-macroglobulin in the present baseline nasal lavage liquids (obtained after diluent challenge) agree with previous demonstrations of increases in plasma proteins in common cold infections [29, 30] and support the present identification of active disease by symptom scores. α_2-Macroglobulin is a large molecule (920 kDa) and its appearance on the mucosal surface reflects the nature of the plasma exudation process, which involves the extravasation of unsieved plasma into the lamina propria and the further movement of this bulk plasma across the basement membrane and between intact but yielding epithelial cells [23]. (As long as plasma exudation processes are ongoing the extracellular matrix of the mucosal tissue will be endowed with the peptides and proteins of a bulk plasma exudate, which thus may determine much of the biologically active milieu of the inflamed nasal mucosa *in vivo*.) The luminal entry of bulk plasma is also a major elimination route for the exudate in airways, where the epithelial lining is structurally uncompromised and functions as a normally tight absorption barrier [23]. It was previously demonstrated that a normal mucosal absorption barrier is maintained during the exudative phase of coronavirus inoculation-induced common cold [24]. The present observation of a potentiation of allergen challenge-induced nasal lavage fluid levels of α_2-macroglobulin may thus reflect microvascular "exudative hyperresponsiveness" in common cold [24], rather than any increased penetration of allergens into the mucosa.

Both IL-8 and ECP were significantly increased on day 4 of symptomatic common cold in this study. The data also demonstrate that these two molecules were not likely to be present in the airway tissue of asymptomatic individuals since they responded to allergen challenge with acute exudation of α_2-macroglobulin, but without IL-8 and ECP. The coappearance of these two molecules in the nasal airways was particularly evident during the induced plasma exudation processes. Plasma contains many binding proteins. Of these, α_2-macroglobulin has attracted attention because it may bind, carry and target cytokines and it is also known to bind ECP [21, 22]. The markedly increased luminal entry of IL-8 and ECP that occurred during the present 10-min periods of plasma exudation may thus be explained by a carrier effect of the extravasated plasma and by the presence of IL-8 and ECP molecules in the extracellular matrix of the mucosa. This possibility is consistent with the present observation that most of the IL-8 and ECP were observed in the lumen after the first exudative challenge, whilst the subsequent allergen challenge, which produced greater exudation of α_2-macroglobulin, caused lower luminal levels of IL-8 and ECP which, thus, could have been depleted in the mucosal tissue by the initial exudation response. The brief period elapsing between challenge and sampling in this study makes it unlikely that allergen-induced recruitment and activation of eosinophils contributed to the elevated ECP levels. Hence, the IL-8 and the ECP, determined a few minutes after allergen challenge may largely reflect the availability of these two molecules in the extracellular matrix of the mucosal tissue.

The marked increase in ECP levels in this study suggests the possibility that the common cold, in part through the inducement of IL-8, attracts and activates eosinophils. Increased traffic of eosinophils to the nasal airway may thus occur, although one previous report could not detect elevated numbers of eosinophil cells in the nasal mucosa in rhinovirus infection [14]. Eosinophil cytolysis appears to be a major mode of activation of airway muc-osal tissue eosinophils *in vivo* [31–33]. If the present ECP levels reflect cytolysis of local eosinophils, owing to the dual nature of this process (both activation and fate) one should not expect to find a simple correlation between eosinophil numbers and ECP levels [31]. Incidentally, as stated exclusively in the results section of the report by FRAENKEL *et al.* [14], these authors have observed a correlation between the severity of common cold symptoms and tissue eosinophil numbers in rhinovirus-infected subjects. This result is of particular interest in relation to the present study where since symptoms were used to define subjects with a common cold.

The present experiments demonstrated that the six subjects who remained asymptomatic after inoculation had significantly (threefold) higher nasal lavage fluid levels of IL-8, as determined well in advance of the inoculation. The IL-8 levels correlated with nasal lavage fluid levels of MPO (this study), suggesting that local neutrophil activity, potentially evoked by IL-8 [6], may contribute to an infection-resistant airway mucosa. Whichever mechanisms are involved, the present *in vivo* data do not support a general validity of recent *in vitro* work [34] demonstrating potent proviral infection properties of IL-8, through antagonism of antiviral properties of interferon-α. Further studies assessing the consistency of high IL-8 production

(this study assessed one timepoint) and delineating actual or surrogate roles of IL-8 in protection against common cold infections seem warranted. The present observations with regard to mucosal effects of allergen challenge suggest the additional possibility that the exudative responsiveness of the nasal mucosa is a factor contributing to the outcome of rhinovirus inoculation. In line with the proposal that mucosal exudation of bulk plasma should primarily be considered a first-line airway defence process [35], the exudative responsiveness (to allergen challenge) was greater in those subjects who a few months later, at the time of inoculation, failed to show common cold symptoms.

In summary, human rhinovirus inoculation-evoked infections in atopic subjects without active allergic disease was shown to be associated with nasal coexudation of interleukin-8 and eosinophil cationic protein, particularly in the acute (20-min) exudation phase induced by allergen challenge. The present data thus suggest the possibility that the plasma exudation process, specifically the microvascular–lamina propria–epithelial crossing of α_2-macroglobulin, may bring interleukin-8 and eosinophil cationic protein to the mucosal surface and, hence, that these latter two molecules are enriched in the extracellular space of the nasal mucosa in atopic subjects exhibiting symptoms of common cold. This study further provides evidence that subjects presenting with high nasal interleukin-8 and myeloperoxidase levels and a strong exudative responsiveness may be protected against rhinovirus-induced infection. Finally, the coappearance of interleukin-8 and eosinophil cationic protein in the present subjects with common cold suggests a mechanism by which rhinovirus infections may provoke eosinophilic airway disease.

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