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Elevated levels of myeloperoxidase, pro-inflammatory cytokines and chemokines in naturally acquired upper respiratory tract infections

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Abstract Upper respiratory tract infections (URTIs) are characterised by a neutrophilic mucosal infiltration. The purpose of this study was to investigate the time course of release of the cytokines/chemokines interleukins (IL) IL-1β, IL-1ra, tumour necrosis factor-α (TNF-α), IL-6, IL-8, interferon-γ (IFN-γ) and monocyte chemotactic protein (MCP-1), soluble intercellular adhesion molecule-1 (sICAM-1), myeloperoxidase (MPO) and bradykinin in nasal secretions of patients with a naturally acquired URTI. A total of 117 healthy adult volunteers were recruited for baseline nasal lavages, 39 of whom developed URTI symptoms within 6 months and returned to our centre within 48 h. Lavages were performed daily during the symptomatic period and 3 weeks thereafter, with symptoms no longer present. Compared to baseline, significantly elevated concentrations of total protein, bradykinin, IL-1β, TNF-α, IL-6, IL-8, MCP-1, IFN-γ, MPO and sICAM-1 were detected in nasal lavage fluids of symptomatic patients, whereas IL-1ra remained unaltered. All studied variables reached baseline 3 weeks after the URTI. Naturally acquired URTI represent a limited, neutrophilic inflammatory reaction, orchestrated by the release of pro-inflammatory cytokines and chemokines.

Keywords Chemokines · Interleukin-1 receptor agonist · Naturally acquired upper respiratory tract infection · Pro-inflammatory cytokines · sICAM-1 or soluble intercellular adhesion molecule-1

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Introduction

An upper respiratory tract infection (URTI) is characterised by rhinorrhoea, nasal congestion and sneezing, sometimes accompanied by fever, a sore throat and malaise. It is the most frequent infectious disease in man and accounts for a large economical burden due to an enormous loss in productivity and high medical costs [29]. About 80% of the nasal infections are caused by viruses, the majority being rhinoviruses [24]. Other species frequently causing acute infectious rhinitis are coronaviruses, (para)influenza viruses, respiratory syncytial virus and adenoviruses [9, 29]. There are over 100 serotypes of rhinoviruses, of which 90% infect their host by binding to epithelial intercellular adhesion molecule-1 (ICAM-1), which belongs to the immunoglobulin superfamily and is constitutively expressed on respiratory epithelial cells [26]. According to Winther et al. [32], an URTI starts in the nasopharynx, involving the pharynx before the nose.

Despite it's frequent occurrence, there is still little detailed knowledge on the pathogenesis of URTIs, especially on the aspect of cellular migration and defence. It is currently believed that the symptoms of an URTI are the result of the host inflammatory response to the virus rather than the direct viral cytotoxic effect [18, 31]. Support for this concept comes from studies in both natural and experimental rhinovirus infections demonstrating an accumulation of nasal kinins, an increase in serum proteins such as albumin and IgG in nasal secretions and a predominantly neutrophilic cellular infiltrate [14, 18, 31]. This virus induced inflammatory response is mediated by a spectrum of inflammatory cytokines. For example, the pro-inflammatory cytokines interleukins (IL) IL-6 and IL-8 were shown to be released by respiratory synctial virus-infected human pulmonary epithelial cells in vitro [2]. Furthermore, increased IL-1β concentrations were reported in nasal lavage fluids from adults experimentally infected with rhinovirus [22]. Finally, Noah and coworkers [19] observed elevated levels of IL-1β, IL-6,

IL-8 and tumour necrosis factor-α (TNF-α) compared to baseline values in nasal lavages during an acute viral rhinitis in children.

In vitro studies have demonstrated the ability of pro-inflammatory cytokines to strongly induce adhesion molecule expression and transendothelial migration of inflammatory cells and to activate various cell populations [10]. Therefore these cytokines may play a central role in the pathogenesis of URTI as regulators of chemotaxis, proliferation and activators of inflammatory cells. In order to obtain more insight into the mechanism underlying the neutrophilic mucosal infiltration during an URTI and, in particular, to determine the role of the pro-inflammatory cytokines in this process, we investigated the time course of cytokine and chemokine release during a naturally acquired URTI.

Subjects and methods

From December 1994 to August 1995, 117 healthy adult volunteers with a history of a common cold in the pursuing months were recruited. In this group, an allergy or other forms of rhinitis were excluded based on a careful medical history and skin prick test for common aero-allergens. Furthermore, systemic or topical drug therapy, except for contraceptives, was an exclusion criterion. Two baseline nasal lavage samples were acquired within 5 days of entry into the study (samples 1 and 2). Volunteers were then instructed to contact the study centre within 48 h, if experiencing any common cold symptoms. 39 volunteers returned to the study centre with clinical symptoms conforming to an URTI. Subjects were considered suitable for continuation of the study, if they had a minimum of two out of six target nasal/pain common cold symptoms (headache, sinus pain, sore throat, muscle aches and pains, blocked nose, runny nose) with an overall severity 'moderate (2) or severe (3)', rated by the patient, using a four point categorical scale. Furthermore, subjects had to have pathological signs of URTI in terms of pharyngeal erythema, determined by clinical investigation. Patients returned to the study site on each consecutive day, at approximately the same time of day (+/– 2 h) for the 4-day symptomatic period in order to provide nasal lavage samples 3–6. A final nasal lavage was performed 3 weeks after the infectious episode (sample 7). All subjects gave their informed consents and the study was approved by the local Ethics Committee of the University of Düsseldorf.

Nasal lavage was performed using 5 ml of saline solution (0.9%) at room temperature instilled into each nostril with the subject in a seated position, with their head reclined at an angle of 45° and soft palate closed. After approximately 10 s, the lavage fluid was expelled into a plastic receptacle held on ice. The fluid was immediately centrifuged at 1200 rpm for 10 min at 4°C. The supernatant was frozen and stored at –80°C.

Cytokine concentrations in nasal lavage fluid were measured by commercial ELISA kits (R&D Systems, Minneapolis, USA). The cytokines measured and the observed test sensitivity for the respective ELISA kits were as follows: IL-1β (0.125 pg/ml), IL-1ra (31.3 pg/ml), IL-6 (0.156 pg/ml), IL-8 (31.3 pg/ml), TNF-α (0.5 pg/ml), IFN-γ (15.6 pg/ml), sICAM-1 (2.1 ng/ml), MCP-1 (15.6 pg/ml) and MPO (1.56 ng/ml). Bradykinin levels were analysed by an established and validated RIA technique (Dr. D. Proud, Johns Hopkins University, Baltimore, USA). Total protein was assessed by spectrophotometry.

Statistical analyses were carried out by the paired non parametric Kruskall-Wallis test to establish significant differences in central tendency between time points of measurement followed by the paired, non parametric Wilcoxon signed-rank test to compare data from the viral rhinitis episode and thereafter with mean baseline values. Correlations between mediator/cytokine concentrations in nasal lavages were determined by Spearman rank correlation coefficient analysis. Differences were considered significant at $P < 0.05$.

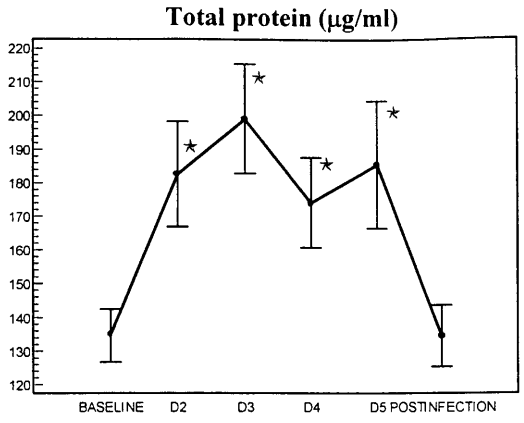
Results

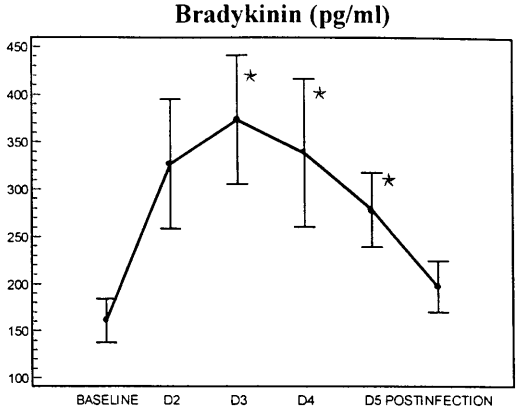
Out of the 117 included healthy individuals, 39 returned to the study centre with symptoms conforming to a common cold. The study population consisted of 17 females and 22 males with an average age of 27.5 years (range 20–48 years). At the final visit, 3 weeks after the end of the URTI, when 38 patients returned, a complete resolution of cold symptoms was observed in 26 subjects. The remaining 12 patients still showed pharyngeal erythema consistent with a new or persisting URTI and were excluded from all analyses involving the 3 weeks post-treatment lavage values.

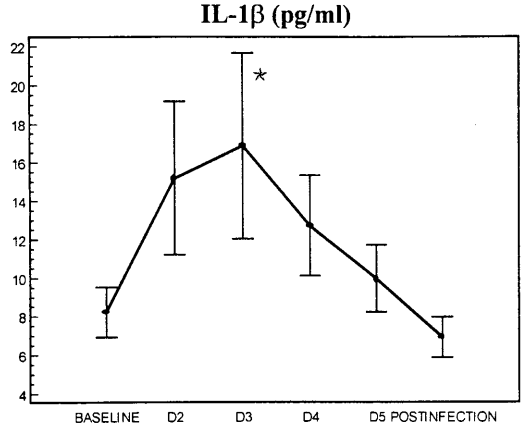
In order to allow for analysis, the values below the ELISA detection limits (i.e. pre-and post-infection concentrations of IFN-γ and sICAM-1) were replaced by half of the detection level of the corresponding parameter. As there was no significant difference between the two baseline nasal lavage samples for any parameter, the mean of both samples is referred to as baseline in the following.

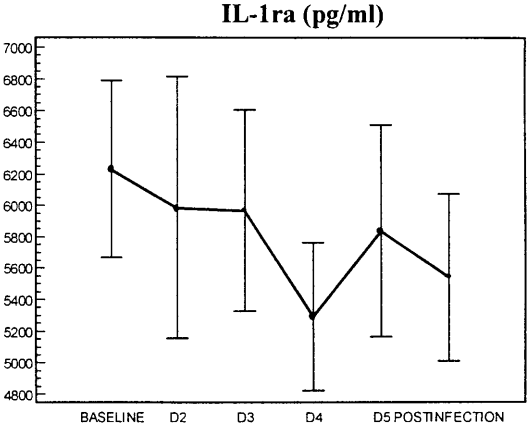
The mean concentrations of all parameters studied at baseline, during the naturally acquired common cold and 3 weeks after the end of the infectious episode are depicted in Fig.1. Compared to baseline, significantly elevated total protein levels were observed during acute illness. This disease-induced increase in total protein concentration renders this parameter unsuitable as a reference for further calculations of concentrations of mediators/cytokines in nasal secretions, as proposed by Reynolds [23]. However, no significant alterations in IL-1ra levels, the naturally occurring IL-1 receptor antagonist, were demonstrated during infection, thus excluding the possibility that increases in other mediators/cytokines are based on increases in the amount of nasal secretions collected. In addition to bradykinin, significantly elevated concentrations of the pro-inflammatory cytokines TNF-α, IL-6 and IL-1β were found in nasal lavage fluids during the days of rhinitis symptoms compared to baseline levels, whereas the concentration of IL-1ra did not show any significant alterations due to infection. Furthermore, significant increases in both chemokines IL-8 and MCP-1 and the mediator MPO were observed during acute URTI. The former significantly correlated to the pro-inflammatory cytokine levels at each time point during the days of rhinitis (Table1), while for MPO, a highly significant correlation with IL-8 levels was observed in that period (day 2: $P < 0.001$, r: 0.72; day 3: $P < 0.001$, r: 0.77; day 4: $P < 0.001$, r: 0.76; day 5: $P < 0.001$, r: 0.72).

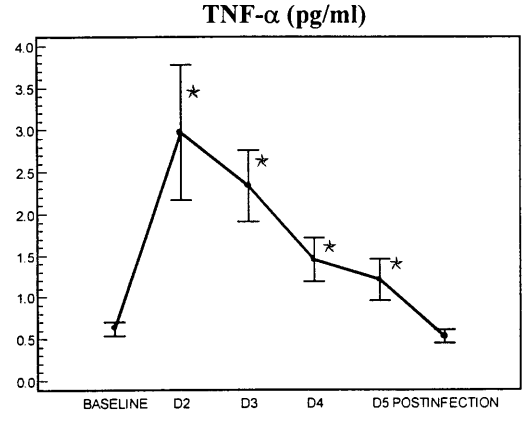
Even though the concentrations of the regulatory cytokine IFN-γ as well as sICAM-1 remained undetectable in all samples at baseline and post-infection, both significantly increased at the onset of common cold symptoms. Moreover, a statistically significant correlation between sICAM-1 and the pro-inflammatory cytokine levels was

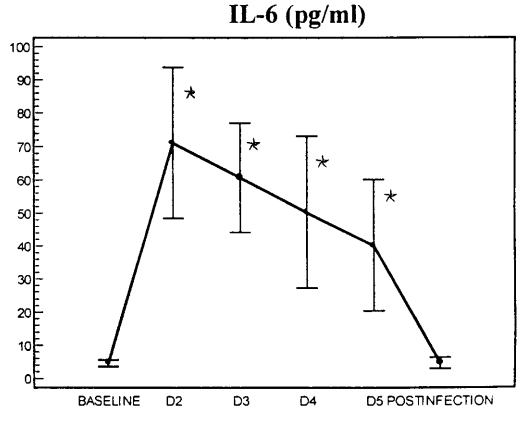

Total protein (μg/ml)
220
210
200
190
180
170
160
150
140
130
120
BASELINE
D2
D3
D4
D5 POSTINFECTION

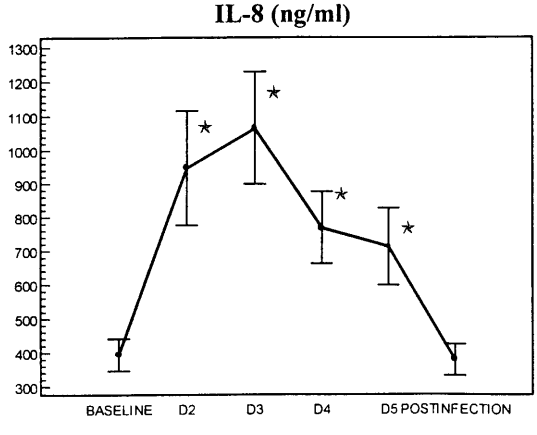

Bradykinin (pg/ml)
450
400
350
300
250
200
150
100
BASELINE
D2
D3
D4
D5 POSTINFECTION

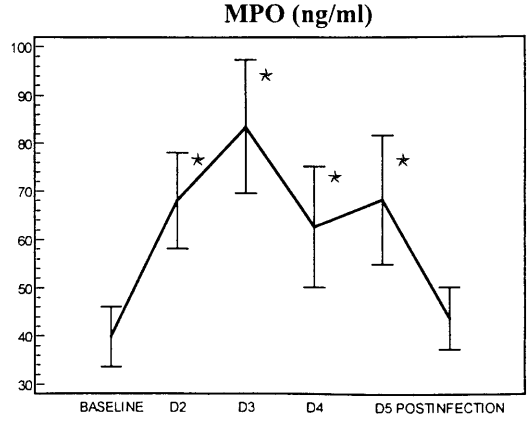

IL-1β (pg/ml)
22
20
18
16
14
12
10
8
6
4
BASELINE
D2
D3
D4
D5 POSTINFECTION

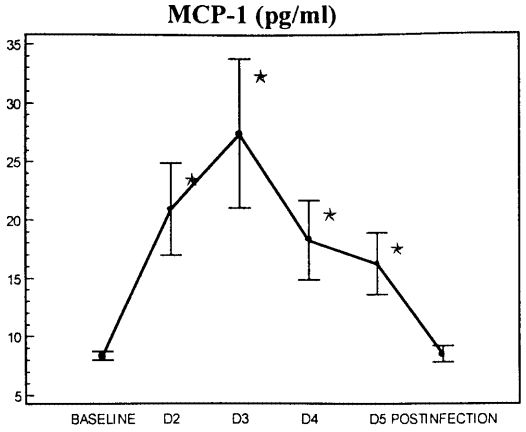

IL-1ra (pg/ml)
7000
6800
6600
6400
6200
6000
5800
5600
5400
5200
5000
4800
BASELINE
D2
D3
D4
D5 POSTINFECTION

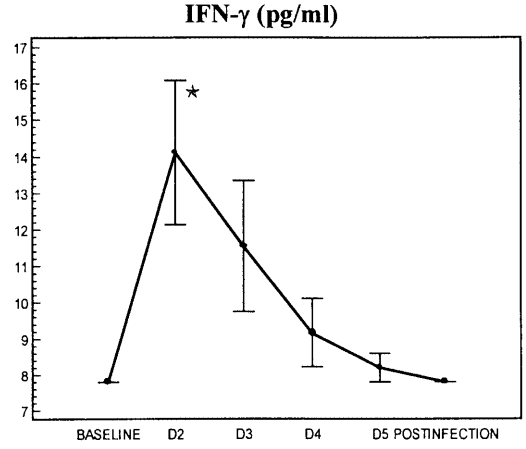

TNF-α (pg/ml)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
BASELINE
D2
D3
D4
D5 POSTINFECTION


IL-6 (pg/ml)
100
90
80
70
60
50
40
30
20
10
0
BASELINE
D2
D3
D4
D5 POSTINFECTION


IL-8 (ng/ml)
1300
1200
1100
1000
900
800
700
600
500
400
300
BASELINE
D2
D3
D4
D5 POSTINFECTION


MPO (ng/ml)
100
90
80
70
60
50
40
30
BASELINE
D2
D3
D4
D5 POSTINFECTION





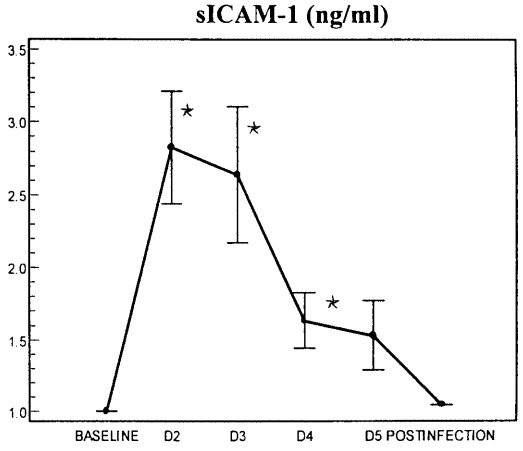


Fig. 1 Time course of cytokine, chemokine and mediator concentrations in nasal secretions during a naturally acquired common cold. Patients (n = 39) were monitored twice before the URTI (mean baseline), daily during the symptomatic period (D2–D5), and once 3 weeks after the infectious episode (post-infection). Data are given as mean and standard error of means, $P < 0.05$* compared to baseline (non-parametric Wilcoxon signed-rank test)

Table 1 Correlation analysis for the different pro-inflammatory cytokines and chemokines or sICAM-1 during a symptomatic naturally acquired common cold (Spearman rank correlation)

		Day 2		Day 3		Day 4		Day 5	
		P	*R*	*P*	*R*	*P*	*R*	*P*	*R*
IL-8	IL-6	< 0.001	0.78	< 0.001	0.75	< 0.001	0.74	< 0.001	0.77
	TNF-α	< 0.001	0.78	< 0.001	0.75	< 0.001	0.76	< 0.01	0.47
	IL-1	< 0.001	0.75	< 0.001	0.77	< 0.001	0.80	< 0.001	0.65
MCP-1	IL-6	< 0.001	0.52	< 0.001	0.66	< 0.001	0.56	< 0.001	0.68
	TNF-α	< 0.001	0.69	< 0.001	0.63	< 0.001	0.56	< 0.01	0.51
	IL-1	< 0.05	0.35	< 0.001	0.56	< 0.05	0.31	< 0.01	0.43
sICAM-1	IL-6	< 0.001	0.64	< 0.001	0.73	< 0.001	0.59	< 0.001	0.51
	TNF-α	< 0.001	0.71	< 0.001	0.73	< 0.01	0.46	< 0.01	0.48
	IL-1	< 0.01	0.44	< 0.001	0.63	Not applicable			

observed during the symptomatic period (Table 1). Finally, the levels of all variables studied returned to baseline in the post-infectious symptom-free period.

Discussion

This study demonstrates the complex and time-limited upregulation of bradykinin, pro-inflammatory cytokines, the chemokines IL-8 and MCP-1, MPO, IFN-γ and sICAM in nasal secretions due to a naturally acquired URTI. In contrast, the concentration of IL-1ra remained unaltered by the infection. Since we intended to focus on pro-inflammatory cytokines as a possible pivot on which the pathogenesis of an URTI hinges, we decided to monitor the inflammatory process throughout the study period by referring to two parameters already shown to increase in nasal secretions during a viral rhinitis, namely total protein and bradykinin [18].

In our study group, we observed a rapid rise in total protein concentrations in nasal lavage fluids during the initial stage of infection. Igarashi and coworkers [14] observed, in experimentally induced rhinovirus type 39 infections, a similar rise in total protein concentrations and

demonstrated the vascular origin of this early increase. Our findings thus substantiate the early increased permeability of the nasal vessels under natural conditions, which presumably plays an important role in the pathogenesis of common colds, as it initiates the resolution of the infection by washing out pathogens and by facilitating the transport of inflammatory cells to the site of infection. In line with Naclerio et al. [18] studying rhinovirus type 39 challenged subjects, we showed a significant rise in bradykinin concentrations in nasal lavage fluids compared to baseline pre-infection values. This rise paralleled the increase in total protein concentrations. Bradykinin is a potent inflammatory mediator which causes vasodilatation, increased vascular permeability and stimulates pain and glandular secretion via neuronal reflexes [18, 29]. When administered intranasally in healthy subjects, symptoms of nasal obstruction, rhinorrhoea and a sore throat are evoked suggesting that this mediator might contribute to common cold symptoms [21]. Indeed, Naclerio and coworkers [18] did show a correlation between increased kinin concentrations during an experimentally induced rhinovirus infection and common cold symptom severity.

Both, IL-6 and TNF-α concentrations rose significantly with the onset of symptoms, reached a plateau level over the ensuing symptomatic days and decreased to baseline concentrations after 3 weeks. The level of the second pro-inflammatory cytokine IL-1β, showed a strong trend towards a rise on day 2 of common cold symptoms compared to mean baseline values, but reached statistical significance on the 3rd symptomatic day only. On the contrary, the concentration of IL-1ra, which is found in high molecular excess in nasal secretions of healthy and as well as allergic subjects [3], remained unaltered during the viral infectious episode. Since IL-1ra binds to IL-1 receptors without effecting a biological response, constant IL-1ra concentrations and increased levels of IL-1β would favour the pro-inflammatory process. These findings of elevated pro-inflammatory cytokine levels during symptomatic colds are in accordance with previous observations during natural and experimental viral respiratory tract infections [19, 22, 33]. The source of these pro-inflammatory cytokines is, at least partly, the respiratory epithelium. In fact, during a naturally acquired viral URTI, Noah and coworkers [19] were able to demonstrate increased transcripts for IL-1β and IL-6 in epithelial cells, and the protein concentrations of these cytokines in supernatants were markedly elevated compared to baseline.

Pro-inflammatory cytokines are potent biological factors that have pleiotropic functions. As mentioned previously, they are known to enhance the expression of adhesion molecules such as E-selectin or ICAM-1 on endothelial cells [25]. These molecules induce the recruitment and transendothelial migration of granulocytes and lymphocytes to the site of inflammation by interacting with their cognate receptors [24]. Moreover, pro-inflammatory cytokines also induce adhesion molecule expression on epithelial cells. In a recent in vitro study using nasal and bronchial epithelial cells, Sethi et al. [25] showed that not only the rhinovirus per se, but also IL-1β and TNF-α, released upon viral infection, could upregulate epithelial ICAM-1 expression. Our findings of significantly elevated concentrations of sICAM-1, in nasal lavage fluids during the infectious episode when compared to baseline indirectly support this upregulating effect. Furthermore, the finding of a close correlation between sICAM-1 and pro-inflammatory cytokine levels during the days of rhinitis symptoms favour their role in inducing epithelial adhesion receptor expression. The increased sICAM-1 levels are likely to be secondary to an upregulated epithelial ICAM-1 expression followed by shedding of the molecule from the cell surface into nasal secretions [20]. In contrast to cell-bound ICAM-1, the exact function of sICAM-1 is not clarified yet. Since approximately 90% of the rhinoviruses infect their host by binding to ICAM-1 expressed on the respiratory epithelial cell surface, sICAM-1 could prevent further cellular infection by binding to the virus in the secretions [26]. In fact, recombinant sICAM-1 has been shown to specifically inhibit human rhinovirus infection of HeLa cells in vitro [17]. Besides, sICAM-1 could serve as a marker of infection, as its concentration increases upon URTI.

In addition to inducing the expression of specific adhesion molecules, pro-inflammatory cytokines may also enhance the preferential migration of cell subsets by inducing the release of chemokines from various cells. In vitro studies have shown their ability to release IL-8 from epithelial- and endothelial cells [5, 27]. Indeed, we found significantly elevated concentrations of this latter chemokine and the neutrophil-derived mediator MPO compared to baseline during the entire symptomatic common cold period. IL-8 is a chemo-attractant cytokine of the CXC-family with preferential specificity for neutrophils [28]. It induces the expression of β2-integrins, namely LFA-1 and Mac-1, on neutrophils, which bind to the endothelium via ICAM-1 and mediate transendothelial migration [4, 28]. In addition, IL-8 is capable of activating recruited neutrophils resulting in the release of their granule content such as MPO. We observed a strong correlation between IL-8 and MPO lavage levels, suggesting that IL-8 acts as a chemotactic factor for neutrophils and that MPO reflects the activation of these cells. Such a finding is in accordance with Douglas and colleagues [11] who found a significant neutrophilic infiltrate in nasal smear and biopsy samples after IL-8 challenge. MPO and other neutrophilic enzymes have been shown to cause severe tissue damage. In fact, a direct relation between MPO concentrations and the severity of common cold symptoms has been observed in children with virus-induced asthma [28]. These correlations between IL-8, MPO and common cold symptom severity were recently confirmed by Turner et al. [30] who observed a direct relation between the magnitude of the rise in IL-8 and the severity of viral rhinitis symptoms in volunteers after rhinovirus type 23 inoculation.

Pro-inflammatory cytokines also stimulate the preferential migration of monocytes to the site of infection. Various in vitro studies have demonstrated the induction of MCP-1 gene expression and secretion by endothelial cells and monocytes upon IL-1β and TNF-α activation [8]. In

vivo, we observed significantly elevated MCP-1 concentrations compared to baseline throughout the entire symptomatic cold period. In addition, MCP-1 levels were found to directly correlate with all three pro-inflammatory cytokine levels on every day of the 4-day viral rhinitis episode. MCP-1 is a member of the CC-family of chemokines, which preferentially attracts and activates monocytes [16]. Matured to tissue macrophages, these cells are involved in antiviral immune responses like phagocytosis and the processing and presentation of viral antigens to T-lymphocytes. Interestingly, airway macrophages were found to specifically bind rhinoviruses in vitro via ICAM-1 ligation and secrete cytokines that have antiviral (IFN-α), but also pro-inflammatory (IL-1β, TNF-α) effects [12]. Based on these in vitro findings, one could hypothese that in addition to their capacity to rapidly dampen and eliminate viral infection, macrophages may also sustain and amplify the host inflammatory response, possibly leading to increased cold symptoms.

In order to activate the recruited macrophages and overcome the viral URTI, a strong cellular immune response has to be induced. Due to their activating and proliferative effects on lymphocytes, pro-inflammatory cytokines are also implicated here. Indeed, in vitro studies have demonstrated the induction of IL-2 receptor expression and the release of IL-2 by T-cells upon stimulation with IL-1β and TNF-α [7]. In the absence of IL-1, no immune response could be observed. Under the influence of IL-2, a T-helper-1 (Th1) lymphocyte response is induced. T-helper lymphocyte responses are classed on the basis of the cytokines produced and their functional effects. Th1 lymphocytes predominantly secrete IL-2 and IFN-γ to activate cellular immune responses, whereas Th2 lymphocytes are characterised by IL-4, IL-5, IL-6 and IL-10 synthesis to induce humoral immune responses [1]. Rhinoviruses per se have been found to induce a favourable environment for Th1 lymphocyte development, as they non-specifically activate peripheral blood T-lymphocytes and natural killer cells to release IFN-γ in vitro [13]. Moreover, CD8+ cytotoxic T-cells were reported to synthesise a T-helper-1 pattern of lymphokines [6]. In our study group of common cold subjects, we found elevated concentrations of IFN-γ at the onset of common cold symptoms indicating the polarisation of the immune response towards Th1-cells. IFN-γ exercises various antiviral activities [7, 15]. It modulates the immune response by promoting the differentiation of naïve helper T-cells towards T-helper-1-cells, essential for cell mediated immunity. In addition, it stimulates cytotoxic T-cell and natural killer cell functions, upregulates MHC class I and II expression, activates macrophages and enhances the expression of certain adhesion molecules on various cells. Interestingly, in contrast to uninfected epithelial cells, the expression of ICAM-1 on rhinovirus-infected cells was recently shown to be downregulated by IFN-γ in vitro [25]. This possibly reflects another strategy of IFN-γ to inhibit further rhinoviral spread.

Our results clearly demonstrate the importance of pro-inflammatory cytokines in the pathogenesis of a naturally acquired URTI. Firstly, they mediate selective chemotaxis and transmigration by inducing chemokine release and upregulating adhesion molecule expression. Secondly, they activate resident and recruited cells, which respond to this activation by releasing various cytokines. This way the inflammatory response is amplified and a Th1 cytokine environment is created that is necessary to overcome the common cold.

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