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Enhanced severity of virus associated lower respiratory tract disease in asthma patients may not be associated with delayed viral clearance and increased viral load in the upper respiratory tract

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Abstract

Background: Viral respiratory infections, particularly human rhinovirus (HRV) infections, are the most common cause of asthma exacerbation. HRV infections usually lead to more severe and longer duration of lower respiratory tract (LRT) symptoms in asthmatics than in otherwise healthy individuals. However, the exact mechanism by which viruses contribute to exacerbation of asthma is unknown.

Objectives: The main objective of our study was to investigate the relationship of the enhanced severity of LRT symptoms to viral dynamics or cytokine responses in the upper respiratory tract (URT).

Study design: Therefore, we conducted a longitudinal study in which asthmatics and healthy controls were followed during natural viral respiratory tract infections.

Results: Our study confirmed that viral respiratory tract infections caused more severe problems of the LRT in asthma patients as compared to healthy controls. However, for all subjects, the severity of LRT symptoms were not related to viral load or prolonged viral shedding in the URT. In addition, we did not detect differences in proinflammatory cytokines in the URT between asthmatics and controls.

Conclusion: Persistence of the virus, as well as viral load in the URT, may not be associated with the induction and/or persistence of asthmatic symptoms.

Keywords: Asthma; Exacerbation; Human rhinovirus (HRV); Respiratory virus dynamics

1. Introduction

Viral respiratory infections, particularly rhinovirus infections, are the most common cause of asthma exacerbation. Johnston et al. reported that viral infections were associated with 80–85% of asthma exacerbations in children (Johnston et al., 1995). Picornaviruses (human rhinoviruses (HRV) and enteroviruses) accounted for about 65% of these virus-induced asthma exacerbations. Also in adult asthma patients, respiratory virus infections are associated with the majority of exacerbations (Nicholson et al., 1993). Corne

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et al. observed that HRV infections usually lead to more severe symptoms and longer duration of lower respiratory tract (LRT) disease in asthmatics than in otherwise healthy individuals (Corne et al., 2002). However, the exact mechanism by which HRV cause exacerbations of asthma is unclear. One postulated mechanism is that the HRV-specific intracellular adhesion molecule 1 (ICAM-1) receptor is up-regulated in asthma patients, therefore increasing their susceptibility to HRV infection (Manolitsas et al., 1994; Yamaya et al., 1999). Another mechanism is suggested by the increased susceptibility to HRV infection of bronchial epithelial cells from asthmatic patients (Wark et al., 2005). The authors suggest that this indicates an abnormal innate immune response by these cells to infection by HRV, resulting in increased viral RNA expression and virus release from cells from asthmatics than from cells from healthy controls. The role of post-infectious immune responses was suggested by studies in patients with allergic rhinitis or asthma; those who had a high IFN-γ–IL-5 ratio (Th1 helper response) tended to have less severe symptoms after an HRV challenge, and shed less virus in the sputum, compared to those who had a low IFN-γ–IL-5 ratio (Th2-helper response) (Gern et al., 2000). The magnitude of the cytokine response (IL-8) in either the sputum or in the upper respiratory tract (nasal secretion), correlated with increased airway hyper-responsiveness (Gern et al., 2000; Grunberg et al., 1997b).

These data suggest that differences in the immune response may be associated with differences in viral replication and severity of illness. Based on these observations, we investigated the relationship between the severity of LRT symptoms observed in asthmatic patients and healthy volunteers, and the cytokine response and viral replication in the upper respiratory tract (URT).

2. Patients and methods

2.1. Subjects

This prospective study, from September 1998 through August 2000, was approved by the local ethical committee. We recruited 44 people with asthma, aged 18–45 years, from general practices. Asthmatics were diagnosed according to the Dutch asthma guidelines and showed a reversibility in peak expiratory flow (PEF) of $\geq$15% and/or forced expiratory flow in 1 s (FEV1) of >9% after administration of a β-agonist (Geijer et al., 2003). All patients were treated as usual with various combinations of inhalation therapy (corticosteroids, β-agonists and parasympathicolytics). All asthmatic subjects had a history of atopy. We also enrolled 44 healthy control subjects (controls were not matched to asthmatic subjects) who did not have a history of asthma, allergic rhinitis, eczema or atopy, nor did their family members. Written informed consent was obtained from participants.

2.2. Procedures

During one season, asthmatic and control subjects daily recorded URT and LRT signs and symptoms, rated from 1 (mild) to 3 (severe). URT symptoms that were scored were defined as runny, stuffy or itchy nose, sneezing, watery/sore eyes, sore throat, hoarse voice and headache. LRT symptoms that were scored included coughing, wheezing, difficult breathing and shortness of breath during effort (Johnston et al., 1995). Patients in the asthma group recorded peak flow each morning and afternoon (peak flow score), using their peak flow instrument (Mini Wright meter; Clement Clarke International London, UK) three times in succession, and recording the highest value. The median baseline score for a subject was the mean of the peak flow score in five consecutive time points around the baseline (week 8). Subjects were asked to contact the investigators if the URT symptoms score totaled 4 or more, and/or if the LRT symptoms score exceeded 5, resulting in a home visit within 48 h. Follow-up visits were performed at days 3–4, days 5–6, after 2 weeks and after 8 weeks (baseline). The duration of URT and LRT symptoms was determined by the number of days with a URT or LRT symptoms score of 1 above the individual baseline score, respectively. The severity of the URT and LRT symptoms was defined as the maximum of URT and LRT symptoms scores, respectively. During each visit a nasal wash (NW) was collected from each nostril as described previously (Grunberg et al., 1997b). Briefly, 10 ml of sterile saline was instilled in each nostril and collected in the same tube. The tube was mixed thoroughly and immediately placed on wet ice. One milliliter of NW was used for virus culture. The remainder was mixed with an equal volume of diluted sputolysin reagent (6.5 mM dithiothreitol in 100 mM phosphate buffer) (Calbiochem-Novabiochem Corporation, San Diego, CA). Both aliquots were then clarified by centrifugation ($1000 \times g$ for 10 min at room temperature). The remaining supernatant fluid was stored at −80 °C until further analysis.

2.3. Respiratory virus detection and quantitation

Conventional viral cultures were performed as described by van Elden et al. (2003). Basically, by inoculating HEP-2C, R-HELA, and tertiary monkey kidney (t-MK) cells (National Institute for Public Health and the Environment (RIVM; Bilthoven, The Netherlands) with 1 ml NW each for the detection of respiratory viruses (parainfluenza viruses 1–3, respiratory syncytial virus (RSV) A and B, influenza viruses, adenovirus and picornaviruses). The cultures were examined twice weekly for 10 days for the development of cytopathic effect (CPE). In CPE-positive cultures, virus was identified by immunofluorescence with monoclonal antibodies (Dako Imagen) for influenza viruses A and B, RSV A and B, adenovirus and parainfluenza viruses 1–3. HRV were distinguished from enteroviruses through acid-lability testing. In shell vial cultures an immunofluorescence test was per-

formed after 2 days of culture, usually before a cytopathic effect was noticed, using the above mentioned monoclonal antibodies (van Elden et al., 2003).

A real-time quantitative RT-PCR was performed on the stored supernatant fluids (nasal wash) for parainfluenza virus 1–4 and picornaviruses (van de Pol et al., 2007). A rhinovirus specific real-time PCR detected all HRV subtypes tested: 1a, 1b, 2–16, 18, 20–24, 26–30, 32–37, 39–67, 70–86, 88–100). Real-time PCR for influenza virus A and B, RSV A and B and human coronaviruses OC43 and 229E were performed as described previously (van Elden et al., 2004, 2003, 2001; Nijhuis et al., 2002). The number of viral RNA copies of influenza A and B, RSV A and B and rhinovirus in the clinical samples was determined by extrapolation from a standard curve generated by amplification of serial dilutions of electron microscopically (EM)-counted virus stocks. To estimate the quantity of human coronaviruses OC43 and 229E, for which no EM-counted virus stocks were available, viral copies were expressed as relative units (RU). Every amplification cycle represents a 2-fold increase in the number of viral RNA copies. The viral load was expressed as 2-fold increase per cycle relative to a baseline value of 2 copies at threshold cycle 36 ($RU = 2^{36\text{-threshold cycle}}$).

2.4. Assays for cytokines

IFN-γ, IL-6, IL-8 (CLB, Amsterdam, The Netherlands) and IL-5 (BioSource International, CA, USA) were measured using commercially available human cytokine ELISA kits according to the manufacturer's recommended protocol. The NW supernatants were assayed in duplicate and the results were expressed in pg/ml. The limits of sensitivity of these assays were as follows: IFN-γ, 1 pg/ml; IL-5, 4 pg/ml; IL-6, 0.2 pg/ml; and IL-8, 1 pg/ml.

2.5. Statistical analysis

Analyses were based only on the first episode of infection of a subject. Differences between asthmatic and control groups were analyzed by Mann–Whitney *U*-test (SPSS 11.0 for Windows). Comparisons of measures of infection and illness (viral load, symptom assessment and peak flow measurements) and cytokine levels between asthmatics and controls were done with the Wilcoxon signed rank test (SPSS 11.0 for Windows). Correlations were determined using the Spearman's rank correlation coefficient (SPSS 11.0 for Windows). An area under the curve (AUC) analysis including both virus load and duration of shedding was performed using the trapezium rule (Matthews et al., 1990). The AUC calculated from the data points from all individuals was then compared with means of the Mann–Whitney test (SPSS 11.0 for Windows).

Table 1
Subject characteristics

	Asthmatic group	Control group
Number of patients	14	14
Sex (male:female)	4:10	5:9
Age (median; range)	38.5 (26–44)	33.5 (21–44)
Smoking	2	4
Influenza vaccination	9	2

3. Results

3.1. Study population and episodes of viral respiratory infection

A total of 44 asthmatics and 44 control subjects were enrolled. Forty-three of these reported 57 episodes of respiratory tract illness. A respiratory virus was detected by PCR in 33 out of 57 (58%) episodes. Viral culture gave no added diagnostic value. There were 14 first episodes each of respiratory virus infection for analysis in asthmatics and controls. Patient characteristics are shown in Table 1.

No significant differences were seen between the number of respiratory tract illnesses caused by a respiratory virus in asthmatics (17/33, 52%) and controls (16/33, 48%) (Table 2). The majority of infections ($n = 20$) were caused by HRV.

Table 2
Virus isolation in nasal wash and corresponding symptoms in upper respiratory tract (URT) and lower respiratory tracts (LRT)

	Asthmatic group $n = 14$ (episodes analyzed)	Control group $n = 14$ (episodes analyzed)
Detected respiratory viruses	17 (14)	16 (14)
- *Rhinoviruses*	8 (6)	12 (10)
- *Influenza virus A/B*	5 (5)	1 (1)
- *RSV A/B*	1 (1)	1 (1)
- *Coronaviruses*	3 (2)	2 (2)
- *Parainfluenza viruses; Enteroviruses; Adenoviruses*	0	0
Days of viral shedding (median, range)	6 (1–16)	4 (1–9)
Viral load, copies/ml (median, range)	20732 (2–62270)	999 (8–262136)
Duration (days) of URT symptoms (range)	9 (5–19)	8 (1–17)
Severity of URT symptoms (range)	8 (4–9)	7 (2–9)
Duration (days) of LRT symptoms (range)	7 (3–17)	3.5 (0–8)[a]
Severity of LRT symptoms (range)	5 (0–9)	0.5 (0–5)[b]

[a] Asthmatic vs. control; $p = 0.03$ (Mann–Whitney *U*-test).
[b] Asthmatic vs. contro. $p = 0.02$ (Mann–Whitney *U*-test).

3.2. Symptoms of viral RTI

The duration and severity of URT symptoms were similar for both the asthmatic and the control group. However, the severity of LRT symptoms was greater in the asthmatics as compared to the controls ($p = 0.02$: Table 2). The duration of symptoms of the LRT was also significantly longer in asthmatic patients ($p = 0.03$). Subgroup analyses performed in the asthmatic population did not indicate significant clinical differences between HRV and other respiratory virus infections, however, the sample size was small.

3.3. Viral dynamics

Using a quantitative real-time PCR for the detection of respiratory viruses we did not find a significant difference between study groups for either the maximum levels of virus produced or the duration of detection of viral RNA (Table 2). In addition for all subjects, neither the peak virus load nor the duration of viral shedding correlated with the duration or severity of LRT symptoms. This lack of correlation was observed with the subgroup analysis limited to HRV infections. The area under the curve plotting virus load against duration of shedding was not statistically different between the study groups ($p = 0.45$). If the dynamics of the virus infections in asthma patients is compared to the peak flow reduction and the LRT symptoms, it seems clear that the

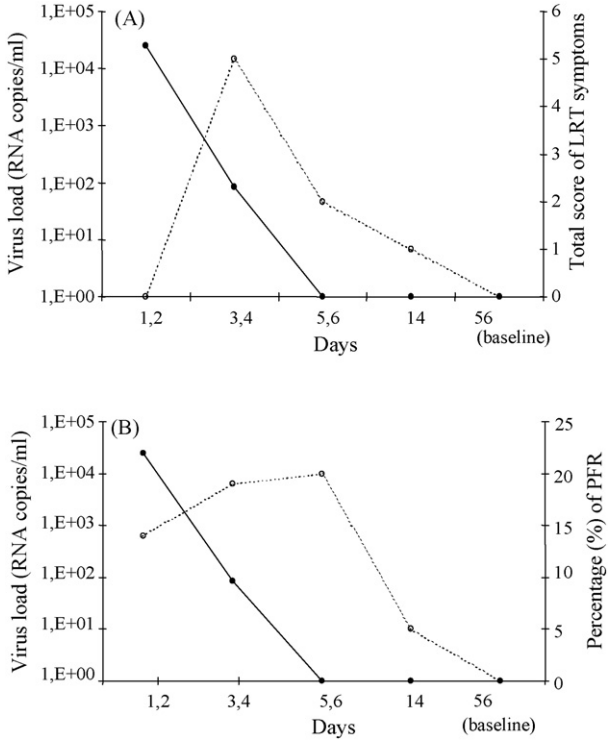

Fig. 1. (A) Median virus load (●) and lower respiratory tract (LRT) symptoms (◯) in asthmatic subjects ($n = 14$). (B) Median virus load (●) and peak flow reduction (PFR) (◯) in asthmatic subjects ($n = 14$). Reduction of peak flow is defined as percentage reduction from personal median baseline score.

Table 3
Median cytokine levels in nasal washes of asthmatic and control subjects

	Baseline		Days 1–2		Days 3–4		Days 5–6	
	Asthmatic	Control	Asthmatic	Control	Asthmatic	Control	Asthmatic	Control
IL-6 (pg/ml) (range)	0 (0–40)	0 (0–16)	12 (0–322)	0 (0–854)	107 (0–975)[a]	124 (0–502)[b]	70 (0–613)	42 (0–353)
IL-8 (pg/ml) (range)	272 (0–1611)	254 (6–4944)	445 (0–2398)	526 (0–31,536)	348 (130–2781)[a]	2921 (41–40,107)[b]	598 (272–3387)	477 (320–52,040)

[a] $p < 0.05$ in comparison to baseline (Wilcoxon signed rank test).
[b] $p < 0.01$ in comparison to baseline (Wilcoxon signed rank test).

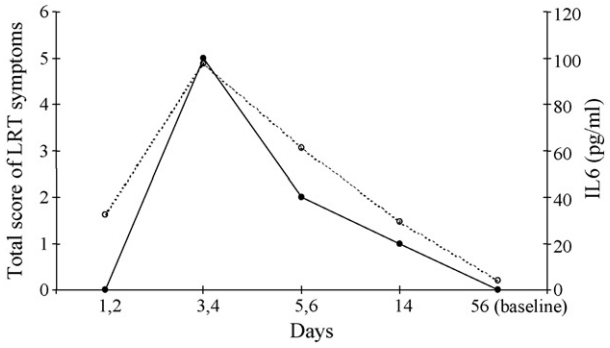

Fig. 2. Median lower respiratory tract (LRT) symptom score (●) and IL-6 concentrations (◯) in asthmatic subjects ($n = 14$).

actual viral infection in the upper respiratory tract precedes the peak flow reduction and LRT symptoms (Fig. 1).

3.4. Inflammatory cytokine response

NW levels of the proinflammatory cytokines IL-6 and IL-8 were significantly increased at days 3–4, as compared to baseline (Table 3). The levels of IL-6 paralleled the LRT symptoms and peak flow reduction and peaked at a time when virus load was declining (Fig. 2). No significant differences in the IL-6 or IL-8 response could be determined between both study groups during signs and symptoms of RTI. The same results were obtained when a subgroup analysis on just the HRV was performed. IL-5 and IFN-γ were not detected in NW of asthmatics and controls.

4. Discussion

The severity of the LRT symptoms in the asthmatics during naturally occurring RTI was greater than controls, confirming a similar study in patients infected with HRV (Corne et al., 2002). However, this difference did not correlate with differences in viral load, duration of viral shedding in the URT, or proinflammatory cytokines in the URT.

Recent studies on virus-related exacerbations of asthma have mainly focused on HRV infection, since HRV is the most frequently detected pathogen in this setting (Gern and Busse, 1999; Johnston et al., 1995; Nicholson et al., 1993). The severity of LRT symptoms in asthmatic patients infected with HRV has been attributed to Th2 dominance in asthma patients and an enhanced Th2-helper response (Papadopoulos et al., 2002; Gern et al., 2000; Konno et al., 1996). Experimental HRV infections suggest that the pattern of cytokine production in asthmatic patients may be responsible for exacerbations related to a HRV infection (Parry et al., 2000; Gern et al., 2000). We were unable to detect IFN-γ or IL-5 in the NW of subjects, which may be related to the presence of DTT in our samples, which blocks detection. This is in line with another observation that both cytokines could not be detected in sputum during acute respiratory virus infections (Pizzichini et al., 1998; Gern et al., 2000). IL-8, a potent chemoattractant for and activator of neutrophils, has also been implicated in virus-related exacerbations of asthma (Grunberg et al., 1997a; Noah et al., 1995; Johnston, 2000; Teran et al., 1997). Although we did see a statistically significant increase in IL-8 over baseline we did not observe a difference in intranasal IL-8 levels between the asthmatic group and the control group.

The limited study population, infrequent sampling and the variety of different viruses hamper interpretation of the results from our study. A subgroup analysis of HRV infections was not possible with the number of events that we observed. Furthermore, sampling of the URT may not reflect viral shedding and cytokine expression in the LRT.

The percentage of viruses detected in symptomatic asthmatics was approximately 60%, similar to previous studies using molecular diagnostics for HRV detection (Nicholson et al., 1993; van Elden et al., 2001). Studies to determine the mechanisms by which viruses cause lower airway disease are hampered by difficulties in sampling lower airways. It has been shown that HRVs can infect the LRT (Papadopoulos et al., 2000). The virus present in the URT may also invade the LRT epithelium in a later stage of the infection thereby directly causing inflammation and bronchospasm, airway obstruction and wheezing. It is also possible that the virus is largely confined to the upper airway and that remote, indirect mechanisms provoke asthma exacerbation.

In conclusion, with the use of quantitative real-time PCR we showed that persistence of the virus, as well as viral load in the upper respiratory tract might not be associated with the induction and/or persistence of lower airway asthmatic symptoms. Elucidation of the local and systemic inflammatory response in virus-induced asthma exacerbations remains a challenge, especially for the development of more sophisticated anti-inflammatory strategies.

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