Increased Systemic Cytokine/Chemokine Expression in Asthmatic and Non-asthmatic Patients with Bacterial, Viral or Mixed Lung Infection

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Abstract
This study was aimed to determine the profiles of serum cytokines (IL-1β, TNF-α, IL-4, IL-5) and chemokines (MCP-1: monocyte chemoattract protein-1 and RANTES: regulated on activation normal T cell expressed and secreted) in individuals with an asthmatic versus a non-asthmatic background with bacterial, viral or mixed acute respiratory infection. Asthmatic (n = 14) and non-asthmatic (n = 29) patients with acute viral, bacterial or mixed (bacterial and viruses) respiratory infection were studied. Patients were also analysed as individuals with pneumonia or bronchitis. Healthy individuals with similar age and sex (n = 10) were used as controls. Cytokine/chemokine content in serum was determined by ELISA. Increased cytokine/chemokine concentration in asthmatic and non-asthmatic patients was observed. However, higher concentrations of chemokines (MCP-1 and RANTES) in asthmatic patients infected by viruses, bacteria or bacteria and viruses (mixed) than in non-asthmatic patients were observed. In general, viral and mixed infections were better cytokine/chemokine inducers than bacterial infection. Cytokine/chemokine expression was similarly increased in both asthmatic and non-asthmatic patients with pneumonia or bronchitis, except that RANTES remained at normal levels in bronchitis. Circulating cytokine profiles induced by acute viral, bacterial or mixed lung infection were not related to asthmatic background, except for chemokines that were increased in asthmatic status.

Introduction
Respiratory viral or bacterial infections can have profound effects on asthma [1]. Viral respiratory infections are found in association with asthma exacerbations [1, 2]. The infections associated with these wheezing events are multiple and include respiratory syncytial virus (RSV), human rhinovirus, metapneumovirus, parainfluenza, coronavirus and other viruses as well as typical and atypical bacteria [3]. Previous studies have shown differences in the bronchoalveolar cytokine profiles between non-asthmatic and asthmatic patients [4]. Several cytokines have been described to play an important role in the pathogenesis of asthma, such as IL-1β, TNF-α, IL-4, IL-5 and chemokines (MCP-1: monocyte chemoattract protein-1 and RANTES: regulated on activation normal T cell expressed and secreted) [2]. In this regard, TNF-α induces pro-inflammatory events, apoptosis and emphysema in lung tissues [5–9]. IL-1 induces releases other cytokines and chemokines and eosinophil accumulation producing neutrophilic inflammation in respiratory disorders [10–13]. In addition, IL-1β can induce increased production of IL-4 and IL-5 that increase IgE production [14, 15] involved in allergic airway inflammation [16–19]. MCP-1 (monocyte chemoattract protein-1) and RANTES (regulated on activation normal T cell expressed and secreted) are important in leucocyte recruitment to lung tissues during lung inflammatory events [1, 2, 20, 21]. However, circulating cytokine response after viral or bacterial respiratory infections in asthmatic and non-asthmatic status has been little studied. Increased circulating blood levels of several cytokines during viral respiratory tract infection have been reported [2]. These cytokines may represent inflammatory markers with different profiles in asthmatic and non-asthmatic patients. In addition to the asthmatic background, the infection type (viral or bacterial) and the site of respiratory infection (upper or lower) could be important to define the circulating cytokine...
profile. Therefore, the aim of this study was to describe changes in the level of several inflammatory (IL-1β, TNF-α) and Th2 (IL-4, IL-5) cytokines and chemokines (MCP-1 and RANTES) in the systemic circulation during acute viral or bacterial infections in patients with asthmatic or non-asthmatic background and their relationship with the respiratory infection site (upper and lower).

Material and methods

Patients. The study population was drawn from outpatient clinics. Male and female patients \( (n = 43) \) presenting clinical diagnosis of acute respiratory infection (ARI) were studied (Table 1). The inclusion criteria were those individuals who had at least one respiratory symptom, such as cough, wheeze, running nose or sneeze, and who were suspected by a professional physician to have respiratory infection. Patients with previous diagnosis of asthma according to the criteria of the Global Initiative for Asthma (GINA) Program [22], but without asthma manifestation (only asthma background), were selected and classified as asthmatic patients in this study \( (n = 14) \). Patients without antecedents of asthma (no asthmatic background) were classified as non-asthmatic patients \( (n = 29) \). No patients were treated with antibiotics, anti-allergic or steroid drugs when blood samples were obtained. Patients with asthmatic background did not present asthma manifestations at least one month previous to the moment of blood sample obtaining. Suggestive clinical and X-rays findings were used for the diagnosis of pneumonia (asthmatic: \( n = 8 \); non-asthmatic: \( n = 9 \)) or bronchitis (asthmatic: \( n = 6 \); non-asthmatic: \( n = 20 \)). Pneumonia criteria were as follows: temperature >38 °C, cough with or without sputum, hemoptysis, pleuritic chest pain, myalgia, dyspnoea, fatigue, rales, rhonchi, wheezing, bronchial breath sounds, dullness to percussion and gastrointestinal symptoms. Regarding chest radiography, the presence of pleural effusion, lobar consolidation or diffuse infiltrates was observed. The physical examination of patients presenting acute bronchitis showed the presence or absence of fever and tachypnea, cough with or without sputum, wheezing, rhonchi and prolonged expiration. Evidence of lung consolidation was absent. Viral (only the presence of studied viruses: asthmatic: \( n = 5 \); non-asthmatic: \( n = 16 \)), bacterial (only the presence of studied bacteria: asthmatic: \( n = 4 \); non-asthmatic: \( n = 7 \)) and mixed (the presence of both studied bacteria and viruses: asthmatic: \( n = 5 \); non-asthmatic: \( n = 6 \)) infection was confirmed by the presence of the agent in specimens from nasopharynx and bronchoalveolar lavage. This study was performed in 43 patients who were obtained from a patient screen \( (161 \text{ patients}) \). Only patients with confirmed presence of studied micro-organisms in both types of samples were selected. Healthy individuals with similar age \( (15–72 \text{ years}) \) and sex \( (n = 10) \) were studied as controls. Controls were individuals without respiratory symptoms, evidence of lung radiography alterations and negative micro-organism results in bronchoscopy and nasal samples. We excluded individuals who had cardiac disease, immunodeficiency and chronic inflammatory disease. In addition, they were negative for studied micro-organisms and had normal levels of IgE, normal chest radiography and non-asthmatic background. Blood samples were obtained from patients at the time when specimens from nasopharynx and bronchoalveolar were obtained. Serum from controls and patients was stored at –70 °C until use. This work was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. The Ethics Committee of Instituto de Investigaciones Clínicas Dr. Américo Negrette and FONACIT (Caracas, Venezuela) approved this study, and written informed consent was obtained from all patients and controls prior to blood collection.

Respiratory virus identification. Viral replication was demonstrated in HEp-2 cell cultures (protocol -520-I; National Institute for Health, USA) [23]. Cells were grown to 50% confluence in Eagle’s minimum essential medium (MEM) containing 7% FBS and 1% antibiotic/antimycotic. Nasopharynx and bronchoalveolar samples from patients were sonicated and centrifuged, and supernatants \( (200 \mu l) \) Table 1 Characteristics and infection type of asthmatic and non-asthmatic patients with acute respiratory infection and controls.

| Parameters | Asthmatic | Non-asthmatic | Controls |
|-----------|-----------|---------------|----------|
| n | 14 | 29 | 10 |
| Age (years): | 17–70; 37.7; 34 | 18–77; 41.9; 43 | 15–72; 36.2; 27 |
| Gender (m/f) | 12/2 | 18/11 | 6/4 |
| Infection type (n) | | | |
| Viral | 5 | 7 | 5 |
| Bacterial | 7 | | |
| Mixed | 6 | | |
| Type of micro-organisms (%): | | | |
| RSV | 42.86 | 34.48 |
| Adenovirus | 14.29 | 31.03 |
| Parainfluenza | 7.14 | 20.69 |
| Influenza | 3.45 | | |
| SP | 21.43 | 20.69 |
| KP | 14.21 | | |
| PA | 7.14 | | |
| EC | 7.14 | 6.90 |
| HBS | 7.14 | 3.45 |
| LP | 7.14 | | |
| SM | 3.45 | | |
| PA | 3.45 | | |

RSV, Respiratory syncytial virus; SP, Streptococcus pneumoniae; EC, Escherichia coli; KP, Klebsiella pneumoniae; PA, Pseudomonas aeruginosa; SA, Staphylococcus aureus; HBS, Hemolytic group A beta Strepoccus; SM, Serratia marcescens; LP, Legionella pneumophila.

*Values were obtained in asthmatic \( (n = 10) \) and non-asthmatic \( (n = 29) \) including viral, bacterial and mixed infections.
were added to HEp-2 cell cultures. Cultures were incubated for 1 h, and then, 300 µl of MEM containing 10% FBS and 1% antibiotic/antimycotic were added and incubated at 37 °C in 5% CO₂ for 96–120 h. Viral cell infection (respiratory syncytial virus, parainfluenza 1, 2 and 3, influenza A and B and adenovirus) was determined by direct immunofluorescence using a commercial kit (Light Diagnostics SimulFluor Respiratory Screen Kit; Chemicon Internacional, Temecula, CA, USA). Infected and uninfected culture (negative control) slides were incubated with FITC-conjugated antibodies against viral antigens for 1 h; after washing, positive fluorescence in nuclei or nuclei/cytoplasm represented viral infection. Positive antigen control slides provided by commercial assay were used as a positive control. Samples were analysed one time to determine the presence of viruses.

**Bacterial identification.** Nasopharynx and bronchoalveolar samples from patients were incubated on McConkey agar, sheep blood agar with kanamycin or chocolate agar and incubated for 14–48 h at 37 °C under aerobic, microaerobic or anaerobic conditions for the bacterial presence. Bacterial species were identified using conventional bacteriological techniques [24, 25]. The presence of serum IgG anti-atypical bacteria (Legionella pneumophila, Coxiella burnetti, Chlamydia trachomatis, Chlamydophila pneumoniae y Micoplasma pneumoniae) was determined using an available commercial kit (Pneumoslide IgG; Vircell, SL, Granada, Spain).

**Quantification of serum IgE and IL-1 β, TNF-α, IL-4, IL-5, MCP-1 and RANTES.** IL-1 β, TNF-α, IL-4, IL-5, MCP-1 and RANTES contents were measured using commercial available ELISA kits (TNF-α, IL-4 and IL-5; Diaclone, Fleming, France; MCP-1; Endogen, Rockford, IL, USA; IL-1 β and RANTES; Alpco Diagnostics, Salem, NH, USA), and the results were expressed as pg/ml. Serum IgE content was determined by ELISA (Calbiochem Inc., San Diego, CA, USA). ELISA analyses were performed by triplicate.

**Statistical analysis.** Values were expressed as mean ± standard deviation. Data analysis was performed using a nonparametric ANOVA (Kruskal–Wallis test) and Dunn’s multiple comparisons test. Two-tailed P values <0.05 were considered statistically significant.
Results

Increased levels of IgE in asthmatic patients \((n = 14)\) compared with non-asthmatic patients \((n = 29)\) and controls \((n = 10)\) were observed (asthmatic: \(380.50 \pm 13.22^*\); non-asthmatic: \(56.80 \pm 9.29\); controls: \(18.49 \pm 5.51\) IU/ml; \(^*P < 0.01\) versus control and non-asthmatic patients). Respiratory syncytial virus, adenovirus, parainfluenza and influenza in studied patients were identified (asthmatic: \(n = 5\); non-asthmatic: \(n = 16\)). Several bacteria were found: *Streptococcus pneumoniae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, Hemolytic group A beta *Streptococcus*, *Serratia marcescens* and *Legionella pneumophila* (asthmatic: \(n = 9\); non-asthmatic: \(n = 13\)). Percentage of infected patient according to asthmatic and non-asthmatic status is shown in Table 1.

In general, the serum levels of IL-1\(\beta\), TNF-\(\alpha\), IL-4, IL-5, MCP-1 and RANTES in patients with acute respiratory infections were found increased when compared with healthy controls. Cytokine expression was similar in asthmatic and non-asthmatic patients, and only chemokines (MCP-1 and RANTES) in asthmatic patients \((n = 5)\) compared with control \((n = 10)\) and non-asthmatic patients \((n = 16)\) were significantly increased \((P < 0.05)\) (Fig. 1). When subgroups were studied according to microorganism type infection, patients infected by virus (asthmatic: \(n = 5\); non-asthmatic: \(n = 16\)) and with mixed infection (bacterium and virus: asthmatic: \(n = 5\); non-asthmatic: \(n = 6\)) showed increased production of cytokines in asthmatic and non-asthmatic patients compared to controls, but only asthmatic patients showed elevated values of chemokines (Figs 2 and 3). Bacterial infection induced systemic increment of cytokines and chemokines in asthmatic \((n = 4)\) and non-asthmatic \((n = 7)\) patients compared with healthy controls; however, TNF-\(\alpha\) and RANTES were found increased only in asthmatic patients (Fig. 4).

![Figure 2](image-url) Expression of cytokine/chemokine in serum of patients with acute viral respiratory infection. Increased levels of interleukin-1 beta (IL-1\(\beta\)), tumour necrosis factor-alpha (TNF-\(\alpha\)) and interleukins 4 and 5 (IL-4 and IL-5) in asthmatic and non-asthmatic patients were observed (A, B, C, D). Higher levels of TNF-\(\alpha\) in non-asthmatic patients than those observed in asthmatic patients were found (B). Monocyte chemoattractant protein-1 (MCP-1) and regulated on activation normal T cell expressed and secreted factor (RANTES) in asthmatic patients were observed increased (E, F). Asthmatic \((n = 5)\); non-asthmatic \((n = 16)\); control \((n = 10)\). Data were analysed using a nonparametric ANOVA (Kruskal–Wallis Test) and Dunn’s multiple comparisons test. Values were expressed as mean ± standard deviation.
In general (values from asthmatic and non-asthmatic patients with ARI), viral and mixed infections were better cytokine/chemokine inducers than bacterial infection (Fig. 5); however, similar inducer effect on IL-4 and IL-5 by all studied infections was observed (Fig. 5C,D). Asthmatic and non-asthmatics patients with pneumonia (asthmatic: \(n=8\); non-asthmatic: \(n=9\)) (Fig. 6) or bronchitis (asthmatic: \(n=6\); non-asthmatic: \(n=20\)) (Fig. 7) showed increment on studied cytokine/chemokine when compared with healthy controls. However, patients with asthmatic background and pneumonia showed MCP-1 and RANTES levels increased compared with control and non-asthmatic patients (Fig. 6E,F), and only asthmatic patients with bronchitis had elevated levels of MCP-1 and RANTES (Fig. 7E,F).

**Discussion**

Asthma is a complex chronic disease of the lung with an incidence growing at all ages. The complexity of this disease is partly due to the environmental insults such as allergens and microbial infections that play differential roles in the pathogenesis. Microbes may play important roles in the exacerbation of asthma [3]. To determine differential circulating cytokine profiles that could be related to the interaction of asthma–microbes, asthmatic and non-asthmatic patients with microbial acute respiratory infection were studied. Patient with and without asthmatic background had similar systemic cytokine response during microbial acute respiratory infection; however, only asthmatic patients had significant increment of chemokines, suggesting a role of asthmatic background in this differential response. As chemokines are important for the recruitment and tissue localization of both immune and structural cells in the lung, they can be relevant in asthma exacerbation [26]. During this study, patients with asthmatic background had no clinical manifestation of asthma, suggesting that increased level of chemokines could be an early manifestation of asthma exacerbation.

Asthma is characterized by airway inflammation, airway wall remodelling and bronchial hyper-responsiveness. Activated T helper type 2 (Th2) lymphocytes, eosinophils
and activated mast cells are the characteristic features to make the difference between atopic versus non-atopic asthma [27–29]. However, the pro-inflammatory and Th2 cytokine responses in asthmatic and non-asthmatic patients after lung microbial infection were similar during this study, suggesting that systemic Th2 and pro-inflammatory responses are common aspects of lung infection. The source of increased levels of cytokine/chemokines in the patient’s circulation after lung infection could involve lung tissues and/or systemic tissues. The respiratory tract is a frequent primary site of microbial infections resulting in the development of acute respiratory distress syndrome. During the lung inflammatory events, the alveolar compartmentalization could be lost, allowing the passage of cytokines to the bloodstream by any other organ to the pulmonary endothelium or inversely from the lung to the bloodstream. Cytokines such as IL-1, TNF-α and IL-8 have important roles in the lung dysfunction [30]. In this study, patients with microbial lung infection had increased serum levels of pro-inflammatory cytokines (IL-1β and TNF-α), Th2 cytokines (IL-4 and IL-5) and chemokines (MCP-1 and RANTES), suggesting a complex immune interaction during microbial respiratory infection. The source of those cytokines may be cells localized in lung tissue. In this regard, epithelial cells, mast cells, basophils, monocyte/macrophages and lymphocytes could secrete diverse types of cytokines and chemokines (IL-1, TNF, IL-4, IL-5, IL-8, MCP-1, RANTES) during lung tissue–microbes interactions [21, 31, 32]. However, it cannot rule out the role of other cells in other tissues.

Potential viral pathogens in acute respiratory infection include respiratory syncytial virus (RSV), human rhinovirus (HRV), human metapneumovirus, human parainfluenza virus (HPIV), human bocavirus, influenza viruses, adenoviruses and enterovirus. Both RSV and HRV have been most commonly linked to the exacerbation of asthma as well as to the pathogenesis [32]. Infection by parainfluenza and influenza viruses has generally been detected in asthma exacerbations [33]. The presence of some of those pathogens (RSV, adenovirus, HPIV and influenza) was found in this study; however, they were not associated with clinical manifestations of asthma. Patients with ARI were
mainly infected by RSV. This virus is involved in serious lower respiratory tract disease in children and can induce the produce of several cytokines (IL-6, IL-1, TNF-α, IL-8 and GM-CSF), chemokines (IL-8, IP-10, MCP-1 and RANTES) and chemokine receptor expressions in vivo and in vitro [34–36]. Accordingly, in this study, serum from RSV-infected patients had increased levels of IL-1β, TNF-α, IL-4, IL-5, MCP-1 and RANTES. As similar serum cytokine/chemokine profile was observed in adenovirus and parainfluenza virus infections, this viral cytokine/chemokine inducer effect probably is a common feature of respiratory virus infection. In addition, infection with RSV could be a risk factor for further lung diseases, as the persistence of cytokines and chemokines in fully recovered RSV-infected patients can provide a substratum for the development of subsequent asthma [37].

Recent studies have begun to introduce typical bacteria such as Streptococcus pneumonia, as well as atypical bacteria such as Chlamydia pneumonia as being responsible for acute wheezing in children [38]. Streptococcus pneumonia was the main pathogen found in bacterial infected patients. This bacterium is the most common cause of pneumonia in the world [39, 40], that when risk factors weaken the protective immunity, pneumococcus can cause serious diseases [40]. Growing evidence indicates that the inflammasome plays a key role in the pathogenesis of acute and chronic respiratory diseases. The inflammasome can be activated by Streptococcus pneumonia, inducing the chronic inflammation of the airways of patients with asthma and chronic obstructive pulmonary disease [41]. This could be a mechanism to induce the bacterial cytokine/chemokine production observed in this study. However, cytokines induced by bacterial respiratory infection and type I interferon induced by virus (mixed infection) can be protective for Streptococcus pneumonia infection [42, 43].

The cytokine/chemokine response pattern observed in this study was not related to the microbial infection type (viral, bacterial or mixed); however, bacterial infection had lower cytokine/chemokine inducer effect. We have no clear explanation for this finding; however, it has been reported that bacteria exert differential effects on eosinophils (important cell in respiratory pathology), resulting in the exacerbation or the inhibition of inflammation in lung infection [44]. As patients with ARI were infected by different bacteria, the serum values observed in bacterial infected patients could represent the result of those responses. Values found in mixed-infected patients (viral
and bacterial) showed similar values than those observed in viral infection, suggesting that the higher viral cytokine inducer effect was involved in cytokine/chemokine production in mixed infection.

Cytokine/chemokines can have harmful effects on the lung regardless of cytokine production sources. In this study, increased expression of IL-1β, TNF-α, IL-4, IL-5, MCP-1 and RANTES in the serum of patients with viral and bacterial respiratory infection could be involved in the clinical manifestations observed in the patients. These cytokines could have deleterious effect in the lung and other tissues. TNF-α can induce the activation of several pro-inflammatory events [5–8] and apoptosis in lung tissues resulting in emphysema [9]. IL-1 induces neutrophilic inflammatory responses in respiratory disorders [10] and releases other cytokines and chemokines [11, 12]. In addition, IL-1β induces eosinophil accumulation, and it is a Th2 and B cell growth factor [13], properties that could be related to the activation of Th2 profile observed in this study, as shown by the increased production of IL-4 and IL-5. These cytokines stimulate B-lymphocytes to produce IgE [14, 15] and increase allergic airway inflammation [16–18]. Increased levels of circulating chemokines found in asthmatic patients could reflect lung chemokine production that may be important in leucocyte recruitment to lung tissues. RANTES is involved in the chemotraction of eosinophils, monocytes and T-lymphocytes, and therefore, it has high relevance in asthma [21]. MCP-1 is involved in the recruitment of regulatory and effector leucocytes (monocytes, lymphocytes and basophils) into tissues [20]. Therefore, chemokines could play an important role in asthma pathogenesis and in ARI. In addition, the increased systemic concentration of those cytokines/chemokines could activate circulating leucocytes with further deleterious effects in the lung [1, 2].

Increased serum concentration of IgE associated with increased production of circulating IL-4 and IL-5 was found in patients with asthmatic background, suggesting a possible role of IgE in the increased levels of those cytokines and in the asthma pathogenesis. In this regard, IgE has a role in the allergic inflammation, involving

Figure 6 Cytokine/chemokine production in asthmatic and non-asthmatic patients with pneumonia. Similar increased levels of interleukin-1 beta (IL-1β), tumour necrosis factor-alpha (TNF-α) and interleukins 4 and 5 (IL-4 and IL-5) in asthmatic and non-asthmatic patients were observed (A, B, C, D). Higher levels of monocyte chemoattractant protein-1 (MCP-1) and regulated on activation normal T cell expressed and secreted factor (RANTES) in asthmatic patients were found (E, F). Asthmatic ($n = 8$); non-asthmatic ($n = 9$); control ($n = 10$). Data were analysed using a nonparametric ANOVA (Kruskal–Wallis test) and Dunn’s multiple comparisons test. Values were expressed as mean ± standard deviation.

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basophil and mast cell degranulation, activation of monocyte/macrophages and stimulation of Th2 cells, inducing local infiltration of IL-4 and IL-5 secreting Th2-cells and amplifying tissue inflammation [19]. Previous reports have shown differences between asthma and other pulmonary diseases regarding cytokine profiles. Bronchoalveolar lavage cytokine profiles between chronic obstructive pulmonary disease (COPD) and asthma have been reported. The profile of cytokine expression in COPD is different from that observed in asthma. Infiltration of eosinophils and Th2-cells involving IL-4, IL-5 and IL-13 productions is usually found in asthma. IL-8, IL-1 and TNF-α play more prominent roles in COPD [4]. In our study, the bronchoalveolar lavage cytokine profile was not analysed; however, circulating cytokine profiles showed no differences in the expression of pro-inflammatory and Th2 cytokine profiles in both asthmatic and non-asthmatic patients, suggesting similar immune response during microbial infection. Only high levels of chemokines in asthmatic patients were observed, suggesting an increased cellular infiltration (monocytes, neutrophils or eosinophils) at tissue level. In this regard, the role of chemokines in microbial-associated asthma exacerbations has been reported [36].

In conclusion, the present study demonstrates that serum cytokine profiles in asthmatic and non-asthmatic patients with viral and/or bacterial respiratory infection were similar. Asthmatic status or the anatomic places of infection (bronchitis or pneumonia) were not relevant in the pro-inflammatory and Th2 cytokine profiles, except that high values of chemokines were observed in asthmatic patients. Bacterial and viral respiratory infections did not induce different circulating cytokine profiles; however, lower cytokine/chemokine inducer effect by bacterial infection was observed. The precise mechanism, regarding the effect of those systemic cytokines in driving microbial respiratory infection processes, remains elusive.
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