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Significant Increase of IL-8 Sputum Levels in Treatment Resistant Severe Asthma Compared with Difficult to Treat Severe Asthma Patients

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Abstract

The spectrum of severe asthma includes different groupings and phenotypes. World Health Organization proposes to differentiate two categories: difficult-to-treat-severe asthma (DTTSA) and treatment-resistant-severe asthma (TRSA). DTTSA includes controllable severe asthma and TRSA includes patient's not achieving adequate levels of control and persisting indicators of severity despite receiving and using maximum levels of inhaled medications. We hypothesized that granulocyte counts from sputum samples could differentiate pathogenic differences between both groups. We analyzed the number of sputum neutrophils and eosinophils in both categories of severe asthma patients admitted for an asthma exacerbation in a respiratory-based hospital. We also compared our results with mild to moderate asthma patients (MMA) and healthy controls (HC). We measured cytokines levels relevant to neutrophil recruitment (IL-8 and IL17-A) and inflammatory cytokines. We found a significant increase in neutrophil numbers in TRSA compared to DDTSA, MMA and HC; moreover, we detected a significant increase of IL-8 levels in sputum samples of TRSA patients. However we found no differences in exhaled FeNO and IL17-A levels among groups.

This study shows that the sputum neutrophilic profile predominates in patients with TRSA, could be useful in distinguishing from DTTSA, and may provide insight into the pathogenic differences between both groups.

Keywords: Severe asthma; Neutrophilia; Inflammation; Cytokines; Sputum; Phenotypes

Abbreviations

TRSA: Treatment Resistant Severe Asthma; DDTSA: Difficult to Treat Severe Asthma; MMA: Mild to Moderate Asthma; FeNO: Exhaled Nitric Oxide

Introduction

Asthma usually responds to Inhaled Corticosteroid (ICs) treatment, with or without the addition of Long-Acting Beta-Agonists (LABAs). However, certain severely affected patients cannot be controlled despite high doses of ICS plus LABAs [1,2]. Severe asthma occurs in approximately 10% of all the subjects with asthmaand is associated with greater morbidity and a disproportionate use of health care resources [3-6].

World Health Organization (WHO) has defined Severe Asthma based in the level of current clinical control and risks characterized by frequent severe exacerbations (or death) and/or adverse reactions to medications and/or chronic morbidity including impaired lung function. They include three different categories: (1) Untreated severe asthma, (2) Difficult-To-Treat Severe Asthma (DTTSA) due to non-adherence and/or accessibility issues and finally (3) Treatment-Resistant Severe Asthma (TRSA). The latter group includes patients whose control was never achieved despite the confirmed use of the highest level of recommended treatment and those that achieve control only with the highest level of recommended treatment including frequent use of systemic corticosteroids [7].

The European Network for Understanding Mechanisms of Severe Asthma (ENFUMOSA) study reported that patients with severe asthma show greater sputum neutrophil counts in comparison with those with mild-to-moderate asthma, suggesting a possible role of neutrophils in the severity of their disease [8,9]. This inflammatory heterogeneity seems to correlate with the identification of a distinct cytokine profile [10,11]. These inflammatory profiles are mainly determined by the involvement of different T helper cell subsets, which contribute to the recruitment and activation of neutrophils [12,13]. Th1 and Th17 cells would be crucial for the development of neutrophilic inflammation in the airways, which may be related to corticosteroid resistance [14]. Expression of both IL-17A and IL-17F have been reported to be high in severe asthma in association with poor lung function, but these cytokines have not been linked to neutrophilic inflammation or clinical parameters [15]. IL-8 is likely a major chemo attractant for neutrophils; however the precise mechanisms by which IL-8 or IL17 are associated with severe asthma have not yet been determined.

The aim of this study was to compare the levels of IL-8 and IL17-A in sputum samples from patients admitted for an asthma exacerbation classified in one of two categories based on WHO criteria: DTTSA and TRSA. We also included in our study patients with Mild to Moderate Asthma (MMA) and Healthy Subjects (HC) for comparison.

Methods

Study design

We conducted a cross-sectional prospective observational study of admissions to a respiratory hospital during a complete year (March 2012-March 2013). This study complied with the Declaration of Helsinki and was approved by the Institutional Ethics Committee of Respiratory Rehabilitation Hospital María Ferrer. Informed consent was obtained for all participants.

Subjects

During the study period we included fifty patients admitted with asthma. Thirty-eight were classified as DTTSA and twelve as TRSA; all according to WHO criteria. DDTSA included patients with severity markers (recurrent admissions, frequent non-programmed visits and use of systemic steroids) due to non-adherence and/or accessibility issues and/or inadequate treatment. In the second group (TRSA) we included patients with persisting indicators of severity despite confirmed use of maximum doses of inhaled therapies and frequent use of systemic steroids. We also included in this study sputum samples from twelve patients with Mild to Moderate Asthma (MMA) and six non-smokers Healthy Controls (HC) between 22 and 60 years old.

Upon admission to the hospital, a baseline questionnaire was completed requesting information on medical history (asthma quality of life questionnaire). All asthma patients underwent spirometry and breathe condensate collection followed by exhaled nitric oxide measure and finally sputum induction.

Samples collection and handling

Sputum samples were collected with the help of physiotherapy man oeuvre in accordance with previous approaches [16]. These samples were processed immediately after collection, mucoid portions of sputum plugs that appeared free of salivary contamination were incubated with 0.1% DTT (dithiothreitol; Sigma, St. Louis, MO, USA) for twenty minutes, filtered through a 40 μm cell strainer (BD Biosciences, Franklin Lakes, USA), and centrifuged at 1500g for 10 min. Cell-free supernatants were collected and stored in aliquots at -80°C pending further cytokine analysis, and cell differential counts were determined from cytospin preparations. Cell counts were expressed as a percentage of nonsquamous cells. A sputum sample was considered inadequate when the percentage of squamous epithelial cells was >5%.Eosinophilic phenotype was defined as ≥3% sputum eosinophil count, while neutrophilic phenotype was considered as ≥60% sputum neutrophil count.

Determination of cytokines in sputum samples by ELISA

We measured IL-8, IL-17A, IL1-β, IL-6 and TNF-α by using enzyme linked immunosorbent (ELISA) assay in according to manufacturer recommendations. We used ELISA kit to measure IL-8, IL1-β, and TNF-α (BD Opt EIATMRyD, whose limits of detection are 0.8pg/ml for IL-8 and IL1-β and 2pg/ml for TNF-α) and IL17-A and IL-6 (ELISA MAXTM Deluxe, whose limits of detection are 2 pg/ml and 4 pg/ml respectively). Measurement of sputum cytokines standards were performed by adding a similar concentration of Dithiothreithol (DTT) to standard and blank wells in order to reach the same level of protein denaturation by DTT as in sputum supernatants.

Statistical analysis

Data were expressed as mean ± SEM. Differences between the groups were evaluated by non-parametric Kruskal Wallis and Dunn´s Multiple comparison test. Differences were considered significant at a level of $p<0.05$ or less. Analysis and graph were performed using Prism 5 software (Graph Pad, San Diego, CA).

Results

Patient characteristics

A total of 62 patients with severe asthma or mild to moderateasthma were included in our study. The characteristics of these patients are shown in Table 1. TRSA patients were older than those with DDTSA or MMA (51.4 ± 3.2 vs40.4 ± 2.7 or 36.2 ± 3.8 years; $p<0.05$) however the age at onset of asthma was similar (15.5 ± 4.5 vs20.3 ± 2.8 or 18.6 ± 3.4 years respectively).

We found differences between both groups of severe asthma patients. Females dominated TRSA group (female/male ratio: 11/1); however among DDTSA subjects we observed female/male ratio of 20/18. The number of previous admissions and the times that patients required mechanical ventilation were significant higher in patients with TRSA respect to DDTSA (3.3 ± 0.3 vs 2.3 ± 0.2 and 1.92 ± 0.33 vs 1.08 ± 0.05 respectively; $p<0.05$). We observed lower FEV1 in TRSA patients compared to DDTSA (775.5 ± 55 vs 911,4 ± 86), however FeNO levels were not different between groups.

		MMA	DTTSA	TRSA
Demographic Dates	N° of patients	12	38	12
	Age average (yrs)	36.2 ± 3.8	40.4 ± 2.7	51.4 ± 3.2*
	Female	8	20	11
	Male	4	18	1
	BMI	27.2 ± 2.7	28.0 ± 1.0	30.4 ± 1.4
Asthma	N° of previous admissions	1.25 ± 0.18	2.3 ± 0.2	3.3 ± 0.3*
	N° of times MV by asthma	0 ± 0.0	1.08 ± 0.05	1.92 ± 0.33*
	N° of awaking in night by asthma	2.6 ± 0.26	2.7 ± 0.16	3.16 ± 0.32
	Onset of asthma age	18.6 ± 3.4	20.3 ± 2.8	15.8 ± 4.5
	FEV1 (ml)	931.7 ± 65.9	911.4 ± 60.4	775.5 ± 86
	FeNO (ppb)	46.5 ± 13.8	39.4 ± 5	37.7 ± 10.8
Smoking Status	Never smoked	5	23	8
	Former smokers	4	8	3
	Active smokers	1	6	0
	Passive smokers	2	1	1

Table 1: General characteristics of asthma patients

Data are presented as mean ± SEM. BMI: Body Mass Index; MV: Mechanical ventilation; FEV1: forced expiratory volume in 1s; FeNO: exhaled nitric oxide. Data are expressed as % mean and SEM (*:p<0.05).

Increase of Neutrophils in patients with TRSA

We found a significant increase in sputum neutrophils in TRSA vs DDTSA, MMA or HC (32.4 ± 6.7 vs 11.3 ± 4.8; 9.8 ± 3.9 or 1.1 ± 0.8 respectively; p<0.05), however we didn´t find significant differences in sputum eosinophils among the three asthma groups (TRSA, DTTSA and MMA) (Figure 1). There was an increase in the percentage of patients with neutrophilic profile in TRSA vs DDTSA (25 vs3 %) respectively; Figure 2.

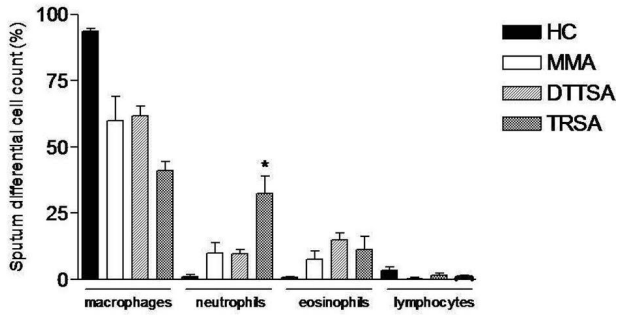

Figure 1: Sputum differential cell count in patients with DDTSA compared with patients with TRSA, MMA and HC. Data are expressed as % mean and SEM (*:p<0.05).

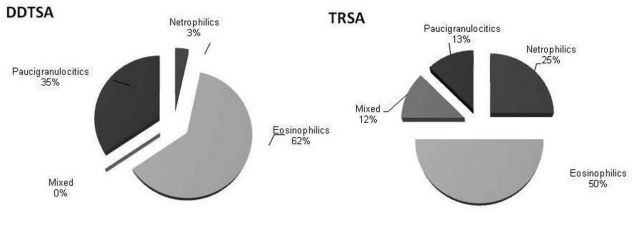

Figure 2: Inflammatory phenotypes: We stratified each group of patients (DDTSA and TRSA) according to % of sputum granulocytes.

Increase in IL-8 in sputum samples of TRSA patients

We analized the levels of major cytokines related to neutrophils recruitment: IL-8 and IL17-A. We show in the Figure 3A a significant increase in IL-8 levels in patients with TRSA compared to DDTSA, MMA or HC (724.8 ± 47.1 pg/ml vs 419.5 ± 80.1pg/ml; 308.3 ± 21.8 pg/ml or 139.0 ± 18.0 pg/ml respectively, p<0.05). No differences were found in IL17-A levels among asthma patients: 13.6 ± 4.6 pg/ml vs14.1 ± 3.3pg/ml and 10.3 ± 3.8 pg/ml respectively; Figure 3B; HC showed non-detectable values.

Analyzing inflammatory cytokines we found a non-significant increase in IL1-β levels in TRSA respect to DTTSA, MMA and HC (135.2 ± 40.1 pg/ml vs 69.8 ± 23.4 pg/ml; 75.9 ± 26.4 pg/ml or 41.3 ± 16.7 respectively), Figure 3C. As we showed in the Figure 3D levels of IL-6 were higher in severe asthma patients (TRSA and DTTSA) respect to MMA patients; however these differences were non-significant. Finally, we analyzed levels of TNF-α (Figure 3E). We detected 7.5 ± 2.3 pg/ml in sputum samples of TRSA group and 5.0 ± 1.5pg/ml in samples of DTTSA group; however MMA and HC showed non-detectable values.

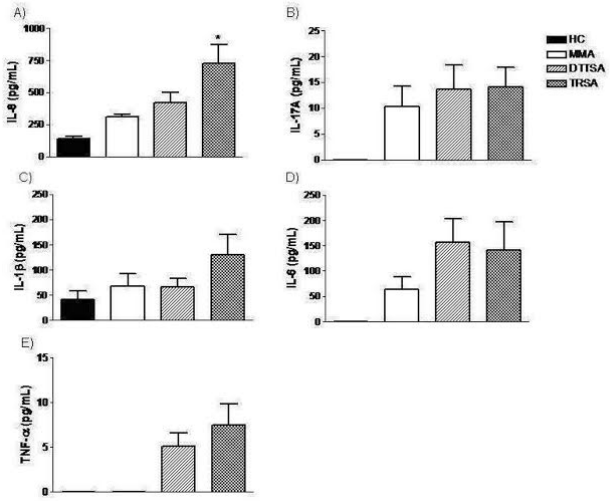

Figure 3: Sputum cytokine levels. We measured by ELISA levels of IL-8 (A) and IL17-A (B), IL1-β (C), IL-6 (D) and TNF-α (E). Data are expressed as mean and SEM (*:p<0.05).

Discussion

Severe asthma affects a small percentage of asthma population; however these patients contribute substantially to the cost and morbidity associated with treating asthma. Moreover the reason for their increased susceptibility remains elusive. They suffer worse health than those with mild or controllable disease. Previous studies showed that neutrophilic asthma may be related to corticosteroid resistance [17]. It is known that corticosteroids inhibit neutrophil apoptosis and contribute to their activation, suggesting that corticosteroid treatment itself could have some role in the development of neutrophilia [18].

It has been previously described that lung neutrophilia could be associated with lower lung function, more air-trapping, thicker airway walls comparing them with non-neutrophilic asthma people, but not with airway hyper responsiveness [19]. Post hoc analysis showed that sputum neutrophilia was greater in a Severe Asthma Research Program (SARP) cluster of individuals who had generally adult-onset and severely obstructive asthma [20].

We observed sputum neutrophilia in asthma patients with both DDTSA and TRSA, with neutrophils being elevated more in TRSA than DDTSA. TRSA subjects had lower FEV1 compared to the DDTSA patients, but no relationship was found between FEV1 and neutrophil count.

The SARP study showed that a high Fraction of Exhaled Nitric Oxide (FeNO) was associated with severe asthma patients [21].

However we detected no differences in FeNO between TRSA and DDTSA patients.

Sputum molecular studies previously showed that neutrophilic inflammation could be associated with up-regulation of IL1-β and TNF-α [22]. According to these results, in our study we also found an increase of both cytokines in TRSA respect to DTTSA, however our results didn´t show significant differences. We also analyzed levels of IL-8 and IL-17A in patients with severe asthma and found a significant increase in IL-8 levels in TRSA vs DDTSA, MMA and HC. Kurashima previously reported increased levels of IL-8 in sputum preceding the exacerbations of asthma [23]. This suggests that perhaps a cytokine profile would be able to distinguish those patients who will develop more severe episodes compared to those who will have more mild exacerbations.

Higher levels of IL-17A and IL-17F in severe asthma were associated with poor lung function, without any relationship with neutrophilic inflammation or clinical parameters [24]. Consistent with this notion, we observed no differences in sputum IL17-A levels between TRSA and DDTSA.

Sputum is a non-invasive technique for the assessment of bronchial inflammation, and for detecting the cellular and inflammatory profile of sputum in patients with asthma. The observations presented in this paper could be important for prophylactic prevention or treatment of severe asthma.

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