Original article

Monocyte chemotactic protein-4 (MCP-4/CCL-13) and CC chemokine receptor 3 (CCR3) in the sputum of asthmatic children

Background: *Monocyte chemotactic protein-4 (MCP-4/CCL-13) is a potent chemoattractant to eosinophils, monocytes and lymphocytes.*
Objective: *We aimed to investigate MCP-4 and its CC chemokine receptor 3 (CCR3) expression on cells of induced sputum during acute asthma exacerbation.*
Methods: *Immunohistochemistry was used to assess MCP-4 and CCR3 expression on induced sputum cells of 30 children during asthma exacerbation and 20 healthy matched controls. Patients were divided into three groups according to exacerbation severity; mild, moderate and severe (n = 10 for each). Patients were followed until quiescence, when sputum was re-examined.*
Results: *MCP-4 and CCR3 were expressed on eosinophils and monocytes. Lymphocytes expressed only MCP-4. The percentages of sputum total cells, eosinophils and lymphocytes expressing MCP-4 and/or CCR3 were significantly higher during asthma exacerbation than in controls and negatively correlated with peak expiratory flow rate, whereas that of monocytes was not. The percentages of sputum total cells, eosinophils, monocytes and lymphocytes expressing MCP-4; and total cells and eosinophils expressing CCR3 were significantly higher in patients with severe than those with mild and moderate exacerbations. When patients were followed till remission, the percentages of sputum cells expressing MCP-4 and CCR3 decreased. Sputum eosinophil percentage correlated positively with the percentage of eosinophils expressing MCP-4 and CCR3 ($r = 0.69$, $p < 0.0001$; $r = 0.62$, $p < 0.001$, respectively). The percentage of sputum eosinophils expressing MCP-4 correlated positively with that of cells expressing CCR3 ($r = 0.95$, $p < 0.0001$).*
Conclusion: *The expression of MCP-4 and CCR3 on sputum cells increases during acute asthma exacerbation and this increase correlates with exacerbation severity, and it decreases during remission. Modification of their expression could be a potential target for asthma therapy.*

Keywords: asthma, CCL-13; CCR3; chemokines; eosinophils; MCP-4; sputum.

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INTRODUCTION

Atopic and non-atopic asthma are characterized by chronic inflammation and local tissue eosinophilia. Atopic asthma is characterized by a 50-fold increase in the number of eosinophils relative to neutrophils in the bronchial mucosa. This is the result of the cumulative and sequential effects of variable increases in selective eosinophil versus neutrophil migration occurring at a number of stages in the life cycle of the eosinophil. Such eosinophils contain an army of chemicals implicated in the damage to airway epithelium and is related to the observed changes in airway function. It is thus likely that understanding the mechanisms of eosinophil recruitment to the airway will offer new therapeutic approaches for the treatment of this disease[1].

The mechanisms that regulate the selective accumulation, activation, and retention of airway eosinophils and T cells are areas of active investigation. Present evidences suggest that recruitment of eosinophils and T cells into sites of inflammation is a multistep process involving leukocyte-endothelial interactions through adhesion molecules and local generation of type 2 T helper (Th2) cytokines and some chemoattractants that direct cell migration from the vascular compartment into the inflamed airways[2].

The effects of Th2 cytokines, such as interleukin (IL)-4, IL-5, IL-9, and IL-13, account for virtually all the pathophysiological manifestations of allergy

and asthma. Moreover, both Th2 cells and the effector cells present in the areas of allergic inflammation, (basophils, mast cells and eosinophils), express chemoattractant receptors. Therefore, chemokine/chemoreceptor interactions are responsible for the recruitment of these effector cells, and play a critical role in the allergen-induced recruitment of Th2 cells in the target tissues of allergic inflammation[3].

Chemokines are a superfamily of proteins that are potent chemoattractants for leukocytes in allergic inflammation. The CC chemokine receptor 3 (CCR3) is the common receptor on eosinophils for cysteine/cysteine (CC) chemokines such as CCL24/eotaxin-2, CCL26/eotaxin-3, CCL13/ monocyte chemotactic protein-4, (MCP-4), MCP-3, and RANTES (Regulated Upon Activation, Normal T Cell Expressed and Secreted), which are important for the release of eosinophils from bone marrow into the circulation and the recruitment of eosinophils from blood into tissues[4]. CCR3 is the receptor that mediates the majority of the eosinophil chemotactic effects. CCR3 is present on cell types associated with allergy such as the eosinophils, basophils, mast cells and CD4 (+) T helper 2 cells[5].

MCP-4 has been shown in vitro to potently induce eosinophil chemotaxis as well as initiate several other pro-inflammatory activities such as integrin activation, lipid mediator biosynthesis and degranulation. Ligand binding and chemotaxis experiments demonstrated that the G-protein coupled-receptor cloned from eosinophils, CCR3, was responsible for producing MCP-4 selectivity profile identical to that of eosinophils. Together these studies strongly suggest a central role for MCP-4/CCR3 interaction in eosinophil trafficking. CCR3 has also been found on in vitro derived Th2 cells and on T-cells co-localizing with eosinophils in diseased tissue, thus revealing a possible pathogenic mechanism for T-cell recruitment into the airways[6].

In asthmatic airways, MCP-4 has been localized immunohistochemically and by in-situ hybridization analysis to eosinophils, macrophages, epithelial and endothelial cells. After allergen challenge, MCP-4 is expressed on the epithelial surface of the airways in sites at which eosinophils accumulate. The established selective chemotactic and activating effects of MCP-4 on eosinophils and the association of eosinophils with asthma activity and severity suggest that MCP-4 is an important mediator in acute asthma[7]. Therefore, this study attempted to determine the percentage of different sputum cells expressing MCP-4 and its receptor CCR3 in induced sputum of asthmatic children during exacerbation and quiescence and in relation to asthma severity. MCP-4 antagonism and/or blockade of CCR3 on eosinophils may represent a highly attractive and innovative strategy for asthma therapy.

METHODS

This follow-up, case-control study comprised 30 asthmatic children as a stratified non-random sample recruited from the Pediatric Allergy and Immunology Clinic and Emergency Department of Ain Shams University Children's Hospital while presenting with an acute asthmatic exacerbation. They were 17 (57%) males and 13 (43%) females, their ages ranged from 5 to 15 years with a mean age of 10.72 ± 3.26 years. It also included 20 healthy children without personal or family history of asthma or other atopic conditions as a control group. They were 12 (60%) males and 8 (40%) females, their ages ranged from 6 to 14 years with a mean age of 10.4 ± 2.44 years. An informed consent was obtained from the parents or caregivers of each child before enrollment.

Children were subjected to thorough history taking and clinical examination. The diagnosis of asthma had been based on the clinical symptoms and signs of episodic wheezing, chest tightness and dyspnea that improved at least partially after bronchodilator therapy. Patients were excluded from the study if they had respiratory infection. None of the patients had chest radiograph or sputum culture evidence of pulmonary bacterial infection and none received antibiotics during the study period.

Patients were studied within 24 hours of the start of an acute asthma exacerbation (defined by increasing cough, wheezing, dyspnea and nocturnal symptoms). Children were classified into three groups according to the severity of acute asthma attacks based on clinical data and the Global Initiative for Asthma Guidelines[8,9]. In this classification, Peak Expiratory Flow Rate (PEFR) was measured using Mini-Wright Peak Flow Meter (Clement Clarke International Ltd., Sales Office Armed House, Edinburgh Way, Harlow, UK). The PEFR of each patient was compared with normal population and % predicted was calculated. Ten children (7 males and 3 females; mean age: 10.75 ± 3.74 years) presented with mild acute asthma exacerbation, 10 children (6 males and 4 females; mean age: 11.5 ± 3.63 years) with moderate exacerbation, and the remaining 10 children (4 males and 6 females; mean age: 10.9 ± 2.38 years) had severe asthma exacerbation.

Based on the criteria of asthma grading[10], the clinical severity of the recruited asthmatics during quiescence was classified into intermittent asthma in four children (13%) and mild and moderate persistent asthma in 26 children (87%).

Patients received quick relief medications to relieve acute bronchoconstriction as oral β_2 agonists (terbutaline; 0.1-0.2 mg/kg/day) in 4 patients (13%), or inhaled β_2 agonists (salbutamol) in 26 patients (87%). Two patients (6.6%) had history of need of short courses of oral corticosteroids for 3-10 days during previous acute exacerbations. As regards long-term controller medications; 10 (33.5%) patients were receiving inhaled corticosteroids, 7 (23%) were receiving sustained-release theophylline (6-18 mg/kg/day), and 10 (33.5%) patients were receiving both drugs. Three children (10%) were not receiving any long-term medications.

Analytical Methods

1. Complete blood counts: applying Coulter Counter T66OA (Coulter Electronics Ltd, UK).
2. Serum total IgE: by ELISA technique (BioCheck, Inc 323 Vintage Park Dr. Foster City, CA 94404).
3. Immunohistochemical analysis of MCP-4/CCL-13 and CCR3 on sputum cells:

- Sputum collection: Subjects under study were selected above 5 years of age so that adequate sputum samples can be obtained spontaneously when required. Sputum was induced only when it could not be produced spontaneously. All subjects were premedicated with inhaled salbutamol given by nebulizer in a dose of (0.15 mg/kg/dose) 30 minutes before sputum induction. To minimize contamination with saliva and postnasal drip, subjects were asked to rinse their mouths and blow their noses before induction and, wherever possible, before expectoration. Subjects inhaled 3% hypertonic saline solution aerosols generated by Farmasol compressor nebulizer. Hypertonic saline was inhaled 10-15 minutes according to the severity of asthma until an adequate volume of sputum was expectorated[11].
- Sputum examination: Sputum sample volumes were recorded. Sputum samples containing <20% of contaminating squamous cells were considered suitable for analysis, they were processed immediately by a modification of the technique described by Pizzichini et al [12]. Sputum was treated by adding 2 mL of Hanks balanced salt solution containing 0.1% dithiothreitol, then vortexed for 2 to 3 min to homogenize the sample. Two mL of phosphate-buffered solution was added to stop the action of dithiothreitol, and then the suspension was centrifuged at 300g for 10 min. The cell pellet was resuspended in phosphate-buffered solution, and total cell counts of leukocytes using hemocytometer were determined. Differential cell counts of eosinophils, macrophages, lymphocytes, and neutrophils were counted microscopically. Eosinophils in sputum were expressed as absolute count and percentage of leukocytes. For immunocytochemistry, coded cytospins were fixed in a solution of acetone: methanol (2:3), air dried, and stored at -20˚C until further assessment of MCP-4, and CCR3.
- Sputum was stained by immunoenzymatic/ immunohistochemical (IHC) analysis techniques that allow the visualization of tissue (cell) antigens. These techniques are based on the immunoreactivity of antibodies and the chemical properties of enzymes or enzyme complexes, which react with colorless substrate-chromogens to produce a colored product[13]. The DAKO LSAB+ kit (DakoCytomation, Inc. 6392 Via Real Carpinteria, California 93013 USA) is based on a modified labeled avidin-biotin (LAB) technique in which a biotinylated secondary antibody forms a complex with peroxidase-conjugated steptavidin molecules. In the assay, endogenous peroxidase activity can be quenched by first incubating the specimens for five minutes in 3% hydrogen peroxide. Then specimens were incubated with an appropriately characterized and diluted rabbit, mouse or goat primary antibody, followed by sequential incubations with biotinylated link antibody and peroxidase-labelled steptavidin. Staining was completed after incubation with substrate-chromagen solution. This was followed by counter staining with hematoxylin. Examining the slides was done as soon as possible. Counting of immunoreactivity among different types of cells (eosinophils, macrophages, lymphocytes, and neutrophils) was done in at least 400 cells (using more than one slide in cases of low total leukocyte counts.

Statistical Analysis

All statistical analyses were carried out using SPSS (Statistical Package for the Social Science) version 9.02 for Windows system. Data were expressed as mean ± standard deviation (SD). As regards parametric data, ANOVA test (F-value) was used for comparison between more than 2 groups and the student's "t" test of significance to compare between 2 groups. Paired-t test was applied to compare between the same group in two repeated

measurements. For non-parametric data Kruskall-Wallis test (H value) was used for comparison between more than 2 groups, and the Mann-Whitney test (Z-value) was used to compare between two groups, whereas, comparison between the same group in two repeated measurements was done via Wilcoxon-signed rank test. The Pearson's correlation coefficient (r) was used to interrelate the numeric variables. P value <0.05 was considered significant.

RESULTS

Total and differential cell counts in the sputum of the studied groups (table 1)

Children during acute asthma exacerbation had significantly higher sputum total cell, eosinophil, lymphocyte, and neutrophil counts than controls; however, their sputum monocyte count was significantly lower than controls ($p<0.01$ for all). During quiescence, sputum monocyte and total cell counts were significantly lower, while sputum lymphocyte and neutrophil counts were significantly higher as compared to controls. Sputum eosinophil counts were statistically comparable ($p>0.05$). Patients during asthma exacerbations had higher sputum total cell, eosinophil and neutrophil counts than during remission. Sputum monocyte and lymphocyte counts were statistically comparable during exacerbation and quiescence.

MCP-4 and CCR3 were expressed on eosinophils and monocytes. Lymphocytes expressed only MCP-4. Percentages of sputum total cells, eosinophils, monocytes, and lymphocytes expressing MCP-4 and percentages of sputum total cells, eosinophils, and monocytes expressing CCR3 were significantly higher in patients during both asthma exacerbation and quiescence than controls ($p<0.01$ for all). Percentage of total and individual sputum cells expressing MCP-4 and CCR3 during asthma exacerbation was significantly higher than in-between attacks ($p<0.01$).

Percentage of sputum cells expressing MCP-4/CCL-13 and CCR3 in relation to severity of acute asthma exacerbations, and asthma grading between exacerbations (tables 2 & 3; fig. 1)

The absolute blood and sputum eosinophil counts were significantly higher in patients with severe asthmatic attacks than those with mild and moderate attacks. Percentage of sputum total cells, eosinophils, monocytes and lymphocytes expressing MCP-4; and total cells and eosinophils expressing CCR3 were significantly higher in patients with severe than those with mild and moderate exacerbation. Monocytes expressing CCR3 were significantly lower in patients with severe attacks than those with mild attacks ($p<0.01$). The percentages of lymphocytes and monocytes expressing MCP-4, and the percentages of total cells and monocytes expressing CCR3 were statistically comparable in the mild and moderate groups (table 2; fig. 1).

Total serum IgE, absolute blood eosinophil count, and the total and differential sputum cell counts were statistically comparable in intermittent and persistent asthma. Patients with mild persistent asthma had higher percentage of total cells and monocytes expressing MCP-4, and eosinophils expressing CCR3 than those with intermittent asthma. Children with moderate persistent asthma had higher percentage of eosinophils expressing CCR3 than those with intermittent asthma (table 3).

Percentage of sputum cells expressing MCP-4/CCL-13 and CCR3 in relation to therapy and asthma variables (table 4; fig. 2 & 3):

The type of controller medication could not be related to the total serum IgE. However, it affected blood and sputum eosinophils counts where the highest count was found in children receiving inhaled steroid therapy. There was a significant difference concerning percentages of monocytes expressing MCP-4 and percentages of total cells expressing CCR3 in response to controller medications (table 4).

There was a significant positive correlation between blood and sputum eosinophils counts ($r = 0.73$; $p<0.05$). Sputum eosinophil percentage was positively correlated to the percentage of eosinophils expressing MCP-4 and CCR3 during acute attacks ($r = 0.69$, $p<0.0001$; $r = 0.62$, $p<0.001$, respectively; fig. 2). Moreover, the percentages of sputum eosinophils expressing MCP-4 and those expressing CCR3 were positively correlated ($r = 0.95$, $p<0.0001$; fig. 3). There were significant negative correlations between PEFR and percentage of sputum total cells, eosinophils, lymphocytes, and monocytes expressing MCP-4 on the one hand and the percentage of total cells and eosinophils expressing CCR3 on the other.

The immunohistochemical staining of MCP-4/CCL-13 and CCR3 in sputum eosinophils during and in-between acute asthmatic attacks are shown in figures 4 and 5 respectively.

Table 1. Total and differential cell counts and percentage of cells expressing monocyte chemotactic protein-4 (MCP-4/CCL-13) and CC chemokine receptor 3 (CCR3) in the sputum of the studied groups. Values are given as mean ± SD.

Variable	Asthma exacerbation (n= 30)	Asthma quiescence (n= 30)	Controls (n= 20)	Asthma exacerbation vs. controls t or z §	Asthma quiescence vs. controls t or z §	Asthma exacerbation vs. quiescence t or z §
Sputum total and differential cell count (cells/10^6 X mL)						
Total count	**4.14 ± 1.62**	**2.26 ± 0.92**	**2.92 ± 0.39**	**3.94 ****	**2.49 ***	**2.4 ***
Eosinophils	**0.48 ± 0.31**	**0.12 ± 0.12**	**0.14 ± 0.84**	**5.04§ ****	**0.78§**	**4.92 ****
Monocytes	**1.37 ± 0.17**	**0.99 ± 0.46**	**2.15 ± 0.28**	**4.64§ ****	**10.9 ****	**1.21**
Lymphocytes	**0.35 ± 0.17**	**0.29 ± 0.19**	**0.16 ± 0.07**	**3.67§ ****	**2.1§ ***	**1.1**
Neutrophils	**1.89 ± 0.86**	**0.85 ± 0.38**	**0.5 ± 0.12**	**8.63 ****	**4.56 ****	**2.21 ***
Percentage of cells expressing Monocyte Chemotactic Protein-4 (MCP-4/CCL-13)						
Total cells	**10.81 ± 4.3**	**3.66 ± 1.06**	**2.36 ± 0.52**	**10.63 ****	**5.79 ****	**8.66 ****
Eosinophils	**19.6 ± 11.5**	**4.57 ± 1.98**	**2.55 ± 0.51**	**5.94§ ****	**5.33 ****	**4.76 ****
Monocytes	**21.47 ± 7.1**	**6.5 ± 2.61**	**2.90 ± 0.72**	**14.05 ****	**7.16 ****	**16.4 ****
Lymphocytes	**15.7 ± 7.17**	**5.1 ± 2.04**	**2.75 ± 0.55**	**9.84 ****	**5.99 ****	**7.92 ****
Percentage of cells expressing CC Chemokine Receptor 3 (CCR3)						
Total cells	**7.77 ± 3.33**	**2.41 ± 0.56**	**1.96 ± 0.35**	**9.49 ****	**3.52 ****	**6.62 ****
Eosinophils	**37.4 ± 15.8**	**8 ± 3.06**	**2.45 ± 0.60**	**12.06 ****	**9.65 ****	**10.6 ****
Monocytes	**8.90 ± 2.19**	**4.63 ± 0.96**	**2.55 ± 0.51**	**15.29 ****	**9.93 ****	**8.6 ****

*: Significant difference ($p < 0.05$); **: Highly significant difference ($p<0.01$); §: Mann-Whitney test

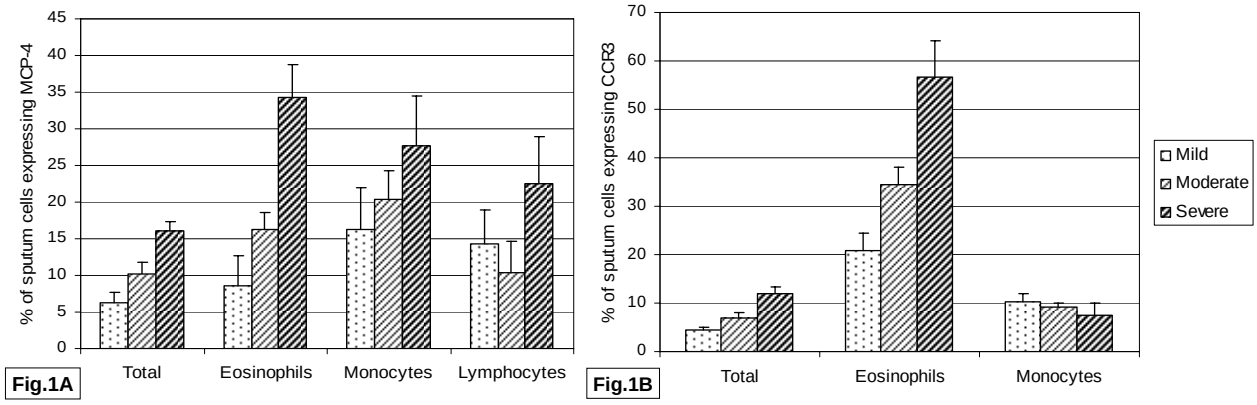

Figure 1. Percentage of sputum cells expressing monocyte chemotactic protein-4 (MCP-4/CCL-13) (fig. 1A) and CC chemokine receptor 3 (CCR3) (fig. 1B) according to the degree of severity of acute asthmatic attacks.

Table 2. Blood eosinophils, serum total IgE and percentage of sputum cells expressing monocyte chemotactic protein-4 (MCP-4/CCL-13) and CC chemokine receptor 3 (CCR3) according to the degree of severity of acute asthmatic attacks. Values are given as mean ± SD.

Variable	Mild exacerbation (n =10)	Moderate exacerbation (n =10)	Severe exacerbation (n = 10)	Mild vs. Moderate t or Z §	Severe vs. Mild t or Z §	Severe vs. Moderate t or Z §
IgE (IU/mL)	331 ± 309.2	804 ± 666.7	2092 ± 1518	1.24 §	1.23 §	1.2 §
Blood eosinophils (cells X 10^9/L)	0.21 ± 0.15	0.33 ± 0.22	0.59 ± 0.21	1.72 §	3.3 § *	2.3 § *
Sputum total and differential cell count (cells/10^6 X mL)						
Total cells	3.54 ± 1.51	3.95 ± 1.57	4.92 ± 1.62	1.2	1.9 *	0.9
Eosinophils	0.22 ± 0.07	0.38 ± 0.15	0.85 ± 0.23	1.92 § *	4.1§ **	3.32 § **
Monocytes	1.17 ± 0.66	1.4 ± 0.55	1.54 ± 0.85	0.75 §	0.11 §	1.1 §
Lymphocytes	0.26 ± 0.14	0.38 ± 0.2	0.41 ± 0.12	0.52 §	0.73 §	1.1 §
Neutrophils	1.88 ± 0.81	1.65 ± 1.04	2.13 ± 0.73	1.4 §	1.2 §	0.1 §
Percentage of cells expressing MCP-4/CCL-13						
Total cells	6.25 ± 1.48	10.2 ± 1.55	15.99 ± 1.42	5.11**	3.5 **	4.02 **
Eosinophils	8.5 ± 4.25	16.2 ± 2.39	34.2 ± 4.54	3.4 § **	3.1 § **	4.4§ **
Monocytes	16.3 ± 5.6	20.4 ± 3.89	27.7 ± 6.77	1.24	3.21 **	2.9 **
Lymphocytes	14.2 ± 4.78	10.4 ± 4.2	22.5 ± 6.35	1.23	4.2 **	4.52 **
Percentage of cells expressing CCR3						
Total cells	4.46 ± 0.47	6.9 ± 1.09	11.96 ± 1.34	3.1	4.1 **	4.58 **
Eosinophils	20.9 ± 3.67	34.5 ± 3.47	56.8 ± 7.35	4.22 § **	5.12 § **	5.44 § **
Monocytes	10.2 ± 1.75	9.1 ± 0.99	7.4 ± 2.63	1.65	3.8 **	0.89

*: Significant difference ($p < 0.05$); **: Highly significant difference ($p<0.01$); §: Mann-Whitney test

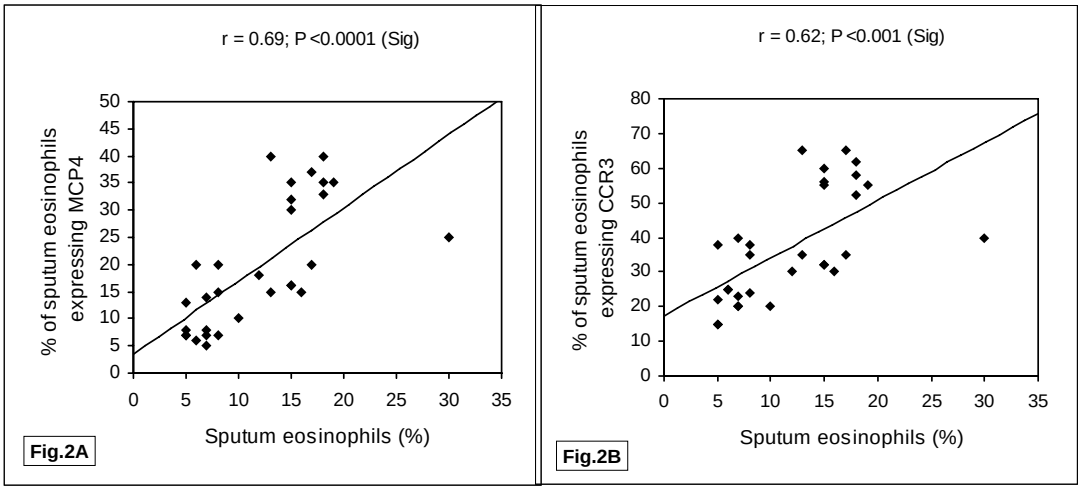

Figure 2. Positive correlations between the percentage of sputum cells expressing monocyte chemotactic protein-4 (MCP-4/CCL-13) (fig. 2A) and CC chemokine receptor 3 (CCR3) (fig. 2B) and the percentage of sputum eosinophils among asthmatic children during acute exacerbations.

Table 3. Blood eosinophils, serum total IgE and percentage of sputum cells expressing monocyte chemotactic protein-4 (MCP-4/CCL-13) and CC chemokine receptor 3 (CCR3) between asthmatic attacks. Values are given as mean ± SD

Variable	Intermittent (no = 4)	Mild persistent (no = 13)	Moderate persistent (no = 13)	Mild persistent vs. intermittent	Moderate persistent vs. intermittent	Moderate persistent vs. mild persistent
				t or z§	t or z§	t or z§
IgE (IU/ml)	821 ± 748	708 ± 537	1823 ±1274	1.1 §	1.44 §	1.3 §
Blood eosinophils (cells X 10^9/mL)	0.11 ± 0.08	0.14 ± 0.1	0.15 ± 0.09	0.33 §	0.5 §	0.41 §
Sputum total and differential cell count (cells/10^6 X mL)						
Total cells	1.95 ± 0.72	2.03 ± 0.8	2.58 ± 1.04	0.51 §	0.56	0.24
Eosinophils	0.09 ± 0.04	0.1 ± 0.05	0.15 ± 0.1	0.09 §	0.21§	0.11§
Monocytes	0.92 ± 0.25	0.9 ± 0.4	1.1 ± 0.57	0.05 §	0.07 §	0.8 §
Lymphocytes	0.21 ± 0.14	0.24 ± 0.2	0.35 ± 0.19	0.07 §	0.24 §	0.34 §
Neutrophils	0.72 ± 0.38	0.78 ± 0.3	0.96 ± 0.39	0.02 §	0.06 §	0.36 §
Percentage of cells expressing MCP-4/CCL-13						
Total cells	2.75 ± 0.96	4.12 ± 1	3.47 ± 0.96	1.93 *	1.6	1.3
Eosinophils	4 ± 1.4	4.62 ± 2.2	4.7 ± 1.9	0.11	0.16	0.04
Monocytes	4.5 ± 2.5	7.6 ± 2.6	6 ± 2.24	2.37 § *	1.8 §	0.58 §
Lymphocytes	5.3 ± 3.3	5.5 ± 1.3	4.6 ± 2.3	0.06 §	0.27 §	0.46 §
Percentage of cells expressing CCR3						
Total cells	2.9 ± 0.5	2.35 ± 0.5	2.3 ± 0.58	0.4	0.31	0.01
Eosinophils	6.7 ± 0.5	8.15 ± 2.8	8.23 ± 3.75	1.9 *	1.95 *	0.51
Monocytes	5.25 ± 0.5	4.54 ± 1.1	4.54 ± 0.9	0.38	0.39	0.05

*: Significant difference ($p < 0.05$); §: Mann-Whitney test

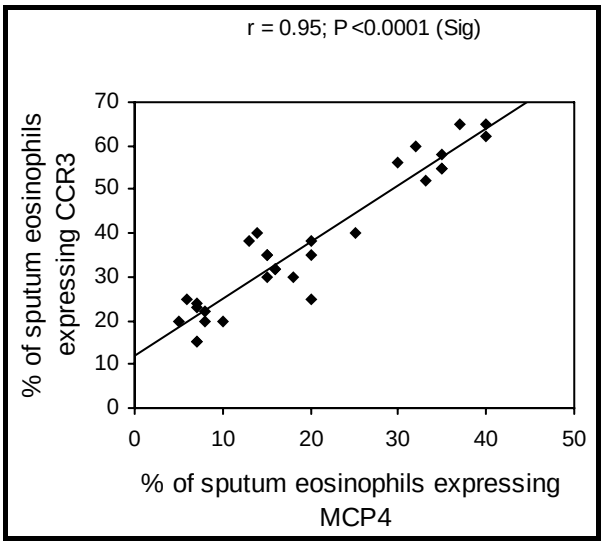

Figure 3. Positive correlation between the percentage of sputum eosinophils expressing monocyte chemotactic protein-4 (MCP-4/CCL-13) and CC chemokine receptor 3 (CCR3) among asthmatic children during acute exacerbations.

Table 4. Blood eosinophils, serum total IgE and percentage of sputum cells expressing monocyte chemotactic protein-4 (MCP-4/CCL-13) and CC chemokine receptor 3 (CCR3) according to therapeutic intervention. Values are given as mean ± SD.

Variable	Patients not receiving controller medication (n =3)	Patients receiving long-acting theophylline (n = 7)	Patients receiving inhaled steroids (n = 10)	Patients receiving both (n = 10)	F or H§
IgE (IU/ml)	948.3 ± 778.6	305 ± 287	1225.8 ± 1060	1028 ± 927	2.66 §
Blood eosinophils (cells X 10^9/mL)	0.28 ± 0.03	0.14 ± 0.13	0.5 ± 0.26	0.46 ± 0.25	10.67 § *
Sputum total and differential cell count (cells/10^6 X mL)					
Total cells	2.93 ± 1.58	3.26 ± 1.75	4.48 ± 1.49	4.67 ± 1.45	5.33 §
Eosinophils	0.28 ± 0.14	0.23 ± 0.09	0.65 ± 0.28	0.58 ± 0.34	9.8 § *
Monocytes	1.27 ± 0.63	1 ± 0.61	1.16 ± 0.5	1.6 ± 0.73	4.12 §
Lymphocytes	0.23 ± 0.14	0.22 ± 0.19	0.4± 0.16	0.4 ± 0.14	8.16 §
Neutrophils	1 ± 0.73	1.8 ± 1.2	2.27 ± 0.8	1.9 ± 0.66	3.54 §
Percentage of cells expressing MCP-4/CCL-13					
Total cells	3.7 ± 2	3.8 ± 1	4 ± 0.5	3.4 ± 0.9	2.3 §
Eosinophils	4.3 ± 0.6	3.6 ± 1	6 ± 3	4.6 ± 1.9	3.2 §
Monocytes	5.3 ± 4	7 ± 2.5	9.6 ± 0.9	5.5 ± 1.9	9.6 § *
Lymphocytes	7.3 ± 2.5	4.4 ± 1	4.8 ± 0.4	5 ± 2.4	1.5
Percentage of cells expressing CCR3					
Total cells	3.2 ± 0.3	2.3 ± 0.1	2 ± 0.2	2.4 ± 0.6	3.3 *
Eosinophils	7.7 ± 2	7.6 ± 1.9	10.6 ± 5.6	7.4 ± 2.3	1.27 §
Monocytes	5.7 ± 1	4.4 ± 1	4.8 ± 0.5	4.5 ± 0.9	1.53

*: Significant difference ($p < 0.05$); §: Kruskall -Wallis test

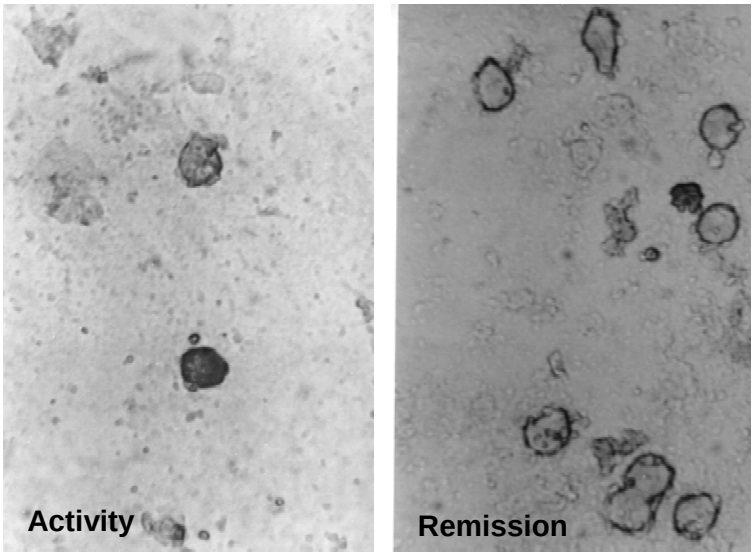

Figure 4. Immunohistochemical staining of MCP-4/CCL-13 in sputum eosinophils of asthmatic children during and between acute exacerbations.

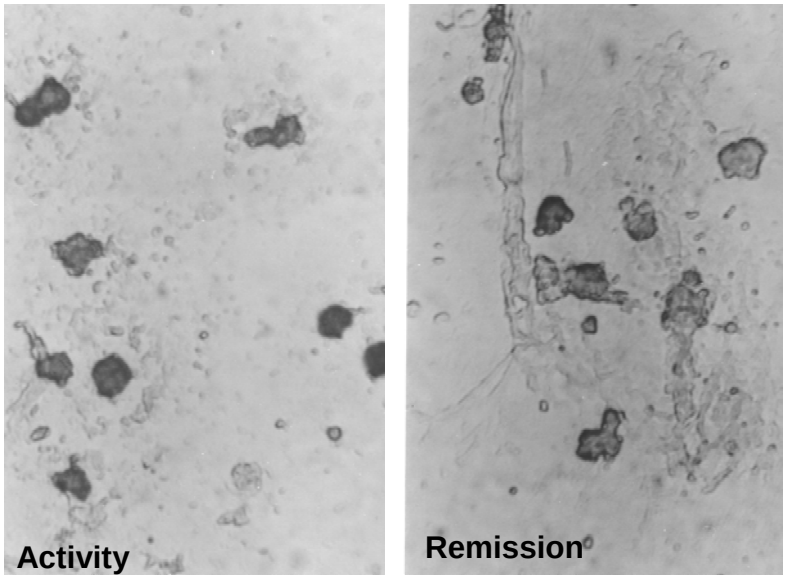

Figure 5. Immunohistochemical staining of CCR3 in sputum eosinophils of asthmatic children during and between acute exacerbations.

DISCUSSION

MCP-4 expression is known to be increased in asthmatic airways where it is induced by proallergic cytokines. Chemokine expression in broncho-alveolar lavage (BAL) and bronchial biopsy specimens has been demonstrated in asthmatic airways, but not thoroughly investigated in induced sputum of asthmatic children. Moreover, the mechanisms responsible for cellular recruitment of eosinophils in the small airways of asthmatic individuals remain to be clarified[1]. Therefore, we sought to determine the expression of MCP-4 and its receptor CCR3 on induced sputum cells of asthmatic children.

During acute asthma exacerbation, the percentage of total and differential sputum cells expressing MCP-4 and CCR3 were significantly higher in comparison to the corresponding values in the stable state and in comparison to controls. These data conform with several other reports[7,13-23]. Taha et al.[18] reported increased MCP-4 immunoreactivity in induced sputum of asthmatic patients in comparison to control subjects. In addition, Kalayci et al.[7] reported that MCP-4 is a systemically expressed biomarker of asthma. MCP-4 mRNA and protein were significantly upregulated in the epithelium and submucosa of bronchial biopsies and in BAL cells of patients with asthma compared with normal subjects[14-17,20]. The results of this study may reflect the up regulation of CCR3 and its ligand, MCP-4, in allergic inflammation, presenting potentially useful markers for the presence of atopic response.

CCR3 had been identified as a major CC chemokine receptor on eosinophils[22]. Its expression had been demonstrated on basophils[13] and Th2-type T cells[20] suggesting possible roles for CCR3 in the genesis and maintenance of allergic inflammation. MCP-4 induces migration of eosinophils through at least two receptors; CCR2b and CCR3, however, CCR3 is the selective receptor on eosinophils and to a lesser extent on lymphocytes for MCP-4[21]. In this respect, Fujisawa and associates[24] proved that MCP-4-induced eosinophil degranulation is mediated only through CCR3. The concordant expression of CCR3 and its ligand in the airway, contributes to bronchial eosinophilia in atopic asthma. MCP-4 might play a role in release of eosinophils from bone marrow into the circulation and the recruitment of eosinophils from blood into the airways to contribute to the small airways and peripheral lung inflammation in asthma. MCP-4/CCR3 may be therefore a major target for anti-inflammatory drug development.

When asthmatic children were followed-up to stability, sustained elevation of the percentage of total and differential sputum cells expressing MCP-4 and CCR3 in comparison to control values was found. This could be due to the ongoing process of sub-clinical allergic inflammation in the lungs of persistent asthmatics during clinical remission. Moreover, patients with mild persistent asthma had higher percentage of total cells and monocytes

expressing MCP-4, and eosinophils expressing CCR3 than those with intermittent asthma. Children with moderate persistent asthma had higher percentage of eosinophils expressing CCR3 than those with intermittent asthma. This might reflect the ongoing inflammatory allergic process with persistent infiltration of airways by inflammatory cells particularly lymphocytes even after subsidence of exacerbations. These cells release mediators that in turn recruit more and more inflammatory cells in a vicious circle manner. Such observations could be indicative of the continued exposure to triggering factors and/or the need for more intensive anti-inflammatory treatment. Therefore, we suggest that MCP-4/CCR3 could be used as markers of the ongoing airway inflammatory activity.

CCR3 and its ligand have been suggested as useful objective markers for the severity of asthma exacerbations. The percentage of sputum total cells, eosinophils, monocytes and lymphocytes expressing MCP-4, and total cells and eosinophils expressing CCR3 were significantly higher in patients with severe than those with mild and moderate exacerbations. Furthermore, CCR3 and MCP-4 expression were observed to be inversely correlated with PEFR. This probably reflects excessive influx of inflammatory cells in acute severe attacks causing injury to bronchial mucosa, mediating bronchospasm and culminating in bronchial obstruction and impaired lung function and in turn the severity of acute exacerbations.

Noteworthy, Kalayci and coworkers[7] found that systemic expression of MCP-4 was significantly higher in patients with chronic stable asthma than in normal subjects, and even higher in matched subjects experiencing acute severe asthma exacerbation. They suggested that a systemically detectable MCP-4 level is an important independent marker of the inflammatory state that occurs in asthma. Furthermore, the observation that patients with an acute exacerbation of asthma had higher plasma levels of MCP-4 than did matched subjects with chronic-stable asthma suggests that the plasma MCP-4 is further increased in active disease or, alternatively, that individuals with higher MCP-4 levels were more prone to suffer severe asthma exacerbation and may benefit from abrogation of MCP-4.

In this study, absolute blood and sputum eosinophil counts were significantly higher in patients with severe than those with moderate and mild asthma exacerbations. Many studies[13,18,21] had suggested a strong association among eosinophil numbers, their state of activation, and the severity of asthma. Eosinophils are thought to be an important effector cell involved in the induction of bronchial mucosal damage by the release of cytotoxic proteins, reactive oxygen metabolites, and proinflammatory and profibrotic cytokines[1].

The positive correlation between the percentages of sputum eosinophils expressing MCP-4 and those expressing CCR3 during exacerbations probably reflects excessive MCP-4 production from bronchial epithelial cells during bursts of bronchial asthma.

In the present study, sputum eosinophil percentage was positively correlated to the percentage of eosinophils expressing MCP-4 and CCR3 during acute attacks. A significant association between the concentrations of MCP-4 and eosinophil numbers in BAL fluid had been reported[19,24]. These findings suggest that MCP-4 regulates eosinophil trafficking into the airways of asthmatic children in a coordinated manner. In contrast, Taha et al [18] did not find such a correlation.

Human blood eosinophils express high levels of CCR3, which is responsible for both migration and degranulation [1]. Liu et al. [25] studied blood and BAL cells that were obtained from allergic subjects 48 hours after segmental bronchoprovocation with antigen. They reported that there was a high expression of CCR3 (nearly 100%) on blood eosinophils and this expression did not change after segmental bronchoprovocation with allergen. Furthermore, expression of CCR3 was significantly correlated with the percentage of eosinophils in BAL fluid at 48 hours. These data provide evidence that as eosinophils are recruited to the airway during an allergic inflammatory response, they demonstrate different phenotypic characteristics manifested, in part, by a different profile of chemokine receptors. These changes might provide insight into the differential regulation of eosinophil recruitment and activation in allergic airway disease.

The effect of inhaled corticosteroid therapy on CCR3 and its ligand was found by some investigators to be highly impressive[8,26]. However, in the current study, the effect of steroids was restricted to the increase in percentage of sputum monocytes expressing MCP-4 and a generalized decrease in total sputum cells expressing CCR3.

Fukakusa et al.[26] demonstrated a dual nature of corticosteroids. Although inhaled corticosteroids down regulates TH 2 cytokines and reduces the number of eotaxin MCP-3, and MCP-4 mRNA-positive cells in the epithelium and subepithelium, they can also up regulate the expression of other

chemokines, including IL-8, IFNγ-inducible protein 10 (IP-10), and MCP-2.

Moreover, the percentage of blood eosinophils and sputum eosinophils were higher in asthmatic patients receiving inhaled steroids, whether alone or with long-acting theophylline, than those not receiving them. The increase in blood and sputum eosinophils may be related to the severity of asthma not to the effect of steroids. Hughes[27] reported that subjects taking inhaled corticosteroids did not differ significantly from those on β2-agonists ± cromolyn with respect to circulating blood eosinophils. Noteworthy, the mechanism of the glucocorticoids effect is multifactorial involving inhibition of both T-cell function and eosinophil recruitment. Inhaled corticosteroids have been shown to decrease the number of eosinophils but to increase neutrophils. Corticosteroids reduce the number of CD3-positive T cells and major basic protein-positive eosinophils[26]. Interpretation of the results of this study may be complicated by the existence of additional variables such as disease state, subject variability and the small number of children recruited in the study. Therefore, further studies involving higher number of patients with similar asthma severity or following up patients before and after treatment with different asthma therapies are recommended.

Eosinophils represent one of the main effector cell populations of allergic airway inflammation and allergic bronchial asthma. Their infiltration correlates with many characteristics of the disease, including airway hyperresponsiveness and increased mucus production. CCR-3 is the principle chemokine receptor involved in eosinophil attraction into inflamed tissue. Therefore, antagonizing CCR-3 could be a novel promising approach toward asthma therapy.

YM-355179 is a novel, selective, and orally available CCR3 antagonist with therapeutic potential for treating eosinophil-related allergic inflammatory diseases. Oral administration of YM-355179 (1 mg/kg) inhibited chemokine-induced shape change of whole blood eosinophils in monkeys. It also inhibited eosinophil degranulation and eosinophil infiltration into airways after segmental bronchoprovocation with eotaxin[5,28]. In addition, blocking CCR3 on eosinophils, with a monoclonal antibody, completely abolished eosinophil responses to these chemokines. The reduction in eosinophil infiltration was accompanied by normalization of airway hyperresponsiveness and prevention of goblet cell hyperplasia, indicating reduced mucus production. Furthermore, antagonizing CCR-3 prevented airway remodeling as defined by subepithelial fibrosis and increased accumulation of myofibrocytes in the airway wall of chronically challenged model[29].

In conclusion, MCP-4 and CCR3 expression on induced sputum cells were up regulated during acute asthma exacerbation and this increase was related to exacerbation severity. MCP-4 and its ligand might contribute to small airways and peripheral lung inflammation in asthma. They are expected to have biological significance in the regulation of eosinophil recruitment into the asthmatic bronchi leading to development of the pathophysiological endpoint of asthma, the airway hyperreactivity. In addition, they seem to be involved in the establishment and maintenance of chronic inflammation of the airways. We recommended the use of induced sputum as a non-invasive technique in studying chemokine expression in children with bronchial asthma. Setting pediatric reference ranges for MCP-4 in biological fluids, and exploring CCR3 expression on different immune cells involved in atopy are worthwhile. Moreover, the effect of MCP-4 and CCR3 antagonism should be considered attractive targets for specific anti-inflammatory therapy.

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