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Original article

Increased expression of T- cell- surface CXCR4 in asthmatic children.

Background: *Signals delivered through the chemokine receptor CXCR4 upon interaction with its ligand, SDF-1 α/β result in the most efficacious chemoattraction of T lymphocytes to the asthmatic airways with the resultant lung inflammation and airway hyperresponsiveness .*
Objective: *The extensive pharmacological and physiological evidence that CXCR4 chemokine receptor influences the allergic airway disease has stimulated us to study the relation between its expression in peripheral blood T lymphocytes and the exacerbation of asthmatic attacks of varying severity.*
Methods: *The chemokine receptor CXCR4 was assayed by flow cytometry in peripheral blood T lymphocytes from 25 asthmatic children, during asthma exacerbation and after complete remission of symptoms and physical signs. The results were compared to those of 30 healthy children.*
Results: *The CXCR4 expression in peripheral blood T lymphocytes was significantly increased in children with acute exacerbations of bronchial asthma as compared to controls (mean ± SD = 62.27 ± 17.57% versus 24.76 ± 6.88%; p<0.001). After remission of acute attacks, the CXCR4 expression decreased significantly as compared to the values during attacks (mean ± SD = 40.90 ± 13.25%), however, the level of expression during quiescence was still significantly higher than the values of the controls (mean ± SD = 40.90 ± 13.25%; p<0.001). The CXCR4 expression was significantly higher in children with acute severe asthma as compared to those with either mild or moderate attacks. During remission, patients with mild intermittent asthma had less expression of CXCR4 when compared to any grade of persistent asthma, while the results were comparable between all groups of persistent asthma of varying severity. A significant positive correlation could link the CXCR4% to the absolute eosinophilic count during acute asthma attacks.*
Conclusion: *CXCR4 is over-expressed in T lymphocytes of asthmatic children. It was found to be related to disease activity and seems to be involved in the establishment and maintenance of chronic inflammation of the airways.*

Key words: Asthma, chemokine receptors, CXCR4, children, T lymphocytes.

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INTRODUCTION

Chemokines are a group of cytokines that are responsible for the influx of blood cells, including T and B lymphocytes, monocytes, neutrophils, eosinophils and basophils in allergic and other inflammatory conditions. They function as G-protein-coupled chemotactic factors which also activate the cells with which they interact. Certain chemokines function within the afferent arm of the immune system, in which antigen is processed and antibody formation initiated and others are active within the effector pathways of cellular immunity and late phase allergic reactions [1]. On the basis of their sequence of arranged cysteine groups, chemokines are categorized into 4 subfamilies: the alpha (CXC), beta (CC), gamma (C) and delta (CX3C) chemokines [2].

The interaction between chemokines and their receptors is an important step in the control of leukocyte migration into sites of inflammation. Chemokines also mediate a variety of effects independent of chemotaxis, including induction and enhancement of Th1 and Th2 associated cytokine responses [3].

One of the typical aspects of airway inflammation is the infiltration of Th2 cells and differentiation of B cells into IgE secretory plasma cells through a complex cascade of events in which adhesion molecules and chemokine receptor CXCR4 plays a crucial role [4].

Studies on the properties of peripheral blood T-cells in adult asthma have suggested that the

properties of these cells reflect those of cells in the bronchial mucosa owing perhaps to recirculation of the activated T-cells into the peripheral blood [5]. Owing to this observation, together with the lack of data on CXR4 expression in peripheral blood T lymphocytes in pediatric asthma we carried out this study to verify the possible changes in its level in relation to asthma activity and severity in children.

METHODS

Study population:

This follow up study comprised 25 asthmatic and 30 healthy children. An informed consent was obtained from their parents before enrollment. Their demographic data were as follows:

A) Patients:

The patients were 25 asthmatic children recruited from the Pediatric Allergy and Immunology Unit, Children's Hospital, Ain Shams University, Cairo, Egypt, during the period from August to October 2002. The diagnosis of asthma was established according to the American Thoracic Society (ATS) criteria[6]. They were 14 males and 11 females. Their ages ranged from 5 to 12 years with a mean value of 7.3 ± 2.4 years.

The patients were evaluated during acute asthmatic attacks and were classified into 10 children with acute mild, 11 with acute moderate and 9 with acute severe asthma. Our patients were followed up and re-evaluated in between attacks. They were subdivided according to the grading of asthma disease into 6 patients with mild intermittent asthma, (MI), 9 with mild persistent asthma (MP), 6 with moderate persistent asthma (OP), and 4 patients with severe persistent asthma (SP). The severity of asthma during and in between attacks was assessed according to GINA guidelines criteria[7].

At the time of initial sampling, 11 children were on corticosteroid therapy (inhaled fluticasone propionate 100-300 μg/day) together with other medications such as β2 agonists and/or theophyllin while 14 children were not receiving steroids.

B) Controls:

This group comprised 30 healthy children. They were 18 males and 12 females. Their ages ranged from 4 to 12 years (mean ± SD = 7.4 ± 2.5years). These children had no personal or family history of atopy.

Study measurements:

Patients were subjected to history taking, clinical examination, plain x-ray chest postero-anterior view, complete blood counting, serum total IgE by ELISA and measurement of CXCR4 by flow cytometry

Blood Sampling:

Five ml of venous blood were drawn from each patient and divided as follows:

a- 3 ml dispensed gently into a tube containing EDTA as anticoagulant (1.2 mg/m1). This anticoagulated blood was used for performing both complete blood counting and CXCR4 analysis.

b- 2 ml were left to clot. Serum separated and collected in sterile clean tubes. Samples were preserved at -20˚C until used for IgE assay.

Flow cytometric analysis: Surface CXCR4 analysis was done on T-lymphocytes by flow cytometry[8] using Coulter EPICS XL (Coulter electronics, Hielaeh FI, USA) by direct immunofluorescence technique. The analysis was performed on whole blood, and lysis of red blood cells was done by adding 3 ml NH4Cl (0.83% buffered with $KHCO_3$, pH 7.2) for 5 minutes at 37˚C. Optimal dilutions of CXCR4 antibody labeled with phycoerythrin (CXCR4-PE) were used along with isotype-matched negative controls. Lymphocytes were electronically gated using light scatter parameters (forward and side scatters). The values were expressed in %. A sample was considered positive when > 20% of the cells showed the marker. The CXCR4 monoclonal antibody was purchased from R&D Systems (Kinley Palace N.E., Minneapolis, MN, USA).

Serum total IgE: Immunoglobulin E was measured by ELISA technique (Medix Biotech Inc., Agenzyme Company, San Carios CA, USA). The value of IgE used for data analysis was the percentage from the highest normal for age[9] as follows: *patient's actual value/ highest normal value for age x100*.

Complete blood counting: This included total and differential leucocytic count, band cells, absolute eosinophilic and basophilic counts using Coulter Counter (Coulter MicroDiff 18, Fullerton CA, USA). Children whose absolute eosinophil counts exceeded 700 were considered to have peripheral blood eosinophilia [9]. Blood sampling of all subjects was performed at the same time daily (10 am) to avoid diurnal variations in eosinophil counts.

Statistical Analysis:

The results were statistically analyzed via a standard computer program (StatView). For non parametric data, Mann-Whitney (U) test was used

for inter-group analysis, and the Spearman correlation coefficient (r) tests were used for intra-group analysis. p values < 0.05 were considered significant.

RESULTS

Results of CXCR4 expression

The percentage of CXCR4 expression on peripheral blood T lymphocytes during acute asthmatic attacks ranged from 30% to 99 % (median = 63.7%; mean ± SD = 62.27 ± 17.57%). These values were significantly higher than the corresponding values of the same patients when studied in between the attacks which ranged from 19% to 73 % (median = 40%; mean ± SD = 40.90 ± 13.25 %). The healthy children had significantly lower CXCR4 expression (range = 16-48%; median = 23%; mean ± SD = 24.76 ± 6.88%) as compared to asthmatics both in acute attacks and in remission (table 1 and figure 1).

Table (1): Comparison between the studied groups in CXCR4 expression on T-cells.

Groups	Mean ± SD	Z	p
Asthmatics in acute attacks vs controls	62.27±17.57 vs 24.76±6.88	6.18	<0.001
Asthmatics in remission vs controls	40.90±13.25 vs 24.76± 6.88	4.68	<0.001
Asthmatics in acute attacks vs asthmatics in remission	62.27±17.57 vs 40.90±13.25	4.80	<0.001

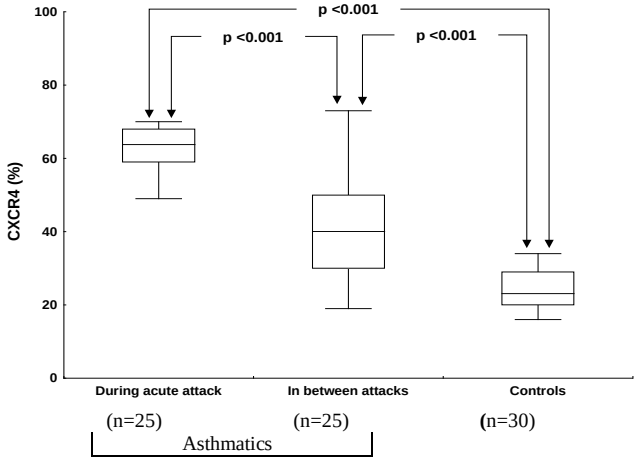


Figure (1): CXCR4 expression in peripheral blood T-lymphocytes in the studied groups.
Horizontal lines indicate the median values and boxes enclose the interquartiles. The ranges are marked as maximum and minimum .

Analysis of CXCR4 results of patient subgroups during acute asthmatic attacks

A significant increase in CXCR4 expression with increased severity of the asthmatic attacks was observed. The CXCR4 expression was significantly higher in children with acute severe asthma as compared to those with either acute mild or moderate asthma. Also, CXCR4% was significantly higher in children with moderate when compared to those with mild attacks (table 2).

Table (2): Variation of CXCR4% results with severity of acute asthmatic attacks.

	Mild (n=7)	Moderate (n=10)	Severe (n=8)
Mean ±SD	42.14 ±10.57	62.67 ±2.34	79.40 ±14.67
Vs mild			
Z		3.37	3.24
p		<0.001	<0.001
Vs Moderate			
Z			3.55
p			<0.001

Analysis of CXCR4 results of patient subgroups during remission

Patients with mild intermittent asthma had significantly lower CXCR4% when compared to any group of persistent asthma. The results of CXCR4 expression were comparable between all groups of persistent asthma of varying severity (table 3).

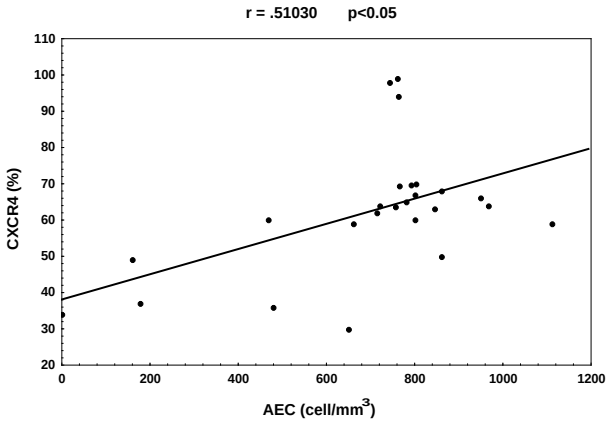
Table (3): Variation of CXCR4% with grades of asthma severity.

	MI (n=8)	MP (n=9)	OP (n=4)	SP (n=4)
Mean ± SD	25.08 ±5.15	41.00 ±6.10	47.00 ±7.74	55.25 ±18.09
Vs MI				
Z		2.23	2.08	2.34
p		<0.05	<0.001	<0.05
Vs MP				
Z			0.53	1.92
p			>0.05	>0.05
Vs OP				
Z				1.91
p				>0.05

MI = Mild intermittent, MP = Mild persistent, OP = Moderate persistent, SP = Severe persistent.

The CXCR4 % was significantly higher in patients with eosinophilia in the peripheral blood as compared to those having normal eosinophil counts during acute asthmatic episodes (mean ± SD = 69.55 ± 13.49 vs 43.57 ± 12.34; p<0.001).

Also, the CXCR4 levels of asthmatic children during acute attacks were positively correlated to the absolute eosinophilic counts (AEC) (figure 2).



AEC: Absolute eosinophilic count.

Figure(2):Positive correlation between CXCR4% and AEC during acute asthmatic attacks.

No significant correlation could be found between the CXCR4% and age, duration of illness, or IgE% during or after remission of acute asthmatic attacks.

DISCUSSION

There is increasing evidence for differential expression of various chemokine receptors on subsets of Th1 and Th2 cells, which might explain their selective migration pattern into inflamed tissues. The Th1 cell subset has been shown to express CXCR 3 and CCR 5, whereas Th-2 subset expresses CCR3, CCR4, CCR8 and CXCR4 receptors[10]. Of these, Th-2 cell-expressed chemokine receptors, CXCR4, and its ligand SDF-1, have been shown to be the most relevant for Th-2-type allergic airway responses [11].

The results presented demonstrate that CXCR4 expression in peripheral blood T lymphocytes was significantly increased in 25 children with acute exacerbation of bronchial asthma as compared to controls. After remission of acute attacks, the CXCR4 expression decreased significantly, however, the levels in between attacks were still significantly higher than the corresponding values of the control group. The persistence of CXCR4 over-expression after remission of exacerbations might be due to the ongoing process of allergic inflammation in the lungs of asthmatics in between attacks as most of our patients had persistent rather than intermittent asthma.

We could not trace basal levels of CXCR4 expression on T cells in children in literature. However, our control values (median =23%) go with previously published data which revealed that only 20% of freshly isolated lymphocytes of healthy adults expressed CXCR4 on their cell surfaces[12].

Our findings are in accordance with an in vitro study in which a single stimulation of peripheral blood mononuclear cells with the allergen Der p resulted in an enhanced surface expression of CXCR4 on about 70% of T cells from atopic adults but not in T cells from non atopic donors[13]. Our results emphasize the in-vivo relevance of their observations and contrasted the proposal that over-expression of CXCR4 in cultured T cells may be a culture induced artifact due to relocation of intracellular CXCR4 in absence of immunologic stimuli[14].

We report a significant increase in CXCR4 expression with increased severity of the asthmatic attacks. This might reflect the excessive trafficking and recruitment of lymphocytes at sites of inflammation through stimulation of CXCR4/SDF-1 axis [15] during acute severe asthmatic attacks.

Patients with mild intermittent asthma had a lower expression of CXCR4 when compared to persistent asthma, while the results of CXCR4 expression were comparable between all categories of persistent asthma of varying severity. This might reflect the ongoing non–stop allergic inflammation inside the airways of children with persistent asthma as compared to the completely reversible changes in those with intermittent asthma.

Our results reflect the importance of CXCR4 up-regulation in induction of acute severe episodes of asthma. This is supported by previously published data which showed that IL-4 up-regulates CXCR4 mRNA expression in $CD4^+$ T lymphocytes with the resultant inflammatory cell recruitment to sites of inflammation[16]. The use of AMD3100 (a specific inhibitor of CXCR4) was found to be associated with a significant reduction in IL-4 and IL-5 levels and a significant increase in IL –12 and IFNγ levels within the lungs of treated allergic mice with the resultant reduction in airway hyperresponsiveness, and overall inflammatory response[17].

The relation between eosinophils and asthmatic inflammation is extensively studied[18]. Neutralizing antibodies to CXCR4 in a mouse model of allergic airway disease reduced lung eosinophilia by half, indicating that CXCR4

mediated signals contribute to lung inflammation and airway hyper-responsiveness[11]. Recently, the functional expression of CXCR4 in eosinophils was reported. SDF-1α was able to induce a strong migratory response comparable to the CCR3/eotaxin system[19]. On the other hand, Gonzalo and associates[11] concluded that the observed decrease in eosinophilia after neutralization of CXCR4 was likely a secondary consequence of reduction of mononuclear cell accumulation in the lung owing to their observation that eosinophils neither express CXCR4 nor respond to SDF-1α.

In our study, the CXCR4% was significantly higher in patients with peripheral blood eosinophilia during acute episodes of asthma as compared to those without eosinophilia. Moreover, a significant positive correlation could link the CXCR4% to the absolute eosinophilic count during acute asthmatic attacks.

We could not establish a link between IgE % and CXCR4 expression in peripheral blood T lymphocytes. This may be explained by the fact that a good number of asthmatics have normal total IgE levels [9] and therefore, the IgE level is not always informative of disease activity although closely related to the pathogenesis.

Taken together, the data presented here demonstrate that CXCR4 is over-expressed in T-lymphocytes of children with bronchial asthma and is closely related to disease activity and persistence of the disease. The realization that Th-2 subsets overexpress specific chemokine receptors in asthma suggests that targeting the correct chemokine receptor may have a significant impact on improvement of atopic asthmatic inflammation.

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