Role of Eosinophil Cationic Protein in Asthma and Confounding Factors.

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Citation

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Abstract

Background: It has been found that activated eosinophils play an important role in the pathogenesis of bronchial asthma.Objective: To clarify the validity of serum Eosinophil Cationic Protein (ECP) as parameter in assessment of asthma since it reflects the pulmonary inflammation in bronchial asthma. Patients and Methods: Serum ECP was determined in 139 asthmatic patients by using enzyme linked immunosorbent assay and compared to control .Results: Serum ECP was very highly significant higher (P<0.00001) in asthmatic patients than in control. There was no overlap between the lower asthmatic limit and higher limit of the control group. In addition, the ECP variation between asthmatic and control was correlated to variations in FEV1 percent predicted (P<0.0001) between both groups. There was an association between serum ECP levels and asthma severity and pattern. A cut – off of 20 µg/l for serum ECP was with sensitivity of 89% and specificity of 88% in detection asthma cases. The area under curve was found to be 0.805, meaning that determination of serum ECP was with a predictive value in detection of asthmatic cases.Conclusion: Serum ECP level may help in discrimination between asthmatic and non asthmatic individuals, also between symptomatic and asymptomatic asthmatic individuals. The residence does not influence serum ECP in our asthmatic patients.

INTRODUCTION

Bronchial asthma is a chronic inflammatory disease of the airway, and the cells mainly responsible for causing this inflammation are eosinophils. When activated eosinophils undergo degranulation causing epithelial damage in the airway, desquamation and increased airway hypersensitivity. Asthma therapy consists of suppressing chronic and persistent airway inflammation. It is, therefore, important to find a marker of disease activity, ideally one that is simple to measure, reliable and inexpensive. As yet no such marker has been found for asthma.

A lot of hypotheses have been suggested to propose totally different mechanisms involved in the pathogenesis of asthma. It has been found that activated Eosinophils play an important role in the pathogenesis of bronchial asthma. Upon activation, Eosinophils undergo de-granulation causing epithelial damage in the airway, desquamation and increased airway hypersensitivity.

Over the last decades and especially during last ten years substantial research work has been carried out to determine changes in serum ECP levels due to different allergic and non-allergic diseases. As a result enough quality work is now available to bridge the link between Eosinophil activity and allergy phenomenon. Serum ECP is now closer to be declared as an established marker of allergy [1]. Many reported studies demonstrate an increase in serum ECP in asthmatic patients as compared to healthy control [2-9]. Amongst the notable studies of Eosinophil activity markers in induced sputum two studies found that ECP levels were significantly positively correlated with the mean weekly total symptom scores [10,11]. The review of literature indicated that serum ECP may serve as objective indicator for clinical activity in the asthma, and point to a possible pathophysiological axis in asthma that is based upon altered airway resistance due to Eosinophils and Eosinophil activity markers [2,12,13]. If it is confirmed that this axis has some role in pathophysiology of asthma then it will open doors to new pharmaceutical research targeted at developing anti-eosinophil activity drugs on the pattern of anti histamine drugs [14]. The studies that mentioned above will eventually establish the importance of serum ECP in diagnosis and management of asthma.

Interleukin -5 (IL-5) , a cytokine that attracts, activates and

prolongs survival of eosinophils, is an important eosinophil-regulating cytokines in the pathogenesis of allergic inflammation and asthma, and its concentration in asthmatics correlates with markers of Eosinophil activation and T Lymphocyte [15]. IL-5 was detectable in induced sputum and its concentration correlated with Eosinophil numbers and ECP [16]. Asthmatic patients with exacerbation tended to have higher levels of IL-5 than stable asthmatics. IL-5 significantly correlated in a positive way with Eosinophil proportion and negatively correlated with FEV1. The correlation between Eosinophil proportion and ECP, and IL-5 suggests that Eosinophils, which may be highly activated , are the source of ECP and IL-5 [16]. We hypothesized that ECP may be used as biological marker for bronchial asthma.

MATERIALS AND METHODS

PATIENTS

Serum ECP was determined in 139 asthmatic patients, 73 (52.5%) of them were symptomatic and 66 (47.5%) were asymptomatic. Ninety one (65.5%) patients of the total (139) were with mild asthma and 48 (34.5%) were with moderate asthma. In addition, 81 (58.3%) patients were from urban and 58 (41.7%) patients were from rural areas. Of the total symptomatic patients, 48 (65.8%) were with persistent asthma { 21 (28.8%) persistent atopic and 27 (37%) persistent non atopic } and 25 (34.2%) were with an intermittent asthma. In addition, 50 matched healthy non asthmatic individuals were included in the study as control and they were recruited from outpatients clinic . The diagnosis of asthma was performed by specialist physician and was established according to National Heart Blood and Lung Institute / World Health Organization (NHLBI/WHO) workshop on the Global Strategy for Asthma [17]. Subjects were considered atopic by positive skin tests to at least one common aeroallergen. Patients were excluded if they were smokers, if they had respiratory infection within the month preceding the study, a rheumatological illness, malignancy, diabetes, heart failure, history of venous embolisms, coronary heart disease and liver or kidney diseases . All patients included in the study they were on Salbutamol inhaler only.

At enrolment, they all underwent full clinical examination, pulmonary function test and blood sampling. Sputum samples were collected from patients when indicated. Normal volunteers were also enrolled in the study as a healthy control. None of them had any previous history of lung or allergic disease and were not using any medication. They had a normal lung function test (FEV1 > 80%) and negative skin allergy test. General stool examinations were performed for all patients and controls to exclude parasitic infections.

The patients were recruited from the outpatient clinic of the Asthma and Allergy Centre in Tikrit. Their age range from 34 to 76 years (58.3 ± 9.4 years). A patient was considered symptomatic when symptoms present at the time of clinical evaluation at the time of study enrollment. While assigned as asymptomatic if asthma symptoms were absent at the time of enrollment. All asymptomatic patients were considered as mild asthma. The sampling performed during the period from December 2004 to May 2005. All samples were collected at the morning following an overnight fasting. The study was approved by the ethics committee of Tikrit University College of Medicine, and written consent was obtained from all participating subjects.

SKIN PRICK TEST

The skin prick tests were performed for all patients and controls and evaluated in accordance with European Academy of Allergy and Clinical Immunology subcommittee on allergy standardization and skin tests using standards allergen panel (Stallergen, France).

DETERMINATION OF SERUM IGE

Total serum IgE was determined by enzyme linked immunosorbent assay kit (Biomaghreb, Arina 2080, Tunisia). The kit was with the ability to detect serum IgE levels above 0.35 unit. The Biomaghreb total IgE microplate ELISA kit is a two site enzyme linked immunosorbent assay for the quantitative determination of IgE. At first mouse monoclonal antibody is immobilized onto the plastic wells. A 100 µl of assay buffer was added into each well. Then 20 µl of standards, control and samples were pipeted into corresponding wells. The plates were covered with plastic film, homogenized at 300 rpm (horizontal shaking) and incubate in an incubator for 90 minutes at 37 °C. The contents were aspirated after incubation and 300 µl of wash solution added to each well. The washing was repeated 3 times. Then 100 µl of conjugate (Anti – IgE alkaline phosphatases) placed in each well. The plate covered and incubated for 90 minutes at 37 °C. The washing of the plates were repeated as above after the incubation. Then 100 µl of chromogen was added to each well and the plates were covered and were incubated for 30 minutes at room temperature in the dark. Then 100 µl of stopping solution (NaOH) was added to each well. Wells contents were

homogenized at 300 rpm and wells were read at 405 nm against blank in microplates EIA reader. The color intensity is directly proportional to the amount of IgE in the sample. The level of unknown IgE was then determined by comparing the optical density with data established using known IgE standards in the same assay system. The concentration of total IgE in the unknown samples may be read directly from the standard curve or EIA microplates reader.

LUNG FUNCTION TEST

Computerized spirometer (Autosphiror, Discom-14, Chest Corporation, Japan) was used for measurement of FEV1 predicted percent of the patients at their enrollment in the study and when indicated according to studies design.

DETERMINATION OF SERUM EOSINOPHIL CATIONIC PROTEIN

Serum ECP was determined by ELISA kit (MBL MESCACUP ECP TEST) from Medical and Biological Laboratories Co. LTD, Japan.

STATISTICAL ANALYSIS

The values are reported as mean ± SD and 95% confidence interval. For statistical analysis between groups paired t test was used . Pearson test was used for correlation analysis. The diagnostic accuracy of sputum and serum ECP were determined by generating a receiver operating characteristic (ROC) curve for each test. The levels of each marker were compared between the study groups and control group, using SPSS computer package. P values of < 0.05 were considered significant.

RESULTS

A 139 asthmatic patients enrolled in the study, with age range from 34 to 76 years (58.3 ± 9.4). Out of the total 92 (66.2%) were males and 47 (33.8%) were females. Mean of baseline serum ECP was very highly significant higher (P<0.00001) in asthmatic patients (36.12 µg/l ± 17.7) than in controls (7.68 µg/l ± 5.63). There was no overlap between the lower asthmatic limit (33.15 g/l) and higher limit of the control groups (10.12 µg/l). In addition, the ECP variation between asthmatics and controls was associated with significant differences in FEV1 (P<0.0001) (Table.1).

Figure 1

Table .1. Serum eosinophilic cationic protein [ECP] and FEV1 in asthmatic patients compared to control subjects.

Group	NO	Eosinophil cationic protein [µg/l]			FEV1 Percent Predicted		
		Mean	SD	95% CI	Mean	SD	95% CI
Asthma	139	36.12	17.76	33.15 – 39.09	81	10	79 – 83
Control	50	7.68	5.63	6.08-9.68	101	3	100 - 102
P value		< 0.0001			< 0.0001		

When asthmatic groups were subdivided according to the presence of symptoms (Table.2), the asymptomatic asthmatics had lower serum ECP (22.47 µg/l ± 15.41) than symptomatic asthma groups (41.32 µg/l ± 16.79; P<0.0001).

Figure 2

Table: .2. Serum eosinophilic cationic protein [ECP, µg/l]and FEV1 in asthmatic patients sub groups

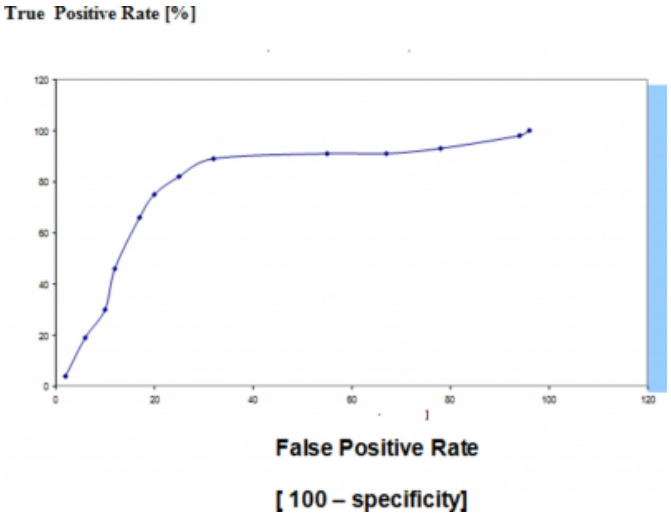
	Group	No.	Eosinophil cationic protein			FEV1 percent predicted		
			Mean	SD	95% CI	Mean	SD	95% CI
Clinical State	Asymptomatic	66	22.47	15.41	18.67-26.27	91	7	89-93
	Symptomatic	73	41.32	16.79	37.39-45.25	78	8	76-80
	P value <		0.0001			0.0001		
Pattern	Persistent	48	41.57	17.75	36.68-46.46	75	8	73-77
	Intermittent	25	25.24	12.02	20.29-30.19	91	5	89-93
	P value <		0.0001			0.0001		
Severity	Mild	91	23.47	12.04	20.96-25.98	90	5	89-91
	Moderate	48	48.51	17.61	43.45-53.57	73	6	71-75
	P value <		0.0001			0.0001		
Residence	Urban	81	30.67	18.25	26.63-34.71	87	8	86-89
	Rural	58	34.59	18.94	29.62-39.51	82	11	79-85
	P value >		NS			NS		

Patients with persistent asthma were with significantly higher (P<0.0001) serum ECP level (41.57 µg/l ± 17.75) than patients with intermittent asthma (25.24 µg/l ± 12.02). Furthermore, serum ECP was significantly lower (P<0.0001) in mild asthma group (23.47 µg/l ± 12.04) than moderate asthma group (48.51 µg/l ± 17.61). This study indicated that there was no significant (P>0.2) difference in serum ECP between urban (30.67 µg/l ± 18.25) and rural (34.59 µg/l ± 18.9) areas asthmatic groups. The relationship between sensitivity and specificity for different serum ECP values

was demonstrated by the ROC curve analysis. (Fig.1).

Figure 3

Fig.1. ROC of Serum Eosinophil Cationic Protein in Asthma



A cut – off of 20 µg/l for serum ECP was with sensitivity of 89% and specificity of 88%. The area under curve was found to be 0.805, meaning that determination of serum ECP was with a predictive value in detection of asthmatic cases.

Asthmatic individuals were with higher serum ECP whether atopic (43.1 µg/l ± 18.33) or non atopic (40.28 µg/l ± 17.49) than pneumonia group (14.6 µg/l ± 12.02), non asthmatics non atopic group (5.96 µg/l ± 2.18) and non asthmatics atopic group (10.85 µg/l ± 4.23). However, asthmatics atopic with higher serum ECP (43.1 µg/l) than asthmatics non atopic (40.28 µg/l), but the difference was statistically not significant (P>0.6). Although, the differences between non asthmatics atopic and non asthmatics non atopic was significant (P<0.01). Asthmatic groups (atopic and non atopic) was highly significant (P<0.0001) higher serum ECP than other groups. (Table.3.).

Figure 4

Table: .3. Effect of confounding factors on serum eosinophil cationic protein (ECP,µg/l) concentrations.

Group	Number	Serum ECP concentration		
		Mean	SD	95% CI
Asthmatic atopic	21	43.1	18.33	34.74-51.46
Asthmatic non atopic	27	40.28	17.49	33.34-47.22
Pneumonia	10	14.6	9.02	8.15-21.05
Non asthmatic Non atopic	10	5.96	2.18	4.4 – 7.52
Non asthmatic atopic	10	10.85	4.23	7.85 – 13.85
Control (Population Control)	50	7.68	5.63	6.08 – 9.68

DISCUSSION

Eosinophil cationic protein (ECP) is an eosinophil granule protein which is highly cytotoxic and is released by activated eosinophils. Concentration of ECP in BALF of asthmatic patients vary with the severity of their disease and ECP concentration in sputum have also been shown to reflect the pathophysiology of the diseases [18,19]. The concentration of serum ECP has recently been found to correlate with ECP concentration in bronchoalveolar lavage fluid (BALF) [2]. Therefore, assessment of serum ECP may be considered to reflect pulmonary inflammation in bronchial asthma [20]. Studies of asthmatic patients, especially adults, have indicated a relationship between the level of serum ECP and the severity and nature of the disease [2,20,21].

The present study showed that the serum ECP levels were significantly elevated in asthmatic subjects as compared to that of healthy controls. This indicated the role of eosinophilic inflammation in the pathogenesis of asthma. Immunohistchemical analysis of ECP revealed that normal subjects had only a few nondegranulated eosinophils deep in the submucosa, whereas all the patients had degranulated eosinophil beneath the basement membrane and among epithelial cells [18], this reinforces our finding. As this study indicated the role of eosinophil inflammation in asthma suggests a significant impact on the management of asthma

with anti – inflammatory medication increasingly being recommended as first line therapy.

It is clear that our results were consistent with previous studies that have shown that higher ECP levels in the serum of asthmatic patients when compared with healthy subjects [13,20,22,23]. Measuring of serum ECP levels have the advantages over eosinophilic count in that it reflects not only the number of cells but also their degree of activation and is therefore a better inflammatory marker [24]. The present study results show a significant higher serum ECP levels in symptomatic asthmatic patients than asymptomatic. Other studies [2,7,9,13,14,20,22-29] reported the same association between serum ECP levels and asthma severity. Contrary, to these studies and to our results, Ferguson et al [21] found that the serum ECP levels in asthmatic children with symptoms was not significantly higher than that for asymptomatic children or that with allergic rhinitis. These differences may be due to the fact that they studied mild cases of asthma; also, 14 out of their 24 patients with symptomatic asthma were receiving inhaled corticosteroids which might have reduced their ECP levels.

This study results suggest that serum ECP levels differ between control subjects and those with all grades of asthma (whether mild or moderate) and between cases with mild and moderate attacks. The absence of any observed significant difference between mild and moderate attacks as reported by Badr El-Din [2] may be due to the small number of cases involved in their study or because the level in inflammation in their study group in mild and moderate cases does not differ significantly.

One of the most critical points in asthma research is the characterization of the relationship between the clinical pattern of the disease and the histological profile of the underlying airways inflammation. Vignola et al [30] reported that epithelial shedding, eosinophil, activated T cell counts in biopsies and ECP in BALF were significantly increased in patients with intermittent asthma by comparison with control subjects and this increase was significantly greater for patients with persistent asthma. These findings suggest that airways inflammation is present in patients with intermittent asthma but to a lesser extent than in patients with persistent asthma [30]. Thus now it is recognized that all asthma even mild asthmatic is associated with inflammation in the airways. This inflammation has been suggested to be responsible for airway reactivity. BALF and bronchial biopsies [18,30] have demonstrated the presence of inflammatory cells even in quiescent asthma. The results of Vignola study [30] show a heterogeneous inflammatory process in intermittent asthma, the profile and magnitude of which present both similarities and differences compared with persistent asthma, depending on cell types and the mediators assessed, as well as the sample studied.

As this study indicated that serum ECP was significantly high in persistent asthma rather than in intermittent asthma and control. This means that eosinophilic inflammation was more potent in persistent asthmatic patients. These results confirm the previously reported studies using BALF and biopsy methodology [18,30,31]. This explanation was accepted for serum ECP, since the concentration of serum ECP has recently been found to correlate with ECP concentration in the bronchial wash and BALF [2]. Thus assessment of serum ECP may be a reflection of pulmonary inflammation in bronchial asthma [20]. The presence of eosinophilic inflammation seems to be of importance since this feature is inconsistently observed in non asthmatic atopic patients [32] and absent in patients with chronic cough [33]. Furthermore, these results indicate that although eosinophils are recruited in intermittent asthma, they are less activated in persistent asthma. The different patterns of eosinophilic activation found in persistent as compared with intermittent asthma, might be important consequences of the integrity of the bronchial mucosa [30].

Almost about half of the asthmatic patients in the present study are symptomatic, giving a high specificity of 89% and 88% sensitivity of serum ECP for asthma diagnosis at a cut – off value of 20 µg/l. Vanto and Koskinen [12] assessed the sensitivity and specificity of serum ECP using cut – off level of 16 µg/l, in the diagnosis of asthma. The diagnostic sensitivity and specificity of serum ECP was found to be 54% and 71% respectively. Recently a reported study indicated a high specificity of 93% but very low sensitivity of serum ECP (24%) for asthma diagnosis using a reference value of 19.1 µg/l [34]. The above two studies were not consistent with our results, may be due to that they might represent different allergen exposure levels or different asthma severity in the two populations, the mountainous area being nearly free from mite exposure. However, an ECP level of 10.3 µg/l in the Naja study [34] gives the value that ensures the highest accuracy (minimal false negative and false positive results). Shields et al [35] calculated the sensitivity and specificity to be 42% and 95% respectively, with a cut – off level of >20 µg/l, using raised eosinophil percentages in bronchial alveolar lavage (>0.86) as an indicator of inflammation instead of asthma diagnosis.

However, the sensitivity still remains too low to be of diagnostic value in population based studies. Our finding indicated that a cut – off value of > 20 µg/l of serum ECP gives accepted specificity and sensitivity (88% and 89% respectively). However, cut – off value of 13 µg/l gives a high sensitivity (93%), but low specificity (42%). Even if the usefulness of the serum ECP in asthma seemed to be of little diagnostic value for reported population based studies in children [14, 34], this study indicated valuable value in the diagnosis of cases on individual levels. Immune system of their study population may contribute to the difference in the finding between our study and that reported by them. The area under ROC curve was found to be 0.805, meaning that a mean serum ECP at a cut off value of > 20 µg/l was accurate for diagnosis of bronchial asthma in 80.5% of cases.

The present study results indicated that there was no significant difference in serum ECP between asthmatic from urban and that from rural areas. Thus residence does not influence the serum levels of ECP in asthmatics as this study indicated. However, the serum ECP in school children living in mountain area of Norway, was with lower serum ECP than that found in this study [34]. This difference was due to the low allergen exposure for their population.

Serum values of biochemical markers show great variance with age and therefore it is important to determine age specific reference value. The manufacturer of the kit (used in this study) reference value for serum ECP based on adult samples are 13 µg/l and value of more than 15 µg/l recorded as elevated. In the present study, 95% percentile was chosen as the upper reference limit value for serum ECP and was calculated in the non atopic, non asthmatic group to be 7.52 µg/l and 13.85 µg/l in atopic non asthmatic subjects. Naja et al [34] reported a reference value of 19.1 µg/l for his asthmatic children involved in their study, which is lower than our reference value. Remes et al [36] using the 97.5% CI the limit, reported 24.7 µg/l in their control group of non atopic subjects aged 7-12 yrs. Fitch et al [37] reported a reference value for serum ECP in non asthmatics, non atopic children of 18.8 µg/l. Another study evaluated reference value of serum ECP in children , finding 24.1 µg/l as the 95% percentile in the 2-4 yr of age and 44.9 µg/l in the group under 2 yr of age, but some atopic children were included [38], thus gave a higher values. However, Krug et al [8] reported a mean of 5.9 µg/l in non asthmatic non atopic children. Melbostand et al [39] reported a mean value of 5.5 µg/l for adult individuals that corresponds well to the present results. Other study reported a mean value of 13.22 µg/l in non asthmatic healthy controls [3]. Since we have excluded all atopic subjects by positive skin prick test and serum IgE level, our control group should constitute a representative group of healthy adults serving as reference group in the age of 15 -56 years for our locality.

In comparison with previous reports from selected patients, the mean serum ECP levels were found to be consistent in both atopic and non atopic [12,22]. However, this value was higher than that reported by others [34,40,41]. This can be partially explained by three factors; firstly, all patients with moderate or severe asthma were treated with ICS which has been shown to decrease serum ECP [24,40-42]. Secondly, most of the asthmatic children in Naja study had mild asthma, whereas this study and most published [2,12,13,23,21,22,39] studies on serum ECP concerns selected patients referred to hospital outpatient clinics suffering from disease symptoms. Thirdly, it has been shown that living at high altitude diminish allergen exposure (mainly allergy to HDM), bronchial responsiveness and serum ECP in asthmatics [34] and therefore improving lung function and reducing airway inflammation [14,40-45].

As found in this study, other reported studies [7,34] found no difference in serum ECP between atopic and non atopic asthmatic subjects. However, Melbostud et al [39] reported that serum ECP values are significantly more elevated in atopic than non atopic asthma, elevated ECP are not specific to atopic asthma. Although atopy do not influence the levels of serum ECP in asthmatic patients, in non asthmatic individuals atopy do affect the serum ECP levels as shown in this study and that reported before [7,34]. In addition, serum ECP levels in individuals with pneumonia was higher than that in individuals without infection irrespective whether they are atopic or non atopic. However, the mean serum ECP in patients with pneumonia is still significantly lower than that in asthmatic patients.

In conclusion, serum ECP level may help in discrimination between asthmatic and non asthmatic individuals, also between symptomatic and asymptomatic asthmatic individuals. This conclusion was reached since there was no overlap between the lower serum limit and upper serum limit for asthmatic patients and control respectively, and for symptomatic asthma group (37.39 µg/l) and asymptomatic asthma group (26.27 µg/l). The residence does not influence serum ECP in our asthmatic patients.

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