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Selected Eosinophil Proteins as Markers of Inflammation in Atopic Dermatitis Patients

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SUMMARY Atopic dermatitis (AD) is an inflammatory skin disease characterized by chronic and recurrent course, beginning primarily in early childhood. The etiopathogenesis of AD has not yet been fully understood, although various types of inflammatory cells including eosinophils may be involved in its pathomechanism. The basic aim of the study was to evaluate the usefulness of selected eosinophil proteins in serum and urine of AD patients, as markers of disease severity. The study also aimed to analyze correlations between the level of examined proteins and parameters such as skin prick test (SPT) results, serum concentration of total IgE, and coexistence of symptoms of other atopic diseases. The study included 30 AD patients and two control groups: 30 patients suffering from chronic urticaria and 30 healthy individuals. The mean level of eosinophil proteins measured in serum and urine of AD patients was higher than that in controls, although a significant difference was only recorded for serum and urine level of eosinophil protein X (EPX). Patients with very severe/severe AD presented higher levels of eosinophil proteins than patients presenting with mild/moderate AD, although no significant difference was found between these two groups. AD patients with positive SPT results and detectable specific IgE in serum, and with coexisting symptoms of other atopic diseases presented with higher mean levels of serum and urine eosinophil proteins than AD cases with negative SPT results and without any symptoms of other atopic diseases. In children suffering from AD, serum eosinophil cationic protein level, EPX level and urine EPX level were higher than those in healthy children, however, without statistical significance. Study results suggested a significant role of eosinophils in the etiopathogenesis of AD. Serum and urine levels of selected eosinophil proteins may serve as an important part of diagnostic approach to AD patients, especially in differentiation of allergic and non-allergic forms of AD. The results are also promising for the usefulness of selected eosinophil proteins in the diagnosis of AD in children, however, thorough analysis on a larger group of patients is needed.

KEY WORDS: atopic dermatitis; eosinophil proteins; airborne allergens

INTRODUCTION

Atopic dermatitis (AD) is an inflammatory, highly pruritic skin disorder, characterized by chronic and recurrent course, and onset usually in early childhood. The etiopathogenesis of AD still remains unclear, although various immune dysfunctions are observed, e.g., elevated serum level of

total IgE (tIgE) antibodies, enhanced production and release of Th2 cytokines in acute phase of AD, and also disturbed function of keratinocytes as a protective barrier (1,2). Many inflammatory cell types are actively involved in the pathogenesis of AD, including eosinophils. Increased expansion, activation and tissue recruitment, together with delayed apoptosis of these cells can be observed in AD. Proinflammatory cytokines and chemokines (RANTES, eotaxin, IL-5, IL-13) produced by antigen presenting cells and T lymphocytes attract eosinophils to the area of inflammation and activate them. Studies show that eosinophils disrupt in tissues and lose their morphological identity, and release numerous mediators, localized initially in specific cytoplasmic granules. Among them, eosinophil cationic protein (ECP) and eosinophil derived neurotoxin/eosinophil protein X (EDN/EPX) are considered as essential markers of eosinophilic activity (2-4). Biologically active concentrations of eosinophil proteins persist in the skin for weeks and they likely play an important role in the inflammatory process, even if intact eosinophil granulocytes are not present.

The most important functions of ECP are associated with its strong cytotoxic features. ECP demonstrates highly destructive action against bacteria, parasites and some mammalian cells. Increased eosinophilic activity, expressed by ECP body fluid level, can be found in numerous diseases, although special attention is drawn to the involvement of this protein in atopic inflammation, particularly in AD (5-8).

EPX shares many features with ECP. Studies have indicated that these proteins show an almost 70% homology regarding amino acid configuration. In contrast to ECP, however, EPX is excreted in significant amounts in the urine. Increased serum/urine level of this protein was detected in patients with asthma, prurigo nodularis and also in patients suffering from AD (5-8).

The aim of the present study was to investigate the usefulness of selected eosinophil proteins detected in blood serum and urine of AD patients as markers of disease severity, evaluation of correlation between serum and urine level of analyzed proteins, and certain clinical and allergologic parameters, e.g, skin prick test (SPT) results, levels of IgE antibodies directed against environmental allergens, and coexistence of other atopic diseases symptoms (asthma, allergic rhinitis). We also aimed to analyze the above correlations in a group of children suffering from AD and to evaluate EPX urine level in AD children as an essential and noninvasive diagnostic tool in this age group.

SUBJECTS AND METHODS

Subjects

Thirty patients suffering from AD diagnosed by use of Hanifin and Rajka criteria (9) were included in the study. There were 22 (73%) female and 8 (27%) male patients, age range 8-60 (mean 24.5) years, including 11 children (7 female and 4 male) with upper age limit of 17 years, mean age 12 years. All patients were hospitalized at Department of Dermatology or attended Allergic Diseases Diagnostic Center, University of Medical Sciences in Poznań.

In addition to AD symptoms, 15 (50%) patients presented with other atopic disease manifestations: atopic asthma in 4 (13.3%), allergic rhinitis in 11 (36.7%) and allergic conjunctivitis in 12 (40%) patients.

Two age- and sex-matched control groups were included in the study: control group A (30 patients suffering from chronic urticaria) and control group B (30 healthy individuals).

Methods

Thorough disease history was taken regarding the onset and course of AD, factors exacerbating the inflammatory state of the skin, perennial disease activity, concomitant symptoms of other atopic diseases, and family history of atopy.

Clinical evaluation of AD patients was based on W-AZS index proposed by Silny *et al.* (10), which grades the severity of pruritus, sleep disturbances together with extensiveness and severity of skin inflammation. The principles of W-AZS have been recently published by Czarnecka-Operacz *et al*. (11), and consecutive study of the usefulness and advantages of W-AZS index in the appraisal of a patient with AD has already been accepted for publication in Acta Dermatovenerologica Croatica. In the group of chronic urticaria patients, both skin lesions and severity of pruritus were evaluated using the scores proposed by Lorette *et al.* (12) and Thomson *et al.* (13).

SPTs were performed in 30 AD patients and control group B using airborne set of allergens by Nexter/Allergopharma (grass pollen, tree pollen, weed pollen, feather, animal dander, moulds and house dust mites *Dermatophagoides (D.) pteronyssinus* and *Dermatophagoides (D.) farinae*). In control group A (chronic urticaria patients), SPTs were only performed if the history suggested the possibility of airborne allergen contribution to the pathogenesis of the disease and in patients pre-

senting additional symptoms indicating airborne allergy. Histamine hydrochloride (1:1000) was used as positive control, and 0.9% saline solution as negative control. Positive SPT result (+++) was defined as an allergen wheal with a mean diameter at least equal to the mean diameter of histamine wheal (14). Laboratory analyses included measurement of total IgE (tIgE) serum level using fluoroenzyme immunoassay (CAP System FEIA, Pharmacia); measurement of antigen specific IgE (asIgE) serum level using fluoroenzyme immunoassay (CAP System FEIA, Pharmacia); the measurement was only performed in selected AD cases where disease severity excluded the patient from SPT; evaluation of ECP serum level using fluoroenzyme immunoassay (CAP System FEIA, Pharmacia); analysis of EPX serum and urine level using EDN ELISA method (MBL Intl., USA): according to literature data on the circadian rhythm of EPX excretion, urine was collected in the morning and then centrifuged and frozen (-70 ºC). In order to formulate uEPX concentration as µg/mmol creatinine, measurement of creatinine urine level was performed using Jaffe's method (15).

The study was approved by the Ethics Committee of the University of Medical Sciences in Poznań (statement number 502/03).

STATISTICAL METHODS

Statistical evaluations were performed by using ANOVA analysis with post-hoc tests by Newmann-Keuls. For the evaluation of correlations, Spearman's rank correlation coefficient was calculated for statistical significance. Statistical analysis was carried out with STATISTICA v. 6.0 and Instat v. 3.0 by GrafPad.

RESULTS

Based on the clinical evaluation of AD patients two subgroups were established: subgroup I of 17 AD patients, W-AZS value <50 points (mild and moderate AD) and subgroup II of 13 AD patients, W-AZS value ≥points (severe and very severe AD). SPT results were positive in 80.8% and negative in 19.2% of AD patients. The sensitizing airborne allergens were predominated by house dust mites and grass pollen allergens; 72% of study patients presented sensitization to numerous groups of allergens (polyvalent allergy), while 28% of patients were sensitized to only one group of allergens (monovalent allergy). In control group B (healthy individuals) SPT results were positive in 26.7% and negative in 73.3% of subjects. The majority of healthy subjects presented sensitization to grass pollen allergens; 25% of healthy individuals sensitized to airborne allergens presented sensitization to numerous groups of allergens (polyvalent allergy) and 75% were sensitized to only one group of allergens (monovalent allergy).

The mean serum ECP level in AD patients was 8.9±9.6 µg/L and was higher than that in control groups A and B (5.2±4.4 µg/L and 5.7±6.6 µg/L, respectively). The difference between groups did not reach statistical significance. The mean serum EPX level in AD patients was 44.4±40.9 ng/mL and also exceeded the serum level of this marker recorded in control groups (23±12.2 ng/mL in control group A and 20.2±14.5 ng/mL in control group B). Statistical analysis yielded a statistically significant difference between AD patients and control group A ($p<0.05$) and control group B ($p<0.001$). Similar results were obtained in relation to the mean urine EPX level: in the group of AD patients it was higher (118.4±62.9 µg/mmol creatinine) than that in control groups A and B (83.7±50.7 µg/mmol creatinine and 65±24.5 µg/mmol creatinine, respectively). There was a statistically significant difference between AD patients and control group A ($p<0.01$) and control group B ($p<0.001$). These results are presented in Table 1.

Table 1. Results of eosinophil protein measurements in AD patients and control groups

	AD patients n=30	Control group A n=30	Control group B n=30	Significance
Mean serum ECP level (µg/L) ±SD	8.9±9.6[a]	5.2±4.4[b]	5.7±6.6[c]	a/b NS a/c NS b/c NS
Mean serum EPX level (ng/mL) ±SD	44.3±40.9[a]	23.7±12.2[b]	20.2±14.5[c]	a/b p<0.05 a/c p<0.001 b/c NS
Mean urine EPX level (µg/mmol creatinine) ±SD	118.4±62.9[a]	83.7±50.7[b]	65.5±24.5[c]	a/b p<0.01 a/c p<0.001 b/c NS

In the group of AD patients serum and urine EPX levels correlated significantly with tIgE serum level. There was no statistically significant correlation between ECP and tIgE serum level.

The results of the eosinophil protein urine level measurement in patients with AD categorized into two subgroups according to disease severity (W-AZS index) were evaluated. In subgroup I of AD patients (mild and moderate AD) the mean serum ECP level was 8.3±7.9 µg/L and was slightly lower than that in subgroup II (severe and very severe AD) (9.9±11.7 µg/L). There was no statistically significant difference between the two groups. Similarly, the mean serum and urine EPX level (Fig. 1) was analyzed in the two subgroups of AD patients, and was found to be lower in subgroup I than in subgroup II (43.9±42.6 ng/mL and 106.7±64.3 µg/mmol creatinine in subgroup I; and 44.8±40.2 ng/mL and 133.6±60.0 µg/mmol creatinine in subgroup II). Statistical analysis showed no difference between these groups.

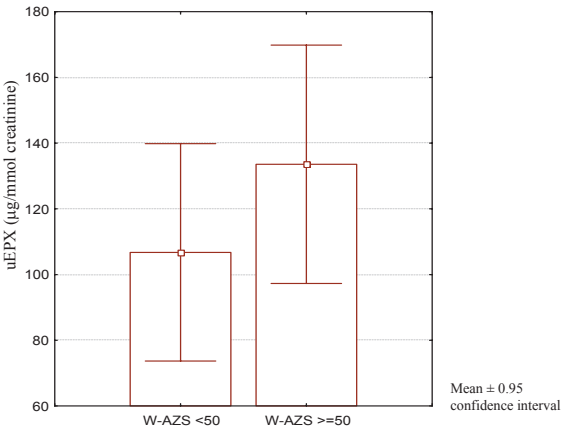

Figure 1. Mean urine EPX level in AD patients categorized into two subgroups according to disease severity

The mean serum ECP level in AD patients presenting symptoms of other atopic diseases was 12.0±12.4 µg/L and exceeded the level of this marker in AD patients with no symptoms of other atopic diseases (5.9±3.7 µg/L), although there was no statistically significant difference between the groups. The mean serum EPX level in AD patients with other atopic diseases was higher than that in the group presenting no signs of additional atopic diseases (59.7±53.1 ng/mL and 28±11.8 ng/mL, respectively), however, with no statistically significant between group difference. Similarly, the mean urine level of EPX in AD patients showing symptoms of other atopic diseases was higher than that in the group free from additional symptoms of atopic diseases, however, without statistically significant between group difference (122.6±70.7 µg/mmol creatinine and 114.1±56.1 µg/mmol creatinine, respectively).

Further analysis referred to the level of eosinophil proteins in serum and urine of AD patients depending on SPT results. In AD patients with positive SPT results and detectable serum asIgE, the mean serum level of ECP was higher than that in patients with negative SPT results, although the difference was not statistically significant (9.7±10.0 µg/L and 5.2±5.8 µg/L, respectively). As shown in Table 2, the mean serum and urine EPX level in AD patients presenting with positive SPT results and detectable serum asIgE was 49.5±42.9 ng/mL and 125.8±64.8 µg/mmol creatinine, respectively, exceeding the serum and urine level of this marker in AD patients with negative SPT results (18.4±9.1 ng/mL; statistically significant difference, $p<0.05$, and 81.3±37.7 µg/mmol creatinine, no statistically significant difference, respectively).

Table 2. Comparison of eosinophil protein levels in AD patients presenting with positive SPT results and detectable serum asIgE and patients with negative SPT results according to selected airborne allergens

	AD patients n=30		Significance
	Patients with positive SPT results and detectable serum asIgE n=25	Patients with negative SPT results n=5	
Mean serum ECP level (µg/L) ±SD	9.7±10.0[a]	5.2±5.8[b]	a/b NS
Mean serum EPX level (ng/mL) ±SD	49.5±42.9[a]	18.4±9.1[b]	a/b $p<0.05$
Mean urine EPX level (µg/mmol creatinine) ±SD	125.8±64.8[a]	81.3±37.7[b]	a/b NS

Evaluation of special interest considered serum and urine level of eosinophil proteins in the group of children suffering from AD in comparison to healthy subjects. The mean serum ECP level was 9.0±9.7 μg/L in AD children and slightly lower in healthy children (8.3±12.5 μg/L), with no statistically significant difference. The mean serum EPX level in AD children was 46.5±52.5 ng/mL, which was higher than the level measured in healthy children (28.2±27.2 ng/mL), however, with no statistically significant difference. The mean level of EPX measured in urine of AD children was 112.2±89.4 μg/mmol creatinine, which was higher than that recorded in healthy children (69.7±16.8 μg/mmol creatinine). There was no significant difference between the two groups. These results are shown in Table 3.

DISCUSSION

Active participation of eosinophil proteins in the etiopathogenesis of AD and their value as markers of disease activity in both adults and children have been the subject of broad investigations performed by several researchers all over the world. ECP has been evaluated as a sensitive measure by several authors. Kägi *et al.* (16), Miyasato *et al.* (17) and Tsuda *et al.* (18) found significantly higher serum levels of ECP in AD patients as compared with control groups. Moreover, according to Tsuda *et al.* (18), serum ECP level significantly correlates with the quantity of «hypodense» eosinophils in peripheral blood, suggesting that elevated serum ECP level may be the consequence of degranulation of circulating activated eosinophils. Amon *et al.* (19) showed a statistically significant correlation between serum ECP level and actual clinical status measured using SCORAD index in a large population of randomly selected AD patients. However, the authors did not observe any pronounced increase in ECP with increasing disease severity, and therefore conclude that determination of serum ECP levels on admission and at discharge may not be a valid tool for routine evaluation in unselected AD patients. In the present study, the mean serum ECP level was higher in the group of 30 AD patients in comparison to control groups, although the difference was not statistically significant. Furthermore, serum ECP level was slightly higher in patients with severe and very severe disease than in those with mild and moderate AD, with no significant between group difference. We found no statistically significant correlation between W-AZS index value and serum ECP level.

In patients presenting allergic type of AD (positive SPT results, detectable serum asIgE), the mean serum level of ECP was distinctly higher than in patients presenting non-allergic type of AD (negative SPT results, serum asIgE not detectable). These results are consistent with literature data. According to Toma *et al*. (20), in severe cases of AD circulating eosinophils are activated to express CD69 antigens on the surface after *in vivo* challenge with airborne allergens. Moreover, Kägi *et al.* (16) measured peripheral eosinophilia, serum ECP and sIL-2R level in AD patients. There was no statistically significant difference between patients presenting allergic and non-allergic type of AD, although serum ECP level was higher in patients with allergic AD.

ECP shares several features with EPX, however, a unique characteristic of EPX is that apart from its presence in human serum it is also excreted in urine. Therefore, as a laboratory parameter for monitoring inflammation in AD, EPX seems to be potentially more suitable and practical than ECP, mainly because there is no need to use complex and time consuming procedures of blood collecting and storage.

In 1994 Ott *et al.* (21) found the serum and urine concentration of EPX to be elevated in patients suffering from AD in comparison to healthy individuals, although the authors failed to demonstrate any significant correlation between these

Table 3. Results of eosinophil protein measurements in serum and urine of children suffering from AD and in healthy individuals

	AD children n=11	Healthy children n=7	Significance
Mean serum ECP level (μg/L) ±SD	9.0±9.7[a]	8.3±12.5[b]	a/b NS
Mean serum EPX level (ng/mL) ±SD	46.5±52.5[a]	28.2±27.2[b]	a/b NS
Mean urine EPX level (μg/mmol creatinine) ±SD	112.2±89.4[a]	69.7±16.8[b]	a/b NS

parameters and the patients' clinical state. Breuer *et al.* (22) examined 40 adult AD patients and acknowledged urine EPX as a valuable marker of disease activity. Urine level of EPX correlated significantly with disease activity as assessed by SCORAD index.

In the present study the mean serum and urine EPX level was significantly higher in AD patients than in control groups. Moreover, in the group of patients with severe and very severe AD, serum and urine EPX exceeded the level of this marker recorded in patients with mild to moderate AD, however, with no statistical significance. There was no statistically significant correlation between serum and urine EPX level and W-AZS index value.

Serum and urine EPX level was higher in patients presenting allergic type of AD than in those with non-allergic type of AD; in case of serum EPX level there was a statistically significant between group difference. Therefore, EPX measured in serum and urine may serve as a useful parameter in differentiating allergic and non-allergic types of AD. These results are consistent with the observations made by Oymar *et al.* (23), who proved a significant difference in urine EPX level between patients sensitized to common environmental allergens and those with no sensitization. According to the authors, urine EPX level distinguishes the two groups of patients better than serum ECP level.

In our study, AD patients presenting symptoms of other atopic diseases had higher levels of serum and urine EPX than AD patients with no other atopic diseases. There was no statistically significant difference between the groups, and studies in a larger group of patients are needed to reach it.

Several authors assessed the value of eosinophil proteins in children suffering from AD. Particularly EPX seems to be a promising parameter in this age group, for its detection is noninvasive, painless and easy.

Pucci *et al.* (24,25) proved the serum and urine EPX level to be significantly higher in AD children than in healthy subjects. Moreover, the authors demonstrated a significant correlation between urine EPX level and subjective symptoms such as skin pruritus and sleep disturbances, suggesting the possible role of eosinophil proteins in the etiopathogenesis of pruritus. In the study evaluating infants and young children with AD, the authors also found a strong positive correlation between immune parameters (serum ECP and urine EPX level) and each of the SCORAD index items, which they consider as the gold standard for assessing disease severity in clinical trials. According to other researchers (23), EPX measured in serum and urine taken together can give more information on the disease severity and may also be a helpful parameter in differentiation of AD children presenting allergic and non-allergic type of the disease. On the other hand, Selnes and Dotterud (26) found no association between serum ECP and AD or allergic rhinitis in children aged 7-12 years. The authors emphasize that the study was performed in an unselected children population; however, in order to evaluate the usefulness of eosinophil proteins, measurements in community medicine and trials in unselected populations are essential. Neither could Wolkerstorfer *et al.* (27) confirm a significant relationship between serum ECP level and clinical activity in children suffering from mild to moderate AD. In the authors' opinion, the lack of correlation between ΔSCORAD and ΔECP may indicate a distinct ongoing eosinophil activity, even if the clinical status has clearly improved. According to our investigations, serum levels of ECP and EPX as well as urine level of EPX were higher in the children suffering from AD than in healthy individuals, however, we failed to obtain a statistically significant difference between these groups. Yet, there is a possibility that increasing the number of study children will allow us to perform a more detailed analysis of the expected relationship, therefore we consider the results obtained as a promising encouragement for further investigations.

CONCLUSIONS

Determination of serum and urine levels of selected eosinophil proteins in AD patients may serve as an important supplementary component in the evaluation of the course of the disease.

In AD patients presenting allergic type of the disease the serum and urine level of eosinophil proteins was higher than that in patients presenting non-allergic type of AD; thus, it may be considered as a helpful marker in differentiating the two types of AD.

Determination of urine EPX level appears to be especially valuable in the diagnosis of AD children, considering its noninvasiveness and simple performance.

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Veramon – against pain, year 1929.
(from the collection of Mr. Zlatko Puntijar)