

Original Article

Concentrations of eosinophil-derived neurotoxin in the blood and urine of patients with allergic diseases

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ABSTRACT

Background: It has been reported that measurements of eosinophil-derived neurotoxin (EDN) may be useful to determine eosinophil activities in allergic diseases.

Methods: Serum, plasma and urine concentrations of EDN in patients with allergic diseases were measured by enzyme-linked immunosorbent assay. Samples of blood and urine were obtained from the same patients when their symptoms were active and when patients were well. The number of eosinophils in peripheral blood was also counted.

Results: The median concentrations of EDN in blood and urine and the number of eosinophils in peripheral blood from patients with active symptoms were all significantly higher compared with values obtained when patients were well. Compared with healthy control subjects, EDN concentrations in the serum, plasma and urine from asymptomatic asthmatic patients, as well as EDN concentrations in the serum and plasma from patients with mild atopic dermatitis and asymptomatic patients with seasonal allergic rhinitis, were significantly higher. Blood and urine concentrations of EDN in patients with active symptoms, but not urine concentrations of EDN from symptomatic patients with seasonal allergic rhinitis, were significantly increased compared with values obtained for healthy control subjects. The concentrations of EDN in the serum and plasma from bronchial asthmatic patients, serum from

patients with atopic dermatitis and plasma from patients with mild atopic dermatitis correlated with the number of eosinophils in peripheral blood; however, the concentration of EDN in the serum correlated with the number of eosinophils from asymptomatic patients with seasonal allergic rhinitis.

Conclusions: Determination of blood and urinary concentrations of EDN is useful to monitor eosinophil activity in allergic diseases.

Key words: allergic rhinitis, atopic dermatitis, bronchial asthma, eosinophil-derived neurotoxin, eosinophil.

INTRODUCTION

Eosinophils are important effector cells in allergic diseases. It can therefore be argued that proper disease monitoring should include an evaluation of the degree of eosinophilic inflammation¹ in addition to symptom recording and function measurements. However, direct assessment of eosinophilic inflammation by means of biopsies or lavage fluid is not practicable in allergic diseases clinics. Thus, less invasive markers of eosinophilic inflammation in allergic diseases have been investigated.

One of the most extensively investigated specific markers of eosinophils is eosinophil cationic protein (ECP). Serum concentrations of ECP have been proposed as a marker of eosinophilic inflammation, especially airway inflammation in bronchial asthma.² However, the clinical usefulness of ECP has been questioned because of problems in sample processing and considerable variability in measured values.³ A standardized, well-validated and generally applicable parameter of eosinophilic inflammation remains to be fully identified.

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In addition to ECP, activated human eosinophils secrete several cationic proteins, such as major basic protein (MBP), eosinophil peroxidase (EPO) and eosinophil-derived neurotoxin (EDN). Eosinophil-derived neurotoxin, also called eosinophil protein X (EPX), is 70% homologous with ECP⁴⁻⁶ and is the only one of the four basic eosinophil granule proteins that can be accurately measured in urine.⁷ In fact, it has been suggested that urinary EDN may be a valid alternative to measurements of eosinophil proteins in serum.⁸

We reported an efficient enzyme-linked immunosorbent assay (ELISA) method for measuring specifically human blood and urinary EDN.⁹ In addition, we have shown that the method may be useful to determine the possible roles of eosinophils in on-going asthma.¹⁰ We have expanded our earlier studies and, in the present paper, demonstrate that measurements of blood and urinary concentrations of EDN are useful for monitoring eosinophil activity in allergic diseases, especially bronchial asthma and atopic dermatitis.

METHODS

Subjects

Subjects were divided into groups of healthy control subjects and patients with allergic diseases, such as bronchial asthma, atopic dermatitis and seasonal allergic rhinitis. The control group comprised 50 subjects (23 males and 27 females) with a median age of 30.0 years (range 13–66 years) and no personal and/or family history of allergic disorders. The bronchial asthma group consisted of 50 patients (30 males and 20 females) with a median age of 58.0 years (range 1–88 years). The atopic dermatitis group consisted of 50 patients (33 males and 17 females) with a median age of 15.0 years (range 1–63 years). The seasonal allergic rhinitis group consisted of 50 patients (18 males and 32 females) with a median age of 41.5 years (range 6–72 years).

The diagnosis of bronchial asthma was performed according to internationally accepted criteria¹¹ after assessing reversible airway obstruction and/or non-specific bronchial hyperresponsiveness to methacholine. Mean screening forced expiratory volume in 1 s (FEV₁) was normal ($90.2 \pm 6.3\%$ of predicted). Bronchial challenge test with methacholine showed a PD₂₀ FEV₁ less than 1 mg in all subjects. All subjects underwent skin prick tests with common airborne allergens, such as house dust, pollens, molds and animal dander, as well

as blood examinations for total IgE levels. All subjects were free of symptoms for the 2 weeks preceding the initial test and had no detectable symptoms at the time of the initial study. In addition to this test, samples of blood and urine were obtained from the same patients when they had active symptoms, such as wheezing and breathlessness on moderate exercise (FEV₁ was $53.8 \pm 5.9\%$ of predicted). Patients had been instructed to take only regular medications before coming to the hospital. They were treated for the attacks by anti-asthma treatments after the second test.

The diagnosis of atopic dermatitis was made according to the clinical and morphological criteria defined by Hanifin and Rajka.¹² Based on the extent of the skin lesions involved (erythema, edema and lichenification) and subjective symptoms, such as pruritus and sleep loss, samples of blood and urine were obtained from the same patients when their conditions were mild and when they were severe.

In the study of seasonal allergic rhinitis, patients with at least a 1 year history of seasonal allergic rhinitis during the Japanese cedar season, skin test sensitivity and IgE test findings positive to Japanese cedar were enrolled. No patients had perennial rhinitis. The study was performed before and during the Japanese cedar pollen season. Each patient was asymptomatic before the pollen season and, during the pollen season, showed active symptoms such as sneezing, rhinorrhea, nasal congestion, itchy nose and/or throat and itchy eyes. By staining, the presence of eosinophils in nasal discharge was confirmed in each patient during the pollen season.

The present study was approved by the Institutional Ethics Committee of Gunma Clinical Research Center for Allergy and Regeneration, Showa Hospital, and written informed consent was obtained from each individual before the study commenced.

Sampling procedures of blood and urine

Because of reports showing circadian variations in urinary EDN¹³⁻¹⁵ and serum EDN,¹⁵ samples of blood and urine were obtained in the afternoon of each test period (13.00–15.00 h). Blood samples for measurement of serum and plasma concentrations of EDN were collected in Venoject-II Tube Autosep tubes (VP-AS076; Terumo, Tokyo, Japan) and in Venoject-II tubes that contained EDTA-3K (VP-TK052; Terumo), respectively, as described previously.⁹ Unless otherwise indicated, samples for the measurement of serum and plasma

concentrations of EDN were incubated at room temperature (20–25°C) for 1 h and for 30 min, respectively. Thereafter, the sera and plasma were separated by centrifugation at 1350 g for 10 min at 4°C and transferred to new plastic tubes. Urine samples were also collected from each individual as a spot sample in the afternoon. After 30 min of collection, urine samples were centrifuged at 1350 g for 10 min at 4°C and transferred to new plastic tubes. Each sample was aliquoted into a new plastic tube and stored at –20°C until assay.

Sandwich ELISA for EDN

We developed an efficient ELISA method for the determination of EDN⁹ that has already been introduced onto the market by Medical and Biological Laboratories Co. Ltd (Nagoya, Japan). All assays were performed in duplicate for each sample and mean values are reported. The concentrations of urinary creatinine (Cr) were determined with an L-type creatinine-F assay system (Wako Pure Chemical, Osaka, Japan). The concentrations of urinary EDN are expressed as µg/mmol Cr to correct for urine volume.

Counting the number of eosinophils

Counting of the number of eosinophils in peripheral blood was performed automatically by the Beckman Coulter counter (Beckman Coulter, Fullerton, CA, USA) and MAXM A/L system (Beckman Coulter).

Statistical analysis

Results of measurements are presented as the median (quartile 1–quartile 3) unless indicated otherwise. The Wilcoxon signed-rank test was used to compare paired data. Differences between each disease group and the control group were analyzed with the Mann–Whitney *U*-test. Correlation coefficients were obtained using the Spearman's Rank correlation procedure. Probabilities less than 5% were considered statistically significant.

RESULTS

Eosinophil-derived neurotoxin concentrations in serum, plasma and urine from patients with allergic diseases when their symptoms were active and when patients were well

Eosinophil-derived neurotoxin concentrations in the blood and urine from patients with allergic diseases

were compared when their symptoms were active and when patients were well. The EDN concentrations in serum (Fig. 1), plasma (Fig. 2) and urine (Fig. 3) from patients with allergic diseases, such as bronchial asthma, atopic dermatitis and seasonal allergic rhinitis, were significantly higher when their symptoms were active compared with when they were well.

Eosinophil-derived neurotoxin concentrations in serum, plasma and urine from patients with allergic diseases, as well as from healthy control subjects

Median (quartile 1–quartile 3) EDN concentrations in the serum, plasma and urine from asymptomatic bronchial asthmatic patients were 41.3 ng/mL (28.5–51.0 ng/mL), 23.4 ng/mL (16.8–29.0 ng/mL) and 75.9 µg/mmol Cr (47.8–106.9 µg/mmol Cr), respectively; those from patients with mild atopic dermatitis were 39.5 ng/mL (26.5–50.0 ng/mL), 19.4 ng/mL (14.3–26.0 ng/mL) and 66.8 µg/mmol Cr (40.8–93.5 µg/mmol Cr), respectively; and those from asymptomatic patients with seasonal allergic rhinitis were 28.0 ng/mL (21.0–35.5 ng/mL), 16.1 ng/mL (12.8–21.5 ng/mL) and 46.6 µg/mmol Cr (35.0–64.3) µg/mmol Cr, respectively. Concentrations of EDN in the serum, plasma and

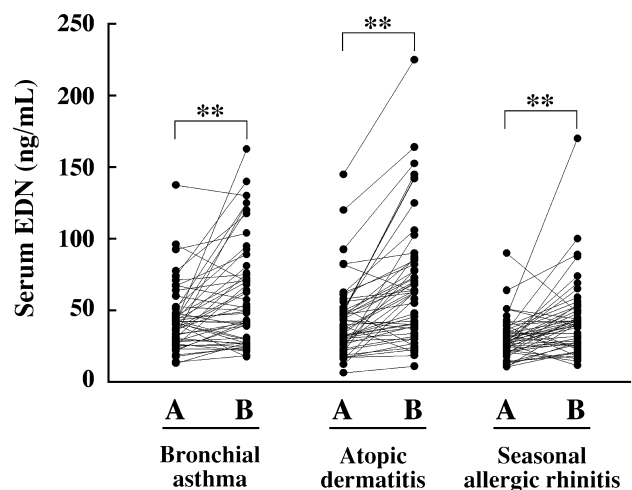


Fig. 1 Eosinophil-derived neurotoxin (EDN) concentrations in serum from patients with bronchial asthma, atopic dermatitis and seasonal allergic rhinitis. The EDN concentrations were measured in patients with bronchial asthma as well as seasonal allergic rhinitis when patients were either asymptomatic (A) or symptomatic (B), and from patients with either mild (A) or severe (B) atopic dermatitis. Concentrations of EDN from patients with allergic diseases were compared when their symptoms were active and when they were well. ***P* < 0.01, **P* < 0.05.

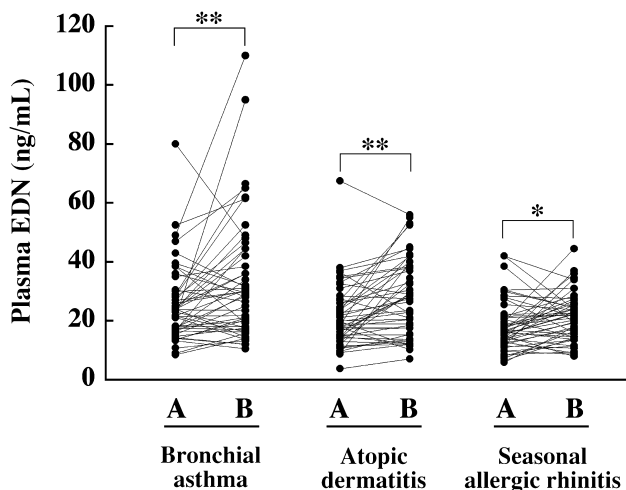


Fig. 2 Eosinophil-derived neurotoxin (EDN) concentrations in plasma from patients with bronchial asthma, atopic dermatitis and seasonal allergic rhinitis. The EDN concentrations were measured in patients with bronchial asthma as well as seasonal allergic rhinitis when patients were either asymptomatic (A) or symptomatic (B), and from patients with either mild (A) or severe (B) atopic dermatitis. Concentrations of EDN from patients with allergic diseases were compared when their symptoms were active and when they were well. ** $P < 0.01$, * $P < 0.05$.

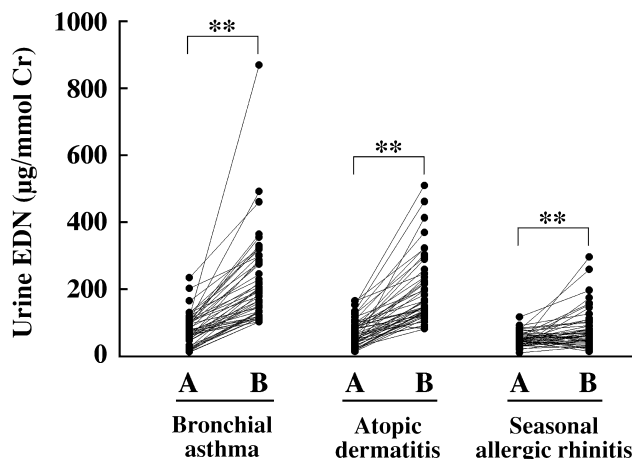


Fig. 3 Eosinophil-derived neurotoxin (EDN) concentrations in urine from patients with bronchial asthma, atopic dermatitis and seasonal allergic rhinitis. The EDN concentrations were measured in patients with bronchial asthma as well as seasonal allergic rhinitis when patients were either asymptomatic (A) or symptomatic (B), and from patients with either mild (A) or severe (B) atopic dermatitis. Concentrations of EDN from patients with allergic diseases were compared when their symptoms were active and when they were well. ** $P < 0.01$.

urine from healthy control subjects were 19.1 ng/mL (16.0–28.5 ng/mL), 13.8 ng/mL (11.3–18.0 ng/mL) and 61.1 $\mu\text{g}/\text{mmol Cr}$ (36.1–80.3 $\mu\text{g}/\text{mmol Cr}$), respectively. The concentrations of EDN in the serum, plasma and urine from asymptomatic asthmatic patients were significantly elevated compared with values obtained for healthy control subjects (Fig. 4). The concentrations of EDN in the serum and plasma from patients with mild atopic dermatitis (Fig. 5), as well as from asymptomatic patients with seasonal allergic rhinitis (Fig. 6), were significantly elevated compared with values obtained for healthy control subjects.

Median (quartile 1–quartile 3) EDN concentrations in the serum, plasma and urine from symptomatic bronchial asthmatic patients were 49.8 ng/mL (29.5–76.0 ng/mL), 28.8 ng/mL (17.8–42.0 ng/mL) and 191.1 $\mu\text{g}/\text{mmol Cr}$ (133.9–280.7 $\mu\text{g}/\text{mmol Cr}$); those from patients with

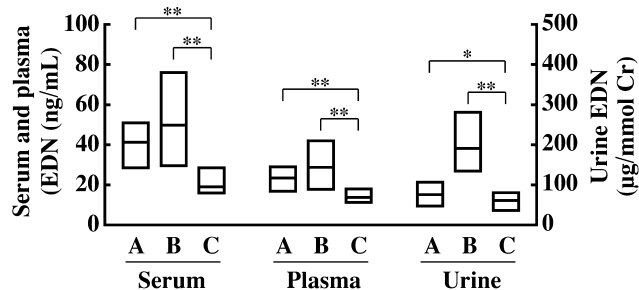


Fig. 4 Eosinophil-derived neurotoxin (EDN) concentrations in serum, plasma and urine from bronchial asthmatic patients when they were either asymptomatic (A) or symptomatic (B) and healthy control subjects (C). Results are presented as the median (quartile 1–quartile 3). ** $P < 0.01$, * $P < 0.05$.

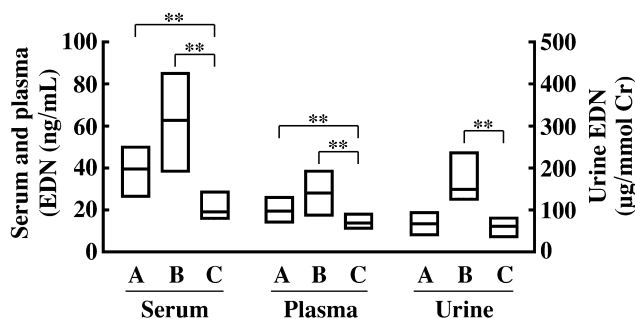


Fig. 5 Eosinophil-derived neurotoxin (EDN) concentrations in serum, plasma and urine from patients with either mild (A) or severe (B) atopic dermatitis and healthy control subjects (C). Results are presented as the median (quartile 1–quartile 3). ** $P < 0.01$.

severe atopic dermatitis were 62.8 ng/mL (38.5–85.0 ng/mL), 28.0 ng/mL (17.5–38.5 ng/mL) and 149.5 µg/mmol Cr (125.6–235.7 µg/mmol Cr), respectively; and those from symptomatic patients with seasonal allergic rhinitis were 41.3 ng/mL (26.5–50.0 ng/mL), 21.8 ng/mL (14.5–25.5 ng/mL) and 65.1 µg/mmol Cr

(44.7–92.3 µg/mmol Cr), respectively. The concentrations of EDN in the serum, plasma and urine, except for EDN concentrations in the urine from symptomatic patients with seasonal allergic rhinitis, were significantly elevated in patients with allergic diseases compared with values obtained for healthy control subjects (Figs 4–6).

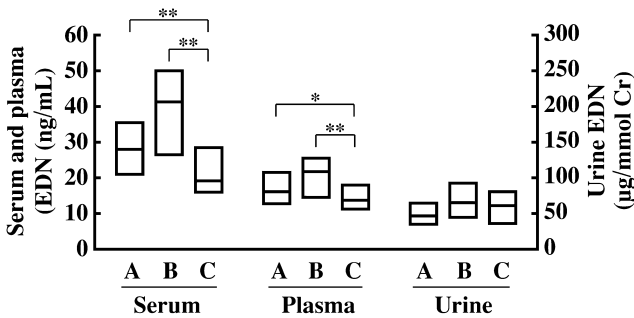
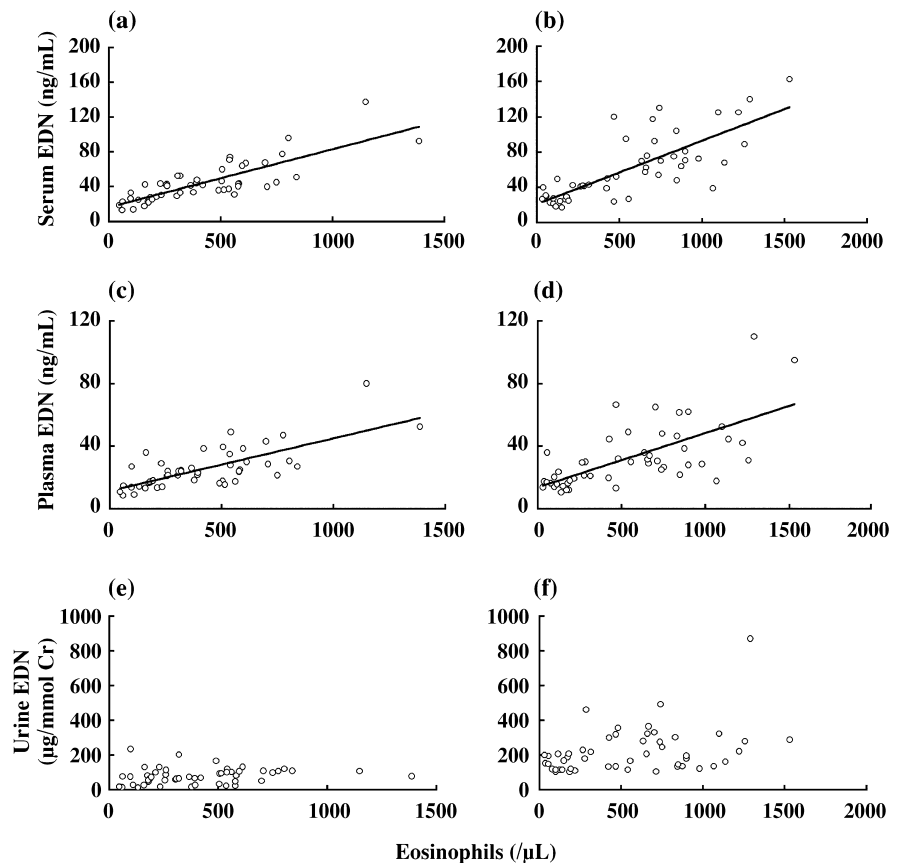


Fig. 6 Eosinophil-derived neurotoxin (EDN) concentrations in serum, plasma and urine from patients with seasonal allergic rhinitis when they were either asymptomatic (A) or symptomatic (B) and healthy control subjects (C). Results are presented as the median (quartile 1–quartile 3). ** $P < 0.01$, * $P < 0.05$.

Number of eosinophils in peripheral blood from patients with allergic diseases when their symptoms were active and when patients were well

The number of eosinophils in peripheral blood was counted in samples from the same patients when their symptoms were active and when they were well. Median values of eosinophils in peripheral blood from asymptomatic and symptomatic bronchial asthma patients were 372 and 510/mm³, respectively; those from patients with mild and severe atopic dermatitis were 464 and 701/mm³, respectively; and those from asymptomatic and symptomatic patients with seasonal allergic rhinitis were 197 and 331/mm³, respectively. The number of

Fig. 7 Relationships in bronchial asthmatic patients when they were either asymptomatic (a,c,e) or symptomatic (b,d,f) between the number of eosinophils in peripheral blood and eosinophil-derived neurotoxin (EDN) concentrations in serum (a,b), plasma (c,d) and urine (e,f). The correlation between serum EDN concentrations and the number of eosinophils in asymptomatic and symptomatic patients was $r = 0.660$, $P < 0.0001$ and $r = 0.605$, $P < 0.0001$, respectively. The correlation between plasma EDN concentrations and the number of eosinophils in asymptomatic symptomatic patients was $r = 0.527$, $P < 0.0001$ and $r = 0.442$, $P < 0.0001$, respectively.



eosinophils in peripheral blood was significantly higher in patients with allergic diseases when their symptoms were active compared with when they were well ($P < 0.05$ in bronchial asthma; $P < 0.01$ in atopic dermatitis and seasonal allergic rhinitis).

Relationships between EDN concentrations and the number of eosinophils in peripheral blood from patients with allergic diseases

As shown in Fig. 7, significant relationships were observed between the concentration of serum EDN/plasma EDN and the number of eosinophils in peripheral blood from asymptomatic patients with bronchial asthma ($r = 0.660$, $P < 0.0001$ and $r = 0.527$, $P < 0.0001$, respectively) as well as symptomatic patients with bronchial asthma ($r = 0.605$, $P < 0.0001$ and $r = 0.442$, $P < 0.0001$, respectively). Significant relationships were observed between serum EDN concentrations and the number of eosinophils in peripheral blood in patients with mild as well as severe atopic

dermatitis ($r = 0.557$, $P < 0.0001$ and $r = 0.431$, $P < 0.0001$, respectively). A significant relationship was also observed between plasma EDN concentrations and the number of eosinophils in peripheral blood in patients with mild atopic dermatitis ($r = 0.454$, $P < 0.0001$; Fig. 8). A significant relationship was observed between serum EDN concentrations and the number of eosinophils in peripheral blood in asymptomatic patients with seasonal allergic rhinitis ($r = 0.520$, $P < 0.0001$; Fig. 9).

DISCUSSION

Concepts have been developed that measurements of eosinophil-derived proteins in serum, especially assessment of serum ECP, may be used to reflect inflammatory activities of eosinophils in the lungs of patients with bronchial asthma.² However ECP is a very sticky and extremely charged protein¹⁶ and the clinical usefulness of ECP has been questioned because of problems in sample processing and the considerable variability of measured values.³

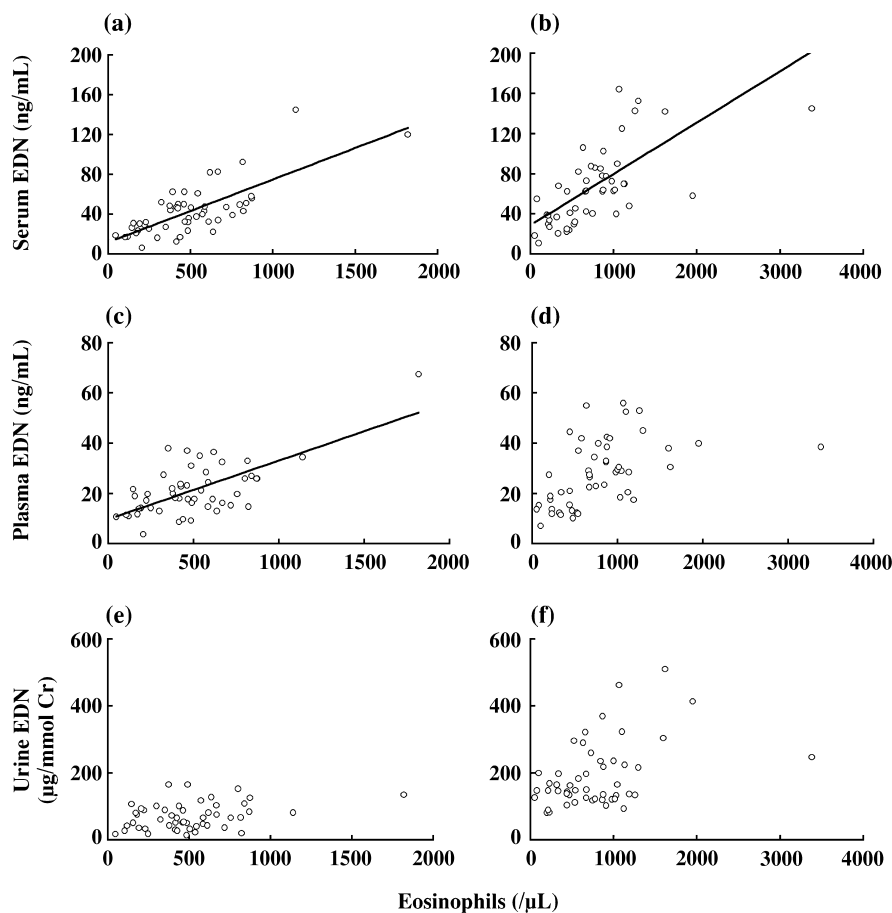


Fig. 8 Relationships in patients with mild (a,c,e) or severe (b,d,f) atopic dermatitis between the number of eosinophils in peripheral blood and eosinophil-derived neurotoxin (EDN) concentrations in the serum (a,b), plasma (c,d) and urine (e,f). The correlation between serum EDN concentrations and the number of eosinophils in patients with mild or severe atopic dermatitis was $r = 0.557$, $P < 0.0001$ and $r = 0.431$, $P < 0.0001$, respectively. The correlation between plasma EDN concentrations and the number of eosinophils in patients with mild atopic dermatitis was $r = 0.454$, $P < 0.0001$.

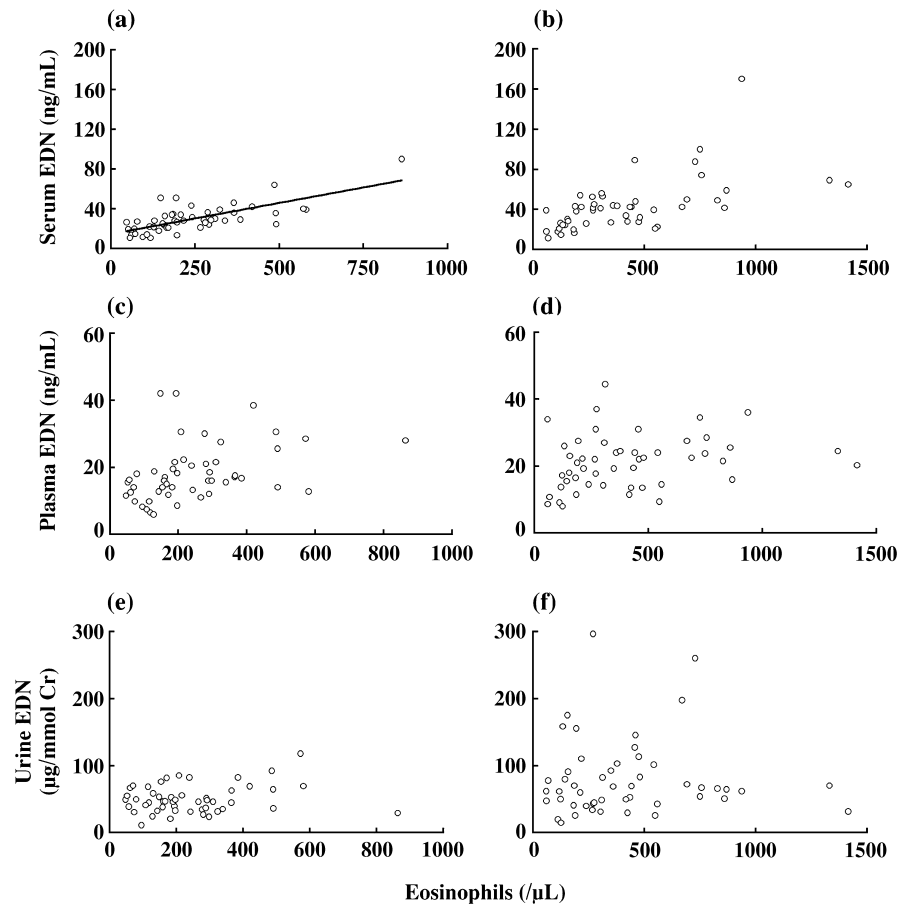


Fig. 9 Relationships in patients with seasonal allergic rhinitis when they were either asymptomatic (a,c,e) or symptomatic (b,d,f) between the number of eosinophils in peripheral blood and eosinophil-derived neurotoxin (EDN) concentrations in serum (a,b), plasma (c,d) and urine (e,f). The correlation between serum EDN concentrations and the number of eosinophils in asymptomatic patients was $r = 0.520$, $P < 0.0001$.

Eosinophil-derived neurotoxin, also called EPX, possesses high sequence homology to ECP⁴⁻⁶ and is the only one of the four basic eosinophil granule proteins that can be measured accurately in urine.⁷ In fact, it has been reported that urinary EDN may be a valid alternative to measurements of eosinophil proteins in serum.⁸ Recent studies have indicated the usefulness of measuring concentrations of urinary EPX in monitoring childhood bronchial asthma^{17,18} and atopic dermatitis in children and adults,¹⁹⁻²¹ and measurement of blood EPX in monitoring seasonal allergic rhinitis.²² However, we are not aware of any report indicating differences in blood and urinary EDN concentrations from patients with allergic diseases.

We have reported an efficient ELISA method for measuring specifically human blood and urinary EDN.⁹ In addition, we have shown that the method may be useful to determine the possible roles of eosinophils in on-going asthma.¹⁰ We have expanded our earlier studies and, in the present paper, have demonstrated that EDN concentrations in blood and urine, as well as the number of eosinophils in peripheral blood, from patients with allergic

diseases were significantly higher when their symptoms were active compared with those when they were well. Concerning EDN concentrations in the blood, the EDN concentrations in the serum were higher than those in the plasma, irrespective of the allergic disease. We could not investigate the mechanisms of this finding in the present study. However, it is well known that sample processing conditions can affect ECP blood levels^{23,24} and serum levels of ECP represent the levels found *in vivo* plus additional proteins released *in vitro* from peripheral blood eosinophils during the coagulation period.^{25,26} So, the same mechanisms may be involved in the case of EDN and further studies are required. In any case, our data support previous reports that urinary EPX may reflect disease activity and that measurements of urinary EPX may reflect inflammatory activities of eosinophils in childhood bronchial asthma,^{17,18} as well as atopic dermatitis in children and adults.¹⁹⁻²¹ However, these studies were performed in a single allergic disease. Therefore, the question remains as to whether there are differences in blood and urinary EDN concentrations among the allergic diseases.

As the next step, we examined differences in blood and urinary EDN concentrations between patients with allergic diseases and healthy control subjects. The concentrations of blood and urinary EDN were increased in bronchial asthmatic patients, irrespective of their condition (symptomatic or asymptomatic), compared with healthy control subjects. Eosinophil-derived neurotoxin concentrations in the serum and plasma from patients with mild atopic dermatitis, as well as asymptomatic patients with seasonal allergic rhinitis, were increased compared with values obtained for healthy control subjects. In addition, EDN concentrations in the blood and urine from patients with active symptoms, except for EDN concentrations in the urine from symptomatic patients with seasonal allergic rhinitis, were significantly increased compared with values obtained for healthy control subjects. There were no differences in urinary EDN concentrations between healthy control subjects and patients with seasonal allergic rhinitis, irrespective of their symptoms. In fact, it has been shown that EPX concentrations in serum and plasma are related to symptoms in patients with seasonal allergic rhinitis.²²

Finally, we examined the relationship between EDN concentrations and the number of eosinophils in peripheral blood from patients with allergic diseases. Eosinophil-derived neurotoxin concentrations in serum and plasma from bronchial asthmatic patients were significantly correlated with the number of peripheral blood eosinophils, irrespective of their clinical symptoms. The EDN concentrations in the serum and plasma from patients with mild atopic dermatitis were significantly correlated with the number of peripheral blood eosinophils. However, in patients with seasonal allergic rhinitis, only EDN concentrations in the serum from asymptomatic patients were significantly correlated with the number of eosinophils in peripheral blood. The reason why EDN concentrations from symptomatic patients did not correlate with the number of eosinophils in peripheral blood remains unknown. However, taken together with the result mentioned above that there were no differences in urinary EDN concentrations between healthy control subjects and patients with seasonal allergic rhinitis irrespective of their symptoms, the degree of eosinophilic inflammation in seasonal allergic rhinitis may be less compared with that in bronchial asthma and atopic dermatitis and measuring the number of eosinophils in the peripheral blood may not be useful in assessing the inflammatory processes of seasonal allergic rhinitis. Further studies are required to clarify this point.

In conclusion, we are the first to report differences in blood and urinary EDN concentrations from patients with allergic diseases. Our findings demonstrate that measurements of blood and urinary EDN concentrations are useful for monitoring eosinophil activity in allergic diseases.

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