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Original article

Utility of serum eosinophil-derived neurotoxin (EDN) measurement by ELISA in young children with asthma

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EDN, eosinophil-derived neurotoxin; ELISA, enzyme-linked immunosorbent assay; MFDS, Ministry of Food and Drug Safety; ECP, eosinophil cationic protein; PBS, phosphate buffer solution; TMB, tetramethylbenzidine; GINA, Global Initiative for Asthma; PPV, positive predictive value; NPV, negative predictive value; MBP, major basic protein; EPO, eosinophil peroxidase; RIA, radioimmunoassay; RSV, respiratory syncytial virus

ABSTRACT

Background: This study was done to compare the efficacy of a recently developed eosinophil-derived neurotoxin (EDN) ELISA kit ("BioTracer™ K® EDN ELISA Kit") to a commercially available EDN ELISA kit ("MBL EDN ELISA Kit") and demonstrate the usefulness of serum EDN measurement in young asthmatic children.

Methods: Forty-eight children with physician-diagnosed asthma (Asthma group) and 31 age-matched normal controls (Control group) were recruited from the Asthma and Allergy Center at Inje University Sanggye Paik Hospital, Seoul, Korea from January 2010 to September of 2012. EDN levels in each serum specimen were measured 2 times using the: 1) BioTracer™ K® EDN ELISA Kit and 2) MBL EDN ELISA Kit at the Inje University Sanggye Paik Hospital laboratory. EDN level measurements in each serum specimen were compared.

Results: EDN measurements from the BioTracer™ K® EDN ELISA Kit correlated well with those from the MBL EDN ELISA Kit: $r = 0.9472$ at the Inje University Sanggye Paik Hospital laboratory. These r values were considered both clinically relevant (i.e., $r > 0.85$) and statistically significant ($p < 0.0001$). EDN measurements from both kits positively correlated with asthma symptom severity ($p < 0.0001$). No serious adverse events occurred during the study.

Conclusions: The BioTracer™ K® EDN ELISA Kit was accurate and useful in measuring EDN levels in young asthma patient serum. Because of our kit's distinct advantages and utility, we suggest this kit can be used for the timely diagnosis, treatment, and monitoring of asthma in asthma patients of all ages, especially those too young to perform pulmonary function tests.

Introduction

The eosinophil is a major player in allergic disease.[1] Therefore, direct measurement of eosinophilic inflammation is needed for diagnosis, treatment, and monitoring of asthma. However, management decisions have traditionally been based on symptoms (non-specific and subjective), airway function, and rescue medication use.[2,3]

The current feeling on eosinophilic inflammation monitoring is that eosinophil counts/percentages provide only a limited understanding of the activity of these cells, whereas, the secretory activity of eosinophils (the product of the concentration of eosinophils and their propensity to release mediators)[4] provides a more accurate and complete picture.

During the past few decades, specific markers have been identified that are currently used to identify the activity and turnover of the eosinophil. The most promising of these markers has been eosinophil-derived neurotoxin (EDN), which has been studied in a number of inflammatory diseases including asthma.[5–8] An enzyme-linked immunosorbent assay (ELISA) kit for measuring EDN has been available for more than ten years (MBL International Corporation, Woburn, MA); however, its utility is limited. The Ministry of Food and Drug Safety (MFDS) in Korea allows this product to be used only for research purposes (MBL Code No 7630)[9] and therefore cannot be used in routine clinical practice such as

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monitoring airway inflammatory markers in asthma patients, especially those too young to participate in lung function tests (i.e., under 7 years of age).

The purpose of this study was to investigate the efficacy and utility of a recently developed EDN ELISA kit (referred to hereafter as "BioTracer™ K® EDN ELISA Kit") for the diagnosis, treatment, and monitoring of asthma patients in Korea. The BioTracer™ K® EDN ELISA Kit was directly compared to a commercially available EDN ELISA kit (referred to hereafter as "MBL EDN ELISA Kit") in Korea.

Methods

Development of EDN antibody

Female BALB/c mice (6–8 weeks old) were injected intraperitoneally with 50–100 μg of natural EDN antigen (Mayo Clinic, Rochester, MN, USA) in Freund's complete adjuvant (Sigma, St. Louis, MO, USA). Booster injections of 50–100 μg of antigen were administered every 2–3 weeks either in incomplete adjuvant or in PBS. After the final injection, antibody formation was checked by eye bleeding. Once verified, 100–200 μg of antibody was injected 3–4 consecutive days before fusion.

Mice were sacrificed by cervical dislocation and briefly immersed in 70% alcohol to sterilize. The spleen was removed, spleen cells were flushed and harvested by centrifuge, then resuspended in serum-free media. Spleen cells were fused with myeloma cells from the P3X63Ag8.653 mouse cell line (Sigma) at a ratio of 1.5:1.1. Hybridomas were selected by ELISA for antibody formation. Monoclonal anti-EDN antibody from the mice was then purified by centrifuge and stored at 4 °C (short-term storage) or at −20 °C (long-term storage).

Sandwich ELISA

The BioTracer™ K® EDN ELISA Kit measured human EDN by sandwich ELISA.[10] This ELISA detects human EDN with a minimum detection limit of 6.0 ng/ml, maximum detection limit of 400 ng/ml, and does not cross-react with eosinophil cationic protein (ECP). The method described by Morioka *et al.*[11] was followed but modified slightly. Briefly, Nunc MaxiSorp 96-well plates (Thermo Fisher Scientific, Waltham, MA, USA) were coated overnight at 4 °C with 100 μl of mouse anti-EDN monoclonal antibody (mAb), diluted in phosphate buffer solution (PBS). The wells were blocked overnight at 4 °C with 200 μl of blocking buffer (1X PBS, 1% bovine serum albumin (BSA), 10% sucrose). Standard EDN was diluted with 50 mM tris pH8.0 containing 0.05% Tween 20 buffer (Sigma–Aldrich, St. Louis, MO, USA), 0.15 M NaCl, and 0.5% BSA (termed assay diluent). The range of measurements was 0.6–40 ng/ml, indicating assay sensitivity was less than 0.6 ng/ml. Between each subsequent step, plates were washed three times in PBS containing 0.05% Tween 20. Samples were then diluted in 50 mM tris pH8.0 containing 0.05% Tween 20, and 0.15 M NaCl. One-hundred microlitre of standards and diluted samples were applied to the plates, and incubated at room temperature for 1 h. After washing, 100 μl of horseradish-peroxidase-labeled mouse anti-EDN mAb was added to the wells and incubated at room temperature for 1 h. After another washing, the peroxidase substrate tetramethylbenzidine (TMB) (Sigma–Aldrich) was added (100 μl/well) and incubated for 10 min at room temperature. Enzyme reactions were stopped with 1 N HCl (100 μl/well). Absorbance was measured at 450 nm by a Micro Plate Reader Infinite 200 PRO (TECAN, Männedorf, Switzerland). Serum EDN was determined from a dose response curve by multiplying the value read from the standard curve by the dilution factor.

Subjects

Forty-eight children with physician-diagnosed asthma (Asthma group) and 31 age-matched normal controls (Control group) were recruited from the Asthma and Allergy Center at Inje University Sanggye Paik Hospital, Seoul, Korea from January 2010 to September of 2012. The Asthma group consisted of 29 boys and 19 girls (mean age, 3.2 years; range, 1.4–5.6 years). All children in this group had previously been diagnosed as having asthma according to the Global Initiative for Asthma (GINA) guidelines.[2] Infantile asthma was originally diagnosed as having more than three wheezing episodes. They had been using bronchodilators on demand during symptomatic periods.

Asthma symptom severity was based on a previously published asthma scoring system for young children.[12] All clinical characteristics were scored on a 3-point ordinal scale (0, 1, and 2), with the total score consisting of five clinical features: (1) respiratory rate (<40 breaths/min, 40–60 breaths/min, >60 breaths/min); (2) wheezing (expiratory or inspiratory wheezing heard with a stethoscope); (3) retraction (subcostal or intercostals muscle retraction); (4) observed dyspnea (observer's impression of the patient's degree of breathlessness); and (5) inspiratory to expiratory ratio (I > E, I = E, I < E). All five characteristics were weighted equally with final clinical scores obtained by summing individual item values for a maximum possible clinical asthma score of 10. Patients were then grouped according to their total score: a score of 0 was considered "symptom free"; scores of 1–3 were considered "Mild"; scores of 4–6 were labeled "Moderate"; and scores of 7–9 were grouped as "Severe". These asthma scores were then compared to serum EDN levels to determine if there were any correlations between them.

Inclusion criteria included symptomatic or asymptomatic asthma. Exclusion criteria included: any antibiotic treatment or other respiratory medicine within 4 weeks of patient enrollment; any health condition that would affect the ability of the patient to give a blood specimen; or any potential for serious adverse events.

The Control group consisted of 18 boys and 13 girls (mean age, 3.1 years; range, 1.3–5.4 years). None had a history of asthma or allergic disease or an identifiable airway infection within the 4 weeks prior to the study.

The primary outcome was EDN level measurements in all patient serum specimens comparing results found with the BioTracer™ K® EDN ELISA Kit to results found in the MBL EDN ELISA Kit at Inje University Sanggye Paik Hospital (Seoul, Korea). All adverse events, including local and systemic reactions, were also recorded.

Legal guardians of all participants gave written informed consent and patient anonymity was preserved using methods approved by the Ethics Committee. This study was approved by the Inje University Sanggye Paik Hospital Institutional Review Board (IRB).

Specimen collection and measurements

Blood specimen collection

BD Vacutainer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) serum separation tubes were used to collect blood specimens. The tourniquet was removed from the arm as soon as blood flowed to prevent hemoconcentration. Care was taken to perform venipuncture in a manner so that the likelihood of any complication following this was minimized. The nurse performing the venipuncture observed universal precautions for the prevention of bloodborne pathogen transmission.

Serum specimen collection

Serum specimens were prepared as described by Peterson *et al.*[13] Briefly, serum was prepared by allowing blood to clot at

25 °C for 1hr, then centrifuged at 1350 *g* for 10 min at 4 °C. Each serum specimen was aliquoted into a new plastic tube and stored at −70 °C until the assay.

Serum EDN measurement

The central laboratory at Inje University Sanggye Paik Hospital in Seoul, Korea was used for serum EDN measurements. Each sample was measured two separate times for EDN level: once by the BioTracer™ K® EDN ELISA Kit and once by the MBL EDN ELISA Kit at Inje University Sanggye Paik Hospital.

Statistical analysis

All demographic data are presented as median [25th percentile, 75th percentile], unless otherwise noted.

As all EDN level data were nonparametric, screening of data for differences in EDN levels between the two groups was performed using the Mann–Whitney U test. Spearman correlation coefficients were used for analyzing correlations of EDN levels between the two kits, BioTracer™ K® EDN ELISA and MBL EDN ELISA. A positive Spearman correlation coefficient r of >0.85 was considered clinically relevant and p values <0.05 were considered statistically significant.

Results

Subject measurements

Demographic data

All demographic data were as follows: Total number of subjects (n), $n = 79$; Age (yr) median [25th percentile, 75th percentile], 3 [1.5, 5]; Male:Female, 47:32.

Human EDN concentration in Asthma and Control groups

Serum samples from the Asthma and Control groups were measured with the BioTracer™ K® EDN ELISA Kit and minimum and maximum EDN levels, 25th percentile, 75th percentile, median, mean and standard deviations were determined (Table 1). All EDN level data were nonparametric; consequently, they are stated as median [25th percentile, 75th percentile] in ng/ml. Using the BioTracer™ K® EDN ELISA Kit, EDN levels for 79 subjects were 50.60[34.05, 83.05]. No serious adverse events occurred during the study.

Correlations between EDN measurements

EDN measurements using the BioTracer™ K® EDN ELISA Kit correlated well with measurements from the MBL EDN ELISA Kit: $r = 0.9611$ at the Inje University Sanggye Paik Hospital laboratory (Fig. 1). This r value was considered both clinically relevant (i.e., $r > 0.85$) and statistically significant ($p < 0.0001$).

Table 1
Serum EDN concentration in asthma and normal controls.

	Asthma group ($n = 48$)	Control group ($n = 31$)
Minimum	37.30	8.000
25% Percentile	50.55	26.70
Median	73.75	31.90
75% Percentile	98.55	34.75
Maximum	168.1	65.00
Mean	77.33	31.51
SD	30.17	12.34
Median + 1SD	103.92	44.24

SD, Standard Deviation.

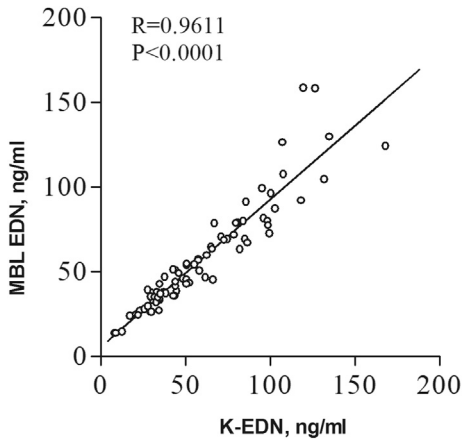

Fig. 1. Correlations between EDN measurements using the BioTracer™ K® EDN ELISA Kit and the MBL EDN ELISA Kit at Inje University Sanggye Paik Hospital laboratory ($r = 0.9472$). These r values were considered both clinically relevant (i.e., $r > 0.85$) and statistically significant ($p < 0.0001$).

Correlations between EDN levels and asthma symptom severity

EDN measurements taken by both kits positively correlated with asthma symptom scores ($p < 0.0001$). 26.7% of subjects were symptom free (score = 0), while 48.9% were in the Mild subgroup (score = 1–3) and 24.4% were in the Moderate subgroup (score = 4–6). There were no patients in the Severe subgroup (score = 7–9).

Standard curve for EDN

A typical standard curve for EDN is shown in Figure 2. EDN concentrations in specimens were determined by multiplying the value read from the standard curve by the dilution factors. EDN measurements ranged from 0.60 to 40.0 ng/ml, indicating that assay sensitivity was less than 0.60 ng/ml.

Assay measurements

Assay precision

Intraassay precision (Table 2) was determined by repetitive measurements (8 times) of 3 different serum specimens. Interassay precision (Table 2) was determined by comparing 3 other different serum specimens from 10 independent assays performed on 10 different days.

Dilution test

Good linearity was noted with 3 different serum specimens that were serially diluted (Fig. 3). Dilution factors were 0.03125, 0.0625, 0.125, 0.25, 0.5, and 1.0.

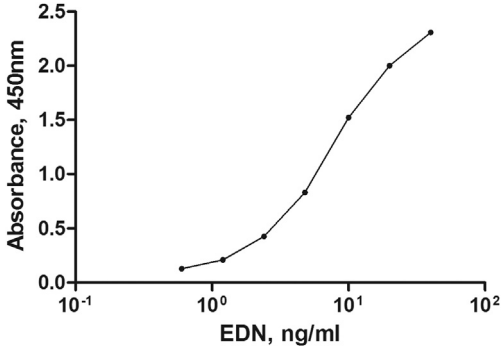

Fig. 2. Standard curve for EDN.

Table 2
Assay precision.

	Sample	Replicates	Mean (ng/ml)	SD (ng/ml)	CV (%)
Intraassay	1	8	0.93	0.04	4.1
	2	8	3.73	0.07	1.9
	3	8	9.02	0.22	2.4
Interassay	4	10	4.67	0.40	8.6
	5	10	14.26	1.04	7.3
	6	10	18.52	1.35	7.3

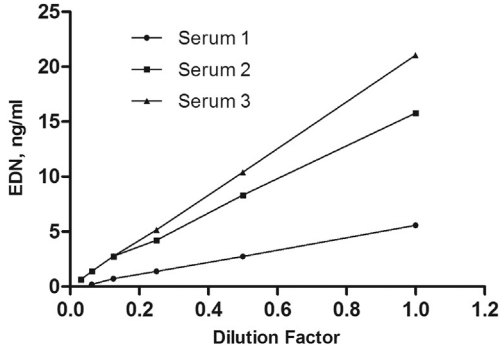

Fig. 3. Dilution test.

Recovery test

Three different specimens supplemented with different amounts of purified EDN (2.0, 4.0, and 8.0 ng/ml, respectively) were used to determine the ratio of recovered/added EDN concentrations, expressed as percentages. As seen in Table 3, recoveries of purified EDN added to serum specimens ranged from 90 to 109%.

Cross-reactivity test

To determine if there was any cross-reactivity with ECP when using the BioTracer™ K® EDN ELISA Kit, two separate tests were conducted. First, as increasing amounts of ECP antigen were added to each well (negative, positive, 0, 0.4, 1.2, 4, 12, and 40 ng/ml), there was no corresponding increase in optical density (O.D), as measured by the BioTracer™ K® EDN ELISA Kit [(O.D1 = 0.058, 0.054, 0.052, 0.054, 0.054, 0.060, 0.054, 0.064) and duplicate results (O.D2 = 0.052, 0.051, 0.054, 0.051, 0.053, 0.053, 0.062, 0.064)]. Second, much larger amounts of ECP antigen (0, 50, 100, 500, 1000, and 10,000 ng/ml) were added to wells with no corresponding increase in O.D, as measured by the BioTracer™ K® EDN ELISA Kit (0.054, 0.058, 0.052, 0.060, 0.078, and 0.083). EDN antigen was then added to each well (0.0, 0.6, 1.2, 2.5, 5.0, 10, 20, and 40 ng/ml) and O.D did increase correspondingly (0.052, 0.104, 0.159, 0.308, 0.577, 1.101, 1.720, and 1.974). The results of these two tests for cross-reactivity were combined in Figure 4.

Table 3
Recovery test.

Sample	Initial EDN (ng/ml)	Added EDN (ng/ml)	Final EDN (ng/ml)	Measured EDN (ng/ml)	Recovered EDN (ng/ml)	Recovery (%)
1	4.0	2.0	6.0	6.1	2.1	105
		4.0	8.0	7.6	3.6	90
		8.0	12.0	11.5	7.5	94
2	5.5	2.0	7.5	7.6	2.1	105
		4.0	9.5	9.7	4.2	105
		8.0	13.5	13.3	7.8	98
3	11.5	2.0	13.5	13.3	1.8	90
		4.0	15.5	15.2	3.7	93
		8.0	19.5	20.2	8.7	109

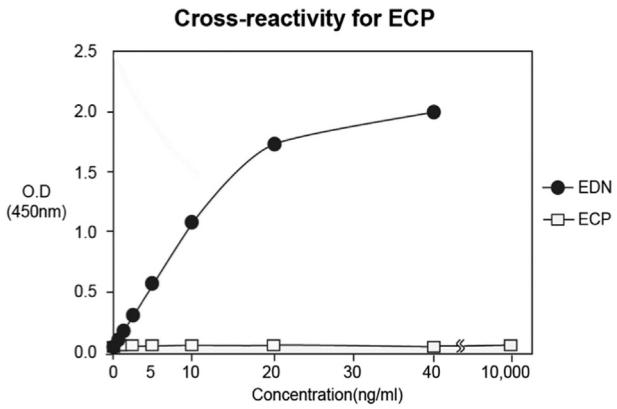

Fig. 4. Cross-reactivity for ECP.

Predictive value of EDN levels for asthma

Predictive value of EDN levels for asthma was based on clinical performance results (Table 4). Using 44.2 ng/ml (median + 1SD) as the cut off for an elevated EDN level compared to those found in Controls, sensitivity was 81.3%, specificity was 87.1%, positive predictive value (PPV) was 90.7%, and negative predictive value (NPV) was 75.0%.

Discussion

A direct comparison of serum EDN levels found using the BioTracer™ K®EDN ELISA Kit and the MBL EDN ELISA Kit showed a clinically and statistically significant correlation. The risks to patients in this study were no greater than those encountered in routine medical practice and were small. Consequently, no serious adverse events occurred during the study. Medical benefits for study subjects included a more accurate understanding of the nature of their underlying eosinophilic inflammation, which could possibly lead to a more effective and timely treating of their asthma. Overall, as this was an opportunity to formally vet the safety and efficacy of the recently developed BioTracer™ K® EDN ELISA Kit for the diagnosis, treatment, and monitoring of asthma, we believe the risk benefit ratio markedly favored the beneficial aspects.

The BioTracer™ K® EDN ELISA Kit has two distinct advantages over the MBL EDN ELISA Kit. First, it has a broader measuring range. Due to the serum specimen dilution being twice what is performed using the MBL Kit (10-fold dilution versus 5-fold dilution), EDN measuring range of the BioTracer™ is 6–400 ng/ml versus 3–200 ng/ml. Theoretically, and in practice, asthma patients may exhibit EDN levels >200 ng/ml, including one patient in this study. Second, because of the greater dilution factor required for the BioTracer™ K® EDN ELISA Kit as stated above, a smaller serum specimen volume from the patient is required (20 μl versus 50 μl). This is highly advantageous in young children because obtaining adequate specimen volume is sometimes prevented by a child's much lower total blood volume, difficulty in vein location, and their unwillingness to cooperate with the venipuncture procedure.

Table 4
Clinical performance.

	Asthma group† (*n* = 48)	Control group (*n* = 31)	Total (*n* = 79)
Positive†	39	4	43
Negative†	9	27	36
Total	48	31	79

Sensitivity: 39/(39 + 9) = 81.3%, Specificity: 27/(27 + 4) = 87.1%, Positive Predictive Value: 39/(39 + 4) = 90.7%, Negative Predictive Value: 27/(27 + 9) = 75.0%.
† Test results: BioTracer™ K® EDN ELISA.

Diagnostic assays should clearly reflect underlying pathophysiology (i.e., airway inflammation) for effective treatment; however, many do not. Eosinophil granule proteins [EDN, ECP, major basic protein (MBP), eosinophil peroxidase (EPO)] are the most strongly implicated in asthma pathophysiology[14] and EDN and ECP are released almost exclusively by eosinophils.[1] Therefore, any change in EDN or ECP level would be a direct reflection of changes in eosinophilic inflammation. In particular, several studies have shown the efficacy of EDN levels as a biomarker for eosinophilic inflammation[5–8] – including studies done by this research group[15–18] – not only for monitoring and treatment, but also for diagnosis (e.g., asthma: PPV = 93%, NPV = 54%, sensitivity = 66%, specificity = 89%).[15] Though eosinophils are important as major effector cells in asthma, eosinophil degranulation and its associated products like EDN may be even more important. It has been suggested that the secretory activity of eosinophils – a combination of the concentration of eosinophils and their tendency to release degranulation products – may be a key marker of disease activity and is more accurately measured by eosinophil degranulation products than by total eosinophil counts.[4] This has been demonstrated in two studies published by our research group[15,17] and one by Kim *et al.*[5] directly comparing EDN, ECP, and total eosinophil counts to disease activity.

To measure EDN quickly, simply, and accurately, a commercial EDN ELISA kit was developed by a Japanese research group,[11] which has been available for some time. EDN ELISA kits have demonstrated accuracy in measuring EDN levels when compared to the gold standard method of radioimmunoassay (RIA).[11,19] Since development, hundreds of studies have used the MBL EDN ELISA kit for quick and accurate measurement of EDN. Indeed, when using the MBL EDN ELISA Kit or BioTracer™ K® EDN ELISA Kit, serum EDN measurement can be completed in under 2.5 h.[11]

Recently, the BioTracer™ K® EDN ELISA Kit was developed by Bio Focus, Co., Ltd, in Korea. Its high accuracy, broad EDN measurement range, and need for a much smaller patient specimen volume would allow EDN measurements to be clinically useful and economically practical for day-to-day diagnosis, treatment, and monitoring of a number of eosinophil-related disorders such as asthma, atopic dermatitis,[11] and post-respiratory syncytial virus (RSV) bronchiolitis recurrent wheezing.[20]

Because of its accuracy, complete lack of serious adverse events when using it, and distinct advantages over the commercially available MBL EDN ELISA Kit, we feel that the BioTracer™ K® EDN ELISA Kit would be a useful and cost-effective tool in the diagnosis, treatment, and monitoring of asthma.

Conflict of interest

The authors have no conflict of interest to declare.

Authors' contributions

CK designed the study. ZC and CK wrote the manuscript. CK, JP, and EK contributed to patient recruitment and data collection. CK and ZC performed data analysis and interpreted the results. All authors read and approved of the final version of the manuscript.

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