Allergology International 66 (2017) 70–74

Contents lists available at ScienceDirect

Allergology International

journal homepage: http://www.elsevier.com/locate/alit

Original article

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

Chang-Keun Kim [a, *], Zak Callaway [a, b], Jin-Sung Park [a], Eunmi Kwon [a]

[a] Asthma and Allergy Center, Department of Pediatrics, Inje University Sanggye Paik Hospital, Seoul, South Korea
[b] School of Biological Sciences, University of Ulsan, Ulsan, South Korea

ARTICLE INFO

Article history:
Received 16 December 2015
Received in revised form
2 May 2016
Accepted 7 May 2016
Available online 18 June 2016

Keywords:
Asthma
Diagnosis
ELISA
Eosinophil-derived neurotoxin
Eosinophils

Abbreviations:
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

* Corresponding author. Asthma & Allergy Center, Inje University Sanggye Paik Hospital, 761-1 Sanggye 7 dong, Nowon Ku, Seoul 139-707, South Korea.
E-mail address: kimck@paik.ac.kr (C.-K. Kim).
Peer review under responsibility of Japanese Society of Allergology.

http://dx.doi.org/10.1016/j.alit.2016.05.008

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