A study for the correlation between eosinophil Derived Neurotoxin (EDN) and asthma.

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Abstract

Background: Eosinophil Derived Neurotoxin (EDN) has been used to assess eosinophil cells activity and to monitor inflammation in asthmatic patients.

Objective: Study the correlation between Eosinophil Derived Neurotoxin (EDN) and asthma.

Materials and methods: Eosinophil Derived Neurotoxin (EDN) was extracted from eosinophils taken from patients with eosinophilic leukemia.This extract was conducted to study its biological effects (Gordon phenomena) and detection of antibodies against it in urine samples of diagnosed asthmatic patients.

Results: One of the two tested rabbits with the extracted EDN test material showed the signs and symptoms of Gordon phenomenon during the second day after injection and continued to show complete paralysis within the fifth day .Patients urine results showed that it contained a higher values of END than the control subjects urine .

Conclusion: Results revealed that EDN is a product that can be used as a monitor for asthma.

Key words: EDN, asthma, ELISA.

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Introduction

Pathophysiological changes of asthma are different , complex and involve interactions of several cellular and humoral mediators [1,2,3].Among the important cells types is the eosinophils.Four different cationic proteins and one neutral protein have been isolated from eosinophil. These products show potency to be a markers of the severity of inflammation [4,5].Major Basic Protein (MBP), constitutes the core of eosinophil granule and showed cytotoxicity to several helminthes, protozoa and bacteria [6,7]. It increases the hyperactivity on bronchial smooth muscle contraction in rabbits [8].

A positive correlation have been observed between the concentration of MBP and the number of thedesquamated epithelial in bronchoalveolar lavage as well as the degree of airway hyperresponsiveness in individuals [9].

MBP may induce airway hyperresponsiveness through its ability to inhibit binding of acetylcholine to muscarinic M2 receptors, resulting in the release of acetylcholine [10].Eosinophil Cationic Protein (ECP) , is a granule protein belongs to RNA–ase superfamily, it shows ribonuclease -3 activity. This protein is rich with arginine and its molecular weight is 18-21 KD [11].

ECP has many biological activities it elicits Gordon phenomenon when injected intrathecally in rabbits [12, 13]. It is able to release histamine from Mast cells [14]. Eosinophil Derived Neurotoxin EDN or eosinophil Protein x (EPX):-_It is a glycosilated single chain with protein with molecular weight of 18-21 KD. It is a member of ribonuclease -A-activity [13, 15]. Although EDN shows high sequence homology to ECP, it has

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2000 fold greater ribonuclease activity than ECP and more neurotoxicity but not cytotoxicity [16]. EPX has been used to assess eosinophil activity and to monitor inflammation in asthmatic patients. [17].Oymer et al (2001) [18], found that atopic children have higher levels of urinary EPX than non atopic with acute asthma.

Asthma inflammatory Mediators can be detected in urine like histamine; leukotriens, eosinophil proteins (ECP, EDN) as well as interleukins like IL-8 [17, 19].There are different methods for the detection of different parameters and mediators useful in asthma diagnosis. Different clinical specimens are suitable for mediators detection such as Blood, Serum, Urine, Sputum and bronchoalveolar lavage. Variety of tests for asthma are available now, some of them are *in vivo* tests like skin prick test or *in vitro* tests as serological tests. So in this study we intended to study the presence of EDN in urine samples of asthmatic patients.

Materials and methods

Patients:One hundred asthmatic patients who attended the clinic of Allergy and Asthma in Ramadi General Hospital , Al-Anbar Governorate, during the period extended from July 2002 to January 2003. Patients were selected randomly from both sexes and their ages were from 10-50 years, and examined by specialized committee to be diagnosed as an asthmatics of different status, some of them were in rest and other were in attack. Skin test was done for all the patients.

Control Group: Twenty four healthy individuals from both sexes were selected resembling the same age groups of patients. They considered as negative control groups as they did not show a history of asthma and atopy after investigation.

Urine Samples:

Clean - catch, Mid – stream urine specimen was collected from each test and control individuals. They were advised to clean their external genetalia with soap and water, then the first stream was discarded and the Mid - stream urine sample (15 ml) was collected in clean dry sterile wide mouth

Eosinophil Derived Neurotoxin or Eosin

Eosinophil Derived Neurotoxin (EDN) was prepared from eosinophils isolated from blood sample withdrawn from eosinophilic leukemia patient as described below : Five milliliters of blood were taken from a patient suffering from eosinophilic leukemia attended Blood cancers center, Baghdad, Guide–lines of specimen collection were also regarded (WHO 1995). Blood film was done from the specimen as soon as possible and stained with Leishman stain and differential cell count was done it showed 45 % Eosinophils. Eosinophils were isolated and purified from blood specimen following method of Fernandez – Botran and Vetricka (2000)[20]: Five milliliters of blood in EDTA tube mixed with equal volume of 3 % dextran solution in normal saline . The mixture shaked gently and left for 45 minutes at room temperature and centrifuged for 5 minutes at 1500 rpm .The supernatant was discarded and the pellet was suspended in an equal volume of sterile normal saline to the starting volume of blood . This suspension was transferred into centrifuge tube and carefully underlayered with 3 ml of Ficoll solution 1.090 gr/ml gravity with clean and sterile syringe and centrifuged at 2000rpm at room temperature for 40 min.The mononuclear cell layer at the interphase was removed and the granulocyte pellet collected. This pellet

washed two times in Hanks balanced salt solution (HBSS).Using percoll powder, a percoll solution of the densities 1.090, 1.095 and 1.100 gr/ml was prepared in clean sterile glass centrifuge tube starting with solution of the lowest density (1.090 gr/ml).The granulocyte suspension as layered carefully on the gradient suspension, centrifugation at 3000 rpm for 30 min.The cell band at 1.095, 1.100 interphase was collected carefully with sterile and dry pasture pipette and washed with HBSS .Contaminating RBCS were lysed by cell pellet resuspension in 4.5 ml of ice cold water for 30 seconds and immediately, tonicity equilibrated with 0.5 ml of HBSS(free of Ca^{++} and Mg^{++}) .Eosinophil pellet was prepared from this suspension by centrifugation at 2000 rpm for 15 min, pellet kept frozen to be used for eosinophil granule extraction (Fernandez – Botran and Vetvicka 2000) [20] .

Release of Eosinophil granules: Eosinophil granules were released from the eosinophil Suspension obtained in the previous steps. Eosinophils pellet dissolved in 10 mls of sterile ice–cold 0.25 molar sucrose solutions. Cells washed once in this solution and precipitated by centrifugation at 2000 rpm for 15 minutes. Cells were resuspended in 10 mls of ice cold 0.25 molar sucrose and broken to release granules by repeated vigorous pipetting through a narrow–bore 10 ml calibrated pipette. Suspension was centrifuged at 10000 rpm in cold centrifuge for 20 minutes to sediment granules as a pellet [12]

Purification of Eosinophil Derived Neurotoxin EDN:Eosinophil granule pellet solubilized in 2 ml of 0.01 molar HCl($pH_{=2}$).This suspension centrifuged at 30000 rpm at $4C^{o}$ for 10 minutes to yield clear granule extract. The supernatant was chromatographed at 4 C^{o} on 1.2×47 cm sephadex G50 column equilibrated with 0.025 molar acetate buffers, pH 4.2. Flow rate was 12 ml/hr and 1.3 ml fractions were collected.Fractions eluting between 27 and 33 ml were pooled, dialyzed overnight against normal saline and centrifuged at 10000 rpm in cold centrifuge for 10 minutes.

Fraction (supernatant) was rechromatographed on a second sephadex G50 column equilibrated with phosphate buffer saline (pH 7.2).

Fractions eluting between 27 and 33 ml were pooled and concentrated on an Amicon filter for 2hours. Protein content of this concentrate was calculated using Lowry *et al*., 1951 (21) method. Concentrated extract was kept frozen at -20.

Identification of Eosinophil Derived Neurotoxin (EDN) by Polyacrylamide Gel Electrophoresis (PAGE): This test was done according to (12, 22).

Biological Test for EDN:

Local breed rabbits which were reared before experiment were used for this purpose. Two rabbits (each was weighing 1.5kg) were injected intracranially with sterile (0.25 ml) of EDN extract containing (250 μg) of protein. Another two rabbits were used as control injected with (0.25 ml) sterile normal saline in the same route.

Intracranial injection of the test and control material was done under high precaution and carefully using surgical theater of small animals in the College of Veterinary Medicine, Baghdad University. The site of injection was prepared properly by clipping and shaving the site of operation, washing with soap and water and disinfections with tincture iodine 2.5%. A burr hole was done in the skull, using sterile stainless steel trephine and injection of (0.25 ml) of the test material was done into the right occipital cavity. The skin closed with sterile silk as single interrupted suture.

The same thing was done for another two rabbits as control, injected with 0.25 ml sterile normal saline (Figure 1).

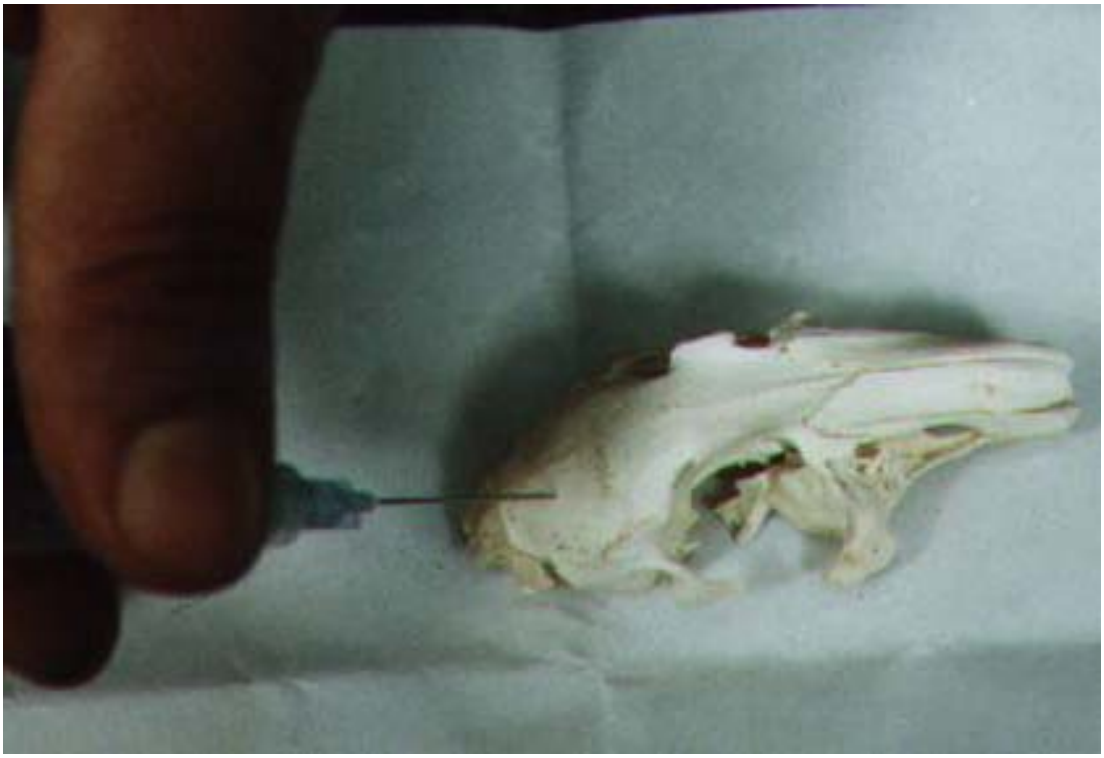

Figure 1: Skull of rabbit to show the site of intracranial injection

Stitches were removed after eight days and animals observed for 10 days [13].

Detection of EDN Antibodies in Urine Samples: Enzyme linked immunosorbent assay (ELISA) test was used for EDN detection in urine samples as described bellow: After 10 fold dilution of extract (EDN) with sterile coating buffer, 100ml of EDN solution was dispensed in each well of the microtiter plate except well A .The plate kept at $4C^{o}$ (in the refrigerator) for overnight. And the test was completed according to (20)using Rabbit Antihuman IgG conjugated with peroxidase, Bio–kit) diluted in specific dilution solution was added to each well except well D. Cut– off value was calculated following method of (AL-Murrani et al 2000) [23].

Statistical Analysis

Data were analyzed using chi square and cross tabulation, following methods of (Daniel 1999) [24] and computer type Pentium4.

Results

One of the two tested rabbits with the extracted EDN test material showed the signs and symptoms of Gordon phenomenon during the second day after injection and continued to show complete paralysis within the fifth day (Figure 2).

Symptoms of Gordon phenomenon were: stiffness mostly in

the fore limbs and dropped to the floor. Complete ataxia later and the animal showed good appetite. Animals were injected with sterile normal saline did not show any sign or symptom or even any abnormality due to post–operative complications (Figure 3).

Figure 2: Positive Gordon test

Figure 3: Negative Gordon test in control rabbit

Detection of extracted EDN by PAGE showed separation of a single band in the same level (line) of the β-lactoglobulin band. This means that the separated test band was a protein of the molecular weight nearly equal to that of the molecular weight of the indicator protein (β-Lactoglobulin, 1800 Daltons).

Trypsinogen band was seen about 6mm before the two above mentioned bands (Figure 4).

Figure 4: Distance traveled by test and Marker proteins IN PAGE.

ELISA test for EDN in Urine

Results showed that there was no significant difference($p > 0.05$) between absorbance mean values for EDN in urine of positive and negative sputum culture groups, (both in attack and rest status of asthma). Control samples showed lower mean values than that of test groups (Table 1).

Table 1: ELISA readings for EDN in urine of the studied group

Age and sex		Attack				Rest			
		10-17year		18-50year		10-17 year		18-50 year	
		Male	Female	Male	Female	Male	Female	Male	Female
Positive sputum	mean	0.081	0.074	0.0907	0.12	0.062	0.076	0.080	0.084
Negative sputum	mean	0.074	0.066	0.093	0.069	0.088	0.076	0.071	0.094

Cut off value =0.050

Discussion

Polyacrylamide gel electrophoresis of eosinophil derived neuroprotein (EDN) extracted from eosinophilic patient showed one band with molecular weight approximately to 18000 Daltons, this result was nearly similar to that gained by (13,15).

Extracted EDN showed Gordon phenomenon in one of the two tested rabbits and this was lower than that of (12), this difference might be due to: Purity of extracted EDN, difference in the breed of the rabbits (in this study local breed rabbits was used) which might be less sensitive to EDN than the New Zeland rabbits were used by (13).
Results obtained showed no significant difference between absorbance mean values of EDN in urine of patients with positive culture and that of patients with negative sputum culture, this might be due to the efficacy of allergens and infective agents in both groups to induce EDN release via eosinophil activation [19]. A number of studies have indicated that the assessment of eosinophil-derived proteins in various body fluids could be used for monitoring disease activity of asthma. Due to the relationship between levels of eosinophil proteins in serum/urine samples and lung function, as well as significant concentration differences between symptomatic and asymptomatic asthmatics, the assessment of eosinophil proteins in serum or urine samples appear to be more appropriate in monitoring disease activity [17, 18].
Different studies mentioned that EDN may be considered as an inflammatory mediator for many reactions in addition to asthma such as endocarditis, entritis and dermatitis [25, 26, 27].

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