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Eosinophil cationic protein (ECP) as a marker of eosinophil activation in autistic children: relation to autoimmunity

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Abstract:
Background: Eosinophil cationic protein (ECP), a cytotoxic protein contained in eosinophils granules, can contribute to various inflammatory and immune responses. Autism may be one of the pediatric autoimmune neuropsychiatric disorders. The exact possible role of eosinophils in autism has not yet been elucidated. The aim of this study was to evaluate serum expression of ECP and its relation to anti-ganglioside M1 brain specific auto-antibodies in autistic children.

Methods: Serum ECP and anti-ganglioside M1 auto-antibodies were measured by ELISA test in 45 autistic children aged between 3-11 years and 45 healthy-matched controls.

Results: Serum levels of ECP were significantly higher in autistic children [median (IQR) = 30.8 (14.1) ug/L] than healthy control children [median (IQR) = 13.6 (11.9) ug/L], P < 0.001. There was a significant positive correlation between serum levels of ECP and results of CARS (r=0.98, P < 0.001). Autistic children had significantly higher percent positivity of serum anti-ganglioside M1 auto-antibodies (62.2%) than healthy controls (4.4%), P < 0.001. Autistic patients with positive serum anti-ganglioside M1 auto-antibodies had significantly higher serum levels of ECP [median (IQR) = 33.8 (25) ug/L] than children with negative serum anti-ganglioside M1 auto-antibodies [median (IQR) = 21.8 (5.5) ug/L], P < 0.001.

Conclusions: Serum ECP levels were elevated in some autistic children and they were significantly associated with the increased levels of serum anti-ganglioside M1 auto-antibodies. Further research is recommend to determine the pathogenic role of ECP in autism and its relation to brain specific auto-antibodies that have been found in some autistic children.

Keywords: Autism, autoimmunity, anti-ganglioside M1 auto-antibodies, eosinophil cationic protein.

Autoimmunity to the central nervous system may play a pathogenic role in some autistic patients as evidenced by the presence of brain-specific auto-antibodies in some autistic children. The reason behind the formation of some brain auto-antibodies in some patients with autism is not fully understood. There is considerable evidence of the role of T-helper cells (Th)1, Th2, Th17, and regulatory T cells in autism [7-10]. Among the cells that can secrete cytokines capable of promoting T-cell proliferation, activation of Th1, or Th2 polarization is the eosinophil. This granulocyte has been implicated in the modulation of both innate and adaptive immune responses. In response to the diverse stimuli, eosinophils are recruited from the circulation to inflammatory foci where they modulate immune responses through an array of mechanisms, such as secretion of cationic proteins and expression of receptors for cytokines, immunoglobulins, complement, and mRNA for a number of

Introduction

Eosinophils are bone marrow-derived granulocytes that have specific granules containing large amounts of toxic materials. The activation of eosinophils results in their degranulation, an upregulation in cytokine production, and an increase of IgE production. The preformed granules within eosinophils contain four major cytotoxic cationic proteins. They include eosinophilic cationic protein (ECP), eosinophil peroxidase, eosinophil-derived neurotoxin, and major basic protein [1]. Eosinophil adhesion to epithelial cells can be an important signal for the activation and degranulation of eosinophils [2]. ECP is the best known of these as it has been assessed and used as a marker in some inflammatory diseases, auoimmune disorders and neurological disorders [1-6].

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parasitic infestation with normal results of stool analysis and normal absolute blood eosinophil counts (30-350 cells per microliter).

The local Ethical Committee of the Faculty of Medicine, Ain Shams University, Cairo, Egypt approved this study. In addition, an informed written consent of participation in the study and its publication was signed by the parents or the legal guardians of the studied subjects.

Study measurements

Clinical evaluation of autistic patients: This was based on clinical history taking from caregivers, clinical examination and neuropsychiatric assessment. In addition, the degree of the disease severity was assessed by using the Childhood Autism Rating Scale (CARS) [24] which rates the child on a scale from one to four in each of fifteen areas (relating to people; emotional response; imitation; body use; object use; listening response; fear or nervousness; verbal communication; non-verbal communication; activity level; level and consistency of intellectual response; adaptation to change; visual response; taste, smell and touch response and general impressions).

Assessment of cognitive function (memory, attention, language, concept formation, problem solving, executive and visuospatial functions) with age-appropriate, translated and validated psychometric instruments that were administered by well-trained psychologists using a set of Arabic norms [25] for a translated Wechsler Intelligence Scale for Children, 3rd edition (WISC-III) [26]. This scale is the most commonly used test to assess cognitive function in the children. Three global measures were examined in the present study. The verbal intelligence quotient (IQ) is derived from different subtests including information, similarities, arithmetic, comprehension, vocabulary and digit span. The performance IQ is derived from different subtests including picture completion, block design, picture arrangement, object assembly and digit symbol. The full-scale IQ is the sum of the verbal and performance IQ. The individual subtests may be particularly useful because each depends on a variety of capabilities and dysfunction of any one could result in a low score on one of the global measures. Cognitive dysfunction is diagnosed when the difference between verbal and performance IQ is more than 15 and / or the result of one or more of the individual subtests is below 7 and/or the full scale IQ is below 70.

***Serum assessment of ECP levels*:**

Venous blood was taken from each subject, centrifuged at 1,000×g for 10 minutes at 4℃, and stored at -70℃. Peripheral blood eosinophil counts were measured with an automated hematology analyzer (Coulter Counter STKS, Beckman Coulter, Fullerton, CA, USA) using blood samples collected in tubes containing EDTA. Serum ECP was

Toll-like receptors. They can initiate antigen-specific immune responses by acting as antigen-presenting cells [11-15].

Gangliosides are a family of sialylated glycosphingolipids expressed in the outer leaflet of the plasma membrane of the cells of all vertebrates. They are abundant in the nervous system and they are involved in neurotransmission [16-19]. Ganglioside M1 is the most abundant ganglioside in neural membranes. It may be an autoantigen through the galactose–galactosamine part of its sugar moiety [20]. In humans, gangliosides elicit a T-cell independent IgM response [21]. Circulating anti-ganglioside M1 auto-antibodies may play an etiopathogenic role in some autoimmune neurological disorders such as neuropsychiatric systemic lupus erythematosus [22]. The exact possible role of eosinophils in autism has not yet been elucidated. Based on the findings of eosinophils in the urine of patients with autoimmune diseases as systemic lupus erythematosus (SLE) and the role of eosinophils in various inflammatory diseases [1-6], this study aimed to evaluate serum expression of ECP and its relation to anti-ganglioside M1 brain specific auto-antibodies in autistic children.

Body Text

Study population

This cross-sectional study was conducted on 45 autistic children. They were recruited from the Pediatric Neuropsychiatric Clinic, Faculty of Medicine of Ain Shams University, Cairo, Egypt, during their follow up visits. Patients were fulfilling the criteria of the diagnosis of autism according to the 4th edition of the Diagnostic and Statistical Manual of Mental Disorders [23]. The autistic group comprised 33 males and 12 females. Their ages ranged between 3 and 11 years (mean ± SD = 5.24 ± 2 years). Patients who had associated neurological diseases (such as cerebral palsy and tuberous sclerosis), metabolic disorders (eg. Phenylketonuria), allergic manifestations or concomitant infection were excluded form the study.

The control group comprised 45 age- and sex- matched apparently healthy children. They included 32 males and 13 females. They were recruited from the Outpatients Clinic, Children's Hospital, Faculty of Medicine, Ain Shams University. They were the sibs of the children attending this clinic because of a minor illness (e.g common cold, tonsillitis and acute bronchitis). The control children were not related to the children with autism, and demonstrated no clinical findings suggestive of neuropsychiatric disorders. Their ages ranged between 3 and 12 years (mean ± SD = 6.8 ± 2.4 years).

All studied children had no clinical evidence of allergic manifestations infections, and immunological diseases or

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presented as mean and standard deviation (SD). In addition, non-parametric data were presented as median and interquartile range (IQR) which is between the 25th and 75th percentiles. Student's t-test was used for comparison of parametric data, while Mann-Whitney test was used for comparison between non-parametric data. Chi-square test was used for comparison between qualitative variables of the studied groups. Spearman's rho correlation coefficient "r" was used to determine the relationship between different variables. For all tests, a probability (P) of less than 0.05 was considered significant. The patient was considered to have an elevated serum ECP level if it was above 28.3 ug/L which was the chosen highest cut-off value (the 95th or the control values as data were non-parametric).

Results:

All studied patients had classic-onset autism. None of the autistic patients had regressive autism, associated neurological diseases (such as cerebral palsy and tuberous sclerosis), metabolic disorders (eg. Phenylketonuria), allergic manifestations or concomitant infections. The degree of the disease severity was assessed by using CARS and according to this scale, children who have scored 30-36 have mild to moderate autism (n=18), while those with scores ranging between 37 and 60 points have a severe degree of autism (n=27). In addition, 30 autistic children had subnormal intellectual function (intelligence quotient below 70); 18 had mild mental retardation (intelligence quotient = 50-69), and 12 had moderate mental retardation (intelligence quotient = 35-49), table 1. None of the healthy control children had a neurocognitive disorder.

measured using a commercial fluoroimmunoassay kit (PharmaciaECP UniCAP System FEIA; Pharmacia Diagnostics, Uppsala, Sweden), with a detection limit of 2 µg/L (27). To increase accuracy, all samples were analysed twice in two independent experiments to assess the interassay variations and to ensure reproducibility of the observed results. There were no discordant data between the results (P > 0.05).

Measurement of serum anti-ganglioside M1 antibodies: This was done by using ELISA kit for the specific measurement of human total anti-ganglioside M1 in cell culture supernates, serum, and plasma (Uscnlife Science and Technology Co., LTD). Monoclonal antibodies specific for ganglioside M1 had been pre-coated onto a microplate. Standards and samples were pipetted into the wells and any ganglioside M1 present was bound by the immobilized antibody. An enzyme-linked polyclonal antibody specific for gaglioside M1 was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solutionwas added to the wells. Color development was in proportion to the amount of ganglioside M1 which was bound in the initial step. The intensity of the color was measured. To increase accuracy, all samples were analyzed twice in two independent experiments to assess inter-assay variations and to ensure reproducibility of the observed results (P > 0.05). No significant cross-reactivity or interference was observed.

Statistical analysis

The results were analyzed by using the commercially available software package (Statview, Abacus concepts, inc., Berkley, CA, USA). The parametric data were

Table 1. Demographic and laboratory data of children with autism and healthy control children

	Children with autism (n=45)	Control group (n=45)
Age (in years): Range	3-11	3-12
Mean ± SD	5.24 ± 2	6.8 ± 2.4
Sex: (Male/Female)	33/12	32/13
Intelligence quotient: Above 70	15	45
50-69	18	
35-49	12	
CARS scores: Mild to moderate (30-36)	18	
Severe (37 -60)	27	
Serum ECP: Range	12.3-108.8	6.2-29.7
(uglL) Median (IQR)	30.8 (14.1)	13.6 (11.9)
Positivity of anti-ganglioside antibodies	62.2%	4.4%

CARS, Childhood Autism Rating Scale; ECP, eosinophil cationic protein; IQ,R, interquartile ranges

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Serum levels of ECP in autistic children and healthy control children

Serum levels of ECP were significantly higher in autistic children [median (IQR) = 30.8 (14.1) ug/L] than healthy control children [median (IQR) = 13.6 (11.9) ug/L], P < 0.001 (figure 1).

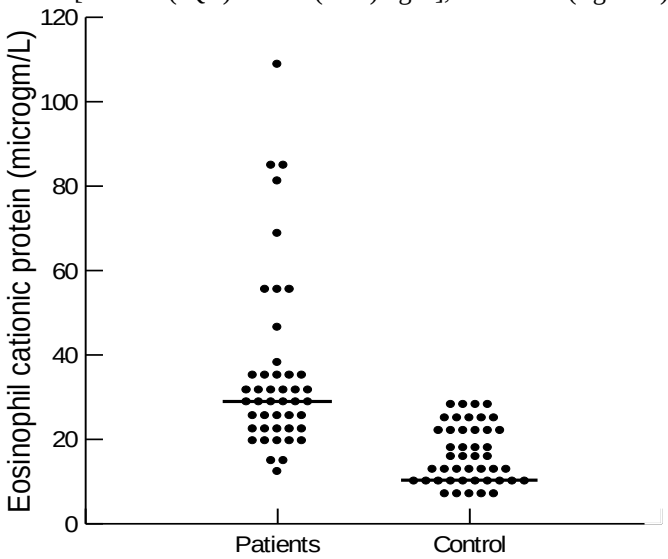

Figure 1. Serum levels of ECP in autistic patients and healthy controls.
ECP, eosinophil cationic protein. Horizontal bars indicate the median values. P < 0.001.

Twenty six autistic children (57.7%) had increased serum levels of ECP.
Children with severe autism had significantly higher serum levels of ECP [median (IQR) = 34.9 (25.3) ug/L] than children mild to moderate autism [median (IQR) = 21.7 (6.1) ug/L] P < 0.001.
There was a significant positive correlation between serum levels of ECP and results of CARS (r=0.98, P < 0.001). On the other hand, there were no significant correlations between serum levels of ECP and the age of autistic children (P > 0.05).

Positive results of serum anti-ganglioside M1 auto-antibodies in autistic children and healthy control children

Twenty eight autistic children (62.2%) had positive results of serum anti-ganglioside M1 auto-antibodies. Autistic children had significantly higher percent positivity of serum anti-ganglioside M1 auto-antibodies than healthy controls (4.4%), P < 0.001.

The association between increased serum levels of ECP and the positive results of serum anti-ganglioside M1 auto-antibodies

Autistic patients with positive results of serum anti-ganglioside M1 auto-antibodies had significantly higher serum levels of ECP [median (IQR) = 33.8 (25) ug/L] than children with negative results of serum anti-ganglioside M1 auto-antibodies [median (IQR) = 21.8 (5.5) ug/L], P < 0.001 (figure 2)..

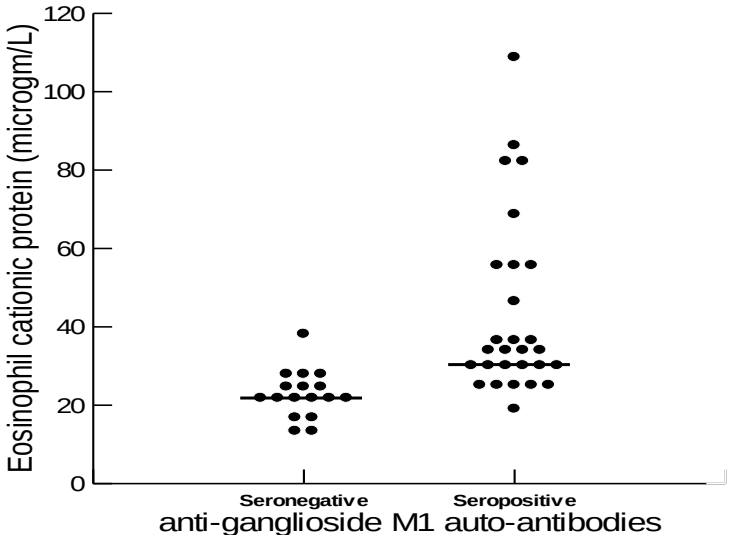

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Figure 2. Serum levels of ECP in autistic patients with positive results of serum anti-ganglioside M1 auto-antibodies and patients with negative results of these auto-antibodies. ECP, eosinophil cationic protein. Horizontal bars indicate the median values. . P < 0.001.

In addition, autistic patients with increased serum levels of ECP had significantly higher frequency of positive results of serum anti-ganglioside M1 auto-antibodies (88.46%) than patients with normal serum levels of ECP (26.3%), P < 0.001 (table 2).

Table 2. The frequency of autistic patients with increased serum levels of ECP in relation to elevated levels of serum anti-ganglioside M1 auto-antibodies.

	Autistic children with elevated serum ECP (n=26)	Autistic children with normal serum ECP (n=19)	X^2 (p)
Positive antineuronal antibodies (n=28)	23 (88.46%)	5 (26.3%)	18 (< 0.001)
Negative antineuronal antibodies (n=17)	3 (11.54%)	14 (73.7%)	

ECP, eosinophil cationic protein

site of inflammation, eosinophils are activated and the four highly cytotoxic cationic protein preformed granules, including ECP are secreted, in addition to chemokines, cytokines, and growth factors through the process of piecemeal degranulation. Regarding cytokines, IL-5 is the most specific to the eosinophil lineage and is responsible for selective differentiation, regulating growth, activation, and survival of eosinophils. In addition to IL-5, ECP is also a key mediator of eosinophil activation.

ECP is RNAse A superfamily, protein rich in arginine residues, which gives a high concentration of positive charges, promoting a strong attraction for molecules negatively charged existing in cell membranes. This property may explain its cytotoxic power in the cell membranes causing the formation of pores or channels on the surface of the membrane, disrupting its lipid structure and possibly facilitating the entry of other cytotoxic molecules. Sensitive assays have been developed for its measurement in biological fluids which have contributed to the understanding of the role of the eosinophils in disease [11,27-29].

In immune-mediated neurological disorders, various antibodies against neuronal tissues have been discovered. Some of these antibodies have been found to correlate with the pathomechanism of the disease [30]. The key to establish the immunopathogenic role for the brain auto-antibodies is to determine their effects on specific brain functions [31].

In our series, 62.2% of autistic children had positive results of serum anti-ganglioside M1 auto-antibodies. Autistic children had significantly higher percent positivity of serum anti-ganglioside M1 auto-antibodies than healthy controls (4.4%), P < 0.001. A previous study reported seopositivity

Discussion

Eosinophil degranulation is a natural physiological phenomenon in vivo. However, excessive degranulation is known to cause epithelial damage via the release of cytotoxic granule proteins such as ECP [1-3]. Eosinophils begin life and reside in the bone marrow for 8 days while undergoing maturation. They subsequently relocate into the peripheral circulation for 8–12 h and finally traffic to specific tissues .Eosinophils are predominantly tissue-dwelling cells with the high affinity for epithelial surfaces [1].

In the current study, serum levels of ECP were significantly higher in autistic children than healthy control children, inspite of the normal eosinophil count. In addition, 57.7% of autistic children had increased serum levels of ECP. Children with severe autism had significantly higher serum levels of ECP than children mild to moderate autism, P < 0.001. There was a significant positive correlation between serum levels of ECP and results of CARS (P < 0.001). These results denote that the increased serum levels of ECP are attributable to increased eosinophil activation in some autistic children, despite of the normal eosinophil count in these patients. This study was the first to investigate serum levels of ECP in autistic children.

In some autistic children there is an imbalance of T-helper (Th)1/Th2 subsets toward Th2, which are responsible for allergic response and production of antibodies. [7]. Allergic reactions are characterized by an infiltration of T helper type 2 (Th2) cells into affected tissues. Upon exposure to an allergen, these cells expand rapidly, and begin secreting large amounts of IL-4, IL-5, and IL-13, resulting in IgE synthesis and eosinophil activation. IL-5 is a potent mediator of eosinophil activation. [27]. Once attracted to the

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study that investigated the relationship between ECP and autoimmunity in autism, so studies should be conducted to investigate eosinophil function in these patients.

There is considerable evidence of the role of increased T-cell proliferation in autism [7,8]. Among the cells that can secrete cytokines and cationic proteins as ECP capable of promoting T-cell proliferation or Th2 polarization is the eosinophil. This cell has been implicated in the modulation of both innate and adaptive immune responses. They can initiate antigen-specific immune responses by acting as antigen-presenting cells [11-15]. It is speculated that autoimmune reaction to neurons in some autistic children might be trigged by cross-reacting antigens in the environment (such as food allergies to certain peptides such as gliadin, cow's milk protein and soy, infectious agents, heavy metals such as mercury) resulting in the release of neuronal antigens. These neuronal antigens may result in induction of autoimmune reactions through the activation of inflammatory cells in genetically susceptible individuals [7,8]. ECP is used as a marker in some auoimmune disorders [5,6].

Thus, increased eosinophil activation in some autistic children may have a role in the induction of the production of brain-specific antibodies in these patients through stimulation of T-cell proliferation or Th2 polarization. The reason behind the increased eosinophil activation in some autistic children may be due to exposure to cross-reacting antigens in the environment in genetically susceptible individuals. [7,8].

Conclusions

Serum levels of ECP were elevated in some autistic children and they were significantly associated with the increased levels of serum anti-ganglioside M1 auto-antibodies. However, these data should be treated with caution until further research is conducted to determine the pathogenic role of ECP in autism and its relation to brain specific auto-antibodies that found in some autistic children. The role of immunotherapy should be studied in autistic children who have positive results of brain specific auto-antibodies.

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of anti-ganglioside M1 antibodies in 74% of Saudi autistic children [32]. The presence of other brain auto-antibodies such as anti-myelin basic protein antibodies and anti-myelin-associated glycoprotein antibodies have been observed in some autistic children [8,33]. In addition, the extracellular mitochondrial DNA could act as an autoimmune trigger because anti-mitochondrial antibodies type 2 were recently reported in blood of some autistic children [34]. Despite of the fact that the origin of autoimmunity in autism is unknown, immune related genes on major histocompatibility complex, which have been associated with some autoimmune diseases, may play a central role in the development of autoimmunity in autism (e.g., HLA-DRB1 and C4B null alleles) [35,36].

Ganglioside M1 is the most abundant ganglioside in the nervous system, in particular at synapses. It is involved in the neurotransmission at the neuromuscular junction [16-19]. Gangliosides are thought to play important roles in memory formation, neuritogenesis, synaptic transmission, and other neural functions [37,38]. The administration of exogenous gangliosides seems to improve nerve regeneration [39]. Indeed, due to their location in the nervous system, gangliosides could be a target molecule in the complex autoimmune response [20]. They may play a pathogenic role in autoimmune neurological disorders. Anti-ganglioside M1 antibodies are commonly found in the sera of patients with autoimmune neurological disorders such as axonal Guillain-Barré syndrome [40,41] and neuropsychiatric systemic lupus erythematosus [22].

Therapy in patients who are seropositive for serum auto-antibodies is directed at reducing the concentration of these antibodies, blocking the effector mechanisms and depleting the monoclonal B cells. The recent availability of a monoclonal antibody suppressing B-cell clones, which is not myelosuppressive and does not cause secondary malignancies, allows for early targeted intervention. Preliminary results suggest that this new line of therapy is well tolerated and is promising in the treatment of some patients. Therefore, the role of this new line of therapy in autistic patients who have increased serum anti-ganglioside M1 antibodies should be studied. [42].

In the present work, autistic patients with positive results of serum anti-ganglioside M1 auto-antibodies had significantly higher serum levels of ECP than children with negative results of serum anti-ganglioside M1 auto-antibodies, $P < 0.001$. In addition, autistic patients with increased serum levels of ECP had significantly higher frequency of positive results of serum anti-ganglioside M1 auto-antibodies (88.46%) than patients with normal serum levels of ECP (26.3%), $P < 0.001$. Thus, there was a significant positive association between the elevated levels of serum ECP and the positivity of serum anti-ganglioside M1 auto-antibodies in autistic children. This is the first

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