Original article

Cellular-mediated and humoral immunity in children with autism

Background: *Autism spectrum disorder (ASD) is a spectrum of behavioral anomalies characterized by impaired social interaction and communication, often accompanied by repetitive and stereotyped behavior. The condition manifests within the first 3 years of life and persists into adulthood. There are numerous hypotheses regarding the etiology and pathology of ASD, including a suggested role for immune dysfunction. While immune system abnormalities have been reported in children with autistic disorder, there is little consensus regarding the nature of these differences which include both enhanced autoimmunity and reduced immune function. It has long been known that extensive interactions occur between the immune system and neuronal system/brain, and that normal neurodevelopment is contingent upon an appropriate interaction with the immune system.* ***Objectives:*** *the aim of the present study was to evaluate the cell mediated and humoral immunity of children with autism through evaluation of the serum antibody levels of immunoglobulin (IgG, IgM, IgA), also we evaluated the T helper and T suppressor cells (CD4 and CD8 T cell subpopulations) and CD4/CD8 ratio in children with autism and compared with the healthy control children.* ***Methods:*** *This study was carried out in the Psychiatry Unit, Department of Pediatrics, Tanta University Hospital. Thirty children with autism (24 males, 6 females) newly diagnosed were included in the study, their age range was (3-9 years) with the mean age of 5±1.8 years. Childhood Autism Rating Scale (CARS) was used for the diagnosis of autism. Diagnosis of autism was based also, on the criteria for the diagnosis of autism that are set out in the Diagnostic and Statistical Manual of Mental Disorders DSM-IV-TR (Fourth Edition, Text Revision). The intial Childhood Autism Rating Scale (CARS) score for these children was ≥30, as children with a CARS score ≥30 were considered to have autism. Intial CARS score range for children with autism was (31-60). The control group consisted of thirty healthy children (10 females, 20 males). Their age range was (2-10 years) and the mean age was 5.3± 2.4 years.* ***Results:*** *Children with autism had significantly lower serum levels of IgG, IgA and IgM compared to the control children p<0.001. Also children with autism had significantly lower numbers of CD4 cells with increased CD8+ T cell subpopulations and decreased CD4+/CD8+ ratio.* ***Conclusion:*** *Children with autism have significantly reduced levels of serum IgG, IgA and IgM compared to the control children, suggesting an underlying defect in the immune function, also the cell-mediated immunity is impaired as evidenced by low numbers of CD4+ cells and increased CD8+ T cell subpopulations and decreased CD4+/CD8+ ratio.*

Key words: autism spectrum disorder (ASD), neurodevelopment, immunity.

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INTRODUCTION

Autism spectrum disorders (ASD) is a heterogenous group of neurodevelopmental disorders, the etiology or etiologies of which remain unknown[1]. The autistic disorder is defined entirely based on the impairment in three areas: 1) Impairment of social interaction, 2) Impairment in communication and, 3) Stereotyped and repetitive behavior[2]. Autism is a lifelong neurodevelopmental disorder characterized by social deficits, impaired verbal and nonverbal communication and the presence of stereotyped behaviors or circumscribed interests[3]. Autism, Rett syndrome together, with Asperger syndrome and pervasive developmental disorder not otherwise specified, referred to as autism spectrum disorders (ASD) that form a spectrum of conditions with varying degrees of impairment that are classified as pervasive developmental disorders in the DSM-IV[4]. The current estimate of prevalence of autism spectrum disorder is approximately 1:150[5]. There are many causes of autism that will likely have varying contributions from genetic and environmental factors[6]. One persistent suggestion has been that an immune dysfunction may contribute to certain forms of autism[7]. Increasing evidence of autoimmune phenomena in individuals with autism could represent the presence of altered or inappropriate immune responses in this disorder,

and this immune system dysfunction may represent novel targets for treatment[8].

There have been numerous findings of altered immune function in autism, numerous attempts at determining susceptibility genes through a number of studies have indicated that multiple genes, including immune related genes, may be associated with autism[9,10]. Studies have highlighted the presence of inflammation in the brain and the activation of microglia as well as evidence for altered peripheral immune function in autism, including increased cytokine levels in the plasma such as interleukin IL-1β, IL-6, and IL-8, elevated levels of complement proteins, decreased cellular activity of natural killer (NK) cells, increased monocyte activation, and a reduced number of CD4+ T cells[11]. In addition, a number of studies have reported abnormal antibody responses to brain and central nervous system proteins, skewed immunoglobulin (Ig) responses, such as decreased total serum IgG levels but increased isotype IgG4, have also been reported in autism[12]. The immune system is made up of several different parts; B-cells produce antibodies or immunoglobulins, T-cells are the cells involved in what is called cellular immunity[13]. The functions of the T-cells are to kill foreign tissue or tissues infected with virus, to produce lymphokines, which are large proteins that regulate other cells of the immune system and to help to enhance the immune response[14]. The complement system is a group of proteins involved as a nonspecific helper to the immune system. The phagocytic cells include cells called macrophages and neutrophils that engulf bacteria and yeast and digest them[15]. Most of the T cells in the body belong to one of two subsets. These are distinguished by the presence on their surface of one or the other of two glycoproteins designated: CD4 and CD8[16]. $CD4^+$ T cells bind an epitope consisting of an antigen fragment lying in the groove of a class II histocompatibility molecule. $CD4^+$ T cells are essential for both the cell-mediated and antibody-mediated branches of the immune system CD4 cells or helper T cells provide protection against different pathogens[17]. $CD8^+$ T cells are cytotoxic T lymphocytes (CTLs). They secrete molecules that destroy the cell to which they have bound[18]. Cell-mediated immunity includes, $CD4^+$ cells that bind to antigen presented by antigen-presenting cells (APCs) like phagocytic macrophages and dendritic cells. The T cells then release lymphokines that attract other cells to the area . The result is inflammation and accumulation of cells and molecules that attempt to wall off and destroy the antigenic material[19,20].

The aim of the present study was to evaluate the immune system including cell mediated and humoral immunity of children with autism in attempt to determine if immune mechanisms are involved in the development of autism, through evaluation of the immunoglobulins (IgG, IgM, IgA) serum levels. Also, the T helper and T suppressor cells (CD4 and CD8 T cell subpopulations) and CD4/CD8 ratio in children with autism, to investigate the possibility that immune abnormalities in some children with autism may involve abnormal distributions of CD4+ and/or CD8+ cells, (suppressor) T cells and compare them with the healthy control children.

METHODS

This study was carried out in Tanta University Hospital, Pediatric Department, Psychiatry Unit. Thirty children newly diagnosed as autism (24 males, 6 females) were included in the study, their age range was (3-9 years), with the mean age of 5±1.8 years. The intial Childhood Autism Rating Scale (CARS) score for these children was ≥30. Children with a CARS score ≥30 were considered to have autism[21]. The intial CARS score range for children with autism was (31-60). Children with CARS score range (31-37) were considered to have moderate autism and their number was 16 children and children with CARS range >37-60 were diagnosed as severe autism, their number was 14 children. The exclusion criteria for all subjects consisted of the presence of children who had clinical features of Fragile X-disorder, tuberous sclerosis and phenylketonuria , severe organic condition, presence of epilepsy or other severe neurological disorder . Children with pervasive developmental disorder-not otherwise specified (PDD-NOS) or Asperger Syndrome were excluded from the study. Children with known endocrine, cardiovascular, pulmonary, liver or kidney disease were excluded from study. The blood samples were taken before the start of any treatment . The control group consisted of thirty healthy children (10 females, 20 males). Their age range was (2-10 years) and the mean age was 5.3± 2.4 years. They had no history or family history of any psychiatric disorder. The control children were matched with the patients as regards to sex and age. All children were included in the study after written informed parental consent had been obtained .The study was approved by the local ethics committee of the Faculty of Medicine Tanta University. Full history was taken including, medical history, family history, birth history, early development and history

of recurrent infections. Also physical examination including complete neurological examination for all children. Mental status examination included the evaluation of social interaction, language and communicative functions. Childhood Autism Rating Scale (CARS) was applied for children with autism to assess its severity. CARS is a diagnostic assessment method that rates children on a scale from one to four for various criteria, ranging from normal to severe, and yields a composite score ranging from non-autistic to mildly autistic, moderately autistic, or severely autistic. The scale is used to observe and subjectively rate fifteen items, these items are: relationship to people, imitation, emotional response, body use, object use, adaptation to change, visual response, listening response, taste-smell-touch response and use, fear and nervousness, verbal communication, non-verbal communication, activity level, level and consistency of intellectual response . This scale was completed by the clinician, based on subjective observations of the child's behavior. Each of the fifteen criteria is rated with a score of :1 normal for child's age, 2 mildly abnormal, 3 moderately abnormal , 4 severely abnormal. Total CARS scores range from a 15 to 60, with a minimum score of 30 serving as the cutoff for a diagnosis of autism[21]. Also hearing test include, Auditory Brain Stem Response (ABR) for children with autism was done. Electroencephalography (EEG) was done for autistic children. Brain Magnetic Resonance Imaging (MRI) was done for autistic children to exclude children with structural lesion or tuberous sclerosis. Complete blood count including cell count for the major immune cell populations i.e., neutrophils, lymphocytes, eosinophils, monocytes and platelets. Other routine laboratory tests, including, liver function, kidney function and fasting blood sugar were done for all children. Serum antibody levels of immunoglobulins (IgG, IgM, IgA) also T helper and T suppressor cells (CD4 and CD8 T cell subpopulations) and CD4/CD8 ratio were assayed in all included children.

Sample Collection:

Ten milliliters of blood single sample from each child was collected in yellow top citrate tubes (BD Biosciences, Franklin Lakes, NJ) according to the study protocol and divided into two equal parts; 5 ml were centrifuged at a rate 3000 round per minute for 10 min to separate the serum which was collected and immediately frozen in 0.5 ml aliquots at -80°C until assayed for Ig levels by ELISA , and the other 5 ml were used as a fresh samples for the procedure of flow cytometry which is performed according to the procedure mentioned below.

Assessment of systemic level of immunoglobulins by ELISA:

Levels of total IgG, IgM and IgA, were determined by enzyme-linked immunosorbent assay (ELISA) using commercially available kits purchased from ALerCHEK Inc. (Portland, ME). Kits were run according to the manufacturer's instructions as described previously . Briefly, serum samples were diluted 1:100,000 (IgG), 1:10,000 (IgM and IgA), or 1:10 (IgE) and added to 96 well plates pre-coated with capture antibody. After 1-hr incubation and subsequent washing, horseradish peroxidase-conjugated detection antibodies were added and TMB (3,3′, 5,5′ tetramethyl benzidine)/peroxide substrate used for development. Data are reported as median mg/mL (IgG, IgM, IgA) . Intra- and inter-assay variability was controlled for using control standards on each plate. The coefficient of variance was less than 10% on any given plate[22].

Flow cytometric analysis

Flow cytometry of lymphocyte subsets was carried out using a lamp-based flow cytometer (Bryte-HS, Bio-Rad, Hercules, Calif.) according to the manufacturer's instructions. White blood cell counting and differentiation were performed using a Symex-SF3000 Coulter counter (Coulter Electronic, Luton, London). Samples were then stained using OptiClone CD4/CD8, immunoglobulin G1-fluorescein isothiocyanate, and immunoglobulin G1-phycoerythrin monoclonal antibodies (Coulter-Immunotech, Miami, Florida). The monoclonal antibodies, 13B8.2 and B9.11, were used to bind specifically to CD4 and CD8 subsets of peripheral blood T lymphocytes, respectively. The determination of positive and negative cells for any combination of reagents was set with directly conjugated antibodies of irrelevant specificity as negative controls. Positive and negative controls were included in each run according to guidelines issued previously . Percentages of $CD8^+$ and $CD4^+$ cells were measured with Coulter software, and CD4/CD8 ratios were calculated[23].

Statistical Analysis

Collected data were coded, analyzed and computed using the Statistical Package for Social Sciences (SPSS) version 10. Results were expressed as mean ±SD, and differences between the means of different variables were tested using the student t-test. Differences were considered significant statistically when $p< 0.05$.

RESULTS

Table (1) represents characteristics of children with autism and the control children. Table (2) and figure (1) show comparison between the mean serum concentrations of the immunoglobulins (IgG, IgM, IgA) in children with autism and the control children. Children with autism had significantly reduced serum levels of IgG, IgM and IgA in comparison to the healthy control children P<0.001. Table (3) and figure (2) show comparison between the mean percentage of the T helper and T suppressor cells (CD4 and CD8) T cell subpopulations and CD4/CD8 ratio in children with autism and the control children. Children with autism had significantly reduced levels of T helper (CD4) T cell subpopulations and increased levels of the T suppressor (CD8) T cell subpopulations and reduced CD4/CD8 ratio in comparison to the control children P<0.001. Figure (3) shows significant negative correlation between the severity of autism and CD4/CD8 ratio, where increased CARS score is associated with decreased CD4/CD8 ratio r = 0.849, p<0.001.

Table 1. Characteristics of Children with Autism and the Control Children.

Number	Children with autism n=30	Control group n=30	t value	P value
Sex: Males/Females	24/6	20/10	---------	---------
Age (years) Range Mean±SD	3-9 5±1.8	2-10 5.3± 2.4	0.65	>0.05

P value significant <0.05

Table 2. Comparison for mean serum concentrations of Immunoglobulins (IgG, IgM, IgA) between children with Autism and the control children.

Serum Immunoglobulins (mg/dl)	Children with Autism	Control Children	t value	P value
IgG Range Mean±SD	950-1087 1009±36	1200-1350 1281±31	4.150	<0.001
IgM Range Mean±SD	70-125 81±10	87-165 143±24	6.355	<0.001
IgA Range Mean±SD	70-105 89±9	107-230 185±42	8.523	<0.001

P value significant <0.05

Table 3. Comparison for mean percentage of CD4, CD8 T Cell Subpopulations and CD4/CD8 ratio between children with Autism and the control children.

T cell subpopulations (%)	Children with Autism	Control Children	t value	P value
T helper cells (CD4) Range Mean±SD	30-41 32±2	39-56 46±6	9.661	<0.001
T suppressor cells (CD8) Range Mean±SD	20-35 21±2	13-23 14±5	6.354	<0.001
CD4/CD8 Ratio Range Mean±SD	1.2-1.7 1.3±0.1	2-3.4 2.3±0.4	9.524	<0.001

P value significant <0.05

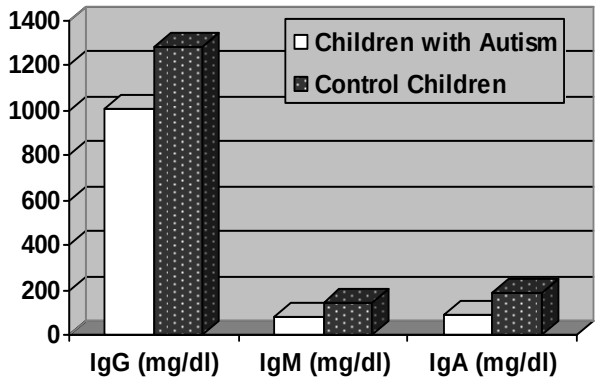


Figure 1. Mean Serum Concentrations of Immunoglobulins (IgG, IgM, IgA) in Children with Autism and the Control Children.

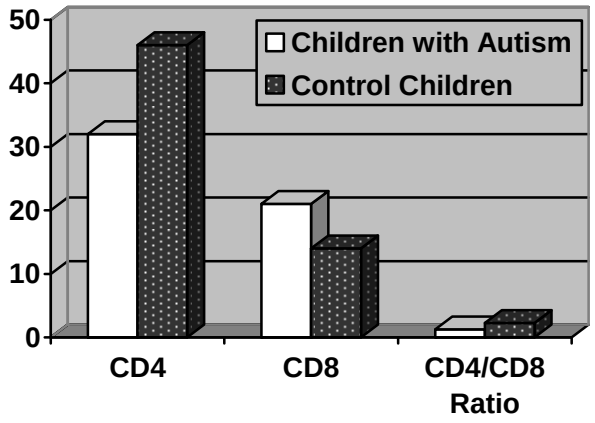
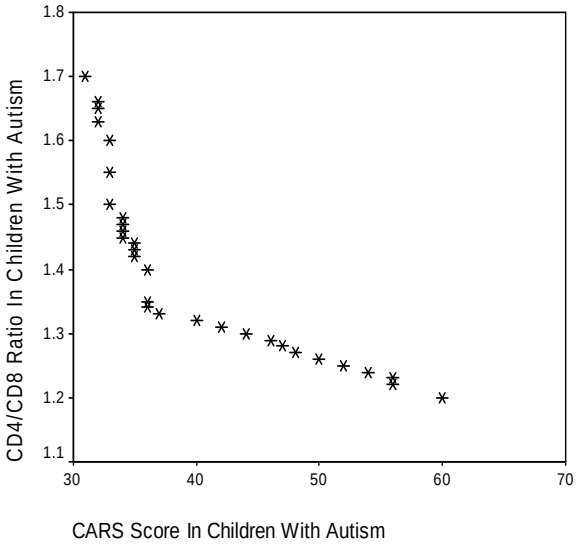


Figure 2. Mean Percentage of The CD4, CD8 T Cell Subpopulations and CD4/CD8 Ratio in Children with Autism and The Control Children.



r=0.849 P value <0.001

Figure 3. Correlation between CARS score and CD4/CD8 Ratio in children with Autism

DISCUSSION

Autism is a generalized or pervasive developmental disorder that affects about five in ten thousands of children worldwide (5/10.000), with a ratio of male/female, 4:1[24,25]. Autism spectrum disorder is of interest neurochemically because it represents a relatively homogeneous disorder with regard to disease development, abnormal cognitive development and intellectual development[26]. A potential etiologic role for immune dysfunction in autism has been suggested, disturbance in the immune function can detrimentally influence early brain development [27].

Our work revealed significantly reduced levels of the serum IgG, IgM and IgA in children with autism compared to the controls, suggesting an underlying defect in immune function with impaired humoral immunity in autism. The results of this study agree with those obtained by Heuer et al.[28], who found significantly reduced plasma levels of immunoglobulins (IgG, IgM, IgA). Immunoglobulin production is the end result of B-cell activation generated during an immune response and decreased levels are indicative of an immune defect[29]. Research has implicated immunological, neurological, genetic, and environmental factors as possible contributors to

this complex disorder, and a correlation between decreased levels of immunoglobulin (Ig) and behavioral outcome had been reported in children with autism[30]. Immune-system abnormalities may be directly related to underlying biologic processes of autism, or these changes may be an indirect reflection of the actual pathologic mechanism , thus identification of the immune defect responsible for reduced immunoglobulin production may provide insight into common affected pathways in neurodevelopment[31].

Our study had also revealed that, children with autism had impaired cell mediated immunity as evidenced by low numbers of CD4 T cells with an increased CD8 T cell with decreased CD4/CD8 ratio in comparison to the control children. A previous study of autistic patients revealed several immune-system abnormalities, including decreased numbers of T lymphocytes; and an altered ratio of helper to suppressor T cells[32]. Our results agree with Ashwood et al.[33]; Who assessed the cellular immune function in 66 children with a confirmed diagnosis of autism and compared them with 73 confirmed typically developing normal controls and had found that, the frequency of CD4+ and CD8+ T cells were significantly reduced in children with autism but we found increased level of CD8+ T cell subset. Our results also agree with the results obtained by Yonk et al.[34]; who had studied the CD4+ and/ CD8+ T cells, and the peripheral blood lymphocytes of 25 autistic children and had found that, autistic children had a significantly lower percentage and number of CD4+ cells, and increased number of CD8+ T cells and a lower percentage and number of total lymphocytes than the normal subjects. Depressed in-vitro response to mitogens and recall antigens and decreased proportions of CD3, CD4 and increased CD8 T cells was reported. Patients with autism have been shown to have decreased intracellular interferon γ and inteleukin (IL)-2 containing CD4 T cells, whereas IL-4 containing are increased[35].

The present work revealed negative correlation between autism severity and CD4/CD8 ratio in autistic children. Evidence of an immune deficiency coupled with severity of behavioral measures would suggest a common defect in both neuro- and immunodevelopment[36]. The interface between the cellular immune system and the nervous system is exceedingly complex with extensive communication occurring between them in health and disease. Immune cells and immune molecules, such as cytokines and chemokines, can modulate brain function, affecting cognitive and emotional processing, and have assorted effects on neuronal tissue, such as the modulation of systemic and CNS responses to infection, injury, and inflammation[37]. Immunological abnormalities in both the innate and adaptive immune system that are manifested by a paradox of immunodeficiency , inflammation and autoimmunity have been reported in autism . Immunological abnormalities include depressed cell-mediated immunity and antibody-mediated immunity, increased production of proinflammatory cytokines and chemokines, and the presence of autoantibodies against various neural tissues and antigens[38]. These neuroimmune interactions begin early during embryogenesis and persist throughout an individual's lifetime, with successful neurodevelopment contingent upon a normal balanced immune response[39].

Defects in all parts of the immune system have been documented in children with autism. Children with autism have significantly reduced serum IgG, IGA and IgM levels compared to the control children, suggesting an underlying defect in immune function. Abnormal CD4/CD8 ratio may represent an important link between inflammatory processes that have reported in some children with autism, and could point to a specific immune basis for the disorder in many subjects. Taken together, these data are suggestive of a link between autism and immune dysfunction and that specific cellular phenotypes or activation status of immune cells may be altered in autism. Thus, identification of the immune defect responsible for reduced immunoglobulins production may provide insight into common affected pathways in neurodevelopment. Further investigation of cellular immunity profiles should be done to find the relationship between immune dysfunction and the progression of behavioral and developmental changes throughout the course of this disorder.

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