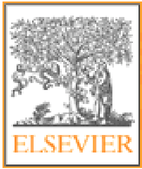

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Antibodies to neuron-specific antigens in children with autism: Possible cross-reaction with encephalitogenic proteins from milk, *Chlamydia pneumoniae* and *Streptococcus* group A

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Abstract

We measured autoantibodies against nine different neuron-specific antigens and three cross-reactive peptides in the sera of autistic subjects and healthy controls by means of ELISA testing. The antigens were: myelin basic protein (MBP), myelin associated glycoprotein (MAG), ganglioside (GM_1), sulfatide (SULF), chondroitin sulfate (CON-SO_4), myelin oligodendrocyte glycoprotein (MOG), _-_-crystallin (_-_-crys), neurofilament proteins (NAFP), tubulin and three cross-reactive peptides, *Chlamydia pneumoniae* (CPP), Streptococcal M protein (STM6P) and milk butyrophilin (BTN). Autistic children showed the highest levels of IgG, IgM and IgA antibodies against all neurologic antigens, as well as the three cross-reactive peptides. These antibodies are specific, since immune absorption demonstrated that only neuron-specific antigens or their cross-reactive epitopes could significantly reduce antibody levels. These antibodies may have been synthesized as a result of an alteration in the blood-brain barrier. This barrier promotes access of pre-existing T-cells and central nervous system antigens to immunocompetent cells, which may start a vicious cycle. These results suggest a mechanism by which bacterial infections and milk antigens may modulate autoimmune responses in autism.

Keywords: Autoantibody, Neuron-specific, Antigen, Autism, Cross-reaction

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1. Introduction

Autism is a developmental disorder described by Kanner in 1943. Its etiopathogenesis is still unknown. A number of factors have been implicated in the pathogenesis of autism, including genetic, environmental, immunological and neurological. Strong lines of evidence suggest that the immune system plays an important role in the development of autism (Yonk et al., 1990; Menage et al., 1992; Fudenberg, 1996, Gupta et al., 1996; 1998; Gupta, 2000).

Immune abnormalities in autism include changes in the numbers and activities of macrophages, T-cells, B-cells and natural killer cells (Warren et al., 1986; 1987; Gupta et al., 1998). In addition, a shift occurs from T-helper-1 to T-helper-2 T-cell type in autism as evidenced by a decrease in the production of interleukin-2 (IL-2) and interferon-_ (IFN- _) and an increased production of interleuken-4 (IL-4) (Gupta et al., 1998). In another study Gupta et al. (1996) reported that patients with autism have elevated IgE levels and an increased incidence of autoantibodies to myelin basic protein (MBP) and neurofilament proteins (NAFP). Singh et al. (1993; 1997) reported MBP and NAFP autoantibodies in 55-70% of children with autism. Considering the regulatory interactions between the nervous system and the immune system, and the detection of MBP and NAFP autoantibodies, it is plausible to propose that drugs and environmental toxins might have detrimental effects on neuroendocrine-immune circuits, resulting in autism (Ballieux, 1992; Kusnecov et al., 1990; Ader et al., 2001).

Toxic chemical exposures such as polychlorinated biphenyl, mercury, lead and others can induce alteration or over-expression of genes involved in regional brain glial fibrillary acidic protein (GFAP) and astroglial glucose regulated protein (GRP). The astroglia cytoskeletal element GFAP, neurotypic and gliotypic proteins or neurofilament triplet are generally accepted as sensitive indicators of neurotoxic effects in mature brains (Morse et al., 1996; Partl et al., 1998; Qian et al., 1999).

Over-expression of the gene results in altering the structural differentiation of astrocytes, and subsequent autoimmune response to neurofilaments and astroglial glucose regulated proteins. Autoantibodies to nervous system antigens are detected in populations exposed to toxic, environmental or occupational chemicals. For example, IgG to neuronal cytoskeletal proteins, neurofilaments and myelin basic protein have been detected in workers exposed to lead or mercury, and in women with silicone breast implants (Vojdani et al., 1994; El-Fawal et al., 1996; 1999). Titers of antibodies against neurofilaments and MBP correlated significantly with blood lead or urinary mercury, the typical indices of toxic exposure. Moreover, the levels of these antibodies correlated with sensorimotor deficits; these antibodies are known to interfere with neuromuscular function (El-Fawal et al., 1999).

Edelson and Cantor (1998; 2000) demonstrated a body burden of neurotoxicants in more than 90% of autistic children. These authors presented evidence for genetic and environmental aspects of a hypothetical process believed to cause immune system injury secondary to exposure to the immunotoxins. “Activation” of the immune system is caused by toxicants leading to the production of autoantibodies against haptens, i.e., the toxic chemicals attached to brain proteins. The subsequent damage may be considered a component in the etiologic process of neurotoxicity in the autistic spectrum.

We detected antibodies against nine different neuron-specific antigens in the sera of children with autism. These antibodies were found to bind with different encephalitogenic molecules, which have sequence homologies with neurological antigens (butyrophilin, a milk protein; *Chlamydia pneumoniae* peptide; and Streptococcus M proteins). Our results suggest a role for antibodies against brain cross-reactive food antigens and infectious agents in the pathogenesis of autistic behavior.

2. Materials and Methods

2.1 Patients

Forty subjects (23 males and 17 females) 3 to 12 years of age (mean 6.4 years), with a diagnosis of autism were sent by different clinicians to our laboratory for immunogical examination. The clinical diagnosis of autism was made according to the DSM-IIIR criteria, established by the American Psychiatric Association, Washington D.C., as well as by a developmental pediatrician, a pediatric neurologist, and/or a licensed psychologist. Blood samples were excluded if their medical histories included head injury, evidence of gliomas, failure to thrive, and other known factors that may contribute to abnormal developmental. For comparison, blood samples from forty healthy, age and sex matched controls were included in this study.

2.2 Neuronal and other Antigens

Myelin basic protein, myelin associated glycoprotein, ganglioside GM_1, _-_-crystallin, sulfatide, chondroitin sulfate and tubulin were purchased from Sigma Chemicals (St. Louis, Missouri). Neurofilament (NAFT) was purchased from Boehringer Mannheim Roche (Indianapolis, Indiana). MBP peptide 87-106, MOG peptides 21-40, 61-80, milk butyrophilin peptide 89-109, Streptococcal M6 peptide, and *Chlamydia pneumoniae* peptide 483 bound to KLH were purchased from Research Genetics (Huntsville, Alabama).

2.3 ELISA Procedure

Enzyme-linked immunosorbent assay (ELISA) was used for testing antibodies against nine different neuron-specific antigens, milk and bacterial peptides in the sera of patients with autism and control subjects. Antigens or peptides were dissolved in methanol at a concentration of 1.0 mg/ml, then diluted 1:100 in 0.1 M carbonate-bicarbonate buffer, pH 9.5, and 50 µl were added to each well of a polystyrene flat-bottom ELISA plate. Plates were incubated overnight at 4°C and then washed three times with 20 mM tris-buffered saline (TBS) containing 0.05% Tween 20, pH 7.4. The nonspecific binding of immunoglobulins was prevented by adding a mixture of 1.5% bovine serum albumin (BSA) and 1.5% gelatin in TBS, and then incubating for 2 h at room temperature, and then overnight at 4°C. Plates were washed as in the above, and then serum samples diluted 1:100 in 1% BSA-TBS were added to duplicate wells and incubated for 2 h at room temperature. Sera from patients with multiple sclerosis, polyneuropathies and other neurological disorders with known high titers of IgG, IgM and IgA against different neurological antigens were used to rule out non-specific antibody activities of inter- and intra-assay variability. Plates were washed, and then peroxidase-conjugated goat anti-human IgG, IgM or IgA antiserum (KPI, Gaithersburg, Maryland) diluted 1:400 in 1% BSA-TBS was added to each well; the plate was incubated for an additional 2 h at room temperature. After washing five times with TBS-Tween buffer, the enzyme reaction was started by adding 100 µl of o-phenylene diamine in citrate-phosphate buffer, pH 5.0 and hydrogen peroxide diluted 1:10,000. After 45 min, the reaction was stopped with 50 µl of 2 N H_2SO_4. The optical density (O.D.) was read at 492 nm by means of a microtiter reader. Several control wells containing all

reagents, but human serum, were used for detecting nonspecific binding.

Coefficients of intra-assay variation were calculated by running five samples eight times in one assay. Coefficients of inter-assay variation were determined by measuring the same samples in six consecutive assays. This replicate testing established the validity of the ELISA assays, determined the appropriate dilution with minimal background, and detected serum IgG, IgM and IgA against different antigens. Two sera from healthy controls, two nonspecific sera from multiple sclerosis patients and two sera from autistic children were used to construct standard control curves. These sera were diluted 1:25, 1:50, 1:100, 1:200 and 1:400. At dilutions of 1:50 to 1:200, the standard curve for multiple sclerosis sera was linear and antibodies from healthy controls were not detected against 12 tested antigens (O.D. <0.1). Hence, antibody detection in autistic sera was performed at 1:100 dilution in the appropriate buffer.

2.4 Absorption of Sera

Three autistic patients' sera containing high levels of IgG and IgM antibodies against MBP and MOG (O.D. in ELISA> 0.7) and three control sera with very low IgG and IgM antibodies against MBP and MOG (O.D. in ELISA< 0.3) were used in inhibition studies.

In different test tubes, 1 ml of each diluted serum was pre-incubated with 100 μg of human serum albumin (HSA), lipopolysaccharide (LPS), MBP, MOG, milk butyrophilin (BTN), *C. pneumoniae* (CPP) and Streptococcal (STM6P) peptides. After mixing the ingredients, the tubes were kept for 1 h in a 37°C water bath, followed by a 1 h incubation at 4°C, and then the tubes were centrifuged at 3,000 g for 10 min. The supernatant was used for the measurement of antibody levels by ELISA and for comparison of optical densities of absorbed serum with nonspecific and specific peptides or proteins.

2.5 Statistical Analysis

A Systat program version 5.2 by Systat Inc. was used for statistical analysis (Mecocci et al., 1995). Normal distribution of data was tested by the Kolmogorov-Smirnov one-sample test. One-way analysis of variance was performed by means of ANOVA and Kruskal-Wallis H test for ranked data. For post-hoc analysis, Tukey's test was employed. Correlations were performed by means of Pearson's test and the nonparametric Spearman's rank test.

3. Results

3.1 Detection of neurologic antibodies

Using ELISA assays, sera from 40 healthy subjects and 40 autistic children were analyzed for the presence of IgG, IgM, and IgA antibodies against nine neuron-specific antigens and three encephalitogenic and cross-reactive proteins. The ELISA results expressed as mean O.D. at 492 nm are summarized in Fig. 1. The O.D. for IgG antibody values obtained with 1:100 dilution of healthy control sera ranged from 0.01 to 0.84, varying among subjects and antigens. The mean ± standard deviation (S.D.) of these O.D. values as shown in Fig. 1 ranged from 0.13 ± 0.09 to 0.23 ± 0.18. The corresponding IgG O.D. values from autistic children's sera ranged from 0.05 to 2.47 and with the mean ± S.D. of IgG values, which ranged from 0.41 ± 0.33 to 0.72 ± 0.65 (Fig. 1). For all 12 antigens, the differences between mean ± S.D. of control sera and autistic children's sera were highly significant ($p < 0.001$). At a cutoff value of 0.3 O.D., levels of IgG antibody against these antigens were calculated in control and patient's sera and found that while 5-22.5% of control sera had IgG values higher than 0.3 O.D., the autistic children's group showed elevated IgG values from 47.5 to 57.5% ($p < 0.001$) (Fig. 2).

Levels of IgM antineuron-specific antigens in sera of healthy controls and autistic children are shown in Fig. 3. These serum IgM antibodies against all 12 different tested antigens were significantly higher in patients than in controls. The mean ± S.D. for controls ranged from 0.12 ± 0.13 to 0.22 ± 0.23 O.D. and for patients ranged from 0.43 ± 0.32 to 0.92 ± 0.63 OD ($p < 0.001$) (Fig.3). When the 0.3 O.D. cutoff point was used, 10 to 20% of controls versus 57.5 to 72.55 % of autistic children's sera showed elevated IgM antibody levels ($p < 0.001$) (Fig. 4). Likewise, IgA antibody levels against these neurological antigens were examined in both groups. Individual and mean ± S.D. data depicted in Fig. 5 showed significant differences between control and patients groups. The mean ± S.D. for IgA antibody levels in controls ranged from 0.10 ± 0.07 to 0.2 ± 0.22 and in patients, from 0.25 ± 0.28 to 0.53 ± 0.52 (Fig. 5) ($p < 0.001$). Percent elevated serum IgA anti-neuronal autoantibodies at the O.D. value of greater than 0.3, were significantly higher in autistic children than in controls. The percent positive for IgA antibodies in controls ranged from 5 to 15% and in patients 20-52.5% ($p < 0.001$) (Fig. 6).

3.2 Assay Variation of IgG, IgM, IgA

Coefficients of intra-assay variations for all three antibodies (IgG, IgM and IgA) against 12 antigens were less than 6%. Coefficients of interassay variations were less than 10%. Individual antibody ELISA values were examined for simultaneous detection of IgG, IgM or IgA antibodies against all tested antigens. We observed an elevation of IgA in 2.5%, IgG in 5%, and IgM in 10% of the control group against all nine neuron-specific antigens and their cross-reactive peptides (Table 1). In comparison, specimens from children with autism were elevated in 27.5% (IgA), 35% (IgG) and 50%(IgM) (Table 1). Furthermore, when elevations in all three isotype antibodies were examined against all twelve

different antigens, none of the control specimens were elevated simultaneously for IgA, IgM and IgG antibodies. However, 20% of specimens from children with autism were elevated simultaneously for IgA, IgM as well as IgG antibodies against all tested antigens (Table 1).

3.3 Absorption of neurologic antibodies using MBP, MOG and their cross-reactive peptides

In order to examine if antibodies against nervous system antigens are specific, we performed an absorption study using nonspecific antigens (LPS, HSA), specific antigens (MBP, MOG) and cross-reactive peptides. The results, summarized in Tables 2 and 3, showed that only specific antigens and their cross-reactive peptides were capable of significantly absorbing IgG and IgM antibodies. The most significant antibody absorption was achieved when specific antigen was used, as expected. For example, anti-MBP positive sera were absorbed up to 67% with MBP (Table 2) and anti-MOG positive sera were absorbed by MOG up to 74% but not with HSA or LPS (Table 3).

4. Discussion

The etiology and pathogenesis of autism is not well understood. The disorder may have a variety of causes including environmental, neural, genetic, immune, and biochemical. Structural abnormalities have been identified in areas of the autistic brain, with a pattern suggesting that a neurodevelopmental abnormality may have occurred (Rodier et al., 1996; Purcell et al., 2001). Immunological research has suggested autoimmunity as a pathogenic factor in autism (Weizman et al., 1982; Singh et al., 1993, 1997). Circulating autoantibodies are produced against brain tissue antigens and hence, aid in the diagnosis of different autoimmune neurological disorders. In animal models of multiple sclerosis (MS), the major targets of autoimmune responses are: MBP, MOG, and α-β-crystallin. MOG is an abundant myelin constituent expressed exclusively by oligodendrocytes or the myelin forming cells. α-β-crystallin is another immunodominant antigen or small heat shock protein in neuronal tissue. Expression of α-β-crystallin in oligodendrocytes is prominent during early development of MS lesions. Therefore, antibodies against MBP, MOG and α-β-crystallin have been used as clinical markers of MS (Genain et al., 1999; Holz et al., 2000; Bajramovic et al., 2000; Brock et al., 2000).

In demyelinating sensorimotor neuropathies, such as Gillain Barre Syndrome (GBS) and chronic inflammatory demyelinating polyneuropathy, IgG, IgM and IgA antibodies have been detected against myelin and acidic glycolipids (GM_1, LM_1, GQ_1, and GD_1b) and sulfatide (Baba et al., 1989; Chabraoui et al., 1993; Isoardo et al., 2001). These antibodies cross-react with *Campylobacter jejuni* since the enterotoxin of *C. jejuni* utilizes GM_1 as its receptor. Therefore, high incidences of antibodies to *C. jejuni* and GM_1 are seen in acute GBS (Kaldor and Speed, 1984; Greunewald et al., 1991). In patients with polyneuropathy associated with paraproteinemias, IgM against MAG and IgM or IgA monoclonal gammopathy are observed (Ropper and Gorson, 1998). Antibodies against sulfatide and chondroitin sulfate are found in the blood of subjects suffering from chronic sensory neuropathy. These antibodies are polyreactive and react with neurons in the surface of dorsal root ganglia where the blood nerve barrier is relatively permeable (Nemni et al., 1993; Fredman et al., 1993). Circulating autoantibodies to neuronal and glial filament proteins have been detected in people exposed to environmental or occupational chemicals (El-Fawal, 1996, 1999), in patients with brain aging, dementia and Alzheimer's disease (Mecocci et al., 1995) and in children with autistic behavior (Singh et al., 1997).

Toxic chemicals (such as polychlorinated biphenyl (PCBs), mercury, lead and others) can induce alteration or over-expression of genes involved in regional brain GFAP and astroglial GRP. Over-expression of these genes results in a change in the structural differentiation of astrocytes and, hence, autoimmune response to neurofilament proteins (Morse et al., 1996; Partl et al., 1998; Qian et al, 2000). Autoantibodies against neurologic antigens in autism have been studied by three different investigators using crude antigens: brain tissue antigens (Weizman et al., 1982); myelin basic protein (Singh et al., 1993); and partially purified preparation of cytoskeletal intermediate filaments (Singh et al., 1997). The high prevalence of these autoantibodies in neurodegenerative and neuropsychiatric disorders has led many investigators to believe that these antibodies reflect an alteration of the blood-brain barrier, which promotes the access of immunocompetent cells to the central nervous system (Mecocci et al., 1995; Morse et al., 1996; Singh et al., 1997; Partl et al., 1998; Qian et al., 2000).

Based on these published findings, we hypothesized that if infectious antigens or toxic chemicals cause the blood-brain barrier to become more permeable, then antibodies (IgG, IgM, IgA) to neurologic antigens or pathogenic peptides should be detectable in the blood of patients with autism. However, we did not investigate whether or not these antibodies can be a risk factor(s) for autism.

To examine this hypothesis, we used twelve different purified protein and synthetic peptides in our highly specific ELISA procedure with low background of smaller than 0.1 O.D. at 492. Another advantage of this assay is the use of a second antibody such as antihuman IgG, IgM or IgA for identification of isotypes, which were not studied by Weizman et al., (1982) or by Singh et al., (1993, 1997). In many autoimmune diseases, including autoimmune neurologic disorders, the isotopes

IgM and IgA autoantibodies are considered to be more pathogenic than the IgG isotype. We found that all three isotype antibodies, alone or in combination, were higher in autistic patients than in healthy controls (Fig. 1-6, Table 1).

This simultaneous elevation of IgG, IgM and IgA antibodies against all nine neurological antigens, indicates that an alteration of the blood-brain barrier promotes the access of immunocompetent cells to many different nervous system antigens. Thus, immune cell reaction to nervous system antigens is not limited to only neuronal and glial filament protein in children with autism as described by Singh et al., (1997).

We used highly purified neurofilament antigen in the ELISA assay and diluted the sera 1:100. We found that only 7.5-10% of controls had IgA, IgG or IgM antibodies against neurofilaments compared to 37.5, 50 and 57.5% of autistic subjects. Our results show a relatively higher percent positive for neurofilament antibodies in autistic patients than in the study by Singh et al., (1997) perhaps because of differences in purity of antigens or dilution of sera.

It is of considerable interest that antibodies to neuron-specific antigens are prevalent in populations exposed to environmental and occupational chemicals and in patients with neurodegenerative diseases in which viruses or other infectious agents are suspected etiological agents. For example, IgG antibodies to MBP, neuronal cytoskeletal proteins and neurofilaments are detected in workers exposed to lead or mercury (El-Fawal et al., 1996). The titer of these antibodies is significantly correlated with blood lead or urinary mercury, which are the typical indices of exposure. Moreover, the level of these antibodies is correlated with the degree of sensorimotor deficits, since these antibodies interfere with neuromuscular function (El-Fawal et al., 1999).

Many infectious agents including: measles (Wakefield, 1998); rubella virus (Chess et al., 1978) and cytomegalovirus (Ivarsson et al., 1990) have long been suspected as etiologic factors in autism. But whether these viruses induce brain autoantibodies has not yet been explored according to Singh et al., (1997).

For this reason, we reviewed the scientific literature, and found that over 60 different microbial peptides have been reported to cross-react with human brain tissue and MBP. Furthermore, these peptides not only have the capability to cross-react with MBP and induce T-cell response, but can also induce experimental autoimmune encephalomyelitis (Gorgan et al., 1999; Bronze and Dale, 1993; Lenz et al., 2001).

Among families with autistic children, it is well known that elimination of milk from the child's diet significantly improves the patient's condition. This clinical finding correlates with laboratory results reported by Stefferl et al., (2000): they found that an encephalitogenic T-cell response to MOG can be either induced or alternatively suppressed as a consequence of immunological cross-reactivity or 'molecular mimicry" with the extracellular IV-like domain of milk protein butyrophilin. All of these clinical laboratory findings shed light on our detection of higher levels of antibodies against milk antigens in autistic sera. Based on the publications by Bronze et al., (1993); Gorgan et al., (1999); Stefferl et al., (2000); and Lenz et al., (2001), we chose Streptococcus synthetic peptide containing the conserved M protein or brain-cross-reactive epitope, a *Chlamydia pneumoniae*-specific peptide and the butyrophilin milk peptide, which modulates the encephalitogenic T-cell response to MOG in experimental autoimmune encephalomyelitis for our cross-reactivity study.

Indeed when we tested IgG, IgM, and IgA antibodies against these three peptides, we found that every single serum with ELISA values higher than 0.3 O.D. against neurological antigens exhibited high levels of antibodies against Streptococcal, *C. pneumoniae* and milk peptides as well (Fig. 1-6, Table 1). Overall, antibodies against these three peptides (first IgM then IgG) were elevated in a higher percentage of controls and experimental sera than the percentage of elevated antibodies against neurological antigens. But, we did not observe even one specimen with a high antibody level against these peptides without having antibody levels against one or all nine tested neuron-specific antigens. These antibodies appear to be specific since in our absorption studies, milk butyrophilin, *C. pneumoniae* and Streptococcal peptide had a similar effect to MBP or MOG in reducing antibody levels from highly positive sera (Tables 2, 3). Based on these findings, we postulate that dietary and infectious antigens play a role in the pathophysiology of autism. It is likely that environmental factors including infection-induced injury causes release of neuronal antigens, which through activation of inflammatory cells, could lead to autoimmune reactions in genetically susceptible individuals. However, only long-term studies can prove the protective versus pathogenic role of these antibodies in children with autism.

For cross-reactive circulating antibodies to become pathogenic, they must cross the blood-brain barrier. It is now known that permeability of the blood-brain barrier increases after major histocompatibilty complex Class I expression (Fabry et al., 1994), activated lymphocyte interaction (Singh et al., 1997) and change in neuronal cell adhesion molecules (Purcell et al., 2001). Based on our review of the literature and our results reported here, we propose that the following chain of events may lead to autism:

> Pre-existing autoreactive T-cells are generated by molecular mimicry as a result of contact with dietary proteins, and viral, bacterial and parasitic antigens, which have sequence homologies or matched motifs of autoantigens.

Toxic chemicals, such as bacterial enterotoxins, viral antigens and metals such as mercury and lead may increase adhesion molecules on brain endothelial cells; toxic chemicals may also increase leukocyte function-associated antigen on activated T-cells.

Pre-existing autoreactive T-cells may transmigrate across the blood-brain barrier and induce activation of local antigen-presenting cells, such as microglia and astrocytes.

Production of IL-2, INF-γ and TNF-α by T-helper-1 autoreactive cells and TNF-α by the antigen presenting cells (astrocytes and microglia) may result in oligodendrocyte damage and demyelination.

As a result of this sequence of events, MBP, MAG, MOG, α-β-crystallin and other antigens are released from neurofilaments and enter the circulation, resulting in immune reactions, such as the formation of plasma cells with the capacity of producing IgG, IgM, and IgA antibodies against neuron-specific antigens.

These antibodies may cross the blood-brain barrier, combine with brain tissue antigens forming immune complexes, thus further damaging the neurological tissue.

This hypothesis may explain the significant difference in levels of anti-neurologic autoantibodies between controls and children with autism. While differentiation between protective versus pathogenic antibodies have yet to be elucidated, we suggest that antibodies against neuron-specific antigens and their cross-reactive epitopes may play a role in the pathogenesis of autism.

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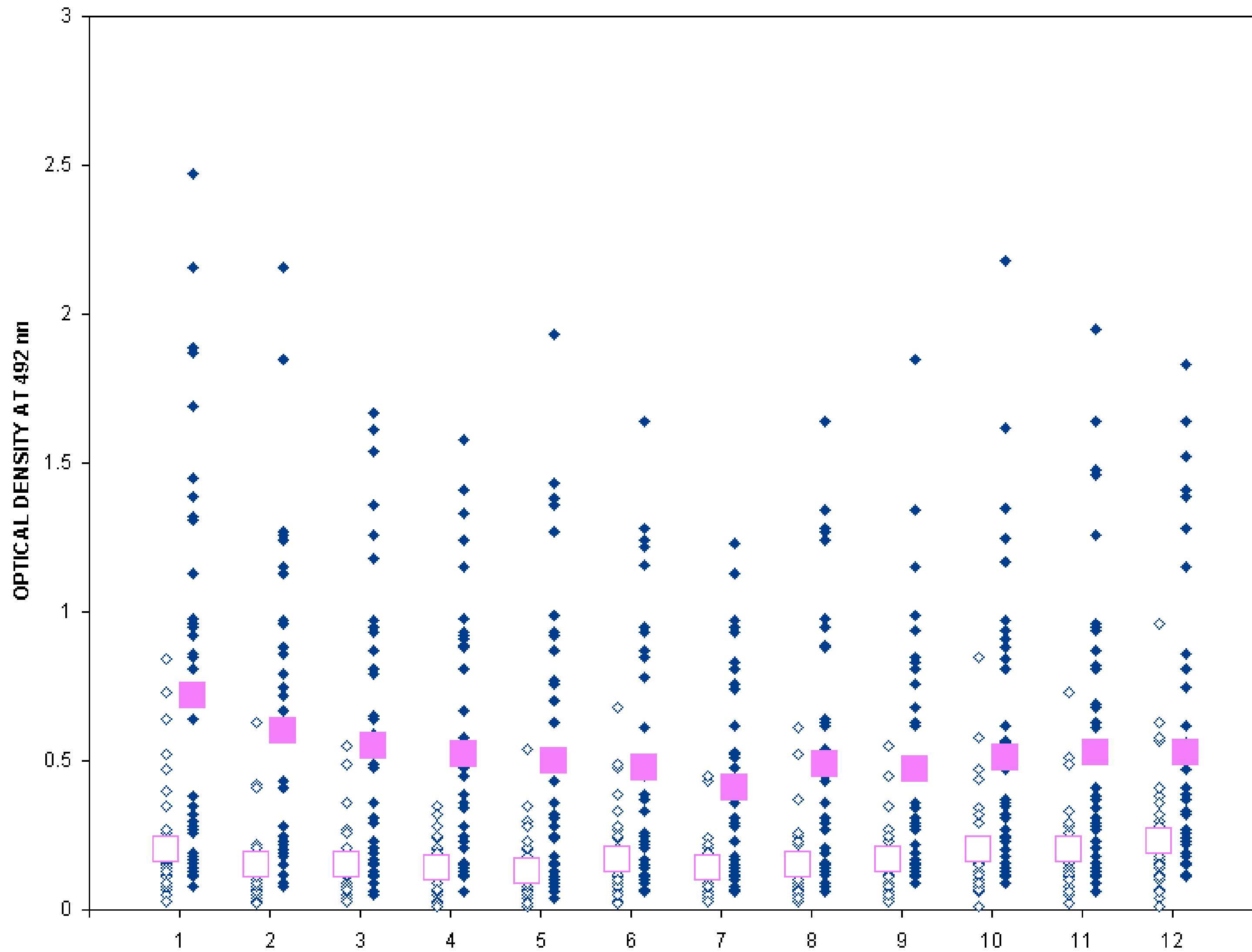

Fig. 1 Scattergram of serum titer of IgG antibody against different neurologic antigens (1-MBP, 2-MAG; 3-GM_1; 4-SULF; 5-$CONSO_4$; 6-MOG; 7-β-CRYS; 8-NAFP; 9-TUBULIN) and their cross-reactive peptides (10-CPP; 11-STM_6P; 12-MILK-BTN) in healthy control subjects □ and patients with autism ■ expressed as optical density in ELISA test.

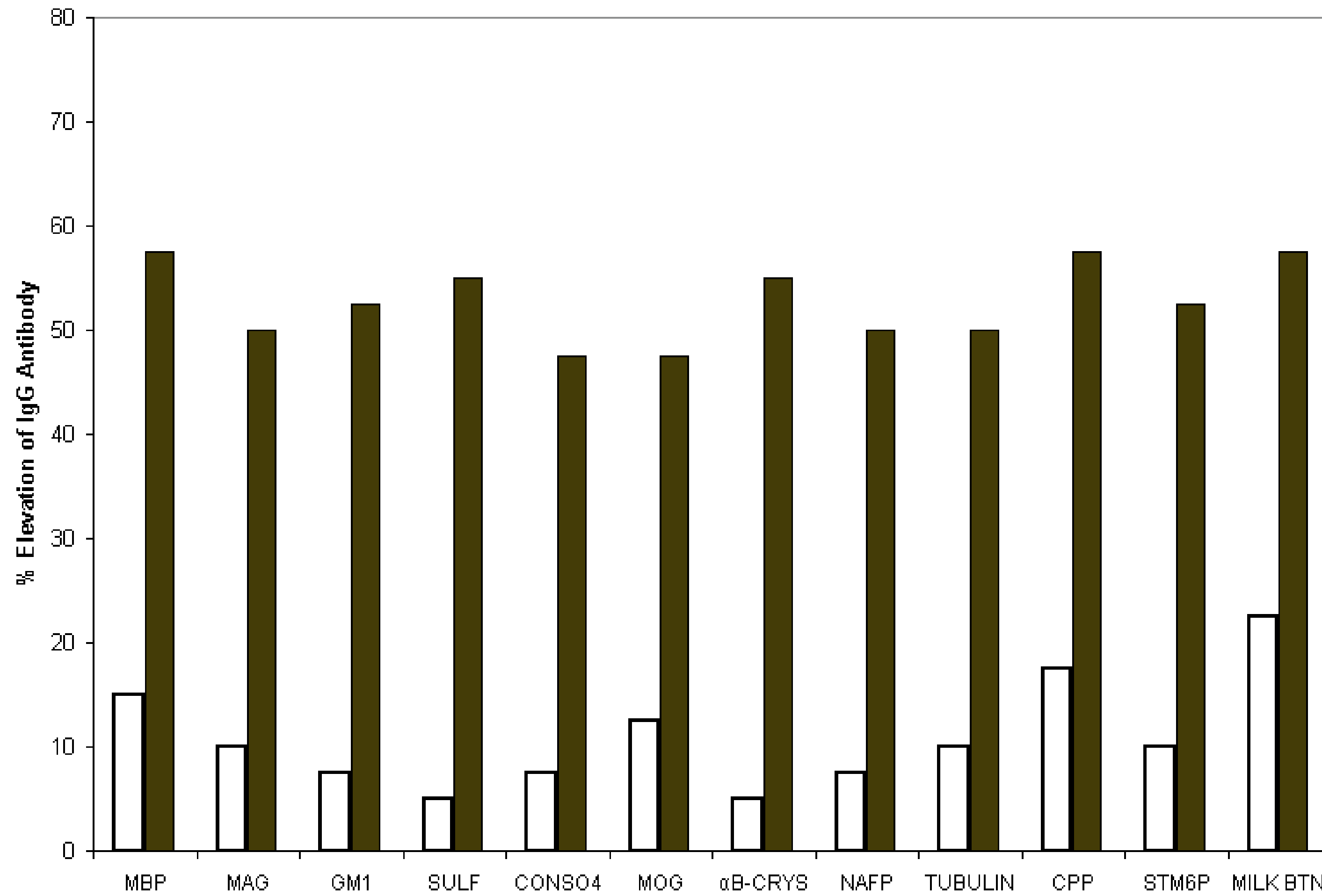

Fig. 2 Percent elevation in IgG antibody against neurologic antigens and their cross-reactive peptides in healthy control subjects ☐ and patients with autism ■ at cut-off point of 0.30 O.D.

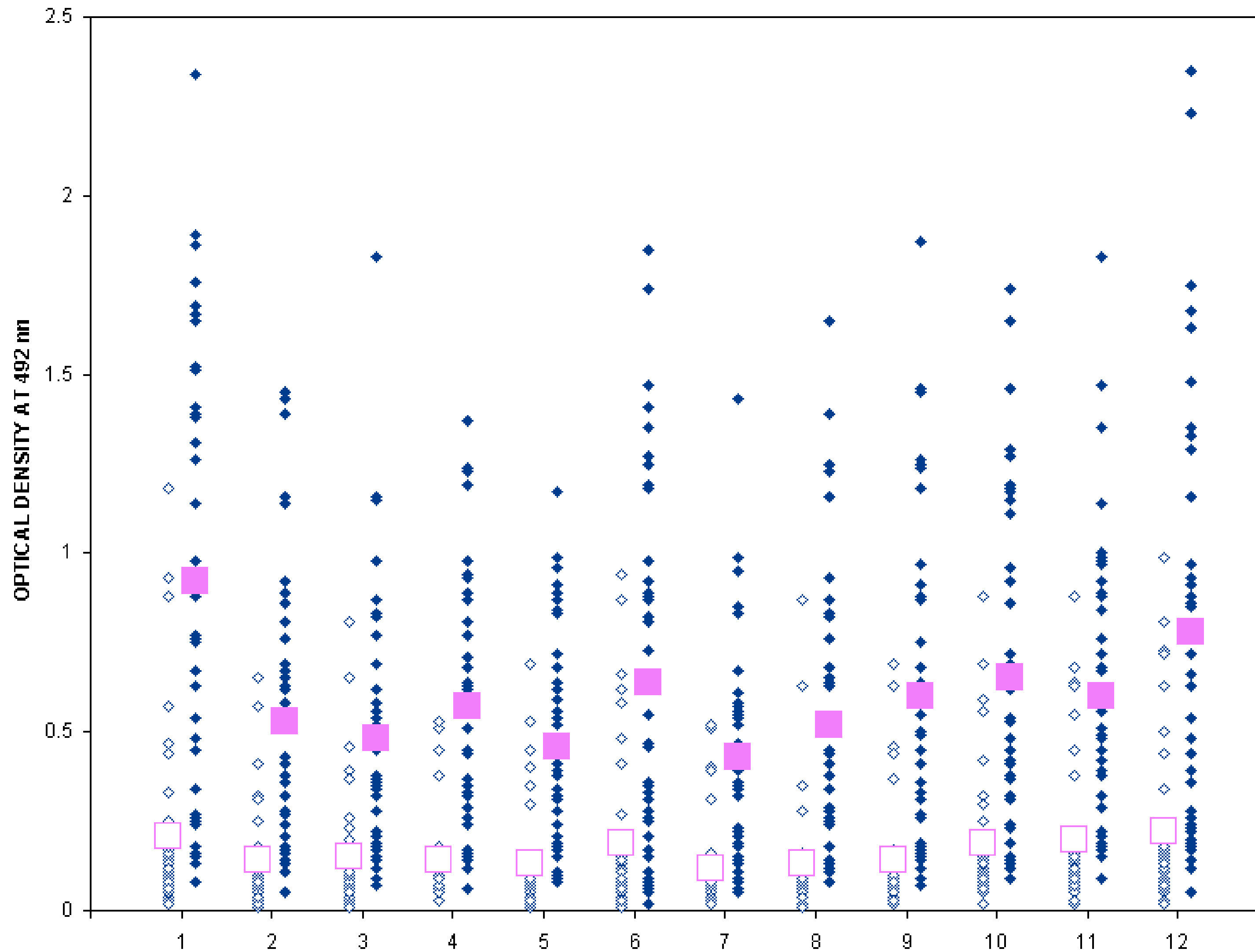

Fig. 3 Scattergram of serum titer of IgM antibody against different neurologic antigens (1-MBP, 2-MAG; 3-GMI; 4-SULF; 5-CONSO$_4$; 6-MOG; 7-β-CRYS; 8-NAFP; 9-TUBULIN) and their cross-reactive peptides (10-CPP; 11-STM$_6$P; 12-MILK-BTN) in healthy control subjects □ and patients with autism ■ expressed as optical density in ELISA test.

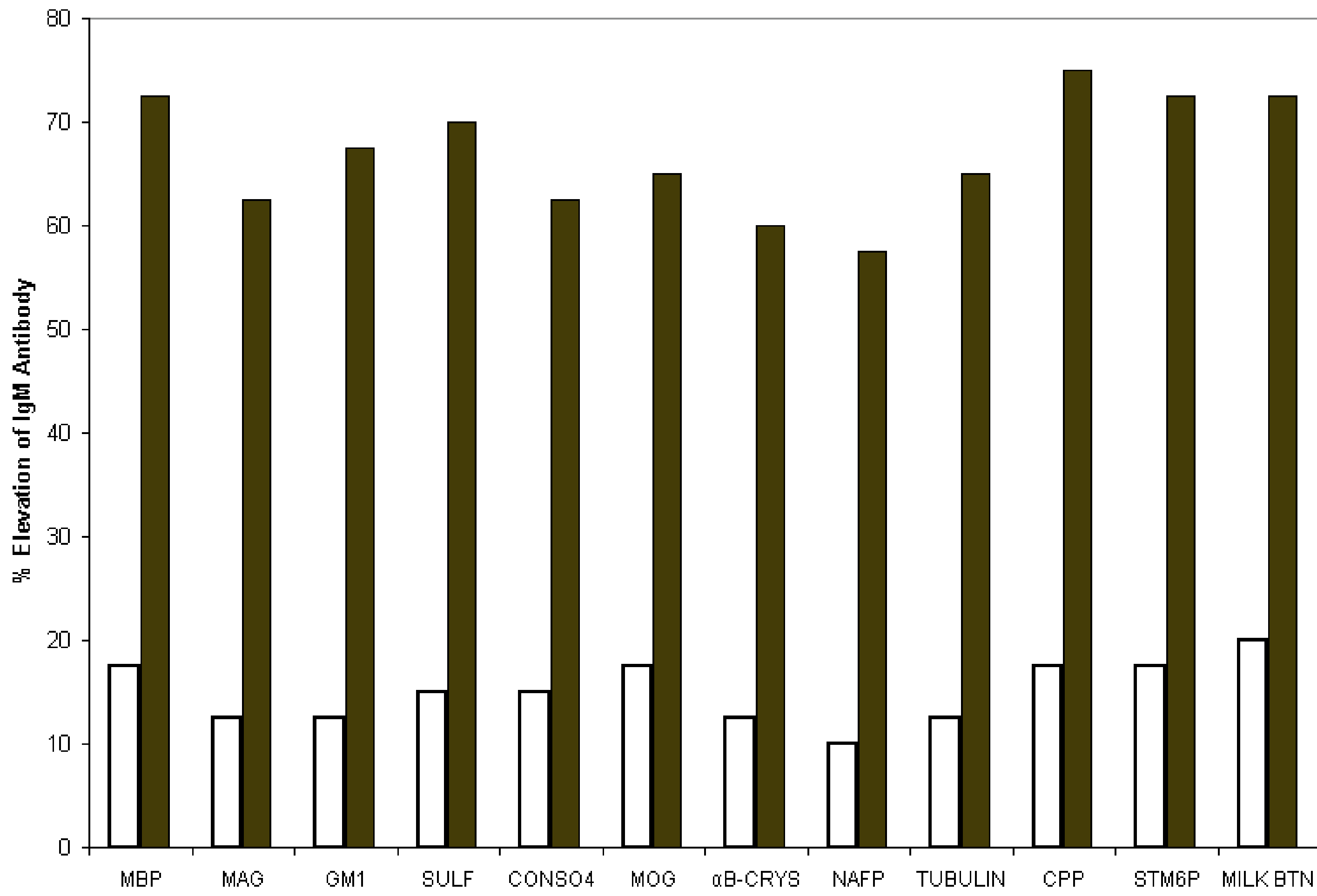

Fig. 4 Percent elevation in IgM antibody against neurologic antigens and their cross-reactive peptides in healthy control subjects □ and patients with autism ■ at cut-off point of 0.30 O.D.

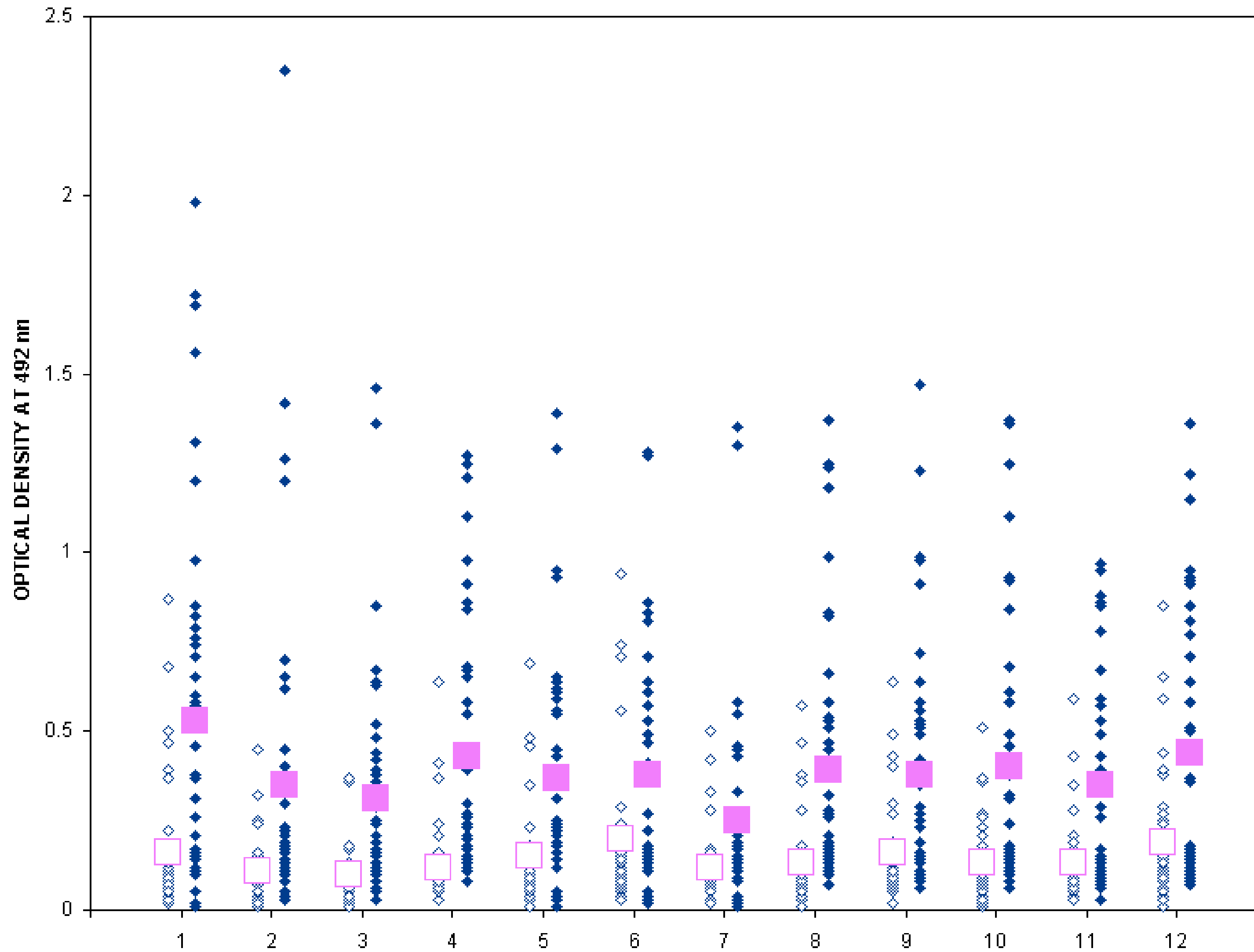

Fig. 5 Scattergram of serum titer of IgA antibody against different neurologic antigens (1-MBP, 2-MAG; 3-GMI; 4-SULF; 5-CONSO$_4$; 6-MOG; 7-β-CRYS; 8-NAFP; 9-TUBULIN) and their cross-reactive peptides (10-CPP; 11-STM$_6$P; 12-MILK-BTN) in healthy control subjects □ and patients with autism ■ expressed as optical density in ELISA test.

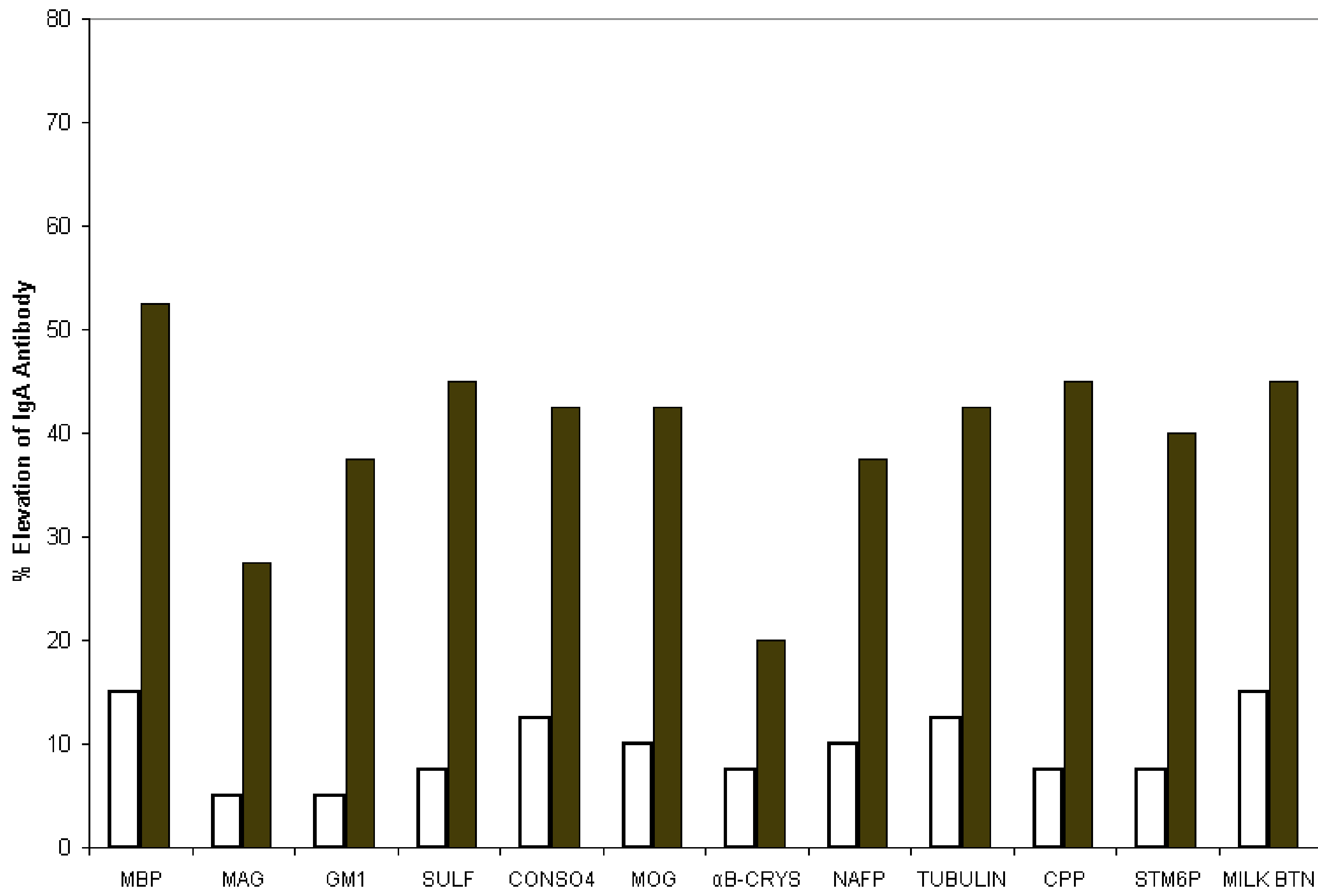

Fig. 6 Percent elevation in IgA antibody against neurologic antigens and their cross-reactive peptides in healthy control subjects ☐ and patients with autism ■ at cut-off point of 0.30 O.D.

TABLE –1 NUMBER AND PERCENTAGE OF SPECIMENS WITH SIMULTANEOUS ELEVATION OF ANTIBODIES AGAINST ALL NEURON-SPECIFIC ANTIGENS AND CROSS-REACTIVE PEPTIDES

	IgG		**IgM**		**IgA**		**IgG + IgM + IgA**	
	Controls	*Patients*	*Controls*	*Patients*	*Controls*	*Patients*	*Controls*	*Patients*
NUMBER	2	14	4	20	1	11	0	8
PERCENTAGE	5	35	10	50	2.5	27.5	0	20
P VALUES	< 0.001		< 0.001		< 0.001		< 0.001	

TABLE –2 SERUM LEVELS OF ANTI-MBP ANTIBODIES EXPRESSED AS O.D. IN ELISA AND PERCENT INHIBITION AFTER ABSORPTION WITH DIFFERENT SPECIFIC AND NONSPECIFIC ANTIGENS

	IgG Levels and % Inhibition after Absorption with:							**IgM Levels and % Inhibition after Absorption with:**						
SAMPLE	HSA	LPS	MBP	MOG	MILK BTN	CPP	STM6P	HSA	LPS	MBP	MOG	MILK BTN	CPP	STM6P
PATIENT 1	1.23	1.15	0.41	0.79	0.83	0.62	0.57	1.4	1.27	0.78	0.92	1.1	0.99	0.95
% INHIBITION	*N.S.*	*N.S.*	*67*	*36*	*33*	*50*	*54*	*N.S.*	*N.S.*	*44*	*33*	*22*	*29*	*32*
PATIENT 2	0.89	0.92	0.33	0.45	0.64	0.39	0.41	1.6	1.65	0.85	0.96	1.25	0.87	0.83
% INHIBITION	*N.S.*	*N.S.*	*63*	*50*	*28*	*57*	*54*	*N.S.*	*N.S.*	*47*	*40*	*22*	*46*	*48*
PATIENT 3	1.52	1.49	0.67	0.82	0.89	0.71	0.96	0.98	1.1	0.44	0.58	0.69	0.54	0.59
% INHIBITION	*N.S.*	*N.S.*	*56*	*46*	*42*	*53*	*37*	*N.S.*	*N.S.*	*55*	*41*	*30*	*45*	*40*
CONTROL 1	0.25	0.24	0.19	0.21	0.18	0.20	0.19	0.19	0.21	0.16	0.18	0.15	0.17	0.20
% INHIBITION	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*
CONTROL 2	0.28	0.27	0.22	0.17	0.23	0.21	0.24	0.22	0.19	0.21	0.17	0.18	0.19	0.18
% INHIBITION	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*
CONTROL 3	0.15	0.17	0.13	0.15	0.14	0.16	0.13	0.15	0.13	0.16	0.15	0.17	0.14	0.16
% INHIBITION	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*

N.S. = non-significant

TABLE – 3 SERUM LEVELS OF ANTI-MOG ANTIBODIES EXPRESSED AS O.D. IN ELISA AND PERCENT INHIBITION AFTER ABSORPTION WITH DIFFERENT SPECIFIC AND NONSPECIFIC ANTIGENS

	IgG Levels and % Inhibition after Absorption with:							**IgM Levels and % Inhibition after Absorption with:**						
SAMPLE	HSA	LPS	MBP	MOG	MILK BTN	CPP	STM6P	HSA	LPS	MBP	MOG	MILK BTN	CPP	STM6P
PATIENT 1	0.83	0.85	0.62	0.31	0.38	0.68	0.63	0.81	0.78	0.66	0.41	0.45	0.64	0.61
% INHIBITION	*N.S.*	*N.S.*	*25*	*63*	*54*	*18*	*24*	*N.S.*	*N.S.*	*19*	*49*	*45*	*21*	*25*
PATIENT 2	0.75	0.71	0.55	0.26	0.27	0.51	0.47	1.15	1.22	0.51	0.48	0.50	0.59	0.55
% INHIBITION	*N.S.*	*N.S.*	*27*	*65*	*64*	*32*	*37*	*N.S.*	*N.S.*	*56*	*58*	*57*	*49*	*52*
PATIENT 3	1.1	1.24	0.67	0.45	0.49	0.72	0.68	1.24	1.15	0.69	0.32	0.43	0.72	0.68
% INHIBITION	*N.S.*	*N.S.*	*39*	*59*	*56*	*35*	*38*	*N.S.*	*N.S.*	*44*	*74*	*65*	*42*	*45*
CONTROL 1	0.28	0.26	0.21	0.19	0.20	0.22	0.24	0.18	0.18	0.14	0.15	0.12	0.19	0.17
% INHIBITION	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*
CONTROL 2	0.22	0.23	0.20	0.15	0.17	0.21	0.19	0.20	0.21	0.22	0.16	0.14	0.18	0.20
% INHIBITION	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*
CONTROL 3	0.27	0.25	0.19	0.1	0.16	0.18	0.20	0.17	0.18	0.15	0.17	0.14	0.19	0.18
% INHIBITION	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*

N.S. = non-significant