

# Immune Response to Dietary Proteins, Gliadin and Cerebellar Peptides in Children with Autism

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The mechanisms behind autoimmune reaction to nervous system antigens in autism are not understood. We assessed the reactivity of sera from 50 autism patients and 50 healthy controls to specific peptides from gliadin and the cerebellum. A significant percentage of autism patients showed elevations in antibodies against gliadin and cerebellar peptides simultaneously. For examining cross-reaction between dietary proteins and cerebellar antigens, antibodies were prepared in rabbits, and binding of rabbit anti-gliadin, anti-cerebellar peptides, anti-MBP, anti-milk, anti-egg, anti-soy and anti-corn to either gliadin- or cerebellar-antigen-coated wells was measured. In comparison to anti-gliadin peptide binding to gliadin peptide at 100%, the reaction of anti-cerebellar peptide to gliadin peptide was 22%, whereas the binding of anti-myelin basic protein (MBP), anti-milk, anti-egg and anti-soy to gliadin was less than 10%.

Further examination of rabbit anti-gliadin (EQVPLVQQ) and anti-cerebellar (EDVPLLED) 8 amino acid (AA) peptides with human serum albumin (HSA) and an unrelated peptide showed no binding, but the reaction of these antibodies with both the cerebellar and gliadin peptides was greater than 60%. This cross-reaction was further confirmed by DOT-immunoblot and inhibition studies. We conclude that a subgroup of patients with autism produce antibodies against Purkinje cells and gliadin peptides, which may be responsible for some of the neurological symptoms in autism.

**Keywords:** Autism; Autoantibodies; Celiac disease; Gliadin and cerebellar peptides; Gluten ataxia

## INTRODUCTION

Autism is a complex developmental disorder with unknown etiology. As with many complex diseases, genetic and environmental factors including diet, infections and xenobiotics play a critical role in the development of autism (Ivarsson *et al.*, 1990; Wakefield *et al.*, 1998; Edelson and Cantor, 2000; Fatemi *et al.*, 2002; Kibersti and Roberts, 2002; Vojdani *et al.*, 2002; Shi *et al.*, 2003). Opioid peptides are considered components in the etiology of autism, and these peptides are available from a variety of food sources. These dietary proteins and peptides, including casein, casomorphins, gluten and gluteomorphins, can stimulate T-cells, induce peptide-specific T-cell responses, and abnormal levels of cytokine production, which may result in inflammation, autoimmune reactions and disruption of neuroimmune communications (Jyonouchi *et al.*, 2001). Peptides from inadequate digestion of casein and gluten apparently are absorbed in excess by autistic children, as reflected by very high levels of urinary peptides (Reichelt *et al.*, 1990; Shattock *et al.*, 1991; Reichelt, 1994; Reichelt and Reichelt, 1997; Whiteley *et al.*, 1999; Horvath *et al.*, 1999; Nelson *et al.*, 2001; Reichelt and Knivsberg, 2003). Excessive peptides from undigested casein and gluten are suspected to exert significant toxicity. Indeed, parents and clinicians report improvement of autistic children

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on gluten- and casein-free diets (Knivsberg *et al.*, 1995; Knivsberg *et al.*, 2001; Reichelt and Knivsberg, 2003). This clinical finding correlates with laboratory results reported earlier by our group in children with autism (Vojdani *et al.*, 2002; 2003). We detected IgG, IgM and IgA antibodies against nine neuron-specific antigens in the sera of children with autism. These antibodies were found to bind with different encephalitogenic molecules that have sequence homologies to a milk protein (Vojdani *et al.*, 2002). Moreover, in a very recent study we showed that infectious agent antigens such as Streptokinase (SK), heat shock proteins and dietary peptides (gliadin and casein) bind to different tissue enzymes and lymphocyte receptors. This binding of infectious agents antigens and dietary peptides to tissue enzymes results in autoantibodies as well as anti-SK, anti-gliadin and anti-casein in a significant percentage of children with autism (Vojdani *et al.*, 2004).

Gluten sensitivity is the most common immune-mediated disease induced by ingestion of gluten peptides in genetically susceptible individuals. This disease encompasses a spectrum of diverse manifestations including celiac disease (Sollid, 2000), dermatitis herpetiformis (Spurkland *et al.*, 1979), gluten-induced neurologic dysfunction (gluten ataxia) (Hadjivassiliou *et al.*, 1998; 2001; 2002), and gluten-related neuropathy (Cooke and Thomas-Smith, 1966). These variants are collectively called gluten sensitive spectrum.

Antibodies against gliadins and gliadin peptides have been described in celiac disease, gluten ataxia (Pratesi *et al.*, 1998; Hadjivassiliou *et al.*, 1998; 2001; 2002), and recently, in children with autism (Vojdani *et al.*, 2003). Gluten ataxia is considered one of the most frequent presentations of gluten sensitivity, which results in neurologic dysfunction. When patients with gluten ataxia were autopsied, perivascular cuffing with inflammatory cells, predominantly affecting the cerebellum, and loss of Purkinje cells were detected. These inflammatory reactions, resulting in Purkinje cell loss, imply that the neurologic insult may be immune-mediated (Kinney *et al.*, 1982; Ritvo *et al.*, 1986; Bürk *et al.*, 2001; Bushara *et al.*, 2001). It is not clear whether such immune-mediated damage is primarily cellular or antibody-driven. In a recent study, investigators assessed the reactivity of sera from patients with gluten ataxia, patients newly diagnosed with celiac disease without neurologic dysfunction, and healthy control subjects (Hadjivassiliou *et al.*, 2002). Using indirect immunocytochemistry on human cerebellar and rat CNS tissue, cross-reactivity of a commercial IgA anti-gliadin antibody with cerebellar tissue was demonstrated. Also, sera from 12 of 13 patients with gluten ataxia stained Purkinje cells strongly (Hadjivassiliou *et al.*, 2002). It was suggested that patients with gluten ataxia have antibodies against Purkinje cells

that cross-react with epitopes on Purkinje cells, and humoral immune responses are involved in the pathogenesis of gluten ataxia. However, this cross-reaction between cerebellar Purkinje cells and gliadin was done by using commercially-available whole rabbit serum made against the gliadin-molecule and not against its immunodominant peptides.

In the current study, we used affinity-purified antibodies made against the gliadin peptide and demonstrated its reactivity against the recombinant brain protein of Purkinje cells. The molecular target for these antibodies is an epitope consisting of 6 amino acids (VPLLED) expressed predominantly in neuroectodermal tissue (Dropcho *et al.*, 1987). This molecular mimicry between gliadin and cerebellar peptide may be a mechanism by which dietary peptides induce autoimmunity in autism.

## MATERIALS AND METHODS

### Patients

Blood samples from fifty subjects (33 males and 17 females), 3–14 years of age (mean 7.2 years), with a diagnosis of autism, were sent to our laboratory by different clinicians for immunological examination. The clinical diagnosis of autism was made according to the DSM-IV-R criteria, established by the American Psychiatric Association (Washington, DC), as well as by a developmental pediatrician, a pediatric neurologist, and/or a licensed psychologist. 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 development. For comparison, serum samples from 50 healthy age- and sex-matched controls with negative ANA titers and no known autoimmune diseases were included. The test requests were properly documented and kept in a confidential file. All persons gave their informed consent and allowed inclusion of their data in the manuscript without disclosure of their identity in the publication.

### Peptides and Proteins

Immunodominant T-cell epitopes of gliadin peptides (PQLQPQNPSQQQPQEQLVPLVQQ, EQVPLVQQ spanning the 6–27 and 20–27 amino acid (AA) sequence of gliadin) and cerebellar peptides (FLEDVPWLEDVDFLEDVPLLED, EDVPLLED spanning 9–30 and 23–30 AA were selected from human cerebellar cDNA library based on enzyme-linked immunoabsorbent assay (ELISA) reactivity with sera of patients with celiac disease. These and unrelated peptide from *Chlamydia trachomatis* HSP-60 peptide LKQIAAHAGKEGAIIFQQVM, HPLC grade, were

synthesized by Bio-Synthesis Inc. (Lewisville, TX). Human myelin basic protein (MBP), crude gliadin, and human serum albumin (HSA), egg, soy and corn proteins were purchased from Sigma (St Louis, MO).

### Antibodies

Antibodies to gliadin and cerebellar peptides, crude gliadin, MBP, milk, egg, corn and soy proteins were prepared in rabbits according to standards and purified by affinity chromatography on protein A-sepharose by Biosynthesis Inc. (Lewisville, TX). The immunization protocol conformed to the guide for the care and use of laboratory animals published by the National Institutes of Health (NIH Publication No. 85-23) and was approved by the institutional animal care and use committee. Affinity-purified antibodies against gliadin and cerebellar peptides were used for demonstration of cross-reactivity between anti-gliadin with cerebellar peptides and anti-cerebellar with gliadin peptides.

### Detection of Antibodies by ELISA

Antigens and 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  $\mu$ l 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 200  $\mu$ l Tris-buffered saline (TBS) containing 0.05% Tween 20, pH 7.4. The non-specific binding of immunoglobulins was prevented by adding a mixture of 1.5% bovine serum albumin (BSA) and 1.5% gelatin in TBS, and incubated overnight at 4°C. Plates were washed as in the above, and then serum samples diluted 1:200 in 1% HSA in TBS containing 1 mg/ml of IgG FC

room temperature. After washing five times with TBS-Tween buffer, the enzyme reaction was started by adding 100  $\mu$ l of paranitrophenylphosphate (PNPP) in 0.1 ml diethanolamine buffer 1 mg/ml containing 1 mM MgCl<sub>2</sub> and sodium azide pH 9.8. The reaction was stopped 45 min later with 50  $\mu$ l of 1 N NaOH. The optical density (OD) was read at 405 nm by means of a microtiter reader. To detect non-specific binding, several control wells contained all reagents except human serum, or wells were coated with different tissue antigens, such as liver and kidney. Human serum and all other reagents were added and used in each assay.

### ELISA for Demonstration of Cross-reactivity between Gliadin and Cerebellar Peptides

Microtiter plates were coated with either gliadin or cerebellar peptides. Similar to the above ELISA method, peptides were first dissolved in methanol, and then all the coating, incubation and blocking steps were followed. Affinity-purified rabbit anti-gliadin or anti-cerebellar peptides, crude wheat gliadin, anti-brain MBP, anti-milk, anti-egg, anti-corn and anti-soy were added at dilution 1:500 in duplicate to micro-well plates coated with either gliadin or cerebellar peptides and incubated for 2 h at room temperature. Plates were washed and then 100  $\mu$ l of alkaline phosphatase-labeled goat anti-rabbit immunoglobulin (KPI, Gaithersburg, MD) optimal dilution of 1:1000 in 1% HSA-TBS was added to each well. Following incubation, washing, and reaction with PNPP substrate, the OD was read at 405 nm as above. Percent binding of gliadin and cerebellar peptides antibodies to different antigens or peptides were calculated based on the following formula:

$$\begin{aligned} & \% \text{ Binding of rabbit antibody binding to different peptides or antigens} \\ & = 100 - \frac{\text{OD of anti-gliadin binding to gliadin-background OD}}{\text{OD of anti-gliadin binding to tested antigen-background OD}} \times 100 \end{aligned}$$

fragments (to avoid reactivity of specific antibodies with rheumatoid factors) were added to duplicate wells and incubated for 2 h at room temperature. Sera from patients with gluten ataxia with known high titers of IgG, IgM, and IgA against gliadin and cerebellar peptides were used in dilutions of 1:200–1:1600 to construct a standard curve.

To rule out non-specific antibody activities, plates were washed, and then alkaline phosphatase goat anti-human IgG, IgM or IgA F(ab')<sub>2</sub> fragments (KPI, Gaithersburg, Maryland) optimal dilution of 1:400–1:2000 in 1% HSA-TBS was added to each well; plates were incubated for an additional 2 h at

For further analysis of peptides responsible for cross-reaction between gliadin and cerebellar, antibodies were prepared in rabbits against gliadin 8 AA EQVPLVQQ, and cerebellar EDVPLLED 8 AA peptides were reacted in ELISA with non-specific protein (HSA), non-specific peptide (*Chlamydia* HSP-60), and with specific gliadin (18 AA) and cerebellar (22 AA) peptides.

### Dot-blot Analysis

The protein blotting transfer membrane manufactured by Millipore (Bedford, MA) was

used according to their instructions. Briefly, the membrane was first immersed in 100% methanol and then in water for 2 min, followed by equilibration with buffer containing 25 mM/1 Tris, 120 mM/1 glycine and 20% methanol, pH 8.6. Gliadin peptide, crude gliadin, cerebellar peptide, brain proteins, egg yolk, and soy were first dissolved in ethanol and diluted to a final concentration of 2 mg/ml in 10 mM Tris, 150 mM NaCl and 0.1% Tween 20 (TBS-T). Five (5  $\mu$ l) or 10  $\mu$ g of each protein or peptide were added to the membrane and kept at room temperature for 4 h. Blots were blocked for 2 h in TBS-T containing 1.5% BSA and 1.5% gelatin. After incubation for 1 h with affinity-purified rabbit anti-gliadin or anti-cerebellar peptides, it was washed and then enzyme-labeled goat anti-rabbit immunoglobulin was added. Visualization of the antibody-antigen reaction was conducted after additional incubation and washing using ECL blot detection reagents (Amersham Life Science), according to manufacturer's instructions.

#### Coefficients of Intra- and Inter-assay Variation

Coefficients of intra-assay variation were calculated by running 5 samples eight times within a single 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. Coefficients of intra- and inter-assay variations for IgG, IgM, and IgA against gliadin and cerebellar were less than 8%.

#### Absorption of Sera with Specific and Non-specific Peptides

Autistic patients' sera containing high levels of IgG, IgM or IgA antibodies against gliadin and cerebellar peptides (OD in ELISA > 1.2) were used in inhibition studies. In different test tubes, 1 ml of each serum sample was pre-incubated with 100  $\mu$ l containing 100  $\mu$ g of either HSA, crude gliadin, gliadin peptide, anti-brain MBP and cerebellar peptides. After mixing, the tubes were kept for 1 h in a 37°C water bath followed by 4 h incubation at 4°C and then centrifuged at 3000g for 10 min. The supernatant was used for measuring antibody levels and comparing the ELISA OD of unabsorbed and absorbed sera with non-specific and specific antigens.

Similar to the absorption of human serum, 1 ml of affinity-purified rabbit anti-gliadin and cerebellar peptides were absorbed with specific and non-specific peptides and antigens, including HSA, cerebellar peptides, anti-brain MBP, crude gliadin,

gliadin peptide, milk, egg, corn and soy. Sera from different absorption conditions were measured for the level of anti-gliadin and anti-cerebellar antibodies using the ELISA method.

#### Statistical Analysis

Statistics on Software S.O.S. version 2 was used for statistical analysis. Normal distribution was tested by the Kolmogorov-Smirnov one-sample test. One-way analysis of variance was performed by means of ANOVA. For *post hoc* analysis, the large sample Z-test was employed. Analysis of population variances was performed using the F-test. P-values were used to determine levels of significance.

## RESULTS

#### Detection of Antibodies against Gliadin and Cerebellar Peptides

Sera from 50 children with autism and 50 healthy controls were measured for simultaneous presence of IgG, IgM and IgA antibodies against gliadin and cerebellar peptides. Results expressed as OD with mean  $\pm$  standard deviation of the means are summarized in Figs. 1 and 2. The OD for IgG antibody levels against gliadin was obtained with 1:200 dilution ("with 1:200 dilution") of healthy control sera ranging from 0.01–0.74, varying among subjects with mean  $\pm$  SD of  $0.17 \pm 0.16$ . The corresponding IgG OD values from autistic children's sera ranged from 0.07–1.45 with mean  $\pm$  SD of  $0.45 \pm 0.42$ . At a cutoff value of 0.3 OD, levels of IgG anti-gliadin were calculated in controls' and patients' sera and while 8 out of 50 (16%) of controls had high IgG gliadin antibody values, the autistic group showed significant IgG elevation in 42% ( $p < 0.0001$ ) (Fig. 1). Levels of IgM anti-gliadin in healthy controls and children with autism were also significantly higher in patients than in controls (Fig. 1). The mean  $\pm$  SD for controls were  $0.14 \pm 0.13$ , and, for the autistic group,  $0.32 \pm 0.33$  ( $p < 0.0003$ ). When the 0.3 OD cutoff point was used, 8% of controls versus 34% of patients' sera showed elevation in IgM antibody levels. Likewise, IgA antibody levels against gliadin peptide were examined. Individual and mean  $\pm$  SD data depicted in Fig. 1 showed highly significant differences between the controls and patients groups. The mean  $\pm$  SD for IgA antibody levels in controls was  $0.17 \pm 0.17$  and in patients  $0.44 \pm 0.49$ . Percent elevated serum IgA anti-gliadin antibodies at OD values greater than 0.3 were significantly higher in patients with autism (36%) than in controls (14%). Concomitant with the increase of IgG, IgM and IgA against gliadin peptide, we observed a statistically significant increase of anti-cerebellar peptide in most autistic sera.

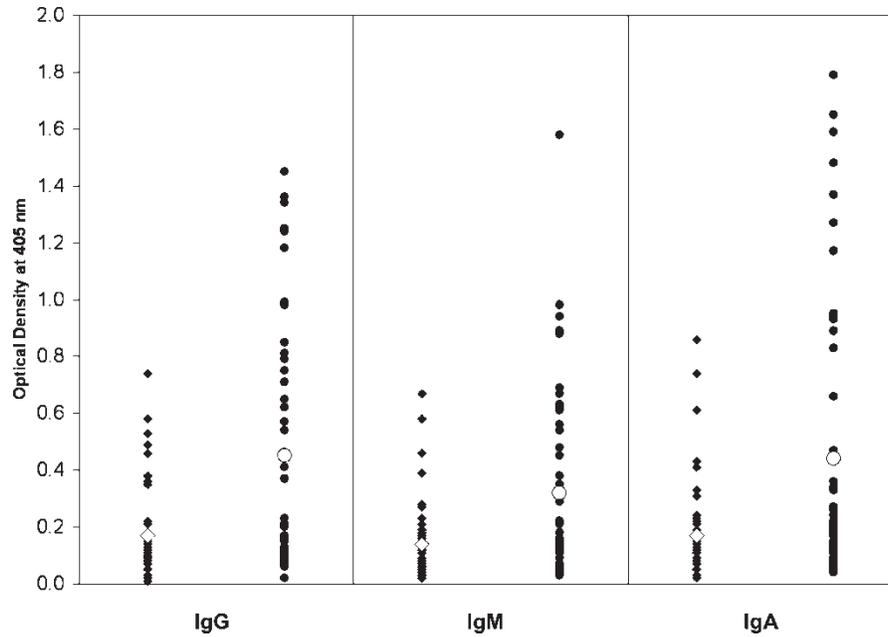


FIGURE 1 Scattergram of serum levels of gliadin antibodies, IgG, IgM, and IgA, in controls  $\blacklozenge$  and in patients with Autism  $\bullet$ , expressed as optical density in ELISA test.

The mean  $\pm$  SD of IgG, IgM and IgA antibodies ranged from  $0.16 \pm 0.11$  to  $0.19 \pm 0.15$  for controls and from  $0.30 \pm 0.32$  to  $0.48 \pm 0.50$  for patients ( $p < 0.003$ ) (Fig. 2).

These values as well as the percent elevation of IgG, IgM, and IgA antibodies against cerebellar peptide were almost identical to antibody levels against gliadin (Fig. 2). At the cutoff of 0.3 OD, the percent elevation for these antibodies in controls were 12–16%, and in patients, 34–38%, an indication of a possible relationship between gliadin and

cerebellar peptides in the production of IgG, IgM, and IgA antibodies.

#### Simultaneous Elevation of Antibodies against Gliadin and Cerebellar Peptides

Calculation of simultaneous elevation of these antibodies in patients' sera was made and is presented in Fig. 3. Data presented in this figure and its analysis showed that 18 out of 21 (85%) of samples positive for gliadin IgG also had significant

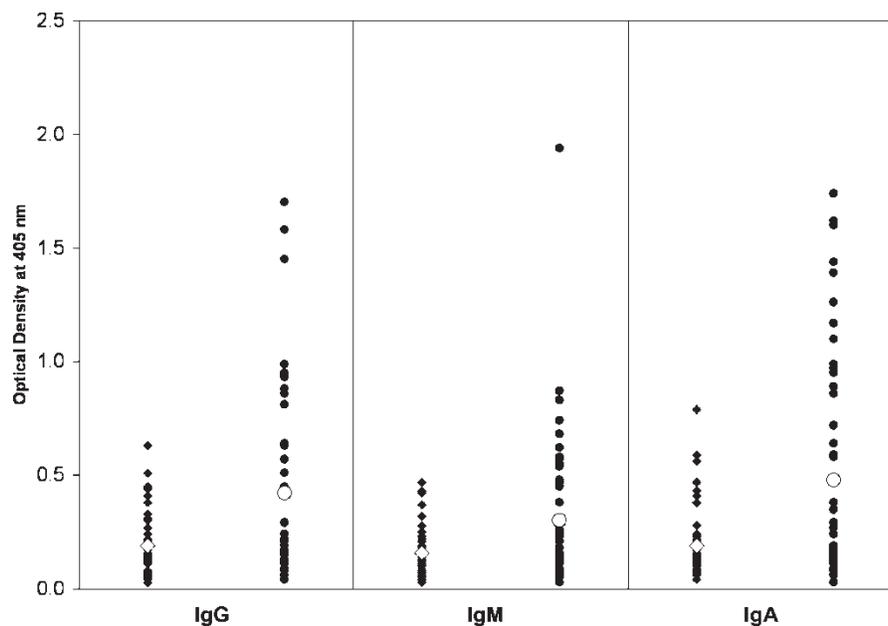


FIGURE 2 Scattergram of serum levels of cerebellar peptide antibodies, IgG, IgM, and IgA, in controls  $\blacklozenge$  and in patients with Autism  $\bullet$ , expressed as optical density in ELISA test.

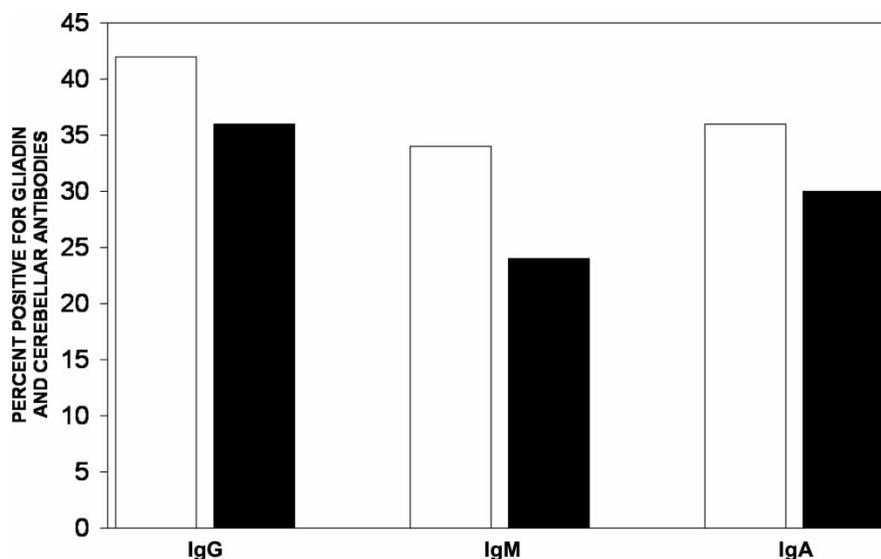


FIGURE 3 Percent positive sera from patients with Autism for IgG, IgM, and IgA antibodies against gliadin □ and cerebellar peptides ■.

elevation in IgG antibody against cerebellar peptides, 3 samples positive for gliadin IgG but negative for cerebellar IgG and 1 specimen positive for cerebellar IgG but negative for gliadin IgG. For IgM antibodies, 12 out of 17 (70%) specimens positive for gliadin had simultaneous elevation in cerebellar antibodies, 5 specimens were positive for gliadin but negative for cerebellar antibodies, and 5 samples were positive for cerebellar but negative for gliadin IgM. In regards to IgA, 15 out of 18 (83%) samples presented simultaneous elevation in antibodies against both gliadin and cerebellar, 3 samples showed positive for cerebellar but negative for gliadin IgM, and, finally, 5 samples were positive for cerebellar IgA but not for gliadin IgA. Moreover, 8 out of 18 (44%) specimens positive for IgA against both gliadin and cerebellar peptides also presented IgM and IgA antibodies against these peptides, simultaneously. These results led us to examine possible antibody specificity and cross-reactivity between gliadin and cerebellar peptides.

#### Demonstration of Cross-reactivity between Gliadin and Cerebellar Peptides

Rabbit anti-gliadin, anti-cerebellar peptides, anti-MBP, anti-milk, anti-egg, anti-soy and anti-corn were reacted with different specific and non-specific peptides and antigens. A degree of antibody binding to different peptides and antigens was measured by indirect ELISA. Rabbit-anti-gliadin peptide binding to gliadin peptide resulted in an OD of 2.65, which was considered as 100% binding (Fig. 4). However, in comparison to anti-gliadin peptide binding to a specific gliadin peptide, binding of anti-crude gliadin, anti-brain MBP, anti-cerebellar,

anti-milk, anti-egg, anti-corn and anti-soy to gliadin peptide-coated plates resulted in ODs of 1.74, 0.22, 0.62, 0.26, 0.18, 0.21 and 0.25 or binding of 65.1, 6.7, 22.2, 8.4, 5.4, 6.5, and 9.2%, respectively (Fig. 4). Using a similar formulation, cerebellar peptides, brain MBP, crude gliadin, gliadin peptide, milk, egg, corn and soy antibodies reaction with cerebellar peptide-coated plates resulted in 100, 11.5, 22.5, 17.4, 55.7, 68, 11.1 and 9.4% binding (Fig. 5).

#### Dot-blot Immuno Detection of Antibody Binding

Affinity-purified rabbit anti-gliadin and cerebellar peptides were examined for their binding capacity to gliadin peptide, crude gliadin, cerebellar peptide, anti-brain MBP, egg yolk and soy antigen. As shown in Fig. 6, strong specific staining was observed using rabbit anti-gliadin with gliadin peptides and crude gliadin or anti-cerebellar reaction with cerebellar peptides or, to a lesser degree, with anti-brain MBP. Cross-reaction between rabbit anti-gliadin and cerebellar peptide or anti-cerebellar peptide with gliadin peptide resulted in less staining of the Dot-Blot. Anti-cerebellar antibodies reacted with egg but not with soy (Fig. 6A), while anti-gliadin antibodies did not react with both egg and soy antigens (Fig. 6B).

#### Finding an Epitope Responsible for Cross-reactivity between Gliadin and Cerebellar Peptides

Rabbit anti-gliadin and cerebellar 8 AA peptide were reacted with HSA, *Chlamydia* HSP-60 peptide and with 18 AA gliadin peptide, as well as with cerebellar 22 AA peptide. Reaction of these antibodies with HSA and unrelated peptide resulted in background OD ranging from 0.05–0.23. But binding of rabbit anti-gliadin 8 AA

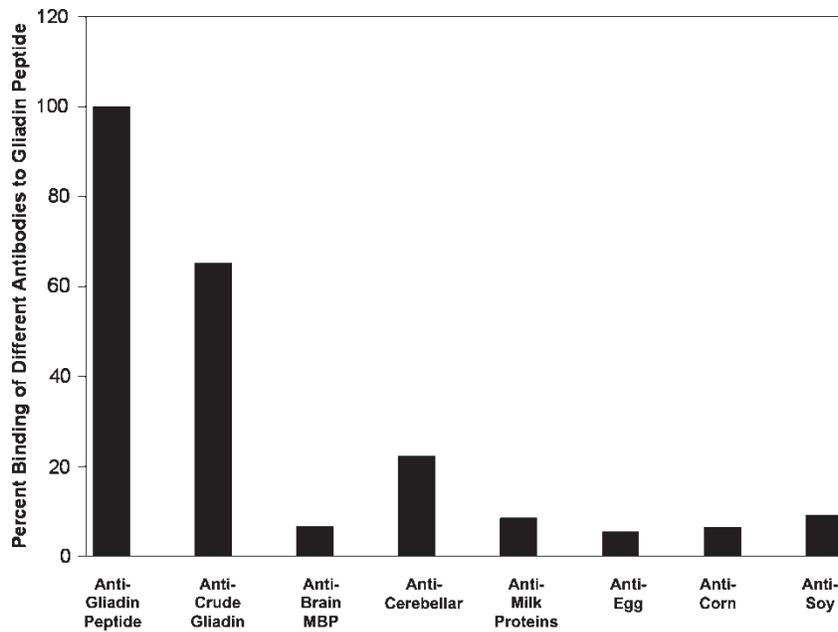


FIGURE 4 Reaction of antibody to: gliadin peptide, crude gliadin, brain, cerebellar, milk, egg, corn, and soy with gliadin peptide coated plates.

peptide resulted in an OD of 2.45 and with cerebellar 22 AA peptide an OD at 1.88. This means that anti-gliadin 8 AA peptide binding to cerebellar was 73.4%. Also, rabbit anti-cerebellar peptide binding to

cerebellar and gliadin peptides resulted in an OD of 2.73 for cerebellar and 1.69 for gliadin. This means anti-cerebellar 8 AA peptide binding to gliadin was 61.9% (Fig. 7).

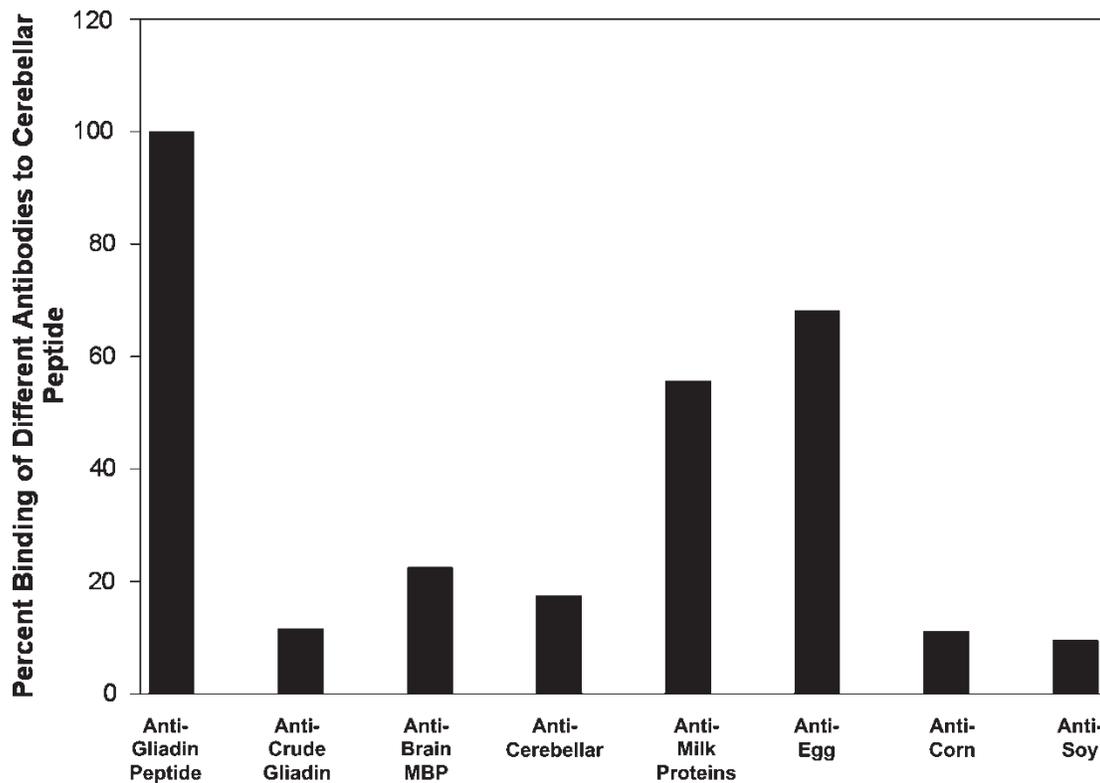


FIGURE 5 Reaction of antibody to: gliadin peptide, crude gliadin, brain, cerebellar, milk, egg, corn, and soy with cerebellar peptide coated plates.

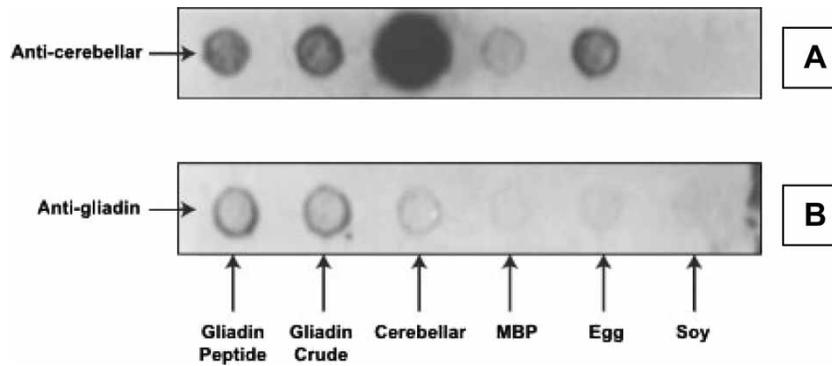


FIGURE 6 Dot-blot immunodetection of anti-cerebellar A and anti-gliadin B binding to gliadin peptide, crude gliadin, cerebellar peptides, brain MBP, egg and soy antigens.

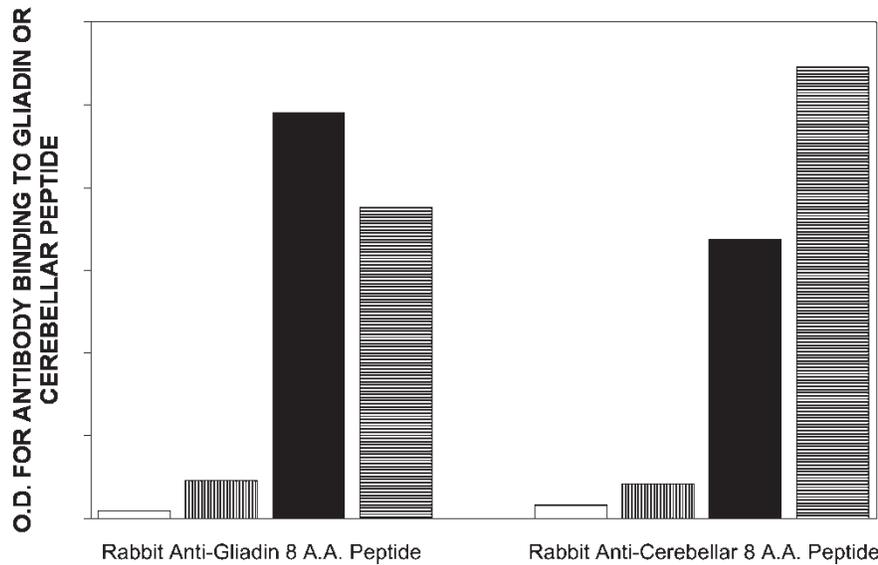


FIGURE 7 Reaction of rabbit anti-gliadin or anti-cerebellar 8 amino acid (AA) peptides with non-specific protein (HSA) □, non-specific peptide ▨, and specific peptides gliadin 18 AA ■ and cerebellar 22 AA ▤ measured by OD in ELISA.

**Absorption of Anti-gliadin and Anti-cerebellar Peptide Positive Human Sera with Non-specific Peptides or Antigens**

To examine whether antibodies against gliadin and cerebellar peptides are specific or cross-reactive, we performed an absorption study with non-specific antigen (HSA) and specific antigens and peptides.

Affinity-purified rabbit anti-gliadin and cerebellar peptides were absorbed using HSA, cerebellar

peptide, MBP, crude gliadin, gliadin peptide, milk, egg, corn and soy proteins. Crude gliadin and gliadin peptides could inhibit anti-gliadin antibodies from 53–63%, followed by cerebellar peptide, which was capable of absorbing between 18–20% of anti-gliadin antibodies (Table I). However, when affinity-purified rabbit anti-cerebellar was absorbed with the above peptides and antigens, the highest percent inhibition was attributed to cerebellar (56–64%),

TABLE I Serum levels of anti-gliadin antibodies expressed as OD in ELISA and percent inhibition after absorption with different specific and non-specific peptides and proteins

Sample	IgG levels and percent inhibition with					IgM levels and percent inhibition with					IgA levels and percent inhibition with				
	HSA	C-Gli	Gli-P	Brain MBP	Cereb	HSA	C-Gli	Gli-P	Brain MBP	Cereb	HSA	C-Gli	Gli-P	Brain MBP	Cereb
Serum 1	1.26	0.92	0.98	0.88	0.92	1.38	1.13	0.81	0.86	0.98	0.96	0.45	0.59	0.78	0.68
% Inhibition	–	27	22.3	30.2	27	–	18.2	41.4	37.7	29	–	53	39.9	19	36.5
Serum 2	1.44	1.08	1.13	1.19	1.11	0.96	0.72	0.64	0.73	0.58	0.88	0.46	0.55	0.73	0.66
% Inhibition	–	25	21.5	17.4	23	–	25	33.4	24	39.6	–	47.7	33	17.1	25

C-Gli = Crude Gliadin; Gli-P = Gliadin peptide; Brain MBP = Brain myelin basic protein; Cereb = Cerebellar peptide.

TABLE II Serum levels of anti-cerebellar antibodies expressed as OD in ELISA and percent inhibition after absorption with different specific and non-specific peptides and proteins

Sample	IgG levels and percent inhibition with					IgM levels and percent inhibition with					IgA levels and percent inhibition with				
	HSA	C-Gli	Gli-P	Brain MBP	Cereb	HSA	C-Gli	Gli-P	Brain MBP	Cereb	HSA	C-Gli	Gli-P	Brain MBP	Cereb
Serum 1	1.8	1.12	1.26	1.21	1.13	1.70	1.62	1.64	1.50	1.28	1.18	0.63	0.89	0.93	0.51
% Inhibition	–	37.8	30	32.8	37.3	–	4.7	3.5	11.8	24.7	–	46.7	24.6	21.2	56.8
Serum 2	1.45	1.09	0.98	1.36	0.87	1.26	1.15	1.09	1.17	0.99	1.38	0.96	0.91	0.91	0.75
% Inhibition	–	24.9	32.4	6.4	40	–	8.7	13.5	7.2	21.4	–	30.5	34.1	34.1	45.7

C-Gli = Crude gliadin; Gli-P = Gliadin peptide; Brain MBP = Brain myelin basic protein; Cereb = Cerebellar peptide.

then egg (53–54%), milk (42–42.5%), crude gliadin (24.9–27.2%), gliadin peptide (18.8–25.5%), MBP (15.8–20%), corn and soy (less than 10%) (Table II).

## DISCUSSION

Since identification and cloning of neuronal protein as the molecular target for autoantibody production in patients with paraneoplastic cerebellar degeneration (Dropcho *et al.*, 1987) and the availability of gluten peptides and demonstration of peptides binding to transglutaminase (Anderson *et al.*, 2000; Vader *et al.*, 2002), we established an ELISA for measuring antibodies against cerebellar and gluten peptides. Using this assay, we investigated the antibody-binding epitopes with similar structure in gliadin and cerebellar peptides by measuring antibodies against these peptides in controls and children with autism. We found that children with autism had significantly higher levels of both gluten and cerebellar peptide antibodies in more than 80% of the cases (Figs. 1 and 2). If gluten antibodies were elevated, cerebellar peptide antibodies were also high. Furthermore, in inhibition studies using specific and non-specific peptides or proteins, we demonstrated that both gluten and cerebellar peptides could absorb the antibodies, against both gluten and cerebellar peptides (Tables I and II). Since antibodies in rabbit and human serum are not pure and may react with many peptides that cross-react with gluten and cerebellar tissue proteins, we prepared affinity-purified rabbit anti-gliadin and anti-cerebellar peptides (Shan *et al.*, 2002; Ellis *et al.*, 2003). In both ELISA and Dot-Blot assay, we showed that antibodies made against gliadin peptides reacted with cerebellar peptides, and antibodies prepared against cerebellar peptides reacted with crude gliadin and gliadin peptides.

For comparison, we also prepared affinity-purified rabbit anti-brain MBP, milk, egg, corn and soy and reacted them against gliadin peptides. Binding of these antibodies to gliadin was less than 10% (Fig. 4). Similar to the above results, while anti-brain MBP, anti-corn and anti-soy binding to cerebellar peptide-coated wells was less than 10%, the binding of rabbit

anti-milk and anti-egg to cerebellar peptide was 55.7% for milk, 68% for egg. These findings indicate a significant antigenic cross-reactivity between egg and milk with cerebellar antigens (Figs. 5 and 6). Since both milk and egg contain phosphoproteins and antibodies against phosphorylcholine (PC) and oxidized low-density lipoprotein (O-LDL) is detected in patients with autoimmune disease and atherosclerosis (Puurunen *et al.*, 1994; George and Shoenfeld, 1997; Shan *et al.*, 2002), we reacted the anti-cerebellar peptide antibodies against PC and O-LDL. Similar to egg, significant immune reaction between anti-cerebellar peptide with PC and O-LDL did occur (data not shown). While investigation of cross-reaction between egg, phospholipids, phosphoproteins and cerebellar peptides require further investigation, several epitopes on  $\alpha$ ,  $\beta$ , and  $\kappa$ -casein, which were identified in cow's milk-allergic patients (Chatchatee *et al.*, 2001) were compared to cerebellar 8 mer peptide. Three out of 8 AA of  $\alpha$ -casein peptide 70–78 (EIVPNSVE) and  $\beta$ -casein peptide 175–182 (LPLPLLQS) were shown to be similar to the cerebellar 15–22 (EDVPLLED) peptide. This epitope similarity between  $\alpha$ ,  $\beta$  casein and cerebellar may explain the reaction of anti-milk with cerebellar peptides.

For further confirmation of the epitope responsible for this cross-reactivity between gliadin and cerebellar peptide, affinity-purified antibody against gliadin 8 mer consisting of EQVPLVQQ and anti-cerebellar 8 mer consisting of EDVPLLED were used in ELISA assay. Anti-gliadin epitopes or anti-cerebellar epitopes reacted almost equally against both gliadin and cerebellar peptides, indicating that this epitope is responsible for cross-reactive antibody production between gliadin and cerebellar peptides (Fig. 7).

These findings clearly confirm the earlier report concerning cross-reaction between gliadin and cerebellar Purkinje cells being responsible for gluten ataxia, but at the molecular level. Similarly to gluten ataxia, we believe that the clinical significance of these antibodies in children with autism should be confirmed by HLA typing and MRI of the brain. In additional studies, we obtained this MRI on 5 children with autism who demonstrated significant elevation in antibodies against gliadin and cerebellar

peptides. Marked right and left cerebellar and vermian atrophy was detected in 3 out of 5 patients. These preliminary results with normal MRI of brainstem and cerebrum but atrophy of the cerebellum should be confirmed and correlated with HLA typing in larger studies.

In our earlier studies we showed that due to molecular mimicry, antibodies to neuron-specific antigens and encephalitogenic proteins from milk (*Chlamydia pneumoniae* and Streptococcus Group (A) were detected in children with autism (Vojdani *et al.*, 2002). We further demonstrated that dietary peptides such as gliadin and casein, bacterial toxins, and xenobiotics binding to lymphocyte receptors and tissue enzymes result in autoimmune reactions in autism (Vojdani *et al.*, 2004). Moreover, we showed that the lymphocyte receptors and tissue enzymes are: dipeptidylpeptidase IV (CD26), dipeptidylpeptidase I (DPPI), aminopeptidase N (CD13), and early lymphocyte activation marker (CD69) (Vojdani *et al.*, 2004).

Based on these findings we proposed that in individuals with predisposing HLA molecules, dietary peptides bind to aminopeptidases and possibly other enzymes, and induce antibodies to dietary peptides and tissue antigens. This autoantibody production and dysfunctional membrane peptidases may result in neuroimmune dysregulation and autoimmunity (Vojdani *et al.*, 2002; 2003; 2004). Collectively, the results of these studies further support dietary intervention, including a gluten-, gliadin- and casein-free diet, in children with autism (Reichelt *et al.*, 1990; Knivsberg *et al.*, 1995; 2001; Reichelt and Knivsberg, 2003).

The antibody production against different tissue enzymes demonstrated in our earlier studies (Vojdani *et al.*, 2003; 2004) further supports the notion of oral peptidase treatment to increase protein and peptide breakdown (Brudnak *et al.*, 2002). And finally, amino acid diets instead of proteins, implemented as early as possible, may prevent the autoimmune reaction to the brain tissue antigens shown in this study. Such a diet may prevent the behavioral effects of dietary proteins and peptides of gliadin and casein and other food antigens. This suggested diet containing amino acids for children with autism warrants further study in double blind fashion.

Identification of gliadin or cerebellar epitope in addition to a gliadin- and casein-free diet, enzyme replacement or amino acid diets may help to design oral tolerance or other immune suppression strategies in patients with autism, celiac disease, gluten ataxia or patients in the early stage of paraneoplastic cerebellar degeneration.

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