

INFECTIONS, TOXIC CHEMICALS AND DIETARY PEPTIDES BINDING TO LYMPHOCYTE RECEPTORS AND TISSUE ENZYMES ARE MAJOR INSTIGATORS OF AUTOIMMUNITY IN AUTISM

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Similar to many complex autoimmune diseases, genetic and environmental factors including diet, infection and xenobiotics play a critical role in the development of autism. In this study, we postulated that infectious agent antigens such as streptokinase, dietary peptides (gliadin and casein) and ethyl mercury (xenobiotic) bind to different lymphocyte receptors and tissue enzyme (DPP IV or CD26). We assessed this hypothesis first by measuring IgG, IgM and IgA antibodies against CD26, CD69, streptokinase (SK), gliadin and casein peptides and against ethyl mercury bound to human serum albumin in patients with autism. A significant percentage of children with autism developed anti-SK, anti-gliadin and casein peptides and anti-ethyl mercury antibodies, concomitant with the appearance of anti-CD26 and anti-CD69 autoantibodies. These antibodies are synthesized as a result of SK, gliadin, casein and ethyl mercury binding to CD26 and CD69, indicating that they are specific. Immune absorption demonstrated that only specific antigens, like CD26, were capable of significantly reducing serum anti-CD26 levels. However, for direct demonstration of SK, gliadin, casein and ethyl mercury to CD26 or CD69, microtiter wells were coated with CD26 or CD69 alone or in combination with SK, gliadin, casein or ethyl mercury and then reacted with enzyme labeled rabbit anti-CD26 or anti-CD69. Adding these molecules to CD26 or CD69 resulted in 28-86% inhibition of CD26 or CD69 binding to anti-CD26 or anti-CD69 antibodies. The highest % binding of these antigens or peptides to CD26 or CD69 was attributed to SK and the lowest to casein peptides. We, therefore, propose that bacterial antigens (SK), dietary peptides (gliadin, casein) and Thimerosal (ethyl mercury) in individuals with pre-disposing HLA molecules, bind to CD26 or CD69 and induce antibodies against these molecules. In conclusion, this study is apparently the first to demonstrate that dietary peptides, bacterial toxins and xenobiotics bind to lymphocyte receptors and/or tissue enzymes, resulting in autoimmune reaction in children with autism.

As with many complex autoimmune diseases, genetic, immune and environmental factors, including diet, toxic chemicals and infections, play critical roles in the development of autism (1,2). Opioid peptides are considered to be part of the etiology of autism, and these peptides are available from a variety of food sources. These

dietary proteins and peptides, including casein, casomorphins, gluten (GLU) 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 (3). In celiac disease

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(CD), a majority of patients who express HLA-DQ2 and/or DQ8 react to a 33-mer peptide and 15 other T-cell stimulatory peptides (4). Transglutaminase is the target of endomysium-specific antibodies in CD patients (5).

A majority of children with autism cannot tolerate wheat and milk proteins or peptides and hence elimination of these peptides from the diets significantly improves their conditions. This clinical finding correlates with laboratory results reported earlier by our group in children with autism (1). We detected IgG, IgM and IgA antibodies against nine specific 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 (1).

Autoantibodies to nervous system antigens are detected in populations exposed to toxic, environmental or occupational chemicals. Titers of antibodies against neurofilaments and myelin basic protein (MBP) correlated significantly with blood lead or urinary mercury, the typical indices of toxic exposure. Moreover, levels of these antibodies correlated with sensorimotor deficits and these antibodies are known to interfere with neuromuscular function (6).

Edelson and Cantor (7) demonstrated a body burden of neurotoxicants in more than 90% of autistic children. These authors presented evidence for genetic and environmental aspects of a proposed process involving immune system injury and autoimmune responses secondary to exposure to immunotoxins.

For a chemical compound to lead to an autoimmune response, it is generally thought that the compound must first become covalently bound to a carrier protein (8). Immune reactions to drugs or their metabolites can develop when a hapten carrier complex interacts with gut-associated lymphoid tissues (GALT) (9). If covalent adducts of drugs or other chemical compounds are formed in GALT, it seems reasonable that they may lead to immune responses and chemically-induced Type I- Type IV allergic reactions (10).

Many infectious agents including measles, Rubella virus and Cytomegalovirus have long been suspected as etiologic factors in autism (11-13). In fact, by reviewing the scientific literature, we found that over 60 different microbial peptides

have been reported to cross-react with human brain tissue and MBP that induce T-cell responses but can also induce experimental autoimmune encephalomyelitis (14-16). Using the recent observation that maternal infection increases risk for schizophrenia and autism in offspring, respiratory infection of pregnant mice (both BALB/c and C57BL/6 strains) with the human influenza virus have resulted in offspring that displayed highly abnormal behavioral responses as adults. As in schizophrenia and autism, these offspring displayed deficits in prepulse inhibition (PPI) in the acoustic startle response. It was concluded that abnormal levels of cytokine production that interfere with neuroimmuno-communications were responsible for abnormal development of the brain (17,18).

In addition, antigens from infectious agents may react or interact with lymphocyte receptors that have digestive function, in the gastrointestinal tract. Such a receptor protein that possesses inherent enzymatic activity is dipeptidylpeptidase IV (DPP IV) or CD26. DPP IV is a serine aminopeptidase with a capacity of cleaving peptides at locations containing amino-terminal dipeptides that have either L-alanine or L-proline at position 2 (19). In addition to enzymatic activity, CD26 concurrently participates in a variety of cellular functions. CD26 has been detected in various tissues and cell types including cells involved in the immune system. Moreover, different T-cell subsets vary in their level of CD26 expression (20). With an upregulation of CD26 molecule, T-cells undergo activation and regulate IL-2 production and receptor expression (21, 22). Due to a key role that the membrane-bound DPP IV plays in T-cell mediated immune responses and cytokine production, this enzyme has been analyzed in several autoimmune diseases, such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) (23, 24). In fact, in patients with autoimmune disease, it was shown that the bacterial protein streptokinase (SK) could bind to DPP IV and induce significant levels of anti-SK and anti-DPP IV antibody production (25).

CD69 is an additional lymphocyte surface marker involved in autoimmune disease (26). CD69 contributes to deletion of autoreactive lymphocytes by inducing apoptosis; thus, abnormal expression of this molecule could be involved in the pathogenesis of autoimmune diseases. In patients

with rheumatoid arthritis (RA), CD69 is expressed on surfaces of T-cells in synovial membranes but not on surfaces of circulating peripheral blood lymphocytes (PBLs). Level of CD69 expression on the synovial T-cells in RA is correlated with disease activity (27, 28). In a different study, autoantibodies to CD69 were reported in the sera of 38.3% of RA patients, 14.5% of SLE, and 4 % of Behcet's disease (29).

Based on the above observations and since so little is known about the range of intestinal immune functions that are shaped by dietary proteins, xenobiotics and infectious agents in autism, we decided to test the hypothesis that infectious agent antigens, dietary peptides and haptenic chemicals may bind to DPP IV (CD 26) and CD69, resulting in autoantibody production and modulation and expression of immune and inflammatory reaction 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 by different clinicians to our laboratory for immunological examination. Clinical diagnosis of autism was made according to the DSM-III-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 conditions that may contribute to abnormal development.

For comparison, serum samples from 50 healthy matched controls with negative anti-nuclear antibody 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 this manuscript without disclosure of their identity in the publication.

Peptides, Proteins and Reagents

Gliadin peptides
 Q Q L P Q P Q Q P Q Q S F P Q Q Q P F ,
 L Q L Q P F P Q P Q L P Y P Q P Q L P Y -

P Q P Q L P Y P Q P Q P F ,
 QQPQQFZPQQPYQPXQPQLGQQQPFPQ,
 gluteomorphin QGQPGYYPTSPQQPGQEQ,
 casomorphin QTQSLVYPPFGPIPNLSP, b-casein
 LHLPLLLQSWMHQPHQPL and CD69 antibody
 binding epitope MECEKNLYWICNKPYK were
 synthesized by Bio-Synthesis Inc. (Lewisville,
 TX). Dipeptidylpeptidase IV (CD26), streptokinase
 (SK), lipopolysaccharide (LPS), human serum
 albumin (HSA), mercury [(o-carboxyphenyl) Thio]
 ethyl mercury sodium salt (Thimerosal) were
 purchased from Sigma (St. Louis, MO).

Binding of Thimerosal to Human Serum Albumin

For this preparation, 100 mg of human serum albumin (HSA) was dissolved in 9 ml of buffer solution containing potassium chloride and sodium borate 0.05 ml/liter and pH was adjusted to 9.4 with 0.1 N NaOH. Then 25 mg of Thimerosal or sodium merthiolate was dissolved in one ml of H₂O and added dropwise to the HSA solution while stirring over a period of one hour. The reaction mixture was stirred overnight, dialyzed against 0.1 M PBS using tubing with a cutoff of 8000 Dalton. Conjugation of ethyl mercury to HSA was confirmed by SDS gel electrophoresis (shift in the HSA band). In addition spectrograph analysis of the conjugate was undertaken. There was a marked increase in absorption from 230 to 260 nm, which indicated that ethyl mercury became covalently linked to the protein carrier (HSA).

Antibodies

Antibodies to CD26 and CD69 were prepared in rabbits according to standard protocols (56) by Cocalico Biological Inc. (Reamston, PA). These polyclonal antibodies were purified by affinity chromatography on protein A-sepharose first and then labeled with horseradish peroxidase (57).

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was used for testing antibodies against different antigens and peptides in the sera of patients with autism and with control patients. 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 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 200 µl 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, then incubating for 2 hrs at room temperature, and then overnight at 4°C. For testing antibodies against ethyl mercury, microplates were coated with 5 µg/well of ethyl mercury HSA conjugate or HSA alone. Plates were incubated at 37°C for 2 hours and then overnight at 4°C. After repeated washing and addition of BSA, gelatin plates were used for determination of antibody levels. Plates were washed as in the above and then serum samples diluted 1:200 in 1% HSA in TBS containing 1mg/ml of IgG Fc fragments (to avoid reactivity of specific antibodies with rheumatoid factors) were added to duplicate wells of HSA alone or ethyl mercury HSA conjugate and incubated for 2 hrs at room temperature. Sera from patients with autoimmune disorders and known high titers of IgG, IgM and IgA against CD26, CD69, gliadin, casein peptides or SK were used in dilutions of 1:200 – 1:1600 to construct a standard curve to rule out non-specific antibody activities. Using washed plates, phosphatase goat anti-human IgG, IgM or IgA F(ab')₂ fragments (KPI, Gaithersburg, Maryland) at optimal dilution of 1:400 – 1:2000 in 1% HSA-TBS was added to each well. Plates were incubated for an additional 2 hrs at room temperature. After washing five times with TBS-Tween buffer, the enzyme reaction was started by adding 100 µl paranitrophenylphosphate in 0.1 ml diethanolamine buffer 1mg/ml containing 1 M MgCl₂ and sodium azide at pH 9.8. The reaction was stopped 45 minutes later with 50 µl of 1 N NaOH. The optical density (O.D.) 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 and other reagents were used.

Coefficients of Intra- and Interassay Variations

Coefficients of intra-assay variation were calculated by running five samples eight times within a single assay. Coefficients of interassay variation were determined by measuring the same samples in six consecutive assays. This replicate testing established the validity of ELISA assays,

determined appropriate dilutions with minimal background and detected serum IgG, IgM, and IgA against different antigens. Coefficients of intra-assay and interassay variations for IgG, IgM, and IgA against CD26, CD69, gliadin, casein peptides, ethyl mercury and SK were less than 12%.

Calculation of Optimal Serum Dilution

Two sera from healthy controls with low levels of antibodies against CD26, CD69, gliadin, casein peptides, and SK, two sera from patients with autoimmune disease, and two sera from autistic children with known high titers of antibodies were used to construct standard control curves. These sera were diluted 1:25 to 1:800. At dilutions of 1:50 – 1:400 the standard curves for samples with autoimmune disease or autism were linear (O.D. 0.4 – 2.2) and antibodies were not detected against these antigens in two tested healthy control sera (O.D. <0.3) or 2 S.D. of the mean. Hence, antibody detection in sera from children with autism was performed at 1:200 dilutions in appropriate buffer.

Possible Binding of SK, Gliadin, Casein Peptides and Ethyl Mercury to CD26 and CD69

Since interaction of CD26 with SK has been shown to be associated with SK and anti-CD26 autoantibodies (36), we sought out the possible binding of other peptides and mercury to CD26 and CD69. A series of ELISA experiments was performed to establish the binding specificity of peptides, SK and mercury to CD26 and CD69. The plates were coated with CD26 or CD69 first and then with 1% BSA or HSA for inhibition of non-specific binding to microplate wells. Gliadin, casein peptides, SK and ethyl mercury were then added. Plates were incubated for 1 hr at 37°C and washed five times for removal of unbound competing antigens. Then, for demonstration of casein, gliadin, SK and mercury binding to CD26 and CD69, purified enzyme labeled rabbit anti-CD26 and CD69 were added to different wells. After proper incubation and washing, binding of these peptides and proteins to CD26 and CD69 was measured by addition of peroxidase substrate and measurement of color development at 492 nm. Binding of dietary peptides, SK and ethyl mercury to CD26 and CD69 was demonstrated by % inhibition in binding of CD26 or CD69 to anti-CD26 and anti-CD69

respectively. This % inhibition of CD26 or CD69 to its specific antibody and different peptides, SK or mercury was calculated by using the following formula:

$$\begin{aligned} & \text{\% binding of} \\ & \text{gliadin to CD26} \\ & \text{and inhibition of} \\ & \text{anti-CD26 binding} \\ & \text{to CD26} \\ & = 100 - \frac{\text{O.D. after addition of peptide} - \text{background O.D.}}{\text{O.D. of CD26 + anti-CD26} - \text{background O.D.}} (100) \end{aligned}$$

Example:

O.D. for CD26 + anti-CD26 = 2.16

O.D. for CD26 + gliadin and anti-CD26 = 1.19

O.D. for background = 0.28

$$\text{\% binding of gliadin to CD26} = 100 - \frac{1.19 - 0.28}{2.16 - 0.28} (100) = 100 - \frac{0.91}{1.88} (100) = 100 - 48 = 52$$

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 (ANOVA) 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

Anti-CD26 and CD69

We investigated whether autoantibodies to CD26 exist in the sera of patients with autism by ELISA using highly purified CD26. As shown in Fig. 1, at a cutoff of 0.3 O.D. or 2 S.D. above the mean and sera dilution of 1:100, IgG, IgM and IgA isotype anti-CD26 autoantibodies were detected in 24 of 50 (48%) for IgG, 20 of 50 (40%) for IgM, and 22 of 50 (44%) for IgA in patient serum samples. In contrast, autoantibodies to CD26 were detected in 14%, 10% and 8% of healthy donors. The mean \pm S.D. for these antibodies in controls ranged from 0.13 ± 0.13 to 0.15 ± 0.14 and in patients, significantly elevated and ranged from 0.34 ± 0.27 to 0.41 ± 0.39 with p-value being highly significant ($p < 0.0001$). Each serum sample was also tested for the presence of anti-CD69 autoantibodies by using the specific CD69 epitope.

Analysis of anti-CD69 IgG, IgM and IgA levels in controls and patients with autism showed significant differences between antibody values and % elevation of these antibodies against CD69 (Fig. 1). The mean \pm S.D. of O.D. values in controls ranged from 0.09 ± 0.09 to 0.11 ± 0.09 and for patients, from 0.27 ± 0.21 to 0.45 ± 0.44 ($p < 0.0001$). Similar to antibodies against CD26, these values for CD69 were the highest for IgA, and then for IgG or IgM levels.

Eight of 50 (16%) or 7 of 50 (14%) of patients showed simultaneous elevation in IgG, IgM and IgA antibodies against CD26 and CD69. This simultaneous elevation of antibodies was not detected in sera of any of the healthy controls (Fig. 1).

Antibodies Against Gluten and Casein Peptides

Having shown that a subpopulation of children with autism exhibited antibodies against CD26 and CD69, we then set out to show that these antibodies are generated in response to dietary peptides, infectious agent antigens (SK) and ethyl mercury. Using similar ELISA methods, the results of IgG, IgM and IgA antibodies against gluten peptides are shown in Fig 2. The O.D. for IgG antibody values with 1:100 dilutions of healthy control sera ranged from 0.01 – 0.84, varying among subjects. The mean \pm S.D. values were 0.17 ± 0.17 . The corresponding IgG O.D. values from autistic children's sera ranged from 0.03 – 1.18 with a mean \pm S.D. of 0.34 ± 0.29 . At a cutoff value of 0.3 O.D., levels of IgG antibody against gliadin peptides were calculated and found that while 6 of 50 (12%) of controls had high IgG values, patients showed IgG elevation in 22 or 44% ($p < 0.0001$). Levels of IgM and IgA anti-gliadin peptides in controls and children with autism are also shown in Fig. 2. Similar to IgG, at 2 S.D. above the mean, these antibodies were significantly higher in patients, 36% for IgM and 46% for IgA, while in controls, 10% were elevated for IgM and 12% for IgA ($p < 0.0001$).

In conjunction with the increase of IgG, IgM and IgA antibodies against gliadin peptides, we observed a statistically significant increase of anti-casein peptide antibodies in patients' sera. The mean \pm S.D. of antibodies against casein peptide for controls was 0.16 ± 0.17 for IgG, 0.16 ± 0.13 for IgM and 0.14 ± 0.09 for IgA antibodies.

Specimen #	IgG						IgM						IgA					
	CD26	CD69	Gli	CA	SK	Hg	CD26	CD69	Gli	CA	SK	Hg	CD26	CD69	Gli	CA	SK	Hg
1	-	-	+	+	+	+	-	+	-	-	+	-	+	+	+	+	+	-
2	⊕	⊕	⊕	⊕	⊕	-	-	-	-	+	-	-	⊕	⊕	⊕	⊕	⊕	+
3	-	-	-	-	-	-	+	+	-	+	+	+	⊕	⊕	⊕	-	+	-
4	-	⊕	⊕	⊕	⊕	-	-	⊕	⊕	⊕	⊕	-	+	⊕	⊕	⊕	⊕	-
5	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
6	-	-	-	-	-	-	+	+	-	-	+	-	⊕	⊕	⊕	⊕	⊕	-
7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	+	-	+	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
9	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	+	-	-	-	+	+	-	-	-	-	+	-
13	⊕	⊕	⊕	⊕	⊕	-	⊕	⊕	⊕	⊕	⊕	+	⊕	⊕	⊕	⊕	⊕	-
14	-	-	-	-	-	-	-	-	+	+	-	-	⊕	⊕	⊕	⊕	-	-
15	+	-	-	+	+	-	-	-	+	+	-	-	⊕	⊕	⊕	⊕	-	-
16	⊕	-	⊕	⊕	⊕	-	+	-	-	+	+	-	⊕	⊕	⊕	⊕	-	-
17	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
19	⊕	⊕	⊕	⊕	⊕	⊕	-	-	-	-	+	-	+	-	+	-	-	-
20	-	-	-	+	+	-	+	-	+	-	+	-	-	-	-	-	⊕	-
21	+	-	-	-	-	+	+	-	+	-	-	-	⊕	⊕	⊕	-	⊕	-
22	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-
23	⊕	⊕	⊕	⊕	⊕	⊕	-	-	-	-	+	+	-	-	-	+	-	-
24	+	+	+	-	-	-	-	-	-	-	+	-	+	+	-	-	-	-
25	⊕	-	⊕	⊕	⊕	⊕	-	-	-	-	-	-	-	-	-	-	-	-
26	-	+	-	+	-	-	+	+	-	+	+	-	-	-	-	-	-	+
27	+	+	+	-	-	+	+	-	-	+	-	-	-	-	-	-	-	-
28	-	-	-	-	-	+	⊕	⊕	⊕	⊕	+	-	+	+	-	-	-	-
29	⊕	⊕	⊕	⊕	-	+	-	-	-	+	+	-	⊕	⊕	⊕	⊕	-	-
30	+	+	+	-	-	-	-	-	+	+	-	-	-	-	+	+	-	-
31	⊕	⊕	⊕	⊕	-	-	+	+	-	-	-	-	+	+	-	-	-	-
32	⊕	⊕	⊕	⊕	⊕	-	⊕	-	⊕	⊕	⊕	⊕	+	+	+	+	+	-
33	+	-	-	-	-	-	+	-	-	-	-	-	+	+	+	-	-	-
34	⊕	⊕	⊕	⊕	-	+	-	-	+	+	+	+	-	+	+	+	-	+
35	-	-	-	-	-	+	-	-	-	-	+	+	-	-	-	-	+	+
36	-	+	-	-	-	-	+	-	-	-	-	-	+	+	-	-	-	-
37	-	-	-	-	-	-	-	+	+	-	-	-	-	-	+	+	-	-
38	⊕	⊕	⊕	⊕	-	-	+	-	-	-	-	-	+	+	-	-	-	-
39	-	-	-	-	+	-	-	-	+	+	-	+	-	-	-	-	-	-
40	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
41	-	-	+	+	-	-	+	-	-	-	-	-	⊕	⊕	⊕	⊕	-	-
42	+	-	+	-	+	+	+	-	+	+	+	+	⊕	⊕	⊕	⊕	⊕	+
43	-	+	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-
44	+	-	-	+	-	-	-	-	+	+	-	-	+	+	-	-	-	-
45	+	-	-	-	+	+	+	+	-	-	-	-	+	-	+	+	+	-
46	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
47	+	-	+	+	-	-	+	+	-	-	-	-	+	+	-	-	-	-
48	-	-	+	-	-	+	⊕	⊕	⊕	-	⊕	⊕	-	-	+	+	-	-
49	-	+	+	+	-	-	-	-	+	-	+	+	-	-	+	+	-	-
50	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

- = negative + = positive ⊕ = multiple positive results

Tab. I. Simultaneous detection of antibodies against CD26, CD69, gliadin (Gli), casein peptides (CA) SK, and ethyl mercury (Hg) in children with autism

The corresponding values in patients with autism were 0.39 ± 0.38 for IgG, 0.40 ± 0.41 for IgM, and the highest value, 0.52 ± 0.52 for IgA antibodies (Fig. 2). Percent elevation of IgG, IgM and IgA antibodies in controls were 10%, 8% and 8%, while 42%, 34% and 42% of patients' sera at the cutoff of 0.3 O.D. showed IgG, IgM or IgA antibodies against casein peptides.

Anti-Streptokinase (SK) Antibody Levels

Analysis of anti-SK IgG, IgM and IgA levels (Fig. 3) shows that while only one or two out of 50

control specimens (2-4%) had elevated antibodies, a significant percent of patients (18%, 48% and 24%) demonstrated IgG, IgM or IgA elevation. The mean \pm S.D. of anti-SK antibodies was significantly elevated in patients over controls with IgA and IgM ($p < 0.0001$) and for IgG ($p < 0.008$) (Fig. 3).

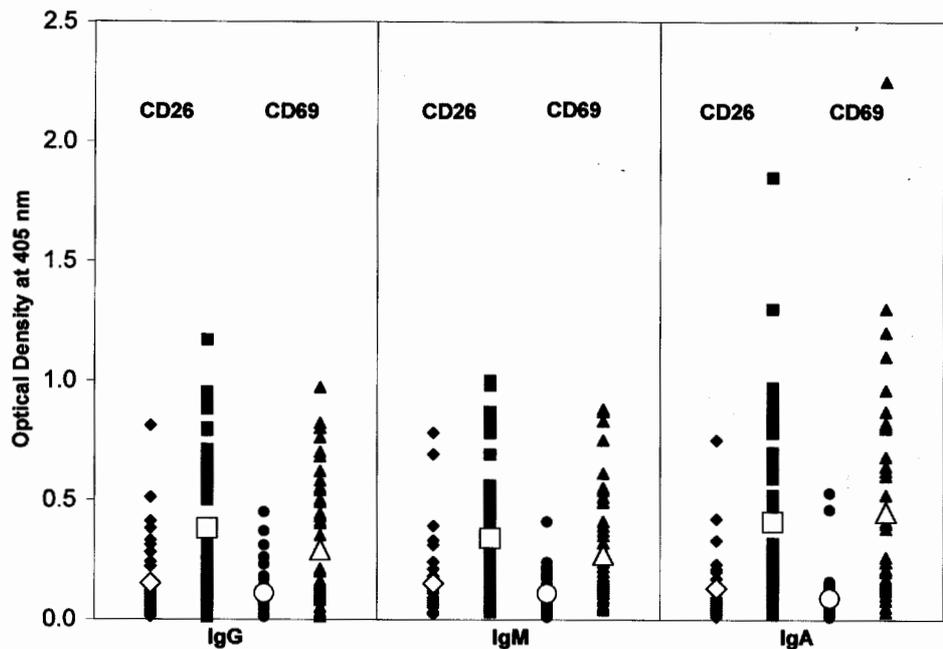
Anti-Ethyl Mercury Antibody Level

Similar to the above determination at a cutoff of 0.30 O.D., levels of IgG, IgM and IgA antibodies against ethyl mercury were calculated in controls

Microwell Coated With:	Peroxidase Labeled Rabbit Anti-:	ELISA O.D. at 492 nm	% Binding of Gliadin, Casein, Streptokinase, or Mercury to CD26 and CD69
BSA + HSA	CD26	0.28	B.G.
BSA + HSA	CD69	0.24	B.G.
BSA + HSA	Gliadin	0.31	B.G.
BSA + HSA	Casein	0.29	B.G.
BSA + HSA	SK	0.33	B.G.
CD26 + BSA + HSA	CD26	2.16	-
CD26 + Gliadin	CD26	1.19	52
CD26 + Casein	CD26	1.34	44
CD26 + SK	CD26	0.72	77
CD26 + Ethyl Mercury	CD26	0.79	73
CD69 + BSA + HSA	CD69	1.92	-
CD69 + Gliadin	CD69	0.97	57
CD69 + Casein	CD69	1.45	28
CD69 + SK	CD69	0.48	86
CD69 + Ethyl Mercury	CD69	0.56	81

Tab. II. Inhibition of anti-CD26 and anti CD-69 by gliadin, casein, streptokinase and ethyl mercury which reflects the binding of these molecules to CD26 and CD69 coated plates. BG= background

Fig. 1. Scattergram of serum titer of IgG, IgM and IgA antibodies against Dipeptidyl peptidase IV (CD26) in healthy control subjects \blacklozenge and autistic patients \blacksquare and CD69 in healthy control subjects \bullet and autistic patients \blacktriangle expressed as optical density in ELISA test.



and patients' sera and found that while one or two out of 50 (2%-4%) of controls had high IgG values, the patients' group showed IgG elevation in 28% and IgM elevation in 30%. In regards to IgA elevation against mercury, none of the controls and only 5 of 50 patients (10%) had increased antibody levels (Fig. 3). Comparison of these antibody values in controls and patients resulted in p values < 0.0001 for IgG and IgM but < 0.004

for IgA. For this measurement, since ethyl mercury was conjugated to HSA, the O.D. of corresponding wells coated with HSA alone were subtracted from the O.D.s of ethyl mercury bound to HSA-coated wells.

Simultaneous Detection of Antibodies Against One or All Tested Antigens

For examination of possible involvement of

Fig. 2. Scattergram of serum titer of IgG, IgM and IgA antibodies against Gliadin peptides in healthy control subjects \blacklozenge and autistic patients \blacksquare and CD69 in healthy control subjects \bullet and autistic patients \blacktriangle expressed as optical density in ELISA test.

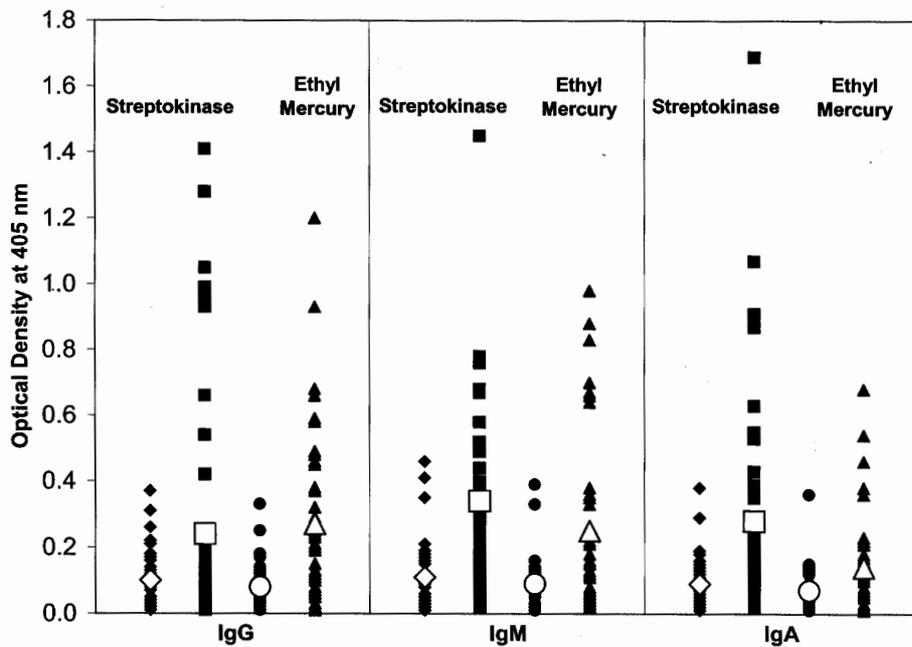
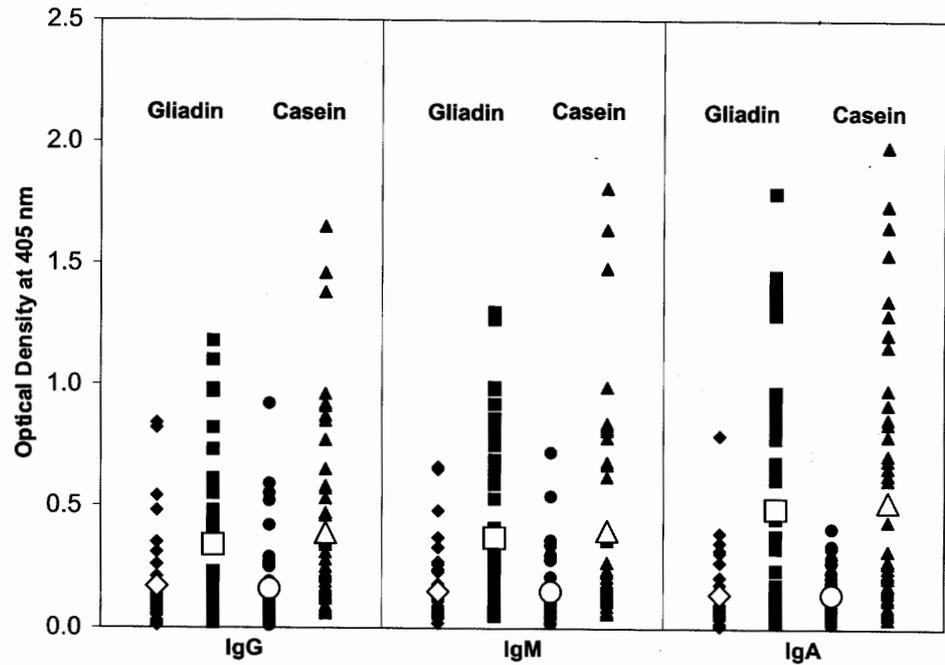


Fig. 3. Scattergram of serum titer of IgG, IgM and IgA antibodies against streptokinase in healthy control subjects \blacklozenge and autistic patients \blacksquare and CD69 in healthy control subjects \bullet and autistic patients \blacktriangle expressed as optical density in ELISA test.

gliadin, casein peptides, SK and ethyl mercury in the production of autoantibodies against CD26, CD69, calculation of simultaneous elevation in these antibodies in patients' sera were made and summarized in Table I. Analysis of data showed that while some patients had elevated IgG, IgM or IgA against one or two out of six tested antigens, different subgroups showed simultaneous elevation

in IgG, IgM or IgA antibodies not only against CD26 or CD69, but also against gliadin (Gli), casein (CA) peptides, SK, ethyl mercury (Hg) or a combination of CD26 or CD69 + Gli + CA, or Gli + CA + SK + Hg (Table I). For example, patients #2, 4, 13, 16, 19, 23, 24, 29, 31, 32, 34 and 38 demonstrated IgG antibody elevation not only against CD69 but also against gliadin, casein, SK

or their combinations. Similarly, patients #4, 13, 28, 32 and 48 demonstrated IgM antibody elevation against CD26 or CD69 in combinations with gliadin, casein and SK; patients #1, 2, 3, 4, 6, 13, 14, 16, 21, 24, 29, 33, 41 and 42 demonstrated IgA antibodies against CD26, CD69 and gliadin, casein or SK. In patient #13, with the exclusion of IgG and IgA against mercury, all other measurements were highly elevated. This suggests that the patient not only reacted against CD26, CD69 but also against gliadin, casein, SK and mercury as well (Table I). These antibodies are not gender specific since patients # 2, 4, 13, 16, 25, 31 and 32 were male and patients # 19, 23, 29, 34 and 35 were female.

Binding of Gliadin, Casein Peptides, SK and Ethyl Mercury to CD26 and CD69

For demonstration of gliadin, casein, SK and mercury binding to CD26 and CD69, polyclonal antibodies raised against CD26 or CD69 and labeled with enzyme were added to different wells coated either with CD26 or CD69. Rabbit anti-CD26 reacted only with wells coated with CD26 that resulted in an ELISA O.D. of 2.16. Adding rabbit anti-CD69 to wells coated with CD69 gave an ELISA O.D. of 1.92. Adding gliadin, casein, SK and mercury to wells coated with either CD69 or CD26 caused 52%, 44%, 77% and 73% inhibition in binding of anti-CD26 to CD26. Similarly, in wells coated with CD69, gliadin, casein, SK and mercury caused 57%, 28%, 86% and 81% inhibition in Anti-CD69 to CD69 coated wells. This reduction in ELISA O.D.s or anti-CD26 or anti-CD69 binding to CD26 or CD69, is an indication of gliadin, casein, SK and mercury binding to CD26 and CD69 (Table II).

DISCUSSION

This is the first analysis that shows autoantibodies to CD26 and CD69 that have been generated in patients with autism. As in an earlier study of patients with systemic autoimmune diseases (25), we demonstrated that most of the anti-CD69 autoantibodies recognized one distinct epitope located at the C-terminus of the CD69 molecule.

Recently, we have suggested that autoimmunity is a pathogenic factor in autism (1). This possible autoimmunity etiology in autism has been supported by detection of antibodies to nine different neuron-

specific antigens and their cross-reactive peptides from milk, *C. pneumoniae* and Streptococcus group A (1, 14, 30). In this manuscript, we suggest a role for dietary proteins (milk and wheat), bacterial toxins (bacterial enterotoxins) and metals, such as mercury, in the pathogenesis of autistic behavior. Based on these findings and earlier reports concerning elevation of anti-CD26 and anti-SK antibodies in RA, SLE and in patients with myocardial infarction after fibrinolytic therapy with SK (21), we examined the possible existence of CD26 and CD69 autoantibodies in blood samples of children with autism.

These antibodies against CD26 and CD69 appear to be specific as a result of our absorption studies. CD26 and CD69 were capable of significantly reducing (up to 63%) the levels of IgG, IgM and IgA anti-DPP IV antibodies from highly positive sera (data not shown). CD69 reduced levels of CD26 antibodies to a less significant degree (up to 29%). These findings lead us to hypothesize that infectious agents, superantigens, including SK, dietary proteins such as gliadin and casein peptides and xenobiotics like ethyl mercury or thimerosal in individuals with predisposing HLA molecules, bind to different enzymes or cell surface receptors and induce autoantibodies against peptides, haptenic chemicals and tissue antigens.

To test this hypothesis, and based on gliadin peptide binding to transglutaminase in brush border of celiac disease (5), we measured anti-gliadin and casein peptides, SK and anti-mercury antibodies in blood samples of children with autism. A significant number of sera samples from children with autism exhibited anti-gliadin and casein peptide and anti-SK and mercury antibodies. We therefore asked the question, what percent of sera from children with autism that are positive for IgA, IgG and IgM antibodies against CD26 and CD69 have concomitant elevation in gliadin casein, SK and mercury antibodies? As shown in Table I, a majority of patients with autism who had elevated CD26 and/or CD69 antibodies also had anti-gliadin, anti-casein, SK and mercury antibodies. These antibodies are not gender specific since they were simultaneously detected in both male and female patients. Similar to SK binding to CD26 shown earlier (21), we postulated that gliadin, casein, SK and mercury binding to CD26 and CD69 is

responsible for anti-gliadin, anti-casein, anti-SK, mercury, CD26 and CD69 autoantibodies production. Incubation of DPP IV plates with a single concentration of either IgA or IgG anti-DPP IV autoantibody in the presence of SK peptide, inhibited binding of both classes of autoantibodies. From these results, SK has been suggested to promote development of DPP IV autoantibodies (21).

We used a similar approach not only by using SK but gliadin, casein, and mercury for further demonstration of binding of these molecules to CD26 and CD69. Using rabbit anti-CD26 and anti-CD69, a series of ELISA experiments were performed to demonstrate binding specificity of SK, gliadin, casein and mercury to CD26 and CD69 coated plates. Results showed that adding HSA to CD26-coated wells and reacting them with rabbit anti-CD26 resulted in non-significant changes in antibody levels. But when HSA was replaced with gliadin, casein, SK or ethyl mercury and rabbit anti-CD26 was added, the O.D.s were reduced, significantly (Table II).

Similar results were obtained with gliadin, casein, SK and ethyl mercury inhibition of anti-CD69 binding to CD69-coated wells. This suggests that binding of gliadin, casein, SK and ethyl mercury to CD26 or CD69 resulted in 44-81% inhibition of rabbit anti-CD69 to CD69 and anti-CD26 to CD69 molecules. In measuring binding of different molecules that were tested against CD26 and CD69, the highest levels were those detected first with SK (77-86%), then ethyl mercury (73-81%), then gliadin (52-57%) and finally, casein, being the lowest (28-44%) (Table II). To demonstrate possible binding of ethyl mercury to DPP IV-coated plates, similar to adding SK, gliadin and casein to DPP IV, CD26 and CD69, ethyl mercury was added to DPP IV-coated wells and then reacted with rabbit anti-CD26 and CD69. Binding of mercury to CD26 and CD69 was demonstrated by a significant inhibition of anti-CD26 and CD69 binding to CD26 or CD69 molecules.

This reactivity of antibodies against CD26 in autism may interfere with its enzymatic function in the GI tract resulting in accumulation of dietary peptides in the blood and possible neuroimmune miscommunication. Furthermore, binding of antibodies to CD26 and CD69 bearing lymphocytes

may result in suppression of immune function, cytokine production and inhibition of autoreactive lymphocyte apoptosis. This abnormal expression of CD26 and CD69 on lymphocytes may further contribute to autoimmune reaction in autism.

In conclusion, to our knowledge, our analyses are the first to clearly demonstrate that dietary peptides, bacterial toxins and xenobiotics bind to lymphocyte receptors and/or tissue enzymes. This results in autoimmune reactions in children with autism. We suggest that these findings provide a mechanism by which environmental factors modulate the immune system and should help us develop preventive and therapeutic methods to reduce dietary peptides, bacterial toxins and toxic chemical-induced autoimmune reaction in autism.

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