

1

Binding of Infectious Agents, Toxic Chemicals, and Dietary Peptides to Tissue Enzymes and Lymphocyte Receptors and Consequent Immune Response in Autism

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Abstract:

Because so little is known about the range of intestinal immune functions that are influenced by dietary proteins, xenobiotics and infectious agents, we decided to test the hypothesis that infectious agents, dietary peptides and haptenic chemicals may bind to DPP IV and other tissue antigens or receptors, resulting in autoantibody production and modulation of immune and inflammatory processes in autism. Similar to many complex autoimmune diseases, genetic predispositions plus environmental factors including infection, xenobiotics and diet play a critical role in the development of autism. In a very recent study, we postulated that infectious agent antigens such as *Streptokinase* (SK), dietary peptides (gliadin and casein) and ethyl mercury (xenobiotic) bind to different lymphocyte receptors and tissue antigens. We assessed this hypothesis first by measuring IgG, IgM and IgA antibodies against CD26, CD69, 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, anti-casein peptide (casomorphin) 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 peptide, casein peptide and ethyl mercury binding to CD26 and CD69. Immune absorption demonstrated that only certain antigens, like CD26, were capable of significantly reducing serum anti-CD26 levels, indicating that they are specific. However, for direct demonstration of SK, gliadin peptide, casein peptide and ethyl mercury binding to CD26 or CD69, microtiter wells were coated with CD26 or CD69 alone or in combination with SK, gliadin and casein peptides 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 anti-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 from gluten and casein and Thimerosal (ethyl mercury) in individuals with predisposing HLA molecules, bind to CD26 or CD69 and induce antibodies against these molecules as well as to lymphocyte receptors and tissue antigens.

INTRODUCTION

Autism was originally thought to be primarily a psychiatric condition. However, further investigation showed that genetic and environmental factors are implicated in the pathogenesis of autism (1-5). Similar to many complex diseases, genetic and environmental factors including infections, xenobiotics, dietary proteins and peptides, play a critical role in the development of autism. The effects of environmental factors on genetic makeup result in immune, gastrointestinal, neurological, biochemical and neuroimmunological abnormalities (1-6). Based on extensive research, which led to publication of three different manuscripts and two review articles (7-11), we postulated that autism is induced by infectious agent antigens, toxic chemicals and dietary proteins. This process begins in the gastrointestinal tract but manifests itself in the brain (Fig. 1).

Many infectious agents, including measles, Rubella, and Cytomegalovirus have long been suspected as etiologic factors in autism (2, 12, 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 myelin basic protein (MBP) that induce T-cell responses but can also induce experimental autoimmune encephalomyelitis (14-16). Recent observations indicate that maternal infection with the human influenza virus increases the risk for schizophrenia and autism in offspring of pregnant mice (both BALB/c and C57BL/6 strains). 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 neuroimmunocommunications were responsible for abnormal development of the brain (17, 18).

In addition, antigens from infectious agents may interact with or impair lymphocyte receptors that have digestive functions in the gastrointestinal tract. Cell surface peptidases such as aminopeptidase N (CD13), aminopeptidase-I or dipeptidylpeptidase-I (DPP I), and dipeptidylpeptidase IV (DPP IV) play a key role in controlling growth and differentiation of many cellular systems including lymphocytes, leukemia or lymphoma cells (19, 20). DPP IV is a serine aminopeptidase with a capacity for cleaving peptides at locations containing amino-terminal dipeptides that have either L-alanine or L-proline at position 2 (21).

DPP IV is also found in human plasma where its structure and enzymatic activity are similar to the enzyme on normal T-lymphocytes, suggesting that plasma DPP IV originates from T-lymphocytes (22). The lymphocyte receptor CD26 is the same protein that binds adenosine deaminase, an enzyme that changes adenosine to inosine and is essential for adequate immune response (22). Some autistic individuals have an allergic response to casein (milk allergy) and in this condition there is a very significant reduction in expression of CD26 on lymphocytes (23). Furthermore, adenosine deaminase alleles with varying enzymatic activities have been identified in a subset of autistics (24). Binding and immune response to the CD26 site on lymphocytes, as well as milk allergy and genetic alleles, may reduce local cellular capacity for metabolism of adenosine, causing a local adenosine excess. If so, then the consequences may include dysregulation of lymphocyte immune functions, interference with cellular signal transduction (perception and response to external messengers), and rate limitation of metabolic sequences that are sensitive to adenosine concentrations. The later consequence could involve metabolism of

methionine via adenosylmethionine and adenosylhomocysteine. This in turn could cause methylation and sulfation deficits as seen in many autistics (22, 25).

Due to the 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) (26, 27). DPP IV is a receptor for SK on rheumatoid synovial fibroblasts via the LTRPA amino acid sequence (28). Binding of SK to DPP IV resulted in the appearance of anti-SK and anti-DPP IV autoantibodies in patients with myocardial infarction treated with SK (29).

Based on these observations and the potent immunogenicity of SK in patients with autoimmune disease, the bacterial protein (SK), heat shock proteins, or others could bind to DPP IV and induce significant levels of anti-SK and anti-DPP IV antibody production.

CD69 is an additional lymphocyte surface marker involved in autoimmune disease (30). 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, CD69 is expressed on surfaces of T-cells in synovial membranes but not on surfaces of circulating peripheral blood lymphocytes. The level of CD69 expression is correlated with disease activity (30, 31).

Autoantibodies to nervous system antigens are detected in populations exposed to toxic, environmental or occupational chemicals. Titers of antibodies against neurofilaments and 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 (32).

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 (33). Immune reactions to drugs or their metabolites can develop when a hapten carrier complex interacts with gut-associated lymphoid tissues (GALT) (34). 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 (35).

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 and gluteomorphins, can stimulate T-cells, induce peptide-specific T-cell responses, and abnormal levels of cytokine production. This stimulation of T-cells may result in inflammation, autoimmune reactions and disruption of neuroimmune communications (9, 36, 37).

We detected IgG, IgM and IgA antibodies against nine neuron-specific antigens in the sera of children with autism. These antibodies are formed either as a result of the binding of dietary peptides to human tissue and cell receptors, or due to epitope similarity between gliadin and casein with myelin oligodendrocyte glycoprotein or with cerebellar peptides.

Based on the above observations, we decided to test the hypothesis that infectious agent antigens (SK and HSP-60), dietary peptides (from gluten, gliadin and casein), and haptenic chemicals

(ethyl mercury) may bind to DPP IV (CD26) and CD69, resulting in autoantibody production, and modulation and expression of immune and inflammatory reactions in autism.

MATERIALS AND METHODS

2.1 Patients

Blood samples from 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. For comparison, serum samples from 50 patients with known mix connective tissue disease and healthy age- and sex-matched controls with negative ANA titers and no known autoimmune diseases were included.

2.2 Enzyme-Linked Immunosorbent Assay (ELISA)

Elisa was used for testing antibodies against different aminopeptidases, gliadin and bacterial antigens in the sera of patients with autism and autoimmune disease, and with control subjects. 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. 50 ml of the antigen or peptide solutions was 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 ml 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. Plates were washed as above, and then serum samples diluted 1:200 in 1% human serum albumin (HSA) in TBS containing 1 mg/ml of IgG FC fragments (to avoid reactivity of specific antibodies with rheumatoid factors) were added to duplicate wells and incubated for 2 hrs at room temperature. Sera from patients with autoimmune disorders with known high titers of IgG, IgM, and IgA against DPP IV, gliadin or HSP 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')₂ 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 hrs at room temperature. After washing five times with TBS-Tween buffer, the enzyme reaction was started by adding 100 ml of paranitrophenylphosphate in 0.1 ml diethanolamine buffer 1 mg/ml containing 1 mM MgCl₂ and sodium azide pH 9.8. The reaction was stopped 45 mins later with 50 ml of 1 N NaOH. The optical density (O.D.) was read at 405 nm by means of a microtiter reader.

2.3 Possible Binding of SK, HSP-60, 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 peptide, 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.

RESULTS AND DISCUSSION:

3.1 Demonstration of SK, HSP-60 and Gliadin Binding to DPP IV, DPP I and CD13

In searching for a mechanism underlying autoimmunity in autism, we postulated that gliadin peptides, heat shock protein (HSP-60) and streptokinase (SK) bind to different peptidases. Binding results in autoimmunity. We assessed this hypothesis in patients with autism and in those with mixed connective tissue diseases. Concomitant with the appearance of anti-gliadin and anti-HSP antibodies, children with autism and patients with autoimmune disease developed anti-DPPI, anti-DPP IV, and anti-CD13 autoantibodies. These antibodies may be synthesized as a result of gliadin and HSP-60 binding to different peptidases since a significant percentage of autoimmune and autistic sera were associated with elevated IgG, IgM or IgA antibodies against all three peptidases, gliadin and HSP-60. These antibodies are specific since immune absorption demonstrated that only specific antigens (i.e., DPP IV absorption of anti-DPP IV significantly reduced IgG, IgM and IgA antibody levels). For direct demonstration of SK, HSP-60 and gliadin peptides binding to DPP IV, microtiter wells were coated with DPP IV and with SK, HSP-60 and gliadin. Finally, they were reacted with rabbit anti-DPP IV, or anti-SK, anti-HSP-60 and anti-gliadin. Addition of SK, HSP-60 and gliadin peptides to DPP IV resulted in 27-43% inhibition of DPP IV anti-DPP IV reaction. Furthermore, addition of anti-SK, anti-HSP-60 and anti-gliadin to DPP IV + peptides caused 18-20% enhancement of antigen-antibody reaction. These results further support binding of SK, gliadin and HSP to DPP IV. From our results we conclude that binding of bacterial superantigens to DPP IV, DPPI or CD13 is responsible for autoantibody production in children with autism and in patients with autoimmune diseases.

3.2 The Role of Heavy Metals and Other Toxic Chemicals in Autism

Xenobiotics have been suspected to contribute to the induction of autoimmunity (33, 34). Many environmental chemicals or drugs are toxic to their hosts, and their detoxification is achieved primarily in the liver. During their metabolism, they may form reactive metabolites, which can then modify cellular proteins to form neoantigens.

Among many toxicants, Thimerosal (merthiolate) or ethyl mercury in vaccines has been associated with immune injuries described in children with autism (38-41). Contrary to many haptens that bind covalently to a single amino acid, such as lysine, metal complexes often consist of a central metal ion composed of four different amino acids, and hence they possess increased complex stability (33). To demonstrate possible binding of ethyl mercury to DPP IV and CD69, in a very recent study we postulated that in addition to infectious agent antigens such as SK, ethyl mercury binds to different lymphocyte receptors and tissue antigens. We assessed this hypothesis first by measuring IgG, IgM and IgA antibodies against CD26, CD69 and SK against

ethyl mercury bound to human serum albumin in patients with autism. A significant percentage of children with autism developed anti-SK and anti-ethyl mercury antibodies, concomitant with the appearance of anti-CD26 and anti-CD69 antibodies. These antibodies are synthesized as a result of SK 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 a direct demonstration of SK and ethyl mercury binding to CD26 or CD29, microtiter wells were coated with CD26 or CD29 alone or in combination with SK or ethyl mercury and then reacted with enzyme labeled rabbit anti-CD26 or anti-CD69. Adding these molecules to CD26 or CD29 resulted in 28-86% inhibition of CD26 or CD29 binding to anti-CD26 or anti-CD69 antibodies. We, therefore, propose that bacterial antigens and thimerosal (ethyl mercury), in individuals with pre-disposing HLA molecules, bind to CD26 or CD69 and induce antibodies against these molecules as well as to lymphocyte receptors and tissue antigens.

3.3 Binding of Dietary Peptides to Different Tissue Enzymes may Promote Development of Peptidase Antibodies in Children with Autism

Some dietary proteins, and their opiate peptides, including casein with casomorphins, and gluten with gluteomorphins, can stimulate T-cells, induce peptide-specific T-cell responses, and cause abnormal levels of cytokine production. This may result in inflammation, autoimmune reactions and disruption of neuroimmune communications (42-45).

A majority of children with autism cannot tolerate milk and wheat proteins or peptides, and, hence, elimination of these peptides from the diet significantly improves their conditions. This clinical finding correlates with laboratory results reported earlier by our group in children with autism (7-11).

We measured IgG, IgM and IgA antibodies against milk peptides and found that every autistic's serum having ELISA values higher than 0.3 O.D. against neurological antigens also had high levels of neurological antigens and antibodies against milk peptides. Similar to milk peptides, antibodies against different gliadin peptides have also been described in celiac disease, gluten ataxia and recently in children with autism (42, 43).

3.4 Cross-reaction Between Gliadin and Cerebellar Purkinje Cells as a Possible Mechanism for Neuroimmune Abnormalities in Autism

One of the most frequent presentations of gluten sensitivity is the neurologic dysfunction called gluten ataxia. Sera from these patients stain cerebellar Purkinje cells. For this reason, sera from 50 patients with autism were measured for the simultaneous presence of IgG, IgM and IgA antibodies against gliadin and cerebellar peptides and compared to healthy controls. Considering only the results that are at least 2 SD above the mean of the controls, 21 (or 42%) of patients with autism had elevated antibody levels against gliadin peptides, while only 6 (or 12%) of control subjects had elevated antibodies against gliadin peptides. In comparison, 18 (or 36%) of patients and 4 (or 8%) of controls demonstrated significantly elevated antibodies against cerebellar peptides. 17 of 21 (80%) patients with autism had simultaneous elevation in anti-gliadin and anti-cerebellar peptides, indicating cross-reaction between gliadin and cerebellar antigen, which results in these antibodies in a majority of gliadin-reactive patients with autism.

Based on this antigenic similarity between milk butyrophilin, casein and gliadin peptides with myelin basic protein, myelin oligodendrocyte glycoprotein, and cerebellar Purkinje cells, a casein- and gliadin-free diet should be recommended for individuals with elevated milk and gliadin IgG, IgM or IgA antibodies.

CONCLUSIONS

From these results, we learn that autoantibodies to different tissue antigens in autism are produced by two different mechanisms of action:

- a. by direct binding of infectious agent antigens or peptides,, dietary proteins or peptides, or by binding of xenobiotics or their metabolites to tissue enzymes or cell receptors
- b. many infectious agents, dietary proteins, and peptides share similar epitopes with different tissue antigens. Therefore, immune responses against the infectious agents or dietary proteins result in autoimmune reactions with different tissue antigens, including brain cells.

Based on these findings, we postulate that dietary and infectious antigens as well as xenobiotics play a role in the pathophysiology of autism.

These antibodies may cross the blood-brain barrier and combine with brain tissue antigens to form immune complexes, thus causing further damage to the neurological tissue. The antibodies, along with toxic biological weaponry, such as arachidonic acid and free radicals, can “chew off” neuron myelin and impair electrical transmission between a muscle and the central nervous system, resulting in neuroimmune disorder.

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