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NEUROTENSIN AND EXTRACELLULAR MITOCHONDRIAL DNA: POTENTIAL BIOMARKERS AND NOVEL TREATMENT TARGETS

Autism spectrum disorders (ASD) are neurodevelopmental disorders characterized by deficits in social interactions, communication, and learning, as well as stereotypic behaviors. There are still few insights for its early detection or treatment. The presence of brain expression of pro-inflammatory cytokines and circulating autoantibodies against brain proteins indicate immune dysfunction. Many ASD children suffer from "allergic-like" problems in response to environmental triggers that could contribute to brain inflammation in at least a subgroup of ASD patients. We recently identified high serum levels of neurotensin, a neuropeptide present both in the brain and gut, and mitochondrial DNA in young children with autism. These molecules could serve as unique biomarkers as well as targets for novel treatments.

BY THEOHARIS C. THEOHARIDES, PHD, MD AND SHAHRZAD ASADI, PHARMD

INTRODUCTION

Autism spectrum disorders (ASD) are pervasive developmental disorders characterized by deficits in social interactions, communication, and learning, as well as repetitive stereotypic behaviors.[42] At least 30% of ASD children present with sudden clinical regression at around 3 years of age.[55,79] There has been an impressive rise in ASD, especially after 1986,[56] with current prevalence estimates being about 1/68 boys.[27,47,10]

Some possible autism susceptibility genes have been identified.[77] However, no single gene or group of genes can explain the meteoric rise in autism incidence. Gene interactions with environmental factors (epigenetic factors) have been suspected as related to the increase in autism prevalence.[35] For instance, a recent paper reported that mothers with one autistic child had a much higher chance of having another if born within one year of the first pregnancy.[16] This finding suggests that factors other than genetics must have been influencing the outcome of the pregnancy during that first year. Such factors could not be genetic because they would then have influenced the outcome regardless of the time from the first pregnancy. More likely contributors include nutrition, stress due to a second child being born within a year of the first, or environmental factors (for example, parents might move to a larger house as their family grows). Unfortunately, in most cases the cause of ASD is said to be unknown.[50]

Interestingly, epigenetic mechanisms may also explain the "epidemic" of immune diseases and allergies.[54] An inappropriate immune response by autistic subjects to antigenic stimuli has been

Abbreviations:
ASD: autism spectrum disorders
BDNF: brain-derived neurotrophic factor
BBB: blood-brain barrier
CGRP: calcitonin-gene related peptide
CRH: corticotropin-releasing hormone
CSF: cerebrospinal fluid
FcεRI: high affinity IgE receptor
GI: gastrointestinal
IFN: interferon
LPS: lipopolysaccharide
M-CHAT: Modified Checklist for Autism in Toddlers
MCP-1: chemoattractant protein-1
MIF: macrophage inhibitory factor
NGF: nerve growth factor
NK cells: natural killer cells
NT: neurotensin
PCB: polychlorinated biphenyl
PDD-NOS: pervasive developmental disorder – not otherwise specified
SP: substance P
TGF-β1: transforming growth factor-beta1
TLR: toll-like receptor
TNF: tumor necrosis factor
UP: urticaria pigmentosa
VEGF: vascular endothelial growth factor
VIP: vasoactive intestinal peptide

Generally, allergic reactions are associated with elevated plasma IgE antibodies. In the absence of elevated serum IgE, "allergic-like" reactions are not always considered "true" allergies. Many ASD children suffer from just such "allergic-like" symptoms.

observed in unaffected siblings, suggesting a particular genetic background influenced by environmental triggers.[64] A number of papers have reviewed family or personal history of children with ASD and reported an association with immune disorders, especially during the third trimester of pregnancy or in the child with ASD.[8,30] Using health records, a nested case-control study of infants with ASD born in California between 1995-1999 reported that the prevalence of maternal psoriasis, asthma, hay fever, and atopic dermatitis during the second trimester of pregnancy correlated with a greater than 2-fold elevated risk of ASD in the children.[20] In a National Survey of Children's Health, similarly, parents of autistic children (n=483) reported more symptoms of allergies, especially food allergies or intolerance, than those of healthy control children (n=84,789).[33] These results, and the presence of autoantibodies against brain proteins in the serum of many autistic children and their mothers, prompt the suggestion that there may be a neuroimmune component[8,30,71] in at least some ASD endophenotypes within the autism spectrum.[59]

"ALLERGIC" SYMPTOMS IN AUTISM: A LITERATURE SURVEY

Generally, allergic reactions are associated with elevated plasma IgE antibodies. In the absence of elevated serum IgE, "allergic-like" reactions are not always considered "true" allergies. Many ASD children suffer from just such "allergic-like" symptoms.[43,68,4] As shown in Table 1, a number of conditions can present with allergic-like symptoms such as chronic idiopathic or chronic autoimmune urticaria, without any of the typical test results for allergies (e.g., elevated serum IgE or positive skin tests).[46]

Table 1: Diseases involving mast cell activation*

1. **Primary**
   a. Mastocytosis
   b. Monoclonal mast cell activation disorder (MMAD)
2. **Secondary**
   a. Allergies
   b. Mast cell activation in inflammation or cancer
   c. Physical urticarias
   d. Chronic autoimmune urticaria
3. **Idiopathic**
   a. Anaphylaxis
   b. Angioedema
   c. Urticaria
   d. Mast cell activation syndrome (MCAS)

*Adapted from[2]

A recent preliminary study of children with ASD (n=245) indicated that the strongest association of autism was with a history of allergies.[63] Another paper reported that increased atopic dermatitis, asthma, rhinitis, high serum IgE, and positive skin tests were present in 70% of Asperger's syndrome patients (n=15) compared to 7% of age-matched healthy controls (n=15).[53] As mentioned earlier, however, not all reports of allergic symptoms in children with ASD implicate allergic IgE antibodies. In a hospital-based case-control study using questionnaires completed by the parents and scored blindly by an allergist, 30% of autistic children (n=30) had a family history of allergic features compared to 2.5% of age-matched "neurologic controls" (n=39) ($p<0.005$); there was no difference, however, in serum IgE or skin prick tests to 12 common antigens between autistic subjects and controls, suggesting non-immune triggers.[9] Another study reported that the prevalence of atopic disorders in subjects with ASD (n=133) was similar to that of the controls, but non-IgE-mediated food intolerance was observed at a significantly higher rate in ASD compared to controls (n=13), again highlighting the likely role of other non-allergic factors.[44] Some colleagues speculate that elevated IgG1 antibodies may be indicative of non-allergic reactions, but even this is controversial. For instance, the significance of increased plasma IgG4 levels in children with autism (n=114) compared to normally-developing children (n=96) in one study is unclear,[25] because high levels of IgG4 antibodies to foods during infancy are associated with tolerance later in life[73] while many ASD children are intolerant to foods.

Unfortunately, one can only test for food allergies against suspected available food "allergens." When anecdotal reports surfaced that some children with ASD present with hives after eating meat, they were greeted with skepticism by some investigators. Nevertheless, as recently reported, this phenomenon turns out to be true due to IgE antibodies specific for the meat carbohydrate epitope galactose-α-1,3-galactose, which result in delayed angioedema and urticaria after eating beef, lamb, or pork.[18]

MAST CELLS

Mast cells are immune cells often attributed with causing allergic reactions. In addition to being necessary for the development of allergic reactions,[70] mast cells are also critical for both innate and acquired immunity[28] as well as inflammation.[69] Functional mast cell-neuron interactions occur in the GI tract[5] and the brain.[62] Given that GI-related symptoms are quite common in ASD patients,[51] especially abnormal intestinal permeability,[11] it is important to note that mast cells are involved in GI inflammation and increased gut permeability.[26]

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In particular, the peptide neurotensin (NT), which is present both in the brain and gut, can trigger mast cell activation and increase vascular permeability.

Diagnostic criteria were just proposed for a new entity called "mast cell activation syndrome" (MCAS), defined as "presenting with signs and symptoms involving the dermis, gastrointestinal tract, and cardiovascular system frequently accompanied by neurologic complaints."[2] A preliminary report indicates that the prevalence of ASD is 10-fold higher (1/10 children) in mastocytosis patients than in the general population (1/100 children).[66] Mastocytosis is a spectrum of disorders with a prevalence of about 1/4000 children, which involves proliferation and activation of mast cells in the skin (urticaria pigmentosa, UP) and other organs,[15] leading to skin reactions, food allergies (often in the absence of positive skin testing), and food intolerance, and notably also behavioral problems.[1,74]

Many substances originating in the environment, the intestine, or the brain can trigger mast cell activation,[70] leading to release of numerous bioactive mediators that include vascular endothelial growth factor (VEGF) as well as cytokines, such as IL-6, IL-8, IL-9, and tumor necrosis factor (TNF). This is potentially destructive because these molecules can cause inflammation and destroy the protective gut-blood and blood-brain barriers. A recent paper reported increased plasma levels in children with ASD of the chemokines RANTES (regulated upon activation, normal T cell expressed and secreted), MCP-1 (macrophage chemoattractant protein-1), and eotaxin,[7] all of which are potent chemoattractants for mast cells.[19,14,39] CSF and microglia of ASD patients have been found to express high levels of MCP-1.[76] In contrast, ASD plasma levels of transforming growth factor-beta1 (TGF-ß1) were low,[6] which is relevant in view of the fact that TGF-ß1 inhibits mast cell function and high affinity IgE receptor (FcεRI) expression.[29] TGF-ß is also an important mediator released by regulatory T cells,[6] and the low plasma TGF-ß levels in autistic patients suggest reduced regulatory T cell function in autism.

Environmental toxins linked to neurodevelopmental damage,[31] such as polychlorinated biphenyl (PCB) and mercury, have been associated with ASD[36,78] and these also activate mast cells.[45,49] A number of rotaviruses have been isolated from 75% of asymptomatic neonates[24] and could activate mast cells since they express viral TLR-3, activation of which by viral double-stranded RNA induces release of IL-6 and TNF without degranulation.[48] Degranulation is defined as the secretion of all granule-stored mast cell mediators. Bacterial lipopolysaccharide (LPS) also activates toll-like receptor-4 (TLR-4) on mast cells and induces selective release of TNF.[75] This unique ability of mast cells to release some mediators selectively without degranulation suggests that their involvement may go unnoticed in many diseases.[72]

In particular, the peptide neurotensin (NT), which is present both in the brain and gut, can trigger mast cell activation[13] and increase vascular permeability.[23] This action is synergistic with corticotropin-releasing hormone (CRH), which is secreted under stress, and induces selective release of VEGF.[12] We recently reported that NT levels are increased in the serum of young children with autistic syndrome as compared to normal, age-matched controls.[3] Unlike NT, however, the somewhat similar peptide substance P (SP) was not elevated (as had been reported previously),[57,58] and ß-endorphin was also not elevated. These results indicate that the NT elevation is specific, since the other related neuropeptides were not increased. Our findings are supported by those from other investigators. One study showed no difference in serum levels of SP, vasoactive intestinal peptide (VIP), calcitonin-gene related peptide (CGRP), or nerve growth factor (NGF) in children with ASD (n=69) and those with mental retardation without ASD (n=60).[58] Another study showed elevated ß-endorphin levels in the CSF of children with infantile autism (n=9), but serum levels were not measured.[61] These results again indicate that our finding of increased serum levels of NT is specific.

Other evidence suggests that mast cell-derived mediators may be released. For instance, TNF levels were elevated in the CSF of patients with ASD, but not in the serum,[17] while serum TNF receptor II was significantly elevated.[82] A trend towards increased production of TNF and IL-6 was noted in whole blood of autistic children.[21] IL-6 expression was elevated in the brains of deceased ASD patients,[52] and it was detected at low levels in the CSF of subjects with autism (n=35) as compared to control subjects with other neurologic disorders. Macrophage inhibitory factor (MIF), a molecule known to increase immunity through different mechanisms, was higher in the plasma of probands with ASD than their unaffected siblings and correlated with severity of ASD symptoms.[32] These results indicate that some inflammatory molecules are elevated in the blood of at least some patients with autism.

OUR THEORY

We theorize that an early insult can cause the gut-blood and blood-brain barriers to break down. This would allow neurotoxic molecules to get to the brain, causing neuroinflammation and impaired processing. Many children on the autism spectrum have tested positive for brain autoantibodies, which supports this theory.

Neurotensin is a neuropeptide that can trigger mast cells. Other agents that can trigger mast cells are environmental and infectious agents and stress triggers. This can disrupt the blood-brain barrier. If mast cell activation occurs during gestation, for example, mast cell-derived mediators could alter gene expression in autism susceptibility genes.

We recently showed that NT can stimulate mast cells to release mitochondrial DNA extracellularly and that such DNA is significantly elevated in the serum of autistic children.[80] This unique process occurs through mitochondrial translocation to the cell surface.[81] Extracellular mitochondrial DNA is a potent trigger of autoimmunity through activation

We theorize that an early insult can cause the gut-blood and blood-brain barriers to break down. This would allow neurotoxic molecules to get to the brain, causing neuroinflammation and impaired processing.

Neurotensin and extracellular mitochondria represent two potential biomarkers that have applicability for children with autism. As we have seen, both neurotensin and extracellular mitochondria have been reported to be increased in the serum of young children with autism.

of TLR. Because mitochondria were bacteria that became symbiotic with eukaryotic cells millions of years ago, mitochondrial components are not supposed to be released outside the cells.

To reiterate these relationships, neurotensin is a neuropeptide that is found both in the brain and in the gut, and it is released very quickly when triggered by stress, infection, or inflammation. After being released, neurotensin contributes to the pathogenesis of autism by stimulating mast cells, both in the brain and in the gut, which in turn secrete two different classes of inflammatory mediators: (1) Molecules such as IL-6, TNF and VEGF that directly disrupt the protective gut-blood and gut-brain barriers, thus allowing toxic molecules to get into the brain; and (2) pieces of mitochondria *outside* of the cell. The mitochondria dumped outside the cell then mimic an infection (think of a fake infectious trigger) and the body mounts an inflammatory response through stimulation of other immune cells to release inflammatory mediators; these both contribute to the further disruption of the gut-blood-brain barriers, and induce gut and brain inflammation.

The secretion of the mitochondrial particles could be a double-edged sword, because if many mitochondria are already damaged (as appears to be the case in many children with autism), then the secretion of the remaining mitochondria outside the cell makes them unavailable to produce needed energy for the developing brain.

WHAT DO WE NEED TO DO?

Neurotensin and extracellular mitochondria represent two potential biomarkers that have applicability for children with autism. As we have seen, both neurotensin and extracellular mitochondria have been reported to be increased in the serum of young children with autism.[81] Because neurotensin can trigger release of mast cells and have neurotoxic actions of its own, while extracellular mitochondria induces an autoimmune response, the following steps should be taken.

1. Measure earlier. We can prognosticate by looking for biomarkers in amniotic fluid or archived blood.

2. To ascertain the full range of conditions for which the two molecules might be able to serve as biomarkers, consider the entire autism spectrum to see if this phenomenon is unique to full-syndrome autism or if it also appears in pervasive developmental disorder – not otherwise specified and Asperger's syndrome.

3. Investigate whether neurotensin has a more potent effect when combined with other triggers (e.g., mercury, viral triggers, stress hormones).

In terms of remediating the effects of neurotensin and extracellular mitochondria, there are three paths of intervention: block mast call activation (e.g., with flavonoids); neutralize mitochondrial DNA or block/antagonize neurotensin; and/or neutralize mitochondria outside the cell so that they don't trigger an inflammatory response.

CONCLUSION

The evidence discussed above does not imply a cause-and-effect relationship. Nevertheless, potential inhibitors of mast cell activation may be useful. Our preliminary evidence indicates that the naturally occurring flavonoid luteolin can inhibit NT-induced mast cell activation and release of extracellular mitochondrial DNA, as well as gut-blood-brain barrier disruption. Luteolin also inhibits microglial production of IL-6,[40] can induce anti-inflammatory changes in glial cells,[22] and can inhibit cytokine release from peripheral blood monocytes from multiple sclerosis patients.[65] Luteolin (*5, 7,* 3′, 4′-tetrahydroxyflavone) is closely related to *7,* 8-dixydroxyflavone, which mimics brain-derived neurotrophic factor (BDNF) and is neuroprotective.[41] Luteolin inhibits maternal IL-6-induced autism-like behavioral deficits in social interaction in mice.[60] Luteolin and the structurally similar quercetin are generally safe[34] and could be useful in treating neuroinflammatory diseases, either alone or as an adjunct to other therapeutic approaches.[67] Unfortunately, luteolin is lipophilic and poorly absorbed after oral administration, with significant liver metabolism.[37,38] Combining this in a formulation with three flavonoids has been done to increase oral bioavailability (see Disclosure). NT receptor antagonists, extracellular mitochondria neutralization by aptamers, and/or prevention of gut-blood-brain barrier disruption could serve as potential treatment approaches.

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DISCLOSURES

The authors declare that they have no competing interests. TCT is the inventor of patent application US 12/534,571 covering the diagnosis and treatment of ASD, as well as the trademarked dietary formulation NeuroProtek (**www.algonot.com**).

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