Autism: Where Genetics Meets the Immune System

Guest Editors: Antonio M. Persico, Judy Van de Water, and Carlos A. Pardo
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Individuals with autism often display immune abnormalities in the form of altered cytokine profiles, autoantibodies, and changes in immune cell function. Are they mere “smoke” or real “fire”? Given what we now know about the cross-talk between the immune and nervous systems, one must ask: are these immune abnormalities simply bystander effects of genetic/genomic variants directly responsible for abnormal neurodevelopment, or is there a sizable subgroup of patients with autism spectrum disorder (ASD), in which a dysfunctional immune system plays a critical mediator role in the pathogenic chain of events leading to the disorder? Is autism one of many genetic/genomic conditions, Down syndrome representing the best-known example, accompanied by immune abnormalities not primarily responsible for changing the trajectory of neurodevelopment? Or could conceivably an unfortunate combination of genetic/genomic susceptibility and environmental factors converging onto the same individual affect neurodevelopment through immune-mediated mechanisms, such as fetomaternal incompatibility, autoimmunity, abnormal neuroinflammation, and so on?

This special issue contains six contributions aimed at addressing different aspects of this question.

The paper by N. Momeni et al. documents significantly elevated plasma levels of factor I among ASD children compared to controls. Factor I is a plasma enzyme responsible for degrading complement factor 3b, which in turn is the major opsonin in the complement system enabling phagocytosis of microbial agents. Higher factor I levels can be interpreted as either primary (i.e., conferring vulnerability toward microbial infections) or as a secondary event part of a broader immunomodulatory response aiming to blunt an inflammatory process. F1 plasma levels suggest that this process could play a more relevant role in males and in small ASD children.

The contribution by S. Rose et al. describes reduced glutathione-mediated redox/antioxidant capacity both inside primary leukocytes and in the plasma of ASD children. Consistently with this intracellular and extracellular imbalance of the glutathione redox status, intracellular production of free radicals is also enhanced, especially in 30% of ASD cases. This and several previous studies provide converging evidence of excessive oxidative stress likely leading to abnormal mitochondrial function in a similar percentage of autistic individuals.

Along similar lines, M. I. Waly et al. direct their attention on the complex array of pathophysiological consequences produced by enhanced oxidative stress. In addition to imbalanced glutathione redox status, reduced methionine synthase activity is particularly interesting, as it results in lower availability of S-adenosyl-methionine and consequently blunted DNA methylation. Curiously, a similar abnormality underlies autistic features also in two entirely different contexts, namely, children exposed prenatally to valproic acid and in Rett syndrome girls, carrying MECP2 mutations.

The review by S. D. Bilbo et al. presents a stimulating hypothesis, linking ASD to inflammatory, allergic, and autoimmune diseases. These “hyperimmune” disorders are
known to share steeply increasing incidence rates over the last decades, as well as significant loading in many families with children with autism. In the authors’ view, they also share an inappropriate activation of the immune system due to biome depletion in postindustrial societies, possibly suggesting preventive strategies based on biome reconstitution.

The paper by C. Onore et al. reports an impressive threefold decrease in EGF plasma levels among small ASD children compared to controls. EGF is involved in wound healing in the skin, in the gastrointestinal, and respiratory systems, as well as in the central nervous system where this neurotrophic factor supports both adult subventricular zone and midbrain dopaminergic neurons, in addition to stem cell proliferation.

A. R. Torres et al. review immune abnormalities in autism, focusing on HLA gene roles as potential contributors to autism pathogenesis through several distinct mechanisms. HLA genes or haplotypes have been found associated with several autoimmune diseases, but strong associations with autism have also been reported.

These contributions, while not providing the ultimate answers, do add some new pieces to the autism puzzle, providing further support to the idea that immune abnormalities may play a pathophysiologically relevant role in a subgroup of families with autistic children. Future studies of the interaction of the immune and central nervous system are needed to clarify in more detail how neuroimmune mechanisms influence the enhancement of the neurodevelopmental disarray and disturbances of neurobehavioral trajectories determined by genetic and environmental factors. The heuristic potential of this line of investigation, both in terms of prevention and clinical therapeutics, ensures continued efforts until a more definitive answer can be given to our initial question, so crucial in today’s autism field.

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Review Article

Is Autism a Member of a Family of Diseases Resulting from Genetic/Cultural Mismatches? Implications for Treatment and Prevention

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Several lines of evidence support the view that autism is a typical member of a large family of immune-related, noninfectious, chronic diseases associated with postindustrial society. This family of diseases includes a wide range of inflammatory, allergic, and autoimmune diseases and results from consequences of genetic/culture mismatches which profoundly destabilize the immune system. Principle among these consequences is depletion of important components, particularly helminths, from the ecosystem of the human body, the human biome. Autism shares a wide range of features in common with this family of diseases, including the contribution of genetics/epigenetics, the identification of disease-inducing triggers, the apparent role of immunity in pathogenesis, high prevalence, complex etiologies and manifestations, and potentially some aspects of epidemiology. Fortunately, using available resources and technology, modern medicine has the potential to effectively reconstitute the human biome, thus treating or even avoiding altogether the consequences of genetic/cultural mismatches which underpin this entire family of disease. Thus, if indeed autism is an epidemic of postindustrial society associated with immune hypersensitivity, we can expect that the disease is readily preventable.

1. Introduction: Autism as a Member of a Large Family of Postindustrial Epidemics Involving a Hyperimmune Response

In this paper, we outline a paradigm that points toward autism as one disease among many other well-known diseases which all share a common origin and, most likely, a common prevention strategy [1]. These emerging, noninfectious diseases are all epidemics of postindustrial culture, which are absent in antiquity in any culture, and absent in present day developing cultures. Members of this family of disease are invariably associated with a hyperimmune response and can have a very high prevalence in postindustrial populations, with prevalence often greater than 0.1% and sometimes greater than 1.0%. These hyperimmune-associated diseases include a wide range of allergic, autoimmune, and inflammatory diseases such as lupus, multiple sclerosis, hay fever, appendicitis, chronic fatigue syndrome, inflammatory bowel disease, asthma, celiac disease, type 1 diabetes, Graves’ disease, some types of eczema, and a wide range of food allergies. Here, based on a variety of evidence, we argue that autism is yet another member of this family of diseases, despite the slowness of the medical community to recognize it as such. Since the pathogenesis of at least one form of autism has been directly linked to autoantibody production [2], and since seven out of every eight cases of severe autism are associated with antineuronal antibodies in the serum [3], this idea has already been demonstrated, at least in part. Further, as described below, autism is associated with a wide range of immune abnormalities. Even though some children with autism appear to have “normal” immune systems, it is argued that adverse immune events early in development might lead to an autistic phenotype.
A primary consideration in this view of autism as a hyperimmune-associated disease is the connection between immunity and brain development in general, and between hyperimmune responses and autism in particular. Although these connections have little bearing on the overall model describing induction of disease by immune hypersensitivity in postindustrial society, the connections provide reason to expect that the developing brain is sensitive to the same postindustrial changes in the immune system which are known to affect virtually all other organs of the human body (e.g., kidneys, pancreas, epidermis, adult nervous tissues, large and small bowel, cardiovascular system, thyroid, lungs, and others). The next four sections will summarize much of what is currently known regarding those very extensive connections.

2. Pervasive Immune System Abnormalities in Autism

Immune system abnormalities exist throughout the body and brain of autistic children. These include evidence of brain specific auto-antibodies, altered T, B, and NK cell responses to antigen, altered cytokine production, an increased incidence of allergies and other autoimmune disorders, and functional changes in brain glial cells (microglia and astrocytes) [4–11]. Microglia are the primary immunocompetent cells of the brain and rapidly respond to any infection, injury or other perturbation of homeostasis via a dynamic process of activation [12]. Notably, increased microglial activation has been observed in several brain regions of autistic patients [13]. Once activated, glia produce a wide number of immune signaling molecules, including cytokines (e.g., interleukin [IL]-1β, tumor necrosis factor [TNF]-α), chemokines (e.g., monocyte chemoattractant protein [MCP]-1), and other inducible factors (e.g., nitric oxide), which may profoundly influence neural function [14].

3. Immune System-Central Nervous System Communication

Beyond its traditional role in host defense and tissue repair, the immune system is now considered a diffuse sensory organ that works in concert with the endocrine, metabolic, and nervous systems to achieve and maintain homeostasis throughout the body [15, 16]. In essence, the immune system serves as an interface between the human body and the environment, coordinating the response of the body to the environment. Immunocompetent cells are located throughout every organ of the body, including the brain, and regular communication occurs between the central nervous system and immune tissues during both health and disease processes. Many excellent reviews have been written on these topics [17–20]. Importantly, bidirectional communication between the brain and immune system has significant consequences for plasticity mechanisms within the brain, including cognition and emotion, which are markedly altered in autism.

4. Glial Cells Direct Normal Brain Development

Microglia are the resident macrophages of the central nervous system, are associated with the pathogenesis of many inflammatory diseases of the brain, and derive from primitive yolk sac macrophage precursors, which are of mesodermal origin and enter the neuroectoderm during embryogenesis [21]. Early in development, microglia are highly mobile and primarily amoeboid, consistent with their role in the phagocytosis of apoptotic cells [22]. The expression of many cytokines within the developing brain, including IL-1β and TGF-β, depends on the presence of amoeboid microglia [23]. Microglia transform into a highly branched, ramified morphology by adulthood in most brain regions. This morphological transition occurs in parallel with neural cell genesis and migration, synaptogenesis, and synaptic pruning, suggesting functions for microglia in each of these processes, though these are just beginning to be explored [24–26]. Oligodendrocytes myelinate axons primarily during the postnatal period, and disruption of their function can be profoundly debilitating as in the case of periventricular leukomalacia leading to cerebral palsy [27]. Astrocytes mediate synapse formation within the developing brain [28], in part via the secretion of extracellular matrix proteins called thrombospondins (TSPs) [29, 30]. Alterations in spine density via a putative TSP mechanism are implicated in neurodevelopmental disorders such as Down’s and Rett Syndrome [31]. Notably, astrocyte maturation marks the end of the perinatal synaptogenic period when the brain is most plastic [32].

5. Long-Term Consequences of Immune Activation during Early Development

Developmental or “fetal programming” is a growing field of science based on evidence that experiences during critical or sensitive periods of perinatal life may modulate or “program” the normal course of development, with the result that adult outcomes, including behavior, are significantly and often permanently altered [33]. Given the many ways in which the immune system is important for normal brain development, the capacity for immune-inducing events to influence the long-term trajectory and function of these processes is likely profound, perhaps more so than at any other stage of life [34]. Notably, there is strong evidence that early-life infection can permanently alter stress reactivity, disease susceptibility, and notably, vulnerability to cognitive and neuropsychiatric disorders, including Alzheimer’s disease, Parkinson’s disease, schizophrenia, and autism [35–38]. One of the most dramatic and direct illustrations of this effect is the induction of autism-like symptoms in mice by stimulation of the maternal immune system during pregnancy [39].

Neuroinflammatory-induced alterations in neurogenesis, migration, axon growth, myelination, synaptogenesis, and dendritic spine density/function are all candidate mechanisms by which long-term changes in behavior might result. Further, the long lifespan of microglia and their
pattern of central nervous system colonization during development suggest that these immune cells may have particular significance in terms of neuroinflammatory effects on developmental programming. Diverse early-life events may have enduring consequences for the brain and behavior of organisms via an influence on these resident immune cells, both by impacting their own intrinsic function as well as their interactions with ongoing developmental processes [40, 41].

6. Approaching Autism as Another Member of the Family of Hyperimmune-Associated Diseases: Addressing the Underlying Causes

The study of individual postindustrial associated diseases has often failed to enlighten researchers as to the underlying cause of diseases. While genetic factors associated with the epidemics of postindustrial society abound, these genetic factors have not changed over many generations, and thus are not responsible for newly emerging epidemics of disease. Almost paradoxically, even when specific triggers are identified, these often cannot be said to cause newly emerging epidemics. For example, peanuts and ragweed pollen are two well-known triggers for allergy, but these substances have been present in the environment for millennia without triggering disease in humans. Similarly, viral infections and potentially some milk proteins [42] have been identified as triggers for particular autoimmune diseases, but these factors have been present for much longer than the diseases they trigger. Thus, as will be described in detail, it is not the genetics of the disease which point toward a cure, and it is generally not identification of the triggers for disease that is most helpful. Rather, we suggest that the most effective and insightful approach toward defeating autism for future generations will be the neutralization of underlying factors in postindustrial culture which render postindustrial humans uniquely susceptible to disease, despite the presence of the same genetics and many of the same triggers as found in preindustrial cultures. These factors have been identified as mismatches between normal human genetics and the environment of postindustrial culture. With that in mind, an examination of the factors in postindustrial culture which trigger the plagues of hyperimmune-associated diseases will be presented.

7. Mismatches between Normal Human Genetics and Postindustrial Culture Leading to Hyperimmune Epidemics: Biome Depletion

If indeed autism fits into the family of hyperimmune-associated diseases plaguing postindustrial culture, then the pathogenesis or origins of these diseases merit detailed analysis in any consideration of autism. As shown in Figure 1, postindustrial culture has several mismatches with normal human genetics (known as “evolutionary mismatches”) which have a profoundly destabilizing effect on the human immune system. This effect is closely intertwined with an impact on metabolism and transport, as will be described later. These mismatches of postmodern culture involve some of the most cherished attributes of postmodern culture, including the widespread use of toilets, water treatment facilities, indoor working environments, soaps and shampoos, modern medicine, and the loss of factors which demand exercise for survival (e.g., human-eating predators, hardship in finding food and procuring shelter for most of the population). These mismatches are not a target for therapy, because reversal of any of these mismatches is untenable. Thus, these mismatches can be described as belonging to the “pretherapeutic zone” in the course of pathogenesis (Figure 1). However, these mismatches lead directly to readily measurable consequences that, in turn, lead to disease if left untended. Fortunately, these direct consequences of mismatches are readily treated or even avoided altogether. Given that these consequences can be treated or avoided, this part of the pathogenesis of hyperimmune disease can be described as the “optimal therapeutic zone” (Figure 1).

In this paper we describe how mismatches between culture and genetics/biology have led to specific consequences which cause immune system hypersensitivity and/or destabilization. All of these consequences are associated with a wide range of diseases that include allergic, autoimmune, and inflammatory diseases.

The single consequence of genetic/culture mismatches that most profoundly impacts hyperimmune-associated disease has been described as a depletion of components from the natural ecosystem of the human body, or simply “biome depletion” (Figure 1). The use of modern medicine, toilets, water treatment facilities, and cleaning agents in our culture has led to a substantial alteration or even loss of living organisms that have coevolved with humans. Three primary components of the biome which are potentially important have been affected [43]: (a) loss of interactions with soil bacteria, (b) alterations of the microbiome, the normal bacteria that are associated with the human body, and (c) loss of helminths, worms that typically inhabit the gut of mammalian species. Substantial evidence points toward all three of these factors as being important in stabilization of the human immune system. Although not widely appreciated, several lines of evidence point very strongly if not conclusively to the idea that helminths in particular are necessary for a stable immune system. This evidence has been reviewed extensively [1, 43] and can be very briefly summarized as follows.

(i) Helminths are found almost ubiquitously in preindustrial humans [44] and in nondomesticated mammals, including chimpanzees [45]. Even when helminths cannot be found, the immune system shows signs of stimulation by helminths [46].

(ii) Helminths produce and secrete a number of molecules which regulate the host immune system [47].

(iii) Addition of helminths to laboratory animals blocks allergic, inflammatory, and autoimmune diseases [47].
Addition of helminths to humans cures inflammatory bowel disease [48] and stops the progression of multiple sclerosis [49].

The effects of biome depletion on the immune system are not necessarily instantaneous because long-term effects of immune interactions with the biome can span decades or, through epigenetics, even generations [1]. Nevertheless, the epidemiology of hyperimmune disease reveals an inverse correlation between helminths and disease.

Humans with helminths and rodents colonized with helminths have profoundly less reactive immune systems than do either without helminths [44, 50, 51].

A long coevolutionary history of helminths and vertebrates, in conjunction with the potent immunoregulatory effects of the former, is consistent with the idea that the human immune system is literally, physically dependent on helminths [1].

We propose that biome depletion is changing the immune system at a population-wide level, which in turn will undoubtedly impact the brain, particularly during sensitive periods of development. Importantly, this model does not compete with any prior hypotheses or known risk factors for neuroinflammatory conditions, but rather complements them. For instance, biome depletion may set the stage for exaggerated levels of neuroinflammation, which interacts with other known risk factors or triggers (psychological or metabolic stress, infection, genetics or epigenetics) in the induction of autism.

8. Mismatches between Normal Human Genetics and Post-Industrial Culture Leading to Hyperimmune Epidemics: Vitamin D Deficiency

Based on the available information outlined above, it seems very likely that biome depletion is by far the most significant and most profoundly influential consequence of genetic/cultural mismatches in postindustrial society. However, other consequences which apparently exacerbate the effects of biome depletion are evident. For example, vitamin D deficiency has reached epidemic proportions in postindustrial society, is known to impact inflammation during early development [52], and, like biome depletion, has been linked to a spectrum of allergic, autoimmune, and inflammatory diseases [53–55]. Further, epidemiology and other circumstantial evidence link vitamin D deficiency to autism [56, 57]. Interestingly, the widely publicized association between rainfall and autism [58] can be accounted for, perhaps more than in part, by decreased exposure to sunshine in areas with high rainfall, and the subsequent impact of lower vitamin D levels.

Vitamin D deficiency is another consequence of postindustrial culture’s mismatches with human biology (Figure 1). Vitamin D production requires exposure of oils on the body’s surface to sunlight. The resulting photochemical reaction and production of vitamin D is greatly reduced
by components of postindustrial culture that reduce exposure to sunlight (e.g., indoor work environments, sunscreen).

The association of vitamin D deficiency with a spectrum of hyperimmune-associated diseases places this consequence of biology/culture mismatch alongside biome depletion as an underlying agent destabilizing immune system function in postindustrial populations. However, vitamin D deficiency is an ancient problem, as indicated by the ancient occurrence of rickets, whereas epidemics of hyperimmune-associated diseases are more recent in nature. Thus, it seems likely that vitamin D deficiency is a contributor to hyperimmune-associated disease, but not sufficient by itself to lead to disease. Further, the prevalence of vitamin D deficiency in postindustrial society is somewhat less than the prevalence of biome depletion: although the prevalence of vitamin D deficiency is substantial, with one study finding 24% of individuals deficient ($\leq 20$ ng/mL) with an additional 34% having levels that were insufficient ($\leq 29$ ng/mL) [59], the prevalence of biome depletion in postindustrial populations is essentially 100%. Thus, it is expected that the impact of vitamin D deficiency on immunity in postmodern culture may be a matter of exacerbating the problems associated with biome depletion more than a significant problem when considered in isolation.

Some mechanisms by which vitamin D might stabilize the immune system have been elucidated. Vitamin D acts as a signaling molecule to enhance immune cell function in the presence of infection and is important for maintaining the normal interface between the microbiome and the immune system [60]. However, the mechanisms by which vitamin D interacts within the body are complex, with the molecule being intertwined either directly or indirectly in virtually all aspects of human biology. The active form of vitamin D binds and activates the Vitamin D receptor, altering the expression of a variety of genes involved in such diverse processes as cell growth and proliferation, bone remodeling, and calcium homeostasis [61]. It has been estimated that vitamin D binds human DNA in more than 2,500 places and changes the expression of more than 200 genes [62]. Thus, while it is known that vitamin D is required for normal immune function, it is likely that vitamin D deficiency could cause a general destabilization of the human biome independent of the immune system, particularly during early development. Fortunately, regardless of the extent to which vitamin D deficiency destabilizes the human biome, dietary supplements are readily available, and thus this consequence of culture/biology mismatch is easily avoided.

9. Mismatches between Normal Human Genetics and Postindustrial Culture Leading to Hyperimmune Epidemics: Other Factors

The above discussion points out two factors, primarily biome depletion and secondarily vitamin D deficiency, which generally destabilize the human immune system. These factors can be identified by their association with a wide range of hyperimmune-associated diseases. Other consequences of postindustrial culture can also be found which are associated with a wide range of disease, but limits in space prevent a complete discussion. For example, chronic uncontrollable psychosocial stress is yet another factor that is generally associated with a range of hyperimmune-associated diseases that include allergy, autoimmunity, and inflammation [63–66]. Such stress derives from a complex range of biology/culture mismatches, including, for example, the loss of physical exercise [67–69] as a requirement for survival in postindustrial culture (Figure 1). As another example of consequences of biology/culture mismatches which destabilizes immune function, deprivation from mother’s milk during infancy (not included in Figure 1) is associated with a wide range of allergic and autoimmune conditions [70–74]. This genetic/culture mismatch, like other mismatches, involves an aspect of postindustrial society which should not be reversed (the survival of infants despite the unavailability of mother’s milk), and thus fits into the “pretherapeutic zone” of Figure 1. The extent to which factors such as uncontrollable stress and lack of mother’s milk can contribute to disease in the presence of an otherwise normal biome remains to be elucidated, although, as outlined above, biome depletion appears to be the single most important factor contributing to the increased incidence of hyperimmune-associated diseases.

10. Metabolism and Transport of Toxins and Hyperimmune-Associated Disease

The connection between metabolism and immunity is important in a consideration of hyperimmune-associated diseases. A variety of enzymes have been identified which are useful in the biotransformation of endogenous (produced by the body or the associated biome) as well as exogenous (pharmaceutical) substrates. This system of enzymes provides the body with a means of processing a wide range of substances, some of which are toxins, and is profoundly affected by the immune system [75]. The metabolic portion of this system is readily divided into two components: Phase I biotransformation includes the oxidative reactions of the microsomal cytochrome P450 monooxygenase system that is expressed in the liver and gastrointestinal tract, and to some extent in a variety of extrahepatic tissues. Phase II biotransformation includes conjugation reactions of substrates with endogenous cofactors such as glucuronate, sulphate, acetate, or glycine.

In addition to Phase I and Phase II biotransformation, the transport of their substrates and byproducts is also an important component of this system. A variety of membrane spanning polypeptides have been identified in the liver, gastrointestinal tract, kidney as well as other tissues in the body, each responsible for the active trafficking of substrates across cell membranes. These transport proteins are in fact regulated by the same pathways that regulate the Phase I and II enzymes, and they can be thought of as being regulated in tandem [76–80].

Immune activation results in a downregulation of Phase I components [81, 82], transporters [83, 84] and potentially
Phase II components in what might be viewed as a tradeoff between immunity and metabolism. In time of duress, when the immune system is geared to inflammation and defense, the energy devoted to metabolism/transport may need to be temporarily diverted to host defense. This is apparently an effective strategy, but as a consequence, chronic immune activation could result in metabolic difficulties as outlined in Figure 1. Unfortunately, vitamin D deficiency is expected to make this problem worse, since vitamin D is required to upregulate both Phase I and Phase II components [85] of metabolism. Further, accumulation of toxins as a result of reduced Phase I, Phase II, or transport activity may also stimulate the immune system, adding further to the propensity for hyperimmune activity.

11. Implications of Autism as a Member of the Family of Hyperimmune-Associated, Postindustrial Diseases

The implications of this model are several-fold and deserve careful consideration. The consideration of some possible assertions that are not supported by this model is probably equally as important.

(i) This model indicates that prophylactic normalization of the biome will result in a profound decrease in the incidence of autism. Results with other hyperimmune-associated diseases have been promising, although a true normalization of the biome as a means of prevention has never been attempted for any disease.

(ii) This model is consistent with the idea that a variety of factors affecting immunity and/or metabolism/transport may strongly influence the incidence of autism.

(iii) There are several potential therapeutic approaches that this model does not suggest which will necessarily be successful. For example, this model does not predict that helminths will necessarily work as a cure for autism. Whether helminths can be used to treat autism will depend on the extent to which autism is reversible and the extent to which ongoing problems with biome stability can be reversed.

(iv) Although porcine whipworms are currently being used to treat the effects of biome depletion in some studies, the model described in this paper does not predict that the porcine whipworm will prevent autism, even prophylactically. (Although the use of porcine whipworms is currently underway for a few clinical studies, the use of porcine whipworms as a means of prophylaxis is probably not economically feasible because the costs of giving the necessary biweekly doses would be prohibitive. This problem is not expected when using helminths adapted to humans, since one dose lasts for years.) It might, but this potentially depends on the extent to which this species recapitulates the presence of a normal biome. Thus, if porcine whipworms do not work, it does not suggest that the hypothesis is wrong.

(v) This model does not suggest that helminths are the only organisms involved in biome depletion. Normalization of the microbiome may also be a critical factor. In the face of modern practices in obstetrics and of widespread antibiotic use, this is an issue that must be considered.

(vi) Pharmaceuticals may never prove adequate. The complexity of the immune system and the effects of biome depletion suggest that efforts to readjust the system using drugs may be naive and overly optimistic. Biome reconstitution may be the easiest and most straightforward way to bring the system into homeostasis. Similarly, metabolic factors may not easily be compensated for by pharmaceuticals. Normalization and regulation rather than compensation and suppression are probably the better approaches.

(vii) Genetics may be relatively unimportant from a clinical perspective because it may not provide a means to prevent or treat disease. Triggers may also be unimportant from a clinical perspective because (a) they may simply be unavoidable, and (b) even if triggers for a particular disease can be identified and avoided, such efforts may simply decrease the prevalence of one disease at the expense of an increased prevalence of another.

12. The Required Approach

Clinical trials are urgently needed to conclusively test a variety of questions. The approach needs to be extensive and systematic rather than timid and piecemeal. The tests required will be on a large scale, although this scale pales in comparison to the current amount of effort and energy being poured into research on allergic, inflammatory, and autoimmune issues. This approach to medicine will not likely be supported by any committee of experts containing members with vested interests in traditional immunological approaches (either in humans or in rodents) or in the pharmaceutical industry. Rather, this approach is much more likely to be appreciated by those individuals with a broad understanding of biology and whose primary interest is in improving health care and, ultimately, the health of the population as a whole. With that in mind, the following questions need to be answered.

(i) Can autism be cured or effectively treated with biome reconstitution, or only prevented by biome reconstitution?

(ii) How important for children is the biome of the mother prior to and during pregnancy, and while breastfeeding? (Does prevention require reconstitution of the mother’s biome?)

(iii) Which helminths or combination of helminths is both safe and effective? Although some concerns dealing with the use of helminths for therapy have
been expressed, these objections are easily dealt with [1]. Several candidate species are well adapted to humans, asymptomatic in low numbers, and thus are obvious candidates for initial and immediate testing. Further, new technologies might be developed which could improve on species that are naturally occurring. For example, irradiation of organisms to achieve sterility and eliminate the possibility of transmission, or cultivation of human-specific helminths in ultraclean and immunodeficient rodents or even in vitro might improve the utility of helminths for widespread medical use.

(iv) What medical conditions such as a suppressed immune system, anemia, or coagulopathy might be contraindications for biome reconstitution?

(v) Does optimal biome reconstitution vary with human genotype or other factors such as age, gender, and body size/composition?

(vi) In addition to the restoration of helminths to the biome, what steps should be taken for restoration and maintenance of the microbiome?

(vii) How will a reconstituted biome affect other areas of medicine, including aging, immunosuppression, infectious disease, cancer biology, and vaccine technology?

13. Is Autism a Postindustrial, Hyperimmune-Associated Epidemic?

The idea that autism is a result of evolutionary mismatches is predicated on the idea that autism is epidemic in postindustrial society. Thus, if the incidence of autism is the same in preindustrial societies as it is in postindustrial societies, then biome depletion is not a factor in the pathogenesis of autism, and biome reconstitution will not affect the incidence of autism. The epidemiology of autism has been hotly debated. Some changes in diagnostic criteria and awareness of autism have certainly affected changes in the reported incidence of autism over time. However, debates regarding the changing diagnosis of autism over the past few decades have little bearing on the idea that biome depletion profoundly influences autism. Rather, the pertinent question is whether the prevalence of autism has changed over the past 150 years as various cultures have transitioned from pre to postindustrial over the course of several generations. Although it may be very difficult if not impossible to estimate the prevalence of autism prior to the industrial revolution in countries that are currently postindustrial, it might still be possible to examine the prevalence of autism in some preindustrial societies. One very recent study by Schieve et al. is particularly informative in this regard: using a phone survey technique to obtain data on 4,690 Hispanic children, a “striking heterogeneity” in the prevalence of autism spectrum disorder between US-born Hispanic children with 2 US-born parents (autism spectrum disorder prevalence 2.39%) and otherwise similar children with 2 foreign-born parents (autism spectrum disorder prevalence 0.31%; \( P = 0.05 \)) that autism is a consequence of evolutionary mismatches in postindustrial society and is consistent with the idea that the parental immune status is important in the development of autism. Further assessment of autism in preindustrial cultures may, however, prove unnecessary; the easiest way to evaluate the idea that autism is related to biome depletion is probably to conduct the experiment. Whether prophylactic biome reconstitution does or does not affect the incidence of autism will answer all questions conclusively, just as the effects of biome reconstitution [49, 87] and vitamin D supplementation [88] on multiple sclerosis, if confirmed by future studies, will effectively end long-standing debates about the etiology of that disease.

The idea that autism is a result of evolutionary mismatches is predicated on the idea that autism often occurs in genetically “normal” individuals. Thus, if autism can be attributed to genetic factors which dictate the presence of disease even in individuals living in a preindustrial society, then biome depletion is probably not a factor in the pathogenesis of autism, and biome reconstitution will probably not affect the incidence of autism. Certainly there are cases where autism is associated with genetic mutations which are deleterious, such as fragile X syndrome, but at present most individuals with autism appear genetically normal [89]. However, it remains unknown what percentage of autism can be attributed directly to genetics, and the idea that biome depletion and other evolutionary mismatches affect mutation rates is speculative and unexplored. Again, the most effective approach to resolving the debate regarding the role of genetics may be to prophylactically reconstitute the biome of a sample population and await the results.

The epidemiology and the genetics of autism are inextricably woven together. It is anticipated that one of two pictures will emerge. Either a postindustrial epidemic of autism with normal genetics will be identified, or autism will be eventually defined as a “normal” part of the human condition, potentially associated with certain genes or combinations of genes that predispose to disease regardless of the environment. In the former view, autism is due to an evolutionary mismatch, whereas in the latter view, autism is due to evolution itself. It is of critical importance not to accept the latter view prematurely since such acceptance may disable efforts at compensating for evolutionary mismatches or discourage work on identifying postindustrial triggers for disease, with potentially tragic consequences. Fortunately, several lines of evidence argue against the idea that autism is a “normal” part of human biology. First, a general consideration of the nature of human evolution argues that evolution is not responsible for autism. The social interactions disrupted by autism are fundamental to survival of not only humans, but also a wide range of mammalian and nonmammalian species, pointing to the ancient evolutionary origins of those interactions. Given the intense selection pressures on early human populations, it seems unlikely that an error rate of 1% in social interactions would have developed and then persisted for many tens of thousands of years. However, the idea that autism was tolerated during human evolution because it is essentially a tradeoff, an inherent “side-effect” of human brain function...
that is required for survival, cannot be ruled out. A second line of evidence pointing in favor of autism as a result of evolutionary mismatch rather than evolution is the study, described above, showing that parental birth in a postindustrial culture seems to predispose offspring to autism [86]. Third, the observations (a) that the immune system is profoundly destabilized by postindustrial culture and (b) that brain development is closely tied with immune function, when considered together, argue strongly for the idea that it is an evolutionary mismatch, not evolution, which is responsible for autism. Indeed, to the extent that human brain development is strongly tied to the immune system, it would be difficult to explain the idea that postindustrial culture and the resulting consequences of evolutionary mismatches such as biome depletion do not profoundly affect brain development. Finally, the strong association of autism with autoantibody production [3] and other immune abnormalities points toward the etiology of autism as straight forward: another epidemic in the long list of hyperimmune associated epidemics caused by biome depletion in postindustrial society. The fact that this particular disease affects primarily the brain rather than other organs or tissues undoubtedly leads to the tremendous complexity in the pathology associated with autism, but this complexity should not blind the medical community to the simple roots that apparently underlie epidemics of all allergic and autoimmune diseases, or to the apparent solutions that can currently be implemented to prevent those diseases [1].

14. Summary/Conclusions

Autism has many of the characteristic features of hyperimmune-associated diseases which result from biome depletion and other consequences of biology/cultural mismatches in postindustrial culture, potentially sharing a common cause and potentially a common solution with those diseases. The identification of triggers for autism (e.g., association with viral infection during pregnancy [90]) parallels that of other diseases in this family. However, the trigger that causes disease is often unrelated to the cultural mismatches with human biology which destabilize the human biome and make the trigger dangerous. The association of autism with genetic and probably epigenetic factors also parallels that of other hyperimmune-associated diseases, but offers little in the hope of prevention, treatment, or a cure.

The ecosystem of the human body, the human biome, is a tightly interwoven collection of factors which includes immunity, brain function, sunlight, hormones, metabolism, transport of metabolites, the microbiome, and helminths. It stands firmly to reason that if one or more of these components are profoundly altered or even deleted, the entire system may become destabilized. This view of ecosystems has long been appreciated by ecologists [91]. Although some hyperimmune diseases might be eliminated if the respective triggers for those diseases are identified and avoided, such as approach cannot be successful in eliminating the overall problem; roughly 50% of children in postindustrial society have some form of chronic health condition [92], many millions are affected by a wide range of allergic, inflammatory, and autoimmune disorders, and inflammation as a result of biome destabilization may play a key role in such common maladies as heart disease and dementia. With that in mind, the need for normalization of the biome cannot be overemphasized, regardless of particular diseases that might be prevented by identifying and avoiding the environmental trigger. Thus, while it is hoped that a trigger that causes autism might be confidently identified in the near future, and new cases of autism might be greatly reduced as a result, it is hypothesized that normalization of the human biome will be necessary to avoid the full milieu of chronic immune-related diseases that plague postindustrial society.

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References


Research Article

Prenatal and Postnatal Epigenetic Programming: Implications for GI, Immune, and Neuronal Function in Autism

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Although autism is first and foremost a disorder of the central nervous system, comorbid dysfunction of the gastrointestinal (GI) and immune systems is common, suggesting that all three systems may be affected by common molecular mechanisms. Substantial systemic deficits in the antioxidant glutathione and its precursor, cysteine, have been documented in autism in association with oxidative stress and impaired methylation. DNA and histone methylation provide epigenetic regulation of gene expression during prenatal and postnatal development. Prenatal epigenetic programming (PrEP) can be affected by the maternal metabolic and nutritional environment, whereas postnatal epigenetic programming (PEP) importantly depends upon nutritional support provided through the GI tract. Cysteine absorption from the GI tract is a crucial determinant of antioxidant capacity, and systemic deficits of glutathione and cysteine in autism are likely to reflect impaired cysteine absorption. Excitatory amino acid transporter 3 (EAAT3) provides cysteine uptake for GI epithelial, neuronal, and immune cells, and its activity is decreased during oxidative stress. Based upon these observations, we propose that neurodevelopmental, GI, and immune aspects of autism each reflect manifestations of inadequate antioxidant capacity, secondary to impaired cysteine uptake by the GI tract. Genetic and environmental factors that adversely affect antioxidant capacity can disrupt PrEP and/or PEP, increasing vulnerability to autism.

1. Introduction

Neurological and behavioral symptoms implicate abnormal brain development as a core pathophysiological feature of autism, but increasing evidence also indicates immune [1–8] and gastrointestinal (GI) abnormalities in a significant subset [1, 8–12]. This triad of dysfunctional systems provides not only a more complete description of autism and autism spectrum disorders (ASDs), but also an important opportunity to consider the mechanisms which could result in the shared involvement of these three particular systems, especially in the context of development. Recent elucidation of the central role of epigenetic regulation of gene expression in development presents a molecular framework within which prenatal and postnatal maturation of neuronal, immune, and GI systems can be viewed.

As all cells possess the same DNA, their differentiation into various cell types reflects stable suppression of some genes and activation of others, accomplished in large part by epigenetic regulation. Such regulation involves reversible modifications of both DNA nucleotides and histone proteins [13]. These modifications or epigenetic marks favor or disfavor formation of nucleosomes, DNA/histone complexes that maintain genes in a compacted, inactive state (heterochromatin). Methylation of DNA is the most fundamental epigenetic mark, enabling binding of proteins containing methylDNA binding domains, such as methyl CpG binding protein 2 (MeCP2). Proteins with methylDNA binding domains recruit other proteins, including those capable of modifying histones at their tail regions [14]. Multiple sites and forms of histone modification (e.g., methylation, acety-
lation, and phosphorylation) make this a highly complex mode of gene regulation, affected by diverse signaling pathways that adjust gene expression to local cellular metabolic conditions [15]. Whereas certain epigenetic marks are to some degree reversible, others are not readily reversed and, once in place, these may be sustained for an entire lifespan and/or be transmitted across generations through germline modifications [16]. Accordingly, epigenetic marking or programming early in development has the potential to exert long-lasting effects [17].

There is considerable evidence that epigenetic programming is highly sensitive to changes in the cellular environment, as broadly defined [18]. Indeed, it appears that epigenetic regulation is a widely utilized adaptive mechanism to allow cells to maintain a favorable metabolic status under different conditions, including differential exposure to physiological substances (e.g., hormones, neurotransmitters, or growth-regulation factors), xenobiotics (e.g., pollutants, toxic chemicals), or even infectious agents (e.g., bacteria or viruses, fungi or parasites) [19]. Epigenetic regulation may facilitate adaptation to changes in the cellular environment through stable alterations of cellular phenotype, potentially resulting in progressive differentiation and maturation during fetal and possibly postnatal development [20].

During prenatal development, nutritional support to the fetus is provided via the transplacental circulation as a function of the available maternal nutriture, with metabolic consequences for both the mother and developing child. During postnatal development, nutritional support is provided by oral food intake into the GI tract: breast-milk- or cow-milk-based formula at first, followed by introduction of other foods, usually in a controlled, gradual manner, but the provision of adequate nutritional resources to support further development of the newborn is not assured. Thus, the prenatal/postnatal transition is a critical juncture in metabolic adaptation. This transition is particularly linked to aerobic metabolism, as the delivery of oxygen via newborn lungs and its ultimate rate of utilization must be balanced by the available antioxidant capacity of body tissues. Epigenetic regulation offers an opportunity for adaptation during this metabolic transition, allowing the level of ongoing aerobic metabolism in each organ system, tissue, and cell type to be maintained in homeostatic equilibrium with antioxidant metabolism.

We use the term postnatal epigenetic programming (PEP) to describe the ongoing adaptive changes in gene expression occurring in response to the transition from fetal to postnatal metabolism, as distinct from prenatal epigenetic programming (PrEP), at term that recognizes the dynamic occurrence of similar changes which occur in utero. While it is clear that autism can result from a variety of genetic and environmental factors, by focusing on factors affecting antioxidant and methylation status in the developing GI tract, immune system, and brain, we hope to illuminate the pathological mechanisms underlying ASDs and other related disorders.

2. Materials and Methods

2.1. Purification of Regulatory T Cells. To purify CD4+ CD25+ regulatory T cells, spleen cells and lymph node cells from C57BL/6 mice were combined as a cell source. The cells were labeled with FITC-conjugated anti-CD24 and anti-CD8 mAbs and with anti-FITC microbeads. After the removal of CD8+ T cells and CD24-expressing B cells using an AutoMACS separator (Miltenyi Biotec, Auburn, CA), regulatory T cells were purified by positive selection of CD25+ cells using PE-conjugated anti-CD25 mAb and anti-PE microbeads. All antibodies except for anti-CD25 mAb were from BD Biosciences (San Jose, CA). Other materials were from Miltenyi Biotec. Purity of CD4+ CD25+ cells was higher than 95%.

2.2. RNA Isolation. RNA was isolated using the RNAqueous-4PCR kit (Ambion, Naugatuck, CT), then treated with DNase to eliminate contaminating genomic DNA, and quantified using an ND-1000 NanoDrop spectrophotometer.

2.3. cDNA Synthesis. cDNA synthesis was performed using the First strand cDNA synthesis (Roche, Nutley, NJ). 1 μg RNA was used as template, along with 1 mM dNTP mix, 60 μM random hexamer primers and dH2O to make a final volume of 25 μL. Samples were denatured at 65°C for 5 minutes and then placed on ice. Transcriptor RT (20 units/μL), Protector RNase inhibitor (40 U/μL), 5x Transcriptor Reverse Transcriptase Reaction Buffer, and dH2O in a final volume of 7 μL were used to bring the final volume to 20 μL. This was followed by incubation at 25°C for 10 min followed by 30 min incubation at 55°C. The reverse transcriptase enzyme was inhibited by incubating at 85°C for 5 min and then cooling on ice.

2.4. Primers. Primers were designed using the Invitrogen OligoPerfect Designer to have approximately 50–60% GC content, an annealing temperature of 60°C, and a length of 20 bases. Glyceraldehyde phosphate dehydrogenase (GAPDH) expression was used for normalization. Primers for EAAT3 (designated EAAC1 in mice) and GAPDH were EAAT3-Forward: 5'-TTACAGCCACCGTGGCAAGC-3’; EAAT3-Reverse: 5’-GGCACGCCACAGCATCGTACG-3’; GAPDH-Forward: 5’-TCTCCACACCTGTTGCAA-3’; GAPDH-Reverse: 5’-CAAGCAACAGGGAGCTGAG-3’.

2.5. qRT-PCR Analysis. qRT-PCR was performed on duplicate samples using the LightCycler 480 from Roche. 5 μL of cDNA was used for qRT-PCR, along with 10 μM sense and antisense primers, 10 μL SYBR Green I Master from Roche, and dH2O in a final volume of 20 μL. The following thermal parameters were used: incubation for 5 min at 95°C, followed by 45 cycles of 95°C for 10 sec, 60°C for 20 sec and 72°C for 30 sec, and lastly a single cycle of 95°C for 5 sec, 1 min at 65°C and 97°C for the melting curve. No template controls were run on each plate and dissociation curves were generated to determine any nonspecific products. Data was normalized to
GAPDH, and the ΔCt second derivative method was used for analysis of EAAT3 expression.

2.6. MS Assay. Brain cortex samples were obtained from thimerosal-treated or untreated C57BL/6 and SJL/J mice, as per the previously published protocol [21]. MS activity in mouse cortex was determined by measuring incorporation of radiolabel from [methyl-14C]methyltetrahydrofolate into methionine, as previously described [22]. Enzyme assays were performed under anaerobic conditions by bubbling nitrogen gas through stoppered vials for 1 hour at 37°C and terminated by heating at 98°C for 2 min after which samples were cooled on ice. Radiolabeled methionine was separated from unreacted radiolabeled methylfolate by passing the reaction mixture through an anion exchange column. The column was washed with 2 mL of water and the aqueous samples were collected and counted. Reported values are normalized to protein content and corrected for the counts observed in control assays in which sample enzyme was omitted.

2.7. GSH Measurement. A 100 μL aliquot of cortex supernatant was incubated with 2 μL of monochlorobimane (25 mmol/L) and 2 μL of glutathione-S-transferase reagent was added, as provided by a commercial kit (Biovision, Mountain View, CA). After a 30 min incubation at 37°C, fluorescence was read at 380 nm excitation and 460 nm emission. GSH content was determined by comparison with values from a standard curve using freshly prepared GSH.

2.8. Statistical Methods. Statistical analysis was carried out using Graph Pad Prism version 5.01. Data was expressed as mean ± standard error of the mean (SEM). Student’s t-test was conducted to determine the differences between individual groups.

3. Results and Discussion

3.1. Antioxidant Capacity and Aerobic Metabolism. Aerobic metabolism is unavoidably accompanied by the risk of oxidative damage, most notably caused by reactive oxygen species (ROS) generated as by-products of mitochondrial oxidative phosphorylation and ATP production. Increased metabolic activity requires higher rates of ATP production and increased ROS formation. Maintenance of redox equilibrium requires inactivation of ROS before these by-products can oxidize cellular components, as well as repair of oxidized biomolecules (proteins, lipids, DNA, and RNA). Catalase, superoxide dismutase and the selenoprotein glutathione peroxidase inactivate ROS, and a wide array of redox-active proteins and enzymes participate in the repair of oxidized molecules and the maintenance of thiols in their reduced state, with glucose-derived NADPH serving as the source of reducing electrons [23, 24].

As elegantly reviewed by Schafer and Buettner [25], the equilibrium between reduced and oxidized forms of glutathione (GSH and GSSG, resp.) is the primary determinant of intracellular redox status. The relatively high concentration of GSH within the cell imposes a pervasive influence on essentially all aspects of metabolism. Although all cell types and tissues rely upon the same core antioxidant mechanisms, variations on this central theme are recognized. For example, the intracellular level of GSH in liver hepatocytes is close to 10 mM [25], whereas in neurons it is 0.2 mM, some 50-fold lower [26].

As the rate of ROS formation must be quantitatively balanced by the available antioxidant capacity, a multitude of mechanisms have evolved to restrict aerobic metabolism when antioxidant status is low and vice versa. One prominent example involves glutathionylation of cysteine residues in complex I of the mitochondrial electron transport chain, which limits the flow of electrons, thereby restricting downstream ROS formation [27]. In glutathionylation, GSGG, the oxidized form of glutathione, reacts with a reduced cysteine, resulting in GSH release and modification of the protein in proportion to the prevailing redox state [28]. This regulatory mechanism not only controls ROS formation but also provides an auxiliary pathway for glutathione reductase activity and production of GSH. Additional intracellular control over the balance between ROS and antioxidant reservoirs is achieved by the capacity for reversal of the glutathionylation of complex I by the action of glutaredoxin 2, in conjunction with the selenoprotein thioredoxin reductase, using NADPH-derived electrons [27].

More than ten studies have reported significantly lower plasma levels of GSH in autism, accompanied by lower levels of cystine [29–39]. Among these studies, the average decrease in GSH was 37%, representing a substantial reduction in antioxidant capacity. Several studies also reported a significant decrease in the GSH to GSSG ratio [8–20, 22–36], indicative of oxidative stress, consistent with other reports of increased biomarkers of oxidative stress [29, 32, 40]. One consequence of persistent oxidative stress, an increase in glutathionylation of complex I of the mitochondrial electron transport chain, provides a potential explanation for the mitochondrial dysfunction frequently reported in autism [7]. Genetic factors affecting mitochondrial function may interact with oxidative stress. For example, SLC25A12, the gene encoding the Ca2+-dependent aspartate-glutamate carrier isoform 1 (AGC1), has been identified as a putative autism susceptibility gene [41]. AGC1 facilitates the supply of NADH for oxidative phosphorylation, and elevated Ca2+ levels will, therefore, increase ROS formation via increased AGC1 activity. Higher Ca2+-dependent AGC1 transport rates were demonstrated in autistic subjects [41], and it has been proposed that dysregulated Ca2+ levels may be a broadly influential factor in causing autism [42].

3.2. Intestinal Absorption of Cysteine. Starting from birth, the systemic availability of cysteine to support GSH synthesis is dependent upon absorption of the food-derived sulfur-containing amino acids cysteine and methionine from the GI tract (Figure 1). Adequate GI uptake of selenium is also critical for maintaining GSH in its reduced state. Intestinal epithelial cells express different transporters on
Figure 1: Absorption and systemic distribution of dietary cysteine and methionine. GI epithelial cells take up cysteine and methionine, with EAAT3 being particularly important for cysteine uptake in the distal ileum. A portion of methionine is converted to cysteine via the methionine cycle and transsulfuration of homocysteine. Absorbed cysteine is taken up by the liver, which releases oxidized cystine. Cystine, but not cysteine, is able to traverse the blood brain barrier and is taken up by astrocytes, which convert it to GSH. Cysteine from astrocyte-derived GSH is available for EAAT3-mediated neuronal uptake, supporting neuronal GSH synthesis.

Among amino acid transporters in GI epithelial cells, EAAT3 (excitatory amino acid transporter 3, EAAC1) is selective for cysteine and was initially cloned from GI epithelial cells [43]. Subsequent studies revealed that EAAT3 is most prominently expressed in the small intestine, especially in the terminal ileum [43, 44], a prominent site of inflammation in subjects with autism [45]. The highest levels were found in crypt cells and lower villus regions, the locus of multipotent stem cells that sustain the epithelial lining of the gut [44]. EAAT3 is a member of a family of Na⁺ - and H⁺ -dependent amino acid transporters named for their ability to transport glutamate and aspartate, but EAAT3 is unique in its preference for cysteine. EAAT3 exists in equilibrium between the endoplasmic reticulum and the plasma membrane, and its transport activity is regulated by the PI3 kinase signaling pathway, similar to insulin regulation of glucose transporters [46]. In resting cells, about 75% of EAAT3 resides inside the cell, where it is inactive, but activation of either PI3 kinase or protein kinase C increases the proportion of active transporters in the plasma membrane. Oxidative stress inhibits EAAT3 transport activity [47], and several cysteine residues have been identified as being critical for this mode of regulation, raising the possibility that they might be targets for glutathionylation. Oxidative stress affecting gut epithelium would, therefore, decrease EAAT3-dependent absorption of cysteine, with the local and systemic consequences of lower GSH levels.

Cells express a multitude of adaptive mechanisms that allow them to maintain homeostatic redox equilibrium, of which the Keap1/Nrf2 system is a prominent example. Nrf2 (nuclear factor erythroid 2-like 2) is a transcription factor protein with a high turnover rate, that is, tightly bound to Keap-1 (Kelch-like ECH-associated protein 1); however, in response to a more oxidative redox state, Nrf2 is released from Keap-1 and moves to the nucleus where it promotes expression of a large number of genes via binding to antioxidant response element (ARE) sites [48]. It has been recently demonstrated that Keap1/Nrf2 mediates up-regulation of EAAT3 in response to oxidative stress [49].

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Similar to autism, GI disorders such as celiac disease and inflammatory bowel disease have an autoimmune component [50], and diets excluding gluten and/or casein (i.e., GF/CF diet) are frequently beneficial in both conditions. Prompted by the recognition that digestion of both gluten and casein yields peptides with opiate activity [51, 52], we recently examined the activity of these peptides on EAAT3-mediated cysteine uptake and redox status in human GI epithelial and neuronal cells and found that they significantly increase the release of glutathione.
Figure 2: Redox and methylation pathways in neurons. The amino acid cysteine is rate-limiting for glutathione (GSH) synthesis and it is provided either by uptake from astrocyte-derived cysteine or by transsulfuration of homocysteine (HCY). The methionine cycle of methylation (lower right) depends upon both dietary methionine (MET) and remethylation of HCY by methionine synthase. Since formation of HCY from S-adenosylhomocysteine (SAH) is reversible and SAH inhibits methylation, decreased methionine synthase activity (e.g., caused by oxidative stress) both augments GSH synthesis and inhibits methylation reactions. Thus, redox status and methylation activity are closely linked. The D4 dopamine receptor carries out a cycle of dopamine-stimulated phospholipid methylation which is completely dependent upon methionine synthase and is therefore also sensitive to oxidative stress. Growth factors promote EAAT3 activity by increasing its location on the cell surface. EAAT3 activity is inhibited by oxidative stress and by food-derived opiate peptides.

3.3. Postnatal Epigenetic Regulation (PEP). Methylation of DNA and histones is fundamental to epigenetic regulation of gene expression, providing factors affecting methylation capacity with an opportunity to affect development. S-adenosylmethionine (SAM) is the donor of methyl groups to more than 200 methyltransferase reactions, including DNA and histone methyltransferases [54], whereas its demethylated form, S-adenosylhomocysteine (SAH), inhibits methylation by virtue of its competition with SAM. The SAM to SAH ratio determines methylation capacity for all methylation reactions, providing an exceptionally broad influence over cellular metabolism.

The SAM/SAH ratio is responsive to the activity of methionine synthase (MS), the folate, and vitamin B12-dependent enzyme that converts homocysteine (HCY) to methionine (MET) as part of the methionine cycle of methylation (Figure 2). Its vitamin B12 (cobalamin) cofactor renders MS activity highly sensitive to oxidative stress; changes in cellular redox status (shifts toward reducing or oxidizing conditions) are able to exert epigenetic effects via their influence on MS and the SAM/SAH ratio. Recognizing this metabolic relationship, we previously proposed a “redox/methylation hypothesis” of autism, linking this neurodevelopmental disorder to oxidative stress induced by xenobiotic exposures [55], and now we apply this perspective to epigenetic programming of GI and immune function.

During oxidative stress associated with GI inflammation, impaired EAAT3 activity will decrease availability of cysteine for GSH synthesis, leading to increased ROS levels, diminished MS activity, a decrease in the SAM/SAH ratio and a global decrease in methylation capacity, altering the pattern of DNA and histone methylation in epithelial cells. The distribution of EAAT3 suggests that this inflammation-induced epigenetic response will be most prominent in crypt stem cells of the terminal ileum, where it can influence both the proliferation and the functionality of the GI epithelium. Notably, the terminal ileum is also a critical location for folate and vitamin B12 absorption [56, 57].

Postnatal infant nutrition is most commonly provided by either human breast milk or bovine milk. Human breast milk is comparatively rich in sulfur-containing amino acids, and colostrum contains 2.5-fold higher levels versus mature milk [58]. A comparison of plasma cysteine levels in 12-week-old infants found significantly higher levels for breast-fed versus formula-fed infants [59]. Thus, breast feeding appears inhibited uptake [53]. Their action was associated with a decrease in GSH levels and the SAM/SAH ratio, along with alteration in the expression of redox-related genes. Important differences, however, were observed between peptides derived from bovine versus human casein, which may contribute to the well-recognized benefits of breastfeeding.
to provide a superior supply of the essential raw materials for synthesis of the antioxidant GSH. As this source of cysteine is made available, EAAT3 and the factors that modulate its activity serve as critical regulators of the extent to which cysteine may be absorbed.

Breast milk contains a number of different growth factors, including insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and transforming growth factor-β1 and β2 (TGF-β1/β2), among others [60, 61]. These factors activate PI3 kinase and mitogen-activated protein (MAP) kinase signaling pathways to achieve their growth-promoting effects, which are associated with increased metabolic activity and its attendant increase in ROS production. We recently demonstrated the ability of IGF-1 and other PI3 kinase-activating growth factors to stimulate EAAT3-mediated cysteine uptake in cultured human neuronal cells, accompanied by an increase in GSH levels, an increased SAM/SAH ratio, and increased DNA methylation [62]. HGF, acting via the MAP kinase pathway, increases expression of the two enzymes which convert cysteine to GSH [63]. Thus, growth factors promote GSH synthesis via both increased cysteine uptake and transcriptional effects, corresponding to short-term and longer-term effects, respectively. These observations imply that breast-milk-derived growth factors can stimulate cysteine uptake and increase GSH in the GI epithelium, coincident with the initiation of postnatal digestion. The extent to which antioxidant resources (i.e., cysteine and GSH) are made available to the rest of the body depends upon the efficiency of this process.

Human breast milk is also rich in selenium, and breast-fed infants have higher selenium levels than formula-fed infants [64]. A significant proportion (up to 30%) of breast milk selenium is in the form of glutathione peroxidase (GPx), which is hydrolyzed to selenocysteine during digestion. The importance of GPx activity in breast milk may be to combat oxidation, especially of vulnerable omega-3 fatty acids. However, GPx appears to also represent an important reservoir for delivery of selenium to the developing infant. As a structural analog of cysteine, breast-milk-derived selenocysteine can also be transported by EAAT3 and contributes to the antioxidant capacity and redox status [65].

As MS activity and the SAM/SAH ratio are responsive to redox status, it is reasonable to propose that the postnatal transition to independent nutrition is associated with epigenetic responses and that growth factor stimulation of cysteine uptake is a key event in this transition, which we call postnatal epigenetic programming or PEP. Although PEP is an ongoing body-wide process affecting the development of all tissues, but we specifically propose that the GI tract plays a uniquely important role as the source of nutritional support for metabolism, and that availability of antioxidant resources is the critical determinant of the level of metabolic activity that may be achieved without cellular damage. Epigenetic regulation of gene expression provides a molecular mechanism by which the functional state of the GI tract can be matched to the ongoing level of metabolic activity. In other words, the supply of antioxidant must be matched to the demand for antioxidant, and the rate of postnatal growth and development must be restricted so as to not exceed the supply provided by the GI tract.

As a developmental disorder in which the availability of GSH is reported to be reduced by almost 40%, autism can be viewed as a syndrome resulting from an imbalance between antioxidant supply and demand—essentially, a state of oxidative stress that interrupts the normal epigenetically based program of development. In the absence of a large-scale prospective study, it is not possible to determine whether the autism-associated GSH deficit is present from birth or begin during postnatal development. The former case would likely reflect a significant maternal deficit in antioxidant resources, and the finding that plasma levels of GSH and the SAM/SAH ratio are significantly decreased in mothers of autistic children [36] is consistent with such a possibility. Under the latter scenario, the imbalance might develop in a progressive manner during postnatal development if escalating metabolic activity and ROS production outstripped the ability of the GI tract to provide sufficient antioxidant, and inhibition of EAAT3-mediated cysteine uptake in response to developing oxidative stress would also exacerbate this self-reinforcing cycle. Postnatal exposure to agents which interfere with antioxidant capacity, including mercury, lead, pesticides, and other xenobiotics, could shift the redox balance sufficiently to initiate developmental regression secondary to impaired methylation and its epigenetic consequences.

Microbes within the GI tract compete for nutrient resources in a normally symbiotic manner, but dysbiosis can intervene when the normal array of organisms is displaced or skewed. GI dysfunction, which is common in autism [8–12], may be associated with a failure of the GI epithelium to absorb antioxidant nutrients, such as cysteine or selenocysteine. Increased availability of these critical antioxidant nutrients for intestinal microbes may encourage the growth of previously restricted microorganisms, while diminishing the growth of others. Once established, the dysbiotic microbial population will decrease availability of antioxidant nutrients for absorption by further competing with the host, perpetuating the antioxidant deficit.

The importance of PEP varies by cell and tissue types, depending upon the extent of the developmental maturation of specific cells and organs at birth. Delaying the development of selected neural networks (e.g., cortex and hippocampus) until after birth allows their programming to be guided by experience, in contrast to other networks (e.g., brainstem respiratory centers) which must function immediately upon birth. Indeed, unique features of human brain allow PEP to be dynamically exploited throughout the lifespan, providing the capacity for memory [66]. In a similar manner, maturation of the immune system is restrained until birth so as to optimize its response to environmental exposures, while at the same time minimizing its potential for autoimmunity. While this perspective encompasses
the triad of neuronal, immune, and GI symptom domains in autism, the prominence of each component may vary among individuals, reflecting, in part, genetic differences in the vulnerability to oxidative stress and impaired methylation.

3.4. Redox Signaling in Human Brain. As previously noted, cysteine availability is limited for GSH synthesis. However, the level of cysteine in cerebrospinal fluid (CSF) is only one tenth the level in blood [67], meaning that the raw material for making antioxidants is much scarcer in the brain. Indeed, the GSH level in neurons is the lowest reported for any cell type [25], despite the fact that the brain utilizes oxygen at a ten times higher rate than other tissues. As illustrated in Figure 2, neurons obtain their cysteine from neighboring astrocytes, which release GSH that is subsequently broken down to cysteine, which is then available for uptake by neurons, depending upon the level of EAAT3 activity, which is controlled by growth factors. Growth factors utilize redox status to regulate neuronal function via the intermediate involvement of MS, a form of “redox signaling.” By allowing more cysteine into cells and increasing GSH synthesis, growth factors increase MS activity and increase DNA methylation. These increases in MS activity and DNA methylation lead to epigenetic changes in gene expression as well as many other metabolic effects through >200 methylation reactions, all affected by the SAM/SAH ratio. The increase in antioxidant level also allows cells to safely increase their oxidative metabolism (that is, their mitochondrial activity); the resultant increase in ATP supports a higher level of neuronal activity. In this way, redox signaling not only regulates gene expression but also controls the level of neuronal function.

A second brain-specific feature that augments redox signaling is a brain-specific limitation in the level of transulfuration activity, which restricts conversion of HCY to cysteine [68]. This unique feature contributes to the low GSH level in neurons, making them more responsive to growth factor-induced EAAT3 activation. Together with the limited availability of extracellular cysteine, this makes epigenetic regulation exceptionally dynamic. Along with other methylation-dependent consequences of redox signaling, the long-term nature of epigenetic effects endows neurons with the ability to create “metabolic memories” that are coordinated with experience via the input of sensory systems. This view is consistent with recent studies identifying the central role of epigenetic changes in learning and memory formation [66, 69]. Compromised function of these redox-dependent systems could contribute to the types of cognitive function deficits and neurodevelopmental delays that are primary to autism.

The restricted availability of GSH in the brain is partly compensated for by the presence of alternate mechanisms for sustaining antioxidant capacity, including an increased dependence upon selenoproteins. The brain has developed the capacity to retain selenium under conditions of scarcity, even when other tissues become depleted. Nonetheless, reliance on and retention of selenoproteins are an insufficient means of maintaining antioxidant defenses under certain conditions, such as exposure to mercury. Selenoproteins are exquisitely sensitive to inhibition by mercury, and any mercury that penetrates the brain will interfere with redox signaling and disrupt normal epigenetic regulation [70, 71]. Very tight binding of mercury by selenoproteins, especially to selenoprotein P, contributes to the long-term retention of mercury in the brain and its accumulation across the lifespan, and it has been proposed that selenoprotein P, which contains ten selenocysteine residues, has evolved to serve a specific role to protect against mercury toxicity [72].

In addition to conversion of HCY to MET, MS carries out a second reaction, providing methyl groups to the D4 dopamine receptor; the D4 receptor, when stimulated by dopamine, subsequently transfers these methyl groups to membrane phospholipids—a unique activity carried out by the D4 dopamine receptor [73]. Genetic variants of the D4 receptor (such as the 7-repeat variant) have been linked to novelty-seeking behavior and are important risk factors for attention-deficit/hyperactivity disorder (ADHD) [74]. Although the role of dopamine-stimulated phospholipid methylation remains incompletely understood, we first proposed that it facilitates attention by modulating the frequency of neural networks, promoting their synchronization [75]; others subsequently confirmed dopamine-stimulated phospholipid methylation to be critically involved in neuronal synchronization during attention [76]. As D4 receptor-mediated phospholipid methylation is absolutely dependent upon MS activity, it is thus also dependent on EAAT3, raising the possibility that ADHD might involve a decrease in MS activity secondary to oxidative stress.

3.5. Redox and the Immune Response. In the past several years, a previously unappreciated role for redox regulation of the immune response has been elucidated, involving extrusion of GSH from activated antigen presenting cells (APCs, e.g., dendritic cells, macrophages, or B cells), followed by release of its cysteine, which is then taken up by T cells [77] This relationship is analogous to the relationship between astrocytes and neurons illustrated in Figure 2. Uptake of this cysteine by naïve CD4+ CD25− effector T cells (Teff) leads to their activation and proliferation, in association with increased GSH synthesis and the epigenetic consequences thereof. Activated Teff cells release cytokines that attract other cells and promote inflammation, as well as stimulate B cell activation and antibody production (Helper T cells). However, the extent of Teff cell activation is limited by CD4+ CD25+ Foxp3+ regulatory T cells (Treg) that compete with Teff cells for available extracellular cysteine, thereby suppressing immune and autoimmune responses [77]. Recognizing this newly described mode of T cell regulation, we hypothesized that EAAT3 might be involved in cysteine uptake. To evaluate this possibility, we used qRT-PCR to determine whether EAAT3 was expressed in murine T cell subsets,
EAAT3 expression normalized to GAPDH

(a)

EAAT3 expression normalized to GAPDH

(b)

Figure 3: Expression of EAAT3 in murine lymphocyte subsets. Spleen and lymph node-derived lymphocytes were separated by magnetic cell sorting, followed by qRT-PCR analysis for EAAT3 (EAAC1) expression. (a) EAAT3 expression was significantly higher ($P < 0.05$) in CD4+ T cells as compared to unseparated lymphocytes (UNSEP), CD8+ T cells, or B cells. (b) EAAT3 expression was significantly higher ($P < 0.05$) in FoxP3+ Treg cells.

Figure 4: GSH levels in frontal cortex of SJL/J and C57BL6/J mice with or without thimerosal treatment. GSH levels were significantly lower ($P < 0.05$) in cortex of SJL/J autoimmune-prone mice compared to C57BL6/J strain mice. GSH levels were not affected by thimerosal treatment.

expression by immune cells provides a mechanism for them to take up cysteine in a competitive manner, and higher EAAT3 expression in Treg cells may underlie the ability of this cell subset to limit the immune response and restrict autoimmunity.

The SJL/J strain of mice has been widely employed as a model for autoimmunity, but these mice also exhibit deficits in GSH, similar to those reported in autistic children. In SJL/J mice, the deficit is due to mutations in the enzymes which synthesize GSH; factors underlying GSH deficits in autism are as yet unknown. In a previous study, we showed that the thiol-reactive compound thimerosal caused growth impairment, as well as neurodevelopmental and neurochemical effects in SJL/J mice, but not in C57BL6/J mice, including upregulated brain levels of EAAT3 [21]. To further characterize the role of redox and methylation status in autoimmunity, we measured both GSH levels and MS activity in the liver and brain of control and thimerosal-treated SJL/J mice, as compared to C57BL6/J mice. GSH levels were significantly lower in SJL/J versus C57BL6/J mice, but not different in thimerosal-treated animals (Figure 5). These results confirm the occurrence of low GSH in the autoimmune-prone SJL/J strain, along with the novel finding that MS activity is reduced, although neonatal exposure to thimerosal did not alter these features. Expression of EAAT3 was significantly lower in CD4+ T-cells from SJL/J mice versus C57BL6/J mice, suggesting that autoimmunity is associated with impaired capacity for cysteine uptake (Figure 6). Together these findings provide support for the roles of oxidative stress and impaired methylation in autoimmunity.

Abnormal immune function, including, but not limited to, increased autoimmunity, has been extensively documented in autism. Of particular relevance are autoantibodies...
targeting brain and neuronal proteins [2, 3, 5] as well as autoantibodies targeting the folate receptor [78–80]. The latter were initially linked to the relatively rare cerebral folate deficiency syndrome [78] but recently were found to be present in a high proportion (75%) of autistic subjects [80], and a casein-free diet greatly reduced the circulating level of anti-folate receptor antibodies [79]. The number of circulating Treg cells was reported to be significantly lower in SJL/J autoimmune-prone mice, with 73% showing abnormally low levels [4]. The Treg cell decrease was more prominent in the more severe cases, as well as among individuals with allergic manifestations such as asthma and atopic dermatitis. Although autistic subjects with low Treg cells did not manifest clinical or laboratory-based signs of autoimmunity (arthritis or arthralgia, elevated erythrocyte sedimentation rate, or leucopenia), the frequency of autoimmunity was higher in their families. The same researchers found elevated markers of oxidative stress (increased plasma F2-isoprostane and/or decreased GPx activity) in 89% of autistic subjects and evidence of antineuronal antibodies in 55%, leading them to suggest a link between oxidative stress and autoimmunity [5]. Notably, expression of Foxp3, the transcription factor which defines Treg cells, is epigenetically regulated, with increased expression when DNA methylation is decreased [81], and Treg cell levels are elevated in male, but not female, SJL mice [82]. As illustrated in Figure 7, these observations suggest that autoimmunity in autism may be related to inflammation in the distal ileum, where impaired activity of EAAT3 in Treg cells can lead to formation of autoantibodies directed against the folate receptor.

Figure 5: Methionine synthase activity in cortex of SJL/J and C57BL6/J mice with or without thimerosal treatment. Methionine synthase activity was measured in the presence of either methylcobalamin (MethylB12) or hydroxocobalamin (HydroxoB12). Activity was significantly lower in SJL/J autoimmune-prone mice compared to the C57BL6/J strain, irrespective of thimerosal treatment. Activity was higher in the presence of methylcobalamin. *P < 0.02 compared to C57BL6/J group, **P < 0.001 compared to C57BL6/J group.

Figure 6: Expression of EAAT3 in CD4+ T-cells from C57BL6/J and SJL/J mice. Spleen and lymph node-derived lymphocytes were separated by FACS analysis cell sorting, followed by qRT-PCR analysis for EAAT3 (EAAC1) expression. EAAT3 expression was significantly higher in CD4+ T-cells from C57BL6/J mice versus autoimmune-prone SJL/J mice (P < 0.05).

4. Conclusions

The prominence of neurological, GI, and immune symptoms in autism suggests a shared mechanism of dysregulation that may relate to the significant deficits in systemic reservoirs of antioxidant GSH reported in this neurodevelopmental disorder. Impaired GI absorption of cysteine, the essential GSH precursor, is proposed as a crucial factor in the pathogenesis of this triad of symptoms, wherein reduced cysteine availability leads to a condition of local and systemic oxidative stress, and subsequent disruption of normal epigenetic regulation of gene expression. In some cases, the extent of impaired cysteine absorption may be severe enough to result in overt GI inflammation, while in other cases the restriction may only alter immune and/or neurological development and function. Awareness of the redox-based linkage between GI, brain, and immune systems serves to illuminate a number of diseases whose origins may reflect the critically important roles of PrEP and PEP. Indeed, adaptive epigenetic responses to changes in redox status are likely to play a critical role in diseases arising across the lifespan, especially those that can be traced to environmental exposures that interfere with antioxidant homeostasis.

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Redox status

Figure 7: Cysteine and selenocysteine uptake in the terminal ileum regulates autoimmunity. EAAT3 transports diet-derived cysteine (CYS) and selenocysteine (Sel-CYS) into GI epithelial cells in the terminal ileum. Within the cell, cysteine is directed to GSH synthesis and selenocysteine is incorporated into selenoproteins, which maintain GSH in its reduced state. EAAT3 activity is regulated by multiple factors, including intracellular redox status, Nrf2-dependent transcription, growth-factor-dependent translocation to the cell surface, and the inhibitory effects of casein and gluten-derived opiate peptides. Systemic availability of cysteine and GSH depends upon these terminal ileum events, as does the local mucosal environment. Immune cells in the terminal ileum can be a source of auto-antibodies, including anti-folate receptor (FR) antibodies, which are commonly present in subjects with autism [80]. Under oxidative stress conditions, EAAT3-mediated uptake of cysteine by Treg cells is impaired, limiting their suppression of naïve CD4+ T cells, and increasing autoimmune responses to antigens present locally, such as the folate receptor.

References


Decreased Levels of EGF in Plasma of Children with Autism Spectrum Disorder

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Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder estimated to affect 1 in 110 children in the U.S., yet the pathology of this disorder is not fully understood. Abnormal levels of several growth factors have been demonstrated in adults with ASD, including epidermal growth factor (EGF) and hepatocyte growth factor (HGF). Both of these growth factors serve important roles in neurodevelopment and immune function. In this study, concentrations of EGF and HGF were assessed in the plasma of 49 children with ASD aged 2–4 years old, and 31 typically developing controls of a similar age as part of the Autism Phenome Project (APP). Levels of EGF were significantly reduced in the ASD group compared to typically developing controls ($P = 0.003$). There were no significant differences in HGF levels in young children with ASD and typically developing controls. EGF plays an important role in regulating neural growth, proliferation, differentiation and migration, and reduced levels of this molecule may negatively impact neurodevelopment in young children with ASD.

1. Introduction

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder characterized by impairments in social interaction and communication, and the presence of restricted behaviors or interests [1]. According to the most current CDC estimate, ASD affects 1 in 110 children in the US [2], yet the pathophysiology of the disorder is largely unknown. Recently, several growth factors have been found to be dysregulated in a substantial proportion of adults with ASD [3].

In the central nervous system, growth factors can regulate the processes of neuronal growth, differentiation, and proliferation, as well as regulating neuronal survival, neuronal migration, and the formation or elimination of synapses [3]. In addition to their central function in regulating neurodevelopment, current literature has also illustrated the dual nature of many growth factors as immune modulators and highlighted their involvement in crosstalk between the immune system and the central nervous system (CNS) [4–7]. Many studies suggest the presence of aberrant immune activity in ASD, in the CNS [8, 9] and in the periphery [10–12], which may be influenced by atypical growth factor activity. Growth factor dysregulation may contribute to ASD pathology by directly affecting CNS development, and/or by augmenting immune function.

Epidermal growth factor (EGF) and hepatocyte growth factor (HGF) are both involved in the growth and proliferation of several cell types, including neurons and glia of the CNS. EGF is present at high levels in the central nervous system (CNS) and plays a critical role in controlling proliferation and differentiation of nervous tissue during neurogenesis [13, 14]. In addition to the function of EGF as a CNS growth factor, it is a central factor in promoting wound healing. EGF is expressed at sites of injury and inhibits the activity of nitric oxide synthase, preventing inflammation [4, 15]. EGF deficiency results in several neurological, gastrointestinal, dermal, and pulmonary abnormalities in animal models [16]. An increased frequency of EGF single nucleotide polymorphisms has been reported in ASD, as well as lower plasma EGF levels in adults with autism [17, 18].

HGF also plays a dual role as a neurological growth factor and an immune modulator. HGF can modulate immune
responses by signaling through the MET receptor on antigen presenting cells, which results in a tolerogenic phenotype with reduced proinflammatory cytokine production and cellular activity [5]. HGF is also essential for normal neurodevelopment, and disruption of HGF signaling results in complex alterations in GABAergic neuron development in the forebrain of animal models [19]. Lower levels of HGF in sera of autistic adults have been described [20], as well as decreased expression of the HGF receptor in postmortem brain samples [21].

To determine if there exists a differential profile for peripheral blood growth factor levels in ASD, we analyzed plasma EGF and HGF in well-characterized young children aged 2–4 years old with a diagnosis of ASD and unrelated typically developing children who were frequency-matched for age. In addition, levels of plasma EGF and HGF were investigated for any associations with clinical behavioral and developmental outcomes.

2. Methods and Materials

2.1. Subjects and Behavioral Assessments. Eighty study participants aged between 2–4 years of age were recruited as part of the APP [22]. Participants consisted of 49 children with ASD (median age of 2.88 years, interquartile range 2.66–3.41 years, 42 males) and 31 typically developing (TD) children (median age of 2.96 years, interquartile range 2.85–3.27, 20 males). Diagnostic instruments included the Autism Diagnostic Observation Schedule-Generic (ADOS-G) [23] and the Autism Diagnostic Interview-Revised (ADI-R) [24]. All diagnostic assessments were conducted or directly observed by trained, licensed clinical psychologists who specialize in autism and had been trained according to research standards for these tools. Inclusion criteria for ASD were taken from the diagnostic definition of ASD in young children formulated and agreed upon by the Collaborative Programs of Excellence in Autism. Inclusion criteria for TD controls included developmental scores within two standard deviations of the mean on all subscales of the MSEL. Exclusion criteria for TD controls included a diagnosis of mental retardation, pervasive developmental disorder or specific language impairment, or any known developmental, neurological, or behavioral problems. TD children were screened and excluded for autism with the Social Communication Questionnaire (scores > 11) (SCQ—Lifetime Edition) [25].

2.2. Measurement of EGF. Peripheral blood was collected in acid-citrate-dextrose Vacutainers (BD Biosciences, San Jose, CA). The plasma fraction was immediately harvested by centrifugation and stored as aliquots at −80°C until the date of assay. Plasma levels of EGF were determined by Human EGF enzyme-linked immunosorbant assay (ELISA) kit (R&D Systems, Minneapolis, MN). Plasma levels of HGF were determined by Human HGF enzyme-linked immunosorbant assay (ELISA) kit (R&D Systems). The assays were performed according to the protocols provided by the manufacturer, and all samples were assayed in duplicate. Optical density was measured on a Wallac Victor3 multilabel-plate reader (PerkinElmer, Boston, MA) at 450 nm.

2.3. Statistical Analysis. Statistical analysis to compare levels of growth factors between ASD and TD groups was conducted with unpaired Student’s t-test. All analyses were conducted with GraphPad Prism statistical software (GraphPad Software Inc., San Diego, CA).

3. Results

Plasma levels of EGF were approximately 3-fold lower in the ASD group (23.1 ± 6.2 pg/mL) compared with the TD group (76.9 ± 20.0 pg/mL) (P = 0.003). Plasma levels of HGF were not statistically different between children with ASD (423.5 ± 20.9 pg/mL) and TD controls (436.6 ± 19.2 pg/mL) (Figure 1).

4. Discussion

In this study, we investigated the levels of EGF and HGF, two growth factors involved in neurodevelopment. Previous studies have found decreased levels of EGF in high functioning adults with autism [18], but no studies have looked at children with ASD who are close to the onset of the disorder. We found that levels of plasma EGF were significantly reduced in young children with ASD as compared with similarly age-matched typically developing control children. This finding is consistent with that seen in adults with high functioning autism and may suggest that a deficiency in EGF is persistent throughout the time course of ASD.

Current research has demonstrated that EGF is involved in growth, differentiation, and maintenance of several tissues including the CNS and the gastrointestinal tract (GI) [14]. Notably, abnormalities of both the CNS and the GI have been reported in ASD [26]. In the CNS, EGF serves as a potent neurotrophic factor, and in vivo studies have demonstrated the effect of EGF in promoting proliferation, differentiation, survival, and migration of multipotent neural progenitor cells and the differentiation of these cells into astrocytes and neurons [27]. In this paper, we were limited to investigating EGF in plasma and not in cerebral spinal fluid. However, EGF rapidly transports across the blood-brain barrier [28], suggesting peripheral EGF levels could be representative of levels in the CNS that may impact neurodevelopment.

In the GI tract, EGF is necessary for normal development of the intestinal mucosa, and mice deficient in EGF receptor suffer from symptoms similar to those of necrotizing enterocolitis, with gradual destruction of villi, become severely malnourished, and typically die before postnatal day 8 [16]. EGF promotes wound healing in animal models of ulcerative colitis [29, 30]. Although GI symptoms affect a large proportion of children with ASD, the exact nature and extent of GI inflammation in ASD are still controversial [31–34]. Interestingly, preliminary investigations in this study show that EGF levels are associated with increased bloating in children with ASD, as well as with raw, standard, and percentile rank scores on the Peabody picture vocabulary
test-III and with the composite and nonverbal development quotient of the Mullen’s test (\(P < 0.05\), data not shown). However, the meaning of this data is not clear and we will validate these findings in a larger replication cohort.

It has been previously reported that HGF was decreased in the plasma of adults with autism [20]; however, we did not find a significant difference between children with ASD and typically developing children for plasma HGF levels. This may be due to age differences between the participants of our study and those in the previous reports. To our knowledge, the relationship between age and serum HGF levels has not been thoroughly established, but there is evidence that levels may decrease with age [35].

Collectively, our data suggest that reduced levels of EGF are present in ASD. The roles of EGF and ASD require further research to better elucidate the relationship between this potent growth factor and ASD pathophysiology.

References


Review Article

HLA Immune Function Genes in Autism

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The human leukocyte antigen (HLA) genes on chromosome 6 are instrumental in many innate and adaptive immune responses. The HLA genes/haplotypes can also be involved in immune dysfunction and autoimmune diseases. It is now becoming apparent that many of the non-antigen-presenting HLA genes make significant contributions to autoimmune diseases. Interestingly, it has been reported that autism subjects often have associations with HLA genes/haplotypes, suggesting an underlying dysregulation of the immune system mediated by HLA genes. Genetic studies have only succeeded in identifying autism-causing genes in a small number of subjects suggesting that the genome has not been adequately interrogated. Close examination of the HLA region in autism has been relatively ignored, largely due to extraordinary genetic complexity. It is our proposition that genetic polymorphisms in the HLA region, especially in the non-antigen-presenting regions, may be important in the etiology of autism in certain subjects.

1. Autism

Leo Kanner first described autism in 1943 [1] after finding 11 children with common symptoms of obsessiveness, stereotypy, and echolalia at Johns Hopkins University. Autism remained an esoteric disorder for several decades until physicians and parents connected these symptoms with an increasing number of patients. It is important to note that the diagnostic criteria have been modified over the years to include a broader category of symptoms, thus increasing the number of children diagnosed with the disorder, now referred to as Autism Spectrum Disorder (ASD) [2]. Currently, the Centers for Disease Control and Prevention (CDC) states that the incidence of ASD is 1 out of 110 children in the United States [3]. The severity of ASD varies greatly with the most severe forms, much like Kanner autism, displaying language regression, seizures, and lower IQ. Altevogt et al. [4] have suggested that autism, or more properly ASD, is not a single disorder, but a collection of similar disorders each with different characteristics and perhaps etiologies.

Even after several decades of research, there is much debate around the world on the etiology of ASD. It is clear that ASD results from abnormal brain development in either the prenatal period or infancy stage of life. Exposure to mercury, maternal viral infections, autoimmune disorders, and the inheritance of certain gene combinations have been implicated in the etiology. Unfortunately, none of these areas have given clear answers as to the etiology. Fortunately, psychologists have made significant strides in treating children and it appears the earlier behavioral treatment starts, the better the outcome. Nevertheless, medical researchers continue to search for the cause(s) of ASD. This paper discusses a possible role for the immune system, and in particular immune function genes in the human leukocyte antigen region (HLA), as a research area that should be more closely investigated.

2. Infections

One of the first areas of interest in the 1960s and 1970s was the search for an infectious agent that might be involved in the etiology of ASD [5]. During this time, there were many case reports in the literature that suggested an association between congenital rubella infection and resultant autistic behaviors. However, after decades of research no definite role
for infectious agents in autism etiology has been confirmed. On the other hand, these endeavors have led to observations that perhaps the immune system was involved in autism, and evidence continues to mount that immune abnormalities are indeed associated with ASD.

3. Familial Studies

Studies indicating familial clustering and the increases of ASD in twins have been interpreted by many as an indication of genetic predisposition. Twin studies show the concordance rates of monozygotic twins at 36–96%, whereas dizygotic twins are 0–24% concordant, resulting in an estimated heritability of autism at >90% [6–8]. Additionally, family studies have shown autism to have familial aggregation with 3–8% of subsequently born siblings either being autistic or showing some form of pervasive developmental disorder (PDD) [9, 10]. This is a 3- to 8-fold increase in risk for siblings over the general population. A more recent study gave an estimate of 18.7% sibling recurrence risk for ASD, a 20-fold increase over the general population [11]. It is important to note, however, that family data should be looked at with great caution, as individuals living in the same household will have similar exposures to microorganisms and environmental chemicals. Taking this into account, a recent paper based on data from twin pairs estimates the genetic heritability for ASD to be 14–67% [12]. However, this idea is somewhat controversial as many in the research community continue to feel that the results from family studies are indicative of a strong genetic etiology [13, 14].

4. Genetics

Many of the early genetic studies involved the examination of microsatellites throughout the human genome in an attempt to find genomic regions that would associate with autism. Overall, this approach was not very fruitful and researchers were associated with ASD. Unfortunately, studies proposing that multiple candidate genes were associated with ASD. Unfortunately, studies proposing candidate genes were often contradictory and proved to be unreliable [15]. One of the most interesting genetic findings in ASD is the association of autism with the MET receptor tyrosine kinase gene located on chromosome 7q31 as MET signaling participates in gastrointestinal repair, immune function, neocortical, and cerebellar growth [16]. It is important to mention that the autism MET associations have been replicated by other research groups [17]. This autism association showed a relative risk of 2.27 which is much lower than the relative risk for HLA gene/allele associations discussed below.

It is very reasonable to believe that deletions or duplications of genetic regions, which can cause lower or higher levels of gene expression, could produce pathological phenotypes. Consequently, newer approaches examining copy number variation (CNV) and microarray analyses of 500,000 SNPs or more have been in vogue for several years in the study of ASD. These newer approaches have identified CNV mutation differences in genes involved in neuronal cell adhesion and ubiquitin degradation as being associated with ASD [18]; however, these results have yet to be replicated by other researchers.

The neurexin-1 gene has been associated with a variety of developmental disorders including ASD [19]. The neurexin-1 gene (2p16.3) is one of the largest genes in the human genome with 24 exons in 1.1 Mb. With two independent promoters there can be over 1,000 neurexin isoforms generated from the 24 exons in different cells or tissue. Another gene of interest is the contactin-associated protein-like 2 (CNTNAP2) gene that was shown to be associated in Old-Order Amish subjects with intractable epilepsy and ASD [20]. Three other groups have now confirmed the involvement of CNTNAP2 in ASD [21–23].

Both the neurexin-1 and the CNTNAP2 genes are involved in synaptic function. Although these approaches have shown a strong association of certain genes with ASD, only a small percentage of subjects with ASD have these mutations. For example, the neurexin-1 gene is found in only about 0.5% of autism cases and 0.2% of controls. The 90% missing inheritance may be largely due to marked genetic heterogeneity, suggesting that different ASD phenotypes should be examined separately [24–26]. Recent genetic research has also associated numerous immune function genes with autism [27–30]. A large study that examined SNP data from several genomewide scans on 3,130 subjects with schizophrenia found that the 5 most significant SNP markers are found across the HLA region [31]. It appears that some of missing inheritance, at least in schizophrenia, was uncovered in the HLA region and we suggest a similar finding will be confirmed/uncovered in autism.

5. Immune Abnormalities in Autism

It is no surprise to see immune gene associations in ASD, as numerous researchers have reported immune abnormalities in autism for over 20 years. It has become increasingly obvious that inflammatory processes are associated with autism. Blood levels of the inflammatory cytokines IL-6, INF-γ, and TNF-α were shown to be elevated in autistic individuals compared to controls [32, 33]. Later, seminal work by Vargas et al. [34] utilized direct morphological analysis and immunohistologic techniques to show an active neuroinflammatory process in the cerebral cortex, white matter, and in particular the cerebellum of ASD patients that was dependent on activation of microglia and astroglia. Cytokine profiling demonstrated that neuroinflammation was accompanied by upregulation of the macrophage chemoattractant protein (MCP-1) and TGF-beta in brain tissue, and that MCP-1 was also upregulated in cerebral spinal fluid [34]. More recent work has directly demonstrated that aside from blood, IL-6, TNF-α, and INF-γ are elevated in ASD brains, along with the other inflammatory cytokines GM-CSF and IL-8 [35]. Most recently, it has been reported that the important inflammatory-associated transcription factor, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is
found in the serum of subjects with autism. upregulated in both blood [36] and brain tissue [37] of autistic individuals. Other immune abnormalities such as autoantibodies started to be reported in 1993 [38] (Table 1). Autoantibodies to myelin basic protein have been noted by at least a couple of researchers [38, 52]. An increase in autoantibody reactivity has been reported against other brain proteins in ASD including nerve growth factor [53], brain endothelium [54], cerebellar proteins [40], and serotonin 5-HT receptors [55] and transglutaminase-2, a protein important in synaptic stabilization [39]. Croonenberghs et al. [56] noted a significant increase in gamma globulin especially of the IgG2 and IgG4 subclasses in children with autism over a control population. Autoantibodies to several uncharacterized brain-specific proteins have been reported in the plasma of autistic individuals. In particular, western blot analysis has shown the presence of IgG autoantibodies targeting a protein of approximately 52 kDa located in the hypothalamus and thalamus [45]. Other autoantibodies targeting three brain proteins of 43–48 kDa located in the hypothalamus have also been observed in the serum of autistic individuals [45]. Autoantibodies targeting cerebellar proteins of 45 and 62 kDa have been associated with ASD [40]. These autoantibodies may be specific to cerebellar Golgi cells, which are GABAergic interneurons [41]. Autoantibodies reactive to human brain proteins in the 36–39 and 61 kDa range have also been found in the sera of mothers of autistic children. [57, 58].

It is important to note that while autoantibodies associated with ASD may be biomarkers, they may not necessarily be pathologic in and of themselves. Central tolerance refers to the process whereby immature lymphocytes are negatively selected based on the ability of their antigen receptors (the B cell receptor or BCR for B cells and the T-cell receptor or TCR for T cells) to recognize self-antigens [59–61]. Until fairly recently, it was believed that most immature lymphocytes recognizing self-antigens (autoimmune repertoire) were normally neutralized by virtue of central tolerance before they could mature, and that peripheral tolerance would insure the removal of any self-reactive lymphocyte escaping central tolerance. Peripheral tolerance refers to the process whereby mature self-reactive lymphocytes which have escaped central tolerance are eliminated, largely by CD95-mediated apoptosis [62]. Thus responses to foreign antigens were viewed as normal, while anti-self responses were considered necessarily pathologic. However, with the realization that many self-reactive lymphocytes survive central and peripheral tolerance, this view has had to be modified [49]. Limited immune responses to self-antigens (autoimmunity) are now understood to be normal and not necessarily pathologic [63].

Upon stimulation of the system with a pathogen, cognate lymphocyte clones representative of the anti-foreign repertoire normally expand and mature, providing a protective immune response. On the other hand, it is the expansion and maturation of those clones representative of the autoimmune repertoire that leads to autoimmune disease. In other words, the developing immune system can be characterized as balancing production between anti-foreign (protective) and anti-self (autoimmune) repertoires. While a beneficial function of naturally occurring, low level autoimmune antibodies, also referred to as natural antibodies (NAs), remains a matter of debate, it appears as if the repertoire of NA is reflective of the susceptibility to develop specific autoimmune diseases [64, 65]. Because central tolerance of T cells depends to a large extent upon the strength of the TCR interaction with an autoantigen or an HLA class I or II complex [59], the NA repertoire will to a large extent depend upon the HLA haplotype, with some haplotypes favoring autoantibodies targeting one antigen over another. For instance, in the case of ASD we have found an association with low level antibody responses to tissue transglutaminase, and that this response appears linked to the HLA-DR3/DQ2 and DR7/DQ2 haplotypes [39]. It is likely that the other autoantibodies noted above as being associated with ASD may be linked to different haplotypes.

Aside from autoantibodies and altered cytokine levels there appear to be other immune abnormalities associated with ASD. It was noted that there were decreased numbers of T-lymphocytes and an altered ratio of suppressor T-lymphocytes to helper T-lymphocytes [66, 67] and altered T-lymphocytes responses in children with autism [68]. Warren et al. [69] reported that subjects with autism had reduced NK cell killing in the standard K562 target cell cytotoxicity assay. This observation of decreased NK cell killing has been repeated by at least a couple of other research teams [28, 70]. One research group [71] observed that monocyte counts and neopterin levels were increased in autistic children compared to gender and age-matched healthy controls suggesting that the immune system was overactivated in the ASD group. Another elegant set of experiments involved the stimulation of cultured monocytes with several toll-like-receptor (TLR) ligands. The monocytes from subjects with ASD had significant increases or decreases in proinflammatory cytokines.

### Table 1: A list of proteins against which autoantibodies have been found in the serum of subjects with autism.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transglutaminase 2</td>
<td>[39]</td>
</tr>
<tr>
<td>45 and 62 kDa proteins in cerebellum</td>
<td>[40, 41]</td>
</tr>
<tr>
<td>Voltage dependent anion channel (VDAC)</td>
<td>[42]</td>
</tr>
<tr>
<td>Hexokinase-1</td>
<td>[42]</td>
</tr>
<tr>
<td>Mitochondrial protein</td>
<td>[43]</td>
</tr>
<tr>
<td>Antimitochondrial DNA auto-antibodies</td>
<td>[43]</td>
</tr>
<tr>
<td>Nuclear proteins</td>
<td>[44]</td>
</tr>
<tr>
<td>52 kDa protein in hypothalamus and thalamus</td>
<td>[45]</td>
</tr>
<tr>
<td>43–48 kDa protein in the hypothalamus</td>
<td>[45]</td>
</tr>
<tr>
<td>Folate receptor</td>
<td>[46]</td>
</tr>
<tr>
<td>Brain-derived neurotrophic factor</td>
<td>[47]</td>
</tr>
<tr>
<td>HSP90</td>
<td>[48]</td>
</tr>
<tr>
<td>Myelin basic protein (MBP)</td>
<td>[38, 49]</td>
</tr>
<tr>
<td>Myelin-associated glycoprotein (MAG)</td>
<td>[50]</td>
</tr>
<tr>
<td>Myelin oligodendrocyte glycoprotein (MOG)</td>
<td>[50]</td>
</tr>
<tr>
<td>Neuron-axon filament protein</td>
<td>[51]</td>
</tr>
<tr>
<td>Glial fibrillary acidic protein</td>
<td>[51]</td>
</tr>
</tbody>
</table>
depending on the particular TLR ligand added to the cell culture media [72].

One large-scale study found that the frequency of autoimmune disorders in the families with autistic children was found to be higher than in control subjects, especially mothers of autistic children [73]. Another group demonstrated that autoimmune diseases were increased significantly in families with ASD compared with those of healthy control subjects [74], suggesting a link between the disorders. Croen et al. [75] showed that maternal psoriasis diagnosed around the time of pregnancy is significantly associated with a subsequent diagnosis of autism in the child. Additionally, they showed a 2-fold increase in risk for a child having ASD if the mother was diagnosed and with asthma or allergies during pregnancy. An association between a family history of type 1 diabetes mellitus (T1DM) and infantile autism as well as a significant association between maternal histories of either rheumatoid arthritis (RA) or celiac disease and ASDs was noted by Atladóttir et al. [76].

6. Autism HLA Genetics

HLA is the name for the major histocompatibility complex (MHC) in humans and HLA and MHC are often used interchangeably in the literature. The HLA region on chromosome 6p21 (about $4 \times 10^6$ bp) is of major interest in basic research as well as medicine as genes/proteins in this region are involved in many biological processes such as histocompatibility, inflammation, ligands for immune cell receptors, and the complement cascade. The HLA region has 20 typical HLA genes and 112 nontypical HLA genes (Table 2) that are inherited together as frozen blocks of DNA called ancestral haplotypes. Complete DNA sequences have been published for 8 of the more common ancestral haplotypes in an effort to expedite basic and disease research [77]. It should be mentioned that smaller haplotypes can also be constructed for genes that are linked. HLA genes also play a role in reproduction, pregnancy maintenance, mate selection, and even kin recognition [78, 79] and so forth. Current research is increasingly demonstrating a role for HLA proteins in neural cell interactions, synaptic function, cerebral hemispheric specialization, central nervous system (CNS) development [80–84], and even neurological disorders [85]. The genes of the HLA region are shown in Figure 1. Shiina et al. [86, 87] have published two excellent reviews on the HLA super-locus. Not only is there extraordinary complexity in the HLA genes, there are extensive haplotype-related transcriptional differences [88]. It has been shown that genetic mechanisms outside of the non-antigen-binding HLA genes in the ancestral haplotype 8.1 (also referred to as COX) are associated with susceptibility to many autoimmune diseases [89]. It is our proposition that HLA genes/proteins should be more carefully examined due to increasing evidence of autoimmune type associations in autism.

### Table 2: Genes and alleles in the HLA region.

<table>
<thead>
<tr>
<th>HLA genes</th>
<th>Number</th>
<th>HLA alleles</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA class I genes</td>
<td>6</td>
<td>HLA class I alleles</td>
<td>2215</td>
</tr>
<tr>
<td>HLA class II genes</td>
<td>12</td>
<td>HLA class II alleles</td>
<td>986</td>
</tr>
<tr>
<td>HLA class I-like genes</td>
<td>2</td>
<td>HLA class I-like alleles</td>
<td>94</td>
</tr>
<tr>
<td>Non-HLA genes</td>
<td>112</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total genes</td>
<td>132</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLA class I genes</td>
<td>A/B/C/E/F/G</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLA class I-like genes</td>
<td>MICA/MICB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLA class II genes</td>
<td>DRA/DRB/DQ/DP/DM/DO</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

7. HLA Associations in ASD

It was suggested over 30 years ago by Stubbs and Magenis [90] that the HLA region might be important in autism. Warren et al. [91] first reported that the HLA ancestral haplotype 44.1 (B44-SC30-DR4) was associated with autism with a relative risk of 7.9. That result was confirmed in a separate case/control population [92]. Interestingly, the individual components of AH 44.1 (A2-B44-SC30-DR4) include a deleted C4B gene and DRβ1*0401, both of which have been shown independently to be significantly associated with ASD [93, 94]. Examination with different genetic markers than those used by Warren suggested that certain HLA haplotypes are associated with autism in Sardinian and Italian families [95, 96].

Warren et al. [97] reported that the shared epitope-binding pocket (DRβ1*0401, *0404, and *0101) in the third hypervariable region of DRβ1 has a strong association with autism. A relative risk of 19.8 for autism was reported for subjects with one of the two extended HLA haplotypes. Both of these haplotypes have many allelic similarities especially the DRβ1*0401.

AH 44.1 (HLA-A2, Cw5, B44, Bf*S, C2*C, C4A3, C4BQ0, DRβ1*0401, DQB1*0301).

AH 62.1 (HLA-A2, Cw3, B15, Bf*S, C2*C, C4A3, C4B3, DRβ1*0401, DQB1*0302).

The shared epitope has been associated with several autoimmune diseases such as rheumatoid arthritis, psoriatic arthritis, and systemic lupus erythematosus [98]. Torres et al. [99] confirmed the association of the HLA-DR4 allele and also found that the DR13 and DR14 alleles occurred less
Figure 1: Gene map of the human leukocyte antigen (HLA) region. The major histocompatibility complex (MHC) gene map corresponds to the genomic coordinates of 29677984 (GABBR1) to 33485635 (KIFC1) in the human genome build 36.3 of the National Center for Biotechnology Information (NCBI) map viewer. The regions separated by arrows show the HLA subregions such as extended class I, classical class I, classical class II, and extended class II regions from telomere (left and top side) to centromere (right and bottom side). White, gray, striped and black boxes show expressed genes, gene candidates, noncoding genes and pseudogenes, respectively. The location of the alpha, beta, and kappa blocks containing the cluster of duplicated HLA class I genes in the class I region are indicated. (Reprinted with permission from the Journal of Human Genetics [87].)

often in subjects with autism, suggesting a possible protective mechanism. Interestingly, the DR13 allele was inherited less frequently than expected from the mothers. Associations with autism and the DR4 allele have since been confirmed in three additional research groups. Lee et al. [99] demonstrated that boys with autism and their mothers had a significantly higher frequency of DR4 than normal control subjects (odds ratios 4.20 and 5.54, resp.), suggesting that a maternal-fetal
immune interaction could be involved in autism. Johnson et al. [100] reported significant transmission disequilibrium for HLA-DR4 (odds ratio 4.67) from maternal grandparents to mothers of children with autism which also suggests a maternal-fetal interaction for HLA-DR4. It has been recently shown in Han Chinese that the HLA-DRβ1 allele frequencies including DR4 are different in subjects with autism versus control subjects [101].

It was reported 20 years ago that subjects with autism had a significant increase in the C4B null allele (C4B gene deletion) compared to control subjects [91]. After this observation, it was also noted that subjects with autism had a significant deficiency in the plasma C4B protein [102]. These initial findings of an increase in the deletion of the C4B gene was supported by examining a new population of subjects with autism [94]. Mostafa and Shehab [103] have recently reported a significant increase in the deletion of the C4B gene in the Egyptian population. They also reported an increased risk when there was a family history of autoimmune diseases in the autism population. Descriptions of several non-antigen-binding HLA genes that are associated with autoimmune diseases are discussed below and listed in Table 3.

8. Non-Antigen-Binding HLA Genes in Class I

Although MICA/MICB genes are structurally similar to classical antigen-binding HLA class I genes, they encode proteins that interact with different T-cell receptors in response to stress as infection or neoplastic transformation. The two genes are located on the centromeric end of the class I region near HLA-B at the border of the class III region (Figure 1) [87]. MICA/MICB proteins are ligands for NKG2D receptors on NK cell and gamma/delta T-cell receptors. Adaptive immunity involves three lymphocyte populations (B cells, alpha/beta T cells, and gamma/delta T cells). Gamma/delta T cells represent a small population of T cells that possesses a T-cell receptor that is distinct from the typical alpha/beta T cell. They are concentrated in the intestinal mucosa and appear to have a prominent role in recognizing small bacterial phosphoantigens and undefined antigens presented by MICA/MICB proteins. Gamma/delta T cells have potent cytotoxic activity and have been considered a link between innate and adaptive immunity. Polymorphisms in MICA/MICB genes have been associated with T1DM, AD, and SLE independent of DRβ1 alleles. There are several genes associated with psoriasis (PSORSI locus). It is unclear how the risk is spread among these genes as they are closely linked. Two genes RNF39 and TRIM39 in the class I region have been associated with Behçet’s disease.

9. Non-Antigen-Binding HLA Genes in Class III

Tumor necrosis factor-alpha (TNF-α) is a proinflammatory, multifunctional cytokine that plays important roles in cell physiology. It is synthesized in numerous cells including macrophages, NK cells, T cells, mast cells, osteoblasts, granulocytes, smooth muscle cells, fibroblasts, and keratinocytes. In the CNS, TNF-α is made in microglia cells, astrocytes, and neurons [141]. In the normal state, TNF-α drives acute and chronic inflammatory responses that leads to the removal of injurious stimuli and the restoration of homeostasis. TNF-α is necessary for neural cell differentiation and neuron maturation and there is evidence that it is critical in the normal brain for proper synaptic function.

There is extensive research that implicates TNF-α as a key mediator in disease progression, inflammation, blood-brain-barrier deterioration, and even cell death [142]. Elevated levels are present in numerous neurological disorders including multiple sclerosis, Alzheimer’s disease, Parkinson’s disease, ischemia, traumatic brain injury, and as mentioned above, ASD [141]. It is unclear if TNF-α contributes to the disease state or the higher concentrations limit neuronal injury. There are three adjacent genes: lymphotoxin alpha and beta (LTA and LTB) and leukocyte-specific transcript-1 (LST1) in the TNF-α block. LTA and LTB are proinflammatory cytokines like TNF-α, and LST1 plays a role in inflammatory and infectious diseases. It has been suggested that vaccine-induced immunity changes with certain haplotypes in these genes [143]. Two other genes important in immune function are adjacent to the TNF-α block. Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-like 1 (NF-κBIL1) on the telomeric side of TNA-α is an inhibitor of NF-κB, an important pleiotropic immune system transcription factor that is upregulated in ASD [144, 145]. In addition, NF-κBIL1 has been associated with several autoimmune diseases (Table 3). The natural cytotoxicity trigger receptor three (NCR3) gene on the centromeric side encodes proteins that are ligands for activating receptors on NK cells that recognize tumor cells [146]. Typical HLA-B and -C proteins are also ligands for KIR receptors on NK cells.

There are numerous heat shock proteins (HSPs), also referred to as chaperones, that assist the folding of newly synthesized proteins as well as those that have been unfolded or denatured [147]. HSPs are also important in cell protective functions and in inhibiting the apoptosis cascade. They are named by their molecular weights (HSP100, HSP90, HSP70, HSP60, and smaller HSPs). Although the proteins appear in normal cellular functions, they are induced to higher levels in trauma, epilepsy, neurodegenerative diseases, and other injuries. An underlying feature among AD, Parkinson’s disease, spinocerebellar ataxia, and other neurodegenerative diseases is the accumulation of misfolded proteins and HSPs are being studied because of their role in folding and refolding proteins [147]. There is intense interest in HSPs as they appear to protect neurons [148] and one must remember that postmitotic neurons are unable to dilute misfolded or aggregated proteins through division.

There are three HSP70 genes (HSPA1L, HSPA1A, and HSPA1B) in the class III HLA region that are adjacent but separate genes. HSP70 proteins have been demonstrated to stimulate IL-6 and TNF-α production, activate microglial cells, and stimulate phagocytosis [148]. HSP70 proteins are also important in autoimmunity by enhancing antigen presentation in both HLA class I and HLA class II systems [147, 149]. Peptides that are associated with HSP70 at the time of
Table 3: Non-classical HLA genes associated with autoimmune diseases.

<table>
<thead>
<tr>
<th>Gene</th>
<th>HGNC gene number</th>
<th>Autoimmune disease</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extended class I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OR2H2</td>
<td>8253</td>
<td>SLE</td>
<td>[104]</td>
</tr>
<tr>
<td>RNF39</td>
<td>18064</td>
<td>Behçet’s disease</td>
<td>[105]</td>
</tr>
<tr>
<td>TRIM39</td>
<td>10065</td>
<td>Behçet’s disease</td>
<td>[105]</td>
</tr>
<tr>
<td>PSORS1 locus</td>
<td>9573</td>
<td>Systemic sclerosis, Psoriasis</td>
<td>[106–111]</td>
</tr>
<tr>
<td>MICA</td>
<td>7090</td>
<td>T1DM, AD, SLE</td>
<td>[112–114]</td>
</tr>
<tr>
<td>MICB</td>
<td>7091</td>
<td>SLE</td>
<td>[104]</td>
</tr>
<tr>
<td>Class I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BAT1–BAT5</td>
<td>13917–21</td>
<td>Alzheimer’s, AIDS</td>
<td>[115, 116]</td>
</tr>
<tr>
<td>NFKBIL1</td>
<td>7800</td>
<td>Sjögren’s syndrome, SLE, RA</td>
<td>[117, 118]</td>
</tr>
<tr>
<td>TNF Block</td>
<td>11892</td>
<td>Alzheimer’s, Psoriasis, Autoimmune hepatitis, Sarcoidosis</td>
<td>[115, 119–121]</td>
</tr>
<tr>
<td>AIF1</td>
<td>352</td>
<td>T1DM</td>
<td>[122]</td>
</tr>
<tr>
<td>HSP70 genes</td>
<td>5232–4</td>
<td>MS</td>
<td>[123]</td>
</tr>
<tr>
<td>Complement genes</td>
<td>1248, 1324, 1323</td>
<td>SLE, myasthenia gravis, T1DM</td>
<td>[124–126]</td>
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<tr>
<td>SKIV2L</td>
<td>10898</td>
<td>SLE</td>
<td>[127]</td>
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<tr>
<td>ATF6B (CREBL1)</td>
<td>2349</td>
<td>SLE</td>
<td>[104]</td>
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<tr>
<td>NOTCH4</td>
<td>7884</td>
<td>Systemic sclerosis</td>
<td>[128]</td>
</tr>
<tr>
<td>C6orf10</td>
<td>13922</td>
<td>SLE</td>
<td>[104]</td>
</tr>
<tr>
<td>BTN1L2</td>
<td>1142</td>
<td>Ulcerative colitis, Sarcoidosis</td>
<td>[129, 130]</td>
</tr>
<tr>
<td>Class II</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAP2</td>
<td>44</td>
<td>Psoriasis</td>
<td>[131]</td>
</tr>
<tr>
<td>PSMB8</td>
<td>9545</td>
<td>Hypersensitivity pneumonitis</td>
<td>[132]</td>
</tr>
<tr>
<td>Class III</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAP1</td>
<td>43</td>
<td>Vitiligo</td>
<td>[134]</td>
</tr>
<tr>
<td>PSMB9</td>
<td>9546</td>
<td>Psoriasis, Vitiligo</td>
<td>[133, 134]</td>
</tr>
<tr>
<td>HLA-DM</td>
<td>4934, 4935</td>
<td>Psoriasis, Antiphospholipid auto-antibodies, RA, SLE, T1DM</td>
<td>[131, 135–138]</td>
</tr>
<tr>
<td>HLA-DO</td>
<td>4936, 4937</td>
<td>common variable immunodeficiency</td>
<td>[139]</td>
</tr>
<tr>
<td>Class II Extended</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSD17B8</td>
<td>3554</td>
<td>SLE</td>
<td>[104]</td>
</tr>
<tr>
<td>DAXX</td>
<td>2681</td>
<td>MS</td>
<td>[140]</td>
</tr>
</tbody>
</table>

Abbreviations: (HGNC) HUGO Gene Nomenclature Committee (http://www.genenames.org/); (SLE) systemic lupus erythematosus; (AIDS) acquired immune deficiency syndrome; (T1DM) type 1 diabetes mellitus; (AD) Addison’s disease; (RA) rheumatoid arthritis; (MS) multiple sclerosis, PSORS1 psoriasis locus genes (CDSN, PSORS1C1, PSORS1C2, CCHCR1, POUSF1, PSORS1C3), TNF Block genes (LTA, TNF, LTβ, LST1), HSP70 genes (HSPA1L, HSPA1A, HSPA1B), Complement genes (C2, C4B, C4A).

T-cell presentation have been shown to be more antigenic and therefore involved in autoimmunity [149, 150].

Another gene that is independently associated with autoimmune diseases outside of non-antigen-binding HLA alleles is allograft inflammatory factor-1 (AIF1). The SNP rs2269475 C > T in AIF1 has been associated with RA. The T allele was significantly higher in the RA patients and there was no significant linkage disequilibrium between the AIF1 SNP and DRβ1 alleles. Anticyclical citrullinated peptide antibodies commonly used to monitor RA were significantly increased in carriers with the T allele [151] but not the C allele.

10. Non-Antigen-Binding HLA Genes in Class II

Although HLA-DM and -DQ proteins have structures like classical antigen-binding HLA proteins, they work in the cytoplasm and not at the cell surface antigen-presenting HLA proteins. DM stabilizes and edits the peptide repertoire presented by DQ proteins by catalyzing CLIP release. The associations of DQ2 with T1DM and celiac disease have been known for several decades; however, the biochemistry behind these associations has not been elucidated. It is now known that DQ2 is a poor substrate for DM and it has been proposed that antigen presentation in the thymus and periphery can be affected by impaired DQ-DM interactions so as to promote autoimmune disease [152]. HLA-DO is another nonclassical class II HLA protein that is involved in the loading of peptides to HLA-DR proteins by modulating the function of HLA-DM [153].

There are two genes in the class II region that encode proteins involved in the transport of antigen from the cytoplasm to the endoplasmic reticulum for binding to class I HLA proteins: transporter associated with antigen-processing 1 and 2 (TAP1 and TAP2). Several research groups have reported associations of TAP2 genes with SLE independent of classical HLA alleles [154]. This interaction is very interesting
as it means that class II genes can affect class I antigen binding.

There are two other genes in the extended class II region that have been associated with autoimmune diseases (Table 3). Hydroxysteroid 17-beta dehydrogenase 8 (HSD17B8) is important in regulating the concentrations of active estrogens and androgens and high levels of estrogen are well known to be associated with SLE and other autoimmune diseases. The second gene, death-associated protein 6 (DAXX), is a very important protein that interacts with a variety of proteins in the nucleus and cytoplasm. Perhaps most importantly, it interacts with the death receptor Fas. Engstrom et al. [155] published a paper describing decreased expression of Fas on CD4+ T lymphocytes but higher serum levels of soluble Fas in ASD.

11. Summary

There is mounting evidence that the immune system plays a role in the pathogenesis of ASD in certain individuals. This evidence comes from several research areas including an increase in proinflammatory cytokines in blood and brain, autoantibodies to numerous antigens, and HLA associations.

Autism HLA associations have been observed across the entire HLA region. For example, in the class I region HLA-A2 has been associated with autism by at least two research groups [156, 157]. In the class II region several researchers have reported autism associations with the DRB1*04 allele [93, 99, 100]. Strong associations also appear in the class III region where the C4B null allele has been associated with autism with relative risks of 4.3 [94] and 4.6 [97], and an odds ratio of 6.3 [103]. The HLA-associated risk is the highest for autism (19.8) when combining two ancestral haplotypes (44.1 and 62.1). Both of these haplotypes have HLA-A2 and DRB1*0401 as well as other genetic similarities; however, these two alleles cannot account for all of the 19.8 risk. Compared to other genetic associations with autism, the HLA associations may be more important than realized, as they have the highest genetically associated risk, that we are aware of for autism. For example the MET gene, one of the most studies genetic regions in autism, has a relative risk of 2.27 [17].

It is our premise that some of the autism missing inheritance may be hidden in the HLA region, both in classical HLA alleles and nonclassical HLA genes, as seen in schizophrenia [31]. For example, the HLA class III region contains clusters of genes such as TNF-α, HSP70, C4A/C4B, and NF-xBIL1 that are seminal in cellular function and are also associated with numerous autoimmune diseases (Table 3).

Acknowledgment

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[47] M. Enberg, M. J. Huentelman et al., “Antibodies to neuron-specific enkephalitogenic proteins from milk, Chlamydia pneumo-


Research Article

Intracellular and Extracellular Redox Status and Free Radical Generation in Primary Immune Cells from Children with Autism

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The modulation of the redox microenvironment is an important regulator of immune cell activation and proliferation. To investigate immune cell redox status in autism we quantified the intracellular glutathione redox couple (GSH/GSSG) in resting peripheral blood mononuclear cells (PBMCs), activated monocytes and CD4 T cells and the extracellular cysteine/cystine redox couple in the plasma from 43 children with autism and 41 age-matched control children. Resting PBMCs and activated monocytes from children with autism exhibited significantly higher oxidized glutathione (GSSG) and percent oxidized glutathione equivalents and decreased glutathione redox status (GSH/GSSG). In activated CD4 T cells from children with autism, the percent oxidized glutathione equivalents were similarly increased, and GSH and GSH/GSSG were decreased. In the plasma, both glutathione and cysteine redox ratios were decreased in autistic compared to control children. Consistent with decreased intracellular and extracellular redox status, generation of free radicals was significantly elevated in lymphocytes from the autistic children. These data indicate primary immune cells from autistic children have a more oxidized intracellular and extracellular microenvironment and a deficit in glutathione-mediated redox/antioxidant capacity compared to control children. These results suggest that the loss of glutathione redox homeostasis and chronic oxidative stress may contribute to immune dysregulation in autism.

1. Introduction

Autism is a behaviorally defined neurodevelopmental disorder that usually presents in early childhood and is characterized by significant impairments in social interaction and communication and by abnormal repetitive hyper-focused behaviors. The prevalence of autism spectrum disorders has increased more than 10-fold in the last two decades, now affecting one in 110 US children, yet the etiology of these disorders remains elusive [1]. Glutathione depletion and oxidative stress have been implicated in the pathology of numerous neurobehavioral disorders including schizophrenia [2], bipolar disorder [3], and Alzheimer’s disease [4]. Accumulating evidence suggests that redox imbalance and oxidative stress may also contribute to autism pathophysiology. Multiple biomarkers of oxidative stress have been identified in blood samples from children with autism [5–12]. Our group has reported a decrease in concentrations of glutathione (GSH) and several of its metabolic precursors, an increase in oxidized glutathione disulfide (GSSG), and a decrease in glutathione redox ratio (GSH/GSSG) in case-control evaluations of plasma and lymphoblastoid cell lines derived from children with autism [13–16]. Recently, several interactive polymorphisms in enzymes regulating glutathione synthesis were found to be more prevalent in children with autism suggesting that the glutathione deficit and predisposition to oxidative stress may be genetically based in some children [17].

Oxidative stress occurs when cellular antioxidant defense mechanisms fail to counterbalance endogenous ROS production and/or exogenous prooxidant environmental exposures. Glutathione (γ-L-glutamyl-L-cysteinylglycine) is a tripeptide that functions as the major intracellular antioxidant and redox buffer against macromolecular oxidative damage. The glutathione thiol/disulfide redox couple (GSH/GSSG) is the predominant mechanism for maintaining the intracellular
microenvironment in a highly reduced state that is essential for antioxidant/detoxification capacity, redox enzyme regulation, cell cycle progression, and transcription of antioxidant response elements (ARE) [18–23]. Subtle variation in the relative concentrations of reduced and oxidized glutathione provides a dynamic redox signaling mechanism that regulates these vital cellular processes [24–27]. For example, in both CNS precursor cells and naive immune cells, intracellular glutathione redox status is the primary determinant modulating the cellular decision to undergo cell cycle arrest, differentiation, or proliferation [27]. A reducing intracellular environment is required for proliferation, while a more oxidized microenvironment favors cell cycle arrest and differentiation. A chronic deficit in the GSH/GSSG redox ratio is considered to be a reliable indicator of oxidative stress and increased vulnerability to oxidative damage from prooxidant environmental exposures [28, 29].

In the extracellular plasma compartment, the cysteine/cystine (thiol/disulfide) redox couple independently provides the ambient redox environment for circulating immune cells. The ambient extracellular cysteine/cystine redox potential has been shown to be more oxidized than the intracellular GSH/GSSG redox potential and is independently regulated [30]. Dynamic shifts in the plasma cysteine/cystine redox potential alter the redox status of cysteine moieties in cell surface proteins to induce conformational changes in protein structure that can reversibly alter function [31, 32]. For example, under oxidizing extracellular conditions, redox-sensitive cysteine residues in the catalytic core of protein tyrosine phosphatases become oxidized and reversibly inactivate enzyme activity depending on the ambient cysteine/cystine redox potential [31, 33, 34]. Extracellular cysteine/cystine redox status is emerging as an important new signal transduction mechanism that can induce posttranslational alterations in downstream redox-sensitive proteins including a variety of enzymes, transcription factors, receptors, adhesion molecules, and membrane signaling proteins resulting in the dynamic modulation of their activity and function [32, 35, 36].

Recent studies have revealed numerous immunologic abnormalities among children with autism including alterations in immune cell proportions [37–40] and shifts in helper T-cell subpopulations after mitogenic stimulation [41, 42]. Peripheral blood mononuclear cells (PBMCs) from individuals with autism have been shown to produce higher levels of proinflammatory cytokines and abnormal levels of regulatory cytokines compared to control PBMCs at baseline and upon mitogenic stimulation [43–46]. Taken together, the immunological studies suggest a role for a dysregulated immune system in autism that potentially could be related to a deficit in glutathione-mediated antioxidant capacity and an oxidized microenvironment in immune cells. To investigate this possibility, we examined whether primary immune cells (PBMCs) from children with autism exhibit decreased intracellular glutathione redox capacity compared to PBMCs from age-matched control children and whether a more oxidized intracellular and extracellular microenvironment is associated with increased production of oxidizing intracellular free radicals. Because immune cells from children with autism have been shown to have abnormal responses to stimulation, we also elected to challenge the PBMCs with immune activators known to promote oxidative stress and measure the resulting intracellular glutathione redox status in activated isolated monocytes and T cells.

2. Subjects and Methods

2.1. Participants. This investigation was conducted on a subset of children from the autism IMAGE (Integrated Metabolic and Genomic Endeavor) study at Arkansas Children’s Hospital Research Institute (ACHRI) that has recruited over 162 case and control families to date. The IMAGE cohort for this study consisted of 43 children diagnosed with autistic disorder and 41 unaffected control children (16 of which were unaffected siblings). The autism case families were recruited locally after referral to the University of Arkansas for Medical Sciences (UAMS), Dennis Developmental Center and diagnosed by trained developmental pediatricians. Children aged 3 to 10 with a diagnosis of autistic disorder as defined by the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV 299.0), the Autism Diagnostic Observation Schedule (ADOS), and/or the Childhood Autism Rating Scales (CARS >30) were enrolled. Children diagnosed with other conditions on the autism spectrum or rare genetic diseases associated with symptoms of autism were excluded from the study. Children with chronic seizure disorders, recent infection, and high-dose vitamin or mineral supplements exceeding the RDA were also excluded because these conditions are potential confounders that could affect redox status. Unaffected siblings and unrelated, neurotypical children aged 3 to 10 with no medical history of behavioral or neurologic abnormalities by parent report made up the comparison group. The protocol was approved by the Institutional Review Board at UAMS, and all parents signed informed consent.

2.2. Materials. Culture flasks, plates, and pipettes were obtained from Corning Life Sciences (Lowell, Mass, USA). RPMI 1640, penicillin/streptomycin, Dulbecco’s phosphate-buffered saline (PBS), fetal bovine serum (FBS), and glutamine were purchased from Life Technologies (Carlsbad, Calif, USA). Carboxy-H2DCFDA (6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, diacetoxymethyl ester) was obtained from Molecular Probes (Carlsbad, Calif, USA). Human Monocyte Isolation Kit II and Human CD4 T cell Isolation Kit II were purchased from Miltenyi Biotec (Bergisch-Gladbach, Germany). Histopaque-1077 and all other chemicals were obtained from Sigma-Aldrich (St. Louis, Mo, USA).

2.3. Isolation of PBMCs and Stimulation of Monocytes and CD4 T Cells. Fasting blood samples (≤20 mL) were collected before 9:00 AM into EDTA-Vacutainer tubes and immediately chilled on ice before centrifuging at 1300 × g for 10 min at 4°C. Aliquots of plasma were stored at −80°C in cryostat tubes until extraction and HPLC quantification. PBMCs were isolated by centrifugation over Histopaque-1077. Red
blood cells were lysed using a brief (15 s) incubation with 1 mL ice-cold water. Approximately, 30 × 10^6 PBMCs were resuspended in RPMI 1640 medium (supplemented with 10% FBS, 1% penicillin/streptomycin, and 2 mM glutamine) at a density of 10^6 cells/mL. Note that because we were unable to obtain 20 mL blood volume from every child, it was not possible to isolate and analyze monocytes and CD4 T cells for all participants. For monocyte stimulation, PBMCs were treated with 0.1 μg/mL lipopolysaccharide (LPS); for T-cell stimulation, PBMCs were treated with 10 ng/mL phorbol 12-myristate 13-acetate (PMA) and 1 μg/mL ionomycin. Cells were placed in a humidified 5% CO_2 incubator at 37°C for 4 hr. Stimulated monocytes and CD4 T cells were then isolated by negative selection using magnetic cell labeling as described by the manufacturer (Miltenyi Biotec, Bergisch-Gladbach, Germany). Using flow cytometry, we determined that ≥75% of isolated monocytes are positive for CD14 and that ≥87% of isolated CD4 T cells are positive for CD4. For HPLC quantification of GSH and GSSG, approximately 2 × 10^6 unstimulated (resting) PBMCs, stimulated monocytes, or stimulated CD4 T cells were pelleted, snap frozen on dry ice, and stored at −80°C.

2.4. Cell Extraction and HPLC Quantification of Intracellular Glutathione and Plasma Cysteine Redox Status. The storage interval at −80°C before extraction was consistently between 1-2 weeks after blood draw and cell isolation to minimize potential metabolite interconversion. The methodological details for intracellular and extracellular GSH extraction and HPLC elution and electrochemical detection have been described previously [15, 16], and metabolite detection does not require derivatization. Although most GSSG is present as a mixed disulfide with other thiols including cysteine, our measurements detect only the free GSSG in plasma. Glutathione and cysteine concentrations were calculated from peak areas of standard calibration curves using HPLC software. Intracellular results are expressed as nanomoles per milligram of protein using the BCA Protein Assay Kit (Pierce, Rockford, Ill, USA), and plasma results are expressed as micromoles per liter.

2.5. Measurement of Intracellular Free Radicals. Carboxy-H2DCFDA (DCF) is a membrane-permeable ROS/RNS-sensitive probe that remains nonfluorescent until oxidized by intracellular free radicals. The intensity of DCF fluorescence is directly proportional to the level of free radical oxidation. Approximately, 10^6 PBMCs were resuspended in 1 mL RPMI 1640 medium supplemented with 10% FBS, 1% penicillin/streptomycin, and 2 mM glutamine in the dark for 20 min with 1 μM DCF at 37°C. Stained cells were washed and resuspended in PBS and analyzed immediately on a Partec CyFlow flow cytometer (Görlitz, Germany) using 488 nm excitation wavelength with 530/30 nm (FL1) emission filter. For each analysis, the fluorescence properties of 10000 cells were collected, and the data were analyzed using the FCS Express software (De Novo Software, Los Angeles, Calif, USA). Intracellular free radical levels are expressed as median fluorescence intensity (MFI) of subject sample DCF fluorescence normalized to DCF fluorescence of a standard PBMC preparation. As an internal control, the standard PBMC preparation was isolated from a 100 mL blood sample from an unaffected healthy adult volunteer, aliquoted and frozen at −180°C in 90% FBS/10% DMSO. An aliquot of the standard PBMC preparation was stained and analyzed with each subject sample. Evaluation of oxidizing free radical production was possible only in those case and unrelated control samples for which sufficient (~20 mL) blood volume was obtained.

2.6. Statistical Analysis. Within the control group, 16 of the 41 unaffected control children were case siblings. There were 27 additional case children without a sibling and 25 additional unrelated control children comprising the total case-control cohort of 84 children. To down-weight the impact of outliers, three metabolites observations were curtailed at the extremes of the distributions for PBMC GSH, PBMC GSSG, and Monocytes GSH/GSSG (see footnote in Table 2). The sibling data are correlated resulting in a combined sample of correlated and uncorrelated data; thus, the assumption of all data being independent is not satisfied for the standard two-sample t-test. To make use of all data from dependent and independent observations, we used the corrected Z-test proposed by Looney and Jones [47]. This statistical approach provides adequate control of Type 1 errors and has more power than a standard Student’s t-test. Because the DCF data compared cases and unrelated controls (without siblings) the standard Student’s t-test was used with significance set at 0.05. Nonparametric intercorrelations (Spearman correlation coefficients) between age and gender and the 7 outcome variables, GSH, GSSG, GSH/GSSG, % oxidized glutathione, cysteine, cystine, and cysteine/cystine were determined with the significance level set at 0.05. Data was analyzed using SAS 9.2 software (SAS Institute Inc, Cary, NC, USA).

3. Results

3.1. Demographics of Study Population. Table 1 lists the demographics of the study population. The only major differences between cases and controls are that the control group was composed of a greater proportion of females and African Americans, whereas the case group had a greater proportion of Asian subjects. Over-the-counter multivitamin supplement use was higher among cases (39.5%) compared

<p>| Table 1: Demographics of study population. |
|------------------------------|----------------|----------------|
|                              | Case children  | Control children |</p>
<table>
<thead>
<tr>
<th></th>
<th>n = 43</th>
<th>n = 41</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age; mean (SD)</td>
<td>5.42 (1.98)</td>
<td>6.16 (2.29)</td>
</tr>
<tr>
<td>Male; n (%)</td>
<td>36 (84)</td>
<td>20 (49)</td>
</tr>
<tr>
<td>White; n (%)</td>
<td>38 (88.4)</td>
<td>31 (75.6)</td>
</tr>
<tr>
<td>Asian; n (%)</td>
<td>2 (4.65)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>African American; n (%)</td>
<td>2 (4.65)</td>
<td>8 (19.5)</td>
</tr>
<tr>
<td>Hispanic; n (%)</td>
<td>1 (2.3)</td>
<td>2 (4.9)</td>
</tr>
<tr>
<td>OTC multivitamin use; n (%)</td>
<td>17 (39.5)</td>
<td>8 (19.5)</td>
</tr>
</tbody>
</table>
Neither age nor gender was significantly correlated with any of the outcome measures. The protein content per 10^6 cells did not differ between case and control children (data not shown).

### 3.2. Decreased Intracellular Glutathione Redox Status in Autism

Table 2 presents the relative intracellular concentrations of GSH, GSSG, the glutathione redox ratio, and the percentage of oxidized glutathione equivalents in resting (unstimulated) PBMCs and in isolated stimulated monocytes and CD4 T cells from children with autism and age-matched control children. The percent oxidized glutathione is expressed in absolute glutathione equivalents as 2GSSG/(GSH+2GSSG). Relative to controls, the intracellular concentration of GSSG and the percent oxidized glutathione were significantly increased (~40%), and the GSH/GSSG ratio decreased (~21%) in PBMCs from children with autism (P < 0.001). After stimulation with LPS, monocytes from children with autism also exhibited significantly decreased GSH/GSSG (~31%, P = 0.003), increased GSSG concentration (~32%, P = 0.01), and 40% higher percent oxidized glutathione (P < 0.001). In mitogen-stimulated CD4 T cells from children with autism, the intracellular GSH concentration was ~33% lower, the GSH/GSSG was ~40% lower (P < 0.001), and the percent oxidized glutathione was ~55% higher than in stimulated CD4 T cells from control children (<0.001). As expected, activation with LPS and PMA both resulted in decreased intracellular GSH levels and GSH/GSSG in isolated monocytes and CD4 T cells compared to resting (unstimulated) PBMCs. Upon stimulation, there was a greater decrease in intracellular GSH and GSH/GSSG in both CD4 T cells and monocytes from children with autism compared to control children.

### 3.3. Decreased Extracellular Glutathione and Cysteine Redox Status in Autism

Table 3 presents the relative concentrations of GSH, GSSG, % oxidized GSH, cysteine, cystine, and the cysteine/cystine redox ratio in the extracellular plasma compartment. Children with autism exhibited a significantly decreased extracellular concentration of GSH (~21%) and GSH/GSSG (~54%) and increased concentration of GSSG and the percent oxidized glutathione (52% and 82%, resp., P < 0.001). Figures 1(a) and 1(b) compare GSH/GSSG and % oxidized glutathione equivalents, respectively, in plasma, T cells, and monocytes from case and control children and graphically demonstrates the consistent decrease in both extracellular and intracellular glutathione redox status among the case children.

The dynamic balance between the reduced and oxidized forms of glutathione can also be expressed as the redox potential or reducing power of the GSH/GSSG redox couple (E_h) and can be calculated from the Nernst equation, E_h = E_0 + R T/nF ln([disulfide]/([thiol 1] * [thiol 2])), where E_0 is the standard potential for the glutathione redox couple (~264 mV), R is the gas constant (8.314 J/Kmol), T is the absolute temperature of analytical measurement (25°C = 298 K), n is 2 for the number of electrons transferred, and F is Faraday’s constant (96,485 coulomb/mol) [48]. The calculated E_h value for the GSH pool in the children with autism is −116 mV, which is 12 mV more oxidized than in the control children, with an E_h value of −128 mV (Table 3).

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Case children</th>
<th>Control children</th>
<th>Corrected Z-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH (nmol/mg protein)</td>
<td>43 25.45 ± 8.16</td>
<td>41 23.35 ± 6.38</td>
<td>2.09 (−1.09, 5.29)</td>
</tr>
<tr>
<td>GSSG (nmol/mg protein)</td>
<td>43 0.90 ± 0.3</td>
<td>41 0.66 ± 0.23</td>
<td>0.24 (0.13, 0.35)</td>
</tr>
<tr>
<td>GSH/GSSG</td>
<td>43 29.58 ± 9.04</td>
<td>41 37.58 ± 10.89</td>
<td>−7.99 (−12.51, −3.48)</td>
</tr>
<tr>
<td>Oxidized GSH (%)</td>
<td>43 0.07 ± 0.02</td>
<td>41 0.05 ± 0.01</td>
<td>0.02 (0.0075, 0.024)</td>
</tr>
</tbody>
</table>

### Table 2: Intracellular glutathione redox status in resting PBMCs and activated monocytes and CD4 T cells.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Case children</th>
<th>Control children</th>
<th>Corrected Z-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH (nmol/mg protein)</td>
<td>18 7.73 ± 3.16</td>
<td>20 8.55 ± 2.5</td>
<td>−0.82 (−2.02, 0.38)</td>
</tr>
<tr>
<td>GSSG (nmol/mg protein)</td>
<td>18 0.62 ± 0.24</td>
<td>20 0.47 ± 0.17</td>
<td>0.14 (0.03, 0.25)</td>
</tr>
<tr>
<td>GSH/GSSG</td>
<td>18 13.31 ± 7.26</td>
<td>20 19.30 ± 6.35</td>
<td>−5.98 (−9.99, −1.97)</td>
</tr>
<tr>
<td>Oxidized GSH (%)</td>
<td>18 0.14 ± 0.05</td>
<td>20 0.10 ± 0.03</td>
<td>0.04 (0.02, 0.07)</td>
</tr>
</tbody>
</table>

### Table 3: Intracellular glutathione redox status in activated monocytes and CD4 T cells.

**GSH:** glutathione; **GSSG:** oxidized glutathione disulfide; **oxidized GSH:** (%)2GSSG/(GSH+2GSSG); **curtailment:** PBMC GSH >45 set = 45 (n = 1); PBMC GSSG >1.75 set = 1.75 (n = 1); Monocytes GSH/GSSG >35 set = 35 (n = 1).
The concentration of cystine, the oxidized form of cysteine, was significantly elevated (∼52%), while the cysteine/cystine redox ratio was significantly decreased (∼31%) in plasma from children with autism (P < 0.001). The $E_0$ value for the cysteine pool can also be calculated from the Nernst equation (see above) where the $E_0$ for cysteine is equal to $-250 \text{ mV} + (30 \text{ mV}) \times \log([\text{GSSG}]/[\text{GSH}]^2)$; $E_0$ for cysteine: $-250 \text{ mV} + (30 \text{ mV}) \times \log([\text{Cys}]_2)/([\text{CySSCy}])^2$.

### 3.4. Elevated Free Radical Production in Autism.

The level of intracellular free radicals was measured in available resting PBMCs from children with autism ($n = 15$) and unaffected control children ($n = 16$) using DCF, an ROS/RNS-sensitive fluorescent probe. Monocytes and lymphocytes were gated based on light scatter properties (size and density) and analyzed separately. Figure 2 presents the median fluorescence intensity (MFI) of lymphocytes from children with autism and unaffected control children (normalized to MFI of the standard PBMC preparation). Gated lymphocytes from children with autism exhibited a significantly higher mean level of intracellular free radicals compared to lymphocytes from control children ($P < 0.05$). No differences in free radical production were observed in gated monocytes from case and control children. Intracellular free radical production was not correlated with age or gender in this cohort.

### 4. Discussion

Oxidative stress is generally defined as an imbalance between oxidant production and endogenous antioxidant defense mechanisms and can be clinically defined in humans by a decrease in the redox status of GSH/GSSG and cysteine/cystine thiol/disulfide redox couples [49]. The relative equilibrium between reduced and oxidized sulphydryl groups defines the ambient redox state. Low glutathione redox status...
has been associated with the pathophysiology of several neurobehavioral disorders including schizophrenia [2, 50], bipolar disorder [3], alcoholism [51], HIV [52], and Alzheimer’s disease [53]. This is the first study to evaluate intracellular glutathione-mediated antioxidant/redox capacity in primary cells from children with autism as well as the extracellular plasma cysteine/cystine redox status. Because these two redox systems are compartmentalized and independently regulated, evaluation of both redox couples provides a complete picture of the primary immune cell microenvironment in children with autism. Supporting and extending our previous findings of decreased plasma and lymphoblastoid cell GSH/GSSG, we now report that both primary immune cell GSH/GSSG and plasma cysteine/cystine redox couples are similarly compromised resulting in a more oxidized immune cell microenvironment in children with autism compared to control children.

Recent evidence supports the notion that subtle fluctuations in ambient redox status may provide an important regulatory mechanism that can dynamically modulate immune cell function. Activation and proliferation of T cells require a reducing intracellular microenvironment, whereas a more oxidized environment promotes cell cycle arrest and blunted responsiveness to immune stimulation [54–57]. For example, a mechanism involving extracellular redox modulation by regulatory T cells (Tregs) was recently elucidated by Yan et al. [35]. Tregs were shown to inhibit the release of cysteine into the immune synapse between dendritic cells and naive T cells, which effectively reduces GSH levels in T cells by eliminating the rate-limiting amino acid for GSH synthesis. A high ratio of reduced to oxidized glutathione is required for cell cycle progression from G1 to S phase and induction of the T-cell proliferative response [55]. Thus, the more oxidized GSH/GSSG redox state of the intracellular glutathione pool in PBMCs and in activated CD4 T cells observed in children with autism (Table 2) would suggest a hyporesponsive phenotype that is less conducive to T-cell activation and proliferation. Consistent with this hypothesis, several recent studies have documented abnormalities in the adaptive immune response in children with autism [44, 58].

A glutathione deficit in T cells has been shown to negatively affect the adaptive immune response and T-cell proliferation by reducing IL-2 receptor turnover and IL-2-dependent DNA synthesis [59, 60]. In monocytes, an oxidized intracellular environment has been shown to alter the cytokine profile and skew the Th1 and Th2 balance [61, 62]. Studies in mice have demonstrated that the intracellular GSH content of antigen presenting cells (APCs) reversibly alters the Th1 and Th2 cytokine response pattern [61]. Specifically, a GSH deficit reduced Th1-associated IFN-γ production and exaggerated Th2-associated IL-4 production. Restoration of GSH restored the Th1 cytokine response and normalized the Th2 response. Consistent with these observations, two independent studies have reported that helper T-cell sub-populations in PBMCs from children with autism are shifted towards T helper 2 (Th2) dominance [41, 42]. Further, a decrease in T-cell IL-2 receptor expression has been reported to be associated with decreased proliferative response after mitogen stimulation in children with autism [58].

The more oxidized GSH/GSSG redox status in plasma and primary immune cells in children with autism (Figure 1) may offer a mechanistic explanation for the abnormal adaptive immune response previously reported in these children. When intracellular oxidative stress exceeds glutathione redox capacity, cells export GSSG into the plasma as a mechanism to restore internal redox homeostasis [49, 63]. The elevated GSSG concentrations in PBMCs (Table 2) suggest that the GSSG export mechanism and intracellular antioxidant capacity were not sufficient to maintain intracellular redox homeostasis and that redox imbalance was chronic in these children. The association between a more oxidized immune cell microenvironment and an abnormal adaptive immune response warrants continued investigation especially in light of the potential reversibility of immune dysfunction with targeted treatment to restore redox homeostasis [15].

The calculated $E_h$ values for the extracellular GSH and cysteine pools (Table 3) in our control population differ somewhat from previously published values. In adults, the plasma glutathione $E_h$ is more reduced at around $-137$ mV, and the plasma cysteine redox couple is more oxidized at $-80$ mV [30, 48]. These discrepancies may reflect methodological differences in sample preparation in that our electrochemical detection does not require derivatization for detection. It is also possible that children (age 3–10 years) may have less reducing capacity than previously reported in adults (age 25–35 years) [48]. Nonetheless, our calculated $E_h$ values are consistent with previous reports that plasma cysteine $E_h$ ($-111$ mV) is more oxidized than that of GSH ($-128$ mV).
Mean intracellular free radical production was higher in primary lymphocytes from children with autism relative to lymphocytes from age-matched control children (Figure 2) and was driven by a subset of 5 (33.3%) children whose lymphocytes exhibited especially high levels of free radicals. Mitochondria are the primary producers and targets of intracellular free radicals, and mitochondrial dysfunction has been postulated to be a contributing factor in the pathogenesis of autism and numerous other neurological disorders [64–67]. In a lymphoblastoid cell model, we previously demonstrated that the GSH/GSSG redox ratio in mitochondria was significantly lower in autism compared to control cells and was associated with a significantly lower mitochondrial membrane potential after nitrosative stress [16]. It is well established that mitochondria are highly concentrated in presynaptic terminals and that loss of redox control can negatively affect the efficiency of neurotransmission and synaptic plasticity [68, 69]. Similarly, mitochondrial localization and redox sigaling at the immunological synapse between lymphocytes and antigen presenting cells are required for immune activation, and excessive ROS can interrupt these signaling pathways [70–72]. A recent study of mitochondrial defects in lymphocytes from children with autism found decreased complex I activity and overreplication of and deletions in mitochondrial DNA compared to control lymphocytes [73]. Based on this evidence, it is plausible to hypothesize that mitochondria may be the source of the increased levels of lymphocyte free radicals observed in the subset of autistic children presented in Figure 2. Consistent with this hypothesis, a recent meta-analysis estimated that mitochondrial dysfunction may affect up to 30% of children with autism [64]. Based on this evidence, further study of mitochondrial function and redox status in lymphocytes from children with autism is warranted.

Relevant to our observations, two recent papers have revealed that an oxidized extracellular cysteine/cystine redox status can initiate a redox signaling cascade that stimulates intracellular mitochondrial ROS production as a mechanism to initiate an inflammatory immune response [74, 75]. The signal transduction from the extracellular to intracellular compartments occurs through oxidative modification of redox-reactive cysteines on cell surface proteins. Exposed cysteine sulfhydryl groups on proteins can be reversibly oxidized to sulfenic acid or disulfide bonds resulting in altered protein structure and function that initiate downstream redox signaling cascades [33, 76, 77]. In an elegant series of experiments, Imhof and Hansen demonstrated that mitochondrial ROS production was significantly increased in cells incubated under extracellular oxidized cysteine/cystine redox conditions [74]. The stimulated intracellular ROS production resulted in the expression of Nrf-2, the transcription factor responsible for initiation of the inflammatory response. Treatment to block the availability of cell surface cysteine thiol groups abrogated mitochondrial ROS production and Nrf-2 expression. Go et al. confirmed and extended these observations by demonstrating that treatment to maintain mitochondrial redox status abrogated ROS production in the presence of oxidized extracellular cysteine/cystine [75]. Although the precise mechanism for the oxidative cysteine/cystine-dependent signaling for mitochondrial ROS production is not yet clear; the authors provide evidence of a possible link to changes in the redox state of cytoskeletal proteins that could be functionally linked to the mitochondrial membrane. Other studies have demonstrated that an oxidized plasma cysteine/cystine redox potential is associated with proinflammatory conditions [78, 79] and can be modulated by diet [80, 81]. These observations support the possibility that the oxidized plasma cysteine/cystine in children with autism may be functionally related to the increase in lymphocyte free radical production observed and contribute to immune cell abnormalities in these children.

In summary, we show for the first time that both the extracellular and intracellular immune cell compartments are more oxidized in children with autism compared to age-matched unaffected control children. Randomized clinical trials will be needed to determine whether treatment to normalize plasma and intracellular redox status will improve immune cell function and possibly the health and behavior in children with autism.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgments

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References


Clinical Study

High Complement Factor I Activity in the Plasma of Children with Autism Spectrum Disorders

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1. Introduction

Autism spectrum disorders (ASDs) are characterized by impairments in social interaction, communication, and repetitive or restricted patterns of interests, or behaviours, and are classified as developmental disorders in DSM-IV [1]. ASD is about 4-5 times more prevalent in boys than in girls. The ratio is estimated to range from 5.5 : 1.4 to 16.8 : 4.0 [2]. Recent research clearly indicates that the underlying causes of autism are neurobiological disorders and combinations of different factors, such as environmental and genetic factors, and abnormality in the communication between neurons, probably associated with an abnormal set of neuropeptides in the brain [3–9].

The symptoms of ASD have been linked with elevated plasma levels of serotonin [10, 11] and opioid [12], abnormal levels of melatonin [13], altered levels of activity of the serine protease prolyl endopeptidase [14], and infectious and immunological factors such as abnormalities of T cells, B cells, natural killer (NK) cells, and of the complement system [15–21].

The complement system comprises a group of proteins which, when activated, provide one of the first lines of defence by promoting lysis and the removal of invading microbes. Activation of the complement system in response to an infection or foreign antigen is achieved via three complement pathways, the classical pathway, which is activated by antigen-antibody complexes, the lectin pathway, which is activated by the interaction of microbial carbohydrates with mannose-binding proteins in the plasma and tissue fluids, and the alternative complement pathway, which is activated by C3b binding to microbial surfaces and to antibody molecules. All of the three pathways converge with the activation of the central C3 component. This leads to a final common pathway with assembly of the C5–C9 components to form a cell surface transmembrane pore (membrane attack complex) [22, 23]. It has been shown by comparison with healthy control children that several differentially expressed proteins are related to the complement system in children with ASD [22]. The alternative pathway consists of six proteins: C3, factor B, factor D, factor H, factor I, and properdin. The plasma
glycoprotein factor I (C3b/C4b inactivator) is a serine protease that acts as a regulator of the complement C3 cascade. Factor I has a molecular weight of about 88 kDa, consists of two disulfide-linked polypeptide chains (50 kDa and 38 kDa, resp.), and is synthesized as a single-chain precursor in the liver [24, 25]. Factor I cleaves C3b and C4b in a reaction, where fl is dependent on various cofactors, such as factor H, C4b-binding protein CR1 and membrane cofactor protein (MCP) [26]. Factor I-mediated cleavage of the α chain of C3b liberates 3 fragments with molecular weights of 68 kDa, 43 kDa, and 2 kDa. Degradation of C3b by fl abrogates the action of this protein in the C3 pathway [27]. Complement C3b is the major opsonin of the complement system which facilitates the phagocytosis process by coating antigens (each of the phagocytes expresses a complement receptor such as CR1, CR3, or CR4 that binds C3b, C4b, or C3bi) [28, 29]. Factor I deficiency can be conferred by a C3 deficiency, since this also increases susceptibility to pyogenic infections by Neisseria meningitides, Haemophilus influenza, and Streptococcus pneumonia and increases the incidence of immune complex diseases due to impaired complement-mediated function [30]. Immune system abnormalities have been associated with autism [15–20], and it has been suggested that children with ASD might have an increased incidence of bacterial inflammation [31]. Immunological aspects of the early onset of autism have recently highlighted the fact that immune dysfunction may occur in some children with autism [31, 32].

Having previously discovered altered levels of the serine protease prolyl endopeptidase in children with ASD [14], the aim of this study was to investigate if an association exists between serine protease fl deficiency and the development of ASD.

2. Materials and Methods

2.1. Participants. Thirty children with ASD and thirty typical control children participated in this study. The ASD group comprised 23 boys and 7 girls with a mean age of 4.5 years (age range 3–9 years). The control group comprised 13 boys and 17 girls, mean age 6.0 years (age range 3–12 years), (Table 1).

Children in the ASD group were recruited from the Autism Rehabilitation Centre at the University of Social Welfare and Rehabilitation Sciences in Tehran, Iran. After having obtained informed consent from the parents, blood samples were collected. All children with ASD were examined by clinical specialists on autism. A child psychiatrist and a child neurologist who was familiar with the core behaviours in autism stated by the American Academy of Pediatrics in its Embargo from 2007 [35]. The control group consisted of typically developed and healthy children showing no signs of neurodevelopmental disorders who were recruited from the same area as the children with ASD. Children who had any kind of infection/infectious disease within two weeks prior to the time of examination were excluded from this study.

The study was approved (MT/1247) by the ethics committee of the Iran University of Medical Sciences, Tehran.

2.2. Procedure

2.2.1. Blood Sample Collection. Blood samples were collected by a paediatric nurse, and those from the children diagnosed with autism were collected under the supervision of a child psychiatrist with special training in the field of childhood psychosis. Venous blood was collected into 3 mL EDTA tubes (Vacutainer System; Becton-Dickinson Inc., Plymouth, UK), and plasma was separated immediately thereafter by centrifugation at 1,300 g for 10 min at 4°C. Thereafter, an inhibitors cocktail (30 μL per 1 mL plasma) was added to the resultant plasma sample (cocktail inhibitor solution: 2.0 M Tris, 0.9 M Na-EDTA, 0.2 M Benzamidine, 92 μM E-64, and 48 μM Pepstatin; Sigma, St. Louis, Mich, USA). The plasma was stored at −80°C.

2.2.2. Assay. Methods based on the hydrolysis of fluorogenic substrates have previously been described by Tsifoglu and Sim [36] and Gupta et al. [15]. The following assay procedure was found to be optimal for assaying fl activity in the plasma. 20 μL of plasma was incubated with 80 μL of buffer (100 mM phosphate buffer, pH 7.5, containing 1 mM EDTA, 1 mM DTT and 1 mM sodium azide) for 10 min at 37°C to reach thermal equilibrium. 100 μL of substrate solution (200 μM Boc-Asp(OBz)-Pro-Arg-7-amino-4-methylcoumarin; Bachem, Bubendorf, Switzerland) in 25 mM phosphate buffer, pH 7.4, was then added, and the mixture was incubated at 37°C for 60 min (see Figure 1). The reaction was inhibited by the addition of 1 mL of 1.5 M acetic acid, and the release of 7-amino-4-methylcoumarin was measured by spectrofluorometer (Hitachi-f 2000; λex: 360 nm; λem: 440 nm; slit width: 2.5). All measurements were carried out randomized and in duplicate. Background fluorescence in the assay was monitored by the use of plasma in the absence of substrate and was subtracted from values obtained in the presence of substrate.

2.3. Data Analysis and Statistics. Plasma fl activity was log-normally distributed, and logarithmic values were, therefore, used when analysing differences between the ASD group and the control group. To adjust for age (dichotomized using the median value, 5 years) and gender, factorial ANOVA was used. A P value < 0.05 was considered statistically significant. Statistica 8.0 (StatSoft©, Tulsa, Okla, USA) was used. Intra- and interassay variability of the plasma fl activity was expressed as the standard error of a single determination (Smethod), using the formula first proposed by Dahlberg [37]

\[
S_{\text{method}} = \sqrt{\frac{\sum d_i^2}{(2n)}},
\]

(1)
Table 1: Age/y, gender, and medication of the participants.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ASD (n = 30)</th>
<th>Controls (n = 30)</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (SD)</td>
<td>4.8 (1.7)</td>
<td>6.1 (2.3)</td>
<td></td>
</tr>
<tr>
<td>Median (range)</td>
<td>4.5 (3–9)</td>
<td>6 (3–12)</td>
<td>0.033</td>
</tr>
<tr>
<td>≤5 years</td>
<td>21</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>&gt;5 years</td>
<td>9</td>
<td>16</td>
<td>0.115</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>23</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Females</td>
<td>7</td>
<td>17</td>
<td>0.017</td>
</tr>
<tr>
<td>Medication</td>
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<tr>
<td>No specific medication</td>
<td>8</td>
<td>30</td>
<td>—</td>
</tr>
<tr>
<td>Risperdal alone or in combination</td>
<td>18</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>Ritalin alone or in combination</td>
<td>4</td>
<td>0</td>
<td>—</td>
</tr>
</tbody>
</table>

*Difference between ASD and controls. Mann-Whitney U-test for age and Fisher's exact test for age category and gender.

Figure 1: Fluorescence intensity of release of 7-amino-4-methyl coumarin as a function of plasma incubation period (mean ± SD; 1 h (974 ± 44.2), 2h (1995 ± 45.2) and 3 h (2374 ± 1.2)).

where $d_i$ is the difference between the $i$:th paired measurement and $n$ is the number of differences. The $S_{method}$ was expressed as the coefficient of variation (%).

3. Results

There was significantly higher activity of plasma fl in the children with ASD (geometric mean (95% confidence limit): 523 (154–1776) when compared with the control group: 361 (135–967; ANOVA $P = 0.015$, adjusted for age and gender; Figure 2).

No statistically significant interactions were found with regard to gender and age, and no significant associations were found between fl activity and age or gender (ANOVA; $P = 0.25$ for gender and 0.42 for the two age groups, Figure 2). In the ASD group, some children with severe autism were under medication with Risperdal to reduce hyperactivity and violent behavior, and a few were under medication with Ritalin to improve attention (Table 1). It would have been ethically questionable to discontinue medication with the purpose of controlling the experimental design. We have correlated the data shown in Figure 2(a) with the type of medication the children in the ASD group were receiving. Although we did not see any clear correlation between medication and distribution for the scatter plot data, it cannot be excluded that some differences in the pattern may be influenced by medication, as has been previously discussed [22].

The values were statistically significantly higher in the children with ASD, and there was a weak association with gender. No statistically significant differences were found, however, between the age groups.

The methodological intra-assay error was small, 0.5%. The interassay methodological error was 13%. We found a significantly higher complement factor I enzyme activity in children with ASD compared to the control group of around the same age. This is, as far as we know, the first report regarding dysfunction of fl activity in children with ASD. Although not statistically significant, males tended to exhibit higher fl activity than females, and the difference between the control group and the ASD group was more convincing amongst the younger children, as shown in Figure 2. Due to fl’s role as a regulating factor in the complement system pathway, an fl abnormality could play a role in the onset of ASD. A defect in this pathway makes the individual more vulnerable to various inflammations. Some reports [21, 24–26] provide increasing evidence of a connection between immunological abnormality and human disease. ASD numbers among the types of disorders that are associated with immune system abnormalities [28, 38]. Our results are consistent with a recent proteomic study of serum from ASD children, where a significant differential expression was shown for proteins related to the complement system [22].

4. Discussion

The aetiology of ASD is still largely unknown despite the fact that many factors such as genetic, environmental, immunological, and neurological aspects are thought to influence
The pathophysiology of ASD is poorly understood. Children with ASD are prone to recurrent viral and bacterial inflammations. There are also some reports of immune system abnormalities in children with ASD [15–20]. An association between ASD and immune system abnormalities together with the vulnerability of the ASD group with regard to inflammatory processes may indicate an impaired mechanism in this system. It is known that phagocytosis is an important part of the body’s defence mechanism. This mechanism requires an active complement system and a functioning C3b protein. C3b is degraded by fI, and abnormal fI activity might cause an abnormal and uncontrolled degradation of C3b protein, resulting in the loss of the phagocytosis function for this particular protein (a function which partly protects the body from invasion of foreign organisms). Altered levels of other serine protease activities, such as that of proline endopeptidase (PEP), have also been found in a group of children with ASD when compared to a control group [14]. The results of the present study, together with our previous findings on altered levels of PEP activity, may indicate a connection between the onset of ASD and serine protease dysfunction. The higher plasma fI activity observed in the male group as compared to that of the female group (Figure 2) is paralleled by a higher occurrence of ASD in male children. Also, the higher plasma fI activity in children younger than six years of age may indicate that the inflammatory process is more active in younger ages or that we may be dealing with two subgroups of children with ASD with different onsets of the disease.

5. Conclusions

The preliminary findings of this study together with our previous report [14] suggest that there may be an association between abnormal serine protease activity and the development of ASD. Further research is needed, however, to establish a possible role of serine proteases in the aetiology of ASD.

Conflict of Interests

The authors declare that they have no conflict of interest.
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References


