ORIGINAL ARTICLE

Elevated plasma concentrations of S100 calcium-binding protein B and tumor necrosis factor alpha in children with autism spectrum disorders

Selin Aktan Guloksuz,[1] Osman Abali,[2] Esin Aktas Cetin,[3] Sema Bilgic Gazioglu,[3] Gunnur Deniz,[3] Abdurrahman Yildirim,[4] Ivana Kawikova,[5] Sinan Guloksuz,[6,7] James F. Leckman[1]

[1]Child Study Center, School of Medicine, Yale University, New Haven, CT, USA. [2]Department of Child and Adolescent Psychiatry, Istanbul University School of Medicine, Istanbul, Turkey. [3]Department of Immunology, Institute of Experimental Medicine (DETAE), Istanbul University, Istanbul, Turkey. [4]Department of Pediatrics, Istanbul Memorial Hospital, Istanbul, Turkey. [5]Department of Pediatrics, School of Medicine, Yale University, New Haven, CT, USA. [6]Department of Psychiatry, School of Medicine, Yale University, New Haven, CT, USA. [7]Department of Psychiatry and Neuropsychology, South Limburg Mental Health Research and Teaching Network, European Graduate School of Neuroscience (EURON), School for Mental Health and Neuroscience (MHeNS), Maastricht University, Maastricht, The Netherlands.

Objective: To investigate plasma concentrations of S100B (a calcium-binding protein derived primarily from the glia) and inflammatory cytokines in children with autism and the relationship between S100B and cytokine concentrations.
Methods: Plasma levels of S100B, tumor necrosis factor alpha (TNF-α), interferon gamma, interleukin (IL)-1β, IL-4, IL-6, IL-10, and IL-17A were measured in 40 unmedicated children with autism and 35 normally developing healthy children. The severity of autism was assessed using the Childhood Autism Rating Scale (CARS).
Results: Concentrations of both S100B and TNF-α were higher in children with autism before and after adjusting for a priori-selected confounders (age, sex, and body mass index). S100B concentrations were higher in children with severe autism compared to children with mild-moderate autism. However, this association remained as a trend after adjusting for confounders. S100B concentrations correlated positively with TNF-α concentrations.
Conclusion: Our findings showing an increase in peripheral concentrations of S100B and TNF-α provide limited support to the hypothesis about the roles of altered immune function and S100B in autism spectrum disorder (ASD). Studies of larger numbers of well-characterized individuals with ASD are needed to clarify the potential role of the immune system in the pathophysiology of this disorder.

Keywords: Autism; inflammation; glia; tumor necrosis factor; cytokine; S100B

Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by deficits in the development of social, communicative, and cognitive skills, accompanied by restricted, repetitive patterns of behavior or interests. The heterogeneous and complex pathoetiology of ASD remains largely unknown. Recently, the role of aberrant immune system functioning in the pathogenesis of ASD has received greater attention.[1-4] Specifically, current evidence suggests that microglial activation, synaptic under-connectivity, and neuroinflammation may contribute to some cases of ASD.[5,6]

Earlier studies have documented prominent microglial activation and increased production of inflammatory cytokines and chemokines, including interferon gamma (IFN-γ), interleukin (IL)-1β, IL-6, IL-12p40, tumor necrosis factor alpha (TNF-α), and chemokine (C-C motif) ligand 2 (CCL2) in the brain tissue and cerebrospinal fluid (CSF) of some individuals with ASD.[3,7] Although findings are not consistent, there is growing evidence indicating elevated plasma levels of pro-inflammatory cytokines, including TNF-α, IFN-γ, IL-1β, IL-6, IL-12, IL-17A, IL-23,[8-15] and decreased plasma levels of the anti-inflammatory cytokines IL-10 and transforming growth factor-beta (TGF-β) in children with ASD as compared to controls.[1,16] Although the phenotypic and neurobiological heterogeneity in ASD might be underlying factors that account for inconsistent findings, it should also be noted that important confounding factors influencing immune markers, such as medication and body mass index (BMI), have not been adequately addressed in previous research.

S100B is a calcium-binding protein. It is glial-specific and is expressed primarily by astrocytes. S100B modulates the cell cycle (e.g., proliferation, differentiation, apoptosis), neurite extension, and energy metabolism in the central nervous system (CNS).[17,18] Although S100B has been rigorously investigated in a number of neurological and psychiatric conditions, including schizophrenia,[18] mood disorders,[19] and Alzheimer's disease,[20] only

a small number of studies have focused on ASD. Higher S100B concentrations have been reported in children with ASD compared to controls and associated with increased severity of ASD.[21] In addition, a recent study addressed risk genes for ASD through copy number variation analysis and identified S100B as one of the genes potentially associated with ASD.[22] High concentrations of S100B may be an indicator of glial cell pathology and ongoing damage. However, the scarcity of longitudinal studies prevents us from making further inferences as to whether elevated concentrations of S100B is a cause or a consequence of ASD.[21]

In this study, we aimed to determine whether there is a difference between circulating S100B and cytokines (IL-1β, IL-4, IL-6, IL-10, IL-17A, IFN-γ, and TNF-α) measured in peripheral plasma samples from unmedicated ASD patients compared to healthy control children (HC) after adjusting for confounding factors.

Methods

Study population

A total of 75 children were enrolled for this cross-sectional study in the summer months (July-August 2013), to reduce the probability of flu infection and seasonal allergy as possible confounders. Forty children with ASD diagnosed according to the DSM-IV criteria (mean age = 7.13± 3.89 years, 10 [25%] girls) were recruited among long-term follow-up outpatients of the Autism Clinic of the Child and Adolescent Psychiatry Department at Istanbul University School of Medicine, Istanbul, Turkey. Of children with ASD, 28 (70%) were diagnosed with autistic disorder, while 12 (30%) were diagnosed with pervasive developmental disorder, not otherwise specified. Following initial screening, the autism diagnosis was reconfirmed according to the DSM-IV criteria through independent clinical interviews and medical chart reviews conducted by two child psychiatrists (SAG and OA) to eliminate diagnostic reliability issues. Thirty-five typically developing HC without any Axis I/II psychiatric disorders or positive family history of ASD were recruited from the Well Child Outpatient Clinic (mean age = 6.75±3.96 years, 13 [37%] girls). Detailed medical and psychiatric interviews - including developmental and family history - of HC and their parents were conducted by a child psychiatrist (SAG) and a pediatrician (AY). The study was approved by the Ethics Committee of Istanbul School of Medicine, Istanbul University, and carried out in accordance with the Declaration of Helsinki. All parents of the participants gave written informed consent before enrollment in the study. Children who were aged 12 years or older also signed the consent forms themselves.

Each participant underwent a physical examination and a medical chart review, including immunization records. Additionally, the parents of each participant were interviewed by medical doctors (SAG and AY) to obtain a detailed medical history. Weight and height were measured to calculate the BMI (18.14±6.39 kg/m² in ASD group; 17.02±4.42 kg/m² in HC group).

All children were unmedicated (including any psychotropic medications) and in good health at the time of study participation. The exclusion criteria were any known neurological disorders (e.g., fragile-X syndrome, tuberous sclerosis), congenital metabolic disorders, chronic immune-related diseases (e.g., asthma, atopic dermatitis), chronic infectious diseases (e.g., tuberculosis), acute infectious disease within the last 4 weeks, immunization within the last 8 weeks, and use of any immune-modulating medication (including non-steroidal anti-inflammatory drugs and aspirin) within the last 4 weeks.

Clinical assessments

Symptom severity in children with ASD was assessed using the Childhood Autism Rating Scale (CARS), which also aims to help screen and differentiate ASD diagnosis from other developmental disorders. CARS is rated on a four-point scale from 1 to 4 (in ½-point increments) on 15 dimensions (relationships with people, imitation, emotional response, body use, object use, adaptation to change, visual response, auditory response, near-receptor response, anxiety, verbal communication, nonverbal communication, activity level, intellectual inconsistency, and general impression), with total scores ranging from 15 to 60.[23] Total scores between 30 and 36.5 indicate mild-moderate autism, whereas total scores of 37 or greater indicate severe autism. The mean CARS score of children with ASD was 39.78±6.73. Of the children with ASD, 17 (42.5%) were rated as having mild-moderate and 23 (57.5%) as having severe autism.

Developmental regression - the loss of previously acquired developmental milestones such as social skills, nonverbal communication, imitation, self-care, simple pretend play, direct eye gaze, and orienting to name[24] - was assessed according to previous medical records and a clinical interview, and was found to be present in 12 (30%) children in the ASD group.

Fasting blood samples from children with ASD and HC were collected into heparin vacuum tubes (BD Biosciences, San Jose, CA, USA) between 8:00 a.m. and 10:00 a.m. and processed immediately after collection by centrifugation at 2,000 g for 10 min. Plasma samples were aliquoted into tubes and stored at -80 °C until analysis. The samples were not exposed to freeze-thaw cycles.

Measurement of IL-17A and S100B

S100B and IL-17A were determined using a commercial sandwich enzyme-linked immunoassay (ELISA) (Human S100B ELISA kit; DiaMetra, Spello, Italy) and human IL-17A ELISA kit, respectively (Diaclone, Bejançon, France). All assays were conducted according to manufacturer protocols. A monoclonal antibody specific for IL-17A and S100B was pre-coated onto a 96-well plate. Supplied standards were used to generate the standard curves. The samples and standards were applied to wells. Unbound protein was removed by washing, and biotin conjugate followed by horseradish peroxidase-conjugated streptavidin were added in a stepwise manner. After color reaction with the substrate, the ELISA plates were immediately read at 450 nm. The limits of detection for

this system were 2.3 pg/mL for IL-17A and 35 pg/mL for S100B.

Measurement of IL-1β, IL-4, IL-6, IL-10, IFN-γ, and TNF-α

A panel for the IL-1β, IL-4, IL-6, IL-10, TNF-α, and IFN-γ cytokines was run using the bead-based Milliplex MAP multiplex technology (Millipore Corporation, Billerica, MA, USA). The analytes were measured according to the manufacturer's instructions. Antibody-labeled beads were resuspended and aliquoted into a pre-washed 96-well filter plate. Plasma samples and controls were mixed with the beads and incubated overnight at 4°C. After overnight incubation, the plate was incubated with biotinylated detection antibodies and streptavidin-phycoerythrin solution and run on the Luminex 200™ instrument (Luminex, Austin, TX, USA). Milliplex Analyst software was used for data analysis. Standard curves were generated for each analyte and the mean fluorescence intensity value was converted into concentration using a five-parameter logistic curve fit. The minimum detectable concentrations for IL1-β, IL-4, IL-6, IL-10, TNF-α, and IFN-γ were 0.6 pg/mL, 4.5 pg/mL, 0.9 pg/mL, 1.1 pg/mL, 0.7 pg/mL, and 0.8 pg/mL, respectively.

Data analysis

Data were analyzed using STATA version 12.0. Associations between group (ASD vs. HC) and immune markers (IL-1β, IL-4, IL-6, IL-10, IL-17A, IFN-γ, TNF-α, and S100B) were analyzed using multiple regressions with immune markers as dependent variables and a dummy variable for the groups serving as an independent variable, and the HC group serving as a reference. In children with ASD, multiple regression procedures were applied to examine associations between immune markers and developmental regression (no developmental regression group serving as reference) as well as autism severity (coded as mild-moderate vs. severe, with mild-moderate serving as reference). As our outcome variables deviated from normality, we applied the distribution-free method of bootstrapping to each regression analysis (with n = 1,000 bootstrap resamples).[25] Analyses were adjusted a priori for age, sex, and BMI. Spearman's rank correlation was applied to determine associations between S100B and cytokine concentrations. Two-sided statistical significance was set at p < 0.05.

Results

Immune marker concentrations are shown in Table 1. Even after controlling for age, sex, and BMI, there were significant elevations in plasma levels of both S100B (Figure 1A) and TNF-α (Figure 1B) in children with ASD as compared to HC (Table 2).

Plasma S100B concentrations in children with severe ASD symptoms were higher than in children with mild-moderate ASD symptoms (B = 128.26, 95%CI = 16.25-240.26, p = 0.02). However, after controlling for age, sex, and BMI, S100B concentrations were no longer significantly different between children with severe ASD and

those with less severe ASD. No other significant differences were found in plasma cytokine concentrations in children with severe ASD compared to children with mild-moderate ASD (data not shown). In addition, there was no association between developmental regression and plasma immune markers (data not shown).

S100B correlated significantly with TNF-α (Spearman's rho = 0.34, p = 0.003). There were no other correlations between S100B and cytokines.

Discussion

A role for immune involvement in ASD has long been hypothesized.[4] Our findings that plasma S100B and TNF-α concentrations were higher in children with ASD confirm the findings of earlier studies and provide additional limited support for this hypothesis. Studies of larger numbers of well-characterized individuals with ASD are needed to clarify the potential role of S100B and the immune system in the pathophysiology of ASD. Based on the work of Careaga et al.,[26] it may be possible to identify immune subphenotypes within the ASD population.[4]

Although S100B has predictive value in estimating neuronal damage and altered blood-brain barrier permeability, and is easily measured in the periphery, little attention has been paid to its potential role as a peripheral marker in ASD.[21] As a result, our finding of elevated circulating levels of S100B is of particular interest. A growing body of evidence suggests the potential use of circulating S100B as a biomarker of global glial activity, which is shown to be altered in several neurological and neuropsychiatric conditions, ranging from CNS traumatic and vascular brain injury[27] to psychiatric conditions, including schizophrenia, mood disorders,[18] and childhood trauma.[28] At higher concentrations in the brain, S100B can also cause neuronal apoptosis by both microglial activation and neurotoxicity.[29,30]

We also observed an association between circulating levels of S100B and TNF-α. Biologically, 80 to 90% of S100B is localized in the CNS and secreted predominantly by astrocytes.[31] S100B synergistically interacts with TNF-α to upregulate receptor for advanced glycation endproducts (RAGE)-mediated nuclear factor-kappa-B (NF-κB) activation, which in turn induces transcription of antiapoptotic genes leading to cell survival at minimal S100B concentrations.[29,30] In addition to its co-regulatory role in RAGE-mediated NF-κB activation,[32] TNF-α-activated NF-κB transcription expression promotes cell survival under physiological conditions. However, when NF-κB transcription is activated by excessive amounts of TNF-α, it leads to production of excitotoxins, reactive oxygen species, and additional proinflammatory cytokines, which, in turn, leads to apoptosis in the absence of active feedback systems.[33,34] Whether and how any of these pathways are involved in ASD will need to be addressed by future experimental studies in mouse models of ASD and confirmed by correlative clinical studies.

Table 1 Plasma concentrations (pg/mL) of immune markers

	ASD	HC
S100B	182.50 (34.61)	48.13 (13.17)
TNF-α	15.59 (7.97)	10.50 (4.77)
IFN-γ	9.20 (8.07)	10.73 (14.03)
IL-1β	0.97 (0.56)	0.89 (0.85)
IL-4	19.22 (22.13)	13.86 (13.58)
IL-6	9.21 (3.93)	13.22 (14.09)
IL-10	49.87 (45.16)	71.84 (126.65)
IL-17A	2.69 (3.50)	3.26 (3.22)

Data presented as mean (standard deviation).
ASD = autism spectrum disorder; HC = healthy children; IFN-γ = interferon gamma; IL = interleukin; TNF-α = tumor necrosis factor alpha.
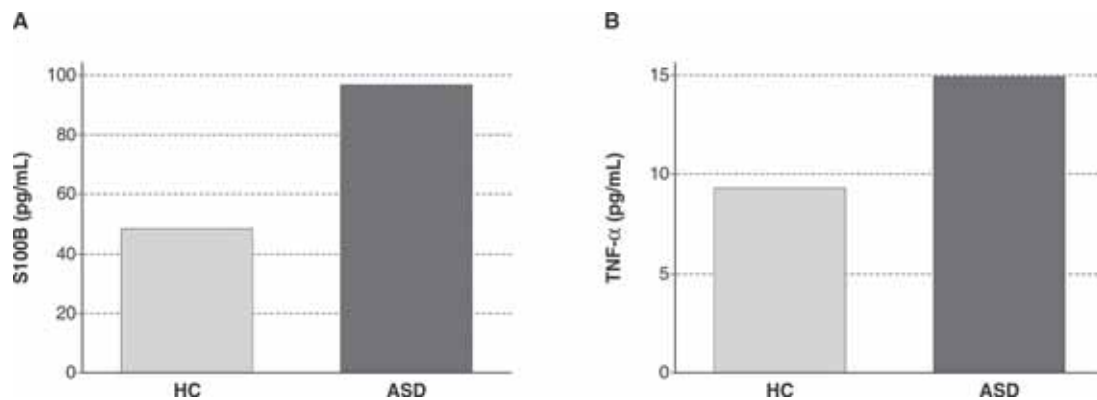

Figure 1 A) Plasma concentrations of S100B in children with autism spectrum disorder (ASD) and typically developing healthy children (HC). B) Plasma concentrations of tumor necrosis factor alpha (TNF-α) in children with ASD and typically developing HC. Bars represent median values.

Table 2 Associations between immune markers and autism spectrum disorder

	B*	SE	95%CI	p-value	B*†	SE	95%CI	p-value
S100B	134.37	34.81	66.14 to 202.59	< **0.001**	139.99	36.99	67.47 to 212.50	< **0.001**
TNF-α	5.08	1.46	2.23 to 7.94	< **0.001**	5.81	1.41	3.06 to 8.57	< **0.001**
IFN-γ	-1.53	2.62	-6.66 to 3.59	0.56	-1.49	2.76	-6.89 to 3.92	0.59
IL-1β	0.08	0.18	-0.27 to 0.42	0.66	0.11	0.17	-0.22 to 0.44	0.52
IL-4	5.36	4.37	-3.20 to 13.91	0.22	5.78	4.40	-2.84 to 14.40	0.19
IL-6	-4.01	2.46	-8.84 to 0.82	0.10	-3.67	2.59	-8.76 to 1.41	0.16
IL-10	-21.97	22.38	-65.83 to 21.89	0.33	-20.13	24.45	-68.06 to 27.79	0.41
IL-17A	-0.57	0.74	-2.03 to 0.89	0.44	-0.71	0.76	-2.21 to 0.78	0.35

95%CI = 95% confidence interval; B = regression coefficient; IFN-γ = interferon gamma; IL= interleukin; SE = standard error; TNF-α = tumor necrosis factor alpha.
* Normally developing healthy children serving as the reference.
† Controlled for age, sex, and body mass index.
Values in bold are significant.

Our findings replicated an earlier study that also reported higher circulating S100B concentrations in children with ASD compared to typically developing children, and an association between ASD severity and S100B.[21] We should note that earlier studies of plasma cytokines have yielded variable results. For example, a recent meta-analysis examined 19 cytokines across 17 studies with a total sample size of 743 individuals with ASD and 592 controls. Although this meta-analysis did not evaluate S100B measurements, it is important to note that our study of Turkish children with ASD failed to confirm the presence of elevated plasma levels of IL-1β, IL-6, or IFN-γ in individuals with ASD. These discrepancies are possibly due to the existence of subsets among patients with ASD. By lumping all of the cases together in a single ASD group, these differences may constitute a major confounding factor. Combined with previous data, our findings lead us to further consider phenotypic and neurobiological heterogeneity in ASD and the stability of immune markers over the course of the disease process. There is a significant need for longitudinal studies to help clarify the nature of the crosstalk between central neural circuits and the immune system, and the putative roles of various molecules in disease progress.

CSF levels of S100B and other cytokines are also of potential interest. However, since astrocytes are the primary source of S100B and plasma levels of S100B have been shown to be a valid marker of CNS trauma,[27] we chose to measure plasma concentrations rather than CSF levels, given the well-defined risks associated with lumbar puncture, which, in our estimation, exceeded the potential benefit. Likewise, noninvasive positron emission tomography and single-photon emission computed tomography using radioligands could be used to determine the regional specificity, if any, of CNS immune activation, including activation of microglial cells.[35] Radioligands - such as [11C]PK11195 and the more specific [11C]PBR-28 - binding to 18 kDa translocator protein (TSPO), which is highly expressed by activated microglia, provide a unique opportunity to trace microglial activation in the brain.[35] However, just as performing lumbar puncture has associated risks, so does the use of radioactive ligands, especially in children.

Several other limitations need to be taken into account when interpreting our findings. Despite the fact that we were able to recruit children with established diagnoses of ASD who were being followed up in the specialty autism clinic of an academic medical center,[36] where patients were observed by trained child psychiatrists and ultimately diagnosed by expert diagnosticians, collecting data by using the Autism Diagnostic Interview Revised (ADI-R) and the Autism Diagnostic Observation Schedule (ADOS) could have provided more detailed information. Unfortunately, neither instrument has been translated or adapted for use in the Turkish medical system thus far; therefore, these clinical ratings were not available for use in this study. Also, although all confirmed cases with fragile X were excluded, rigorous genetic testing was not performed, so this sample might have included children with the fragile X premutation.

In conclusion, its limitations notwithstanding, this study shed some light on the possible role of immune activation in ASD, as reflected in our findings of an increase in peripheral levels of S100B and TNF-α. To further elucidate the role of immune molecules in individuals with ASD, prospective longitudinal studies investigating a broad set of immune markers, both in serum and CSF, in large samples - with sufficient power to allow control for confounders - are needed.

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Disclosure

The authors report no conflicts of interest.

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