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Associations of impaired behaviors with elevated plasma chemokines in autism spectrum disorders

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ABSTRACT

A role for immune dysfunction has been suggested in autism spectrum disorders (ASD). Elevated levels of chemokines have been detected in the brain and CSF of individuals with ASD but, to date, no study has examined chemokine levels in the plasma of children with this disorder. In the current study, we determined whether there were differential profiles of chemokines in the plasma of children with ASD compared to age-matched typically developing controls and children with developmental disabilities other than ASD. Increased MCP-1, RANTES and eotaxin levels were observed in ASD children compared with both control groups ($p<0.03$), and increased chemokine production was associated with higher aberrant behavior scores and more impaired developmental and adaptive function.. Elevated MCP-1, RANTES and eotaxin in some ASD children and their association with more impaired behaviors may have etiological significance. Chemokines and their receptors might provide unique targets for future therapies in ASD.

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1. Introduction

Autism spectrum disorders (ASD) are a heterogeneous group of neurodevelopmental disorders that manifest during early childhood and are characterized by stereotyped interests and impairments in social interaction and communication (APA, 2000). Recent epidemiologic studies have suggested that ASD is diagnosed in approximately 1% of children (Kogan et al., 2009; MMWR, 2009) yet little is known about the etiology and underlying neuropathology, and there are no clear biological markers for these disorders. Evidence of immune dysfunction has been observed in many individuals with ASD, including marked activation of microglia, increased levels of pro-inflammatory cytokines in brain tissue, CSF and plasma, and increased production of cytokines by peripheral blood mononuclear cell (PBMC) cultures (Ashwood et al., 2008, 2009, 2010, in press-a,b; Ashwood and Wakefield, 2006; Enstrom et al., 2009a; Enstrom et al., 2010; Jyonouchi et al., 2001; Molloy et al., 2005; Onore et al., 2009; Vargas et al., 2005; Zimmerman et al., 2005).

Chemokines are a class of structurally similar proteins that are important in the development of lymphocytes, including their recruitment and trafficking to specific tissue compartments. In addition to acting as key chemotactic factors in the immune system, chemokines and their receptors play a pivotal role in dictating the movement of leukocytes into the CNS in both healthy and diseased individuals as illustrated in multiple sclerosis, Alzheimer's and Parkinson's disease (Mélik-Parsadaniantz and Rostène, 2008). In ASD, levels of the chemokines macrophage chemoattractant MCP-1 and eotaxin were found to be elevated in astrocytes in the anterior cingulate gyrus, with pronounced elevation of MCP-1 also noted in the cerebellum and in brain tissue homogenates (Vargas et al., 2005). A 12-fold increase in MCP-1 and eotaxin was also noted in CSF of ASD children when compared with controls (Vargas et al., 2005). Expression of mRNA transcripts for the chemokines macrophage inflammatory protein (MIP)-1β and 10 kDa interferon-inducible protein IP-10 was elevated in the temporal cortex of ASD individuals (Garbett et al., 2008), whereas gene expression of the chemokines MIP-1β and 'regulated upon activation normal T-cell expressed and secreted' (RANTES) were shown to be increased in the peripheral cells of ASD children (Enstrom et al., 2009a). However, to date, no study has addressed whether protein levels of chemokines are increased in the sera or plasma of children with ASD.

To examine if there exists a differential profile for peripheral blood chemokine levels, we analyzed plasma chemokine levels in young children with a diagnosis of ASD, typically developing children, and children with developmental disabilities other than ASD who were frequency-matched for age. Moreover, plasma chemokine levels were

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investigated for any associations with clinical behavioral and developmental outcomes.

2. Materials and methods

Participants in the study were recruited in conjunction with the population-based case-control CHARGE (Childhood Autism Risk from Genetics and Environment) study. The study design, recruitment and assessment protocols have been described in detail elsewhere (Hertz-Picciotto et al., 2006; Ashwood et al., 2008; Enstrom et al., 2009a). One hundred and seventy five children were investigated in this study and were frequency-matched for age; all children were medication-free and in good health at the time of blood draw. Participants included 80 children with ASD (median age 3.6, interquartile range (IOR) 3.0–4.5; 67 males), 58 typically developing (TD) controls (median 3.6, IOR 2.8–4.3; 39 males) and 37 children with developmental disability other than autism (DD) (median 3.5, IOR 3.0–4.0; 27 males). The study was approved by the UC Davis Institutional Review Board and complied with all requirements regarding human subjects. Informed consent was obtained from the legal guardian of each participant. ASD diagnosis was confirmed using gold standard assessment with the Autism Diagnostic Interview-Revised (ADI-R) and the Autism Diagnostic Observation Schedule (ADOS), with all assessments administered by qualified clinicians trained to research reliability on both measures. Children from the TD and DD groups were screened for autism traits using the Social Communication Questionnaire (SCQ) and no child in the TD or DD groups scored above the cut-off for ASD. Of the children with DD, 5 have an identified genetic disorder or chromosome abnormality including Cornelia de Lange syndrome (n = 1), Down syndrome (n = 3) and Chromosome 22 Ring (n = 1). The remaining children with DD have global developmental delay of unknown etiology. Adaptive function was assessed by parental interview using the Vineland Adaptive Behavior Scales (VABS) in ASD and control groups. Additional measures of cognitive ability were determined using the Mullen Scales of Early Learning (MSEL) and aberrant behaviors using the Aberrant Behavior Checklist (ABC) in ASD and control groups.

2.1. Chemokine analysis

For each subject peripheral blood was collected in acid-citrate-dextrose Vacutainers (BD Biosciences; San Jose, CA), centrifuged at 2300 rpm for 10 min and the plasma harvested. Plasma was aliquoted and stored at −70 ºC until chemokine levels were measured. The chemokines MCP-1, MIP-1α, MIP-1β, RANTES, eotaxin and IP-10 were assessed with human bead immunoassays (Biosource, Camarillo, CA) using Luminex technology. Plasma chemokine concentrations were measured according to the manufacturer's instructions and using chemokine standards they provided. The sensitivity of this assay allowed the detection of chemokine concentrations within the following ranges: MCP-1 15–9500 pg/ml; MIP-1α 6–40,000 pg/ml; MIP-1β 5–11,600 pg/ml; RANTES 3–16,300 pg/ml, eotaxin 15–9000 pg/ml; and IP-10 5–4400 pg/ml. Concentrations obtained below the level of detection (LOD) of the method were calculated as LOD/2 values for statistical comparisons. Values obtained from the reading of samples that exceeded the upper limit of the sensitivity method were further diluted and cytokine concentrations calculated accordingly. Plasma aliquots had not undergone any previous freeze/thaw cycle.

Results were generally not normally distributed and were naturally log-transformed for statistical analysis. Statistical analysis of data was performed using analysis of covariance (ANCOVA), so that adjustments could be made for age and gender. Evaluations of the relationship between the levels of chemokine and psychological measures among children with autism were determined by Spearman's rank correlations (rho). *P* values were corrected for multiple comparisons using the Benjamini–Hochberg False Discovery Rate and considered statistical significant if $p<0.05$ after corrections were applied. All analyses were performed using the Statistical Analysis System, version 9.1 (SAS Institute Inc, Cary, NC).

3. Results

Levels of MCP-1, RANTES and eotaxin were significantly higher in children with ASD compared with TD and DD controls (Table 1). After adjusting for child's age and gender, levels of MCP-1 in children with ASD (median 139.7, IQR 86–254.7 pg/ml) were significantly increased compared with TD (median 90.5,IQR 62–246.6 pg/ml; $p = 0.027$) and DD controls (median 84, IQR 56.7–119.1 pg/ml; $p = 0.011$). Plasma levels of RANTES were approximately two-fold higher in children with ASD (median 7967, IQR 4295–19,100 pg/ml) when compared with TD (median 4040, IQR 2341–9008 pg/ml; $p = 0.001$) and with DD controls (median 4170, IQR 2431–5271 pg/ml; $p = 0.005$). Observed levels of eotaxin in children with ASD were significantly higher (median 42.8, IQR 30.9–56.6) compared with TD (median 30.1, IQR 18.1–42.6 pg/ml; $p = 0.013$) and with DD (median 30, IQR 19.7–44.1 pg/ml; $p = 0.022$). In addition, levels of MIP-1β were significantly elevated in children with ASD (median 81.4, IQR 46.8–196.6 pg/ml) compared with DD (median 35.6, IQR 25.4–86.2 pg/ml; $p = 0.01$). There was also a trend for increased MIP-1α levels in children with ASD (median 123.7, IQR 72.9–321.8) compared to DD (median 96.5, IQR 25.4–86.2, $p = 0.059$) but this did not reach statistical significance after correction for multiple comparisons. The levels of chemokines were not significantly different between TD and DD diagnostic groups.

We then examined whether there were associations between chemokine levels and clinical behavioral variables among ASD and control participants. Using ASD specific assessments, impairments in communication were associated with increased RANTES levels ($r = 0.256$, $p = 0.041$) and MIP-1α ($r = 0.292$, $p = 0.019$) in children with ASD as assessed by ADOS module 1. Increased RANTES was also associated with greater impairments in parent reported composite scores of behavior patterns ($r = 0.215$, $p = 0.05$) in children with ASD based on ADI-R assessment, .Moreover, we found significant associations between chemokine levels of MCP-1, RANTES and eotaxin and behaviors as assessed by the ABC, VABS and MSEL such that increased levels were associated with more impaired behaviors on the ABC or decreased cognitive and adaptive functions on the MSEL and VABS (specific associations with domains assessed are listed in Table 2). More impaired ABC scores for lethargy, stereotypy and hyperactivity were associated with elevated levels of eotaxin and RANTES. Worse cognitive scores of visual reception, fine motor skills and expressive language were associated with higher eotaxin, RANTES and MCP-1 levels. The chemokines eotaxin and RANTES were associated with

Table 1
Comparison of plasma chemokine levels in children with autism spectrum disorders (n = 80), typically developing controls (n = 58) and children with developmental disabilities other than autism (n = 37). Data are presented as median and (interquartile ranges). *$p<0.05$ compared with typically developing controls; #$p<0.05$ compared with developmentally delayed controls.

	Autism spectrum disorder	Non-autism controls	
		Typically developing	Developmental delays
MCP-1	**139.7*#** (86–254.7)	90.5 (62–246.6)	84 (56.7–119.1)
MIP-1α	123.7 (72.9–321.8)	125.3 (49.8–406.7)	96.5 (56.9–136.6)
MIP-1β	**81.4#** (46.8–196.6)	85.1 (12.7–192.3)	35.6 (25.4–86.2)
RANTES	**7967*#** (4294–19,100)	4040 (2341–9008)	4170 (2430–5271)
Eotaxin	**42.8*#** (30.9–56.6)	30.1 (18.1–42.6)	30 (19.7–44.1)
IP-10	8.5 (5–14.1)	7.5 (5–13.9)	7.7 (6.0–13.5)

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Table 2
Association analysis of MCP-1, RANTES, eotaxin with behavioral outcome measures as assessed by the Aberrant Behavior Checklist (ABC), Mullen Scales of Early Learning (MSEL), Vineland Adaptive Behavior Scales (VABS) using Spearman's rank correlations demonstrated there were significant correlations between chemokine levels and the severity of impairments in behavior in participants enrolled in this study. For ABC as aberrant behaviours increase the chemokine levels increase resulting in a positive correlation. The VABS and MSEL are measures of adaptive and cognitive function and the higher the values the better the skills; chemokines were associated with more impaired scores and resulted in a negative correlation.

	RANTES		Eotaxin		MCP-1	
	r	*p* value	r	*p* value	r	*p* value
Aberrant behavior checklist						
Subscale I: irritability	0.137	0.101	0.062	0.459	0.028	0.733
Subscale II: lethargy	0.217	0.008	0.203	0.013	0.091	0.266
Subscale III: stereotypy	0.241	0.003	0.159	0.050	0.105	0.198
Subscale IV: hyperactivity	0.243	0.003	0.222	0.007	0.093	0.265
Subscale V: inappropriate speech	0.124	0.130	0.111	0.175	0.023	0.783
Mullen scales of early learning						
Visual reception	−0.175	0.027	−0.254	0.001	−0.225	0.004
Fine motor	−0.166	0.036	−0.232	0.003	−0.201	0.011
Receptive language	−0.151	0.056	−0.199	0.012	−0.135	0.089
Expressive language	−0.173	0.028	−0.248	0.001	−0.153	0.050
Vineland adaptive behavior scales						
Communication	−0.192	0.015	−0.224	0.004	−0.126	0.112
Daily living skills	−0.136	0.086	−0.271	0.001	−0.167	0.034
Socialization	−0.193	0.014	−0.259	0.001	−0.109	0.171
Motor skills	−0.074	0.351	−0.194	0.013	−0.107	0.178

worse adaptive behaviors of communication and socialization whereas eotaxin and MCP-1 were associated with worse daily living scores.

4. Discussion

The major finding of this study was that the production of MCP-1, RANTES and eotaxin was significantly higher in children with ASD compared with TD and DD controls. These perturbations were linked to impairments in core features of ASD, with associations observed between levels of RANTES and greater impairments on assessments of communication and behavior on the ADOS and ADI-R measures. In addition, we found significant associations between increased MCP-1, RANTES and eotaxin levels and more aberrant behaviors or impairments in cognitive and adaptive function as assessed by the ABC, MSEL and VABS, respectively. It is currently unclear how altered chemokine levels affect behaviors during childhood and these novel, preliminary observations need further investigation. However, a clear pattern has emerged over several studies that shows altered levels of immune mediators are associated with increased impairments in behaviors (Ashwood et al., 2008; Ashwood et al., 2010; Grigorenko et al., 2008; Tsuchiya et al., 2007; Enstrom et al., 2009b; Onore et al., 2009) and suggests that a dysregulated immune response is related to behavioral and cognitive impairments in children with ASD.

Our results are consistent with data from other studies that showed an increase in protein levels of MCP-1 and eotaxin in brain specimens from individuals with ASD (Vargas et al., 2005) or transcripts for chemokines in the brain or blood of ASD children (Garbett et al., 2008; Enstrom et al., 2009a). Chemokines and their receptors play key functions in directing the trafficking and movement of mononuclear cells in the CNS. For example, MCP-1 mediates the recruitment of myeloid cells to sites of injury or inflammation and is increased in brain ischemia, Alzheimer's disease and experimental autoimmune encephalomyelitis (Grammas et al., 2006; Kim et al., 1995; Rebenko-Moll et al., 2006). Chemokines are expressed in the developing brain and have been shown to regulate neuronal cell migration, proliferation and neuronal cell differentiation as well as being involved in the communication between neurons and microglia (Biber et al., 2008; Patterson, 2009). However, due to the high degree of promiscuity between several chemokines and their receptors, and the fact that neurons express many chemokine receptors, it is difficult to determine the exact roles for specific chemokines during neurodevelopment. As such, the pathologic significance of chemokines in children with ASD is not clear. However, the reported differential production of chemokines in the brain, CSF, and/or plasma in the context of their known affect on neuronal activity, suggests potential chemokine involvement in aberrant neuronal development leading to altered early brain development and function.

Our preliminary observations are the first to show differences in plasma chemokine levels in young children with ASD and lead us to hypothesize that altered chemokine levels and responses may be implicated in the pathophysiology of ASD. The development of new therapies that target chemokine receptors is a promising focus for many neurological diseases and may also have relevance to ASD. Our findings support the importance of further study of the biological impact of chemokines and their association with behavioral and cognitive impairments in children with ASD.

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