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Plasma cytokine profiles in Fragile X subjects: Is there a role for cytokines in the pathogenesis?

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Abstract

Background—Fragile X syndrome (FXS) is a single-gene disorder with a broad spectrum of involvement and a strong association with autism. Altered immune responses have been described in autism and there is potential that in children with FXS and autism, an abnormal immune response may play a role.

Objectives—To delineate specific patterns of cytokine/chemokine profiles in individuals with FXS with and without autism and to compare them with typical developing controls.

Methods—Age matched male subjects were recruited through the M.I.N.D. Institute and included: 19 typically developing controls, 64 subjects with FXS without autism and 40 subjects with FXS and autism. Autism diagnosis was confirmed with ADOS, ADI-R and DSM IV criteria. Plasma was isolated and cytokine and chemokine production was assessed by Luminex multiplex analysis.

Results—Preliminary observations indicate significant differences in plasma protein levels of a number of cytokines, including IL-1alpha, and the chemokines; RANTES and IP-10, between the FXS group and the typical developing controls ($p < 0.01$). In addition, significant differences were observed between the FXS group with autism and the FXS without autism for IL-6, Eotaxin, MCP-1 ($p < 0.04$).

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Conclusions—In this study, the first of its kind, we report a significantly altered cytokine profile in FXS. The characterization of an immunological profile in FXS with and without autism may help to elucidate if an abnormal immune response may play a role and help to identify mechanisms important in the etiology of autism both with and without FXS.

Keywords

Autism; Fragile X; cytokines; chemokines

Introduction

Fragile X syndrome (FXS) is a single-gene disorder with a broad spectrum of involvement including cognitive and behavioral impairments of varying degrees associated with distinct physical features. One behavioral phenotype of FXS is also characterized by autistic symptoms, including social and communication deficits, and stereotypic behavior. From the most recent studies, approximately 25–33% of children with FXS have autism (Kaufmann et al., 2004; Rogers et al., 2001) whereas Pervasive Developmental Disorder - not otherwise specified (PDD-NOS) occurs in an additional 30%, and approximately 2 to 6% of children with autism have FXS (Estecio et al., 2002; Hagerman and Hagerman, 2002; Harris et al., 2008; Reddy, 2005; Wassink et al., 2001).

Fragile X syndrome is nearly always caused by an expansion (>200) of a CGG trinucleotide repeat in the 5' untranslated region (5'UTR), followed by methylation and silencing of the fragile X mental retardation 1 (*FMR1*) gene, with subsequent deficiency or absence of *FMR1* protein (FMRP). It is the absence of FMRP, important for normal brain development that causes FXS (Irwin et al., 2000; Weiler and Greenough, 1999). Since lack of FMRP leads to immature dendritic spines, it is thought that FMRP is involved in synaptogenesis, especially in the cerebral cortex, cerebellum and hippocampus, and, more specifically, in synaptic plasticity (Hagerman, 06).

Although the etiology of idiopathic autism without FXS is unknown, other causes of autism are associated with known gene defects including neuroligin, neuorexin or SHANK protein (Hagerman et al., 2008). In addition, multiple genetic association studies have implicated genes that are relevant to the function of the immune system including human leukocyte antigens (HLA) complement C4, MET tyrosine kinase pathway, serine and threonine kinase C gene PRKCB1, macrophage inhibitory factor (MIF), Reelin and PTEN (reviewed in Enstrom et al., 2009). Moreover, altered immune responses have been described in autism (Ashwood et al., 2006) and it is possible that an abnormal immune response may play a role in children with FXS and autism. For example immune abnormalities such as increased frequency of infections, particularly otitis media and sinutitis infections, especially in early childhood, have been described in at least a subgroup of boys with FXS (Hagerman and Hagerman, 2002). An increased susceptibility to infections in FXS may underlie a dysfunctional immune response which might also play a role in increased autism susceptibility in these patients. Persistent gastrointestinal (GI) symptoms, such as loose stools have also been described in FXS and are consistent with similar reports of GI symptoms and increased mucosal immune activation in a subset of children with autism (Ashwood et al., 2003; Ashwood et al., 2004; Hagerman, 1987; Hagerman and Hagerman, 2002). Several patients studied with FXS, demonstrated a transient hypogammaglobulinemia particularly IgG subclass 1 and 3 (Hagerman and Hagerman, 2002). Interestingly, in autism, reduced total IgG levels have been observed (Heuer et al., 2008) and are directly correlated with worsening in aberrant behaviors. Immune dysfunction may also be present in some carriers of the permutation of FXS, as women who are carriers have a higher rate of

hypothyroidism and fibromyalgia related to autoimmune dysfunction (Coffey et al 2008), however, immune dysregulation has not been studied in individuals with FXS.

Cytokines are key mediators of cell-cell communication in the immune system. Within the nervous system, cytokines play important roles in conveying signals between cells, with neurons and glia not only able to produce different cytokines but can also respond to different cytokines through a diverse array of cytokine receptors expressed on the cell surface. Cytokines have assorted effects on neuronal tissue such as the modulation of systemic and central nervous system (CNS) responses to infections or injury and can modulate brain function affecting cognitive and emotional processing. The cytokine milieu has been shown to directly affect neural tissue function and development, especially the pro-inflammatory cytokines such as IL-1, IL-6, IL-12, IFN γ and TNF α , which have pleiotropic effects in the CNS including in neurodevelopment. Abnormal immune profiles in FXS may play a role in the development of cognitive deficits and may also lead to behaviors characteristics of autism. In this study we assessed specific cytokine and chemokine profiles in individuals with FXS who have autism spectrum disorders and those that do not have autism spectrum disorders and compared these cytokine profiles to the profiles seen in typical developing controls.

Materials and Methods

Study Participants

This study included 123 male subjects who were recruited through the M.I.N.D. Institute: 64 subjects with FXS without autism spectrum disorders (FXS, mean age 10.3 years, range 2.5–26.6 years), 40 subjects with both FXS and autism spectrum disorder (FXS+AU, mean age 11.6 years, range 2.6–28 years) and 19 typically developing (TD) controls (mean age 15.1 years, range 4.0–28.9 years). Informed consent was obtained from each participant, and the study was approved by the UC Davis Institutional Review Board.

DNA analysis

To confirm the presence of FXS a blood sample was obtained from each subject (FXS, FXS +AU and TD controls) for the determination of CGG repeat number in the *FMR1* gene. Genomic DNA was isolated from peripheral blood leukocytes using standard methods (Puregene Kit; Gentra Inc., Minneapolis, MN). PCR analysis were performed on all the subjects using primer c and f and the FastStart Taq DNA Polymerase (Roche Diagnostics, Indianapolis, IN) as described in Tassone et al (2008).

For Southern blot analysis, 5–10 μ g of isolated genomic DNA was digested with EcoRI and NruI. Probe hybridization used the *FMR1*-specific dig-labelled StB12.3. Details were as previously described (Tassone et al., 2008). Analysis and measurement of trinucleotide allele size, as well as the determination of the methylation status were determined using an Alpha Innotech Fluor Chem 8800 Image Detection System (Alpha Innotech, San Leandro, CA).

Clinical evaluation and assessment measures for autism

A complete medical evaluation, including medical history, psychological testing and physical examination was conducted on each subject including controls. Individuals were confirmed to have autism spectrum disorder or using the Autism Diagnostic Interview-Revised (ADI-R), which is a standardized, semi-structured, investigator-based interview for caregivers of individuals with autism or pervasive developmental disorders, and using the Autism Diagnostic Observation Schedules (ADOS) which is a semi-structured, standardized assessment of the child in which the researcher observes the social interaction,

communication, play, and imaginative use of materials for children suspected of having autism spectrum disorders. Each participant was discussed by the clinical team, who participated in the evaluation of the child and agreement was reached regarding the diagnosis of autism spectrum disorder, with the additional use of the DSM IV-TR criteria for autism spectrum disorders. Sibling controls were not used in this study.

Cognitive and Adaptive measures

The Wechsler Intelligence Scale for Children – Fourth edition (WISC-IV, WPPSI-III) or WASI for cognitive testing and the Vineland Adaptive Behavior Scales (VABS) were used for all children with FXS with and without autism and for all control subjects included in this study. Controls were screened for autistic spectrum disorders traits using Social Communication Questionnaire (SCQ). Only participants that were within the normal ranges based on SCQ and VABS testing were considered as typically developing controls for this study.

Cytokine analysis

Blood was collected in citrate containing tubes from all participants. Plasma was collected following centrifugation, and was aliquoted and stored frozen at -80°C prior to cytokine analysis (within 3 months of collection). Blood was only collected from patients who had no evidence of fever, or recent chronic or acute infections and/or known immune related diseases. Cytokine analysis was performed on the collected plasma samples using multiplex assays (Millipore) and read on a Luminex100™ platform. Samples were prepared according to the manufacturer's recommendation and cytokine and chemokine levels were assessed by Luminex™ multiplex analysis. The cytokines/chemokines analyzed were interleukin (IL)-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, granulocyte macrophage colony stimulating factor (GM-CSF), interferon gamma (IFN γ), tumor necrosis factor alpha (TNF α), eotaxin, monocyte chemoattractant protein (MCP)-1 α , regulated upon activation, normal T-cell expressed, and secreted (RANTES), macrophage inflammatory protein (MIP)-1 α and 10 kDa interferon-gamma-induced protein (IP-10), detection limits for these cytokines/chemokines were 2.4, 0.3, 1.2, 1.3, 2.2, 0.2, 1.1, 2.4, 0.9, 0.3, 34.9, 0.8, 1.0, 0.8, 5.0, 0.5, 1.5, 5.4, 5.4, 1.8, 10.7 and 3.6 pg/mL, respectively. Intra and inter-plate/assay variations of cytokine levels, using representative samples run on all plates, were less than 5%. The cytokine analyst was blinded to the case or control status of each sample.

Statistical analysis

Robust summary statistics, including median and median absolute deviation (MAD), were used to summarize plasma protein levels of cytokines and chemokines. These provide better summary of the raw data by moderating the effects of a few outlying observations. Subjects in the three groups were matched on age to be in the range of 2.5–30 years. However, the observed average age was different between FXS and control ($p = 0.009$). Because of this we used an analysis of covariance (ANCOVA), adjusted for age, to compare between the three groups (FXS+AU, FXS, and TD control). This analysis was applied to a subset of cytokines and chemokines that have complete data or had the majority (>75%) of cytokine values that fell above the detection limit (DL) per group (Table 1). After graphical analysis, the ANCOVA was applied to log-transformed plasma cytokine levels to better satisfy statistical model assumptions, such as normality. We also used logistic regression to adjust for age and found that age was not a significant confounder.

Results

Plasma protein levels of the cytokines IL-1 α , IL-6, IL-12 (p40) and IFN γ and the chemokines eotaxin, MCP-1 α , RANTES, MIP-1 α and IP-10 were measured with sufficient accuracy and reproducibility above the detection limit (DL). These cytokines were compared among the three groups FXS+AU, FXS and TD controls. The results are summarized in Table 1 where median levels (and median absolute deviations) are provided for each protein. Observed median levels of cytokines IL-1 α and IL-12 (p40) are highest in the FXS+AU group compared to FXS and are lowest in the control group. For IL-1 α , significant differences were observed between FXS+AU relative to control ($p = 0.003$) and FXS compared to control ($p = 0.009$) but not between FXS and FXS+AU. Observed levels of IL-12 (p40) in FXS+AU were elevated relative to controls ($p = 0.04$). Plasma IL-6 levels were elevated in FXS compared to FXS+AU ($p = 0.007$) but neither group was significantly different from controls.

The median levels of the chemokines; eotaxin, MCP-1 α , RANTES and IP-10 were lower in both the FXS+AU and FXS groups compared to controls ($p < 0.05$). Observed levels of eotaxin, MCP-1 α and RANTES were significantly different for FXS+AU compared to FXS. Details are summarized in Table 1 and Figure 1, where we have chosen to display the four most salient plasma cytokines/chemokines ranked by their p -values.

For the remaining analytes, there were substantial measurements below the detection limits in the control group, with the majority (9 of 13) only having between 0% to 26% of values that were readable (data not shown). Thus comparison of plasma protein levels of these cytokines and chemokines between the FXS groups and control group was not feasible. However, with the exception of IL-1 β and IL-8, all remaining 11 cytokines were present above the detection limit significantly more in both the FXS+AU and FXS groups compared to the control group when analyzed using Fisher's exact test ($p < 0.02$, data not shown).

Discussion

Cytokine profiles in subjects with FXS have not previously been examined. The major findings of this study are that the levels of cytokines and chemokines in subjects with FXS with and without autism spectrum disorders are significantly different from the levels determined in typically developing controls. These data suggest that there are significantly distinct profiles of cytokines and chemokines in participants with FXS without autism spectrum disorders compared with controls (IL-1 α , IP-10, RANTES) and in FXS participants with autism spectrum disorders when compared to controls (IL-1 α , IL-12p40, eotaxin, IP-10, MCP-1 α , RANTES). In addition, there were differences in several of the cytokines measured between individuals with FXS and autism spectrum disorders and those with FXS without autism spectrum disorders with increased IL-6, eotaxin, MCP-1 α and decreased RANTES being seen in the latter group.

In this study we observed an increase in IL-1 α in FXS participants with and without autism spectrum disorders compared with TD controls. Neuro-immune interactions are extensive, begin during early neurodevelopment and continue throughout life, with the immune system supporting many aspects of neural function. A dysregulation in the immune system could result in increased sensitivity to neurologic damage from a variety of sources including infection and exposure to xenobiotics, particularly during early development. Pro-inflammatory cytokines such as IL-1, IL-6, and TNF α can directly modulate responses in the CNS, and can alter neurodevelopment and subsequently may impact on behavior (Gilmore et al., 2005; Shi et al., 2003; Mehler and Kessler 1998). For example, IL-1, can alter proliferation, cell survival, cell death, neurite outgrowth, and gene expression in

neurons (Mehler and Kessler 1998; Gadiant and Patterson 1999). Aberrant immune response during critical periods of neurodevelopment could therefore lead to changes in early brain development and produce neurological dysfunction characteristic of autism. While a cytokine-mediated mechanism has yet to be conclusively established in neurodevelopmental disorders such as autism or FXS, evidence of adverse immunological functioning in these disorders suggests that neuro-immune interactions may be altered and that they may impact on neurodevelopmental and early brain development.

The potential role of the immune system in autism spectrum disorders has been addressed in several studies. These include reports of neuroglial activation and neuroinflammation in the CNS, with elevated cytokine responses including IL-6 and TNF α in the brains of individuals with autism spectrum disorders (Garbett et al., 2008; Vargas et al., 2005), as well as the increased presence of plasma antibodies reactive to neuronal tissue in children with autism (Cabanlit M, 2007; Connolly, 2006; Singer HS, 2006; Wills et al., 2009). Several researchers have demonstrated increased proinflammatory cytokines profiles and a skewing of the ratio of T_H1 cytokines such as IFN γ and IL-12 compared to T_H2 cells cytokines such as IL-4, in the plasma of individuals with autism spectrum disorders (reviewed in Enstrom et al., 2009). In contrast, another study, using stimulated PBMC showed a bias towards a T_H2 response (Gupta et al., 1998); however, no un-stimulated PBMC cultures were analyzed for comparison and the apparent skewing in cytokines may have been due to a hyporesponse of T_H1 cells as a result of prior activation *in vivo* rather than differentially increased T_H2 responses. Overall no consensus has yet been reached as to the exact nature of the cytokine profile in autism and discrepancies among the studies may be due in large part to the subjects chosen, i.e. whether they had a full autism spectrum diagnosis, age of subjects, use of siblings as controls, gender of subjects, as well as the analytical techniques, power of the statistical analysis and what tissue/specimens were used. No previous study has looked at immune dysfunction in FXS however, increased susceptibility to infections and GI issues have been described in FXS boys (Hagerman and Hagerman, 2002) and may suggest that immune dysfunction occurs in a subgroup of individuals with FXS. Whether the single genetic change seen in FXS could lead to altered common signaling pathways that affect both neuronal and immune pathways and whether the extent of this immune dysfunction leads to an increased susceptibility for autism in these patients is not clear but warrants further investigation.

It is currently not known whether altered cytokine levels are active determinants in the development of neuropathology in FXS, or accompanying phenomena secondary to the onset of disease. The findings present here provide a framework for further longitudinal studies to investigate changes in cytokine levels over the lifetime of the FXS. A key question remains regarding the concentration of cytokines at birth and/or in early development in FXS subjects with or without autism spectrum disorders. Future studies evaluating cytokine levels during critical periods of neurodevelopment would be essential towards addressing the potential role of cytokines as a biological component in FXS.

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Figure 1.

Distribution of IL-1 α , IL-6, RANTES and IP-10 among FXS, FXS+AU and typically developing controls (IL-1 α : FXS+AU vs. control $p = 0.003$, FXS vs. control $p = 0.009$; IL-6: FXS+AU vs. FXS $p = 0.007$; RANTES: FXS vs. control $p < 0.001$; IP-10: FXS+AU vs. control $p < 0.001$; FXS vs. control $p < 0.001$). y-axis represents cytokine levels in log scale (Log (pg/ml)). Horizontal box lines are 25%, 50% (median) and 75% of the distribution with whiskers marking 1.5 times the interquartile range. Open circles indicate potential outliers. Analysis results with or without these two data points did not differ.

Cytokine/Chemokine levels in participants with fragile X syndrome without autism spectrum disorders (FXS), fragile X syndrome with autism spectrum disorders (FXS+AU), and typically developing control groups. Cytokine/Chemokine levels were determined by Luminex analysis and are shown as pg/ml.

Table 1

Cytokine/ Chemokine	FXS+AU			FXS			Control		ANCOVA comparisons		
	Med ¹	MAD ²		Med	MAD		Med	MAD	FXS+AU vs control	FXS vs control	FXS+AU vs FXS
IL-1 α	783.0	485.2		731.2	460.8		440.8	255.9	0.003	0.009	0.453
IL-6	13.3	7.6		26.7	18.9		21.6	12.0	0.128	0.685	0.007
IL-12 (p40)	450.5	219.3		354.7	149.2		334.8	105.8	0.042	0.169	0.306
IFN γ	13.3	8.3		14.1	8.0		24.2	9.9	0.709	0.405	0.552
eotaxin	18.3	5.7		22.5	8.0		31.4	15.0	0.036	0.552	0.032
MCP-1 α	35.0	12.1		43.9	18.3		57.9	20.3	0.020	0.524	0.016
RANTES	180.0	35.1		150.5	34.2		204.1	60.5	0.021	<0.001	0.038
MIP-1 α	229.9	110.5		167.5	63.2		187.7	40.8	0.087	0.722	0.059
IP-10	57.8	21.7		64.3	16.6		129.8	40.2	<0.001	0.001	0.139

¹Median level

²Median absolute deviation