Cerebrospinal Fluid and Serum Markers of Inflammation in Autism

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Systemic immune abnormalities have no known relevance to brain dysfunction in autism. In order to find evidence for neuroinflammation, we compared levels of sensitive indicators of immune activation: quinolinic acid, neopterin, and biopterin, as well as multiple cytokines and cytokine receptors, in cerebrospinal fluid and serum from children with autism, to control subjects with other neurologic disorders. In cerebrospinal fluid from 12 children with autism, quinolinic acid ($P = 0.037$) and neopterin ($P = 0.003$) were decreased, and biopterin ($P = 0.040$) was elevated, compared with control subjects. In sera from 35 persons with autism, among cytokines, only tumor necrosis factor receptor II was elevated compared with controls ($P < 0.02$). Decreased quinolinic acid and neopterin in cerebrospinal fluid are paradoxical and suggest dysmaturation of metabolic pathways and absence of concurrent infection, respectively, in autism. Alternatively, they may be produced by microglia but remain localized and not expressed in cerebrospinal fluid. © 2005 by Elsevier Inc. All rights reserved.

Introduction

Various immune abnormalities have been reported in children with autism. However, there has been no direct or causal link between these observations in the peripheral immune system and neuropathologic findings in autism postmortem brain tissues [1,2]. Serum immunoglobulin abnormalities and the presence of autoantibodies against central nervous system and viral antigens, altered T-cell functions and abnormalities in their associated cytokines, decreased serum complement levels, and differences in levels of inflammatory cytokines such as tumor necrosis factor-α have all led to hypotheses proposing that autism may result from immune deficiency, chronic viral persistence, or autoimmunity [3]. However, it is difficult to interpret these findings with respect to the pathogenesis of autism for several reasons: most studies have failed to relate immune abnormalities to the clinical features of the patients; previous studies were not longitudinal with respect to age, clinical progression, or potential normalization of immunologic differences with maturation; and pertinent findings were observed only in subsets of children with autism. More importantly, it is not clear that the immune findings in peripheral blood in autism correlate with immune-mediated pathology within the central nervous system. If the peripheral immune findings are truly relevant to the pathogenesis of autism, there should be evidence of immune activation in the central nervous system, and it should be reflected in markers of inflammation in cerebrospinal fluid.

To find evidence of immune activation in the central nervous system in children with autism, markers of inflammation in cerebrospinal fluid were measured in this study: quinolinic acid; neopterin and the related pterin, biopterin; and inflammatory cytokine levels (interleukins1β, 2, 4, 5, 6, interferon-γ, tumor necrosis factor-α, and interleukin-12) and counter-regulatory cytokine levels (interleukins 1ra and 10, transforming growth factor-β,

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Table 1. Patient groups

Groups	n	Ages (average, range)	Sex (M/F)	Diagnoses (n)
Autism Group 1 (CSF and serum)	12	6.1 (33 mo-10 yr)	10/2	Autism, moderate to severe (see Table 2) Macrocephaly (2); epilepsy (absence, 1); EEG abnormal (4; 3 with slowing, 1 with spikes); abnormal chromosomes (2; 1qh+; 5-6 translocation).
Autism Group 2 (Serum)	35	7.8 (2.8-43 yr)	32/3	Autism, moderate to severe (32); pervasive developmental disorder (1); Asperger syndrome (1); high functioning autism (1); epilepsy (3); bipolar disorder (2); abnormal chromosomes (1; XYY); history of developmental regression (18).
Control Group 1 (CSF)	15	7.5 (2-14 yr)	6/9	Headaches, seizures, myoclonus, developmental delay, chorea
Control Group 2 (CSF)	12	9.3 (1.3-17 yr)	6/6	Seizure disorders (2), acute lymphocytic leukemia in remission (4), pseudotumor cerebri (4), resolved viral meningitis (1), and transverse sinus thrombosis (1)
Control Group 3 (Serum)	23			
3A: Other neurologic disorders	11	8.9 (3.3-16 yr)	10/1	ADHD, seizures
3B: Typically developing siblings of children with autism	10	9.3 (3-18 yr)	5/5	
3C: Unrelated normal children	2	5.6, 11.6 yr	1/1	

Abbreviations:
ADHD = Attention-deficit hyperactivity disorder
CSF = Cerebrospinal fluid
EEG = Electroencephalogram

soluble tumor necrosis factor receptors I and II). The correlation between cerebrospinal fluid and serum cytokine levels was also assessed. Typically developing siblings and age-matched patients with other central nervous system diseases were used as control subjects.

Quinolinic acid, an excitotoxic kynurenine [4,5], is a sensitive marker of acute activation of microglia, the major innate immune cells in the central nervous system, corresponding to peripheral tissue macrophages [6]. Increased neopterin production occurs in monocyte-macrophage lineage cells after activation of the enzyme guanosine triphosphate cyclohydrolase I by interferon γ, endotoxin, or tumor necrosis factor-α [7]. Both quinolinic acid and neopterin, as well as increased cytokines produced by microglial cells, are sensitive markers of inflammation in cerebrospinal fluid [8]. Total biopterin consists mostly of the reduced form, tetrahydrobiopterin, an essential cofactor for the biosynthesis of catecholamines and serotonin, hydroxylation of phenylalanine, and nitric oxide production. Interestingly, reduced levels of both biopterin and neopterin have been reported in cerebrospinal fluid in autism [9]. As opposed to neopterin, biopterin does not increase with infections [7,10].

Many inflammatory cytokine levels are elevated transiently after acute infections or at the onset of autoimmune diseases. Upon production of inflammatory cytokines, physiologic counter-regulatory factors are also produced. These counter-regulatory factors (such as soluble receptors) tend to be elevated for longer periods, as is well illustrated in the biology of tumor necrosis factor and its soluble receptors [11]. Thus serum and cerebrospinal fluid

levels of these counter-regulatory factors may be used as markers of chronic inflammation in various diseases [12,13]. These markers were measured in the present study to detect possible immune-mediated inflammation in the central nervous system in children with autism.

Subjects and Methods

Study Subjects

All children with autism fit the diagnostic criteria of the Diagnostic and Statistical Manual-IV, which were confirmed by the Autism Diagnostic Interview-Revised [14]. The Institutional Review Board of The Johns Hopkins Medical Institutions approved the study. Parents gave written consent, and children assented if they were able to do so. Regression was defined as loss of previously acquired language and social interaction skills, and was based on family reporting; it was not a selection criterion. Cerebrospinal fluid was obtained by lumbar puncture from 8 to 10 AM in 12 children with moderate to severe autism (Autism Group 1, Tables 1 and 2), fasting, with conscious sedation using intramuscular ketamine, midazolam, and atropine [15] lasting 20-30 minutes, and with continuous monitoring of vital signs. No evidence could be found in the literature that these anesthetic agents increase levels of inflammatory markers in the cerebrospinal fluid. Cerebrospinal fluid and venous blood samples were obtained simultaneously from Autism Group 1 and sent for immediate analysis of routine laboratory tests or stored ($-80°C$) up to 2 years for measurements. For the studies of

Table 2. CSF results: Autism Group 1

Subject No.	Age	Sex	Age at First Symptoms	Regression	Cell Count (wbc/rbc)	Protein (mg/dL)	Glucose (mg/dL)	Protein EP	Immuno EP
1	6.8 yr	M	24 mo	28 mo	2-0	29	63	Normal	Normal
2	2.7 yr	M	22 mo	22 mo	3-0	25	54	ND	ND
3	4.3 yr	M	30 mo	30 mo	2-5	31	52	ND	ND
4	8.3 yr	M	13 mo	None	2-19	33	59	ND	ND
5	4.4 yr	F	15 mo	18 mo	2-0	24	47	ND	Normal
6	4.9 yr	M	15 mo	15 mo	2-2	28	55	ND	ND
7	4.9 yr	M	24 mo	24 mo	2-0	27	50	Normal	Normal
8	6.5 yr	M	18 mo	18 mo	0-0	24	51	Normal	Normal
9	10.0 yr	M	18 mo	18 mo	0-2	29	ND	Normal	Normal
10	6.3 yr	M	23 mo	23 mo	0-0	30	52	Normal	ND
11	6.3 yr	F	30 mo	None	1-0	22	54	Normal	Normal
12	3.8 yr	M	24 mo	24 mo	1-0	10	47	ND	Normal

Abbreviations:
EP = Electrophoresis
ND = Not determined
rbc = Red blood cells
wbc = White blood cells

quinolinic acid, neopterin, and biopterin, measurements in cerebrospinal fluid (but not serum) were compared with those from 15 control subjects (Control Group 1) without histories of inflammatory disorders stored in our laboratory. For the study of cytokines in cerebrospinal fluid, a second set of samples was obtained (Control Group 2) from 12 children examined in the pediatric neurology clinic, obtained at the time of medically indicated lumbar punctures.

Serum samples alone were also obtained from 35 autistic subjects (Autism Group 2; Table 1) for measurement of cytokine-related markers. A total of 23 control sera were also obtained from 11 children with other neurologic disorders, 10 typically developing siblings of autistic children, and 2 unrelated control children (Control Group 3; Table 1).

Methods

Quinolinic acid concentrations were quantified in cerebrospinal fluid from Autism Group 1 and Control Group 1 by a modified gas chromatography technique [16,17] using a 15-m DB5 analytical column, mass spectrometry (Hewlett-Packard 5988), and [²H₃]quinolinic acid as internal standard, after freeze-drying the samples, derivatization to dihexafluoroisopropanol ester, and extraction into heptane. The 12-point standard curve had a dynamic range from 0 pg to 10 ng/100 μL.

Neopterin and biopterin were measured in cerebrospinal fluid in Autism Group 1 and Control Group 1 by high-performance liquid chromatography as previously described [4,7].

Cytokines and their soluble receptor levels were measured in cerebrospinal fluid from Autism Group 1 and Control Group 2; and in serum from Autism Groups 1 and 2 and Control Group 3, by high sensitivity enzyme-linked immunosorbent assays. OptEIA Reagent Sets (BD Pharmingen, San Diego, CA) were used for measuring interferon-γ, tumor necrosis factor-α, interleukins 1β, 4, 5, and 6. Matched antibody pairs and standards from R & D systems, Minneapolis, Minnesota were used for soluble tumor necrosis factor receptors I and II. Intra- and inter-sample variations of cytokine levels were less than 5%.

Statistical Methods

The data were analyzed using nonparametric tests owing to the small groups of subjects with unequal variance (Mann-Whitney and Kruskal-Wallis tests). Results are expressed as median and Exact Significance (2-tailed).

Results

Cerebrospinal Fluid Clinical Laboratory Testing

No overt signs of inflammation were detectable using conventional markers of central nervous system inflammation in Autism Group 1 (Table 2); and Control Group 1 or 2 (data not shown): cell counts, total protein, and glucose were normal in all subjects. Cerebrospinal fluid protein electrophoresis (n = 6) and immunoelectrophoresis (n = 7) were normal in all patients in whom they were measured in Autism Group 1 (Table 2). There were no abnormal immunoglobulins or oligoclonal bands, and the cerebrospinal fluid immunoglobulin G index (cerebrospinal fluid/serum) was normal.

Cerebrospinal Fluid Quinolinic Acid, Neopterin, and Biopterin (Fig. 1)

In cerebrospinal fluid from Autism Group 1 compared with Control Group 1, the median for quinolinic acid was
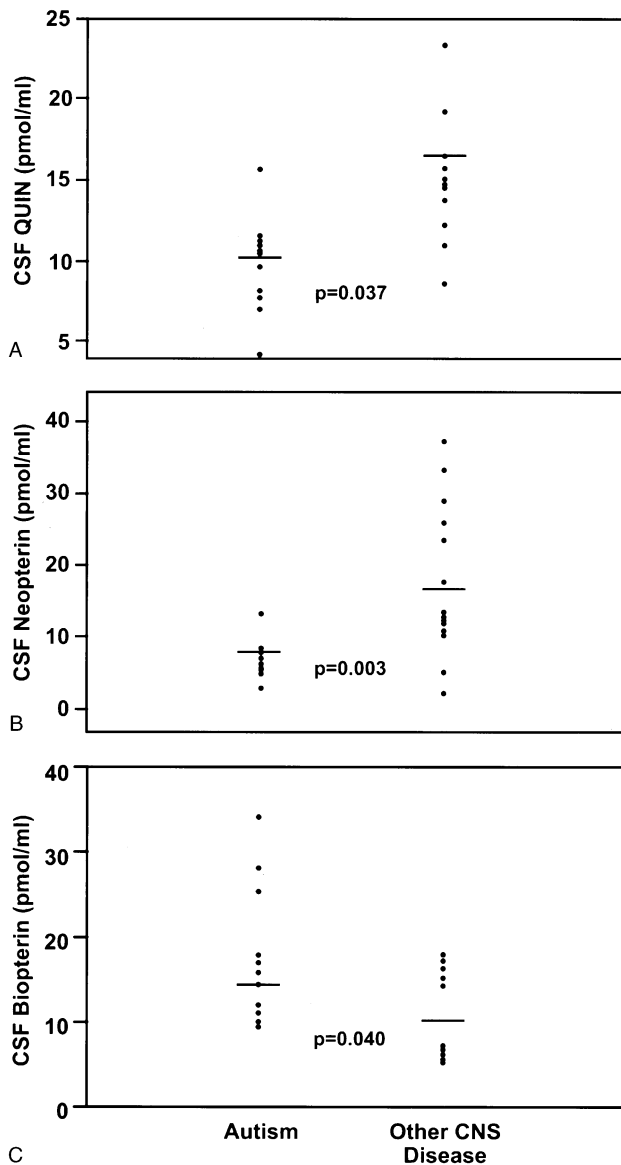
Figure 1. Cerebrospinal fluid quinolinic acid, neopterin, and biopterin from 12 subjects from Autism Group 1 and Control Group 1. Medians differed significantly in all three determinations; quinolinic acid (QUIN) (A) and neopterin (B) were lower than in control subjects with other neurologic disorders, whereas biopterin (C) was greater (Mann-Whitney test).

10.4 pmol/mL compared with 16.8 pmol/mL ($P = 0.037$); neopterin was 8.18 pmol/mL, compared with 16.8 pmol/mL ($P = 0.003$); and biopterin was 16.2 compared with 10.1 ($P = 0.040$). The three subjects in Autism Group 1 (Table 2, numbers 3, 5, and 7) in whom biopterin levels were greater than control subjects were young (4.3-4.9 years).

Cerebrospinal Fluid Cytokines (Fig. 2)

Interleukins 1β, 2, interferon-γ, and transforming growth factor-β were not detected using high sensitivity enzyme-linked immunoassay in either matched cerebrospinal fluid or serum in Autism Group 1. Interleukin-1ra (n

= 2) and interleukin-6 (n = 3) were detected at low levels in cerebrospinal fluid in Autism Group 1, and in Control Group 2 (n = 1 for interleukin-1ra, n = 3 for interleukin-6), at or near the detection limits for the assays. All cytokines except interleukin-2 were detected in cerebrospinal fluid and sera from several subjects from Control Group 1 at similar or greater values; however, there were too few determinations to make reliable statistical comparisons. The median (range) of cerebrospinal fluid soluble tumor necrosis factor receptor I levels was 61.2 (16.2-472.3) pg/mL in Autism Group 1 and 27.2 (17.9-239.8) pg/mL in Control Group 2 ($P = 0.092$). Levels of soluble tumor necrosis factor receptor I in autism cerebrospinal fluid were widely variable. In seven patients in Autism Group 1, simultaneous serum and cerebrospinal fluid samples yielded no significant correlation between serum and cerebrospinal fluid levels of soluble tumor necrosis factor receptors I or II.

Serum Cytokines (Fig. 3)

As in the cerebrospinal fluid, soluble tumor necrosis factor receptors I and II, interleukins-6 and 1ra were consistently detectable in all the serum samples (Autism Group 2 and Control Group 3), most likely as a result of their relatively long half-lives [11,12]. Other cytokine levels were variable and not detectable in all patients.
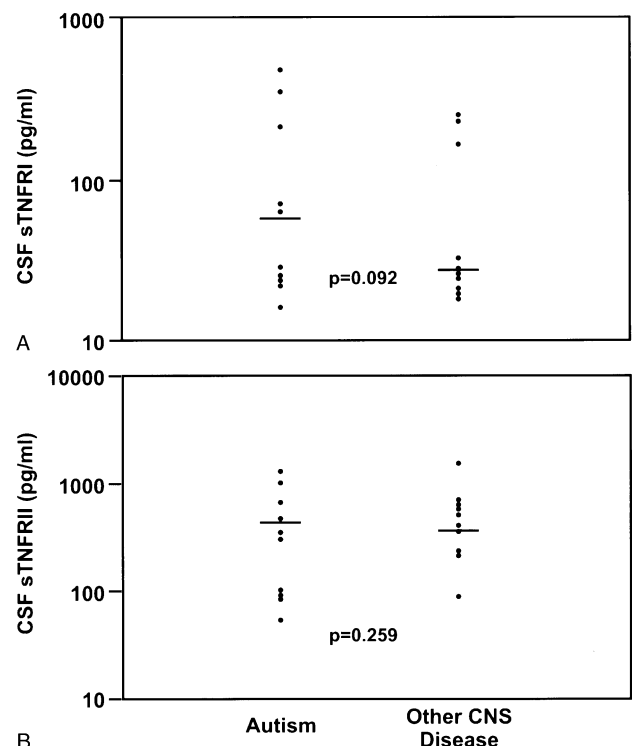

Figure 2. (A) Cerebrospinal fluid soluble tumor necrosis factor receptor I (sTNFRI) and (B) soluble tumor necrosis factor receptor II (sTNFRII) from 12 children in Autism Group 1 and 12 in Control Group 2. A trend toward greater median soluble tumor necrosis factor I was observed for subjects with autism compared with control individuals (P = 0.092); there was no difference for soluble tumor necrosis factor II (Mann-Whitney test).
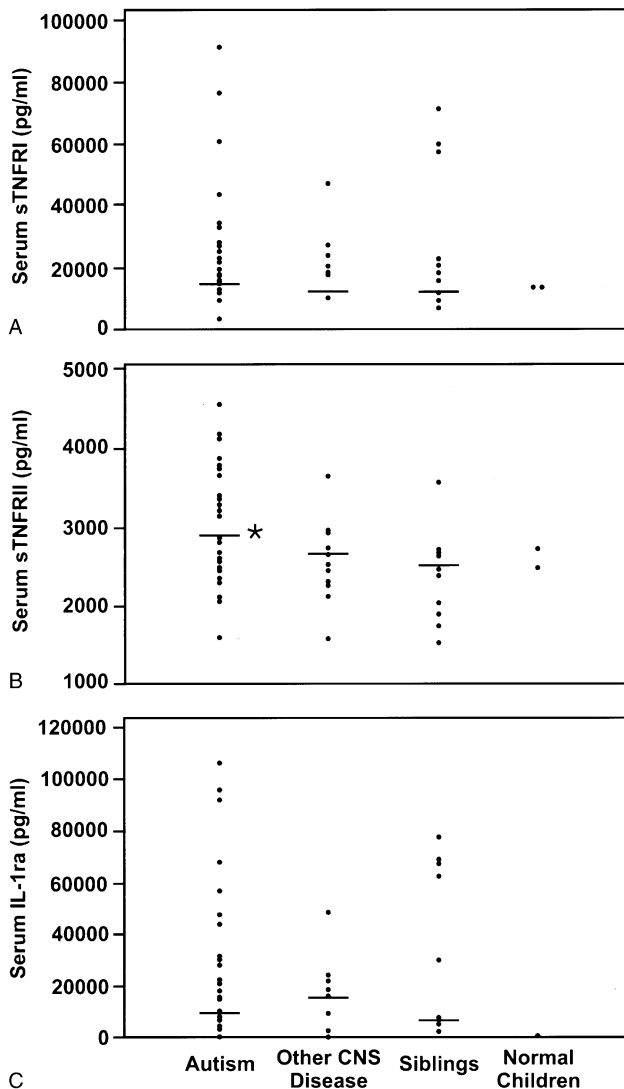
Figure 3. (A) Serum soluble tumor necrosis factor I (sTNFRI), (B) soluble tumor necrosis factor II (sTNFRII), and (C) interleukin-1ra (IL-1ra) in 35 persons with autism compared with 24 control subjects (other neurologic disorders, normal siblings and unrelated children). Differences were significant (*) only for soluble tumor necrosis factor II in the group with autism, compared both with other neurologic disorders and with normal siblings (P < 0.02, Kruskal-Wallis test).

There was no significant difference in serum soluble tumor necrosis factor receptor I levels between Autism Group 2 (1913; 238-9134 pg/mL; median, range) and Control Group 3A (other central nervous system diseases, 1758; 914-4661) or Control Group 3B (normal siblings, 1854; 598-7080) pg/mL. Levels of soluble tumor necrosis factor receptor II in Autism Group 2 (2937; 1674-4610 pg/mL) were significantly greater than both Control Group 3A (2584; 1636-3657 pg/mL) and Group 3B (2447; 1576-3597 pg/mL; P < 0.02 by Kruskal-Wallis test). As in the cerebrospinal fluid, serum levels of soluble tumor necrosis factor receptors I and II revealed a wide distribution.

Median interleukin-6 levels in Autism Group 3 sera (27; 5.4-629.1 pg/mL) did not differ from either Control Groups 3A (29.7; 10.8-181.6) or 3B (24.3; 16.2-237.6)

and were elevated in only two subjects with autism (100.6 and 629.1 pg/mL). Serum interleukin-1ra levels in subjects with autism (9063; 208-106,514 pg/mL) also were not significantly different compared with Control Groups 3A (18,491; 360-48,303) or 3B (18,568; 2612-77,275 pg/mL), and levels were widely distributed.

Discussion

Despite several lines of investigation that suggest immunologic factors are important for the pathogenesis of autism [18-20], we were unable to find any direct evidence or surrogate markers of neuroinflammation, using our methods, in the cerebrospinal fluid of 12 children with moderate to severe autism. On the contrary, cerebrospinal fluid quinolinic acid and neopterin, both sensitive indicators of inflammation in the central nervous system, were significantly decreased in the children with autism compared with control subjects. However, values from neurologic controls may have been moderately increased as a result of some degree of neuroinflammation associated with their diseases, while those in the subjects with autism may have been normal. While we would not expect long-term storage of the cerebrospinal fluid specimens to affect quinolinic acid, neopterin, or biopterin, this could have affected the detection of cytokines.

Cerebrospinal fluid biopterin levels have been reported to be normal [21] or even decreased in children with autism [9]. In contrast, the median cerebrospinal fluid biopterin level in the autism patients described in the present report was higher than controls, and these subjects were not taking dietary supplements of biopterin. These results do not support a conventional inflammatory process in the cerebrospinal fluid and may be consistent with reduced or dysregulated immune activity in the central nervous system in autism.

A trend was observed in this study toward elevated cerebrospinal fluid levels of soluble tumor necrosis factor receptor I, and serum levels of soluble tumor necrosis factor receptor II and interleukin-1ra in patients with autism, measures that may indicate chronic inflammation. However, elevated cerebrospinal fluid levels of the cytokines and receptors did not correlate with serum levels in our subjects when matched serum and cerebrospinal fluid samples were examined. Furthermore, in these cerebrospinal fluid samples there was no evidence of inflammation in routine laboratory analyses, or in the levels of quinolinic acid or neopterin. No significant elevation of inflammatory cytokines with short half-lives was observed.

Many cytokine measurements in cerebrospinal fluid by high sensitivity enzyme-linked immunosorbent assay may be low or undetectable in normal subjects. This finding presents difficulty in statistical analyses, because at levels below the limits of detection differences in cytokines in cerebrospinal fluid may be indistinguishable between autistic subjects and control subjects. There are additional difficulties inherent in studies of cerebrospinal fluid in-

flammatory markers in autism. First among these is acquiring cerebrospinal fluid from age-matched control subjects without infectious or degenerative disorders that might affect inflammatory or other related measures. Secondly, the heterogeneity inherent in the autism spectrum disorders, along with their associated medical disorders, likely influences results of this and other research studies. Stress, conscious sedation, and other factors may all affect the results of these studies. The relevance of findings in serum to those in cerebrospinal fluid is uncertain, and one should not assume that they are related, in autism or other conditions, until clearly demonstrated. For example, although a previous report of abnormal serum cytokines in autism [22] may imply an immune basis for this heterogeneous group of disorders, no evidence for this was found in the subjects of the present study.

There was ascertainment bias for developmental regression in the subjects, due both to the investigators' interest and parents' concern for their autistic children's loss of skills. However, we found no differences in our results between those with and without histories of regression.

The sensitivity of enzyme-linked immunosorbent assays for low levels of cytokines in cerebrospinal fluid was an important limiting factor in this study. Vargas et al. [2] have subsequently used cytokine protein arrays to measure cytokines, chemokines, and growth and differentiation factors in cerebrospinal fluid and brain tissue [23]. In their recent study, which included cerebrospinal fluid samples from six of the subjects in Autism Group 1 (Table 2, Subject numbers 5, 6, 8, 9, 11, 12), Vargas et al. found a unique proinflammatory profile of cytokine expression [2], consistent with activation of the innate immune system within the central nervous system. However, they did not measure tumor necrosis factor receptors I or II. It is of note that increased production of inflammatory cytokines by peripheral blood mononuclear cells with a surrogate stimulus of innate immunity has been reported in a subset of autistic children, indicating innate immune abnormalities in these children [24,25]. Also, although the finding of elevated tumor necrosis factor receptor I in cerebrospinal fluid in the present study was not statistically robust, it may reflect chronic neuroinflammation, from activation of both microglia and astroglia, a finding that was recently observed in postmortem autism brain tissue [2].

The low levels of quinolinic acid and neopterin we observed in cerebrospinal fluid may reflect an inability of the brain in autism to mount a "typical" inflammatory response. The (low) quinolinic acid and (elevated) biopterin levels documented herein may also reflect delayed maturation of immune activity in the brain in autism. For example, fetal brain cultures cannot produce more than minute amounts of quinolinic acid because of low enzyme activity [26,27]; likewise, the kynurenine pathway may be functionally immature in the central nervous system in autism. In contrast, cerebrospinal fluid biopterin levels are normally relatively high (as in our sample) at birth and

then decrease with age, whereas neopterin levels are greater than biopterin at birth and do not change [28-30]. Elevated levels of neopterin and biopterin have been found in the urine of children with autism [31], indicating that these pathways are functional and may be activated in the peripheral immune system, although they may be limited in the central nervous system. Because the pathways for biopterin and neopterin are closely related, our finding of decreased neopterin in cerebrospinal fluid, in the presence of increased biopterin, suggests that concurrent or chronic infection (which readily stimulates neopterin) is an unlikely cause of the neuroinflammation in the subjects of this study.

Another explanation for low levels of quinolinic acid in cerebrospinal fluid might be failure of the kynurenine pathway to form quinolinic acid from tryptophan, a pathway that is developmentally dependant on the activation of indoleamine-2,3-dioxygenase in the central nervous system [27]. In the liver, the analogous enzyme to indoleamine-2,3-dioxygenase is tryptophan 2,3 dioxygenase, for which a genetic polymorphism with decreased activity recently has been associated with autism [32]. Similar polymorphisms for indoleamine-2,3-dioxygenase have not been described. The kynurenine pathway for quinolinic acid is linked to the pathway for production of serotonin, because this neurotransmitter is also generated from tryptophan by tryptophan-5-hydroxylase, an enzyme that is also developmentally regulated [33]. This reaction is dependent on the availability of tryptophan as a precursor, and on biopterin as a cofactor [34]. Serotonin is an important neurotrophic factor in the developing brain [35], and its synthesis in the central nervous system is deficient in children with autism [36]. Therefore, dysregulated synthesis of both quinolinic acid and serotonin in autism may be related through these two developmentally regulated pathways, perhaps due to abnormal ontogenic patterns of gene transcription and enzyme expression that would affect cell signaling at an early stage of development. Our findings of low levels of quinolinic acid and neopterin in cerebrospinal fluid may therefore appear paradoxical, given recent evidence for neuroinflammation in the brain in autism. However, they are consistent with both a state of delayed maturation in the case of quinolinic acid as well as absence of concurrent infection with respect to neopterin.

In addition to abnormal developmental patterns of gene expression and absence of current infection, it is also possible that quinolinic acid and neopterin are generated in autism brain by microglial activation [2], but are not reflected in the cerebrospinal fluid because the choroid plexus, meninges, and ependyma are intact, and the microglial reaction remains compartmentalized. Disruption of the blood-brain barrier by antigen-antibody or cellular immune reactions characteristic of the adaptive immune system would allow diffusion of these markers into the cerebrospinal fluid. However, there is now evidence that such reactions do not appear to be present in the

brain in autism [2]. Therefore, the situation in autism may be analogous to findings in Huntington disease [37], which is characterized by localized microglial activation but no elevation of quinolinic acid in cerebrospinal fluid [4]. Further studies of neuroinflammation in autism brain and cerebrospinal fluid should help to clarify these atypical patterns.

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