BRIEF REPORT

High Nitric Oxide Production in Autistic Disorder: A Possible Role for Interferon-γ

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Background: *Neuroimmune regulation abnormalities have been implicated in the pathophysiology of autistic disorder. Nitric oxide (NO) is involved in immune reactivity and is known to affect brain neurodevelopmental processes. Recent evidence indicates that NO, and cytokines involved in NO production, may be high in children with autism. The purpose of this study was to verify that plasma NO is high in children with autism and determine whether this elevation is related to plasma levels of cytokines involved in NO production.*

Methods: *The metabolites of NO, nitrite, and nitrate (NOx), along with the cytokines interferon-γ (IFN-γ), tumor necrosis factor-α, and interleukin-1β, were measured in plasma of 29 children with autism (mean age ± SD = 6.1 ± 2.8 years) and 27 age- and gender-matched healthy comparison subjects using commercially available assay kits.*

Results: *Plasma levels of NOx were significantly higher in the autistic subjects (p = .006); plasma levels of the cytokines did not differ between groups. NOx and IFN-γ levels were positively correlated in the autistic subjects (r = .51; p = .005).*

Conclusions: *These results confirm that plasma NO is high in some children with autism and suggest that this elevation may be related to IFN-γ activity.*

Key Words: Autism, nitric oxide, interferon, cytokines, immunology, pathophysiology

Autistic disorder (autism) is a lifelong neurodevelopmental disorder characterized by deficits in social interaction and communication, along with a markedly restricted repertoire of activity and interests. Despite more than 50 years of investigation, the etiology and pathophysiology of autism remain unknown in the majority of cases.

Nitric oxide (NO) is a free radical gas generated from L-arginine by the enzyme nitric oxide synthase (NOS). Numerous mammalian cells produce and secrete NO, and it has many physiologic roles such as vasodilation (Gruetter et al 1979), neurotransmission (Garthwaite et al 1989), and macrophage-mediated cytotoxicity (Hibbs et al 1988). In neuronal cultures, NO has also been shown to mediate glutamate neurotoxicity (Dawson et al 1991). Because NO is very labile, its production is often measured by determining the levels of its stable metabolites, nitrite and nitrate (NOx), in biological fluids (Marletta et al 1988).

Three isoforms of NOS have been described. Two of these are constitutively expressed, endothelial (eNOS) and neuronal (nNOS), whereas the third is a high-output inducible enzyme (iNOS) found in activated immune cells such as macrophages (Lyons et al 1992), as well as glial cells (Simmons and Murphy 1992). The induction of iNOS is mediated primarily by the cytokine interferon-γ (IFN-γ), in combination with tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), or endotoxin (Kilbourn and Belloni 1990; Nussler et al 1992).

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The hypothesis that NO production is high in autistic patients (Lombard 1998; Johnson 2001) has recently been demonstrated (Zoroğlu et al 2003; Söğüt et al 2003). Higher plasma NOx levels were found in children with autism compared with control children (Zoroğlu et al 2003). In addition, higher NOx levels were found in red blood cells, along with enzymatic evidence of NO-related oxidative stress, in plasma samples of children with autism compared with control children (Söğüt et al 2003).

To date, it is unknown which enzymatic form of NOS is responsible for the elevated production of NO in autism. In vitro studies of peripheral blood mononuclear cells from children with autism showed heightened production of the cytokines IFN-γ, TNF-α, and IL-β compared with control children (Croonenberghs et al 2002a; Jyonouchi et al 2001). Given the role of these cytokines in the induction of iNOS, it is reasonable to hypothesize that iNOS is involved in the elevated NO production in autism. Further support for iNOS involvement comes from the recent finding of higher blood monocyte counts and plasma neopterin levels in children with autism compared with healthy comparison subjects (Sweeten et al 2003a). IFN-γ drives increased neopterin production in macrophages and monocytes during periods of cell-mediated immune activation (Huber et al 1984). Both neopterin and NOx have been shown to be concurrently elevated in the urine of patients with immune-related disorders, including multiple sclerosis (MS), rheumatoid arthritis, and acquired immunodeficiency syndrome (AIDS; Giovannoni et al 1999).

To verify whether NO production is high in autism and to determine whether NO production is related to levels of cytokines involved in iNOS induction, we measured plasma levels of NOx, IFN-γ, TNF-α, and IL-β in a group of children with well-characterized autism and age- and gender-matched healthy comparison subjects.

Methods and Materials

The study was approved by the Indiana University Institutional Review Board (IRB). Written informed consent or assent was obtained from the legal guardians and subjects

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Table 1. Demographic Data

Variables	Autistic Children	Healthy Subjects	t or χ^2	df	p
Number of Subjects	29	27			
Mean Age	6.1 ± 2.8	6.5 ± 2.5	$t = -.55$	54	.58
Age Range	2–12	2–10			
Gender	4F, 25M	4F, 23 M	$\chi^2 = .012$	1	.91

Age results are in years. Mean age results are shown as mean ± SD. All study subjects were Caucasian. F, female; M, male.

according to IRB protocol. Twenty-nine subjects with autism (4 girls, 25 boys; mean age ± SD = 6.1 ± 2.8 years; age range 2–12 years) were recruited from the Christian Sarkine Autism Treatment Center at the James Whitcomb Riley Hospital for Children in Indianapolis, Indiana. All subjects met DSM-IV (American Psychiatric Association 1994) criteria for a diagnosis of autistic disorder as determined by a board certified child and adolescent psychiatrist (DJP or CJM). The Autism Diagnostic Interview—Revised (ADI-R; Lord et al 1994) was administered to confirm the diagnosis. The Autism Diagnostic Observation Schedule—Generic (Lord et al 2000) was not performed to confirm the ADI-R and clinical diagnosis.

Twenty-seven age-, race-, and gender-matched healthy comparison subjects (4 girls and 23 boys; mean age ± SD = 6.5 ± 2.5 years; age range 2–10 years) were recruited from the surrounding community via newsletters and flyers. All subjects were medication-free for at least 5 weeks and were given a physical examination to screen for evidence of immune activation such as elevated temperature, infection, inflammation, or malignancy before blood draw.

Blood was drawn into ethylenediamine tetraacetate tubes between 7 AM and 10 AM after overnight and morning fasting of approximately 12 hours. Plasma samples were obtained and frozen at −40°C within 30 min. These samples had been used in a previous investigation of immune function in autism (Sweeten et al 2003a). Plasma NOx levels were determined using a total NO assay kit using the Griess Reaction (R&D Systems, Minneapolis, Minnesota). The intraassay coefficient of variation for NOx measurements in our laboratory is 3.1%. High-sensitivity enzyme linked immunosorbent assay (ELISA) kits were used to measure plasma IFN-γ (Amersham Pharmacia Biotech, Piscataway, New Jersey), TNF-α, and IL-1β (R&D Systems). The intraassay coefficient of variation was < 10% for each of these ELISAs. All assays were performed in duplicate by technicians blinded to diagnosis. For statistical analysis, independent sample t tests were used to determine numeric group differences. Correlations were calculated using Pearson correlation coefficients. Chi-square analysis was performed on binomial data such as gender. Two-tailed tests with $p < .05$ were considered significant. All statistical analysis was performed using SPSS statistical software (SPSS, Chicago, Illinois).

Results

There were no demographic differences between the two groups (Table 1). Plasma levels of NOx were significantly higher in the children with autism (mean = 48.8 ± 12.1 μmol/L) compared with the healthy subjects (mean = 40.9 ± 8.3 μmol/L; $t = 2.86$, $df = 54$, $p = .006$; Figure 1). There were no group differences in levels of any of the plasma cytokines that were measured (Table 2). In the autistic subjects there was a positive correlation between plasma levels of NOx and IFN-γ ($r = .51$; $p = .005$). No other significant correlations were found.

Discussion

The high plasma levels of NOx found in this study replicate the results of Zoroğlu et al (2003). Factors that were not controlled for in the study, including genetics, metabolism, activity level, and diet, can influence NO production. The positive correlation between plasma NOx and IFN-γ in the autistic children, however, suggests that the heightened NO production may be related to IFN-γ–mediated up-regulation of iNOS. The higher production of NO seen in the autistic subjects may be a result of a cell-mediated immune response, as in an earlier study high blood monocyte counts and plasma neopterin levels were found in children with autism (Sweeten et al 2003a). Although plasma levels of IFN-γ, TNF-α, and IL-β did not differ between the groups, this could be a methodologic issue because more sensitive techniques have detected increased production of these cytokines in autistic compared with control subjects in vitro (Croonenberghs et al 2002a; Jyonouchi et al 2001) but not in serum samples (Croonenberghs et al 2002a).

Perhaps the high NO production detected in this study is secondary to immune activation related to autoimmunity in some autistic subjects. NO production is commonly increased in autoimmune states (Belmont et al 1997; Giovannoni 1998), and

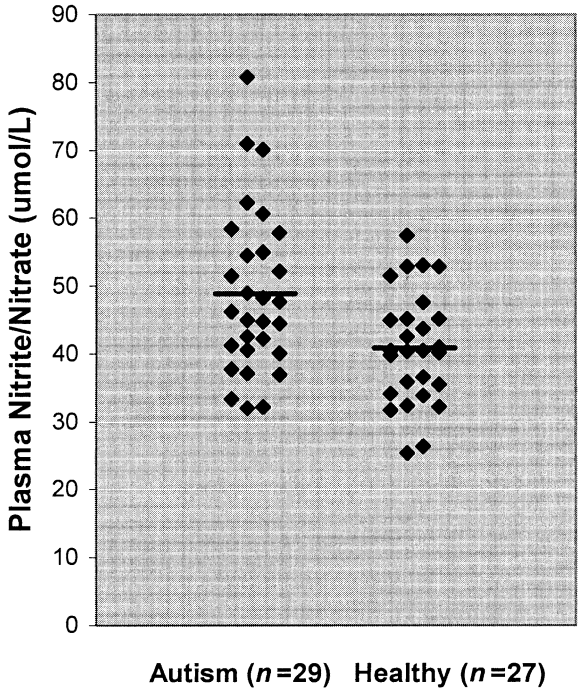

Figure 1. Higher plasma nitrite and nitrate levels in children with autism compared with healthy comparison children ($t = 2.86$, $df = 54$, $p = .006$). The horizontal lines indicate mean levels.

Table 2. Plasma Cytokine Levels in Autistic and Healthy Comparison Children

Variables	Autistic Children ($n = 29$)	Healthy Subjects ($n = 27$)	*t* Score	*df*	*p*
IFN-γ (pg/mL)	.77 ± .57	.86 ± .46	−.63	54	.53
TNF-α (pg/mL)	2.57 ± 1.05	3.11 ± 2.09	−1.20	37.7	.24
IL-1β (pg/mL)	.67 ± 1.34	.32 ± .25	1.33	54	.35

Results are shown as mean ± SD. IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β.

preliminary studies show an increased family history of autoimmune disease in probands with autism and related disorders (Comi et al 1999; Sweeten et al 2003b). Increased familial autoimmunity is commonly found in probands with various autoimmune disorders (Broadley et al 2000; Prahalad et al 2002).

Additional evidence of autoimmunity exists in autistic subjects, such as an increased number of DR+ (activated) T-cells (Plioplys et al 1994b; Warren et al 1995), decreased or abnormally distributed CD4:CD8 T-cell ratios (Plioplys et al 1994b; Warren et al 1986), and elevated levels of immunoglobulins in blood (Croonenberghs et al 2002b). In the central nervous system (CNS), perivascular lymphocytic cuffs have been described in various brain regions of three autistic subjects examined postmortem (Bailey et al 1998; Guerin et al 1996), but not in others. Perivascular lymphocytic cuffs are common in the brains of patients with MS (Tanaka et al 1975). Increased levels of autoantibodies reacting against a neurofilament subunit from cerebellar tissue (Plioplys et al 1994a) or endothelial cells of the brain (Connolly et al 1999) provide additional evidence for possible CNS immune activation in some cases of autism. These findings related to autoimmunity are complemented by results of immunogenetic studies in autism (Torres et al 2002; Warren et al 1991).

Immune activation can increase NO levels in the blood and in the brain. In MS, elevated NOx levels have been detected both in the blood and cerebrospinal fluid (Giovannoni 1998). In disease states such as systemic lupus erythematosus (SLE) and human immunodeficiency virus infection, elevated serum NOx levels have been found (Belmont et al 1997; Zangerle et al 1995), along with evidence of increased NO production in the brain during their related neurologic conditions, cerebral SLE, and AIDS dementia (Adamson et al 1996; Brundin et al 1998). It is therefore possible that NO production is increased in the brain of some autistic subjects, but this remains unknown.

As an intercellular messenger, NO has multiple roles in the development and function of the CNS. Neurite growth is under the regulation of NO (Hess et al 1993; Hindley et al 1997), and NO appears to play an important role in synaptogenesis (Lizasoain et al 1996; Truman et al 1996). Long-term potentiation in the hippocampus and long-term depression in the cerebellum are mediated by NO (Schuman and Madison 1991; Shibuki and Okada 1991); NO also can induce neuronal release of several neurotransmitters from brain slices (Lonart et al 1992; Zhu and Luo 1992) and is important for memory formation (Holscher and Rose 1992).

In conclusion, the results of this study provide additional evidence that NO production is high in children with autism and suggest that this elevation may be secondary to IFN-γ mediated up-regulation of iNOS. If NO production is abnormally increased in the brain, it could disrupt normal brain synaptic connections and neurodevelopment, possibly contributing to the underlying pathophysiology of autism.

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