PARENTAL AGE AND MATERNAL ANTIBODIES TO FETAL HUMAN BRAIN IN AUTISM

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Abstract
The etiology of autism remains unknown however the role of the immune system has recently come into question. Specifically, transfer of maternal autoantibodies to neural antigens may occur in utero in autism. These antibodies may be associated with clinical symptoms of regression and related to maternal and paternal age. Western immunoblotting was used to identify protein bands against human fetal brain tissue present more frequently in serum of mothers of children with autism (n=107) compared to mothers of controls children (n=100). Subsequently, associations between band status and clinical features of families were investigated among the autism cases. One protein band of 36 kDa in size was found more commonly in autism vs. control mothers (p=0.034). Presence of this band was associated with a maternal history of allergies (OR=0.194, p=0.045) and suggestive of an association with the child having combined social and language regression (OR=4.84, p=0.054) and. Maternal and paternal ages at child’s birth were both higher and more tightly clustered in the 36 kDa positives, though this finding was not statistically significant. With adjustment for maternal age at time of serum sample and total number of gestations, maternal history of allergies remained statistically significant. These findings suggest a possible link between this antibody and a more severe regressive form of autism.
1. Introduction

Autism is a pervasive developmental disorder characterized by social detachment, language impairment, and repetitive stereotyped behavior. Current estimates of the prevalence of autism in range from 6.7 to 9.0 cases per 1000 with a 3-4:1 male to female ratio (Fombonne E 2003; Williams JG 2006; Autism and Developmental Disabilities Monitoring Network Surveillance Year 2000 Principal Investigators 2007; Autism and Developmental Disabilities Monitoring Network Surveillance Year 2002 Principal Investigators 2007). Consistent with the phenotypic heterogeneity, research into the etiology of autism suggests the disorder to arise from a complex interplay of genetic factors, with a potential role for the environment. With an estimated heritability of at least 90% (Freitag C 2007), genetics in autism is under great scrutiny. The most recent twin study reports an MZ twin concordance rate of 60-92% and 0-10% in DZ twins (Bailey A 1995). Sibling recurrence risk, similar to the DZ twin concordance rate, lies between 2-6% (Bailey A 1995; Szatmari P 1998) representing a 100-fold increase in risk of autism compared to the general population (Bailey A 1995). Several studies reporting familial aggregation of phenotypic similarities to the individual with autism also support the strong contribution of genetics to this disorder (Pickles A 1995; Piven J 1997; Risch N 1999; Szatmari P 2000; Lainhart JE 2002). Approximately 10-15% of autism cases are comorbid with single gene or cytogenetic disorders, with tuberous sclerosis, Fragile X, and duplication of chromosome 15q11-13 occurring most frequently (Veenstra-Vanderweele J 2004; Xu J 2004; Freitag 2007). Linkage and candidate gene studies have identified various regions conferring susceptibility to autism, reviewed in detail by Freitag (Freitag C 2007), with the most compelling evidence for 2q, 7q, and 17q. Chromosome 7, containing the RELN and engrailed 2 genes, both critical in early CNS development, has also gained support from a meta-analysis (Badner JA 2002). A large
linkage study of multiplex families was recently reported by the Autism Genome Project Consortium, with results implicating a role for chromosome 11p12-13 in autism (Autism Genome Project Consortium, Szatmari et al. 2007). Ongoing investigations into environmental contributors continue as well, with in utero viral infection (such as rubella, CMV, herpes simplex), toxin exposures (medications, alcohol), or perinatal factors as leading contenders (Hertz-Picciotto I 2006; Kolevzon A 2007). Though the etiology of autism remains elusive, advances in molecular biology coupled with clinical observations have led researchers to investigate the potential role of immunologic processes in the origin of this disease.

Studies of the immune system in autism are not new. As early as 1971, a link between autism and a family history of autoimmune disease was reported (Money J 1971). The late 1970’s saw the beginnings of investigation into the (dys)function of the immune system in individuals with autism (Stubbs EG 1976; Stubbs EG 1977). Subsequent research over the past 30 years revealed abnormalities in both the humoral and cellular components of the immune system, as well as signs of neural inflammation identified post-mortem, presence of anti-neural autoantibodies in serum and pro- and anti-inflammatory cytokines in CSF, and autoimmune related genes in individuals with autism and their family members (for review see Cohly HH 2005; Pardo CA 2005; Ashwood P 2006). Motivated by earlier reports and continued clinical observation, a recent study demonstrated an increased frequency of autoimmune disorders in relatives of children with autism as compared to relatives of typically developing children (Comi AM 1999). Further, antibodies reactive to neural tissue were isolated from sera of mother’s of children with autism (Warren RP 1990; Dalton P 2003; Zimmerman AW 2006). Evidence of an active, chronic neuroinflammatory process was identified in assessment of post-mortem brains of individuals
with autism (Vargas DL 2005). These data led researchers to posit a novel theory of etiology for some cases of autism: the in utero transfer of maternal antibodies to the fetus may contribute to the development of autism (Dalton P 2003).

This study seeks to identify antibodies reactive against fetal human brain that are present statistically more frequently in the serum of mothers of children with autism than that of control mothers. Subsequently, associations will be sought between maternal serum status of the identified antibody and clinical features of the child with autism (social, language, or motor regression), in addition to family specific data such as maternal and paternal age at birth of the child, parity, history of autism or autoimmune disorders, maternal history of allergies and/or medication use.

The prevalence of regression in autism is estimated at 15.6-50%, with variability in reports depending on the definition of regression and the sampling methods of a given study (Ozonoff S 2005). One difficulty in defining regression is differentiating loss of an acquired skill or behavior from developmental plateauing where the child fails to gain additional skills (Ozonoff S 2005; Landa R 2006; Mitchell S 2006; Bernabei P 2007). Loss of social, language, or motor skills, if they occur, are generally seen between 12 and 24 months of age (Ozonoff S 2005). While the possibility of recall bias has been raised in retrospective assessment of a child’s development, one study confirmed the validity of parental report (Davidovitch M 2000).

As age is known to correlate positively with the production of autoantibodies (Prelog M 2006), older mothers would be expected to have more of the anti-neural autoantibodies under
investigation. Similarly, increased exposure to semi-allogeneic fetuses with increases in parity would likely stimulate production of alloantibodies as well (Girardi G 2006). A recent review of studies on parental age suggests an increased risk of autism in offspring with either advanced maternal (>35 years) or paternal age (>40 years)(Kolevzon A 2007). As the mechanism of these associations has not been elucidated, maternal and paternal ages will be considered here.

2. Methods

2.1. Study participants

Volunteers were recruited between December 2005 and September 2006 from the Baltimore/Washington DC metropolitan area via institutional review board (IRB) approved posters placed throughout communities, advertisements in autism-related publications, and from patient families affiliated with the Kennedy Krieger Institute at Johns Hopkins University, a referral center for pediatric developmental disabilities and the site of the study. The study was approved prior to subject recruitment by the Johns Hopkins Medicine IRB. All subjects hailed from the Eastern U.S. and provided informed consent prior to participation in the study. Cases (mothers of children with autism; n=107) were included following confirmation of a diagnosis of Autistic Disorder in accordance with DSM-IV(American Psychiatric Association 1994) criteria and taking into account findings from the Autism Diagnostic Interview-Revised (ADI-R)(Lord C 1994) and the Autism Diagnostic Observation Schedule-Generic (ADOS-G)(Lord C 2000). In five cases, three sets of twins and two sibling pairs, data was available on two children with autism per one mother. In these instances, one child was randomly chosen for inclusion in the study. Control mothers (n=100) were excluded if positive for a family history of autism spectrum
or autoimmune disorder. Case and control mothers were frequency matched on maternal age and lifetime number of gestations.

2.2. Laboratory techniques

Serum samples from case and control mothers were collected in vacuum tubes containing no preservatives, allowed to clot, then immediately centrifuged and frozen for later use. Frozen human fetal cortex (17 weeks gestation) was obtained from the University of Maryland Brain Bank. Tissue was homogenized and centrifuged for collection of the supernatant fraction, which was then stored at -80 °C. Western immunoblotting using human fetal brain tissue exposed to serum from subjects was conducted in order to identify the number of, peak height, and area under the curve of protein bands present in maternal serum against human fetal brain. Details of brain tissue preparation and Western immunoblotting have been described previously (Singer HS 2006).

2.3. Clinical and family history data

Clinical data on regression, pregnancy course, and family history of autoimmune and developmental disorders was collected through retrospective chart review and interviews with mothers. Interviews were conducted either in person or by telephone and investigators were blinded to anti-neural antibody status. Regression inquiries encompassed social, language and motor skills. Social regression was defined as acquisition and subsequent loss of one or more of the following: direct eye contact during communication, interest in interactive games, pretend or imaginative play, reaching for a caregiver, or interest in interaction with parents, siblings, relatives, or peers. Language regression was characterized as loss of most words previously used
in a meaningful manner. Deficit in a formerly mastered motor skill qualified as motor regression. In assessment of regression, care was taken to differentiate between true regression, or loss of an acquired skills or behavior, and developmental plateauing, or failure to reach normal developmental milestones. Due to the time of onset of regression children with autism, those under the age of 2 years were excluded from relevant analyses.

2.4 Statistics

Two-tailed Fisher exact tests were used to compare serum reactivity to fetal human brain between case and control groups. Protein bands identified significantly more frequently among mothers of children with autism were used to divide the children with autism into two groups, those mothers positive for and those negative for a given serum protein. Associations between serum protein status and developmental or family history were analyzed with unadjusted and adjusted (for maternal age at serum sample and parity) odds ratios using logistic regression. All analyses were conducting using STATA 9.0 (College Station, Texas).

3. Results

3.1 Serum studies

Thirty-one protein bands ranging in size from 22 to 213 kDa and reactive against human fetal tissue were identified. Of these, one protein band of 36 kDa in size was found more frequently in autism than control mothers (p=0.034).

3.2 Clinical and family history

Children of mothers positive for the 36 kDa band were compared to those negative for this protein. No significant differences were identified in baseline characteristics of the two groups.
(Table 1). Between group differences were assessed for maternal and paternal age at the child’s birth, total number of pregnancies before the birth of the child, maternal autoimmune disorders, and presence of language and/or social regression (Table 2). Due to the small sample size and limited number of mothers with serum positive for the 36 kDa band, several categories of clinical or family history data lacked the variability necessary to assess them statistically (Table 3). Of those analyzed, no statistically significant differences were identified based on child’s sex, age at social or language regression, maternal parity, or maternal history of medication use. Maternal history of allergies conferred a decreased odds of having the 36 kDa protein (OR=0.19, p=0.045). Presence of combined social and language regression was suggestive of an association with the 36 kDa band, with an odds ratio of 4.84 (p=0.054). While not statistically significant, maternal and paternal ages at child’s birth were both higher and more tightly clustered in the 36 kDa positives (maternal age median=36.48, mean=34.65, SD=5.04; paternal age median=36.73, mean=36.52, SD=4.77) than the negatives (maternal age median=32.09, mean=32.20, SD=5.08; paternal age median=33.62, mean=34.41, SD=5.60)(Figure 1). Following adjustment for known confounders of maternal age at time of serum sample and number of gestations, maternal history of allergies remained statistically significant with an OR of 0.19 (p=0.044).

4. Discussion

While the 36 kDa band reactive to human fetal brain was identified more frequently in maternal serum of mothers of children with autism than controls, the reasons remain unidentified. This association appears to be negatively associated with the odds of a maternal history of allergies. A suggestion of an association was additionally seen with combined social and language regression in the affected children. These findings appeared despite low power, however, correction for
multiple comparisons negates any statistical significance. Molloy et al. (Molloy, Kaddache et al. 2005) recently linked regression in autism to regions of chromosome 7q and 21q. Interestingly, the CXADR gene, part of the immunoglobulin superfamily, is located on chromosome 21. Whether presence of the 36 kDa antibody is associated with function of this gene remains to be investigated. As for allergies, Croen et al. (2005) demonstrated a positive association between maternal history of allergies during pregnancy and autism spectrum disorder diagnosis in the offspring. The interpretation of a protective effect of the 36 kDa antibody in association with a maternal history of allergies is uncertain, but might be associated with a protective immune mechanism with respect to type I hypersensitivity reactions. Suggestion of an association between advanced maternal or paternal age and presence of this antibody are likely attributable to the known increase in autoantibody production with age and the collinearity by age within a couple.

This study achieved a relatively large sample size considering the patient population under recruitment. Additionally, restricting enrollment to children diagnosed with Autistic Disorder and not other forms of Pervasive Developmental Disorder, such as Asperger’s syndrome, Pervasive Developmental Disorder-Not Otherwise Specified, Rett syndrome, or Childhood Disintegrative Disorder, contributed to homogeneity in the sample, decreasing the baseline variation between groups, making them most comparable, and minimizing residual confounding.

The main limitation of this study is lack of power due to small sample size. Specifically, only 10 mothers were positive for the 36 kDa band, contributing to instability in the OR estimates. Lack of variability on particularly characteristics made statistical analysis difficult. The initial study
design intended comparison of clinical data and family characteristics between both the cases and the controls. Recruitment of 100 to each group yielded an estimated power of 0.832 to detect an effect size of 0.25. However, appropriately matching the groups was exceedingly difficult. Mothers were frequency matching on age at the time the sample was drawn in addition to lifetime number of gestations as these are both presumed confounders of autoantibody production. However, to then draw comparisons between clinical characteristics of an index child and a control child, groups would additionally need to be matched on maternal age at birth of the child and number of gestations prior to that pregnancy. Matching on all four of these variables was not possible, thus the analysis was limited to comparisons within the case group stratified by 36 kDa band status. This reduced the sample size to 97 with great loss in power to detect differences in clinical or family characteristics. In regards to interpretation of the findings, maternal history of asthma, allergies, or medication use was not specifically identified as occurring during pregnancy with the child with autism. It is possible that these symptoms/prescriptions reflect on time between childbirth and sample attainment and had no bearing on the pregnancy of interest.

Future studies of a larger sample, perhaps with additional assays to investigate specific anti-neural antibodies suspected a prior, would be warranted considering the suggestion of statistically significant findings demonstrated here. Considering the likely multi-factorial etiology of autism, combining candidate gene and autoantibody investigations would be useful to delineate associations between these two areas of autism research.
Acknowledgements

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References


### Table 1. Characteristics of families of children with autism

<table>
<thead>
<tr>
<th></th>
<th>36 kDa positive</th>
<th>36 kDa negative</th>
<th>p-value&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number (percentage)</td>
<td>10 (9.35)</td>
<td>97 (90.65)</td>
<td>N/A</td>
</tr>
<tr>
<td>Percent male</td>
<td>80.00%</td>
<td>91.75%</td>
<td>0.235</td>
</tr>
<tr>
<td>Number maternal gestations</td>
<td>2.80 (1.55)</td>
<td>3.13 (1.50)</td>
<td>0.540</td>
</tr>
<tr>
<td>Child’s age at time sample drawn (yrs)</td>
<td>9.35 (4.07)</td>
<td>8.24 (4.47)</td>
<td>0.435</td>
</tr>
<tr>
<td>Maternal age at time sample drawn (yrs)</td>
<td>43.99 (6.55)</td>
<td>40.35 (6.28)</td>
<td>0.085</td>
</tr>
<tr>
<td>Paternal age at time sample drawn (yrs)</td>
<td>45.87 (5.63)</td>
<td>42.68 (6.42)</td>
<td>0.120</td>
</tr>
</tbody>
</table>

<sup>a</sup>2-sided Fisher’s exact test used for percent male; 2-sided t-test with unequal variances for all remaining

### Table 2. Associations of clinical and family factors by 36 kDa band status

<table>
<thead>
<tr>
<th></th>
<th>Unadjusted OR (95% CI)</th>
<th>p-value</th>
<th>Adjusted OR&lt;sup&gt;a&lt;/sup&gt; (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male sex</td>
<td>0.36 (0.07, 1.99)</td>
<td>0.241</td>
<td>0.39 (0.07, 2.23)</td>
<td>0.287</td>
</tr>
<tr>
<td>Number gestations at birth of child with autism</td>
<td>0.88 (0.58, 1.33)</td>
<td>0.549</td>
<td>1.00 (0.37, 2.73)</td>
<td>0.995</td>
</tr>
<tr>
<td>Language regression only</td>
<td>2.00 (0.21, 19.05)</td>
<td>0.547</td>
<td>2.63 (0.26, 26.80)</td>
<td>0.414</td>
</tr>
<tr>
<td>Social and language regression</td>
<td>4.84 (0.98, 23.99)</td>
<td>0.054</td>
<td>4.44 (0.87, 22.61)</td>
<td>0.073</td>
</tr>
<tr>
<td>Age at social regression</td>
<td>1.00 (0.90, 1.12)</td>
<td>0.956</td>
<td>0.96 (0.86, 1.09)</td>
<td>0.606</td>
</tr>
<tr>
<td>Age at language regression</td>
<td>1.01 (0.90, 1.14)</td>
<td>0.876</td>
<td>0.97 (0.85, 1.11)</td>
<td>0.660</td>
</tr>
<tr>
<td>Maternal age at childbirth</td>
<td>1.10 (0.96, 1.26)</td>
<td>0.154</td>
<td>1.03 (0.86, 1.24)</td>
<td>0.739</td>
</tr>
<tr>
<td>Paternal age at childbirth</td>
<td>1.07 (0.95, 1.20)</td>
<td>0.254</td>
<td>1.03 (0.91, 1.18)</td>
<td>0.635</td>
</tr>
<tr>
<td>Maternal family history of autism diagnosis</td>
<td>0.86 (0.10, 7.44)</td>
<td>0.890</td>
<td>0.84 (0.09, 7.59)</td>
<td>0.880</td>
</tr>
<tr>
<td>Maternal family history of symptoms of autism</td>
<td>2.31 (0.54, 9.97)</td>
<td>0.260</td>
<td>2.55 (0.56, 11.57)</td>
<td>0.224</td>
</tr>
<tr>
<td>Paternal family history of symptoms of autism</td>
<td>1.60 (0.30, 8.60)</td>
<td>0.581</td>
<td>2.37 (0.40, 14.14)</td>
<td>0.342</td>
</tr>
<tr>
<td>Maternal medication use</td>
<td>1.02 (0.28, 3.76)</td>
<td>0.975</td>
<td>0.89 (0.23, 3.39)</td>
<td>0.862</td>
</tr>
<tr>
<td>Maternal history of allergies</td>
<td>0.19 (0.04, 0.96)</td>
<td>0.045</td>
<td>0.19 (0.04, 0.96)</td>
<td>0.044</td>
</tr>
</tbody>
</table>

<sup>a</sup>Adjusted for maternal age and complete maternal gestations
Table 3. Characteristics of families of children with autism

<table>
<thead>
<tr>
<th></th>
<th>Positive</th>
<th>Negative</th>
<th>Unknown</th>
<th>Variability in 36 kDa status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Social and language regression</td>
<td>51</td>
<td>56</td>
<td>0</td>
<td>Yes</td>
</tr>
<tr>
<td>Social regression only</td>
<td>17</td>
<td>90</td>
<td>0</td>
<td>Yes</td>
</tr>
<tr>
<td>Language regression only</td>
<td>6</td>
<td>101</td>
<td>0</td>
<td>Yes</td>
</tr>
<tr>
<td>Motor regression</td>
<td>3(^a)</td>
<td>104</td>
<td>0</td>
<td>No, all 36 kDa positive</td>
</tr>
<tr>
<td>Maternal family history</td>
<td>61</td>
<td>0</td>
<td>46</td>
<td>No, all 36 kDa positive</td>
</tr>
<tr>
<td>of autoimmune disorders</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal family history</td>
<td>12</td>
<td>94</td>
<td>1</td>
<td>Yes</td>
</tr>
<tr>
<td>of autism diagnosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paternal family history</td>
<td>7</td>
<td>88</td>
<td>12</td>
<td>Limited</td>
</tr>
<tr>
<td>of autism diagnosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal history of asthma</td>
<td>8</td>
<td>97</td>
<td>2</td>
<td>Limited</td>
</tr>
</tbody>
</table>

\(^a\) All 3 cases with motor regression also demonstrated social and language regression.

Figure 1. Parental ages at birth of child by 36 kDa band status