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Detection of autoantibodies to neural cells of the cerebellum in the plasma of subjects with autism spectrum disorders

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ABSTRACT

Autism spectrum disorders (ASD) are a group of heterogeneous, behaviorally defined disorders characterized by disturbances in social interaction and communication, often with repetitive and stereotyped behavior. Previous studies have described the presence of antibodies to various neural proteins in autistic individuals as well as post-mortem evidence of neuropathology in the cerebellum. We examined plasma from children with ASD, as well as age-matched typically developing controls, for antibodies directed against human cerebellar protein extracts using Western blot analysis. In addition, the presence of cerebellar specific antibodies was assessed by immunohistochemical staining of sections from *Macaca fascicularis* monkey cerebellum. Western blot analysis revealed that 13/63 (21%) of subjects with ASD possessed antibodies that demonstrated specific reactivity to a cerebellar protein with an apparent molecular weight of approximately 52 kDa compared with only 1/63 (2%) of the typically developing controls ($p = 0.0010$). Intense immunoreactivity, to what was determined morphologically to be the Golgi cell of the cerebellum, was noted for 7/34 (21%) of subjects with ASD, compared with 0/23 of the typically developing controls. Furthermore, there was a strong association between the presence of antibodies reactive to the 52 kDa protein by Western blot with positive immunohistochemical staining of cerebellar Golgi cells in the ASD group ($r = 0.76$; $p = 0.001$) but not controls. These studies suggest that when compared with age-matched typically developing controls, children with ASD exhibit a differential antibody response to specific cells located in the cerebellum and this response may be associated with a protein of approximately 52 kDa.

1. Introduction

Autism spectrum disorders (ASD) are a group of heterogenous, behaviorally defined disorders characterized by disturbances in social interaction and communication (verbal and non-verbal) and often with repetitive and stereotyped behavior which is typically apparent between 2 and 3 years-of-age. The prevalence of ASD has been estimated at 1 in 150 in the total population and affects approximately four times as many males as females (MMWR, 2007). The potential exists for numerous etiologies, including those of a genetic and/or environmental nature, which could contribute to the development of ASD.

Immune system-related irregularities associated with ASD involve the innate as well as the adaptive arms of the immune system. Immune findings in ASD are varied and include increased numbers of circulating monocytes, decreased natural killer cell lytic activity, abnormal cytokine, and immunoglobulin levels and decreased peripheral lymphocyte numbers, and responsiveness (Ashwood et al., 2006; Croonenberghs et al., 2002; Molloy et al., 2006; Stubbs and Crawford, 1977; Sweeten et al., 2003; Trajkovski et al., 2004; Warren et al., 1987, 1990; Yonk et al., 1990). The presence of several putative autoantibodies to various elements of the nervous system has also been reported in ASD, including antibodies directed against myelin basic protein (MBP), brain serotonin receptor, neurofilament proteins, brain endothelial cell proteins, heat shock protein as well as autoantibodies directed against epitopes within the cerebellum (Cabanlit et al., 2007; Connolly et al., 1999; Evers et al., 2002; Plioplys et al., 1994; Silva et al., 2004; Singer et al., 2006; Singh et al., 1998, 1997a,b, 1993; Todd et al., 1988; Vojdani et al., 2002). It is important to note that many

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of these autoantibodies are not unique to individuals with ASD, nor are they found in all subjects with ASD. MBP self-reactive antibodies, for example, are often identified in individuals with multiple sclerosis (Egg et al., 2001).

The neuropathology of ASD is still in its infancy (Amaral et al., 2008). Yet, cerebellar irregularity is one of the most consistent findings in the brains of subjects with ASD. For example, in a review by Brambilla et al. of the 24 post-mortem cases of autism reported in which the cerebellum was studied, 19 demonstrated a lower number of Purkinje cells (Brambilla et al., 2003). The lack of cells is most commonly observed in the cerebellar hemispheres. Other abnormalities observed in the cerebellum or cerebellum-related brain regions, such as changes overtime in the inferior olive, deep cerebellar nuclei, and increases in white matter have also been described in post-mortem tissue (Bailey et al., 1998; Kemper and Bauman, 1998). Interestingly, very little information is available with respect to the other neuronal cell types found in the cerebellum, although Yip et al., recently described reduced GAD 67 mRNA in the basket and stellate cells of the molecular layer in post-mortem autism tissue (Yip et al., 2007).

Previous studies describing the presence of antibodies to various neural proteins as well as neuropathological indications for a reduced number of Purkinje cells led us to examine plasma from a cohort of extremely well-characterized children with ASD as well as age-matched typically developing and developmentally delayed controls using a two-pronged approach. First, to look for the presence of specific autoantibodies to brain tissue, we examined plasma from children with ASD and controls for reactivity to human brain protein extracts using Western blot analysis. Second, to identify specific autoantibodies that were directed to neural structures using immunohistochemistry, plasma of subjects with ASD were examined for their ability to bind to sections from the *Macaca fascicularis* monkey cerebellum. These approaches enabled us to determine both the apparent molecular weight and the cellular location of the target molecule(s).

2. Methods and materials

2.1. Subjects/sample collection

The study protocol followed the ethical guidelines of the most recent Declaration of Helsinki (www.cgmh.org.tw/intr/intr1/c0040/web/C/Declaration%20of%20Helsinki.pdf), and was approved by the Institutional Review Boards of the UC Davis School of Medicine and the State of California, and all subjects enrolled in the study had written informed consent provided by their parents and assented to participate if developmentally able. Subjects for this study were enrolled through the MIND (Medical Investigations of Neurodevelopmental Disorders) Institute clinic. The MIND clinic sample population consisted of children diagnosed on the autism spectrum (ASD) ($n = 63$) and their siblings ($n = 25$). There were two separate control populations: one consisted of age-matched typically developing children ($n = 63$); the other contained children who are developmentally delayed but do not have ASD ($n = 21$) (Table 1). A diagnosis of ASD was confirmed in all subjects using the autism diagnostic interview-revised (ADI-R) and the autism diagnostic observation schedule (ADOS) (DiLavore et al., 1995; Joseph et al., 2002; Lord et al., 2001, 1997). Final autism case status is defined as meeting criteria on the communication, social interaction, and repetitive behavior domains of the ADI-R with onset prior to 36 months and scoring at or above the social plus communication cut-off for autism on the ADOS module 1 or 2.

Table 1
Demographics of study subjects for Western blot analysis

Study population	Median age (mos.)	Range (mos.)	Gender	
			Male	Female
ASD ($N = 63$)	53	30–184	60	3
Typically developing controls ($N = 63$)	68	25–168	48	15
Developmental delay ($N = 21$)	37.5	30–57	17	4
Siblings of ASD subjects ($N = 25$)	61	21–160	13	12

The ADI-R was used to define onset of ASD. The ADI-R provides a standardized, semi-structured interview, and a diagnostic algorithm for the DSM-IV and the ICD-10 definitions of autism (Association, 1994) (Steinhausen and Erdin, 1992). The social communication questionnaire was used to screen for characteristics of ASD among the subjects with developmental disabilities, the typically developing controls, and the siblings of subjects with ASD. Children who scored above the screening cut-off were fully assessed using the ADI-R and ADOS.

Blood samples for Western blot were obtained from the 172 individual subjects at the time of assessment. A number of these subjects were also analyzed by immunohistochemical staining, including 34 children diagnosed with ASD, 23 age-matched typically developing controls, 14 siblings of children with ASD and 11 children diagnosed with developmental delay but not ASD (Table 2).

2.2. Antibodies and reagents

Human adult cerebellum protein medley (BD Bioscience Clontech, Palo Alto, CA) was used to screen for potential autoantibodies in the plasma by Western blot. Human kidney protein was used as negative tissue control (BD Bioscience Clontech, Palo Alto, CA). Horseradish peroxidase-conjugated goat anti-human IgG was used as a secondary antibody (Zymed, San Francisco, CA), and SuperSignal Chemiluminescent Substate was used to develop the blot (Pierce, Rockford, IL).

2.3. Immunoblotting

To determine the incidence of plasma containing antibodies that were reactivity to human brain extracts, SDS–PAGE was performed using 12% Tris–HCl Mini Ready gels (Bio-Rad, Hercules, CA). A final concentration of 300 μg/mL of adult human brain extracts from the cerebellum, extracts from human kidney, and 5 μL of magic mark protein standard were loaded into the gel and electrophoresed for 1 h and 30 min at a constant current of 30 mA. After gel electrophoresis, proteins were transferred at 30 V overnight to a nitrocellulose membrane, dried, and stored at 4 °C. For immunoassay, the membranes were blocked with 5% milk in PBS buffer containing 0.05% Tween 20 (PBST) for 1 h at room temperature. Blots were incubated in plasma from subjects with ASD ($n = 63$), TD controls ($n = 63$), typically developing siblings ($n = 25$), and DD subjects ($n = 21$) diluted 1:500 in 5% milk and PBS-Tween for 1 h at room temperature. They were washed five times for 5 min duration with PBST followed by 1 h incubation with HRP-conjugated goat anti-human IgG diluted 1:10,000. To visualize the signal, blots were developed using a SuperSignal Chemiluminescent Substate according to the manufacturer's instructions. Strips were imaged and analyzed using a FluorChem 8900 imager and AlphaEaseFC imaging software. (AlphaInnotech Corporation, San Leandro, CA.) Since multiple blots were used in this project, a reference subject, who demonstrated reactivity to all bands noted in this study, was run as a standard control on each blot.

Cerebellum from a healthy adult male macaque monkey was prepared by suspending 1.0 g of fresh tissue in 10 mL of 20 mM