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# Immunoglobin Reactivity in Autism and Rett's Syndrome

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**Abstract.** Blood samples were obtained from 17 patients with autism (8 – 23 years of age; 16 males and 1 female). B cell numbers as measured by anti-B1 antibodies were normal. B cell function (proliferation and in vitro IgG and IgM synthesis in response to pokeweed mitogen) was normal. Quantitative serum immunoglobulins (IgG, IgA and IgM) were normal. When tested against Western blots prepared from normal, human cerebellar tissue, there was an increased incidence of IgG anti-210K neurofilament subunit reactivity (41 vs. 7% in 348 controls; $p < 0.001$). IgM anti-210K reactivity occurred in 53% of the patients (22% in 111 controls; $p < 0.05$) with an overall incidence of anticerebellar Western blot banding of 88% (23% in controls; $p < 0.001$). IgG or IgM reactivity against front cortex Western blots was not observed. Similar investigations performed on 8 girls with Rett's syndrome failed to reveal any abnormalities.

## Introduction

Autism is a syndrome characterized by social and communicative deficits of early onset accompanied by abnormal behaviors. There are many biomedical causes underlying autistic symptomatology [1], but in the majority of cases no clear etiology is ascertained.

Immune system abnormalities have been associated with autism. Lymphocyte abnormalities have included inhibition of macrophage migration in response to human myelin basic protein [2], reduced mitogen-induced lymphocyte blastogenesis [3 – 5], decreased numbers of T lymphocytes with altered ratios of helper to suppressor T cells [5], and decreased natural killer cell activity [6].

Abnormalities in the circulating immune system have also been described in autism. There have been reports of defective antibody response to rubella vaccine [7] and the presence of circulating antibodies to serotonin receptors [8] and to neurofilament axonal proteins [4].

This study was undertaken in an attempt to better define possible circulating antibody abnormalities in autism. We specifically investigated B cell function and searched for the presence of circulating anticentral nervous system (CNS) antibody reactivity. For comparison, girls with Rett's syndrome were also investigated.

## Materials and Methods

Blood samples were obtained from a total of 17 patients with autism. There were 16 males and 1 female. The age range was from 8 to 23 years with a mean age of 17. The diagnosis of autism conformed to the DSM III-R criteria for autism. There were no identified biomedical causes of autism in any of the studied population. Parental signed consent was obtained prior to phlebotomy. This study was approved by hospital ethics review committees. Simultaneously drawn blood samples from healthy young adults served as controls for the lymphocyte stimulation studies.

Blood samples were obtained from 8 Rett's syndrome girls with an age range of 2 – 15 years and a mean age of 8. The heparinized blood samples were obtained at different geographic areas in the United States and Canada and courier delivered to the laboratory for analysis. In all cases, a blood sample from a healthy young adult accompanied the Rett's syndrome sample, in the same package, to control for handling and shipping differences. In all cases, blood samples were received and processed less than 24 h from the time when they were drawn. Peripheral blood lymphocytes (PBL) were separated on a Ficoll-Hypaque density gradient [9]. After washing, 200 $\mu$l of cells were plated at a concentration of $5 \times 10^5$ cells/ml in 96-well microplates in RPMI-1640 media with 10% fetal calf serum and *L*-glutamine. Triplicate wells were cultured in the presence or absence of pokeweed mitogen in concentrations of 1:10 and 1:40 at 37 °C in 5% $CO_2$. After 7 and 14 days, the supernatants were collected and quantitative IgG and IgM determinations performed using a Pandex nephelometer.

PBL cell surface phenotype was determined by indirect immunofluorescence. Briefly, $10^6$ isolated PBL were incubated with saturating amounts of murine monoclonal antibody. After washing, cells were then incubated with FITC-conjugated goat anti-mouse, isotype-specific immunoglobulin. The percentage of fluorescent positive cells was determined from a 2-parameter analysis of at least $10^4$ lymphocytes on a gated lymphocyte population. The green fluorescence intensity was detected at 488 nm with a laser power of 500 mW using a Coulter Epics V flow cytometer (Coulter Electronics, Hialeah, FL, USA). Murine monoclonal antibody B1 (pan B cell marker) was obtained from Coulter Immunology (Hialeah).

Quantitative serum immunoglobulins were performed in the biochemistry laboratories of The Hospital for Sick Children using a Beckman array nephelometer. Immunoglobulin concentration normative data from the biochemistry laboratories was used for comparison.

Brain samples were obtained at the time of autopsy from neurologically normal young adults who had died from non-neurologic causes. They were kindly provided by Dr. J. Deck of the neuropathology service at the Toronto General Hospital. The time of autopsy was no later than 12 h after the time of death. Routine neuropathologic examination was normal. The brain samples were stored at – 80 °C until being used.

Western blots were made by standard techniques [10]. Homogenates of frontal cortex or cerebellum (taken from the cerebellar hemispheres) were homogenized and boiled for 2 min in 2.5% sodium dodecyl sulphate (w/v), 7% 2-mercaptoethanol (v/v) in TBS (50 m*M* Tris-HCl, 200 m*M* NaCl, pH 7.4) and the proteins separated as a curtain by polyacrylamide gel electrophoresis through a 15% acrylamide gradient. The gel loading was 10 $\mu$g protein/mm track width. The separated polypeptides were electrophoretically transferred to a cellulose nitrate sheet. To detect specific antibody binding 3 mm wide strips from the blot were first incubated 30 min in 10% normal horse serum (NHS) in phosphate-buffered saline (PBS; 0.1 *M* phosphate buffer, 0.15 *M* NaCl, pH 7.4) to block nonspecific binding sites, then overnight in the serum sample diluted 1:100 gave optimal visualization of immunoreactive bands without significant background staining. After two 15-min washes in PBS, the blot strips were incubated for 2 h in horse radish peroxidase conjugated rabbit anti-human IgG or anti-human IgM (Dako Inc.) diluted 1:100 in 10% NHS. Antibody binding was detected by washing the blot

twice for 15 min in PBS and then for 15 min in 0.5 mg/ml 4-chloro-1-naphthol-0.01% (v/v) hydrogen peroxide [11]. The apparent molecular weights of antigenic polypeptide bands in kiloDaltons (K) were estimated from prestained molecular weight standards (BRL Inc.) which were blotted concomitantly. Frontal cortex and cerebellar blots in which the serum sample was replaced by 10% NHS revealed no bands. MabN210 is a murine monoclonal antibody which recognizes the 210K subunit of neurofilaments [12]. Frontal cortex and cerebellar blots in which mabN210 was substituted for the serum, and the second antibody replaced by horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dako Inc.) diluted 1:100 in 10% NHS, consistently revealed an immunoreactive band at 210K.

For statistical analysis, $\chi^2$ and Fisher's exact probability tests were used.

Simultaneously drawn blood samples from healthy young adults served as controls for the pokeweed mitogen stimulations. For comparison of IgG anti-CNS reactivity the results from a study of 248 children were used [13]. For the IgM results a comparison group of 111 normal young adults was used.

## Results

In all cases of autism and Rett's syndrome, B cell numbers as measured by anti-B1 antibodies were normal. B cell function (proliferation and in vitro IgG and IgM synthesis in response to two different concentrations of pokeweed mitogen) was also normal. Quantitative determinations of serum IgG, IgM and IgA concentrations were normal.

When tested against Western blots prepared from normal, human cerebellar tissue, there was an increased incidence of IgG anti-210K neurofilament subunit reactivity in the autistics (41 vs 7% in 348 controls; $p < 0.001$). Representative Western blots are illustrated elsewhere [13, 14]. Immunoreactive IgG banding against other cerebellar molecular weight epitopes was not observed. Western blots prepared from frontal cortex revealed no IgG-reactive banding. In a previously reported investigation of anti-CNS antibody reactivity in adults using identical techniques, the incidence of IgG anti-210K cerebellar reactivity in 18 patients with cerebellar ataxia was 17% [14].

IgM anti-210K cerebellar reactivity occurred in 53% of the autistics and in only 22% of 111 controls ($p < 0.05$). IgM banding at other molecular weights was also observed with an overall incidence of 88% (23% in controls; $p < 0.001$). IgM reactivity against frontal cortex Western blots was not observed. No IgM immunoreactivity was detected in the Rett's syndrome patients.

Of the autistic patients 6 (35%) had both IgG and IgM anti-210K cerebellar reactivity. These were all males with ages of 8, 12, 17, 18, 20, and 22 years (mean age of 19 years) and only 1 was taking a medication (methylphenidate). One (6%) had only IgG anti-210K cerebellar reactivity. This was a 14-year-old male who was taking pimozide. Three (18%) had only IgM anti-210K cerebellar reactivity. These were all males of ages 10, 16 and 18 years (mean age of 16 years) and none were taking medications. Seven (41%) had neither IgG or IgM anti-210K cerebellar reactivity. These included one female of 19 years of age and the remainder were males of 9, 14, 16, 17, 22 and 23 years of age (mean age of 17 years). Of these 4 were taking medications (1 was taking haloperidol, 1 carbamazepine and 2 thioridazine).

There was no correlation between any of the positive results and medication intake or age distribution. There was no correlation between the positive results and the presence of epilepsy since only 1 of the 17 autistics was being treated for seizures. In no case was there any clinical indication of cerebellar dysfunction.

No IgG or IgM immunoreactivity was detected in the Rett's syndrome patients when the serum samples were screened against blots prepared either from frontal cortex or cerebellum.

## Discussion

Although antibodies recognizing serotonin receptors have been reported in 1 autistic child [8], a screening investigation of 20 patients with autism did not detect anti-CNS antibody reactivity [15]. However, the techniques that were used in this study were substantially different from those that we used. A membrane preparation made from human frontal cortex was used, not whole CNS tissue homogenates. Immunoreactivity against many CNS antigenic determinants, including cytoskeletal components such as neurofilaments, was not screened for.

Our results indicate that there is a significantly increased incidence of circulating anti-210K neurofilament immunoreactivity in autism. This finding is not specific for autism since it has been described in Creutzfeldt-Jakob disease [16, 17], Kuru [16], Parkinson's disease [18], the opsoclonus-myoclonus syndrome of childhood [19], and in normals [13, 14]. Furthermore, claims have been made that anti-210K immunoreactivity for both IgG and IgM classes of antibodies can be detected in virtually all normal individuals [20]. However, in our hands consistently the incidence of IgG anti-210K reactivity is low. In a study of 257 adults the incidence was 6% [14]. In a subsequent investigation of 358 children, the incidence was 3% when screened against Western blots prepared from frontal cortex, and 7% when screened against blots prepared from cerebellum [13]. For IgM, in 111 normal adults, the incidence of anti-210K reactivity was 22%. Thus our results of anti-210K reactivity in autism are statistically highly significant, but not specific for autism. It should be noted that none of our autistic patients had any clinical evidence of cerebellar dysfunction.

We did not detect immunoreactivity against frontal cortex blots which are also rich in neurofilaments. It is possible that there may be another substance in cerebellar tissue comigrating at the same molecular weight as the 210K neurofilament subunit. Alternatively, antigenic epitopes on the 210K neurofilament subunit may be revealed in processing cerebellar tissue, an event which may not take place in preparing frontal cortex immunoblots. Nevertheless, a consistent finding was the fact that there was a high incidence of anticerebellar immunoreactivity.

The cerebellar specificity of our findings is particularly intriguing in light of a report suggesting cerebellar abnormalities in autism using brain-imaging techniques [21, 22]. These results are controversial and have not been confirmed by other investigators [23–25]. None of our studied autistic patients had brain MRI scans performed. It is possible that subsets of autistics may have differing neuropathologic findings on imaging studies. Ongoing anticerebellar immunoreactivity may play a role in the genesis and/or modification of cerebellar circuitry in autism.

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