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Serum autoantibodies measured by immunofluorescence confirm a failure to differentiate PANDAS and Tourette syndrome from controls

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ABSTRACT

PANDAS and some cases of Tourette syndrome (TS) have been proposed to be post-streptococcal movement disorders in which antibodies produced against group A β-hemolytic streptococcus cross react against brain epitopes. Attempts to identify disease specific anti-striatal antibodies in the serum of affected patients have focused on the use of Western immunoblotting and ELISA methodologies. In this study, immunohistochemical techniques were used to identify serum anti-striatal antibody reactivity. In positive samples, double staining with anti-GFAP (glial) and anti-MAP2 (neuronal) was used to establish localization of the immunofluorescence. No significant differences in immunofluorescence or localization were identified in patients with PANDAS ($n=30$) and TS ($n=30$) as compared to controls ($n=30$). IF reactivity did not correlate with tic severity or elevated titers of antistreptococcal antibodies. Further comparisons showed no correlation between autoreactivity determined by immunofluorescence and the presence of previously measured immunoblot reactivity against human caudate or putative antigens (pyruvate kinase M1 and aldolase C). These results confirm an inability to distinguish patient populations by antibody measurements and raise further concerns about the presence of an autoimmune mechanism in PANDAS and TS.

1. Introduction

The list of proposed post-streptococcal disorders affecting the central nervous system has expanded from solely Sydenham's chorea (SC) to include other movement disorders such as tics[1], Tourette syndrome (TS)[2], Pediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcal infection (acronym PANDAS)[3], dystonia[4], myoclonus[5], Parkinsonism[6], paroxysmal dystonic choreoathetosis[7], motor stereotypies[8], and encephalitis lethargica[9]. In each disorder it is hypothesized that antibodies produced against group A β-hemolytic streptococcus (GABHS) cross react against brain epitopes, i.e., anti-streptococcal antibodies become antineuronal antibodies, through the process of molecular mimicry. Based on this speculation, one of the criteria for confirming a post-streptococcal immune status has been the identification of anti-basal ganglia antibodies as measured by either immunofluorescent histochemical (IF) staining, ELISA, or Western blotting techniques. Historically, the IF methodology is the most renowned, having been used to distinguish patients with Sydenham's chorea and often favored for its ability to localize putative immunoglobulins at a specific target site[10]. Studies using ELISA and immunoblotting have resulted in conflicting results when performed in children with PANDAS and Tourette syndrome[2,11,12], generating controversy in the literature[11,13,14].

Despite its frequent citation in Sydenham's chorea, evaluations using immunofluorescent histochemical reactivity in other childhood movement disorders have been relatively limited. Children followed in an Attention Deficit Hyperactivity Disorder (ADHD) and associated neurodevelopmental disorders clinic were more likely to have positive IF screening if they had coexisting movement disorders including TS, tics, and choreiform movements[1]. About one-third of the children with TS, investigated by IF, showed positive immunofluorescence[15], as were a small number of patients identified to have serum anti-basal ganglia antibodies on indirect immunoblot[2]. IF studies on TS subjects using frozen rat striatum as the epitope showed a greater total amount of antineuronal antibodies, but findings were not significant when children and adolescents were compared to controls[16].

Even fewer studies using the IF methodology of Husby have been published in children with PANDAS. Approximately two-thirds of twenty-two children and adolescents with this disorder had positive detectable staining at a 1:10 serum dilution[17], as did a small number of TS cases possessing positive putative antibodies determined by immunoblotting assays[12].

The goal of this study is to expand the investigation of immunofluorescent histochemical antibody staining in children with TS and PANDAS, and, if the staining is positive, to investigate whether the

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antibody is bound to neuronal or glial elements. Further, results of IF assays were compared to antibody reactivity previously determined in a subset of these subjects by Western immunoblotting against human caudate and several putative antigens[11] and the presence of antistreptococcal antibodies. We hypothesized that if antineuronal antibodies are involved in PANDAS, and if IF histochemistry is a more sensitive technique, then these studies would identify associations not demonstrable using ELISA or Western blot approaches[11].

2. Materials and methods

2.1. Subjects

Initial serum obtained from 30 children with the diagnosis of PANDAS and 30 children with Tourette syndrome, participating in a two-year multi-center longitudinal study by the Tourette Syndrome Study Group[11], was used in this investigation. Demographic and clinical background information on these subjects is shown in Table 1. Cases with PANDAS met the criteria as proposed by Swedo et al.[3]. Tourette syndrome subjects met the diagnostic criteria of the *Diagnostic and Statistical Manual of Mental Disorders* (4th ed.)[18]. The diagnosis of ADHD was made by parent completed DuPaul ADHD scale (DuPaul, 1991;[19]) and Obsessive Compulsive Disorder (OCD) by clinical evaluations and use of a Children's Yale–Brown Obsessive Compulsive Scale (CYBOCS) Score >15 (Scahill et al., 1997;[20]). Control subjects included 30 children without symptoms of TS, OCD, or ADHD, and no lifetime personal history for the patient, or any first-degree relative, of a chronic tic disorder, TS, OCD, or ADHD. No ethnic or racial groups were excluded from the study. Serum samples were aliquoted and stored at −80 °C. Only four samples had been previously thawed for prior Western immunoblotting and ELISA studies[11].

2.2. Basal ganglia antibody indirect immunofluorescence

A double stain using indirect immunofluorescence was performed on 20 μm sections obtained from a snap-frozen block of human striatum. Sections were obtained from a 48-year-old previously healthy female who died from an accident without head trauma. Slides were fixed in 100% methanol for 10 min, washed for 5 min in distilled water, and again for 10 min in PBS. Each slide was then overlaid with blocking solution (3% normal rabbit serum, 0.4% Triton X-100 in PBS) and incubated in a dark, damp chamber at room temperature for 1 h. After washing, each slide was overlaid with two primary antibodies at once; both 1:50 human serum (from either a control, TS patient, or PANDAS patient) and a specific mouse antibody (either anti-glial fibrillary acidic protein (GFAP; glial marker) diluted 1:1000 or anti-microtubule associated protein 2 (MAP2; neuronal marker) (Chemicon International, Temecula, CA)) diluted 1:100 with 3% normal rabbit serum and 0.1% Triton X-100 in PBS. To determine background fluorescence, negative controls were run using PBS as primary. Slides were incubated with primary antibodies for 48 h at 4 °C in a dark, damp chamber.

After washing, two secondary antibodies were added; both rabbit anti-human IgG/FITC (Dako Cytomation, Carpinteria, CA) and rabbit anti-mouse IgG/Texas Red (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:250 with 3% normal rabbit serum and 0.1% Triton X-100 in PBS, and incubated as above for 24 h. After washing in PBS, MOWIOL mounting medium (Calbiochem, La Jolla, California) was applied to each slide and specimens were held at 4 °C until viewing.

Analysis was done using a Zeiss LSM 510 laser scanning confocal microscope (Carl Zeiss Microimaging, Thornwood, NY).

All sections were analyzed by an expert experienced neuropathologist (CPV) who was blinded to the diagnosis. Immunoreactivity was observed visually and scored as positive or absent based on the morphology of the stained section. Positive fluorescence was recorded in only nucleated cells, and colocalization was determined if the glial or neuronal process stained was attached to a cell body.

2.3. Statistical analyses

Statistical analyses were performed by chi-square analysis using SPSS software, version 15.0 (SPSS Inc, Chicago, IL) to test the null hypothesis that IF reactivity is similar among three diagnostic groups (control, TS and PANDAS). Analyses were also performed to determine group differences in terms of immunofluorescent colocalization, association with tic severity, and presence of antistreptococcal antibodies. Upper limits of normal for ASO and antiDNase B were based on values defined by Kaplan[21] for children less than 12 years of age, and using 200 Todd units for children 13 years and above. In a post-hoc analysis, multiple logistic regression analyses were performed to determine whether positive IF reactivity combined with the presence of specific bands previously identified[11] against human caudate at molecular weights 60, 45, or 40 kDa (bands proposed to be abnormal in TS and PANDAS) and against specific putative proteins (pyruvate kinase M1 and aldolase C) predicts the diagnosis of PANDAS or TS. These backward logistic regression analyses compared PANDAS to controls, TS to controls, and PANDAS to TS using the covariates: 1) positive IF; 2) band at 40 kDa; 3) band at 45 kDa; 4) band at 60 kDa; 5) positive PK M1 reactivity; and 6) positive aldolase C reactivity. A p-value of <0.05 was considered significant.

Table 1
Sample population demographics

	PANDAS ($n=30$)	TS ($n=30$)	Control ($n=30$)
Age $m\pm$SD, (range)	11±2 years, (7–14)	11±2 years, (9–14)	13±3 years, (8–17)
Gender	22 M, 8 F	25 M, 5 F	17 M, 13 F
YGTSS	34.5±24.7, (0–74)	30.2±16.9, (0–60)	n/a
TTS	17.7±10.9, (0–34)	16.9±8.4, (0–30)	n/a
Motor	11.1±5.9, (0–19)	11.1±5, (0–19)	
Vocal	6.2±6.5, (0–18)	5.8±4.9, (0–12)	
OCD	15	4	0
ADHD			
Inattentive	7	8	0
Hyperactive/impulsive	4	1	0
Combined	4	3	0
ASO titer $m\pm$SD[a]	133±112	125±148	61±18
Number exceeding ULN[b]	4	8	0
AntiDNase B titer[a]	358±369	350±374	181±130
Number exceeding ULN[b]	4	6	3

YGTSS, Yale Global Tic Severity Score; TTS, Total Tic Score from YGTSS; OCD, Obsessive Compulsive Disorder; ADHD, Attention Deficit Hyperactivity Disorder; M, male; F, female; ULN, upper limits of normal.

[a] Antistreptococcal data available on 29/30 PANDAS, 30/30 TS, and 9/30 controls.

[b] For individuals aging 6–12 years, defined by Kaplan et al., 1998[21], for individuals 13 years and older, a value of 200 was used to determine ULN.

3. Results

3.1. IgG reactivity to striatal tissue

Positive IgG IF reactivity to striatal tissue was observed in control ($n=7$), TS ($n=12$), and PANDAS ($n=11$) groups (see Fig. 1 and Table 2). A three-group chi-square analysis showed no statistical difference.

3.2. Colocalization with glial and neuronal markers

Positive reactivity to striatal tissue colocalizing with the glial marker (GFAP) was identified in sera from children with PANDAS ($n=6$), TS ($n=7$), and controls ($n=4$) (see Fig. 1 and Table 2). A three-group chi-square analysis showed no significance (degrees of freedom=2). Reactivity colocalizing to the neuronal marker (MAP2) was identified