Antibody binding to neuronal surface in Sydenham chorea, but not in PANDAS or Tourette syndrome

F. Brilot, PhD
V. Merheb, BSc
A. Ding, BSc
T. Murphy, MD
R.C. Dale, PhD

Address correspondence and reprint requests to Dr Fabienne Brilot, Neuroimmunology Group, Institute for Neurosciences and Muscle Research, The Kids Research Institute at the Children's Hospital at Westmead, University of Sydney, Australia
fabienb@chw.edu.au

ABSTRACT

Objective: To test the hypothesis that Sydenham chorea (SC) immunoglobulin G (IgG) autoantibodies bind to specific neuronal surface proteins, whereas IgG from patients with pediatric autoimmune neuropsychiatric disorders associated with streptococcal infection (PANDAS) or Tourette syndrome (TS) do not bind to neuronal surface proteins.

Methods: We used live differentiated SH-SY5Y cells, which have neuronal and dopaminergic characteristics. Using flow cytometry, we measured serum IgG cell surface binding in patients with SC (n = 11), PANDAS (n = 12), and TS (n = 11), and compared the findings to healthy controls (n = 11) and other neurologic controls (n = 11). In order to determine the specificity of binding to neuronal antigens, we also used a non-neuronal cell line, HEK 293.

Results: The mean IgG cell surface binding was significantly higher in the SC group compared to all other groups ($p < 0.001$). By contrast, there was no difference between the PANDAS or TS groups and the controls. Using the non-neuronal HEK-293 cells, there was no significant difference in IgG cell surface binding between any groups.

Conclusions: Serum autoantibodies that bind to neuronal cell surface antigens are present in SC, but not in PANDAS or TS. These findings strengthen the hypothesis that SC is due to a pathogenic autoantibody, but weaken the autoantibody hypothesis in PANDAS and TS. *Neurology*® 2011;76:1508-1513

GLOSSARY

ASO = anti-streptolysin-O; **DSM-IV** = *Diagnostic and Statistical Manual of Mental Disorders*, 4th edition; **HC** = healthy children; **IgG** = immunoglobulin G; **MFI** = mean fluorescence intensity; **OCD** = obsessive-compulsive disorder; **OND** = other neurologic disease; **PANDAS** = pediatric autoimmune neuropsychiatric disorders associated with streptococcal infection; **PMA** = phorbol-12-myristate 13-acetate; **RA** = retinoic acid; **SC** = Sydenham chorea; **TH** = tyrosine hydroxylase; **TS** = Tourette syndrome.

Sydenham chorea (SC) is the principal neurologic manifestation of acute rheumatic fever, a poststreptococcal autoimmune disorder. SC is characterized by chorea and neuropsychiatric features such as obsessive-compulsive disorder (OCD). Reports of the presence of serum autoantibodies and the therapeutic benefit of plasma exchange suggest that SC may be due to a pathogenic antibody response.¹⁻⁴ Additionally, an outbreak of streptococcal pharyngitis associated with tic disorders led to the hypothesis that some cases of Tourette syndrome (TS) and OCD are precipitated by streptococcus infections. This led to the term pediatric autoimmune neuropsychiatric disorders associated with streptococcal infection (PANDAS).⁵

Autoantibody detection and the identification of their brain antigens in SC and PANDAS has been the object of scrutiny for some time.^{1,6-9} Most studies in SC using immunohistochemistry and Western blotting have reported elevated antibody binding.^{1,4,7} However, in PANDAS and TS, there have been inconsistent results with positive^{4,10,11} and negative findings.^{9,12}

It is increasingly recognized that pathogenic autoantibodies typically bind to cell surface neuronal antigens that are involved in cell function.¹³⁻¹⁵ Therefore, to define pathogenic anti-

Supplemental data at
www.neurology.org

e-Pub ahead of print on March 16, 2011, at www.neurology.org.

From the Neuroimmunology Group (F.B., V.M., A.D., R.C.D.), Institute for Neuroscience and Muscle Research, The Kids Research Institute at the Children's Hospital at Westmead, University of Sydney, Australia; and Departments of Pediatrics and Psychiatry (T.M.), University of South Florida, Tampa.

Study funding: Supported by the American Tourette Syndrome Association. R.D. and F.B. received funding from the University of Sydney postdoctoral fellowship scheme.

Disclosure: Author disclosures are provided at the end of the article.

Table Clinical characteristics of patients with Sydenham chorea, PANDAS, and Tourette syndrome

Disorder	Sydenham chorea (n = 11)	PANDAS (n = 12)	Tourette syndrome (n = 11)
Male:female	2:9	10:2	9:2
Mean age, y (range)	12 (4–15)	9 (6–15)	10 (6–15)
Movement disorder	Chorea (11/11)	Motor and vocal tics (12/12)	Motor and vocal tics (11/11)
Movement disorder onset	Subacute (11/11)	Abrupt and dramatic (10/12) ^a	Gradual onset (11/11)
Course of disease	Monophasic (11/11)	Relapsing-remitting (7/12) ^a ; complete remission between exacerbations (5/12)	Waxing and waning but no remission (11/11)
Psychiatry	Obsessive-compulsive behavior (3/11); emotional lability (5/11)	Acute onset emotional change (8/12) ^a ; obsessive-compulsive disorder (5/12); separation anxiety (5/12); emotional lability (7/12)	Obsessive-compulsive disorder (4/11); attention-deficit disorder (4/11); depression (1/11)
Associated symptoms	Dysarthria (7/11); carditis (5/11)	Motoric hyperactivity (5/12) ^a ; altered concentration or learning (5/12) ^a ; abrupt personality change (7/12) ^a ; nighttime fears/sleep disorder (5/12) ^a ; frequent urination (4/12)	Mild learning difficulties (1/11)
Evidence of streptococcal infection at time of serum study	Positive ASOT ^b (11/11); positive throat culture (3/11)	Positive ASOT ^b (12/12); positive throat culture (2/12)	Positive ASOT ^b (5/11); positive throat culture (0/12)

Abbreviations: ASOT = anti-streptolysin-O titer; PANDAS = pediatric autoimmune neuropsychiatric disorders associated with streptococcal infection.

^a Feature association with postinfectious exacerbation. Clinical characteristics of patients with PANDAS as previously described and published.⁵

^b Positive ASOT defined as >240 IU/mL.¹⁶

bodies, it is necessary to use live cells expressing antigens in their native conformational state. We used a live neuronal cell line and FACS to compare cell surface IgG binding between children with SC, PANDAS, TS, and controls. We showed that cell surface IgG binding is present in patients with SC, but not in PANDAS or TS.

METHODS **Patients and controls.** The following patients and controls were recruited between 2000 and 2009.

SC (n = 11). All patients fulfilled criteria for SC and had positive streptococcal serology (table). Serum samples were taken acutely during active chorea, and before the use of any immune therapy.

PANDAS (n = 12). Between 2000 and 2009, over 300 patients with TS were seen by R.C.D. Among these patients, 12 patients who best fulfilled the criteria for PANDAS were recruited for this study.⁵ Serum samples were taken during acute exacerbations of their tic disorders which were associated with a clinical pharyngeal infection plus evidence of streptococcal infection.⁵ The onset of disease, clinical course, and clinical characteristics are described in the table. The main differentiating feature of PANDAS was the abrupt and dramatic onset of symptoms associated with streptococcal infection (present in 10 of 12 patients) or the relapsing-remitting course associated with streptococcal infections (present in 7 of 12 patients) (table). Although all 12 patients with PANDAS had one or more clinical exacerbations associated with streptococcal infections, only 7 had multiple exacerbations and a relapsing-remitting course as previously described.⁵ All patients with PANDAS had elevated acute anti-streptolysin-O (ASO) titers >240 IU/mL (mean 960, range 400–2,200).¹⁶ All convalescent ASO titers were reduced during remission. The 7 patients with a relapsing-remitting course had further elevation of ASO titer associated with clinical relapse.

TS (n = 11). All patients with TS fulfilled *DSM-IV* criteria. All had active tic disorders at the time of serum sampling, but none had the PANDAS phenotype. The 11 patients were otherwise selected randomly from approximately 300 patients seen in the tic clinic by R.C.D. during this time period.

Healthy children (HC, n = 11, 6 male, mean age 11 years, range 9–13 years). These children were healthy with no infectious or neurologic disorders.

Other neurologic diseases (OND, n = 11, 6 male, mean age 6.0 years, range 2–15 years). These children had noninflammatory neurologic disorders including epilepsy and developmental syndromes.

All serum samples had IgG concentration measured by nephelometry (BN ProSpec, Siemens), and IgG values were within the normal range (6.2–14.4 g/L). The IgG mean and range did not significantly differ between any patient and control groups.

Standard protocol approvals, registrations, and patient consents. Ethics approval for this study was granted by the Children's Hospital at Westmead ethics committees (HREC 2007/035 and SSA 07/CHW/58), and written informed consent from all patients was obtained.

Cell-based assay for detection of IgG binding to cell surface neuronal antigens. FACS was recently used to demonstrate IgG binding to cell surface antigens in CNS immune-mediated diseases.^{13,17} We used FACS analysis to detect antibody binding of patient serum IgG to the surface of differentiated human SH-SY5Y cells and to HEK293 as a non-neuronal control cell line. SH-SY5Y cells were differentiated with 10 μ M retinoic acid (RA) for 3 days (Sigma-Aldrich), or 10 μ M RA for 3 days followed by 80 nM phorbol-12-myristate 13-acetate (PMA) (Sigma-Aldrich) for 3 days.¹⁸ The cells were then harvested using versene (Invitrogen), washed in PBS supplemented with 2% FBS (PBS/FBS), and then resuspended at a density of 100,000 cells/mL. A total of 50,000 of SH-SY5Y/RA and HEK293 cells were incubated with serum at a 1:100 dilution in V-bottom plate (Corning) for 1 hour at RT. Cells were then

Figure 1 Differentiation of SH-SY5Y cells with retinoic acid (RA)

Treatment of cells with 10 μ M RA for 3 days increased formation of neurite-like extensions (A) and expression of basal ganglia marker tyrosine hydroxylase (TH) (B). Representative data from at least 3 different experiments are shown.

washed 3 times with 200 μ L PBS/FBS and incubated with Alexa Fluor 488-conjugated goat antihuman IgG secondary antibody (Invitrogen) for 1 hour at RT. Cells were washed 3 times with PBS/FBS and then resuspended in 50 μ L PBS/FBS before analysis. Before acquisition, viability dye 7-AAD (BD Biosciences) was added to the cells to exclude dead cells. In addition, differentiated SH-SY5Y cells were also fixed and permeabilized with 1% PFA and 0.1% TritonX for 10 minutes at RT to analyze the binding of patient sera to intracellular antigens. In this case, cells were washed in PBS/FBS with 0.02% saponin. A total of 10,000 events/well were recorded on a BS LSRII instrument equipped with a high-throughput sampler (BD Biosciences). Data analysis was performed using Flow Jo software and Excel (Microsoft). Binding was expressed as mean fluorescence intensity (MFI). An MFI greater than mean + 3 SD of the control samples was considered positive. Each experiment was performed 3 times.

Statistical analysis. The Mann-Whitney *U* test was used to compare antibody titer between patients and controls.

RESULTS SH-SY5Y cells were differentiated by treatment with RA into neuron-like cells as shown by upregulation of neuronal marker tyrosine hydroxylase (TH) and outgrowth of neurites (figure 1, A and B). Flow cytometry allowed quantification of IgG cell surface binding. We used a cutoff of the healthy control mean + 3 SD (MFI 41.6) (figure 2A). Using this cutoff, 6/11 children with SC had elevated IgG binding, compared to 0/12 PANDAS, 0/11 TS, 0/11 HC, and 0/11 OND controls. The mean MFI in the SC group (48.44 ± 21.4) was significantly elevated compared to the PANDAS group (18.1 ± 5.3), TS group (16.62 ± 4.21), HC (22.27 ± 6.5), and OND controls (19.2 ± 4.6) ($p < 0.001$). The mean

MFI of PANDAS and TS groups were not different from the 2 control groups. RA followed by PMA treatment produces the same degree of neurite outgrowth and TH upregulation, and gave similar IgG cell surface binding results compared to RA differentiation alone (figure e-1 on the *Neurology*[®] Web site at www.neurology.org, $p < 0.001$).

When we fixed and permeabilized RA-differentiated cells, there was no difference in results obtained; the only group to show any significant binding was the SC group with 6/11 sera above threshold (mean SC MFI 105.9 ± 70.2 , $p < 0.05$, figure 2B). Interestingly, the 6 patients above the threshold (healthy control mean + 3 SD, MFI 78.3) were identical in experiments on live (figure 2A) and fixed and permeabilized cells (figure 2B). There was no correlation of total serum IgG levels with neuronal binding in any experiment.

We performed the same experiments using non-neuronal HEK293 cells. No serum from any groups showed positive binding above threshold, or elevated mean MFI (figure 2C), suggesting that the IgG surface binding in SC samples was directed to a neuronal-specific cell surface antigen.

DISCUSSION Our study used a quantitative method to demonstrate elevated IgG cell surface binding in SC. Previous studies have shown IgG binding to live neurons using immunohistochemistry, and have defined lysoganglioside as an important and potentially pathogenic antigen assuming a mo-

Figure 2 Immunoglobulin G (IgG) binding in patients and controls

IgG binding of healthy controls (HC), other neurologic controls (ONC), Sydenham chorea (SC), pediatric autoimmune neuropsychiatric disorders associated with streptococcal infection (PANDAS), and Tourette syndrome (TS) to retinoic acid (RA)-differentiated live (A) and RA-differentiated fixed/permeabilized (B) neuroblastoma SH-SY5Y, or non-neuronal control live HEK 293 cells (C). Binding to cells was determined by flow cytometry. Representative histograms and data from at least 3 different experiments are shown. Binding is expressed as mean fluorescence intensity (MFI); threshold was defined as mean of HC + 3 SD. $p < 0.001$ and $p < 0.05$ vs control group.

lecular mimicry hypothesis.³⁻⁵ Our article strengthens the hypothesis that patients with SC have IgG binding to neuronal specific cell surface antigens. We found that differentiation of the SY-SH5Y cells asso-

ciated with increased dopaminergic characteristics increased the IgG cell surface binding. In addition, permeabilizing the cells and revealing intracellular antigens did not alter the results. Indeed, the same

samples were positive for IgG binding, suggesting that the important antigen in SC is expressed on the cell surface. In this study, we have not proposed which antigen is involved in IgG cell surface binding. Previously proposed antigens in SC include lysoganglioside, tubulin, and glycoytic enzymes,^{6,8,19} although it is possible that the dominant pathogenic antibody is yet to be defined.

We aimed to determine whether patients with PANDAS or TS had similar cell surface IgG binding. The patients with PANDAS were recruited from a clinical service for children with TS. The patients in the PANDAS group were selected as they all had one or more exacerbations associated with streptococcal infections, although only 7 had multiple relapses associated with streptococcal infections and a relapsing-remitting course.⁵ All serum samples were taken during postinfectious clinical deteriorations associated with positive streptococcal serology. There was no evidence of elevated cell surface IgG binding in any patient with PANDAS or TS. These findings suggest that the antibody repertoire in PANDAS and TS is different from that of SC.

It is possible that the sensitivity of our assay is too low to demonstrate IgG cell surface binding in PANDAS or TS. It is also likely that the differentiated SH-SY5Y cells do not express all potential surface and intracellular epitopes found in the brain. Our findings contradict previous reports that have shown elevated lysoganglioside IgG binding in PANDAS and SC compared to controls.⁴ Alternatively, it is possible that different immune processes other than autoantibody production are important in the clinical fluctuations of PANDAS and TS, as recently described.^{20,21}

Using a quantitative method we have strengthened the hypothesis that SC is mediated by an autoantibody that binds to neuronal cell surface antigens. By contrast, we have failed to demonstrate a similar finding in PANDAS and TS, therefore weakening the autoantibody hypothesis in these conditions.

DISCLOSURE

Dr. Brilot has received research support from the Tourette Syndrome Association, USA, the Brain Foundation Australia, and the Trish Multiple Sclerosis Foundation Australia. V. Merheb and A. Ding report no disclosures. Dr. Murphy has received research support from the Tourette Syndrome Association, USA; serves on scientific advisory boards for the Tourette Syndrome Association and ThinkPANDAS; receives publishing royalties for *Handbook of Child and Adolescent Obsessive-Compulsive Disorder* (Lawrence Erlbaum, Inc., 2007); and receives research support from Otsuka Pharmaceutical Co., Ltd., Endo Pharmaceuticals (Indevus), Forest Laboratories, Inc., Janssen, Shire plc, the NIH, the CDC, the Tourette Syndrome Association, the National Alliance for Research on Schizophrenia and Affective Disorders, and the Robidoux Foundation. Dr. Dale serves on a scientific advisory board for the Brisbane Children's Hospital; may receive publishing royalties for *Autoimmune and Inflammatory Disorders of the Nervous System in Children* (MacKeith Press, 2009); has received speaker honoraria from Biogen Idec; and has received research support from the Tourette Syndrome Association, USA, the Brain Founda-

tion Australia, and the Trish Multiple Sclerosis Foundation Australia.

Received July 25, 2010. Accepted in final form November 23, 2010.

REFERENCES

- Church AJ, Cardoso F, Dale RC, Lees AJ, Thompson EJ, Giovannoni G. Anti-basal ganglia antibodies in acute and persistent Sydenham's chorea. *Neurology* 2002;59:227–231.
- Garvey MA, Snider LA, Leitman SF, Werden R, Swedo SE. Treatment of Sydenham's chorea with intravenous immunoglobulin, plasma exchange, or prednisone. *J Child Neurol* 2005;20:424–429.
- Kirvan CA, Swedo SE, Kurahara D, Cunningham MW. Streptococcal mimicry and antibody-mediated cell signaling in the pathogenesis of Sydenham's chorea. *Autoimmunity* 2006;39:21–29.
- Kirvan CA, Swedo SE, Snider LA, Cunningham MW. Antibody-mediated neuronal cell signaling in behavior and movement disorders. *J Neuroimmunol* 2006;179:173–179.
- Swedo SE, Leonard HL, Garvey M, et al. Pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections: clinical description of the first 50 cases. *Am J Psychiatry* 1998;155:264–271.
- Dale RC, Candler PM, Church AJ, Wait R, Pocock JM, Giovannoni G. Neuronal surface glycolytic enzymes are autoantigen targets in post-streptococcal autoimmune CNS disease. *J Neuroimmunol* 2006;172:187–197.
- Husby G, van de Rijn I, Zabriskie JB, Abidin ZH, Williams RC Jr. Antibodies reacting with cytoplasm of subthalamic and caudate nuclei neurons in chorea and acute rheumatic fever. *J Exp Med* 1976;144:1094–1110.
- Kirvan CA, Swedo SE, Heuser JS, Cunningham MW. Mimicry and autoantibody-mediated neuronal cell signaling in Sydenham chorea. *Nat Med* 2003;9:914–920.
- Singer HS, Hong JJ, Yoon DY, Williams PN. Serum autoantibodies do not differentiate PANDAS and Tourette syndrome from controls. *Neurology* 2005;65:1701–1707.
- Church AJ, Dale RC, Lees AJ, Giovannoni G, Robertson MM. Tourette's syndrome: a cross sectional study to examine the PANDAS hypothesis. *J Neurol Neurosurg Psychiatry* 2003;74:602–607.
- Pavone P, Parano E, Rizzo R, Trifiletti RR. Autoimmune neuropsychiatric disorders associated with streptococcal infection: Sydenham chorea, PANDAS, and PANDAS variants. *J Child Neurol* 2006;21:727–736.
- Morris CM, Pardo-Villamizar C, Gause CD, Singer HS. Serum autoantibodies measured by immunofluorescence confirm a failure to differentiate PANDAS and Tourette syndrome from controls. *J Neurol Sci* 2009;276:45–48.
- Blaes F, Fuhlhuber V, Korfei M, et al. Surface-binding autoantibodies to cerebellar neurons in opsoclonus syndrome. *Ann Neurol* 2005;58:313–317.
- Graus F, Saiz A, Dalmau J. Antibodies and neuronal autoimmune disorders of the CNS. *J Neurol* 2010;257:509–517.
- Graus F, Saiz A, Lai M, et al. Neuronal surface antigen antibodies in limbic encephalitis: clinical-immunologic associations. *Neurology* 2008;71:930–936.
- Kaplan EL, Rothermel CD, Johnson DR. Antistreptolysin O and anti-deoxyribonuclease B titers: normal values for

- children ages 2 to 12 in the United States. *Pediatrics* 1998; 101:86–88.
17. Brilot F, Dale RC, Selter RC, et al. Antibodies to native myelin oligodendrocyte glycoprotein in children with inflammatory demyelinating central nervous system disease. *Ann Neurol* 2009;66:833–842.
 18. Presgraves SP, Ahmed T, Borwege S, Joyce JN. Terminally differentiated SH-SY5Y cells provide a model system for studying neuroprotective effects of dopamine agonists. *Neurotox Res* 2004;5:579–598.
 19. Kirvan CA, Cox CJ, Swedo SE, Cunningham MW. Tubulin is a neuronal target of autoantibodies in Sydenham's chorea. *J Immunol* 2007;178:7412–7421.
 20. Kawikova I, Grady BP, Tobiasova Z, et al. Children with Tourette's syndrome may suffer immunoglobulin A dysgammaglobulinemia: preliminary report. *Biol Psychiatry* 2010;67:679–683.
 21. Martino D, Dale RC, Gilbert DL, Giovannoni G, Leckman JF. Immunopathogenic mechanisms in Tourette syndrome: a critical review. *Mov Disord* 2009;24:1267–1279.

***Neurology*[®] Launches Subspecialty Alerts by E-mail!**

Customize your online journal experience by signing up for e-mail alerts related to your subspecialty or area of interest. Access this free service by visiting <http://www.neurology.org/cgi/alerts/etoc.xhtml> or click on the “E-mail Alerts” link on the home page. An extensive list of subspecialties, methods, and study design choices will be available for you to choose from—allowing you priority alerts to cutting-edge research in your field!

Guideline Examines Effective Treatments for Painful Diabetic Neuropathy

The AAN has published “Treatment of Painful Diabetic Neuropathy,” an evidence-based guideline that identifies the most effective treatments for pain from nerve damage in people with diabetes. The guideline was published in the April 11, 2011, online issue of *Neurology*[®]. To access the guideline, summaries for clinicians and patients, and tools, visit www.aan.com/gol/practice/guidelines. For information on an audio conference explaining the guideline on Thursday, May 12, 2011, at 12:00 p.m. ET, visit www.aan.com/view/audiocon. The audio conference is only \$25 for AAN members; \$99 for the public; and free for students, residents, and fellows.