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Identification of Pyruvate Kinase as an Antigen Associated with Tourette Syndrome

Janice W. Kansy^{a,*}, Liliya Katsoyich^b, Kevin S. McIver^c, Jennifer Pick^a, John B. Zabriskie^d, Paul J. Lombroso^b, James F. Leckman^b, and James A. Bibb^a

^a Department of Psychiatry, The University of Texas Southwestern Medical Center, Dallas, Texas, United States of America

^b Child Study Center, Yale University, School of Medicine, New Haven, Connecticut, United States of America

^c The Department of Microbiology, University of Texas Southwestern Medical Center, Dallas, Texas, United States of America

^d Laboratory of Clinical Microbiology and Immunology, The Rockefeller University, New York, New York, United States of America

Abstract

Immune responses to β -hemolytic streptococcal infections are hypothesized to trigger tic disorders and early-onset obsessive-compulsive disorder (OCD) in some pediatric populations. Here we identify the M1 isoform of the glycolytic enzyme, pyruvate kinase (PK) as an autoimmune target in Tourette syndrome and associated disorders. Antibodies to PK reacted strongly with surface antigens of infectious strains of streptococcus, and antibodies to streptococcal M proteins reacted with PK. Moreover, immunoreactivity to PK in patients with exacerbated symptoms who had recently acquired a streptococcal infection was 7-fold higher compared to patients with exacerbated symptoms and no evidence of a streptococcal infection. These data suggest that PK can function as an autoimmune target and that this immunoreactivity may be associated with Tourette syndrome, OCD, and associated disorders.

Keywords

autoimmunity; group A β -hemolytic streptococci; obsessive-compulsive disorder; PANDAS; Tourette syndrome; pyruvate kinase

1. Introduction

Tic disorders, obsessive-compulsive disorder (OCD), and related conditions affect as many as 3% of children and adolescents (Costello et al., 1996; Flament et al., 1988; Kadesjo and

*Corresponding author. Department of Psychiatry, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd, Dallas, TX 75390, United States. Tel.: +1 214 648 4171; fax: +1 214 648 1293. E-mail address: janice.kansy@utsouthwestern.edu (J. W. Kansy).

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Gillberg, 2000; Leckman, 2002; Mason et al., 1998; Valleng-Basile et al., 1994). The factors that contribute to the pathogenesis of these disorders are poorly defined. The hypothesis that infections can modulate the clinical appearance of tic disorders dates from the 1800s (Kushner, 1999). The past decade has seen the reemergence of the hypothesis that post-infectious immune mechanisms account for at least some cases of Tourette syndrome (TS) and OCD.

It is well known that group A β -hemolytic streptococci (GABHS) can trigger immune-mediated diseases (Bisno, 2000; Carapetis et al., 1999; Stollerman, 1997). Rheumatic fever (RF), one of the most well recognized examples of a delayed non-suppurative complication of GABHS infection, usually occurs a few weeks to several months after streptococcal infection among susceptible persons. RF typically involves the heart, joints, and central nervous system. The central nervous system manifestations usually take the form of chorea (Sydenham's chorea). However, some patients with RF also display motor or phonic tics, obsessive-compulsive (OC) symptoms, or features suggesting attention-deficit/hyperactivity disorder (ADHD) (Allen et al., 1995; Mercadante et al., 1997; Swedo et al., 1989). On the basis of these associations, Swedo et al. (Swedo et al., 1998) proposed that pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections (PANDAS) represent a distinct clinical entity that includes cases of TS and OCD. This has proved to be a controversial hypothesis (Kurlan and Kaplan, 2004). For example, one study using an administrative regional database of more than 500,000 privately insured individuals linked recent GABHS infections with an increased risk of tic disorders and OCD (Mell et al., 2005). Another study reported that over 90% of TS patients that tested positive for anti-basal ganglia antibodies had serological evidence of a recent streptococcal infection (Church et al., 2003), and two other studies showed that TS patients' sera possessed elevated streptococcal antibody titers (Müller et al., 2001; Müller et al., 2000). However, these results have not been confirmed by other reports (Loiselle et al., 2003; Luo et al., 2004; Morshed et al., 2001; Singer et al., 1998).

It has also been reported that patients with tic disorders and/or OCD have responded positively to antibiotic treatment, antibiotic prophylaxis, and plasma exchange treatment (Mell et al., 2005; Perlmutter et al., 1999; Perrin et al., 2004; Snider et al., 2005). Some children and adults with TS have significantly increased titers of antineural antibodies (Kiessling et al., 1993; Morshed et al., 2001; Singer et al., 1998), and IgG from children with TS bound neurons in the caudate nucleus of human postmortem brains (Singer et al., 1998). A number of proteins of varying size have been identified as possible antigenic target proteins in the sera of tic disorder and early-onset OCD patients. Two studies have identified a protein of 60 kDa as a possible target (Church et al., 2003; Hoekstra et al., 2003), while a third study showed numerous proteins with different molecular weights as contributing to changes in TS antibody repertoires (Wendlandt et al., 2001). Most recently three neuronal glycolytic proteins, pyruvate kinase, aldolase and enolase have been suggested to be potential autoantigens in a group of patients with post-streptococcal movement and psychiatric disorders (Dale et al., 2006).

Following a series of preliminary studies characterizing the antibodies found in pediatric patients with tic disorders and early-onset OCD, we identified the M1 isoform of the glycolytic enzyme, pyruvate kinase (PK) as a potential target. Anti-PK antibodies reacted with GABHS surface antigens, and antigenic M protein antibodies reacted with PK. These findings suggest that PK may serve as an autoimmune antigen associated with streptococcal infections in patients with Tourette syndrome and associated disorders. To test this hypothesis we screened four sets of patients' sera: (1) symptom exacerbations plus a recently acquired GABHS infection, (2) symptom exacerbations and no GABHS infection, (3) no symptom exacerbations but a recently acquired GABHS infection and (4) no symptom exacerbations and no GABHS infection. Age-matched controls with and without recent GABHS infections were also screened. The results of these studies indicate that GABHS infections in a substantial subset

of pediatric patients with a predisposition to tics and OCD symptoms result in the production of antibodies that react with PK.

2. Materials and methods

2.1. Patients

Specimens used in this study were collected from children, aged 7–17 years, with a chronic tic disorder, OCD, or both and healthy children without these disorders who participated in a prospective longitudinal study. A total of 77 children (46 cases and 31 controls) were assessed at baseline and followed prospectively for periods ranging from 4 to 24 months (Leckman et al., 2005; Luo et al., 2004). All patients were followed at the Yale Child Study Center Tic Disorder-Obsessive-Compulsive Disorder Specialty Clinic. Expert clinicians using DSM-IV criteria made all psychiatric diagnoses based on all available information. Exclusion criteria included an intelligence quotient of <75 ; serious medical illness; major sensory handicaps (e.g., blindness, deafness); major neurologic disease (including a seizure disorder); head trauma resulting in loss of consciousness; current (past 6 months) psychiatric disorder that could interfere with participation, such as major depression; psychosis; and autism or another pervasive developmental disorder. All parents provided written informed consent after the study was described to them in detail. A separate assent form was used to ensure the informed participation of the child and adolescent subjects.

When a family entered the study, information concerning the patient was collected in a two-stage process, as previously described (Findley et al., 2003; Leckman et al., 2005; Lin et al., 2002; Luo et al., 2004). The first stage consisted of the collection of information concerning symptoms associated with TS and OCD according to a self-and-family report (Robertson et al., 1999) based on the tic inventory, ordinal severity scales of the Yale Global Tic Severity Scale (YGTSS) (Leckman et al., 1989), and the symptom checklist and ordinal scales of the Yale-Brown Obsessive Compulsive Scale (Y-BOCS) (Goodman et al., 1989; Scahill et al., 1997). The semi-structured interview used to assess and diagnose PANDAS cases was based on that used by the Pediatrics and Developmental Neuropsychiatry Branch of the National Institute of Mental Health Intramural Program (Swedo et al., 1989). In a second stage of assessment, an experienced clinician reviewed these symptom ratings with the child and the parent to ensure their accuracy and validity. Comorbid psychiatric diagnoses were made with all available information, including data collected with the Schedule for Affective Disorders and Schizophrenia for School-Age Children (Kaufman et al., 1997). The Human Investigation Committee at Yale University School of Medicine approved each of these studies, and all parents provided informed consent.

2.2. Ratings of symptom severity

Tic symptom severity was rated with the tic portion of the YGTSS (YGTSS_{TIC}). Obsessive-compulsive symptom severity was rated with the Children's Yale-Brown Obsessive Compulsive Scale (CY-BOCS). Exacerbation criteria were previously described (Lin et al., 2002). In the case of tic symptoms, exacerbations were identified when the current monthly YGTSS rating exceeded the previous monthly rating by 9 points (Δ YGTSS_{TIC} score > 9) and the current YGTSS_{TIC} score exceeded 19. For OC symptoms, exacerbations were identified when the current monthly CY-BOCS rating exceeded the previous monthly rating by 7 points (Δ CY-BOCS score > 7) and the current CY-BOCS score exceeded 16. Finally, when considering the summed total of tic and OC symptoms, we identified as exacerbation points where the Δ score (YGTSS_{TIC} + CY-BOCS) was greater than 14 and the current score (YGTSS_{TIC} + CY-BOCS) exceeded 33.

2.3. Sera analyses

This study was part of a larger, ongoing, 24-month prospective longitudinal study. In addition to the monthly clinical assessments, serum specimens were collected at regular 4-month intervals. If an exacerbation of tic or OC symptoms was detected, two additional assessments were performed (one as soon as possible after the detection of the exacerbation and another 2 months later). Each of these assessments also included a collection of serum.

Blood samples obtained from patient and control subjects were analyzed for several markers related to GABHS infection and/or neuropsychiatric disorders. Serum or plasma was collected by centrifugation and stored in aliquots at -80°C .

Plasma or serum obtained from each blood sample was assessed for anti-streptolysin O (ASO) and anti-DNase B (ADB) titers with standard methods (Johnson et al., 1996), and values were expressed as Todd units and standard units, respectively. A newly acquired GABHS infection was defined according standard criteria. The criteria required two-tenths of a $\log_{10}$ increase in either the ASO or ADB titer between 2 consecutive blood samples (Kaplan et al., 1998; Kaplan et al., 1971). This value corresponds to a 1.6-fold increase in titer. Titers of <50 units were treated as 50 ($\log_{10} = 1.70$). ASO and ADB titers ranged from 30 to 400 and 40 to 960, respectively.

2.4. Screening of patients' sera

Two sets of patients' sera were used in this study, the first, a subset of presumptive four PANDAS TS cases, and four age-matched controls was used to screen tissue lysates from rat brain cortex, hippocampus, cerebellum, brainstem, striatum, and heart muscle by immunoblot analysis (Fig. 1). For each patient, two separate serum samples were compared, one collected prior to and one collected during an exacerbated episode of tics. The interval between the pre-exacerbation visit and the exacerbation visit was $2.5 (\pm 0.4)$ months (standard deviation). This subset was used for the identification of PK as a potential antigenic target.

Once PK was identified, we screened a second set of sera from patients and age-matched controls. These patients were either positive or negative for exacerbated symptoms and either did or did not have a recently acquired GABHS infection. We also screened patients' sera obtained during exacerbation of tic, or tic and OC symptoms.

2.5. Reagents

PK from rabbit muscle was purchased from ICN Biochemicals, Inc. (Costa Mesa, CA, United States). Goat anti-pyruvate kinase antibody was from Chemicon International (Temecula, CA, United States). Mouse anti-dopamine- and cyclic AMP-regulated phosphoprotein, relative molecular mass 32,000 (DARPP-32) antibody has previously been characterized (Bibb et al., 2000). The secondary antibodies (rabbit anti-goat, goat anti-human and goat anti-rabbit peroxidase-conjugated IgGs) were from Pierce Biotechnology (Rockford, IL, United States). Normal goat serum was purchased from Vector Laboratories, Inc. (Burlingame, CA). M5, M6 and M24 streptococcal antibodies have previously been described (Bronze and Dale, 1993) and were provided by Dr. James B. Dale, University of Tennessee, Memphis, TN, United States. Complete EDTA-free protease inhibitor cocktail was purchased from Roche (Indianapolis, IN, United States). Enhanced chemiluminescence (ECL) immunoblot detection reagents were from Amersham Biosciences (Piscataway, NJ, United States).

2.6. Immunoblot analysis

Rat brain and peripheral tissues were rapidly dissected, homogenized by sonication, and boiled in 1% sodium dodecyl sulfate (SDS), 50 mM NaF. Samples were resolved on 10% polyacrylamide SDS gels after quantitation using a BCA protein reagent assay kit (Pierce

Biotechnology, Rockford, IL, United States) with bovine serum albumin as a standard. Immunoblot analyses were conducted following electrophoretic transfer of the proteins to nitrocellulose membranes (0.2 μ m) (Whatman). Membranes for immunoblotting with human sera (diluted 1:200) were incubated overnight to reduce background. TS patients' sera were preabsorbed with PK by incubation of the sera (diluted 1:200 in 10 ml final volume) with 3 ng purified PK at 4°C for two hours; the sera were then used to blot the membranes. Antibodies to PK and the M proteins were used at dilutions of 1:5,000 and 1:200, respectively. Horseradish peroxidase-conjugated secondary antibodies, anti-rabbit, anti-goat and anti-human were used at dilutions of 1:8,000. Immunoreactive bands were visualized using the chemiluminescent reagent, ECL. Results were quantitated by densitometry using NIH Image software on laser-scanned x-ray film exposures that provided optimal linearity of signal intensity. Membranes were re-probed by stripping the primary and secondary antibodies in a solution of 0.06 M Tris-HCl, pH 8.0, 0.2% SDS, 0.1M β -mercaptoethanol for one hour at 60°C. Before use, the membranes were screened ECL to ensure no residual signals were present. Whole cell and extracellular surface streptococcal proteins were isolated as previously described (McIver and Myles, 2002).

2.7. Protein fractionation

Rat striatal tissue (two hemispheres) was rapidly dissected, frozen, then resuspended in 0.5 ml 10 mM HEPES, pH 7.4, 10 mM NaCl, 1 mM KH_2PO_4 , 5 mM NaHCO_3 , 1 mM CaCl_2 , 0.5 mM MgCl_2 (CLB buffer) containing protease inhibitors. Cells were lysed by dounce homogenization and cell debris was removed by centrifugation at 7,000 g for 5 min followed by further centrifugation of the supernatant at 26,500 g for 30 min. The pellet from this centrifugation constituted the membrane fraction. The supernatant (400 μ l at approximately 3 mg/ml) was applied to a Mono Q HR 5/5 FPLC column (Amersham Pharmacia Biotech, Piscataway, NJ). The column was washed with CLB buffer and proteins were eluted from the column with a linear 0.1 to 1.0 M gradient of NaCl. Twenty-five 1.0 ml fractions were collected and aliquots were immunoblotted to detect reactivity with TS patient serum. Animal experiments were performed in accordance with the US National Institutes of Health Guidelines and approved by the Animal Resources Center Committee at UT Southwestern Medical Center.

2.8. Immunohistochemistry

Male C57BL/6 mice were anesthetized with chlorohydrate and quickly perfused through the ascending aorta with 4% paraformaldehyde in phosphate buffered saline for 5 min. Brains were removed and post-fixed in 4% paraformaldehyde at 4°C overnight. Brains were then cryoprotected in 30% sucrose at 4°C overnight followed by freezing at -80°C. Forty-five micrometer thick sagittal slices were obtained using a Leica CM 3050S Cryostat. Sections were slide-mounted then washed twice with phosphate buffered saline followed by a one-hour incubation in 3% normal donkey serum in 0.3% TritonX-100, phosphate buffered saline. Slides were incubated overnight with primary antibodies, goat anti-PK (1:500 dilution) and mouse anti-DARPP-32 (1:5,000 dilution). Slides were washed 3 times with phosphate buffered saline and incubated with secondary antibody (Jackson Laboratories Cy-2 donkey anti-mouse (DARPP-32) and Cy-3 donkey anti-goat (PK)) diluted 1:200 in phosphate buffered saline for two hours then dehydrated and coverslipped. Brain sections were imaged using a confocal Zeiss LSM 510 microscope with objective settings of X20 and X63.

3. Results

3.1. Identification of PK as antigen in TS patients' sera

Sera from four TS patients and four age-matched controls were used to screen tissue lysates from rat brain cortex, hippocampus, cerebellum, brainstem, striatum, and heart muscle by

immunoblot analysis (Fig. 2A). For each patient, two separate serum samples were compared, one collected prior to and one collected during an exacerbated episode of tics. The interval between the pre-exacerbation visit and the exacerbation visit was 2.5 (± 0.4) months (standard deviation). For two of the four TS patients, exacerbation of TS symptoms corresponded to the clear detection of a protein with a molecular weight of approximately 60 kDa as shown for one of these patients in Fig. 2A. The 60 kDa protein was detected in all regions of the brain, with highest levels in the striatum, hippocampus, and cortex. Additionally, this protein was detected in rat heart muscle lysate.

To further characterize this potential autoimmune target, brain tissue was fractionated into membrane and cytosolic components (Fig. 2B). The 60 kDa protein occurred almost exclusively in the soluble fractions of all brain tissues analyzed. The soluble proteins from acutely dissected striatum were further fractionated by anion exchange liquid chromatography and immunoblotted using the same TS exacerbated serum used in Fig. 2 (Fig. 3A). The majority of the 60 kDa protein eluted from the column in fraction 5 and produced a distinct ultraviolet absorbance peak (280 nm) on the corresponding chromatogram. The 60 kDa protein was visible as the major Coomassie blue stained protein in an SDS-PAGE gel (Fig. 3A).

The 60 kDa protein was analyzed by MS/MS mass spectrometry and identified as the M1 isozyme of the glycolytic enzyme, pyruvate kinase (M_R 57,818) (Fig. 4). To confirm the identity of the protein, the membrane from the FPLC fractionation (Fig. 3A) was re-probed with an antibody specific for PK (Fig. 3B). A single 60 kDa protein was detected in the soluble protein extract loaded onto the Mono Q column and in fraction 5, confirming the identity of this protein as PK.

3.2. Tissue Distribution and Immunohistochemical Analyses of PK in Rodent Brain

The distribution and relative abundance of PK in different regions of the brain and peripheral tissue was evaluated by immunoblot analysis of rat tissue lysates using a polyclonal antibody to PK (Fig. 5A). PK was detected in all regions of the brain with the highest levels in the ventral striatum (nucleus accumbens), cortex, and cerebellum. Moderate levels were detected in striatum, brain stem, hippocampus, and hypothalamus, as well as skeletal muscle.

For immunohistochemical analyses, mouse dorsal striatum tissue was co-stained for the striatal-specific protein, DARPP-32 and PK, and images were captured by confocal microscopy (Fig. 4B). Coronal sections of striatal tissue showed intense staining for DARPP-32 (red) in somata (1 and 2), dendrites, axons, and terminals of neurons in the caudate and putamen, in agreement with previous immunohistological and immunocytochemical studies of this protein (Bibb et al., 2001; Bibb et al., 2000; Ouimet et al., 1984). PK (green) colocalized with DARPP-32, showing intense staining in all cell types within the striatum. Less intense staining was also observed in the neuronal tracks (white matter of the corpus callosum).

3.3. Immunoreactivity Between PK and the Streptococcal M Proteins

To assess reactivity between PK and proteins associated with streptococcal bacteria, cytoplasmic or extracellular surface protein extracts were prepared from four clinically isolated infectious strains of GABHS. Streptococcal serotypes are determined by the more than 80 antigenically distinct M proteins displayed on the surface of the bacterium, and the use of genetic probes indicate that there are many more as yet uncharacterized (Cunningham, 2000). Two of the serotypes used in this study included the serotype M18 strain MGAS8232, which was isolated from a rheumatic fever patient (Smoot et al., 2002), and the serotype M3 strain MGAS315, which was isolated from a patient undergoing toxic streptococcal shock (*i.e.*, septicemia) (Musser et al., 1991). The anti-PK antibody exhibited reactivity to each of the extracts with pronounced increase in detection of proteins extracted from the bacterial

extracellular surface (Fig. 6A). Interestingly, the strongest reactivity was detected with the extracellular proteins of the pathogenic strain MGAS315. In agreement with these findings, it has previously been shown that TS patients have increased titers against M12 and M19 proteins (Müller et al., 2001). While PK was not selectively detected, glycolytic enzymes have been shown to either occur on the surface of streptococci or be secreted and contribute to pathogenesis (Fontân et al., 2000). These data demonstrate that there are epitopes on the surface of strains of streptococcus, which are similar to antigenic epitopes of PK. Immediately below the immunoblot is the control blot using normal goat serum in place of the PK antibody. There is very little if any reactivity detected even at film exposure times 10 fold higher than blots with the primary anti-PK antibody.

To further evaluate streptococcal surface antigens for the ability to elicit an immune response to pyruvate kinase, rabbit polyclonal antibodies raised against purified preparations of the M5, M6, and M24 proteins were used to immunoblot PK (Fig. 6B). Antibodies to all three serotype M proteins reacted with PK in a concentration-dependent manner with the antibody to M5 being the most sensitive. For comparison, enolase, another glycolytic protein, was immunoblotted with the antibodies to the M proteins (Fig. 6B). Antibodies to the M proteins showed a much lower level of reactivity to enolase as compared to PK. Identical blots using normal rabbit serum in place of the primary antibody are shown below each panel and labeled Con. No signal was detected in these controls even at film exposure times 30 fold longer than those using the anti-M protein antibodies. To further evaluate reactivity, cytoplasmic lysates from different rat brain regions were immunoblotted with the anti-M protein antibodies. The antibody against M24 (Fig. 6C), as well as those against M5 and M6 (data not shown), detected a 60 kDa protein that corresponded to a protein band of the same molecular weight as detected with an anti-PK antibody. Control blots using normal rabbit and normal goat sera in place of anti-M24 and anti-PK antibodies, respectively, are shown in the lower panel. No signal was detected in these blots at film exposures 7 fold greater than blots with the primary antibodies.

3.4. Screening of Patients' Sera for Reactivity to Pyruvate Kinase

The prevalence of anti-PK antibodies was next assessed by immunoblot analyses in a cohort of patients with TS with or without OCD. Examples of immunoblots of rat striatal cytosolic extract and pure PK with a set of one patient's sera (pre-exacerbation, exacerbation and post-exacerbation) are shown in the first three panels of Fig. 7A. Reactivity to PK was observed in sera obtained during and following an exacerbation of symptoms but only slightly prior to the exacerbation. When the patient's serum (exacerbation) was preabsorbed with PK prior to the immunoblot analysis, the 60 kDa protein bands in the cytosolic extract and in the purified PK sample were greatly reduced in intensity (TS, ex+pk). Additionally, immunoblots probed with anti-PK antibody showed that the 60 kDa bands in the cytosolic extract and purified PK appear in the same relative positions as the bands detected with the patient's serum (Anti-pk).

To further explore the association between streptococcal infections and TS/OCD, sera from patients with streptococcal infections and exacerbations of TS or TS/OCD symptoms were compared to controls for reactivity to PK (Fig. 7B). Sera from TS or TS/OCD patients without streptococcal infections showed no greater reactivity to PK than age-matched controls. However, reactivity to pyruvate kinase increased 6–7 fold when symptoms of TS or TS/OCD were co-morbid with streptococcal infections compared to the other patients' sera. In summary, these data indicate that streptococcal infections result in the production of antibodies that react to PK, and could be the result of an immune response to proteins on the surface of the group A streptococcus that are antigenically similar to PK.

4. Discussion

Since the PANDAS hypothesis (Swedo et al., 1998), several studies have been conducted to evaluate the affects of streptococcal infections upon the etiology of TS and associated disorders (Church et al., 2003; Hoekstra et al., 2003; Singer et al., 1998; Wendlandt et al., 2001). PK has not been previously reported to be involved in the epidemiology of TS. However, in agreement with the findings reported here, serum from PANDAS patients has been reported to selectively recognize proteins in the supernatant fraction of human caudate (Singer et al., 2004). TS patient sera have previously been shown to detect several protein species including a 60 kDa protein in immunoblots of human postmortem brain tissue lysates (Church et al., 2003; Singer et al., 1998; Wendlandt et al., 2001). A 60 kDa protein was similarly recognized by Sydenham's chorea patients' sera (Church et al., 2002) and recently identified as PK (Dale et al., 2006). Antibodies to PK were the most predominant of three glycolytic proteins identified as antigenic targets in the sera of patients with Sydenham's chorea. The other antigenic target proteins were aldolase and enolase. Moreover, the sera of a patient with dementia and striatal hypermetabolism selectively detected a 60 kDa protein (Léger et al., 2004). However, a separate study identified a 60 kDa protein isolated from a neuroblastoma cell line and detected by TS patient sera as the human heat shock protein (hsp60) (Hoekstra et al., 2003). Finally, one study indicated that monoclonal antibodies derived from Sydenham's chorea patients recognized neurolipids and invoked Ca^{2+} signaling cascades (Kirvan et al., 2003).

PK is a key cytosolic regulatory enzyme in the glycolytic pathway and has been implicated in several brain-related metabolic disorders including phenylketonuria and hypertryptophanemia. It is also known that dendritic cells presenting PK M1/M2 isozyme peptide can induce experimental allergic myositis in BALB/c mice (Kawachi et al., 2001). Tissue damage during myocardial ischemia/reperfusion can be reduced by the administration of pyruvate (Bassenge et al., 2000), and pyruvate, being a scavenger of hydrogen peroxide thereby reduces excessive production of free radicals, a process that has been implicated in the pathology of several neurodegenerative diseases including Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis.

It is unclear if immunoreactivity to PK is casually related to TS or serves as a novel sero-indicator of the disorder. Antibodies to neuronal targets have been implicated in the pathology of several neurological disorders including Sydenham's chorea (Kirvan et al., 2003), Guillain-Barre syndrome (Ang et al., 2004) and multiple sclerosis (MS) (Gran et al., 1999; Levin et al., 2002). Furthermore, antibodies to the intracellular heterogeneous nuclear ribonuclear protein-A1 have been shown to cause a neurological disease related to MS (Levin et al., 2002), and a recent study has shown an increase in serum levels of the cytokines IL-12 and TNF α in TS (Leckman et al., 2005).

Future studies are needed to determine the relevance, if any, to alterations in the number of parvalbumin-positive GABAergic interneurons in the cortex and basal ganglia in TS patients (Kalanithi et al., 2005) and the presence of PK in these cell types. Additionally, it has recently been reported that, in a small number of cases, mutations in the SLITRK1 gene may be associated with TS (Abelson et al., 2005). Mutations in this gene result in impaired dendritic neuronal growth in neuronal cultures. It would be of interest to determine if alterations in the expression levels of PK may also be playing a role in these cells. The ability to predict exacerbations of TS/OCD through monitoring antibody titers would allow early medical treatment (Luo et al., 2004; Perrin et al., 2004).

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Fig. 1. Study Design

Initially 4 cases and 4 controls were studied extensively to isolate a candidate protein present on both Group A β -hemolytic streptococci (GABHS) and basal ganglia neurons and recognized by sera obtained from a putative case of PANDAS. Subsequently, four distinct groups of cases and two groups of controls were screened (colored boxes).

Fig. 2. Immunoblot Analysis of Rat Tissue Lysates Probed with Serum from an Individual with a Presumptive Diagnosis of PANDAS

(A) The indicated tissue lysates (100 μ g/lane) were blotted for reactivity to antibodies in the sera of a Tourette Syndrome (TS) patient prior to (TS, pre) and during an exacerbation of symptoms (TS,+ex). The middle right panel shows the same tissue blotted with sera from an age-matched control. The right panel shows the same tissues stained with Coomassie blue. (B) Immunoblot of lysates (100 μ g/lane) from the indicated tissue samples fractionated into cytosolic (cyt) or membrane-associated compartments (mem). The positions of the 60 kDa protein and size standards are indicated.

Fig. 3. Immunoblots Using Sera from an Individual with a Presumptive Diagnosis of PANDAS to Isolate the 60 kDa Protein

(A) FPLC fractionation of rat striatal cytosolic extract by anion exchange chromatography. An immunoblot of eluted fractions using the same TS patient's sera obtained during an exacerbation of symptoms is shown. The arrow denotes the location of the 60 kDa protein. Load represents the sample loaded on the column (100 μ g). The isolated 60 kDa protein from fraction 5 is shown in the Coomassie wet gel (Frac. 5 stained). The chromatogram showing the elution profile from the column is shown in the middle of the figure. (B) The membrane with the FPLC fractions shown in Fig. 3A re-probed using anti-PK antibody.

Fig. 4. HPLC/MS/MS Analysis of the 60 kDa Digested Protein and Amino Acid Sequence of PK
(A) Total ion HPLC spectrum from retention time 14 to 39 min is shown for the 60 kDa protein after digestion with trypsin. The MS spectrum at the retention time of 28.73 min from the mass range 400 to 1500 (m/z) is shown in the lower left and the MS/MS spectrum for the parent ion 820.84 is shown in the right panel. The peptide GVNLPGAAVDLPAVSEK was identified for these data. HPLC/MS was carried out in a LCQ Deca XP (ThermoFinnigan, Palo Alto, CA) coupled with a capillary HPLC system (Agilent 1100 capillary pump, Agilent Technologies, San Jose, CA) and nanospray source. The resulting spectra were searched for protein candidates in the NCBI nonredundant protein sequence database with Sonar search engine software (Genomics Solutions Inc., Lansing, MI). **(B)** The amino acid sequence of PK is shown with the peptides identified from the MS analyses highlighted in bold. Amino acids that differ between the M1 (v,f,r and l) and M2 isoforms (i,y, l and q) of PK are underlined.

Fig. 5. Tissue Distribution of PK in Brain and Peripheral Tissue, and Immunocytochemical Staining for PK in Striatal Tissue

(A) Immunoblot of rat brain and peripheral tissue lysates (100 μ g total protein per lane) using anti-PK antibodies. Quantification of the immunoblot is shown below the autoradiogram. (B) Representative coronal sections from striatal tissue were immunostained for DARPP-32 (red) in panels (1) and (2) and PK (green) in panels (3) and (4). Merged images are shown in (5) and (6). The panels on the left are 200X and the panels on the right are 630X magnifications. CPu and cc denote caudate putamen and corpus callosum, respectively.

Fig. 6. Reactivity of Streptococcal Proteins with Anti-PK Antibody

(A) Proteins from the M serotypes 18, 12, 6 and 3 (with designated strain numbers) were fractionated into cytosolic (cyt) and extracellular membrane associated fractions (ext) (10 μ g total protein/lane) and immunoblotted using anti-PK antibody. Purified PK (1 μ g) was loaded in the first lane (left). The lower panel in A shows a control blot under identical conditions but with goat serum (1:200 dilution) in place of the primary antibody (film exposed time is 10 fold longer than in upper panel). (B) Immunoblots of purified PK (0.5 and 1.0 μ g) using anti-M5, -M6, and -M24 antibodies. Enolase was also screened for reactivity under the same conditions used for PK. The two panels in the figure labeled Con are controls using normal rabbit serum (diluted 1:200) in place of the primary antibodies (film exposure times are 30 fold longer than

in the upper panels). (C) Lysate proteins (100 µg/lane) from the indicated rat brain tissues were immunoblotted with anti-M24 antibody (left panel) and anti-PK antibody (right panel). The panels immediately below each are controls showing identical blots but normal rabbit serum (1:200 dilution) and normal goat serum (1:200 dilution) were used in place of the primary antibodies anti-M-24 and Anti-pk, respectively (film exposure times are 7 fold longer than in the upper panels).

Fig. 7. Analysis of Patient and Control Sera for Immunoreactivity to PK

(A) Immunoblots of cytosol (cyt) proteins extracted from rat striatum (100 μ g) and purified pyruvate kinase (pk) (0.5 μ g) were screened using sera from a TS/OCD patient prior to (TS, pre), during (TS, ex) and following (TS, post) exacerbation of symptoms. Panel TS, ex+pk is an immunoblot of the cytosol and pk samples using the TS, ex serum preabsorbed with PK. The panel labeled Anti-PK shows an immunoblot of the cytosol and pk samples probed with anti-PK antibody. (B) Quantitation of immunoblots using sera from 21 patients with TS or TS and OCD, and 11 age-matched controls with and without GABHS infections is shown. Immunoblots were quantitated with the highest value of reactivity set at 1. The average reactivity for each set is plotted. For controls: -GABHS n=5 and +GABHS n=6 (total

controls=11). For TS patients' sera: +symptoms (sym)-GABHS n=8, -sym+GABHS n = 6, +sym+GABHS n=2, -sym-GABHS n=5 (total patients=21). Error bars indicate standard deviation.