Antineural antibody in patients with Tourette's syndrome and their family members

Chin-Bin Yeh[1], Ching-Hsing Wu[2], Hui-Chu Tsung[2], Chia-Wei Chen[2], Jia-Fwu Shyu[1,2,]*
& James F. Leckman[3]

[1]*Department of Psychiatry, Tri-Service General Hospital, Taipei, Taiwan;* [2]*Department of Biology & Anatomy, National Defense Medical Center, National Defense University, No. 161, Sec. 6, Minquan E. Rd., Neihu District, Taipei City, Taiwan, 114;* [3]*Child Study Center, Children's Clinical Research Center, Department of Pediatrics, Yale University, New Haven, CT, USA*

Summary

It has been proposed that antineural antibodies were present in patients with Tourette's syndrome (TS) and other neuropsychiatric disorders. The purpose of our study was to investigate the presence of antineural antibodies in the individuals with Tourette's syndrome and the family members of TS patients. The sera of four TS patients with no current streptococcal infection, their tic-free family members including father, mother and sibling, and a age-matched control group who were tic free were assayed for antineural antibodies directed against rat tissue and neurons in primary cell culture. There were prominent antineural antibodies present in TS patients and their first-degree family members, but not in the control group. Western blotting showed proteins of about 120 kDa in their sera that were not present in the sera of controls. The preliminary results of our study suggest the importance of genetic vulnerability in the immunological pathophysiology of tic disorders. Future studies should investigate the interactions of genetics, environment, infectious agents, and immunity on symptom expression in families with tic disorders.

Introduction

Previous studies have proposed that antineural antibodies in patients with tic disorders and related conditions, such as obsessive-compulsive disorder [1–7]. Swedo and colleagues proposed the existence of a group of patients with pediatric antoimmune neuropsychiatric disorders associated with streptococcal infection, or "PANDAS" [8].

This hypothesis was based on the observation that some patients with Sydenham's chorea (SC) present with tics, obsessive-compulsive disorder (OCD), and/or attention-deficit hyperactivity disorder (ADHD) [2, 9–11]. SC is thought to be a late autoimmune sequela of streptococcal infection [12, 13]. Swedo and colleagues [8] suggested that these neuropsychiatric symptoms may share a common etiology. Some case reports and cross-sectional studies have demonstrated the presence of antineuronal antibodies in patients with SC, tic disorders, and obsessive-compulsive disorder. It was thought that the autoantibody was induced by streptococcal infection, and that this antibody attacked the basal ganglion, resulting in the above mentioned neuropsychiatric symptoms [6, 14, 15].

Contrary to the above findings, several studies have investigated the controversial concept of PANDAS [5, 7, 16–19]. The existence of an autoimmune mechanism underlying those neuro-psychiatric disorders has not yet been proven, although a novel treatment based on this theory produced dramatic effects [20]. In the first longi-tudinal prospective study [21], there was no association between streptococcal infection and any exacerbation of disease severity among patients with tic or obsessive-compulsive disorder over a 2-year follow-up period. These observations confound the theory of a relationship between infections and antibodies in the pathogenesis of TS and related neuropsychiatric disorders. These days, the etiologies of many diseases, including diabetes and multiple sclerosis, point to an auto-immune mechanism [22–25]. Moreover, the results of previous studies indicate that both genetic and environmental factors play important roles [26]. As we know from the biopsychosocial model of the etiology of neuropsychiatric disorders, genetic vulnerability seems to be the nexus for the inter-action of biological and psychological factors [27]. Family studies are designed to explore genetic vul-nerability to autoimmune-related disorders and/or heritable diseases. In the past, research has focused on the manner of inheritance, or the genetic linkage, with very few contributions coming from association studies. There is growing evidence that both autoimmune and genetic factors are involved in the pathogenesis of TS [28–32] although a study tried but failed to identify such a marker by investigating choline in the red blood cells of TS twins and their parents [33]. Therefore, it is considered a trait marker that is indicative of a genetic susceptibility to Tourette's syndrome [33, 34]. Instead of a single approach or multiple genetic approaches to test the theory of autoim-mune stimulation by infections, the investigation of proteins and their interactions should be the next step upon which to focus. In the meantime, the immunological deficits underlying these dis-eases are gradually being identified. Therefore, we are more interested in the underlying etiology as a "trait marker", such as the immunological mech-anism that might be inherited and explained as the vulnerability factor. The hypothesis we proposed is that genetic vulnerability is crucial for the pathogenesis of tic-related disorders. The aim of our study was to investigate the role of antineuronal antibodies among the family mem-bers of patients with Tourette's syndrome.

Methods and materials

Subjects

Four outpatients were recruited and met the DSM-IV criteria for TS. The children and their parents gave their informed consent after the explanation of the process of study and were evaluated for acute respiratory tract infection. The antistreptolysin O antibody titer was monitored if any infection was suspected in the previous 2 months. The ASO titer was assayed with Rate Nephelometry (Beckman; Conneticut, US). The patients were interviewed by an experienced psychiatrist to evaluate the severity of their tics and obsessive-compulsive symptoms. Comorbid neuropsychiatric disorders were also identified with DSM-IV based on the information from the interviews with children and their parents. We also asked the parents to complete the parents' report version of the Yale Global Tic Severity Scale (YGTSS) before the interview. We then arranged another session to interview the parents with the Mini-International Neuropsychiatric Interview and DSM-IV, and their sibling with Kiddi-SADS. At the same time, we collected blood samples from the patients and their parents and/or siblings. The samples were sent for further assay to a laboratory where the laboratory personnel were unaware of the patients' background data. No comparisons were made until all investigations were completed. The control group was four healthy young adults and four age matched children with no current or past history of tics or other neuropsychiatric disorder. They were also evaluated had no family history of tics and related problems.

Brain sections and protein preparations from rats

The brains of male Wistar rats were sectioned into the striatum, cortex, midbrain, hippocampus, cer-ebellum, spinal cord, brain stem, olfactory bulb, etc (Figure 1). After the addition of lysis buffer (10% sodium dodecyl sulfate (SDS), Na_3VO_4, Tris–HCl; Sigma Chemical Co., St Louis, MO, USA), the samples were grounded, heated to 100 °C for 5 min, centrifuged at $16,000 \times g$ for 30 min, and then the upper layer was removed for protein assay.
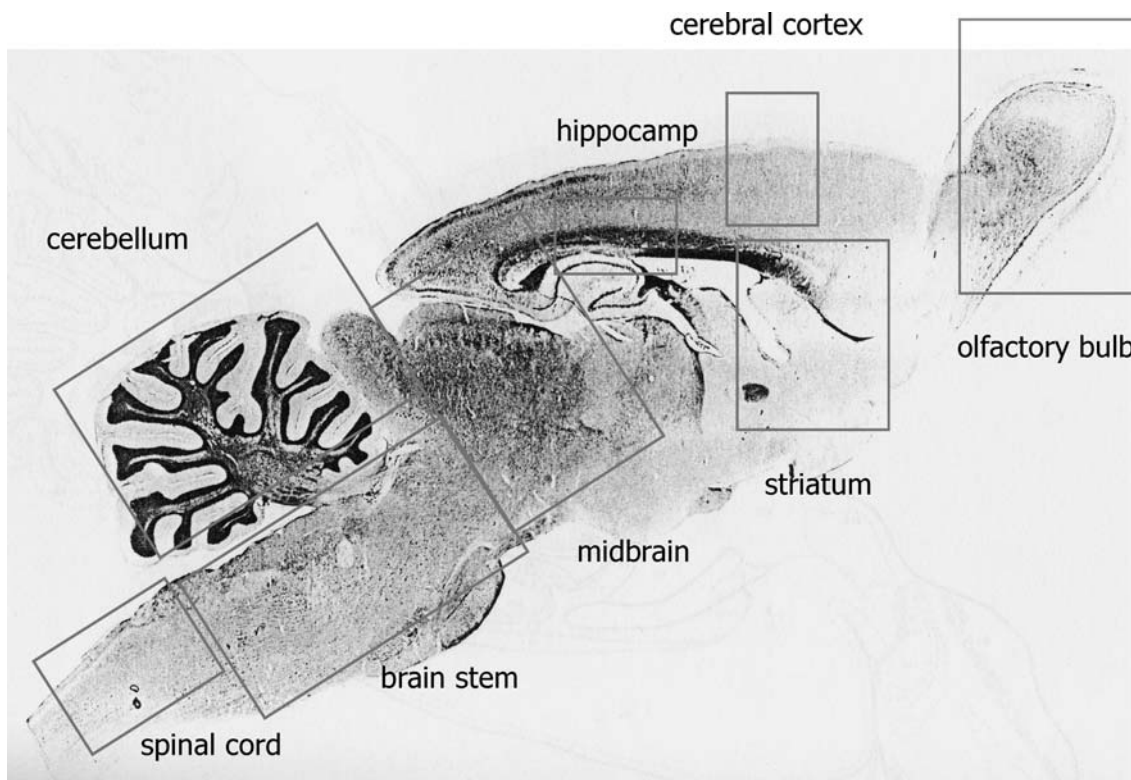
Figure 1. Brain sections of rats.

Western blotting

Equal amounts of protein from different brain areas were resolved by SDS-polyacrylamide gel (8%) electrophoresis (PAGE), and then transferred to nitrocellulose (NC) membrane. After the transfer process was completed, the NC membrane was carefully dyed with Ponceau S. Skimmed milk powder (10%, w/v) was used to inhibit non-specific binding at room temperature. After 6 h, primary antibody (serum samples taken from TS patients) was added at a dilution of 1:2000, and the membrane incubated at 4 °C overnight. The NC membrane was then washed three times with a solution of Tris–base, NaCl, and Tween-20 (TBST) for 10 min each time, before the addition of the secondary antibody (mouse anti-human IgG conjugated with horseradish peroxidase (HRP) and diluted 1:7500; Jackson Company, West Grove, Pennsylvania, USA) and incubated at room temperature for 90 min. The membrane was then washed three times with TBST, for 10 min each time. Finally, the membrane was added into enhanced chemiluminescence (ECL) solution and sandwiched between two plates for autoradiography.

Immunohistochemical analysis

Wistar rats weighing 250 g were perfused with 0.9% normal saline to clean out the blood. The tissues of the rats were fixed with 2% PLP (lysine, 8% paraformaldehyde, m-periodate). The rat brains were immersed in 30% sucrose solution and stored at 4 °C until the tissue sank to the bottom. The brain tissues were placed on a block made from aluminum foil, and the tissue was embedded with a cryomatrix (OCT compound) for instant freezing for cryotomy (Leica 3060). The brain tissue was cut into thicknesses of 30 μm and washed with TBS (Tris–base, NaCl) three times, for 10 min each. The tissue was then washed in PBS with 1% H_2O_2 for 30 min. Samples were then washed thrice with TBS, each time for 10 min, and then transferred to a solution of 10% normal horse serum containing 0.1% Triton X-100 for

10 min at room temperature. Primary antibody (sera of Tourette's syndrome patients diluted 1:100) was added, and the samples were incubated at 4 °C overnight. The next day, the tissues were washed three times, each for 10 min, before the addition of secondary antibody. The secondary antibody (rabbit anti-human IgG conjugated with fluorescein isothiocyanate (FITC) and diluted 1:200; Sigma Company) was added and the samples incubated at room temperature for 60 min, then washed with TBS three times, each for 10 min, and observed with a fluorescence microscope (Leica DMIRE2).

Primary neuronal cell culture

Striatal tissue was dissected from the fetal ganglionic eminence of 14-day-old Sprague-Dawley rat embryos. The striata were carefully dissected out under a dissecting microscope, according to the procedures described by Smart and Sturrock [32, 35]. They were then placed into another dish containing ice-cold phosphate-buffered saline (PBS) to thoroughly remove blood vessels and membranes from the striatal tissues.

The tissues were cut into approximately 1 mm^2 pieces and then incubated in sterile PBS containing 0.25% trypsin for 30 min at 37 °C. The tissues were centrifuged for 5 min at 600×g. The cells were resuspended in RPMI 1640 medium containing 10% fetal bovine serum, 5% penicillin/streptomycin, 5% L-glutamine. The cultures were incubated at 37 °C in 5% CO$_2$.

Deionized water was used to prepare RPMI 1640 medium (Biochrom KG, Germany); 4 g NaHCO$_3$ was added and the pH adjusted to 7.2–7.3, before the solution was sterilized through a 0.2 μm filter. Before the experiment was commenced, the following were added to the medium: (1) 10% fetal calf serum and the solution incubated at 56 °C for 30 min to inhibit any interference by complement; (2) 1% L-glutamine; and (3) 1% penicillin and 0.1 mg/ml streptomycin, which were mixed heterogeneously before use.

Immunocytochemical staining of primary neuronal cell culture

The cultured cells were fixed immediately onto discs with −20 °C methanol for 5–10 min (the cells were not allowed to dry out). The methanol was then removed and the cells washed briefly, with the addition of blocking buffer containing 10% normal goat serum for 40 min at room temperature. The cells were then incubated with primary antibodies (sera from patients with TS or healthy controls; or anti-MAP-2 antibody (Chemicon International, Temecula, CA, USA) or 24 h at 4 °C. Samples were washed thrice for 10 min each with PBS, then reacted with secondary antibody (anti-human IgG conjugated with FITC, or goat anti-mouse IgG conjugated with Cy3) (Jackson Company, West Grove, Pennsylvania, USA) for 1 h at room temperature. Samples were again washed thrice for 10 min each with PBS, and then reacted with 4′,6-diamidino-2-phenylindole (DAPI) for 15 min at room temperature. The cells were then examined with a confocal laser scanning microscope (Zeiss, LSM510).

Results

Group characteristics

Patients with TS were diagnosed as comorbid with either OCD or ADHD. There was an identical twin pair in our study and both were diagnosed with TS and comorbid ADHD. Their family members had no current and past history of tics or other neuropsychiatric disorders. All the subjects recruited into our study were not currently infected with *Streptococcus*. The demographic data are shown in Table 1.

Immunohistochemistry

Selective binding of TS serum was observed in specific brain regions of rats. There was no specific binding of sera from normal healthy controls to the cerebral cortex or striatum. In contrast, binding of the sera from patients with Tourette's syndrome and their family members was observed in pyramidal and dendrite neurons, forebrain, midbrain, hypothalamus, and cortex. However, sera did not bind to the corpus callosum (Figure 2). Staining of similar density was seen in the brain sections when treated with sera from the family members of TS patients. However, there was almost no staining in these brain regions when treated with sera from the control group.

Table 1. Demographic data and summary of the analysis of patients with Tourette's syndrome and their family members compared with a control group.

	Age/ gender	Current diagnosis	Past tic or OCD history	Immunoreactivity in rat nervous tissue	Western blot (kDa)	Age of tic onset	YGTSS-motor total score	YGTSS-phonic total score
C1	10/M	No	No	–	–	NA	0	0
C2	12-/M	No	No	–	–	NA	0	0
C3	12/M	No	No	–	–	NA	0	0
C4	14/M	No	No	–	–	NA	0	0
C5	26/M	No	No	–	–	NA	0	0
C6	28/M	No	No	–	–	NA	0	0
C7	27/M	No	No	–	–	NA	0	0
C8	25/M	No	No	–	–	NA	0	0
P1	10/M	TS	+	+	120,150	5	44	19
F1	41/M	No	–	+	120, 150	0	0	0
M1	39/F	No	–	+	120, 150, 170	0	0	0
S1	7/M	No	–	+	120, 170	0	0	0
P2	12/M	TS, ADHD	+	+	120, 150	5	63	62
F2	45/M	No	–	+	150	0	0	0
M2	46/F	No	–	+	120, 190	0	0	0
S2	12.3/M	TS, ADHD	+	+	120, 170	8	60	60
P3	12/M	TS	+	+	120, 170, 180	10	62	45
F3	45/M	No	–	+	150	0	0	0
M3	41/F	No	–	+	100, 170, 180	0	0	0
P4	14/M	TS, OCD	+	+	120, 170	5	40	40
F4	42/M	Alcohol abuse	–	+	170, 180	0	0	0
M4	40/F	No	–	+	150	0	0	0
S4	12/F	No	–	+	120	0	0	0

P, F, M, and S denote patients, father, mother, and siblings of patients with Tourette's syndrome, respectively; C denotes the control group. P2 and S2 are twin brothers with Tourette's syndrome comorbid with ADHD.

Western blotting

A protein of about 120 kDa was identified in various regions of the rat brain using sera from TS patients but not in normal control (Figure 3). A similar banding pattern was also observed with the sera of the family members of TS patients, The Western blot showed that the high molecular weight proteins were identified in various regions of rat brain using serum from the individuals with Tourette's syndrome and their family members but not in normal control group, but not with that of the control group (Figure 5 and Table 1).

Primary neuronal cell culture

In vitro immunocytochemical staining showed immunofluorescence in neuronal cells treated with the sera of TS patients and their family members.

This neuronal cell straining was confirmed by double immunofluorescence labeling with neuron-specific anti-MAP2 antibody (Figure 4). We also added dopamine-neuron-specific tyrosine hydroxylase and choline acetyltransferase to detect choline, but neither showed significant staining.

Discussion

The results of our study show that antineural antibodies run in the families of patients with TS. The molecular weights of the proteins visualized on western blots probed with the sera of TS patients and their families were above 110 kDa, whereas those visualized with the sera of normal controls were about 60 kDa [36]. Western blot analyses were designed to identify possibly relevant antigen. Previous studies mentioned that antibodies to heat
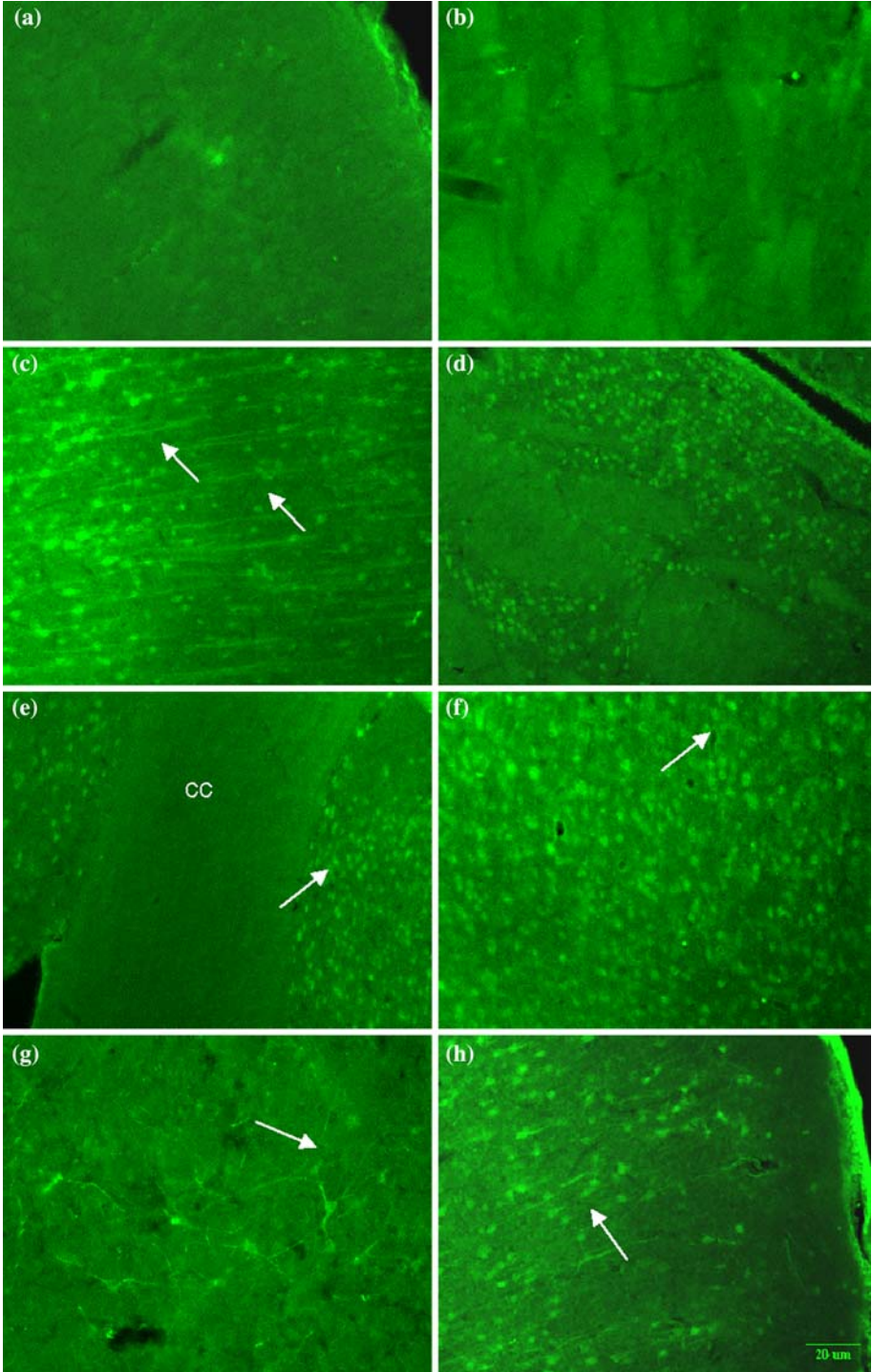
Figure 2. Selective binding of TS sera to rat neurons is observed in specific regions of rat brains. There was no specific binding of sera from normal healthy controls to the cerebral cortex (a) or striatum (b). In contrast, binding of the sera from patients with Tourette's syndrome and their family members was observed in the following rat brain sections: pyramidal and dendrite neurons (c, d), forebrain (e), midbrain (f), hypothalamus (g), and cortex (h). However, sera did not bind to the corpus callosum (cc) in (e).
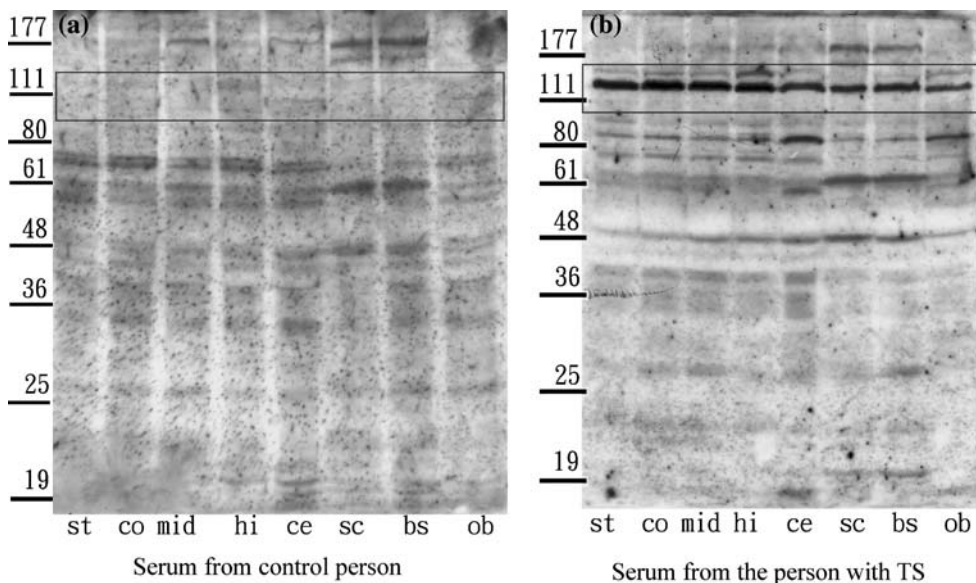
Figure 3. The Western blot showed that a protein of about 120 kDa was identified in various regions of rat brain using serum from a Tourette's case (b) but not in the normal control (a).

shock protein 60 are known to be present in virtually all individuals [37] in Western blot analyses. Anti-60 kDa binding of seroreactivity against neuronal antigen was found significantly just above

threshold more frequently in tic disorder patient when compared to healthy control, autistic disorder [38]. Thus, it suggested that the anti-60 kDa might be not a specific pathogentically relevant
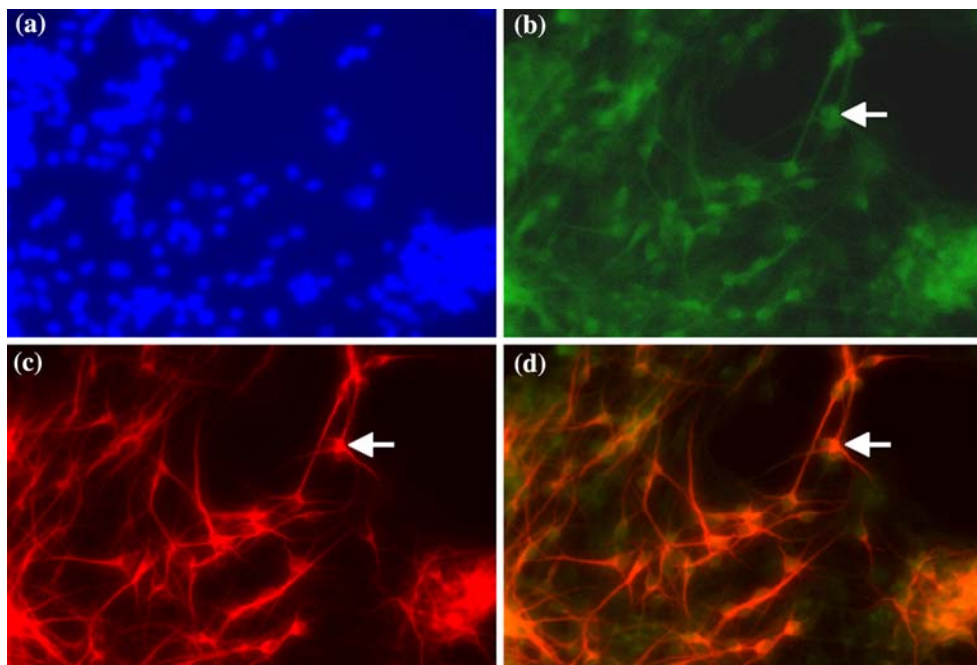

Figure 4. In vitro immunocytochemical staining further confirmed the selective binding of TS serum to striatal neurons. (a) DAPI stain showing the nuclear labeling of the culture neuron cells. (b) Using serum from Tourette's patient as the first antibody and FITC conjugated secondary goat antibody. (c) Using neuron specific anti-MAP antibody as first antibody and rhodamine conjugated secondary goat antibody. (d) Overlay of figure (b) and (c).

autoantigen in the individuals with Tourette's syndrome. In immunohistochemical studies, the antineuronal antibodies identified proteins in the neuronal tissues of rats, and these were distributed more specifically in the striatum than in the brain stem, cortex, or paraventricular regions. In contrast to the sera of TS patients and their families, there was very limited staining in the rat neuronal tissue with the sera of the control group. We also used a neuron-specific antibody to confirm that the antibodies present in the sera of TS patients and their families were directed against neuronal cells in primary striatum neuronal cell cultures. Compared with previous studies, our results indicated that there were antineural antibodies in the sera of TS patients, even in the absence of significant anti-streptolysin O antibody titers or other signs of streptococcal infection.

Husby and colleagues were the first to describe antineuronal antibodies that putatively arise in acutely ill SC patients in response to group A β-hemolytic streptococci (GABHS) infection [9]. In 1993, Swedo and colleagues, using similar methods, showed that 91% (10/11) of SC patients tested positive for antineuronal antibodies, but 50% (9/18) of healthy controls were also positive. This technique was also used to study individuals with acute, chronic, or remote rheumatic chorea, compared with 40 controls; antineuronal antibodies were found in 100%, 93%, and 44% of the SC patients, respectively [6]. Most studies involving antineuronal antibodies have been in those patients with tics with *Streptococcus*-related symptoms. There were very few studies which have considered antineuronal antibodies in patients with Tourette's syndrome, the clinical symptoms of which do not correlate with streptococcal infection.The most recent study compared the PANDA group and the uncomplicated active Group A streptococcal infection children and found that the presence of antibrain antibody could not be explained merely by the history of GABHS infection [32]. The sera from clinical OCD cases showed antibodies directed against caudate and putamen at a rate significantly higher than that of clinical controls, providing evidence of basal ganglia involvement in OCS without *Streptococcus* infection [4]. Anti-basal ganglia antibodies were positive in 65% of a group of 65 patients with atypical movement disorders, but were very rare in healthy adults and adults with idiopathic dystonia. An autoimmune mechanism was suggested underlyng the cases of atypical movement disorders [39]. Anti-60 kDa binding occurred significantly more frequently in unselected patients with tic disorders (67.1%) than in those with autistic disorders or OCD, or in healthy controls [36]. There was also no significant correlation between antineuronal antibody and anti-streptolysin O antibody titers [18]. Compared with those studies, the preliminary results of our study suggest the presence of antineuronal antibodies among TS patients and their families, even in the absence of streptococcal infection. In comparison, no control subject was positive for antineuronal antibodies. The relationship between antineuronal antibodies and streptococcal infection warrants further study.

Our study demonstrates the presence of antineuronal antibodies not only in TS patients, but also in their family members. These family members either lacked lifelong tic symptoms or were in remission from tic symptoms at the time of the study. The molecular weights of the proteins detected by TS sera on Western blots were consistently higher than that detected by control sera. These results suggest that these antineuronal antibodies occur in the sera of TS patients and their families, but not in the sera of the control group, and detect a protein with a molecular weight of about 120 kDa. Antineuronal antibodies were also found in patients' families, which is consistent with our hypothesis. To the best of our knowledge, no other study has previously investigated the presence of antineuronal antibodies in the families of TS patients. However, we did not know any reason that both mother and father of patient should have binding if genetic susceptibility. Possibly family members share some sort of environmental trigger for pathology with only those with lack of resistance genes showing clinical symptoms. It is hard to make conclusion that the family members of the case with TS shared the same constitution with the cases of TS by the results of our study since they might have the similar exposure at home. The antineural antibodies might be secondary to the factors including unknown infection, toxin although there was no significant antistreptococcal O antibody titer among them. This finding emphasizes the importance of genetic and immunological vulnerability in the pathogenesis of tic symptoms.
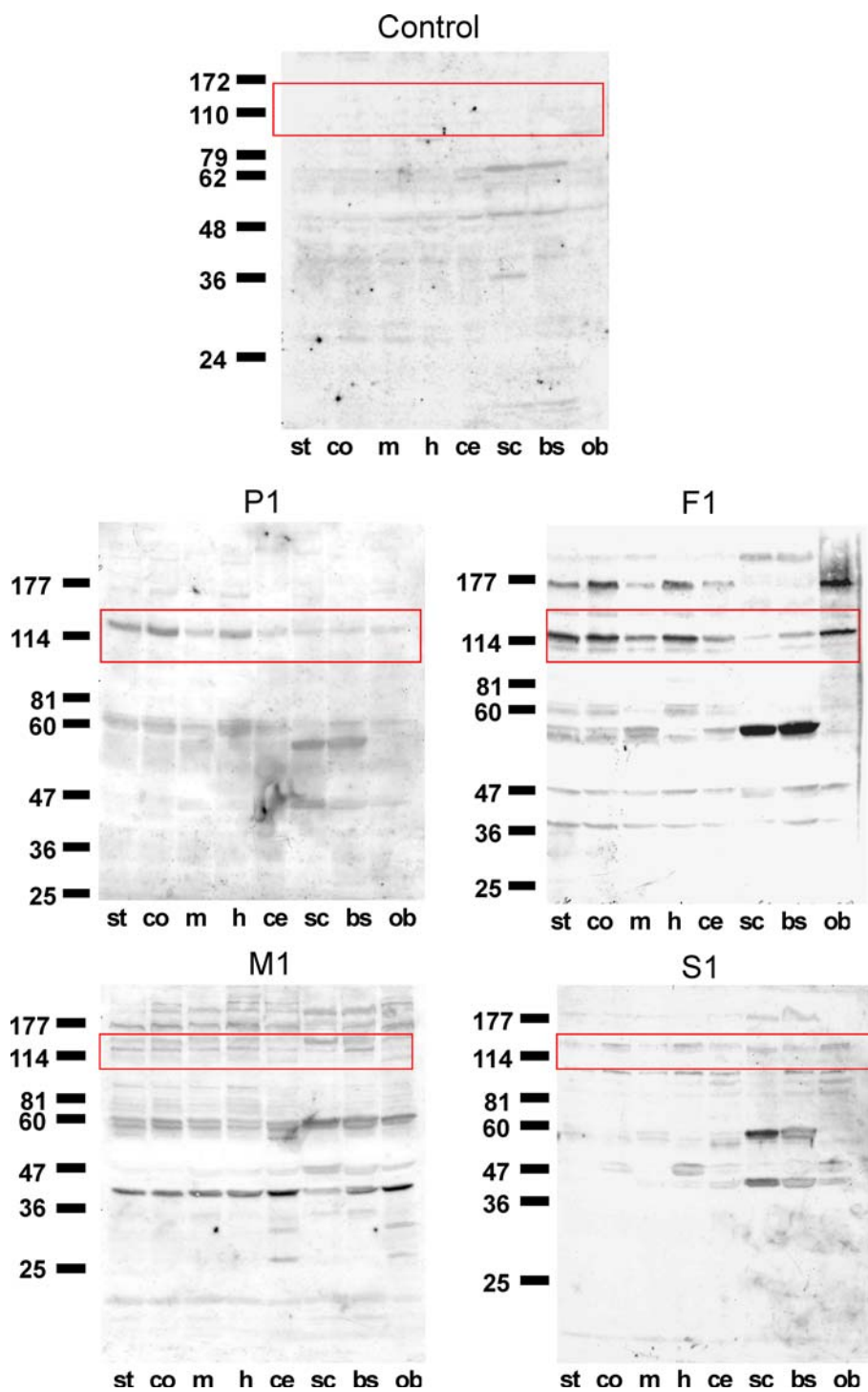
Figure 5. Western blotting analysis of a patient with Tourette's syndrome and his family members. The high molecular weights of the proteins (about 120 kDa) were identified in various regions of the rat brain by using the sera of the individuals with Tourette's syndrome and their family members (P1–patient with TS, F1–Father of the TS patient, M1–Mother of the TS patient, S1–Sibling of the TS patient), but not in the sera of normal control (control).

The antineuronal assay used in previous studies was performed with either human brain sections [9] or a neuroblastoma cell culture [40]. The IgG antibody fraction from the sera of TS patients was isolated by protein A affinity chromatography [40]. The postmortem human brain might have been exposed to a hypoxic process or medication. It complicated the identification of the specific antigen in the brain. Moreover, the neuroblastoma cell culture might not contain the pathogenic neuronal antigenic structure in the brain of the individuals with TS. In our study, we used two separate substrates including rat brain tissue from several brain areas, and cultured neuronal cells from rat embroyos. Also, both Western blotting and fluorescence microscopy has been done. The proteins used in the Western blotting and immunohistochemical studies were extracted from the brains of adult male Wistar rats. Primary neurons were cultured from striata dissected from 14-day-old Sprague-Dawley rat embryos [35]. The results of the cell culture study showed good resolution, even without image analysis. Compared to the human basal ganglia they used in the previous studies, it suggests that the fresh striata of rat embryos and the brains of rats are reactive to the antineuronal antibodies of TS patients, when our method is used.

Interestingly, the family members of TS patients exhibited antineuronal antibodies in their sera, but had no tic symptoms. In a large family study, almost 40% of patients with PANDAS had one or more first-degree relatives with a history of motor or phonic tic [41]. However, in our study, no family member had a history of tic. Our sample may not represent the general population of individuals with tic-related disorders. Meanwhile, the number of our patient and their family was relatively small. We did not assay the family member of the children in the control group for the antineural antibody although they was evaluated no tic history or related problem. Using longitudinal and family studies, future research should address the interactions between genetics, environment, infectious agents, stress, immunity, and symptom expression in children with tics or obsessive-compulsive disorders. The molecular characterization of antigens should also be undertaken in future studies.

We believed that the occurrence of antineuronal antibodies in the sera of TS patients and their families strengthens the view that immune-mediated mechanisms are involved in the development of Tourette's syndrome. To explore the role of antibodies in the sera of TS patients, animal models of TS have been established in which the antibodies of TS patients are transferred into the brains of rats by microinfusion [15]. The involuntary movements seen in rats treated with intrastriatal microinfusions of TS sera or gamma immunoglobulins (IgG) are similar to those observed in TS. Another immunohistochemical analysis also confirmed the presence of IgG selectively bound to striatal neurons in TS [40]. The preliminary results of our study should provide a basis for future directions in research into the underlying pathophysiology of TS, in a model of genetic susceptibility to autoimmune conditions, and their relationship to environmental triggers.

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