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Striatal Microinjection of Sydenham Chorea Antibodies: Using a Rat Model to Examine the Dopamine Hypothesis

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Abstract We hypothesized that injection of anti-basal antibodies from patients with Sydenham's chorea into rats' striatum will induce behavioral and histological changes. Antibodies from eight Sydenham's chorea patients and eight age-matched controls were injected into the left caudate of 16 rats. Apomorphine- and amphetamine-induced rotations were performed on days 10 and 17, respectively, followed by immunohistochemical studies. Antibodies from patients with Sydenham's chorea, but not controls, bound to a ~50-kDa molecule in the striatum extract; immunohistology staining demonstrated specific binding to cellular component(s) in rats' striatum. Contrary to our hypothesis, we could not detect in the rats injected with Sydenham's chorea antibodies changes in rotational behavior or immunohistochemistry staining for dopaminergic or GABAergic markers. Injection of small quantities of anti-neuronal antibodies present in patients with Sydenham's chorea into rat striatum is insufficient to alter motor behavior or cause detectable cellular changes.

Keywords Anti-neuronal antibodies · Animal model · Dopamine · Rotational behavior · Striatum

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Background

Sydenham's chorea (SC), a manifestation of rheumatic fever, is a disabling neuropsychiatric disorder involving the basal ganglia, presenting weeks or months after group A streptococcal infection (Giedd et al. 1995; Cardoso et al. 1997). Clinical characteristics of SC encompass disturbances in the motor system as well as psychiatric abnormalities. While chorea is the most common movement disorder (Ben-Pazi et al. 2003; Teixeira et al. 2003; Dale et al. 2004), behavioral changes can also appear (Asbahr et al. 2005; Walker et al. 2005). Despite the previous perception that SC is a self-limiting disease (Nausieda et al. 1980), it was found to persist in half of the cases (Cardoso et al. 1999; Tumas et al. 2007) and may have a relapsing course (Walker et al. 2007).

Clinical motor and psychiatric symptoms are attributed to an immune-mediated basal ganglia neurotransmitter imbalance (Naidu and Narasimhachari 1980). PET and MRI studies demonstrate inflammatory process involving the caudate nuclei and the putamen (Emery and Vieco 1997; Aron 2005; Moreau et al. 2005); anti-inflammatory immunosuppressive treatments, such as steroids, are effective in shortening the chorea period (Jordan and Singer 2003; Garvey et al. 2005; Paz et al. 2006; Walker et al. 2007). The characteristic chorea movements are attributed to neurotransmitter changes such as decreased GABA and acetylcholine activity and increased dopaminergic activity in basal ganglia (Mitchell et al. 1989; Breakefield et al. 2008). Anti-dopaminergic medications (e.g., haloperidol) and drugs increasing GABA effect (e.g., valproic acid) are the gold standard treatment in SC (Jordan and Singer 2003).

SC is thought to be an antibody-mediated disorder caused by "molecular mimicry" of antibodies directed against group A beta hemolytic streptococcus antigens which might cross-

react with epitopes on neurons (Jordan and Singer 2003). Despite evidence confirming the presence of antibodies, there is an ongoing debate regarding the role of anti-basal ganglia antibodies in the pathophysiology of SC. Church et al. found a specific anti-neuronal antibody profile in patients with SC (Church et al. 2002); however, other studies failed to replicate these results (Singer et al. 2003). Additional studies demonstrated that antibodies isolated from SC patients' sera induce intracellular changes (Kirvan et al. 2003, 2007; Rakhilin et al. 2004; Teixeira et al. 2005). Thus, the role of anti-neuronal antibodies in the pathogenesis remains unknown and there is no evidence for a causal relationship between the anti-neural antibodies and clinical manifestations; antibodies may be a mere byproduct of basal ganglia inflammation rather than the cause of the disorder.

In this study, we hypothesized that antibodies generated in children with SC induce basal ganglia inflammation and are responsible for abnormal movement and behavioral changes. We checked whether SC-extracted antibodies injected stereotactically into rats' brains are capable of inducing cellular changes in the basal ganglia leading to changes in motor activity and behavioral patterns.

Methods (Details in a Supplementary File)

Participants Sera were collected from eight children with acute SC and from eight age-matched controls (Table 1). The study was approved by Shaare Zedek Medical Center's Helsinki committee and all parents signed an informed consent. Gama-immunoglobulin (IgG) fraction was isolated from the pool of SC patients sera and from the pool of the normal controls sera (4 ml containing 0.5 ml from each of the participants) by ammonium sulfate precipitation. The resulting IgG concentration was 935 mg/l for the SC sera pool and 874 mg/l for the control pool (the normal range for IgG values in serum is 340–1240 mg/l). Additional sera from a SC patient (8-year-old female) and from age-matched control (7-year-old female) were used for Western blot and immunohistochemistry.

Western Blot Analysis Western blot was performed on cell extracts from rat striatum, a human neuroblastoma cell line (SH-SY-5Y), and rat liver cells.

Animals and Stereotactic Injection Six microliters of the IgG fraction from the SC or control sera pools was injected using a stereotactic frame under chloral hydrate anesthesia to Wistar male. Two microliters was injected per site at a rate of 1 μl/min to three locations of the rats' left striatum: AP +1.7 Lat 2.5 Ven. −4.5, AP +0.2 Lat 3.5 Ven. −5.5, and AP −0.9 Lat 4.0 Ven. −4.5 (coordinates relative to the bregma).

Behavioral Tests in Rats Amphetamine- and apomorphine-induced rotational behavior. On day 10, rats were injected with D-amphetamine which releases synaptic dopamine, and on day 17 with apomorphine, a dopamine agonist. The rotational behavior was measured by a Rotameter for 60 min after D-amphetamine injection and for 45 min after apomorphine injection. *Spontaneous exploratory forelimb use test.* A blinded investigator assessed forelimb use during explorative activity in a transparent plexiglas cylinder for 5 min. Forelimb-use asymmetry was determined by independent use of the left or right forelimb for contacting the wall during rearing (Schallert et al. 2000; Kells et al. 2008).

Immunohistochemistry Naïve rats—Frozen brain of Wistar male rats tissues were cryosectioned. Following blocking, sections were exposed to IgG from SC or control serum overnight and then exposed to biotinylated anti-human IgG and to 488 Alexa-conjugated streptavidin.

Stereotactically Injected Rats—Rats were sacrificed on day 17 after injection and brain sections from three animals per group were exposed to biotinylated anti-human IgG antibodies and then to 488 Alexa-conjugated streptavidin. Sections were incubated with the primary antibody overnight and were incubated with an anti-mouse biotinylated secondary antibody followed by DAB staining.

Table 1 Clinical details of children with Sydenham's chorea and controls

#	Dx	Duration	Gender	Age	#	Dx	Gender	Age
S1	Acute SC	3M	M	6	C1	Broken arm	M	6
S2	Acute SC	3W	M	6	C2	Thalassemia	F	8
S3	Acute SC	2M	F	7	C3	Pseudotumor cerbri	M	9
S4	Acute SC	4M	F	9	C4	S/P teratoma	F	13
S5	Acute SC	2W	F	12	C5	S/P Hodgkin	F	14
S6	Acute SC	2W	F	12	C6	Sinusitis	F	14
S7	Acute SC	2W	M	17	C7	Abdominal pain	F	14
S8	Acute SC	2W	F	19	C8	Catatonia	M	17

S SC, *C* control, *W* weeks, *M* months, *Dx* diagnosis, *Duration* time from chorea until blood samples were obtained

Results

Western blot analysis with the IgG from the SC patient disclosed binding to multiple liver proteins. Incubation of gel separated lysates from rat liver, human neuroblastoma cell line and rat striatum, with IgG from the SC patient and the control, revealed the presence of anti-tissue antibodies in the sera of the SC patient. The SC IgG interacted with multiple components in the liver lysate while IgG from the healthy child did not interact with these constituents. In addition, the sera of the SC patient contained antibodies directed against neuronal antigens since it recognized a ~50-kDa molecule present both in the neuroblastoma cell line and in the striatum of normal rats. The IgG fraction of an age-matched control child did not contain antibodies against these neuronal antigens (see figure in the Electronic Supplementary Material).

In order to verify the presence of anti-neuronal antibodies in sera of SC patients, we examined the binding pattern of human IgG to rat brain sections that were incubated with IgG from the same SC patient and from the control. Assessment of these brain sections that were stained with anti-human IgG antibodies revealed detectable binding of human IgG to the striatum in all three sections (Fig. 1a, b), while other brain areas such as the cortex and cerebellum did not contain a measurable quantity of human IgG. In contrast, none of the brain sections that were incubated with IgG from controls contained any areas which stained with anti-human IgG antibodies (Fig. 1c, d).

With the intention of demonstrating the effect of the SC-associated anti-neuronal antibodies on the activity of the brain, we used a stereotactic frame to inject 16 rats into their left striatum with IgG from sera either from SC patients (eight rats) or from controls (eight rats) (Table 1). We recorded the rats' rotational behavior 10 and 17 days following injection of D-amphetamine and apomorphine, respectively. Quantitative rotational behavior is presented as the number of complete rotations to the left (ipsilateral to the stereotactic injection site) minus the number of complete rotations to the right (contralateral to the stereotactic injection site) (Sulzer et al. 1995; Gilgun-Sherki et al. 2003). There was no significant difference in the rotational behavior between the SC IgG injected group (ipsi-contra=−34±93) and the group injected with IgG from controls (−20±69; two-tailed t test, p=0.76). There was also no difference in the apomorphine-induced rotational behavior test 17 days post-injection. Both SC and control IgG treated groups rotated ipsilateral to the lesion side (5.5±14 and 5.5±15, respectively, two-tailed t test, p=0.99). Although during the cylinder test SC IgG injected rats tended to raise their contralateral forelimb (0.9±4.6) and rats injected with control IgG tended to use their ipsilateral forelimb (−0.75±3.7), the difference between the two groups was not significant (two-tailed t test, p=0.48). Since we detected the presence of anti-brain antibodies in brain sections of rats that were incubated with SC IgG, we stained brain sections from rats 17 days after striatal microinjections of SC IgG or control IgG. These brain

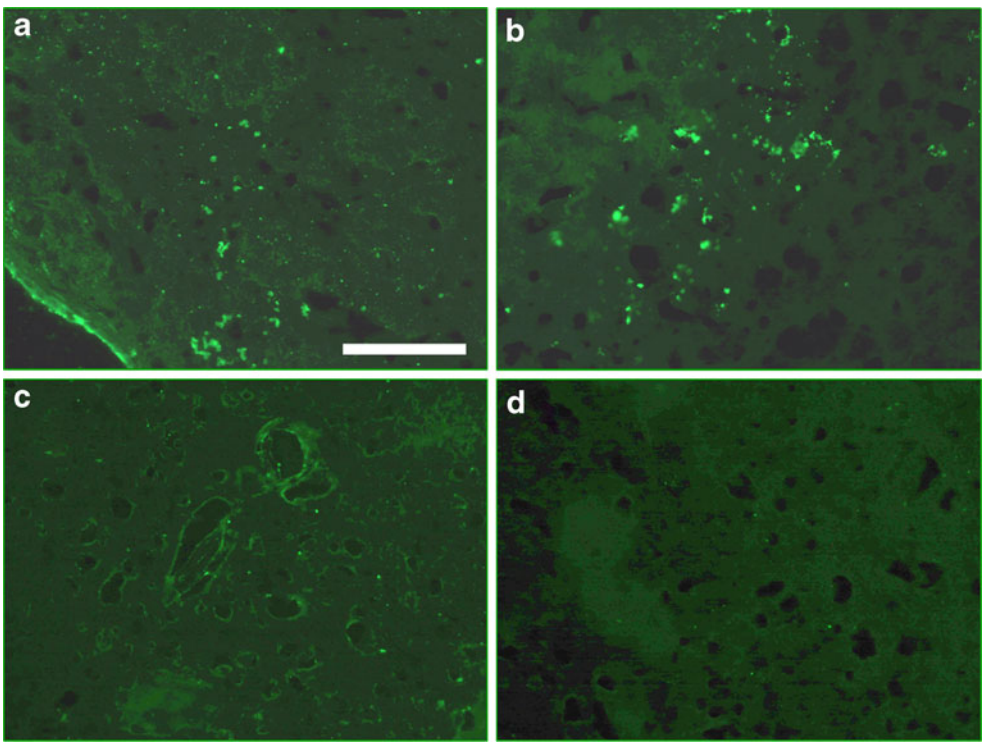

Fig. 1 Immunostaining for human IgG in the basal ganglia of rats incubated with IgG extract from Sydenham's chorea (SC) patients and controls. Sydenham's chorea (SC) IgG, but not control IgG, binds to the rats' striatum. Exposure to SC's IgG leads to specific striatal staining (**a**–**b**), while control extract (**c**–**d**) does not. *Scale bar*=100 μm

sections did not reveal any brain bound human antibodies following immunohistochemistry staining. Nor was any difference detected in tyrosine hydroxylase (TH) and GAD65/67 staining between the two groups 17 days after stereotactic microinjection.

Discussion

Our study revealed that the sera of an SC patient contained antibodies which reacted with liver molecules and with molecules that are expressed in the brain and in neuronal tissues. Such anti-tissue antibodies are not present in the sera of the healthy child since its IgG did not bind to either the liver or the neuronal components. Although previous studies did not report the presence of antibodies against non-neuronal antigens in SC patients (Church et al. 2002), our finding is not surprising since non-neuronal tissues such as skin, joints, and endocard are known to be affected in rheumatic fever. The identity of these non-neuronal molecules is not yet published and further studies are required to find out whether they have a role in the disease.

We have demonstrated that the SC patient produced anti-neuronal antibodies against a ~50-kDa protein. These antibodies were not present in samples from a healthy child. In the initial stages of this study, we performed Western blots with sera from a different group of eight children: acute SC (n=3), persistent SC (n=1), recurrent SC (n=1), antiphospolipid syndrome (n=1), psychogenic chorea (n=1), and abrupt onset of tics (n=1). Western blots were extremely sensitive and revealed multiple bands against neuroblastoma and Hela extracts. In addition to the multiple bands, we detected a ~50-kDa band in all blots that were incubated with sera of children with acute and persistent SC (4/4). This ~50-kDa band was not detected in blots that were incubated with the serum of the relapsing SC patient or with sera of three children with non-rheumatic movement disorders (data not shown). The presence of anti-neuronal antibodies against a ~50-kDa antigen corroborates the results of Kirvan et al. (2007) who detected anti-tubulin antibodies at ~55 kDa and of Church et al. (2004) who detected anti-basal ganglia antibodies which bound to three antigens (~40 kDa, ~45 kDa, and ~60 kDa). We did not detect clear bands against ~40 kDa and ~60 kDa components among the multiple bands in the five out of eight children with SC nor in the single child with acute SC. Our data further corroborates the need to identify the ~50-kDa antigen, common to human SH cells and rat striatum, in human brain extracts and to investigate its role in the pathophysiology of SC.

We demonstrated that antibodies in SC patients are directed against cellular striatal components. This non-random distribution may explain the clinical motor and psychiatric symptoms of SC. The striatum mediates emotional processes, cognitive aspects, and motor performance; consequently, even a small lesion or injury impacts on a wide range of motor and non-motor functions (Haber 2003).

Despite the presence of antibodies directed against molecules in rat basal ganglia in the sera of SC patients, we did not detect changes in the apomorphine and amphetamine behavioral tests performed in rats 10 and 17 days after stereotactic injection with SC IgG. In addition, we did not detect any loss or change in the dopaminergic and GABAergic cells of the basal ganglia of rats 17 days following stereotactic injection with SC IgG despite the use of pool of antibodies from several patients intensifying the binding to the antigen. Our data resemble the negative results reported in pediatric autoimmune neuropsychiatric disorders associated with streptococcal infection (PANDAS), a closely related post-streptococcal neuroimmune mediated disease (Loiselle et al. 2004). These negative results may have stemmed from the low quantities (6 μl; ~5 mg of IgG) of IgG that was infused into the striatum. Behavioral changes were documented in rats following continuous infusion of sera from children with Tourette syndrome (Hallett et al. 2000; Taylor et al. 2002). We hypothesize that if SC is mediated by anti-basal ganglia antibodies, the activation and/or blockage of the disease eliciting molecules in the basal ganglia cells may require continuous exposure to relatively high concentrations of pathologic antibodies.

In summary, our study revealed the presence of anti-tissue and anti-neuronal antibodies specific to striatal components in sera of SC patients. Single stereotactic microinfusion of these antibodies in small quantities is insufficient to induce detectable binding, cellular changes, and motor behavioral modification in the injected rats.

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