Mimicry and autoantibody-mediated neuronal cell signaling in Sydenham chorea

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Streptococcus pyogenes–induced acute rheumatic fever (ARF) is one of the best examples of postinfectious autoimmunity due to molecular mimicry between host and pathogen. Sydenham chorea is the major neurological manifestation of ARF but its pathogenesis has remained elusive, with no candidate autoantigen or mechanism of pathogenesis described. Chorea monoclonal antibodies showed specificity for mammalian lysoganglioside and *N*-acetyl-β-D-glucosamine (GlcNAc), the dominant epitope of the group A streptococcal (GAS) carbohydrate. Chorea antibodies targeted the surface of human neuronal cells, with specific induction of calcium/calmodulin-dependent protein (CaM) kinase II activity by monoclonal antibody 24.3.1 and sera from active chorea. Convalescent sera and sera from other streptococcal diseases in the absence of chorea did not activate the kinase. The new evidence implicates antibody-mediated neuronal cell signaling in the immunopathogenesis of Sydenham chorea and will lead to a better understanding of other antibody-mediated neurological disorders.

GAS is an important human pathogen responsible for a number of significant infections but is most commonly associated with pharyngitis. ARF is an autoimmune sequela of streptococcal pharyngitis, when an immune response against streptococcal antigens initiates events resulting in an inflammatory response against the heart, joints or brain. Sydenham chorea is recognized as the principal neurologic manifestation of ARF, and can occur alone or in combination with other symptoms including carditis, arthritis and erythema marginatum[1]. A resurgence of ARF has been seen in the United States since 1985, and ARF continues unabated in developing nations[2]. Whereas the postinfectious immune responses leading to inflammatory heart disease in ARF have been characterized, the mechanisms of streptococcal-induced central nervous system (CNS) dysfunction in Sydenham chorea have not been defined[3–8].

Sydenham chorea is characterized by involuntary movements and neuropsychiatric disturbances, including obsessive-compulsive symptoms, hyperactivity and emotional lability[9]. Sydenham chorea can develop in 10–30% of ARF cases and may be the only manifestation of ARF, presenting as late as 6 months after the initiating streptococcal pharyngitis[10,11]. Reports of therapeutic benefit from immunomodulatory therapies such as plasma exchange suggest that Sydenham chorea may be caused by a pathogenic antibody response[12].

Immune cross-reactivity between GAS and host tissues is well established with regard to molecular mimicry[4,13]. Considerable evidence has shown that cross-reactive immune responses against GAS and host antigens lead to rheumatic carditis[4,6]. Although the precise mechanisms of pathogenesis in Sydenham chorea are unknown, it has been proposed that an antecedent GAS infection induces a cross-reactive immune response directed against neuronal determinants in the brain[8]. The basal ganglia are responsible for motor function and

have been implicated as a target of post-streptococcal immune responses by clinical and magnetic resonance imaging data[14,15]. In previous studies, streptococcal-specific antibodies in Sydenham chorea reacted with neuronal antigens in human basal ganglia cross-sections, and neuronal-specific antibody titers were associated with both severity and duration of choreic episodes, suggesting that CNS dysfunction is immunologically mediated[16,17]. Streptococcal cell wall components may evoke cross-reactive antibodies, as brain-specific antibody reactivity was absorbed from Sydenham chorea sera with cell wall preparations of rheumatogenic streptococcal strains. However, the identity of the brain antigens and the mechanism by which autoantibodies might mediate CNS dysfunction in Sydenham chorea were not determined. The work reported here uses monoclonal antibodies derived from a patient with Sydenham chorea to identify the GAS and brain antigens as well as a mechanism by which brain-specific autoantibodies might have a role in the disorder.

Our study suggests that antibodies against streptococci and brain in Sydenham chorea may produce CNS dysfunction through neuronal signal transduction, and identifies potential molecular targets of the brain-specific antibodies as well as a mechanistic role for antibodies in the pathogenesis of Sydenham chorea. The evidence supports the hypothesis that cell signaling of neuronal cells by antibody may lead to Sydenham chorea. Identification of streptococcal and neuronal antigen(s) as well as a pathogenic mechanism of the antibody response in Sydenham chorea will provide a more complete understanding of disease pathogenesis.

Sydenham chorea antibodies target brain-derived ganglioside

To identify antigen targets and study cross-reactive antibodies against streptococci and brain, human hybridomas were derived

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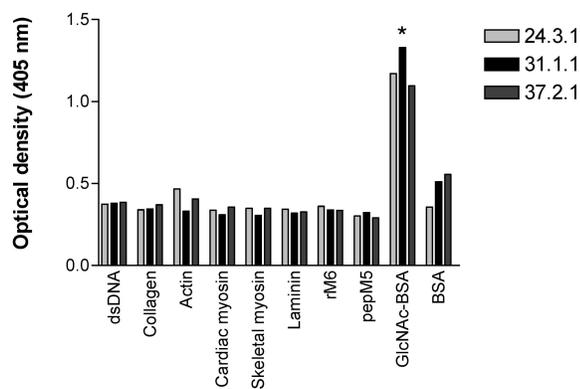
Figure 1 Reactivity of chorea monoclonal antibodies with streptococcal and mammalian autoantigens. *, $P = 0.005$. rM6, full-length recombinant streptococcal M6 protein; pepM5, fragment of streptococcal M protein. Chorea monoclonal antibodies showed significant reactivity only with GlcNAc-BSA.

from a Sydenham chorea patient. Human hybridomas established from fusions of peripheral blood mononuclear cells with the K6H6/B5 cell line resulted in three monoclonal antibodies (24.3.1, 31.1.1 and 37.2.1) that reacted strongly with glutaraldehyde-fixed, whole-cell type 5 *S. pyogenes*[18]. Monoclonal antibody reactivity was subsequently localized to GlcNAc, the immunodominant epitope of the GAS-specific carbohydrate that is a major constituent of the GAS cell wall (**Fig. 1**). Chorea antibody reactivity was significantly ($P = 0.005$) greater with the group A carbohydrate than with double-stranded DNA, collagen, actin, human cardiac myosin, skeletal myosin and laminin. Because of the reactivity of Sydenham chorea antibodies with GlcNAc, we searched for structurally related host molecules found in brain. The group A carbohydrate epitope is a structural isomer of many neural glycoconjugates, including gangliosides and related glycosphingolipids[19]. Sydenham chorea antibody recognition of gangliosides was of particular interest as gangliosides are highly enriched in the CNS, exhibiting both distinctive developmental patterns and differential distribution within brain[20]. Mimicry between GlcNAc and ganglioside antigen may not be readily apparent. Because gangliosides such as monosialoganglioside GM1 and the group A carbohydrate epitope have little structural similarity or identity, the mimicry between the two molecules would be classified as 'dissimilar', as we have defined in previous studies of mimicry between GlcNAc and peptide structures[18,21,22] (**Supplementary Fig. 1** online). In addition, gangliosides participate in a variety of functions mediated at the cell surface, including signal transduction[23]. The carbohydrate moiety of gangliosides is exposed on the cell surface, making it available for antibody-mediated attack.

To assess the specificity of the GlcNAc-binding monoclonal antibodies from Sydenham chorea, we tested the chorea antibodies against nine gangliosides, two cerebrosides, streptococcal M protein and bovine serum albumin (BSA) in competitive-inhibition ELISAs. Analysis of chorea antibodies by competitive-inhibition ELISA indicated that binding of antibodies to immobilized GlcNAc-BSA was inhibited by soluble gangliosides in a dose-dependent manner, confirming the cross-reactivity between GlcNAc and gangliosides (**Table 1**). Lysoganglioside GM1, a CNS ganglioside that influences neuronal signal transduction[24], was the strongest inhibitor of Sydenham chorea antibody reactivity to GlcNAc-BSA. The concentration of lysoganglioside GM1 required for 50% inhibi-
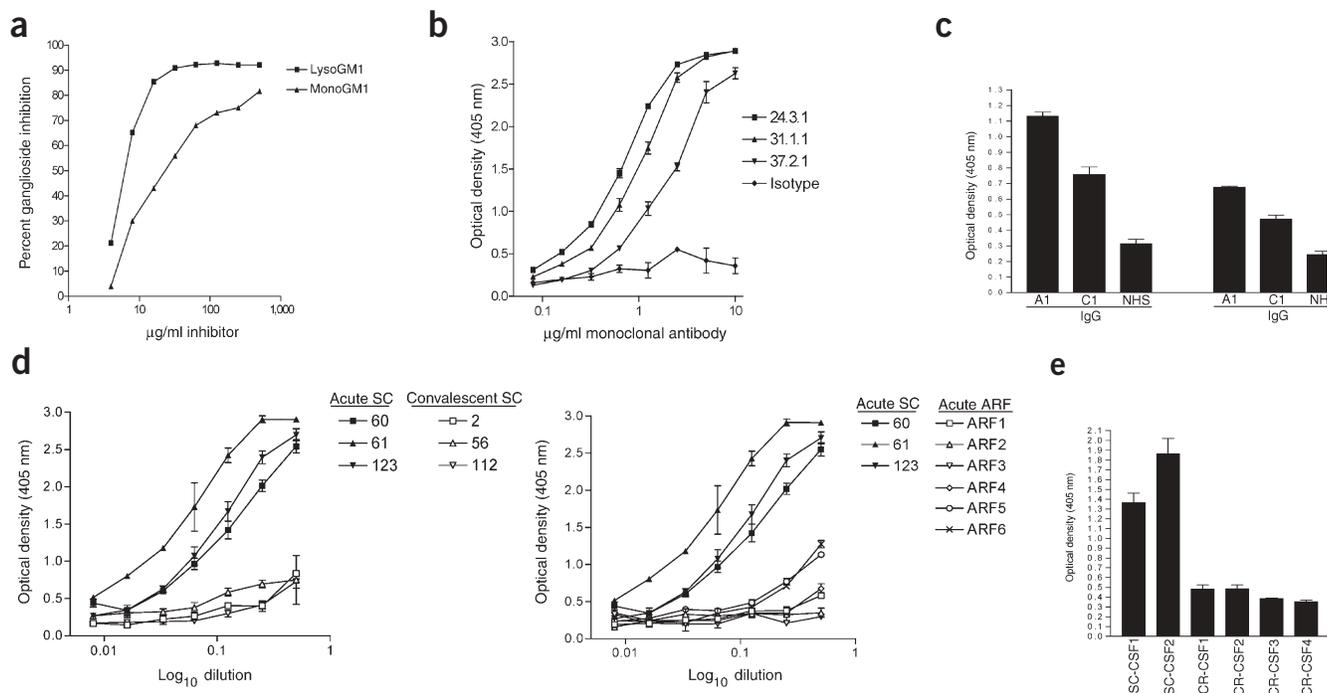
tion of antibody 24.3.1 binding to GlcNAc-BSA was significantly less (6 µg/ml; $P < 0.05$) than for antibodies 31.1.1 and 37.2.1 (9.9 µg/ml and 11.5 µg/ml, respectively), indicating that antibody 24.3.1 had the greatest specificity or avidity for lysoganglioside GM1 (**Table 1**). Lysoganglioside GM1 was a more potent inhibitor of antibody 24.3.1 binding to GlcNAc-BSA than monosialoganglioside GM1 when inhibition curves were compared (**Fig. 2a**). The differences in avidity among the chorea monoclonal antibodies for lysoganglioside GM1 were 24.3.1 > 31.1.1 > 37.2.1. To confirm chorea monoclonal antibody recognition of lysoganglioside GM1, we tested our antibodies by direct ELISA. Dose-response curves showed that all three chorea-derived monoclonal antibodies reacted strongly with lysoganglioside GM1, whereas the isotype control did not (**Fig. 2b**). Therefore, lysoganglioside GM1 is a specific and potent inhibitor of chorea monoclonal antibody binding to streptococcal carbohydrate *in vitro*.

To determine the clinical relevance of Sydenham chorea monoclonal antibody recognition of lysoganglioside GM1, acute and convalescent sera from which Sydenham chorea monoclonal antibodies were derived were tested for lysoganglioside GM1 reactivity. Lysoganglioside GM1–specific IgG and IgM activity in acute Sydenham chorea serum was significantly higher ($P < 0.01$ and $P < 0.05$, respectively) than that of convalescent serum or age-matched normal human sera (**Fig. 2c**). Significantly higher lysoganglioside GM1 antibody concentration was also found in additional Sydenham chorea acute sera but not in matched convalescent sera, or sera from patients with ARF but not Sydenham chorea ($P < 0.0001$; **Fig. 2d**). Acute Sydenham chorea cerebrospinal fluid (CSF) samples had significantly ($P = 0.002$) elevated levels of lysoganglioside GM1–specific IgG compared with control CSF, indicating that antibodies to lysoganglioside GM1 were present intrathecally during active disease (**Fig. 2e**). Therefore, increased levels of lysoganglioside GM1–specific antibodies correlated with active chorea. Additional competitive-inhibition analysis showed that binding of acute chorea sera to GlcNAc-BSA was strongly inhibited by lysoganglioside GM1 (**Table 2**). Three of the acute chorea sera tested were inhibited with concentrations of lysogan-

Table 1 Inhibition of chorea monoclonal antibodies binding to streptococcal group A carbohydrate

Inhibitor (µg/ml)[a]	Antibody		
	24.3.1	31.1.1	37.2.1
Lysoganglioside GM1	6	9.9	11.5
Asialoganglioside GM1	>500	>500	>500
Monosialoganglioside GM1	24	75	125
Monosialoganglioside GM2	>500	201	>500
Monosialoganglioside GM3	>500	389	>500
Disialoganglioside GD1a	>500	>500	>500
Disialoganglioside GD1b	254	210	500
Trisialoganglioside GT1b	>500	>500	>500
Type III gangliosides	>500	>500	>500
Galactocerebroside	>500	>500	>500
Lactocerebroside	>500	>500	>500
M protein	>500	>500	>500
BSA	>500	>500	>500

Competitive-inhibition ELISA of monoclonal antibody reactivity to bound GlcNAc-BSA. [a]Concentration required to produce 50% inhibition of monoclonal antibody reactivity with GlcNAc-BSA.

Figure 2 Lysoganglioside GM1–specific reactivity of chorea monoclonal antibodies, sera and CSF. (**a**) Lysoganglioside GM1 (LysoGM1) inhibited monoclonal antibody 24.3.1 binding to GlcNAc-BSA compared with monosialoganglioside GM1 (MonoGM1). (**b**) All three Sydenham chorea monoclonal antibodies reacted with lysoganglioside GM1. (**c**) Lysoganglioside GM1–specific antibodies in acute (A1) or convalescent (C1) sera from which chorea monoclonal antibodies were derived, and in normal human sera (NHS; $n = 3$). $P < 0.01$ for IgG and $P < 0.05$ for IgM. (**d**) Acute chorea sera (Acute SC) had significantly higher levels of lysoganglioside GM1–specific IgG than either matched convalescent sera (Convalescent SC; $P < 0.0001$) or ARF sera without Sydenham chorea (Acute ARF; $P < 0.0001$). (**e**) Lysoganglioside GM1–specific IgG in CSF from acute chorea or control CSF. SC-CSF, acute chorea CSF; CT-CSF, control CSF.

glioside GM1 similar to those required to produce 50% inhibition of antibody 24.3.1 binding to GlcNAc-BSA. Therefore, the chorea monoclonal antibodies appear to be representative of antibodies found during acute Sydenham chorea in both sera and CSF.

In order to investigate monoclonal antibody neuronal cell surface binding, we reacted Sydenham chorea antibodies with the catecholamine-secreting human neuroblastoma SK-N-SH cell line, used extensively as a model of neuronal cell function. The Sydenham chorea monoclonal antibodies deposited on the neuronal cell surface, with antibody 24.3.1 exhibiting the greatest intensity of binding (**Fig. 3a**, left panel). A 9-O-acetyl-disialoganglioside GD3

Table 2 Lysoganglioside GM1 inhibition of acute chorea sera binding to GlcNAc-BSA

Acute serum	Lysoganglioside GM1 (μg/ml)[a]
60	7.3
61	6.9
101	79.4
118	11.4
123	108.3
A1	20.3

Competitive-inhibition ELISA of serum antibody reactivity by inhibitors to bound GlcNAc-BSA. Sera from patients 60, 61 and 118 were inhibited with concentrations of lysoganglioside GM1 similar to that required to produce 50% inhibition of monoclonal antibody 24.3.1 binding to GlcNAc-BSA. [a]Concentration required to produce 50% inhibition of serum reactivity with antigen.
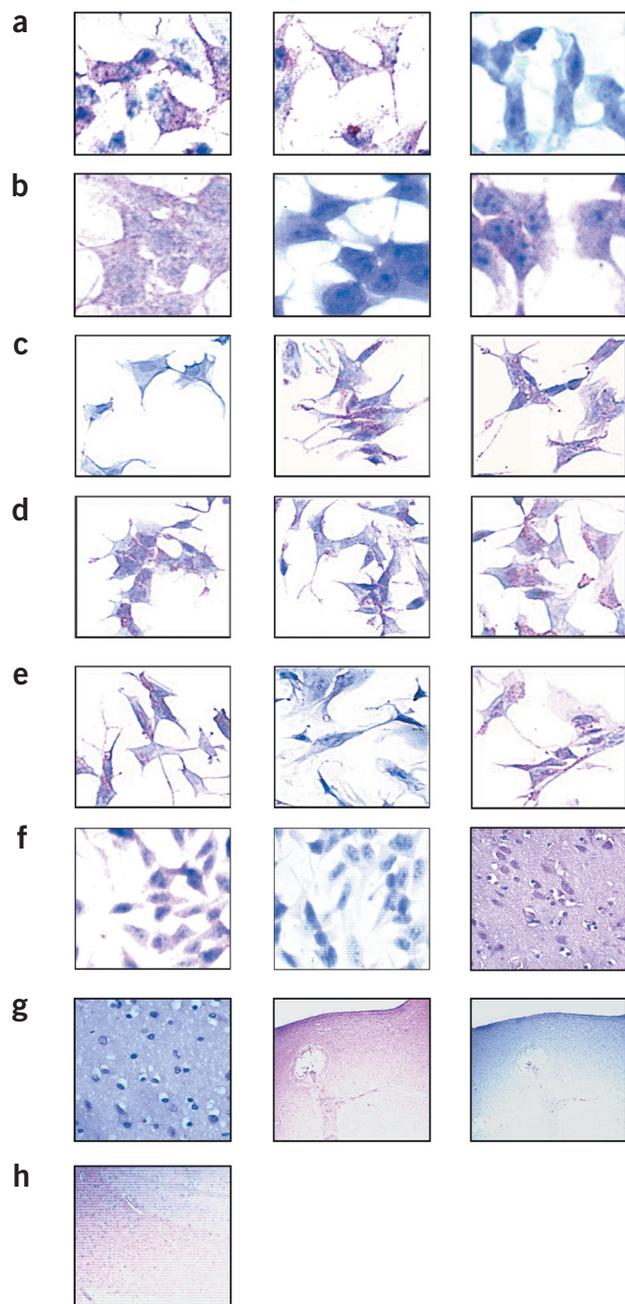
(O-GD3)-specific monoclonal antibody also showed strong binding to the cell surface and served as a positive control for the presence of ganglioside (**Fig. 3a**, middle panel). The IgM isotype control showed no staining (**Fig. 3a**, right panel). To determine the specificity of Sydenham chorea monoclonal antibody reactivity to cell surface antigen, antibody 24.3.1 was tested for inhibition with various gangliosides. In the absence of inhibitor, antibody 24.3.1 labeled the SK-N-SH surface (**Fig. 3b**, left panel). Lysoganglioside GM1 completely abolished antibody 24.3.1 reactivity with the neuronal cells (**Fig. 3b**, middle panel). In contrast, lysoganglioside GM1 did not inhibit cell surface binding of the O-GD3-specific monoclonal antibody (**Fig. 3b**, right panel). All other gangliosides tested, including monosialoganglioside GM1, were unable to block cell surface binding of antibody 24.3.1 (**Fig. 3c–e**). GlcNAc-BSA inhibited antibody 24.3.1 binding to neuroblastoma cells (**Fig 3e**, middle panel), whereas BSA alone did not block chorea antibody 24.3.1 binding (**Fig 3e**, right panel). Acute chorea sera strongly labeled the neuroblastoma cell surface (**Fig. 3f**, left panel), and lysoganglioside GM1 inhibited cell surface recognition of the chorea sera in a manner similar to inhibition of antibody 24.3.1 cell surface binding (**Fig. 3f**, middle panel). Identical results were obtained using caudate putamen tissue derived from human basal ganglia. Strong reactivity of antibody 24.3.1 with the caudate putamen sections (**Fig. 3f**, right panel) was completely blocked by lysoganglioside GM1 (**Fig. 3g**, left panel). Acute chorea serum IgG reacted with brain tissue (**Fig. 3g**, middle panel); IgG deposition was inhibited by lysoganglioside GM1 (**Fig. 3g**, right panel). Acute chorea CSF reacted with the human caudate putamen tissue and showed differential binding,

indicating specificity for antigen in the basal ganglia (**Fig. 3h**). Lysoganglioside GM1 specifically inhibited antigen-antibody interactions of antibody 24.3.1 and chorea acute serum and was a potent and specific inhibitor of Sydenham chorea antibody reactivity to neuroblastoma cells as well as to human brain tissue.

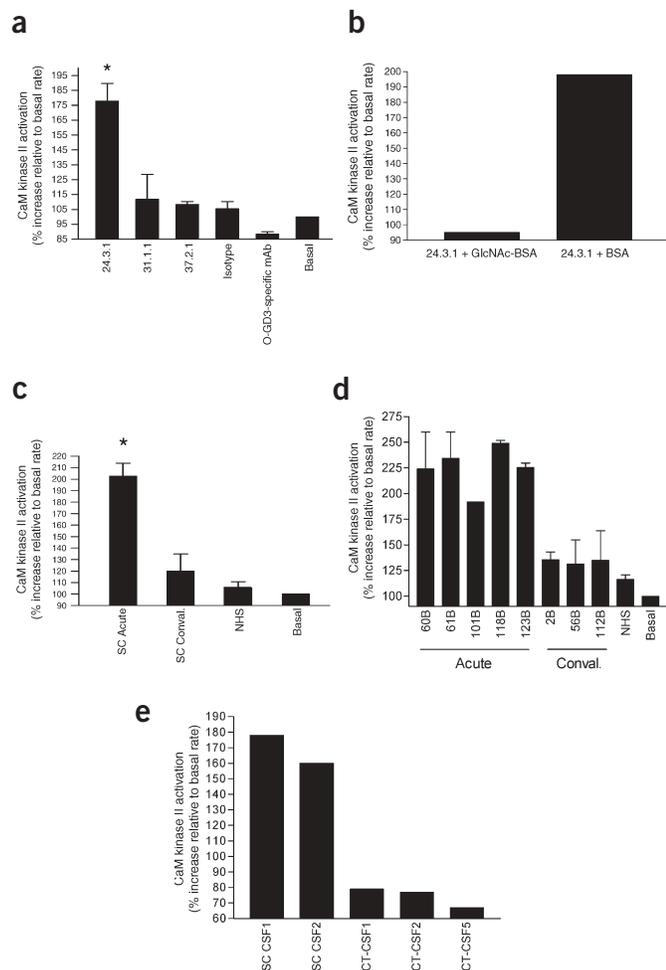## CaM kinase II induction by Sydenham chorea antibodies

The recognition of gangliosides and neuronal cell surface determinants by the monoclonal antibodies, as well as the total inhibition of cell and tissue recognition by lysoganglioside GM1, suggests that Sydenham chorea antibody binding could lead to intracellular signaling. CaM kinase II is highly abundant in brain tissue and is of particular interest because of its function in behavior[25], learning[26],

memory[27], and neurotransmitter synthesis and release[28]. Antibody-mediated activation of CaM kinase II was analyzed to determine whether signal transduction is a potential mechanism of the chorea monoclonal antibodies. Monoclonal antibody 24.3.1 significantly activated CaM kinase II to 76% above the basal level ($n = 3$; $P < 0.05$; **Fig. 4a**). Over 90% of this activity was calcium dependent and was completely abrogated by the addition of autocamtide-2-related inhibitory peptide, a specific inhibitor of CaM kinase[29]. Monoclonal antibodies 31.1.1 and 37.2.1 and the isotype-control antibody did not activate CaM kinase II above the basal rate. Increased avidity or specificity of antibody 24.3.1 for lysoganglioside GM1 over the other two monoclonal antibodies may be responsible for activation of CaM kinase II by antibody 24.3.1 (**Table 1**). The O-GD3-specific monoclonal antibody did not induce kinase activity above the basal rate, suggesting that the ability to activate CaM kinase II is not a property common to all ganglioside-specific antibodies. Antibody 24.3.1–induced CaM kinase II activity was completely inhibited by GlcNAc-BSA but not by BSA alone (**Fig. 4b**), which supports cell-surface inhibition data (**Fig. 3c–e**) and shows that the CaM kinase II activity was the result of an antibody-mediated mechanism. Acute Sydenham chorea serum, from which the monoclonal antibodies were derived, induced CaM kinase II activity to 102% above the basal rate, whereas pooled normal human sera did not activate the kinase ($n = 3$; $P < 0.05$; **Fig. 4c**). Most importantly, convalescent serum obtained 3 months after the acute serum and in the absence of chorea showed no significant increase in CaM kinase II activity ($n = 3$; $P =$ not significant). The testing of an additional five acute and three matched convalescent Sydenham chorea sera (**Fig. 4d**), as well as two acute Sydenham chorea CSF samples (**Fig. 4e**), revealed a similar pattern of CaM kinase induction. Sydenham chorea acute sera did not produce significant activation of cyclic adenosine monophosphate–dependent protein kinase or protein kinase C (data not shown). No significant increase in CaM kinase II activity was seen in six sera from patients with active ARF without Sydenham chorea (0–61.5% above basal rate) and seven streptococcal pharyngitis sera (0–47% above basal rate; data not shown). The ability to induce CaM kinase II activity correlated with increased ganglioside-specific antibody concentrations (**Fig. 2**).

The induction of CaM kinase II by monoclonal antibody 24.3.1 paralleled the CaM kinase II activity of acute Sydenham chorea sera and CSF. Because antibody 24.3.1 has features of acute Sydenham chorea sera, it will be a valuable tool to further identify cell surface antigen(s) responsible for CaM kinase II induction as well as downstream effects of kinase activation and their relationship to pathogenesis.

Figure 3 Chorea antibodies recognize neuroblastoma cell surface and caudate putamen. (**a–e**) Staining with monoclonal antibody 24.3.1 (left), O-GD3-specific antibody (middle) and isotype control (right). (**b**) Monoclonal antibody 24.3.1 without inhibitor (left), 24.3.1 inhibited by lysoganglioside GM1 (middle) and lysoganglioside GM1 inhibition of O-GD3-specific antibody (right). (**c**), Lysoganglioside GM1 inhibited 24.3.1 (left) but not monosialoganglioside GM1 (middle) or asialoganglioside GM1 (right). (**d**) Inhibition of 24.3.1 binding by disialoganglioside GD1a (left), disialoganglioside GD1b (middle) and trisialoganglioside GT1b (right). (**e**) Type III gangliosides (left). GlcNAc-BSA inhibited 24.3.1 (middle), whereas BSA alone did not (right). (**f–h**) Chorea sera (left) was inhibited by lysoganglioside GM1 (middle). Human caudate putamen was labeled with 24.3.1 (right). (**g**), Lysoganglioside GM1 inhibited 24.3.1 (left). Chorea serum reactivity to caudate putamen (middle) was inhibited by lysoganglioside GM1 (right). (**h**), Acute chorea CSF reacted to a specific region within human caudate putamen tissue (left).

Figure 4 Induction of CaM kinase II by Sydenham chorea monoclonal antibodies and sera. (**a**) Monoclonal antibody 24.3.1 significantly ($P < 0.05$) induced CaM kinase II activity above the basal rate, whereas other monoclonal antibodies (mAb) did not. (**b**) GlcNAc-BSA abolished monoclonal antibody 24.3.1-induced CaM kinase II activity in neuroblastoma cells, whereas BSA alone had no effect. (**c**) Acute serum from the monoclonal antibody source significantly ($P < 0.05$) activated CaM kinase II. SC, Sydenham chorea sera; NHS, normal human sera. (**d**) CaM kinase II activity was induced by additional acute Sydenham chorea sera. Conval., convalescent. (**e**) Acute chorea CSF (SC-CSF) induced CaM kinase II. CT-CSF, control CSF.

Previous reports support our hypothesis. Ganglioside-specific antibodies can affect the physiologic homeostasis of neuronal cells. Antibodies to human monosialoganglioside GM1 induced calcium flux in neuroblastoma cells through modulation of L-type voltage-gated calcium channels[30]. Only monosialoganglioside GM1–specific antibodies of the IgM isotype could affect intracellular signaling, suggesting that the pentameric structure of IgM is required to promote cross-linking of cell surface antigens that potentially elicit cell signaling. Although our data showed that the chorea monoclonal antibodies and sera have greater specificity and avidity for lysoganglioside GM1, we do not know the exact antigen in brain. Pathogenic antibodies in Sydenham chorea may bind directly to gangliosides or indirectly cause aggregation of neuronal receptors by binding to gangliosides, which trigger a signal transduction cascade. Ganglioside-specific monoclonal antibodies regulate signal transduction in neuronal cells through cross-linking of lipid raft-associated proteins[31]. Similar mechanisms may explain how antibodies in Sydenham chorea, such as 24.3.1, induce neuronal dysfunction by CaM kinase II activation.

Antibodies to gangliosides, including monosialoganglioside GM1–specific antibodies, have been reported in other neurologic disorders including Guillain-Barré syndrome (GBS), a postinfectious, antibody-mediated disease[32,33,34]. In contrast with Sydenham chorea, GBS is characterized predominantly by nerve conduction block[35,36], and studies with sera from patients with Miller-Fisher syndrome, a variant of GBS, have shown the presence of ganglioside GQ1b–specific antibodies capable of complement-mediated cytotoxicity at the motor nerve terminal[37,38]. Localization of Sydenham chorea monoclonal antibodies to the neuronal cell membrane indicated potential antibody-induced, complement-mediated cytotoxicity. Sydenham chorea monoclonal antibodies were incapable of mediating complement-dependent killing of human neuronal cell lines, however, indicating that the monoclonal antibodies mediate their effects through other mechanisms.

Our data indicate molecular mimicry between GAS and the brain and identify the molecular targets of antibody cross-reactivity as well as the humoral mechanisms in Sydenham chorea. Our study has linked antibodies in Sydenham chorea to neuronal cell surface antigens and the induction of kinase activity. Although monoclonal antibodies 31.1.1 and 37.2.1 reacted with the neuronal cell surface and basal ganglia in a manner similar to antibody 24.3.1, they did not activate CaM kinase II. The stronger avidity of antibody 24.3.1 for ganglioside may explain its ability to activate CaM kinase II.

Antibodies in Sydenham chorea, such as monoclonal antibody 24.3.1, may promote signal transduction that leads to the release of excitatory neurotransmitters. Although the consequence of CaM kinase II activation in the development of Sydenham chorea is not entirely clear, it has been postulated that the clinical manifestations of Sydenham chorea arise from an imbalance of neurotransmitters

DISCUSSION

Altering neuronal cell function through signal transduction is a mechanism by which neuronal-specific autoantibodies may produce neurologic dysfunction in Sydenham chorea. In our study, monoclonal antibody 24.3.1 activated CaM kinase II in human neuroblastoma cells, and kinase activation was abolished by a specific CaM kinase II inhibitor. The ability of antibody 24.3.1 to activate CaM kinase II was similar to acute Sydenham chorea sera, which induced kinase activity at even greater levels than antibody 24.3.1. The convalescent sera, obtained in the absence of chorea, did not activate CaM kinase II significantly above normal levels. Induction of CaM kinase II by acute Sydenham chorea CSF indicated that the ability to stimulate CaM kinase II is present intrathecally. The data support the hypothesis that antibody-induced signal transduction is associated with clinical symptoms. Sera from acute ARF without Sydenham chorea or from acute streptococcal pharyngitis were not capable of producing significant increases in CaM kinase II activity, but did show low levels of CaM kinase II induction, suggesting that a threshold concentration of antibody may be required for induction of cell signaling. The higher avidity of Sydenham chorea antibody 24.3.1 for lysoganglioside GM1, together with evidence that significantly higher levels of lysoganglioside GM1-specific antibody were present in Sydenham chorea acute sera and CSF, suggests that avidity, specificity and concentration of antibody to ganglioside may be triggers for cell signaling.

or inflammation within the basal ganglia. Although gangliosides have been implicated as a cell surface target antigen, additional mediators of intracellular signaling may be identified. CaM kinase activation has recently been associated with increased dopamine release in brain slices[39]. In our studies, preliminary data show that Sydenham chorea monoclonal antibody 24.3.1 elicited [^{3}H]dopamine release from SK-N-SH cells (data not shown), which may be particularly applicable, as Sydenham chorea patients have been treated with dopamine receptor blockers such as halperidol[40].

In summary, our study revealed a new mechanism of action by antibodies against streptococci and brain, which may produce the neurological effects that characterize Sydenham chorea. Monoclonal antibodies and acute Sydenham chorea sera identified gangliosides as autoantigens in the disorder and showed that autoantibody-mediated signal transduction by CaM kinase II may function as the mechanism of immunopathogenesis in Sydenham chorea. Our data may provide insight into other confounding neurologic and psychiatric disorders that may be related to Sydenham chorea, including childhood obsessive-compulsive disorder, Tourette syndrome and attention deficit and hyperactivity disorders[41,42]. The antibody-mediated cell signaling mechanism we have described may be applicable to other antibody-mediated neuropsychiatric disorders that affect children worldwide.

METHODS

Patient and hybridoma production. We obtained peripheral blood from a 14-year-old female diagnosed with ARF, according to approved research protocol by the University of Oklahoma Institutional Review Board. Informed consent was obtained from all human subjects. We stimulated peripheral blood mononuclear cells with whole GAS membranes in Iscove's modified Dulbecco's medium (IMDM; Gibco-BRL), fused them with the K6H6/B5 cell line (CRL 1823; American Type Culture Collection) and selected on IMDM with hypoxanthine, aminopterin and thymidine as previously described[18]. We cloned hydridomas by limiting dilution to produce Sydenham chorea monoclonal antibodies of the IgM κ-light chain class. We maintained clones in DMEM with 10% FCS under standard cell culture conditions. We purified monoclonal antibodies by affinity chromatography using Kappalock resin (Zymed) according to the manufacturer's instructions.

Cell lines. We obtained the human SK-N-SH (HTB-11) neuroblastoma line from the American Type Culture Collection. Cells were routinely cultured with F12-DMEM (Gibco-BRL) media containing 10% FCS, 1% penicillin and streptomycin and 0.1% gentamycin at 37 °C in 5% CO_2.

Antigens. Pepsin fragment of streptococcal M5 protein, GlcNAc-BSA, M5 streptococcal membranes and human cardiac myosin were made as previously described[18,21,22]. Recombinant M6 protein was a gift from V. Fischetti (Rockefeller University). We purchased all other antigens from Sigma.

ELISA. We coated 96-well polyvinyl, Immunolon-4 microtiter plates (Dynatech Laboratories) with 10 μg/ml of antigen or 20 μg/ml lysoganglioside GM1 overnight at 4 °C. Plates were reacted with monoclonal antibody, sera or CSF overnight at 4 °C. We detected antibody binding using specific alkaline phosphatase–conjugated secondary antibody (Sigma). We developed plates with 2 mg/ml *p*-nitrophenylphosphate-104 substrate (Sigma) at 100 μl/well. Optical density was quantified at 405 nm in an Opsys MR microplate reader (Dynex Technologies).

Competitive-inhibition ELISA. We conducted the competitive inhibition ELISA in triplicate as previously described[5]. We prepared 1 mg/ml inhibitor solutions and serially diluted them from 500 to 4 μg/ml. We mixed inhibitors with an equal volume of 10 μg/ml of monoclonal antibody or 1:500 dilution of serum and incubated at 37 °C for 30 min and overnight at 4 °C. We added 50 μl of antibody-inhibitor mixture to 96-well microtiter plates coated with 10 μg/ml GlcNAc-BSA and incubated overnight at 4 °C. The remainder of the assay was performed as described above.

Immunohistochemistry. We plated four-well chamber tissue culture slides with 5×10^4 SK-N-SH cells per chamber overnight[3,5,7]. We added monoclonal antibodies to individual chambers and incubated at 37 °C in 5% CO_2 for 1 h, followed by addition of buffered formalin. Cell surface–bound antibody was detected with specific biotin-conjugated secondary antibody (Jackson ImmunoResearch) and alkaline phosphatase–conjugated strepavidin (Jackson ImmunoResearch). We detected antibody binding using Fast Red Substrate (Biogenex) and counterstained cells with Mayer's hematoxylin (Biogenex). For inhibition experiments, cells were formalin-fixed before monoclonal antibody binding. For tissue staining, we incubated monoclonal antibody, sera and CSF with tissue for 1 h at room temperature and developed as previously described[5].

Protein kinase assays. We plated SK-N-SH cells at 1×10^7 overnight under standard cell culture conditions and preincubated with F12 media supplemented with 2 mM $CaCl_2$, 3 mM KCl and 0.2 mM $MgCl_2$ for 30 min at 37 °C in 5% CO_2. We incubated cells with monoclonal antibodies, sera or CSF in a final volume of 15 ml of media. The basal control was incubated with supplemented F12 medium alone. We incubated cells and antibodies for 30 min, mechanically dislodged cells from the flask and centrifuged them. We solubilized cell pellets in 0.3 ml of protein extraction buffer with proteinase inhibitors and subjected them to homogenization. Protein kinase activity was measured using CaM kinase assay system, cyclic adenosine monophosphate–dependent protein kinase assay system, and protein kinase C assay system (Promega) according to the manufacturer's instructions. In brief, 5 μl of cell lysate was incubated with 50 μM peptide substrate and [γ-^{32}P]ATP for 2 min at 30 °C. We spotted the sample onto the capture membrane and washed in 2 M NaCl, followed by 2 M NaCl with 1% H_3PO_4. Radioactivity retained on the membrane was determined by scintillation counting. We assessed calcium-independent CaM kinase II activity by adding EGTA to the assay. We did CaM kinase II inhibition by adding 10 μM autocamtide II–related inhibitory peptide (Calbiochem) to the reaction mixture before adding the cell lysate[43]. We did competitive-inhibition ELISAs with 3 mg/ml GlcNAc-BSA. Inhibition with lysoganglioside GM1 was not done because of cell toxicity[44]. We determined the specific activity of the enzyme in pmol per min per μg for each sample and presented the results as percentages of the basal rate.

Statistics. Bar graphs were analyzed by two-tailed *t* test. Dose-response curves were analyzed by two-way analysis of variance (ANOVA).

Note: Supplementary information is available on the Nature Medicine website.

COMPETING INTERESTS STATEMENT
The authors declare that they have no competing financial interests.

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