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Tubulin Is a Neuronal Target of Autoantibodies in Sydenham's Chorea[1]

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Sydenham's chorea is a CNS disorder and sequela of group A streptococcal infection where deposition of Abs in brain may result in movement and neuropsychiatric abnormalities. We studied human mAbs 24.3.1, 31.1.1, and 37.2.1 derived from chorea and selected for cross-reactivity with group A streptococci and brain Ags. Our novel findings reveal that Sydenham's chorea mAbs target a 55-kDa brain protein with an N-terminal amino acid sequence of MREIVHLQ corresponding to β-tubulin. Chorea mAb specificity for purified brain tubulin was confirmed in ELISA and Western immunoblot, and significant levels of anti-tubulin IgG were found in acute chorea sera and cerebrospinal fluid. Lysoganglioside G_{M1} inhibited binding of chorea mAbs to tubulin and mAb reactivity with human caudate and putamen brain sections was blocked by anti-tubulin mAb. The chorea mAbs labeled both intra- and extracellular Ags of a neuronal cell line providing evidence suggesting mimicry between intracellular brain protein tubulin and extracellular lysoganglioside. In addition, chorea mAb 24.3.1 and acute chorea sera induced calcium/calmodulin-dependent protein kinase II activity in human neuronal cells. Nucleotide sequence analysis of the chorea mAb V_H genes revealed that mAb 24.3.1 V_H gene was encoded by the V_H1 germline gene family which encodes other anti-ganglioside V_H genes associated with motor neuropathies. mAb recognition of tubulin and the neuronal cell surface with initiation of cell signaling and dopamine release supports an emerging theme in autoimmunity whereby cross-reactive or polyreactive autoantibodies against intracellular Ags recognize cell surface epitopes potentially leading to disease. ***The Journal of Immunology,*** **2007, 178: 7412–7421.**

Although polyreactive Abs were discovered almost 20 years ago with the advent of mAbs, the role these Abs may play in immunopathogenesis has not been recognized. The Ags identified for polyreactive Abs were generally intracellular cytoskeletal proteins or DNA (1, 2). However, work on cross-reactive or polyreactive Abs has progressed, and it is becoming increasingly clear that they may cause disease by cross-reaction with extracellular Ags at the cell surface (3, 4). The reaction with the cell surface or entrapment in the extracellular matrix may lead to inflammatory responses targeting the tissue for autoimmune disease. The theme emerging from our work on cross-reactive Abs in autoimmunity and infection is that the Abs recognize intracellular and extracellular Ags where at the cell surface these Abs can lead to cytotoxicity or altered cell signaling in disease.

In this study, we focus on autoantibodies from acute rheumatic fever (ARF)[3] which follows a group A streptococcal infection and is an autoimmune inflammatory disease affecting the heart, joint, skin, or brain (5, 6) In susceptible individuals, an immune response to streptococcal Ags appears to initiate events that result in development of the different clinical manifestations of ARF. It has been well-documented that group A streptococci promote the generation of cross-reactive or polyreactive Abs through molecular mimicry between streptococcal and host Ags (5, 7). Cross-reactive Abs, particularly against the group A carbohydrate Ag, may play a role in the clinical manifestations of ARF (3, 4).

The group A carbohydrate is a structural component of the streptococcal cell wall and has been implicated in provoking Abs that cross-react with a variety of host tissues (3, 8–10). Elevated anti-streptococcal Ab responses against the group A streptococcal carbohydrate Ag persisted in patients with ARF and rheumatic heart disease and is supporting evidence for the hypothesis that anti-carbohydrate Abs may play a role in disease (11). *N*-acetyl-β-D-glucosamine (GlcNAc) is an epitope of the group A carbohydrate and our previous work suggests that Abs against GlcNAc may play a role in injury to the valve surface endothelium in rheumatic carditis and in altered signal transduction in the brain in Sydenham's chorea (SC) (3, 4, 12). The group A carbohydrate consists of a polyrhamnose core in alternating 1,2- and 1,3-linkages and it has been suggested that the terminal *O*-linked GlcNAc residue is important in the induction of cross-reactive Abs due to its structural similarity to many host glycoconjugates (13, 14). Importantly, previous studies using murine and human mAbs from patients with ARF identified the GlcNAc epitope as well as group A streptococcal M protein as cross-reactive streptococcal Ags capable of cross-reactivity with host α-helical proteins including myosin, tropomyosin, keratin, vimentin, and laminin. The cross-reactivity demonstrated that Abs were capable of binding Ags of different molecular compositions (1, 3, 10, 15–17). Although molecular mimicry between GlcNAc and cytoskeletal proteins has

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[3] Abbreviations used in this paper: ARF, acute rheumatic fever; SC, Sydenham's chorea; GlcNAc, *N*-acetyl-β-D-glucosamine; CaM kinase II, calcium-calmodulin-dependent protein kinase II; PVDF, polyvinyl difluoride; O-DG3, 9-*O*-acetyl-disialoganglioside G_{D3}.

been characterized in heart disease, similar cross-reactivity with α-helical proteins has not been investigated in rheumatic streptococcal neurological disorders.

Immune responses to GlcNAc and host Ags may be important in development of streptococcal neurological disorders and in the generation of anti-GlcNAc/anti-neuronal Abs found in patients with disease. SC is the neurological manifestation in group A streptococcus-induced acute rheumatic fever (5). This CNS disorder develops in 10–30% of rheumatic fever cases and is characterized by both involuntary movements and neuropsychiatric disturbances (18, 19). Sera from SC patients were shown to react with neuronal cytoplasmic Ags of the striatum, indicating that Abs in SC may recognize intracellular protein Ags (20). In addition, brain-specific reactivity of SC sera was adsorbed with membranes of rheumatogenic streptococcal strains. Recently, we have shown that human mAbs from SC were reactive with GlcNAc and brain-derived lysoganglioside G_{M1} and were capable of directing calcium-calmodulin-dependent protein kinase II (CaM kinase II) activation in human neuronal cells, however, Ab recognition of host intracellular proteins has not been established (4).

In an effort to gain a more precise understanding of chorea Ab specificities, mAbs derived from SC were studied for reactivity with brain proteins and were found to react with the cytoskeletal protein tubulin in addition to GlcNAc, group A streptococci, and gangliosides. Acute SC sera and cerebrospinal fluid showed elevated levels of tubulin-specific IgG in contrast to matched SC convalescent sera and sera from ARF without SC. The mAbs strongly reacted with basal ganglia in addition to labeling the extracellular surface of human neuronal cells. Nucleotide sequence analysis of SC mAb V_H and V_L genes revealed that they were encoded by the same V gene families as V genes encoding anti-ganglioside Abs associated with motor neuropathies (21). In summary, our data shows that autoantibodies in SC not only signal and bind to the cell surface but recognize the intracellular α-helical protein tubulin.

Materials and Methods

Patient and hybridoma production

Peripheral blood was obtained from a 14-year-old female diagnosed with rheumatic fever according to the Jones criteria. Patient presented with chorea and mild carditis with an anti-streptolysin O titer of 1250 at the time of acute disease. Sera were obtained from the patient during the acute phase of disease and 3 mo later during convalescence. PBMC were obtained from whole blood by Histopaque-1077 Hybri-Max (Sigma-Aldrich) gradient and stimulated with group A streptococcal membranes for one week in IMDM (Invitrogen Life Technologies) containing 10% human AB serum. Cells were fused with K6H6/B5 hybridoma fusion partner, a nonsecreting human × mouse heterohybridoma line (ATCC CRL 1823; American Type Culture Collection) using polyethylene glycol 1000 as previously described (10). Hybridomas were allowed to proliferate for 1 wk and cell culture supernatants were subsequently tested for Ig production. Subsequently, hybridomas were cloned by limiting dilution and then subcloned twice to produce B cell hybridoma clones 24.3.1, 31.1.1, and 37.2.1. Isotype analysis showed that all three mAbs were IgM/κ class. Clones were maintained in DMEM with 10% FCS under standard cell culture conditions.

Affinity chromatography

Hybridoma lines were grown in T300 cm^3 flasks and exhausted cell culture supernatants of the three anti-GlcNAc cell lines (24.3.1, 31.1.1, and 37.2.1) were used for mAb purification. Particulate material was removed from the supernatants by centrifugation before column purification and the pH of the supernatants was adjusted to pH 7.0. Ig from hybridoma supernatants was absorbed to a 1-ml column of Kappalock affinity matrix (Zymed Laboratories) and washed with 5 column volumes of PBS before elution. mAb was eluted from the column using 0.5 M acetic acid (pH 3.0) and the fractions were immediately neutralized with 0.2 ml of 1 M Tris-Cl (pH 9.0). Fractions were pooled and dialyzed overnight in 4 L of PBS (pH 7.2) at 4°C. Purified mAbs were filter sterilized and treated with 0.001% gentamicin. Protein concentration of the purified mAbs was determined by Branford assay (Bio-Rad). To ascertain the relative purity of the purified mAbs, 50 μl of each sample was separated on a 7% SDS-PAGE both in the presence and absence of DTT and stained with Coomassie blue. mAbs were stored at 4°C until use.

Cell lines

The human SK-N-SH (ATCC HTB-11) neuronal cell line was obtained from the American Type Culture Collection. The NT2 precursor human neuronal cell line (hNT) was purchased from Stratagene. Cells were routinely cultured with F12-DMEM (Invitrogen Life Technologies) medium containing 10% FCS (HyClone), 1% penicillin and streptomycin, and 0.1% gentamicin at 37°C, 5% CO_2.

Reagents

The following Ags were used in this study to determine the fine specificity of human mAbs 24.3.1, 31.1.1, and 37.2.1: actin, asialoganglioside G_{M1}, BSA, lysoganglioside G_{M1}, and monoganglioside G_{M1} were purchased from the Sigma-Aldrich. Tubulin consisting of both α and β subunits was purchased from ICN Pharmaceuticals. Pepsin cleavage products of streptococcal M5 protein (pepM5), GlcNAc-BSA, and serotype M5 whole streptococcus were prepared as previously described (1, 15). The anti-9-*O*-acetyl-disialoganglioside G_{D3} (O-DG3) mAb was obtained from Sigma-Aldrich and anti-β-tubulin mAb was purchased from ICN Pharmaceuticals.

BALB/c mouse brain extracts

Whole brains were harvested from 6-wk-old BALB/c mice and homogenized in a buffer containing a mixture of protease inhibitors (Roche Diagnostics) followed by centrifugation for 45 min at 21,000 × *g*. The brain lysate was stored in aliquots at −80°C until use.

Western blots

A total of 70 μg of BALB/c brain extract or 20 μg of purified tubulin were separated on a 7% SDS-PAGE using a 5% stacking gel and electroblotted to polyvinyl difluoride (PVDF) membranes (Bio-Rad) overnight at 4°C in Tris-glycine buffer. The blot was blocked with 5% dry milk in PBS for 1 h at room temperature and individual lanes were separated into strips and incubated with mAbs or sera diluted 1/100 overnight at 4°C. The strips were washed and incubated with peroxidase-conjugated goat anti-human IgM or IgG secondary Ab for 1 h. The blot was visualized using a solution of 4-chloro-naphthol (Sigma-Aldrich) and hydrogen peroxide as previously described (1). The bands developed from the immunoblot were compared with the relative position of separated proteins on Coomassie blue-stained immobilized PVDF strips. The relative masses of proteins were determined in kilodaltons using prestained molecular mass protein standards (Bio-Rad).

Protein sequencing

An approximate 55-kDa protein from BALB/c brain extracts was removed from Coomassie blue-stained PVDF and submitted for amino acid sequencing by Edman degradation performed at Protein Sequencing Laboratory (University of Oklahoma Health Sciences Center). The N-terminal amino acid sequence obtained was analyzed by fastA genetic database search.

ELISA

96-well Immunolon 4 microtiter plates (Dynatech Laboratories) were coated with 10 μg/ml of each Ag (tubulin, GlcNAc-BSA, and BSA) in 0.015 M carbonate buffer (pH 9.6) overnight at 4°C. To test reactivity with group A streptococci, whole M type 5 streptococci were fixed to polyvinyl chloride plates with glutaraldehyde as previously described (1). Plates were blocked with 1% BSA in PBS for 1 h. Primary Ab was detected with alkaline phosphatase-conjugated goat anti-human secondary Ab specific to either IgG or IgM as appropriate (Sigma-Aldrich) diluted 1/500 in 1% BSA-PBS. Plates were further incubated for 60 min at room temperature and washed with PBS. Plates were developed with 1 mg/ml *p*-nitrophenyl phosphate colorimetric substrate (Sigma-Aldrich) and the OD was determined at 405 nm in an Opsys MR microplate reader (Dynex Technologies).

Competitive inhibition ELISA

Competitive inhibition ELISA was performed in triplicate as previously described (3, 8). Inhibitors were prepared as 1 mg/ml solutions in PBS (pH 7.2) and serially diluted from 500 to 4 μg/ml. The diluted inhibitors were mixed with an equal volume of 10 μg/ml of each mAb and incubated at 37°C for 30 min followed by an overnight incubation at 4°C. The mAb-inhibitor mixture was added to 96-well microtiter plates coated with 10

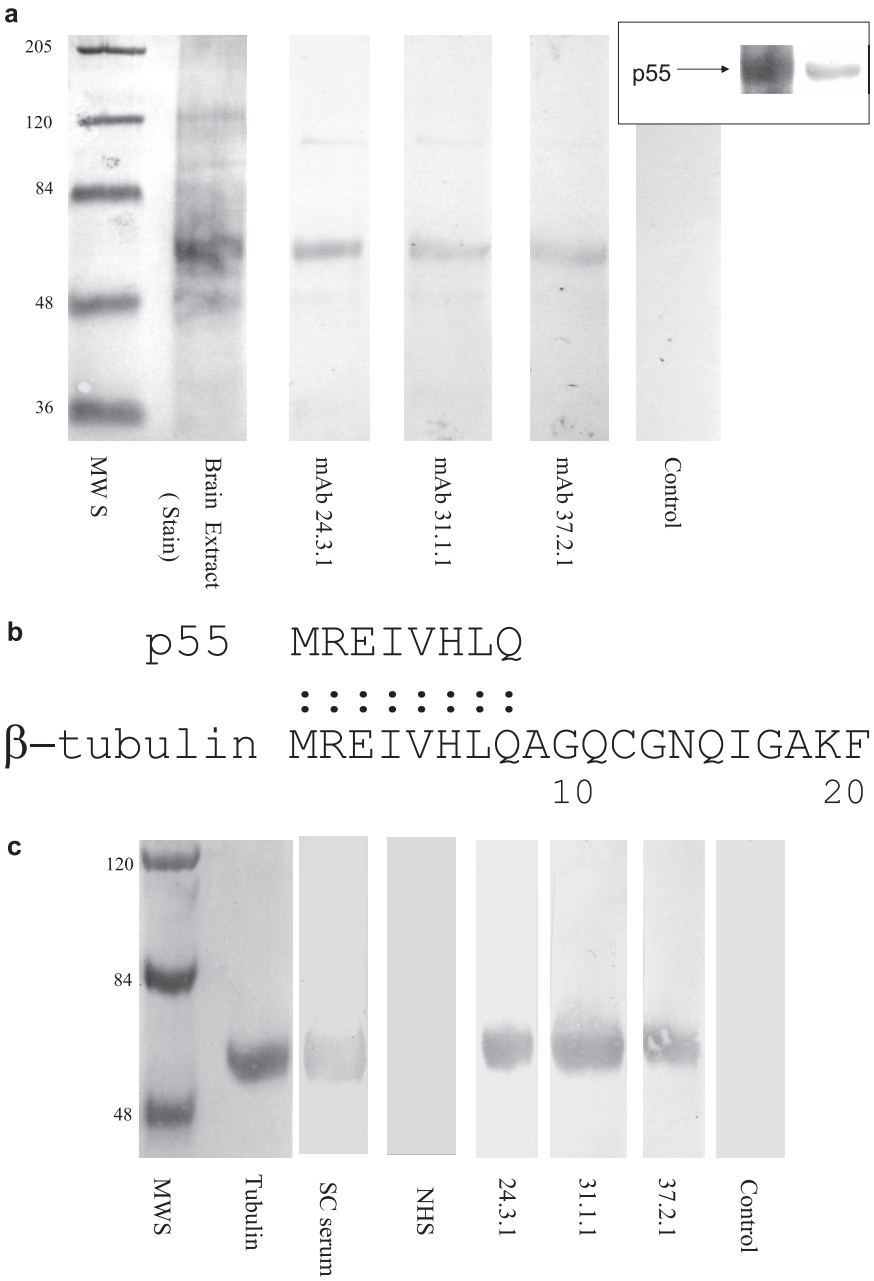

FIGURE 1. *a–c*, SC mAb recognition of brain-derived tubulin. *a*, SC mAbs 24.3.1, 31.1.1, and 37.2.1 recognized a 55-kDa protein separated from BALB/c brain homogenate by SDS-PAGE. Control blot was treated only with secondary peroxidase-labeled Abs. *Inset*, Magnified image of p55 from brain homogenate and mAb 24.3.1 reactivity to the protein. *b*, The 55-kDa protein recognized by chorea mAbs was excised from Coomassie blue-stained PVDF membrane and subjected to N-terminal sequencing by Edman degradation. The sequence of p55 was analyzed by fastA genetic database search. p55 was found to have 100% identity in an 8-aa overlap with the N terminus of β-tubulin. The human and murine tubulin amino acid sequence is conserved. *c*, Purified tubulin separated by SDS-PAGE was immobilized to PVDF membrane and used in immunoblot analysis. SC patient serum from which the mAbs were derived and all three SC mAbs bound to purified tubulin whereas pooled age-matched normal human sera (NHS) exhibited no binding. MWS, molecular mass standards.

μg/ml GlcNAc-BSA or tubulin in bicarbonate buffer. Samples were allowed to incubate overnight at 4°C. The remainder of the assay was performed as described above. Percentage of inhibition was calculated as: 100 × (1-(A405 inhibitor + mAb/A405 PBS + mAb)). Maximal (100%) reactivity was determined by mAb-PBS incubation without inhibitors.

Immunohistochemistry

For formalin-fixed tissues, human caudate/putamen tissue was fixed overnight in 10% buffered formalin, sectioned (5-μm thick), and mounted onto Microprobe ProbeOn Plus slides. Mounted tissue was baked for 60 min and deparaffinized followed by rehydration using graded ethanol. The tissue was then washed four times in PBS and blocked with a protein blocker for 15 min at room temperature followed by four additional washes in PBS. The sections were incubated with 20 μg of affinity purified SC mAbs, as well as 20 μg/ml anti-tubulin mAb (Sigma-Aldrich), anti-ganglioside *O*-acetyl G_{D3} mAb (Sigma-Aldrich), or pooled normal human IgM that served as an isotype control (Sigma-Aldrich) for 1 h at room temperature in a humidified chamber. Slides were washed and incubated for 30 min at room temperature with biotin-conjugated goat anti-human IgM or biotin-conjugated goat anti-mouse IgG/IgM polyclonal Abs diluted 1/500 in PBS (Jackson ImmunoResearch Laboratories). Tissues were further incubated with alkaline phosphatase-conjugated streptavidin (Jackson ImmunoResearch Laboratories) at 1/500 dilution as before. Ab binding was detected using Fast Red Substrate (Biogenex) and tissue was counterstained with Mayer's hematoxylin (Biogenex).

For intracellular Ab binding, NT2 neuronal cells (Stratagene) were plated at a concentration of 5×10^4 cells/well in 4-well chamber cell culture slides overnight under standard tissue culture conditions. Cells were washed three times with PBS and permeabilized with ice-cold acetone at room temperature for 5 min followed by additional 30-min incubation with fresh acetone at 4°C. Slides washed three times in PBS were allowed to air dry. Purified mAbs 24.3.1, 31.1.1, 37.2.1, anti-tubulin mAb, anti-*O*-acetyl G_{D3} mAb, or pooled human IgM at concentration of 20 μg/ml were incubated with the cells for 1 h in humidified chamber. Slides were washed three times in PBS and the remainder of the experiment was conducted as described in the previous section above for tissue sections.

For extracellular Ab binding, 4-well chamber tissue culture slides were plated with 5×10^4 cells/chamber NT2 neuronal cells overnight. The cells were washed three times with medium containing no FCS and 50 μg of purified test mAb in 200 μl of F12 medium were added to individual chambers and allowed to incubate at 37°C 5% CO_2 for 1 h. At the end of the incubation, cells were washed three times with F12 medium. The slides were blocked with 5% goat serum in F12 medium for 15 min and washed three additional times. One milliliter of a 0.2 M paraformaldehyde-lysine-HCl

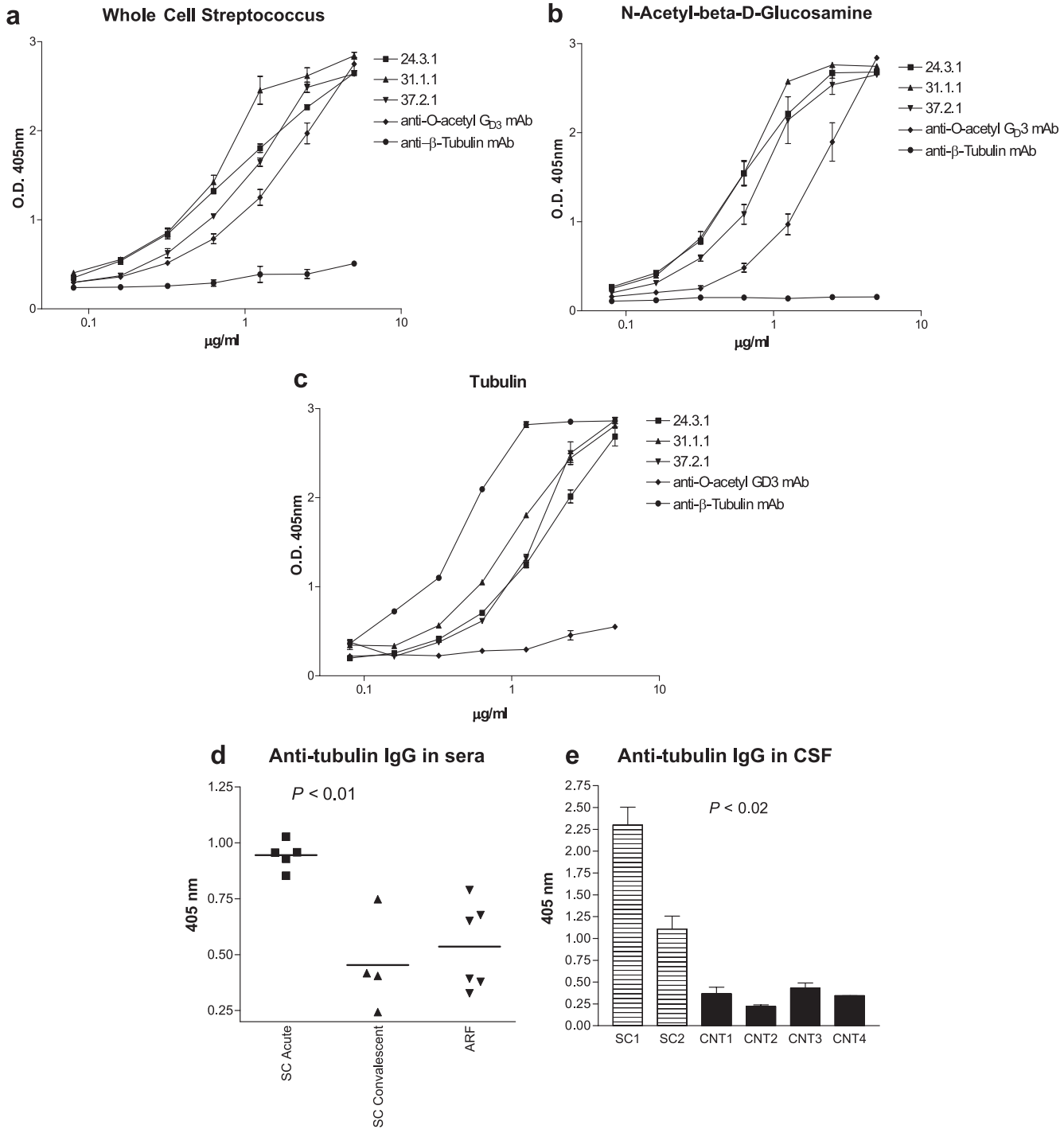

FIGURE 2. *a–e*, Dose-response curves demonstrating Ag reactivity of human chorea mAbs to tubulin and streptococcal Ags. The three chorea mAbs are shown to have high activity for both (*a*) whole group A streptococci (M5 serotype) and (*b*) GlcNAc the carbohydrate Ag of group A streptococci. The commercial anti-ganglioside mAb also reacted with streptococcal Ags while the anti-β-tubulin mAb shows no binding. *c*, Anti-β-tubulin mAb and SC mAbs, but not the anti-ganglioside mAb, bind to purified tubulin. *d*, SC acute sera show elevated reactivity of tubulin-specific Ab in comparison to matched convalescent sera or sera from acute rheumatic fever without chorea. Sera was tested at a 1/800 dilution to determine difference in IgG reactivity. *e*, Acute chorea cerebrospinal fluid show significantly increased levels of anti-tubulin IgG than control cerebrospinal fluid all tested at a 1/50 dilution. Sera dot plot was analyzed by one-way variance (ANOVA) ($p < 0.01$) and individual groups were analyzed by the Mann-Whitney two-tailed *t* test with SC acute sera vs SC convalescent sera ($p < 0.05$) and SC acute sera vs ARF sera ($p < 0.005$). The cerebrospinal fluid bar graph was analyzed by unpaired two-tailed *t* test ($p = 0.02$).

solution (22) without periodate was added to each chamber and allowed to incubate for 15 min at 37°C. The slides were washed four times in PBS and the remainder of the assay was conducted as above.

CaM II assay

SK-N-SH cells were plated at 1×10^7 in T75 cm^2 flasks overnight under standard cell culture conditions. Media was removed and the SK-N-SH cells were incubated with F12 medium with no FCS and supplemented with 2 mM $CaCl_2$, 3 mM KCl, and 0.2 mM $MgCl_2$ for 30 min at 37°C, 5% CO_2. Medium was removed and the cells were incubated with 50 μg/ml mAb 24.3.1, 31.1.1, 37.2.1, and isotype control in 15 ml of the same medium. The basal control was incubated with supplemented F12 medium alone. Cells and Abs were incubated for 30 min. Media or Abs were removed and cells washed with 10 ml of ice-cold PBS. Cells were mechanically

Table I. *Ganglioside inhibition of SC mAb binding to brain-derived tubulin*[a]

Inhibitor (μg/ml)[b]	mAbs			
	24.3.1	31.1.1	37.2.1	Anti-β-tubulin
Lysoganglioside G_{M1}	17.7	15.8	16.5	>500
Monosialoganglioside G_{M1}	72.8	74.6	78.7	>500
Asialoganglioside G_{M1}	60.2	109.7	110.8	>500
Actin	>500	>500	>500	>500
pepM5	>500	>500	>500	>500
BSA	>500	>500	>500	>500

[a] Competitive inhibition of mAb reactivity by inhibitors to bound tubulin in ELISA. The amount of the three gangliosides needed to inhibit 50% of chorea mAb binding to immobilized tubulin.

[b] Micrograms per milliliter required to produce 50% inhibition of mAb reactivity with tubulin bound to the ELISA plate.

dislodged from the flask and centrifuged at 1,500 rpm for 10 min at 4°C and subjected to homogenization. Cell homogenates were centrifuged at 21,000 × *g* for 20 min to separate insoluble material from the lysate. Protein concentrations of the cell lysates were determined by Branford assay (Bio-Rad). CaM kinase II activity was measured using the calcium/calmodulin-dependent protein kinase 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. The sample was spotted onto the capture membrane and washed in 2 M NaCl followed by 2 M NaCl plus 1% H_3PO_4. The amount of radioactive label (cpm) retained on the membrane was determined by scintillation counting. Calcium-independent CaM kinase II activity was assessed by the addition of EGTA to the assay. The specific activity of the enzyme as picomoles per minute per microgram was determined for each sample and the results presented as percentages of the basal rate.

Cloning and sequencing of Ig V regions

RNA was extracted from hybridoma cells producing mAbs 24.3.1, 37.2.1, and 31.1.1 using TriReagent (Molecular Research Center) and resuspended in diethylpyrocarbonate-treated water (Invitrogen Life Technologies). RNA was treated with DNase I (Invitrogen Life Technologies), and RT-PCR was performed using Superscript III One-Step RT-PCR System with Platinum *Taq* DNA Polymerase (Invitrogen Life Technologies), using a human Ig-Primer Set from Novagen. Reactions were performed on a GeneAmp PCR System 2700 (Applied Biosystems) using the following cycling profile: 45°C for 30 min (first-strand cDNA synthesis reaction); denaturation at 94°C for 2 min, followed by 50 cycles of 94°C for 15 s, 50°C for 30 s, and 68°C for 1 min, and a 5-min extension at 68°C. RT-PCR amplification products were separated on a 1.25% modified TAE (40 mM Tris-acetate, 0.1 mM Na_2 EDTA, pH 8.0) agarose gel, and DNA bands were excised and purified using the Montage DNA gel extraction kit (Millipore). Purified DNA products were cloned into the pCR2.1-TOPO TA vector (Invitrogen Life Technologies) and transformed into TOP10 competent *Escherichia coli* cells (Invitrogen Life Technologies). Plasmid DNA was isolated from transformed cells using Wizard DNA Minipreps (Promega). Restriction enzyme digestion using *Eco*RI (Invitrogen Life Technologies) was performed to verify the presence of the insert. Sequencing was performed using M13 forward and reverse primers at the University of Oklahoma Health Sciences Center Laboratory for Genomics and Bioinformatics. Nucleotide alignments were performed using the basic local alignment search tool analysis program (National Center for Biotechnology Information), IMGT/V-QUEST (International Immunogenetics Information System), and WUR MUSCLE multiple alignment analysis program (www.bioinformatics.nl/tools/muscle.html).

Statistical analysis

Values of *p* were calculated by the Mann-Whitney two-tailed *t* test for comparison of individual groups and one-way ANOVA for comparison of more than two groups for sera samples. Cerebrospinal fluid samples were analyzed by unpaired student *t* test.

Results

Reactivity of SC mAbs with brain-derived tubulin

Because anti-GlcNAc Abs are known to recognize cytoskeletal proteins (3, 10, 23), we tested chorea mAbs 24.3.1, 31.1.1, and 37.2.1 for reactivity with proteins of the CNS. Murine whole brain cell lysates were separated by SDS-PAGE and immunoblot analysis revealed that all three chorea mAbs recognized a 55-kDa protein (Fig. 1*a* and *inset*). To determine the identity of the cross-reactive protein, the 55-kDa band was excised from the blot and subjected to protein sequence analysis. The resultant peptide sequence MREIVHLQ was found to be identical with the N terminus of β-tubulin (Fig. 1*b*). To confirm the identity of the 55-kDa protein as tubulin, immunoblot analysis was performed using purified tubulin and all three chorea mAbs bound strongly to tubulin (Fig. 1*c*). In addition, the chorea patient serum from which the mAbs were derived also reacted with tubulin in comparison to age-matched normal control sera. Chorea mAb reactivity with tubulin was of particular interest as tubulin is especially abundant in the brain.

To determine whether the anti-GlcNAc mAbs derived from chorea did indeed react with both streptococcal and protein Ags, indirect ELISA was performed to confirm Western blot and Ag panel results as well as to determine the avidity of mAbs for the specific Ags. Commercial anti-O-DG3 and anti-β-tubulin mAbs were used as controls for cross-reactivity. The chorea mAbs bound strongly to whole group A streptococci (Fig. 2*a*) and GlcNAc-BSA (Fig. 2*a*) but not BSA alone (data not shown) in comparison to the commercial anti-β-tubulin mAb which showed no reactivity for streptococcal Ags (Fig. 2, *a* and *b*). Surprisingly, the anti-O-DG3 mAb also bound to streptococcal Ags, but with lower avidity than

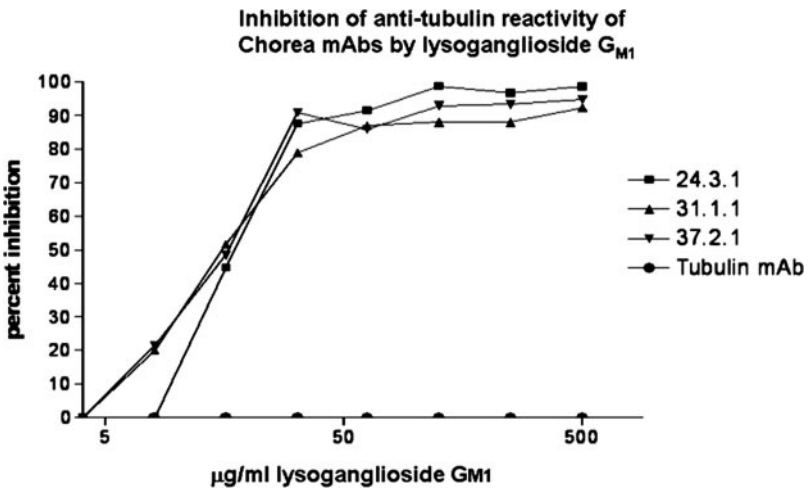

FIGURE 3. Lysoganglioside G_{M1} inhibited binding of chorea mAbs to brain-derived tubulin in a dose-dependent manner. In contrast, the β-tubulin mAb was not inhibited.

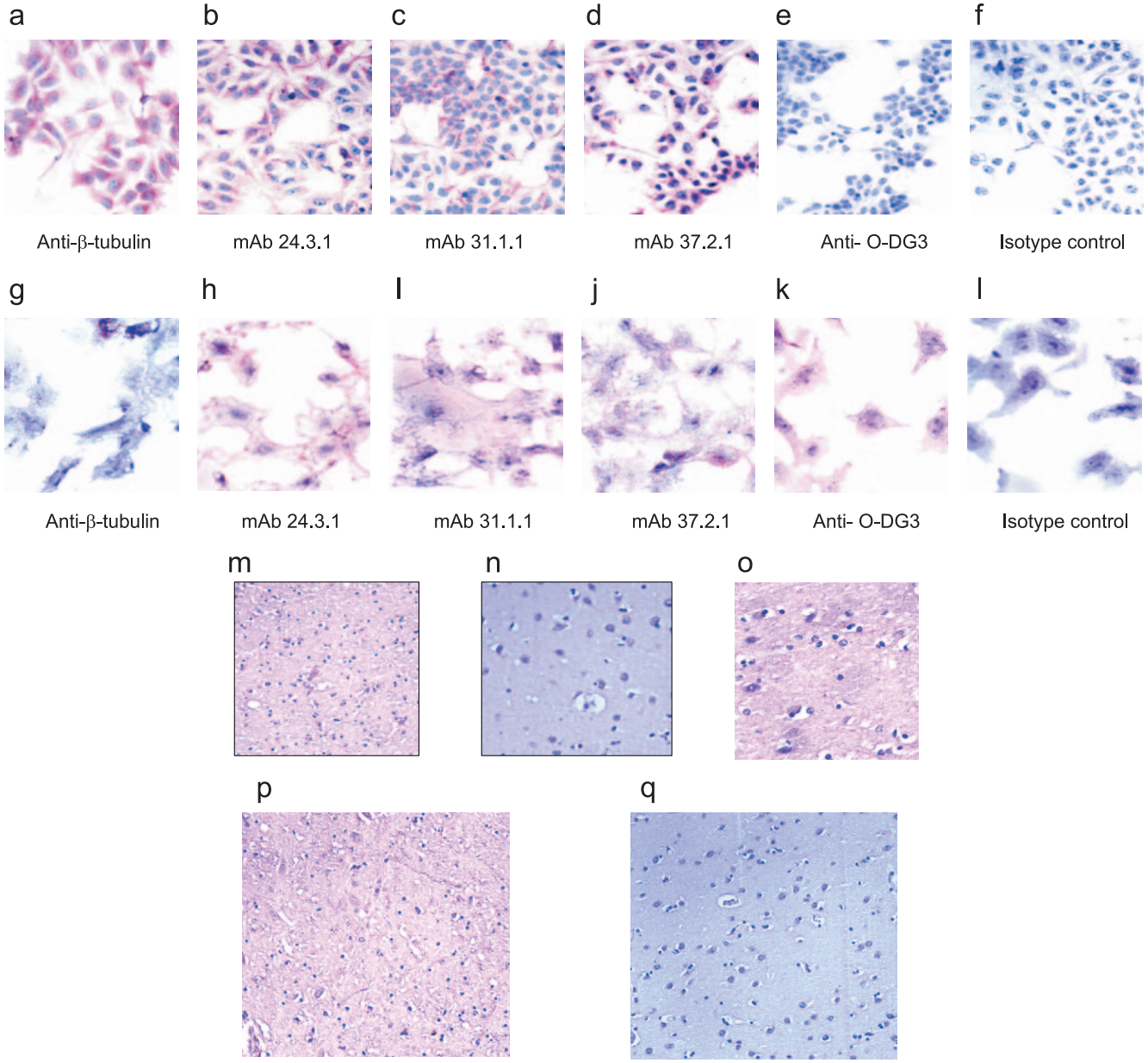

FIGURE 4. *a–q*, Chorea mAb reactivity with intra- and extracellular Ags of NT2 neuronal cells. Intracellular reactivity: *a*, anti-β-tubulin mAb labeling of intracellular tubulin as determined by Fast Red immunohistochemistry in acetone-permeabilized cells followed by counterstaining with hematoxylin; nucleus of cell as shown is blue; *b–d*, chorea mAbs 24.3.1, 31.1.1, and 37.2.1 demonstrate intracellular cytoplasmic binding similar to the anti-β-tubulin mAb; *e–f*, the anti-*O*-DG3 or isotype control mAbs did not react with the neuronal cell cytoplasmic constituents (blue color); mAb cell surface labeling: *g*, anti-β-tubulin mAb shows no reactivity with the NT2 cell surface in contrast to the chorea mAbs 24.3.1, 31.1.1, and 37.2.1 *h–j*, The anti-*O*-DG3 mAb strongly labeled the neuronal cell surface (*k*) and the isotype control is negative (*l*). *m*, mAb 24.3.1 reacted strongly with human caudate-putamen tissue and (*n*) lysoganglioside G_{M1} completely blocked mAb 24.3.1 binding to tissue but did not inhibit the anti-β-tubulin mAb (*o*). To confirm brain tissue Ag recognition by mAb 24.3.1, caudate-putamen tissue was blocked with either anti-actin or anti-tubulin mAb before treatment with mAb 24.3.1. mAb 24.3.1 demonstrated strong binding to anti-actin mAb-treated tissue (*p*), however, tissue reactivity by mAb 24.3.1 was completely abolished by pretreatment of the tissue with the anti-tubulin mAb (*q*).

the chorea mAbs. Dose-response curves showed that all three SC-derived mAbs strongly reacted with tubulin while the O-DG3 mAb did not (Fig. 2*c*). In addition, rheumatic carditis-derived IgM mAb 3B6 did not react with tubulin when compared in the ELISA with mAb 24.3.1 similar to the O-DG3 mAb as shown in Fig. 2*c*. To determine the potential clinical relevance of our findings, we tested five patient sera for anti-tubulin reactivity. Significantly higher anti-tubulin IgG Ab reactivity was found in SC acute sera compared with matched convalescent sera or sera from six patients with ARF without SC (Fig. 2*d*). Acute SC cerebrospinal fluid samples were also found to have significantly ($p < 0.02$) elevated levels of anti-tubulin IgG in comparison to control cerebrospinal fluid, indicating that anti-tubulin Abs were present intrathecally during active disease (Fig. 2*e*). Therefore, increased levels of anti-tubulin IgG Abs correlated with active chorea, and the specificities of Abs found during acute SC in both sera and cerebrospinal fluid were similar to human chorea mAbs.

Previously, we have shown that chorea mAbs and acute SC sera were cross-reactive with GlcNAc and lysoganglioside G_{M1}, a CNS ganglioside known to influence neuronal signal transduction. To demonstrate the specificity of the anti-GlcNAc mAbs from SC, the mAbs were tested against lysoganglioside G_{M1} and two structurally

related gangliosides, monosialoganglioside G_{M1}, and asialoganglioside G_{M1}, as well as serotype 5 streptococcal M protein, and BSA in competitive-inhibition ELISAs against immobilized tubulin. Analysis of chorea mAbs by competitive inhibition ELISA indicated that mAb binding to tubulin was inhibited by soluble gangliosides, confirming the cross-reactivity between tubulin and gangliosides (Table I). Lysoganglioside G_{M1} was the most potent inhibitor of SC mAb binding to tubulin and inhibited tubulin recognition by chorea mAbs in a dose-dependent manner (Fig. 3). Therefore, lysoganglioside G_{M1} is a specific and potent inhibitor of chorea mAb binding to brain-derived tubulin in vitro. In contrast, the commercial tubulin mAb showed no reactivity to any of the gangliosides (Table I and Fig. 3) and could only be inhibited by soluble tubulin (data not shown). Our data show that the chorea mAbs possess a unique Ag reactivity profile in comparison to the other anti-tubulin and anti-ganglioside mAbs tested.

To investigate mAb reactivity to both intracellular and extracellular Ags, SC mAbs were reacted with the NT2 human neuronal cell line, a precursor line for the induction of normal human neurons. The SC mAbs (Fig. 4, *b–d*) reacted with intracellular Ags of acetone-fixed NT2 neuronal cells in a pattern similar to that seen for the commercial anti-β-tubulin mAb (Fig. 4*a*). The anti-O-DG3 mAb and isotype control showed no intracellular staining (Fig. 4, *e* and *f*). To confirm that chorea mAbs reacted with extracellular Ag, NT2 cells were reacted with mAbs and then fixed with paraformaldehyde to identify cell surface binding. All three chorea mAbs deposited on the neuronal cell surface with mAb 24.3.1 exhibiting the greatest intensity of binding (Fig. 4, *h–j*). The anti-O-DG3 mAb also demonstrated strong binding to the cell surface and served as a positive control for the presence of ganglioside (Fig. 4*k*). Neither the anti-β-tubulin mAb nor isotype control reacted with the neuronal cell surface (Fig. 4, *g* and *l*).

Further studies investigated mAb reactivity with human brain tissue. SC mAb 24.3.1 reacted to caudate-putamen tissue sections derived from human basal ganglia (Fig. 4*m*) and lysoganglioside G_{M1} completely abolished mAb 24.3.1 reactivity with the basal ganglia (Fig. 4*n*). In contrast, lysoganglioside G_{M1} did not inhibit tissue binding of the β-tubulin mAb (Fig. 4*o*). To demonstrate mAb 24.3.1 specificity for brain tubulin, caudate-putamen sections were blocked with either anti-actin mAb or anti-β-tubulin mAb before incubation with mAb 24.3.1. The chorea mAb 24.3.1 showed strong reactivity with the tissue treated with the anti-actin mAb (Fig. 4*p*), however, mAb 24.3.1 deposition on caudate-putamen tissue was completely blocked by β-tubulin-specific mAb (Fig. 4*q*), indicating that chorea mAb 24.3.1 recognized tubulin. In summary, the chorea mAbs demonstrated unique binding to both intra- and extracellular Ags of human neuronal cells. mAb 24.3.1 reactivity with human caudate putamen tissue could only be abolished with lysoganglioside G_{M1} inhibitor or anti-β-tubulin blocking Ab indicating the distinctive cross-reactivity of the chorea mAbs.

Induction of CaM kinase II by chorea mAb

Abs from SC have been shown to induce neuronal cell signaling via activation of CaM kinase II. Ab-mediated CaM kinase II activation was induced by SC acute sera, but not convalescent sera, suggesting that increased CaM kinase II activity is associated with the active disease state. CaM kinase II is highly abundant in the brain and has a broad spectrum of neuronal targets including tubulin (24). To assess the ability of mAbs to direct neuronal cell signaling, the three chorea mAbs, anti-β-tubulin, and anti-O-DG3 mAbs were tested for CaM kinase II activation in the SK-N-SH human neuronal cell line (Table II). Only chorea mAb 24.3.1 was capable of inducing CaM kinase while, neither the anti-tubulin, anti-O-DG3, nor isotype control mAbs significantly activated CaM kinase above the basal rate. In studies of other neuronal cell lines, SC mAb 24.3.1 was capable of activating CaM kinase above the basal rate in the human NT2 and murine Neuro 2a cell lines similar to that seen in the SK-N-SH cell line (data not shown). The ability to mediate neuronal cell signaling was a property unique to chorea mAb 24.3.1 and is likely to be due to amino acid sequence differences of the H and L chain variable regions affecting avidity and specificity of the mAb.

Table II. *mAb-induced CaM kinase II activation in human neuroblastoma cells*[a]

mAb	Relative Increase in CaM Kinase Activity above Basal Rate (%)
24.3.1	77.8
31.1.1	11.8
37.2.1	8.5
O-DG3	0.0
Tubulin	31.0
Isotype	5.5
Acute serum	102.0
Convalescent serum	20.0

[a] The relative percent difference between samples and basal rate measured as specific activity (picomoles per microgram per minute). mAb 24.3.1 induced significant ($p < 0.05$) increase in CaM kinase II activity in comparison to other mAbs. Acute patient serum of the mAb source induced CaM kinase II activity to 102% above the basal rate in comparison to 20% activity of matched convalescent serum.

Nucleotide sequence analysis of chorea mAbs

Previous studies have shown that mAbs from rheumatic heart disease and uncomplicated streptococcal pharyngitis recognize the GlcNAc epitope of the group A carbohydrate as well as multiple cytoskeletal autoantigens including human cardiac myosin and cytokeratin (3, 9, 10, 25, 26). Although the chorea mAbs reacted with both GlcNAc and tubulin, they did not recognize the cytoskeletal Ag myosin, indicating that Abs in SC may represent a unique population of anti-streptococcal/anti-GlcNAc Abs (4). Nucleotide sequence analysis of SC mAb V_H and V_L genes revealed that the H chain variable genes were distinct from V_H genes previously studied in rheumatic carditis (25).

Germline genes most closely homologous to genes encoding chorea mAbs IgV_H were genes $V_H1–8$ (mAb 24.3.1) and $V_H3–64$ (mAbs 31.1.1 and 37.2.1). L2 (mAb 24.3.1) and A27 (mAbs 31.1.1 and 37.2.1) encoded the IgV_L genes. Nucleic acid and amino acid sequence homology comparisons to germline genes are shown in Tables III and IV. Chorea mAb 24.3.1 H chain V region gene had 99% homology with the germline $V_H1–8$, 100% homology with $D_H4–17$ and 97.3% homology with J_H4. The differences from germline were in framework 1 (a single nucleotide difference near CDR1, resulting in a coding change from a threonine to an alanine) and in the joining segment, J_H4 (a silent mutation not resulting in

Table III. *V_H gene usage and homology of mAb 24.3.1, 31.1.1, and 37.2.1 V_H genes*[a]

Hybridoma	V_H	Nucleic Acid Homology	Amino Acid Homology	D_H	J_H
24.3.1	$V_H1–8$	99	99	D4–17	J_H4
31.1.1	$V_H3–64$	91	91	D4–17	J_H6
37.2.1	$V_H3–64$	90	90	D3–16	J_H6

[a] Hybridoma cell lines are listed with the most closely homologous reported germline variable, diversity, and joining segments and percentage of homology to germline V region gene segments. Nucleotide sequences are listed with the GenBank database under accession numbers DQ768422 (24.3.1 V_H), DQ779565 (31.1.1 V_H), and DQ779566 (37.2.1 V_H).

Table IV. *V_L gene usage and homology of mAb 24.3.1, 31.1.1, and 37.2.1 V_L genes*[a]

Hybridoma	V_L	Homology	J_L
24.3.1	L2	99	Jκ2
31.1.1	A27	100	Jκ3
37.2.1	A27	100	Jκ3

[a] Hybridoma cell lines are listed with the most closely homologous reported germline variable and joining segments and percentage of homology to germline V region gene segments. Nucleotide sequences are listed with the GenBank database under accession numbers DQ768423 (24.3.1 V_L), DQ778307 (31.1.1 V_L), and DQ778308 (37.2.1 V_L). The L-chain V regions of 31.1.1 and 37.2.1 are identical.

a coding change). The mAb 24.3.1 L chain V region had 99.7% homology with the germline L2 gene and 97.3% homology with Jκ2. The 24.3.1 V_L had a mutation in the CDR2 region, changing an arginine to a glycine. There was also a nucleotide transversion from an adenine to a cytosine in Jκ2.

SC mAb 31.1.1 and 37.2.1 V_H genes shared closest homology with the germline H chain V_H3–64 gene (91 and 90%, respectively). For both 31.1.1 and 37.2.1, most differences occurred in the sequence after CDR-1, in frameworks 2 and 3. SC mAb 31.1.1 H chain shared usage of diversity gene segment D4–17 with 24.3.1 H chain. SC mAb 37.2.1 V_H used the same joining gene segment (J_H6) as 31.1.1 V_H, but used D3–16 instead of D4–17. The nucleotide sequences of the 31.1.1 and 37.2.1 V_L genes were identical with that of the germline A27 and Jκ3 genes.

The chorea mAb H chains were encoded by different germline genes than those encoding known cross-reactive anti-streptococcal mAbs. The V_H genes of 24.3.1, 31.1.1, and 37.2.1 were distinct from anti-streptococcal mAbs 3.B6, 1.C8, 1.H9, and 5G.3 from rheumatic heart disease, and mAb 10.2.3 from tonsillar B cells following streptococcal pharyngitis (25, 27). However, the chorea-derived V_L gene encoding mAbs 31.1.1 and 37.2.1 and the rheumatic carditis-derived mAb 3.B6 V_L was the germline A27 L chain variable gene. The 3.B6 VL had 99% nucleotide homology with 31.1.1 and 37.2.1 V_L and 86% amino acid homology with translational differences in the framework region. The L chain V regions of 31.1.1 and 37.2.1 were identical. Chorea Ab variable gene nucleotide sequences are listed with the GenBank database. The V_H gene sequences are listed under accession numbers DQ768422 (24.3.1), DQ779565 (31.1.1), and DQ779566 (37.2.1). L chain variable gene nucleotide sequences are listed under accession numbers DQ768423 (24.3.1), DQ778307 (31.1.1), and DQ778308 (37.2.1).

The chorea mAbs were encoded by some of the same germline gene families encoding anti-ganglioside Abs associated with motor neuropathies (21, 28). Weng et al. (21) reported anti-lysoganglioside-G_{M1} Abs derived from four different patients with sensory and motor neuropathies (polyneuropathy and motor neuropathy-amylotrophic lateral sclerosis syndrome) which had H chain V regions encoded by the V_H1 gene family. The V_H1 gene family also encoded the V_H of chorea mAb 24.3.1. Chorea mAbs 31.1.1 and 37.2.1 V_L genes shared 98% nucleic acid sequence homology with human anti-ganglioside Ab B5G10K V_L which is also encoded by the A27 germline gene (21). In addition, chorea mAb 24.3.1 V_L shares 93% nucleotide sequence homology with anti-lysoganglioside Ab R6B8K, derived from a patient with polyneuropathy, which is also encoded by germline L2 (21).

Discussion

The theme that emerges in autoimmunity is that polyreactive autoantibodies recognize intracellular cytoplasmic or cytoskeletal Ags and extracellular cell surface Ags which may lead to disease through cytotoxicity (3, 29), altered cell signaling (4), or some other inflammatory mechanism. Although our human cross-reactive mAbs are IgM, they have provided important clues to the IgG specificities of Abs in sera from disease. Our study has revealed that SC mAbs are cross-reactive with intracellular tubulin, extracellular lysoganglioside G_{M1} and the GlcNAc epitope of streptococcal group A carbohydrate. In addition, the demonstration of Ab-mediated cell signaling by mAb 24.3.1 as well as by serum IgG from SC suggests a role for the Abs in disease (4, 30). The data support the hypothesis that cross-reactive Abs may contribute to the unique pathological manifestations of ARF as well as other autoimmune disorders.

The SC mAbs investigated in our study were shown to bind an immunodominant 55-kDa brain extracted protein identified as β-tubulin by immunoreactivity and amino acid sequence analysis. The reaction of anti-GlcNAc chorea mAbs with tubulin is in accordance with previous studies that have shown anti-GlcNAc Abs cross-reacted with cytoskeletal proteins keratin and myosin and that anti-GlcNAc mAbs recognized both carbohydrate and peptide epitopes (9, 10). Because the purified tubulin consisted of both α and β tubulin, we do not know whether chorea sera or cerebrospinal fluid IgG reacted with one or both subunits.

Our results are consistent with reports of Ab reactivity with human basal ganglia in SC. The chorea mAbs were shown in our study to react with intracellular cytoplasmic tubulin of the neuronal cell which did not extend to the nucleus indicating that the mAbs did not bind DNA or nuclear proteins. Previous studies link streptococcal Ags to the development of a cross-reactive Ab response against intracellular proteins in SC. Husby et al. (20) found that SC patient sera strongly reacted with cytoplasmic, but not nuclear, Ags in human caudate and subthalamic nuclei as well as cerebral cortex neurons. The anti-brain reactivity was found to correlate with both severity and duration of choreic symptoms and could be abolished by adsorption with group A streptococcal membranes. In a complementary study, Bronze and Dale (31) found that rabbits immunized with rheumatogenic group A serotypes M5, 6, and 12 produced antisera cross-reactive with CNS Ags in Western blots. Reactivity to specific brain Ags was inhibited by preincubation with streptococcal M protein, however, the neuronal Ags were not identified.

Chorea mAbs were reactive with both GlcNAc and mammalian tubulin in ELISA in contrast to the anti-β-tubulin and anti-O-DG3 mAbs, which were monospecific. Competitive inhibition studies showed that the tubulin reactivity of the chorea mAbs was blocked by three gangliosides with lysoganglioside G_{M1} the most potent inhibitor. Gangliosides have been shown to interact with integral and peripheral proteins found at the cell surface. Interestingly, it has been recently reported that monosialoganglioside G_{M1} and tubulin can associate at the cell membrane in cerebellar granule cells (32). The chorea mAbs appear to be specific for GlcNAc, lysoganglioside G_{M1}, and tubulin because keratin, actin, pepM5, or BSA did not react with the chorea mAbs. Previous studies have demonstrated that anti-GlcNAc mAbs from rheumatic heart disease and uncomplicated pharyngitis were cross-reactive with other cytoskeletal and microbial proteins including epidermal keratin, human cardiac myosin, and streptococcal recombinant M6 and pepM5 (3, 25, 27). In contrast, SC mAbs appear to have unique Ag reactivity. Different Ag cross-reactive profiles of GlcNAc-specific mAbs in ARF indicate that there may be different subsets of anti-GlcNAc Abs that may contribute to the different clinical manifestations of ARF. The question of chorea Ab binding to different regions within the CNS is of great importance. More testing will be done in the future to determine whether chorea Abs recognize only

neurons from the basal ganglia or can bind to cells from additional cortical areas.

The fact that tubulin is equally abundant in many tissues leads to the question of why brain is targeted and not other tissues. Although we can only speculate, the chorea Abs may be targeted to Ags present only on the surface of neuronal cells thereby overstimulating nerve tracts in the caudate putamen region leading to choreic movement disorder. We do not know for certain the identity of such brain-specific Ags but they could be cell surface lysoganglioside G_{M1} or associated with lysoganglioside G_{M1} in lipid rafts. Chorea Abs capable of neuronal cell signaling may be targeted to the brain due to reactivity with cell surface lysoganglioside G_{M1}. In contrast, the brain specificity could be related to receptors which we have not yet identified. The mechanism of how the Abs cross the blood brain barrier is unclear but the chorea Abs themselves may alter the BBB to allow entry or infection itself may alter permeability of the fenestrated endothelial layer protecting the brain.

Mimicry between gangliosides and tubulin has been classified as "dissimilar" as defined previously in studies of mimicry between GlcNAc and peptide structures (8–10). Cross-reactive Abs in ARF that are capable of recognizing both extra- and intracellular Ags may be directly associated with pathogenesis of disease. The anti-GlcNAc mAb 3.B6 from rheumatic carditis recognizes extracellular laminin and intracellular cardiac myosin and is cytotoxic for human endothelial cells. These data provide a mechanism of how Abs in carditis might cause valvular damage (3). SC mAbs are cross-reactive with lysoganglioside G_{M1} presumably at the cell surface associated with lipid rafts and tubulin inside the cell. mAb 24.3.1 directs CaM kinase II activation in human neuronal cells that may cause the neurological manifestations of SC by increasing dopamine release to the synapse. Despite the ability of mAb 24.3.1 to strongly bind to the cell surface, mAb 24.3.1 is not cytotoxic for neuronal cells (4, 30). Identification of cross-reactive Abs which recognize dissimilar epitopes is not limited to ARF. Investigation of cross-reactive Ab responses in the neuropsychiatric disorders of systemic lupus erythematosus demonstrated that Abs cross-reactive with dsDNA and subunits of the cell surface *N*-methyl-D aspartate receptor induce apoptosis of neurons in the hippocampus and lateral amygdala resulting in a behavioral disorder (29, 33). A key finding of the study indicated that a breech of the blood brain barrier was required to allow pathogenic serum Abs access to the CNS and cause disease (33). Therefore, disruption of the blood brain barrier maybe a necessary event in the pathogenesis of Ab-mediated neurological disorders including SC.

Finally, the nucleotide sequence analysis of the V_H and V_L genes of the chorea mAbs established that the V genes encoding signaling mAb 24.3.1 were different from those encoding the mAbs 31.1.1 and 37.2.1. In addition, the chorea mAbs had different specificities than those reported for mAbs from rheumatic carditis. Including the chorea-derived mAbs, V_H and V_L genes of all of the mAbs derived from ARF were a heterogeneous group of V_H and V_L genes without any common or restricted group or sequence. Some of the genes reported herein displayed a few mutations in or near the CDRs. Because the IgG specificities of the IgM mAbs are found in the serum and cerebrospinal fluid of patients, it would be expected that the B cells could class switch from IgM to IgG and develop somatic mutations. Because there is little known about cross-reactive IgG, we will be interested to generate IgG-producing hybridomas in the future to see whether they are related to the IgM Abs. Nucleotide sequence analysis of SC mAb V_H and V_L genes revealed that the H chain variable genes were distinct from V_H genes studied in rheumatic carditis (25), and that they were encoded by the same V gene families as other anti-ganglioside Ab V genes associated with motor neuropathies (21). The investigation of the chorea mAb V genes has provided our initial view of sequences from autoantibodies which mediate cell signaling.

The relevance of our human chorea-derived mAbs includes their association with a potentially pathogenic Ab-mediated signaling mechanism and their recognition of both intracellular and extracellular Ags related to both autoimmunity and infection. Clearly, the autoantibodies which characterize the disease are multipotent and demonstrate recognition of the autoantigen targets lysoganglioside and tubulin.

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Disclosures

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