Brain Human Monoclonal Autoantibody from Sydenham Chorea Targets Dopaminergic Neurons in Transgenic Mice and Signals Dopamine D2 Receptor: Implications in Human Disease

Carol J. Cox, Meenakshi Sharma, James F. Leckman, Jonathan Zuccolo, Amir Zuccolo, Abraham Kovoor, Susan E. Swedo and Madeleine W. Cunningham

*J Immunol* 2013; 191:5524-5541; Prepublished online 1 November 2013; doi: 10.4049/jimmunol.1102592

http://www.jimmunol.org/content/191/11/5524

Supplementary Material

http://www.jimmunol.org/content/suppl/2013/11/01/jimmunol.1102592.DC1

**Why The JI?**

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

**References**

This article cites 84 articles, 29 of which you can access for free at: http://www.jimmunol.org/content/191/11/5524.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Brain Human Monoclonal Autoantibody from Sydenham Chorea Targets Dopaminergic Neurons in Transgenic Mice and Signals Dopamine D2 Receptor: Implications in Human Disease

Carol J. Cox,* Meenakshi Sharma,† James F. Leckman,*§,¶ Jonathan Zuccolo,* Amir Zuccolo,* Abraham Kovoor,‡ Susan E. Swedo,§,¶ and Madeleine W. Cunningham*

How autoantibodies target the brain and lead to disease in disorders such as Sydenham chorea (SC) is not known. SC is characterized by autoantibodies against the brain and is the main neurologic manifestation of streptococcal-induced rheumatic fever. Previously, our novel SC-derived mAb 24.3.1 was found to recognize streptococcal and brain Ags. To investigate in vivo targets of human mAb 24.3.1, VH/VL genes were expressed as functional chimeric human VH 24.3.1-mouse C-region IgG1* autoantibody. Chimeric human–mouse IgG1* autoantibody colocalized with tyrosine hydroxylase in the basal ganglia within dopaminergic neurons in vivo in VH 24.3.1 Tg mice. Both human mAb 24.3.1 and IgG1* in Tg sera were found to react with human dopamine D2 receptor (D2R). Reactivity of chorea-derived mAb 24.3.1 or SC IgG with D2R was confirmed by dose-dependent inhibitory signaling of D2R as a potential consequence of targeting dopaminergic neurons, reaction with surface-exposed FLAG epitope-tagged D2R, and blocking of Ab reactivity by an extracellular D2R peptide. IgG from SC and a related subset of streptococcal-associated behavioral disorders called “pediatric autoimmune neuropsychiatric disorder associated with streptococci” (PANDAS) with small choreiform movements reacted in ELISA with D2R. Reaction with FLAG-tagged D2R distinguished SC from PANDAS, whereas sera from both SC and PANDAS induced inhibitory signaling of D2R on transfected cells comparably to dopamine. In this study, we define a mechanism by which the brain may be altered by Ab in movement and behavioral disorders. The Journal of Immunology, 2013, 191: 5524–5541.

Sydenham chorea (SC) is well established as the major neuropsychiatric syndrome of Streptococcus pyogenes–induced rheumatic fever (1) and is characterized by involuntary movements and neuropsychiatric disturbances, including obsessive-compulsive symptoms and hyperactivity (2). Although SC is associated with streptococcal pharyngitis (3, 4), tics and obsessive-compulsive disorder (OCD) appear to be associated with streptococcal and other types of infections (5–9), as well as with autoantibodies against neuronal Ags (10–14). Movement, behavioral, and neuropsychiatric disorders affect millions of children worldwide. Studies suggesting that infection and autoantibodies might contribute to the pathogenesis of some groups of movement and behavioral disorders began with studies of SC and its relationship to streptococcal infection and autoantibodies against the brain in streptococcal-induced rheumatic fever (15–19). However, actualization of this hypothesis was not recognized until recently when it was discovered that SC was treatable by plasmapheresis, which correlates with removal of autoantibodies against the brain (20, 21). Other childhood diseases, including some groups of OCD or tics, have also been related to streptococcal infections, with subsequent development of autoantibodies (10, 13, 20, 22–25). These disorders may be identified as pediatric autoimmune neuropsychiatric disorder associated with streptococci (PANDAS) (22) or pediatric acute-onset neuropsychiatric syndrome in the presence of other types of infections (23, 26).

The basal ganglia has been implicated as a target of poststreptococcal immune responses (25, 27). Streptococcal-specific Abs in SC were shown to react with neurons in human basal ganglia, and neuronal-specific Ab titers were associated with both severity and duration of choreic episodes (15, 16). Husby et al. (16) found that chorea patient sera reacted strongly with cytoplasmic, but not neuronal-specific Ab titers were associated with both severity and duration of choreic episodes (15, 16). Husby et al. (16) found that chorea patient sera reacted strongly with cytoplasmic, but not neuronal-specific Abs, which correlated with neuronal reactivity correlated with both severity and duration of choreic symptoms. Immunomodulatory therapies, such as plasma exchange, provide strong evi-

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1102592
dence that SC is caused by a pathogenic Ab response (20). Other groups of neuropsychiatric and movement disorders, including PANDAS, may also be associated with an autoantibody response against the brain (13, 28) and abnormal brain magnetic resonance imaging (29). Improvement was also seen in these cases after plasmapheresis (21, 30).

The hypothesis that SC and its pathogenesis may develop as a result of signaling autoantibodies directed against neuronal cell proteins in the brain was delineated by studies using SC-derived human mAbs. SC mAb cross-reactivity was first associated with the group A streptococcal carbohydrate epitope N-acetyl-β-D-glucosamine (GlcNAc) and neuronal surface Ag lysosangioside GS1 and α helical intracellular brain protein tubulin (10, 11). The cross-reactivity between GlcNAc and α helical peptide structures has been studied extensively (31–33). Chorea-derived human anti-streptococcal mAb 24.3.1 also induced increased calcium-calmodulin dependent protein kinase II (Cam kinase II) activity in a human neuronal cell line (10), and intrathecal passive transfer of mAb 24.3.1 induced elevated tyrosine hydroxylase (TH) in rat brains (12). mAb 24.3.1 exhibited stronger avidity for neuronal Ags, which may explain its ability to signal neuronal cell proteins, but the extracellular targets responsible for the signaling were not defined. SC mAb specificities were similar to the neuronal-specific IgG in sera or cerebrospinal fluid from active chorea (10). Ab-mediated cell signaling leading to excess dopamine release was suggested to be due to signaling autoantibodies directed against neuronal cells, but the extracellular targets responsible for the signaling were not defined. SC mAb specificities were similar to the neuronal-specific IgG in sera or cerebrospinal fluid from active chorea (10). Ab-mediated cell signaling leading to excess dopamine release was suggested to be due to signaling autoantibodies directed against neuronal cells, but the extracellular targets responsible for the signaling were not defined. SC mAb specificities were similar to the neuronal-specific IgG in sera or cerebrospinal fluid from active chorea (10).

To gain further insight into in vivo functional Ab targets that may be involved in the mechanisms of SC and related CNS disorders, we created transgenic (Tg) mice expressing chorea-derived human mAb 24.3.1 H chain (HC) and L chain (LC) V-region (VH and VL) genes as part of a chimeric (human V-gene/mouse C-region) IgG1 Ab. Mice Tg for mAb 24.3.1 V genes were validated by characteristic cross-reactive anti-neuronal Ab specificities in serum and of mAbs produced from lymphocytes of Tg mice. Chimeric 24.3.1 Tg Ab penetrated the brain and dopaminergic neurons in the basal ganglia of Tg mice, most likely in the substantia nigra or ventral tegmental area. However, not all neurons targeted were dopaminergic, and a significant portion of them were located in the cortex. Furthermore, human mAb 24.3.1 was similar to sera from SC and other movement and neuropsychiatric disorders, which had elevated IgG to human dopamine D2 receptor (D2R). SC-derived mAb 24.3.1 and SC IgG reacted with FLAG-tagged D2R, whereas PANDAS IgG did not. However, human chorea mAb 24.3.1, sera from the human SC mAb B cell donor, and PANDAS sera all induced inhibitory signaling of D2R, which may be the consequence of Ab targeting of dopaminergic neurons. Our novel findings suggest that signaling autoantibodies may play a role in chorea and other movement and behavioral disorders.

Materials and Methods

**Human blood samples**

Blood samples from SC and PANDAS patients and healthy controls were obtained from the National Institute of Mental Health (S.E.S.), the Child Study Center, Yale University (J.F.L.), and the University of Oklahoma Health Sciences Center (M.W.C.). SC was diagnosed using Jones Criteria. PANDAS was diagnosed using the published National Institute of Mental Health criteria (22) and included patients displaying fine piano playing movements of the fingers and toes in addition to obsessive-compulsive symptoms (24). Attention deficit hyperactivity disorder (ADHD), most importantly, in vivo in the brain. The 24.3.1 chimeric transgenes was tested by transient transfection into mouse myeloma cells, as described below.

**Generation of Tg mice**

Rearranged Ig HC and LC V genes of human mAb 24.3.1 were cloned previously, and the VH and VL sequences are published (11). Tg constructs were designed to produce a chimeric Ab (human V-gene/mouse C-region) Ab when expressed in Tg mice.

24.3.1 HC Tg construct. A 2.8-kb BamHI-EcoRI fragment of plasmid pG11-LcC-M (provided by Dr. Brett Aplin, University of Melbourne, Victoria, Australia) was excised and religated. This contains IgG promoter, leader, and enhancer regions, as well as a unique Sall restriction site for cloning a V-gene insert (36). The mAb 24.3.1 rearranged VH gene, previously cloned into a PCR vector, was amplified using the following primers: 24HCTg5′, 5′-CGCGTCGACTTCTGCCTTTCTGTTTCCAAGGGGTCATTAGGTGCAGCTGGTGTGATGGTGACAGTGGAGGAGCCATCGGGGAAATTAGGTGATGACGAGCAGCATCAGACAGACCGGTTGGACGAGCACTGACTGATGGCATGAGG-3′ and 24HCTg3′, 5′-GGCCGTGCACGAGCTCTGCCAGACCGAGGAGCTGAGG-3′. These primers contain consensus splice acceptor or donor sequences plus Sall restriction sites (bold type for cloning). The 456-bp amplified PCR product was digested with Sall (underlined) and cloned into the corresponding Sall site in the modified Tg vector, creating a 3.2-kb plasmid. Verification of the presence of the 24.3.1 VH insert was done by diagnostic restriction enzyme digestion, as well as by sequencing of PCR amplification products. A mouse HC construct containing the IgG1 constant regions and membrane exons was kindly provided by Dr. Chris Goodnow (John Curtin School of Medical Research, The Australian National University, Canberra ACT, Australia). Experiments with this vector were performed previously (37); parts of this vector were the same as pG11-LcC-M. A 9.5-kb region of the IgG1 plasmid, flanked by HindIII restriction sites, contains the Syl1 region, the constant and hinge exons, and the membrane exons. Primers were designed to amplify this region and the upstream regions, based on the published genomic sequence (GenBank). Three regions (including the cloned 24.3.1 VH gene) were amplified and sequenced after PCR amplification of these regions, as previously described (38), to create the 24HCTg Tg construct.

24.3.1 LC Tg construct. The LC Tg vector, pSV-LcCΔ4HS, generously provided by Dr. Brett Aplin (University of Melbourne, Melbourne, VIC, Australia), is an 11.4-kb plasmid containing a 6.4-kb region with the LC promoter and leader sequences, the Sall restriction site for cloning VH1 gene, and the mouse κ C-region. Amplification primers, TgLK-F5 (5′- CGGCTGTGTAAGAATCCACTGCTGTTTCCAAGGAAATTTAGGTGATGACGAGCAGCATCAGACAGACCGGTTGGACGAGCACTGACTGATGGCATGAGG-3′) and TgLK-R4 (5′- CCCCCCTGAACCGGCTTATCAATGATCTCTTCTGTTTCCAAGGAGCCATCGGGGAAATTAGGTGATGACGAGCAGCATCAGACAGACCGGTTGGACGAGCACTGACTGATGGCATGAGG-3′), which spanned this region, were designed based on known plasmid and insert DNA sequences; this 6.5-kb fragment was amplified by PCR and subsequently cloned into the PCR cloning vector, pCR2.1 TOPO-TA (Invitrogen), creating the LKTA vector. Verification of the presence and orientation of insert was done by diagnostic restriction enzyme digestion, as well as by sequencing of PCR amplification products. The 24.3.1 VH gene was amplified using primers 24LCTg5′ (5′-CGCGTGACGACCGGTGATGACGAGCAGCATCAGACAGACCGGTTGGACGAGCACTGACTGATGGCATGAGG-3′) and its copy number. Expression of the chimeric human V-gene/mouse C-region Ab from the 24.3.1 transgenes was tested by transient transfection into mouse myeloma cells, as described below.
were coinjected, and litters consisting of a total of 65 mice were born. Screening for incorporation of transgene(s) was done by tail DNA PCR using mAb 24.3.1 V-gene–specific primers. Nine founder mice were generated that were either HC (VH) only or double-integration positive Tg (VH+VL). Southern hybridization was performed to confirm correct orientation of the transgene(s) in the genome and to determine copy number. Sequencing of PCR products confirmed that the inserted VH and VL genes in founder animals were correct and unmutated. Sera from founders were collected and screened by ELISA to verify expression of the chimeric IgG1 Ab. Founders were maintained at Xenogen and backcrossed to C57BL/6 mice. Tg mice were shipped to the University of Oklahoma Health Sciences Center for experimental studies and housed at the University of Oklahoma Health Sciences Center Animal Facility. Negative littermates or C57BL/6 wild-type mice (Taconic) were used as experimental controls. All experiments on animals were performed in accordance with relevant institutional (National Institutes of Health and University of Oklahoma Health Sciences Center) guidelines and regulations as set by the University of Oklahoma Health Sciences Center Institutional Animal Care and Use Committee.

DNA isolation and purification

Small-scale plasmid DNA isolation was performed using a Wizard Plus Miniprep DNA Purification system (Promega). EndoFree Plasmid Maxi kit (QIAGEN) and PureYield Plasmid MidiPrep and Maxiprep systems (Promega) were used for medium- and large-scale plasmid DNA preparation, cloning, cell transfections, and transgenesis microinjection. Genomic DNA from mouse tail tissue samples was purified using the Easy-DNA isolation kit (Invitrogen). All kits were used following the manufacturers’ instructions. DNA constructs used for microinjection were further purified by dialysis against TE buffer (10 mM Tris, 0.25 mM EDTA [pH 7.5]) using Millipore V-series mixed cellulose ester microdialysis membranes (0.025 μm) following the transgene preparation protocol supplied by Xenogen. Concentration and purity of DNA samples were determined by spectrophotometric analysis at 260/280 nm using a Synergy HT Multidetection microplate reader (Bio-Tek) with KC4 software.

PCR

PCR amplification of DNA was performed using 0.1–1 ng plasmid template DNA or 50–400 ng genomic template DNA and 0.5 μM 5′ and 3′ oligonucleotide primers, using Platinum Taq PCR Supermix, High Fidelity (Invitrogen). Amplification was performed using a Gene-Amp PCR System 2700 (Applied Biosystems). Cycling parameters were dependent on the primers and template being used, but followed this general profile: 94°C for 1 min, followed by 30–45 cycles of 94°C for 15–30 s, 50–65°C for 15–30 s, 72°C for 1 min/kb, and then 10 min at 72°C. PCR amplification products were separated by electrophoresis on 1% (w/v) Tris HCl–borate–EDTA agarose gel. The remainder of the amplification products (for cloning or direct sequencing) were purified by column filtration or gel extraction, stained 1% Tris HCl–borate–EDTA agarose gel. The remainder of the PCR amplification protocol provided. Contaminating DNA was removed by treatment with DNase I (1 U/μg DNA) (Invitrogen), per the manufacturer’s protocol. RT-PCR was performed using the Superscript III One-step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen), using either gene-specific primers or Ig-Primer Sets (human or mouse) from Novagen. Reactions were performed on a Gene-Amp 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, 68°C for 1 min, and a 5-min extension at 68°C. Results of amplification were assessed by visualizing an aliquot of the RT-PCR products (10 μl each) on an ethidium bromide–containing agarose gel and visualized under UV light, as per standard molecular biological procedures.

DNA cloning

Molecular weight markers, restriction endonucleases, and DNA-modifying enzymes (Promega, Invitrogen, New England Biolabs) were used according to the manufacturers’ instructions. Insert DNA and/or plasmids for cloning were digested with appropriate restriction enzymes and size separated by agarose gel electrophoresis. Digested DNA samples were separated on 0.8–1.25% modified TAE (40 mM Tris-acetate, 0.1 mM Na2 EDTA [pH 8]) agarose gels, and DNA bands were excised and purified using the Montage Gel Extraction Kit (Millipore) or the QiAgquick Gel Extraction Kit (QIAGEN). DNA samples were concentrated using Microcon YM-50 centrifugal filter units (Millipore) if necessary. PCR amplification products (for cloning or direct sequencing) were purified by the above-mentioned gel-extraction methods or by Montage PCR filter units (Millipore). Small DNA products (<1 kb), which were cloned into the pCR2.1-TOPO TA vector (Invitrogen), were transformed into One Shot TOP1. Chemically Competent Escherichia coli cells (Invitrogen), following the provided protocols. Inserts cloned into Tg or other vectors were ligated using T4 DNA ligase (Invitrogen), following the recomended insert/vector DNA ratios. Larger DNA inserts and DNA fragments cloned into large Tg vector constructs with a propensity for recombination were transformed into One Shot OmniMax 2 T1 Phage–resistant cells (Invitrogen) or SURE cells (Stratagene), following the suppliers’ protocols. Growth and propagation of transformed cells and selection of positive transformants were performed following standard molecular biological protocols.

Oligonucleotide primers

Primers were designed according to known sequence, using the Primer 3 (v0.4.0) primer design program (SourceForge) and were synthesized by Invitrogen. Commercial universal forward and reverse primers for M13 (Promega) were also used.

DNA sequencing

Sequencing was performed by 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 (http://www.bioinformatics.nl/tools/muscle.html).

Transient transfections

Sp2/0 mouse myeloma cells were maintained in culture in IMDM (Life Technologies) containing 10 IU/ml penicillin/streptomycin, (Life Technologies), 10 μg/ml gentamicin (Sigma), and 10% (v/v) heat-inactivated FCS (HyClone). Cells were fed 18 h prior to transfection. A total of 10–15 μg each DNA species to be transfected and 1.5 μg the plasmid were linearized separately by restriction enzyme digestion and purified using Amicon Micropure–EZ centrifugal filter devices (Millipore). Electroporation was performed using parameters previously published for Sp2/0 cells (39). Each linearized DNA sample was added to 1.6 × 107 Sp2/0 cells, and samples were incubated on ice for 10 min. The cells were then electroporated at 960 μF, 2 kV/cm using Gene Pulser Xcell (Bio-Rad). After an additional 10-min incubation on ice, each set of cells was transferred to a plastic media dish containing 3 ml prewarmed IMDM with 20% FCS. Cells were cultured at 37°C overnight, after which the supernatants were collected for ELISA, and transfectants were collected and stored in TRI Reagent (Molecular Research Center) at −80°C for subsequent RNA extraction and RT-PCR.

Southern hybridization

Southern blotting was performed to determine orientation and copy number of transgenes. Purified genomic DNA from Tg mice and control C57BL/6 wild-type mice was provided by Xenogen. Hybridization was done using Stratagene’s Illuminator Chemiluminescent Detection System, following the Southern blot procedure described in the accompanying manual. Probes were purified PCR products of the variable regions from the 24.3.1 HC or LC (24HCTg5′–3′ and 24LCTg5′–3′).

RNA isolation and RT-PCR

Total RNA was isolated from whole spleen or tissue culture cell lines using TRI Reagent (Molecular Research Center, Cincinnati, OH) following the protocol provided. Contaminating DNA was removed by treatment with DNase I (1 U/μg DNA) (Invitrogen), per the manufacturer’s protocol. RT-PCR was performed using the Superscript III One-step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen), using either gene-specific primers or Ig-Primer Sets (human or mouse) from Novagen. Reactions were performed on a Gene-Amp 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, 68°C for 1 min, and a 5-min extension at 68°C. Results of amplification were assessed by visualizing an aliquot of the RT-PCR products (10 μl each) on an ethidium bromide–containing 1% Tris HCl–borate–EDTA agarose gel. The remainder of the amplification products were purified by column filtration or gel extraction, as described above. Purified RT-PCR products were either cloned into plasmid vectors or submitted for sequencing.

Treatments to open blood–brain barrier

To open the blood–brain barrier (BBB) and stimulate Tg B cells, Tg and non-Tg littermates were immunized with streptococcal cell wall and LPS, following the method of Kowal et al. (40). Serum was collected before and after immunization and analyzed by capture ELISA using anti-mouse IgG1. Animals were sacrificed at the end of the experiments, brains were collected and placed in formalin for histopathological analysis and immunohistochemical staining, and serum was collected for ELISA. Spleens were collected from Tg mice and used for RNA extraction/RT-PCR, FACs analysis, and hybridoma production. Peripheral lymph nodes and bone marrow were harvested for FACS analysis.

ELISAs

Ninety-six–well polyvinyl, Immunolon-4 microtiter plates (Dynatech Laboratories) were coated with 10 μg/ml Ag overnight at 4°C. Plates were reacted with sera or hybridoma supernatant overnight at 4°C. Ab binding

Downloaded from http://www.jimmunol.org/ on guest on October 30, 2017
was detected using alkaline phosphatase conjugated to specific secondary Ab or to streptavidin. Plates were developed with 1 mg/ml p-nitrophenylphosphate-104 substrate (Sigma) at 50 μl/well. OD was quantified at 405 nm in an Opys MR microplate reader (Dynex Technologies) or a Synergy HT Multidetection microplate reader (Bio-Tek) using KC4 software. Capture ELISAs were performed using purified mouse anti-mouse IgG1 (Igh-4a) mAb (BD Pharmingen) or goat anti-mouse IgG1 (Sigma) as the coating Ab. Biotin-conjugated mAbs (mouse anti-mouse IgG1, mouse anti-mouse IgG2a, IgG2b [Igh-4b], rat anti-mouse IgG1 [BD Pharmingen]) diluted 1/5000 in 1% BSA-PBS (or 1/500 for anti-IgG1) were used as secondary Ab where appropriate. Biotinylated conjugates were further incubated with ExtrAvidin-Alkaline Phosphatase (Sigma) at the same dilution. Ag panel ELISA testing included ds- and ssDNA (Invitrogen), human dopamine receptor D2a (D2R long isoform; PerkinElmer) (41), and rat anti-mouse Ig receptor D2L (Permin) at 41 °C overnight, and then analyzed on a FACSCalibur automated four-color benchtop flow cytometer (BD Biosciences) at the University of Oklahoma Health Sciences Center Flow and Image Cytometry Laboratory. Dead and irrelevant cell populations were excluded by setting gates on the basis of forward and side scatter profiles. Stained cells were analyzed initially to estimate the percentage of cells expressing B220, a marker present on cells committed to the B lineage. B cell subpopulations were identified by anti-B220-PerCP-Cy5.5 in combination with fluorescently labeled Abs against the other cell surface markers, anti-CD23 and anti-CD21, to characterize the cells with regard to maturation level and location (i.e., marginal zone or follicular). Computer analysis was done using Summit v 4.3 software (Dako Colorado).

Competitive-inhibition ELISA with D2R peptide

Competitive-inhibition ELISA was performed in duplicate, as previously described (32, 44). Concentration of mAbs was 100 ng/ml. Peptide inhibitors were prepared as 1-10 μg/ml solutions in PBS (pH 7.2) and serially diluted from 500 to 1 μg/ml. The diluted inhibitors were mixed with an equal volume of each mAb and incubated at 37 °C for 1 h, followed by an overnight incubation at 4 °C. The mAb-inhibitor mixture was added to 96-well microtiter plates coated with human dopamine receptor D2a (PerkinElmer) at 10 μg/ml in bicarbonate buffer. Samples were allowed to incubate overnight at 4 °C. Ab binding was determined using alkaline phosphatase–conjugated anti-human IgG (γ-chain specific) F(ab’2) fragment (Sigma) was used for detection. To determine the D2R Ab titer, sera were diluted 1/100 in 1% BSA in PBS buffer and subsequently diluted 2-fold, and ELISA was performed as described above. For the D2R Ab titers, tests were performed separately three times, and the titration was done in duplicate in each assay. Titers were determined as the highest dilution with OD value of 0.10 to 2.0. Controls used included serum alone, secondary Ab conjugate alone, and 1% BSA alone.

Human D2R peptide sequences

D2R peptides were synthesized by Immunokontact (AMS Biotechnology, Lake Forest, CA) and purified by HPLC. The sequences and designations are as follows: DRD2 E1.1, MDPFLNLSWYDDDLERQNVSR and DRD2 E1.2, SRPENGSGDKAPHRYYYA. Coated peptide was a previously described human cardiac myosin peptide: FTRLKEALEKSEARRKERRTVLMLERVSLS (45, 46).

Flow cytometry

Lymphocytes from spleen, bone marrow, and lymph nodes were fluorescently labeled and analyzed by FACS. Spleens, lymph nodes, and bone marrow from Tg mice and non-Tg littermates were harvested, and single-cell suspensions were prepared. Spleens were pushed through autoclaved screens to homogenize tissue. Isolation of spleen cells and cells from peritoneal lymph nodes was performed using Histopaque-1083 B (Sigma). Bone marrow was isolated from mouse femurs, as follows: heads of femurs were cut off and discarded, and bones were placed in 0.6 ml centrifuge tubes, each with a hole in the end made by an 18-gauge needle. Each tube was placed in a 1.5-ml centrifuge tube and centrifuged for 30 s. RBCs in the collected bone marrow were removed by treatment with the Mouse Erythrocyte Lysing Kit (R&D Systems), following the manufacturer’s protocol. Cells from spleen, bone marrow, and lymph nodes were resuspended at 1.0 × 106 cells/ml in 500 μl PBS stain buffer (Pharmingen) and incubated for 30 min with the appropriate biotinylated or directly fluorescein-labeled Ab for FACS analysis. Cells were washed with PBS stain buffer after incubations; sequential incubations were performed for cell samples that were stained with more than one fluorochrome. All incubations were conducted on ice in the dark. Wash steps were carried out by adding 1 ml PBS stain buffer to each tube, followed by centrifugation at 1000 rpm for 5 min at 4 °C in a Beckman Coulter Allegra 25R centrifuge. Cells were labeled with Abs coupled to biotin (anti-mouse IgG1, anti-mouse IgG1) or one of the following fluorochromes: PerCP-Cy5.5 (anti-mouse B220), PE (anti-mouse CD23), or allophycocyanin (anti-mouse CD11c). Biotin-conjugated Abs were also incubated with streptavidin-FTTC. All labeled Abs were purchased from Pharmingen. Isotype-control Abs (Biotin Mouse IgG2a, κ, Biotin Mouse IgM, κ, PerCP-Cy5.5 Rat IgG2a, κ, PE-Rat IgG2a, κ, allophycocyanin Rat IgG2a, κ) were used at the same concentrations as the Abs of interest. After labeling, cells were resuspended in 500 μl 1× fixative containing 1% formaldehyde (R&D Systems). Labeled cells were stored in the dark at 4 °C overnight and then analyzed on a FACS Calibur automated four-color benchtop flow cytometer (BD Biosciences). The Journal of Immunology 5527

Immunohistochemistry

Colocalization studies were performed using paraaffin-embedded Tg mouse brain tissues. Brain sections were tested to determine whether Tg Ab was detected in vivo by B cells deposited in the brains of Tg mice. Formalin-fixed mouse brains were cut, mounted on slides, and stained following previously described protocols (11, 44). Briefly, tissue was fixed overnight in 10% buffered formalin, sectioned (5-μM thick), and mounted onto Microprobe probe-On Plus slides. Tissue was baked for 60 min and deparaffinized, followed by rehydration using graded ethanol. Paraffin-embedded Ag retrieval was performed using citrate buffer (10 mM sodium citrate, 0.05% Tween 20 [pH 6]). Slides were heated for 20 min and allowed to cool to room temperature. Slides were then washed twice in PBS (pH 7.4) and blocked with a protein blocker for 15 min at room temperature, followed by two additional washes in PBS. Immunohistochemistry by double immunostaining used Abs conjugated to FITC and tetramethyl rhodamine isothiocyanate (TRITC). For localization of catalase and GABA (1:100) biotinylated anti-GABA (Abcam) mAb was used (5 μg/ml; BD Pharmingen) in combination with FITC-conjugated streptavidin (1:20; Invitrogen). For immunostaining of dopaminergic neurons, rabbit polyclonal Ab to TH (Sigma) was used as the primary Ab (1:100), with TRITC-conjugated sheep anti-rabbit polyclonal Ab as the secondary Ab (1:100). Tissue staining by primary Abs was done by overnight incubation at 4 °C, and fluorescently labeled secondary Abs were incubated on tissues for 1 h at room temperature in a humidified chamber in the dark on the following day. Washes were done in PBS (pH 7.4). For positive control for anti-mouse IgG1 staining, mouse brain tissue was incubated with IgG1 Ab (mouse mAb to neuron-specific enolase; III in [Abcam]) (1:500) for 2 h, followed by washes and incubation with biotin-labeled anti-mouse IgG1 and streptavidin-FTTC, as previously described. Mouse liver tissue was used as negative control for TH Ab staining. Prior to viewing, slides were treated with Prolong Gold Anti-fade reagent with DAPI (Invitrogen), per the manufacturer’s recommended protocol, to reduce photobleaching and stain nuclei. Slides were viewed with an Olym- pus BX41 microscope equipped with DAPI, FITC, and TRITC filters, and micrographs were imaged with a SPOT InSight 2 Firewire Camera. Images were merged using SPOT Software V. 4.6 (Diagnostic Instruments). Colocalization studies were performed on paraaffin-embedded normal mouse brain tissue sections using SC-derived human mAbs 24.3.1, 37.2.1, and 31.1.1 (100 ng/ml). Detection of human mAbs was by FITC-conjugated goat anti-human IgM (1:50; Jackson ImmunoResearch). Incub- ation with media only (DMEM supplemented with 10% FCS) was performed as a control.
hybridoma fusion partner, a nonsecreting human × mouse heterohybridoma line (ATCC CRL-1823: American Type Culture Collection), which were selected using hypoxanthine, aminopterin, and thymidine–supplemented media and allowed to proliferate in TH medium, after which cell culture supernatants were tested for Ig production and specific Ag binding. Subsequently, hybridomas were cloned by limiting dilution at <1 cell/well and then subcloned twice to produce B cell hybridoma clones. Clones were maintained in DMEM with 10% FCS under standard cell culture conditions.

Screening of hybridomas

Hybridoma supernatants were screened by capture ELISA for isotype (IgG1, IgK) and allotype (IgG1a, IgG1b) of Ab and by Ag panel ELISA for antigenic specificity. V-gene cDNA sequence analysis was performed on each hybridoma clone. Total RNA was extracted from 5 × 10⁶ hybridoma cells using TRI Reagent (Molecular Research Center). RT-PCR was performed using mAb 24.3.1 V-gene specific primers, Mouse Ig-Primers (Novagen), and primers specific to γ and κ constant regions. The resulting RT-PCR products were purified and sequenced.

cAMP assays

Assays were done using A9-L-hD2 S.C.18 (ATCC CRL-10225), a fibroblast cell line expressing human D2R. The untransfected A9 cell line (ATCC CCL-1.4) was used as a control. Cells were grown and isolated for the assay following the published procedure of Tang et al. (47). Briefly, cells were plated in 12-well culture plates and grown to confluence (~1 × 10⁵ cells/well) in IMDM supplemented with 10% FBS (Life Technologies) at 37˚C. Prior to assays, cells were washed three times with prewarmed DMEM supplemented with 0.1% ascorbic acid and 20 mM Tris (pH 7.4) and preincubated in 400 μl the same medium for 10 min at 37˚C. Incubations were started by adding 100 μl the above-mentioned prewarmed DMEM containing test drugs (i.e., dopamine, 10 μg/ml [Sigma]), human serum (1:50), or hybridoma cell supernatant. Incubations were done for 20 min at 37˚C. Overnight treatment with pertussis toxin (100 ng/ml; Sigma) was done as a control. cAMP assay was performed using a cAMP Direct Immunoassay Kit (BioVision), following the manufacturer’s protocol, and a standard curve from 3.12 to 200 pmol was run on each plate. Concentration of cAMP in samples was calculated from the standard curve using GraphPad Prism 5 or KC4 software.

Ab-binding experiments using FLAG epitope-tagged D2R transfectants

HEK293T cells were cultured in 96-well plates in DMEM with 10% FBS (Sigma), 50 μU/ml penicillin, and 50 μg/ml streptomycin. Transient expression of D2R was achieved by transfecting cells with a plasmid vector (pcDNA/Zeo; Invitrogen) containing a FLAG-tagged D2L receptor cDNA construct using Lipofectamine LTX (Invitrogen) transfection reagent. Control cells were transfected with the empty vector. Forty-eight hours posttransfection, cells were fixed using 4% w/v formaldehyde (15 min at 4˚C) in PBS. Cells were blocked for 45 min with 3% BSA in PBS before incubation with the indicated Abs (1 h, 20˚C) in the blocking buffer. The M2 anti–FLAG-tagged mouse mAb (Sigma) was used at a dilution of 1:250 as a positive control. The affinity-purified human mAbs were used at a concentration ~3 μg/ml. Human sera from PANDAS and SC patients and normal healthy controls were used at 1:100 dilution. Cells were washed three times with PBS at 4˚C and then incubated (1 h, 20˚C) with the appropriate HRP-conjugated anti-mouse or anti-human secondary Abs (Jackson Immunoresearch; 1:5000 dilution in blocking buffer). HRP-catalyzed chemiluminescence was generated using SuperSignal West Femto Chemiluminescent Substrate (Pierce) and measured using a GloMax Microplate Luminometer (Promega). To normalize the signal for cell number, cells were incubated with ethidium bromide (1 μM in PBS, 5 min), and fluorescence intensity was measured using a SpectraMax Microplate Reader (Molecular Devices; excitation, 530 nm; emission, 590 nm). Data are reported as the ratio of luminescence/ethidium bromide fluorescence intensity.

Statistical analysis

For ELISA and cAMP assays, the effect of multiple treatment groups was evaluated by one-way ANOVA using GraphPad Prism 5 software. Differences of the means was evaluated by the Tukey test. For human sera ELISA data (Fig. 7), analysis was performed using Mann–Whitney and Kruskal–Wallis tests.

Results

Tg mice

To further investigate in vivo targets of human mAb 24.3.1 from SC, Tg mice expressing HC and LC V-region genes of anti-streptococcal/anti-brain human mAb 24.3.1 were generated by pronuclear microinjection of HC and LC Tg vector constructs into C57BL/6 mouse embryos. The human V L and V H genes were cloned into Tg vectors containing upstream regulatory (promoter, leader, and enhancer) and constant regions of murine Igκ (Fig. 1A) and murine IgG1 allotype a (Fig. 1B). The HC construct also contained the murine IgG1 membrane exons. Tg constructs were coinjected into C57BL/6 mouse embryos by Xenogen Biosciences-Caliper Life Sciences (Cranbury, NJ). Litters were screened with mAb 24.3.1 V-gene–specific primers for incorporation of the gene(s). The chimeric VH4 transgene IgG1a allotype allowed detection of expression in the C57BL/6 background (b allotype) by anti-allotypic Ab. Tg mice produced were primarily single Tg (containing VH4 only).

Expression of transgene(s) in mice resulted in production of a chimeric (human V-gene/mouse IgG1α C-region) Ab. The Tg24.3.1-IgG1α Ab was expressed in serum at 1–4 μg/ml, with the highest levels observed when the double Tg (VH4+VL4) was expressed (Fig. 2). Expression levels were monitored at monthly intervals. Fig. 2 shows levels in representative 6-mo-old Tg mice.

FACS analysis determined B cell surface expression of Tg24.3.1-IgG1α chimeric Ab. Splenic B cells from Tg mice expressed similar levels of IgG1α and endogenous IgG1β (data not shown), indicating no allelic exclusion. In bone marrow, some Tg mice had B cells that expressed more than twice as many chimeric IgG1α receptors as IgG1β receptors. Previous work suggests that Tg mice expressing Ig genes have reductions in relative B cell numbers in spleen, bone marrow, and peripheral blood and accelerated maturation and more rapid emigration of B cells due to earlier, more synchronous expression of the Tg HC (48).

![FIGURE 1. Schematic representation of DNA constructs used to generate 24.3.1 Tg mice. (A) LC construct. The Vκ construct contains the human mAb 24.3.1 Sall-flanked VJ region in addition to Ig promoter (Ps) and leader (Lκ) sequences and the mouse κ C region (Cκ). (B) HC construct. Human mAb 24.3.1 VDJ region flanked by Sall restriction sites was cloned into a mouse IgG1a construct that was described previously (37). Restriction sites: B, BamHI; C, ClaI; N, NotI; S, Switch region; M, membrane region.](http://www.jimmunol.org/ by guest on October 30, 2017)
Chimeric IgG1 Ab expressed in Tg mice demonstrated cross-reactive anti-streptococcal/anti-brain Ab specificities in Tg sera and Tg mAbs similar to human mAb 24.3.1

To determine whether binding specificities of chimeric Tg 24.3.1-IgG1 Ab matched those recognized by human mAb 24.3.1, sera from Tg mice were reacted with a panel of streptococcal and brain Ags. Single VH 24.3.1 and double VH+VL 24.3.1 Tg serum IgG1 Ab retained specificity to brain Ags lysoganglioside and tubulin, as well as streptococcal group A carbohydrate epitope GlcNAc and streptococcal wall membrane Ag (Fig. 3A, 3B). These sera did not react with ssDNA, dsDNA, or BSA, indicating the specificity of the Tg Abs. Fig. 3A shows double Tg serum reactivity, and single VH Tg sera are shown in Fig. 3B. Furthermore, mAbs were produced from splenic B cells of VH 24.3.1 Tg mice. Thirteen Tg B cell hybridoma clones producing strong anti-lysoganglioside binding by the Tg IgG1 Ab were selected. VH transgene expression in the hybridomas was verified by RT-PCR and subsequent cDNA sequencing through the V(D)J junction. All thirteen Tg-derived VH 24.3.1 mAbs (IgG1a) were similar and retained specificities to tubulin, lysoganglioside, and GlcNAc; three representative clones are shown in Fig. 3C. The specificities found in Tg mouse sera or Tg hybridomas expressing the 24.3.1 VH transgene were very similar to the human chorea-derived mAb 24.3.1. Twelve mAbs, including three mAbs shown, did not react with ssDNA or dsDNA. One 24.3.1 Tg-derived mAb (66-4.8) was unique in that it strongly recognized dsDNA (OD = 0.6). None of the Tg-derived mAbs reacted with BSA. These data further confirm the validity of the VH 24.3.1 Tg Ab in the Tg mice.

Nucleotide sequence analysis of Tg B cell hybridoma–derived VH genes showed similarities in LC VH gene usage, with some clones preferentially using Vκ 21E and Vκ 1 (bb1) LC genes previously described in mouse anti-peptide-DWEYSWVL/SN/idsDNA cross-reactive autoantibodies associated with lupus and neurologic symptoms (49, 50). Five Abs used the Vκ 21E subgroup (21.12) LC gene, and two used the Vκ 1 (bb1) LC gene (Table 1). VH-derived mAbs encoded by the 21.12 LC gene (66-1.23, 66-2.5, 66-6.11, 66-4.8, and 66-4.12) had >95% homology with mouse mAbs (16-9, 19-43, and 37g) that reacted with the DWEYS peptide [associated with neuropsychiatric lupus (50)] and with multiple autoantigens, including dsDNA, cardiolipin, and fibrinogen. Vλ LCs pairing with our Tg VH 24.3.1 have often been repeated in other reported autoantibody specificities, especially systemic lupus erythematosus (49).

To summarize, our chimeric Tg 24.3.1-IgG1 Ab was expressed in B cells and sera of Tg mice. We expected that our Ab would originate from the sera and penetrate the brain. Therefore, we conducted experiments to determine whether the Tg 24.3.1-IgG1 Ab could target brain tissues. The studies described below show that Tg 24.3.1-IgG1 could be detected in serum, expressed by B cells, and deposited in brain. Therefore, the Tg 24.3.1-IgG1 Ab expressed in B cells and released from plasma cells in serum penetrated the brain and was taken up by neuronal cells in vivo.

In vivo chimeric Tg Ab localized in dopaminergic neurons in basal ganglia of Tg VH24.3.1 mice

To determine where chimeric Tg 24.3.1-IgG1 Ab (IgG1a) was localized in the brain in vivo, we investigated tissue sections of brains from Tg VH24.3.1 mice. Anti-IgG1a allotypic Ab detected Tg 24.3.1-IgG1 within neuronal cell bodies in the basal ganglia.
BRAIN AUTOANTIBODY TARGETS DOPAMINERGIC NEURONS

Table I. V<sub>k</sub> gene usage in Tg mAbs derived from 24.3.1 V<sub>H</sub> Tg mouse

<table>
<thead>
<tr>
<th>Hybridoma</th>
<th>V&lt;sub&gt;k&lt;/sub&gt;</th>
<th>J&lt;sub&gt;e&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>66-1.9</td>
<td>1 (bb1)</td>
<td>2 (86%)</td>
</tr>
<tr>
<td>66-1.23</td>
<td>21.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2 (98%)</td>
</tr>
<tr>
<td>66-1.24</td>
<td>1 (bb1)</td>
<td>2 (92%)</td>
</tr>
<tr>
<td>66-2.5</td>
<td>21.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2 (97%)</td>
</tr>
<tr>
<td>66-2.11</td>
<td>21.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2 (94%)</td>
</tr>
<tr>
<td>66-4.8</td>
<td>21.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2 (95%)</td>
</tr>
<tr>
<td>66-4.12</td>
<td>21.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4 (91%)</td>
</tr>
</tbody>
</table>

Hybridoma cell lines are listed with the most closely homologous reported germ-line variable and joining segments.

*Percentage homology to germline sequence is noted in parentheses. Sequencing was performed on at least three replications with different primer combinations.

<sup>b</sup> V<sub>k</sub>21E subgroup.

**FIGURE 4.** Chimeric Tg24.3.1 V<sub>H</sub> IgG<sub>1a</sub> Ab–targeted dopaminergic neurons in Tg mouse brain in vivo. Colocalization of Tg 24.3.1 IgG<sub>1a</sub> (anti-IgG<sub>1a</sub> Ab, green) and TH (anti-TH Ab, red) by immunofluorescent staining of mouse brain tissue. TH is a marker for dopaminergic neurons (51, 52). Chimeric Tg24.3.1-IgG<sub>1a</sub> Ab colocalized in TH-rich dopaminergic neurons in vivo (Fig. 4A–C). Fig. 4A–F show Tg 24.3.1-IgG<sub>1a</sub> Ab within dopaminergic neurons in brains of Tg V<sub>H</sub>24.3.1 mice; enlarged images are also shown (Fig. 4D–F). Similar results were observed in five of seven Tg V<sub>H</sub>24.3.1 mice, whereas no IgG<sub>1a</sub> was detected in brains of non-Tg mice (Fig. 4G). Fig. 4H shows TH Ab staining in non-Tg brain, and Fig. 4I shows the merged image for Fig. 4G and Fig. 4H. Secondary Ab controls were negative in all experiments (Fig. 4J, 4K). In Fig. 4L, DAPI-stained brain denotes the basal ganglia region where colocalized fluorescence (Fig. 4A–F) was observed. IgG<sub>1a</sub>-positive neuronal cells were also observed in the cortex of Tg mouse brain and did not stain with anti-TH Ab (Fig. 4N, 4O). Therefore, our data suggest that our Tg autoantibody localized in vivo in both dopaminergic and nondopaminergic neurons in the basal ganglia within the substantia nigra/ventral tegmental area and cortex, respectively. The presence of D2R on other types of neurons could explain our results (53–55). DAPI-stained brain (Fig. 4M) indicates the region where IgG<sub>1a</sub>–FITC staining in the cortex was observed. No IgG<sub>1a</sub> staining was seen in the hippocampus (Fig. 4Q, 4R). Fig. 4P shows DAPI staining of hippocampus.

Our Tg24.3.1-IgG<sub>1a</sub> Ab colocalized in dopaminergic neurons in the substantia nigra or ventral tegmental area, which are part of the basal ganglia. Dopaminergic neurons originating in the substantia nigra/ventral tegmental area and terminating in the dorsal striatum play a critical role in basal ganglia functions (56–59). The substantia nigra and the adjacent ventral tegmental area, both intrinsic nuclei of the basal ganglia, contain dopaminergic neurons that project into the striatum. Nigrostriatal axon terminals release dopamine into the striatum. D2Rs are expressed on the dopaminergic neurons in the substantia nigra and ventral tegmental area, as well as on neurons in the striatum (55, 58, 60, 61). However, dopaminergic neuronal cell bodies are only seen in the substantia nigra and ventral tegmental area, and the axons of these cells project into the striatum. All dopamine receptor subtypes are expressed in dopamine neurons.
FIGURE 5. Tg24.3.1-IgG1a and human SC mAb 24.3.1 reacted and colocalized with D2R. (A) Reactivity of 24.3.1 Tg mAbs and Tg mouse serum (IgG1a) with human D2R in the ELISA using anti-mouse IgG1a. Tg mAbs reacted similarly to human mAb 24.3.1 with D2R. Other human chorea-derived mAbs 37.2.1 and 31.1.1 reacted less with D2R than mAb 24.3.1. The p value was obtained by the Tukey test using one-way ANOVA. (B) Chimeric Tg24.3.1 VH IgG1a Ab colocalized with anti-D2R in Tg mouse brain in vivo. Colocalization of Tg 24.3.1 IgG1a (anti-IgG1a Ab) and rabbit polyclonal anti-D2R Ab by immunofluorescent staining of mouse brain tissue. Top row, Brain sections of Tg mouse, showing FITC-labeled anti-mouse IgG1a (left panel), TRITC-labeled anti-D2R Ab (center panel), and merged image (right panel) (original magnification ×20). Middle row, Enlarged images from top row. Bottom row, Secondary Ab controls: FITC-conjugated streptavidin (1:20; left panel) and TRITC-conjugated sheep anti-rabbit Ab (1:100; right panel) were negative. (C) Human SC-derived mAb 24.3.1 (IgM) colocalized with anti-D2R in normal mouse brain tissue sections. (Figure legend continues)
the striatum, but D1Rs and D2Rs are most abundant (58). D2Rs are also expressed by striatal interneurons (54). D2Rs have presynaptic and postsynaptic localizations and functions (54, 55, 61). The basal ganglia location of the Tg24.3.1-IgG1α–targeted dopaminergic TH+ neurons was determined based on the presence of TH as a marker for dopaminergic neurons in the substantia nigra/ventral tegmental area of the basal ganglia. Dopaminergic (TH+) neuronal cell bodies are absent from the striatum.

Additional colocalization studies were performed on normal mouse brain tissue to determine whether SC-derived human mAb 24.3.1 (IgM) reacted with dopaminergic neurons. As shown in Supplemental Fig. 1, mAb 24.3.1 binds to TH-rich dopaminergic neurons as expected, because dopaminergic neurons were targeted in the Tg mice (Fig. 4). The right panels in Supplemental Fig. 1 are merged images. Human mAbs 37.2.1 and 31.1.1 were observed to bind dopaminergic neurons (Supplemental Fig. 1). Media controls and secondary Ab controls were negative in all experiments. Thus, both the chorea-derived mAb 24.3.1 and the Tg expressed 24.3.1 Ab bind to dopaminergic neurons (Fig. 4, Supplemental Fig. 1).

Tg serum Ab and IgG1α Tg-derived mAbs reacted with human D2R

To investigate the possibility that the Tg-derived Abs, as well as the chorea-derived human mAb 24.3.1, might bind to human D2R, a receptor present on dopaminergic neurons (54, 55), human mAb 24.3.1, Tg sera, and Tg-derived mAbs were tested for reactivity with human D2R. Single- and double-Tg mouse serum and Tg hybridoma mAbs (IgG1α) exhibited strong reactivity (OD > 2.0) with human D2R (Fig. 5A). All 13 Tg-derived mAbs exhibited reactivity with D2R; three of those tested are shown in Fig. 5A. Strong reactivity with D2R was seen for chorea-derived human mAb 24.3.1, from which the VH and VL genes were derived and expressed in the Tg mice (Fig. 5A). Tg sera, as well as Tg-derived mAbs, reacted with D2R similar to mAb 24.3.1. Reactivity of chorea-derived mAbs 37.2.1 and 31.1.1 with D2R was considerably lower than seen with mAb 24.3.1.

To determine whether our chimeric Tg24.3.1-IgG1α Ab colocalized with D2R, we treated the Tg brain sections with rabbit polyclonal anti-D2R Ab and anti-IgG1α Ab to detect the Tg24.3.1 Ab. As shown in Fig. 5B, chimeric Tg24.3.1-IgG1α Ab colocalized with anti-D2R (merged images shown in right panels) in Tg mouse brain. Secondary Ab controls were negative.

To compare binding of Tg24.3.1-IgG1α Ab after expression in Tg mice with direct binding of human mAb 24.3.1 to brain tissues, normal mouse brain tissue sections were reacted with SC-derived human mAb 24.3.1 and rabbit polyclonal Ab to D2R. As shown in Fig. 5C, human mAb 24.3.1 colocalized with anti-D2R similar to Tg 24.3.1-IgG1α Ab (merged images, right panels). Media controls and secondary Ab controls were negative.

Chorea-derived mAb 24.3.1 strongly reacted with FLAG epitope-tagged D2R transfectants on the cell surface and D2R extracellular peptide sequence

To further confirm dopamine receptor binding of our human chorea-derived mAb 24.3.1, we examined the binding of the Ab to HEK293T cells transiently expressing a D2R construct that contained a FLAG epitope tag at the extracellular N terminus (62, 63). Transient expression of FLAG-tagged D2R is evidenced by the enhanced binding of the anti-FLAG Ab specifically to the cells transiently transfected with cDNA for the D2R construct (Fig. 6A). Enhanced binding of human chorea-derived mAb 24.3.1 to cells transiently expressing the D2R construct is observed relative to untransfected cells that do not express D2R. Chorea-derived mAbs 31.1.1 and 37.2.1 exhibited significantly enhanced binding to the FLAG-tagged D2R–expressing cells but were less reactive compared with mAb 24.3.1 (Fig. 6B). Thus, all three SC mAbs bind significantly to D2R (Fig. 6B).

To determine whether IgG in SC and PANDAS sera reacted with extracellular D2R surface exposed with a FLAG tag, human sera from SC and PANDAS patients and normal control sera were tested in the FLAG assay. We found that all SC sera tested reacted consistently with extracellular D2R in the FLAG epitope-tagged transfected cell line, whereas all PANDAS sera were negative (Fig. 6C). Our data correlate well with other studies that found SC IgG positive and PANDAS IgG negative by FACS analysis of HEK293 cell lines expressing D2R (64). Table II compares results from the FLAG-tagged D2R assay with other D2R assays, including ELISA and signaling through D2R in the cAMP assay (cAMP results described below indicate that both SC and PANDAS sera signal through D2R, whereas only the SC IgG binds to the FLAG-tagged D2R).

To investigate the reactivity of human SC mAb 24.3.1 with D2R, mAb 24.3.1 was reacted in ELISA with several peptides that we synthesized from the D2R extracellular region; the most reactive peptide was chosen for a competitive-inhibition ELISA. Chorea mAbs 24.3.1, 31.1.1, and 37.2.1 all reacted most strongly with D2R peptide E1.1 (OD 1.0) and were tested further to determine whether binding to whole D2R could be inhibited in vitro by blocking with D2R E1.1 in competitive-inhibition ELISA. D2R peptide E1.1 was a strong inhibitor of SC mAb 24.3.1, 31.1.1, and 37.2.1 binding to D2R and inhibited D2R recognition by chorea mAbs in a dose-dependent manner (Fig. 6D, right panel, Fig. 6E). A commercial anti-human D2R mAb, specific for the region included in the D2R E1.1 peptide (mAb 1B11; Sigma), was compared with mAb 24.3.1 in the competitive-inhibition ELISA (Fig. 6D, left panel). A previously characterized peptide from human cardiac myosin (45, 46) did not strongly inhibit binding of either anti-D2R mAb 1B11 or chorea mAb 24.3.1 to whole D2R in ELISA (Fig. 6D, control peptide). In Fig. 6D, the y-axis scale is different for the two Abs shown; the condition “no peptide” shows an OD of 2 with the commercial D2R E1.1 Ab and an OD ~1 with the SC mAb 24.3.1. Fig. 6D shows that the two Abs differ significantly in the inhibition curves. A higher concentration of inhibitor peptide D2R E1.1 (~62 μg/ml) was required to reach ~50% inhibition of cross-reactive SC mAb 24.3.1, whereas <1 μg/ml peptide produced ~50% blocking of the commercial positive-control D2R E1.1 peptide Ab. Neither Ab was significantly inhibited with 500 μg/ml of a “control peptide”: 25% inhibition was seen for the SC mAb 24.3.1, and 12% inhibition was observed for the commercial positive-control anti-D2R E1.1 peptide Ab. Inhibition of both mAbs was significantly greater with the D2R E1.1 peptide (Fig. 6D). In addition, extracellular D2R peptide E1.2 did not exhibit strong inhibition, and it behaved similarly when reacted with the commercial anti-D2R Ab and SC mAb 24.3.1 (Fig. 6D). Two
FIGURE 6. Human SC mAb 24.3.1 reacted with FLAG epitope-tagged D2R at the cell surface and with a D2R peptide. (A) Quantification of the relative levels of cell surface binding of anti-FLAG M2 Ab to HEK293T cells transfected with cDNA for the FLAG-tagged D2R construct or HEK293T cells transfected with an empty vector (untransfected cells). White bars represent HEK293T cells treated with control media (no Ab), and black bars represent HEK293T cells treated with anti-FLAG M2 Ab (mean ± SD, n = 5). (B) Quantification of the relative levels of cell surface binding of human chorea-derived SC mAbs to HEK293T cells transfected with cDNA for the FLAG-tagged D2R construct (black bars) or with empty vector (untransfected cells, white bars) (mean ± SD, n = 5). All of the chorea-derived SC mAbs showed significant binding to D2R transfectants compared with cells transfected with empty vector. (C) Human SC sera reacted with FLAG epitope-tagged D2R at the cell surface. Quantification of the relative levels of cell surface binding of human PANDAS sera (P), SC sera (SC), and sera from normal healthy controls (N) to HEK293T cells transfected with cDNA for the FLAG-tagged D2R construct (black bars) or with empty vector (untransfected cells, gray bars) (mean ± SD, n = 4). Four SC sera tested (SC1–4). (Figure legend continues)
D2R peptides in the ELISA. Tg mAbs expressing human mAb 24.3.1 HC (VH) were tested in the direct ELISA for reactivity with D2R peptides E1.1 and E1.2 and behaved similarly when reacted with the commercial mouse anti-D2R mAb and our chorea-derived human mAb 24.3.1. Control peptide and extracellular D2R peptide E1.2 did not strongly inhibit binding below the graphs. (D) Dose-response inhibition of human SC mAbs 24.3.1 and 31.1.1 binding to D2R by D2R peptide E1.1. Tg mAbs showed specificity for peptide D2R E1.1. Media control (negative) not shown. (E) Dose-response inhibition of two other human SC mAbs 31.1.1 and 37.2.1 by D2R peptide E1.1. Dose-dependent inhibition of human SC mAbs 31.1.1 and 37.2.1 by extracellular D2R peptide E1.1 (p < 0.001). D2R peptide E1.1 did not show significant inhibition compared with E1.2. There was a significant difference between the inhibition observed for peptide E1.1 versus E1.2 (Fig. 6F). Although all three SC mAbs reacted similarly with the D2R peptide, they differed with regard to their signaling and binding properties of D2R and did not react with D2R with the same intensity in the ELISA.

other SC mAbs, 31.1.1 and 37.2.1, produced from the same individual, also showed similar peptide reactivity and inhibition patterns (Fig. 6E). D2R peptide E1.2 did not show significant inhibition compared with E1.1. There was a significant difference between the inhibition observed for peptide E1.1 versus E1.2 (mAb 24.3.1 and mAb 31.1.1, p < 0.001; mAb 37.2.1, p < 0.01) (Fig. 6D, 6E). Tg mAbs also reacted with D2R peptides in the direct ELISA and showed stronger specificity to D2R E1.1 peptide compared with D2R E1.2 peptide (Fig. 6F). Although all three SC mAbs reacted similarly with the D2R peptide, they differed with regard to their signaling and binding properties of D2R and did not react with D2R with the same intensity in the ELISA.

IgG in SC and PANDAS with fine choreiform movements reacted with D2R

To determine whether autoantibodies against D2R were elevated in sera from humans with SC and related disorders, we used ELISA to investigate a small group of well-characterized human sera from SC, PANDAS, TS, and OCD. Our PANDAS (22) group represents children who had acute-onset OCD and/or tics, as well as fine choreiform piano-playing movements (fingers and toes), following a streptococcal infection, which may indicate a type of PANDAS. Human sera were titrated in the ELISA against the D2R membrane Ag. As shown in Fig. 7A, IgG reactivity with D2R was significantly elevated in SC and in a PANDAS group with fine choreiform movements.

Table II. Summary of immunoreactivity of human SC and PANDAS sera

<table>
<thead>
<tr>
<th>Human Sera ID No.</th>
<th>D2R ELISA IgG Titer</th>
<th>cAMP</th>
<th>D2 FLAG</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SC1</td>
<td>8,000</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>SC2</td>
<td>16,000</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>SC3</td>
<td>16,000</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>SC4</td>
<td>32,000</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>SC5</td>
<td>16,000</td>
<td>Positive</td>
<td>Not tested</td>
</tr>
<tr>
<td>SC6 (mAb donor)</td>
<td>32,000</td>
<td>Positive</td>
<td>Not tested</td>
</tr>
<tr>
<td>SC7</td>
<td>16,000</td>
<td>Positive</td>
<td>Not tested</td>
</tr>
<tr>
<td>SC8</td>
<td>16,000</td>
<td>Positive</td>
<td>Not tested</td>
</tr>
<tr>
<td>PANDAS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P1</td>
<td>5,120</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>P2</td>
<td>8,000</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>P3</td>
<td>8,000</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>P4</td>
<td>128,000</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>P5</td>
<td>8,000</td>
<td>Positive</td>
<td>Not tested</td>
</tr>
<tr>
<td>P6</td>
<td>16,000</td>
<td>Positive</td>
<td>Not tested</td>
</tr>
<tr>
<td>P7</td>
<td>32,000</td>
<td>Positive</td>
<td>Not tested</td>
</tr>
<tr>
<td>Normals</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N1</td>
<td>16,000</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>N2</td>
<td>2,000</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>N3</td>
<td>8,000</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>N4</td>
<td>2,000</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>N5</td>
<td>16,000</td>
<td>Negative</td>
<td>Not tested</td>
</tr>
<tr>
<td>N6</td>
<td>2,000</td>
<td>Negative</td>
<td>Not tested</td>
</tr>
<tr>
<td>N7</td>
<td>4,000</td>
<td>Negative</td>
<td>Not tested</td>
</tr>
</tbody>
</table>

showed significant (p < 0.001 or p < 0.05) binding to D2R transfectants compared with cells transfected with empty vector. Four PANDAS sera tested (P1–4) were negative and did not show significant binding. Normal control sera tested were negative. *, **, ***, #, and ## in (A)–(C) denote p values shown below the graphs. (D) D2R peptide E1.1 significantly inhibited binding of human SC mAb 24.3.1 to D2R in a dose-dependent manner. Left panel, Dose-dependent inhibition of anti-D2R mAb 1B11 binding to D2R by extracellular D2R peptide E1.1 (p < 0.001). Right panel, Significant (p < 0.001) inhibition of chorea-derived mAb 24.3.1 binding to D2R by D2R peptide E1.1. Control peptide and extracellular D2R peptide E1.2 did not strongly inhibit binding and behaved similarly when reacted with the commercial mouse anti-D2R mAb and our chorea-derived human mAb 24.3.1, p < 0.001, D2R E1.1 versus E1.2. Media control (negative) not shown. (E) Dose-response inhibition of two other human SC mAbs 31.1.1 and 37.2.1 by D2R peptide E1.1. Dose-dependent binding inhibition of human SC mAbs 31.1.1 and 37.2.1 to D2R by extracellular D2R peptide E1.1 (p < 0.001). D2R peptide E1.1 did not show significant inhibition compared with E1.1 (mAb 31.1.1, p < 0.001; mAb 37.2.1, p < 0.01). D2R peptide E1.2 did not strongly inhibit. Extracellular D2R peptide E1.2 did not strongly inhibit. Media control (negative) not shown. (F) SC Tg Vq24.3.1 mAbs reacted with D2R peptides in the ELISA. Tg mAbs expressing human mAb 24.3.1 HC (Vq1) were tested in the direct ELISA for reactivity with D2R peptides E1.1 and E1.2. Tg mAbs showed specificity for peptide D2R E1.1. Media control (negative) not shown. p < 0.001, D2R E1.1 versus D2R E1.2.
choreiform movements but not in TS, OCD, ADHD, or normal age-matched subjects. The dot plot shows normal controls with an anti-D2R mean titer of 6,000, whereas the mean titer in SC was 17,600 (~3-fold increase). In PANDAS, the mean titer was 13,449 (~2-fold the normal mean). Our ELISA data suggest that auto-antibodies against D2R were associated more significantly with movement disorders, such as SC or PANDAS with fine choreiform movements, and not with TS, OCD, or ADHD. Fig. 7B shows IgG reactivity of SC and PANDAS IgG with D2R compared with dopamine D1 receptor (D1R). When anti-D2R was compared with anti-D1R in SC and PANDAS, the $p$ values were significantly different ($p = 0.001$ and $p < 0.0001$, respectively; Fig. 7B). Fig. 7C used the same data as shown in Fig. 7B, but it shows OD values at 1:500 serum dilution rather than titers for comparison.

Chorea-derived human mAb 24.3.1 signaled human D2R and inhibited adenylate cyclase activity in D2R transfectants

To study Ab activation of the D2 inhibitory receptor, D2R-expressing transfectants were reacted with human mAbs, and changes in adenylate cyclase were measured as cAMP levels in cell lysates. Chorea-derived human mAb 24.3.1, which reacted with the human D2R membrane Ag in the ELISA, signaled D2R and inhibited cAMP in the D2R-expressing transfectants, comparable to activation of D2R by dopamine. Fig. 8A shows D2R transfectants after...
treatment with dopamine and human chorea-derived mAbs 24.3.1, 31.1.1, and 37.2.1. Transfectants treated with 1 μM dopamine as a positive control showed 53% inhibition of cAMP compared with untreated transfectants, which is consistent with results of previous studies that showed ~50% inhibition (47). Human chorea-derived mAb 24.3.1–treated D2R-transfected cells showed a reduction in cAMP that was comparable to that of dopamine stimulation. Although D2R transfectants incubated with chorea-derived mAb 31.1.1 also showed a small reduction in cAMP (~18%), it was significantly less than with mAb 24.3.1, and chorea-derived mAb 37.2.1 did not activate D2R (0% for mAb 37.2.1) because cAMP was not significantly reduced. As expected, a receptor-mediated decrease in cAMP levels was not inhibited in cells preincubated with pertussis toxin. D2Rs are coupled to G proteins of the Gi/o subfamily, which are pertussin toxin sensitive; consequently, pertussin toxin treatment blocked D2R-mediated decreases in cAMP (47, 65). Control nontransfected A9 cells showed no significant differences in cAMP levels in untreated and treated cells (Fig. 8B). Thus, human chorea-derived mAb 24.3.1 showed a significant activation of D2R compared with the same isotype and

![Figure 8](http://www.jimmunol.org/)

**FIGURE 8.** Human mAb 24.3.1 signaled human D2R in transfected D2R cells by inhibiting adenylate cyclase activity comparable to dopamine. Inhibition of adenylate cyclase by receptor activation in human D2R-transfected cell lines (A9-hD2R) was quantified by cAMP levels measured in cell lysates. Incubation of the D2R cell line with chorea-derived mAbs 24.3.1, 31.1.1, and 37.2.1. Results are shown from cAMP direct-competition immunoassay. (A) D2R-transfected cells treated with mAb 24.3.1 showed a 53% decrease in cAMP, which was comparable to dopamine-treated cells (p < 0.001). Cells treated with pertussis toxin are also shown; this treatment was used as an experimental control, because D2R-mediated activation and inhibitory signaling with concomitant decreases in cAMP can be blocked by preincubation of cells with pertussis toxin. (B) Unresponsive control (nontransfected) A9 cells treated with dopamine and mAbs. (C) Dose-response curve showed cAMP levels in D2R-transfected cells following treatment with mAb 24.3.1 at 1, 5, 10, 25, and 50 ng and unresponsive control (nontransfected) A9 cells after same treatment. Assays were performed in triplicate. The p value was obtained by the Tukey test using one-way ANOVA. The following concentrations of mAbs were tested: 24.3.1, 90 ng/ml; 31.1.1, 100 ng/ml; and 37.2.1, 100 ng/ml. ns, not significant.
concentrations of other chorea-derived mAbs. Fig. 8C shows a dose-response curve of D2R transfectants treated with 1, 5, 10, 25, or 50 ng of mAb 24.3.1, whereas nontransfected A9 cells were unresponsive after the same treatment. The data show that mAb 24.3.1 inhibitory signaling through D2R is dose dependent. mAbs derived from Tg24.3.1-IgG1a mice were tested for D2R signaling, but no reduction in cAMP was seen (data not shown). Lack of D2R signaling by Tg mAbs may be due to mouse LC pairing with our human/mouse Tg V_{iH} 24.3.1–IgG1a, which may affect the epitope recognition or avidity required for signaling. Because Tg 24.3.1-IgG1a Ab localized in dopaminergic neurons in vivo, and human mAbs and Tg mAbs all recognized D2R, the data strongly suggest that D2R on neurons may be one of the Ab targets in vivo, as was shown in our colocalization studies on Tg mouse brain tissue (Fig. 5B).

Inhibitory signaling of adenylate cyclase activity in D2R transfectants by human SC sera

In support of the hypothesis that SC mAb 24.3.1 and Tg 24.3.1-IgG1a Ab came from chorea donor sera with D2R signaling, acute serum from the mAb 24.3.1 human B cell donor was shown to have an elevated serum anti-D2R IgG titer of 32,000 by ELISA, which is ~5-fold greater than the normal mean (6,000), and the acute serum showed strong signaling inhibition of D2R comparable to dopamine (Fig. 9A). Assay results, shown in Fig. 9A, illustrate the inhibition as a decrease in cAMP levels in transfected cell lysates of human D2R transfectants by the mAb 24.3.1 donor serum but not in control A9 cells (Fig. 9B). Results are shown after treatment with dopamine, anti-D2R serum, normal serum, and mAb 24.3.1. As shown in Fig. 9A, transfectants treated with mAb donor sera (SC6) showed significantly decreased cAMP levels (40% decrease) relative to untreated cells, and this was comparable to the inhibitory response of mAb-treated transfected cells (43%). Transfectants treated with another SC sera (SC7), with a serum anti-D2R IgG titer of 16,000, also showed a similar decreased (~40%) cAMP level. Additionally, CAMP levels were significantly decreased \( p < 0.001 \) in transfectants treated with five other SC sera with anti-D2R titers of 8,000 or 16,000. Dopamine-treated D2R transfectants showed a 48% reduction in cAMP levels, whereas levels in transfected cells treated with normal serum (anti-D2R titers of 2,000, 8,000, or 16,000) were not significantly inhibited (Fig. 9A). Control nontransfected A9 cells (Fig. 9B) showed no significant differences in CAMP levels between treated and untreated cells. Our results suggest that the high level of anti-D2R Abs detected in ELISA (32,000) in the human SC mAb 24.3.1 donor sera and other human SC sera signaled the human D2R, with a concomitant reduction in adenylate cyclase (cAMP) activity. Normal sera with an anti-D2R titer of 2,000, 8,000, or 16,000 did not signal D2R. Our results illustrate how the human chorea donor sera elicited the same inhibitory signaling response as did mAb 24.3.1 in D2R-transfected cells, whereas normal sera with comparable anti-D2R ELISA titers did not.

Inhibitory signaling of adenylate cyclase activity in D2R transfectants by human PANDAS sera

Using the D2R-transfected cell line, human PANDAS sera with elevated anti-D2R Ab show decreased cAMP levels. Inhibition of adenylate cyclase by receptor stimulation in human D2R-transfected cell lines (A9-D2R) was quantified by cAMP levels measured in cell lysates. Incubation with human SC mAb 24.3.1 Ab donor serum, other SC sera, and normal healthy control sera. Results are shown from cAMP direct-competition immunoassay. (A) D2R-transfected cells treated with SC Ab donor serum (SC6; anti-D2R titer 32,000) and SC serum 7 (SC7; anti-D2R titer 16,000) showed a 40% decrease in cAMP levels comparable to dopamine (48%); and mAb 24.3.1–treated cells (43% decrease); \( p < 0.0001 \). CAMP levels were also significantly decreased \( p < 0.001 \) in transfectants treated with five other SC sera (SC1, SC2, SC3, SC5, SC6) with anti-D2R titers of 8,000 and 16,000. CAMP level was not reduced significantly in transfected cells treated with normal serum (anti-D2R titers 2,000, 8,000, or 16,000). (B) Control (nontransfected) A9 cells were unresponsive. Assays were performed in triplicate. The \( p \) values were obtained by the Tukey test using one-way ANOVA. Concentration of mAb used was 24.3.1–90 ng/ml. ns, Not significant.

![FIGURE 9](http://www.jimmunol.org/)

D2R transfectants treated with human SC sera with elevated anti-D2R Ab show decreased cAMP levels. Inhibition of adenylate cyclase by receptor stimulation in human D2R-transfected cell lines (A9-D2R) was quantified by cAMP levels measured in cell lysates. Incubation with human SC mAb 24.3.1 Ab donor serum, other SC sera, and normal healthy control sera. Results are shown from cAMP direct-competition immunoassay. (A) D2R-transfected cells treated with SC Ab donor serum (SC6; anti-D2R titer 32,000) and SC serum 7 (SC7; anti-D2R titer 16,000) showed a 40% decrease in cAMP levels comparable to dopamine (48%); and mAb 24.3.1–treated cells (43% decrease); \( p < 0.0001 \). CAMP levels were also significantly decreased \( p < 0.001 \) in transfectants treated with five other SC sera (SC1, SC2, SC3, SC5, SC6) with anti-D2R titers of 8,000 and 16,000. CAMP level was not reduced significantly in transfected cells treated with normal serum (anti-D2R titers 2,000, 8,000, or 16,000). (B) Control (nontransfected) A9 cells were unresponsive. Assays were performed in triplicate. The \( p \) values were obtained by the Tukey test using one-way ANOVA. Concentration of mAb used was 24.3.1–90 ng/ml. ns, Not significant.
difference in cAMP levels. Our results suggest that sera from SC, as well as from other streptococcal-associated neuropsychiatric sequelae, such as PANDAS with fine piano playing choreiform movements, signal the human D2R.

Discussion

Little is known about the targets of autoantibodies in the brain in movement or behavioral disorders, and the effects of autoantibody targeting dopaminergic neurons or their dopamine receptors is virtually unknown. Studies suggest that anti-streptococcal Abs target the brain in human disease or their animal models and may lead to altered behaviors or movements (10, 13, 15, 16, 35, 64–68). Two recent studies (35, 64) suggested that Abs in SC recognize the D2R in humans, and studies (35, 66, 67) in rats and mice linked behavioral changes with streptococcal exposure and D2R. Our studies and those of other investigators used different methodologies to detect autoantibodies against D2R (64). Table II summarizes our human sera studies in three immunoassays, as shown in Figs. 6, 7, 9, and 10. The SC-derived human mAb studies are found in Figs. 5–8 and Supplemental Fig. 1, and the Tg Ab studies are found in Figs. 2–6. Collectively, the studies suggest that D2R is the target of autoantibodies produced in SC and PANDAS. Abs against D2R measured by ELISA recognize both extracellular and intracellular epitopes, whereas functional Ab signaling or extracellular binding assays would recognize only extracellular domains of the receptor. Our peptide, which inhibited mAb 24.3.1, was an extracellular epitope at the exposed N terminus of D2R.

To test the hypothesis that autoantibodies from movement and behavioral disorders target neurons and possibly D2R in the brain, we developed a novel Tg mouse expressing an SC-derived brain autoantibody. The Tg autoantibodies exhibited cross-reactive anti-neuronal specificities, in particular D2R reactivity, and targeted dopaminergic neurons in vivo. We show in our study that a human SC-derived Ab, with defined specificity and with a defined VH-region gene linked to a mouse IgG1 C-region gene, targeted neurons in the Tg mouse brain that was most likely the substantia nigra/ventral tegmental area of the basal ganglia. Only the substantia nigra and ventral tegmental area contain dopaminergic neuronal cell bodies that project long axonal extensions into the striatum. Dopaminergic neuronal cell bodies are not observed in the striatum. The Tg24.3.1-IgG1a construct was found within the striatum. Dopaminergic neuronal specificities, in particular D2R reactivity, and targeted neurons in the Tg mouse brain that was most likely the substantia nigra/ventral tegmental area of the basal ganglia. Only the substantia nigra and ventral tegmental area contain dopaminergic neuronal cell bodies that project long axonal extensions into the striatum. Dopaminergic neuronal cell bodies are not observed in the striatum.

We have suspected for some time that mAb 24.3.1 signaled a functional receptor on the surface of neuronal cells. Our current results from our Tg mouse studies reveal that chimeric Tg24.3.1 Ab deposited in neurons in Tg brain tissues and colocalized with D2R. Our new data link the signaling of D2R with autoantibodies in SC and other diseases for which treatment with D2R blocker
drugs are effective (68–72). Our studies published in Brimberg et al. (35) demonstrated reactivity of SC and PANDAS IgG in ELISA, whereas studies by Dale et al. (64) demonstrated anti-D2R Ab in SC using cell-based flow cytometry assays. Neither of these studies investigated anti-D2R Ab function/signaling or localization in brain tissues. This study extends these findings to demonstrate the anti-D2R Ab targeting of neurons in basal ganglia and cortex.

Previous studies (10, 12, 13) showed that SC and PANDAS IgG, as well as chorea-derived mAb 24.3.1, activated CaM kinase II in a human neuronal cell line, leading to TH activation and subsequent dopamine release. TH is the rate-limiting enzyme in dopamine synthesis and was increased in the brain of Lewis rats after intrathecal infusion of mAb 24.3.1 (12). Dopamine is the main catecholamine neurotransmitter in the CNS and is synthesized from tyrosine via TH and stored in vesicles in axon terminals. Dopaminergic signaling is a balance between dopamine release and uptake by the presynaptic terminal. The five dopamine receptor types are divided into two families: D1-like (stimulatory) and D2-like (inhibitory), both of which act through G-protein-coupled protein receptors (73, 74). Dopamine plays a pivotal role in modulating the functioning of the basal ganglia brain circuitry (56, 59, 73), and abnormalities in the functioning of this neurotransmitter have been implicated in disorders like SC (75).

Our Tg24.3.1-IgG1a construct was expressed in mouse sera and penetrated the brain targeting dopaminergic neurons (Fig. 4). Our focus was on Tg Ab expression and targeting of neuronal cells. To open the BBB, we used LPS and streptococcal cell walls in the mice to promote Ab penetration of the brain. Requirements for BBB penetration were established previously by Kowal et al. (40) where tubulin is intracellular and is the most abundant protein in the brain. Tubulin was reported to be associated with post-synaptic density (82). Our previous studies also showed that human mAb 24.3.1 activates CaM kinase in a human neuronal SKNSH cell line (10). CaM kinase activation was reported in association with heterodimers of dopamine receptors containing both D1 and D2 (83). A recent study of SC reports that the ratios of Abs against both D1R and D2R correlated directly with the Universidade Federal de Minas Gerais Sydenham’s Chorea Rating Scale for symptoms of SC (84). In this study, we compared the reactivity of SC and PANDAS sera against D1R and D2R. Although anti-D1R was much less reactive than was anti-D2R IgG, it was significantly elevated in the sera from SC and PANDAS and will be investigated further in future studies.

Cross-reactivity of anti-neuronal autoantibodies alone may not lead to disease, as suggested by the results with normal sera and other mAbs, but when the avidity of the Ab causes signaling of a receptor it may be a risk factor in the development of movement or neuropsychiatric symptoms. The similarity in Ag specificity, but differences in signaling, of D2R by the human chorea-derived mAbs 24.3.1, 37.2.1, and 31.1.1 illustrates this principle that cross-reactivity by itself may not necessarily lead to signaling of a receptor. Thus, our studies of mAb 24.3.1 in comparison with the two other chorea-derived human mAbs show their differences in signaling, avidity (10), and perhaps recognition of different epitopes in the CaM kinase assay (10), as well as in the D2R-signaling assay (Fig. 8).

The molecular basis for the cross-reactivity of mAb 24.3.1 with D2R and two other neuronal Ags may be due, in part, to Abs against the group A streptococcal carbohydrate Ag GlcNac and its cross-reactivity with l-lysoganglioside (10) and tubulin (11). Lysoganglioside is associated with lipid rafts in the membrane, whereas tubulin is intracellular and is the most abundant protein in the brain. Tubulin was reported to be associated with post-synaptic density (82). Our previous studies also showed that human mAb 24.3.1 activates CaM kinase in a human neuronal SKNSH cell line (10). CaM kinase activation was reported in association with heterodimers of dopamine receptors containing both D1 and D2 (83). A recent study of SC reports that the ratios of Abs against both D1R and D2R correlated directly with the Universidade Federal de Minas Gerais Sydenham’s Chorea Rating Scale for symptoms of SC (84). In this study, we compared the reactivity of SC and PANDAS sera against D1R and D2R. Although anti-D1R was much less reactive than was anti-D2R IgG, it was significantly elevated in the sera from SC and PANDAS and will be investigated further in future studies.

Cross-reactivity of anti-neuronal autoantibodies alone may not lead to disease, as suggested by the results with normal sera and other mAbs, but when the avidity of the Ab causes signaling of a receptor it may be a risk factor in the development of movement or neuropsychiatric symptoms. The similarity in Ag specificity, but differences in signaling, of D2R by the human chorea-derived mAbs 24.3.1, 37.2.1, and 31.1.1 illustrates this principle that cross-reactivity by itself may not necessarily lead to signaling of a receptor. Thus, our studies of mAb 24.3.1 in comparison with the two other chorea-derived human mAbs show their differences in signaling, avidity (10), and perhaps recognition of different epitopes in the CaM kinase assay (10), as well as in the D2R-signaling assay (Fig. 8).

The molecular basis for the cross-reactivity of mAb 24.3.1 with D2R and two other neuronal Ags may be due, in part, to Abs against the group A streptococcal carbohydrate Ag GlcNac and its cross-reactivity with l-lysoganglioside (10) and tubulin (11). We know from extensive studies (31) that Abs against α helical or specific peptide sequences containing aromatic or nonpolar residues are cross-reactive with GlcNac. It is notable that the peptide most strongly recognized (Fig. 6) by mAb 24.3.1 has many nonpolar and aromatic amino acids in sequence. The previous study by Shikhman et al. (31) used alanine substitutions to prove that the presence of these nonpolar and aromatic residues contributed to the cross-reactivity between dissimilar molecules.
We do not completely understand the basis of the discrepancy in lack of binding of PANDAS sera to FLAG-tagged extracellular D2R expressed in transfected HEK293T cells (extracellular N-terminal octapeptide DYKDDDDK [FLAG]-tagged D2$_2$ isoform) (63) compared with PANDAS sera signaling through D2R expressed on live mouse fibroblast A9 L-hD2 S.C.18 cells (A9 L cell line hD2 subclone #18, ATCC CRL-10225) (Fig. 10A). The differences could be explained if PANDAS and SC Abs bind different extracellular epitopes on D2R. It is conceivable that the FLAG tag may interfere with the binding of PANDAS Abs in HEK293T cell experiments. Alternatively, differences in post-translational modifications of D2R in different cell lines also could result in interference in PANDAS Abs binding one cell line but not the other.

In summary, based on several pieces of evidence, our findings support recognition of D2R by mAb 24.3.1. We demonstrated mAb 24.3.1 binding to a FLAG-tagged dopamine D2R, as well as induction of cAMP signaling in vitro in D2R-transfected cells. Although mAb 24.3.1 identified a peptide epitope of D2R, we cannot be certain of the in vivo target. Our Tg24.3.1-IgG1 Ab mouse model translated to human SC by evidence of in vivo expression of chorea-derived human V$_5$ Ab that targeted dopaminergic neurons. In addition, human sera IgG from SC and PANDAS induced inhibitory signaling of the D2R in transfected cells. Reactivity with D2R may be present in SC and PANDAS that displays small choreiform piano playing movements (22). Finally, our evidence supports the hypothesis that in brain disorders, such as SC and PANDAS/pediatric acute-onset neuropsychiatric syndrome, Ab-mediated D2R signaling on dopaminergic neurons could contribute to alteration of the central dopamine pathways and development of movement and neuropsychiatric symptoms.

Acknowledgments

We thank Dr. Chris Goodnow for the IgG1 HC vector and Dr. Brett Aplin for the Igs LC vector. We also thank Janet Baker, Adita Blanco, and Kathy Alvarez for expert technical assistance. We acknowledge Dr. David Dyer and Dr. Allison Gillaspy and the University of Oklahoma Health Sciences Center Laboratory for Genomics and Bioinformatics for DNA sequencing, as well as Jim Henthorn and the University of Oklahoma Flow and Imaging Cytometry Laboratory for FACS analysis. We thank the dedicated parents of children with movement and behavioral disorders for support. We also thank Dr. Shu Man Fu (University of Virginia College of Medicine, Charlottesville, VA) for critical review of the original manuscript and Dafna Lotan and Dr. Daphna Joel (Tel Aviv University, Tel Aviv, Israel) for helpful discussions.

Disclosures

M.W.C. serves as Chief Scientific Officer of Moleculara Labs, which provides testing of anti-neuronal Abs in children with neuropsychiatric and movement disorders. M.W.C. and C.J.C. declare a financial interest in Moleculara Labs. M.W.C. serves as Chief Scientific Officer of Moleculara Labs. We do not completely understand the basis of the discrepancy in lack of binding of PANDAS sera to FLAG-tagged extracellular D2R expressed in transfected HEK293T cells (extracellular N-terminal octapeptide DYKDDDDK [FLAG]-tagged D2$_2$ isoform) (63) compared with PANDAS sera signaling through D2R expressed on live mouse fibroblast A9 L-hD2 S.C.18 cells (A9 L cell line hD2 subclone #18, ATCC CRL-10225) (Fig. 10A). The differences could be explained if PANDAS and SC Abs bind different extracellular epitopes on D2R. It is conceivable that the FLAG tag may interfere with the binding of PANDAS Abs in HEK293T cell experiments. Alternatively, differences in post-translational modifications of D2R in different cell lines also could result in interference in PANDAS Abs binding one cell line but not the other.

In summary, based on several pieces of evidence, our findings support recognition of D2R by mAb 24.3.1. We demonstrated mAb 24.3.1 binding to a FLAG-tagged dopamine D2R, as well as induction of cAMP signaling in vitro in D2R-transfected cells. Although mAb 24.3.1 identified a peptide epitope of D2R, we cannot be certain of the in vivo target. Our Tg24.3.1-IgG1 Ab mouse model translated to human SC by evidence of in vivo expression of chorea-derived human V$_5$ Ab that targeted dopaminergic neurons. In addition, human sera IgG from SC and PANDAS induced inhibitory signaling of the D2R in transfected cells. Reactivity with D2R may be present in SC and PANDAS that displays small choreiform piano playing movements (22). Finally, our evidence supports the hypothesis that in brain disorders, such as SC and PANDAS/pediatric acute-onset neuropsychiatric syndrome, Ab-mediated D2R signaling on dopaminergic neurons could contribute to alteration of the central dopamine pathways and development of movement and neuropsychiatric symptoms.

Acknowledgments

We thank Dr. Chris Goodnow for the IgG1 HC vector and Dr. Brett Aplin for the Igs LC vector. We also thank Janet Baker, Adita Blanco, and Kathy Alvarez for expert technical assistance. We acknowledge Dr. David Dyer and Dr. Allison Gillaspy and the University of Oklahoma Health Sciences Center Laboratory for Genomics and Bioinformatics for DNA sequencing, as well as Jim Henthorn and the University of Oklahoma Flow and Imaging Cytometry Laboratory for FACS analysis. We thank the dedicated parents of children with movement and behavioral disorders for support. We also thank Dr. Shu Man Fu (University of Virginia College of Medicine, Charlottesville, VA) for critical review of the original manuscript and Dafna Lotan and Dr. Daphna Joel (Tel Aviv University, Tel Aviv, Israel) for helpful discussions.

Disclosures

M.W.C. serves as Chief Scientific Officer of Moleculara Labs, which provides testing of anti-neuronal Abs in children with neuropsychiatric and movement disorders. M.W.C. and C.J.C. declare a financial interest in Moleculara Labs.