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Self-antigen tetramers discriminate between myelin autoantibodies to native or denatured protein

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AUTHOR CONTRIBUTIONS

K.C.O. performed the initial analysis of ADEM serum samples and coordinated specimen collection. K.A.M. generated the MOG tetramer, performed most of the RIA experiments and generated the MOG transfectant. D.A.H. and K.W.W. coinitiated and cosupervised the entire project, and K.W.W. conceived the tetramer approach. P.L.D. and T.C. compiled and analyzed clinical data. Other authors contributed specimens, clinical information or key reagents.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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Abstract

The role of autoantibodies in the pathogenesis of multiple sclerosis (MS) and other demyelinating diseases is controversial, in part because widely used western blotting and ELISA methods either do not permit the detection of conformation-sensitive antibodies or do not distinguish them from conformation-independent antibodies. We developed a sensitive assay based on self-assembling radiolabeled tetramers that allows discrimination of antibodies against folded or denatured myelin oligodendrocyte glycoprotein (MOG) by selective unfolding of the antigen domain. The tetramer radioimmunoassay (RIA) was more sensitive for MOG autoantibody detection than other methodologies, including monomer-based RIA, ELISA or fluorescent-activated cell sorting (FACS). Autoantibodies from individuals with acute disseminated encephalomyelitis (ADEM) selectively bound the folded MOG tetramer, whereas sera from mice with experimental autoimmune encephalomyelitis induced with MOG peptide immunoprecipitated only the unfolded tetramer. MOG-specific autoantibodies were identified in a subset of ADEM but only rarely in adult-onset MS cases, indicating that MOG is a more prominent target antigen in ADEM than MS.

The role of autoantibodies in the pathogenesis of human demyelinating diseases of the central nervous system (CNS) is an important, unresolved issue. In animal models, autoantibodies that recognize epitopes on the surface of myelin or myelin-producing oligodendrocytes can enhance demyelination[1,2]. A monoclonal antibody (8–18C5) against myelin oligodendrocyte glycoprotein (MOG) induces severe demyelination in mice and rats with mild experimental autoimmune encephalomyelitis (EAE) but does not induce disease in healthy animals because the antibody cannot gain access to the CNS parenchyma[1,2]. MOG is a minor component of myelin but is localized on the outer surface of the multilamellar myelin structure and is thus accessible to antibodies, whereas more abundant antigens such as myelin basic protein are inaccessible in intact myelin[1]. In the marmoset primate model of EAE, immunization with MOG induces a chronic demyelinating disease with pathological features reminiscent of MS (ref. 3). In mouse models, however, severe demyelination is observed in the absence of antibodies and B cells[4], indicating that autoantibodies are not required for demyelination in all species.

These elegant studies in animal models have shown the demyelinating potential of autoantibodies to myelin surface proteins, but their role in the pathogenesis of human inflammatory demyelinating diseases such as MS and acute disseminated encephalomyelitis (ADEM) is far less certain. MOG has been extensively studied as a potential target antigen for autoantibodies in MS (refs. 5–8), but the presence and involvement of such autoantibodies is controversial. Although oligoclonal IgG is often found in the cerebrospinal

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fluid (CSF) of individuals with MS (ref. 9), the specificities of these locally produced antibodies and their role in disease progression are not known. A substantial fraction of individuals with neuromyelitis optica, a variant of MS in which the optic nerves and spinal cord are primarily affected, were recently shown to have autoantibodies against the aquaporin-4 water channel localized on astrocyte foot processes at the blood-brain barrier[10].

Unlike MS, ADEM typically has a rapidly progressive clinical presentation that includes encephalopathy[11]. The disease course is usually self limiting, although in a minority of cases relapses may occur. The pathogenic relationship between MS and ADEM is unclear, and it remains to be determined whether ADEM and MS are related in the spectrum of demyelinating diseases or whether they are entirely separate entities. Approximately 1 of 400 recipients of a CNS-derived rabies vaccine contaminated with myelin basic protein[12] developed encephalomyelitis, the occurrence of which was strongly correlated with the presence of serum autoantibodies to myelin basic protein[13]. It is important to distinguish vaccine-related forms of ADEM, which are the direct result of immunization with myelin proteins, from most ADEM cases, which often follow an infection[14,15]. In some cases, no clear antecedent history of infection or vaccination is present.

The evidence for an autoimmune process is less conclusive for postinfectious forms of ADEM than vaccine-related cases. Although a modest proliferative T-cell response to myelin basic protein has been observed in some individuals with postinfectious forms of ADEM (refs. 16–18), the diagnosis is still primarily based on the exclusion of CNS infection or other causes of acute demyelination. The presence of autoantibodies to MOG or other myelin surface antigens has not been investigated in ADEM.

Extensive studies in EAE models have shown that only antibodies that recognize folded MOG protein are pathogenic, whereas antibodies that solely bind to denatured protein or short synthetic peptides fail to induce demyelination[19,20]. Commonly used techniques only permit detection of autoantibodies directed against linear epitopes on denatured proteins (western blot) or do not adequately discriminate between denatured and folded proteins (ELISA)[5,21]. We have developed a new method for the identification of autoantibodies and have used it to investigate their role in human demyelinating diseases of the CNS.

RESULTS

Design of tetrameric antigens for RIA

Solution-phase binding of antibodies to monomeric radiolabeled autoantigens is a powerful technique for identifying prediabetic individuals[22,23] but only enables detection of relatively high-affinity autoantibodies. In animal models of antibody-mediated autoimmunity, such as hemolytic anemia, low-affinity autoantibodies to membrane proteins can be highly pathogenic *in vivo* because antigen clustering enables bivalent antibody binding[24]. We reasoned that increasing the valency of a target antigen could permit detection of rare and/or low-affinity antibodies by enabling bivalent binding.

We designed a series of multimeric antigens based on the ability of the streptavidin monomer to spontaneously assemble into a tetrameric structure. The extracellular domain of human MOG and a control member of the immunoglobulin (Ig) superfamily (human CD2) were each connected to a monomer of streptavidin via a flexible linker (illustrated for MOG in Fig. 1a). The difference in the molecular weight of MOG and CD2 tetramers enabled inclusion of CD2-SA as an internal control in immunoprecipitation experiments.

To provide the native folding environment for the MOG Ig domain, we used an *in vitro* translation system with endoplasmic reticulum microsomes isolated from a mouse

hybridoma cell line[25]. The signal peptide used for endoplasmic reticulum targeting was efficiently cleaved, and both MOG and CD2 fusion proteins were glycosylated (Fig. 1b). Addition of biotin during translation enabled the formation of SDS-stable tetramers (Fig. 1b), which were immunoprecipitated by the conformation-sensitive antibody 8–18C5 (Supplementary Fig. 1 online). Tetramers with an N- or C-terminal position of MOG relative to streptavidin (MOG-SA and SA-MOG, respectively) were equally effective in detecting autoantibodies (Fig. 1a,c) in the serum of ADEM individual 1724, excluding the possibility that antigen binding was the result of an artificial epitope at the junction of MOG, linker and streptavidin. The CD2 control tetramer was not precipitated by ADEM or control sera (Fig. 1c).

Detection of MOG autoantibodies in human demyelinating disease

We sought to determine if autoantibodies to conformational determinants of MOG are present in serum or CSF in different human inflammatory demyelinating diseases. We reproducibly identified IgG autoantibodies in the serum of 13 of 69 individuals with ADEM or ADEM with relapse (Fig. 2). We only detected IgM antibodies to MOG (anti–MOG IgM) in one relapsing-ADEM sample, which also contained IgG antibodies to MOG (anti–MOG IgG) (Table 1). One of the MOG^+ samples was obtained 272 d following diagnosis and another 211 d following a relapse (721 d following initial diagnosis), suggesting that such autoantibodies can persist for extended periods of time. We found that MOG autoantibodies were absent in individuals with viral encephalitis ($n = 58$), underscoring the difference between autoimmune processes and viral CNS infections that also cause inflammation.

Analysis of paired serum and CSF from eight ADEM individuals revealed a correlation between the presence of antibodies to MOG in the periphery and CNS (Table 1). In each case, we detected anti–MOG IgG or IgM in both compartments or not at all. We observed binding to the MOG tetramer in 2 of 35 control CSF samples. These individuals were diagnosed with neurosarcoidosis or fungal meningitis (Supplementary Table 1 online), but matched serum samples were not available.

We also performed a comprehensive analysis of individuals with different subtypes of MS (Table 1). We detected IgG autoantibodies to MOG in only 1 of 109 serum and 0 of 68 CSF samples from individuals from North America with adult-onset MS. Most individuals were untreated at the time of serum collection (Supplementary Table 2 online). Also, MOG autoantibodies were not detected in sera from 32 patients with clinically isolated syndrome who had an initial demyelinating event and had not yet progressed to definite MS, as defined by the McDonald criteria. One Japanese individual with adult-onset MS and one individual with pediatric MS had detectable serum anti–MOG IgG. The MOG^+ pediatric individual was from a minority population (South Asian). No additional clinical data were available for the Japanese MS individual. Both of these cases were from ethnic groups with a lower incidence of classical MS than populations of European ancestry.

Distinguishing antibodies to folded versus unfolded protein

Immunoglobulin domains are stabilized by a conserved disulfide bond. We examined whether antibody binding was dependent on MOG conformation by cleaving this disulfide bond with dithiothreitol (DTT) at 70 °C. Although the tetrameric structure was preserved, the antigen was no longer immunoprecipitated by any of the ADEM sera tested (Fig. 3a). Antibody binding did not require the N-linked glycan, as ADEM sera and the 8–18C5 antibody bound to a MOG mutant in which Asn 31 was substituted by Asp (Fig. 3b). Also, nonglycosylated MOG extracellular domain refolded from *Escherichia coli* inclusion bodies

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(rMOG) efficiently competed with tetramers for antibody binding (Fig. 3c). Autoantibodies in ADEM sera therefore require proper folding of MOG, but not the N-linked glycan, for binding.

In mice immunized with the encephalitogenic MOG peptide (35–55), we detected serum antibodies to rMOG by ELISA. The RIA, however, revealed that these antibodies were directed solely against unfolded protein (Fig. 3d). The pathogenic 8–18C5 antibody showed substantially stronger binding to native rather than denatured tetramer (Fig. 3d), and a newly generated MOG monoclonal antibody bound to folded but not unfolded tetrameric MOG (Supplementary Fig. 1). This RIA can therefore simultaneously identify strictly conformation-sensitive antibodies (ADEM sera), as well as antibodies against unfolded self-antigen (MOG peptide immunized mice), and may thus help to clarify the role of antibodies in autoimmune diseases.

Comparison of tetramer RIA to other assays

The tetramer was superior to monomer for detection of autoantibodies in ADEM serum because, in ADEM individual 1724, more than 25% of the MOG tetramer was immunoprecipitated at serum dilutions ranging from 1:50 to 1:800, compared to less than 5% of monomer (Fig. 4a,b). The increase in signal with the tetramer was far greater than the four-fold change in signal per protein unit, indicating that multivalent binding enabled detection of a greater number of antibody-antigen interactions (Fig. 4b).

To determine whether autoantibodies identified by tetramer RIA would also bind MOG on the cell surface, we transfected Jurkat cells with a construct encoding full-length human MOG fused to green fluorescent protein (GFP). We used cells transfected with only GFP as controls. To enable sensitive detection of autoantibodies, we used a three-step staining procedure with a biotinylated secondary antibody to human IgG and streptavidin-phycoerythrin (PE). Although antibodies to MOG were detected by the FACS assay, the sensitivity of this assay was lower than the tetramer RIA. Antibody binding by FACS was close to background levels at a 1:400 dilution of serum from ADEM individual 1724 (Fig. 4c), whereas the RIA was positive at a serum dilution of 1:3,200 (Fig. 4b).

We then used the FACS assay to test a panel of sera at a dilution of 1:50 (Fig. 4d). Six of eight ADEM sera that were positive by tetramer RIA were also positive by FACS, as was the only RIA-positive pediatric MS sample (Supplementary Fig. 2 and Supplementary Table 3 online). One ADEM sample was negative by RIA but positive by FACS, possibly because of the use of the Ig domain in the tetramer versus the full-length protein in the cell-based assay. All tested sera from MS, clinically isolated syndrome and control donors were negative by FACS and RIA (Supplementary Table 4 online).

The MOG ELISA was suitable for detection of monoclonal antibodies but had only very limited ability to detect polyclonal antibodies in serum (Supplementary Fig. 1). The tetramer RIA detected MOG antibodies in 10 of 47 ADEM samples tested in both assays, but only one of the RIA-positive samples was identified by the ELISA (Fig. 4f). In addition, some control samples gave high levels of background binding by ELISA (Supplementary Fig. 1 and Supplementary Table 5 online), possibly resulting from the partial denaturation of the antigen following immobilization on plastic. Antibodies present in some serum samples may have cross reacted with conserved elements of the Ig fold in the extracellular domain of MOG.

Clinical features of ADEM individuals with MOG autoantibodies

In the ADEM cases studied here, individuals with MOG autoantibodies had a higher incidence of bilateral motor and sensory symptoms than unilateral symptoms, whereas the

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reverse was observed in the MOG-negative group (Supplementary Table 6 online). The relatively small number of cases in the MOG-positive group prevented definitive assessment of statistical significance. These initial findings may, however, be useful in defining hypotheses to pursue in future analyses of a larger patient population.

DISCUSSION

These results show the presence of circulating conformation-dependent autoantibodies to a myelin surface antigen in individuals with ADEM. It seems that ADEM represents more than one pathogenic entity, and our approach permitted the identification of a subset of individuals with evidence for antibody-associated autoimmunity. In contrast, MOG autoantibodies were only identified in serum or CSF of rare MS cases and were not detected in individuals with clinically isolated syndrome.

The RIA described here offers a number of advantages over previously described techniques for autoantibody detection. Multimerization of the antigen enables identification of antibodies that have a low binding affinity or are present at low concentrations. *In vitro* translation with endoplasmic reticulum microsomes allows rapid expression of radiolabeled proteins that require disulfide bond formation, glycosylation and chaperone-assisted folding. The assay can be multiplexed by combining multiple tetramers that differ in molecular weight, such as the MOG-SA and CD2-SA tetramers described here. The assembled streptavidin tetramer is resistant to a variety of denaturing agents, permitting selective unfolding of the antigen domain for parallel assessment of conformation-sensitive and conformation-insensitive antibodies with the same technique. FACS analysis with a MOG-GFP transfectant confirmed the presence of MOG antibodies in most tetramer RIA-positive ADEM individuals. Comparisons of different assays, however, showed that tetramer RIA was substantially more sensitive than monomer RIA, ELISA or FACS. Our method permits characterization of autoantibodies that are difficult to detect with classical techniques, and could be applied to the study of antibodies in a variety of autoimmune diseases.

Tetrameric RIA offers a high level of specificity, as antibodies were rarely detected at low levels in control sera. In contrast, studies using western blotting or ELISA techniques have reported antibodies to denatured MOG or MOG peptides in healthy individuals and individuals with non-CNS autoimmune conditions[8]. One study claimed that IgM antibodies to MOG as measured by western blotting represent a marker for prediction of clinically definite MS (ref. 5), but other studies have failed to confirm these findings[6,7]. In general, radiolabeled antigens provide a more quantitative and objective readout than assays with enzymatic substrates. In spite of the sensitivity of this new technique, autoantibodies to folded MOG protein could not be detected in the serum or CSF of most individuals with MS. We recently observed anti-MOG antibodies in the CNS tissue of patients with MS (ref. 26), consistent with the hypothesis that myelin-reactive B cells are synthesizing autoantibodies in the CNS but not CSF or peripheral immune compartments.

A role for MOG autoantibodies in the pathogenesis of MS has been extensively discussed, based on the ability of such autoantibodies to induce demyelination in animal models. A recent study examined binding of serum IgG to MOG-transfected Chinese hamster ovary (CHO) cells by FACS (ref. 27). Binding was expressed as a ratio of fluorescence with MOG-transfected and nontransfected cells. Serum samples from 2 of 36 individuals with clinically isolated syndrome and 1 of 35 individuals with relapsing-remitting MS gave a binding ratio of 1.5–1.75 at a high serum concentration (1:10 dilution). The FACS staining we observed with ADEM samples was substantially brighter—binding ratio ranging from 2.41 to 89.9—at a five-fold greater serum dilution. Our new data show that MOG is a more

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prominent target autoantigen in a subgroup of ADEM individuals than in individuals with adult-onset MS.

ADEM is heterogeneous in terms of clinical presentation and responsiveness to particular treatment approaches[11,14,28], and our data suggest that this heterogeneity is at least in part the result of different mechanisms of pathogenesis. The response of some ADEM individuals to plasmapheresis or intravenous immunoglobulins suggests that pathogenic autoantibodies may play an important role in certain individuals[29,30]. The assay we have developed makes it possible to prospectively determine whether individuals with MOG autoantibodies benefit from plasmapheresis or other approaches that target autoantibodies, B cells or plasma cells.

METHODS

MOG and CD2 streptavidin tetramers

We generated the expression constructs by PCR using the extracellular domains of human MOG (wild-type or N-glycan–deficient [Asn31-Asp]) or CD2 joined by a flexible 11–amino acid linker (GSGMGMGMGMM) to the 127–amino acid core domain of streptavidin. We used the murine H-2K^b signal sequence for endoplasmic reticulum targeting. We ligated antigen-SA DNA constructs into a modified pSP64 vector for generation of RNA transcripts using the RiboMax T7 large-scale RNA production kit (Promega). We performed *in vitro* translation using endoplasmic reticulum microsomes isolated from a murine hybridoma cell line[25] in rabbit reticulocyte lysate (Promega) supplemented with ^{35}S-labeled methionine (final activity 0.3 μCi/μl, Amersham), unlabeled amino acids minus methionine (Promega, final concentration 20 μM each), 2% SUPERase-In RNAse inhibitor (Ambion), 160 μM biotin (Sigma) and 8 ng/μl RNA. Translation of CD2-SA took place in 250 μl aliquots, each for ten immunoprecipitation reactions. MOG-SA translations were 180 μl per 10 immunoprecipitations.

Proteins were translated at 30 °C under reducing conditions for 1 h, followed by 2 h under oxidizing conditions (addition of oxidized glutathione (Sigma) to a final concentration of 4 mM) to enable formation of disulfide bonds. We washed the endoplasmic reticulum membrane fraction with cold stopping buffer (PBS, 10 mM iodoacetamide (Sigma), 160 μM biotin), and we released the proteins by addition of NP-40 (Pierce) to 0.5%. Tetramers of CD2 and MOG were combined and filtered (Spin-X 0.2 micron, Corning). To produce unfolded antigen, we cleaved the disulfide bond in the MOG Ig domain by addition of 10 mM DTT, 0.5% SDS and 160 μM biotin to solubilized membranes, followed by heating at 70 °C for 15 min.

We stored 5 μl of the combined antigen at 4 °C as a reference, and the remainder was cleared against protein A–Sepharose CL4B (Amersham) in 500 μl of RIA buffer (PBS, 1% BSA, 0.1% Tween-20, 160 μM biotin) to reduce nonspecific binding. We filtered the cleared antigens to remove Sepharose beads and increased the volume with RIA buffer. We added 25 μl of labeled antigen to serum diluted in RIA buffer to a final volume of 250 μl. For competition experiments, monomeric MOG was produced in *E. coli* and refolded[26], and purified aldolase was purchased from Amersham. We incubated unlabeled proteins with serum in 250 μl of RIA buffer for 3 h before addition of labeled tetrameric antigen. The final concentration of DTT in immunoprecipitations with denatured MOG was 0.22 mM, and an equal concentration of DTT was added to parallel immunoprecipitations with folded MOG tetramer. We carried out immunoprecipitation reactions with CSF using 200 μl of CSF and 50 μl of RIA buffer.

Antibody binding took place overnight at 4 °C on an orbital rotator. Immunoglobulins and bound radiolabeled antigens were isolated by incubation with 20 μl of protein A–Sepharose CL4B beads or anti-IgM (Medix Biochemica) coupled to Sepharose 4 (Amersham) for 90 min. We washed the Sepharose beads twice with 1 ml of RIA buffer, and eluted the bound proteins at 4 °C in 10 μl of 50 mM 3-(cyclohexylamino)-1-propanesulfonic acid, pH 11.5. We filtered the supernatant into 10 μl of lithium dodecyl sulfate (LDS) gel–loading buffer (Invitrogen) containing 160 μM biotin, 100 mM sodium phosphate pH 6.0, 100 mM DTT and protease inhibitors (1 mM EDTA, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin A (Roche)). When digestion with endoglycosidase H (New England Biolabs) was performed, we added 1,000 units of the enzyme and incubated the samples at 37 °C for 2 h. Samples were then loaded onto 4–12% NOVEX Bis-Tris gels and run in MOPS buffer (Invitrogen). Proteins were electro-transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad), and dried membranes were exposed to a phosphor imager screen (Molecular Dynamics, Amersham) for quantification of incorporated ^{35}S-methionine. We normalized the amount of tetrameric MOG and CD2 isolated in immunoprecipitation reactions to the input quantity of each antigen.

All sera that precipitated greater than 10% of MOG-SA were included in three subsequent immunoprecipitation experiments, and the mean of these measurements was taken. The threshold for positive samples was set at 10.75%, 4 s.d. above the mean of healthy control donors. For some samples, the volume of serum was insufficient to allow analysis of both IgG and IgM antibodies or inclusion in all analyses.

Serum and CSF samples

Patient and control serum and CSF samples were collected at the Partners MS Center at the Brigham and Women's Hospital, Boston, and at other international neurological centers that care for adults and/or children with MS and demyelinating diseases. Each site collected samples using a protocol approved by their Institutional Review Board. Informed consent was obtained from all subjects.

Details of serum and CSF samples, and methods for flow cytometry, ELISA, induction of experimental autoimmune encephalomyelitis and generation of monoclonal antibodies can be found in the Supplementary Methods online.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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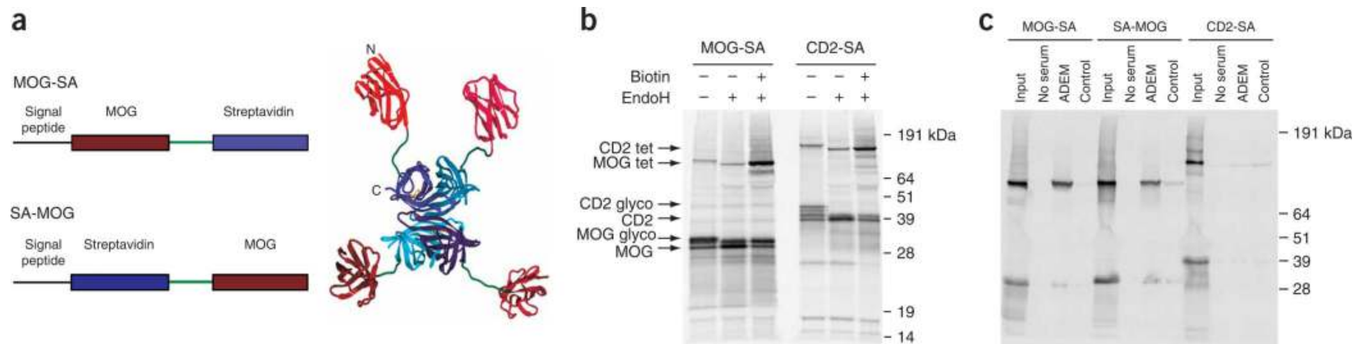

Figure 1. Generation of tetrameric antigens for RIA

(**a**) Design of antigen-streptavidin fusion proteins. The extracellular Ig domain of MOG was connected to the N terminus (MOG-SA) or C terminus (SA-MOG) of the streptavidin monomer via a flexible linker. Crystal structures of MOG and streptavidin (PDB numbers PY9 and 1SW) were used to model the MOG-SA structure (right; MOG domains in red, streptavidin domains in blue). The N and C termini of the streptavidin monomer are solvent exposed on the same face, which enabled attachment of the antigen at either site. (**b**) Assembly of MOG-SA and CD2-SA fusion proteins into tetramers. ^{35}S-labeled proteins were expressed in an *in vitro* translation system with endoplasmic reticulum microsomes to enable folding in a native environment. Both proteins were glycosylated, indicated by a decrease in molecular weight following digestion with EndoH (lanes 2 and 5). Addition of biotin during translation yielded tetramers stable during SDS-PAGE (lanes 3 and 6). (**c**) Specific immunoprecipitation of MOG tetramers by autoantibodies in serum from an ADEM individual (1724). Immunoprecipitation reactions were performed with MOG-SA, SA-MOG and CD2-SA tetramers using no serum, ADEM serum or a control serum at a dilution of 1:100. Data were standardized by calculating the percent of radiolabeled tetramer isolated in the immunoprecipitation as compared to the amount of input radiolabeled protein.

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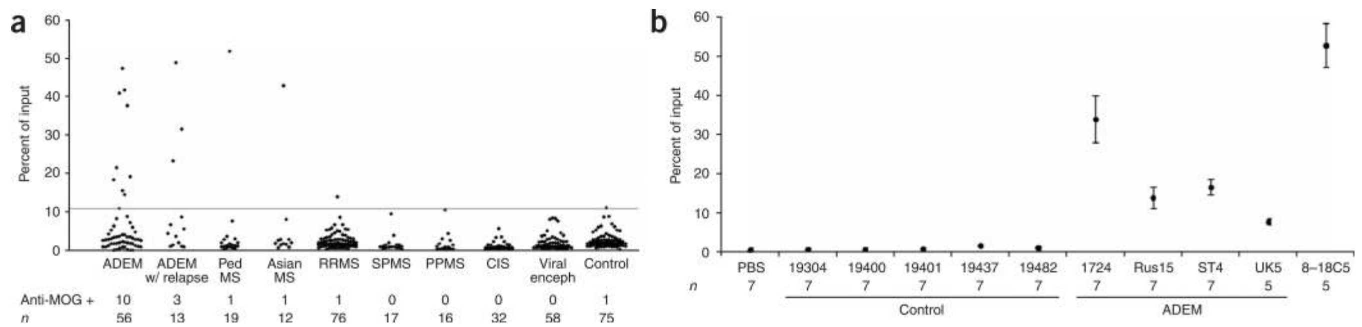

Figure 2. Analysis of MOG autoantibodies in CNS diseases

(**a**) The tetramer assay was used to examine serum samples from cases of ADEM ($n = 56$), ADEM with relapse ($n = 13$), different forms of MS (pediatric MS, $n = 19$; Asian MS, $n = 12$; relapsing-remitting MS, $n = 76$; secondary progressive MS, $n = 17$; primary progressive MS, $n = 16$) and clinically isolated syndrome ($n = 32$). Sera from cases of viral encephalitis ($n = 58$) and healthy individuals ($n = 75$) were used as controls. Each sample was assayed at a dilution of 1:100. Samples that precipitated >10.75% of the input MOG tetramer were considered positive. This threshold is 4 s.d. above the mean of healthy donors. No sample precipitated the CD2 control tetramer. (**b**) Results obtained in the MOG tetramer RIA were reproducible. Mean percentages and s.d. of immunoprecipitated MOG tetramer are indicated for 5–7 repeat measurements for sera from five healthy controls and four ADEM cases. Results with the 8–18C5 monoclonal antibody (positive control) are also shown.

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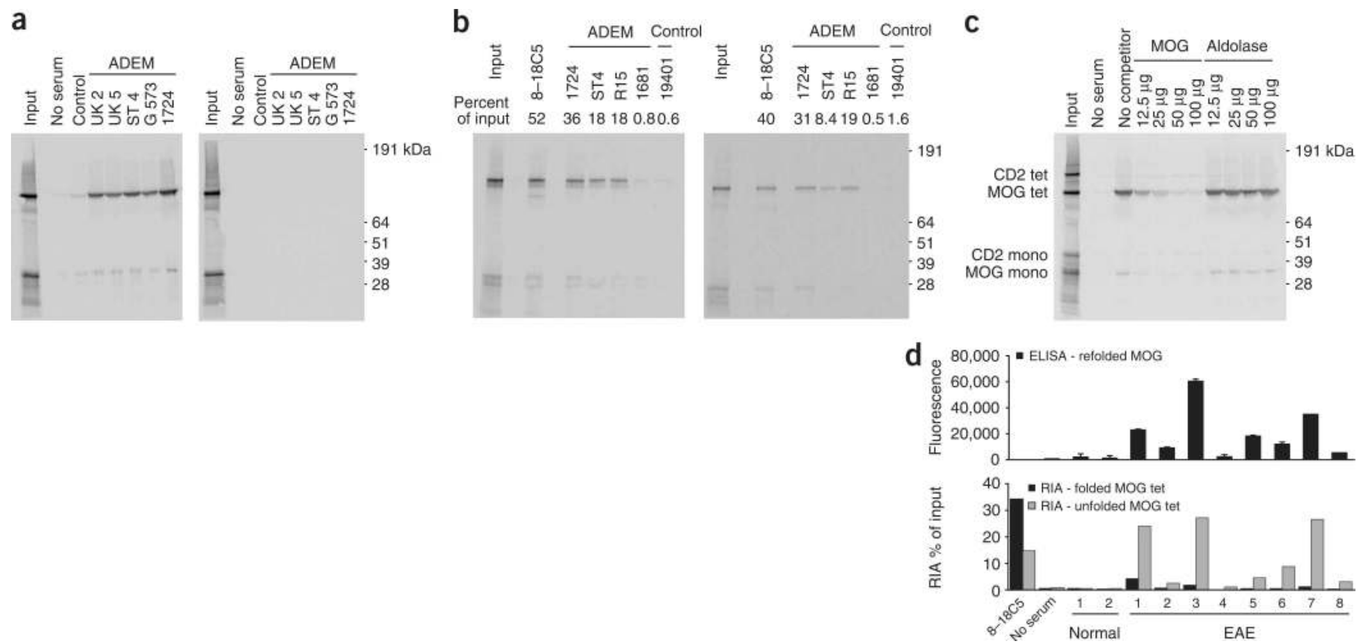

Figure 3. The tetramer RIA permits discrimination of antibodies directed against conformation-dependent and conformation-independent epitopes

(**a**) ADEM autoantibodies against MOG are strictly conformation dependent. The tetrameric structure was maintained but the Ig domain of MOG was unfolded by cleavage of the MOG disulfide bond with DTT (heating at 70 °C for 10 min) (right). DTT was added to the same final concentration in immunoprecipitation reactions with untreated tetramer (left). (**b**) MOG binding by ADEM autoantibodies and the 8–18C5 monoclonal antibody did not require the N-linked glycan. The N-linked glycan was removed by mutation of MOG Asn 31 to Asp (left, wild-type; right, glycan-deficient). Immunoprecipitations were performed with 8–18C5 monoclonal antibody (50 ng) and ADEM and control sera. (**c**) Competition for autoantibody binding to MOG tetramers. Serum from an ADEM individual (1724) was incubated for 3 h with the indicated amounts of unlabeled recombinant MOG monomer or control protein (aldolase), before addition of radiolabeled MOG tetramer. (**d**) Antibodies from mice immunized with MOG (35–55) peptide bound to unfolded but not folded MOG. Sera from two control mice and eight mice that had been immunized with MOG (35–55) peptide in complete Freund's adjuvant (CFA) were analyzed by ELISA using purified, refolded MOG extracellular domain expressed in *E. coli* (top) or by RIA using folded and unfolded MOG tetramer (bottom). The RIA showed that serum antibodies from MOG (35–55)-immunized mice bound to unfolded but not folded MOG. Seven of the eight immunized mice had clinical EAE with a score of 2 or 3; only one mouse did not develop EAE (#6).

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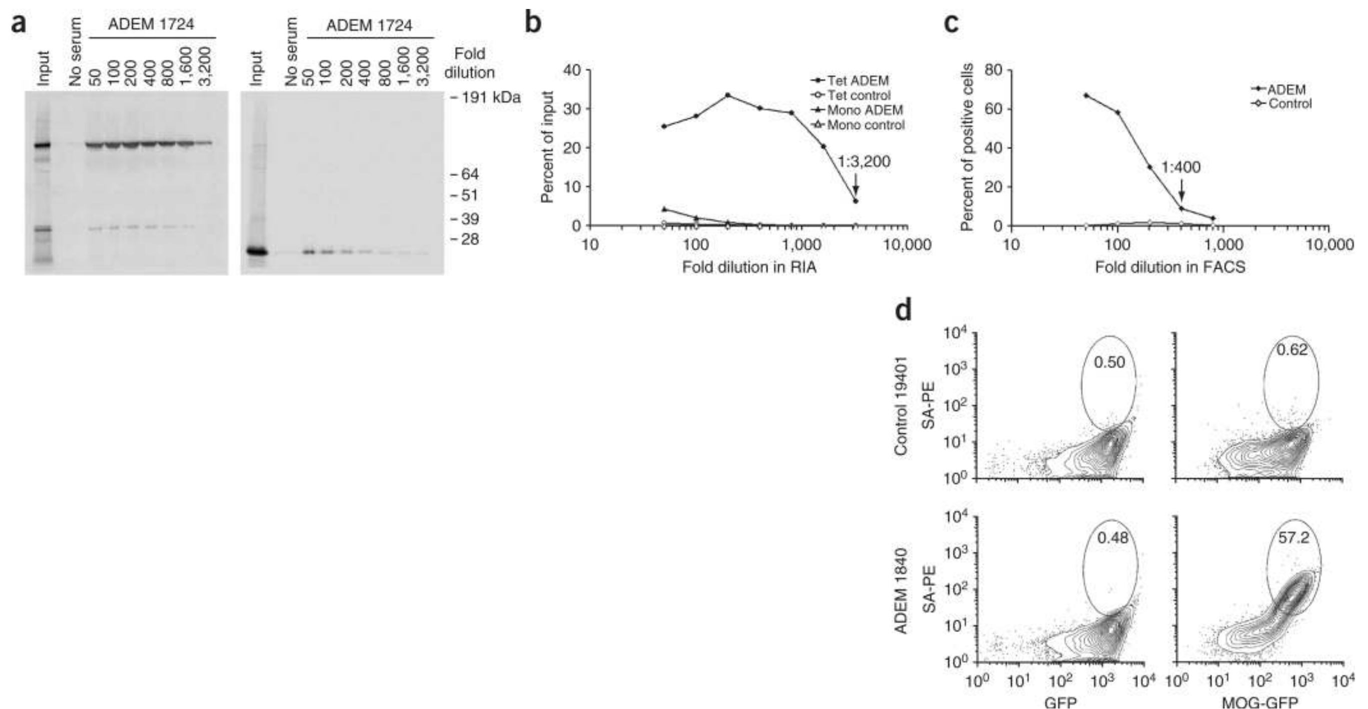

Figure 4. Comparison of tetramer RIA to other autoantibody assays

(**a**) ^{35}S-labeled MOG-SA tetramers (left) and MOG monomers (right) were compared in immunoprecipitation experiments using serial dilutions of serum from ADEM individual 1724. (**b**) Quantification of immunoprecipitation with tetramer or monomer as percentage of input radiolabeled protein. Tetrameric and monomeric versions had the same number of radiolabeled methionine residues per chain because the linker was retained in the monomeric form. (**c**) Antibodies in the serum of ADEM individual 1724 also bound to MOG on the surface of cells transfected with a MOG-GFP fusion protein. The ADEM serum was positive at a serum dilution of 1:400; no staining was observed at any dilution with the control serum (from individual 19437). (**d**) Example of FACS analysis. Jurkat cell lines transfected with MOG-GFP (right) and a control GFP vector (left) were stained with a control serum (from individual 19401) and an ADEM serum (from individual 1840) at a 1:50 serum dilution. Bound antibodies were detected with biotinylated antibodies to human IgG and streptavidin-PE. The IgG^{+} gates were centered on the GFP-bright population and set such that < 0.5% of live cells from the GFP vector control cell line were within the gate. For the ADEM sample, the intensity of antibody staining correlated with the level of MOG-GFP expression. Primary FACS data for all positive sera are shown in Supplementary Figure 2 online.

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Table 1

Serum and CSF samples

Serum samples		Number of samples	Median age (range)	Percent female	Anti–MOG IgG	Anti–MOG IgM
ADEM	Without relapse	56	8 (0.8–28)	41.2	10/56	0/26
	With relapse	13	9 (4–41)	41.7	3/13	1/3
Multiple sclerosis	Pediatric	19	13 (5–17)	63.2	1/19	0/4
	Asian	12	NA	NA	1/12	nd
	Relapsing-remitting	76	40 (18–74)	84.2	1/76	0/12
	Secondary progressive	17	57 (43–70)	64.7	0/17	nd
	Primary progressive	16	55 (35–75)	31.3	0/16	nd
Clinically isolated syndrome		32	43 (22–54)	87.5	0/32	0/28
Viral encephalitis		58	NA	NA	0/58	0/20
Control		75	40 (22–64)	60	1/75	0/32

Paired ADEM serum and CSF samples (% of MOG precipitated)

		Individual						
		1724	1840	1715	1700	1690	1726	1681
Serum	IgG	48.82	18.32	7.11	5.54	3.6	3.11	2.05
	IgM	43.36	1.2	0.86	0.91	0.91	0.7	nd
CSF	IgG	12.4	11.75	0.22	0.24	0.22	0.49	0.27
	IgM	18.5	0.93	0.34	0.36	0.36	0.28	0.5

All CSF samples

	Number of samples	Anti–MOG IgG	Anti–MOG IgM
ADEM	8	2/8	1/8
Asian MS	11	1/11	0/11
Adult-onset MS	68	0/68	0/68
Other	54	3/54	0/54

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NA, not available. nd, not determined. Diagnoses of ‘other’ CSF samples are detailed in Supplementary Table 1.