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(54) Title: A METHOD FOR DIAGNOSIS AND PROGNOSIS OF MULTIPLE SCLEROSIS SUBTYPES

(57) Abstract: This invention provides methods of assessing autoantibodies to specific epitopes of myelin components (e.g. to conformational epitope of myelin/oligodendrocyte glycoprotein) for the diagnosis and/or prognosis of multiple sclerosis.



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A METHOD FOR DIAGNOSIS AND PROGNOSIS OF MULTIPLE SCLEROSIS SUBTYPES

CROSS-REFERENCE TO RELATED APPLICATIONS

5 This application claims benefit of U.S. Provisional Application No. 60/674,354,
filed on April 21, 2005, which is herein incorporated by reference in its entirety.

**STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY
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15 **FIELD OF THE INVENTION**

This invention pertains to multiple sclerosis (MS). In particular this invention provides improved diagnostics and prognostics for diagnosing, staging, or predicting disease outcome for a patient having multiple sclerosis.

20 BACKGROUND OF THE INVENTION

Multiple sclerosis (MS) designates a group of heterogeneous, immune-mediated demyelinating disorders of the central nervous system (CNS). There is currently no paraclinical investigation that accurately predicts clinical course, prognosis, or pathological subtypes for individual patients. MS pathogenesis is complex and multifactorial, with a strong genetic component likely acting in concert with environmental exposure(s). In addition to the well-established major histocompatibility complex association, recent full genome screening of MS families supports the role of several unidentified genes each with modest effect. Previous studies support the hypothesis that humoral immunity plays a role in MS pathogenesis, and the heightened incidence of antibodies associated with

autoimmune disorders observed in MS families suggest that genetic factors may control susceptibility to develop humoral autoimmunity. Anti-myelin antibodies are of particular importance to study in MS, since they reflect CNS-specific humoral responses.

Whereas mediation of tissue damage by anti-myelin antibodies can be unequivocally demonstrated in the disease model experimental allergic encephalomyelitis (EAE), the pathophysiological significance of such antibodies in humans is uncertain because they are frequently detected in sera of both MS-affected and control subjects. This may indicate that regulatory mechanisms that would normally prevent autoantibodies to gain access to the CNS, for example suppressive T cell responses, are defective in MS patients. An additional possible explanation is that autoantibodies to CNS constituents are functionally heterogeneous, and qualitatively differ in MS from that in other subjects as a result of genetic polymorphisms and/or exposure of the immune system to antigenic determinants specifically associated with pathogenicity. Unlike T helper cells responses, which require antigen processing and presentation and are thus restricted to short antigenic peptides, antibodies most often target additional determinants on proteins that are defined by their tertiary structure. Studies of antibody repertoire specificity that accounting for the complexity of humoral responses in outbred populations are needed in order to elucidate their pathogenic properties in disorders like MS.

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SUMMARY OF THE INVENTION

Using combinatorial Fab fragments libraries, we have characterized the diversity of autoantibody responses against myelin/oligodendrocyte glycoprotein (MOG) during EAE in the *C. jacchus* marmoset, a non-human primate in which pathogenic autoantibodies are obligatory for the formation of MS-like demyelinating plaques. Several discrete, tertiary structure-dependent determinants have been defined on the surface-exposed, extracellular domain of MOG, and distinct populations of native polyclonal antibodies have been characterized based on their ability to bind to short linear peptides or structurally defined epitopes of MOG. Our results strongly suggest that pathogenicity is correlated with recognition of the structural determinants of MOG, in agreement with previous studies of murine monoclonal antibodies.

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Directly relevant to MS pathophysiology, we found that conformational epitopes recognized by marmoset antibodies appear commonly within the anti-MOG antibody repertoires of MS sera. Recombinant *C. jacchus* Fab fragments that define structural epitopes of MOG recognized by autoantibodies in humans now afford refined studies of pathogenic autoantibody responses in MS.

Thus, without being bound to a particular theory, we believe that development of pathogenic humoral immunity in MS is controlled at least in part at the genomic level, and that comprehensive serum autoantibody profiling in MS families defines distinct clinical phenotypes.

Thus, in various embodiments, this invention contemplates methods utilizing detection/quantification of autoantibodies to specific epitopes of myelin components (e.g. to conformational epitope of myelin/oligodendrocyte glycoprotein (MOG)) for the definitive diagnosis, and/or staging or typing, and/or prognosis of multiple sclerosis.

Thus, in one embodiment, this invention provides a method of diagnosing or evaluating the prognosis of multiple sclerosis (MS) or allergic encephalomyelitis (EAE) in a mammal. The method typically involves detecting the presence or quantity of an antibody in the mammal specific for a conformational epitope of myelin/oligodendrocyte glycoprotein (MOG) where the presence or increased concentration of the antibodies indicates the presence of a particular stage of multiple sclerosis or the increased likelihood of the development of a more severe form of the disease. In certain embodiments, the detecting comprises obtaining a biological sample comprising serum or cerebrospinal fluid from the mammal. In certain embodiments, can involve screening for a plurality of antibodies specific for different conformational epitopes of the myelin/oligodendrocyte glycoprotein. In certain embodiments, the antibody specific for a conformational epitope of myelin/oligodendrocyte glycoprotein is an antibody that specifically binds to an epitope specifically bound by an antibody comprising a polypeptide sequence selected from the group consisting of SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:27, SEQ ID NO:29, SEQ ID NO:31, SEQ ID NO:33, SEQ ID NO:35, and SEQ ID NO:37. The detecting can, optionally involve a competitive assay using a competitive binder an antibody comprising a CDR3 comprising a peptide sequence as shown in Table 2 (SEQ ID NOs:1-12). In certain embodiments, the

detecting involves a competitive assay using as a competitive binder an antibody specific for a conformational epitope of myelin/oligodendrocyte glycoprotein is an antibody that specifically binds to an epitope bound by an antibody comprising a polypeptide sequence selected from the group consisting of SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:27, SEQ ID NO:29, SEQ ID NO:31, SEQ ID NO:33, SEQ ID NO:35, and SEQ ID NO:37. In certain embodiments, the detecting comprises a competitive assay using as a competitive binder an antibody specific for a conformational epitope of myelin/oligodendrocyte glycoprotein where the antibody comprises a polypeptide sequence selected from the group consisting of SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:27, SEQ ID NO:29, SEQ ID NO:31, SEQ ID NO:33, SEQ ID NO:35, and SEQ ID NO:37. The mammal can be a human (e.g. a human with a preliminary diagnosis of multiple sclerosis) or a non-human mammal.

In another embodiment, this invention provides a method of evaluating the risk of progressing to a severe form of multiple sclerosis and/or the extent of central nervous system damage in a mammal. The method typically involves obtaining a biological sample comprising serum or cerebrospinal fluid from the mammal; and detecting the proportion of autoantibodies specific for a conformational epitope to those specific for a linear MOG epitope or a linear epitope of another myelin protein; where an increased ratio of conformational specific antibodies indicates an increased likelihood or progressing to a severe form of the disease and/or increased central nervous system damage. In certain embodiments, detecting the proportion comprises detecting binding of autoantibodies to a MOG conformational epitope and to a MOG linear peptide. In certain embodiments, detecting the proportion comprises determining the ratio of MOG-peptide-specific to rMOG-specific antibodies. In certain embodiments, the detecting comprises screening for a plurality of antibodies specific for different conformational epitopes of the myelin/oligodendrocyte glycoprotein. The antibodies specific for a conformational epitope of myelin/oligodendrocyte glycoprotein include, but are not limited to an antibody that specifically binds to an epitope bound by an antibody comprising a polypeptide sequence selected from the group consisting of SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:27, SEQ ID NO:29, SEQ ID NO:31, SEQ ID NO:33, SEQ ID NO:35, and SEQ ID NO:37. In certain embodiments, the

detecting comprises a competitive assay using a competitive binder an antibody comprising a CDR3 comprising a peptide sequence as shown in Table 2 (SEQ ID NOs:1-12). In certain embodiments, the detecting comprises a competitive assay using as a competitive binder an antibody specific for a conformational epitope of myelin/oligodendrocyte glycoprotein is an antibody that specifically binds to an epitope bound by an antibody comprising a polypeptide sequence selected from the group consisting of SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:27, SEQ ID NO:29, SEQ ID NO:31, SEQ ID NO:33, SEQ ID NO:35, and SEQ ID NO:37. In certain embodiments, the detecting comprises a competitive assay using as a competitive binder an antibody specific for a conformational epitope of myelin/oligodendrocyte glycoprotein where the antibody comprises a polypeptide sequence selected from the group consisting of SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:27, SEQ ID NO:29, SEQ ID NO:31, SEQ ID NO:33, SEQ ID NO:35, and SEQ ID NO:37. The mammal can be a human (e.g. a human with a preliminary diagnosis of multiple sclerosis) or a non-human mammal (e.g. a test/model animal).

This invention also provides a method of treating a patient having a preliminary diagnosis of multiple sclerosis. The method typically involves obtaining a biological sample comprising serum from the patient; and detecting autoantibodies specific for a conformational epitope to those specific for a linear MOG epitope or a linear epitope of another myelin protein; and prescribing a more aggressive treatment regimen when the ratio is elevated (e.g. as compared to that observed in healthy patients and/or in patients having a mild or non-progressive form of the disease).

Also provided is a method of diagnosing definite multiple sclerosis in patients with a first episode of demyelination in the central nervous system. The method typically involves measuring antibodies against specific myelin constituents where the presence and/or quantity of such antibodies indicates a definite diagnosis of multiple sclerosis. In certain embodiments, the myelin constituent comprises MOG and/or GalC. In certain embodiments, the antibodies are specific for a conformational epitope of MOG and/or a conformational epitope of GalC.

In still another embodiment this invention provides a method of determining the form of multiple sclerosis. The method typically involves measuring a plurality of antibodies against specific myelin constituents where the presence or level of certain members of the plurality indicate the form or stage of multiple sclerosis. In certain

5 embodiments, the myelin constituent comprises MOG and/or GalC. In certain embodiments, the detecting comprises detecting the presence or quantity of an antibody specific for a conformational epitope of myelin/oligodendrocyte glycoprotein (MOG). The detecting can comprise screening for a plurality of antibodies specific for different conformational epitopes of the myelin/oligodendrocyte glycoprotein and/or GalC. In

10 certain embodiments, the antibody specific for a conformational epitope of myelin/oligodendrocyte glycoprotein is an antibody that specifically binds to an epitope bound by an antibody comprising a polypeptide sequence selected from the group consisting of SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:27, SEQ ID NO:29, SEQ ID NO:31, SEQ ID NO:33,

15 SEQ ID NO:35, and SEQ ID NO:37. In certain embodiments, the detecting comprises a competitive assay using a competitive binder an antibody comprising a CDR3 comprising a peptide sequence as shown in Table 2 (SEQ ID NOs:1-12). In certain embodiments, the detecting comprises a competitive assay using as a competitive binder an antibody specific for a conformational epitope of myelin/oligodendrocyte glycoprotein is an antibody that

20 specifically binds to an epitope bound by an antibody comprising a polypeptide sequence selected from the group consisting of SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:27, SEQ ID NO:29, SEQ ID NO:31, SEQ ID NO:33, SEQ ID NO:35, and SEQ ID NO:37. In certain embodiments, the detecting comprises a competitive assay using as a competitive binder an antibody specific

25 for a conformational epitope of myelin/oligodendrocyte glycoprotein where the antibody comprises a polypeptide sequence selected from the group consisting of SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:27, SEQ ID NO:29, SEQ ID NO:31, SEQ ID NO:33, SEQ ID NO:35, and SEQ ID NO:37

30 In certain embodiments, this invention provides a method of predicting disease outcome in patients with a first episode of demyelination in the central nervous system or with definitive multiple sclerosis. The method typically involves measuring antibodies

against specific myelin constituents where the presence or increasing concentrations of such antibodies indicates a progressively negative outcome. In certain embodiments, the myelin constituent comprises MOG and/or GalC. In certain embodiments, the antibodies are specific for a conformational epitope of MOG and/or GalC. The method can, optionally,
5 involve measuring the antibodies at two or more times. In certain embodiments, the two or more times comprise a first time at initial presentation or diagnosis of the disease and a second time at least two months later.

This invention also provides methods of estimating the time within the history of an individual patient when MS disease will transform from benign to progressive. The
10 methods typically involve measuring a plurality of antibodies against specific myelin constituents where presence or level of certain members of the plurality indicate the imminence of transformation of MS from benign form to a progressive form. In certain embodiments, the myelin constituent comprises MOG and/or GalC. In certain
15 embodiments, the measuring comprises detecting the presence or quantity of an antibody specific for a conformational epitope of myelin/oligodendrocyte glycoprotein (MOG). In certain embodiments, the measuring comprises screening for a plurality of antibodies specific for different conformational epitopes of the myelin/oligodendrocyte glycoprotein. In certain embodiments, the antibody specific for a conformational epitope of
20 myelin/oligodendrocyte glycoprotein is an antibody that specifically binds to an epitope bound by an antibody comprising a polypeptide sequence selected from the group consisting of SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:27, SEQ ID NO:29, SEQ ID NO:31, SEQ ID NO:33, SEQ ID NO:35, and SEQ ID NO:37. In certain embodiments, the method involves
25 measuring the antibodies at two or more times. In certain embodiments, the two or more times comprises a first time at initial presentation or diagnosis of the disease and a second time at least two months later.

Also provided are recombinant proteins consisting essentially of a MOG extracellular domain and a truncation at the C-terminus, wherein the protein is soluble in an aqueous buffer at neutral pH. In certain embodiments, the protein is a protein selected from
30 the group consisting of Rat MOG 1-117, Rat MOG 1-125, human MOG 1-118, and human MOG 1-125.

In addition, this invention provides an assay for detecting antibodies to conformational epitopes of MOG in a mammal. The assay typically involves providing a serum or CSF sample from the subject; and contacting antibodies in the sample with two or more recombinant proteins as described herein where specific binding of one or more of the recombinant proteins to the antibodies indicates the presence of one or more antibodies to conformational epitopes of MOG in the mammal. In certain embodiments, the two or more proteins are independently selected from the group consisting of Rat MOG 1-117, Rat MOG 1-125, human MOG 1-118, and human MOG 1-125.

In further embodiments the present invention provides methods of diagnosing or evaluating the prognosis of multiple sclerosis (MS) in a mammal, the method comprising: detecting the presence or quantity of an antibody in the mammal that specifically binds a rat MOG1-125 protein; wherein the presence and/or elevation of an the antibody is an indicator of PP-MS in the mammal. In some embodiments, the methods are performed in the context of a differential diagnosis for multiple sclerosis.

Also provided by the present invention are methods of diagnosing or evaluating the prognosis of multiple sclerosis (MS) in a mammal, the method comprising: detecting the presence or quantity of an antibody in the mammal that specifically binds α -GalC, wherein elevated levels of the antibody as compared to those levels observed in a healthy mammal is an indicator of a relapsing remitting form of multiple sclerosis (RR-MS) and/or a secondary progressive form of multiple sclerosis (SP-MS). In some embodiments, the methods are performed in the context of a differential diagnosis for multiple sclerosis. In some embodiments, the quantity of the antibody is compared to a mean or a median quantity of the antibody in healthy mammals. In some embodiments, the quantity of the antibody is at least 2-fold, 5-fold, or 10-fold of the mean or median quantity of the antibody in healthy mammals.

Also provided by the present invention are methods of diagnosing or evaluating the prognosis of multiple sclerosis (MS) in a mammal, the method comprising: detecting the presence or quantity of an antibody in the mammal that specifically binds α -GalC, wherein elevated levels of the antibody as compared to those levels observed in a healthy mammal is an indicator of a relapsing remitting form of multiple sclerosis (RR-MS) and/or a secondary progressive form of multiple sclerosis (SP-MS). In som embodiments, the methods are

performed in the context of a differential diagnosis for multiple sclerosis. In some embodiments, the method comprises determining quantitative ratios of Ig concentrations (IgG, IgM and other subtypes). In some preferred embodiments, the detecting comprises obtaining a biological sample comprising serum or cerebrospinal fluid from the mammal.

5 In some embodiments, the detecting comprises screening for a plurality of antibodies specific for different epitopes of the myelin/oligodendrocyte glycoprotein and/or the α -GalC. In some preferred embodiments, the mammal is a human. In some particularly preferred embodiments, the mammal is a human with a preliminary diagnosis of multiple sclerosis. In some embodiments, the detecting comprises a liquid phase assay. The present
10 invention further provides methods of treating a patient having a preliminary diagnosis of multiple sclerosis, the method comprising: obtaining a biological sample comprising serum from the patient; performing the screening methods described above; and prescribing a more aggressive treatment regimen when the indicators are positive for a primary progressive form of multiple sclerosis (PP-MS).

15 Additionally, the present invention provides methods of diagnosing definite multiple sclerosis in patients with a first episode of demyelination in the central nervous system, the method comprising: measuring antibodies against specific myelin constituents; where the presence of such antibodies indicates a definite diagnosis of multiple sclerosis. In some embodiments, the myelin constituent comprises MOG. In other embodiments, the myelin
20 constituent comprises GalC. In some preferred embodiments, the antibodies are specific for a conformational epitope of MOG. In further preferred embodiments, the antibodies are specific for a conformational epitope of Galc.

The present invention also provides methods of determining the form of multiple sclerosis, the methods comprising: measuring a plurality of antibodies against specific
25 myelin constituents; where presence or level of certain members of the plurality indicate the form or stage of multiple sclerosis. In some embodiments, the myelin constituent comprises MOG. In other embodiments, the myelin constituent comprises GalC. In some embodiments, the detecting comprises detecting the presence or quantity of an antibody specific for a conformational epitope of myelin/oligodendrocyte glycoprotein (MOG). In
30 some embodiments, the detecting comprises screening for a plurality of antibodies specific for different conformational epitopes of the myelin/oligodendrocyte glycoprotein. In some embodiments, the detecting comprises a competitive assay using as a competitive binder an

antibody specific for an epitope of myelin/oligodendrocyte glycoprotein and/or α GalC and an antibody that specifically binds to an epitope bound by an antibody described herein.

Further more the present invention provides methods of predicting disease outcome in patients with a first episode of demyelination in the central nervous system or with
5 definitive multiple sclerosis, the method comprising: measuring antibodies against specific myelin constituents; where the presence or increasing concentrations of such antibodies indicates a progressively negative outcome. In some embodiments, the myelin constituent comprises MOG. In further embodiments, the myelin constituent comprises GalC. In some preferred embodiments, the antibodies are specific for a conformational epitope of MOG. In
10 further embodiments, the antibodies are specific for a conformational epitope of GalC. In some preferred embodiments, the methods comprise measuring the antibodies at two or more times. In some embodiments, the two or more times comprises a first time at initial presentation or diagnosis of the disease and a second time at least two months later.

The present invention also provides methods of estimating the time within the
15 history of an individual patient when MS disease will transform from benign to progressive, the method comprising: measuring a plurality of antibodies against specific myelin constituents; where presence or level of certain members of the plurality indicate the imminence of transformation of MS from benign form to a progressive form. In some embodiments, the myelin constituent comprises MOG. In further embodiments, the myelin
20 constituent comprises GalC. In some embodiments, the measuring comprises detecting the presence or quantity of an antibody specific for a conformational epitope of myelin/oligodendrocyte glycoprotein (MOG). In further embodiments, the measuring comprises screening for a plurality of antibodies specific for different conformational epitopes of the myelin/oligodendrocyte glycoprotein. In some preferred embodiments, the
25 antibody specific for a conformational epitope of myelin/oligodendrocyte glycoprotein is an antibody that specifically binds to an epitope bound by an anti-MOG and/or an anti-GalC antibody described herein. In some embodiments, the methods comprise measuring the antibodies at two or more times. In a subset of these embodiments, the two or more times comprise a first time at initial presentation or diagnosis of the disease and a second time at
30 least two months later.

Also provided by the present invention are recombinant proteins consisting of a MOG extracellular domain and a truncation at the C-terminus, wherein the protein is soluble in an aqueous buffer at neutral pH. In some preferred embodiments, the protein is selected from the group consisting of Rat MOG 1-117, Rat MOG 1-125, human MOG 1-118, and human MOG 1-125. In some embodiments the recombinant proteins are included in kits, wherein the kits further comprise instructions for using the recombinant proteins to diagnose a subject as having a subtype of MS (e.g., RR-MS).

Furthermore the present invention provides cell lines comprising cells transfected with a nucleic acid encoding MOG. In some embodiments the cell line comprises cells transfected with a nucleic acid encoding a membrane bound form of MOG, while in other embodiments the cell line comprises cells transfected with a nucleic acid encoding a soluble form of MOG. In some preferred embodiments the cell line comprises cells transfected with a nucleic acid encoding an alpha1 isoform of MOG, while in other embodiments the cell line comprises cells transfected with a nucleic acid encoding a beta1 isoform of MOG.

In more recent embodiments, the present invention provides methods of diagnosis of a relapsing remitting form of multiple sclerosis (RR-MS) or a secondary progressive form of multiple sclerosis (SP-MS) in a subject, the method comprising: comparing a level of antibodies in a biological sample from a subject that bind to galactocerebroside (alpha-GalC) to a control level of antibodies that bind to alpha-GalC, wherein an elevated level of the antibodies in the sample as compared to the control level indicates that the subject has an increased likelihood of having a relapsing remitting form of multiple sclerosis (RR-MS) or a secondary progressive form of multiple sclerosis (SP-MS). In some embodiments, the control level is a threshold established by detecting levels of antibodies that bind to alpha-GalC in biological samples from healthy subjects. In some preferred embodiments, the biological sample from the subject comprises serum or cerebrospinal fluid. In some embodiments, the level of antibodies that bind to alpha-GalC is detected by measuring binding of the antibodies to alpha-GalC immobilized on a solid surface. In some embodiments, the level detected is a binding ratio calculated by the ratio of signal over background (e.g., signal: noise ratio). In some preferred embodiments, the subject is a human. In a subset of these embodiments, the subject is a human with a preliminary diagnosis of multiple sclerosis. In further embodiments, the level of antibodies that bind to alpha-GalC is detected by a liquid phase assay. In some particularly preferred

embodiments, the level of antibodies that bind to alpha-GalC is detected at a first time point and at a second time point, wherein the second time point is at least about three months (at least 6 months, 9 months, 12 months, 15 months, 18 months or 24 months) after the first time point. In some particularly preferred embodiments, the methods further comprise
5 utilizing magnetic resonance imaging (MRI) to assess the subject.

Moreover, the present invention provides methods of diagnosis of a relapsing remitting form of multiple sclerosis (RR-MS) or a secondary progressive form of multiple sclerosis (SP-MS) in a subject, the method comprising: comparing a level of antibodies in a biological sample from a subject to a control level, wherein the antibodies bind to a myelin
10 oligodendrocyte glycoprotein (MOG) isoform expressed on a eukaryotic cell surface, wherein the expressed MOG comprises a conformational epitope of MOG, and wherein an elevated level of the antibodies in the biological sample as compared to the control level indicates that the individual has an increased likelihood of having a relapsing remitting form of multiple sclerosis (RR-MS) or a secondary progressive form of multiple sclerosis (SP-
15 MS). In some embodiments, the eukaryotic cell is a mammalian cell (such as CHO, COS, HeLa), an insect cell or a yeast cell. In some embodiments, the eukaryotic cell has been transfected with a nucleic acid encoding an alpha isoform (such as alpha1) of MOG or a beta isoform (such as beta1) of MOG. In some preferred embodiments, binding of Fab M3-31 or M26 identifies the conformational epitope of MOG. In some embodiments, the
20 control level is a median or a mean level of antibodies that bind to the MOG isoform expressed on the eukaryotic cell surface in biological samples from healthy subjects. In some preferred embodiments, the subject is a human. In some particularly preferred embodiments, the subject is a human with a preliminary diagnosis of multiple sclerosis. In some preferred embodiments, the level of the antibodies is detected by FACS analysis. In
25 some particularly preferred embodiments, the level of antibodies that bind to a myelin oligodendrocyte glycoprotein (MOG) isoform expressed on a eukaryotic cell surface is detected at a first time point and at a second time point, wherein the second time point is at least about three months (at least 6 months, 9 months, 12 months, 15 months, 18 months or 24 months) after the first time point. In some particularly preferred embodiments, the
30 methods further comprise utilizing magnetic resonance imaging (MRI) to assess the subject.

The present invention also provides methods of assessing multiple sclerosis (MS) risk in a subject, the method comprising: comparing a level of antibodies in a biological

sample from a subject to a control level, wherein the antibodies bind to a myelin oligodendrocyte glycoprotein (MOG) isoform expressed on a eukaryotic cell surface; wherein the expressed MOG comprises a conformational epitope of MOG, wherein an elevated level of the antibodies as compared to the control level indicates that the subject

5 has an increased likelihood of having a clinically isolated syndrome (CIS) indicative of an increased risk of developing MS. In some embodiments, the eukaryotic cell is a mammalian cell (such as CHO, COS, HeLa), an insect cell or a yeast cell. In some embodiments, the eukaryotic cell has been transfected with a nucleic acid encoding an alpha isoform (such as alpha1) of MOG or a beta isoform (such as beta1) of MOG. In some preferred

10 embodiments, binding of Fab M3-31 or M26 identifies the conformational epitope of MOG. In some embodiments, the control level is a median or a mean level of antibodies that bind to the MOG isoform expressed on the eukaryotic cell surface in biological samples from healthy subjects. In some preferred embodiments, the subject is a human. In some particularly preferred embodiments, the subject is a human with a preliminary diagnosis of

15 multiple sclerosis. In some preferred embodiments, the level of the antibodies is detected by FACS analysis. In some particularly preferred embodiments, the level of antibodies that bind to a myelin oligodendrocyte glycoprotein (MOG) isoform expressed on a eukaryotic cell surface is detected at a first time point and at a second time point, wherein the second time point is at least about three months (at least 6 months, 9 months, 12 months, 15

20 months, 18 months or 24 months) after the first time point. In some particularly preferred embodiments, the methods further comprise utilizing magnetic resonance imaging (MRI) to assess the subject.

The present invention further provides methods of assessing severity of multiple sclerosis in a subject having a relapsing remitting form of multiple sclerosis (RR-MS), the

25 method comprising: detecting cumulative concentration of antibodies in a biological sample from a subject that bind to a plurality of recombinant MOG proteins, wherein the biological sample from the subject has a high titer reactivity to at least one of the plurality of the MOG proteins, and wherein the extent of elevation in the cumulative concentration as compared to a control level is indicative of the severity of RR-MS in the subject. In some preferred

30 embodiments, the subject is a human. In some preferred embodiments, the plurality of the MOG proteins comprises recombinant human MOG₁₁₈ (rhMOG₁₁₈), recombinant human MOG₁₂₅ (rhMOG₁₂₅), and rat MOG₁₂₅ (ratMOG₁₂₅). In some embodiments, the control

level is established with cumulative concentration of antibodies in a control biological sample that bind to the plurality of recombinant MOG proteins, wherein the control biological sample is from subjects with zero or lowest degree of disability as measured by extended disability status scale (EDSS). In other preferred embodiments, the control level is established with cumulative concentration of antibodies that in a control biological sample that bind to the plurality of recombinant MOG proteins, wherein the control biological sample is from subjects with zero or lowest multiple sclerosis severity score (MSSS). In some embodiments, the cumulative concentration is measured by detecting binding of antibodies in the biological sample from the subject to rhMOG₁₁₈, rhMOG₁₂₅, and ratMOG₁₂₅ immobilized on a solid support. In some particularly preferred embodiments the cumulative concentration of antibodies in a biological sample from a subject that bind to a plurality of recombinant MOG proteins is detected at a first time point and at a second time point, wherein the second time point is at least about three months (at least 6 months, 9 months, 12 months, 15 months, 18 months or 24 months) after the first time point. In some particularly preferred embodiments, the methods further comprise utilizing magnetic resonance imaging (MRI) to assess the subject.

DEFINITIONS

The terms “polypeptide”, “peptide” and “protein” are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residues is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers.

The term “antibody” refers to a polypeptide substantially encoded by an immunoglobulin gene or immunoglobulin genes, or fragments thereof which specifically bind and recognize an analyte (antigen). The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as the myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively. An exemplary immunoglobulin (antibody) structural unit comprises a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one “light”

(about 25 kD) and one “heavy” chain (about 50-70 kD). The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms variable light chain (VL) and variable heavy chain (VH) refer to these light and heavy chains respectively

5 Antibodies exist e.g., as intact immunoglobulins or as a number of well-characterized fragments produced by digestion with various peptidases. Thus, for example, pepsin digests an antibody below the disulfide linkages in the hinge region to produce $F(ab)_2$, a dimer of Fab which itself is a light chain joined to VH-CH1 by a disulfide bond. The $F(ab)_2$ may be reduced under mild conditions to break the disulfide linkage in the hinge
10 region, thereby converting the $F(ab)_2$ dimer into an Fab' monomer. The Fab' monomer is essentially a Fab with part of the hinge region (see, Fundamental Immunology, Third Edition, W.E. Paul, ed., Raven Press, N.Y. 1993). While various antibody fragments are defined in terms of the digestion of an intact antibody, one of skill will appreciate that such fragments may be synthesized de novo either chemically or by utilizing recombinant DNA
15 methodology. Thus, the term antibody, as used herein, also includes antibody fragments either produced by the modification of whole antibodies, those synthesized de novo using recombinant DNA methodologies (e.g., single chain Fv), and those found in display libraries (e.g. phage display libraries).

 The term “specifically binds”, as used herein, when referring to a binding agent
20 (e.g., protein, nucleic acid, antibody, etc.), refers to a binding reaction that is determinative of the presence binding agent in a heterogeneous population of proteins and other biologics. Thus, under designated conditions (e.g. immunoassay conditions in the case of an antibody, or stringent hybridization conditions in the case of a nucleic acid), the specified ligand or antibody binds to its particular “target” (e.g. a protein or nucleic acid) and does not bind in a
25 significant amount to other molecules.

 As used herein, an epitope that “preferentially binds” to an antibody or a polypeptide is a term well understood in the art, and methods to determine such specific or preferential binding are also well known in the art. A molecule is said to exhibit “preferential binding” if it reacts or associates more frequently, more rapidly, with greater duration and/or with
30 greater affinity with a particular cell or substance than it does with alternative cells or substances. An antibody “preferentially binds” to a target if it binds with greater affinity,

avidity, more readily, and/or with greater duration than it binds to other substances. For example, an antibody that preferentially binds to a MOG epitope is an antibody that binds this MOG epitope with greater affinity, avidity, more readily, and/or with greater duration than it binds to other MOG epitopes or non-MOG epitopes. It is also understood by reading
5 this definition that, for example, an antibody (or moiety or epitope) that preferentially binds to a first target may or may not preferentially bind to a second target. As such, "preferential binding" does not necessarily require (although it can include) exclusive binding.

The term a "conformational epitope", e.g. when referring to a conformation epitope of a MOG, refers to region of the subject protein that is specifically recognized by an
10 antibody and that introduces secondary or tertiary structure into the subject protein. This is as distinguished from "linear epitope" that refers to a region of the protein that does not introduce secondary structure (e.g. bends, helices, etc.). A conformational epitope can be identified by any of a number of methods known to one of skill in the art. For example, when a conformational epitope is "denatured" i.e. the conformation is altered and/or
15 linearized, binding by the conformational epitope specific antibody is diminished or eliminated. In contrast, "denaturation" of a linear epitope will not substantially alter binding by antibodies specific to that epitope.

A "MOG conformational epitope antibody" refers to an antibody that specifically binds a conformational epitope of a MOG protein.

20 As used herein, methods for "diagnosis" refer to methods that assist in making a clinical determination regarding the presence, or nature, of multiple sclerosis, and may or may not be conclusive with respect to the definitive diagnosis.

As used herein, the singular form "a", "an", and "the" includes plural references unless indicated otherwise. For example, "an" antibody includes one or more antibodies.

25 As used herein, the term "control" refers to subjects or samples that provide a basis for comparison for experimental subjects or samples. For instance, the use of control subjects (e.g., age-matched healthy subject) or samples permits determinations to be made regarding likelihood of a disease, such as MS and/or a subtype of MS. A control may be established by determining the mean or median level of a certain indicator in healthy
30 subjects. A control may also be a threshold established from levels of a certain indicator in healthy subjects. For example, a threshold may be established by a mean + 3 x SD (standard

deviation), wherein the mean is the mean binding in the sample from healthy subjects. Level of binding may be expressed as binding ratio calculated as the ratio of signal over background.

5 A high titer reactivity of a sample means that the sample shows detectable binding to a target after certain level of dilution (for example, at least about 1/400 dilution, at least about 1/800 dilution, at least about 1/1000, at least about 1/1600, at least about 1/2000, at least about 1/3200 dilution). For example, to determine a high titer reactivity, samples having a binding ratio (calculated as the ratio of signal over background) no less than 3 after 1/3,200 dilution of each sample are identified; and their mean binding ratio at 1/800 dilution
10 may be used as the cut-off for high titer reactivity.

A "subject" is a mammal, more preferably a human. Mammals also include, but are not limited to, farm animals (such as cows), sport animals, pets (such as cats, dogs, and horses), primates, mice and rats.

15 As used herein, cumulative concentration of antibodies that bind to a plurality of proteins means the total or the sum of antibody concentrations of each antibody that preferentially binds to each protein.

As used herein, the terms "sample" and "biological sample" refers broadly to all types of samples obtained from humans and other animals, including but not limited to, body fluids such as urine, blood (also serum or plasma), fecal matter, cerebrospinal fluid
20 (CSF), semen, and saliva, as well as solid tissue. These examples are not to be construed as limiting the sample types applicable to the present invention.

As used herein, the terms "MOG" and "myelin oligodendrocyte glycoprotein" refer to a human gene (*e.g.*, *Homo sapiens* - GENBANK Accession No. Z48051; and Roth et al., Genomics, 28:241-250, 1995) and its gene product, as well as its mammalian counterparts
25 (preferably rodent or primate), including wild type and variant products. The coding region of the human MOG alpha1 isoform is set forth as SEQ ID NO:41, while the human MOG alpha1 isoform protein sequence is set forth as SEQ ID NO:42 (GENBANK Accession No. U18798). The coding region of the human MOG beta1 isoform is set forth as SEQ ID NO:43, while the human MOG alpha1 isoform protein sequence is set forth as SEQ ID
30 NO:44 (GENBANK Accession No. U18801). However, the present invention is not limited to the alpha1 and beta1 MOG isoforms. In some embodiments, the present invention

comprises one isoform of the group consisting of alpha1, alpha2, alpha3, beta1, beta2, beta3 and beta4 (See, e.g., Roth et al., Genomics, 28:241-250, 1995; and Pham-Dinh et al., Genomics, 29:345-352, 1995, both herein incorporated by reference). In other embodiments, the present invention comprises a fusion protein comprising a MOG
5 extracellular domain and a heterologous (non-MOG) transmembrane domain (and optionally a heterologous cytoplasmic tail and/or affinity tag). MOG variants that differ from the wild type MOG sequences in less than 1% of their residues, are also contemplated to be suitable for use in the methods and compositions of the present invention. In some
10 embodiments, the variants consist of one or two amino acid substitutions, deletions, or substitutions.

As used herein, the term "instructions for using said kit" includes instructions for using the reagents contained in the kit for the diagnosis of a multiple sclerosis subtype (e.g., RRMS or SP-MS) or propensity (e.g., CIS) by assessing GalC and/or MOG-reactive
15 antibodies in a sample from a subject. In some embodiments, the instructions further comprise the statement of intended use required by the U.S. Food and Drug Administration (FDA) in labeling *in vitro* diagnostic products. The FDA classifies *in vitro* diagnostics as medical devices and requires that they be approved through the 510(k) or analyte specific reagent (ASR) procedure. Information required in an application under 510(k) includes: 1)
20 The *in vitro* diagnostic product name, including the trade or proprietary name, the common or usual name, and the classification name of the device; 2) The intended use of the product; 3) The establishment registration number, if applicable, of the owner or operator submitting the 510(k) submission; the class in which the *in vitro* diagnostic product was placed under section 513 of the FD&C Act, if known, its appropriate panel, or, if the owner or operator
25 determines that the device has not been classified under such section, a statement of that determination and the basis for the determination that the *in vitro* diagnostic product is not so classified; 4) Proposed labels, labeling and advertisements sufficient to describe the *in vitro* diagnostic product, its intended use, and directions for use. Where applicable, photographs or engineering drawings should be supplied; 5) A statement indicating that the
30 device is similar to and/or different from other *in vitro* diagnostic products of comparable type in commercial distribution in the U.S., accompanied by data to support the statement; 6) A 510(k) summary of the safety and effectiveness data upon which the substantial

equivalence determination is based; or a statement that the 510(k) safety and effectiveness information supporting the FDA finding of substantial equivalence will be made available to any person within 30 days of a written request; 7) A statement that the submitter believes, to the best of their knowledge, that all data and information submitted in the premarket notification are truthful and accurate and that no material fact has been omitted; 8) Any additional information regarding the *in vitro* diagnostic product requested that is necessary for the FDA to make a substantial equivalency determination. Additional information is available at the Internet web page of the U.S. FDA.

The following abbreviations may be used herein: AM: Acute monophasic; CIS: Clinically isolated syndrome; CNS: Central nervous system; EAE: Experimental allergic encephalomyelitis; GalC: Galactocerebroside; α -GalC: Antigalactocerebroside; HC: Healthy control; HR: Hazard ratio; MBP: Myelin basic protein; MOG: Myelin/oligodendrocyte glycoprotein; MRI: Magnetic resonance imaging; MS: Multiple sclerosis; PP: Primary-progressive; rMOG: Recombinant rat myelin/oligodendrocyte glycoprotein (extracellular domain); RR: Relapsing-remitting; RT: Room temperature; SP: Secondary-progressive.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the conformational requirements of MOG-specific Fab clones. Means of triplicate or quadruplicate values. (*) PepMOG designates a mixture of overlapping 20mer peptides spanning the entire sequence of rMOG. For comparison, representative reactivity of rMOG-immune serum Abs is also shown (Serum).

Figures 2A-C illustrate the results of competition ELISAs with representative Fab fragments. Figure 2A: M26Biotin is displaced from rMOG by itself (O), M38 ($\diamond$), and M45(X) but not by M3-8 ($\square$), M3-24 (Δ), or M3-31 (inverted triangle). Figure 2B: M3-24Biotin is displaced only by itself (Δ). Figure 2C: M3-31Biotin is displaced by itself (inverted triangle), M38 ($\diamond$), and M45 (X), and also by high concentrations of M26 (O). Highlighted in red are the Fab fragments that tightly cluster within the major immunogenic region of MOG. IGHV and IGKV gene usage is indicated in the legend.

Figure 3 illustrates binding of recombinant Fab fragments to MOG in situ on *C. jacchus* CNS myelin. Fluorescent light micrographs of *C. jacchus* corpus callosum showing oligodendrocytes and myelinated fibers stained with the biotinylated Fab fragment M26 (Left). Specificity of the staining was confirmed by signal quenching after coincubation
 5 with rMOG (Right). Arrows indicate groups of aligned oligodendrocyte cell bodies.

Figure 4 shows correlations between anti-MOG Ab epitope recognition and neuropathological phenotypes. (Upper) Perivascular mononuclear cell infiltrates in brain white matter of representative rMOG- (Left) and PepMOG-immunized marmosets (Right). Note the large size of the infiltrate and the broad area of demyelination in the rMOG-
 10 immunized animal, and the lack of demyelination after PepMOG-immunization. Luxol Fast Blue/periodic acid Schiff, x200. (Lower) The specificities of serum anti-MOG Abs in these animals were analyzed before (Serum) and after (PepMOG-depleted) removal of Abs binding to PepMOG (see Materials and Methods in Examples). rMOG reactivity is clearly retained after removal of the peptide-reactive Abs from rMOG-immune serum (Left),
 15 indicating the presence of separate subsets of Abs that react either with linear peptides or conformational rMOG. In marked contrast, no reactivity to MOG remains after removal of PepMOG-specific Abs from the animal immunized with PepMOG (Right), demonstrating that conformation-dependent Abs were not produced. Identical results were obtained in the other rMOG- and PepMOG-immunized monkeys.

20 Figure 5 shows the results of competition between marmoset Fab fragments and human anti- MOG Abs. Affinity-purified serum anti-MOG Abs from patient AA with MS are displaced by M3-8 ($\square$) and M3-24 (Δ) or a combination of and M3-8 and M3-24 ($\blacktriangledown$).

Figure 6 illustrates neuropathology of EAE induced in *C. jacchus* by active immunization with whole rMOG (top), MOG aa21-40 (middle), and adoptive transfer of a
 25 MOG aa20-40-reactive T cell clone (bottom).

Figure 7 shows lesion load in the entire neuraxis (brain, optic nerves and spinal cord) of MOG-peptide and rMOG-immunized marmosets, respectively (mean $\pm$ SD).

Figures 8A and 8B illustrate fractionation of MOG-specific serum Ig by affinity-chromatography. Ig binding to MOG-peptides was removed from serum using MOG
 30 peptide-Sepharose columns, and acid eluted. Flow through fractions (depleted of all Ig binding to MOG-peptides), and eluted fractions (containing the peptide-binding Ig) were

tested by ELISA for IgG reactivity to rMOG and MOG-peptides, respectively (insets).
Figure 8A: rMOG-immune serum: reactivity to rMOG is still detected after removal of peptide-binding IgG, indicating the presence of IgG binding to strictly conformational determinants (red). Note that the MOG peptide binding IgG also recognize rMOG (blue).

- 5 Figure 8B: MOG-peptide immune serum: removal of peptide-binding IgG results in the complete loss of reactivity to rMOG.

Figure 9 illustrates the detection of macrophages (HAM56), IgG and activated complement (C9neo) by immunostaining of EAE lesions from rMOG- (n=4) and MOG peptide-immunized marmosets (n=9). IgG cells designate cells positively stained for IgG, likely plasmocytes. A total of 84 lesions were examined and the percentage of positive
10 lesions is shown. Data are mean $\pm$ SEM.

Figure 10 illustrates competition of Fab fragments against native anti-MOG IgG, and the 8.18.C5 antibody. Constant concentrations of biotinylated, purified *C. jacchus* anti-MOG IgG (animal 318-97) were incubated with increasing concentrations of individual Fab
15 fragments (red, blue), combination of both Fabs (green), or the non-biotinylated anti-MOG IgG themselves (black diamonds). Y-axis: % of MOG-bound biotinylated IgG. X-axis: log of concentration of competitor. In contrast to *C. jacchus* Fabs, 8.18.C5 fails to compete with purified marmoset anti-MOG IgG (black circles).

Figure 11 shows the staining of MOG-transfected COS cells (top panel) and
20 fibroblast cell line CCL-153 (middle panel) with biotinylated M26 Fab. Right panel: an untransfected cell line.

Figure 12 illustrates the transfer of human IgG in MBP-immunized marmosets. Top panel: transfer of IgG from an MS serum reactive to MOG. Large subpial infiltrate with underlying demyelination in the spinal cord (LFB/PAS). Bottom panel: spinal cord of an
25 animal transferred with IgG from a control, unreactive serum. Subpial infiltrate with intact underlying myelin (H&E).

Figure 13 shows the percentage of sera testing positive for MOG and MBP antibody in the different clinical phenotypes of MS. The number of patients studied for each MS subtype is given in parentheses on the X axis (MBP reactivity was assessed in only 17 of
30 the controls). Results were replicated independently by two different technicians in the laboratory.

Figure 14 shows serum reactivity (IgG) to rMOG, MBP, and MOG-derived 20mer peptides in patient CIS 5 presenting with transverse myelitis, positive brain and cervical spine MRI, and Gd+ enhancement. Note the lack of reactivity to MBP in this patient.

Figure 15 Anti-GalC antibody ELISA. Top panel, validation using marmoset sera: from left to right in succession, naïve control, animals immunized with adjuvant mixture alone (CFA), rMOG (all negative), and time course of appearance of anti-GalC IgG in animals immunized with whole white matter. The animal with very high titers (*) had chronic EAE and was sampled after 3 relapses. A rabbit polyclonal anti-GalC antibody is used as positive control (far right). Bottom panel, human sera from six individual patients with MS. Sera were diluted 1:100. Results are means of duplicate wells, corrected for background values for each patient, which ranged from 0.05 to 0.12 OD units.

Figure 16A shows sequential studies of IgG reactivity to MOG, MBP and GalC in patient DM. Figure 16B shows time-dependent variation in titers and epitope recognition of rMOG-specific IgG in a patient with SPMS. Note the low level of reactivity to MBP. Results are for sera diluted 1:100 and background corrected. Serial measurements for each patient were performed in a single assay plate

Figure 17 illustrates fractionation of MOG-specific antibodies. C designates the fraction containing conformation-dependent antibodies, and L the fraction containing antibodies that recognize linear MOG peptides.

Figure 18 shows the inverse correlation between the ratio of MOG/peptide- (AbPep) to rMOG-reactive IgG, and clinical severity of MOG-induced marmoset EAE (marmoset expanded scale, 0-45 points 82). Antibody measurements were performed quantitatively using serial serum dilutions and a standard curve for marmoset IgG.

Figure 19 shows the results of passive transfers in MBP-immunized marmosets. Left, large confluent demyelinating infiltrates in a recipient of peptide-depleted, rMOG-purified Ig. Right, typical lesion in a recipient of MOG-peptide-specific Ig. Note minimal demyelination. LFB/PAS.

Figure 20A and 20B, show neuropathology of rMOG1-125- and MOG peptide-induced EAE. High power views (x 200) of cervical spinal cord sections stained with LFB. Panel A: typical inflammatory infiltrate in a marmoset immunized with MOG aa21-40 (368-

94). Note contiguity with the subpial space (upper right corner) and the limited amount of demyelination. Panel B: perivascular, inflammatory infiltrate in deep periventricular white matter (V=blood vessel) with pronounced concentric demyelination, characteristic of rMOG1-125-immunized animals (J2-97). Such lesions were never found in MOG peptide-immune *C. jacchus*. Note myelin vacuolation (arrows) in both MOG peptide-induced EAE (A) and at the lesion edges in rMOG1-125-induced EAE (B).

Figure 21A-F show fine specificities of unfractionated sera and anti-MOG-P-depleted sera from representative animals of groups I and II. The left panels show reactivity of whole sera at a dilution of 1:200. The right panels show residual reactivity after removal of anti-MOG-P antibodies by affinity-chromatography. Panels A and B: Antibody specificities in rMOG1-125-immunized monkeys (n=4, mean +/- SEM), demonstrating that strong reactivity against rMOG1-125 is retained after removal of all MOG peptide-specific antibodies. Panels C-F: Representative experiments for individual animals immunized with individual or all MOG-derived peptides (aa21-40, 199-94; pepMOG, 39-95). Panels G and H: reactivity of a pool of MOG peptide-immune sera (animals 252-93, Tx245-90, 14-91, Tx75-92, Tx256-93): The MOG-reactivity is completely removed in all animals immunized with MOG-derived peptides by passage on pepMOG columns, indicating that this immunization regimen does not induce conformation-dependent antibodies. Compare to A and B, rMOG1-125-immune animal.

Figure 22A-D show reactivity of affinity purified anti-MOG antibody fractions with native MOG. Immunohistochemical staining (brown) of normal brain tissue from an unimmunized *C. jacchus*. Panels A and B: anti-MOG-C and anti-MOG-P from an rMOG1-125-immune serum pool; Panel C: anti-MOG-P from a MOG peptide-immune serum pool; Panel D: naïve *C. jacchus* serum. Consecutive sections showing corpus callosum (cc) and adjacent gray matter (gm) at 200x magnification.

Figure 23 shows T cell proliferation against rMOG1-125 in rMOG1-125- and MOG peptide-immune animals. Mean +/- SEM.

Figure 24A-F show immunohistochemical characterization of CNS lesions. Representative lesions from an rMOG1-125-immunized animal (J2-97, left) and an animal immunized with MOG aa21-40 (199-94, right). From top to bottom, staining (brown) for macrophages (HAM56, Panels A and B); IgG (Panels C and D); C9neo (Panels E and F).

IgG depositions were predominantly found in rMOG1-125-immunized animals (Panel C) compared to MOG peptide-immune animals (Panel D). Activation of complement (C9neo) was a characteristic of rMOG1-125-induced EAE (Panel E) and was not found in MOG peptide-immune animals (Panel F). Original magnification 600x.

5 Figure 25 shows alignment of human (SEQ ID NO:38), marmoset (SEQ ID NO:39), and rat (SEQ ID NO:40) MOG proteins.

Figure 26 shows staining of MOG-transfected CHO cells with the murine monoclonal anti-MOG 8.18.C5 and human serum (bottom left).

10 Figure 27 shows the incidence of positive MOG-CHO staining in healthy controls (HC), clinically isolated syndromes (CIS), relapsing remitting MS (RRMS), secondary progressive (SP) MS, and primary progressive (PP) MS. BRN: binding ratio normalized to background (untransfected CHO cells).

15 Figures 28A and 28B show binding ratios and frequencies of α -GalC IgG responses in human MS and HCs. Figure 28A: α -GalC IgG binding ratios for each disease subgroup. Solid lines (-) denote mean binding ratios; dashed line (- -) denotes threshold of detection (mean binding ratio of HC 1 3 SD. Figure 28B: Frequencies of anti-GalC IgG seropositivity in human sera.

20 Figure 29A-E show immunostaining of HOG cells with affinity-purified human α -GalC IgG. Figure 29A: Affinity purified anti-GalC IgG (1006-GalC) at 30 μ g/mL. Figure 29B: Positive control (rabbit anti-GalC antiserum) at 1:50 dilution. Figure 29C: Staining with serum of 1006-GalC at dilution 1:50. Figure 29D and 29E: Negative controls: fluorescein isothiocyanate-labeled antihuman and anti-rabbit IgG, respectively.

25 Figure 30 illustrates the time course of α -GalC and α -myelin protein IgG responses in immunized *C. jacchus*. Serum dilutions, 1:100. (. . .) denotes onset of clinical signs; (- ∇ -) denotes anti-MBP positivity; (-*-) denotes anti-rMOG positivity; (-■-) denotes anti-GalC positivity. Significant levels for median onset post-immunization (pi) of antibody positivity were determined by a Cox proportional hazard model.

30 Figure 31A shows the percentage of sera testing positive for MOG and MBP antibody in the different clinical phenotypes of MS. The number of patients studied for each MS subtype is given in parentheses on the X axis (MBP reactivity was assessed in only 17

of the controls). Results were replicated independently by two different technicians in the laboratory.

Figure 31B illustrates the linearity of our standard Ig curves to calculate antibody concentrations. Note that the sensitivity of the assay is in the picogram range.

5 Figure 32 illustrates the reactivity of human serum in healthy control and PPMS subjects against rat MOG 1-125. No difference was observed between the two groups when using human MOG 1-125.

Figure 33 illustrates IgG response to MOG in various MS subtypes. Top panel: Mean reactivity to ratMOG1-125 (ELISA) in control and MS subjects at increasing serum
10 dilutions. Bottom panel: Quantitative evaluation of mean concentration of MOG-specific IgG in the same MS and control groups. Note the robustness of the assay since similar values are derived from each serum dilution. Note also the major differences between PPMS and other groups.

Figure 34 Antibody reactivities against rhMOG125 and TT in solution-phase
15 LiPhELIA.

Figure 35 Antibody reactivity against TT in ELISA and LiPhELIA.

Figure 36 Antibody reactivities of *C. jacchus* marmosets immunized with rat MOG, HWM or MOG peptides in ELISA and LiPhELIA.

Figure 37: Reactivity of 8.18c5 and four marmoset, M3-24, M26, M3-31, derived
20 Fab fragments against rhMOG125 in ELISA (gray bars) and LiPhELIA (black bars). Results are expressed as mean binding ratios of three experiments; error bars denote SD. Identical amounts of antibody within the linear range in ELISA were assayed per monoclonal in ELISA and LiPhELIA, respectively.

Figure 38A-D: Cell-based (hMOGcme) assay. Figure 38A: FACS staining of
25 MOG-transfected CHO cells with anti-MOG 8-18C5 (0.5 µg/ml). Figure 38B: FACS staining of MOG-transfected CHO cells with anti-MOG 8-18C5 (0.5 µg/ml). Figure 38C: Positive control (RRMS serum diluted 1:10) displaying a clear shift for MOG transfected-CHO staining (filled curve) when compared to non transfected-CHO cells (non-filled curve). Figure 38D: Mean binding ratio (BR) calculated with the FITC geometrical mean
30 (Gmean) of the positive control based on nine independent FACS assays (+/- SEM).

Figure 39A: Results of IgG antibodies against MOG-transfected CHO cells in the different subgroups of patients and control studied by FACS. Binding ratio normalized (BRN) relates to the geometric mean (Gmean) of MOG-transfected CHO cells divided by the Gmean of non-transfected CHO cells and normalized to the value of a positive control.

- 5 The difference of IgG binding against MOG-transfected CHO cells is significantly increased in CIS ($P < 0.001$), RRMS ($P < 0.01$) and SPMS ($P < 0.05$) when compared to HC or PPMS. Figure 39B: Mean BRN values and median age compared in each subgroup. A linear regression analysis shows a positive correlation between the median age of each subgroup and the mean binding ratio to MOG-transfected CHO cells ($p < 0.05$, $r^2 = 0.9279$).
- 10 HC = healthy controls; CIS = clinically isolated syndrome; RRMS = remitting-relapsing multiple sclerosis; SPMS = secondary progressive multiple sclerosis; PPMS = primary progressive multiple sclerosis.

Figure 40A-F: Staining of the CHO-MOG cells with monoclonal, rat MOG1-125-immune marmoset-derived Fab antibody fragments. Note that all the Fab fragments were
 15 selected for their ability to bind to rat MOG1-125 as presented in ELISA wells.

Figure 41: Time course of serum IgG directed against hMOGcme in marmoset EAE. Results are from eleven EAE *C. jacchus* marmosets immunized with human white matter, three of which were killed before onset of clinical disease. First occurrence of serum IgG directed against hMOGcme is compared with time of clinical onset of EAE in a Kaplan–
 20 Meier survival plot.

Figure 42A-C: Selective epitope presentation on hMOGcme. Figure 42A: Binding to hMOG125 by ELISA compared with binding to hMOGcme by FACS in the CIS cohort ($n = 36$). By linear regression analysis, there is no correlation between the results of these two methods (P not significant, $r^2 = 0.00023$, Spearman r ; straight line is the linear regression
 25 curve; dotted line indicates 95% confidence interval) even when clear serum reactivity is present in both assays. BRN, BR normalized. Figure 42B and 42C: Pre-absorption of serum on either hMOGcme (Left) or hMOG125 (Right), followed by testing by FACS (B, hMOGcme) or ELISA (C, hMOG125). Pre-absorption on hMOG125 or hMOGcme only altered the reactivity in the corresponding system of detection.

30 Figure 43A-D: Monoclonal reagents define distinct epitopes on MOG. Serial two-fold dilution series of the mouse anti-ratMOG monoclonal antibody 8.18c5 (Figure 43A),

and the marmoset derived anti-ratMOG Fabfragments M26 (Figure 43B), M3-24 (Figure 43C) and M3-8 (Figure 43D) against rhMOG118 (-●-), rhMOG125 (-■-) and ratMOG125 (-◆-). While 8.18c5 and M26 bind equally well to all three MOG preparations, M3-24 is reactive exclusively with the 125 amino acid long preparations regardless of the species origin. In contrast, M3-8 is species-dependent and exclusively reactive to ratMOG125.

Figure 44: Scatter plot of ELISA reactivity of MS patients and healthy controls against rhMOG118, rhMOG125 and ratMOG125 Differences between 164 healthy controls (open symbols) and 325 MS patients (solid symbols) for the three MOG preparations, rhMOG118 (●, ○), rhMOG125 (■, □) and ratMOG125 (◆, ◇). Results are expressed as IgG concentrations in µg/mL serum, horizontal lines represent means. In all groups the distributions were skewed in favor of low IgG concentrations with the means between the medians and 75th percentile values. Differences between HC and MS were not significant for each antigen ($p>0.05$; Mann-Whitey U test). In contrast, anti-ratMOG125 IgG concentrations were significantly lower compared to the anti-rhMOG118 and anti-rhMOG125 responses for both HC and MS ($p<0.001$; Kruskal-Wallis test and Dunn's post hoc test).

Figure 45: Dilution series of high binding samples. Two-fold serial serum dilutions starting at 1/200. Results are expressed as binding ratios (OD_{MOG} / OD_{BSA}); error bars represent SD. Thus samples with high IgG concentrations ($>95^{th}$ percentile) can be diluted beyond dilutions of 1:2,000 retaining ELISA reactivity against rhMOG118 (-●-), rhMOG125 (-■-) or ratMOG125 (-◆-).

Figure 46A-C: Correlation of anti-MOG IgG concentration to disease disability in high-titer RRMS samples. Lack of correlation of the magnitude of combined reactivity against rhMOG118, rhMOG125 and ratMOG125 (expressed as anti-MOG IgG concentration) with EDSS in all 192 RR-MS patients tested ($p=0.2358$, Pearson's $r=-0.086$) (Figure 46A). In contrast, positive correlation in the subset of RRMS patients that were identified with high-titer anti-MOG reactivity, to current disability at blood draw by EDSS ($p=0.0221$, $r=0.4386$) (Figure 46B) and projected disability by MSSS (18) ($p=0.0031$, $r=0.5671$) (Figure 46C).

Figure 47: Persistent anti-MOG reactivity in serial samples. Anti-rhMOG118 (top panel), anti-rhMOG125 (middle panel) and ratMOG125 (bottom panel) reactivity of six

RRMS for whom longitudinal samples were drawn every 3 months. -●- and -▼- denote samples with high-titer reactivity vs. -□-, -◇-, -△- and -X-. Results are expressed as specific IgG concentrations (μg/mL). The magnitudes of reactivity vary with the limits of the assay, but high-titer samples remain high and vice versa.

5 Figure 48A-D: Figure 48A and 48B provide the amino acid (SEQ ID NO:41) and cDNA (SEQ ID NO:42) sequences for the alpha-1 form of MOG. Figure 48C and 48D provide the amino acid (SEQ ID NO:43) and cDNA (SEQ ID NO:44) sequences for the beta-1 form of MOG.

10

DETAILED DESCRIPTION

This invention pertains to diagnostics and prognostics for evaluation and/or treatment of multiple sclerosis. Human multiple sclerosis (MS) and the related disease model experimental allergic encephalomyelitis (EAE) are autoimmune disorders of the central nervous system characterized by destruction of myelin and axons. Antibodies to myelin are known to occur in multiple sclerosis.

Antibodies against certain myelin constituents, including myelin oligodendrocyte glycoprotein (MOG), and galactocerebroside (GalC), directly create myelin damage in experimental allergic encephalomyelitis (EAE) models. These antibodies, and others as well, can be detected in serum and cerebrospinal fluid of animals with EAE, and MS patients using established techniques, for example ELISA. However, because these techniques also detect antibodies in control subjects, simple screening for anti-MOG antibodies appeared to offer little diagnostic and/or prognostic value.

It was a surprising discovery of this invention that there exist certain classes of myelin autoantibodies in serum and cerebrospinal fluid and that the presence and/or quantity of these classes of antibodies is strongly correlated with the severe and progressive forms of multiple sclerosis. Accordingly this invention provides sensitive and specific assays (e.g. ELISA) systems to measure these antibodies and these assays provide effective diagnostics and/or prognostics for MS.

In particular, it was determined that autoantibodies against MOG are more frequently detected in severe, chronic progressive forms of MS. In primary progressive

forms, incidence approximates 100% whereas in beginning MS, it is around 30-40%. Autoantibodies against MOG are present in a significant proportion (50%) of patients during a first clinical attack corresponding to a demyelinating event (clinically isolated syndrome, which does not meet criteria for a diagnostic of MS). These antibodies persist in
5 worsening forms of MS, but decrease or disappear with improvement or stabilization. They also appear to disappear following treatment with disease modifying therapies. It is believed that these dynamic patterns of specific anti-myelin antibodies in MS have not been previously recognized, and are not observed in the case of antibodies directed against another antigen of myelin, myelin basic protein.

10 In addition, we have discovered that autoantibodies against MOG segregate into several categories according to epitope recognition, including epitopes that are strictly conformational, and epitopes corresponding to linear, short peptides. Autoantibodies against conformational epitopes of MOG, and not those against linear peptides, are pathogenic in the marmoset model of EAE. The severity of EAE correlates with titers of
15 autoantibodies against conformational epitopes of MOG, and not the titers of antibodies directed against linear peptides.

Autoantibodies to MOG in humans also segregate into strictly conformational, and linear peptide dependent classes.

In addition, it was discovered that autoantibodies against GalC appear late in the
20 course of EAE in marmosets, and are also associated with chronic disease.

In one embodiment, this invention provides methods that involve measuring autoantibodies against MOG that have specificity restricted to conformational determinants of this protein in human. This was possible because we isolated antibody clones that represent these specificities and are able to use them as reagents in specific competition
25 ELISA systems. The presence and/or level of such autoantibodies indicate the presence and/or prognosis and/or stage of multiple sclerosis.

This invention also provides methods that involve measuring the proportions of antibodies against conformational MOG epitopes and of those against the linear epitopes, or of those against other proteins. These methods are useful to assess the risk of developing
30 severe forms of MS and/or the extent of central nervous system tissue damage (brain atrophy). This can be accomplished practically in ELISA (or other assay) systems that do

not require physical separation of the different classes of antibodies. Such assays have direct application to prognosis and clinical management of MS patients.

In other embodiments, this invention contemplates methods that involve detecting antibodies against myelin constituents, including, but not limited to MOG, Galc, and other
5 antigens in the blood and/or cerebrospinal fluid, for example, at regular intervals (e.g. initial presentation/diagnosis of the disease, at least one month later, at least 2 months later, at least 3, 4, or 6 months later), in order to: 1) Help diagnose definite MS in patients with a first episode of demyelination in the central nervous system. 2) Predict disease outcome for such patients, and also for patients with definite MS. 3) Help define the time within the
10 history of individual patients when MS disease will transform from benign to progressive, severe forms which corresponds to major disability and brain atrophy. and 4) Diagnose the primary progressive forms of MS, when diagnosis cannot be ascertained by other means of evaluation (e.g., clinical, electrophysiological, MRI, standard cerebrospinal fluid studies, or others).

15 It was a discovery of ours that two or more repetitive tests can help physicians in the diagnosis, prognosis, and therefore therapeutic management, of MS patients. We also discovered that the presence and persistence of certain antibodies to myelin (for example, conformation-dependent binding antibodies as assessed by specific competition with newly developed recombinant marmoset Fab fragments), or antibody associations (one, two, or
20 more), represent new means for clinicians to assess the risk of individual patients to develop severe MS. Furthermore, we believe such measurements can be used in MS as paraclinical marker(s) of tissue destruction (myelin damage, axonal loss, scarring), as suggested by the findings of high prevalence and persistence in severe forms of disease.

One particular relevant clinical index is described in Example 3. As described
25 therein, the ratio of MOG-peptide-specific over rMOG-specific antibodies is predictive of the severity of clinical EAE in the marmoset. Thus it appears to be an extremely useful index for evaluating MS patients.

As illustrated in Figure 8, it can be difficult to distinguish these different antibody fractions by ELISA where the ligand (MOG peptide) is attached to a substrate. The
30 difference in epitope recognition, however, is important and translates into functional heterogeneity (e.g., pathogenic potential), since marmosets immunized with the linear

peptides develop an attenuated EAE phenotype compared to rMOG-immunized animals, despite the apparent induction of similar T cell responses. We have also observed that disease severity in rMOG-induced marmoset EAE is inversely proportional to the ratio of serum concentrations ($\mu\text{g/ml}$) of MOG peptide/rMOG-reactive IgG.

5

DIAGNOSTICS/PROGNOSTICS FOR MS

As indicated above, it was a discovery of this invention that anti-MOG autoantibodies and/or anti-GalC antibodies, more preferably antibodies directed against the conformation epitope(s) of MOG are particularly useful as measures of existence and/or stage and/or prognosis of multiple sclerosis in a mammal (e.g. a human or a non-human mammal).

Thus in various embodiments, this invention provides diagnostic and/or prognostic assays for multiple sclerosis that involve detecting and/or quantifying antibodies directed against (specific to) one or more epitopes of MOG and/or GalC, more preferably detecting antibodies specific to one or more conformational epitopes of MOG.

Typically the methods involve providing a biological sample from the mammal (e.g. human) that is to be screened. The biological sample is one that would typically be expected to contain anti-MOG antibodies (e.g. cerebrospinal fluid, blood, or blood fractions (e.g. serum). The sample can be "acute" or processed (e.g. diluted, fractionated, etc.). The sample is then screened for the presence and/or quantity/concentration of one or more of the antibodies in question (e.g. MOG conformational epitope antibodies).

Any of a variety of methods can be used to identify/quantify the antibodies in question. Such methods include electrophoretic methods, mass spectrometric methods, various immunoassays, and the like. Thus, for example, the target antibodies (e.g. MOG structural epitope antibodies) can be identified by fractionation methods, e.g. using affinity columns as described in Example 2.

In certain embodiments, any of a number of well recognized immunological binding assays (see, e.g., U.S. Patent Nos. 4,366,241; 4,376,110; 4,517,288; and 4,837,168) are well suited to detection or quantification of the antibodies identified herein.. For a review of the general immunoassays, see also Asai (1993) Methods in Cell Biology Volume 37:

Antibodies in Cell Biology, Academic Press, Inc. New York; Stites & Terr (1991) Basic and Clinical Immunology 7th Edition.

Where it is desired to specifically detect conformational epitopes, assays that preserve the conformational epitope(s) of the protein are preferred. In “solid phase” ELISA systems, antigens may be “nonspecifically” bound to the plate and their structure or appearance may be altered to some extent. Thus, in certain preferred embodiments, a liquid phase assay is utilized. For example, we have created a liquid phase assay employing biotinylated MOG1-118, MOG1-125, MBP, and MOG peptides; after incubation of these antigens with serum, antibodies are captured by Protein G and immunocomplexed antigen detected by streptavidin, and/or other methods, of antibody capture such as protein L, anti-Fc, protein A/G coupled to agarose or sepharose, etc.

In immunized marmosets specific antibodies can be detected readily, whereas in humans the frequencies appear lower than that observed with a classical ELISA. Without being bound to a particular theory, it is believed that the liquid phase assay only detects certain subgroups of antibodies (for example those with higher affinity and those reactive to conformational, but not linear epitopes). However, in view of the enhanced specificity for conformational epitopes, these assays will greatly enhance the interpretation of antibody profiling studies in patients with MS and clinically isolated syndromes (CIS, first clinically detectable demyelinating event).

We have generated a panel of novel recombinant proteins (e.g., rat MOG1-117, rat MOG1-125, human MOG1-118, human MOG1-125) that correspond to rat and human MOG extracellular domains with various truncations at the C-terminus. These proteins are soluble at mg/ml concentrations in aqueous buffers at neutral pH, unlike various previously available proteins. Most important, combined use of these recombinant MOG “variants” permits direct, one-step identification of epitope specificities that correspond to the conformational epitopes of MOG within the primate and human polyclonal repertoires (e.g., this avoids fractionation steps) (see, e.g., Table 1).

Table 1. Identification Of Structural Target Epitopes Using Recombinant MOG Variants

Modified MOG peptide	Epitope		
	M3-8	M3-24	M26
Rat MOG 1-117	+	-	+
Rat MOG 1-125	+	+	+
Human MOG 1-118	-	-	+
Human MOG 1-125	-	+	+

In certain embodiments, the anti-MOG and/or anti-GalC antibodies can be detected using protein and/or lipid/glycolipid microarrays comprising a plurality of MOG and/or GalC epitopes. Such arrays provide a powerful technique to allow one-step characterization of many antibody specificities (see, e.g., Robinson et al. (2002) *Biotechniques* Dec Suppl: 66-69; Liotta et al. (2003) *Cancer Cell* 3(4): 317-325; Bacarese et al. (2002) *Biotechniques* Dec Suppl: 24-9; Delechanty and Ligler (2003) *Biotechniques* 34(2): 380-385, and the like). Such methods are particularly suitable for measuring epitope spreading of antibody responses.

The assays of this invention are scored according to standard methods well known to those of skill in the art. The assays of this invention are typically scored as positive where there antibodies to one or more target epitopes (e.g. MOG conformational epitopes) are detected and/or quantified. In certain embodiments, the detection is with respect to one or more positive and/or negative controls. In certain embodiments, the "signal" is a detectable signal, more preferably a quantifiable signal (e.g. as compared to background and/or negative control).

It is noted that antibodies that bind to conformational epitopes of MOG are known to those of skill in the art (see, e.g., the Examples, herein, Sequences provided herein, and von Büdingen et al. (2002) *Proc Natl Acad Sci USA*, 99: 8207-8212). Proteins encoding such epitopes can readily be used in various assays (e.g. immunoassays) to detect and/or quantify anti-MOG antibodies, anti-GalC antibodies, and/or conformational epitope antibodies. Using such antibodies, and peptide/nucleic acid sequences for MOG (see, Figure 31)

provided herein, conformational epitopes can readily be identified and cloned using standard epitope mapping methods known to those of skill in the art. It is also noted that the foregoing assays and those illustrated herein in the Examples are intended to be illustrative and not limiting. Using the teaching provided herein numerous other assays will
5 be available to one of ordinary skill in the art.

KITS

In another embodiment, this invention provides kits for the screening procedures and/or diagnostic and/or prognostic procedures described herein. Screening/diagnostic kits typically comprise one or more reagents that specifically bind to the target that is to be
10 screened (e.g. ligands that specifically bind to MOG conformational epitope antibodies). The reagents can, optionally, be provides with an attached label and/or affixed to a substrate (e.g. as a component of a protein array), and/or can be provided in solution. In certain embodiments, the kits comprise nucleic acid constructs (e.g. vectors) that encode one or more such ligands to facilitate recombinant expression of such. The kits can optionally
15 include one or more buffers, detectable labels, or other reagents as may be useful in a particular assay.

In addition, the kits optionally include labeling and/or instructional materials providing directions (i.e., protocols) for the practice of the methods described herein. Thus, for example, in certain embodiments, preferred instructional materials describe the detection
20 of MOG conformational epitope antibodies for the diagnosis, staging, and/or prognosis of multiple sclerosis and/or CIS.

While the instructional materials typically comprise written or printed materials they are not limited to such. Any medium capable of storing such instructions and communicating them to an end user is contemplated by this invention. Such media include,
25 but are not limited to electronic storage media (e.g., magnetic discs, tapes, cartridges, chips), optical media (e.g., CD ROM), and the like. Such media may include addresses to internet sites that provide such instructional materials.

EXAMPLES

The following examples are offered to illustrate, but not to limit the claimed invention.

5

EXAMPLE 1

Molecular Characterization Of Antibody Specificities Against Myelin/Oligodendrocyte Glycoprotein In Autoimmune Demyelination

Multiple of the central nervous system (CNS) that is thought to be mediated by autoaggressive immune responses against myelin antigens (reviewed in Hohlfeld (1997) Brain 120: 865–916). Extensive investigations have addressed the respective roles of T and B cell responses against myelin antigens in experimental allergic encephalomyelitis (EAE), a disease model for MS. It is now recognized that, whereas myelin-reactive T cell responses are essential to disease pathogenesis, auto-Abs may play a major role as effectors of tissue damage (Hohlfeld (1997) Brain 120: 865–916; Bauer et al.(2001) Glia 36: 235–243; Brosnan and Raine (1996) Brain Pathol. 6: 243–257; Cross et al. (2001) J. Neuroimmunol. 112: 1–14). Myelin/oligodendrocyte glycoprotein (MOG) is a surface-exposed protein of myelin that has been identified as a prime target for demyelinating auto-Abs in several species (Genain et al. (1995) J. Clin. Invest. 96: 2966–2974; Linington et al. (1987) J. Immunol. 139: 4016–4021). Anti-MOG auto-Abs mediate a characteristic vesicular transformation of compact myelin in acutely demyelinating lesions, a neuropathological feature which has also been documented in human MS (Genain et al. (1999) Nat. Med. 5:170–175).

Despite these advances, the significance of polyclonal Ab responses against MOG measured in humans remains unclear. Anti-MOG Abs seem to be equally prevalent in the peripheral blood of affected patients and healthy controls (Karni et al. (1999) Arch. Neurol. 56: 311–315; Xiao et al. (1991) J. Neuroimmunol. 31: 91–96), and precise definition of the disease-relevant Ab epitopes of MOG is lacking. Similarly, the pathogenic significance of humoral responses directed against MOG has not been established with certainty for all EAE models (von Bu et al. (2001) J. Clin. Immunol. 21: 155–170). Indeed, these findings raise the possibility that the MOG-specific humoral response may be heterogeneous in

terms of their potential to mediate demyelination. Analyses of the fine specificities of anti-MOG Abs in EAE and MS have mainly been conducted with short peptides derived from the amino acid sequence of MOG (Meslehy et al. (2002) *Neurobiol. Dis.* 9: 160–172; Haase et al. (2001) *J. Neuroimmunol.* 114: 220–225; Ichikawa et al. (1996) *Int. Immunol.* 8: 1667–1674). This approach cannot provide an understanding of the full complexity of anti-MOG humoral responses, because it does not account for epitopes that depend on the tertiary structure of the folded protein. Similarly, whereas molecular studies have independently established that CNS-specific clonal expansion of B cells occurs in MS (Qin et al. (1998) *J. Clin. Invest.* 102: 1045–1050; Owens et al. (1998) *Ann. Neurol.* 43: 236–243; Colombo et al. (2000) *J. Immunol.* 164: 2782–2789; Baranzini et al. (1999) *J. Immunol.* 163: 5133–5144), the antigenic specificities of these responses have not been identified. The use of systems that permit analysis of gene usage and individual Ab specificities should facilitate characterization of humoral responses against myelin autoantigens.

Here, we used a combinatorial Ab library of Fab fragments to characterize the humoral immune response against MOG in the common marmoset, an outbred primate species that develop an MS-like, Ab-mediated form of EAE after immunization with MOG (Genain and Hauser (1996) *Methods* 10: 420–434). We have observed that the recombinant MOG-specific Ab fragments use a limited repertoire of heavy (H)- and light (L)-chain genes and identify epitopes of MOG with specificities that are strictly conformation-dependent. The conformational epitopes of MOG defined by these Fab fragments are consistently targeted by the humoral repertoire in all outbred marmosets studied to date. Furthermore, we show that MOG-immune marmosets do not develop demyelinating EAE unless their humoral repertoire includes conformation-dependent Abs, a finding that underscores the relevance of this Ab subgroup in disease pathogenesis.

MATERIALS AND METHODS

Animals and Induction of EAE.

All *Callithrix jacchus* marmosets used in this study were maintained in a primate colony at the University of California, San Francisco, and were cared for in accordance with all guidelines of the local Institutional Animal Care and Usage Committee. EAE was induced by active immunization with either 50 µg of recombinant protein corresponding to the extracellular domain of rat MOG_{aa1–125} (rMOG) expressed in *Escherichia coli* and

- purified to homogeneity following published procedures (Amor et al. (1994) J. Immunol. 153: 4349–4356) or a mixture of 100 µg each of overlapping synthetic 20-mer peptides corresponding to the sequence of MOGaa1–120 (Research Genetics, Huntsville, AL). Peptides were purified >95% by HPLC, and purity was confirmed by mass spectrometry.
- 5 Antigens were dissolved in 200 µl of PBS, emulsified with an equal volume of complete Freund's adjuvant, and injected intradermally as described (Genain et al. (1995) J. Clin. Invest. 96: 2966–2974). Animals were killed under deep phenobarbital anesthesia 4–70 days after the onset of clinical signs of EAE. Brain and spinal cord were dissected and fixed in 4% para-formaldehyde, and serial sections of the entire neuraxis were processed for routine
- 10 histology.

Table 2. MOG-Fab IGHV and IGKV subgroup usage and H/κL-CDR3 motifs

HV	H chain CDR3	GENBANK No.	KV	κL chain CDR3	GENBANK No.	% of clones	Clone ID
1	CARDVNFGNYFDY (SEQ ID NO:1)	AF393235	3	CQQYSSWPPTF (SEQ ID NO:2)	AF393236	73	M26
1	CARDRGMGNYFDY (SEQ ID NO:3)	AF393237	3	CQQYSSWPLTF (SEQ ID NO:4)	AF393238	13	M38
1	CARDATRILADVLDY (SEQ ID NO:5)	AF393239	3	CQQYSSWYTF (SEQ ID NO:6)	AF393240	8	M45
3	CARAWRLSARAGYFDY (SEQ ID NO:7)	AF393229	1	CQQHYSTPLTF (SEQ ID NO:8)	AF393230	2	M3-8
3	CILSDTGAFDV (SEQ ID NO:9)	AF393231	3	CQQYSSWYTF (SEQ ID NO:10)	AF393232	2	M3-24
3	CTGAGPTYFYFDY (SEQ ID NO:11)	AF393233	1	CQQGYTTPTVTF (SEQ ID NO:12)	AF393234	2	M3-31

Amino acid sequences of CDR3 motifs are deduced from cDNA. For complete H- and L-chain sequences please refer to GENBANK.

15 **Construction of a Combinatorial IgG-Fab Library from a MOG-Immunized *C. jacchus* Marmoset.**

The system used to generate the combinatorial library involved the phage display vector pCOMB3H (provided by C. F. Barbas III, The Scripps Research Institute, La Jolla,

CA). This system permits the construction of a cloning product containing L and H chains flanked by SfiI restriction sites for directional cloning (Barbas et al. (2001) Phage Display: A Laboratory Manual (Cold Spring Harbor Lab. Press, Plainview, NY)). Bone marrow and spleen cells were obtained from an rMOG-immunized *C. jacchus* that was killed after onset of clinical EAE. RNA was extracted with the Trizol reagent (Invitrogen) and first strand synthesis was performed with Superscript II reverse transcriptase (Invitrogen). In brief, three steps of PCR reactions were necessary to generate cloning inserts containing the Fab portions of *C. jacchus* IgG. (For a detailed description of these PCR steps, see Barbas et al. (2001) Phage Display: A Laboratory Manual (Cold Spring Harbor Lab. Press, Plainview, NY)). First, all known marmoset H-chain variable region (IGHV) and SfilκL -chain variable region (IGKV) genes, as well as H-chain IgG CH1-domain and SfilκL chain C-region (IGKC) genes, were amplified in separate reactions (for primer sequences see itsa.ucsf.edu/claudeg/primers.htm website and supporting information published on the PNAS web site). The template for IGHC and IGKC was a marmoset Fab library previously constructed in pCOMB3H, to include an SfiI restriction site on the 3' end of IGHC and the pelB leader sequence with IGKC. In the second step, IGHV was joined with IGHC (H-chain assembly), and IGKV with IGKC (SfilκL-chain assembly). Third, the SfilκL chain (IGKV-IGKC-pelB) was joined with the H chain (IGHV-IGHC-SfiI) to yield an ~1,460-bp cloning product containing SfiI-κL chain-pelB-H chain-SfiI. Finally, the cloning product and pCOMB3H were digested with SfiI (Roche Molecular Biochemicals) and purified. Equal amounts of pCOMB3H and *C. jacchus* VL/VH DNA were ligated with T4 ligase (Roche Molecular Biochemicals) and electroporated into electrocompetent XL1-Blue cells (Stratagene) with a Bio-Rad GenepulserII (2.5 kV, 200 ohms, 25 μF). The complexity of the obtained *C. jacchus* IgG-pCOMB3H library was ~1x10⁷ recombinants. Infective phagemid particles were generated by rescue with the helper phage VCSM13 (Stratagene).

Screening of the *C. jacchus* IgG Fab Library with rMOG.

Approximately 1012 Fab-expressing phagemids were incubated (37°C, 1 h) in ELISA wells coated with rMOG (1 μg per well). In the first round of the selection process (panning), wells were washed 5 times with PBS containing 0.05% Tween20 (PBS-T), bound phagemid eluted with trypsin (500 μg per well), and eluted phagemid used to infect XL1-Blue cells. After incubation at 37°C overnight, phagemids were precipitated and

resuspended in PBS containing 1% BSA and submitted to the panning process 3 more times with increasing washing stringency (second round, 10 times; third round, 15 times; fourth round, 15 times). Enrichment of rMOG-specific Fab fragments was confirmed by measuring bound phagemid from each panning round in rMOG-coated ELISA wells with an anti-M13, horseradish peroxidase-conjugated Ab (Amersham Pharmacia Biotech).

DNA Sequence Analysis.

Phagemid DNA was extracted with the Qiagen (Valencia, CA) MaxiFilter kit and digested with SpeI and NheI for removal of the gIII protein gene, which permitted expression of soluble Fab fragments. SpeI_NheI-digested DNA was gel-purified, religated with T4 ligase, and transformed into XL1-Blue cells. Sixty randomly picked, Fab-expressing clones from the last panning round were grown in Superbroth containing 100 µg/ml of carbenicillin for minipreps, plasmid DNA was extracted with the Qiagen MiniPrep kit, and DNA was sequenced at the University of California, San Francisco, Genomics Core Facility by automated fluorescent chain termination sequencing. Sequences of both H- and L chains were aligned with MEGALIGN (DNASTar, Madison, WI).

Expression of Soluble Fab Fragments.

Fab-expressing clones representing all IGHV-IGKV combinations and H chain complementarity determining region (CDR) 3 motifs (Table 2) were grown in 3 liters of Superbroth until OD₆₀₀ > 1.2, and expression was induced with 1 mM IPTG. After overnight incubation at 30°C, bacteria were lysed by sonication in 30 ml of PBS and Fabs were purified from the soluble fraction over a protein L column (Pierce) following the manufacturer's protocol. Where desired, purified Fab fragments were biotinylated with a sulfo-Nhydroxysuccinimide (NHS) biotinylation reagent (Pierce) following the manufacturer's instruction. Unreacted sulfo-NHS biotin was removed by extensive dialysis against PBS.

Purification of Serum Anti-MOG Abs and Fractionation of Ab Specificities.

rMOG-reactive fractions of serum Abs were purified on 1-ml prepacked N-hydroxysuccinimide (NHS)-Sephacrose columns reacted with 200 µg of rMOG, following the manufacturer's instructions (Amersham Pharmacia Biotech). rMOG Sepharose columns were loaded with *C. jacchus* immune sera, diluted 1:5 in PBS, extensively washed with

PBS, and bound Abs were eluted in 0.1 M glycine buffer, pH 2.2. For human sera, the protein G-reactive fraction (IgG) was extracted before purification by rMOG-affinity chromatography. To separate the Ab fractions binding to linear peptides from those binding to structural determinants of rMOG, MOG-peptide-reactive Ab fractions were removed
5 from serum by repeated passes ($n = 5$) on 1-ml PepMOG (mixture of overlapping 20mer peptides spanning the entire sequence of rMOG) columns, which were synthesized by reacting 5.5 mg of PepMOG (500 μ g per peptide) with NHS-Sepharose. Where desired, purified Abs were biotinylated as described previously.

Epitope Specificities of MOG-Reactive Ab and Recombinant Fab Fragments.

10 Maleic anhydride-activated ELISA plates (Pierce) were coated with 1 μ g per well of rMOG or PepMOG, blocked with PBS-T/3% BSA, and washed with PBS-T. Samples were added in PBS-T/3% BSA as follows: rMOG- or PepMOG-immune *C. jacchus* serum, or fractions thereof (after removal of peptide-specific Abs), 1:200; monoclonal Fab fragments, 1 μ g per well. After incubation at 37°C for 1 h, wells were washed with PBS-T, and
15 appropriate secondary Ab added in PBS-T/3% BSA [serum and serum fractions: anti-monkey IgG horseradish peroxidase (HRP) 1:6,000, Sigma; Fab fragments: protein L-HRP 1:5,000, Pierce] for 1 h at 37°C. After a final wash with PBS-T, plates were developed with tetramethylbenzidine (Pierce) and read at 450 nm.

Competition Assays.

20 Competition experiments were designed to examine the ability of Fab fragments to compete against each other and against native *C. jacchus* anti-MOG Abs for binding to rMOG. First, the amount of biotinylated Ab or Fab necessary to achieve 50% saturation of rMOG (50–100 ng per well) adsorbed on Ni-coated ELISA plates (Pierce) with biotinylated anti-MOG Abs or MOG-specific Fab was determined. To study competitive displacement,
25 nonbiotinylated Fabs or native Abs were added to MOG-coated wells at increasing concentrations (10^{-12} to 10^{-5} M) in the presence of the 50% saturation concentrations of the biotinylated reagent. After overnight incubation at 4°C, wells were washed and incubated with a streptavidin-peroxidase conjugate (Invitrogen; 1:1,000 in PBS-T/3% BSA, 20 min at room temperature). Bound Abs were detected with tetramethylbenzidine. Competition
30 experiments with human anti-MOG Abs were performed with a similar protocol, in Immunosorp ELISA wells (Nunc) coated with rMOG (500 ng per well) in PBS. A constant

concentration of unlabeled, MOG-affinity-purified Abs was incubated in the presence of increasing concentrations of the M26 and M3-8 Fab fragments overnight at 4°C. Bound human anti-MOG Abs were detected with an Fc-specific, alkaline phosphatase-conjugated anti-human IgG (Sigma, 1:5,000; this Ab was not cross-reactive with Fab fragments), and
5 plates were developed with para-nitrophenol phosphate and read at 405 nm. Displacement was quantitated as the ratio of OD in the presence of competition over that in the absence of competition X 100 (%).

Immunohistochemistry.

Paraformaldehyde-fixed paraffin embedded sections of *C. jacchus* brain (7 µm)
10 were deparaffinized, hydrated, and treated with a citrate-based antigen unmasking solution (Vector Laboratories) at high temperature for 20 min. Sections were blocked with 3% normal goat serum (Sigma) in PBS for 1 h at 37°C, washed with PBS-T, and incubated with biotinylated MOG-specific Fab (2.8 µg/ml) for 2 h at 37°C. Additional experiments were performed with the same dilutions of Fab fragments in the presence of rMOG to
15 demonstrate specificity of binding. After incubation with the alkaline phosphatase (AP)-conjugated avidin complex [Vectastain ABC-AP (Vector Laboratories), 30 min, room temperature], fluorescence was revealed by the VectorRed AP substrate (Vector Laboratories), and slides were counterstained with hematoxylin.

RESULTS

20 Ig Gene Usage of Recombinant MOG-Specific Fab Fragments.

Sixty randomly chosen, MOG-specific Fab-encoding clones were sequenced. The IGHV subgroup usage in this library was limited to IGHV1 and IGHV3, and IGKV usage to IGKV1 and IGKV3. Ninety-four percent (57 clones) of all clones were composed of IGHV1-IGKV3 (representative clones are designated M26, M38, and M45), and 6% were
25 IGHV3-IGKV1 (M3-8, M3-31; 2 clones) or IGHV3-IGKV3 (M3-24; 1 clone). Sequences corresponding to contact residues (CDRs) showed considerable diversity, with variability in the H-CDR3 motifs (Table 2).

Recombinant Fab Fragments Exclusively Recognize Structural Epitopes of MOG.

Polyclonal Ab populations present in serum of rMOG-immunized marmosets have been shown to recognize a broad repertoire of specificities, including linear epitopes
5 corresponding to short peptide sequences contained within MOGaa1–125 (12, 22). Surprisingly, however, none of the recombinant Fab fragments studied showed binding to any of these linear, extended epitopes or to PepMOG (Figure 1). Additional testing with an array of 60 overlapping 12-mer peptides confirmed these results. Thus, the MOG-specific Fab fragments selected from the combinatorial library exclusively recognized conformation-
10 dependent epitopes.

Diversity of Structural Ab Epitopes of MOG.

We performed competition experiments between Fab fragments representing all H-L chain combinations to understand the diversity of structural epitopes of rMOG targeted by the recombinant Fab fragments. Increasing concentrations of nonbiotinylated Fab fragments
15 were allowed to compete in rMOG-coated ELISA wells with individual Fab fragments labeled with biotin. Figure 2A illustrates the binding of a fixed amount of biotinylated M26 Fab (M26Biotin, IGHV1-IGKV3) in the presence of increasing concentrations of all other representative Fab fragments. Despite the variability in the CDR motifs (Table 2), all Fabs encoded by IGHV1-IGKV3 (M26, M38, and M45) recognize a similar epitope of rMOG. In
20 contrast, no competition was observed between M26Biotin and M3-8, M3-31(IGHV3-IGKV1), or M3-24 (IGHV3-IGKV3). Figure2B illustrates a similar experiment with M3-24Biotin as the displaced Fab, which shows no competition with any of the other Fab fragments. These results indicate that the M3-24 Fab defines an epitope of rMOG that is distinct from that recognized by M26, M38, and M45. A similar lack of competition was
25 observed for the M3-8Biotin Fab, suggesting that this IGHV3-IGKV1 combination defines another, unique conformational epitope. Subtle conformational features on exposed surfaces of MOG may be responsible for a microheterogeneity within the Ab binding sites. We found that M38 and M45 could displace the Fab M3-31Biotin, whereas only weak displacement by M26 occurred at significantly higher concentrations (Figure 6C).
30 Noncompetitive inhibition (e.g., steric interference) may play a role in this case and may explain the lack of displacement observed for the reverse experiment (e.g., M26Biotin vs.

unlabeled M3-31, shown in Figure 6A). Whether the M26 and M3-31 Fab fragments define similar or separate epitopes cannot be currently resolved. Nonetheless, these experiments identify at least three distinct conformational epitopes accessible on rMOG. All Fab fragments encoded by IGHV1- κ IGKV3 seem to recognize similar or closely associated epitopes on a single, major immunogenic region of MOG, which may be partially overlapped by M3-31.

Relevance of Antigenic Specificities Defined by Combinatorial Fab Fragments.

We next examined the ability of the recombinant Fab fragments to displace native anti-MOG Abs from *C. jacchus* serum, which represent a polyclonal mixture of Ab-specificities against linear determinants, structural determinants, or both. Biotinylated, affinity-purified anti-MOG Abs were incubated in the presence of increasing concentrations of Fabs. Figure 2 shows representative experiments in which the M26 and M3-8 Fabs were allowed to compete against native, polyclonal auto-Abs from rMOG-immunized marmosets. Combinations of both M26 and M3-8 Fab fragments showed an additive effect for displacement, a finding that supports our hypothesis that the epitopes recognized by the M26 and M3-8 Fab fragments are topographically distinct (Figure 3). Importantly, the representative Fabs derived from a single animal in this study efficiently displaced serum Abs from four genetically distinct marmosets. To verify that the Fab fragments were capable of binding to exposed epitopes of MOG on myelin sheaths, we confirmed by immunofluorescence that the recombinant Fab fragments were capable of binding to the MOG protein in situ in CNS white matter. Figure 3 Left shows strong staining of oligodendrocytes and staining of myelinated fibers in *C. jacchus* corpus callosum with the biotinylated M26 Fab fragment. Specificity was confirmed by the ability to completely quench the fluorescent signal by addition of rMOG (Figure 3 Right). Identical results were obtained with the M3-8 Fab fragment.

In Vivo Pathogenicity of Conformational Versus Linear Epitope- Specific Anti-MOG Abs.

To understand further how epitope recognition influences Ab pathogenicity, we examined the binding characteristics of serum anti-MOG Abs in marmosets immunized with either rMOG (n = 4) or PepMOG (n = 2) before and after removal of the PepMOG-reactive fractions. Consistent with previous experience (Genain and Hauser (1996) Methods

10: 420–434), rMOG-immunized animals developed severe neurological signs corresponding to multifocal, widespread inflammatory infiltrates accompanied by prominent demyelination (Figure 4). In contrast, animals immunized with PepMOG exhibited reduced disease burden with little or no demyelination. Importantly, we found that the repertoire of MOG-reactive Abs in this group was strictly restricted to linear epitopes, as removal of PepMOG-reactive Abs completely abolished reactivity to MOG. However, the sera from each of the rMOG-immune animals contained residual reactivity against whole rMOG after the complete removal of peptide-specific Abs (Figure 4), indicative of the presence of immunogenic structural epitopes. Thus, the conformation-dependent Abs are only present in rMOG-immunized animals and seem to be responsible for the extensive demyelination observed in lesions of rMOG-induced EAE.

Marmoset Fab Fragments Delineate Structural Determinants of the MOG Ab Response in Humans.

We examined the ability of recombinant marmoset Fab fragments to displace affinity-purified anti-MOG Abs from the sera of three patients with MS (AA, DM, and WS), who were previously shown to be MOG-reactive by ELISA. We found that the M3-8 fragment was able to compete with anti-MOG Abs from all three patients and also found competition with M3-24 for patient AA. Furthermore, similar to marmosets, the combination of M3-8 and M3-24 showed an additive effect (Figure 5, representative experiment; patient AA). These results indicate that the targets for MOG-specific Abs in humans include conformation-dependent epitopes that are identical to those in marmosets.

DISCUSSION

In this report we provide information regarding the molecular complexity of pathogenic auto-Ab responses against exposed domains of MOG in an outbred species. Previous studies reporting the effects of passive transfer of certain Abs in rodent and marmoset systems (Genain et al. (1995) *J. Clin. Invest.* 96: 2966–2974; Brehm et al. (1999) *J. Neuroimmunol.* 97: 9–15), and of MOG-DNA vaccination in SJL mice (Bourquin et al. (2000) *Eur. J. Immunol.* 30: 3663–3671), have shown that conformation-dependent anti-MOG Abs are capable of inducing demyelination. In contrast, whether Abs directed at linear determinants of MOG have demyelinating properties has not been unequivocally demonstrated (Ichikawa et al. (1996) *Int. Immunol.* 8: 1667–1674; Adelman et al. (1995) *J.*

Neuroimmunol. 63: 17–27). Data obtained from two PepMOG-immunized animals in this study suggest that the presence of MOG-peptide-specific Abs is not associated with widespread demyelination. Indeed, EAE in these animals was reminiscent of the disease phenotype produced by adoptive transfer of MOG-reactive T cells (Villoslada et al. (2001) Eur. J. Immunol. 31: 2942–2950). Similar EAE phenotypes could also be reproduced in animals immunized with groups of individual peptides that contain the marmoset immunodominant MOG-T cell epitopes (n = 7) (von Budingen et al. (2001) J. Clin. Immunol. 21: 155–170). Taken together, these results suggest that Abs against linear peptides are not pathogenic in marmosets and that recognition of conformational features of MOG is a prerequisite for Ab pathogenicity.

Peptide-specific anti-MOG Abs are part of the MOG-immune repertoire in EAE and can be detected in the serum of healthy controls and patients with MS (. Karni et al. (1999) Arch. Neurol. 56: 311–315; Xiao et al. (1991) J. Neuroimmunol. 31: 91–96). However, because of the stringent conditions applied during the panning process, it is likely that the conformational epitopes of rMOG define binding sites for Abs of higher affinity than MOG-peptide Abs, which were not found in the Fab library. Similar differences in affinity have been described in the case of a different antigen (Sachs et al. (1972) Proc. Natl. Acad. Sci. USA 69: 3790–3794; Jemmerson and Blankenfeld (1989) Mol. Immunol. 26: 301–307). Nonetheless, we show that the Fab fragments specifically bound to native MOG in situ in brain tissue, indicating that our combinatorial approach had yielded Ab fragments that correctly define structural features of MOG that are exposed in vivo. The relevance of these Ab fragments is underlined further by our finding that the monospecific Fab fragments are capable of displacing a significant portion of the polyclonal, native anti- MOG Abs in several marmosets, despite the genetic heterogeneity present between outbred individuals. Thus, despite the fact that our library may not exhaustively include all Ab specificities present in the polyclonal, MOG-specific humoral repertoire, we propose that the MOG-specific Fab fragments represent epitope specificities with demyelinating potential.

Accurate definition of the determinants of MOG that are targets of demyelinating Abs in humans will be of critical importance. Qualitative differences in epitope recognition may be present among anti-MOG Ab populations that are frequently detected in patients with MS and healthy controls (Haase et al. (2001) J. Neuroimmunol. 114: 220–225; Reindl et al. (1999) Brain 122: 2047–2056; Sun et al. (1991) J. Immunol. 146: 1490–1495). For

example, T cell mimicry between viral antigens and MOG peptides has been reported (Mokhtarian et al. (1999) J. Neuroimmunol. 95: 43–54), but in the absence of exposure of B cells to the whole MOG polypeptide, may only induce production of MOG peptide-specific Abs. These auto-Abs would be detected in standard Ab assays, although they may not be pathogenic. We show here that Ab fragments that define structural determinants of MOG in *C. jacchus* can be used to specifically detect the presence of MOG-specific idiotypes directed against identical determinants in humans. Although in marmosets the M26 and M3-8 Fab fragments seem to represent important specificities, in serum from MS patients the M3-8 and M3-24 Fabs have thus far been shown to compete against native anti-MOG Abs. Additional experiments are proceeding to identify human Abs capable of competing with the remaining marmoset Fabs.

Clonal B cell expansion with restricted usage of IGHV germline genes in CNS lesions and cerebrospinal fluid of patients with MS has been reported (Qin et al. (1998) J. Clin. Invest. 102: 1045–1050; Owens et al. (1998) Ann. Neurol. 43: 236–243; Colombo et al. (2000) J. Immunol. 164: 2782–2789; Baranzini et al. (1999) J. Immunol. 163, 5133–5144). In this context, it was of interest to find that a limited number of H- (IGHV1 and IGHV3) and kappa L-chain (IGKV1 and IGKV3) subgroup genes was used in the marmoset MOG-specific Ab repertoire. However, we also found that diverse CDR-encoding gene rearrangements were used to target only three epitopes of MOG. Therefore, the current study extends beyond prior molecular analyses of Ig gene usage, which have not identified target antigens for the clonally expanded immune responses. Competition experiments also demonstrated that the *C. jacchus* Fab fragments define antigenic determinants of MOG that are commonly targeted in all marmosets, regardless of H- and L-chain usage.

The critical importance of MOG to autoimmune demyelination is a consequence of its restricted expression in the CNS (Gardinier et al. (1992) J. Neurosci. Res. 33: 177–187), its exposed extracellular domain at the outermost lamellae (Brunner et al. (1989) J. Neurochem. 52: 296–304), its high level of encephalitogenicity in multiple species, and tendency to induce pathogenic auto-Ab responses directed against the myelin sheath (reviewed by von Bu et al. (2001) J. Clin. Immunol. 21: 155–170). The finding reported here that related conformational features of MOG are targets of auto-Abs in marmosets and humans highlights the value of nonhuman primate models for dissection of auto-Ab responses relevant to the pathophysiology of CNS tissue damage in MS.

EXAMPLE 2

The Use of Epitope Specific Antibodies For Diagnosis and Prognosis of Multiple Sclerosis

5

RESULTS

A. Complexity of Autoantibody Responses Against MOG in Outbred Species

1. Repertoire heterogeneity.

In marmosets immunized with the extracellular domain of MOG (aa1-125, rMOG),
10 mapping of rMOG-specific antibody specificity in sera and CSF using short peptides
revealed 2 immunodominant regions, MOG aa13-21 (100% of animals) and MOGaa63-75
(85%). Additional reactive peptides were identified at residues aa28-35 and aa40-45. Some
of the B cell epitopes in marmosets match the location of T cell epitopes (Brok et al. (2000)
J. Immunology, 165(2):1093-1101; von Büdingen et al. (2001) J. Clin. Immunol.,
15 21(3):155-170; Mesleh et al. (2002) Neurobiol Dis., 9(2):160-172), as has been shown for
MOG in rodents (Ichikawa et al. (1996) J. Immunol., 157:919-926), and for an
immunodominant epitope of MBP in humans (Wucherpfennig et al. (1997) J. Clin. Inves.,
997: 100(5):1114-1122). These linearly defined epitopes include aa residues that are located
on accessible regions of the molecule, according to predictive models for the structure of
20 MOG (Mesleh et al. (2002) Neurobiol Dis., 9(2):160-172). Epitope diversity in the antibody
repertoires against MOG in outbred species is confirmed by studies of MOG reactivity in
macaque monkeys (de Rosbo et al. (2000) J. Neuroimmunol. 110:83-96), and humans (de
Rosbo et al. (1997) Eur. J. Immunol., 27(11):3059-69; Lindert et al. (1999) Brain
122(Pt11):2089-2100).

25

2. Functional Heterogeneity Within Anti-MOG Antibodies.

Pathogenic properties of autoantibodies can be directly demonstrated in
experimental systems that use combinations of adoptive transfer of T cells, and passive
transfer of autoantibodies with demyelinating properties (Genain et al. (1995) J. Clin.
Invest. 96: 2966-2974; Lassmann et al. (1988) Acta Neuropathol. (Berl) 75: 566-576; ;

Schluesener et al. (1987) J Immunol 139(12):4016-4021). *C. jacchus* marmosets do not develop severe EAE associated with prominent demyelination after immunization with MBP, or adoptive transfer of MBP- or MOG-specific T cell clones (Genain et al. (1994) J. Clin. Invest. 94: 1339-1345; Villoslada et al. (2001) Eur. J. Immunol. 31: 2942-2950), in contrast to immunization with whole white matter, or with rMOGaa 1-125. Non-demyelinating EAE can be converted to fully demyelinating disease by passive transfer of rMOG-, or whole white matter-reactive IgG, indicating that these preparations contain pathogenic autoantibodies.

To investigate whether pathogenicity is dependent on epitope recognition, we have studied the clinical and pathological phenotypes of EAE induced in *C. jacchus* with 20 mer linear peptides encompassing the most frequently recognized epitopes within human rMOG. Active immunizations with 100 µg of MOG aa21-40, combinations of equal amounts of peptides spanning the reactive T cell epitopes (aa20-40, aa63-72, aa91-110), or combination of each of 11 overlapping peptides corresponding to the entire sequence of human rMOG, reproducibly induced mild, chronic EAE (n=2 per group), although a severe clinical course associated with a large solitary cervical cord lesion was observed in one animal immunized with a mixture of all the overlapping peptides. In all other animals, the mild clinical phenotype correlated with small inflammatory infiltrates, accompanied by sparse demyelination (Figure 6).

Reminiscent of adoptive transfer EAE in this species (Villoslada et al. (2001) Eur. J. Immunol. 31: 2942-2950), pathology remained scarce (<10 infiltrates within the entire neuraxis, Figures 6 and 7) and mostly confined to the cervical spinal cord. Despite the production of robust T cell responses against the immunizing peptides that paralleled those measured in rMOG-immune animals, no combination of peptides was capable of reproducing the protracted, multifocal disease associated with prominent demyelination that typically results from immunization with whole rMOG in this species.

3. Fractionation of polyclonal MOG-reactive autoantibody populations.

MOG peptide- and rMOG-reactive antibodies were separated by affinity chromatography on Sepharose columns containing MOG peptides covalently bound to Sepharose. In rMOG-immune marmosets, serum antibodies appeared to contain one fraction that recognized both linear MOG peptides and the whole rMOG polypeptide, and a second

fraction that exclusively recognized conformational determinants (Figure 8A, red bars). ELISA of the bound material after elution demonstrated that this second fraction contained antibodies that are capable of binding to rMOG, in addition to MOG peptides (Figure 8-A, blue bars). By contrast, all sera from animals immunized with 20 mer overlapping peptides of rMOG (individually or in combination, n=9), only contained antibodies binding to the immunizing peptides. The MOG peptide-specific antibodies present in these animals did recognize rMOG (Figure 8B, green bars), but not its conformational determinants, as shown by removal of all reactivity after depletion on the MOG peptide columns. (Figure 8B, red bars).

These data demonstrate that MOG-reactive autoantibodies in marmosets are heterogeneous in terms of epitope recognition, and may be directed against 3 different classes according to their binding characteristics to conformational rMOG, linear rMOG-derived peptides, or both. As illustrated in Figure 8, it is not possible to distinguish these different antibody fractions by ELISA or other standard antibody detection methods using whole serum. The difference in epitope recognition appears to translate into functional heterogeneity (e.g., pathogenic potential). Immunohistochemical analysis in marmosets immunized with the linear peptides showed lesion patterns that were strikingly different from those in rMOG-immune animals. Whereas macrophage infiltration was equally present in both forms of EAE, Ig and complement deposition were uniformly absent in the peptide-immune animals (Figure 9). We have also observed that disease severity in rMOG-induced marmoset EAE is inversely proportional to the ratio of serum concentrations ($\mu\text{g/ml}$) of MOG peptide/rMOG-reactive IgG (Tanuma et al. (2001) *J. Neuroimmunol.*, 118:60, and Figure 18).

These findings are in agreement with studies of the properties of murine monoclonal antibodies (Brehm et al. (1999) *J. Neuroimmunol.*, 97:9-15), and studies of EAE in C57/Bl6 wild type and B cell KO mice immunized with either whole rMOG or MOG peptide aa35-55 which is immunodominant in rodents (Lyons et al. (1999) *Eur. J. Immunol.*, 29(11):3432-3439; Svensson et al. (2002) *Eur J Immunol.*, 32(7):1939-46; Lyonset al. (2002) *Eur J Immunol.*, 32(7):1905-1913; Albouze-Abo et al. (1997) *Eur J Biochem.*, 246(1):59-70). It is noteworthy however, that severe demyelinating EAE can clearly be induced by active immunization with linear peptides of MOG in several rodents strains (Bernard et al. (1997) *J. Mol. Med.*, 75(2):77-88; Slavin et al. (1998) *Autoimmunity*,

28(2):109-120; Amor et al. (1994) J. Immunol., 153:4349-4356; Tsunoda et al. (2000) Brain Pathology, 10(3):402-418; Ichikawa et al. (1996) International Immunology, 8(11):1167-1674). The apparent discrepancy may reflect differences in strain/species susceptibility, and supports an hypothesis that genetic background may selectively determine factors of disease pathogenesis that translate into variation in the clinical and pathological phenotype of CNS demyelinating disease. Our combined observations that MOG peptide-specific antibodies do not appear to be demyelinating in marmosets, but can be detected in situ in active lesions of EAE and MS (Genain et al. (1999) Nature Medicine, 5:170-175), also suggest that certain autoantibodies are cross-reactive to both linear and conformational determinants. As will be discussed below, similar heterogeneity exists within anti-MOG humoral responses in humans. Pathogenicity of the different classes of antibodies to MOG has not been clearly defined.

Without being bound by a particular theory, we believe that autoantibodies restricted to selective determinants of MOG cause demyelination in human MS, and influence clinical course. The complex patterns of serum antibody responses are correlated with disease phenotype and their pathogenic potential.

B) Molecular and Structural Complexity of MOG-Specific Antibody Repertoires in Outbred Species.

The molecular diversity of MOG-specific *C. jacchus* antibody repertoires was analyzed using phage-displayed combinatorial libraries of Fab fragments from bone marrow and spleen obtained from animals immunized with rat rMOG (after they had developed antibody responses and clinical EAE), using sequence information previously obtained for the variable regions of H chains (VH) and the k L chains (Vk) (von Büdingen et al. (2001) Immunogenetics, 53:557-563; von Büdingen et al. (2002) Proc. Natl. Acad.Sci. USA, 99(12):). Sequence analysis of clones from the first library showed predominant usage of *C. jacchus* VH1 and VkIII rearrangements (94% of all combinations), while VH3/VkI and VH3/VkIII represented the remaining combinations. Six H-CDR3 motifs and 5 different kL-CDR3 were identified, with the greatest degree of variability in the H-CDR3 motifs. A second rMOG-immune library has been constructed from a genetically distinct marmoset, and is currently being analyzed. Data from 30 clones of this library appear to confirm the predominant usage of VH1, VH3 and VkIII. Soluble, recombinant Fab fragments were

expressed from selected Fab-producing clones, purified on protein L- affinity columns, and analyzed for their binding properties by ELISA. In contrast to whole serum or antibody fractions from immune *C. jacchus*, all Fab fragments representative for the VH/Vk rearrangements failed to show binding to any of 20 mer overlapping linear peptides spanning the sequence of rMOG (Figure 1), or to a panel of 96 overlapping peptides corresponding to the sequence of MOG aa1-120.

The availability of these monoclonal, recombinant Fab fragments from *C. jacchus* afforded to design competition experiments, where displacement of one biotinylated Fab fragment by other Fabs is measured. This technique affords to study the diversity of epitopes available for antibody binding on rMOG at the structural level. All Fab fragments encoded by VH1/VkIII rearrangements were shown to fully compete with each other, even though these clones utilized different H-CDR3 motifs. The M3-8 and M3-24 Fab fragments only competed against themselves, while the M3-31 was shown to compete against both M38 and M45. M26 only weakly displaced M3-31 at high concentrations, suggesting partial overlap between the epitopes defined by those 2 Fab fragments. These data suggest that the diversity of humoral responses to MOG observed at the nucleic acid sequence level does not translate into the same degree of diversity for structural antigenic specificities. Three different conformational antibody epitopes have been identified so far, suggesting the existence of a limited number of accessible binding sites on the intact rMOG polypeptide. Work is in progress to similarly characterize additional MOG-immune Fab libraries and will provide a complete definition of anti-MOG antibody repertoire in *C. jacchus*.

C) Relevance of Combinatorial Studies to Native Autoantibody Repertoires

The biological relevance of the randomly rearranged recombinant Fab fragments was tested by measuring their ability to compete with biotinylated rMOG-specific IgG fractions purified by affinity chromatography from serum of marmosets with rMOG EAE. Fab fragments M26 and M3-8 efficiently displaced these polyclonal antibodies, indicating that the combinatorial approach produced antibody specificities that reflected the mature, rMOG-driven IgG repertoire (Figure 10). Similar conclusions have been derived from other studies using combinatorial antibody technology (Barbas et al. (2001) Phage Display. A laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press). Importantly, displacement of native, rMOG-immune serum IgG could be demonstrated for 4

unrelated animals, indicating that the epitope specificities captured by the combinatorial approach correspond to specificities that are commonly targeted in marmosets, despite their outbred genetic characteristics.

In contrast to *C. jacchus* recombinant Fabs, the murine monoclonal antibody 8.18.C5 was not capable of displacing MOG-immune *C. jacchus* IgG. We also observed that none of the recombinant *C. jacchus* Fab fragments studied to date, were capable of competing for binding with the 8.18.C5 antibody. Although this antibody recognizes rat, mouse, human and marmoset MOG, and is capable of inducing demyelination in *C. jacchus* (Genain et al. (1995) J. Clin. Invest. 96: 2966-2974) and other species (Linnington et al. (1988) Am J Pathol., 130(3):443-454; Schluesener et al. (1987) J Immunol 139(12):4016-4021), our results indicate that the epitope defined by 8.18.C5 is not part of the natural *C. jacchus* repertoire against MOG.

D) Relevance of *C. jacchus* Studies to Pathogenic Antibody Responses in Humans

1. Competition between *C. jacchus* Fab fragments and MS serum antibodies.

Competition experiments were extended to Ig (IgG and IgM) present in the serum of patients with MS. rMOG-specific Igs were affinity purified on MOG-Sepharose columns, and tested for their ability to displace recombinant *C. jacchus* Fab fragments from rMOG in ELISA wells. To date, we have identified 3 patients in a group of 6 who displayed Ig reactivity to rMOGaa1-125 containing conformation-dependent antibodies that can be displaced by the *C. jacchus* Fab M3-8, and/or the M3-24 Fab (Figure 5). A fourth patient exhibited antibodies against linear peptides, but not conformational determinants, of MOG (please also refer to Table 3, below).

These experiments underscore the value of studies of *C. jacchus* antibody repertoires as the first practical tools to define the target epitopes of MOG for pathogenic antibody responses in MS, and demonstrate that certain conformation-dependent epitopes of MOG are unique to primate species. The structural diversity of human anti-MOG antibodies readily be further assessed by approaches similar to those described for *C. jacchus*.

2. *C. jacchus* Fab fragments recognize human MOG in situ.

C. jacchus Fab fragments were tested for their ability to bind to MOG under conditions that mimic exposed epitopes of the MOG molecule in vivo. First, we confirmed that these fragments could bind to CNS myelin sheaths and oligodendrocytes by

5 Immunohistochemistry in sections of *C. jacchus* and CNS brain (von Büdingen et al. (2002) Proc. Natl. Acad.Sci. USA, 99(12)). Second, we generated several cell lines transfected with a plasmid encoding for the full-length sequence of human MOG (aa1-218), including COS cells, a human fibroblast cell line, and the human oligodendroglioma cell line TC 620. All *C. jacchus* Fabs tested to date are capable of binding to transfected, but not to untransfected

10 cells.

3. Demyelinating potential of MOG-specific autoantibodies present in humans.

In preliminary experiments, we have performed passive antibody transfers in two MBP-immunized marmosets with IgG purified from patients with MS that tested positive

15 for anti-MOG antibodies by ELISA. Similar to homologous IgG transfers, administration of these human antibody fractions in *C. jacchus* readily induced large EAE lesions with prominent demyelination (Figure 12). These experiments indicate that the *C. jacchus* passive antibody transfer system is suitable to assess pathogenicity of human anti-MOG antibodies.

20 E) Reactivity to Myelin Antigens in MS

Specific ELISA systems have been developed in the laboratory to assess reactivity of human sera to MBP, rMOG, MOG-derived peptides and GalC (IgG and IgM).

1. rMOG-, MOG peptide- and MBP-reactive autoantibodies.

We have examined a series of 33 age-matched controls (including older subjects to

25 better match the age distribution of progressive MS), 27 patients with relapsing remitting (RR) MS, 26 with secondary progressive (SP) MS, and 41 with primary progressive (PP) MS, for serum reactivity to rMOG and MOG-derived peptides. While the frequency of anti-MOG antibodies is sensitively higher in the controls (54%) but still consistent with previous work by others (Reindl et al. (1999) Brain, 122:2047-2056; Lindert et al. (1999) Brain

30 122(Pt11):2089-2100; Egg et al. (2001) Mult Scler., 7(5):285-289), the data confirm the

high prevalence of anti-MOG antibodies in the SPMS and PPMS (85% and 93% respectively) and the high prevalence of anti-MBP antibodies in SPMS (Figure 13).

Similar to studies of *C. jacchus* and macaque monkeys (Mesleh et al. (2002) Neurobiol Dis., 9(2):160-172; de Rosbo et al. (2000) J Neuroimmunol., 110(1-2):83-96),

5 linear epitopes recognized by human anti-MOG antibodies showed great diversity and variability between individual patients. In a total of 16 rMOG-reactive patients studied for fine mapping with overlapping 20mer peptides (10 relapsing remitting and 6 secondary progressive), the most frequently recognized motifs included aa21-40, aa31-50, aa51-70, aa71-90, and aa101-120. We also observed a significant proportion of patients whose serum

10 did not react with whole rMOG, but reacted to linear peptides. Analysis of serum reactivity using an extensive panel of 96 overlapping peptides corresponding to MOGaa1-120, and portions of the C-terminus of human MOG, confirmed this diversity of linear epitopes. No distinctive pattern of epitope reactivity in relation to clinical phenotype was apparent in this small sample. These results are in agreement with previous studies of antibody reactivity to

15 MOG in MS (de Rosbo et al. (1997) Eur. J. Immunol., 27(11):3059-3069; Haase et al. (2001) J Neuroimmunol., 114(1-2):220-225), and confirm diversity in human anti-MOG antibody repertoires.

2. Detection of conformation-dependent MOG autoantibodies in human MS.

20 The presence of antibodies that exclusively recognize conformational determinants of MOG within complex polyclonal antibody responses is difficult to assess using standard detection techniques. These antibodies can be physically separated from those recognizing linear determinants. However, the technique is not suitable to distinguish between antibodies that are strictly conformational, and those that may recognize both

25 conformational determinants and linear peptides, because there likely are antibodies that bind to both linear peptides and rMOG (this Section, Figure 8). We have taken advantage of specific competition assays using biotinylated recombinant, monoclonal *C. jacchus* Fab fragments that exclusively define conformational epitopes to investigate the presence of conformation-dependent antibodies within the repertoire of patients that tested positive for

30 anti-rMOG antibodies. To date, we have examined in detail the fine specificities of anti-MOG antibodies (IgG and IgM) in six individuals with RRMS or SPMS. Data presented in

Table 3 summarize these findings, and also show that IgG and IgM antibodies can be independently involved in the MOG-specific human humoral response.

Table 3. Reactivity of MS sera to rMOG, MOG derived peptides, and competition experiments with the conformation-dependent *C. jacchus* Fabs

Subject	IgG (ELISA)		IgM (ELISA)		Competition with <i>C. jacchus</i> Fab		
	rMOG	PepMOG	rMOG	PepMOG	M3-8	M26	M3-24
1	+	+	+	+	Yes	No	ND
2	-	+	ND	ND	No	No	No
3	+	+	+	+	Yes	No	Yes
4	+	+	+	+	Yes	No	ND
5	-	-	-	-	No	No	No
6	+	+	-	-	No	No	ND

PepMOG designates one or more reactive peptides within MOGaa1-120. ND, not done.

Based on these findings, it is possible to envision that responses to MOG in humans segregate into subtypes depending on the pattern of epitope recognition. Of interest is the relative pathogenic potentials of these different human antibody populations. Heightened incidence of anti-MOG antibodies in our cohorts of SPMS and PPMS patients suggest that these antibodies are consistently associated with late forms, or progressive forms of disease typically characterized by severe disability and CNS atrophy.

3. Reactivity to MOG in patients with clinically isolated syndromes

We have studied the sera of 8 patients that presented with a clinically isolated syndrome (CIS) associated with MRI abnormalities (Table 4). Six of these subjects displayed strong serum reactivity to MOG and/or MOG peptides. These data indicate that anti-MOG antibodies are a prominent part of the immune response during the first detectable clinical event in MS, in agreement with previous studies of early relapsing remitting MS (Reindl et al. (1999) Brain, 122:2047-2056).

An ongoing collaboration between the neuroimmunology laboratory and the UCSF MS and MRS Centers has begun to analyze correlations between serologic measurements (anti-myelin antibodies), and clinical and MRI phenotypes. Results for the first 11 CIS patients studies are shown below (Table 4).

5

Table 4. Clinical, MRI, And Serologic Characteristics Of CIS Patients

ID	Clinical Presentation	Resolved at sampling	EDSS at sampling	T2 Lesion Load (cm3)	# of T2 Lesions	# of Gd+ Lesions	α-MBP	α-rMOG	α-MOG peptides
1	Myelitis	improved	2.0	2.077	16	0	-	+	-
2	Myelitis	improved	1.5	0.117	3	0	-	+	-
3	Brainstem	improved	1.5	0.035	2	0	-	+	+
4	Brain Stem	No	1	2.432	16	0	+	-	-
5	Myelitis	No	3	0.656	13	4	-	+	+
6	Optic neuritis	improved	1.5	0.622	9	0	-	-	+
7	Myelitis	Yes	0	0.387	5	1	+	+	ND
8	Myelitis	improved	1.5	0.252	5	1	-	-	ND
9	Myelitis		2.0	3.811	19	0	-	+	-
10	Myelitis		0	4.534	>40	0	-	-	-
11	Brain Stem		0	1.807	6	0	-	+	-

These data, although limited to a small number of patients that does not permit statistical analysis, very clearly indicate that anti-MOG antibodies are a prominent part of the immune response during the first detectable clinical event in MS. This finding is in agreement with previous studies of early relapsing remitting MS (Reindl et al. (1999) Brain, 122:2047-2056). A recently published study analyzed the prognostic significance of these antibodies in patients presenting with CIS, using a longitudinal design. This supports our assertion that antibody measurements can be used for prognosis in CIS patients, and in particular for predicting conversion to a diagnosis of clinical MS (Berger et al. (2003) N. Engl. J. Med, 349:139-135).

ELISA systems for specific detection of anti-GalC antibodies have been developed for marmosets and humans, and are routine in the laboratory. Extensive studies of reactivity to GalC have not yet been performed. Preliminary results indicate that anti-GalC antibodies are present in 67% of our SPMS cohort, and only 1 of the CIS patients studied, which
5 suggests that these antibodies are associated with late and severe forms of MS.

5. Time course of autoreactive immune responses in MS.

Previous work suggests that T cell autoreactivity to myelin antigens may vary in time, or may remain stable in given individuals. With the exception of some reports (Bielekova et al. (2000) *Nature Medicine*, 10:1167-1175), there is generally no obvious
10 correlation between reactivity and occurrence of MS relapses (Pender et al. (2000) *J Immunol.*, 165(9):5322-5331; Tuohy et al. (1998) *Immunol. Rev.*, 164:93-100; Goebels et al. (2000) *Brain* 123 Pt 3:508-518; Meinl et al. (1993) *J Clin Invest.*, 92(6):2633-43; Hellings et al. (2002) *J Neuroimmunol.*, 126(1-2):143-460; Lovett-Racke et al. (1997) *J Neuroimmunol.*, 78(1-2):162-171). The time-dependency of autoantibody responses has not
15 been systematically investigated in CNS demyelinating disorders, and available data are derived from cross-sectional studies (Reindl et al. (1999) *Brain*, 122:2047-2056). We had the opportunity to study serum samples from a few patients on repeated occasions for anti-myelin antibody reactivity. One patient (treated with interferon- β 1-a), showed a pronounced decrease in serum reactivity to MOG, and GalC over the course of 5 years (both IgG and
20 IgM), and had no attack during this period. No change in anti-MBP antibody titers was observed, indicative that the change in MOG reactivity was not related to overall immune down-regulation. A second patient sampled on 3 occasions within a period of one year, similarly displayed time-dependent variability in MOG-specific IgG titers and peptide reactivity (Figure 16). These preliminary results are consistent with studies of antibody
25 reactivity in other autoimmune disorders showing that antibody reactivity may fluctuate in time. According to a recent review of diagnostic criteria, detection of antibodies upon two phlebotomies at 6 weeks interval is required for diagnosis of anti-phospholipid syndrome (Wilson et al. (1999) *Arthritis Rheum.*, 42(7):1309-1311).

METHODS

1. Analysis of Repertoire of Antibodies Against MOG

Sera from the CIS and MS patients tested for reactivity to MOGaa1-125 (rMOG), overlapping MOG peptides, MBP, and control antigens are studied using ELISA systems already developed in the laboratory. An aliquot of CSF is also included in these analyses, where a lumbar is required for clinical care of the patients. For individuals who display reactivity to MOG or MOG peptides (IgG and/or IgM) by ELISA, autoantibodies will be further characterized as follows:

Separation of conformation dependent and non-conformation dependent Ig fractions.

The MOG-reactive fraction in sera from MOG-seropositive patients (all Igs) are depleted from the peptide-reactive fractions by a pass on Sepharose columns coupled with MOG-peptides, and further purified on human rMOG-Sepharose affinity columns (Figure 17). For the preparation of MOG-Sepharose affinity columns with the desired specificity, 200 µg of human rMOG, or MOG-derived 20mer peptides (200 µg each) is reacted with NHS-Sepharose pre-packed in 1ml columns, following the manufacturer's instructions (Amersham Pharmacia). The column is ready for use after inactivation of unreacted NHS groups and washing. Serum is slowly loaded and, after extensive washing (PBS), bound antibody is eluted in buffer at pH 2.2 and immediately neutralized by addition of Tris buffer.

This protocol permits the isolation of conformation-binding (designated "C") and linear peptide-binding antibody ("L") fractions. Fractions are analyzed by SDS-PAGE/Western blotting and ELISA to confirm purity and antigenic specificity and Ig class. A second pass on the columns may be necessary to achieve >95% purity

In vitro binding studies.

A pre-requisite for pathogenicity is that antibodies be capable of binding to exposed epitopes of MOG in situ on CNS myelin. To test this property of antibody fractions, we use flow cytometry. A human fibroblast cell line (CCL-153), COS cells, and a human oligodendroglioma cell line have been stably transfected with the human MOG gene cloned in a tetracycline-regulated expression vector (see, e.g., Figure 11). A similar method with a

mouse fibroblast transfected cell line has been successfully employed to characterize the conformational binding specificities of murine monoclonal anti-MOG antibodies (Brehm et al. (1999) J. Neuroimmunol., 97:9-15). Surface binding of Igs was measured by flow cytometry on MOG-expressing cells and control, untransfected cells. Cells are washed and
5 blocked with 3% normal goat serum, then incubated with the purified, biotinylated human Igs (using a commercial biotinylation kit), or unlabeled Igs and protein A/G-biotin. Fluorescence is detected using fluorescent-labeled streptavidin. Additional controls are performed in each experiment using an irrelevant Ig, or protein A/G-biotin, in the absence of Ig.

10 **Competition between human antibodies and recombinant *C. jacchus* Fab.**

The ability of affinity purified, MOG-specific human antibodies to compete with the monoclonal Fab fragments that define the epitope specificities of demyelinating *C. jacchus* antibody responses are assessed in competition experiments using ELISA plates coated with
15 rMOG. Fab fragments that represent structural epitope specificities isolated from *C. jacchus*, are expressed in soluble form and purified over protein L-affinity columns. The purity of these preparations was confirmed by PAGE (single band at ~55 kDa depending of the Fab, and reducing to L and truncated H chain under reducing conditions). Increasing concentrations of unlabeled, MOG-specific Fabs are incubated in the presence of affinity-
20 purified Ig fractions. Bound Ig is detected using an Fc-specific, conjugated anti-human IgG antibody (see, e.g., Section C, Figure 5).

2. General Material and Methods.

Human MOGaa1-118, ratMOGaa1-125, rat MOGaa1-117 were produced in a transformed *E. coli* strain available in the laboratory. The plasmid encodes for residues aa1-
25 125, with additional residues derived from the vector (MRGS at the N-terminus; SEQ ID NO:45) and a polyhistidine (RSQSHHHHHH; SEQ ID NO:13) tag for affinity purification at the C-terminus. rMOG is purified Ni-NTA-agarose columns using a standard protocol, yielding highly pure MOG as ascertained by SDS PAGE (major band at 15.9 kDa and a very minor band at ~32 kDa corresponding to a dimer).

Native human MBP purified by the method of Deibler (Deibler et al. (1972) Prep. Biochem., 2:139-165) is available for these studies. Galactocerebroside (1-0-Galactosyl-N-Acetyl-Sphingosine, C₄₈H₉₃NO₈) from bovine spinal cord purified (>98%) by thin layer chromatography is purchased from commercial sources. Panels of synthetic peptides are
5 available as follows: MOG peptides, 20mer overlapping peptides overlapping the sequence of the extracellular, Ig-like domain of MOG (aa1-120). A panel of 96 overlapping peptides (15mers offset 3 and 12mers offset 1 for immunodominant epitopes in marmosets and humans) encompassing the same domain of MOG, and several peptides located in the transmembrane regions of the protein that have recently been shown to be potential targets
10 for MOG-directed T cell responses (Weissert et al. (2002) J Immunol., 169(1):548-556). Synthetic MBP peptides are also available to extend these if needed.

ELISA systems.

Sera are separated from blood, properly aliquoted for analysis and antibody fractionation, and stored at -80°C until use.

15 Human ELISA for MOG, MOG-peptides, and MBP:

These ELISAs are routine in the laboratory. Maxisorp plates are coated with 100 µl of 1 µg/ml antigen, washed and blocked with 3% bovine serum albumin. Serum is added at 3 dilutions. Second antibody is AP-labeled anti-IgG (Fc-specific), or anti-IgM (both 1:5,000), and color is developed with pNPP and read at 405 nm.

20 ELISA for GalC:

This ELISA is adapted from previously published studies (Ichioka et al. (1988) Neurochem Res., 13(3):203-207). GalC is sonicated and heated at 65°C for 10 min and plated at a concentration of 5% on polystyrene ELISA plates (100 µl/well). After blocking, 1:100 to 1:1,000 dilutions of sera are added and incubated for 1 hr. at 37°C.
25 Secondary antibody is anti-human IgG (Fc portion), 1:6,000, labeled with PE. The technique is identical to standard ELISAs with protein antigens, except that Tween is omitted from washes. Color development is performed by adding TMB substrate, and plates are read at 450 nm. Positive control is provided by a delipidized whole rabbit antiserum directed against GalC.

Quality control and quantitative measurements of antibody concentrations.

Standard curves: titers and actual concentrations of autoantibodies are obtained routinely. Standard curves are constructed using serial amounts of purified human IgG and included on each ELISA plate. Three serum dilutions are analyzed in duplicates, in order to establish an accurate determination of concentration. ELISA readings are analyzed in semi-quantitative (dilution titer) and quantitative (concentration) fashion. Criteria for positivity are: titer equal or greater than 1:100, concordant duplicate measurements, and signal greater than twice the background, with background less than 0.150 OD. The methods currently established in the laboratory detect IgG and IgM in separate assays, due to differences in processing and background for these individual Ig subtypes. A method for simultaneous detection of IgG and IgM is in development.

In addition to usual negative and positive control wells for antigen and secondary antibody, each assay can include control antigens (candida, measles, and/or tetanus toxoid), and negative and positive reference sera that have each been aliquoted in frozen single use vials. These assay systems show <1% intrassay and <5% interassay variability. An ongoing protocol conducts regular analysis of myelin and other antibody reactivity by ELISA in a separate cohort of control subjects (n=10, non MS), in order to control for other sources of variability (such as seasonal infections, for example).

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EXAMPLE 3

The Ratio Of MOG-Peptide-Specific Over rMOG-Specific Antibodies Is Predictive Of The Severity Of Clinical EAE

Figure 18 demonstrates that the ratio of MOG-peptide-specific over rMOG-specific antibodies is predictive of the severity of clinical EAE in the marmoset. Thus it appears to be an extremely useful index for evaluating MS patients:.

As illustrated in Figure 8, it is not possible to distinguish these different antibody fractions by ELISA or other standard antibody detection methods. The difference in epitope recognition may translate into functional heterogeneity (e.g., pathogenic potential), since marmosets immunized with the linear peptides develop an attenuated EAE phenotype

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compared to rMOG-immunized animals, despite the apparent induction of similar T cell responses. We have also observed that disease severity in rMOG-induced marmoset EAE is inversely proportional to the ratio of serum concentrations ($\mu\text{g/ml}$) of MOG peptide/rMOG-reactive IgG.

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EXAMPLE 4

Pathogenic Properties Of rMOG- And MOG Peptide-Specific Antibodies

Passive antibody transfer experiments have been done in marmosets immunized with MBP that received homologous marmoset affinity-purified Ig fractions, either rMOG-specific (2 animals), rMOG-specific depleted from linear peptide-specificities (hence only conformation-dependent, 2 animals), and MOG peptide-specific (linear 20mer peptides, 2 animals). Neuropathological examination obtained after antibody transfer revealed the presence of intra-parenchymal perivascular infiltrates with demyelination in all cases. However, reminiscent of the differences between rMOG-induced and MOG peptide induced EAE in marmosets, both the lesion burden and the extent of demyelination were much more pronounced in recipients of conformation dependent or rMOG-specific antibodies compared to animals receiving MOG peptide-specific antibodies (see Figure 19).

These experiments provide direct confirmation that conformational antibodies and linear peptide-specific antibodies, although both recognizing rMOG, have strikingly different pathogenic potential. Although limited, the demyelination present in recipients of MOG peptide-specific Ig appears to be more severe than what has previously been observed in MBP-induced EAE or in MBP-immunized marmosets receiving control IgG, which have never been observed to develop pathology beyond rare subpial inflammatory infiltrates. These findings suggest that the MOG peptide-specific antibodies could also be pathogenic.

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EXAMPLE 5

Epitope Recognition On The MOG Protein Differentially Influences Antibody Effector Functions And Disease Phenotype In Autoimmune Demyelination

In *C. jacchus* marmosets, immunization with myelin/oligodendrocyte glycoprotein (MOG, rMOG1-125) produces a disseminated and demyelinating, multiple sclerosis (MS)

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like form of experimental allergic encephalomyelitis (EAE), with antibody responses against conformational and linear epitopes of MOG. By comparison, fewer, focal lesions mostly confined to subpial tissue of spinal cord and brainstem, less demyelination and autoantibodies that strictly target linear epitopes, characterize EAE induced with short MOG-derived peptides. To understand the basis for these phenotypic differences, we characterized effector mechanisms of pathogenicity associated with anti-MOG antibody subpopulations. Both linear and conformation-dependent antibodies were capable of binding to MOG in situ on myelin sheaths as demonstrated by immunohistochemistry. However, while macrophage/microglial activation was observed in both forms of EAE, IgG deposition and complement activation were only observed in lesions of rMOG1-125-immune marmosets. These findings indicate that polyclonal anti-MOG antibody repertoires in primates are highly heterogeneous in terms of pathogenicity, and that epitope recognition is a determinant factor of antibody effector functions and spatial dissemination of inflammatory demyelinating disease. Because marmoset and human anti-MOG repertoires target identical epitopes, this information offers critical insight for understanding the significance of antibody responses frequently detected in MS.

INTRODUCTION

Myelin/oligodendrocyte glycoprotein (MOG)-induced experimental allergic encephalomyelitis (EAE) in the common marmoset (*C. jacchus*) is a multifocal disease of central nervous system (CNS) white matter that closely approximates human multiple sclerosis (MS) (1-3). Myelin-directed T cell reactivity is obligatory for disease development in marmosets as in all EAE models, however involvement of anti-MOG antibodies is necessary for development of the typical MS-like neuropathological phenotype (4). Sensitization of rodents with immunodominant peptides of MOG gives rise to restricted antibody responses and usually suffices to induce severe EAE. Not unexpectedly however, a broader heterogeneity of epitopes within MOG antibody responses is found in higher mammals. Preliminary work suggests that diversity is present within the antiMOG antibody repertoire in humans (5, 6) and primates (7) and may underlie certain differences in the biological properties of autoantibodies (8). However, the relationship between anti-MOG antibody specificity and effector functions remains largely unexplored.

Structurally, antibodies against MOG can be differentiated on the basis of their ability to recognize either linear or conformational, tertiary structure-dependent epitopes (8). There is limited information on the respective pathogenicity of these antibody subgroups, which may differ depending on the species studied. Preliminary observations of marmoset EAE suggest that immunization with linear 20mer MOG-derived peptides induces a form of EAE that is clearly different from that typically induced in this species by immunization with the entire extracellular domain of MOG (aa1-125, rMOG1-125) (7, 8). Despite these discrepancies, standard ELISA methods detect antibody-reactivity against rMOG1-125-and against linear MOG-epitopes at similar titers in both forms of MOG-induced marmoset EAE, as is also the case for rodents (9-11). Thus, pathogenic properties and effector functions of the different antibody subtypes cannot be understood unless these antibody populations are isolated and separately studied. Such information is needed to facilitate the interpretation of findings of anti-MOG antibody reactivity in man, which in MS and control subjects has been reported with varying frequencies depending on the study, the method of detection and the form of MOG antigen used (6, 12, 13).

The recent cloning of recombinant MOG-reactive antibodies present in the marmoset immune repertoire has revealed monoclonal antibody specificities that define several distinct conformational, surface exposed epitopes of MOG, which are also present in the human antibody repertoires. We have therefore taken advantage of this model system to characterize the immunopathogenicity of anti-MOG antibodies according to their epitope recognition. First we confirmed the association existing between presence of circulating antibodies with linear or structural anti-MOG specificity and neuropathological features of disease in a large series of animals immunized with linear MOG-derived peptides in comparison with the stereotyped pathology encountered in rMOG1-125EAE (14). We consistently found markedly reduced burden and dissemination of demyelinating lesions in these MOG peptide-immune animals despite the fact that MOG peptide-immune animals could clearly proceed to develop severe lethal EAE associated with large solitary destructive lesions. Second, we demonstrate that purified antibodies that recognize either conformational or linear epitopes are both capable of binding to MOG in situ in normal marmoset white matter. Third, the pathogenic functions of these different antibodies were elucidated by immunohistochemical characterization of inflammatory infiltrates. Lesion pathogenesis in MOG peptide-induced EAE appeared strikingly different from rMOG1-

125-induced disease. IgG deposition and complement activation were only observed in the latter with concomitant presence of antibodies against conformational determinants of MOG. However, both types of lesions were characterized by prominent macrophage infiltration. These findings are the first comprehensive analysis of the pathogenicity of polyclonal, native MOG antibody populations representative of specificities that can be found in an outbred species. We formally demonstrate that while both linearly and conformationally defined antibodies may contribute to macrophage recruitment and activation, complement mediated demyelination is exclusively linked to in situ deposition of conformation-dependent antibodies. This information is crucial to the interpretation of MOG antibody responses in MS in context of the heterogeneity of pathology observed for this disease.

MATERIALS AND METHODS

Antigens

A recombinant protein corresponding to the sequence of the extracellular domain of rat MOG (rMOG1-125) was expressed and purified to homogeneity as fusion protein with a His6-Tag in *E. coli* following published procedures (15). A panel of 11 synthetic overlapping linear 20mer peptides corresponding to the sequence of the extracellular domain of rat MOG (aa1-120), and the C-terminus peptide of rMOG1-125 (WINPGRSRSHHHHH; SEQ ID NO:46) were synthesized using standard solid phase chemistry (Research Genetics, Huntsville, AL) and purified > 95% by HPLC. Purity was confirmed by mass spectrometry.

Animals, Immunization and Characterization of EAE

C. jacchus marmosets used in this study were maintained in a primate colony at the University of California, San Francisco and were cared for in accordance with all guidelines of the Institutional Animal Care and Usage Committee (IACUC). Marmosets were actively immunized with either 50 µg of rMOG1-125 (Group I), or 100 µg of MOGderived 20 mer peptides (Group II, individual peptides or combinations, please also refer to Table 5) dissolved in phosphate buffered saline and emulsified with complete Freund's adjuvant (CFA) as previously described (1). The peptides, or combinations of peptides were selected

according to previous mapping studies that have characterized the immunodominant T cell and antibody epitopes of rMOG1-125 in marmosets (14, 16)

- EAE was assessed by daily clinical examination and animals were observed for a total of 12 to 140 days (marmoset expanded scale, score 0 to 45 (17). At the end of the
- 5 observation period, euthanasia was performed under deep pentobarbital anesthesia by intracardial perfusion with 4% para-formaldehyde, and the entire neuraxis obtained and examined in serial consecutive sections (2 mm each). Five μ m, paraffin-embedded sections were stained with Luxol Fast Blue /Periodic Acid Schiff (LFB/PAS) or used for immunohistochemical analysis. Pathologic findings were graded according to separate
- 10 inflammation and demyelination scores: Inflammation score: 0, no inflammation present; +, rare (1-3) inflammatory infiltrates/average whole section; ++, moderate numbers (310) of inflammatory infiltrates/section; +++, widespread parenchymal infiltration by inflammatory cells, with numerous large confluent lesions. Demyelination score: 0, no demyelination; +, rare (1-3 lesions/section) foci of demyelination; ++, moderate (3-10 lesions/section)
- 15 demyelination; +++, extensive demyelination with large confluent lesions.

Table 5. Characteristics of EAE in Marmosets

Animal ID	Immunogen	Max. clinical score	No. of lesions	Inflammation	Demyelination
U004-99	rMOG1-125	14	326	+++	+++
U009-99	rMOG1-125	8	135	+++	+++
Cj72-88	rMOG1-125	5.5	67	+++	+++
J2-97	rMOG1-125	29	124	+++	+++
199-94	aa21-40	8	4	+++	+
368-94	aa21-40	10	4	++	+
39-95	pepMOG	19	4	+++	+
65-92	pepMOG	9	33	++	+ ^a
252-93	aa1-40	5	6	++	+
TX245-90	aa1-40	10	8	+++	+
14-91	aa21-40, 51-90	12	8	+	+
TX75-92	aa51-90	10	13	++	+
Tx256-93	aa81-120	9	3	+	+

Groups I (rMOG1-125- animals U004-99, U009-99, Cj72-88, J2-97) and II (MOG peptide)-immune marmosets. pepMOG denotes a mixture of 11 20mer peptides overlapping by 10 amino acids (aa) and spanning the sequence of MOG aa1-120.

^aDemyelination was found with the grade indicated in all lesions except in animal 65-92, in which only 18 of 33 (55%) lesions were demyelinated.

Fractionation and purification of antibodies from immune *C. jacchus* sera

Sera were collected from each animal at euthanasia, and stored at -20 °C until use.

The respective fractions of serum antibodies with binding specificities for linear peptide or conformational epitopes were separated by affinity chromatography. Sera or pools of sera

from animals in groups I and II were repeatedly passed over columns containing a mixture of the 11 20mer overlapping peptides spanning MOGaa1-120 (pepMOG) covalently linked to sepharose. Bound material containing the MOG peptide-reactive fraction (anti-MOG-P) was eluted with glycine buffer pH 2.5, immediately brought to neutral pH with 1 M Tris
5 buffer (pH 8.0) and extensively dialyzed against PBS. Thus, in these experiments antibody reactivity found in flowthrough fractions (if present) could not represent any epitope of MOG directed against a linear feature, and was considered to represent conformation-dependent MOG-epitopes (anti-MOG-C). The binding characteristics of all eluted and flow
10 through fractions were analyzed by ELISA. Anti-MOG-C if present were further affinity-purified by passing pepMOG column flowthrough fractions over sepharose columns containing covalently linked rMOG1-125, followed by elution, neutralization and dialysis as described above. In addition to characterization of fine specificity by ELISA, the ability of purified anti-MOG-P and anti-MOG-C to bind to native marmoset MOG in situ was
15 determined by immunohistochemistry as described below using antibody fractions biotinylated with a sulfo-NHS biotinylation reagent following the manufacturer's instruction (Pierce). Unreacted sulfo-NHS biotin was removed by extensive dialysis against PBS.

Epitope specificity

Epitope specificities of whole unfractionated sera, fractionated sera, or affinity-
20 purified antibodies were determined by ELISA. Plastic wells (Pierce, Maleic Anhydride plates) were coated with rMOG1-125 or MOG-derived 20mer peptides. Control wells contained no antigen, the recombinant glutathione-S-transferase (GST) from *E. coli*, and the (His)6 C-terminal peptide of rMOG1-125. Wells were blocked with PBS containing 0.05% Tween20 (PBS-T) and 3% bovine serum albumin (BSA), and the following samples were
25 added in blocking buffer and incubated for 1 hour at 37 °C: 1. whole immune serum, 1:200; 2. Three µg/ml of affinity purified anti-MOG-P antibodies; 3. Group I (rMOG1-125), or Group II (MOG peptide-immune) sera depleted of anti-MOG-P antibodies, 1:200. Next, a horseradish peroxidase labeled anti-monkey IgG (A0170, Sigma) was added, and after incubation for 1 hour, wells were developed with tetramethylbenzidine (TMB, Pierce) and
30 read at 450 nm.

Immunohistochemistry

Sections of *C. jacchus* brain were de-paraffinized, hydrated, and treated with a citratebased antigen unmasking solution (Vector Labs, Burlingame, CA) at high temperature for 20 minutes. Endogenous peroxidase activity was blocked by incubation of sections in 0.3 % H₂O₂ in methanol for 30 minutes. Sections were blocked with 5% normal goat serum (Sigma, St. Louis, MO) in PBS-T or 5 % for 1 hour at 37°C, washed with PBS-T, and incubated with the following primary antibodies in blocking buffer: 1. Mouse anti-human C9neo (IgG1, Novocastra; 1:25) for staining of the terminal membrane attack complex (MAC); 2. mouse anti-human HAM56 (IgM, Accurate Chemicals; 1:20), pan-macrophage/microglia marker; 3. mouse anti-human IgG (IgM, DAKO; 1::25). After incubation for 1 hour at 37°C and washes with PBS-T, the appropriate biotinylated secondary antibodies were applied and incubated for another hour at 37°C (rabbit antimouse IgG1 (Zymed); goat anti-mouse IgM (Vector)). Slides were rinsed again, incubated with the Vectastain Elite ABC Kit (Vector) and stained with 3,3'-diaminobenzidine (DAB, Vector). All slides were counterstained with hematoxylin and permanently mounted. Biotinylated anti-MOG-P (from rMOG1-125- and MOG peptideimmune animals; 7 µg/ml and 20 µg/ml resp.) and anti-MOG-C (10 µg/ml) were used to characterize their ability to bind to native, full length MOG expressed in situ by oligodendrocytes in marmoset CNS.

T cell-proliferative responses

Peripheral blood mononuclear cells (PBMC) were isolated from blood samples obtained at euthanasia by centrifugation over a Ficoll gradient, and rested overnight in AIM-V media (Invitrogen). 1x10⁵ PBMC/well were incubated in triplicates in the presence of 10 µg/ml antigen (rMOG1-125, individual MOG-derived peptides) or without antigen (negative control) in 200 µl AIM-V and pulsed with 0.5 µCi ³H-thymidine after 48 hours. After an additional 18 hours, wells were harvested and ³H-thymidine incorporation was measured in a beta-counter. The stimulation-indices (SI) were calculated as the ratio of stimulated/control wells.

Statistics

The following quantitative and qualitative parameters were analyzed for each animal of groups I (n=4) and II (n=9): 1. Total lesion load by counting the number of lesions in 20-24 sections stained with LFB covering the entire neuraxis. 2. Immunohistochemical patterns

of staining by examining 2-4 sections of brain and spinal cord from group I (n=4, 24 -82 lesions per animal), and all the sections containing lesions from animals in group II. We compared the percentages of lesions positive for each marker, with positivity defined as follows: HAM56, > 10 stained cells/lesion; IgG (cellular distribution, B cells), > 2 stained cells/lesion in the immediate perivascular vicinity; IgG (parenchymal distribution), clearly positive staining above background along fibers within lesions and not associated with cells; C9neo, clearly positive staining above background. Comparisons between the two groups were performed using a two-tailed, un-paired Student's t-test.

RESULTS

10 **Clinical and neuropathological characteristics of MOG peptide- and rMOG1-125-induced EAE**

All animals in the study developed clinical EAE, with variable severity observed in both groups I and II. Table 5 recapitulates the clinical and neuropathological phenotypes of EAE induced with the various immunogens. The course of MOG peptide-induced EAE tended to be progressive over time, with more rapid progression in the 2 animals immunized with pepMOG (the mixture of all peptides, #39-95 and 6592). rMOG1-125-induced EAE was either rapidly progressive or relapsing-remitting, as previously described in animals observed chronically. It is noteworthy that overall severity of disease was not associated with a particular immunization regimen: both animals J2-97 (rMOG1-125-immune) and 39-95 (pepMOG-immune) developed hyperacute EAE symptoms requiring immediate euthanasia.

Neuropathologically, the most remarkable difference between animals in the 2 immunization groups was a tremendously reduced white matter lesion burden in group II (Table 5, Figure 7: group I: 163 +/- 56.3 lesions (mean +/- SEM); group II: 9.2 +/- 3.1, p=0.0012). Second, in contrast with the multifocal disease that we and others have consistently observed in many rMOG1-125-immune marmosets (typically involving optic nerves, spinal cord, brain hemispheres, and brainstem with perivascular intraparenchymal distribution 14), the distribution of lesions in MOG peptide-immune animals was mainly restricted to brainstem and spinal cord with a pattern of subpial space infiltration reminiscent of MOG-peptide-induced EAE in mice (18, 19) (Figure 20).

Animal 39-95 developed a large hemorrhagic lesion in the left optic tract and nerve. Only one of the pepMOG-immunized animals developed inflammatory lesions within the cerebral white matter (#65-92), none of which showed evidence of demyelination. The third major neuropathological difference between the two groups was that the extent of demyelinated areas was reduced in lesions of MOG peptide-induced EAE compared with those of rMOG1-125-EAE, in the presence of roughly similar degrees of inflammation in most animals (Table 5). The demyelination in MOG peptide-induced EAE did not extend beyond the margin of inflammatory infiltrates, in contrast to the protracted and expanding lesions of rMOG1-125-induced EAE (Figures 4 and 21). The most abundant pattern of demyelination in lesions of MOG peptide-induced EAE was myelin vacuolation, a feature that is present at the periphery of expanding lesions in rMOG1-125-induced marmoset EAE (3) (Figure 20A).

Epitope specificities of antibody responses

As expected from previous studies, all monkeys developed serum antibodies that reacted to both rMOG1-125 and linear peptides as shown by standard ELISAs of unfractionated serum (Figure 21, left panels). To separately characterize conformational and linear specificities, the following selected sera were depleted from pepMOG-reactive antibodies: group I (rMOG1-125-immune): J2-97, 72-88, U004-99, U009-99; group II: pepMOG-immune: 39-95, 65-92; MOG aa21-40-immune: 199-94, 368-94; a pool of equal amounts of sera from animals 14-91, 75-92, 252-93, 245-90, 256-93. The results from representative animals and the pooled sera from group II are shown in the right panels of Figure 21.

Complete depletion of sera from anti-MOG-P antibodies was achieved after 3-5 passes over the pepMOG columns, as shown by the lack of binding to individual peptides (Figure 21, right panels). Depletion from anti-MOG-P- resulted in complete loss of reactivity to rMOG1-125 in each of the animals immunized with MOG peptides (Figure 21 D, F, H), regardless of the sequence of the immunizing peptides. By contrast, sera from rMOG1-125-immune animals always retained reactivity against whole rMOG1-125 after being depleted from anti-MOG-P antibodies (Figure 21B).

Anti-MOG-P and anti-MOG-C antibodies from animals of both groups were eluted from the respective affinity columns. Only anti-MOG-P displayed binding to MOG

peptides, as did the respective sera from which they were purified. These antibody fractions were also capable of binding to rMOG1-125 in vitro in the ELISA system.

Incubation of normal marmoset CNS sections with anti-MOG-P antibodies showed that these antibodies strongly stained white matter, as did anti-MOG-C antibodies (Figure 22). This was observed regardless of the immunization regimen used to produce anti-MOG-P antibodies (e.g., rMOG1-125 or MOG peptides) (Figure 22). No significant reactivity to either recombinant GST expressed in *E. coli* or the (His)₆ C-terminal peptide was detected in any of the antibody fractions. Together, these findings demonstrated that: 1. Both linearly defined (anti-MOG-P) and conformational (anti-MOG-C) antibodies are capable of binding to MOG in situ, thus epitope recognition per se does not appear to be the determining factor for antibody binding to MOG embedded in intact myelin sheaths. 2. Anti-MOG-C antibodies that were isolated after depletion of pepMOG-specific antibodies were not directed against the C-terminal peptide of rMOG1-125 or against bacterial contaminants in the rMOG1-125-preparation used to synthesize the columns for affinity-chromatography.

15 **MOG-specific T cell proliferative responses**

Circulating T cell proliferative responses to rMOG1-125 were observed in PBMC of all animals at euthanasia. The magnitude of these responses was similar in MOG peptide-immune animals and rMOG1-125-immune animals (10 +/- 3.1 vs. 12.7 +/- 5.8, NS, Figure 23). T cell proliferative responses mapped to 20mer peptides corresponding either to the immunodominant T cell epitopes in rMOG1-125-immune marmosets 7 or to the immunizing peptide(s) in MOG peptide-immune animals.

Immunohistochemical characterization of lesions

Results of immunostaining experiments are summarized in Figures 24 and 9. Macrophage infiltration was a consistent feature of inflammatory infiltrates in all animals, as indicated by staining for HAM56 (Figure 24A and B). Pronounced IgG deposition was found in rMOG1-125-immune animals, either in the immediate perivascular vicinity or deeper within the white matter parenchyma (Figure 24C), in agreement with previous findings (20). In sharp contrast, lesions that showed IgG deposition were observed in only 2 animals immunized with MOG-peptides (39-95 and one in 252-93). This involved a single hemorrhagic lesion in both cases, which raises the possibility that this was the result of

exsudation of blood into the lesion. In addition to parenchymal deposition, IgG could also be detected in cells present in close vicinity of blood vessels in rMOG1-125-immune animals (B cells or plasmocytes, Figure 24C). Some of the lesions found in MOG peptide-immune animals also showed IgG positive cells, though much less frequently.

- 5 Quantitatively, the differences in IgG deposition and IgG positive cells between rMOG1-125-and MOG peptide-immune animals were significant ($p=0.003$ and $p=0.038$ respectively, Figure 9). Highly significant differences were also observed for C9neo deposition, which was prominently observed in rMOG1-125-immune animals (56 % positive of 204 analyzed lesions), but was uniformly absent from any of the 83 lesions
10 analyzed in MOG peptide-immune animals (0%, $p<0.0001$, Figure 24 E, F, and Figure 9).

DISCUSSION

- T cell responses directed against one or several immunodominant linear peptides of MOG have been demonstrated to be powerful inducers of CNS inflammation and, in some EAE models, demyelination. The humoral responses against this encephalitogen however,
15 appear to be much more complex in terms of determinant recognition and participation in lesion pathogenesis. The respective pathogenic potentials of antibodies directed against either linear or conformational determinants of MOG are not firmly established in all EAE models, and have not been investigated in primate species which share with humans the most complex antibody responses. Some studies of the murine anti-MOG antibody
20 repertoire (19, 21, 23) suggest that the recognition of conformational determinants of MOG may be an important requirement for pathogenicity. However, according to some (24) but not all (11) investigations, Lewis rats immunized with MOG aa35-55 can develop multifocal demyelinating disease, despite the demonstration that these animals do not develop conformation-dependent anti-MOG antibodies. This implies that MOG peptide-
25 specific antibodies may be pathogenic in the rat, as seems to be the case in several mouse strains (10, 25, 26).

- Regardless of these apparent species-specific differences, a role for humoral mechanisms of demyelination in human MS is even less clear than in EAE, although suggested by the presence of intrathecal immunoglobulin (Ig) synthesis (27) clonal
30 expansion of B cells (28-30) and complement activation (31). Anti-MOG antibody deposition has been recently demonstrated in context of a characteristic pattern of myelin

disintegration in actively demyelinating lesions of both MS and marmoset EAE (2, 3). Despite one in vitro study that provides clues to the nature of exposed determinants of MOG in humans (5), to what extent the recognition of conformational determinants of MOG by B cells and/or antibodies influences the expression of MS phenotypes in humans remains
5 largely unknown. We therefore designed the current studies to systematically investigate functional properties of anti-MOG antibodies according to epitope recognition in an outbred model of EAE that closely approximates the diversity of humoral responses and neuropathology encountered in MS (2, 3).

Mapping studies of anti-MOG antibody responses conducted in primate EAE and
10 MS have generally paralleled these of T cell reactivity, using short peptides derived from MOG (5, 6, 9, 32, 33). As shown here and by others (24) antibodies that are directed against linear epitopes can also bind to whole protein antigens, however standard techniques of antibody measurement do not discriminate between these antibodies and those that exclusively recognize conformational determinants of target antigens. We fractionated sera
15 by affinity-chromatography to ensure complete distinction between antibody responses against strictly conformational and linear MOG epitopes, and demonstrate that marmosets immunized with MOG-derived peptides develop a restricted population of antibodies against the linear sequences. These animals fail to develop antibodies that are exclusively conformation-dependent, in contrast to rMOG1-125-immunized marmosets. We took
20 advantage of this finding to formally demonstrate a link between antibody epitope recognition and differential expression of EAE phenotypes.

It is important to note that the differences between rMOG1-125- and MOG peptide-EAE predominantly involved patterns of disease dissemination and demyelination, and not severity of EAE. Animals in both groups developed either severe, rapidly progressive
25 disease or mild to moderate forms, as can be expected in this outbred species. However, MOG peptide-immunized animals showed reduced disease burden and reduced, albeit significant demyelination compared to rMOG1-125-immune animals. Demyelinating lesions in the former animals were mostly observed in spinal cord and brain stem, and not in cerebral hemispheres where they typically occur after rMOG1-125-immunization (7, 8).
30 This pattern of pathology was a consistent feature of marmoset MOG peptide-EAE regardless of the choice of immunizing peptide within the extracellular domain of MOG, likely indicating that the observed differences were not a consequence of T cell epitope

immunodominance. Rather, we propose that the recognition of conformational determinants of MOG was the basis for certain pathogenic properties of antibodies and/or B cells, which together with T cell responses resulted in MS-like multifocal and prominent demyelination.

The subpial localization of demyelinating infiltrates in MOG peptides-immunized marmosets is strikingly similar to CNS pathology observed in C57/Bl6 mice immunized with MOG peptide 35-55 (18, 19, 34). Our findings are in partial agreement with studies of mice lacking B cells, which fail to develop EAE after immunization with rMOG1-120 (19). Passive transfer of whole serum from wild type mice in these animals indicates that the MOGaa35-55 peptide-immune serum is less efficient than rMOG1-120-immune serum in restoring the EAE phenotype of wild type mice immunized with rMOG1-120 (23). This could mean that the rMOG1-120 immune mouse serum contained certain pathogenic antibodies that are not present in MOG aa35-55-immune animals, as is the case for marmosets, however this was not investigated. In addition, these rodent studies have not addressed the question of disease dissemination, a major finding of the current work which clearly shows a link between antibody determinant recognition and density and distribution of CNS lesions. The presence of conformation dependent antibodies (anti-MOG-C) appears strictly associated with disease in a typical MS-like distribution (brain hemispheres, optic nerve and spinal cord), whereas linear-dependent antibodies are clearly associated with focal disease mostly restricted to brain stem and spinal cord in most animals. Possible biological explanations for these differences include differential binding affinity, or as discussed below different effector functions of anti-MOG-P and anti-MOG-C antibodies. It is also possible that the density of expression of MOG molecules, and/or presentation of its accessible epitopes on myelin sheaths differ within the different parts of the CNS, thus influencing lesion dissemination and location.

Certain immunopathological similarities appear to exist between MOG peptide- and rMOG1-125-induced EAE. The lesion pattern observed in the former includes evidence of myelin vacuolation, which is also present at the edge of lesions in rMOG1-125-induced EAE (3). This phenomenon has previously been shown to result for example, from exposure of the intact myelin sheath to a variety of toxic soluble substances such as TNF- α (35) and triethyl tin sulfate (36) and could also be an effect of MOG-specific antibodies. Thus, a pathogenic role cannot be ruled out for anti-MOG-P antibodies that are induced in marmosets by immunization with either rMOG1-125 or MOG-derived peptides.

Antibodies specific for MOG aa21-40 have been detected in close association with disintegrating myelin membranes in lesions of rMOG1-125-induced marmoset EAE (2, 3), thus it is possible that anti-MOG-P antibodies play a pathogenic role in sustaining myelin-destruction by binding to epitopes newly exposed during active demyelination. Future
5 studies of passive transfer of anti-MOG-P or anti-MOG-C in MBP sensitized animals should unequivocally determine which antibodies are capable of initiating certain patterns of demyelination.

Rat rMOG1-125 is ~ 90% homologous to *C. jacchus* MOG1-125 (37) and is a well established encephalitogen in this species (39). rMOG1-125 and native *C. jacchus* MOG
10 share identical conformational antibody epitopes, as demonstrated by immunohistochemical studies of marmoset brain conducted with monoclonal conformation-dependent Fab-fragments directed against rMOG1-125 (8). Both anti-MOG-P (from rMOG1-125- and MOG peptide-immune animals), and anti-MOG-C were able to recognize native MOG in situ in normal CNS white matter. It is thus highly unlikely that the differences in
15 neuropathology observed in our animals were due to amino acid substitutions between marmoset and rat MOG. Similarly, we found that anti-MOG-C antibodies (which were purified by complete removal of any linear specificity within MOG aa1-120 followed by purification on rMOG1-125 columns), did not bind to the (His)6-tagged C-terminal portion of MOG1-125, which is not present in native MOG expressed in marmoset CNS white
20 matter.

Whether or not anti-MOG-P and anti-MOG-C bind to myelin with different affinities in vivo, parenchymal IgG deposition was only observed in rMOG1-125-immunized marmosets, while it was noteworthy that macrophage infiltration and activation was present to a similar extent in both MOG peptide- and rMOG1-125-induced EAE. It is
25 well known that macrophage infiltration and microglial activation can occur independently of stimulation by IgG via Fcγ-receptors, for example through activation by inflammatory mediators such as interferon-γ. Nevertheless, the recognition of structural epitopes of MOG not only could influence antibody binding in vivo but could also result in different effector mechanisms for antibody pathogenicity. Our results demonstrate that the C9 component of
30 the lytic complex is only detected in brain tissue from rMOG1-125-immunized animals, and is absent from lesions of MOG peptide-induced EAE. This suggests that antibodies against conformation-dependent MOG epitopes, and not those against the linear epitopes are

capable of activating lytic complement pathways, thus potentially augmenting the destructive potential of these antibodies. Numerous studies of EAE (22, 29, 40) and MS (31, 41) support a role for complement in lesion pathogenesis, however there is no practical marker to detect this type of pathology. The knowledge of what antibodies within the MOG repertoire have specific pathogenic properties is of considerable importance for future studies of humoral autoimmunity in MS.

Another intriguing finding of the current study was the absence of epitope-spreading to structure-dependent MOG-determinants in animals immunized with MOG-derived peptides. This suggests that exposure of complex, discontinuous determinants to B cells did not occur de novo in the context of myelin destruction in these animals, at least during an observation period of up to 140 days. These considerations have important implications for understanding molecular mimicry triggered by pathogens in terms of their capacity to induce pathogenic antibody responses. T cell mimicry between microbial peptides and myelin-antigens has been demonstrated in many experimental systems and can occur via direct sequence homology (42, 43) or bystander mechanisms (44). The current study provides strong evidence that humoral immunity against conformational determinants of MOG is a major modifying factor that could be responsible for disease dissemination within the CNS. Therefore, the presence of complex structural determinants similar to those of MOG would be required to provide the basis for molecular mimicry leading to such pathogenic antibody responses.

In summary, we present here the first comprehensive analysis of functional heterogeneity of the MOG-specific antibody repertoire in an outbred species that share complexity and similar structural epitopes with humans (8). . The notorious heterogeneity of clinical presentations of MS has stimulated recent investigations demonstrating distinct neuropathological subtypes, some of which clearly involve humoral mechanisms of tissue damage (41). We now demonstrate that antibody responses to a single target molecule of myelin are sharply dichotomized in terms of pathogenic and functional properties. Our observations bear important implications for the interpretation of anti-MOG antibody serotypes in humans and will be essential to guide the choice of future therapies antagonizing pathogenic antibody responses in MS.

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EXAMPLE 6

Characterization Of The Antibody Repertoire Against Native, Glycosylated MOG

To further characterize the antibody repertoire against native, glycosylated MOG, we successfully expressed the alpha 1 form of protein (major isoforms, human) after transfecting CHO cells. These cells retain stable expression for about 10 passages. We have used them to confirm binding of the marmoset antibodies and more importantly, studies of human anti-MOG reactivity. A significant percentage of human sera studied to date are binding to the MOG-transfected, and not to untransfected cells. Figures 26 and 27 illustrate these findings.

EXAMPLE 7

Antibody Responses Against Galactocerebroside Are Potential Stage-Specific Biomarkers In Multiple Sclerosis

Galactocerebroside (GalC), the major glycolipid of central nervous system (CNS) myelin, is a known target for pathogenic demyelinating antibody responses in experimental allergic encephalomyelitis (EAE), the animal model of multiple sclerosis (MS). α -GalC IgG were quantified from sera of MS patients and in EAE animals by a newly designed

immunosorbent assay. We report, for the first time, a significant difference in serum α -GalC IgG titers between relapsing-remitting (RR)-MS patients and healthy controls (HC) ($p < 0.001$). The frequencies of α -GalC antibody positive subjects (α -GalC titers $\geq$ mean HC titers + 3 SD) are also significantly elevated in RR-MS compared to HC (40 % vs. 0 %; $p = 0.0033$). There was a trend towards antibody positivity in secondary-progressive MS. Immuno-affinity purified α -GalC IgG from human serum bind to cultured human oligodendrocytes, indicating that the ELISA detects a biological relevant epitope. Corroborating these findings, α -GalC antibody responses in marmoset EAE were similarly found to be specifically associated with the relapsing-remitting forms and not the per-acute or progressive forms, in contrast to other anti-myelin antibodies ($p = 0.0256$). Thus, α -GalC antibodies are MS-specific, unlike antibodies against myelin proteins. In addition, when present, α -GalC antibodies identify mostly relapsing-remitting and to a lesser extent secondary-progressive subtypes of MS, and appear to be an indicator of ongoing disease activity. This novel assay is a suitable and valuable method to increase accuracy of diagnosis and disease staging in MS.

INTRODUCTION

Multiple sclerosis (MS) is a chronic immune-mediated inflammatory demyelinating disease of the central nervous system (CNS) characterized by heterogeneity in clinical presentation and underlying pathological mechanisms.(1) There is currently no easy paraclinical marker to diagnose MS subtypes and predict disease course accurately without lengthy periods of clinical follow-up.

Several myelin autoantigens may serve as targets for the autoaggressive attack in MS -- for example, myelin protein myelin/oligodendrocyte glycoprotein (MOG), expressed on the outermost lamellae of the myelin sheath and thus readily accessible to the immune machinery; and a major CNS myelin glycolipid, galactocerebroside (GalC), which accounts for 32% of the myelin lipid content. Both MOG and galactocerebroside are highly encephalogenic in various models of experimental autoimmune encephalomyelitis (EAE), the prototypic animal model for MS.(2-4) Furthermore, passive antibody transfers in myelin basic protein (MBP)-primed animals (5-9) and in vitro models have demonstrated the demyelinating properties of antigalactocerebroside (α -GalC) and α -MOG antibodies.(10-13) Antibody responses against these myelin targets are thus factors that

potentially regulate disease phenotype expression in the context of established CNS inflammation.

The pathogenic involvement of antimyelin antibodies in human MS is less well established, because antibody titers against the myelin proteins do not unequivocally differ
5 between control populations and patients with MS.(14-19) However, regardless of pathogenicity, antimyelin antibodies have recently been proposed as predictive disease markers.(20)

Here, we examined whether α -GalC antibodies could serve as disease markers in MS. We demonstrate for the first time that significantly elevated titers of α -GalC
10 antibodies are specifically found in relapsing-remitting (RR)-MS, and not in early or progressive forms of the disease. In strong support of our clinical observations, longitudinal assessment of galactocerebroside reactivity during the course of relapsing EAE in marmosets indicates that appearance of antibodies against galactocerebroside is delayed with respect to disease onset.

15 MATERIAL AND METHODS

Patients and controls

Sixty-five consecutive patients seen in our MS center, 51 meeting the diagnostic criteria for clinically definite MS,21 were recruited for this study: 20 with RR-MS,15
20 secondary-progressive (SP)-MS, and 16 primary-progressive (PP)-MS (Table 6). In addition, 14 patients had a clinically isolated syndrome (CIS), ie, a single clinical attack suggestive of CNS demyelination. Twenty volunteers served as healthy controls (HCs). Both untreated patients and patients treated with IFN- β and glatiramer acetate were included in this study, but those treated with glucocorticoids within 3 months or on immunosuppressive therapy within 6 months of phlebotomy were excluded.

25

Table 6. Characteristics Of Patients With MS And HCS

Variable		HC	CIS	RR-MS	SP-MS	PP-MS
N		20	14	20	15	16
Gender	female	10	9	15	9	10
	male	10	5	5	6	6
Age (years)	median	51.0	36.0	43.0	45.0	51.0
	range	28-71	23-50	25-61	37-60	40-65
Disease duration (moths)	median	n.a.	n.a.	120	120	48
	range	n.a.	n.a.	9-266	24-384	9-216
EDSS	median	n.a.	1.5	2.0	5.5	4.5
	SD	n.a.	0.9	1.5	1.5	1.2

NA, Not applicable.

* $P < .05$ if compared with HC and $P < .01$ if compared with PP-MS

(ANOVA with Bonferroni correction for multiple comparisons).

5 $P < .001$ and $P < .01$ if compared with SP-MS or PP-MS, respectively

(Kruskal-Wallis test with Dunn posthoc test for multiple comparisons).

Blood was drawn by venipuncture and clotted serum stored at -40°C . Informed consent was obtained from the patients and HCs, and the study was conducted in accordance with Institutional Review Board approval.

10 **Animals**

C. jacchus marmosets were cared for in accordance with the guidelines of the Institutional Animal Care and Usage Committee. EAE was induced by immunization with 100 mg human white matter homogenate as described (22). Plasma samples were obtained from EDTA-anticoagulated blood at baseline and at intervals of 2 to 4 weeks and stored at -
 15 40°C . The animals were scored every other day for the development of clinical signs and disability using a previously published scale (22).

Anti-GalC ELISA

Bovine brain-derived galactocerebroside (Matreya, Pleasant Gap, Pa) was dissolved in chloroform-methanol (2:1). For coating, galactocerebroside was air-dried, stepwise resuspended in 65°C hot ethanol (50% vol/vol) at a final concentration of 50 µg/mL, with 100 µL added to wells of Polysorb 96-well microtiter plates (Nunc, Rochester, NY), and incubated uncovered overnight at room temperature (RT) for solvent evaporation. Plates were washed with ddH₂O and blocked with 1% BSA (A7030; Sigma, St Louis, Mo) in PBS (ELISA buffer) for 2 hours at RT. After washing with PBS and ddH₂O, 100 µL of either human serum samples, diluted 1:40 in ELISA buffer, or *C. jacchus* samples, diluted 1:100, were incubated in triplicate overnight at 4°C. Background binding of each sample was controlled for on blocked wells without coated antigen. After washing, specific antibody binding was detected by an alkaline phosphate-labeled goat-antihuman IgG (A9544; Sigma) or by a horseradish peroxidase-conjugated rabbit-antimonkey IgG (A2054; Sigma), diluted in ELISA buffer and incubated for 1 hour at RT. For human sera, binding was detected by reading the OD at 405 nm in a microplate reader (SpectraMax; Molecular Devices, Sunnyvale, Calif) after incubation with paranitrophenyl phosphate (Moss, Pasadena, Md) for 30 minutes in the dark at RT. The marmoset assay was developed with 3,3',5,5'-tetramethylbenzidine (Pierce, Rockford, Ill) for 15 minutes at RT and the OD read at 450 nm wavelength.

For specificity and sensitivity controls, a polyclonal rabbit antbovine galactocerebroside antiserum (G9152; Sigma) was used and antibody binding detected by a horseradish peroxidase-labeled goat-antirabbit IgG (A0545; Sigma). Quenching experiments were performed by overnight pre-incubation with solubilized galactocerebroside; galactocerebroside was air-dried and resuspended in 65°C hot ethanol at 200 µg/mL and further diluted in ELISA buffer to a final concentration of 2 µg/mL.

Anti-myelin protein antibody ELISA.

C. jacchus antibodies against human MBP and recombinant rat (r)MOG, amino acids 1-125 (23) were coated to microtiter plates (Maxisorb; Nunc) overnight with 1 µg antigen per well. After washing and blocking with 3% BSA in PBS plus .05% Tween for 1 hour at 37°C, marmoset samples were incubated for 1 hour at 37°C and diluted 1:100 in 3%

BSA in PBS plus .05% Tween. Antibody binding was detected by a peroxidase-labeled rabbit-antimonkey IgG for 1 hour at 37°C.

Statistical analysis

To express the results of the galactocerebroside assay, a signal-to-background binding ratio was calculated as the ratio of OD (signal) over OD (background). Positive controls, ie, a human sample with strong binding signal, and negative controls, ie, ELISA buffer only, omitting serum, were included on each plate. For human samples, samples above the mean binding ratio 1.3 SD for the HC group were considered positive. In the marmoset assay, samples were considered positive for a binding ratio above 3 with OD_{GalC} >0.1 and greater than 3-fold the baseline (unimmunized) sample. Statistical analysis was conducted by using STATA 7.0 and GraphPad Prism 3.0. Categorical variables were compared by using the χ^2 test, continuous variables by using ANOVA, and ordinal variables by using the Kruskal-Wallis test. The Bonferroni method and the Dunn test were used to determine differences in between groups. Survival analysis was used to assess time-dependent variables. Because the binding ratios are not normally distributed, the binding ratio was transformed by using an inverse ratio to generate a normal distribution for parametric analysis.

Antibody affinity purification

Human serum was diluted in 10 mmol/L sodium phosphate buffer, pH 7.0 (SP buffer), and IgG was purified over a protein G column (HiTrap HP; Amersham, Piscataway, NJ). Bound IgG was eluted with 100 mmol/L glycine-HCl, pH 2.7, and dialyzed against the sodium phosphate buffer. For immunoaffinity purification of α -GalC antibodies, galactocerebroside was dissolved at 5.0 mg/mL in 65°C hot methanol and hydrophobically bound to a FF-octyl column (HiTrap; Amersham) as previously described. (24) The IgG fraction was applied to this column and bound IgG eluted and dialyzed into PBS as described.

Immunohistochemistry

The human oligodendrocytoma cell line HOG (kind gift of Dr Glyn Dawson), known to express galactocerebroside, (25) was grown in monolayers. Cells were trypsinized and plated at a density of 20,000 cells/well onto chamber glass slides (Nunc);

fixed in icecold methanol; blocked with 2% BSA and 2% FBS in PBS; and stained with human serum (1:50), rabbit antiserum (1:50), or 1006-GalC (30 ug/mL), respectively, diluted in 1% BSA-PBS for 1 hour at RT and developed with fluorescein isothiocyanate-labeled anti-IgG secondary antibodies (F3512 for human, F9887 for rabbit; Sigma). Control
5 slides omitting the first antibodies were included.

RESULTS

Validation of the α -GalC assay

The assay was validated by a rabbit antiserum reactive to bovine galactocerebroside, with reactivity detectable to a titer of 1:12,800. Preincubation of the rabbit antiserum with
10 galactocerebroside solubilized in ELISA buffer (maximal solubility concentration, 2 μ g/mL in aqueous buffer) led to an 85% reduction in signal, proving specificity of the assay. A mAb reactive against MOG (8.18- C5) did not react with the coated galactocerebroside, confirming the purity of the antigen. In serial dilutions of total IgG purified on protein G from either the human positive control or pooled immune *C. jacchus* sera, the threshold of
15 detection was 6.25 ug IgG per well. The interplate and intraplate coefficients of variation were 15% and 4%, respectively.

Detection of alpha-GalC IgG in patients with MS

Quantitatively, significant differences in anti-GalC antibody titers were found between HC and RR-MS ($P < .001$) as well as between patients with CIS and RR-MS ($P < .05$; ANOVA with Bonferroni correction for multiple comparisons; Figure 28A). There was
20 a trend suggesting a difference for the antibody titers between SP-MS and HC ($P = .092$). In contrast, there were no significant differences for anti-GalC reactivity among the HC, CIS, and PP-MS subgroups. Even if the 2 patients with the highest binding ratios in the RR-MS group were excluded from the calculations, the difference in the reciprocal binding ratio
25 compared with the HC group remained highly significant ($P < .01$).

The threshold for positivity was 3.23 and is indicated in Figure 28A (dashed line). The frequencies of patients with RR-MS identified as anti-GalC antibody-positive by this analysis were significantly higher compared with HC (40% vs 0%; $P = .0033$; Fisher exact test with Bonferroni correction for multiple independent comparisons). Again, there was a
30 trend observed for anti-GalC antibody positivity in SP-MS compared with HC (26.7% vs

0%; $P = .026$; not significant after correction for multiple independent comparisons). Other pairwise comparisons were not significant (Figure 28B).

Immunoaffinity purification of α -GalC IgG and immunohistochemistry

To assess the specificity and biological relevance of the ELISA assay, serum of 1
5 patient demonstrating a high anti-GalC response (#1006) was subjected to immunoaffinity
purification of anti-GalC IgG. From 50 mL serum, 190 μ g anti- α -GalC IgG (1006-GalC)
was extracted by a custom made galactocerebroside column. 1006-GalC reacted in the
ELISA with a detection limit 62.5 ng specific IgG per well (0.625 μ g/mL), and the signal
could be quenched by soluble galactocerebroside (45% signal reduction). These
10 galactocerebroside-purified IgGs showed staining of the human oligodendrocytoma cell line
HOG identical to the control rabbit anti-GalC specific antiserum (Figure 29A and B). These
results unequivocally show that galactocerebroside specific antibodies purified from human
serum are cell surface binding on oligodendrocytes, and indicate that the newly implemented
ELISA assay system likely detects biologically relevant antibodies.

15 Detection of α -GalC IgG responses in marmoset EAE

Sequential sera of 20 animals immunized with human white matter homogenate
were studied. Because of the outbred nature of the animals, the clinical course of EAE is not
uniform: 9 animals displayed a RR-EAE disease course, and 2 animals did not remit during
attacks but progressively worsened over time (similar to a PP course). Six animals were
20 euthanized at onset of the first attack, termed acute monophasic (AM), and 2 of these had a
peracute disease course rapidly progressing to a score of 4. An additional 3 animals were
euthanized before the onset of clinical disease, at the time when pleocytosis was present in
the cerebrospinal fluid, demonstrating presence of CNS inflammation. Clinical information
is summarized in Table 7).

25 Antibodies against rMOG and MBP were detected in all but 1 of the animals
regardless of their disease course, including the preclinical animals (Table 7). In contrast,
anti-GalC antibodies were detected only in animals with RR-EAE, and not during the first
attack of AM-EAE, even in the severely affected animals or in animals displaying a
progressive course (Table 7). However, this could have resulted from the overall shorter
30 observation period for these animals (median, 28 and 60 days postimmunization vs 70 days

postimmunization for RR-EAE; Table 7). The anti-GalC antibody response appeared significantly later compared with antibody responses against the myelin protein rMOG and MBP in RR-EAE: median time lapse between immunization and appearance, 70 days for α -GalC vs 45 days for α -rMOG and 27 days for α -MBP ($P = .0256$; log rank test for equivalence of survival functions). A Cox proportional hazard model showed that the hazard ratios (HRs) for α -rMOG and α -MBP antibody responses were significantly different from the HR for α -GalC (HR α -rMOG = 5.56, $P = .013$; HR α -MBP = 12.76, $P = .001$; Figure 30), indicating that α -GalC antibodies occurred most distant from immunizations and thus onset of EAE in these animals.

10

Table 7. Characteristics Of *C. jacchus* Marmoset EAE And Antibody Status

Animal	Disease Course	Onset (day PI)	Sacrifice (day PI)	Max Score (day PI)	anti-GalC IgG	First Day Detect (PI)	anti-MOG IgG	First Day Detect (PI)	anti-MPB IgG	First Day Detect (PI)
326-91	RR	14	120	4 (120)	+	23	+	63	+	23
106-90	RR	16	112	3 (96)	+	80	+	53	+	25
91-92	RR	16	112	2 (20)	+	81*	+	53*	+	27*
378-85	RR	16	112	2 (40)	+	26*	+	26*	+	56*
u062-02	RR	43	97	3 (84)	+	96	+	36	+	36
185-99	RR	7	86	2 (56)	+	70	+	15	+	28
U050-01	RR	21	86	2 (57)	+	70	+	45	+	1
U057-02	RR	32	82	1.5 (73)	+	62	+	62	+	19
U050-00	RR	21	78	3 (38)	+	78	+	29	+	29
Median $\pm$ SD	RR	21 $\pm$ 11	98 $\pm$ 16	2.0 $\pm$ 0.8 (97 $\pm$ 16)		70 $\pm$ 25		42 $\pm$ 17		
							+			
127-93	CP	16	68	4 (56)	-	-	+	54*	+	
U052-01	CP	21	52	3 (40)	-	-	+	36	+	
U025-00	AM	21	61	2 (57)	-	-	+	42	+	
U023-00	AM	16	31	2 (28)	-	-	+	28	+	
273-93	AM	12	28	3.5 (24)	-	-	+	28*	+	
274-93	AM	16	28	2 (20)	-	-	+	30*	+	
U021-99	AM	18	23	1.5 (23)	-	-	+	20	+	
346-92	AM	17	22	3 (22)	-	-	+	23	+	
Median $\pm$ SD	AM	16 $\pm$ 3	28 $\pm$ 15	2.0 $\pm$ 0.8 (23 $\pm$ 14)		-	+	28 $\pm$ 8		
U053-01	Preclinical	NA	38	0	-	-	+	35	+	25
U061-02	Preclinical	NA	31	0	-	-	-	-	+	31
U030-00	Preclinical	NA	23	0	-	-	+	13	+	20

NA, Not applicable; PI, postimmunization.

*Monthly blood draw only.

- 5 P<.001 for timing of euthanasia and maximal clinical scores between RR-EAE vs AM, respectively (2-tailed t-test); maximal clinical score or time of clinical onset were not significantly different (P=.69 and .39, respectively; 2-tailed t-test)
- Statistical analysis provided in Figure 30.

DISCUSSION

We present here a reproducible solid-phase assay for detection of galactocerebroside-specific IgG in human sera. These specific IgG were purified by means of a galactocerebroside immunoaffinity chromatography column and were shown to retain the ability to bind to a galactocerebroside epitope expressed on human oligodendrocytes and in vitro by ELISA. The assays previously described to measure such antibodies in MS (18,19,26) identified differences between controls and MS for cerebrospinal fluid, but not serum, even with undiluted serum in a solid-phase radioimmunoassay.(18) The most likely explanation for the differences we find between HC and MS is the stratification for MS subgroups, which was not examined in previous studies.(18,19) Indeed, comparing all our 65 patients with MS as 1 group with HC showed no significant difference in the frequency of antibody-positive patients.

The current α -GalC IgG assay is performed in serum at dilutions of 1:40 and above, which is considerably easier to access than cerebrospinal fluid and can be repeated multiple times. Most significant, serum α -GalC are specific for MS, because they are not encountered in any of the controls, and practically never if at all in CIS. Although other neurological diseases were not examined, this finding at least indicates that, unlike for myelin proteins like MOG, serum positivity helps to distinguish patients with MS from healthy individuals. The intergroup differences are very significant, despite the relatively small number of subjects studied. The 65 patients were chosen randomly in consecutive order of presentation, and α -GalC measurements were performed in a blind fashion. In addition, we could rule out any confounding variable for age, sex, or disease duration.

These observations imply that α -GalC antibodies can help stratify different MS subgroups, namely RR-MS, a novel finding with high clinical relevance. Patients with CIS by definition have had 1 apparent clinical attack, whereas patients with RR-MS are characterized by disease dissemination in time and space. A high proportion of CIS who present with brain magnetic resonance imaging (MRI) abnormalities will proceed to develop RRMS, (27,28) and indeed, for many of those patients, subclinical MS or minor attacks may have been present for a considerable period. Thus it can be envisioned that detection of α -GalC antibodies may permit staging of MS forms according to time from the first demyelinating event. Because these antibodies appear to be characteristic of

established MS, their detection in patients with early MS and CIS could potentially help correct and achieve an earlier diagnosis of definite MS than with conventional criteria. The antimyelin protein antibodies, on the other hand, have recently been described as potential predictors of early conversion in patients with CIS.(20)

5 Critical for interpretation of our clinical findings in the absence of longitudinal measurements in human MS was the study of chronic relapsing marmoset EAE, which best approximates MS complex pathophysiology. Specifically, we found that the α -GalC responses occurred distinctly after disease onset only in animals with RR forms of EAE (Table 7; Figure 30). This was in contrast to the α -rMOG and α -MBP responses that
10 occurred in all animals tested, in some cases even before clinical onset. These findings are in line with results from 2 other EAE models (29,30) in which α -GalC antibodies were also present in the early chronic stage of guinea pig EAE (29) and occurred after the clinical onset and after the development of α -MBP antibodies.(30) Reactivity against rMOG was not tested in either of these studies. Although the pathophysiological explanation for the
15 delayed antibody response to galactocerebroside in MS and EAE is not known, several mechanisms may be postulated. First, glycolipids are not classic, MHCrestricted T-cell antigens but may elicit a TH1 response via CD1 presentation.(31,32) CD1 expression has been demonstrated on astrocytes within MS lesions.(33) Glycolipid antigens may be presented to T cells only once detached from the membrane bilayer, yet the degradation of
20 myelin glycolipids by macrophages takes considerably longer than the breakdown of myelin proteins.(34) Second, lipids as such may be haptens and have to be attached to carrier proteins to elicit an immune response.(4) It has been proposed that MOG may serve as a carrier protein interacting with galactocerebroside within the cell membrane.35 These possibilities all may also explain the low titers of α -GalC antibodies, which are
25 considerably lower in human beings compared with titers of antibodies against myelin proteins (15), as in EAE models.(29) Antibodies reactive against galactocerebroside may have demyelinating properties, at least experimentally in vitro (10,12,13) and in vivo.(6,9,36)

 Although our study did not aim at proving any functional disability associated with
30 the presence of α -GalC antibodies in human beings, it is interesting to note that 40% of RR-MS cases studied have detectable α -GalC reactivity, which could potentially be indicative

of a particular RR-MS group in terms of disease course and severity. In addition, a lesser proportion of patients with SP-MS than with RR-MS appear to be α -GalC antibody-positive. This could mean that α -GalC autoantibodies predominate during a yet to be defined window of time that overlaps between RR-MS and SP-MS, with a tendency to decrease during the neurodegenerative stage of SP-MS. Future studies with larger numbers of subjects and longitudinal measurements are needed to address whether these antibody responses are associated with clinical (Expanded Disability Status Scale, progression rate, treatment response) or paraclinical (magnetic resonance imaging burden of lesion) parameters, and to establish their prognostic significance. In conclusion, we have demonstrated that α -GalC antibodies are a predominant phenomenon of RR-MS, and that in a primate disease model, the α -GalC response occurs significantly later than α -myelin protein responses. This galactocerebroside assay is available as a paraclinical investigation, in combination with MRI. In line with other recent reports on humoral immunity in MS and EAE, (20,37) these novel findings continue to underscore the value of α -myelin antibody assessment—both protein and glycolipid— as biomarkers that can be used for MS diagnostics, staging, and prognosis.

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EXAMPLE 8

Marmoset Derived Anti-Myelin Fab Fragments That Uniquely Stain Within Multiple Sclerosis Lesions

Myelin/oligodendrocyte glycoprotein (MOG) is a minor protein of CNS myelin and a target for pathogenic antibodies in experimental allergic encephalomyelitis (EAE) and multiple sclerosis.

Four monoclonal marmoset Fab fragments (Fabs) directed against (r) rat MOG125, two of which compete with naturally occurring human anti-MOG antibodies, and the mouse monoclonal antibody 8.18c5 were characterized for binding in various assay systems.

The most striking difference was the ability of the Fabs to stain degraded MOG in the center of MS-like EAE lesions, while 8.18c5 only bound to intact white matter. Additionally, the Fabs failed to react to human rMOG125 in solution as opposed to 8.18c5, indicative of reactivity to epitopes that are only exposed on degraded MOG. Further characterization of the fine epitope usage by Fabs and 8.18c5 by ELISA with human and rat rMOG, immunostaining of MOG-transfected cells and immunoblotting with denatured and native rMOG125 is underway.

Because the monoclonal Fabs that specifically stain degraded MOG cross-react with circulating antibodies found in MS patients, these reagents are contemplated to be useful as markers of endogenous MOG degradation in MS lesions.

This observation is very important. A classification based on immunopathology of MS lesions was proposed in 2000 by Lucchinetti et al. (Ann Neurol., 2000). They argued that type II lesions (and their type I) was characterized by loss of MOG, because they used the 818C5 and similar antibodies to stain tissues. The MOG protein as demonstrated here, is not lost in the marmoset EAE lesion which is the closest to "type II" MS lesions, mediated by antibody and complement. Rather, MOG has lost its original conformation and a different epitope is presented.

Note also, that 1. 818C5 does not cross-react with any of the marmoset Fabs previously described in WO 2004/034031 by Genain et al.; and 2. M3-24 was found to be displaceable by human MS IgG, therefore demonstrating that the MOG epitope it defines can be found in the repertoire of patients.

It can be inferred, that presence of antibody specificities like that of M3-24, or other that specifically stain lesions of EAE (MS), in patients indicate that such epitope has been exposed. This can only happen if the patient's endogenous myelin has been degraded and exposed to the immune system, short of a coincidental mimicry, which is unlikely.

We are researching if similar patterns of lesion staining can be found in MS brain, at the moment, and are finding out what exactly the Fab stain (oligos, myelin in macrophages, etc.). We have also produced antibodies against the CDR3 of the Fabs, or the Fabs themselves. Such antibodies can be used to find in humans whether they have these specific CDR3 bearing antibodies in their repertoire. Another way to track these is through competition experiments with labeled Fabs (WO 2004/034031 by Genain et al.), and/or RT/PCR-TaqMan.

The methods used to stain the lesions have been described in von Budingen et al., Proc Natl Acad Sci USA, 99:8207-8212, 2002.

25

EXAMPLE 9

Restriction Elements Of The Anti-Myelin Oligodendrocyte Glycoprotein Antibody Response In Primate Experimental Allergic Encephalomyelitis

These were obtained and sequenced from a second library constructed from an animal immunized with Rat rMOG 1-125 produced in *E. coli*.

30

GENBANK DQ005534 - *Callithrix jacchus* M6B MOG-specific
immunoglobulin heavy chain Fab fragment mRNA, partial cds.

VQLQESGAEVKKPGASARRSPARSSGYTFTTYSINWVRQPPGQGLEWMGGIDPE

5 YGSTSQTQKFQGRVTMTADTSTSTAYMELSSLRPEDTAVYYCASDGVAGVEYF

DYWGQGALVTS

(SEQ ID NO:47)

1 caggtgcagc tgcaggagtc aggggctgag gtgaagaagc ctggggcttc cgctagaagg

61 tctcctgcaa ggtcttctgg atacaccttc accacctatt ctatcaactg ggtgcgacag

10 121 cccctggac aagggctcga gtggatggga ggtattgac ctgaatatgg tagtacaagc

181 tacacacaga agttccaggg cagagtcacc atgaccgcgg acacgtccac gagcacagcc

241 tacatggagc tgagcagcct gagacctgag gacacggccg tgtattactg tgcgagtgat

301 ggggttgctg gcgttgaata ctttgactac tggggccagg gggccctggt cacctct

(SEQ ID NO:48)

15

GENBANK DQ005535 - *Callithrix jacchus* M7D MOG-specific
immunoglobulin heavy chain Fab fragment mRNA, partial cds.

VQLVESGAEVKQPGASVKVSKASGYTFTRYGMHWVRQAPGQGLEWMGWINT

NTGGTGAYAQKFQGRVTMTRDASTSTAYMELSSLRPEDTAVYYCATLSYYFDYW

20 GQGTLVTS

(SEQ ID NO:49)

1 gaggtgcagc tgggtggagtc tggggctgag gtgaagcagc ctggggcctc cgtgaaggtc

61 tcctgcaagg cttctggata caccttcacc aggtatggta tgcactgggt gcgacaggcc

121 cctggacaag ggctcgagtg gatgggatgg atcaacacca aactgggtgg cacaggctac

25 181 gcacagaagt tccagggcag agtcacatg accagggacg catccacgag cacagcctac

241 atggaactga gcagcctgag acctgaggac acggccgtgt attactgtgc gaccctctca

301 tattactttg actactgggg ccaggggacc ctggtcacct etc

(SEQ ID NO:50)

GENBANK DQ005536 - *Callithrix jacchus* M4A MOG-specific
immunoglobulin heavy chain Fab fragment mRNA, partial cds.

VQLVQSGAEVKKPGASVKVSCKASGYTFTSYVLNWVRQPPGQGLEWMGGIDPE

YGSTDYSQKFQGRVTMTADTSTSTAYMELSSLRPEDTAVYYCARYIAARSFYD

5 WGQGTTLVTV

(SEQ ID NO:51)

1 caggtgcagc tgggtgcagtc tggggctgag gtgaagaagc ctggggcctc cgtgaaggtc

61 tcctgcaagg cttctggata caccttcacc agctatgttc tcaactgggt gcgacagccc

121 cctggacaag ggctcgagtg gatgggaggt attgatcctg aatatggtag tacagactac

10 181 tcacagaagt tccagggcag agtcaccatg accgcagaca cgtccacaag cacagcctac

241 atggagctga gcagcctgag acctgaggac acggccgtgt attactgtgc gcggtatata

301 gcagctcgtt cctttgacta ctggggccag gggaccctgg tcaccgtc

(SEQ ID NO:52)

15 GENBANK DQ005537 - *Callithrix jacchus* M12E MOG-specific
immunoglobulin heavy chain Fab fragment mRNA, partial cds.

VQLVQSGGGLVKPGGSMRLSCAASGFTFSNYGMHWVRQAPGKGLEWVSAIGTS

GDTYYTDSVKGRFTISRDNKNSLYLQMNSLRAEDTAVYYCARSSIAPLDYWG

QGTLVTV

20 (SEQ ID NO:53)

1 gaggtgcagc tgggtgcagtc tgggggtggc ttgggttaagc cagggggatc catgagactc

61 tcctgtgcag cctctggatt caccttcagt aactatggca tgcactgggt ccgccaggct

121 ccaggaaagg ggctggagtg ggtctcagct attggtacta gtggtgacac atactacaca

181 gactccgtga agggccgatt caccatctcc agagacaacg ccaagaattc gctgtatctg

25 241 cagatgaaca gcctgagagc cgaggacacg gccgtgtatt actgtgagc atcctcaata

301 gcacctcttg actactgggg ccaggggacc ctggtcaccg tc

(SEQ ID NO:54)

30 GENBANK DQ005538 - *Callithrix jacchus* M6B MOG-specific
immunoglobulin light chain Fab fragment mRNA, partial cds.

LVMTQSPSSLSASVGDRVTISCHVSQTISNRLAWYQQKPGKVPKLLIYEASTLQS

GVPSRFSGSGSGTDFTLTISLQPEDAATYYCQKHDSAPLTFGQGTKLEIK

(SEQ ID NO:55)

1 gagctcgtga tgaccagtc tccatcctct ctgtctgcat ctgtgggaga cagagtcact
 5 61 atttcttgtc atgttagtca gaccattagt aaccgggttg cctgggtatca gcagaaacca
 121 gggaaagtgc ctaaactcct gatctatgag gcatccacct tgcaatctgg ggtcccatct
 181 cggttcagtg gcagtggatc cgggacagat ttactctca ccataagcag cctgcaacct
 241 gaagatgctg cgacttatta ctgtcagaag catgacagtg ctccactcac tttcggccaa
 301 gggaccaaac tggagatcaa a

10 (SEQ ID NO:56)

GENBANK DQ005539 - *Callithrix jacchus* M7D MOG-specific
 immunoglobulin light chain Fab fragment mRNA, partial cds.

LTLTQSPALVSVTPGDKVTISCKASQDIDNDIYWYQQKPGDAPIFIIQEATTLVSGI

15 PPRFSGSGYGTDFTLTIDNIEPEDAAYYFCQDDNFPYTFGQGTRLEIK

(SEQ ID NO:57)

1 gagtcacac tcacgcagtc tccggcattg gtgtcagtga ctccaggaga caaagtcacc
 61 atctcctgca aagccagcca agacattgat aatgatattt actgggtatca acagaaacca
 121 ggagacgctc ctattttcat tatacaagaa gctactactc tggtttctgg aatcccaccg
 20 181 cgattcagtg gcagtgggta tgggacagat ttaccctca caattgataa catagaacct
 241 gaggatgctg cgtattactt ctgtcaacaa gatgataatt tcccgtacac tttcggccaa
 301 gggaccaggc tggagatcaa a

(SEQ ID NO:58)

25 GENBANK DQ005540 - *Callithrix jacchus* M4A MOG-specific immunoglobulin
 light chain Fab fragment mRNA, partial cds.

LTLTQSPATLSLSPKETATLSCRASQSVRSYLAWYQQKPGQAPRLLIYGASTRAT

GIPARFSGSGSGTDFTLTISLQPEDFAVYYCQQYSSWPFTFGPGTKVEIK"

(SEQ ID NO:59)

30 1 gagtcacac tcacgcagtc tccagccacc ctctctttgt ccccaaaaga aacagccacc

61 ctctcctgca gggccagtca gagggttaga agctacttag cctggtacca gcagaaacct
 121 gggcaggctc ccaggctcct catctatggt gcatccacca gagccactgg catcccagcc
 181 aggttcagcg gcagtgggtc tgggacagac ttcactctca ccatcagcag cctggagcct
 241 gaagattttg cagtttatta ctgtcagcag tatagcagct ggccatttac ttttggcccc
 5 301 gggaccaaag tggaaatcaa a
 (SEQ ID NO:60)

GENBANK DQ005541 - *Callithrix jacchus* M12E MOG-specific
 immunoglobulin light chain Fab fragment mRNA, partial cds

10 LVLTQSPATLSLSPKETATLSCRASQSVSSYLAWYQQKPGQAPRLLIYGASTRAT
 GIPARFSGSGSGTDFTLTISSLEPEDFAVYYCQQYSSWPTFGQGTKLEIK
 (SEQ ID NO:61)

1 gagctcgtgt tgacacagtc tccagccacc ctctctttgt ctccaaaaga aacagccacc
 61 ctctcctgca gggccagtca gagggttagt agctacttag cctggtacca gcagaaacct
 15 121 gggcaggctc ccaggctcct catctatggt gcatccacca gagccactgg catcccagcc
 181 aggttcagcg gcagtgggtc tgggacagac ttcactctca ccatcagcag cctggagcct
 241 gaagattttg cagtttatta ctgtcagcag tatagcagct ggcccacttt cggccaaggg
 301 accaagctgg agatcaaa
 (SEQ ID NO:62)

20

These antibodies and the sequences disclosed herein are contemplated to be suitable for use in various embodiments of the present invention, including but not limited to defining further MOG epitopes. These antibodies are contemplated to compete with human antibodies for binding to MOG, expression of which can be detected in subject by PCR or
 25 specific anti-peptide CDR3 or anti-Fab antibody (e.g., to indicate the existence of various classes of anti-MOG antibodies). Thus, in certain embodiments, the antibody specific for a conformational epitope of myelin/oligodendrocyte glycoprotein is an antibody that specifically binds to an epitope specifically bound by one or more of the antibodies described above. The detecting can, optionally involve a competitive assay using a
 30 competitive binder an antibody comprising a CDR3 comprising a peptide sequence above,

and the like. Additionally, such epitopes are contemplated to be suitable targets for autoantibody inhibitors for characterization and/or treatment of MS. Further sequences suitable for use in various embodiments of the present invention are listed below.

5 SEQ ID NO:14

GENBANK AF393235

Callithrix jacchus M26 MOG-specific immunoglobulin heavy chain Fab
fragment mRNA, partial cds.

```

10 1 caggtgcagc tgggtgcagtc tggggcggag gtgaagaagc ctggggcctc cgtgaaggtc
    61 tcctgcaagg cttctggata caccttcacc agctatgcta tcagctgggc gcgacagccc
    121 cctggacaag ggctcgagtg gatgggagct tttgatacctg aatatggtag tacaacctac
    181 gcacagaagt tccagggcag agtcacatg accgcagaca cgtccacaag cacagcctat
    241 atggagctga gcagcctgag acctgaggac acggccgtgt attactgtgc gagagatgtt
    301 aacttcggta actatcttga ctactggggc caggggaccc tggtcaccgt ctcctcagcc
15 361 tccaccaaga acccagatgt cttccccctg

```

SEQ ID NO:15

Translation of GENBANK AF393235

QVQLVQSGAEVKKPGASVKVSKASGYTFTSYAISWARQPPGQG

```

20 LEWMGAFDPEYGSTTYAQKFQGRVTMTADTSTSTAYMELSSLRPEDTAVYYCARDVNF
    GNYFDYWGGQTLVTVSSASTKNPDVFPL

```

SEQ ID NO:16

GENBANK AF393237

25 *Callithrix jacchus* M38 MOG-specific immunoglobulin heavy chain Fab
fragment mRNA, partial cds

```

    1 caggtgcagc tgggtgcagtc tggggctgag gtgaagaagc ctggggcctc cgtgaaggtc
    61 tcctgcaagg cttctggata caccttcacc agctatgcta tcagctgggt gcgacagccc
    121 cctggacaag ggctcgagtg gatgggaggt gttgatacctg aatatggtag tacaacctac
30 181 acacagaagt tccagggcag agtcacatg accacagaca cgtccacaag cacagcctac
    241 atggagctga gcagcctgag acctgaagac acggccgtgt attactgtgc gagagatcgc
    301 ggtatgggga attactttga ctactggggc caggggaccc tggtcaccgt ctcctcagcc
    361 tccaccaaga acccagatgt cttccccctg

```

35 SEQ ID NO:17

Translation of GENBANK AF393237

QVQLVQSGAEVKKPGASVKVSKASGYTFTSYAISWVRQPPGQG

LEWMGGVDPEYGGTTYTQKFQGRVTMTTDTSTSTAYMELSSLRPEDTAVYYCARDGM

GNYFDYWGQGTLLVTVSSASTKNPDVFPL

SEQ ID NO:18

GENBANK AF393239

- 5 *Callithrix jacchus* M45 MOG-specific immunoglobulin heavy chain Fabfragment mRNA, partial cds.

1 caggtgcagc tgggtgcagtc tggggcgagg gtgaagaagc ctggggcctc cgtgaaggtc
61 tcttgcaagg cttctggata caccttcacc agctatggta tgcagtgggt gcgacaggcc
121 cctgaacaag ggctcgagtg gatgggatgg atcaatacca aactgggtgg cacaagctac
10 181 gcacagaagt tccagggcag agtcaccatg accagggacg catccacgag tacagcctac
241 atggagctga gcagcctgag acctgaggac acggccgtgt attactgtgc gagagatgca
301 acacgtatac tagcggacgt tcttgactac tggggccagg ggaccctggt caccgtctcc
361 tcagcctcca ccaagaaccc agatgtcttc

- 15 SEQ ID NO:19

Translation of GENBANK AF393239

QVQLVQSGAEVKKPGASVKVSKASGYTFTSYGMQWVRQAPQQG

LEWMGWINTNTGGTSYAQKFQGRVTMTRDASTSTAYMELSSLRPEDTAVYYCARDATR

ILADVLDYWGQGTLLVTVSSASTKNPDVF

20

SEQ ID NO:20

GENBANK AF393229

Callithrix jacchus M3-8 MOG-specific immunoglobulin heavy chain Fab fragment mRNA, partial cds.

- 25 1 caggtgcagc tgcaggagtc agggggaggc ttggttcagc ccgggggggtc cctgagactc
61 tcttggtcgg cctctgaatt caccttcagt aactactaca tgagctgggt ccgccaggct
121 ccaggggaagg ggctggagtg ggtctcatat attagttatg atggtggtag cacgtactac
181 gcagactccg tgaagggccg attcaccatc tccagagaca acgccaagaa ctcgctgtat
241 ctgcagatga acagcctgag agccgaggac acggccgtgt attactgtgc gagagcgtgg
30 301 cggctgtcgg ctagagctgg gtactttgac tactggggcc aggggaccct ggtcaccgtc
361 tcttcagcct ccaccaagaa cccagatgtc

SEQ ID NO:21

Translation of GENBANK AF393229

- 35 QVQLQESGGGLVQPGGSLRLSCAASEFTFSNYYMSWVRQAPGKG

LEWVSYSISYDGGSTYYADSVKGRFTISRDNKNSLYLQMNSLRAEDTAVYYCARAWRL

SARAGYFDYWGQGTLLVTVSSASTKNPDV

SEQ ID NO:22

GENBANK AF393231

Callithrix jacchus M3-24 MOG-specific immunoglobulin heavy chain

Fab fragment mRNA, partial cds

1 gaggtgcagc tggaggagtc tgggggaggc ttggttcagc ccgggggggtc cctgagactc
 5 61 tcctgtgctg cctctggatt caccttcagt gactactacg tgaactgggt ccgccagact
 121 ccggggaagg gccagagtg ggtaggtttt attagaaaca aagccaatgg tgggacagcg
 181 gaatacgccg cgtctgtgaa aggccgattc accatctcaa gagatgattc aaagaactcg
 241 ctgtatctgc aaatgagcgg cctgaaaacc gaggacacgg ccgtatatta ctgtatacta
 301 tcggatacgg gcgcttttga tgtatggggc caagggacca tggtcaccgt ctcttcagcc
 10 361 tccaccaaga acccagatgt cttccccctg

SEQ ID NO:23

Translation of GENBANK AF393231

EVQLVESGGGLVQPGGSLRLSCAASGFTFSDDYYVNWVRQTPGKG

15 PEWVGFI RNKANGGTA EYAASVKGRFTISRDDSKNSLYLQMSGLKTEDTAVYYCILSD
 TGAFDVWGQGTMTVSSASTKNPDVFPL"

SEQ ID NO:24

GENBANK AF393233

20 *Callithrix jacchus* M3-31 MOG-specific immunoglobulin heavy chain

Fab fragment mRNA, partial cds

1 caggtgcagc tgcaggagtc agggggaggc ttggcaaagc ctggggggtc cctgagactc
 61 acctgtgctg cctctggatt caccttcagt gactactgga tgagctgggt ccgccaggct
 121 ccaggggaagg ggttggagtg ggttggagaa attaatacctg atgggggtag aacaaactac
 25 181 aaagacttcg tgaaaggccg attcaccatc tccagagaca acgccaagaa cacactttat
 241 ctgcaattga acagccttaa aaccgaggac acagccatct attactgtac tggagctggg
 301 cccacatatt actttgacta ctggggccag gggaccctgg tcaccgtctc ctcagcctcc
 361 accaagaacc cagatgtctt ccccctgaca

30

SEQ ID NO:25

Translation of GENBANK AF393233

QVQLQESGGGLAKPGGSLRLTCAASGFTFSDDYWMSWVRQAPGKG

LEWVGGEINPDGGRITNYKDFVKGRFTISRDNKNTLYLQLNSLKTEDTAIYYCTGAGPT

35 YYFDYWGQGTTLVTSSASTKNPDVFPLT

SEQ ID NO:26

GENBANK AF393236

Callithrix jacchus M26 MOG-specific immunoglobulin light chain Fab

fragment mRNA, partial cds.

```

1 gagctcgtga tgactcagtc tccagccacc ctgtctttgt ctccagggga aagagccacc
61 gtctcctgca gggccgggtca gagggttagt tactacttag cctggtacca gcagaaacct
121 gggcaggctc ccaggctcct catctatggt gcatccacca gagccactgg catcccagcc
5 181 aggttcagcg gcagtaggtc tgggacagac ttcactctca ccatcagcag cctggagcct
241 gaagattttg cagtttatta ctgtcagcag tatagcagct ggccaccac tttcggccaa
301 gggaccaagc tggagatcaa acgagctgtg gctgcgccgt ctgtcttcat cttcccgcca
361 tctgaggatc aggtgaaatc tggaactgcc

```

10 SEQ ID NO:27

Translation of GENBANK AF393236

ELVMTQSPATLSLSPGERATVSCRAGQSVSYLLAWYQQKPGQAP

RLLIYGASTRATGIPARFSGSRSGTDFTLTISLEPEDFAVYYCQYSSWPPTFGQGT

KLEIKRAVAAPSVFIFPPSEDQVKSGTA"

15

SEQ ID NO:28

GENBANK AF393238

Callithrix jacchus M38 MOG-specific immunoglobulin light chain Fabfragment mRNA, partial cds.

```

20 1 gagctcgtga tgactcagtc tccagccacc ctctctttgt ctccaaaaga aacagccacc
61 ctctcctgca gggccagtc gagggttagt agctacttag cctggtacca gcagaaacct
121 gggcaggctc ccaggctcct catctatggt gcatccacca gagccactgg catcccagcc
181 aggttcagcg gcagtggtc tgggacagac ttcactctca ccatcagcag actggagcct
241 gaagattttg cagtttatta ctgtcagcag tatagcagct ggccactcac tttcggccaa
25 301 gggaccaagc tggagatcaa acgagctgtg gctgcgccgt ctgtcttcat cttcccgcca
361 tctgaggatc aggtgaaatc tggaactgcc

```

SEQ ID NO:29

Translation of GENBANK AF393238

30 ELVMTQSPATLSLSPKETATLSCRASQSVSSYLLAWYQQKPGQAP

RLLIYGASTRATGIPARFSGSGSGTDFTLTISRLEPEDFAVYYCQYSSWPLTFGQGT

KLEIKRAVAAPSVFIFPPSEDQVKSGTA

SEQ ID NO:30

35 GENBANK AF393240

Callithrix jacchus M45 MOG-specific immunoglobulin light chain Fab fragment mRNA, partial cds.

```

1 gagtcacac tcacgcagtc tccagtcacc ctctctttgt ctccaaaaga aacagccacc
61 ctctcctgca gggccagtc gagggttaga agctacttag cctggtacca gcagaaacct

```

121 gggcaggtc ccaggtcct catctatggt gcatccacca gagccactgg catcccagcc
 181 aggttcagcg gcagtgggtc tgggacagac ttcactctca ccatcagcag cctggagcct
 241 gaagattttg cagtttatta ctgtcagcag tatagcagct ggtacacttt cggccaaggg
 301 accaagctgg agatcaaacg agctgtgggt gcgcgctctg tcttcactct cccgccatct
 5 361 gaggatcagg tgaaatctgg aactgccact

SEQ ID NO:31

Translation of GENBANK AF393240

ELTLTQSPVTLSPKETATLSCRASQSVRSYLAWYQQKPGQAP

10 RLLIYGASTRATGIPARFSGSGSTDFTLTISSLEPEDFAVYYCQYSSWYTFGQGTK
 LEIKRAVAAPSVFIFPPSEDQVKSGTAT

SEQ ID NO:32

GENBANK AF393230

15 Callithrix jacchus M3-8 MOG-specific immunoglobulin light chain Fab
 fragment mRNA, partial cds.

1 gagctcacac tcacgcagtc tccatcctcc ctgtctgcat ctgtaggaga cagagtcacc
 61 atcacttgcc gggcgagtca ggacattaga gggtatttag cctgggtatca acagaaacca
 121 gggaaatctc ctaggcttct gatctattct gcatctactt tgcaaactgg ggttccctct
 20 181 cggttcagtg gcagtagatc tgggacagac tacactctca ccatcagcag cctgcagtct
 241 gaagatgtgg caacttatta ttgtcaacag cattacagta ctccactcac ttccggccaa
 301 gggaccaagc tggagatcaa acnagctgtg gctgcgccgt ctgtcttcat cttccgcga
 361 tctgaggatc aggtgaaatc tggaactgcc

25 SEQ ID NO:33

Translation of GENBANK AF393230

ELTLTQSPSSLSASVGDRVTITCRASQDIRGYLAWYQQKPKGKSP

RLLIYSASTLQTGVPSRFSRSGTDYTLTISSLQSEDVATYYCQQHSTPLTFGQGT

KLEIKXAVAAPSVFIFPPSEDQVKSGTA"

30

SEQ ID NO:34

GENBANK AF393232

Callithrix jacchus M3-24 MOG-specific immunoglobulin light chain
 Fab fragment mRNA, partial cds

35 1 gagctcgtga tgacgcagtc tccagccacc ctctctttgt ccccaaaaga aacagccacc
 61 ctctcctgca gggccagtca gagtgtaga agctacttag cctgggtacca gcagaaacct
 121 gggcaggtc ccaggtcct catctatggt gcatccacca gagccactgg cataccagcc
 181 aggttcagcg gcagtgggta tgggacagac ttcactctca ccatcagcag cctggagcct
 241 gaagattttg cagtttatta ctgtcagcag tatagcagct ggtacacttt cggccaaggg

301 accaagctgg agatcaaacg agctgtgggt gcgccgactg tcttcattctt cccgacatct
 361 gaggatcagg tgaaatctgg aactgccact

SEQ ID NO:35

5 Translation of GENBANK AF393232

ELVMTQSPATLSLSPKETATLSCRASQSVRSYLAWYQQKPGQAP
 RLLIYGASTRATGIPARFSGSGYGTDFTLTISSLEPEDFAVYYCQQYSSWYTFGQGTK
 LEIKRAVAAPTVMFIFPTSEDQVKSGTAT"

10 SEQ ID NO:36

GENBANK AF393234

Callithrix jacchus M3-31 MOG-specific immunoglobulin light chain
 Fab fragment mRNA, partial cds.

1 gagctcgtga tgactcagtc tccatcctcc ctgtttgcat ctataggaga cagagtcacc
 15 61 attacttgcc gggcgagtca gaacattaga agtaatttag cctgggtatca acagaaacca
 121 ggaaaaactc ctaggctcct gatctatgat gcatctagtt tgcaacctgg gattccctct
 181 cggttcagtg gcagtggatc tgggacatat tacactctca ccatcagcag cctgcagtct
 241 gatgatcttg ccacttatta ctgtcaacaa gggtatacta ctccagtcac ttccggccaa
 301 gggaccaagc tggagatcaa acgagctgtg gctgcgccgt ctgtcttcat cttcccgcca
 20 361 tctgaggatc aggtgaaatc tggaactgcc

SEQ ID NO:37

Translation of GENBANK AF393234

ELVMTQSPSSLFASIGDRVTITCRASQNIRSNLAWYQQKPGKTP
 25 RLLIYDASSLQPGIPSRFSGSGSGTYITLTISLQSDDLATYYCQQGYTTPVTFGQGT
 KLEIKRAVAAPSVFIFPPSEDQVKSGTA

EXAMPLE 10

Additional Data

30 Figure 31A incorporates additional results. We have examined 33 controls
 (including older subjects to better match the age distribution of progressive MS), 27 RRMS,
 26 SPMS, and 41 PPMS. While the frequency of anti-MOG antibodies is high in the
 controls (54%), the data confirm the high prevalence of anti-MOG antibodies in the SPMS
 and PPMS (85% and 93% respectively) and the high prevalence of anti-MBP antibodies in
 35 SPMS.

Data were obtained by ELISA using recombinant rat MOG 1-125 produced in *E. coli* as the antigen. Figure 31B illustrates the linearity of our standard Ig curves to calculate antibody concentrations.

5

EXAMPLE 11

Differential Reactivity Of Human IgG To Rat And Human MOG 1-125 Recombinant Proteins (Produced In *E. coli*)

The reactivity of serum from PPMS patients and controls (HC) was assessed using both the rat and human recombinant MOG 1-125 proteins. In contrast to the significant
10 difference detected between PPMS and HC when using rat MOG1-125, no difference was observed when using the human MOG 1-125 in identical ELISA reactions (see, e.g., Figure 32). This, together with the differential binding of Fab fragments to these different polypeptides, indicates that rat MOG1-125 is a suitable reagent for detection of an epitope of MOG that may be uniquely present in PPMS. The antibodies against these epitopes could
15 be directly involved in MS pathogenesis, and/or uniquely arise in this form of MS.

In any event, this discovery highlights the usefulness of rat MOG as a reagent to detect presence of antibodies directed against this epitope, in the absence of human MOG. Similarly, the rat MOG1-125 is contemplated to be a useful reagent for characterization of mimics, peptides, proteins, small molecules or other compounds that bind to or inhibit
20 binding of antibodies specific for this epitope. Such molecules can readily be identified through binding studies with the monoclonal Fab fragments described herein. In the current assay conditions, it is likely that overlap of reactivity observed in human sera with the different recombinants of MOG is due to some degree of cross-reactivity to several epitopes, which cannot be easily separated in complex mixtures of polyclonal antibodies
25 such as those found in serum. The identification of ligands specific for unique epitopes of MOG is particularly useful for studies of fine epitope specificity of MOG antibodies in humans, and for understanding of their clinical significance or value as paraclinical biomarkers.

30

EXAMPLE 12**Quantitative Differences Of IgG Titers In The Various MS Subtypes**

Using direct quantitation of IgG against MOG as described above, we found that antibody titers in certain patients with PPMS were markedly elevated (~10 times),
5 compared to healthy controls and subjects with other forms of MS. This is an important finding, as these differences cannot be observed using other methods to measure antibodies, or if serum is tested at a low dilution. We have observed that anti-MOG antibodies (as measured by ELISA against rat MOG1-125), could be commonly detected at serum dilutions of 1:6,000 and greater in PPMS.

10 It can be inferred that the quantitative ratios of Ig concentrations (IgG, IgM and other subtypes) against the different antigens can be helpful to diagnose the different MS subtypes and stages.

EXAMPLE 13

15 **Differential Reactivity Against Solid-Phase And Liquid-Phase Myelin/Oligodendrocyte Glycoprotein In MS And EAE**

Myelin protein myelin/oligodendrocyte glycoprotein (MOG) is a potent encephalitogenic antigen in experimental allergic encephalomyelitis (EAE), the animal model of multiple sclerosis (MS), and a target for pathogenic demyelinating antibodies. In
20 humans however, circulating anti-MOG antibodies as detected by current methods do not appear to be specifically associated with disease, as these antibodies can be found in healthy individuals.

This example pertains to the validation of a novel, non-isotopic assay for detection of serum antibodies that bind to MOG in aqueous solution and to compare the binding
25 characteristics of MOG presented in free soluble form vs. adsorbed on a solid support.

The extracellular portion of human MOG (rhMOG125) was expressed in *E. coli*. A protein soluble in non-denaturing buffer was purified to homogeneity, and biotinylated. Sera of 37 MS patients and 13 healthy control subjects (HC), all reactive to rhMOG125 in solid-phase ELISA, were tested for reactivity using the biotinylated rhMOG125 in solution
30 followed by capture of immunocomplexes on platebound protein G and detection by

peroxidase-labeled streptavidin. Biotinylated tetanus toxoid (TT) was used as a positive control. To analyze binding properties of the soluble and plate-adsorbed proteins, a panel of marmoset monoclonal Fab fragments (Fabs), the murine monoclonal antibody 8.18.C5 and plasma from 15 *C. jacchus* marmosets known to contain polyclonal reactivity against native
5 MOG in human white matter (HWM), recombinant ratMOG1-125, or linear 20-mer MOG peptides were used.

No reactivity against solution-phase rhMOG125 was detected in humans, in contrast to high anti-TT reactivity. By that method and to ELISA which measured equal reactivity against plate-adsorbed rhMOG125 and TT ($p < 0.001$, Pearson's correlation). In marmosets,
10 sera from MOG peptide immune animals which do not contain conformational anti-MOG antibodies reacted poorly to solution-phase rhMOG125 unlike sera of ratMOG- or HWM-immune marmosets which showed equal reactivity against rhMOG125 in either the solution- or solid-phase assays ($p < 0.001$, SNK T-test). Using the 8.18.C5 and Fabs which have monoclonal single epitope specificities, we identified patterns of reactivity that
15 indicate that the differences between solid and liquid phase systems are due to detection of separate individual epitopes by each technique, in addition to other epitopes detected in both. It is also likely that overall, antibody affinity against soluble rhMOG125 is higher than that against the solid support-adsorbed protein. Conclusions: Our observations are in line with two recent studies that failed to detect anti-myelin antibodies in liquid phase. However,
20 we are able to provide a rationale justification of this phenomenon for the first time, using the same antigen (rhMOG125) that seems to undergo structural changes or multimerization, resulting in differential epitope exposure and/or masking of epitopes situated on hydrophobic surfaces. Because pathogenic antibodies that bind to solid phase MOG can be easily demonstrated experimentally, it is incorrect to equate the lack of detection in liquid
25 phase with the lack of antibody pathogenicity in humans. Antibody assays employing solution phase rhMOG125 may not be suitable for serum antibody studies in MS most likely because of the biological nature of this membrane-bound, water insoluble antigen.

INTRODUCTION

There is growing evidence that anti-myelin antibodies play an important role in the
30 pathogenesis of multiple sclerosis (MS) namely antibodies directed against myelin/oligodendrocyte glycoprotein (MOG) an exposed antigen mostly expressed in

central nervous system (CNS) myelin. In the disease model experimental allergic encephalomyelitis (EAE), passive anti-MOG antibody transfer in primed animals induces demyelination, a hallmark of MS-plaque pathology 1-4. In active immunization experiments, the extracellular domain of MOG is highly encephalogenic and leads to
5 demyelinating disease, even when produced recombinantly in E.coli, i.e. non-glycosylated. In MS, there is controversy about the pathogenic involvement of anti-MOG antibodies. Because anti-MOG responses have been detected not only in MS patients, but also in healthy control (HC) donors and patients with other inflammatory neurological diseases 5-8. It is important to recognize however, that different recombinant (r)MOG forms of the
10 extracellular domain variable in length have been used in different assay systems, i.e. enzyme linked immunosorbent assays (ELISA), Western blots, or cell based spot-ELISA. Not only do these methodological differences influence the ability to detect antibodies, but they may also measure antibodies with very different biological properties: it has recently been demonstrated that epitope recognition may critically influence antibody pathogenicity
15 (Brehm, Briethaupt, von Budingen). It is thus becoming increasingly important to define anti-MOG antibody reactivity at the molecular and epitope level, to understand which technique is best to provide this information, and to understand the potential value of these paraclinical measures.

Recently, a study supported the notion of anti-myelin antibodies as prognostic tools
20 in MS, demonstrating that patients with clinically isolated syndromes (CIS) positive for anti-MOG and anti-MBP IgM in serum will suffer from the second MS-defining relapse more rapidly 9.

Regardless of their pathogenic involvement, serum autoantibodies have been regarded as prognostic factors in a number of autoimmune diseases, e.g. insulindependent
25 Diabetes mellitus (IDDM) or systemic Lupus erythematoses (SLE). In IDDM it is consensus to assess patients' antibody status by means of solution-phase based radio-immuno-assays (RIA) rather than ELISA 10. Two recent studies have addressed the question whether RIA would be superior to ELISA in testing anti-MOG antibodies in MS11, 12, and could not detect elevated antibody titers against MBP 11 or glycosylated
30 fulllength MOG 12 in either MS or HC sera.

Here we present an easy-to-perform liquid-phase based enzyme linked immune assay (LiPhELIA) that instead of 35S-labelling requires only antigen biotinylation. We fail to detect specific anti-MOG binding in MS sera, but provide evidence for the lack of binding by polyclonal binding data of the marmoset EAE and monoclonal anti-MOG antibodies.

MATERIAL AND METHODS

Human Subjects

Thirteen healthy control (HC) sera and 37 sera of patients seen in our MS center were included in this study; they were identified from a larger cohort of patients and HC to sample a broad range of anti-rhMOG125 (aa 1-125, extracellular domain prepared in *E. coli*, see below) as measured by ELISA. Individuals treated with cortico-steroids within three months or on immunosuppressive therapy within six months of blood draw were excluded. All patients met criteria for clinically definite MS13. 17 patients had a relapsing-remitting (RR) disease course, 10 patients had secondary-progressive (SP)-MS and 10 patients primary-progressive (PP)-MS. The patients' characteristics are summarized in Table 8. Blood was drawn by venipuncture and clotted serum stored at -40°C. Informed consent was obtained from the patients and HC, and the study was conducted in accordance with Institutional Review Board approval.

Table 8. Clinical Demographics Of Multiple Sclerosis (MS) Patients And Healthy Control (HC) Volunteers.

	Multiple sclerosis	Healthy controls
N	37	13
RR-MS (%)	17 (46)	
SP-MS (%)	10 (27)	
PP-MS (%)	10 (27)	
Gender (m/f)	12/25	4/6
Age (yr, median)	45	55
Range	25 – 67	33 - 73
EDSS (median)	3.0	
range	1.0 – 8.5	
Disease duration (m, median)	117	
Range	4 – 348	

Gender and age distributions between MS and HC are not significantly different (χ^2 test and two-tailed T test, respectively).

5 Animals

C. jacchus marmosets were cared for in accordance with the guidelines of the Institutional Animal Care and Usage Committee. EAE was induced by immunization with either 100 mg of human white matter homogenate (HWM), 50 μ g rat rMOG125 or 100 μ g of 20mer MOG-peptides as previously described^{14,15}. Plasma samples were obtained from
10 EDTA-anticoagulated blood at baseline, and 4-9 weeks post immunization (p.i.) and stored at -40°C.

Antigens and antibodies

For recombinant human myelin/oligodendrocyte glycoprotein (rhMOG) an expression vector was constructed; the cDNA encoding for the extracellular domain of

rhMOG spanning the amino acids 1-125 (rhMOG125) was amplified using pfu-Polymerase (Promega) from a custom-made human brain cDNA library using the following primers to create a Nco I restriction site at the 5'-end and a Bgl II site at the 3'-end, respectively: 5'-CGG GGA CCA TGG GGC AGT TCA GAG TGA TAG GAC CAA GAC A-3' (SEQ ID NO: 63) and 5'-ATC CAT GAG ATC TAG GAT CTT CTA CTT TCA ATT CCA TTG CTG CC-3' (SEQ ID NO:64). After digestion with Nco I and Bgl II (NEB) and agarose-gel extraction of DNA (QIA-Quick, Qiagen) the target gene was ligated into the Nco I/Bgl II digested and gel purified pQE60 plasmid (Qiagen) using the Clonables kit (Novagen) and DNA purified by Miniprep (QIA-Spin, Qiagen). The construct was sequenced at the UCSF Genomics Core Facility and correct and in-frame insertion of the rhMOG125 target gene ascertained. M15 cells (Qiagen) conditioned for chemical transformation according to the manufacturer's protocol were transformed for protein expression.

Transformed M15 cells were expanded in LB medium supplemented with carbenicillin and kanamycin and induced by IPTG according to the manufacturer's protocol. rhMOG125 was extracted from the cytoplasmic fraction of the *E. coli* by cell lysis under gentle native conditions using the B-PER in PBS reagent (Pierce). Purification was carried out under non-denaturing conditions in 20 mM sodium phosphate (SP), 500 mM NaCl, 10 % glycerol and 0.05 % sodium deoxycholate (DOC), pH 8.0 (MOG-buffer) using a Ni-NTA FPLC (HiTrap, Amersham), through the pQE60's carboxy-terminal His-tag. Protein was eluted by linear gradient of 250 mM imidazole, 20 mM SP, 1 M NaCl, 25 % glycerol, 0.05 % DOC, pH 8.0. The eluted protein was dialyzed into MOG-buffer, containing 15 % glycerol, purity confirmed to be >95 % by 10-20 % SDS-PAGE.. Bacterial endotoxin contaminations were below detection limits of 0.06 EU/mL as determined by the Pyrogen Plus kit (LAL, BioWhittaker).

Recombinant rat MOG, amino acids 1-125 (rat rMOG125) was produced in *E. coli* and 20mer linear MOG-peptides with 10-mer overlaps were purified as described previously (HCvB, EJI 2004). Tetanus toxoid (TT) was obtained from Wyeth-Ayerst as formulated for vaccination and dialyzed extensively against PBS.

The mouse anti-rat MOG monoclonal antibody 8.18C5 (IgG) was a gift of Dr. Chris Linington. The marmoset Fab-fragments (Fabs) designated M26, M3-24, and M3-31 derived from ratMOG-immunized animals were generated in our laboratory as described

previously 16. These Fabs were chosen according to their binding characteristics that define separate epitopes on the ratMOG on a solid support, and none compete with the 8.18C5-defined epitope.

Solid-phase enzyme-linked immunosorbent assay (ELISA)

5 rhMOG125, diluted to 5 µg/mL in PBS, and TT were coated at 100 µL/well in 96-well microtiter plates (Maxisorb, Nunc). After incubation at 4°C overnight plates were washed 3-times in PBS and 3-times in ddH₂O followed by 2 hrs blocking at room temperature (RT) with 200 µL/well of 1 % bovine serum albumin (BSA, A7030, Sigma) in PBS, supplemented with 0.05 % Tween-20 (PBS-T) (ELISA buffer). After washing 3-times
10 with PBS-T and 3 times ddH₂O serum or purified antibodies were diluted in ELISA buffer and 100 µL/well incubated in duplicates for 90 min at RT. Human sera were diluted 1:200 for rhMOG125 and 1:600 for TT, and marmoset sera studied serial dilutions beginning at 1:1,000; 8.18c5 and Fabs were diluted to 100 ng/well. Duplicate wells without antigens were used as individual background controls. After washing as above, bound human serum
15 antibodies were detected by alkaline phosphatase (AP) labelled anti-human-IgG (A9455, Sigma), marmoset serum antibodies and Fabs by peroxidase (HRP)-conjugated anti-monkey-IgG (A2054, Sigma) and 8.18c5 by HRP-conjugated anti-mouse IgG (A9044, Sigma), diluted in ELISA buffer. After incubation for 1 hr at RT plates were washed as above. For human sera, binding was detected by reading the optical density (OD) at 405 nm
20 in a microplate reader (SpectraMax, Molecular Devices, Sunnyvale, CA) after incubation with para-nitrophenyl phosphate (pNPP, Moss, Pasadena, MD) for 30 minutes in the dark at RT. The marmoset sera, Fabs and 8.18c5 were developed with 3,3',5,5'-tetramethylbenzidine (TMB, Pierce, Rockford, IL) for 15 minutes at RT and the OD read at 450 nm wavelength.

25 A signal-to-background binding ratio was calculated as the ratio of OD (signal) over OD (background). Positive controls, i.e. human sample with strong binding signal, and negative controls, i.e. ELISA buffer only omitting serum, were included on each plate.

Liquid-phase enzyme-linked immuno-assay (LiPhELIA)

TT and rhMOG125 were biotinylated at their primary amines via N-
30 hydroxysuccinimide (NHS-PEO4 biotin, Pierce) according to the manufacturer's protocol in

a 20:1 ratio for 30 min at RT. Unreacted biotin was eliminated by extensive dialysis against PBS (TT) or MOG-buffer (rhMOG125), respectively. A specific activity >10 biotin/mol was confirmed by the 2-Hydroxyazobenzen-4'-Carboxylic Acid/Avidin method (HABA/Avidin, Pierce). 2.5 ng/well of the biotinylated proteins were diluted in 0.1 % BSA-PBS-T and mixed with sera, Fabs or 8.18c5 in inert polypropylene vials or 96-well plates. In the case of sera, the diluent was supplemented with a protease inhibitor cocktail (Roche) and 1 mM PMSF (Sigma). Human sera were adjusted as for the ELISA. After overnight incubation at 4°C on a rocker 50 µL/well of the antigen-antibody mixtures were transferred to 96-well microtiter plates (Maxisorb, Nunc), that were previously coated with 1 µg/well Protein G' (Sigma) or 2 µg/well Protein L (Pierce) for Fab binding, respectively overnight at 4°C, washed with PBS and ddH₂O and blocked with Superblock (Pierce) according to the manufacturer's recommendations. After incubation for 1 hr at 4°C on a rocker, plates were washed with PBS-T and ddH₂O and bound immunocomplexed biotinylated protein detected by 50 µL/well HRP-conjugated streptavidin (50 ng/mL, Pierce), diluted in 1 % BSA-PBS-T. Plates were incubated for 1 hr at 4°C, washed as above and developed with TMB for 15 minutes at RT and the OD read at 450 nm wavelength. Background controls included biotinylated antigens omitting antibodies and antibodies omitting antigens. For sera results were expressed as binding ratio as described for ELISA.

Competitive inhibition assays and affinity measurements

Competition assays were performed in both ELISA and LiPhELIA according to the protocol of Friguet et al. with modifications 17. First, the concentration of rhMOG125 for a 50% of maximum binding was determined for ELISA and LiPhELIA. For ELISA measurements, 8.18c5 and Fabs were incubated in solution with rhMOG125 (biotinylated) in concentrations ranging from 1.0 nM to 1000 nM overnight at 4°C. These antigen-antibodies mixtures were then transferred to 96-well microtiter plates with rhMOG125-coated and background wells. The ELISA was performed as outlined above to detect free, not immunocomplexed antibody. In the LiPhELIA 8.18c5 and Fabs were incubated with a fixed concentration of biotinylated rhMOG125 (hot antigen) and varying concentrations of unlabelled rhMOG125 (cold antigen) ranging from 33 nM to 3300 nM in solution overnight and the assay performed as outlined above. In both assays, specificity controls omitting soluble rhMOG125 or cold antigen, respectively, were included.

Additional controls included wells with soluble rhMOG125 (ELISA) or cold antigen (LiPhELIA).

Dissociation constants (KD) were calculated as described 17 taking into account the OD without soluble rhMOG125 (ELISA) or cold antigen (LiPhELIA) (ODno Ag), the OD
5 with added soluble rhMOG125 or cold antigen (ODAg) and the concentration of the added soluble rhMOG125 or cold antigen (concAg). The KD were expressed as 10^{-7} M by the equation: $(ODno\ Ag)/(ODno\ Ag - ODAg) - 1 \times concAg$.

Statistical analysis

Statistics were performed using GraphPad Prism 3.0 software. Binding ratios were
10 shown to be normally distributed for both ELISA and LiPhELIA, and groups were compared by unpaired two-tailed T-test, multiple comparisons were analysed with one-way ANOVA followed by Student-Newman-Keuls (SNK) T-test. Correlations between ELISA and LiPhELIA were assessed by Pearson's correlation. Sample demographics were analyzed by unpaired two-tailed T-test and χ^2 -test where appropriate.

15 RESULTS

ELISA positive human sera do not bind to rhMOG125 in solution

The fifty human serum samples were chosen from a larger cohort according to anti-rhMOG125 binding ratios ranging from negative – defined as a binding ratio below 2 over background – to highly positive (ratio >20). Anti-TT binding ratios used as controls
20 exhibited a similar distribution in this cohort, reflecting the individual (Fig. 1A).

Conversely, in LiPhELIA none of the human serum samples bind to solutionphase rhMOG125, but reactivity to soluble TT remains unchanged (Fig. 34B). There was a high inter-assay correlation for anti-TT reactivity between ELISA and LiPhELIA ($r = 0.7$, $p < 0.001$, Pearson's correlation). The LiPhELIA for TT also appears more sensitive than the
25 ELISA approximately 80 times (50% binding: 1:600 vs. 1:16,200, Fig. 35). The detection cutoff in the ELISA is 1:5400, while it is 1:145,800 in LiPhELIA.

Several factors and conditions are addressed as reason for the lack in antirhMOG125 reactivity in LiPhELIA; to rule out detrimental protease-activity in human sera, maximal protease inhibitory activity is applied to the antigen antibody mixtures. To exclude

additional serum factors inhibiting the binding of human antibodies to soluble rhMOG125, IgG of four patients with high binding ratios in the ELISA were purified by means of individual Protein G spin columns. While anti-TT is present in both assays, rhMOG125 could only be detected in the ELISA. As apparent in Figure 35, the binding ratio follows rather an inversely u-shaped curve than the typically observed sigmoidal curve. Two samples with high anti-rhMOG125 reactivity in ELISA are tested in serial dilutions in LiPhELIA without observing improvement of reactivity. To exclude steric hindrance a batch of rhMOG125 was applied to a mild biotinylation protocol achieving biotinylation at less than 2 mols biotin/rhMOG125 as assessed by the HABA-assay; LiPhELIA was performed with 4 samples using this batch with identical results.

Marmoset EAE antibodies against linear MOG epitopes are not reactive in LiPhELIA, but ELISA ELISA and LiPhELIA differentially detect anti-MOG antibodies according to recognition of linear and conformational antigenic determinants

Five marmosets per group were immunized with either conformational (ratMOG, HWM) or linearized (MOG peptides) encephalitogenic MOG antigen and the antibody responses evaluated 4-9 weeks after immunization. The ELISA assay measured identical anti-rhMOG125 responses regardless of the immunizing antigen, e.g., MOG peptides, ratMOG or HWM (serum dilutions 1:1,000 and 1:4,000, n.s., SNK T-test). In contrast, in LiPhELIA, sera from MOG peptide immune marmosets, which contain only antibodies directed against linear determinants of MOG as demonstrated by fractionation studies (von Budingen, 2004), reacted poorly against rhMOG125 as compared to the sera from ratMOG- or HWM-immune animals ($p < 0.001$, SNK T-test for serum dilutions 1:1,000 and 1,4000, respectively, Fig. 36). Animals immunized with ratMOG are known to mount a polyclonal response that includes both linear and conformational specific determinants, while those immunized with HWM only produce conformational IgG antibodies (). When comparing in ELISA and LiPhELIA, reactivity patterns of ratMOG- or HWM-immune animals were almost identical (Fig. 36).

Following removal of all antibodies that bind to ratMOG from ratMOG- and HWM-immune sera as detected by ELISA, no reactivity could be detected in these samples by LiPhELIA. The latter experiments indicate that these sera did not contain antibodies against

determinants of MOG that are selectively detected in LiphELIA, and also eliminate the possibility of steric hindrance between ELISA-exposed and LiphELIA-exposed determinants. Rather, it is likely that ELISA detects a broader range of exposed determinants of MOG than LiphELIA.

5 **Studies of monoclonal antibody binding reveal differential presentation of rhMOG125 epitopes in ELISA and LiPhELIA**

The binding of the murine monoclonal antibody 8.18c5 that is directed against native rat cerebellar glycoproteins, and is known to recognize human MOG, and the 3 marmoset derived Fabs directed against recombinant rat MOG were used in order to refine
10 our understanding of exposed individual epitopes in LiphELIA and ELISA systems. None of these monoclonal species recognize any linear MOG epitope, as shown in previous studies (Brehm, von Budingen). We have established the binding characteristics of the marmoset Fabs M26, M3, which can be summarized as follows: 1., M26 , ... Represent different structurally defined epitopes as demonstrated by competition experiments. 2., none
15 of the marmoset fragments, or polyclonal anti-MOG marmoset IgG, compete with the 8.18.C5 antibody, indicating that these respective antibodies detect different structural epitopes. 3., the Fabs bind differentially to marmoset white matter in areas of lesions and normal appearing white matter:. While all four monoclonals show comparable anti-rhMOG125 reactivity in ELISA at equilibrium concentrations, the individual binding
20 reactivity patterns were markedly different in LiPhELIA, with the exception of 8.18c5 and Fab M-24, which showed reactivity comparable to that in ELISA (TTEST). For M26 and M3-31, binding to rhMOG125 in LiPhELIA was markedly reduced. (numbers). Because the binding for M3-24 was identical in ELISA and LiPhELIA this finding eliminates the possibility that differences observed with the other antibodies were due to technical
25 artifacts, in particular the use of Protein G vs. Protein L in the different assay systems.

The observed differences in binding to soluble vs. solidphase rhMOG125 are evident when calculating the respective antibody affinities as determined by the method of Friguet et al.¹⁷ Affinities were measured in ELISA against soluble antigen to determine the affinity against solid-phase rhMOG125 and in LiPhELIA against soluble antigen to
30 determine the affinity against solution-phase rhMOG125 (Table 9). The measured affinities are in the range of 10^{-6} to 10^{-8} M and are 10- to 35-fold greater in LiPhELIA as compared

to ELISA (Table 9). It appears that a KD of approximately 10^{-6} M may be the threshold for efficient epitope detection in liquid-phase, as the magnitude of binding for M26 and M3-31 is greatly reduced in LiPhELIA vs. ELISA, whereas the binding for 8.18c5 and M3-24 remain largely unchanged (Fig. 36).

5

Table 9. Differential Reactivity Of Monoclonal Marmoset Fab Antibody Fragments To Various Forms Of The MOG Polypeptide Antigen (Conformational Epitopes)

	KD (M)			Fold Difference
	ELISA	LiPhELIA		
8.18c5	0.28×10^{-7}	4.14×10^{-7}		14.6
M3-24	0.21×10^{-7}	4.43×10^{-7}		20.9
M26	0.31×10^{-7}	9.92×10^{-7}		31.7
M3-31	2.77×10^{-7}	19.79×10^{-7}		7.2

The Rat and Human truncated recombinants are produced in *E. coli* and reactivity measured in ELISA. The CHO-MOG polypeptide is expressed as full length Human MOG (aa 1-218),
 10 and binding to cell surface measured by FACS.

DISCUSSION

A number of solid-phase assays have been implemented to measure serum antibodies against MOG 5-7,9. Despite variable results due to different techniques and MOG preparations used, the frequency of anti-MOG positive HC is consistently high,
 15 resulting in a low specificity of the test. Thus, efforts have been undertaken to develop more specific assays. In IDDM liquid-phase based RIA have proven to be more sensitive than ELISA with comparable specificities for the three autoantigens of interest¹⁰. We describe here an easy-to-perform antibody assay based on biotinylated, rather than radioactively labeled MOG in solution. While the assay proves its technical feasibility in
 20 humans with highly sensitive detection of anti-tetanus toxoid IgG (Fig. 34B, 35), it fails to detect antibodies against rhMOG125 in either MS patients or HC that are antibody positive by means of solidphase ELISA (Fig. 34A, 34B). As such antibodies are readily detectable in the marmoset EAE model, this failure in the humans is not attributable to technical issues of the assay or the biotinylation.

Prior to this study two groups have encountered identical problems attempting to measure antibodies directed against soluble MBP or MOG, respectively^{11,12}. O'Connor et al. have used in-vitro translated and 35S-methionine glycosylated MBP in a RIA. They failed to detect anti-MBP IgG in MS patients, even if the patients were anti-MBP positive by ELISA¹¹. In contrast, elevated anti-MBP antibody levels could be detected by RIA in 4 of 6 patients with para-infectious acute demyelinating encephalomyelitis (ADEM) after rabies vaccinations with the Semple-strain. The Semple vaccine is propagated in mouse or goat brain cultures and may hence be contaminated with CNS antigens; anti-MBP antibodies have been described in such patients by ELISA¹⁸. Lampasona et al. have used the full-length human MOG, i.e. not only the extracellular portion, and labeled it similarly to O'Connor¹². Anti-MOG antibody binding, IgG or IgM, could not be detected in 146 human sera, 87 of which MS patients. In contrast, a strong antibody response was detectable in rabbits or mice immunized with various MOG peptides¹².

Our study and these previous ones have in common that human MS or HC sera did not react with myelin antigens in solution. In contrast, sera of animals immunized with MOG showed strong reactivity with soluble antigens. Moreover, sera of humans immunized with tetanus toxoid (this study) or involuntarily immunized with MBP during rabies vaccination¹¹ were capable of strongly binding the respective antigen in solution.

Discrepancies between antigen-antibody interactions in solution versus in solid-phase have been a long standing focus of research. Monoclonal antibodies appear to bind preferentially to coated antigens than to the identical antigen exposed in solution¹⁹. Several mAbs did not even recognize their specific antigens in solution at all^{20,21}. This is especially noteworthy as animals are immunized with antigen in solution, but specific hybridoma in general are screened for by ELISA. It appears that coating to polystyrene may partially unfold proteins and reveal buried or hidden epitopes not accessible in the antigen's native form in solution^{19, 22}. However, proteins do not fully denature to become linear polypeptides¹⁹. It was shown that ELISA may promote binding of low-affinity antibodies due to the excess of coated antigen (500 ng per well in our ELISA) whereas solution-phase assays because of the small amount of antigen used (2.5 ng per well in LiPhELIA) rather detect antibodies of higher affinity in an antibody concentration dependent fashion.²³

Under polyclonal conditions, results are not unequivocal and depend largely on the physiology of the individual antigen in question; for the IDDM-related antigens IA-2, GAD and insulin the correlations between ELISA and RIA results are high 10, whereas antibodies directed against another putative diabetes antigen ICA69 were only detectable by Western
5 Blot, but not at all by RIA 24. In contrast, neutralizing antibodies against interferon- (IFN) _ in MS patients are best identified by a capture ELISA²⁵⁻²⁷, while ELISA with coated IFN_ may lead to false positive and false negative results²⁵.

Möller et al. on the contrary have described that in CSF of MS patients antibodies against myelin-associated glycoprotein (MAG) were only detectable by solid-phase, but not
10 liquid-phase RIA²⁸. Interestingly, in the antiphospholipid syndrome (APS), anticardiolipin antibodies (aCL), that in the presence of a co-factor, α_2 -glycoprotein I (α_2 -GPI), cause thrombosis and fetal loss, were shown to bind only to α_2 -GPI coated onto nitrocellulose or onto oxygenized polystyrene plates ^{29,30}. Solution based assays failed to detect these antibodies resulting in false negative test results. It was argued, that oxygen charged the
15 polystyrene plates negatively leading to presentation of cryptic α_2 -GPI epitopes ²⁹. Pathophysiologically, it was discussed that membrane-bound α_2 -GPI may be become altered by, for instance negatively charged phospholipids during apoptosis and hence attract aCL leading to thrombosis and hence clinical APS.²⁹.

These seemingly unrelated studies have in common, that antibodies against antigens
20 biologically present in solution, such as IFN β or insulin, may be ideally detected by solution-based assay systems, whereas antibodies against membrane-bound and lipid-attached antigens, such as α_2 -GPI or MAG may be best evaluated by coating ELISA. The latter however, increases the likelihood to bind low-affinity antibodies, inevitably lowering the specificity of the assay ²³. Since MOG is a membrane-protein expressed on the
25 outermost layer of the myelin sheath, i.e. in a lipid-rich environment, and since it is highly insoluble in aqueous solutions, biologically relevant epitopes may not be correctly presented in solution based assays such as ours or the RIA reported by Lampasona et al.¹². Additionally, it may be argued that in analogy to α_2 -GPI coated MOG is presented in a way that resembles partially denatured or degraded MOG in the inflammatory milieu of a MS
30 CNS lesion. In contrast, it was shown by crystallography that soluble mouseMOG¹¹⁷ may form dimers that hide the encephalitogenic MOG³⁵⁻⁵⁵ epitope³¹ thus potentially blocking antibody epitopes in liquid-phase assays.

In this study marmosets immunized with 20-mer MOG peptides did not mount a sufficient antibody response against the soluble rhMOG125 as opposed to ratMOG- or HWM-immunized animals (Figure 36). In contrast, Lampasona et al. reported elevated anti-MOG titers of rabbits immunized with MOG35-55 or MOG125 peptides 12. This
5 discrepancy may be explained by the potent T-cell antigen MOG35-55 in rodents or the usage of full-length MOG in the RIA. We have previously shown that immunization with 20mer MOG peptides does not result in sufficient IgG deposition and complement activation in the marmoset EAE model 15. This may be reflected by low affinity anti-MOG antibodies that readily bind to solid-phase rhMOG125, but fail to bind to soluble antigen as
10 efficiently (Figure 36). In contrast, by virtue of the monoclonal fab fragments derived from one animal immunized with ratMOG16 we can demonstrate a diverse recognition of MOG epitopes; while M3-24 appears to be specific for an epitope whose presentation is not dependent on the molecule's conformation, M26's and M3-31's target epitope seems fully unmasked only by coated rhMOG125 (Figure 36). The decreased binding of M26 and M3-
15 31 in LiPhELIA may arguably be explained by a lower affinity (Table 8). How much this may be factor in human sera cannot be determined as affinities cannot be assessed in polyclonal sera. Indirect evidence against this may be provided by the fact, that serum anti-MOG antibodies can be consistently detected in fairly high starting dilutions of 1:200 – 1:1,000 (this study) 5-7,9,32 as opposed to 1:10 – 1:100 for insulin 22 and 1:20 for IFN27.

20 In conclusion, we have presented a liquid-phase assay for the rapid and safe detection of serum antibodies against rhMOG125. While marmosets immunized with conformational MOG mount an antibody response detectable in the LiPhELIA, sera of MS patients and HC fail to bind to soluble rhMOG125. This failure may be explained by the presence of low-affinity serum antibodies, but is more likely because of the unphysiological
25 presentation of rhMOG125 in solution and thus incorrect exposure of relevant epitopes. Liquid-phase based assays may not be the correct assay systems to assess the presence of anti-MOG antibodies in humans. ELISA on the other hand may result in low specificity. Future research has to focus on the development of additional test systems, including functional tests that will increase the specificity and enhance the significance of anti-MOG
30 antibody testing in MS.

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EXAMPLE 14

Serum IgG Reactivity Against MOG-Transfected Cells Is Selectively Increased In Early Multiple Sclerosis

Serum IgG reactivity against membrane-associated myelin/oligodendrocyte glycoprotein (MOG) was measured by flow cytometry of MOG-transfected CHO cells in multiple sclerosis (MS) subjects. Compared to healthy controls, MOG specific IgG were increased in clinically isolated syndromes ($p < 0.001$), relapsing-remitting MS ($p < 0.01$) and secondary progressive ($p < 0.05$). In contrast, this increase was not observed for primary progressive MS. Thus, antibodies directed against the "native", conformationally folded and glycosylated MOG may play a role in subtypes of MS that are predominantly associated with inflammation, and/or represent a useful biomarker of this stage of disease.

INTRODUCTION

Myelin oligodendrocyte glycoprotein (MOG) is one a target myelin antigen (Ag) for both humoral and cellular CNS-directed immune responses. The full-length glycoprotein contains 218 amino acids and two predicted transmembrane domains. It is post-transcriptionally processed, as suggested by its apparent electrophoretic mobility, and presence of potential sites for N-glycosylation (Asn 31), O-glycosylation (Ser xx), isoprenylation and myristoylation (Mesleh, 2002). Encephalogenic properties of MOG are

believed to result in part, from the extracellular location of its IgV-likelike domain on the outermost myelin lamellae, which makes it an exposed target accessible to an initial autoimmune attack on compact myelinated axons (4). Antibodies directed against MOG have been shown to directly induce demyelination in EAE models (6, 7) In context of a first
5 demyelination event, anti-MOG antibodies have been proposed to predict early conversion to clinically definite MS (8)

A common problem encountered in human studies of humoral immunity against MOG is that accurate detection of Ab depends on the conformation of the antigens used for detection. Previous studies, either in MS or EAE, have not fully characterized the structural
10 features of the proteins used as Ab targets in vitro and have reported conflicting findings, in part because of the different folded forms of this poorly water-soluble antigen. The existing assays detecting anti-MOG Abs use various MOG preparations, recombinant, native-purified, or in vitro-translated proteins of variable length including short peptides (4, 8–11). None of these methods takes into account the specific tertiary structure of the folded MOG
15 as it is presented to the immune system in vivo, e.g., in association with a hydrophobic, lipid-rich bilayer membrane environment. Therefore, these techniques are likely to fail to detect reactivity against epitopes displayed by native MOG expressed in situ on myelin sheaths, as it would exist upon an initial immune response.

Here, we describe a cell-based assay that specifically measures Abs directed against
20 conformationally folded, cell membrane expressed human MOG (designated hMOGcme), and evaluate the relative incidence of these anti-native MOG Abs in serum of humans and *Callithrix jacchus* marmosets with MS-like EAE.

METHODS

Patients. Ninety-two patients with clinically definite MS (Poser criteria) (24), 36
25 patients with CIS, and 37 healthy controls were recruited from the UCSF outpatient clinics, neurology wards and multiple sclerosis treatment trials. Blood was obtained by venipuncture after informed consent in full compliance with the Institutional Review Board, and clotted serum stored at -40°C until use. Patients were classified as relapsing-remitting (RR, n=35), secondary progressive (SP, n=33), and primary progressive (PP, n=24) MS by
30 clinical history (24) Clinically isolated syndrome (CIS, n=36) was defined by a first clinical attack with no history of previous neurological symptoms and with brain magnetic

resonance imaging (MRI) findings consistent with demyelinating disease. Age, gender, disease duration and disability state [Expanded Disability Status Score (EDSS)] (Kurtzke, Neurology, 33:1444-1452, 1983) were recorded at time of sampling. HCs were chosen to match sex and age of the CIS group. The median age, disease duration, and EDSS were higher for the SPMS group than for the RRMS and CIS groups. Patient characteristics are shown in Table 11 below. All RRMS patients and 19 out of 33 SPMS patients were treated with interferon-beta. Two of the PPMS patients were treated with mitoxantrone and monthly pulsed steroids, respectively.

Animals. *C. jacchus* marmosets were cared for in accordance with the guidelines of the Institutional Animal Care and Usage Committee. EAE was induced by immunization with 100 mg of human white matter, which contains native, membrane embedded MOG homogenized in complete Freund's adjuvant as described (26). Plasma was obtained from EDTA-anticoagulated blood at baseline and 2- to 4-week intervals and stored at -40°C. The animals were scored every other day for the development of clinical signs by using a published scale (27).

Preparation of MOG-Transfected Cells. Chinese hamster ovary (CHO) cells were transfected with a full-length construct corresponding to the major alpha-1 form of human MOG, as described (28) elsewhere. CHO cells were cultured in T225 flask (Costar), in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 1x Glutamax, 1mM Sodium pyruvate and 50 µg/ml gentamycine. G418 (500 µg/ml, Gibco) was added to the medium of transfected cells (CHO-MOG). Cells were used for FACS analysis when a confluence of 80-90% was reached, after surface-expression was verified by immunofluorescence. Briefly, 30,000 cells were smeared on a slide and fixed with methanol 100% for 5min at -20°C. After blocking with PBS containing 2% bovine serum albumin and 2% FCS for 30 min, cells were incubated 1 hour at 37°C with the mouse monoclonal anti-MOG antibody 8-18C5 (5 µg/ml, gift of Dr. C Linington). Fluorescence was revealed after 1-hour incubation at 37°C by a goat anti-mouse IgG FITC antibody (Sigma). Negative controls were done with secondary Ab alone

Serum IgG Reactivity. Serum from patients and controls were diluted 1:10 in FACS buffer consisting of PBS, Na azide 0.1%, FCS 2%. Cells were trypsinized, diluted in FACS buffer and plated in a 96 well plate (Costar) at a density of 200,000 per well. After blocking

in FACS buffer containing 10% FCS for 15 min at 4°C, cells were washed and human serum (1:10) was added for 1-hour incubation at 4°C. After washing, cells were incubated with a secondary goat anti-human IgG FITC (Caltag) at the recommended concentration for a 30 minutes incubation at 4°C. After a final wash, cells were resuspended in FACS buffer
5 containing Propidium Iodide (Molecular Probes) at 2 µg/ml and gently shaken. FACS tubes were kept on ice and analyzed by gating the selected live cell population (10,000 cells) within one hour of harvesting by trypsinisation. Each FACS experiment included an internal positive control using the anti-MOG 8-818C5 antibody (0.5 µg/ml) and rabbit anti-mouse FITC (Dako) as a secondary antibody. For each sample, the geometric mean intensity
10 (Gmean) of FITC (WINMDI 2.8 software) was measured for MOG-transfected CHO cells and compared with that of ntCHO cells. The BR was calculated as the Gmean for MOG-transfected CHO cells divided by the Gmean for ntCHO. To compare different assays, for each sample the BR was normalized to that of a human positive control (RRMS 1158) included in each experiment.

15 For studies in marmosets, MOG-transfected CHO cells were incubated for 1 h at 4°C with marmoset serum diluted 1:100. FITC-conjugated Ab against whole monkey IgG (Sigma) diluted at 1:100 was used as secondary Ab and incubated 30 min at 4°C. FACS analysis was performed as described above. IgG binding against hMOGme was considered positive when the BR (Gmean preimmunization/Gmean time point tested) was greater than
20 1.5.

Differential Reactivity of Monoclonal Fab Fragments. Recombinant Fabs were derived from a *C. jacchus* marmoset immunized with rMOG1–125 produced in *Escherichia coli* (rMOG125) (12). Fabs were diluted in FACS buffer at 0.5 µg/ml and added to ntCHO or MOG-transfected CHO cells. FITC-conjugated Ab against whole monkey IgG (Sigma)
25 diluted at 1:100 was used as secondary Ab for 30 min at 4°C. FACS was performed as described above.

ELISA Assay. hMOG125 expressed in *E. coli* was coated overnight on polystyrene microtiter plates at 0.5 µg per well (Maxisorb, Nunc). After washing and blocking with 1% BSA in PBS containing 0.05% Tween (BSA-PBS-T) for 2 h at room temperature, sera
30 (1:200) were diluted in BSA-PBS-T and added to the plate. Ab binding was detected by an alkaline phosphatase AP-labeled goat-anti-human IgG (Sigma) for 1 h at room temperature.

Plates were developed with para-nitrophenyl phosphate (Moss, Pasadena, MD) for 30 min in the dark at room temperature and read at 405 nm in a microplate reader (SpectraMax, Molecular Devices). Results were expressed as BR (e.g., signal over BSA background).

Preabsorption of Sera on hMOGme and hMOG125. To further validate the cell-based assay and eliminate the possibility of nonspecific binding effects from either MS serum or transfection procedures, we conducted binding experiments after preincubation of serum with the respective antigens. For hMOGme preabsorption, 5×10^6 MOG-transfected CHO cells and ntCHO cells were separately incubated with serum diluted 1:10 for 1 h at room temperature with gentle agitation. After centrifugation at 900xg for 2 min, supernatant were collected and preabsorption was repeated three times in total with fresh cells. After the final preabsorption, supernatants were centrifuged at 3,600xg for 5 min and collected for subsequent experiments. For hMOG125 preabsorption, ELISA plates were coated with either 1 μ g BSA or 0.5 μ g hMOG125 overnight and blocked in 1% BSA in PBS plus 0.05% Tween for 2 h, then sera were incubated 1 h. Supernatants were collected and preabsorption was repeated eight times in total with fresh hMOG125. After the final preabsorption step, supernatants were collected as above.

Marmoset Fab Binding Assay. We also analyzed the binding of monoclonal, recombinant Fab antibody fragments derived from marmosets immunized with the non-glycosylated extracellular domain of rat MOG (aa 1-125). Briefly, cells (CHO and MOG-transfected CHO cells) were plated at 200,000 cells/well in a 96 well plate. Cells were washed 1X, blocked (FACS buffer with FBS 10%) for 15min then washed again before adding the first antibody. Controls included: 1) 8-18C5 added to some wells at two different concentration (0.5 and 0.05 μ g/ml); and 2) Serum from CJ UO-50-01 (human white matter –HWM- immunized) tested (1:100 dilution) at day 0 (before induction of EAE) and at euthanasia. Fab fragments included Fabs designated as M3-8, M26, M3-24, M3-31, which were all cloned by combinatorial technique from rat MOG1-125-immune marmosets. All these antibodies were selected by their ability to recognize rat MOG1-125 (non-glycosylated recombinant protein produced in *E. coli*) as presented on solid support in an ELISA well (von Budingen, Proc Natl Acad USA, 2002). Fabs were diluted at 0.5 and 0.05 μ g/ml and added to CHO / CHO-MOG cells. Cells were incubated 1 hour on ice and after 3 washes, the pellet wash resuspended in FACS buffer. FITC-conjugated Ab anti-

mouse F(ab')₂ diluted at the recommended dilution (1:10) was added to the cells incubated with 8-18C5. FITC-conjugated Ab against whole monkey IgG diluted at the recommended dilution (1:100) was added to the other wells (CJ UO-50-01 and Fab). After incubation with the secondary Ab (30min on ice) cells were washed 3X and resuspended in FACS buffer containing PI (2 µg/ml). The tubes were kept on ice and flow cytometry was performed within the next two hours.

Data analysis. For each sample, the geometric mean (Gmean, Winmdi software 2.8 for PC) intensity of FITC was measured for each batch of CHO-MOG cells and compared to that of non-transfected cells, and a binding ratio (BR-CHO-MOG) was calculated as Gmean of transfected over Gmean of non-transfected cells. In order to compare different assays, the BR-CHO-MOG of each sample was normalized to those of a positive control (# IG-1158-001) included in each experiment (BRN-CHO-MOG). Statistical analyses were performed using ANOVA and Kruskal-Wallis with Dunn's Multiple Comparison for inter-patient group differences linear regression tests (Prism 3.0).

15 RESULTS

Chinese Hamster Ovary (CHO)-MOG Assay (MOGcme) Validation. FIG. 38A and 38B show high levels of MOGcme expression, as demonstrated by staining of MOG-transfected CHO cells with the monoclonal anti-MOG Ab 8-18C5. Detection of hMOGcme-specific Abs with this cell-based assay was sensitive because a concentration of less than 1 ng/ml of 8-18C5 produced a binding ratio (BR) greater than 1.5. Staining with a positive control serum (patient 1158) is shown in FIG. 38C. This control was used in each assay to normalize for interassay variability and minimize experimental errors such as variation in surface expression of MOG. The mean (+/- SEM) BR to hMOGcme of this control from nine independent experiments was 1.96 +/- 0.145 (FIG. 38D), and the interassay coefficient of variation was 22%. The intraassay coefficient of variation (quadruplicate) was 3.2%. In each assay analyzing human serum, the binding against nontransfected CHO (ntCHO) cells was used as background control.

Characterization of Exposed Epitopes of hMOGcme. We analyzed the binding properties of monoclonal, recombinant marmoset Fab Ab fragments produced against the nonglycosylated extracellular domain of recombinant rat MOGaa1-125 (rMOG125). Four Fabs, designated M3-8, M26, M3-24, and M3-31, were selected by their ability to recognize

rMOG125 in ELISA, and because they recognize distinct conformationally defined epitopes (12). The M3-31 and M26 Fabs strongly stained the MOG-transfected CHO cells identical to 8-18C5 (0.5 µg/ml) (FIG. 40A and 40B). On the contrary, no binding was observed for the two other Fabs, M3-24 and M3-8 (FIG. 40C and 40D). These results indicate that very specific epitopes of MOG are expressed on the MOG-transfected CHO cells and do not overlap with the other epitopes displayed by rMOG125 on solid ELISA support. The observation that only three of five monoclonal reagents tested bind to the transfected cells also renders unlikely a nonspecific binding effect.

IgG Reactivity in Human Serum. Compared to age-matched healthy controls (HC), the titers of IgG directed against the native, membrane bound hMOG expressed on the CHO-MOG cell surface was significantly increased in CIS ($P < 0.001$). Increased titers were also present in RRMS and SPMS subtypes as compared to HC (RRMS: $P < 0.01$; SPMS: $P < 0.05$) as shown in FIG. 41. The differences were also significant when comparing CIS, RRMS and SPMS to PPMS (CIS compared to PPMS: $P < 0.001$; RRMS compared to PPMS: $P < 0.01$; SPMS compared to PPMS: $P < 0.05$). No statistical difference was found between PPMS and HC (PPMS compared to HC: P not significant), or between the CIS, RRMS, or SPMS subtypes when paired comparisons were made. In addition, in this cohort no difference was found between MS patients in relation to treatments. However, we found that the linear regression comparing the mean BRN-CHO-MOG with the median age of each group was statistically significant ($p < 0.05$, $r^2 = 0.9279$) and showed a correlation between young age and high IgG titers against CHO-MOG.

IgG Reactivity in Marmoset EAE. Eleven *C. jacchus* marmosets immunized with human white matter were tested for plasma reactivity against hMOG_{me} on MOG-transfected CHO cells. For these serial studies, the first time point of Ab detection (i.e., serum conversion) was compared with the appearance of the first clinical signs of EAE. Three animals (U30-00, UO61-02, and UO53-01) were killed before onset of neurological deficits (preclinical disease), but exhibited CNS inflammation and blood-brain-barrier breakdown as demonstrated by cerebrospinal fluid pleocytosis (mean cerebro-spinal fluid mononuclear cells 173 per µl; range 80–340 per µl). Serum reactivity against hMOG_{me} was consistently detected in the earliest blood sample obtained after immunization (mean $\pm$ SD = 14 $\pm$ 2 days; range = 13–18 days) (Table 11) and was clearly present in each animal before the appearance of any clinical sign (mean $\pm$ SD = 21 $\pm$ 9 days; range = 16–

43 days). The difference between time of appearance of serum IgG reactivity to hMOGme and appearance of clinical signs was highly significant ($P < 0.0001$) as shown in FIG. 41. Reactivity was not detected in preimmune plasma. Comparison of ELISA and CHO-MOG Binding. Table 10 shows the differential binding of the four marmoset monoclonal Fab fragments as assessed by the various antibody-binding systems. Differential reactivity of monoclonal marmoset Fab antibody fragments to various forms of the MOG polypeptide antigen (conformational epitopes) is shown. The Rat and Human truncated recombinants are produced in *E. coli* and reactivity measured in ELISA. The CHO-MOG polypeptide is expressed as full length Human MOG (aa 1-218), and binding to cell surface measured by FACS.

Table 10. Differential Reactivity of Monoclonal Marmoset Fabs

MOG polypeptide	Epitope			
	M3-8	M3-24	M26	M3-31
Rat MOG 1-117	+	-	+	+
Rat MOG 1-125	+	+	+	+
Human MOG 1-118	-	-	+	+
Human MOG 1-125	-	+	+	+
CHO-MOG	-	-	+	+
Competition with human anti-MOG IgG	+	+	-	not done

Table 11. Time Course of *C. jacchus* marmoset EAE and Serum IgG Binding Against hMOGcme

Animal No.	Clinical Onset Dpi	Death dpi	Max Clinical Score (dpi)	antihMOGcme Ab dpi
UO62-02	43	97	3 (84)	18
UO50-01	21	86	2 (57)	14
UO50-00	21	78	3 (38)	14
UO52-01	21	52	3 (40)	18
UO25-00	21	61	2 (57)	14
UO57-02	32	82	1.5 (73)	18
UO21-99	18	23	1.5 (23)	13
UO23-00	16	31	2 (28)	15
UO30-00	Preclinical	23	0	13
UO61-02	Preclinical	31	0	14
UO53-01	Preclinical	38	0	16
Median SD	21 $\pm$ 9	52 $\pm$ 28	2 $\pm$ 1 (49 $\pm$ 22)	14 $\pm$ 2

dpi, day post immunization

- 5 Comparison of Human IgG Binding on hMOG125 and hMOGcme. The serum binding characteristics observed in a cohort of patients (CIS, RRMS, SPMS and PPMS) and controls were tested by ELISA using recombinant human MOGaa1-125 (hMOG125) and compared with MOGcme reactivity by FACS on MOG-transfected CHO cells. Although some CIS patients displayed high reactivity against hMOG125, unlike for
- 10 hMOGcme reactivity, there was no statistically significant difference between CIS and HCs. By linear regression and comparison test, results from these two assays showed no correlation (P not significant), indeed suggesting that different epitopes are detected by both assays (FIG. 42A).

Table 12. Clinical characteristics of different groups of patients and healthy controls.

Group	Nos.	F/M ratio	Median age, yr (range)	Median disease duration, yr (range)	Median EDSS (range)
CIS	36	24/12	32 (18-49)	0.3 (0.1-3)	2.0 (0-4)
RRMS	35	21/14	41 (19-61)	2.4 (0.6-22.2)	2.5 (0-6.5)
SPMS	33	19/14	46 (36-60)	11 (2-32)	6.0 (2.5-8.5)
PPMS	24	16/08	54 (40-65)	6.3 (0.8-18.8)	4.5 (2.5-8)
HC	37	24/13	35 (21-63)	-	-

CIS = clinically isolated syndrome; RRMS = relapsing-remitting MS; SPMS = secondary progressive MS; PPMS = primary progressive MS; HC = healthy controls.

5 Specificity of MOG and Differential MOG-Epitope Binding in Human

Serum. To discriminate the epitopes displayed by hMOG125 from those displayed on hMOGcme on MOG-transfected CHO cells, we performed a series of preabsorption experiments with two sera, both representative of early and inflammatory forms of MS: the positive control used in our cell-based assay (RRMS 1158) and a CIS patient displaying a high reactivity to both hMOGcme and hMOG125 (CIS 008). Preabsorption against ntCHO cells served as a control in the hMOGcme assay (FACS of MOG-transfected CHO cells), and preabsorption against 1% BSA served as a control for the hMOG125 ELISA assay. Compared with these controls, preabsorption of either sample on hMOG125 did not affect hMOGcme reactivity. Preabsorption on hMOGcme resulted in a decrease in BR (31% for CIS 008 and 24% for RRMS 1158), when tested with hMOGcme expressed on the MOG-transfected cells (FIG. 42B). Similarly, when tested on hMOG125, no change in reactivity occurred when samples were preabsorbed on the MOG-transfected cells (hMOGcme). On the contrary, the samples preabsorbed on hMOG125 displayed a decrease in BR, by 21% and 57%, respectively, when tested on hMOG125 (Fig. 42C). These experiments unequivocally demonstrate that hMOGcme and hMOG125 display separate epitopes of the MOG protein.

KEY POINTS

1. The CHO-MOG cell system measures antibodies against the membrane-embedded, glycosylated human MOG that are different from those detected by the ELISA methods. Both kinds of antibodies may be relevant to MS pathophysiology and/or serve as
5 biomarkers of disease. For example, ELISA antibodies against rat MOG 1-125 are elevated in primary progressive MS, whereas this difference is not observed when using ELISAs with human MOG1-125 or human MOG 1-118 as antigen, respectively. Moreover, antibodies directed against CHO-MOG predominate in CIS and RRMS, and in patients that are younger than those of the other MS groups. Importantly, the control subjects in the
10 present study were age-matched with the CIS group, and yet did not display increased CHO-MOG reactivity, indicating that the heightened response in CIS (and likely RRMS and SPMS), is disease-specific and not related to age.

2. There are common epitopes within the set of epitopes presented by the non-glycosylated, rat MOG-1-125 as displayed on a solid support such as an ELISA plate at
15 neutral pH, and the number of epitopes presented on native, membrane-bound human full length MOG expressed on recombinant CHO cells. It is possible that the affinity of the recombinant Fab fragments may differ between these two different targets.

3. The panel of monoclonal Fab fragments directed against MOG can be used to discriminate the different epitopes of the MOG antigen depending on how they are exposed
20 to antibody binding (e.g., ELISA, cell-based systems or liquid phase systems).

4. These properties of the monoclonal Fab fragments (and others similarly derived from a primate system, including humans) can be similarly used to determine what MOG epitope(s) human antibodies (IgG and IgM) bind to, taking advantage of the differential reactivities towards the various systems, and of competition experiments in which these
25 fragments (labeled with biotin or other ligand suitable for detection) are displaced by the human IgG.

5. Similar to the ratios of MOG peptide/conformational MOG antibody binding, the relative binding of human antibodies in the various systems where MOG epitopes are differentially presented can be used to further define the fine specificity of anti-MOG
30 antibodies in a complex polyclonal responses such as the one observed in serum, CSF or other biological fluids of humans.

DISCUSSION

MOG is probably the major myelin target studied in MS. Methods (ELISA, western blot, liquid-phase assay) performed to test Ab reactivity against MOG commonly use linear peptide, protein or partially refolded glycosylated MOG. The exact conformation of MOG displayed in these assay systems is difficult to assess and control and may result in the display of some, or partially aberrant MOG epitopes that are not exposed under physiological conditions in vivo. The disease relevance of these Abs is therefore uncertain, as apparent from somewhat conflicting results in previous reports (2, 8–11, 13–16). We show here that Abs against native glycosylated MOG expressed on mammalian cells are commonly detected in MS serum with high sensitivity (<1 ng). Specificity is established by the selective binding of three of five monoclonal anti-MOG Abs tested in this system (murine 8-18C5 and marmoset Fabs M3-31 and M26). Thus, as is the case for these mAbs (9, 12, 13) the hMOGcme assay measures Abs that bind to conformational epitopes of MOG.

Our results show that there is no correlation in the CIS cohort between serum reactivity against hMOG125 (solid-phase ELISA) and hMOGcme (MOG-transfected CHO cells), and preabsorption demonstrates that there is no cross-reactivity between epitopes of MOG displayed in these two different assays (FIG. 42). This finding also argues, along with the significant signal quenching in the cell-based assay achieved only by preabsorption on hMOGcme (MOG-transfected cells), and not by preabsorption on either hMOG125 or ntCHO cells, against a nonspecific “sticky” effect of MS serum. The hMOGcme assay is unique because it allows the testing of IgG reactivity directed against epitopes presented by the native glycosylated and conformational structure of MOG as it is expressed on intact myelin sheath or oligodendrocytes and subject to membrane lipid protein interactions, which have been shown to be critical for maintenance of myelin structure and epitope exposure (17). Analysis of reactivity against hMOGcme on the MOG-transfected CHO cells in the different MS clinical subtypes showed a very prominent response in CIS, RRMS, and to a lesser degree SPMS, compared with HC and PPMS. Thus it is contemplated that there is a humoral immune response specifically directed against intact MOG expressed on myelin oligodendrocytes in those groups of patients. The predominance of hMOGcme-specific Abs in CIS suggests that these Abs represent early stages of the immune response against intact (as opposed to degraded) myelin, and may represent a marker of inflammatory

phases of disease related to blood-brain-barrier opening and/or molecular mimicry. These results are in partial agreement with a recent report showing an increased Ig response directed against ex vivo glycosylated-native-MOG in first demyelinating events (14). RRMS is the most common MS subtype that includes ~85% of the patients at initial presentation. The secondary progressive pattern is known to follow RRMS in ~50% of the cases after 10 years of disease activity (18, 19). Thus, these two subtypes might be considered as a continuous process starting with a common pathophysiological origin. Although we did not include a group of other neurological disorders in the current study, we have compared MS clinical subtypes among themselves and clearly demonstrated that the hMOGcme-specific Ab response is restricted to early forms. It is of importance to note that the PPMS cohort does not show elevated serum IgG against hMOGcme and that significant differences in antibody status also exist between SPMS and PPMS, which implies that the serum level of these Abs or lack thereof is not solely related to a progressive course of disease. The HC subjects were age-matched with the CIS group, indicative that the heightened response in CIS is disease-specific and not related to a younger age in this cohort. The lack of heightened Ab responses against hMOGcme in PPMS is in contrast with the increased IgG reactivity against recombinant rMOG125 and against neurons in this disease subtype (20). Abs against galactocerebroside, the major myelin glycolipid, are not found in PPMS but are associated mostly with established relapsing-remitting and secondary progressive forms (21). It is thus becoming increasingly apparent that Ab responses against myelin antigens may follow patterns that reflect a combination of underlying cause, antigen exposure, and secondary immune responses. These patterns of humoral reactivity, rather than a classification based on clinical criteria (RRMS, SPMS, or PPMS) can be exploited to refine our understanding of disease stage, cause, and prognosis.

The high prevalence of hMOGcme-reactive Abs in CIS (contemporary of the first clinically apparent event for MS), is in sharp contrast to other antimyelin Abs, such as those directed against glycolipids that predominate in established MS (21). This observation has two important implications: first, it suggests that hMOGcme-reactive Abs may be implicated in the early pathogenesis of disease. Engagement of membrane-embedded MOG by the mAb 8-18C5, which as shown in the current study binds hMOGcme with a high affinity, has been shown to induce MOG phosphorylation in oligodendrocytes, leading to pronounced morphological changes with potentially demyelinating effects (22). Second,

and/or alternatively, our findings also suggest that hMOGcme-reactive Abs may be useful to help diagnose MS at its earliest stages. Accordingly, the hMOGcme assay was used to study the time course of the Ab response against hMOGcme in marmoset EAE induced by immunization with human white matter. In these animals, serum reactivity against

- 5 hMOGcme was always detected before clinical onset, contrary to anti-myelin basic protein and antigalactocerebroside Abs that occur at later stages (21). Because the immunizing antigen contained native MOG similar in conformation to hMOGcme, these findings imply that the hMOGcme-reactive Abs are the ones that initiate and/or first result from active demyelination. Regardless of whether they are pathogenic in and of themselves,
- 10 hMOGcme-reactive Abs clearly represent a valuable biomarker for disease activity and, at least in the MS model, subclinical disease.

- It is of great interest to note that the marmoset Fabs M3-31 and M26, which were obtained from an animal with overt clinical signs of EAE immunized with rMOG125 and had an established anti-MOG Ab response, are the only ones among those tested that
- 15 recognize hMOGcme. In our previous studies of human MS, using a highly specific competition assay between human serum IgG and marmoset Fabs we found that Fabs M3-24 and M3-8 can compete with serum IgG from patients with established MS, but we have so far not been able to demonstrate any competition between human IgGs and either M3-31 or M26 (12). Although further studies are needed to examine whether IgG purified from
- 20 patients with a CIS does compete with Fabs M3-31 and M26, these data indicate that the epitopes defined by these two Fabs are the ones targeted by early humoral responses in MS, whereas the ones defined by M3-8 and M3-24 may be part of the Ab response at a later stage.

- Taken together, our results indicate that analogous to certain serologic markers that
- 25 are predictive of type I diabetes (23) anti-hMOGcme Abs could be used in humans as a biomarker to diagnose MS (e.g., or specific MS subtypes) or MS risk (e.g., prognosis or relative risk of subject to develop MS). Further in combination with other Ab profiling techniques (8, 21) anti-hMOGcme Abs are contemplated to benefit neurologists, and individuals with suspected or established MS.

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EXAMPLE 15

High Titer Antibodies Against MOG As Biomarkers For Disease Severity In MS

Serum samples of 325 MS patients and 164 healthy controls (HC) were tested by a quantitative high-throughput ELISA and correlated to clinical characteristics of MS. Three
10 recombinant MOG preparations have been used as antigens because they expose unique immunodominant epitopes identified by monoclonal reagents. Overall the anti-MOG IgG concentrations are indistinguishable between HC and MS for all three antigens with distributions skewed towards low titer responses resulting in low sensitivity and specificity. In less than 15 % of samples high-titer reactivity can be identified; in RR-MS patients with
15 such high-titer reactivity the anti-MOG titer correlates with current and prospected disability ($p=0.02$, $r=0.44$ and $p=0.003$, $r=0.57$, respectively). Although high-titer antibodies can also be found in controls, the significance and novelty of those quantitative studies have helped to identify for the first time a subpopulation of RR-MS patients where anti-MOG antibodies may play a pathogenic role and correlate with the degree of disability. Hence
20 they are contemplated to be clinically relevant biomarkers.

INTRODUCTION

With regards to MS, there is a line of evidence suggesting the pathogenic involvement of autoantibodies. A large proportion of MS lesions stain positive for IgG deposition and complement activation (Type II lesions) (3). In a comparative study MOG
25 reactive antibodies could be detected within the disintegrating myelin sheath of demyelinating MS lesions with a pattern similar to marmoset EAE lesions (4, 5). Additionally, plasma exchange was shown to be effective in those patients with Type II lesions underscoring the pathogenic importance of serum antibodies in a proportion of MS patients (6). In addition to possible pathogenicity, certain anti-myelin antibodies have been
30 proposed as prognostic markers in early MS (7). However, to date the detection of serum

MOG-Abs with various techniques and using different MOG preparations has resulted in inconsistent results and limited reproducibility. Additionally, sensitivity and specificity of these assays were consistently low (8-14).

MATERIAL AND METHODS

5 Antigens. For the production of recombinant human myelin/oligodendrocyte glycoprotein (rhMOG) an expression vector was constructed from a custom made human brain cDNA library. The cDNA encoding for the extracellular domain of rhMOG spanning the aminoacids 1-125 (rhMOG125) was amplified using pfu-Polymerase (Promega, Madison, WI) with the following primers to create a Nco I restriction site at the 5'-end and
10 a Bgl II site at the 3'-end, respectively: 5'-CGGGGACCAT GGGGCAGTTC AGAGTGATAG GACCAAGACA-3' (SEQ ID NO:65) and 5'-TAGCTTGAGA TCTTCCAGGG CTCACCCAGT AGAAAGG-3' (SEQ ID NO:66). After enzymatic digestion the target gene was ligated into the pQE60 plasmid (Qiagen, Valencia, CA), the construct sequenced at the UCSF Genomics Core Facility and correct and in-frame insertion
15 of the rhMOG125 target gene ascertained. M15 cells (Qiagen) conditioned for chemical transformation according to the manufacturer's protocol were transformed for protein expression, expanded in selective LB medium and induced by IPTG according to the manufacturer's protocol.

 Secondly, a shorter rhMOG protein, spanning the amino acids 1-118 (rhMOG118)
20 was created by usage of a different 3'-end primer: 5'-ATCCATGAGA TCTAGGATCT TCTACTTTCA ATTCCATTGC TGCC-3' (SEQ ID NO:67). This shorter rhMOG preparation (by 7 amino-acids) was expressed in *E.coli* as above. Both rhMOG preparations, rhMOG125 and rhMOG118, were extracted from the cytoplasmic fraction of the *E. coli* by cell lysis under gentle native conditions using the B-PER in PBS reagent (Pierce
25 Biotechnology, Rockford, IL). Purification was carried out under non-denaturing conditions in 20 mM sodium phosphate (SP), 500 mM NaCl, 10 % glycerol and 0.05 % sodium deoxycholate (DOC), pH 8.0 (MOG-buffer) using a Ni-NTA FPLC (HiTrap, Amersham, Piscataway, NJ), through the pQE60's c-terminal His-tag. Different affinity chromatography columns were used for each preparation in order to avoid cross
30 contamination. Proteins were eluted by a linear gradient of 250 mM imidazole, 20 mM SP, 1 M NaCl, 25 % glycerol, 0.05 % DOC, pH 8.0. The eluted proteins were dialyzed into

MOG-buffer, containing 15 % glycerol, purity confirmed to be >95 % by 10-20 % SDS-PAGE. Bacterial endotoxin contaminations were below detection limits of 0.06 EU/mL as determined by the Pyrogen Plus kit (Cambrex, East Rutherford, NJ).

Recombinant rat MOG, amino acids 1-125 (ratMOG125) was produced in *E. coli*
5 and purified as described previously (15).

Antibodies. The murine monoclonal anti-rat MOG IgG 8.18C5 against native form in cerebellar glycoproteins was a gift of Dr. Chris Linington (16). The marmoset Fab-fragments (Fabs) designated M26, M3-24, and M3-8 derived from one ratMOG125-immunized animal were generated by the inventors as described (15).

10 Patients. 325 MS patients meeting the diagnostic criteria for clinically definite MS (17) were recruited for this study. 36 % of these were treated with either interferon beta (IFN β) or glatiramer acetate (GA) at the time of sampling. Patients treated with glucocorticoids within three months or on immunosuppressive therapy within six months of phlebotomy were excluded. 192 patients suffered from relapsing-remitting (RR) MS, 69 had
15 secondary-progressive (SP) MS, and 64 primary-progressive (PP) MS. Blood was drawn by venipuncture and clotted serum stored at -40°C. 164 volunteers served as healthy controls (HC). Informed consent was obtained from the patients and HC, and the study was conducted in accordance with Institutional Review Board approval. The clinical characteristics of the patients and HC and their statistical differences are summarized in
20 Table 13.

Table 13. Clinical Characteristics of Human Subjects

Subject	No.	Sex (M/F)	Age		Duration (m)		EDSS	
			m	Range	M	Range	m	range
HC	164	61/103	42 ^b	16-72				
MS	325	119/206						
RRMS	192	57/135 ^a	40 ^c	16-68	45	0-419	2.0 ^c	0.0-6.5
SPMS	69	30/39	48	22-69	150 ^d	22-498	6.0	2.0-8.5
PPMS	64	32/32	52	32-67	82 ^e	1-309	4.0	1.0-8.5

m = median

^a p<0.05 versus PPMS (χ^2 test with Yates' continuity correction)^b p<0.001 versus PPMS (Kruskal-Wallis test with Dunn's posthoc test)5 ^c p<0.001 versus SPMS and PPMS (Kruskal-Wallis test with Dunn's posthoc test)^d p<0.001 versus RRMS and PPMS (Kruskal-Wallis test with Dunn's posthoc test)^e p<0.05 versus RRMS (Kruskal-Wallis test with Dunn's posthoc test)

High-throughput ELISA. All three rMOG preparations were coated on the same

10 384-well microtiter plate (Maxisorb, Nunc, Rochester, NY; 0.5 ug of rhMOG125 and rhMOG118 and 1.0 ug of ratMOG125 were coated in 50 uL PBS per well overnight. Optimal protein concentrations for coating were determined in preliminary experiments. Control wells were coated with BSA. To quantify the antibody reactivity a human IgG standard curve was created by coating human IgG (I4506, Sigma, St. Louis, MO) in two-

15 fold dilutions, spanning the linear detection range as determined previously. After washing with PBS and ddH₂O plates were blocked for 2 hrs with 1 % BSA in PBS supplemented with 0.05 % Tween 20 (PBS-T). After washing with PBS-T and ddH₂O, human sera were added at three dilutions of 1:200, 1:400 and 1:800 and incubated for 90 min. Standard curve wells were kept in 1 % BSA-PBS-T. A positive control known to be reactive to all

three rMOG preparations, and a negative control omitting serum were included in every plate.

After washing, bound antibodies were detected by an alkaline phosphate labeled anti-human IgG (A9544, Sigma). After incubation for 60 min and subsequent washing, plates were developed with para-nitrophenyl phosphate (pNPP, Moss, Pasadena, MD) for 30 min and the optical density (OD) read at 405 nm wave length in a microplate reader (SpectraMax, Molecular Devices, Sunnyvale, CA). All samples were tested in a blinded fashion in duplicates, standard curves in quadruplicates; incubation was at room temperature, except coating which was at 4 °C. Sample handling and ELISA procedures were performed by a robotic workstation (Biomek FX, Beckman Coulter, Fullerton, CA). Inter- and intraplate variability were below 20 % and 15 %, respectively.

MOG-ELISAs for the monoclonal reagents were performed without standard curves in 96-well Maxisorb plates adhering to the protocol as outlined above. 8.18c5 was detected by an anti-mouse IgG (A9044, Sigma) and the fabs by Protein L (Pierce). Both were peroxidase labeled, hence developed by 3,3',5,5'-tetramethylbenzidine (Pierce) (Pierce) and the OD read at 450 nm wavelength after 15 min.

Data processing and statistical analysis. The ODs were corrected for the individual background (BSA-well) binding and the amount of specific IgG bound to the well interpolated from the on-plate standard curve averaged for the dilutions whose respective ODs fell within the linear portion of the Ig standard curve. A template in Microsoft Excel was created for computer-assisted data processing, internal quality control management and IgG quantification. Additionally, results were expressed as the signal-to- background binding ratio, calculated as the ratio of OD (signal) over OD (background) where applicable, as were results for 8.18c5 and the fabs. For the assessment of high-titer reactivity, samples above to 95th percentile of IgG concentrations were tested in serial dilutions up to 1/3,200 in a separate experiment; those with BRs above 3 at 1/3,200 dilution (i.e. signals against MOG that were three-fold above the BSA signal) were identified and their mean BR at 1:800 dilution defined as the cut-off for high-titer reactivity. This procedure was done for all three MOG preparations.

Statistical analysis was conducted using Prism 4.0 (GraphPad, San Diego, CA). Categorical variables were compared using the χ^2 -test, continuous variables using ANOVA

and ordinal variables the Kruskal-Wallis test. The Student-Newman-Keuls (SNK) method and Dunn's test were used to determine differences in between groups, respectively.

Correlations were described by either the Pearson (parametric) or the Spearman (non-parametric) r . Whenever possible datasets were transformed to generate a normal

5 distribution for parametric analysis by applying the natural logarithm $\ln(y)$.

RESULTS

The three MOG preparations expose distinct immunodominant epitopes. Despite a 90 % sequence homology between rhMOG125 and ratMOG125, and a mere 7 amino acid difference between rhMOG118 and rhMOG125 there is strong evidence that each of the

10 antigenic preparations used in this assay display immunodominant epitopes or combinations thereof that are unique to each of the preparations. The monoclonal abs (monoclonal anti-rat MOG antibody 8.18c5 and ratMOG125-immune marmoset Fabs M26, M3-24 and M3-8), define at least three epitopes; as 8.18c5 and M26 bind equally well to all antigens, they define epitopes commonly exposed on all three MOG preparations (FIG. 43A and 43B).

15 The second epitope is unique to rhMOG125 and ratMOG125, but not exposed on rhMOG118, hence species-independent, but dependent on the length of the protein; it is defined by the marmoset FabM3-24 (FIG. 43C). Thirdly, the FabM3-8 recognizes an epitope uniquely exposed on ratMOG125, but on neither of the human proteins, hence this epitope is species-dependent, but length-independent (FIG. 43D). In this context it was

20 previously shown that the fabs do not inhibit each other's binding or binding of 8.18c5. Because of these findings reactivity in human sera were tested against all three antigens.

Anti-MOG reactivity in HC and MS cohorts. FIG 44 summarizes the quantitative results for antibodies against the 3 different MOG recombinants in 325 MS and 164 HC samples with the highthroughput ELISA. The results are expressed as serum concentrations

25 of anti-MOG IgG by interpolation of the ODs of serial serum dilutions to the IgG standard curve plated in each assay. It appears that IgG concentrations below 5.0 $\mu\text{g/mL}$ are over-represented, resulting in a skewed distribution (75th percentile < 4.5 $\mu\text{g/mL}$ for all groups). There were no statistically significant differences between the specific IgG concentrations of the entire MS cohort and the HC samples for any MOG recombinant ($p > 0.05$, Mann-

30 Whitney U test). However, the reactivity against ratMOG125 was significantly lower

compared to either of the human MOG antigens for both the MS and HC cohorts ($p < 0.001$, Kruskal-Wallis test with Dunn's post-hoc test).

If the MS samples are stratified for their subtypes, the skewed deviation of the IgG concentration and the significantly lower reactivity against ratMOG125 are apparent for all subgroups. In addition, there are significant differences for the mean anti-rhMOG125 and anti-ratMOG125 IgG concentrations between PP-MS and HC, but not the other subgroups and not for rhMOG118 ($p < 0.05$ Kruskal-Wallis test and Dunn's post hoc analysis for all comparisons). Thus it is contemplated that the M3-24 epitope specific to rhMOG125 and ratMOG125, is more predominantly recognized in PP-MS patients than in HC. Importantly, age, gender, disease duration, or treatment could be excluded as confounding factors in any of the subgroups or HC.

Identification and characteristics of subjects with high-titer anti-MOG IgG. Because of the skewed distribution in favor of low serum concentration antibodies we sought to identify high-titer (and thus most likely higher affinity) samples and asked whether these would be the pathophysiologically relevant autoantibodies. An advantage of the quantitative assay with its multiple serial dilutions is that it is capable of identifying signal behavior in relation to increasing dilutions. For instance, samples with reasonable reactivity at 1:200 that only slowly declines may result in an only marginally higher IgG concentration than samples with high reactivity at 1:200 that wears off dramatically once further diluted. It is therefore important to specifically identify samples with high-titer reactivity and to differentiate these cases from those that show reactivity only at lower dilutions. Using this approach, we found that samples above the 95th percentile, when subjected to a preliminary dilution series, could be diluted beyond 1:3,200 and remain positive in the ELISA (i.e., $BR \geq 3$ in FIG. 45). From this series the mean BRs for the three antigens at 1:800 are deduced and used as cut-off values. Applying this to the 1:800 serum dilution of all samples tested, those above the respective cut-off BR were deemed high-titer. Applying this cut-off 8.5 %, 5.5 % and 2.4 % of HC and a similar proportion of MS samples, 8.0 %, 8.6 % and 0.9 %, were identified for the three antigens rhMOG118, rhMOG125 and ratMOG125, respectively.

Fine epitope specificity of the high-titer samples. Further analysis of these high-titer samples reveals that the majority of the samples have high-titer reactivity against only one

of the MOG preparations (57.7 % and 60.0 % for HC and MS). For the HC samples rhMOG118 was the predominant antigen (10/15 samples reactive), while for MS both rhMOG118 and rhMOG125 for were the antigens of predominant specificity (11/24 and 12/24, respectively, as shown in Table 14). For both groups, HC and MS, ratMOG125 was the sole antigen detected only in 2/15 and 1/24 of the samples, respectively. This low specificity to the ratMOG125 epitopes is reflected by the small number of samples that have high-titer reactivity against all three MOG preparations: 1/26 for HC and 1/40 for MS, respectively. Also, if samples were high-titer reactive to two of the MOG preparations, this was almost exclusively against the combination of rhMOG118 and rhMOG125 (9/10 for HC and 14/15 for MS). None of these frequencies were significantly different between HC and MS, or between the MS subgroups ($p > 0.05$, χ^2 test).

Table 14. Reactivity Against Three Different MOG Preparations In High Titer Samples

Subjects	One Antigen			Two Antigens			Three Antigens
	rhMOG ₁₁₈	rhMOG ₁₂₅	ratMOG ₁₂₅	rhMOG ₁₁₈ rhMOG ₁₂₅	rhMOG ₁₁₈ ratMOG ₁₂₅	rhMOG ₁₂₅ ratMOG ₁₂₅	rhMOG ₁₁₈ rhMOG ₁₂₅ ratMOG ₁₂₅
HC	57.7 (15) 66.7	20.0	13.3	38.5 (10) 90.0	0.0	10.0	3.8 (1)
MS	60.0 (24) 45.8	50.0	4.2	37.5 (15) 93.3	0.0	6.7	2.5 (1)
RRMS	59.3 (16) 50.0	43.7	6.3	37.0 (10) 100	0.0	0.0	3.7 (1)
SPMS	71.4 (5) 40.0	60.0	0.0	28.6 (2) 50.0	0.0	50.0	0.0 (0)
PPMS	50.0 (3) 33.3	66.7	0.0	50.0 (3) 100	0.0	0.0	0.0 (0)

Percentage of samples with identified high-titer reactivity to have high-titer reactivity exclusively to one MOG preparation, two of the MOG preparations used or all three MOG preparations respectively. Numbers in parentheses represent actual numbers. Additionally the specificity of the high-titer reactivities are depicted as percentages. There are no
5 significant differences between HC and MS or any of the MS subgroups ($p > 0.05$, χ^2 test).

These observations are supported by the positive correlation of rhMOG118 reactivity with the respective reactivity to rhMOG125 ($p < 0.0193$, Pearson $r = 0.518$) and lack of correlation between ratMOG125 reactivity and reactivity to either rhMOG118 or
10 rhMOG125 ($p = 0.8819$, Pearson $r = 0.0697$, and $p = 0.1295$, Pearson $r = 0.5129$, respectively). These results taken together underscore the findings of unique epitope exposure as defined by the mAbs (FIG. 45) and are contemplated to be indicative of a shared immunodominant epitope between rhMOG118 and rhMOG125 that is not exposed on ratMOG125. As
15 outlined above, the differences between rhMOG118 and rhMOG125 reactivity are not due to technical shortfalls as both antigens coated equally well to the ELISA plates. Overall, the anti-MOG reactivity in humans with high-titer antibody responses appears to be diverse and not exclusively directed against epitopes that are commonly exposed, as a substantial number of high-titer reactive samples are only reactive to one MOG preparation. It should be stressed that samples non-responsive to one MOG preparation, but strongly reactive to
20 one of the others may have not been detected in assays employing only one antigen.

Correlation of anti-MOG IgG to present and prospected disability. In neither the entire MS cohort, nor in any of the subgroups did the anti-MOG titers correlate with the degree of disability as measured by the extended disability status scale (EDSS) (depicted for RR-MS in FIG. 46A). In contrast, for the RR-MS samples with high-titer reactivity, the
25 cumulative anti-MOG IgG concentrations (e.g., the sum of the IgG concentrations against rhMOG118, rhMOG125 and ratMOG125) positively correlated with the EDSS at blood sampling ($p = 0.0221$, Pearson $r = 0.4386$; FIG. 46B). However, only the cumulative IgG concentration resulted in this correlation, but not any of the IgG concentrations against either rhMOG118, rhMOG125 or ratMOG125 alone. This could either indicate that only
30 poly-specific anti-MOG responses targeting numerous MOG epitopes lead to disability

accumulation, or could be due to insufficient statistical power to identify the prevailing MOG epitopes. For the observation presented here the power was calculated to be 0.638.

Additionally to the EDSS correlation, in this cohort there is also a correlation between the cumulative anti-MOG IgG concentrations and the Multiple Sclerosis Severity Score (MSSS) that serves as a predictor of disease activity and projected disability based on the current EDSS and the disease duration in a given patient relative to a large reference cohort ($p=0.0031$, Pearson $r=0.5671$; FIG. 46C) (18). Importantly, since the disease duration alone did not correlate with the anti-MOG titers ($p=0.8142$, Pearson $r=0.0495$), a higher anti-MOG IgG was in fact associated with a higher MSSS score, indicative of a more accelerated disease progression.

For six RR-MS patients, two of which were identified with high-titer anti-MOG reactivity, samples drawn in 3-month intervals were analyzed in one ELISA assay. The results demonstrate consistent anti-MOG responses over a period of 18 months with variations within the variability limits of the assay (FIG. 47). Importantly, the two samples identified as high-titer responders did not lose their high reactivity, while none of the four other samples showed increased titers over time. These results indicate that the correlations reported here were relevant and not simply due to chance at blood sampling (e.g. elevated total IgG due to infection) and raise confidence that blood-sampling at one time-point is sufficient.

It is noteworthy, that clinical follow-up data was collected prospectively for a median time of 57 months after blood sampling (range 24-114) for 83 of the 192 RR-MS patients. 14 of these 83 patients were identified to have high-titer anti-MOG reactivity. However, prospective progression measures could not be correlated to the anti-MOG titers in this small cohort of patients. There was, though a weak positive association between the cumulative IgG concentration and the prospective progression rate (Pearson $r=0.193$), which due to the small sample size and the resulting very low power of 0.095 did not reach statistical significance.

DISCUSSION

Numerous studies have addressed the assessment and clinical implications of serum antibodies against MOG in MS (7-14,19, 20). The results are not unequivocal, but vary widely with anti-MOG frequencies between 0 % and 78 % (14, 19); for the most part this is

due to the different assay techniques used. When identical assays were applied, the variations were caused by the different MOG preparations used. Consistently, low specificities were reported for the majority of assays (8, 9, 11, 19, 21). Hence, prior to the development of the present invention, anti-MOG responses were not a reliable tool for the diagnosis of MS.

The high prevalence of anti-MOG antibodies in healthy individuals is somewhat surprising given that MOG is almost exclusively expressed and present in the CNS (1) and in animal models naïve animals are consistently negative for anti-MOG antibodies (reviewed in (2)). Studies conducted on peptide levels however, suggest that B cell epitopes are shared with bacterial pathogens and the human milk protein butyrophilin (molecular mimicry) (22, 23). It has been suggested that anti-MOG antibodies appear as a bystander phenomenon secondary to the myelin damage (8), but may be persistent in contrast to a transient anti-MOG response in other inflammatory CNS diseases where myelin damage also occurs (9). In any case, anti-MOG mAbs confer demyelination when passively transferred in a variety of primed animals (24-26). In vitro these antibodies exhibit demyelinating properties and are contemplated to activate the complement cascade (27).

The present invention however, employs several features that exploit the variability of anti-MOG antibodies. First, three different preparations of recombinant MOG have been used. Despite only minor differences in terms of peptide homology we are able to identify distinct immunodominant epitopes. This was shown by monoclonal antibodies (FIG. 43) and also by the lack of correlation between the ratMOG125 and either rhMOG118 or rhMOG125 for the human high-titer samples. It is noteworthy, that we can exclude different efficacy of coating of the MOG recombinants as the reason for the differential reactivity in human samples, because similar binding curves to all three antigens were observed for 8.18c5 (FIG. 43A). Hence, the inventors contemplate that in most cases the human anti-MOG response is directed against certain and distinct epitopes, but only infrequently against the entire span of MOG epitopes, a notion reported previously for antibody responses against insulin (28). This may explain why studies employing even slightly different MOG preparations yielded unequivocal results, at least in an ELISA format.

The studies conducted during development of the present invention indicate that anti-MOG reactivity in humans is significantly less frequently directed against the epitopes

defined by M3-8 (ratMOG125 only, FIG. 43D) and the common MOG epitopes defined by 8.18c5 and M26 (FIG. 43A and 43B). In particular, if the latter was a frequently identified epitope identical reactivities against all three MOG preparations would be expected. The identification and fine epitope characterization of pathogenic epitopes is thus of utmost importance as either pathogenic anti-MOG autoantibodies, naturally occurring anti-MOG antibodies as well as non-pathogenic antibodies directed against linear MOG epitopes are found in humans and are not discerned by current assays (29), as was reported for insulin antibodies (30).

There was little reactivity against ratMOG125 both in the entire cohort tested as well as in the high-titer samples (FIG. 44). However, we have shown previously that human anti-MOG antibodies compete with ratMOG125 specific Fabs (M3-24 and M3-8) and thus mount an immune response against these epitopes (15). In contrast, human anti-MOG IgG were not reactive to the common MOG epitopes defined by 8.18c5 and M26 (15).

Secondly, our results are not expressed as ODs derived from only one dilution, which may introduce false positive results and may lead to inconsistent plate to plate comparisons, but as serum concentrations of specific IgG. For the calculation of these concentrations all three dilutions are taken into account, hence reflecting titers of antibodies much better than with a single dilution. Expression of results relative to standard curves has been regarded as a valuable means to reduce inter-laboratory variation (31).

Thirdly, rather than employing a cut-off value derived from a control group, we identify samples with true high-titer reactivity. Because of the high frequency of anti-MOG antibodies in healthy individuals, the identification of high-titer responders is contemplated to be biologically more meaningful than the mere description of sample numbers above the control response. Less than 15 % of samples were identified as high titer reactive, which is comparable to other autoantigens (32;33). An important finding of our study was the association to disability in RR-MS patients producing high-titer anti-MOG IgG (FIG. 46). The concentrations of IgG against all three MOG preparations used in our assay not only correlated to the EDSS at the time-point of blood-sampling, (i.e. the disability already acquired), but also to a algorithmic measure of disease activity and projected disease severity, the MSSS (18). Since the MSSS takes into account the EDSS accumulated over the course of the disease duration, it is worth emphasizing that the anti-MOG titers were not

correlated to the disease durations in this study. Hence, in a subset of patients anti-MOG responses can be used as predictive biomarkers, thus validating the clinical applicability of our high-throughput assay. This correlation cannot be found in the entire MS cohort or the RRMS samples, underscoring the importance to identify and discern high titer samples from low titer samples, which may represent naturally occurring polyspecific antibodies not contributing to the disease pathophysiology. It however, implies that from the large initial number of 192 RR-MS patients only 14 % could be used for this analysis. This may be the reason why only one of the previous anti-MOG antibodies studies has reported a comparable clinical correlation for anti-MOG positive samples in 262 MS patients tested of which 14 % were deemed anti-MOG positive by comparison to the HC samples (12), in contrast most studies have tested considerably smaller numbers of MS patients (9). As described herein, the correlation of the anti-MOG response and EDSS was associated with progressive forms of MS, but not the relapsing-remitting forms (12).

We could not identify any correlation to progression markers in a subset of 154 MS patients, 83 of which RR-MS, with prospective clinical information available. This is most likely because of the heterogeneous nature of the followup information – some patients were examined and information recorded in regular intervals, others were only seen sporadically – and because of a lack of statistical power (e.g, only 14 RR-MS patients were identified with high-titer reactivity). It is therefore extremely important to continue with the systematic screening for high titer anti-MOG responses in MS patients. We envisage that a larger initial screening cohort and well defined, homogeneous follow up measures, such as time to reach landmark EDSS scores or algorithms for disability accumulation (34), may provide sufficient statistical power to identify clinically meaningful and applicable correlations. If only the baseline samples from the placebo arms of the pivotal MS treatment trials were used, 514 RR-MS samples could be screened, high titer samples identified and correlated to the already available and well characterized 2-year follow-up data with an estimated type II error below 0.05 (35-38); the sample size would more than double if the treatment arms were included which would also result in even longer follow up data available. In order to establish the anti-MOG response as a valuable and much needed prognostic marker for MS, which would be more cost-effective, less invasive and clinically better applicable than magnetic resonance imaging (39), this concerted effort is highly warranted. For comparison, in insulin-dependent diabetes mellitus (IDDM) even larger

sample numbers of 1,200-10,000 per study were needed to identify the prognostic value of autoantibodies in healthy children and relatives of patients, as less than 5 % of samples showed high titer reactivity against the respective antigens (40-42).

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It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent
5 applications cited herein are hereby incorporated by reference in their entirety for all purposes. In particular, Provisional Application No. 60/418,001, filed on October 11, 2002, U.S. Application No. 10/683,451 filed on October 10, 2003 (published as U.S. 2005/0009096), and International Application No. PCT/US2003/032349 (published as WO 2004/034031) are herein incorporated by reference in their entirety.

10

CLAIMS

What is claimed is:

1. A method of diagnosis of a relapsing remitting form of multiple sclerosis
5 (RR-MS) or a secondary progressive form of multiple sclerosis (SP-MS) in a subject, said method comprising:
comparing a level of antibodies in a biological sample from a subject that bind to galactocerebroside (alpha-GalC) to a control level of antibodies that bind to alpha-GalC, wherein an elevated level of said antibodies in the sample as compared to the control level
10 indicates that the subject has an increased likelihood of having a relapsing remitting form of multiple sclerosis (RR-MS) or a secondary progressive form of multiple sclerosis (SP-MS).
2. The method of claim 1, wherein the control level is a threshold established by detecting levels of antibodies that bind to alpha-GalC in biological samples from healthy
15 subjects.
3. The method of claim 1, wherein the biological sample from the subject comprises serum or cerebrospinal fluid.
- 20 4. The method of claim 1, wherein the level of antibodies that bind to alpha-GalC is detected by measuring binding of said antibodies to alpha-GalC immobilized on a solid surface.
5. The method of claim 4, wherein the level detected is a binding ratio
25 calculated by the ratio of signal over background.
6. The method of claim 1, wherein the subject is a human.
7. The method of claim 1, wherein the subject is a human with a preliminary
30 diagnosis of multiple sclerosis.

8. The method of claim 1, wherein the level of antibodies that bind to alpha-GalC is detected by a liquid phase assay.

9. The method of claim 1, wherein the level of antibodies that bind to alpha-GalC is detected at a first time point and at a second time point, wherein the second time point is at least about 6 months after the first time point.

10. A method of diagnosis of a relapsing remitting form of multiple sclerosis (RR-MS) or a secondary progressive form of multiple sclerosis (SP-MS) in a subject, said method comprising:

comparing a level of antibodies in a biological sample from a subject to a control level, wherein said antibodies bind to a myelin oligodendrocyte glycoprotein (MOG) isoform expressed on a eukaryotic cell surface, wherein the expressed MOG comprises a conformational epitope of MOG, and wherein an elevated level of said antibodies in the biological sample as compared to the control level indicates that the individual has an increased likelihood of having a relapsing remitting form of multiple sclerosis (RR-MS) or a secondary progressive form of multiple sclerosis (SP-MS).

11. The method of claim 10, wherein the eukaryotic cell is a mammalian cell.

12. The method of claim 10, wherein the eukaryotic cell is a Chinese hamster ovary (CHO) cell.

13. The method of claim 10, wherein the eukaryotic cell has been transfected with a nucleic acid encoding an alpha isoform of MOG.

14. The method of claim 13, wherein the alpha isoform of MOG is an alpha 1 isoform of MOG.

15. The method of claim 10, wherein the eukaryotic cell has been transfected with a nucleic acid encoding a beta isoform of MOG.

16. The method of claim 10, wherein the conformational epitope of MOG is identified by binding of Fab M3-31 or M26.
17. The method of claim 10, wherein the control level is a median or a mean
5 level of antibodies in biological samples from healthy subjects that bind to the MOG isoform expressed on the eukaryotic cell surface.
18. The method of claim 10, wherein the subject is a human.
- 10 19. The method of claim 10, wherein the subject is a human with a preliminary diagnosis of multiple sclerosis.
20. The method of claim 10, wherein the level of said antibodies is detected by FACS analysis.
- 15 21. A method of assessing multiple sclerosis (MS) risk in a subject, said method comprising:
comparing a level of antibodies in a biological sample from a subject to a control level, wherein said antibodies bind to a myelin oligodendrocyte glycoprotein (MOG)
20 isoform expressed on a eukaryotic cell surface; wherein the expressed MOG comprises a conformational epitope of MOG, wherein an elevated level of said antibodies as compared to the control level indicates that the subject has an increased likelihood of having a clinically isolated syndrome (CIS) indicative of an increased risk of developing MS.
- 25 22. The method of claim 21, wherein the eukaryotic cell is a mammalian cell.
23. The method of claim 21, wherein the eukaryotic cell is a Chinese hamster ovary (CHO) cell.
- 30 24. The method of claim 21, wherein the eukaryotic cell has been transfected with a nucleic acid encoding an alpha isoform of MOG.

25. The method of claim 24, wherein the alpha isoform of MOG is an alpha 1 isoform of MOG.
26. The method of claim 21, wherein the eukaryotic cell has been transfected
5 with a nucleic acid encoding a beta isoform of MOG.
27. The method of claim 21, wherein the conformational epitope of MOG is identified by binding of Fab M3-31 or M26.
- 10 28. The method of claim 21, wherein the control level is a median or a mean level of antibodies in biological samples from healthy subjects that bind to the MOG isoform expressed on the eukaryotic cell surface.
29. The method of claim 21, wherein the mammal is a human.
- 15 30. The method of claim 21, wherein the level of antibodies is detected by FACS analysis.
31. A method of assessing severity of multiple sclerosis in a subject having a
20 relapsing remitting form of multiple sclerosis (RR-MS), said method comprising:
detecting a cumulative concentration of antibodies in a biological sample from a subject that bind to a plurality of recombinant MOG proteins, wherein the biological sample from the subject has a high titer reactivity to at least one of the plurality of said MOG proteins, and wherein the extent of elevation in the cumulative concentration as compared to
25 a control level is indicative of the severity of RR-MS in the subject.
32. The method of claim 31, wherein the subject is a human.
33. The method of claim 31, wherein the plurality of said MOG proteins
30 comprises recombinant human MOG₁₁₈ (rhMOG₁₁₈), recombinant human MOG₁₂₅ (rhMOG₁₂₅), and rat MOG₁₂₅ (ratMOG₁₂₅).

34. The method of claim 31, wherein the control level is established with cumulative concentration of antibodies in a control biological sample that bind to the plurality of recombinant MOG proteins, wherein said control biological sample is from subjects with zero or lowest degree of disability as measured by extended disability status
5 scale (EDSS).

35. The method of claim 31, wherein the control level is established with cumulative concentration of antibodies that in a control biological sample that bind to the plurality of recombinant MOG proteins, wherein said control biological sample is from
10 subjects with zero or lowest multiple sclerosis severity score (MSSS).

36. The method of claim 31, wherein the cumulative concentration is measured by detecting binding of antibodies in the biological sample from the subject to rhMOG₁₁₈, rhMOG₁₂₅, and ratMOG₁₂₅ immobilized on a solid support.

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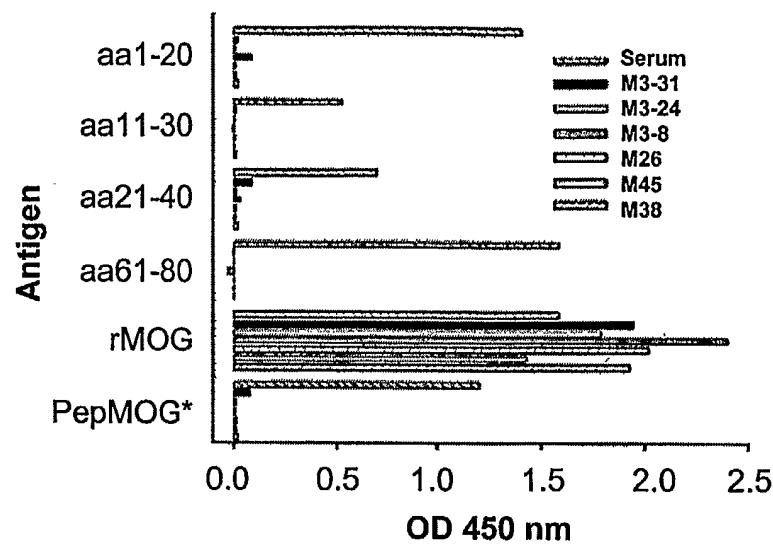


Fig. 1

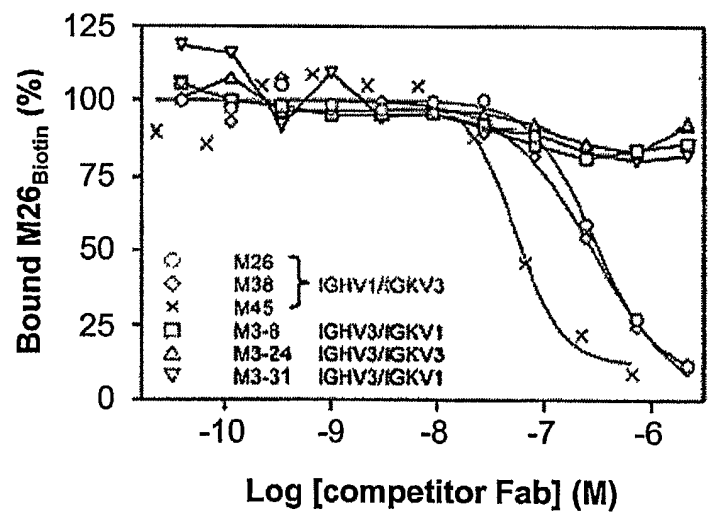
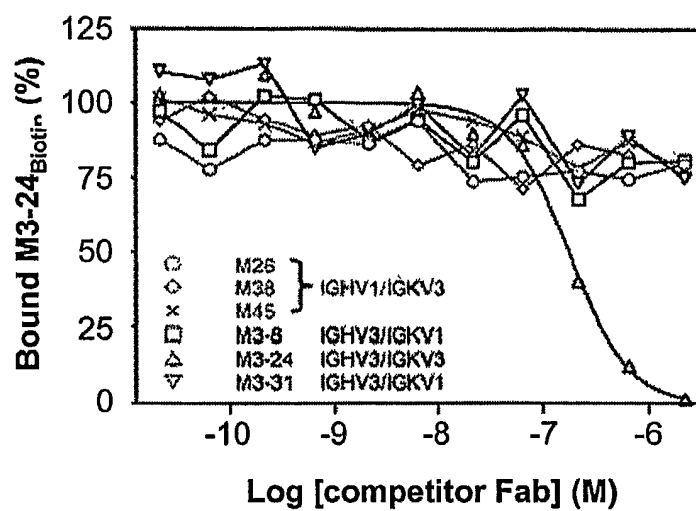
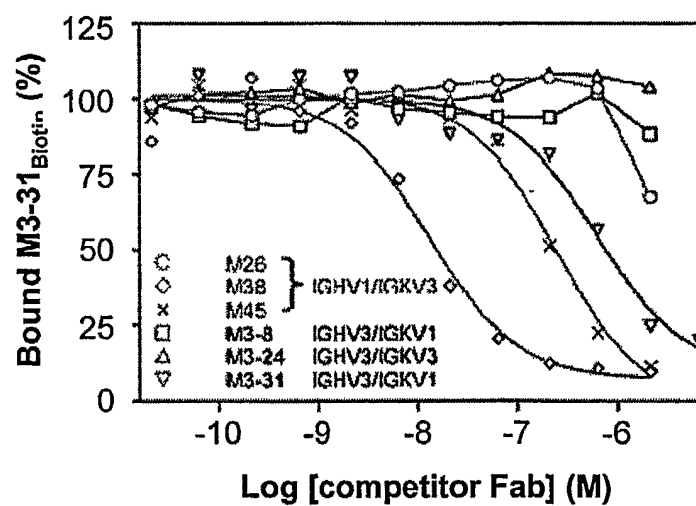


Fig. 2A

**Fig. 2B****Fig. 2C**

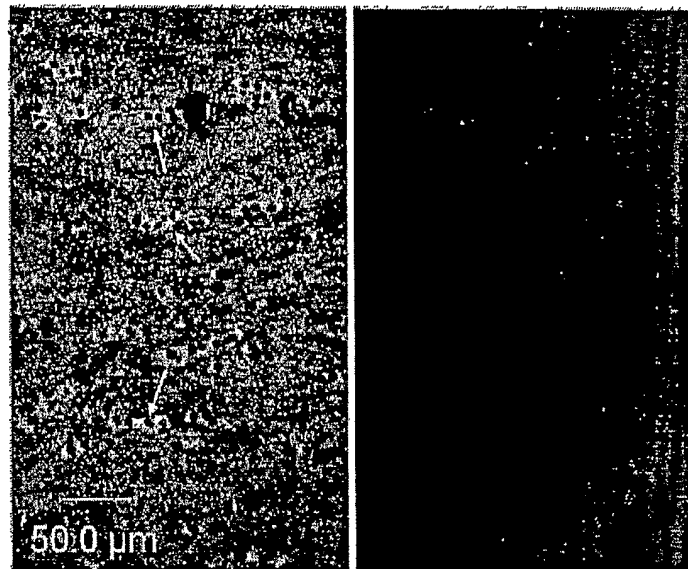


Fig. 3

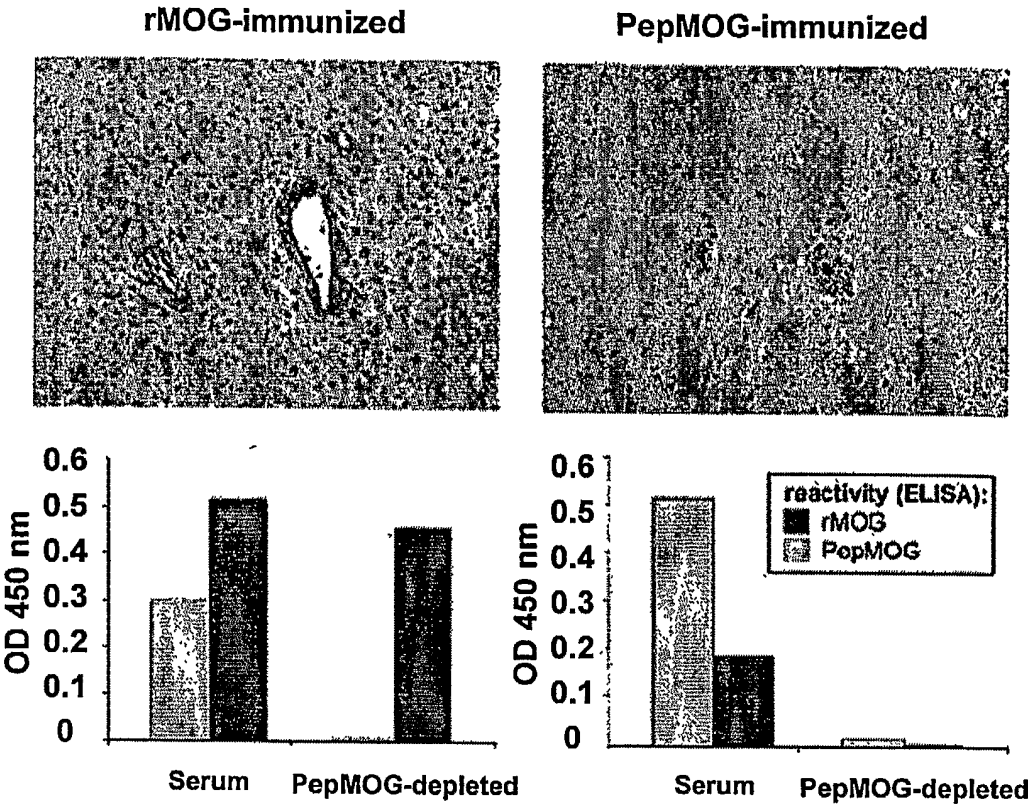


Fig. 4

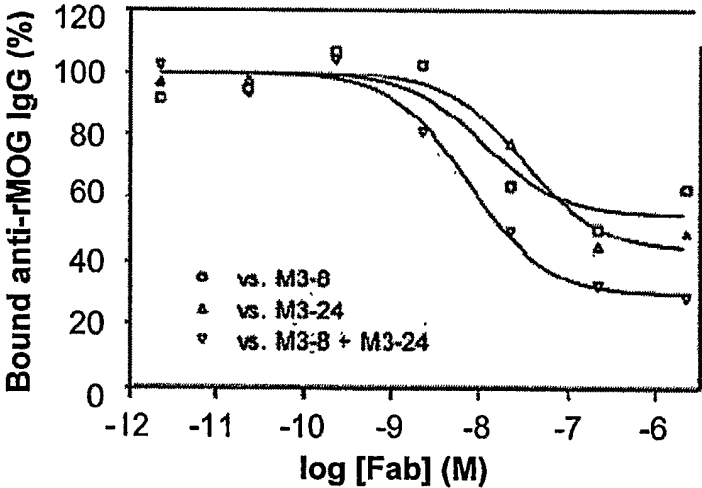


Fig. 5

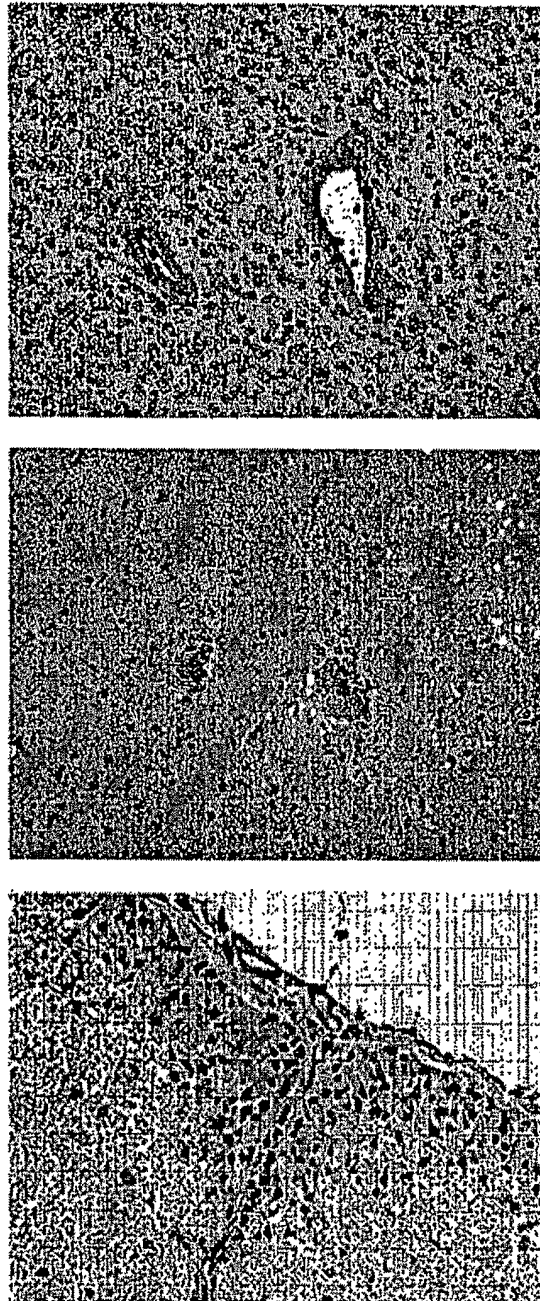
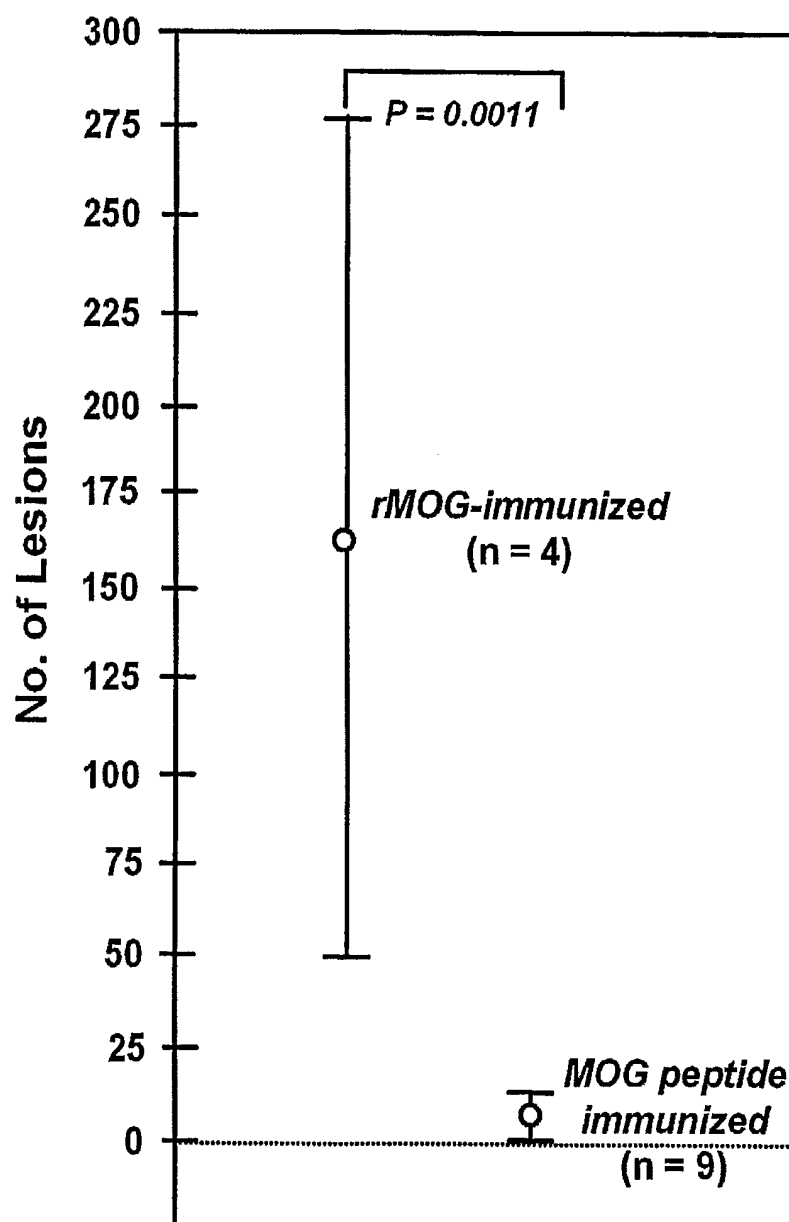
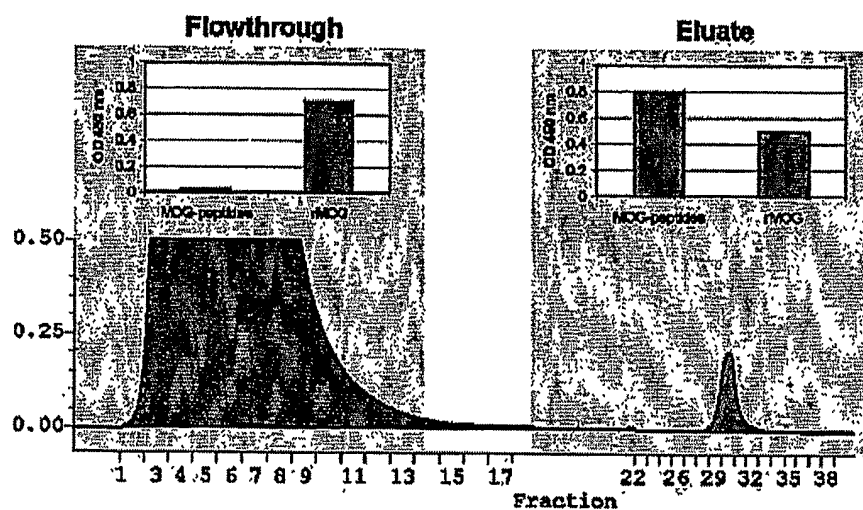
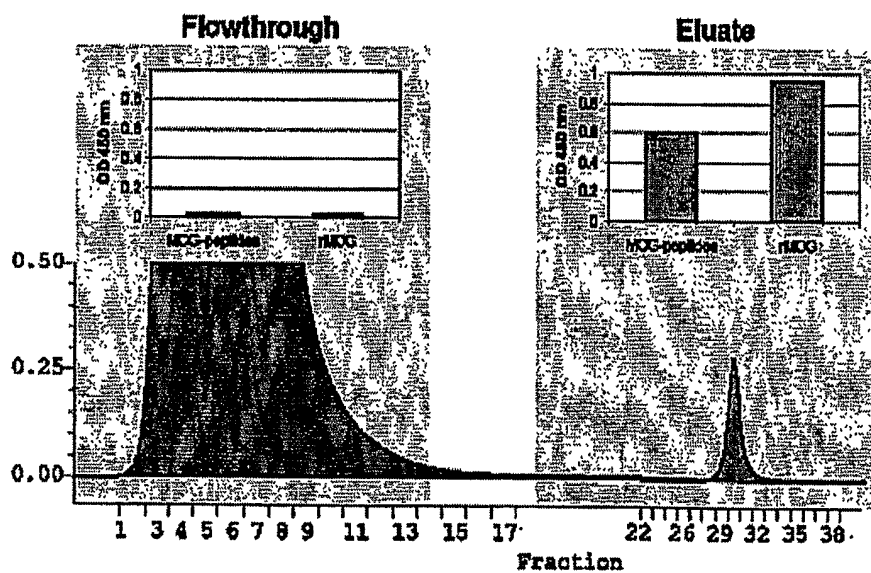


Fig. 6

5/40

**Fig. 7**

**Fig. 8A****Fig. 8B**

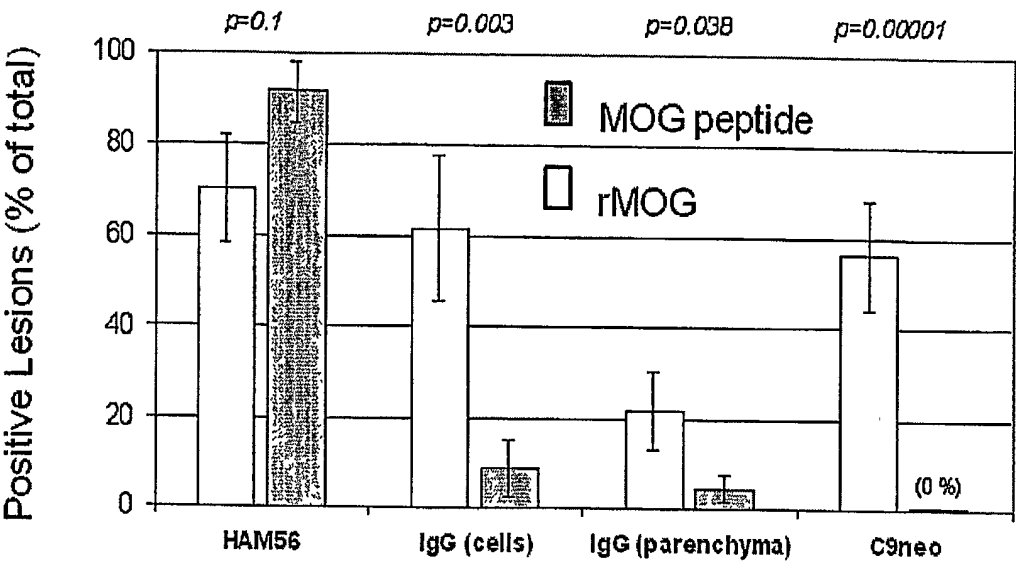


Fig. 9

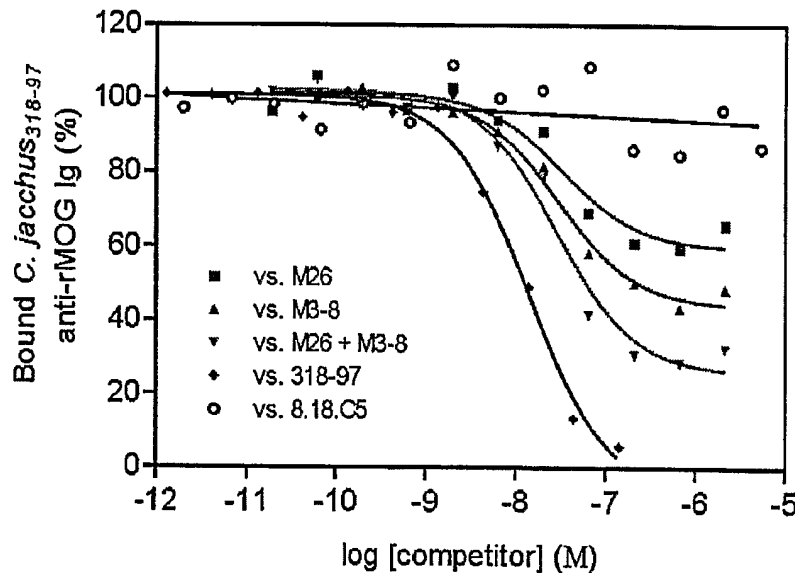


Fig. 10

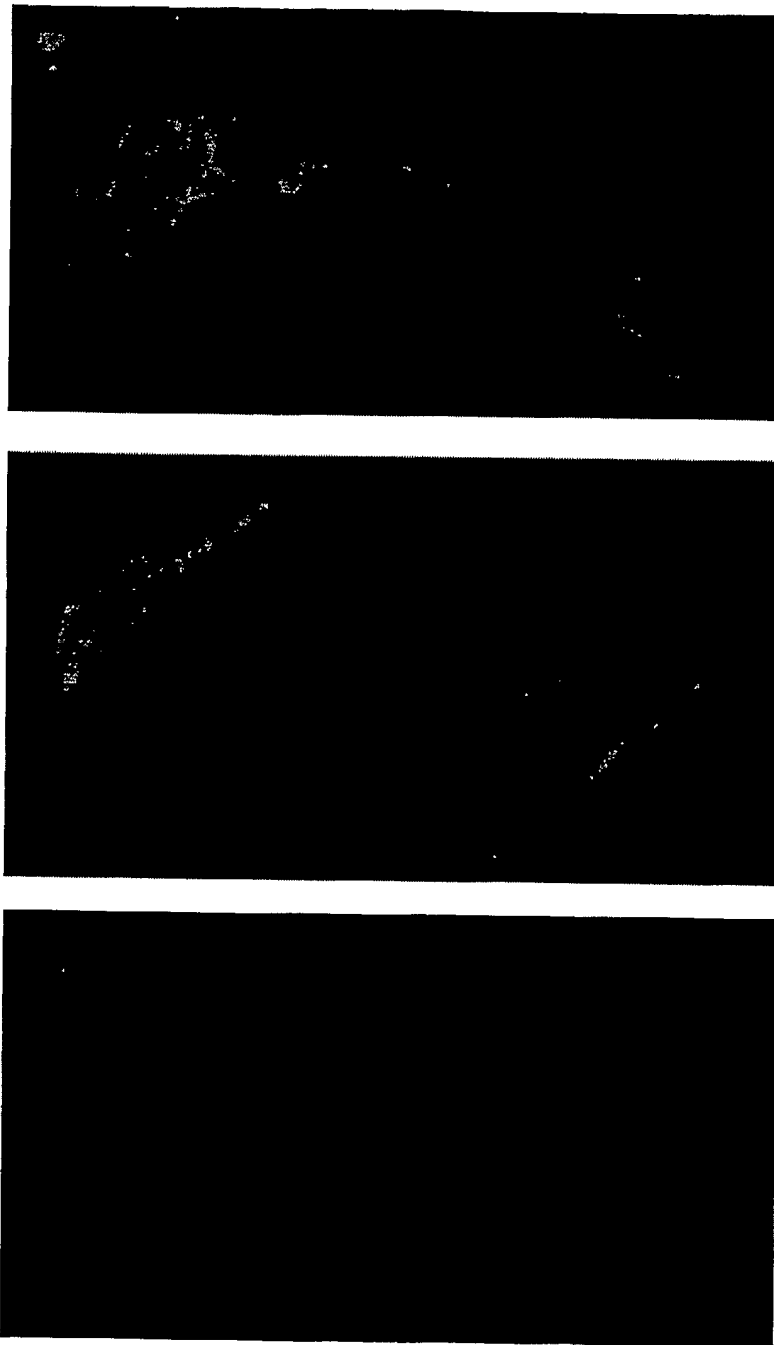


Fig. 11

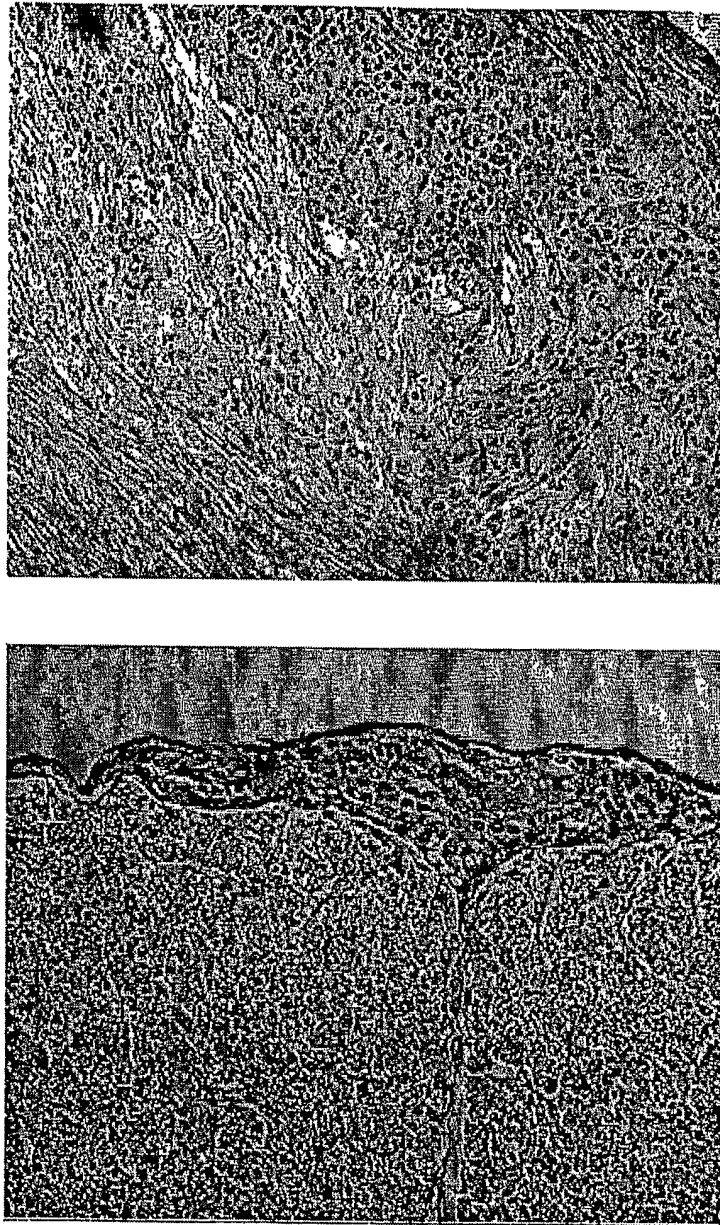


Fig. 12

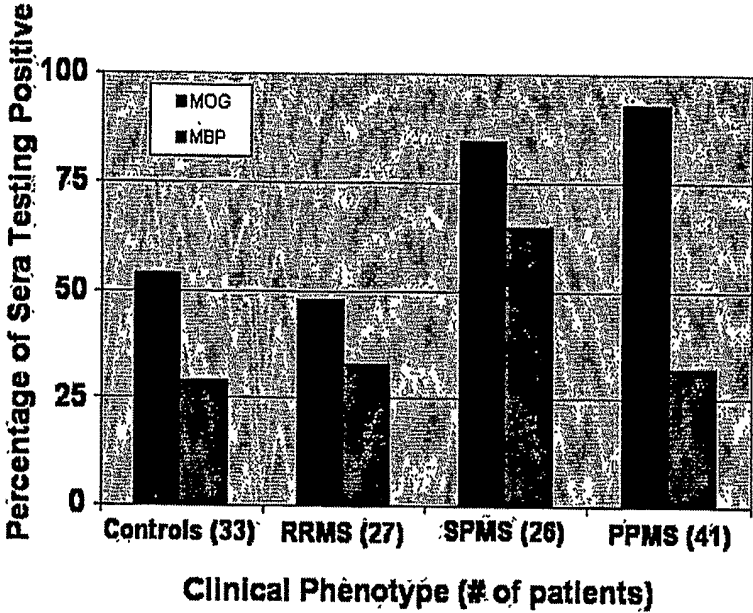


Fig. 13

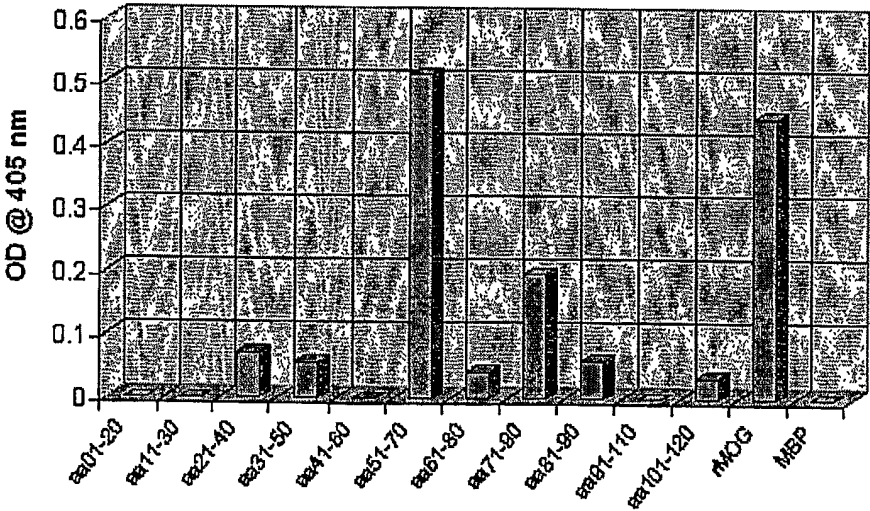
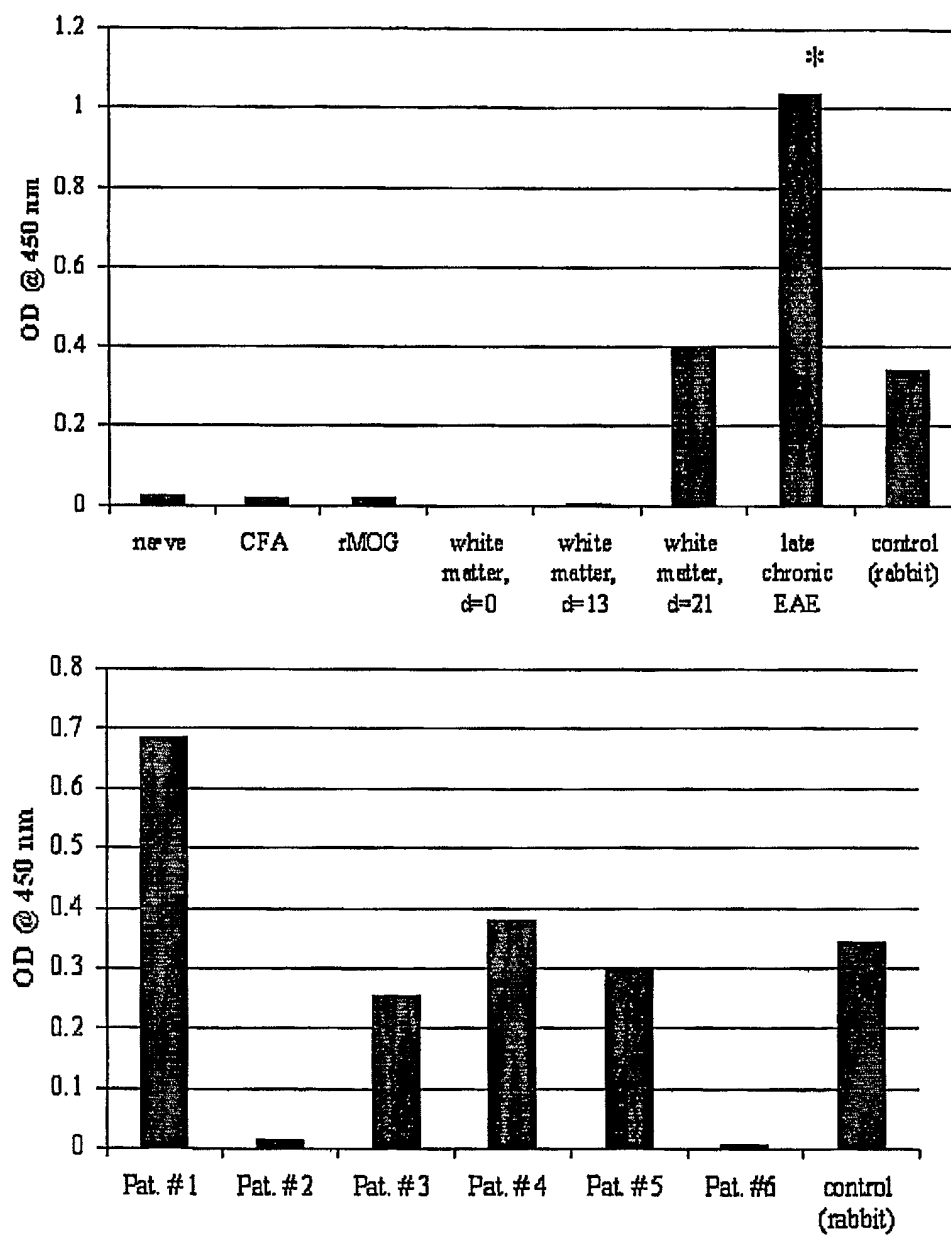


Fig. 14

**Fig. 15**

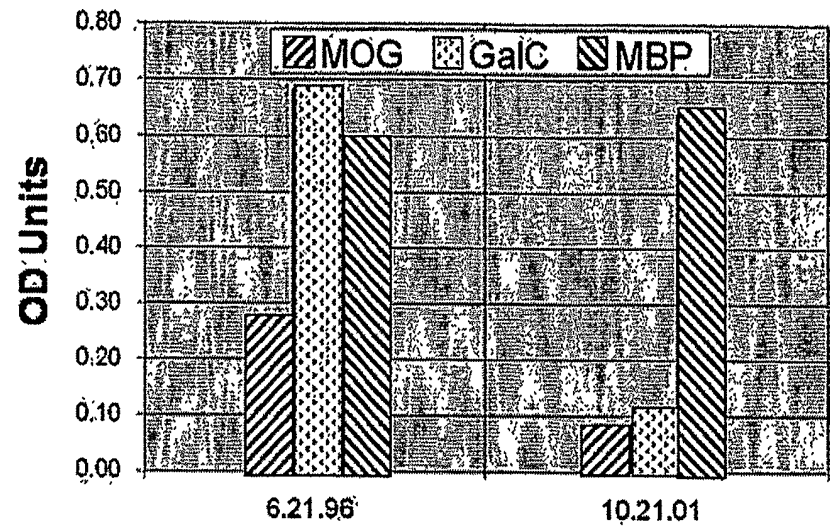


Fig. 16A

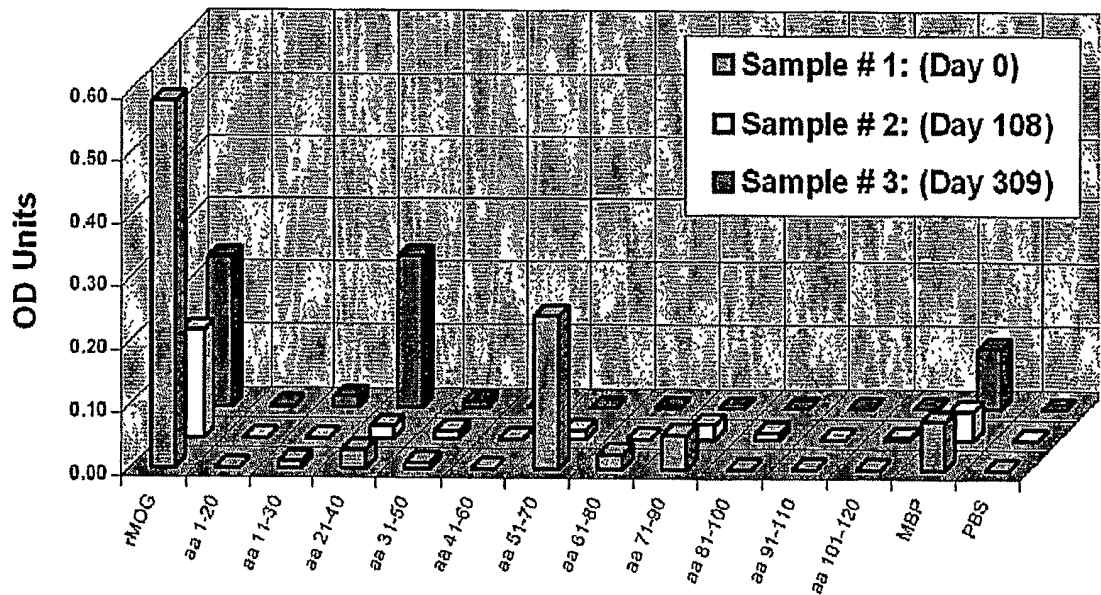
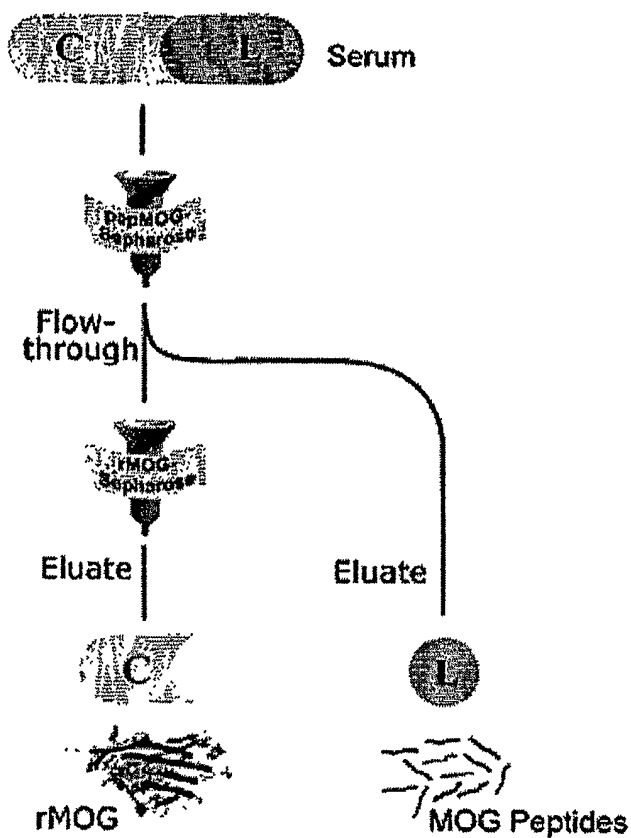
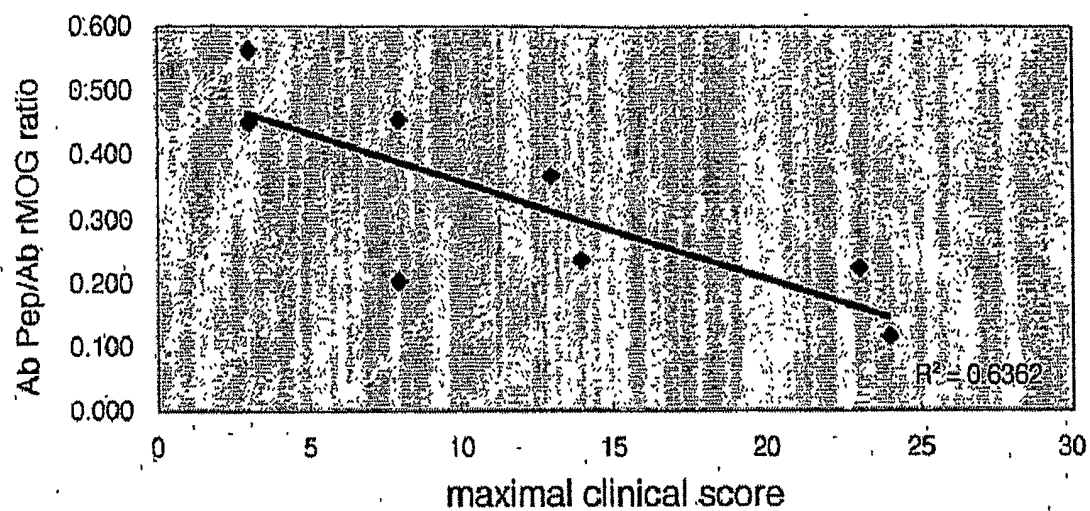
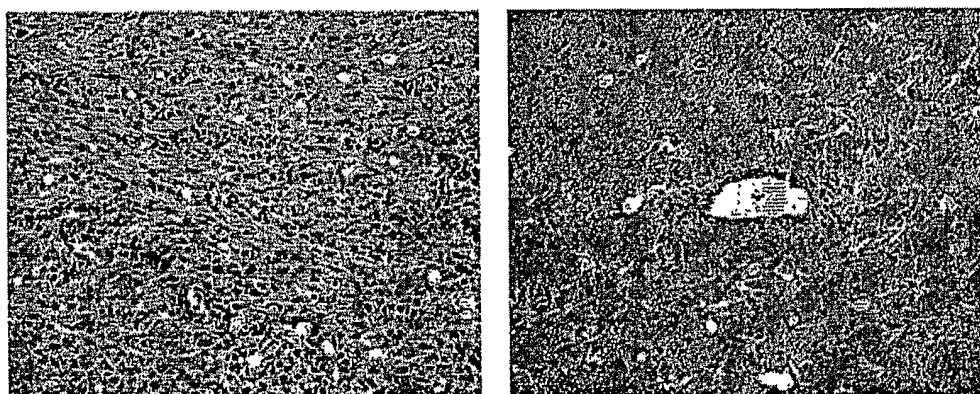


Fig. 16B



- Epitope Mapping
- In Vitro Studies
- Demyelinating Potential

Fig. 17

**Fig. 18****Fig. 19**

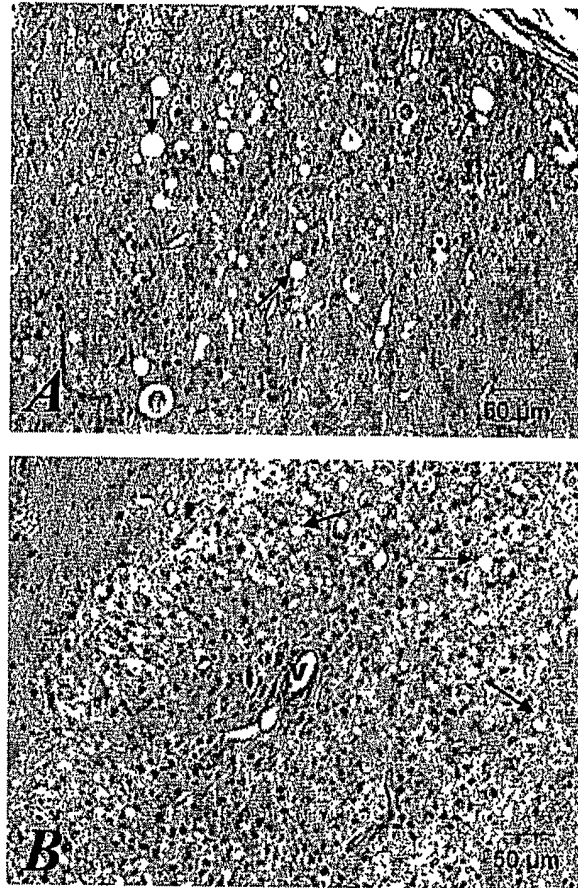


Fig. 20

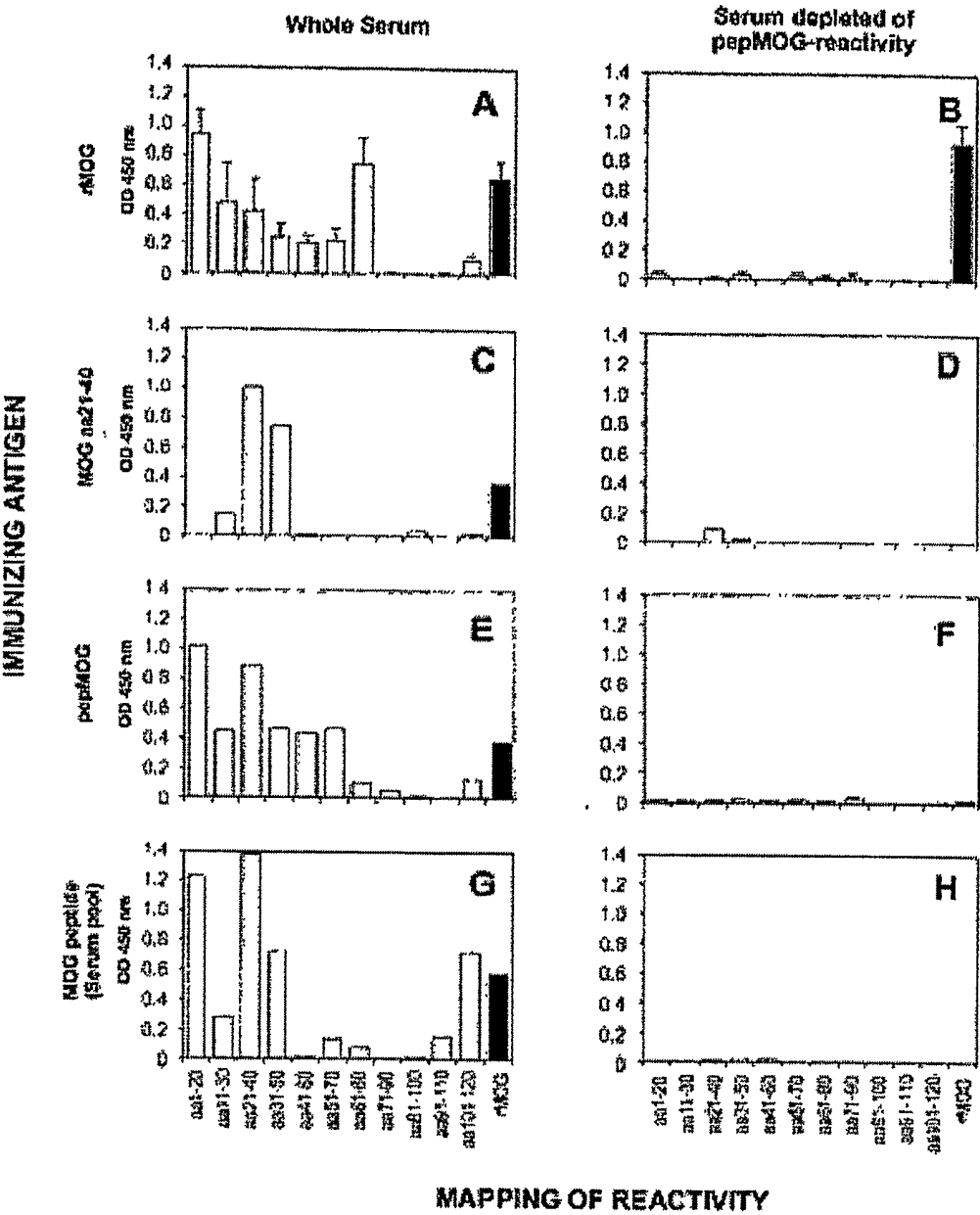
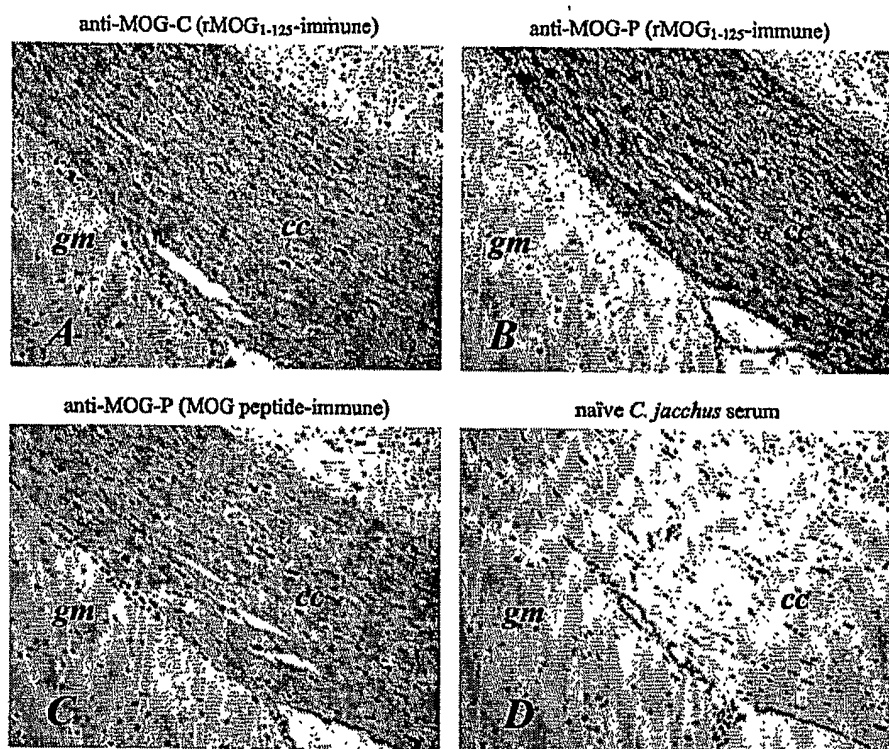
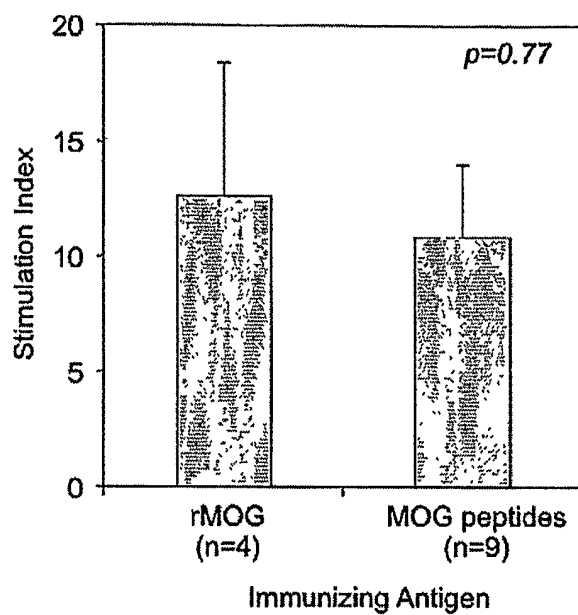


Fig. 21

**Fig. 22**

**Fig. 23**

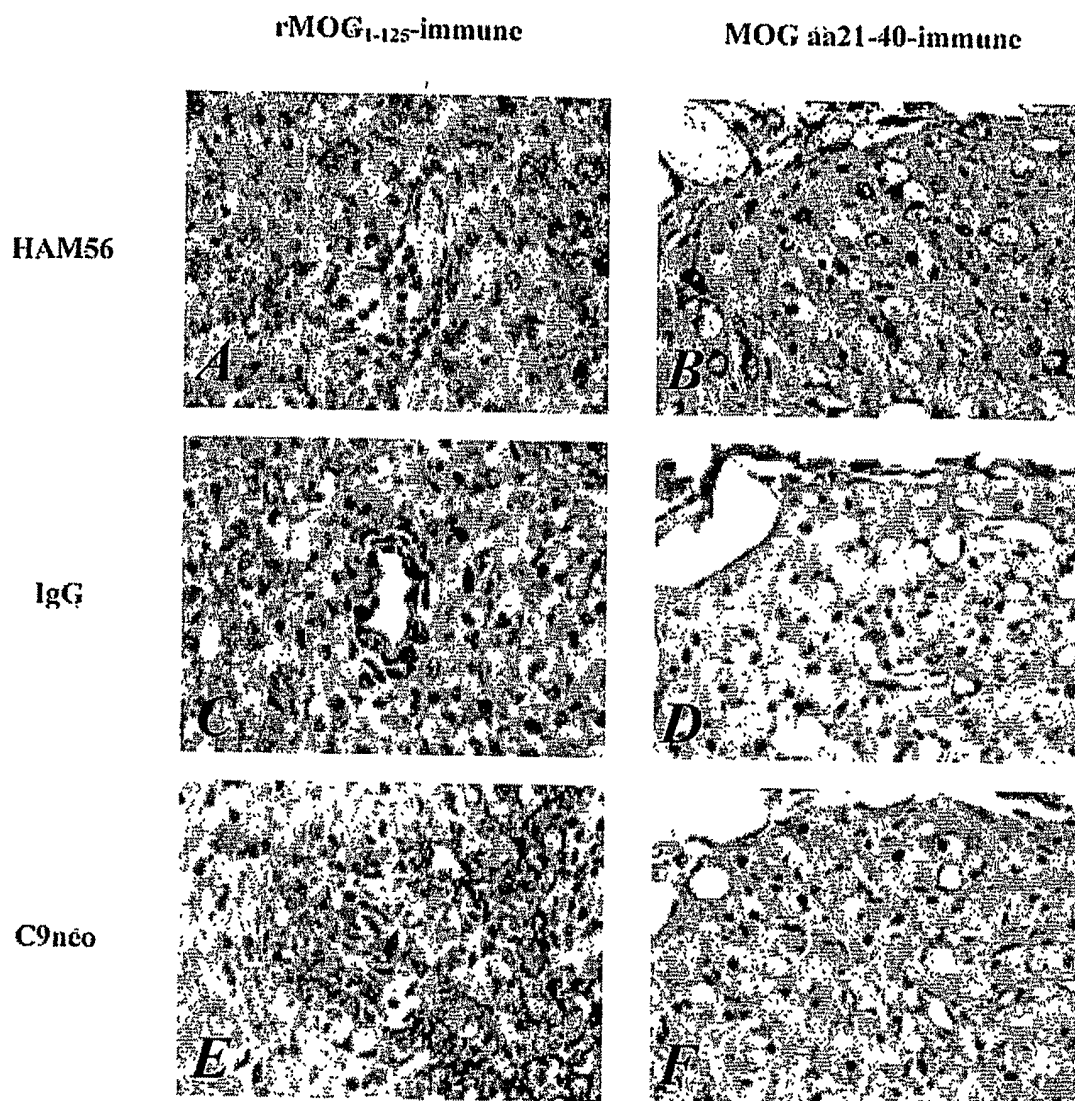


Fig. 24

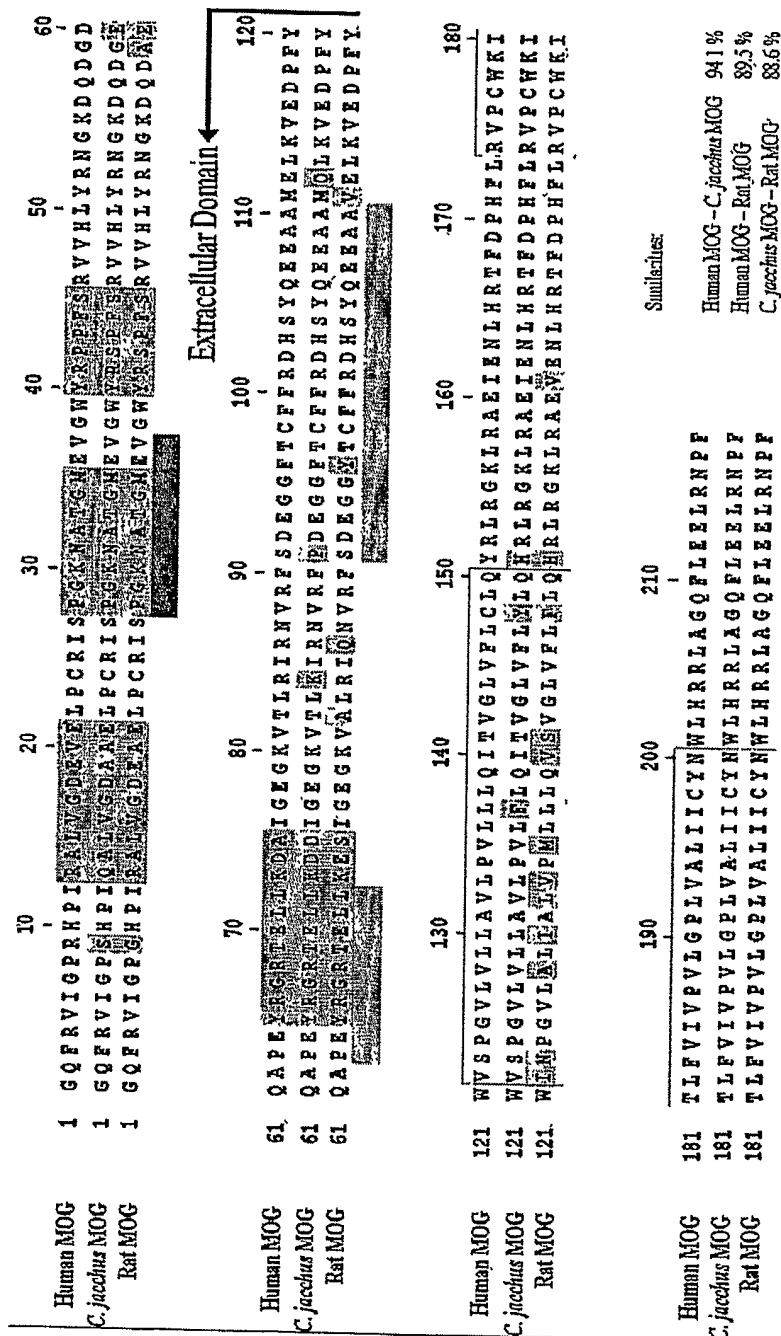


Fig. 25

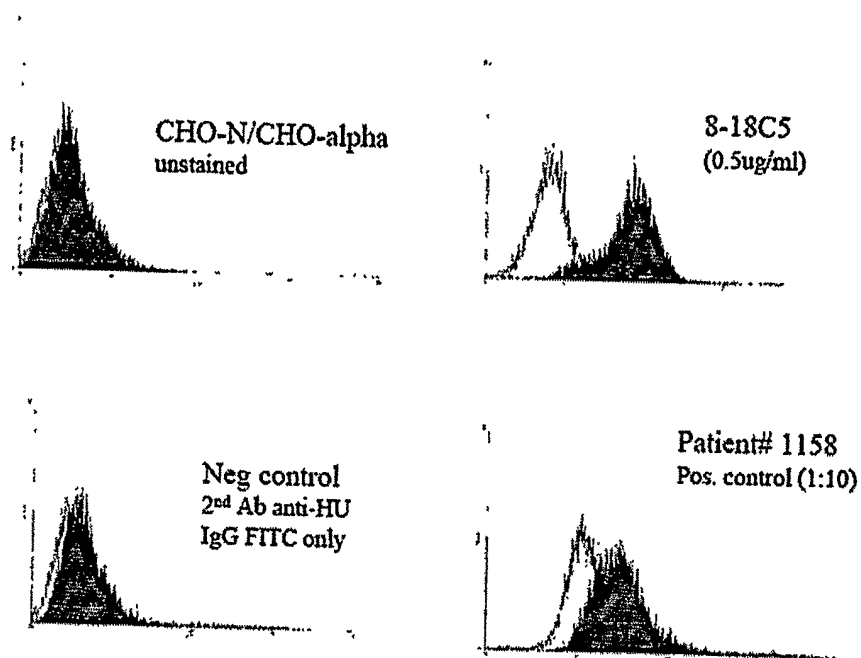
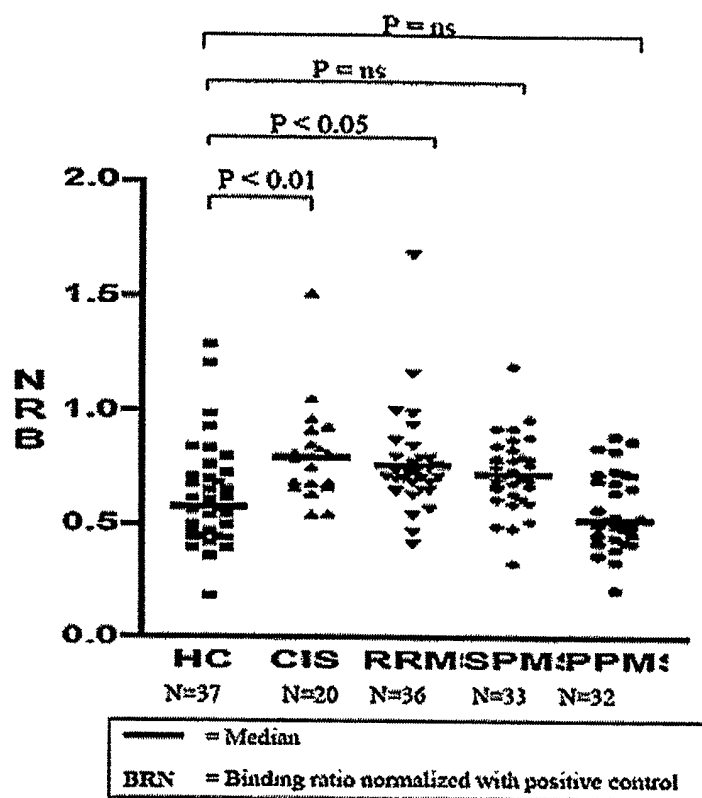
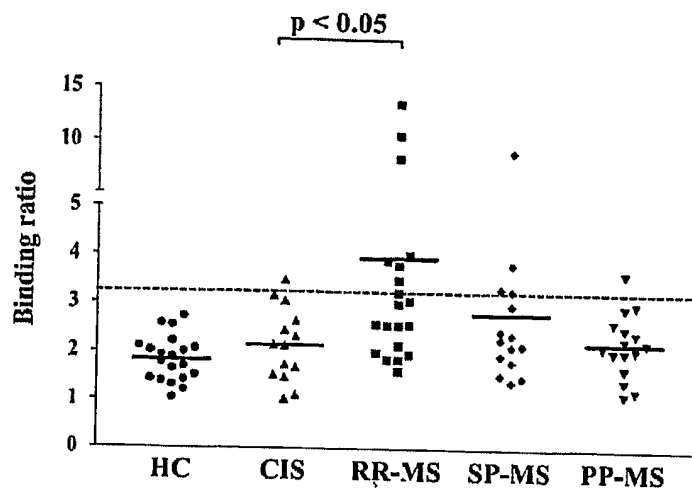
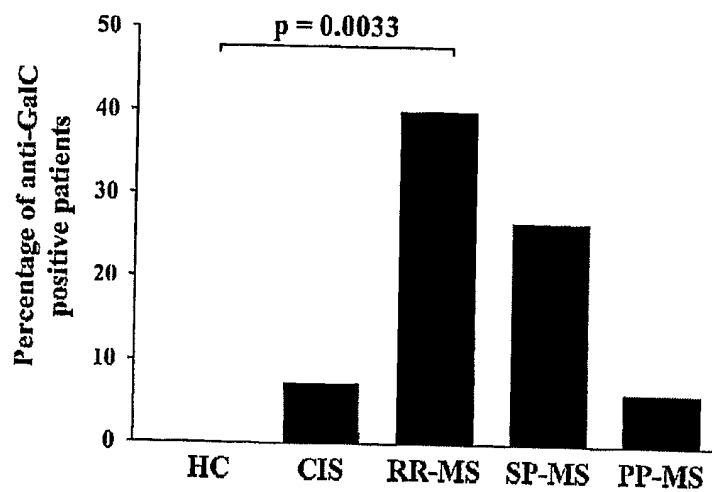
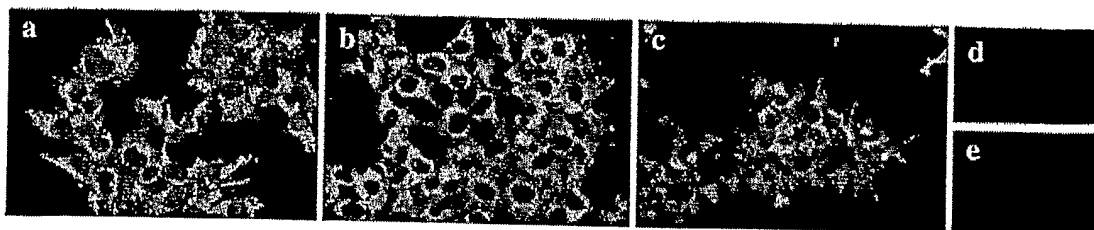
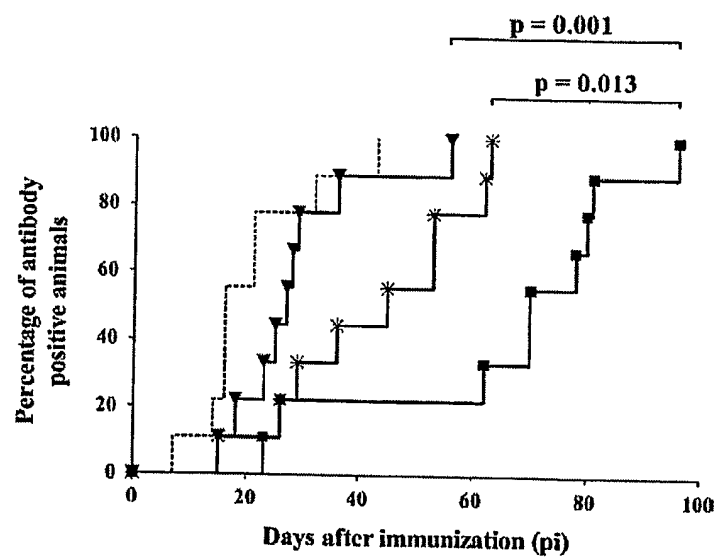


Fig. 26

*Fig. 27*

**Fig. 28A****Fig. 28B**

**Fig. 29****Fig. 30**

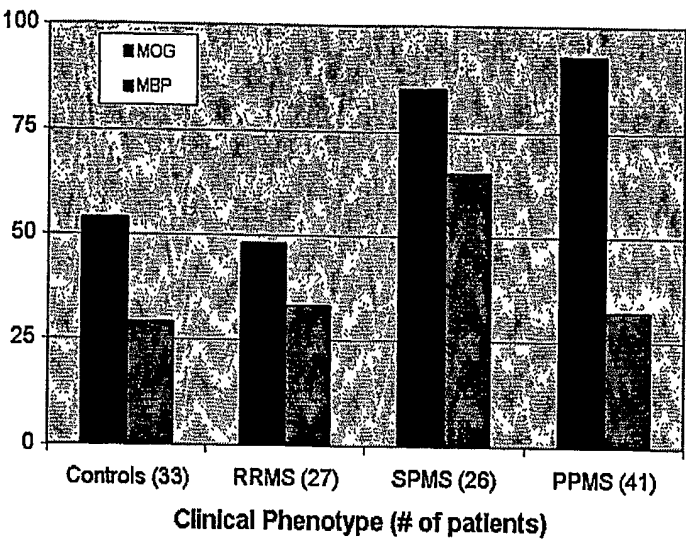


Fig. 31A

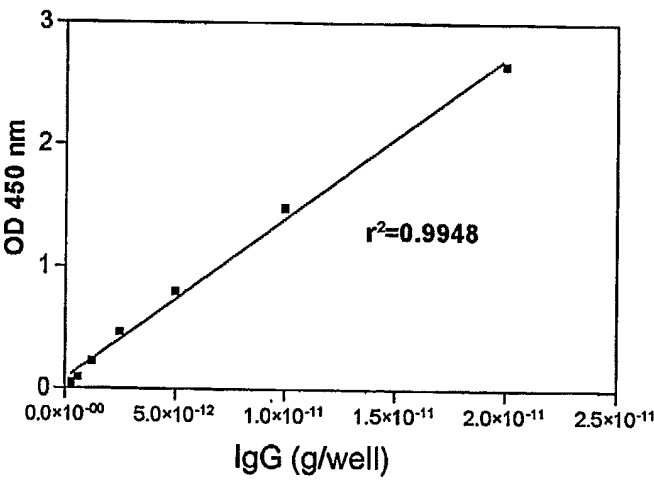
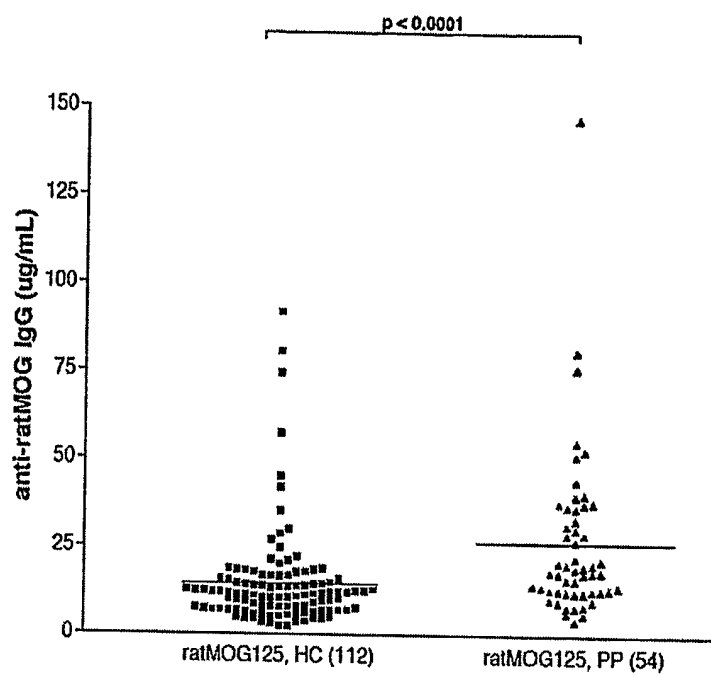


Fig. 31B

**Fig. 32**

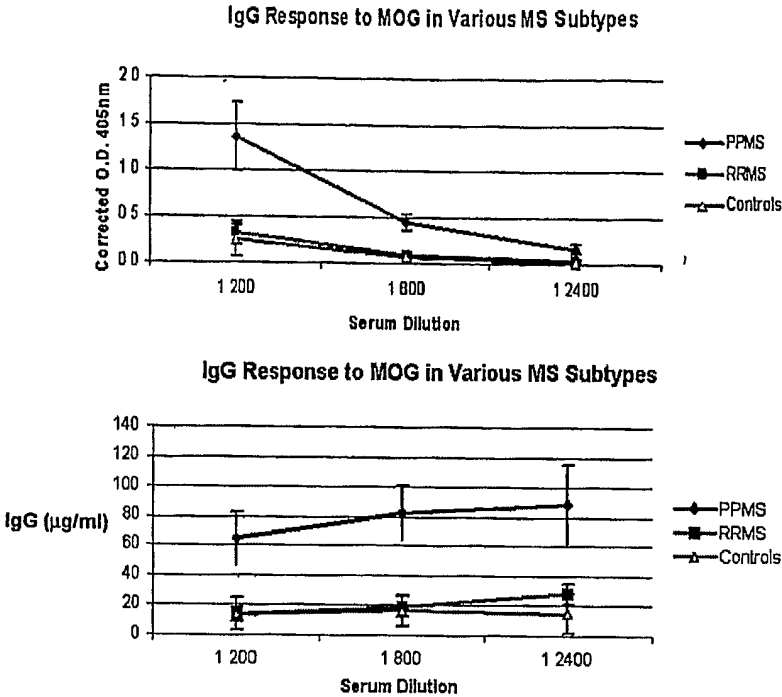


Fig. 33

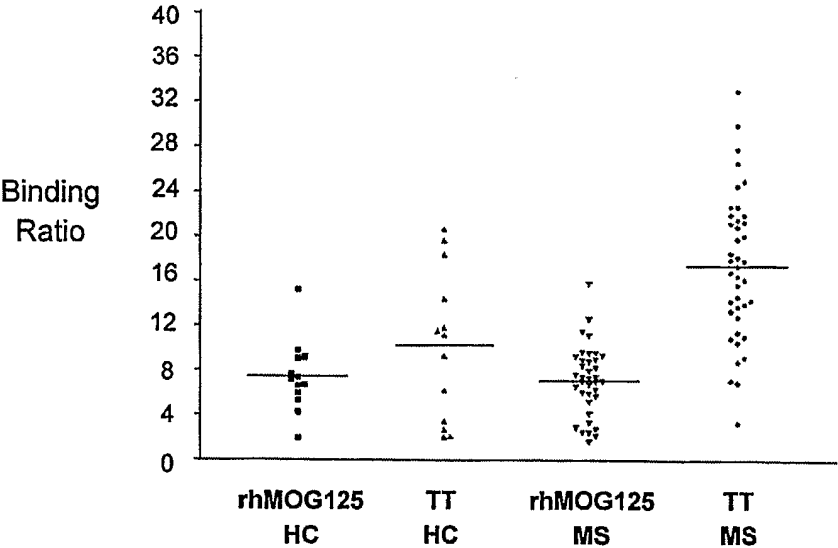


Fig. 34A

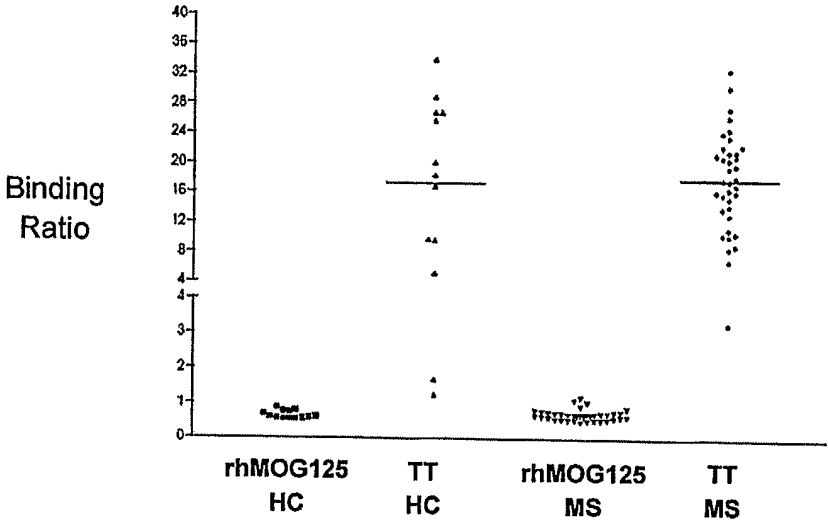


Fig. 34B

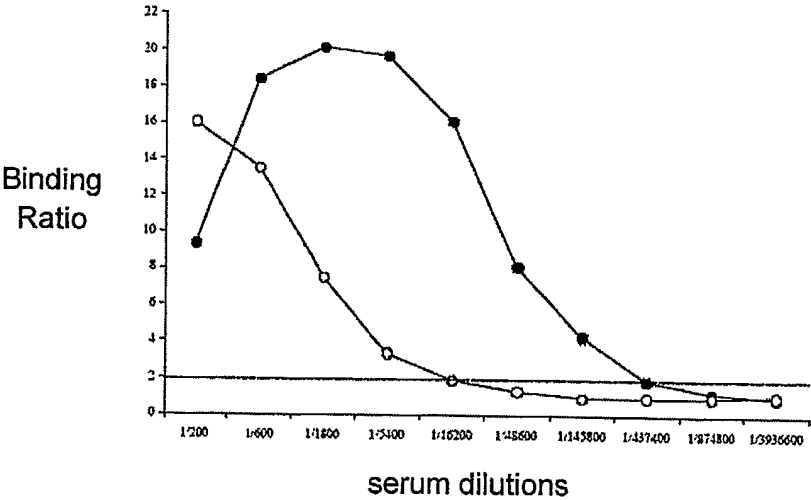


Fig. 35

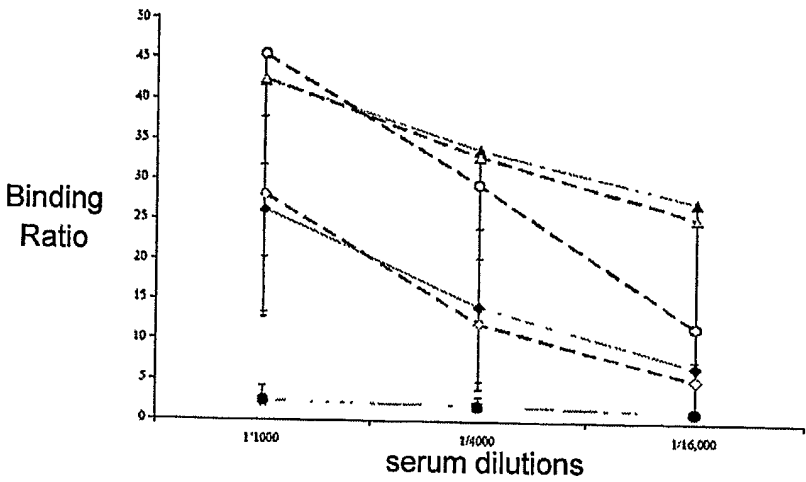


Fig. 36

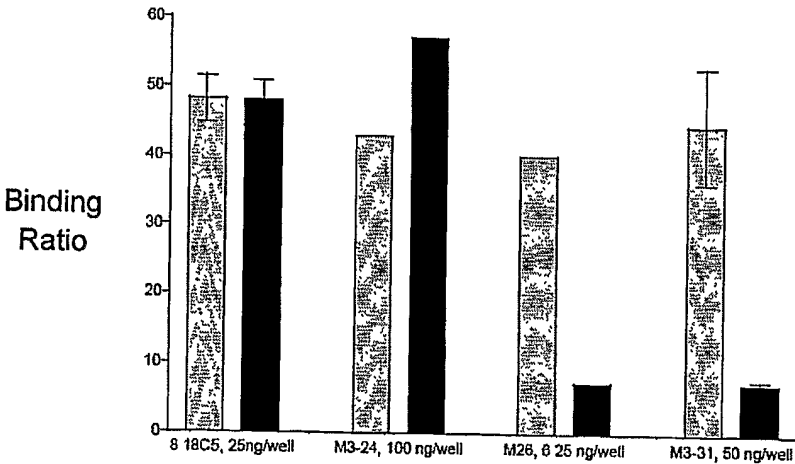
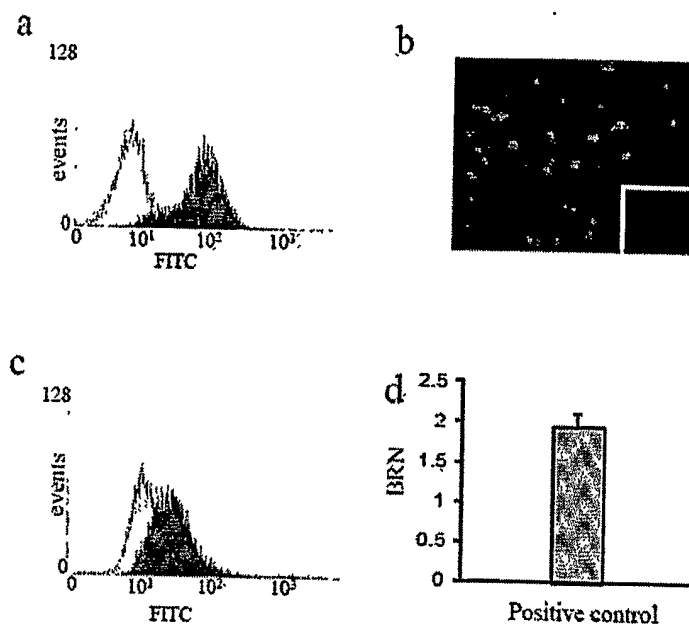


Fig. 37

**Fig. 38**

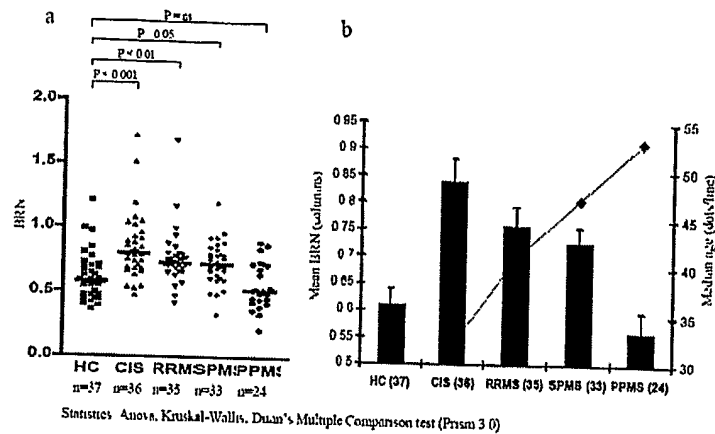


Fig. 39

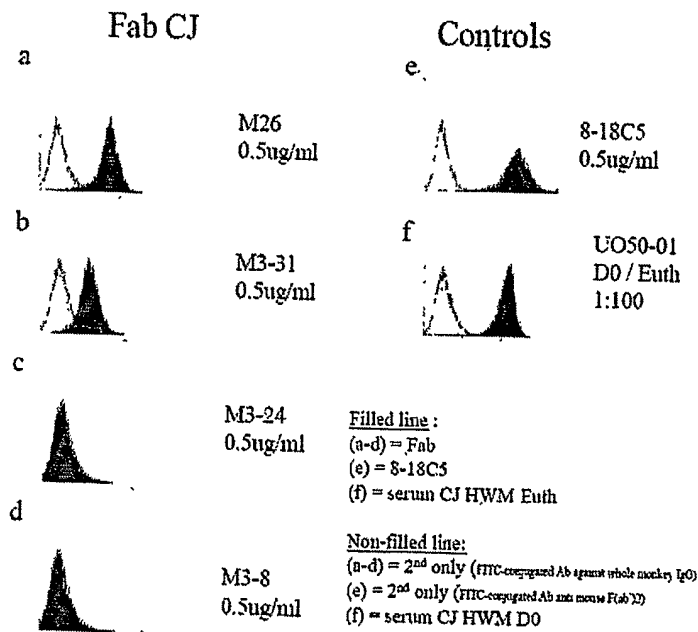


Fig. 40

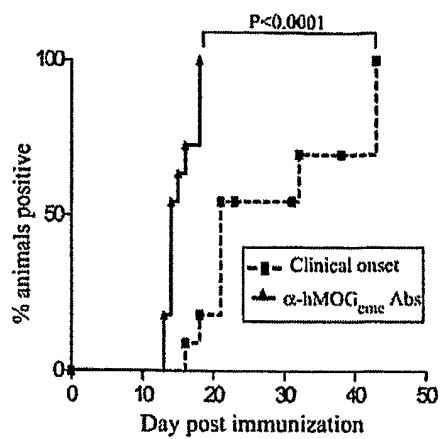
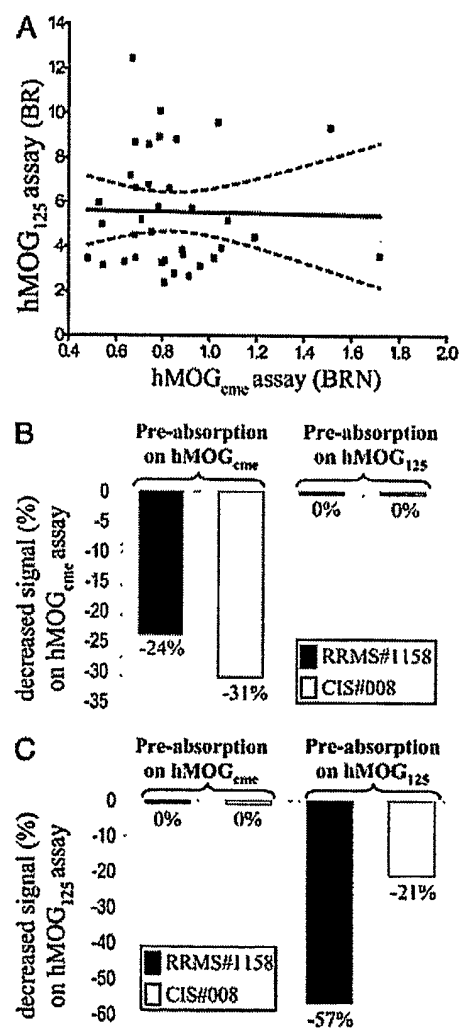
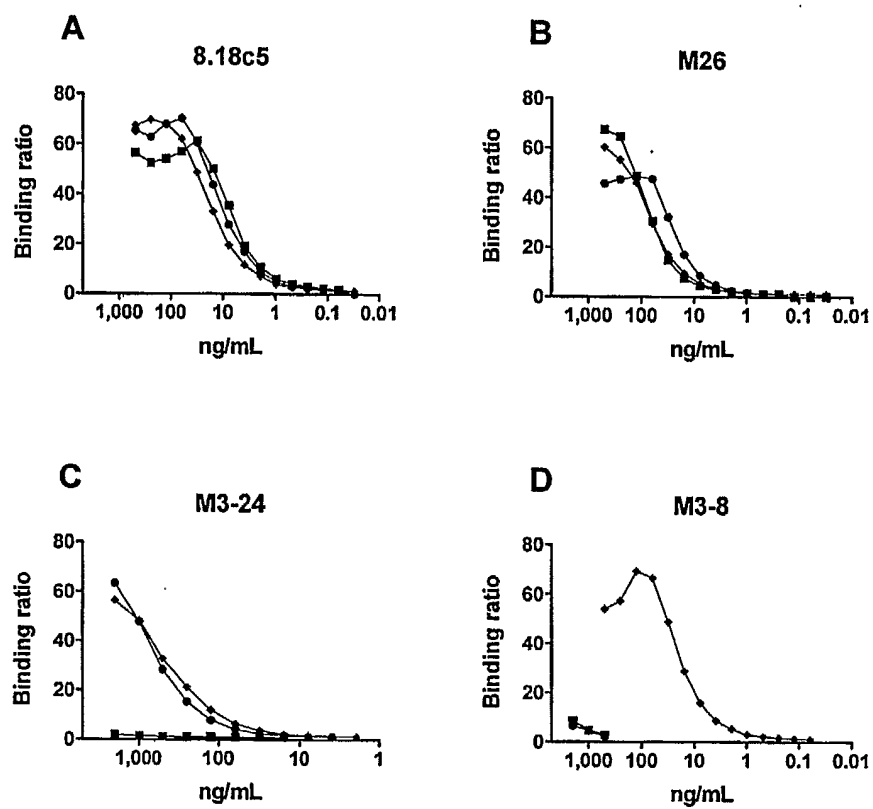


Fig. 41

**Fig. 42**

**Fig. 43**

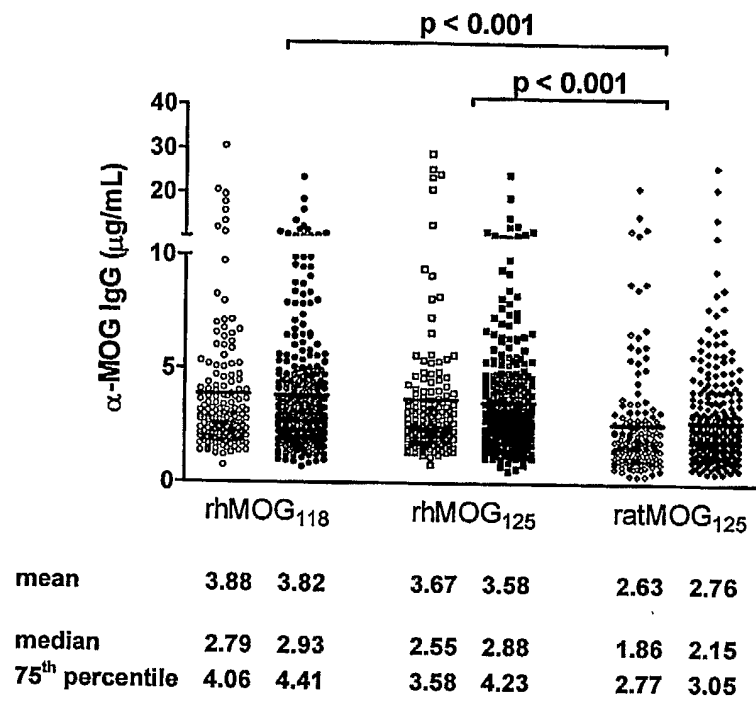


Fig. 44

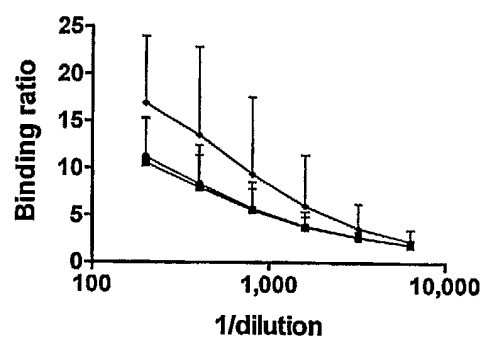
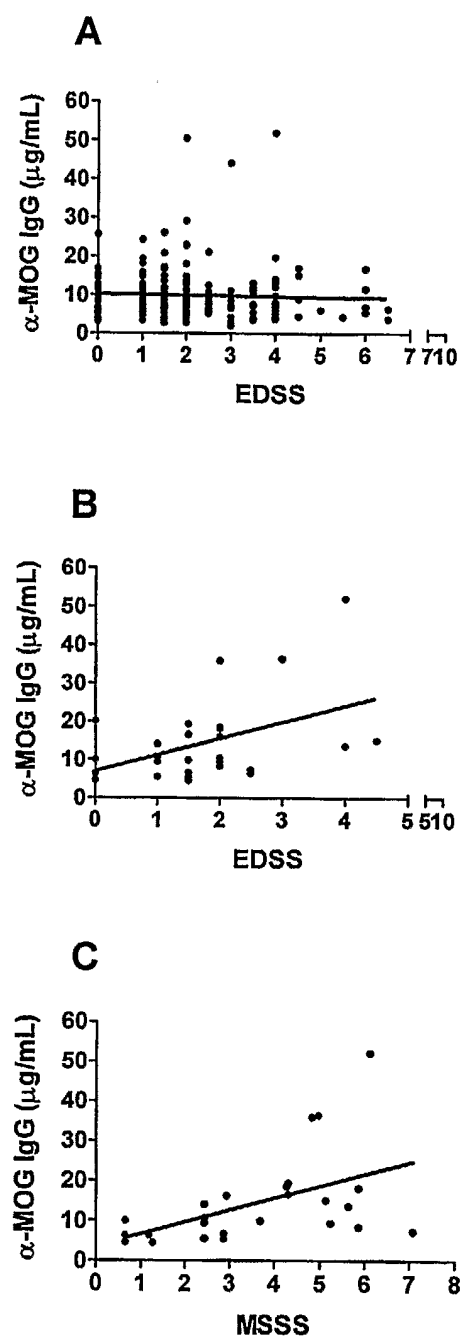


Fig. 45

**Fig. 46**

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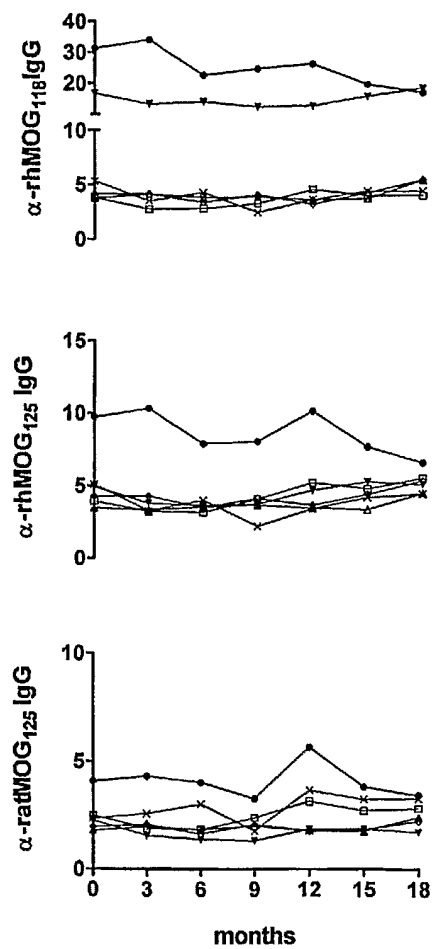
**Fig. 47**

FIG. 48A MOG alpha1 isoform protein (SEQ ID NO:41)

```

1 MASLSRPSLP SCLCSFLLLL LLQVSSSYAG QFRVIGPRHP IRALVGDEVE LPCRISPGKN
61 ATGMEVGWYR PPFSRVVHLY RNGKDQDGDQ APEYRGRTTEL LKDAIGEGKV TLRIRNVRFs
121 DEGGFTCFFR DHSYQEEAAM ELKVEDPFYW VSPGVLVLLA VLPVLLQLIT VGLVFLCLQY
181 RLRGKLRAEI ENLHRTFDPH FLRVPCWKIT LFVIVPVLGP LVALIICYNW LHRRLAGQFL
241 EELRNPF

```

FIG. 48B MOG alpha1 isoform cDNA (SEQ ID NO:42)

```

1 atggcaagct tatcgagacc ctctctgcc agctgcctct gctccttct cctcctcctc
61 ctctcccaag tgtcttcag ctatgcaggg cagttcagag tgataggacc aagacaccct
121 atccgggctc tggtcgggga tgaagtggaa ttgccatgtc gcatatctcc cgggaagaac
181 gctacaggca tggaggtggg gtggtaccgc ccccccctct ctagggtggg tcatctctac
241 agaaatggca aggaccaaga tggagaccag gcacctgaat atcggggccg gacagagctg
301 ctgaaagatg ctattggtga gggaaagggtg actctcagga tccggaatgt aaggttctca
361 gatgaaggag gtttcacctg cttcttcgga gatcattctt accaagagga ggcagcaatg
421 gaattgaaag tagaagatcc tttctactgg gtgagccctg gaggctggg tctcctcgcg
481 gtgctgcctg tgctcctcct gcagatcact gttggcctcg tcttctctg cctgcagtac
541 agactgagag gaaaacttcg agcagagata gagaatctcc accggacttt tgatccccac
601 tttctgaggg tgccctgctg gaagataacc ctgtttgtaa ttgtgccggg tcttggaacc
661 ttggttgctt tgatcatctg ctacaactgg ctacatcgaa gactagcagg gcaattcctt
721 gaagagctac gaaacccttt ctga

```

FIG. 48C MOG beta1 isoform protein (SEQ ID NO:43)

```

1 MASLSRPSLP SCLCSFLLLL LLQVSSSYAG QFRVIGPRHP IRALVGDEVE LPCRISPGKN
61 ATGMEVGWYR PPFSRVVHLY RNGKDQDGDQ APEYRGRTTEL LKDAIGEGKV TLRIRNVRFs
121 DEGGFTCFFR DHSYQEEAAM ELKVEDPFYW VSPGVLVLLA VLPVLLQLIT VGLVFLCLQY
181 RLRGKLRAEI ENLHRTFDPH FLRVPCWKIT LFVIVPVLGP LVALIICYNW LHRRLAGQFL
241 EELLFHLEAL SG

```

FIG. 48D MOG beta1 isoform cDNA (SEQ ID NO:44)

```

1 atggcaagct tatcgagacc ctctctgcc agctgcctct gctccttct cctcctcctc
61 ctctcccaag tgtcttcag ctatgcaggg cagttcagag tgataggacc aagacaccct
121 atccgggctc tggtcgggga tgaagtggaa ttgccatgtc gcatatctcc cgggaagaac
181 gctacaggca tggaggtggg gtggtaccgc ccccccctct ctagggtggg tcatctctac
241 agaaatggca aggaccaaga tggagaccag gcacctgaat atcggggccg gacagagctg
301 ctgaaagatg ctattggtga gggaaagggtg actctcagga tccggaatgt aaggttctca
361 gatgaaggag gtttcacctg cttcttcgga gatcattctt accaagagga ggcagcaatg
421 gaattgaaag tagaagatcc tttctactgg gtgagccctg gaggctggg tctcctcgcg
481 gtgctgcctg tgctcctcct gcagatcact gttggcctcg tcttctctg cctgcagtac
541 agactgagag gaaaacttcg agcagagata gagaatctcc accggacttt tgatccccac
601 tttctgaggg tgccctgctg gaagataacc ctgtttgtaa ttgtgccggg tcttggaacc
661 ttggttgctt tgatcatctg ctacaactgg ctacatcgaa gactagcagg gcaattcctt
721 gaagagctac tcttcacctt ggaagccctc tctggctaa

```