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Die differentielle humorale Immunantwort gegen Myelin bei entzündlichen demyelinisierenden Erkrankungen des ZNS

Habilitationsschrift

Zur Erlangung der venia legendi
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Dr. med. Til Menge
Düsseldorf, den 27. Januar 2011

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Einleitung

Dem Nervensystem wird eine Rolle als so genanntes immunprivilegiertes Organ zugesprochen, da eine Überwachung durch das Immunsystem physiologischerweise nicht im selben Maße stattfindet wie in den übrigen Körperorganen und das Nervensystem kein lymphatisches Gewebe hat [1]. Dennoch wird das Zentralnervensystem (ZNS) kontinuierlich von Immunzellen durchwandert, die auch im nicht aktivierten Zustand über die Bluthirnschranke transmigrieren können [2;3]. Kommt es zu einer wie auch immer gearteten Störung dieser Homöostase, wandelt sich das ZNS in einen Schauplatz reich an aktiven Immunprozessen, die im Falle einer Reaktion gegen *Selbst* auch autoimmuner Natur sein können [4;5]. Einer der Hauptangriffspunkte solch einer autoaggressiven Reaktion sind myelinische Bestandteile [6-8].

Myelin, das im ZNS von Oligodendrozyten gebildet wird, umgibt spiralförmig als lipidreiche Biomembran Axone, um sie elektrisch zu isolieren und dadurch die Nervenleitgeschwindigkeit zu erhöhen [9]. Eine Vielzahl von neurologischen Erkrankungen können zu einer Demyelinisierung führen. Eine häufige, typische Erkrankung ist die Multiple Sklerose (MS). Im derzeit gängigen pathogenetischen Konzept kommt es zu einer autoimmunologischen lokalen Inflammation, in deren Folge zu einer anfangs noch reversiblen Demyelinisierung und im weiteren Verlauf zu einem zumeist irreversiblen Axonschaden [10;11]. Eine derartige Pathologie kann scheinbar wahl- und regellos in der gesamten Neuroachse des ZNS, also Hirn und Rückenmark, vonstatten gehen, was sich in der alternativen Diagnosebeschreibung Enzephalomyelitis disseminata widerspiegelt. Die MS ist eine chronische Erkrankung, die zumeist schubartig verläuft und deren Auslöser nicht bekannt ist [10;12]. Dem gegenüber steht die akute disseminierte Enzephalomyelitis (ADEM), die post- oder parainfektiös vor allem bei Kindern auftritt, zumeist vollständig remittiert und per definitionem ein einmaliges Ereignis ist [13;14]. Experimentell lassen sich beide Erkrankungen im Tiermodell der experimentellen autoimmunen Enzephalomyelitis (EAE) untersuchen [15-17]. Aus der EAE ist bekannt, dass Autoantikörper (AAK) gegen myelinische Antigene an der Pathogenese beteiligt sind [18]. Derartige AAK lassen sich auch bei Patienten mit MS oder ADEM finden, wobei die Datenlage hier nicht eindeutig ist und sich die Hoffnung, durch eine geeignete

Auswahl der AAK prognostische oder gar diagnostische Biomarker zu finden, bisher nicht erfüllt hat [19-21].

Die vorliegende Arbeit wird zunächst den gegenwärtigen Stand des Wissens im Hinblick auf AAK gegen myelinische Antigene bei MS, ADEM und EAE zusammenfassen und diskutieren. Desweiteren werden eigene Arbeiten zu dem Thema erläutert und in Kontext mit dem gegenwärtigen Wissenstand gestellt. Zusammenfassend wird zum Abschluß ein Ausblick auf die zukünftige Richtung dieser Forschung unternommen.

1.1. Myelin – Physiologie und Biologie

Myelin wird im ZNS von Oligodendrozyten und im peripheren Nervensystem von den Schwannzellen produziert [22-24]. Als Biomembran dient es der elektrischen Isolierung der Axone, windet sich spiralförmig und hochorganisiert in mehreren Lagen um diese (Abb. 1A), periodisch unterbrochen von den Ranvier'schen Schnürringen. Dies ermöglicht die saltatorische Reizleitung, die die Nervenleitgeschwindigkeit erheblich erhöht.

Myelin ist lipidreich und besteht nur zu einem Viertel aus Proteinen. Die Lipidkomponente besteht aus 25% Cholesterin, 20% Galactozerebrosid, 5% Galactosulfatid und aus 50% weiteren Phospholipiden [25]. Der Proteinanteil besteht zu fast gleichen Teilen aus myelinbasischem Protein (MBP) und Proteolipid-Protein (PLP). Beide Proteine liegen innerhalb der Membran und haben nur geringe extrazelluläre Anteile. Insbesondere sind sie nicht auf der äußersten Spiralschicht der Myelinscheide exprimiert, so dass ihnen eher eine organisierende Funktion zugesprochen wird (Abb. 1B). Zahlreiche weitere Proteine, zumeist Glykoproteine kommen in nur geringen Anteilen vor, von denen einige spezifisch für das ZNS sind (Abb. 1B).

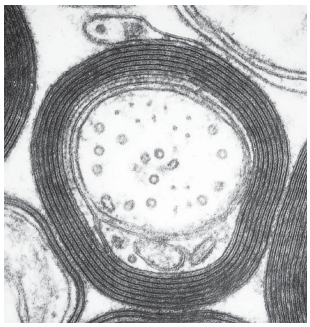

Abb. 1A

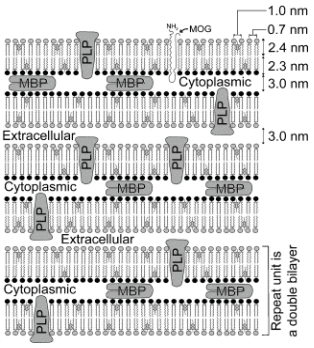

Abb. 1B

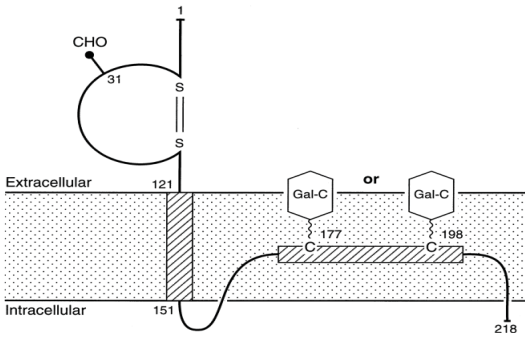

Abb. 1C

Abbildung 1: **(A)** Elektronenmikroskopische Aufnahme eines axial getroffenen myelinisierten Axons; bei 12 Uhr Ansicht des Oligodendrozyten; davon ausgehend spinnt sich zwiebelschalenartig die Myelinschicht um das Axon. Zur Verfügung gestellt von Dr. Cedric S. Raine, New York, USA. **(B)** Schematische Darstellung der Lipiddoppelschicht der Myelinmembran und der darin eingelagerten transmembranösen Proteine. MOG ist als einziges derartiges Protein nur an der äußersten Membranschicht exprimiert und ragt mit seinem N-terminalen Anteil nach extrazellulär (aus [26]). **(C)** Schematische Darstellung der strukturellen Interaktion von MOG und GalC (aus [27]).

1.2. Myelin-Oligodendrozyten Glykoprotein

Eines dieser ZNS-spezifischen Glykoproteine ist das Myelin-Oligodendrozyten Glykoprotein (MOG), das nur knapp 0,1-0,5 % der Myelinproteine ausmacht. Es wird ausschließlich im ZNS exprimiert [27-31] und zwar an der äußersten Lamelle der spiralförmigen Myelinscheide (Abb. 1B). MOG hat eine große extrazelluläre Domäne mit interner Disulfidbrücke, einer immunglobulinartigen Domäne und einer Glykosylierungsstelle [30;32;33]. Wie viele andere myelinische Proteine ist es stark hydrophob. Ursache hierfür ist wahrscheinlich der hohe Lipidgehalt des Myelin, der zu einer stark hydrophoben Umgebung führt. Die Funktion von MOG ist bisher nicht bekannt. Die Expression als membranständiges Protein mit einem nach intrazellulär reichenden C-Terminus läßt an eine Signaltransduktion denken, wobei bisher kein entsprechender intrazellulärer Bindungspartner identifiziert werden konnte. Wahrscheinlich handelt es sich daher um ein Adhäsionsmolekül oder ein Molekül mit zytoarchitektonischer Funktion, indem es bei Stimulation das Wachstum der Myelinscheide begrenzt [27]. Allerdings sind MOG-defiziente Mäuse phänotypisch gesund und bilden insbesondere keine oligodendrozytischen Tumoren aus [34]. In Kontrast dazu haben MBP- oder PLP-defiziente Mäuse bzw. Mäuse mit Mutationen in den MBP- oder PLP-Genen eine Myelinisierungsstörung (Dysmyelinisierung) und sind zumeist schwer krank oder gar nicht erst lebensfähig [35].
Im Kontext dieser Arbeit ist MOG interessant, weil es trotz seines überaus geringen Anteils am Myelin ein starkes enzephalitogenes Antigen ist [18]. Dies geht aus tier-experimentellen Daten hervor, die unten weiter diskutiert werden. Welchen Einfluß MOG auf die Pathogenese humaner demyelinisierender Erkrankungen hat, ist nicht abschließend geklärt und zum Teil Fragestellung der hier besprochenen eigenen Arbeiten.

1.3. Galaktozerebrosid

Rund ein Fünftel der Lipidkomponente des Myelins werden von Galaktozerebrosid (GalC) gebildet, das hauptsächlich im Nervensystem vorkommt und zur Gruppe der Glycosphingolipide gehört. Strukturell besteht GalC aus einem Ceramid, an das glykosidisch die Monohexose Galaktose gebunden ist [25;36;37].

Die Wichtigkeit von GalC auf die Myelinphysiologie und Membranintegrität wird durch den M. Krabbe verdeutlicht, einer autosomal rezessiv vererbten Speichererkrankung, bei der es zum einem Funktionsausfall der Galactosylceramidase kommt, so dass GalC nicht mehr abgebaut werden kann [38;39]. Histopathologisch kommt es zu Demyelinisierung. Die Rolle von GalC als Zielantigen bei autoimmunen demyelinisierenden Erkrankungen wird weiter unten besprochen. Von Bedeutung ist die aus theoretischen Überlegungen heraus angenommene lokalisatorische Nähe von MOG und GalC in der Myelinmembran (Abb. 1C) [27].

1.4. Autoimmunität

Das Immunsystem dient der Abwehr exogener infektiöser Agenzien, im wesentlichen Bakterien, Viren und andere Mikroorganismen. Dafür stehen das angeborene und das adaptive Immunsystem zur Verfügung. Durch komplexe Selektionsmechanismen während der Heranreifung von Immunzellen des adaptiven Immunsystems wird verhindert, dass das Immunsystem gegen *Selbst* reagiert. So durchlaufen T- und B-Lymphozyten zunächst eine positive Selektion, bei der nur funktions- bzw. interaktionsfähige Zellen propagiert werden, und im weiteren eine negative Selektion, bei der solche Zellen eliminiert werden, die mit zu hoher Affinität gegen körpereigene Antigene reagieren [40].

Die Pathogenese von Autoimmunerkrankungen wird als multifaktoriell angesehen. Dabei scheint eine genetische Disposition eine wesentliche Rolle zu spielen. Eine wichtige genetische Determinante ist der MHC-Komplex (MHC = *Major Histocompatibility Complex*), der notwendig für die Antigenpräsentation und -erkennung im Rahmen der adaptiven Immunität ist. Bei einem suszeptiblen Individuum treffen dann wahrscheinlich mehrere exogene (Umwelt-) Faktoren, wie zum Beispiel virale Infektionen, aufeinander, die zur Entstehung einer autoreaktiven Immunreaktion führen. Mehrere Hypothesen zur möglichen Auslösung einer Autoimmunität sind vorherrschend [41]. Zum einen kann es durch die genannte Infektion zu einem direkten Zell- oder Gewebeuntergang kommen, wodurch Autoantigene freigesetzt werden, die im weiteren die Gewebedestruktion unterhalten [42]. Zum anderen können sich Epitope des infektiösen Pathogens und des Zielorgans ähneln. Dadurch kann das Immunsystem nicht mehr zwischen *Fremd* und

Selbst unterscheiden; es bleibt auch nach erfolgreicher Beendigung der initialen Infektion aktiviert und reagiert dann gegen *Selbst* (molekulares Mimikry) [43-46]. Derartige Homologien sind für zahlreiche infektiöse Pathogene und Autoantigene beschrieben worden [47;48]. Desweiteren können Viren im Gewebe persistieren und zu einer chronischen Aktivierung des Immunsystems mit Reaktion gegen *Selbst* führen [41]. Schließlich können auch durch die so genannte *bystander activation* inaktive autoreaktive T-Lymphozyten im Rahmen einer infektionsbedingten Aktivierung des Immunsystems aktiviert werden und gegen *Selbst* reagieren [49].

1.5. Demyelinisierende Erkrankungen des Zentralnervensystems

Das Spektrum entzündlicher demyelinisierender Erkrankungen des ZNS kann eingeteilt werden nach dem klinischen Verlauf, dem Schweregrad und der Dauer der Erkrankung [10;50]. Die Multiple Sklerose (MS) ist die prototypische Erkrankung der entzündlichen demyelinisierenden Erkrankungen des ZNS. Seltenere Formen, so genannte *MS-Varianten*, können eingeteilt werden in fulminante Verlaufsformen, monophasische Verlaufsformen und auf bestimmte neuroanatomische Strukturen beschränkte Syndrome [50;51]. Zu den fulminanten Verlaufsformen gehören unter anderem die Marburg-Variante der MS, die konzentrische Sklerose Baló und die Schildersche Erkrankung (diffuse disseminierte Sklerose). Bei den monosymptomatischen Verlaufsformen handelt es sich um die isolierte transverse Myelitis oder die Optikusneuritis. Bei wiederkehrenden (rekurrenten) transversen Myelitiden oder Optikusneuritiden oder der Kombination aus beiden, der so genannten Neuromyelitis optica (NMO, Devic-Syndrom), handelt es sich um neuroanatomisch begrenzte Syndrome, denen nach derzeitigem Kenntnisstand eine andere Pathogenese als bei der MS zugrunde liegt [52].

Die akute disseminierte Enzephalomyelitis (ADEM) erfährt eine gewisse Sonderstellung in diesem in Abbildung 2 dargestellten Kontinuum von Schweregrad und Dauer der Erkrankung. Eine ADEM verläuft fast ausschließlich monophasisch und ist zumeist gekennzeichnet durch einen fulminanten Verlauf mit para- oder postinfektiösem Beginn [13].

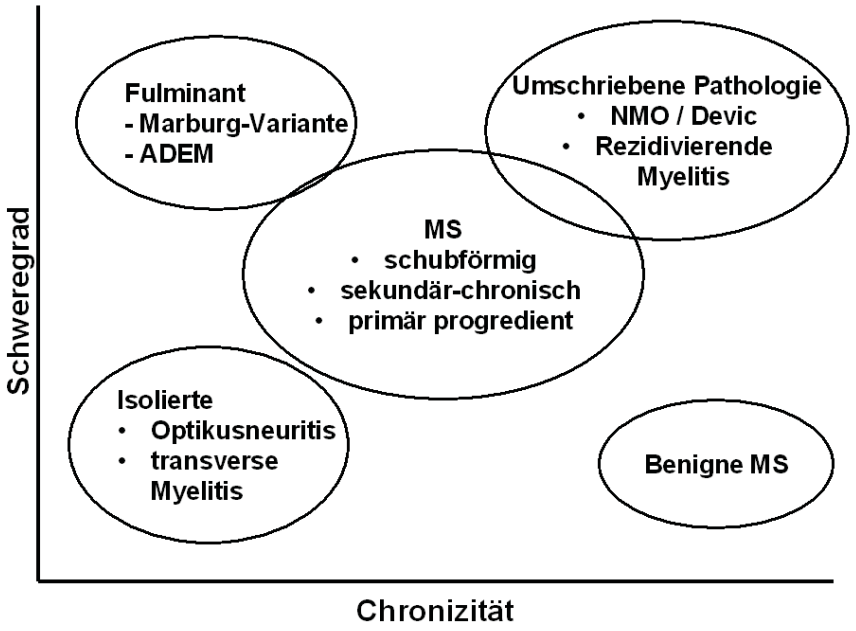

Abbildung 2: Schematische Darstellung von Schweregrad und Dauer der demyelinisierenden ZNS-Erkrankungen als Kontinuum dieser Variablen (modifziert nach [50;53]).

Diese Erkrankung ist per definitionem selbstlimitierend und die Demyelinisierung zum größten bzw. zum wesentlichen Teil reversibel [14]. A priori lassen sich die Erkrankungen häufig nicht eindeutig voneinander trennen, so dass die Angaben zur Chronifizierung bzw. zur Prognose uneindeutig sind [54;55]. Surrogatmarker oder Biomarker von diagnostischem oder prognotischem Wert gibt es bis dato noch nicht.

1.6. Die Multiple Sklerose

Die MS ist die häufigste Erkrankung in diesem Kontinuum der demyelinisierenden Erkrankungen des ZNS (Abb. 2). Es handelt sich um eine chronisch progrediente Erkrankung autoimmuner Genese. Der französische Neurologe J. M. Charcot gilt als Erstbeschreiber der MS [56]. Die MS ist die häufigste neurologische Erkrankung des jungen Erwachsenenalters und ist nach Trauma die zweithäufigste Ursache bleibender Behinderungen des jungen Menschen. In Deutschland sind schätzungsweise 100.000 bis 120.000 Personen an der MS erkrankt. Frauen sind knapp zweimal häufiger betroffen als Männer. Ein Erkrankungsgipfel liegt zwischen dem 20. und 30. Lebensjahr. Inzidenz und Prävalenz sind in Äquatornähe und in südlichen Breitengraden deutlich geringer als in nördlichen Breiten [10;11;57]. Auch

wenn ein Erkrankungsbeginn selten im Kindes- oder hohen Erwachsenenalter ist, wird in letzter Zeit vermehrt auch die pädiatrische MS untersucht [58;59].
Zur Diagnosestellung werden der Nachweis einer zeitlichen und einer räumlichen Dissemination der Demyelinisierung gefordert [60]. Dies geschieht entweder klinisch durch den Nachweis eines demyelinisierenden Ereignisses in Anamnese und klinisch-neurologischem Befund oder paraklinisch mittels Kernspintomographie (MRT) des ZNS. Der Nachweis bestimmter, so genannter Barkhof-Kriterien im MRT erhöht die diagnostische Sensitivität für das Vorliegen einer MS, wenn diese auch nicht spezifisch sind [60-62]. Ebenfalls nicht spezifisch, aber pathognomonisch für die MS ist der Nachweis von (persistierenden) oligoklonalen Banden im Liquor, deren Genese trotz intensiver Bemühungen weiterhin unklar bleibt [63-66].

Es gibt keine für die MS alleinig spezifischen, jedoch einige recht typische Symptome, vor allem wenn sie in den klinischen Kontext, z.B. Alter oder Komorbiditäten gesetzt werden. Häufige neurologische Initialsymptome sind einseitige Visusminderung bei Optikus-Neuritis, paretische und/oder ataktische Gangstörungen und Störungen der Sensibilität (häufig in Begleitung von Parästhesien). Bei einem Teil der Patienten finden sich neuropsychologische Auffälligkeiten. Der Krankheitsverlauf der MS ist höchst variabel und individuell nur schwer vorhersagbar. Bei den meisten Patienten beginnt die Krankheit schubförmig, bei etwa 10% der Patienten verläuft sie von Beginn an schleichend, also primär chronisch progredient. Rund die Hälfte der primär schubförmige Erkrankungen gehen im Median nach zehn Jahren in einen sekundär chronisch-progredienten Verlauf über, was dann nach gegenwärtigem Kenntnisstand Ausdruck einer im Vordergrund stehenden Neurodegeneration ist [10;11;57].

Die Ätiologie der MS ist multifaktoriell zu sehen. Zum einen gibt es eine genetische Verbindung mit bestimmten HLA-Antigenen, nämlich DRB1*1501 und DQB2*0602 [67;68]. Desweiteren sind kürzlich allelische Polymorphismen in den Genen für den Interleukin (IL)-2 und den IL-7-Rezeptor mit der MS assoziiert worden [67;69]. Zu den beeinflussenden Umweltfaktoren zählen vor allem Infektionen; gegenwärtig gibt es zahlreiche Hinweise auf eine pathogenetische Beteiligung des Epstein-Barr Virus (EBV) [70-73]. Aber auch der immunmodulierenden Wirkung von Vitamin D, das durch Sonnenexposition gebildet wird, wird eine Rolle bei der Entstehung der MS zugesprochen [12;74]. Für den jeweils partiellen Einfluß der genetischen Faktoren,

von Infektionen und weitere Umweltfaktoren spricht die Erkrankungskonkordanz bei eineiigen Zwillingen, die nur ca. 30 % beträgt [75].

1.7. Die experimentelle autoimmune Enzephalomyelitis

Zahlreiche Aspekte der MS lassen sich im Tiermodell, der experimentellen autoimmunen Enzephalomyelitis (EAE), untersuchen. 1925 bzw. 1930 wurden erste derartige Versuche veröffentlicht, bei denen Kaninchen mit menschlichem Rückenmark bzw. Rhesusaffen mit ZNS-Homogenaten immunisiert wurden [76;77]. Eine EAE lässt sich entweder durch aktive Immunisierung mit bestimmten Antigenen oder durch Übertragung von aktivierten Autoantigen-spezifischen T-Lymphozyten immunisierter Tiere auf nicht immunisierte Tiere im Sinne einer passiven Immunisierung auslösen. Die klinischen und histopathologischen Aspekte der EAE ähneln der humanen Erkrankung MS in wesentlichen, sind aber nicht identisch mit ihr [16;17;78;79]. Bei den Antigenen handelt es sich um zumeist um ZNS-Myelinbestandteile in immunologischen Adjuvanzien, wie z. B. komplettem Freund´schen Adjuvans. Diese Antigene können entweder singulär als gereinigtes oder rekombinantes Protein, als enzephalitogenes Peptid oder als biologisches Antigengemisch, wie z.B. als Homogenat von Rückenmark oder weißer Substanz (human white matter homogenate, HWM) verwendet werden. Typische Antigene sind rekombinantes MOG, das MOG-Peptid der Aminosäuren 35-55, MBP oder PLP. Eine EAE kann in einer Vielzahl von Tieren, zumeist in Nagetieren, aber auch in höheren Wirbeltieren, wie nicht-menschlichen Primaten ausgelöst werden [18;80]. Klassische Modelle sind die MOG-Peptid-induzierte EAE in der C57BL/6-Maus [81] sowie die PLP-Peptid-induzierte EAE in der SJL-Maus [82], wobei das zweite Modell einem schubförmigen Verlauf entspricht. Bei diesen Tierlinien handelt es sich um Inzuchtstämme, die jeweils durch einen bestimmten genetischen Haplotypen das Antigen erkennen [18;80]. Im Gegensatz dazu steht das auch im Kontext der hier vorgestellten Forschung verwendete EAE-Modell des nicht-menschlichen Primaten des Neue Welt Affen Calithrix jacchus (*C.jacchus*) [83]. Diese verwendeten Tiere sind nicht inzüchtig gezüchtet, so dass eine Vielzahl von Haplotypen der HLA-Äquivalente vorhanden sind [84]. In *C.jacchus* Affen ist die EAE durch verschiedene Antigene induzierbar und folgt dann unterschiedlichen klinischen Verlaufsformen [83-85]. Ein passiver

Transfer von T-Lymphozyten oder anti-Myelin Antikörpern führt in diesen EAE-Modell ebenfalls zu einer Demyelinisierung [85].
GalC ist ebenfalls ein demyelinisierendes Enzephalitogen; bei aktiver Immunisierung in Kombination mit MBP kann es in Meerschweinchen eine demyelinisierende EAE induzieren, während MBP alleine nur eine Enzephalitis induziert [86;87]. Auch ein passiver Transfer von anti-GalC Antikörpern führt zu einer demyelinisierenden EAE in Ratten und Mäusen [88;89] und zu einer Demyelinisierung in vitro [90-92].

1.8. Das pathogenetische Konzept von MS und EAE

Die Erkrankung der MS beginnt wahrscheinlich lange, bevor sie klinisch im Sinne eines Schubes apparent wird. Mutmaßlich kommt es auf dem Boden eines bestimmten HLA-Typus zu einer Aktivierung und Propagierung bis dato inaktiver potentiell autoreaktiver Immunzellen. Dabei sind quasi alle Kompartimente des Immunsystems, also sowohl zelluläre als auch molekulare Komponenten des angeborenen und des adaptiven Immunsystems involviert. Die erstmalige Aktivierung des Immunsystems mit möglicher Reaktion gegen ZNS-Antigene erfolgt systemisch, also nicht wie ursprünglich gedacht ausschließlich im ZNS, sondern in Milz und (regionalen) Lymphknoten [93].
Das auslösende Ereignis der MS ist weiterhin unklar; bei der EAE ist es die Immunisierung, also Inokulation mit einem oder mehreren immunogenen / enzephalitogenen ZNS-Antigenen wie oben beschrieben [80]. Bei der MS kommt es am wahrscheinlichsten durch Infektion mit einem oder mehreren Mikroben, deren Epitope eine immunologische Homologie zu ZNS- bzw. myelinischen Epitopen haben, zu einer chronischen Aktivierung des Immunsystems [94;95]. Diese Theorie der kontinuierlichen Aktivierung durch Homologie wird, wie oben (1.4.) beschrieben, molekulares Mimikry genannt. Entscheidend ist, dass die Homologie nicht auf die Aminosäurensequenz oder auf die Struktur des Epitopes beschränkt sein muss, sondern ggf. auch so degeneriert sein kann, dass es zumindest zu einer Aktivierung eines autoreaktiven T-Zellrezeptors durch das infektiöse und das ZNS-eigene Antigen kommt [10;96]. Eine weitere Möglichkeit ist, dass ein bestimmtes mikrobielles Agens zu einer milden Enzephalitis führt, in deren Verlauf geringe Mengen von ZNS-Antigenen freigesetzt und in systemische Immunzentren, vor allem

regionale Lymphknoten sequestriert und dort entsprechend prozessiert werden. Durch eine konsekutive Infektion mit einem nicht unbedingt artverwandten infektiösen Agens kommt es zu einer Reaktivierung der initial ausgebildeten Lymphozytenklone, die dann unmittelbar gegen ZNS-Antigene reagieren können. Für diese Hypothese gibt es ein elegantes Tiermodell [97]. Zusätzlich lässt sich die Sequestrierung von ZNS-Antigenen in die lokalen Lymphknoten im EAE-Modell des *C.jacchus* verfolgen [98;99]. Die Hypothese des molekularen Mimikrys ist gut durch den Nachweis humaner kreuzreagierender T-Lymphozytenklone untermauert, die nicht nur durch EBV, sondern auch MBP zur Proliferation aktiviert werden können [96]. Beide Hypothesen bedienen sich also einer exogen induzierten, fehlgeleiteten positiven Selektion potentiell autoreaktiver Lymphozyten. Generell gilt, dass die Generierung autoreaktiver T- und B-Lymphozyten nicht auf Autoimmunerkrankungen beschränkt ist, sondern in jedem Individuum erfolgen kann, wobei es unter normalen Bedingungen durch komplexe Mechanismen negativer Selektion und peripherer Toleranz nicht zu einem Ausreifen dieser Zellen kommen sollte [100-102]. Dennoch können im peripheren Blut von gesunden, nicht autoimmun erkrankten Individuen beispielsweise autoreaktive B-Lymphozyten und Autoantikörper nachgewiesen werden, die dann meist polyreaktiv und niedrig-affin sind und als natürlich vorkommende Autoantikörper (NAA) physiologische Aufgaben haben [103-105].

Aktivierte T- und B-Lymphozyten können zur immunologischen Überwachung über die physiologischerweise geschlossene Blut-Hirn-Schranke (BHS) in das ZNS transmigrieren, so dass das ZNS keinesfalls ein vollständig immunpriviligiertes Organ ist, wie urspünglich angenommen [1;106-108]. Der Vorgang der transendothelialen Migration über die BHS wird von einem komplexen Zusammenspiel aus zellulären Adhäsionsmolekülen, Chemokinen und Chemokinrezeptoren sowie Matrixmetalloproteinasen reguliert [109]. Die BHS wird durch eine induzierte Expression von Adhäsionsmolekülen und Matrixmetalloproteinasen zusätzlich durchlässig für die Migration weiterer Immunzellen, so dass auch Zellen des angeborenen Immunkompartiments, z.B. Makrophagen und dendritische Zellen, in das ZNS einwandern können. Durch Aktivierung der Mikroglia entsteht eine weitere Population antigenpräsentierender Zellen, die ebenfalls zur Unterhaltung dieses Circulus vitiosus beitragen. Wenn es nun durch die oben beschriebenen Ereignisse zu einer Aktivierung und klonaler Expansion myelinspezifischer, autoreaktiver T- und B-Lymphozyten kommt, werden diese nach Einwanderung in das ZNS dort ihr

Zielantigen erkennen [110]. Sowohl T-Lymphozyten vom CD4-Helferzelltyp als auch zytotoxische CD8-positive Zellen werden durch Mikrogliazellen reaktiviert und setzen proinflammatorische (Th)1-Zytokine frei. Dies führt entweder direkt oder durch Anlockung weiterer Effektorzellen, vor allem Makrophagen, zur Myelinscheiden-destruktion. Einige dieser Reaktivierungs- und Reifungsprozesse finden unter Umständen in pseudofollikulären Strukturen innerhalb des ZNS statt, die fast ausschließlich bei progredienten Formen der MS in situ nachweisbar waren [111-113]. Die T-Lymphozyten vermittelte reziproke Stimulation von Mikroglia und Makrophagen führt zu einer gesteigerten Phagozytose und einer vermehrten Produktion von Zytokinen und freien Radikalen, wie z. B. TNFα, Lymphotoxin und Stickstoffmonoxid, die ebenfalls zur Demyelinisierung beitragen und einen axonalen Schaden hervorrufen.

Myelinspezifische Autoantikörper tragen ebenfalls zur Gewebedestruktion bei; sie können die Komplementkaskade aktivieren, was die Bildung des sogenannten Membran-Angriffs-Komplexes (C5b-9) zur Folge hat und zur Auflösung der Zielstruktur führt [114]. Histopathologisch gelang der Nachweis von IgG-Ablagerungen und aktiviertem Komplement in über 50 % der untersuchten MS-Läsionen [114]. Ultrastrukturell konnten sogar Antikörper, die gegen MOG gerichtet sind, innerhalb von sich desintegrierendem Myelin nachgewiesen werden [115]. Erst seit kurzem wird zunehmend die tragende Rolle der B-Lymphozyten im Kontext der MS-Pathogenese besser verstanden [116-118]; B-Lymphozyten sind nämlich nicht nur Vorläufer von Antikörper-prodzierenden Plasmazellen, sondern können als potente antigenpräsentierende Zellen und durch Sekretion pro-inflammatorischer und/oder regulatorischer Zytokine (im wesentlichen IL-10) aktiv eine Entzündungsreaktion orchestrieren und mit den weiteren Immunzellen interagieren.
Neben der inflammatorischen Demyelinisierung kommt es bereits früh im Krankheitsverlauf zu einer Akkumulation axonalen Schadens, der letztlich die irreversiblen Behinderungen bei der MS verursacht [119]. Es bleibt allerdings ungeklärt, ob die axonale Destruktion primär eintritt, d. h. eine direkte Folge zellulärer und humoraler Schädigungsmechanismen ist, oder sekundär aufgrund der vorangegangenen Demyelinisierung entsteht.
Hinzuweisen ist auf die Janusköpfigkeit der lokalen Entzündung im ZNS; während diese vor allem destruktiv ist, gibt es aber auch Hinweise, dass bestimmte Mediatoren, vor allem in der Frühphase der Erkrankung, regenerative / remyelinisierende

Funktionen haben können [120-123].

Ferner sind die pathogenetischen Abläufe von MS und EAE nicht völlig identisch. In den meisten EAE-Modellen überwiegt eine läsionäre Infiltration von CD4+ T-Lymphozyten, während die MS-Läsion reich an oligoklonalen CD8+ zytotoxischen T-Lymphozyten ist und sich im Liquor, aber auch in den Läsionen zahlreiche oligoklonale B-Lymphozyten mit antigengereiften B-Zellrezeptoren nachweisen lassen [124].

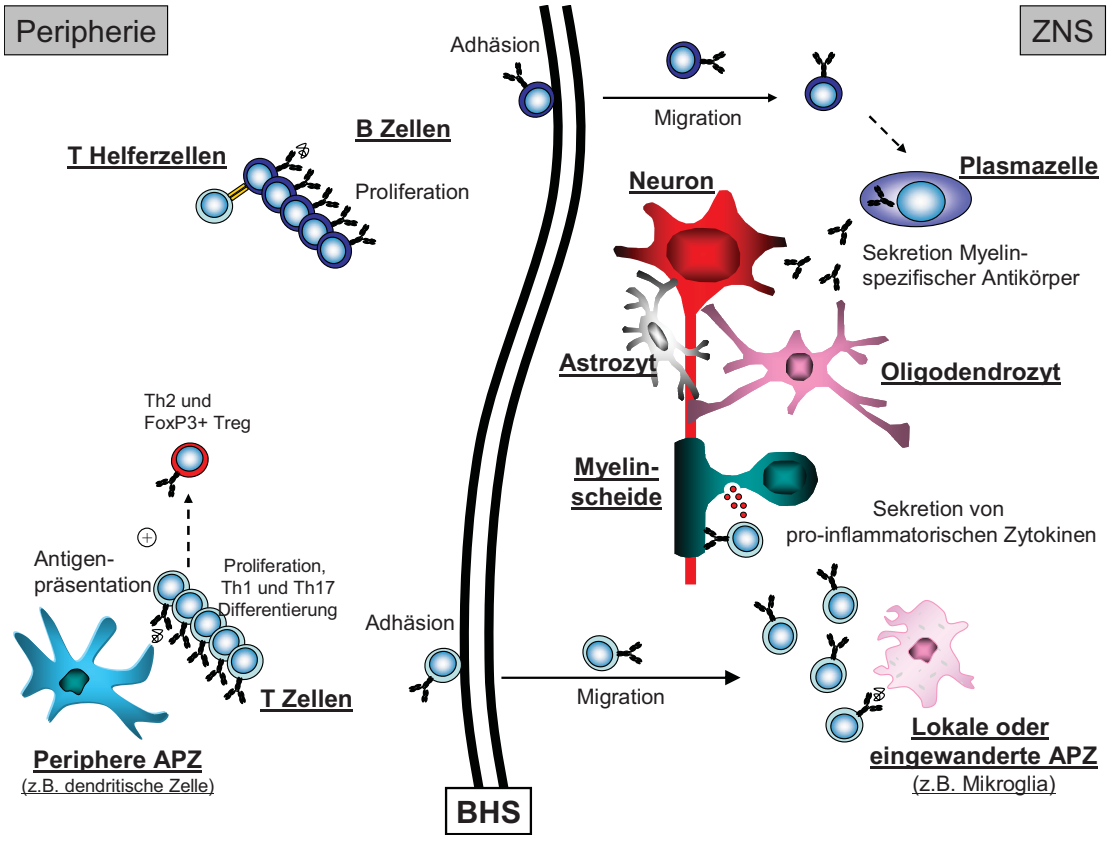

Abbildung 3: Immunpathogenetisches Konzept der MS

Schematische Darstellung der drei Kompartmente des systemischen Immunsystems (links), der Bluthirnschranke (BHS) (mittig) und des ZNS (rechts) als distinkte Orte der MS-Pathogenese (modifiziert aus [125]). APZ – Antigenpräsentierende Zelle.

1.9. Autoantikörper gegen myelinische Bestandteile

Schon lange ist aus den EAE-Modellen bekannt, dass die immunisierten Tiere eine humorale Immunantwort gegen das immunisierende Antigen durchmachen und Serumantikörper produzieren [18;80;85]. Interessanterweise können diese im Sinne eines *epitope spreading* auch gegen Antigene gebildet werden, mit denen nicht immunisiert worden ist [126;127]. Es kommt also zu einer Ausweitung der Immunantwort auf distinkte Antigene, die nicht den initial krankheitsinduzierenden entsprechen, sondern die unter Umständen erst im Verlauf der Erkrankung freigesetzt wurden [128]. Für die EAE im *C.jacchus* Affen ist dies eindrücklich gezeigt worden. Die Tiere wurden mit dem rekombinanten Fusions-protein MP4 immunisiert, das ein Konstruct aus MBP und PLP ist. Diejenigen Tiere, bei denen histopathologisch demyelinisierende Läsionen nachweisbar waren, entwickelten Antikörper gegen MOG, obwohl damit nicht immunisiert worden war [127].

Zusätzlich können auch durch den passiven Transfer von myelinspezifischen Autoantiköpern, z.B. monoklonale Antiköper gegen MOG oder GalC, in entsprechend vorbereitete Tiere eine EAE mit demyelinisierende Läsionen induziert werden [85;89;129;130].
Somit liegt es nahe, dass der Stellenwert von myelinspezifischen Autoantikörpern auch bei Menschen untersucht wird [20;53]. Für die Beteiligung von Autoantikörpern an der Pathogenese der MS gibt es eine Vielzahl von indirekten Hinweisen:

- Die intrathekale Antikörpersynthese und der Nachweis oligoklonaler Banden im Liquor, wenn auch diese für die MS nicht spezifisch sind [131;132];
- der Nachweis von im ZNS affinitätsgereiften Immunglobulinen [133;134;135];
- die histopathologische Beschreibung von IgG-Ablagerungen und Komplementaktivierung in über der Hälfte der aktiv demyelinisierenden MS-Läsionen (Typ II Läsionen) [114;136];
- der Nachweis von myelinspezifischen Autoantikörpern von Plasmazellen aus MS-Liquores [64];
- die elektronenmikroskopisch nachgewiesene Ablagerung von MOG-reaktiven IgG Antikörpern genau am Ort der Myelinscheidenzersetzung in MS-Läsionen [115;137];

- das signifikant bessere Ansprechen auf Plasmaseparation von Patienten mit histologisch nachgewiesenen Typ II Läsionen, deren Schübe sich unter Steroidpulstherapie nicht hinreichend zurückbilden [138].

Neben dem Verständnis zur Beteiligung von Antikörpern an der Pathogenese der MS ist es immer schon ein weiteres Forschungsziel gewesen, MS-spezifische Antikörper zu identifizieren, um diagnostische als auch prognostische Marker sowie Marker zur Klassifizierung der MS-Subtypen zu etablieren. Die Suche nach dem auslösenden Antigen bzw. dem zugehörigen spezifischen Autoantikörper wird allerdings erschwert durch die Tatsache, dass der erstmalige Nachweis von Autoantikörpern nicht zwangsläufig mit dem Beginn der Erkrankung vergesellschaftet ist, sondern auch zu einem späteren Zeitpunkt im Verlauf der Erkrankung im Sinne des oben genannten *Epitope spreading* auftreten kann. Zusätzlich müssen myelinspezfische Autoantiköper nicht zwangsläufig pathologisch sein, sondern können sogar eine remyelinisierende Wirkung entfalten [139;140].

1.10. Das Dilemma der anti-MOG Antikörper

MOG ist das einzige myelinische Autoantigen, das alle bekannten pathogenetischen Voraussetzungen einer Demyelinisierung erfüllt. Es wird an der Oberfläche der Oligodendrozyten exprimiert und ist damit für eine Erkennung durch spezfische Autoantikörper unmittelbar verfügbar [141]. Zum zweiten ist MOG spezifisch für das ZNS, da es im Gegensatz zu anderen enzephalitogenen myelinischen Antigenen, wie MBP oder PLP, nicht im peripheren Nervensystem exprimiert wird [28;30]. Zum dritten können monoklonale Antikörper gegen MOG, z.B. die murinen monoklonalen Antikörper 8.18c5 oder Z12 [129;130;142;143], oder affinitätsgereinigte anti-MOG Antikörper eine Demyelinisierung auslösen, wenn sie passiv in MBP-immunisierte Tiere transferiert werden [85;144]. Die aktive Immunisierung mit MBP führt zwar zu einer Enzephalomyelitis, jedoch ohne nennenswerte Demyelinisierung [85;129;144]. Durch den zusätzlichen passiven intravenösen Transfer von MOG-spezfischen Antikörpern kommt es zu einer dramatischen Zunahme der klinischen Behinderung, deren histopathologisches Korrelat eine Kombination von Komplement-vermittelter Zelllyse und direkten Antikörper-

abhängigen zytotoxischen Effektormechanismen sind [145;146]. Zusätzlich kommt es zu einer demyelinisierenden EAE in transgenen Mäusen, deren B-Lymphozyten den pathogenen monoklonalen 8.18c5 produzieren [147]. Auch in vitro lassen sich die demyelinisierenden Effekte von MOG-spezifischen Antikörpern nachweisen [92;148]. Desweiteren korrelieren die Titer der Serumantikörper gegen MOG, nicht aber gegen MBP in Meerschweinchen mit chronisch schubförmiger EAE mit der Stärke der in vivo demyelinisierenden Serumaktivität dieser Tiere [149]. Und schließlich induziert die extrazelluläre Domäne von MOG in der EAE zugleich eine ausgeprägte T- und eine B-Lymphozytenantwort [146;150]. Bemerkenswerterweise produzieren *C.jacchus* Affen hochtitrige anti-MOG Antikörper, wenn sie mit HWM immunisiert wurden, auch wenn MOG nur in sehr geringen Mengen im Immunisat vorkommt [83;85].

Somit gibt es eine Vielzahl konsistenter und kongruenter Daten, die die pathogene Rolle von anti-MOG Antikörpern bei der demyelinisierenden EAE sehr gut belegen (als Übersicht besprochen in [18]). Allerdings ist es komplex, die pathogenen anti-MOG Antikörper zu identifizieren [151-153]. In einem Panel muriner monoklonaler anti-MOG Antikörper können pathogene und nicht-pathogene Antikörper nicht mittels ELISA oder Western Blot unterschieden werden [151]. Der nicht pathogene monoklonale Antikörper reagiert allerdings nicht mit nativen, auf Fibroblasten-exprimiertem MOG [151]. Interessanterweise reagieren die pathogenen Antikörper der MOG-immunisierten Mäuse im wesentlichen gegen ein immundominantes, nicht-lineares Epitop, wie durch Mutationsstudien für diese monoklonale Antikörper gezeigt werden konnte [154]. Im Serum von MOG-immunisierten Tieren lassen sich mittels ELISA auch dann erhöhte anti-MOG Antikörpertiter nachweisen, wenn diese nicht per se pathogen, also demyelinisierend sind [153]. Dabei zeigte sich, dass nur Mäuse, die mit humanem rekombinanten MOG immunisiert wurden, pathogene anti-MOG Antikörper produzieren; Mäuse hingegen, die mit rekombinantem Ratten-MOG oder einer humanen MOG-Variante, die im immundominanten Epitop die Aminosäuresequenz der Ratte trägt, immunisiert wurden, produzierten nicht-pathogene anti-MOG Antikörper [153].

Bei Menschen ist die Datenlage nicht so eindeutig. Seit der Klonierung von humanem MOG 1995 sind fast 30 Studien durchgeführt worden, die die Prävalenzen

von Serumantikörpern gegen MOG beschreiben [155]. Diese Prävalenzen variieren erheblich und lagen in einer eigenen, unveröffentlichten Literaturrecherche zwischen 0 % und 59 %, während die Prävalenzen gesunder Kontrollen zwischen 0 % und 34 % lagen (Abb. 4).

Diese Diskrepanzen erklären sich durch

- die Verwendung verschiedener MOG-Präparationen,
- die Anwendung unterschiedlicher Antikörperbestimmungsmethoden
- die individuelle Definition von Grenzwerten eines Antikörpernachweises [20].

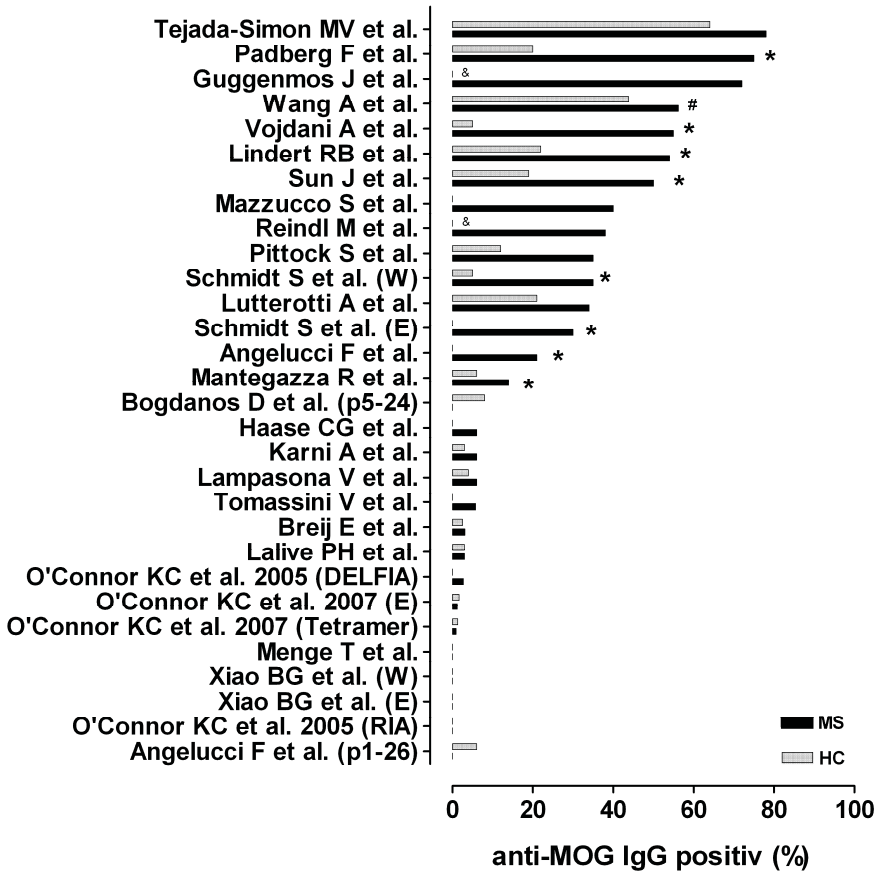

Abbildung 4: Zusammenstellung der Prävalenzen von anti-MOG IgG Antikörpern bei Patienten mit MS (schwarz) und Kontrollen (grau). Die relevanten Originalarbeiten wurden via PubMed durch die Suchbegriffe „myelin oligodendrocyte glycoprotein“, „antibodies“, „multiple sclerosis“ identifiert und die jeweiligen Prävalenzen daraus extrahiert. * weisst auf einen signifikanten Unterschied zwischen Patienten und Kontrollen hin; § zeigt eine fehlende Kontrollgruppe an. # weisst daraufhin, dass die MS-Patienten prä hoc, also zu einem Zeitpunkt untersucht wurden, als sie klinisch assymptomatisch waren und die Diagnose noch nicht bestand.

Auf folgende Referenzen bezieht sich die Abbildung (von oben nach unten): [156;158-180];[158;171]. Einige weitere Studien fanden keinen Eingang in die Abbildung, da keine anti-MOG Prävalenzen, sondern Reaktivitäten oder Titer angegeben wurden: [181-185].

In frühen Studien wurde MOG biochemisch aufgereinigt [156] und im ELISA getestet. Im weiteren wurde rekombinant in E.coli produziertes MOG verwendet, wobei es sich hierbei stets um die extrazelluläre Domäne, also den N-terminalen Anteil bis maximal zur Aminosäure 125 handelt. Die Länge des rekombinanten Proteins ist von wesentlicher Bedeutung, wie eigene und weiter unten diskutierte Untersuchungen belegen. Da MOG eine Glykolysierungsstelle trägt, E.coli aber Proteine nicht glykolysieren können, wurde MOG auch in eukaryoten Systemen exprimiert [157-159]. Dabei konnte gezeigt werden, dass die Zuckerseitkette keinen wesentlichen Anteil an der humoralen Immunität von MOG hat [159].

Eine Vielzahl von verschiedenen Untersuchungsmethoden ist angewandt worden, wobei direkte Methodenvergleiche erst in neuerer Zeit veröffentlicht wurden [159]. Techniken wie ELISA und Western Blot propagieren den Nachweis niedrig-affiner Antikörper und liefern dadurch unter Umständen falsch-positive Ergebnisse. Zusätzlich sind die Resultate der Western Blots nur semiquantitativ. Für beide Methoden gilt, dass die Zielantigene konformationell alteriert werden; durch das Western Blot-Verfahren wird MOG zunächst denaturiert und im weiteren wahrscheinlich partiell renaturiert. Durch das Binden von MOG an die Polystyren-Oberfläche der ELISA-Platten wird es ebenfalls partiell denaturiert, und es werden unter Umständen kryptische Epitope freigelegt [180]. Auch aus diesem Grund ist von mehreren Gruppen MOG in seiner nativen Form in verschiedenen Zellsystemen durch Transfektion überexprimiert worden, um mittels Durchflußzytometrie (FACS) die Antikörperantworten gegen natives MOG zu bestimmen [159;174;179;186]. Auch mit dieser Methode gelingt es nicht, eine spezifische Antikörperantwort zu definieren und anti-MOG Antikörper als diagnostische Biomarker zu implementieren. Generell liegen die Seroprävalenzen hierbei niedriger als bei ELISA und Western Blot; so konnten in der ersten derartigen Studie nur bei einem von 17 untersuchten MS-Patienten Antikörper gegen natives, auf Fibroblasten exprimiertes humanes MOG nachgewiesen werden [174]. Interessanterweise scheint MOG, das in Lösung genommen wird, keine für die MS relevanten Epitope zu präsentieren; denn durch Löslichphasenmethoden mit monomerem oder tetramerem MOG konnten keine anti-MOG Antikörper bei Patienten mit MS nachgewiesen werden [158;176;180].

Der Großteil der genannten Studien beschreibt die Prävalenz von Serumantikörpern gegen MOG (Übersicht bei [20]). Es wurden zu einem geringen Teil aber auch anti-MOG Antikörper im Liquor von Patienten und Kontrollen bestimmt. Dabei finden sich

niedrigere Antikörperprävalenzen als im Serum [168]; es gibt Hinweise, dass anti-MOG Antikörper intrathekal produziert werden, wenn so genannte anti-MOG Indices in Analogie zum IgG-Index errechnet werden [168;184]. Bei anti-MOG Antikörpern, die biochemisch aus MS-Läsionen eluiert wurden, konnten höhere Affinitäten zum Zielantigen, also zu MOG, gemessen werden als bei anti-MOG Antikörpern in Liquor oder Serum [158]. Deswegen wurde diskutiert, ob derartige potentiell pathogene anti-MOG Antikörper nicht in Liquor oder peripherem Blut nachweisbar sind, weil sie hoch-affin an ihr Zielantigen binden [20;158;187].

Durch Kristallisationsstudien mit MOG ist das spezifische Epitop des demyelinisierenden monoklonalen Antiköpers 8.18c5 charakterisiert worden. Es handelt sich um ein diskontinuierliches Epitop mit einer dreidimensionalen Struktur, das an der der Flüssigphase zugewandten Seite liegt [154;188]. Zudem ist bekannt und anerkannt, dass anti-MOG Antikörper, die gegen lineare MOG-Epitope gerichtet sind, kein oder allenfalls ein sehr geringes demyelinisierendes Potential haben [150;152;187]. Aus diesem Grund ist die klinische Aussagekraft der zahlreichen Arbeiten, die MOG-Peptide als Zielantigen verwenden, zu hinterfragen [18;20].

1.11. Anti-MOG Antikörper als Biomarker

Wegen der geringen Spezifität haben anti-MOG Antikörper bisher keinen Einsatz als diagnostische Biomarker in der klinischen Routine finden können [20;187]. In einer rezenten Studie wurden aus der rund 7 Millionen Personen umfassenden Serumbank der US-Armee 126 MS-Fälle und 252 entsprechende Kontrollen isoliert und Serum, das vor der Diagnosestellung abgenommen worden war, auf IgG und IgM gegen $rhMOG_{125}$ mittels ELISA untersucht [163]. Nach Korrektur für diverse Risikofaktoren, einschließlich einer abgelaufenen EBV-Infektion, waren weder der anti-MOG IgG- noch der anti-MOG IgM-Status prädiktiv für die Entwicklung einer MS. Eine weitere Arbeit hat ebenfalls Blutproben von 25 MS-Patienten, die 7,3 Jahre bis 2 Monate vor Erstmanifestation abgenommen worden waren, akquirieren können und auf IgG und IgM gegen natives MOG und $rhMOG_{125}$ im Western Blot untersucht [183]. Auch in dieser Studie war der anti-MOG Serostatus nicht prädiktiv für die Entwicklung einer MS.

Aus der Vielzahl der in Abb. 4 aufgeführten Studien konnte nur eine Studie eine Assoziation der anti-MOG Titer mit dem Grad der klinischen Behinderung beschreiben [172]; 262 Patienten mit MS waren untersucht worden. Anti-MOG Prävalenzen waren am höchsten bei Patienten mit chronischen Verlaufsformen. Bei diesen korrelierten die anti-MOG Titer bzw. die Reaktivität mit dem EDSS bei Blutabnahme [172]. Eigene, bisher nicht veröffentlichte und noch nicht unabhängig validierte Daten zeigen eine Assoziation zwischen ausschließlich hoch-titrigen anti-MOG IgG und dem Grad der Behinderung gemessen als EDSS bei der Blutabnahme (Abb. 5). Im Unterschied zu der zitierten Studie von Mantegazza et al. war in unserer Studie diese Assoziation nur bei Patienten mit schubförmigen Verläufen nachweisbar.

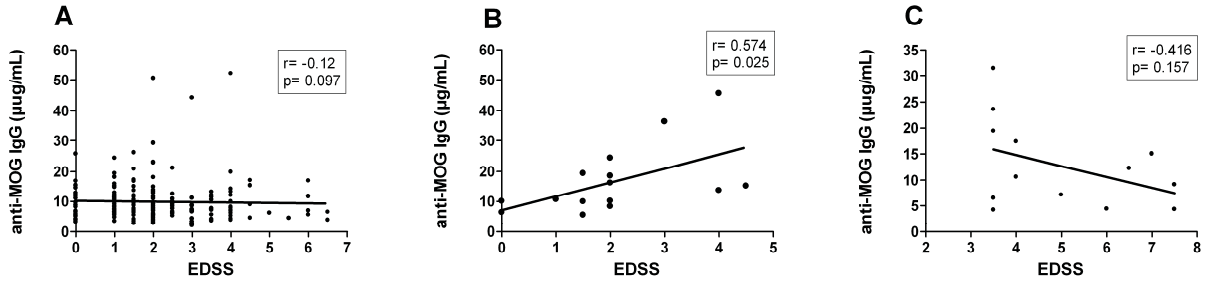

Abbildung 5: Assoziation zwischen dem quantitativen anti-MOG Titer und dem EDSS. Während eine Assoziation in der gesamten Kohorte von 192 Patienten mit schubförmiger MS nicht besteht **(A)**, lässt sich diese bei 15 Patienten mit so genannten hoch-titrigen anti-MOG IgG-Antikörpern im Serum nachweisen (r = 0,574; p = 0,025; Spearman r) **(B)**. Im Gegensatz dazu findet sich die Korrelation derartiger hoch-titriger anti-MOG IgG Antikörper und dem EDSS bei Patienten mit chronischen MS-Verlaufsformen (SPMS und PPMS) nicht **(C)**. Antikörper wurden gegen drei MOG-Isoformen ($rhMOG_{118}$, $rhMOG_{125}$ und $rMOG_{125}$) im ELISA roboterassistiert im 384-Well ELISA-Format in drei Verdünnungen gemessen und mit einer Standardkurve verglichen. Die Ergebnisse wurden als Kombination aus den drei Bestimmungen quantitativ als µg/mL anti-MOG IgG beschrieben. Als hoch-titrig galten Antikörper, deren Bindungsquotienten auch in der geringsten Verdünnung über Grenzwerten lagen, die durch die Seren mit Reaktivitäten oberhalb des jeweiligen 95. Perzentils definiert wurden [189].

Im Jahre 2003 wurde durch Thomas Berger et al. die Rolle von anti-MOG Serumantikörpern als prognostischer Biomarker untersucht [190]. Der Nachweis von anti-MOG IgM im Serum mittels Western Blot prädisponierte in der untersuchten Patientenkohorte von 103 Patienten mit einem klinisch isolierten Syndrom (KIS) zu einem signifikant früheren zweiten, gemäß den Poser-Kriterien MS-definierenden

Schub. Der zusätzliche Nachweis von anti-MBP IgM verkürzte das Zeitintervall bis zum zweiten Schub abermals. Allerdings konnte dieser Biomarker in vier weiteren Studien nicht validiert werden, wie aus Tabelle 1 ersichtlich ist [191-194]. Wesentlich ist dabei die Studie von J. Kuhle et al. [195]. Zum einen wurde eine im Rahmen einer klinischen Therapiestudie prospektiv gesammelte und systematisch charakterisierte, quantitativ umfangreiche Patientenkohorte untersucht. Zum zweiten waren Western Blot-Protokoll und das verwendete rekombinante humane MOG identisch mit dem in der Studie von T. Berger et al. verwendeten.

	N	Follow-up (Monate, Median)	2. Schub (CDMS, %)	Liquor patholo-gisch (%)	MRT patholo-gisch (%)	Anti-MOG IgM (%)	Anti-MOG IgM prädiktiv
Berger T, 2003 [190]	103	51 (12-96)	63,1	100	100	62,1	**+**
Lim ET, 2005 [191]	47	12 (3-12)	25,7	n.a.	72,3	55,3	**-**
Rauer S, 2006 [192]	45	60 (21-106)	62,2	100	93,3	51,1	**(+)**
Kuhle J, 2007a [193]	462	24	32,5	85	100	54,4	**-**
Pelayo R, 2007 [194]	114	46,7 [&] (± 21,2)	55,3	100	100	54,4	**-**
Kuhle J, 2007b [195]	55	32,9 (12-77)	58,2	76,6	86,2	78,2	**(+)**
Tomassini V, 2007 [177]	51	36	51	n.a.	100	54,9	**+**
Greeve I, 2007 [196]	39	>6	56,4	100	100	76,9	**+**
Menge T, 2007 [197]	38	73,7 [&] (± 27,6)	39,5	70	54,1	n.a.	**-**

Tabelle 1: Zusammenschau der Arbeiten über anti-MOG IgM Serumantikörper als prädiktiver Biomarker für die zukünftige Diagnose einer gesicherten MS bei Patienten mit KIS. CDMS – klinisch definitive MS mit Diagnosestellung nach dem zweiten klinischen Ereignis. [&] - Mittelwert und Standardabweichung.

Eine Studie konnte eine Assoziation zwischen dem Nachweis von anti-Myelin Antikörpern und einer erhöhten Krankheitsaktivität im Sinne eines signifikant früheren zweiten Schubes bestätigen, wenn auch hier weniger die anti-MOG Antikörper, sondern vielmehr die anti-MBP Antikörper ausschlaggebend waren [177]. Demgegenüber stand eine Studie aus der Schweiz an 39 Patienten mit KIS, die zwar

das prädiktive Potential von anti-MOG IgM unterstrich, aber keinen zusätzlichen Effekt durch die Bestimmung von anti-MBP IgM nachweisen konnte [196]. Zwei weitere Studien bestätigten zwar nicht den Nutzen von anti-Myelin Antikörper als prognostische Antikörper, zeigten aber zumindest eine Assoziation zum Krankheitsverlauf [192;195].

Auch eigene präliminäre Daten konnten den prognostischen Ansatz von Berger et al. nicht bestätigen, wenn Seren von 38 KIS-Patienten im anti-MOG ELISA untersucht und die IgM-Antwort quantitativ gemessen wurden (Abb. 6).

Neuere Studien zur Relevanz von anti-MOG Antikörpern bei Kindern mit MS und Patienten mit ADEM werden zusammen mit einer eigenen Arbeit im folgenden diskutiert [159;198-201].

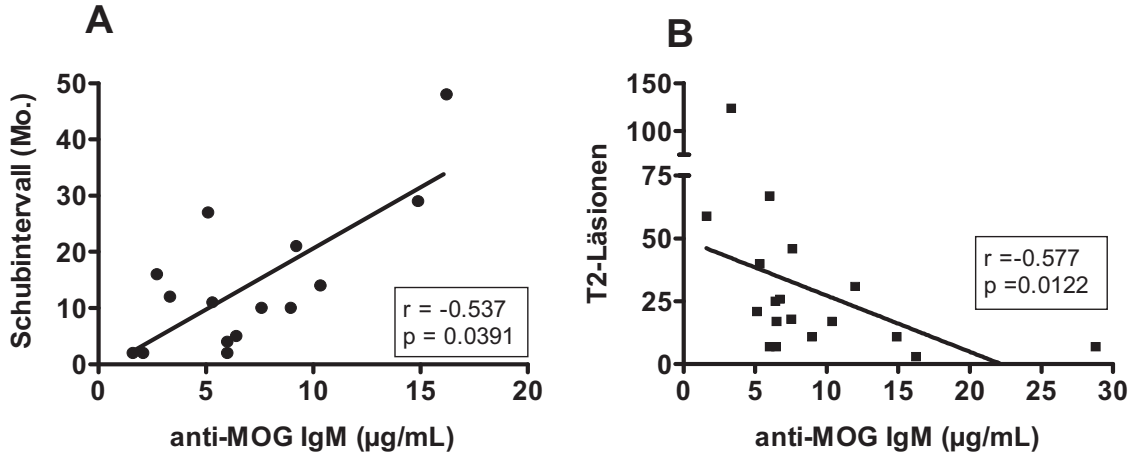

Abbildung 6: **Quantitative anti-MOG IgM Bestimmung bei 38 Patienten mit KIS.** Die Titer korrelieren invers mit dem Zeitinterval bis zum zweiten, krankheitsdefinierenden Schub **(A)** und mit der T2-Läsionslast im MRT zum Zeitpunkt des ersten Schubes **(B)**. Die Analyse der IgM-Antwort gegen MOG erfolgte in Analogie zur Bestimmung der IgG-Antwort (Abb. 5) [189]; lediglich der Sekundärantikörper war gegen humanes IgM gerichtet [197]

1.12. Zusammenfassung

In der EAE als etabliertes Tiermodell der MS gibt es vielfach reproduzierte Daten, dass MOG ein starkes demyelinisierendes Enzephalitogen ist. In Tieren, in denen eine EAE induziert wurde, lassen sich zumeist hoch-titrige anti-MOG Antikörper nachweisen, teilweise auch, wenn die Tiere gar nicht mit MOG

immunisiert wurden. Definierte Antikörper gegen MOG sind sowohl in vivo in der EAE als auch in vitro demyelinisierend. Gleiches gilt für GalC. Allerdings sind nicht alle mit gängigen Labormethoden nachweisbaren anti-MOG Antikörper pathogen.

In der MS ist die Situation dagegen bisher nicht ausreichend aufgeklärt. Anti-MOG Serumantikörper können sowohl bei Patienten mit MS als auch bei Kontrollen erhöht gemessen werden, so dass sie nicht als diagnostische Biomarker genutzt werden können. Auch als prognostische Biomarker in bereits diagnostizierten Patienten mit einem ersten demyelinisierenden Ereignis konnten sie bisher nicht etabliert werden. Die Diskrepanz der publizierten Daten und die erhebliche Varianz der Ergebnisse sind im wesentlichen technisch-experimenteller Natur, da unterschiedliche MOG-Isoformen, verschiedene Messmethoden und selbstdefinierte Grenzwerte von den verschiedenen Arbeitsgruppen verwendet wurden. Dabei ist aus den tierexperimentellen Daten bekannt, dass MOG durch geringefügige Änderungen der Aminosäuresequenz oder der biophysikalischen Umgebungseigenschaften bedeutende strukturelle Alterationen erfahren kann, die die immunologischen Charakteristika des Antigens grundlegend verändern. Zusätzlich ist die humorale Immunantwort gegen Myelinbestandteile bei der MS durch indirekte Beweise gut belegt und somit sicherlich nicht ein technisches Artefakt.

Eigene Arbeiten

Die vorliegenden Originalarbeiten sind im Kontext der anti-Myelin-Antikörper bei MS und *C.jacchus* EAE einzuordnen. Sie beschäftigen sich mit den Fragen nach der Pathogenität von anti-Myelin-Antikörpern und deren Etablierung als Biomarker sowie der immunologischen Relevanz unterschiedlicher Epitope bzw. struktureller Alterationen im Kontext der humoralen Immunantwort. Die Arbeiten werden in einer inhaltlichen Abfolge vorgestellt und diskutiert, auch wenn sie unter Umständen in einer anderen chronologischen Reihenfolge veröffentlicht wurden.

2.1. Pathogenität konformationsabhängiger anti-MOG Antikörper in der EAE des *C.jacchus* Marmoset Affen

von Büdingen HC, Hauser SL, Ouallet JC, Tanuma N, Menge T, Genain CP: Epitope recognition on the myelin/oligodendrocyte glycoprotein differentially influences disease phenotype and antibody effector functions in autoimmune demyelination. *Eur. J. Immunol.* 2004;34:2072-83 [150].

In dieser Arbeit haben wir uns die Vorteile des EAE-Modells im *C.jacchus* Marmoset-Affen zunutze gemacht; wie eingangs dargelegt, sind die verwendeten Labortiere nicht inzüchtig und haben daher keine identische Immunantwort nach Immunisierung. Zusätzlich ist aus vorausgegangenen Studien der Arbeitsgruppe von Claude Genain bekannt, dass es anders als in niedrigen Wirbeltieren im *C.jacchus* Affen zu einer demyelinisierenden EAE ausschließlich durch MOG kommt, während andere Antigene, z.B. MBP, lediglich zu einer Inflammation führen [84;85]. Aus eigener Erfahrung wissen wir, dass die unterschiedlichen Immunisierungsprotokolle sich nicht im klinischen Phänotypus manifestieren. Unabhängig davon, ob ein Tier mit MOG in HWM, der extrazellulären Domäne von rekombinantem Ratten-MOG, Aminosäuren 1-125 ($rMOG_{125}$) oder linearen 20 Aminosäure langen Peptiden der MOG-Sequenz immunisiert wurde, bleibt die klinische Ausbildung der EAE hinsichtlich Erkrankungsbeginn sowie Schwere und Akuität der Erkrankung im wesentlichen gleich (unveröffentlichte Daten; Abb. 7). Die Immunantwort auf T- und B-Lymphozytenebene gegen MOG ist dabei heterogener und auf mehr Epitope verteilt als bei der EAE der niederen Wirbeltiere [18].

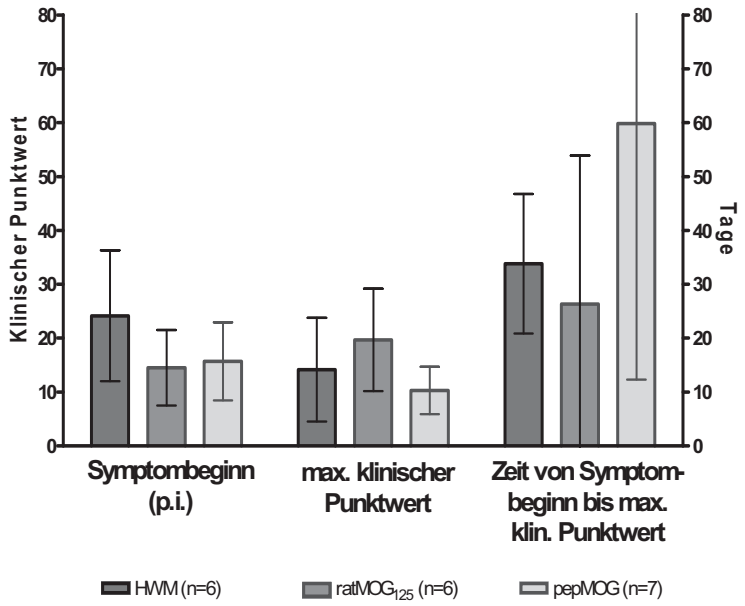

Abbildung 7: Einfluß von drei unterschiedlichen enzephalitogenen MOG-Präparationen auf den klinischen Phänotyp der EAE im *C.jacchus* Affen. Statistisch signifikante Unterschiede ließen sich mittels Kruskal-Wallis-Test für die einzelnen Parameter nicht nachweisen.

Ziel der Studie war es, die Immunpathogenität von anti-MOG Antikörpern im Kontext der differentiellen funktionellen Immunantwort nach Immunisierung zu untersuchen. Dafür wurde anti-MOG Serumantikörper von *C.jacchus* Affen nach ihrer Epitopreaktivität stratifiziert und ihre pathogene Wirkung histopathologisch analysiert. Zunächst wurden vier Tiere mit $rMOG_{125}$ und neun Tiere mit verschiedenen, 20 Aminosäuren langen Peptiden der MOG-Sequenz (pepMOG) aktiv immunisiert. Der klinische Verlauf der EAE war in beiden Gruppen heterogen und nicht signifikant unterschiedlich. Im Gegensatz dazu fand sich in der neuropathologischen Analyse ein deutlicher und signifikanter Unterschied hinsichtlich der so genannten Läsionslast der weißen Substanz; während sich in der $rMOG_{125}$-immunisierten Gruppe im Mittel 163±56,3 Läsionen fanden, waren es in der pepMOG-immunisierten Gruppe nur 9,2±3,1 Läsionen (p=0,0012, unabhängiger T-Test). Zusätzlich waren die Läsionen der pepMOG-immunisierten Tiere vor allem auf den Hirnstamm und das Rückenmark beschränkt.

In der weiteren immunhistochemischen Aufarbeitung waren in beiden Gruppen deutliche Makrophageninfiltrationen in den entzündlichen Läsionen nachweisbar. Im Gegensatz dazu waren aber der Nachweis von IgG-Ablagerungen im ZNS-Parenchym, IgG-positive Zellen in den Läsionen (also B-Zellen oder Plasmablasten) und Komplementaktivierung (Nachweis des Komplementspaltproduktes C9neo) fast ausschließlich bei $rMOG_{125}$-immunisierten Tieren nachweisbar. In der quantitativen Auswertung war dies jeweils signifikant niedriger in den pepMOG-immunisierten

Tieren (Abb. 8). In beiden Gruppen waren Antikörper gegen $rMOG_{125}$ und lineare MOG-Peptide mittels ELISA nachweisbar. Die Proliferation von T-Lymphozyten des peripheren Blutes auf $rMOG_{125}$ war ebenfalls in beiden Gruppen gleich.

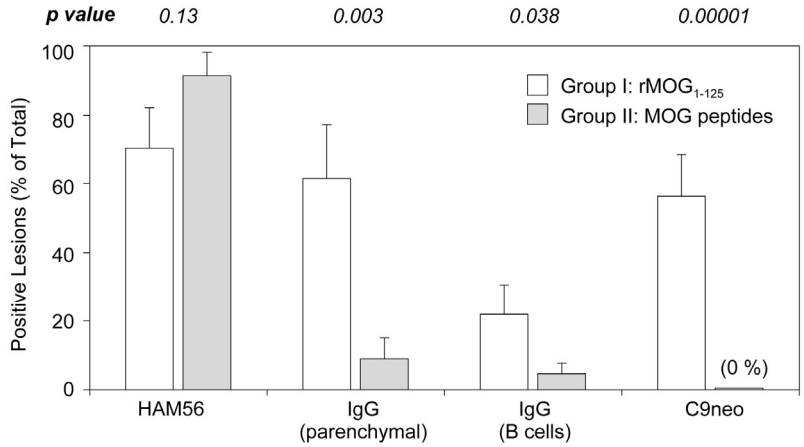

Abbildung 8: Semiquantitativer Vergleich von Läsionen von *C.jacchus* Affen, die entweder mit $rMOG_{125}$ (weiß) oder pepMOG (grau) immunisiert wurden. Die Dichte eingewanderter Makrophagen (HAM56+) war nicht unterschiedlich, wohl aber das Ausmaß von parenchymaler IgG-Ablagerungen, die B-Lymphozyten-Dichte und im besonderen die Komplementaktivierung (fehlender Nachweis von C9neo in pepMOG-immunisierten Tieren) [150].

In einem zweiten Versuchsansatz wurden dann aus den Seren der immunisierten Tiere anti-MOG Antikörperfraktionen hergestellt. Mittels Affinitätsreinigung über selbst hergestellte Chromatographiesäulen, die entweder mit $rMOG_{125}$ oder einem Gemisch der genannten linearen MOG-Peptide beschichtet waren, wurden fraktioniert zunächst diejenigen Antikörper isoliert, die gegen lineare MOG-Epitope binden (anti-MOG-P), und dann jene, die ausschließlich gegen die konformationellen Epitope auf $rMOG_{125}$, also solche mit einer Tertiärstruktur, binden (anti-MOG-C). In der anti-MOG-P Fraktion der pepMOG-immunisierten Tiere waren Antikörper gegen $rMOG_{125}$ im ELISA nicht mehr nachweisbar. Diese Tiere haben also ausschließlich eine humorale Immunantwort gegen lineare MOG-Epitope. Interessanterweise banden aber sowohl anti-MOG-C als auch anti-MOG-P Antikörper in vitro an natives MOG im Corpus callosum von nicht-immunisierten *C.jacchus*. Somit sind beide Epitopklassen (linear vs. konformationell) in situ exprimiert und können von beiden Gruppen von anti-MOG Antikörpern (anti-MOG-P vs. anti-MOG-C) erkannt werden.

In einem abschließenden Experiment wurden diese beide Antikörperfraktionen für einen passiven Antikörpertransfer verwendet; affinitätsgereinigtes IgG von naiven *C.jacchus* Affen diente als Kontrolle. Je zwei Tiere pro Gruppe wurden mit MBP immunisiert. Dadurch kam es zu einer Störung der BHS-Integrität, aber zu keiner klinischen Manifestation einer EAE. Mittels ELISA gegen MBP wurde die Bildung von anti-MBP Serumantikörpern in den Tieren überwacht und bei deren erstmaligen Nachweis je 250 µg der fraktionierten anti-MOG Antikörper intravenös transferiert. Bei der semiquantitativen histologischen Aufarbeitung waren bei den beiden Tieren, die anti-MOG-C Antikörper erhalten hatten, eine höhere Anzahl von Läsionen, die alle deutliche demyelinisierend waren, nachweisbar. Die beiden Tiere, die anti-MOG-P Antikörper erhalten hatten, hatte zahlenmäßiger weniger Läsionen mit nur geringer Demyelinisierung. Die Tiere, die die Kontrollantikörper erhalten hatten, waren keinerlei entzündliche Läsionen nachweisbar.

Diese Studie erbringt den Beweis, dass sowohl Antikörper, die gegen lineare MOG-Epitope gerichtet sind, als auch solche, die gegen tertiäre, konformations-abhängige Epitope reagieren, potentiell pathogen sind, in dem sie Makrophagen in die Läsionen rekrutieren und aktivieren können. Aber nur die konformations-abhängigen anti-MOG Antikörper sind in der Lage, zu einer Dissemination der ZNS-Läsionen, zu einer Komplementaktivierung und schließlich zu einer Demyelinisierung beizutragen. Interessanterweise kommt es in Tieren, die mit pepMOG immunisiert wurden, nicht zu einem humoralen *Epitope spreading* mit Ausweitung der Antikörperantwort auch auf konformationsabhängige MOG-Epitope. Die Bildung demyelinisierender anti-MOG Antikörper ist also ausschließlich von der initialen Immunisierung mit MOG in seiner Tertiärstruktur abhängig. Zusätzlich von Bedeutung ist, dass trotz der differentiellen Pathogenese der klinische Phänotyp und der Nachweis der anti-MOG Antikörper mittels ELISA in beiden Gruppen nicht zu unterscheiden war. Übertragen auf die MS würde dies bedeuten, dass trotz einer unterschiedlichen Pathogenese die MS am klinischen Verlauf und durch Nachweis von anti-MOG Serumantikörpern nicht zu differenzieren ist.

Mittlerweile ist durch die Arbeitsgruppe von Dr. Christopher Linington auch in der murinen EAE gezeigt worden, dass pathogene anti-MOG Antikörper gegen konformationsabhängige Epitope gerichtet sind und deren Spezifität im wesentlichen auf ein dominantes Epitop beschränkt ist [154].

2.2. Differentielle Exposition immundominanter Epitope in Abhängigkeit der physikochemischen Umgebung von MOG

Menge T, von Büdingen HC, Lalive PH, Genain CP: Relevant antibody subsets against MOG recognize conformational epitopes exclusively exposed in solid-phase ELISA. *Eur J Immunol.* 2007;37:3229-39 [180].

Das Ziel dieser Arbeit war es, die Heterogenität der konformationsabhängigen MOG-Epitope, die bei der MS und der *C.jacchus* EAE immundominant sind, weiter zu untersuchen. Wie in der zuerst diskutierten Arbeit gezeigt werden konnte, hilft der ELISA nicht, zwischen pathogenen und nicht pathogenetisch relevanten anti-MOG Antikörpern zu unterscheiden [150]. Aus diesem Grunde fragten wir, ob eine Testmethode, die MOG in Flüssigphase verwendet, besser geeignet ist, diese Antikörperfraktionen in einem polyklonalen Gemisch, wie zum Beispiel Serum, von einander zu unterscheiden. Das Rational ist, dass Testmethoden, die Antigene in Flüssigphase einsetzen, wie zum RIA (radioimmuno assay), preferrentiell höher affine Antikörper detektieren. Die EAE im *C.jacchus* Affen diente abermals als Modell bzw. Vergleichsgruppe, da aus der unter 2.1. zitierten vorherigen Arbeit unserer Arbeitsgruppe die differentielle humorale Immunantwort und insbesondere die Fraktion der pathogenen anti-MOG Serumantikörper unter den verschiedenen Immunisierungsprotokollen bereits näher charakterisiert war [150].

Zunächst wurde ein Flüssigphasentest entwickelt, der zum einen keine Radioaktivität zum Antikörperbindungsnachweis benötigte und der zum zweiten die identische MOG-Isoform wie der ELISA als Zielantigen verwendete. Letzteres war nötig, um direkt und unmittelbar die Bindung von Antikörpern im ELISA gegen gebundenes MOG, also gegen ein Festphasenantigen, mit der Bindung im Flüssigphasentest vergleichen zu können. Dadurch können biochemische Einflußfaktoren gering gehalten und die Aussagekraft der Ergebnisse erhöht werden. Arbeiten zweier anderer Arbeitsgruppen hatten nämlich Antigenpräparationen für ihre Flüssigphasentests verwendet, die nicht im ELISA zu verwenden waren [158;176]. Rekombinantes humanes (rh) MOG_{125} wurde zunächst via seinem primären Amin biotinyliert und so dann in sehr geringen Dosen (im nanomolaren Bereich) mit anti-MOG Antikörpern bzw. Serum in Lösung inkubiert. Antigen-Antikörper-Komplexe wurden mittels einer mit Protein G beschichteten ELISA-Platte immobilisiert. Derartig gebundenes

biotinyliertes rhMOG$_{125}$ konnte mittels Streptavidin-HRP sichtbar gemacht werden. Die Höhe des Farbumschlages korreliert mit der Menge an gebundenem Immunkomplex. Als Positiv- bzw. Methodenkontrolle dient biotinyliertes Tetanus Toxoid (TT). Die Methode wurde in Analogie zum RIA *Liquidphase Enzyme Linked Immuno-Assay* (LiPhELIA) genannt.

Zunächst wurden Seren von 37 Patienten mit MS und von 13 gesunden Kontrollen sowohl mittels ELISA als auch mittels LiPhELIA auf Antikörper, die gegen rhMOG$_{125}$ oder TT reagieren, untersucht. Während unterschiedlich ausgeprägte Reaktionen gegen rhMOG$_{125}$ und TT im ELISA nachweisbar waren, reagierte keine der Proben gegen lösliches rhMOG$_{125}$ im LiPhELIA. Hingegen waren die Antikörperantworten gegen TT nicht unterschiedlich zwischen ELISA und LiPhELIA (Abb. 9).
Je fünf Seren von *C.jacchus* Affen, bei denen eine EAE durch aktive Immunisierung entweder mit HWM, rekombinantem Ratten-MOG$_{125}$ oder linearen MOG-Peptiden (pepMOG) induziert worden war, wurden ebenfalls im ELISA und im LiPhELIA untersucht. Im ELISA waren die Antikörperbindungskurven nach Immunisierungsschemata nicht zu unterscheiden. Im LiPhELIA hingegen gaben die Seren pepMOG-immunisierter Tiere kein Signal; Antikörper gegen lösliches rhMOG$_{125}$ wurden in diesen Tieren also nicht produziert.
Die fehlende Reaktivität der menschlichen Seren gegen lösliches rhMOG$_{125}$ ließ sich jedoch nicht ausschließlich dadurch erklären, dass Menschen mutmaßlich nur Antikörper gegen lineare MOG-Epitope produzieren. Denn aus einem Panel von monoklonalen Antikörpern (8.18c5) bzw. Fab-Fragmenten (M26, M3-8, M3-24, M3-31), von denen bekannt ist, dass sie ausschließlich konformationsabhängige MOG-Epitope erkennen [202], reagierten einige ebenfalls nicht im LiPhELIA. Bei diesen monoklonalen Antiörpern und Fab-Fragmenten wurden zusätzlich die Antikörper-Affinitäten im ELISA gemessen. Es zeigte sich, dass eine fehlende Reaktivität gegen lösliches rhMOG$_{125}$ nicht mit einer niedrigen Affinität vergesellschaftet war. Da gemutmaßt worden war, dass lösliches MOG wegen seiner hydrophoben Eigenschaften Dimere bzw. Multimere bildet, wurde ein nativer Western Blot entwickelt, in dem zunächst in Lösung di- bzw. trimerisiertes rhMOG$_{125}$ auf eine Nitrozellulose-Membran gebunden wurde und mit verschiedenen poly- und monoklonalen anti-MOG Antikörpern inkubiert wurde. Unabhängig von der Reaktivität gegen lösliches rhMOG$_{125}$ reagierten alle getesteten Antikörper im Western Blot,

so dass die immundominanten Epitope nicht durch Multimerisierung des Proteins verdeckt werden.

Entscheidender Bedeutung kam ein abschließendes Experiment zu, in dem Seren von *C.jacchus* Affen, die mit MP4 immunisiert worden waren, differentiell im ELISA und im LiPhELIA untersucht wurden. MP4 ist ein rekombinantes Fusionsprotein, bestehend aus MBP und PLP [127]. Nach aktiver Immunisierung entwickeln einige der *C.jacchus* Affen demyelinisierende Läsionen, und es lassen sich im Sinne des *Epitope spreadings* Serumantikörper gegen MOG mittels ELISA nachweisen [127]. Drei Seren von Tieren mit histopathologisch nachgewiesener ZNS-Demyelinisierung wurden untersucht. Während sich Antikörper gegen gebundenes rhMOG$_{125}$ mittels ELISA in den Seren aller Tiere nachweisen ließen, konnte eine Reaktion gegen lösliches rhMOG$_{125}$ im LiPhELIA nicht gemessen werden.

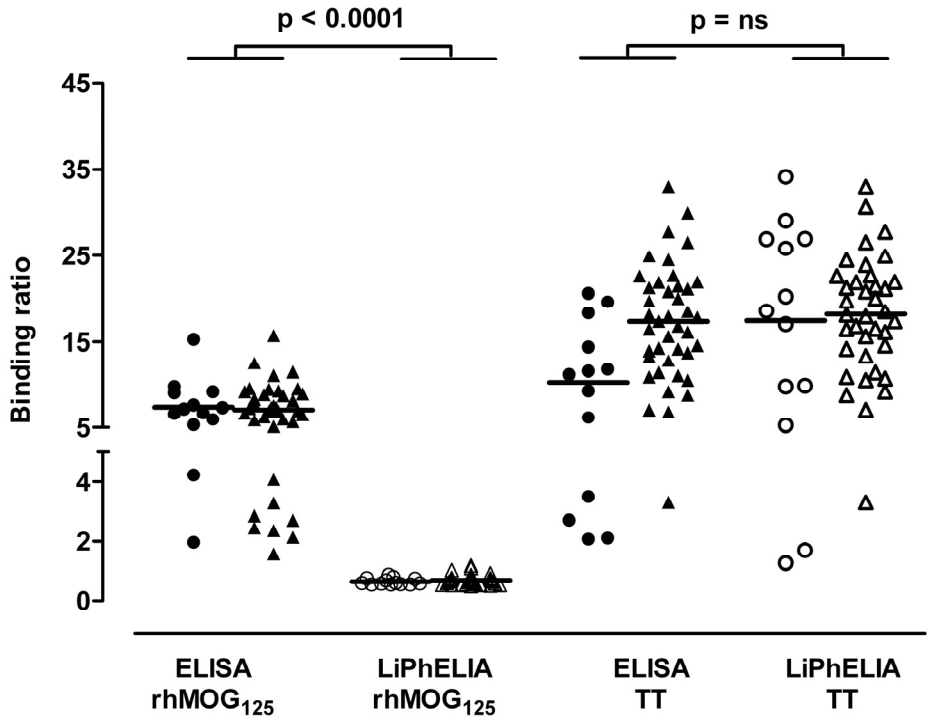

Abbildung 9: IgG-Reaktivität gegen rhMOG$_{125}$ und TT in 13 gesunden Kontrollen (●,○) und 37 Patienten mit MS (▲,Δ), gemessen im ELISA (▲,●) und im LiPhELIA (Δ,○). Bemerkenswert ist der vollständige Verlust der anti-MOG Reaktivität im LiPhELIA, der kein technisches Artefakt sein kann, da beide Gruppen gleich gut gegen TT reagieren [180].

Diese Studie belegt, dass in löslichem MOG immundominante Epitope für eine Antikörperbindung nicht frei verfügbar bzw. zugänglich sind. Ursächlich hierfür ist am ehesten die Tatsache, dass MOG als Bestandteil der fettreichen und wasserarmen

Myelinscheide genuin hydrophob ist und somit in Lösung eine unphysiologische Tertiärstruktur einnimmt. Im Gegensatz dazu werden höchstwahrscheinlich bei der Bindung von MOG an das Polystyren der ELISA-Mikrotiterplatte zusätzlich kryptische Epitope freigesetzt. Darüberhinaus propagiert der ELISA die Bindung auch niedrig-affiner Antikörper, die gegen lineare Epitope reagieren. Die fehlende Bindung bestimmter anti-MOG Antikörper gegen die lösliche Isoform lässt sich hingegen nicht mit niedriger Affinität, Epitopblockade durch Multimerisierung oder die ausschließliche Reaktivität gegen lineare MOG-Epitope erklären. Da eindeutig pathogene anti-MOG Antikörper (die Fab-Fragmente, Sera von MP4-immunisierten Tieren) keine Reaktivität im LiPhELIA haben, andere aber schon (Sera von HWM-immunisierten Tieren, der monoklonale Antikörper 8.18c5), muss konstatiert werden, dass Testmethoden, die lösliches MOG als Zielantigen verwenden, nicht dazu geeignet sind, pathogene bzw. krankheitsrelevante Antikörper gegen MOG verlässlich zu identifizieren.

Ähnliche Schlussfolgerungen sind in zwei weiteren Arbeiten gezogen worden [158;176]. Unsere Studie verwendete jedoch als einzige das identische Antigen für sowohl den Flüssigphasen- als auch den Festphasentest, so dass nur hier ein direkter Vergleich der Ergebnisse und eine unmittelbare Interpretation der konformationsabhängigen Immunogenität von MOG zulässig ist. KC O'Connor et al. hingegen verwendeten ein in-vitro Translationssystem, in dem sie ein Dimer von MOG_{121} exprimieren und posttranslational modifizieren ließen. Das ELISA-Äquivalent (Delfia) verwendete $rhMOG_{121}$ [158]. Lampasona et al. ließen das gesamte MOG-Molekül in einem ex-vivo eukaryoten Translationssystem exprimieren. Als Festphasentest diente ein Western Blot mit $rhMOG_{124}$ als Festphasenantigen [176].

2.3. Distinkte Exposition immunologischer Epitope auf nativem, membrangebundenen MOG

Lalive PH, Menge T, Delarasse C, Della Gaspera B, Pham-Dinh D, Villoslada P, von Büdingen HC, Genain CP: Antibodies to native myelin oligodendrocyte glycoprotein are serologic markers of early inflammation in multiple sclerosis. *Proc Natl Acad Sci U S A.* 2006; 103:2280-5 [179].

Um noch weiter die mögliche pathogene Rolle der anti-MOG Serumantikörper von Patienten mit MS zu untersuchen, wurden in dieser Studie die anti-MOG Reaktivitäten gegen natives, glykosyliertes, membrangebundenes MOG und gegen $rhMOG_{125}$ untersucht. Abermals dienten das *C.jacchus* EAE-Modell und die aus diesem Modell gewonnenen monoklonalen Fab-Fragmente als Kontrollen [202]. Durch den Vergleich der Ergebnisse der menschlichen Sera mit den Seren der Marmoset-Affen sollen Rückschlüsse auf die Immunpathogenität von nativem humanen MOG und die Immunantwort gegen dessen Epitope gezogen werden.

Zunächst wurden CHO Zellen mit einem Genkonstrukt aus der Gensequenz des gesamten humanen MOG im Transfektionsvektor pCDNA-III stabil transfiziert. Mit diesen Zellen wurde ein durchflußzytometrischer Test (FACS) etabliert zur Bestimmung von Serumantikörpern gegen natives, zellmembrangebundenes humanes MOG. Dafür wurden sowohl MOG-transfizierte als auch mit dem Leervektor transfizierte Zellen mit Serum gefärbt und eine Bindung mit fluoreszenzmarkiertem Sekundärantikörper detektiert. Die Ergebnisse wurden als Bindungsquotient (*binding ratio*) beschrieben. Dieser wurde gegen eine humane Positivkontrolle normalisiert, um die inter-experimentelle Variabilität zu eliminieren. Als Vergleich diente der bereits etablierte und in den beiden Arbeiten zuvor verwendete ELISA mit $rhMOG_{125}$ als Zielantigen.

Es wurden Seren von 92 Patienten mit MS, 36 Patienten mit einem klinisch isolierten Syndrom (KIS) und 37 gesunden Kontrollen untersucht. Die Kohorte der MS-Patienten bestand aus 35 Patienten mit einer schubförmigen MS (RRMS), 33 Patienten mit einer sekundär-chronisch progredienten MS (SPMS) und 24 Patienten mit einer primär-progredienten MS (PPMS). Zusätzlich wurden serielle Serumproben von elf *C.jacchus* Affen, die mit HWM zur Induktion einer EAE immunisiert worden

waren, und die in unserer Arbeitsgruppe generierten anti-MOG Fab-Fragmente M26, M3-24, M3-8, M3-31 untersucht.

Verglichen mit den gesunden Kontrollen wurden Serumantikörper gegen natives humanes MOG signifikant häufiger bei Patienten mit KIS ($p < 0.001$) und Patienten mit RRMS ($p < 0.01$) detektiert und zu einem geringeren, aber noch signifikanten Anteil auch bei Patienten mit SPMS ($p < 0.05$), aber nicht Patienten mit PPMS. Im Gegensatz dazu fanden sich keine Unterschiede bei den Serumantikörpern gegen $rhMOG_{125}$, die im ELISA untersucht worden waren. Entsprechend waren zwischen diesen Antikörperpopulationen keine Korrelationen der Bindungsstärken nachweisbar. Dieser Hinweis, dass auf $rhMOG_{125}$ und nativem, membrangebundenen MOG unterschiedliche bzw. distinkte immundominante Epitope vorliegen, konnte durch ein Absorptionsexperiment bestätigt werden. Aus zwei Seren mit guter Antikörperreaktivität in beiden Testverfahren wurde durch wiederholte Exposition an das entsprechende Antigen die spezifischen Antikörper entfernt. Während erwartungsgemäß die Reaktivität gegen das adsorbierte Antigen im entsprechenden Testverfahren sank, blieb es im Vergleich im jeweils anderen Testverfahren mit dem jeweils anderen MOG-Antigen unverändert. Wenn also ein Serum mehrfach hintereinander auf Mikrotiterplatten inkubiert, an die $rhMOG_{125}$ gebunden war, war die Antikörperreaktion gegen $rhMOG_{125}$ hinterher im Vergleich zum Ausgangswert deutlich erniedrigt, während sich nur ein marginaler Unterschied im FACS (Bindung an natives MOG) fand. Ein zusätzlicher Hinweis, dass die beiden MOG-Antigene distinkte immunogene Eigenschaften besitzen, wurde durch die Analyse der monokonalen Fab-Fragmente erbracht. Während eine Bindung der Fab-Fragmente im ELISA nachweisbar war, reagierten die Fabs M3-24 und M3-8 nicht gegen das native, membrangebundene MOG im FACS, so dass die spezifischen Epitope von M3-24 und M3-8, die auf Polystyren-gebundenem $rhMOG_{125}$ vorhanden sind, nicht auf nativem MOG exprimiert oder aber verborgen werden.

In einem weiteren Experiment wurden longitudinal gesammelte *C.jacchus* Serumproben im FACS analysiert und der erstmalige Nachweis von anti-MOG Antikörpern gegen das erstmalige Auftreten von Symptomen der EAE aufgetragen (Abb. 10). In einer Kaplan-Meier-Analyse ließ sich belegen, dass Serumantikörper gegen natives MOG signifikant früher nachweisbar waren als der klinische Beginn der EAE.

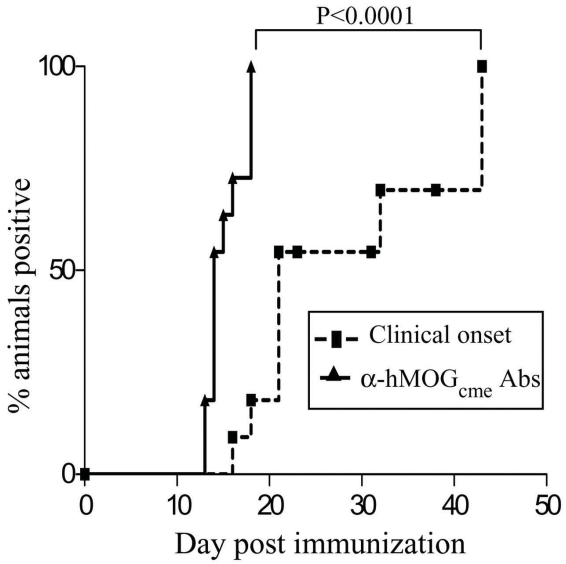

Abbildung 10: IgG Antwort gegen natives MOG im *C.jacchus* EAE-Modell. In der Kaplan-Meier Kurve sind die klinische Erstmanifestation von 11 Tieren mit EAE und der Zeitpunkt der Serokonversion gegen natives MOG aufgetragen. Die Antikörperantwort liegt dabei signifikant vor der klinischen Manifestation [179].

Drei wichtige Schlüsse können aus dieser Studie gezogen werden. Zum ersten scheinen die Epitope, die auf nativem, glykosylierten, physiologisch in vivo exprimierten MOG vorhanden sind, frühe Ziele von pathologischen anti-MOG Antikörpern zu sein. Denn anti-MOG Serumantikörper von HWM-immunisierten *C.jacchus* Affen sind fast ausschließlich gegen konformationelle Epitope gerichtet und können im passiven Antikörpertransfer eine demyelinsierende EAE übertragen [85]. Zum zweiten scheinen diejenigen Antikörper, die nicht gegen MOG in Festphase, also Mikrotiterplatten-gebundenes MOG, sondern im FACS gegen natives MOG reagieren, eine pathogene Rolle in der Frühphase der MS mit vorherrschender entzündlicher Aktivität zu spielen. Schließlich scheint der zellbasierte FACS-Test ein praktikables Verfahren zu sein, um frühzeitig serologische Serummarker zu detektieren, die eine autoimmune demyelinisierende Erkrankung des ZNS anzeigen und zumindest im EAE-Modell des *C.jacchus* bereits vor klinischer Manifestation der EAE nachweisbar sind. In der klinischen Routine allerdings läßt sich dies wegen der zu geringen Spezifität der Antikörper nicht umsetzen, wie auch zwei weitere Studien bestätigt haben [159;186].

2.4. Anti-MOG Antikörper als spezifische Marker demyelinisierender ZNS-Erkrankungen des Kindesalters

Lalive PH, Häusler MG, Maurey H, Mikaeloff Y, Tardieu M, Wiendl H, Schroeter M, Hartung HP, Kieseier BC, Menge T: Highly reactive anti-MOG antibodies differentiate demyelinating diseases from viral encephalitis in children. *Mult Scler.*, im Druck [201].

In dieser Studie wurden die zuvor untersuchten Testverfahren zur Bestimmung von Antikörpern gegen differentielle MOG-Epitope auf eine Kohorte von pädiatrischen Patienten angewendet. Ziel der Studie war es zu untersuchen, ob die humorale anti-MOG Immunantwort bei Kindern spezifisch für bestimmte entzündliche Erkrankungen des ZNS ist und gegen welche Epitope die Immunantwort gerichtet ist. Dabei wurden Seren junger Patienten mit pädiatrischer MS und akuter disseminierter Enzephalomyelitis (ADEM), also demyelinisierenden Erkrankungen des ZNS, untersucht und verglichen mit Seren von gesunden Kindern und Kindern mit viraler Enzephalitis, also einer nicht-demyelinisierenden entzündlichen Erkrankung des ZNS. Zusätzlich zur anti-MOG Reaktivität wurde der EBV-Serostatus erhoben, um zu untersuchen, ob Kreuzreaktivitäten zwischen EBV und MOG bestehen. Wie in der Einleitung ausgeführt, gibt es zahlreiche Hinweise, dass EBV eine gewisse Rolle in der Pathogenese der MS spielt, am wahrscheinlichsten durch molekulares Mimikry.

Insgesamt konnten 53 Seren von Kindern für diese Studie multizentrisch rekrutiert werden. Elf Kinder hatten die Diagnose einer ADEM, 22 Kinder waren an MS erkrankt; sieben Kinder waren mit einer viralen Enzephalitis diagnostiziert. Zusätzlich wurden 13 gesunde pädiatrische Kontrollen untersucht. Serumantikörper gegen $rhMOG_{125}$ wurden im ELISA und LiPhELIA und gegen natives, membrangebundenes MOG mittels FACS bestimmt. Zusätzlich wurden Antikörper gegen MBP mittels ELISA gemessen. Antikörper gegen die drei EBV-Antigene EA, EBNA und VCA wurden in einem kommerziellen ELISA bestimmt. Ein positiver anti-MOG Antikörperstatus wurde durch die gesunden Kontrollen definiert, wobei Bindungsquotienten, die über dem Mittelwert plus zwei Standardabweichungen der gesunden Kontrollen lagen, als positiv erachtet wurden.

Sowohl im ELISA als auch im FACS hatten signifikant mehr Patienten mit demyelinisierenden ZNS-Erkrankungen einen positiven Antikörperstatus gegen

rhMOG$_{125}$ bzw. natives MOG verglichen mit den Kontrollen oder Patienten mit viraler Enzephalitis (rhMOG$_{125}$: 27.3 % vs. 5.0 %; p=0.045, bzw. natives MOG: 12.1 % vs. 0.0 %; p=0.049; χ^2-Test; Abb. 11). Wenn beide Testverfahren kombiniert wurden, ließ sich eine anti-MOG Immunantwort in 30,3 % der Patienten mit demyelinisierenden ZNS-Erkrankungen, aber nur in 5 % der nicht-deymelinisierenden Kontrollen nachweisen. Auch dieser Unterschied war signifikant (p=0.0049; χ^2-Test). Der Großteil der Antikörper positiven Proben wurde als „hoch reaktiv" bezeichnet, weil die Bindungsquotienten mindestens fünffach über der individuellen Hintergrundbindung lagen. Im Gegensatz dazu war ein anti-MBP Antikörperstatus nicht mit einer Demyelinisierung assoziiert. Der anti-MOG Antikörperstatus war nicht assoziiert mit Alter, Geschlecht, oligoklonalen Banden im Liquor, EDSS oder EBV-Serostatus. Serumantikörper gegen MOG-Epitope des löslichen rhMOG$_{125}$ waren erneut mittels LiPhELIA nicht nachweisbar.

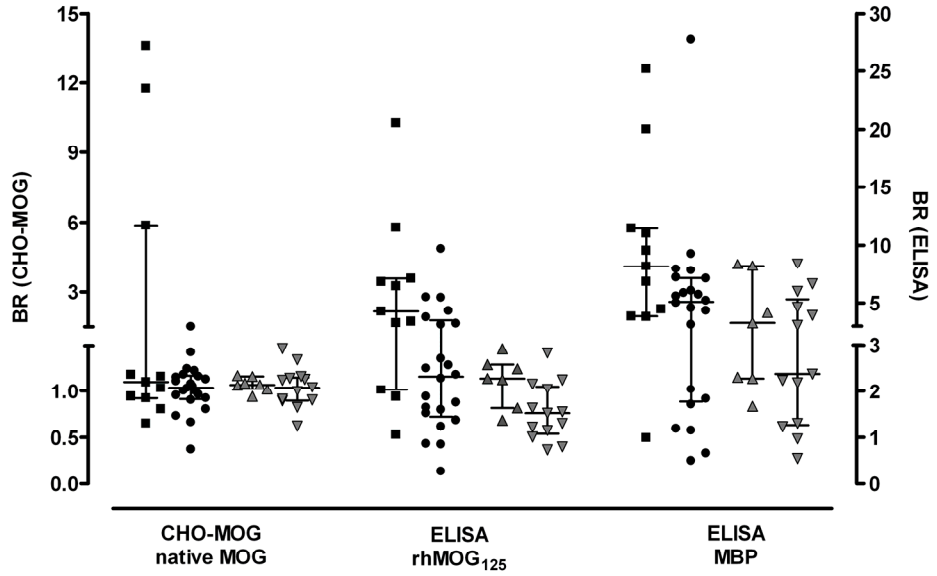

Abbildung 11: Streudiagramm der IgG-Reaktivitäten gegen natives MOG (links), rhMOG$_{125}$ und MBP (rechts) von 11 Kindern mit ADEM (■), 22 Kindern mit MS (●), 7 Kindern mit viraler Enzephalitis (▲) und 13 pädiatrischen Kontrollen (▼). Bemerkenswert sind die teils recht hohen Reaktivitäten (hoher Bindungsquotient) der Kindern mit demyelinisierenden Erkrankungen [201]

Diese Studie lieferte mehrere wichtige Erkenntnisse. Zum einen ist bei Kindern die humorale Immunantwort gegen MOG sehr spezifisch und findet sich fast ausschließlich bei Patienten mit einer demyelinisierenden ZNS-Erkrankung. Dies ist in deutlichem Gegensatz zu den Ergebnissen, die aus erwachsenen Kohorten

gewonnen werden konnte und in der Einleitung ausgeführt wurden (Abb. 4). Beispielsweise wurden in einer neueren Studie bei 32,9 % der gesunden Kontrollen anti-MOG Serumantikörper nachgewiesen [20;163]. Allerdings ist die anti-MOG Immunantwort wie auch bei den Erwachsenen nicht sensitiv für demyelinisierende ZNS-Erkrankungen, da anti-MOG Antikörper nur bei 30,3 % der Kinder mit MS oder ADEM nachweisbar waren.

Zum zweiten ist auffällig, dass die anti-MOG Antikörper fast vollständig hoch reaktiv sind, was wahrscheinlich einem hohen Titer entspricht. Auch dies steht im Gegensatz zu unseren Erfahrungen mit erwachsenen MS-Patienten; so lag der Bindungsquotient des FACS im Mittel für die positiven Proben rund fünffach über dem mittleren Bindungsquotienten der KIS-Patienten der zuvor besprochenen Studie [179]. Desweiteren sind die Ergebnisse dieser Studie eindrücklich kongruent mit den Ergebnissen anderer Arbeitsgruppen, die ebenfalls die Prävalenzen von anti-MOG Serumantikörpern bei Kindern mit MS oder ADEM untersucht haben [159;198-200]; bemerkenswerterweise lagen Prävalenzen, Sensitivität und Spezifitäten in einem vergleichbaren Rahmen. Ebenfalls bemerkenswert ist, dass die Testmethoden und Patientenkohorten zwar ähnlich, aber nicht identisch mit den anderen Studien waren.

Somit erbringt diese Studie Hinweise, dass bei Kindern die humorale Immunantwort gegen MOG hoch reaktiv und spezifisch für Demyelinisierung ist, während sich dieser Effekt mit zunehmendem Alter, d.h. bei Erwachsenen zu verlieren scheint. Die niedrige Sensitivität schließt eine Beteiligung der anti-MOG Antikörper an der Pathogenese demyelinisierender ZNS-Erkrankungen nicht aus, unterstützt aber die gängige Hypothese der Heterogenität dieser Erkrankungen. Vom klinischen Gesichtspunkt ist die routinemäßige Bestimmung der anti-MOG Serum-antikörper bei Kindern mit einer entzündlichen Erkrankung des ZNS wegen der niedrigen Sensibilität nicht sinnvoll. In bestimmten unklaren Fällen könnte eine der-artige Bestimmung aber nützlich sein, wenn der Verdacht einer demyelinisierenden Erkrankung laborchemisch erhärtet werden soll.

2.5. Die humorale Immunantwort gegen Galaktozerebrosid als verlaufsabhängiger Marker von MS und EAE

Menge T, Lalive PH, von Büdingen HC, Cree B, Hauser SL, Genain CP: Antibody responses against galactocerebroside are potential stage-specific biomarkers in multiple sclerosis. *J Allergy Clin Immunol.* 2005;116:453-9 [203].

Wie eingangs beschrieben, gibt es neben MOG noch weitere myelinische Antigene, die potentiell enzephalitogen sind bzw. ein demyelinisierendes Potential haben. Eines davon ist das Glykolipid Galaktozerebrosid (GalC), das recht ZNS-spezifisch und dort integraler Bestandteil der Myelinscheide ist. Anti-GalC Antikörper wirken in vitro und im passiven Antikörpertransfer der EAE demyelinisierend. Das Ziel dieser Studie war daher zu untersuchen, ob anti-GalC Serumantikörper spezifische Antikörper der MS sind und als Biomarker der Erkrankung dienen können. Komplementär zu der Untersuchung einer Kohorte von MS-Patienten und Kontrollen wurde das *C.jacchus* EAE-Modell als Kontrolle verwendet.

Zunächst wurde ein ELISA entwickelt und implementiert, um anti-GalC Antikörper bestimmen zu können. Da GalC ein Lipid ist, mußten gängige ELISA-Protokolle, bei denen Proteine als Zielantigen dienen, modifiziert werden. Zunächst wurde ein Lösungsprotokoll für GalC entwickelt, das kompatibel mit der weiteren Verwendung des GalC auf Polystyren-Platten ist; es mußten die üblicherweise zur Lösung von Fetten verwendeten organischen Lösungsmittel reduziert werden. Desweiteren wurden verschiedene Polystyren-Platten auf ihre Bindungskapazität von GalC untersucht. Dann mußten jegliche Detergenzien, wie zum Beispiel Tween-20, aus Puffern und Waschlösungen eliminiert werden, die das gebundene GalC unmittelbar wieder von der Polystyren-Platte abgewaschen hätten. Dieser ELISA wurde durch einen kommerziellen, polyklonalen anti-GalC Antikörper validiert, der bis zu einer Verdünnung von 1:12.800 titriert werden konnte und dessen Reaktivität mit dem gebundenen GalC durch Vorinkubation mit löslichen GalC reduziert werden konnte.

Sera von 51 Patienten mit MS (20 RRMS, 15 SPMS, 16 PPMS), 14 Patienten mit KIS und von 20 gesunden Kontrollen wurden sodann in diesem ELISA untersucht. Seropositivität wurde definiert als ein Bindungquotient (BR), der über dem mittleren BR plus drei Standardabweichungen der gesunden Kontrollen lag. Zudem wurden

serielle Blutproben von 20 *C.jacchus* Affen, die mit HWM-immunisiert worden waren, untersucht. Neun der Tiere hatten einen schubförmigen EAE-Verlauf, zwei Tiere verschlechterten sich progressiv ohne Besserung; bei sechs Tieren wurde das Experiment nach dem ersten Schub beendet; bei drei weiteren Tieren wurde das Experiment vor dem Auftreten klinischer Symptome, aber nach Nachweis einer Liquorpleozytose vorzeitig beendet.

In 40 % der Patienten mit RRMS ließen sich anti-GalC Serumantikörper nachweisen; dieser Unterschied war signifikant gegenüber der Prävalenz von 0 % bei gesunden Kontrollen. Auch die Stärke der Antikörperreaktivität war bei RRMS signifikant höher als bei den gesunden Kontrollen. In den anderen MS-Subgruppen und bei den KIS-Patienten fanden sich keine Unterschiede im Vergleich zu den gesunden Kontrollen. Zum Beweis der Spezifität dieser Antikörper und deren biologischen Relevanz wurden aus dem Serum einer Patientin mit RRMS, die eine hohe Antikörperreaktivität im ELISA aufwies, mittels einer selbst hergestellten Chromatographiesäule affinitätsgereinigt. Die gewonnene Antikörperfraktion färbte die humane, GalC exprimierende Oligodendrogliom-Zelllinie HOG in einem vergleichbaren Färbemuster wie der kommerzielle polyklonale anti-GalC Antikörper.
Die alle zwei Wochen ab dem Zeitpunkt der Immunisierung gewonnenen Blutproben der 20 *C.jacchus* Affen wurden neben der Reaktivität gegen GalC auch auf Antikörper gegen $rMOG_{125}$ und MBP untersucht und diese Ergebnisse im Zeitverlauf zusammen mit der klinischen Erstmanifestation aufgetragen (Abb. 12). Dabei zeigte sich, dass eine Serokonversion mit Nachweis von anti-GalC Antikörpern signifikant später als das Auftreten von anti-MOG oder anti-MBP Antikörpern eintritt. Somit kann anti-Galc Antikörpern ein verlaufsspezifisches Auftreten zugesprochen werden.

Diese Studie erbringt mehrere wichtige Erkenntnisse. Zunächst gelingt der Nachweis von Serumantikörpern gegen GalC bei Menschen auf einfache Weise in einem modifizierten Lipid-ELISA. Desweiteren ist die humorale Immunantwort gegen GalC bei der MS spezifisch, denn es lassen sich keine anti-GalC Antikörper in der Kohorte der gesunden Kontrollen nachweisen. Dies steht im Kontrast zur bereits beschriebenen Immunantwort gegen MOG, die zumindest beim Erwachsenen nicht spezifisch für die MS ist (Abb. 4) [20;53]. Allerdings ist die Sensitivität der anti-GalC Immunantwort bei der MS nicht hoch, sondern lag in dieser Studie bei 25,5 % für alle MS-Patienten. Somit muss die routinemäßige Verwendung von anti-GalC Antikörpern als diagnostischer Biomarker im klinischen Alltag bezweifelt werden.

Allerdings ist bemerkenswert, dass die anti-GalC Immunantwort sowohl beim Menschen als auch in der Primaten-EAE im *C.jacchus* Affen spezifisch für eine etablierte Erkrankung ist. Bei der MS findet sich eine signifikante Prävalenz mit signifikant erhöhter Reaktivität nur bei Patienten mit RRMS, nicht aber mit KIS. In der EAE treten Antikörper gegen GalC erst im Verlauf der Erkrankung auf, lange nach der klinischen Manifestation. Über die Gründe kann nur spekuliert werden; denkbar ist, dass GalC zwar in einer demyelinisierenden Läsion aus der Zellmembran gelöst wird, dann aber als Hapten fungiert, also ohne Bindung an ein Trägermolekül keine Immunantwort auslösen kann. MOG wäre solch ein denkbares Trägerprotein [29]. Darüberhinaus kann GalC nicht in klassischer Weise den spezifischen Rezeptor von T-Lymphozyten aktivieren sondern tut dies via CD1 [204].

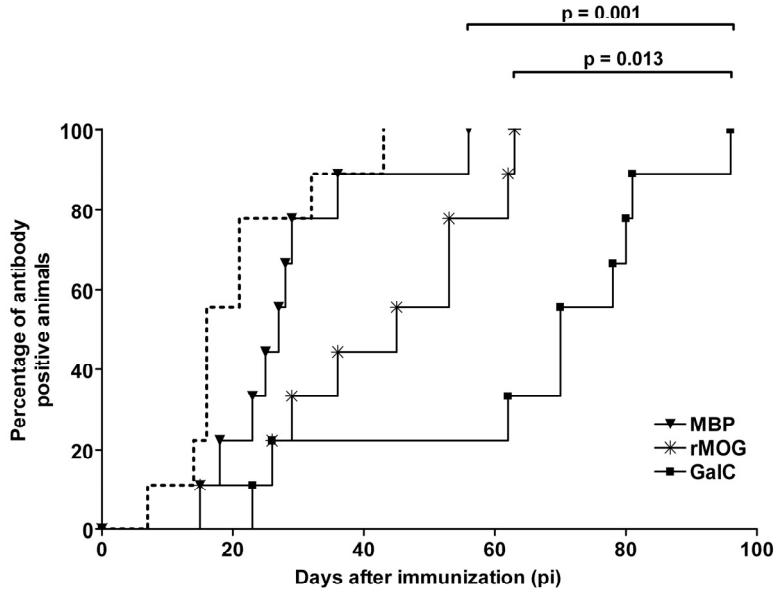

Abbildung 12: Kaplan-Meier Kurve der klinischen Erstmanifestation im *C.jacchus* EAE-Modell im Vergleich zum Nachweis verschiedener anti-Myelin Serumantikörper. Anti-GalC IgG Serumantikörper treten erst signifikant nach der klinischen Manifestation und nach dem Nachweis von anti-MBP und anti-MOG Antikörpern auf (Cox-Modell) [203]

Zusammenfassung und Ausblick

Die MS ist eine chronisch entzündliche, demyelinisierende Erkrankung des ZNS autoimmuner Genese. Das auslösende Ereignis der MS ist noch nicht genau verstanden. Im Tiermodell der MS, der EAE, ist dies die aktive Immunisierung mit einem enzephalitogenen myelinischen Autoantigen oder der passive Transfer von gegen Myelin gerichteten Autoantikörpern in suszeptible Tiere. Pathogenetisch handelt es sich bei der MS um eine heterogene Erkrankung, bei der alle Bestandteile des angeborenen und des erworbenen Immunssystems beteiligt sein können. Histopathologisch finden sich in über der Hälfte von MS-Läsionen Antikörperablagerungen zusammen mit einer Komplementaktivierung, was auf eine prädominante Rolle von Antikörpern bei der MS hinweist. In der EAE können spezifische Antikörper gegen myelinische Antigene zu einer Demyelinisierung führen. Eines der Zielantigene ist MOG, das zwar nur einen sehr geringen Anteil der Proteinfraktion von Myelin ausmacht, im Tiermodell aber enzephalitogen ist und demyelinisierend wirkt.
Aus diesem Grunde liegt es nahe, die Rolle von MOG als Zielantigen und von spezifischen Antikörpern gegen MOG bei der MS zu untersuchen. Zahlreiche Studien haben die Frage nach Prävalenz, Sensitivität und Spezifität von IgG-Serumantikörpern gegen MOG addressiert und divergente Ergebnisse geliefert. Der Versuch, Serumantikörper gegen MOG und/oder andere myelinische Antigene als Biomarker zu etablieren, war auch deswegen bisher noch nicht erfolgreich. Die wichtigsten Gründe für die Divergenz der Studien zur humoralen anti-MOG Immunantwort sind vor allem die unterschiedlichen MOG-Präparationen und Testverfahren, die verwendet wurden.

Die fünf hier vorgestellten eigenen Originalarbeiten leisten im Kontext von rezenten Arbeiten anderer Gruppen einen wesentlichen Beitrag, um die Immunogenität von MOG detailierter zu verstehen und die funktionellen Implikationen unterschiedlicher MOG-Epitope besser erklären zu können. Die erste Arbeit entschlüsselt das Dilemma, dass in der klinischen Routine gängige Testverfahren pathogene und nicht-pathogene anti-MOG Antikörper nicht diagnostisch trennen können. Vielmehr liefert diese Studie den funktionellen Beweis, dass nur Antikörper, die gegen konformationelle Epitope binden, demyelinisierend sind. Interessanterweise spiegelt sich dieser pathogenetische Unterschied im klinischen Phänotyp der *C.jacchus* EAE nicht wider. Dies mag der Grund sein, warum auch bei der MS bisher keine klinischen

Korrelationen zu anti-MOG Antikörpern gelungen sind. Allerdings war bei den meisten Studien die Kohortengröße zu gering, um die notwendige statistische Aussagekraft zu generieren. Im weiteren Verlauf ist dann von der Arbeitsgruppe um C. Linnington gezeigt worden, dass pathogene murine monoklonale anti-MOG Antikörper ebenfalls ausschließlich gegen konformationelle Epitope reagieren [154]. Somit macht es wenig Sinn, MOG-Epitope auf Peptidebene zu definieren [151].

Mit der zweiten und dritten Studie wird belegt, dass jedes Testverfahren zur Bestimmung von Antikörpern gegen MOG (ELISA, LiPhELIA, FACS) preferentiell unterschiedliche Antikörperfraktionen detektiert und zusätzlich MOG in den unterschiedlichen Testverfahren verschiedene immunologische Eigenschaften annimmt, zum Beispiel kryptische Epitope exponiert (gebundene Phase des ELISA) oder immundominante Epitope blockiert (lösliche Phase des LiPhELIA). Diese Epitope sind distinkt voneinander, so dass verschiedene Testverfahren unterschiedliche Ergebnisse liefern. Es verwundert also nicht, dass in den bisherigen Studien zu anti-MOG Antikörpern bei Menschen aufgrund der unterschiedlichen technischen Gegebenheiten nicht vergleichbare Ergebnisse generiert wurden [20]. Unsere Arbeiten liefern zusätzlich zum ersten Mal den Nachweis, dass Testverfahren, die lösliches MOG verwenden, technisch nicht unzulänglich sind, sondern die für pathologische anti-MOG Antikörper relevanten Epitope nicht exponieren. Zusammen mit anderen Arbeiten kristallisiert sich durch unsere Studie zu nativem MOG [179] heraus, dass die Bestimmung von Antikörpern gegen natives MOG am ehesten krankheitsrelevant sind.

Dies läßt sich auch aus der vierten vorgestellten Studie schlußfolgern, wobei erstaunt, dass Kinder mit demyelinisierenden ZNS-Erkrankungen deutlich stärker gegen MOG reagieren als Erwachsenen. Ferner bemerkenswert ist die hohe Spezifität der humoralen anti-MOG Immunantwort im Kindesalter verglichen mit Erwachsenen und die kongruente Datenlage im Vergleich zu ähnlichen Arbeiten anderer Forschungsgruppen [159;198-200]. Wie auch in anderen Arbeiten kann keine Assoziation zwischen einer EBV-Infektion und dem Nachweis von anti-MOG Antikörpern nachgewiesen werden [200].

Schließlich fügt sich die fünfte Studie mit der Beschreibung von anti-GalC Antikörpern als mögliche Marker einer etablierten Erkrankung ein, da sich derartige Antikörper nicht im Serum von Patienten mit einer Erstmanifestation (KIS) nachweisen lassen. Wie schon in den anderen Studien hat die Zuhilfenahme des

EAE-Modells im *C.jacchus* Affen erheblich zum Verständnis und der Interpretation der jeweiligen Daten beigetragen. Der Vorteil dieses EAE-Modells gegenüber anderen EAE-Modellen in Nagetieren ist die differentielle Induzierbarkeit einer EAE durch unterschiedliche, wohldefinierte MOG-Präparationen und die Kenntnis, dass ausschließlich MOG im *C.jacchus* Affen zu einer demyelinisierenden EAE führt. Dadurch können pathologische von nicht-demyelinisierenden anti-MOG Antikörpern getrennt und diese Fraktionen auf die zu testenden Hypothesen angewendet werden.

Zusammenfassend läßt sich feststellen, dass anti-MOG Antikörper auch bei der MS eine relevante Rolle spielen und dass durch die beschriebenen eigenen Arbeiten eben diese Rolle näher charakterisiert worden ist. Der unmittelbare Nachweis demyelinisierender Eigenschaften von humanen Antikörpern gegen MOG ist allerdings zum gegenwärtigen Zeitpunkt noch nicht erbracht worden. Dies mag an der Tatsache liegen, dass die genauen pathogenen Epitope noch nicht identifiziert worden sind. Die hier vorgestellten eigenen Arbeiten haben diese Bestrebung ein gutes Stück vorangebracht. Ein zukünftiges Ziel wird sein, die immundominanten und für den Menschen krankheitsrelvanten Epitope weiter zu charakterisieren. Idealerweise wird die klinische Relevanz von anti-MOG Antikörpern, die spezifisch für diese Epitope sind, an einer großen Kohorte klinisch und paraklinisch gut charakterisierter MS-Patienten validiert. Der endgültige Beweis erfolgt dann durch den passiven Transfer affinitätsgereinigter anti-MOG Antikörper aus Patienten in ein EAE-Modell. Hier bietet sich wegen der guten Homologie der Immunsysteme das *C.jacchus* Affenmodell an. Histopathologisch müssen sich demyelinisierender Läsionen, idealerweise mit Ablagerung von humanen Antikörpern in den Läsionen, nachweisen lassen. Dann kann versucht werden, genau diese anti-MOG Antikörper als Biomarker zu implementieren, wobei es sich am ehesten um einen für die Therapieentscheidung wegweisenden Marker handeln würde. Denn die hier beschriebene niedrige Sensitivität der bisherigen anti-MOG Antikörper wird weiterhin niedrig bleiben. Aber es kann postuliert werden, dass mit Kenntnis der pathogenen MOG-Epitope sepzifische Immuntherapien entwickelt werden. Denkbar wäre eine Immunabsorption genau dieser Antikörper, eine Blockade der Epitope zum Beispiel durch spezifische Fab-Fragmente oder eine Blockade der komplementären Rezeptoren auf autoreaktiven B-Lymphozyten mit dem Ziel, diese Zellen in Anergie zu bringen.

Literatur

1. Galea, I, Bechmann, I, and Perry, VH**,** What is immune privilege (not)? *Trends Immunol.* 2007. 28: 12-18.
2. Hickey, WF**,** Migration of hematogenous cells through the blood-brain barrier and the initiation of CNS inflammation. *Brain Pathol.* 1991. 1: 97-105.
3. Hickey, WF, Hsu, BL, and Kimura, H**,** T-lymphocyte entry into the central nervous system. *J.Neurosci.Res.* 1991. 28: 254-260.
4. McFarland, HF and Martin, R**,** Multiple sclerosis: a complicated picture of autoimmunity. *Nat.Immunol.* 2007. 8: 913-919.
5. Bhat, R and Steinman, L**,** Innate and adaptive autoimmunity directed to the central nervous system. *Neuron* 2009. 64: 123-132.
6. Kerlero, dR, Milo, R, Lees, MB, Burger, D, Bernard, CC, and Ben Nun, A**,**
7. de Rosbo, NK and Ben Nun, A**,** T-cell responses to myelin antigens in multiple sclerosis; relevance of the predominant autoimmune reactivity to myelin oligodendrocyte glycoprotein. *J.Autoimmun.* 1998. 11: 287-299.
8. Diaz-Villoslada, P, Shih, A, Shao, L, Genain, CP, and Hauser, SL**,** Autoreactivity to myelin antigens: myelin/oligodendrocyte glycoprotein is a prevalent autoantigen. *J.Neuroimmunol.* 1999. 99: 36-43.
9. Bradl, M and Lassmann, H**,** Oligodendrocytes: biology and pathology. *Acta Neuropathol.* 2010. 119: 37-53.
10. Sospedra, M and Martin, R**,** Immunology of multiple sclerosis. *Annu.Rev.Immunol.* 2005. 23: 683-747.
11. Compston, A and Coles, A**,** Multiple sclerosis. *Lancet* 2008. 372: 1502-1517.
12. Hauser, SL and Oksenberg, JR**,** The neurobiology of multiple sclerosis: genes, inflammation, and neurodegeneration. *Neuron* 2006. 52: 61-76.
13. Menge, T, Hemmer, B, Nessler, S, Wiendl, H, Neuhaus, O, Hartung, HP et al.**,** Acute disseminated encephalomyelitis: an update. *Arch.Neurol.* 2005. 62: 1673-1680.
14. Menge, T, Kieseier, BC, Nessler, S, Hemmer, B, Hartung, HP, and Stüve, O**,** Acute disseminated encephalomyelitis: an acute hit against the brain. *Curr.Opin.Neurol.* 2007. 20: 247-254.
15. Steinman, L and Zamvil, SS**,** How to successfully apply animal studies in experimental allergic encephalomyelitis to research on multiple sclerosis.

Ann.Neurol. 2006. 60: 12-21.

16. Gold, R, Linington, C, and Lassmann, H. Understanding pathogenesis and therapy of multiple sclerosis via animal models: 70 years of merits and culprits in experimental autoimmune encephalomyelitis reserach. *Brain.* 2006. 129: 1953-1971.
17. Krishnamoorthy, G and Wekerle, H**,** EAE: an immunologist's magic eye. *Eur.J.Immunol.* 2009. 39: 2031-2035.
18. von Büdingen, HC, Tanuma, N, Villoslada, P, Ouallet, JC, Hauser, SL, and Genain, CP**,** Immune responses against the myelin/oligodendrocyte glycoprotein in experimental autoimmune demyelination. *J.Clin.Immunol.* 2001. 21: 155-170.
19. Bielekova, B and Martin, R**,** Development of biomarkers in multiple sclerosis. *Brain* 2004. 127: 1463-1478.
20. Reindl, M, Khalil, M, and Berger, T**,** Antibodies as biological markers for pathophysiological processes in MS. *J.Neuroimmunol.* 2006. 180: 50-62.
21. Berger, T and Reindl, M**,** Multiple sclerosis: Disease biomarkers as indicated by pathophysiology. *J.Neurol.Sci.* 2007.
22. Morell,P. and Quarles,R.H.**,** Myelin Formation, Structure and Biochemistry. In Siegel,G.J., Agranoff,B.W., Albers,R.W., Fisher,S.K., and Uhler,M.D. (Eds.) *Basic Neurochemistry*. Lippincott Williams & Wilkins, Philadelphia, PA 1999, pp 70-90.
23. Quarles, RH**,** Myelin sheaths: glycoproteins involved in their formation, maintenance and degeneration. *Cell Mol.Life Sci.* 2002. 59: 1851-1871.
24. Simons, M and Trotter, J**,** Wrapping it up: the cell biology of myelination. *Curr.Opin.Neurobiol.* 2007. 17: 533-540.
25. Podbielska, M and Hogan, EL**,** Molecular and immunogenic features of myelin lipids: incitants or modulators of multiple sclerosis? *Mult.Scler.* 2009. 15: 1011-1029.
26. Ohler, B, Graf, K, Bragg, R, Lemons, T, Coe, R, Genain, C et al.**,** Role of lipid interactions in autoimmune demyelination. *Biochim.Biophys.Acta* 2004. 1688: 10-17.
27. Johns, TG and Bernard, CC**,** The structure and function of myelin oligodendrocyte glycoprotein. *J.Neurochem.* 1999. 72: 1-9.
28. Linnington, C, Webb, M, and Woodhams, PL**,** A novel myelin-associated

glycoprotein defined by a mouse monoclonal antibody. *J.Neuroimmunol.* 1984. 6: 387-396.

29. Bernard, CC, Johns, TG, Slavin, A, Ichikawa, M, Ewing, C, Liu, J et al., Myelin oligodendrocyte glycoprotein: a novel candidate autoantigen in multiple sclerosis. *J.Mol.Med.* 1997. 75: 77-88.
30. Gardinier, MV, Amiguet, P, Linington, C, and Matthieu, JM, Myelin/oligodendrocyte glycoprotein is a unique member of the immunoglobulin superfamily. *J.Neurosci.Res.* 1992. 33: 177-187.
31. Pagany, M, Jagodic, M, Bourquin, C, Olsson, T, and Linington, C, Genetic variation in myelin oligodendrocyte glycoprotein expression and susceptibility to experimental autoimmune encephalomyelitis. *J.Neuroimmunol.* 2003. 139: 1-8.
32. Della, GB, Pham-Dinh, D, Roussel, G, Nussbaum, JL, and Dautigny, A, Membrane topology of the myelin/oligodendrocyte glycoprotein. *Eur.J.Biochem.* 1998. 258: 478-484.
33. Kroepfl, JF, Viise, LR, Charron, AJ, Linington, C, and Gardinier, MV, Investigation of myelin/oligodendrocyte glycoprotein membrane topology. *J.Neurochem.* 1996. 67: 2219-2222.
34. Delarasse, C, Daubas, P, Mars, LT, Vizler, C, Litzenburger, T, Iglesias, A et al., Myelin/oligodendrocyte glycoprotein-deficient (MOG-deficient) mice reveal lack of immune tolerance to MOG in wild-type mice. *J.Clin.Invest* 2003. 112: 544-553.
35. Baumann, N and Pham-Dinh, D, Biology of oligodendrocyte and myelin in the mammalian central nervous system. *Physiol Rev.* 2001. 81: 871-927.
36. Dupree, JL and Popko, B, Genetic dissection of myelin galactolipid function. *J.Neurocytol.* 1999. 28: 271-279.
37. Coetzee, T, Suzuki, K, and Popko, B, New perspectives on the function of myelin galactolipids. *Trends Neurosci.* 1998. 21: 126-130.
38. Suzuki, K and Suzuki, K, The twitcher mouse: a model for Krabbe disease and for experimental therapies. *Brain Pathol.* 1995. 5: 249-258.
39. Suzuki, K, Globoid cell leukodystrophy (Krabbe's disease): update. *J.Child Neurol.* 2003. 18: 595-603.
40. Development and survival of lymphocytes. In Janeway,C.A., Travers,P., Walport,M., and Shlomchik,M.J. (Eds.) *Immunobiology*. Churchill Livingstone,

Edinburgh 2001, pp 221-293.

41. Fujinami, RS, von Herrath, MG, Christen, U, and Whitton, JL**,** Molecular mimicry, bystander activation, or viral persistence: infections and autoimmune disease. *Clin Microbiol.Rev.* 2006. 19: 80-94.
42. Stohlman, SA and Hinton, DR**,** Viral induced demyelination. *Brain Pathol.* 2001. 11: 92-106.
43. Fujinami, RS and Oldstone, MB**,** Molecular mimicry as a mechanism for virus-induced autoimmunity. *Immunol.Res.* 1989. 8: 3-15.
44. Oldstone, MB**,** Molecular mimicry and immune-mediated diseases. *FASEB J.* 1998. 12: 1255-1265.
45. Stebbins, CE and Galan, JE**,** Structural mimicry in bacterial virulence. *Nature* 2001. 412: 701-705.
46. Karlsen, AE and Dyrberg, T**,** Molecular mimicry between non-self, modified self and self in autoimmunity. *Semin.Immunol.* 1998. 10: 25-34.
47. von Herrath, MG, Holz, A, Homann, D, and Oldstone, MB**,** Role of viruses in type I diabetes. *Semin.Immunol.* 1998. 10: 87-100.
48. Wekerle, H and Hohlfeld, R**,** Molecular mimicry in multiple sclerosis. *N.Engl.J.Med.* 2003. 349: 185-186.
49. Tough, DF and Sprent, J**,** Viruses and T cell turnover: evidence for bystander proliferation. *Immunol.Rev.* 1996. 150: 129-142.
50. Poser, CM and Brinar, VV**,** The nature of multiple sclerosis. *Clin.Neurol.Neurosurg.* 2004. 106: 159-171.
51. Hu, W and Lucchinetti, CF**,** The pathological spectrum of CNS inflammatory demyelinating diseases. *Semin.Immunopathol.* 2009. 31: 439-453.
52. Wingerchuk, DM, Lennon, VA, Lucchinetti, CF, Pittock, SJ, and Weinshenker, BG**,** The spectrum of neuromyelitis optica. *Lancet Neurol.* 2007. 6: 805-815.
53. Lalive, PH**,** Autoantibodies in inflammatory demyelinating diseases of the central nervous system. *Swiss.Med Wkly.* 2008. 138: 692-707.
54. Poser, CM and Brinar, VV**,** Disseminated encephalomyelitis and multiple sclerosis: two different diseases - a critical review. *Acta Neurol.Scand.* 2007. 116: 201-206.
55. Wingerchuk, DM and Lucchinetti, CF**,** Comparative immunopathogenesis of acute disseminated encephalomyelitis, neuromyelitis optica, and multiple sclerosis. *Curr.Opin.Neurol.* 2007. 20: 343-350.

56. Charcot, M**,** Histologie de la sclerose on plaque. *Gaz Hop* 1868. 554-558.
57. Wiendl,H. and Kieseier, B.**,** *Multiple Sklerose - Klinik, Diagnostik und Therapie*. Kohlhammer Verlag, Stuttgart 2010.
58. Yeh, EA, Chitnis, T, Krupp, L, Ness, J, Chabas, D, Kuntz, N et al.**,** Pediatric multiple sclerosis. *Nat.Rev.Neurol.* 2009. 5: 621-631.
59. Venkateswaran, S and Banwell, B**,** Pediatric multiple sclerosis. *Neurologist.* 2010. 16: 92-105.
60. Polman, CH, Reingold, SC, Edan, G, Filippi, M, Hartung, HP, Kappos, L et al.**,** Diagnostic criteria for multiple sclerosis: 2005 revisions to the "McDonald Criteria". *Ann.Neurol.* 2005. 58: 840-846.
61. Barkhof, F, Filippi, M, Miller, DH, Scheltens, P, Campi, A, Polman, CH et al.**,** Comparison of MRI criteria at first presentation to predict conversion to clinically definite multiple sclerosis. *Brain* 1997. 120 (Pt 11): 2059-2069.
62. Charil, A, Yousry, TA, Rovaris, M, Barkhof, F, De Stefano, N, Fazekas, F et al.**,** MRI and the diagnosis of multiple sclerosis: expanding the concept of "no better explanation". *Lancet Neurol.* 2006. 5: 841-852.
63. Link, H and Huang, YM**,** Oligoclonal bands in multiple sclerosis cerebrospinal fluid: an update on methodology and clinical usefulness. *J.Neuroimmunol.* 2006. 180: 17-28.
64. von Büdingen, HC, Harrer, MD, Kuenzle, S, Meier, M, and Goebels, N**,** Clonally expanded plasma cells in the cerebrospinal fluid of MS patients produce myelin-specific antibodies. *Eur.J.Immunol.* 2008. 38: 2014-2023.
65. Awad, A, Hemmer, B, Hartung, HP, Kieseier, B, Bennett, JL, and Stuve, O**,** Analyses of cerebrospinal fluid in the diagnosis and monitoring of multiple sclerosis. *J.Neuroimmunol.* 2010. 219: 1-7.
66. Cross, AH and Wu, GF**,** Multiple sclerosis: Oligoclonal bands still yield clues about multiple sclerosis. *Nat.Rev.Neurol.* 2010. 6: 588-589.
67. Hafler, DA, Compston, A, Sawcer, S, Lander, ES, Daly, MJ, De Jager, PL et al.**,** Risk alleles for multiple sclerosis identified by a genomewide study. *N.Engl.J.Med.* 2007. 357: 851-862.
68. Barcellos, LF, Oksenberg, JR, Begovich, AB, Martin, ER, Schmidt, S, Vittinghoff, E et al.**,** HLA-DR2 dose effect on susceptibility to multiple sclerosis and influence on disease course. *Am.J.Hum.Genet.* 2003. 72: 710-716.
69. Gregory, SG, Schmidt, S, Seth, P, Oksenberg, JR, Hart, J, Prokop, A et al.**,**

Interleukin 7 receptor alpha chain (IL7R) shows allelic and functional association with multiple sclerosis. *Nat.Genet.* 2007. 39: 1083-1091.

70. Giovannoni, G, Cutter, GR, Lunemann, J, Martin, R, Munz, C, Sriram, S et al., Infectious causes of multiple sclerosis. *Lancet Neurol.* 2006. 5: 887-894.
71. Ascherio, A and Munger, KL, Environmental risk factors for multiple sclerosis. Part I: the role of infection. *Ann.Neurol.* 2007. 61: 288-299.
72. Thacker, EL, Mirzaei, F, and Ascherio, A, Infectious mononucleosis and risk for multiple sclerosis: a meta-analysis. *Ann.Neurol.* 2006. 59: 499-503.
73. Ascherio, A, Munger, KL, Lennette, ET, Spiegelman, D, Hernan, MA, Olek, MJ et al., Epstein-Barr virus antibodies and risk of multiple sclerosis: a prospective study. *JAMA* 2001. 286: 3083-3088.
74. Ascherio, A and Munger, KL, Environmental risk factors for multiple sclerosis. Part II: Noninfectious factors. *Ann.Neurol.* 2007. 61: 504-513.
75. Compston, A and Coles, A, Multiple sclerosis. *Lancet* 2002. 359: 1221-1231.
76. Koritschoner, R and Schweinburg, F. Induktion von Paralyse und Rückenmarksentzündung durch Immunisierung von Kaninchen mit menschlichem Rückenmarksgewebe. *Z Immunitätsf Exp Therapie* 42, 217-283. 1925.
77. Rivers, TM, Sprunt, DH, and Berry, GP, Observations on the attempts to produce acute disseminated allergic encephalomyelitis in primates. *J.Exp.Med* 1933. 58: 39-53.
78. Goverman, J and Brabb, T, Rodent models of experimental allergic encephalomyelitis applied to the study of multiple sclerosis. *Lab Anim Sci.* 1996. 46: 482-492.
79. Hohlfeld, R, Biotechnological agents for the immunotherapy of multiple sclerosis. Principles, problems and perspectives. *Brain.* 1997. 120: 865-916
80. Martin, R and McFarland, HF, Immunological aspects of experimental allergic encephalomyelitis and multiple sclerosis. *Crit Rev.Clin.Lab Sci.* 1995. 32: 121-182.
81. Mendel Kerlero, dR and Ben Nun, A, Delineation of the minimal encephalitogenic epitope within the immunodominant region of myelin oligodendrocyte glycoprotein: diverse V beta gene usage by T cells recognizing the core epitope encephalitogenic for T cell receptor V beta b and T cell receptor V beta a H-2b mice. *Eur.J.Immunol.* 1996. 26: 2470-2479.

82. Tuohy, VK, Lu, Z, Sobel, RA, Laursen, RA, and Lees, MB**,** Identification of an encephalitogenic determinant of myelin proteolipid protein for SJL mice. *J.Immunol.* 1989. 142: 1523-1527.
83. Massacesi, L, Genain, CP, Lee-Parritz, D, Letvin, NL, Canfield, D, and Hauser, SL**,** Active and passively induced experimental autoimmune encephalomyelitis in common marmosets: a new model for multiple sclerosis. *Ann.Neurol.* 1995. 37: 519-530.
84. Genain, CP and Hauser, SL**,** Creation of a model for multiple sclerosis in Callithrix jacchus marmosets. *J.Mol.Med.* 1997. 75: 187-197.
85. Genain, CP, Nguyen, MH, Letvin, NL, Pearl, R, Davis, RL, Adelman, M et al.**,** Antibody facilitation of multiple sclerosis-like lesions in a nonhuman primate. *J.Clin.Invest* 1995. 96: 2966-2974.
86. Raine, CS, Traugott, U, Farooq, M, Bornstein, MB, and Norton, WT**,** Augmentation of immune-mediated demyelination by lipid haptens. *Lab Invest* 1981. 45: 174-182.
87. Moore, GR, Traugott, U, Farooq, M, Norton, WT, and Raine, CS**,** Experimental autoimmune encephalomyelitis. Augmentation of demyelination by different myelin lipids. *Lab Invest* 1984. 51: 416-424.
88. Fierz, W, Heininger, K, Schaefer, B, Toyka, KV, Linington, C, and Lassmann, H**,** Synergism in the pathogenesis of EAE induced by an MBP-specific T-cell line and monoclonal antibodies to galactocerebroside or a myelin oligodendroglial glycoprotein. *Ann.N.Y.Acad.Sci.* 1988. 540: 360-363.
89. Morris-Downes, MM, Smith, PA, Rundle, JL, Piddlesden, SJ, Baker, D, Pham-Dinh, D et al.**,** Pathological and regulatory effects of anti-myelin antibodies in experimental allergic encephalomyelitis in mice. *J.Neuroimmunol.* 2002. 125: 114-124.
90. Saida, T, Saida, K, and Silberberg, DH**,** Demyelination produced by experimental allergic neuritis serum and anti-galactocerebroside antiserum in CNS cultures. An ultrastructural study. *Acta Neuropathol.* 1979. 48: 19-25.
91. Raine, CS, Johnson, AB, Marcus, DM, Suzuki, A, and Bornstein, MB**,** Demyelination in vitro. Absorption studies demonstrate that galactocerebroside is a major target. *J.Neurol.Sci.* 1981. 52: 117-131.
92. Menon, KK, Piddlesden, SJ, and Bernard, CC**,** Demyelinating antibodies to myelin oligodendrocyte glycoprotein and galactocerebroside induce

degradation of myelin basic protein in isolated human myelin. *J.Neurochem.* 1997. 69: 214-222.

93. Menge,T., Hemmer,B., Nessler,S., Zhou,D., Kieseier,B., and Hartung,H.P., Immunopathogenesis of Multiple Sclerosis: Overview. In Zhang (Ed.) *Immune Regulation and Immunotherapy in Autoimmune Disease*. Springer, Berlin 2007, pp 171-185.
94. Oldstone, MB, Molecular mimicry, microbial infection, and autoimmune disease: evolution of the concept. *Curr.Top.Microbiol.Immunol.* 2005. 296: 1-17
95. Benoist, C and Mathis, D, Autoimmunity provoked by infection: how good is the case for T cell epitope mimicry? *Nat.Immunol.* 2001. 2: 797-801.
96. Wucherpfennig, KW and Strominger, JL, Molecular mimicry in T cell-mediated autoimmunity: viral peptides activate human T cell clones specific for myelin basic protein. *Cell* 1995. 80: 695-705.
97. Merkler, D, Horvath, E, Bruck, W, Zinkernagel, RM, Del la Torre, JC, and Pinschewer, DD, "Viral deja vu" elicits organ-specific immune disease independent of reactivity to self. *J.Clin.Invest* 2006. 116: 1254-1263.
98. Cserr, HF and Knopf, PM, Cervical lymphatics, the blood-brain barrier and the immunoreactivity of the brain: a new view. *Immunol.Today* 1992. 13: 507-512.
99. van Zwam, M, Huizinga, R, Melief, MJ, Wierenga-Wolf, AF, van Meurs, M, Voerman, JS et al., Brain antigens in functionally distinct antigen-presenting cell populations in cervical lymph nodes in MS and EAE. *J.Mol.Med.* 2009. 87: 273-286.
100. Shlomchik, MJ, Craft, JE, and Mamula, MJ, From T to B and back again: positive feedback in systemic autoimmune disease. *Nat.Rev.Immunol.* 2001. 1: 147-153.
101. Dalakas, MC, B cells in the pathophysiology of autoimmune neurological disorders: a credible therapeutic target. *Pharmacol.Ther.* 2006. 112: 57-70.
102. Tiller, T, Tsuiji, M, Yurasov, S, Velinzon, K, Nussenzweig, MC, and Wardemann, H, Autoreactivity in human IgG+ memory B cells. *Immunity.* 2007. 26: 205-213.
103. Cohen, IR, Biomarkers, self-antigens and the immunological homunculus. *J.Autoimmun.* 2007. 29: 246-249.
104. Lutz, HU, Homeostatic roles of naturally occurring antibodies: an overview. *J.Autoimmun.* 2007. 29: 287-294.

105. Zhou, ZH, Tzioufas, AG, and Notkins, AL**,** Properties and function of polyreactive antibodies and polyreactive antigen-binding B cells. *J.Autoimmun.* 2007. 29: 219-228.
106. Hickey, WF**,** Basic principles of immunological surveillance of the normal central nervous system. *Glia* 2001. 36: 118-124.
107. Becher, B, Bechmann, I, and Greter, M**,** Antigen presentation in autoimmunity and CNS inflammation: how T lymphocytes recognize the brain. *J.Mol.Med.* 2006. 84: 532-543.
108. Bechmann, I, Galea, I, and Perry, VH**,** What is the blood-brain barrier (not)? *Trends Immunol.* 2007. 28: 5-11.
109. Engelhardt, B and Ransohoff, RM**,** The ins and outs of T-lymphocyte trafficking to the CNS: anatomical sites and molecular mechanisms. *Trends Immunol.* 2005. 26: 485-495.
110. Hemmer, B, Nessler, S, Zhou, D, Kieseier, B, and Hartung, HP**,** Immunopathogenesis and immunotherapy of multiple sclerosis. *Nat Clin Pract Neurol* 2006. 2: 201-211.
111. Magliozzi, R, Columba-Cabezas, S, Serafini, B, and Aloisi, F**,** Intracerebral expression of CXCL13 and BAFF is accompanied by formation of lymphoid follicle-like structures in the meninges of mice with relapsing experimental autoimmune encephalomyelitis. *J.Neuroimmunol.* 2004. 148: 11-23.
112. Corcione, A, Casazza, S, Ferretti, E, Giunti, D, Zappia, E, Pistorio, A et al.**,** Recapitulation of B cell differentiation in the central nervous system of patients with multiple sclerosis. *Proc.Natl.Acad.Sci.U.S.A* 2004. 101: 11064-11069.
113. Magliozzi, R, Howell, O, Vora, A, Serafini, B, Nicholas, R, Puopolo, M et al.**,** Meningeal B-cell follicles in secondary progressive multiple sclerosis associate with early onset of disease and severe cortical pathology. *Brain* 2007. 130: 1089-1104.
114. Lucchinetti, C, Bruck, W, Parisi, J, Scheithauer, B, Rodriguez, M, and Lassmann, H**,** Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination. *Ann.Neurol.* 2000. 47: 707-717.
115. Raine, CS, Cannella, B, Hauser, SL, and Genain, CP**,** Demyelination in primate autoimmune encephalomyelitis and acute multiple sclerosis lesions: a case for antigen-specific antibody mediation. *Ann.Neurol.* 1999. 46: 144-160.
116. Meinl, E, Krumbholz, M, and Hohlfeld, R**,** B lineage cells in the inflammatory

central nervous system environment: migration, maintenance, local antibody production, and therapeutic modulation. *Ann.Neurol.* 2006. 59: 880-892.

117. Fraussen, J, Vrolix, K, Martinez-Martinez, P, Losen, M, De Baets, MH, Stinissen, P et al., B cell characterization and reactivity analysis in multiple sclerosis. *Autoimmun.Rev.* 2009. 8: 654-658.
118. Menge, T, Budingen, HC, Dalakas, MC, Kieseier, BC, and Hartung, HP, [Targeting B cells in multiple sclerosis : Current concepts and strategies.]. *Nervenarzt* 2009. 80: 190-198.
119. Trapp, BD, Peterson, J, Ransohoff, RM, Rudick, R, Mork, S, and Bo, L, Axonal transection in the lesions of multiple sclerosis. *N.Engl.J.Med.* 1998. 338: 278-285.
120. Kerschensteiner, M, Gallmeier, E, Behrens, L, Leal, VV, Misgeld, T, Klinkert, WE et al., Activated human T cells, B cells, and monocytes produce brain-derived neurotrophic factor in vitro and in inflammatory brain lesions: a neuroprotective role of inflammation? *J.Exp Med.* 1999. 189: 865-870.
121. Kerschensteiner, M, Stadelmann, C, Dechant, G, Wekerle, H, and Hohlfeld, R, Neurotrophic cross-talk between the nervous and immune systems: implications for neurological diseases. *Ann.Neurol.* 2003. 53: 292-304.
122. Hohlfeld, R, Kerschensteiner, M, Stadelmann, C, Lassmann, H, and Wekerle, H, The neuroprotective effect of inflammation: implications for the therapy of multiple sclerosis. *Neurol.Sci.* 2006. 27 Suppl 1: S1-S7.
123. Hohlfeld, R, Kerschensteiner, M, and Meinl, E, Dual role of inflammation in CNS disease. *Neurology* 2007. 68: S58-S63.
124. Babbe, H, Roers, A, Waisman, A, Lassmann, H, Goebels, N, Hohlfeld, R et al., Clonal expansions of CD8(+) T cells dominate the T cell infiltrate in active multiple sclerosis lesions as shown by micromanipulation and single cell polymerase chain reaction. *J.Exp.Med.* 2000. 192: 393-404.
125. Menge, T, Weber, MS, Hemmer, B, Kieseier, BC, von Büdingen, HC, Warnke, C et al., Disease-modifying agents for multiple sclerosis : recent advances and future prospects. *Drugs* 2008. 68: 2445-2468.
126. Tompkins, SM, Fuller, KG, and Miller, SD, Theiler's virus-mediated autoimmunity: local presentation of CNS antigens and epitope spreading. *Ann.N.Y.Acad.Sci.* 2002. 958: 26-38.
127. McFarland, HI, Lobito, AA, Johnson, MM, Nyswaner, JT, Frank, JA, Palardy,

GR et al., Determinant spreading associated with demyelination in a non-human primate model of multiple sclerosis. *J.Immunol.* 1999. 162: 2384-2390.

128. Vanderlugt, CL and Miller, SD, Epitope spreading in immune-mediated diseases: implications for immunotherapy. *Nat.Rev.Immunol.* 2002. 2: 85-95.
129. Schluesener, HJ, Sobel, RA, Linington, C, and Weiner, HL, A monoclonal antibody against a myelin oligodendrocyte glycoprotein induces relapses and demyelination in central nervous system autoimmune disease. *J.Immunol.* 1987. 139: 4016-4021.
130. Linington, C, Bradl, M, Lassmann, H, Brunner, C, and Vass, K, Augmentation of demyelination in rat acute allergic encephalomyelitis by circulating mouse monoclonal antibodies directed against a myelin/oligodendrocyte glycoprotein. *Am.J.Pathol.* 1988. 130: 443-454.
131. Kabat, EA, Wolf, A, and Bezer, AL, The rapid production of acute disseminated encephalomyelitis in rhesus monkeys by injection of heterologous and homologous brain tissue with adjuvants. *J.Exp.Med.* 1947. 85: 117-129.
132. Reiber, H, Ungefehr, S, and Jacobi, C, The intrathecal, polyspecific and oligoclonal immune response in multiple sclerosis. *Mult.Scler.* 1998. 4: 111-1117.
133. Baranzini, SE, Jeong, MC, Butunoi, C, Murray, RS, Bernard, CC, and Oksenberg, JR, B cell repertoire diversity and clonal expansion in multiple sclerosis brain lesions. *J.Immunol.* 1999. 163: 5133-5144.
134. Owens, GP, Kraus, H, Burgoon, MP, Smith-Jensen, T, Devlin, ME, and Gilden, DH, Restricted use of VH4 germline segments in an acute multiple sclerosis brain. *Ann.Neurol.* 1998. 43: 236-243.
135. Qin, Y, Duquette, P, Zhang, Y, Talbot, P, Poole, R, and Antel, J, Clonal expansion and somatic hypermutation of V(H) genes of B cells from cerebrospinal fluid in multiple sclerosis. *J.Clin.Invest* 1998. 102: 1045-1050.
136. Storch, MK, Piddlesden, S, Haltia, M, Iivanainen, M, Morgan, P, and Lassmann, H, Multiple sclerosis: in situ evidence for antibody- and complement-mediated demyelination. *Ann.Neurol.* 1998. 43: 465-471.
137. Genain, CP, Cannella, B, Hauser, SL, and Raine, CS, Identification of autoantibodies associated with myelin damage in multiple sclerosis. *Nat.Med.* 1999. 5: 170-175.
138. Keegan, M, Konig, F, McClelland, R, Bruck, W, Morales, Y, Bitsch, A et al., Relation between humoral pathological changes in multiple sclerosis and

response to therapeutic plasma exchange. *Lancet* 2005. 366: 579-582.

139. Warrington, AE and Rodriguez, M**,** Remyelination-promoting human IgMs: developing a therapeutic reagent for demyelinating disease. *Curr.Top.Microbiol.Immunol.* 2008. 318: 213-239.
140. Rodriguez, M and Lennon, VA**,** Immunoglobulins promote remyelination in the central nervous system. *Ann.Neurol.* 1990. 27: 12-17.
141. Brunner, C, Lassmann, H, Waehneldt, TV, Matthieu, JM, and Linington, C**,** Differential ultrastructural localization of myelin basic protein, myelin/oligodendroglial glycoprotein, and 2',3'-cyclic nucleotide 3'-phosphodiesterase in the CNS of adult rats. *J.Neurochem.* 1989. 52: 296-304.
142. Linington, C, Engelhardt, B, Kapocs, G, and Lassman, H**,** Induction of persistently demyelinated lesions in the rat following the repeated adoptive transfer of encephalitogenic T cells and demyelinating antibody. *J.Neuroimmunol.* 1992. 40: 219-224.
143. Morris-Downes, MM, McCormack, K, Baker, D, Sivaprasad, D, Natkunarajah, J, and Amor, S**,** Encephalitogenic and immunogenic potential of myelin-associated glycoprotein (MAG), oligodendrocyte-specific glycoprotein (OSP) and 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) in ABH and SJL mice. *J.Neuroimmunol.* 2002. 122: 20-33.
144. Lyons, JA, Ramsbottom, MJ, and Cross, AH**,** Critical role of antigen-specific antibody in experimental autoimmune encephalomyelitis induced by recombinant myelin oligodendrocyte glycoprotein. *Eur.J.Immunol.* 2002. 32: 1905-13
145. Piddlesden, SJ, Lassmann, H, Zimprich, F, Morgan, BP, and Linington, C**,** The demyelinating potential of antibodies to myelin oligodendrocyte glycoprotein is related to their ability to fix complement. *Am.J.Pathol.* 1993. 143: 555-564.
146. Iglesias, A, Bauer, J, Litzenburger, T, Schubart, A, and Linington, C**,** T- and B-cell responses to myelin oligodendrocyte glycoprotein in experimental autoimmune encephalomyelitis and multiple sclerosis. *Glia* 2001. 36: 220-234.
147. Litzenburger, T, Fassler, R, Bauer, J, Lassmann, H, Linington, C, Wekerle, H et al.**,** B lymphocytes producing demyelinating autoantibodies: development and function in gene-targeted transgenic mice. *J.Exp Med.* 1998. 188: 169-80
148. Kerlero, dR, Honegger, P, Lassmann, H, and Matthieu, JM**,** Demyelination induced in aggregating brain cell cultures by a monoclonal antibody against myelin/oligodendrocyte glycoprotein. *J.Neurochem.* 1990. 55:583-587.

149. Linington, C and Lassmann, H**,** Antibody responses in chronic relapsing experimental allergic encephalomyelitis: correlation of serum demyelinating activity with antibody titre to the myelin/oligodendrocyte glycoprotein (MOG). *J.Neuroimmunol.* 1987. 17: 61-69.

150. von Büdingen, HC, Hauser, SL, Ouallet, JC, Tanuma, N, Menge, T, and Genain, CP**,** Frontline: Epitope recognition on the myelin/oligodendrocyte glycoprotein differentially influences disease phenotype and antibody effector functions in autoimmune demyelination. *Eur.J.Immunol.* 2004. 34: 2072-2083.

151. Brehm, U, Piddlesden, SJ, Gardinier, MV, and Linington, C**,** Epitope specificity of demyelinating monoclonal autoantibodies directed against the human myelin oligodendrocyte glycoprotein (MOG). *J.Neuroimmunol.* 1999. 97:9-15.

152. Mathey, E, Breithaupt, C, Schubart, AS, and Linington, C**,** Commentary: Sorting the wheat from the chaff: identifying demyelinating components of the myelin oligodendrocyte glycoprotein (MOG)-specific autoantibody repertoire. *Eur.J.Immunol.* 2004. 34: 2065-2071.

153. Marta, CB, Oliver, AR, Sweet, RA, Pfeiffer, SE, and Ruddle, NH**,** Pathogenic myelin oligodendrocyte glycoprotein antibodies recognize glycosylated epitopes and perturb oligodendrocyte physiology. *Proc.Natl.Acad.Sci.U.S.A* 2005. 102: 13992-13997.

154. Breithaupt, C, Schafer, B, Pellkofer, H, Huber, R, Linington, C, and Jacob, U**,** Demyelinating myelin oligodendrocyte glycoprotein-specific autoantibody response is focused on one dominant conformational epitope region in rodents. *J.Immunol.* 2008. 181: 1255-1263.

155. Hilton, AA, Slavin, AJ, Hilton, DJ, and Bernard, CC**,** Characterization of cDNA and genomic clones encoding human myelin oligodendrocyte glycoprotein. *J.Neurochem.* 1995. 65: 309-318.

156. Xiao, BG, Linington, C, and Link, H**,** Antibodies to myelin-oligodendrocyte glycoprotein in cerebrospinal fluid from patients with multiple sclerosis and controls. *J.Neuroimmunol.* 1991. 31: 91-96.

157. Gaertner, S, de Graaf, KL, Greve, B, and Weissert, R**,** Antibodies against glycosylated native MOG are elevated in patients with multiple sclerosis. *Neurology* 2004. 63: 2381-2383.

158. O'Connor, KC, Appel, H, Bregoli, L, Call, ME, Catz, I, Chan, JA et al.**,** Antibodies from inflamed central nervous system tissue recognize myelin

oligodendrocyte glycoprotein. *J.Immunol.* 2005. 175: 1974-1982.

159. O'Connor, KC, McLaughlin, KA, De Jager, PL, Chitnis, T, Bettelli, E, Xu, C et al., Self-antigen tetramers discriminate between myelin autoantibodies to native or denatured protein. *Nat.Med.* 2007. 13: 211-217.
160. Tejada-Simon, MV, Hong, J, Rivera, VM, and Zhang, JZ, Skewed autoantibody reactivity to the extracellular domain of myelin oligodendro-cyte glycoprotein in multiple sclerosis. *Immunology* 2002. 107: 403-410.
161. Padberg, F, Haase, CG, Feneberg, W, Schwarz, MJ, and Hampel, H, No association between anti-myelin oligodendrocyte glycoprotein antibodies and serum/cerebrospinal fluid levels of the soluble interleukin-6 receptor complex in multiple sclerosis. *Neurosci.Lett.* 2001. 305: 13-16.
162. Guggenmos, J, Schubart, AS, Ogg, S, Andersson, M, Olsson, T, Mather, IH et al., Antibody cross-reactivity between myelin oligodendrocyte glycoprotein and the milk protein butyrophilin in multiple sclerosis. *J.Immunol.* 2004. 172: 661-8
163. Wang, H, Munger, KL, Reindl, M, O'Reilly, EJ, Levin, LI, Berger, T et al., Myelin oligodendrocyte glycoprotein antibodies and multiple sclerosis in healthy young adults. *Neurology* 2008. 71: 1142-1146.
164. Vojdani, A, Vojdani, E, and Cooper, E, Antibodies to myelin basic protein, myelin oligodendrocytes peptides, alpha-beta-crystallin, lymphocyte activation and cytokine production in patients with multiple sclerosis. *J.Intern.Med.* 2003. 254: 363-374.
165. Lindert, RB, Haase, CG, Brehm, U, Linington, C, Wekerle, H, and Hohlfeld, R, Multiple sclerosis: B- and T-cell responses to the extracellular domain of the myelin oligodendrocyte glycoprotein. *Brain* 1999. 122: 2089-2100.
166. Sun, J, Link, H, Olsson, T, Xiao, BG, Andersson, G, Ekre, HP et al., T and B cell responses to myelin-oligodendrocyte glycoprotein in multiple sclerosis. *J.Immunol.* 1991. 146: 1490-1495.
167. Mazzucco, S, Mata, S, Vergelli, M, Fioresi, R, Nardi, E, Mazzanti, B et al., A synthetic glycopeptide of human myelin oligodendrocyte glycoprotein to detect antibody responses in multiple sclerosis and other neurological diseases. *Bioorg.Med.Chem.Lett.* 1999. 9: 167-172.
168. Reindl, M, Linington, C, Brehm, U, Egg, R, Dilitz, E, Deisenhammer, F et al., Antibodies against the myelin oligodendrocyte glycoprotein and the myelin basic protein in multiple sclerosis and other neurological diseases: a

comparative study. *Brain* 1999. 122 (Pt 11): 2047-2056.

169. Pittock, SJ, Reindl, M, Achenbach, S, Berger, T, Bruck, W, Konig, F et al., Myelin oligodendrocyte glycoprotein antibodies in pathologically proven multiple sclerosis: frequency, stability and clinicopathologic correlations. *Mult.Scler.* 2007. 13: 7-16.
170. Schmidt, S, Marrosu, GM, Kolsch, H, Haase, CG, Ferenczik, S, Sokolowski, P et al., Genetic variations and humoral immune responses to myelin oligodendroglia glycoprotein in adult phenotypes of X-linked adrenoleukodystrophy. *J.Neuroimmunol.* 2003. 135: 148-153.
171. Angelucci, F, Mirabella, M, Frisullo, G, Caggiula, M, Tonali, PA, and Batocchi, AP, Serum levels of anti-myelin antibodies in relapsing-remitting multiple sclerosis patients during different phases of disease activity and immunomodulatory therapy. *Dis.Markers* 2005. 21: 49-55.
172. Mantegazza, R, Cristaldini, P, Bernasconi, P, Baggi, F, Pedotti, R, Piccini, I et al., Anti-MOG autoantibodies in Italian multiple sclerosis patients: speci-ficity, sensitivity and clinical association. *Int.Immunol.* 2004. 16:559-565.
173. Bogdanos, DP, Smith, H, Ma, Y, Baum, H, Mieli-Vergani, G, and Vergani, D, A study of molecular mimicry and immunological cross-reactivity between hepa-titis B surface antigen and myelin mimics. *Clin.Dev.Immunol.* 2005. 12: 217-24
174. Haase, CG, Guggenmos, J, Brehm, U, Andersson, M, Olsson, T, Reindl, M et al., The fine specificity of the myelin oligodendrocyte glycoprotein autoantibody response in patients with multiple sclerosis and normal healthy controls. *J.Neuroimmunol.* 2001. 114: 220-225.
175. Karni, A, Bakimer-Kleiner, R, Abramsky, O, and Ben Nun, A, Elevated levels of antibody to myelin oligodendrocyte glycoprotein is not specific for patients with multiple sclerosis. *Arch.Neurol.* 1999. 56: 311-315.
176. Lampasona, V, Franciotta, D, Furlan, R, Zanaboni, S, Fazio, R, Bonifacio, E et al., Similar low frequency of anti-MOG IgG and IgM in MS patients and healthy subjects. *Neurology* 2004. 62: 2092-2094.
177. Tomassini, V, De Giglio, L, Reindl, M, Russo, P, Pestalozza, I, Pantano, P et al., Anti-myelin antibodies predict the clinical outcome after a first episode suggestive of MS. *Mult.Scler.* 2007.
178. Breij, EC, Heijnen, P, van der, GA, Teunissen, CE, Polman, CH, and Dijkstra, CD, Myelin flow cytometry assay detects enhanced levels of antibodies to

human whole myelin in a subpopulation of multiple sclerosis patients. *J.Neuroimmunol.* 2006. 176: 106-114.

179. Lalive, PH, Menge, T, Delarasse, C, Della, GB, Pham-Dinh, D, Villoslada, P et al., Antibodies to native myelin oligodendrocyte glycoprotein are serologic markers of early inflammation in multiple sclerosis. *Proc.Natl.Acad.Sci.U.S.A* 2006. 103: 2280-2285.
180. Menge, T, von Büdingen, HC, Lalive, PH, and Genain, CP, Relevant antibody subsets against MOG recognize conformational epitopes exclusively exposed in solid-phase ELISA. *Eur.J.Immunol.* 2007. 37: 3229-3239.
181. Khalil, M, Reindl, M, Lutterotti, A, Kuenz, B, Ehling, R, Gneiss, C et al., Epitope specificity of serum antibodies directed against the extracellular domain of myelin oligodendrocyte glycoprotein: Influence of relapses and immunomodulatory treatments. *J.Neuroimmunol.* 2006. 174: 147-156.
182. Langkamp, M, Hornig, SC, Hornig, JB, Kirschner, M, Pridzun, L, and Kornhuber, ME, Detection of myelin autoantibodies: evaluation of an assay system for diagnosis of multiple sclerosis in differentiation from other CNS diseases. *Clin.Chem.Lab Med.* 2009. 47: 1395-1400.
183. Chan, A, Decard, BF, Franke, C, Grummel, V, Zhou, D, Schottstedt, V et al., Serum antibodies to conformational and linear epitopes of myelin oligodendrocyte glycoprotein are not elevated in the preclinical phase of multiple sclerosis. *Mult.Scler.* 2010. 16: 1189-1192.
184. Klawiter, EC, Piccio, L, Lyons, JA, Mikesell, R, O'Connor, KC, and Cross, AH, Elevated intrathecal myelin oligodendrocyte glycoprotein antibodies in multiple sclerosis. *Arch.Neurol.* 2010. 67: 1102-1108.
185. Jaskiewicz, E, Michalowska-Wender, G, Pyszczek, A, and Wender, M, Recombinant forms of myelin antigens expressed on Chinese hamster ovary (CHO) cells as a tool for identification of autoantibodies in serum of multiple sclerosis patients. *Folia Neuropathol.* 2010. 48: 45-48.
186. Zhou, D, Srivastava, R, Nessler, S, Grummel, V, Sommer, N, Bruck, W et al., Identification of a pathogenic antibody response to native myelin oligodendrocyte glycoprotein in multiple sclerosis. *Proc.Natl.Acad.Sci.U.S.A* 2006. 103: 19057-19062.
187. Amor, S and Giovannoni, G, Antibodies to myelin oligodendrocyte glycoprotein as a biomarker in multiple sclerosis--are we there yet? *Mult.Scler.* 2007. 13:

1083-1085.

188. Breithaupt, C, Schubart, A, Zander, H, Skerra, A, Huber, R, Linington, C et al., Structural insights into the antigenicity of myelin oligodendrocyte glycoprotein. *Proc.Natl.Acad.Sci.U.S.A* 2003. 100: 9446-9451.
189. Menge, T, Büdingen, HC, Lalive, PH, and Genain, CP. Quantitative Titers of anti-MOG Antibodies Correlate to Disease Severity in Relapsing-Remitting Multiple Sclerosis. *Neurology*. 2006. 66 (Suppl 2): A311.
190. Berger, T, Rubner, P, Schautzer, F, Egg, R, Ulmer, H, Mayringer, I et al., Antimyelin antibodies as a predictor of clinically definite multiple sclerosis after a first demyelinating event. *N.Engl.J.Med.* 2003. 349: 139-145.
191. Lim, ET, Berger, T, Reindl, M, Dalton, CM, Fernando, K, Keir, G et al., Anti-myelin antibodies do not allow earlier diagnosis of multiple sclerosis. *Mult.Scler.* 2005. 11: 492-494.
192. Rauer, S, Euler, B, Reindl, M, and Berger, T, Antimyelin antibodies and the risk of relapse in patients with a primary demyelinating event. *J.Neurol.Neurosurg.Psychiatry* 2006. 77: 739-742.
193. Kuhle, J, Pohl, C, Mehling, M, Edan, G, Freedman, MS, Hartung, HP et al., Lack of association between antimyelin antibodies and progression to multiple sclerosis. *N.Engl.J.Med.* 2007. 356: 371-378.
194. Pelayo, R, Tintore, M, Montalban, X, Rovira, A, Espejo, C, Reindl, M et al., Antimyelin antibodies with no progression to multiple sclerosis. *N.Engl.J.Med.* 2007. 356: 426-428.
195. Kuhle, J, Lindberg, RL, Regeniter, A, Mehling, M, Hoffmann, F, Reindl, M et al., Antimyelin antibodies in clinically isolated syndromes correlate with inflammation in MRI and CSF. *J.Neurol.* 2007.
196. Greeve, I, Sellner, J, Lauterburg, T, Walker, U, Rosler, KM, and Mattle, HP, Anti-myelin antibodies in clinically isolated syndrome indicate the risk of multiple sclerosis in a Swiss cohort. *Acta Neurol.Scand.* 2007. 116:207-10.
197. Menge, T, Lalive, PH, Tintore, M, von Büdingen, HC, Villoslada, P, and Genain, CP. Quantitative anti-MOG Serumtiter sind nicht prädiktiv für Krankheitsaktivität und -progression beim ersten Ereignis der Multiplen Sklerose - Ergebnisse einer ELISA-Studie. Akt Neurol. 2007. 34 (Suppl. 2).
198. Brilot, F, Dale, RC, Selter, RC, Grummel, V, Kalluri, SR, Aslam, M et al., Antibodies to native myelin oligodendrocyte glycoprotein in children with

inflammatory demyelinating central nervous system disease. *Ann.Neurol.* 2009. 66: 833-842.

199. McLaughlin, KA, Chitnis, T, Newcombe, J, Franz, B, Kennedy, J, McArdel, S et al., Age-dependent B cell autoimmunity to a myelin surface antigen in pediatric multiple sclerosis. *J.Immunol.* 2009. 183: 4067-4076.
200. Selter, RC, Brilot, F, Grummel, V, Kraus, V, Cepok, S, Dale, RC et al., Antibody responses to EBV and native MOG in pediatric inflammatory demyelinating CNS diseases. *Neurology* 2010. 74: 1711-5.
201. Lalive, PH, Häusler, MG, Maurey, H, Mikaeloff, Y, Tardieu, M, Wiendl, H et al., Highly reactive anti-MOG antibodies differentiate demyelinating diseases from viral encephalitis in children. *Mult.Scler.* 2011. Im Druck
202. von Büdingen, HC, Hauser, SL, Fuhrmann, A, Nabavi, CB, Lee, JI, and Genain, CP, Molecular characterization of antibody specificities against myelin/oligodendrocyte glycoprotein in autoimmune demyelination. *Proc.Natl.Acad.Sci.U.S.A* 2002. 99: 8207-8212.
203. Menge, T, Lalive, PH, von Büdingen, HC, Cree, B, Hauser, SL, and Genain, CP, Antibody responses against galactocerebroside are potential stage-specific biomarkers in multiple sclerosis. *J.Allergy Clin.Immunol.* 2005. 116: 453-459.
204. Beckman, EM, Porcelli, SA, Morita, CT, Behar, SM, Furlong, ST, and Brenner, MB, Recognition of a lipid antigen by CD1-restricted alpha beta+ T cells. *Nature* 1994. 372: 691-694.

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Originalarbeiten

Im folgenden angehängt finden sich die fünf hier diskutierten Originalarbeiten:

1. von Büdingen HC, Hauser SL, Ouallet JC, Tanuma N, Menge T, Genain CP: Epitope recognition on the myelin/oligodendrocyte glycoprotein differentially influences disease phenotype and antibody eff.ector functions in autoimmune demyelination. *Eur. J. Immunol.* 2004;34:2072-83

2. Menge T, von Büdingen HC, Lalive PH, Genain CP: Relevant antibody subsets against MOG recognize conformational epitopes exclusively exposed in solid-phase ELISA. *Eur J Immunol.* 2007;37:3229-39.

3. Lalive PH, Menge T, Delarasse C, Della Gaspera B, Pham-Dinh D, Villoslada P, von Büdingen HC, Genain CP: Antibodies to native myelin oligodendrocyte glycoprotein are serologic markers of early inflammation in multiple sclerosis. *Proc Natl Acad Sci U S A.* 2006; 103:2280-5.

4. Lalive PH, Häusler MG, Maurey H, Mikaeloff Y, Tardieu M, Wiendl H, Schroeter M, Hartung HP, Kieseier BC, Menge T: Highly reactive anti-MOG antibodies differentiate demyelinating diseases from viral encephalitis in children. *Mult Scler.*, im Druck.

5. Menge T, Lalive PH, von Büdingen HC, Cree B, Hauser SL, Genain CP: Antibody responses against galactocerebroside are potential stage-specific biomarkers in multiple sclerosis. *J Allergy Clin Immunol.* 2005;116:453-9.

Frontline:

Epitope recognition on the myelin/oligodendrocyte glycoprotein differentially influences disease phenotype and antibody effector functions in autoimmune demyelination

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Preliminary observations of humoral immunity against the myelin oligodendrocyte glycoprotein (MOG) in experimental allergic encephalomyelitis (EAE) and human multiple sclerosis (MS) suggest that a subset of anti-MOG autoantibodies directed against conformational epitopes is of pathogenic predominance. Here, we provide proof that in marmoset EAE, autoantibodies reactive against conformational epitopes of MOG are not only responsible for aggravating demyelination, but also an essential factor for disease dissemination in space within the central nervous system, a hallmark for typical forms of human MS. In terms of effector mechanisms, IgG deposition and complement activation occur exclusively in association with presence of these conformational antibodies, while microglial/macrophage activation appears to be a common immunopathological finding regardless of the fine determinant specificity of anti-MOG antibodies. These findings highlight for the first time the complex heterogeneity of function and pathogenicity in the polyclonal anti-MOG antibody repertoire of outbred species. Because the linear and conformational antibody determinants of MOG are shared between marmosets and humans, these results are directly relevant to understanding effector mechanisms of organ damage in MS.

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1 Introduction

Myelin oligodendrocyte glycoprotein (MOG)-induced experimental allergic encephalomyelitis (EAE) in the common marmoset (*Callithrix jacchus)* is a multifocal disease of central nervous system (CNS) white matter that closely approximates human multiple sclerosis (MS) [1, 2]. 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 [3]. Not unexpectedly, a broader heterogeneity of epitopes within MOG antibody responses is found in primates compared to rodents (reviewed in [4]). Although limited preliminary observations suggest that epitope recognition may underlie differences in antibody effector functions in humans [5], these relationships remain largely unexplored.

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Abbreviations: MOG: Myelin oligodendrocyte glycoprotein **CNS:** Central nervous system **MS:** Multiple sclerosis **$rMOG_{1–125}$:** Recombinant extracellular domain of MOG **pep-MOG:** Mixture of 20mer peptides spanning the extracellular domain of MOG **Anti-MOG-P:** Antibodies against peptide epitopes of MOG **Anti-MOG-C:** Antibodies against conformational epitopes of MOG **aa:** Amino acids **MPB:** Myelin basic protein

Structurally, antibodies against MOG can be differentiated on the basis of their ability to recognize either linear or conformational, tertiary structure-dependent epitopes [6]. Standard ELISA methods cannot separately detect antibody reactivity against conformational and linear epitopes of MOG, thus pathogenic properties and effector functions of these different antibody populations cannot be understood unless they are isolated and separately

studied. Such information is needed in order to properly interpret anti-MOG antibody reactivity in MS, which has been reported with varying frequencies depending on the study, the method of detection and the protein used [7–14].

Cloning of 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 human antibody repertoires [6]. Here, we have taken advantage of this model system to characterize the immunopathogenicity of anti-MOG antibodies according to their epitope recognition. Using combinations of active immunizations and passive antibody transfers of affinity-purified antibody fractions in combination with immunohistochemical analysis, we found evidence that both linear and conformation-dependent anti-MOG antibodies are potentially pathogenic by contributing to macrophage recruitment and activation in EAE lesions. However, conformation-dependent antibodies appear to be required for dissemination of CNS pathology and complement deposition in lesions. These results have important implications for interpretation of anti-MOG antibody reactivity in health and disease.

2 Results

2.1 Clinical and neuropathological characteristics of EAE induced with MOG peptides or $rMOG_{1–125}$

Table 1 recapitulates the clinical and neuropathological phenotypes of EAE induced with either the recombinant extracellular domain of MOG ($rMOG_{1–125}$; group I) or linear MOG peptides (group II). The course of MOG peptide-induced EAE tended to be progressive, with more rapid progression in the two animals immunized with a mixture of 20mer peptides spanning the extracellular domain of MOG (pepMOG; animals 39–95 and 65–92). $rMOG_{1–125}$-induced EAE was either rapidly progressive or relapsing-remitting (not shown), 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 ($rMOG_{1–125}$-immune) and 39–95 (pepMOG-immune) developed hyperacute EAE symptoms requiring immediate euthanasia.

Neuropathologically, the first remarkable difference between animals in the two immunization groups was a tremendously reduced white matter lesion burden in group II (group I: 163±56.3 lesions; group II:

Table 1. Characteristics of EAE in groups I ($rMOG_{1–125}$-immune, gray shading) and II (MOG peptide-immune) marmosets[a)]

Animal ID	Immunogen	Max. clinical score	No. of lesions	Inflammation	Demyelination
U004–99	$rMOG_{1–125}$	14	326	+++	+++
U009–99	$rMOG_{1–125}$	8	135	+++	+++
Cj72–88	$rMOG_{1–125}$	5.5	67	+++	+++
J2–97	$rMOG_{1–125}$	29	124	+++	+++
199–94	aa 21–40	8	4	+++	+
368–94	aa 21–40	10	4	++	+
39–95	pepMOG	19	4	+++	+
65–92	pepMOG	9	33	++	+[b)]
252–93	aa 1–40	5	6	++	+
TX245–90	aa 1–40	10	8	+++	+
14–91	aa 21–40, 51–90	12	8	+	+
TX75–92	aa 51–90	10	13	++	+
Tx256–93	aa 81–120	9	3	+	+

a) pepMOG denotes a mixture of 11 20mer peptides overlapping by ten aa and spanning the sequence of MOG aa 1–120.
b) Demyelination was found with the grade indicated in all lesions except in animal 65–92, in which only 18 of 33 (55%) lesions were demyelinated.

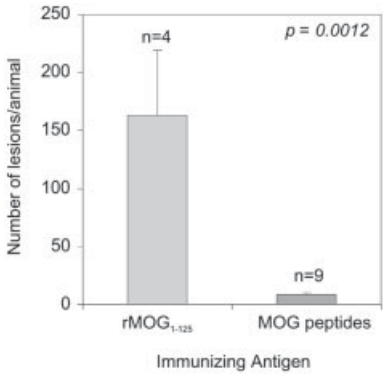

Fig. 1. Average CNS lesion load in $rMOG_{1–125}$- and MOG peptide-immunized marmosets; means ± SEM.

9.2±3.1 lesions; mean ± SEM, p=0.0012; Table 1, Fig. 1). Second, in contrast with the multifocal disease that we and others have consistently observed in many $rMOG_{1–125}$-immune marmosets (typically involving optic nerves, spinal cord, brain hemispheres, and brainstem with perivascular intraparenchymal distribution [1]), 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 [15, 16] (Fig. 2). 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 (animal 65–92), none of which showed evidence of demyelination (not shown). The third difference was that the extent of demyelinated areas was reduced in lesions of MOG peptide-induced EAE compared with those of $rMOG_{1–125}$-induced EAE, in the presence of roughly similar degrees of inflammation (Table 1). 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 $rMOG_{1–125}$-induced EAE (Fig. 2). The most abundant pattern of demyelination in lesions of MOG peptide-induced EAE was myelin vacuolation (Fig. 2A), a feature that is present at the periphery of expanding lesions in $rMOG_{1–125}$-induced marmoset EAE [17] (Fig. 2B).

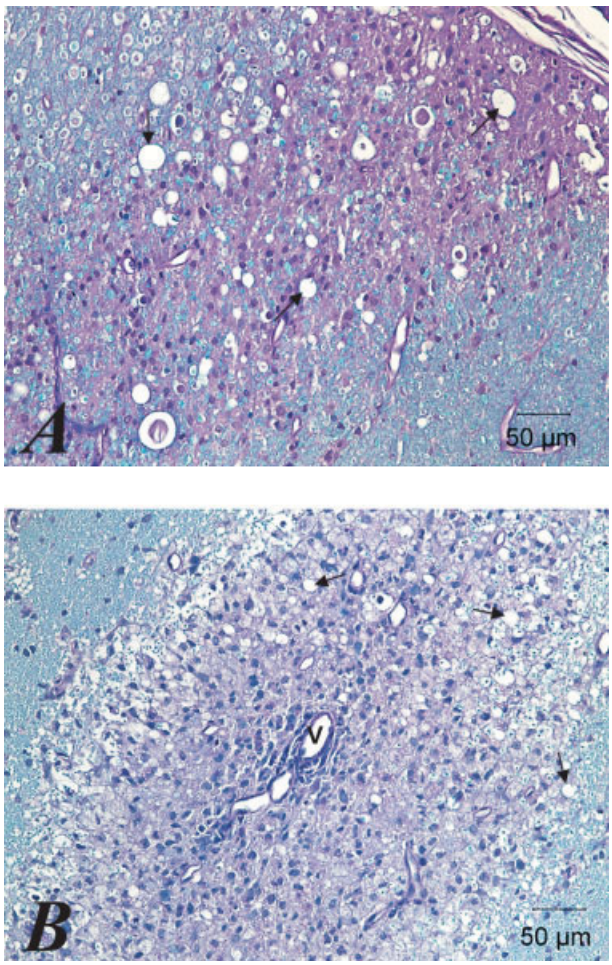

Fig. 2. Neuropathology of $rMOG_{1–125}$- and MOG peptide-induced EAE. High-power views (×200) of cervical spinal cord sections stained with LFB. (A) Typical inflammatory infiltrate in a marmoset immunized with MOG aa 21–40 (368–94). Note contiguity with the subpial space (upper right corner) and the limited amount of demyelination. (B) Perivascular, inflammatory infiltrate in deep periventricular white matter (V = blood vessel) with pronounced concentric demyelination, characteristic of $rMOG_{1–125}$-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 $rMOG_{1–125}$-induced EAE (B).

2.2 Epitope specificities of antibody responses

All monkeys developed serum antibodies that reacted to both $rMOG_{1–125}$ and linear peptides as shown by standard ELISA of unfractionated serum (Fig. 3, left panels). To separately characterize conformational and linear specificities, the following selected sera were depleted from pepMOG-reactive antibodies: group I ($rMOG_{1–125}$-immune): J2–97, 72–88, U004–99, U009–99; group II: pepMOG-immune: 39–95, 65–92; MOG amino acids (aa) 21–40-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 Fig. 3.

Complete depletion of sera from the MOG peptide-reactive antibody fraction (anti-MOG-P) was achieved

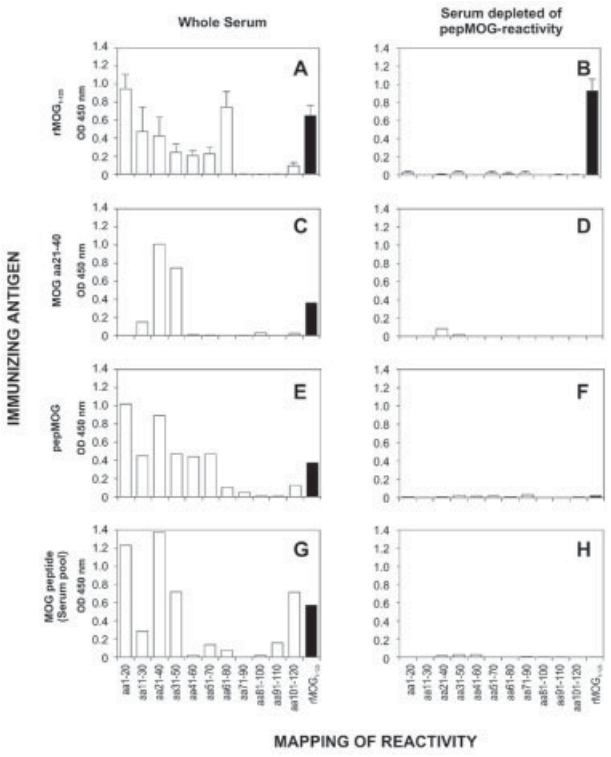

Fig. 3. Fine specificities of unfractionated sera and anti-MOG-P-depleted sera from representative animals of groups I and II. The left panels show reactivity of whole sera at a dilution of 1:200. The right panels show residual reactivity after removal of anti-MOG-P antibodies by affinity chromatography. (A, B) Antibody specificities in $rMOG_{1–125}$-immunized monkeys (n=4, means ± SEM), demonstrating that strong reactivity against $rMOG_{1–125}$ is retained after removal of all MOG peptide-specific antibodies. (C–F) Representative experiments for individual animals immunized with individual or all MOG-derived peptides (aa 21–40, 199–94; pepMOG, 39–95). (G, 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, B), $rMOG_{1–125}$-immune animals.

after three to five passes over the pepMOG columns, as shown by the lack of binding to individual peptides (Fig. 3, right panels). Depletion from anti-MOG-P resulted in complete loss of reactivity to $rMOG_{1–125}$ in each of the animals immunized with MOG peptides (Fig. 3D, F, H), regardless of the sequence of the immunizing peptides. By contrast, sera from $rMOG_{1–125}$-immune animals always retained reactivity against whole $rMOG_{1–125}$ after being depleted from anti-MOG-P antibodies (Fig. 3B).

Anti-MOG-P and/or the fraction of antibodies reactive against conformation-dependent epitopes of MOG (anti-MOG-C) 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 $rMOG_{1–125}$ *in vitro* in the ELISA system (not shown). 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. This was observed regardless of the immunization regimen used to produce anti-MOG-P antibodies (*e.g.* $rMOG_{1–125}$ or MOG peptides) (Fig. 4). No significant reactivity to either recombinant glutathione S-transferase (GST) expressed in *Escherichia coli* or the $(His)_6$ C-terminal peptide was detected in any of the antibody fractions (not shown). 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 $rMOG_{1–125}$ or against bacterial contaminants in the $rMOG_{1–125}$ preparation used to synthesize the chromatography columns.

2.3 MOG-specific T cell proliferative responses

Circulating T cell proliferative responses to $rMOG_{1–125}$ were observed in peripheral blood mononuclear cells (PBMC) of all animals at euthanasia. The magnitude of these responses was similar in MOG peptide-immune animals and $rMOG_{1–125}$-immune animals (10±3.1 *vs.* 12.7±5.8; not significant; Fig. 5). T cell proliferative responses mapped to 20mer peptides corresponding either to the immunodominant T cell epitopes in $rMOG_{1–125}$-immune marmosets [4] or to the immunizing peptide(s) in MOG peptide-immune animals (not shown).

2.4 Immunohistochemical characterization of lesions

Results of immunostaining are summarized in Fig. 6, 7. Macrophage infiltration was a consistent feature of inflammatory infiltrates in all animals, as indicated by staining for HAM56 (Fig. 6A, B). Pronounced IgG deposition was found in $rMOG_{1–125}$-immune animals, either in

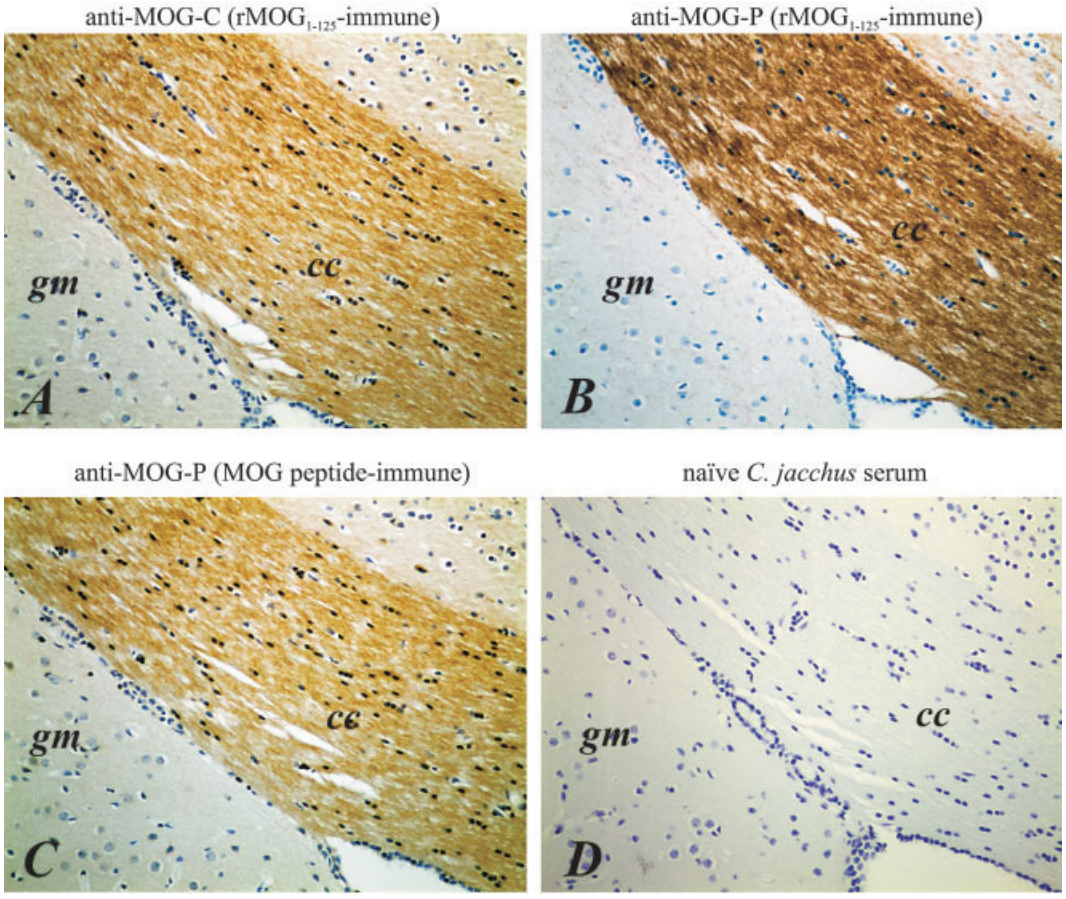

Fig. 4. Reactivity of affinity-purified anti-MOG antibody fractions with native MOG. Immunohistochemical staining of normal corpus callosum (cc) from an unimmunized *C. jacchus*. (A, B) Anti-MOG-C and anti-MOG-P from an $rMOG_{1–125}$-immune serum pool; (C) anti-MOG-P from a MOG peptide-immune serum pool; (D) naive *C. jacchus* serum; gm, gray matter.

the immediate perivascular vicinity or deeper within the white matter parenchyma (Fig. 6C). In sharp contrast, lesions that showed IgG deposition were observed in only two animals immunized with MOG peptides (39–95 and one in 252–93). This involved a single hemorrhagic lesion in both cases (not shown), which raises the possibility that this was the result of exudation 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 $rMOG_{1–125}$-immune animals (B cells or plasmocytes; Fig. 6C). Some of the lesions found in MOG peptide-immune animals also showed IgG-positive cells, though much less frequently (not shown). Quantitatively, the differences in IgG deposition and IgG-positive cells between $rMOG_{1–125}$- and MOG peptide-immune animals were significant (p=0.003 and p=0.038, respectively; Fig. 7). Highly significant differences were also observed for C9neo deposition, which was prominently observed in $rMOG_{1–125}$-immune animals (56% positive of 204 lesions analyzed), but was uniformly absent from any of the 83 lesions analyzed in MOG peptide-immune animals (0%; $p<0.0001$; Fig. 6E, F; 7).

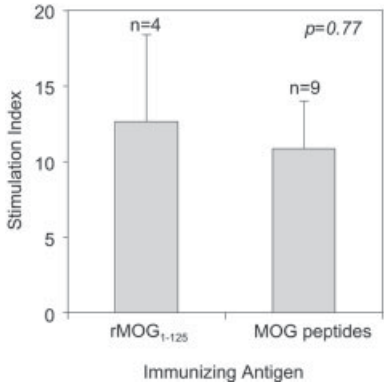

Fig. 5. T cell proliferation against $rMOG_{1–125}$ in $rMOG_{1–125}$- and MOG peptide-immune animals; means ± SEM.

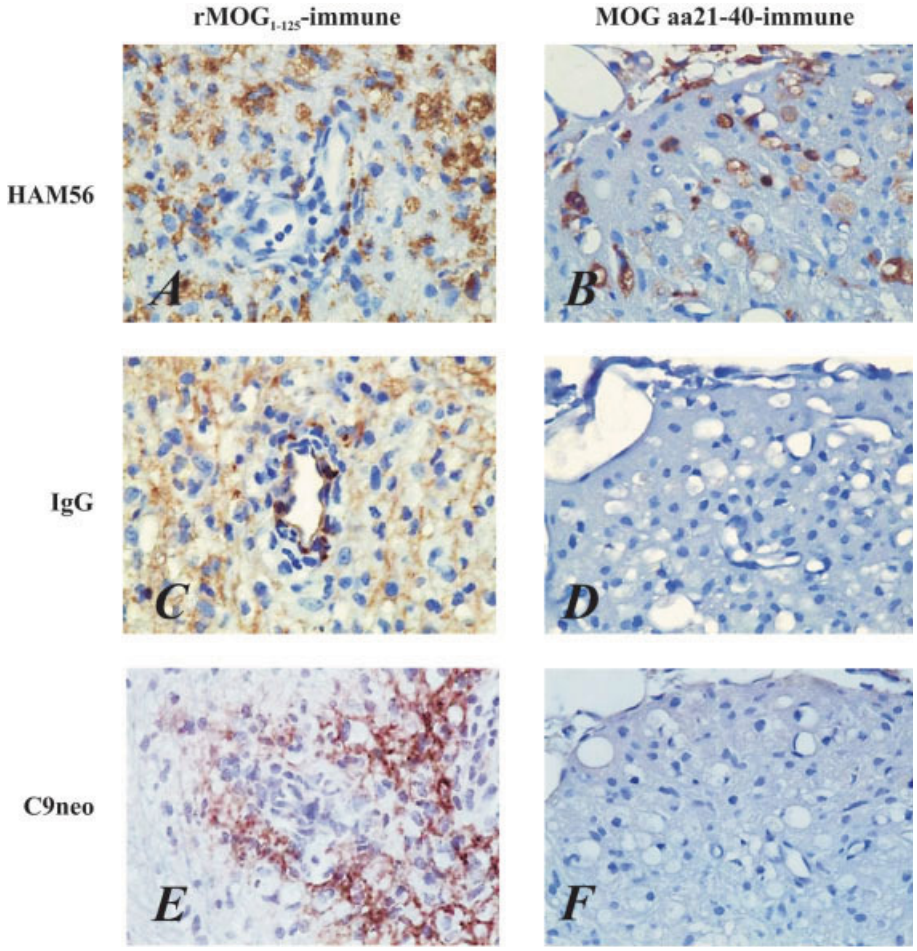

Fig. 6. Immunohistochemical characterization of CNS lesions. Representative lesions from an $rMOG_{1–125}$-immunized animal (J2–97, left) and an animal immunized with MOG aa 21–40 (199–94, right). From top to bottom: staining for macrophages (HAM56; A, B); IgG (C, D); C9neo (E, F). Activation of complement (C9neo) was a characteristic of $rMOG_{1–125}$-induced EAE and was not found in MOG peptide-immune animals. Original magnification 600×.

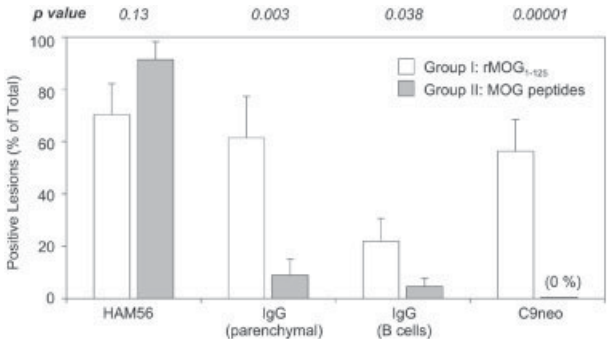

Fig. 7. Quantitative analysis of HAM56, IgG and C9neo in CNS lesions. Values on y axis represent the percentages of positive lesions for each marker ($rMOG_{1–125}$-immune animals: 204 lesions, open bars; MOG peptide-immune animals: all visible lesions (n=83), closed bars; means ± SEM).

2.5 Anti-MOG antibody transfer experiments in myelin basic protein-immunized animals

All animals developed an immune response against myelin basic protein (MBP) as assayed by ELISA (not shown); however, none of the animals showed obvious signs of clinical EAE during the observation period. Neuropathologically, the three groups markedly differed: animals that received anti-MOG-P/C (U015–99 and 437–99) developed 135 and 35 inflammatory lesions, respectively (Fig. 8A, B; Table 2), mostly located in the cerebral white matter, with pronounced demyelination, highly similar to lesions found in $rMOG_{1–125}$-immune animals; animals that received anti-MOG-P (U026–00 and U029–00) developed 35 and 1 inflammatory lesions, respectively. All lesions in the latter animals showed significantly less demyelination and were characterized mostly by perivascular cuffing with mononuclear cells (Fig. 8C, D;

Table 2. Passive antibody transfer of fractionated anti-MOG antibodies in MBP-immunized marmosets

Animal ID	Antibody transferred	No. of CNS lesions	Demyelination
437–99	Anti-MOG-P/C	135	+++
U015–99	Anti-MOG-P/C	35	++
U026–00	Anti-MOG-P	35	+
U029–00	Anti-MOG-P	1	+
118–99	Naive Igκ	0	–
335–97	Naive Igκ	0	–

Table 2). Most of the lesions in animal U026–00 were located in the cerebral hemispheres and were reminiscent of the lesions found in the pepMOG-immunized animal 65–92 (not shown), whereas the 1 lesion found in animal U029–00 was located in the brain stem and thus shared an important characteristic with most of the MOG peptide-immunized animals; no lesion was detectable in the CNS of either animal that received naive *C. jacchus* Igκ (not shown).

3 Discussion

Studies of the murine anti-MOG antibody repertoire [18–22] and recent crystallization studies of MOG/anti-MOG antibody complexes [23, 24] indirectly suggest that recognition of conformational determinants of MOG may be an important requirement for pathogenicity. However, according to some [25] but not all [26] investigations, Lewis rats immunized with MOG aa 35–55 can develop multifocal demyelinating disease despite the demonstration that these animals do not develop conformation-dependent anti-MOG antibodies. This implies that MOG peptide-specific antibodies may be pathogenic in the rat, as seems to be the case in several mouse strains [27].

A role for humoral mechanisms of demyelination in human MS is even less clear than in EAE, although suggested by the presence of intrathecal Ig synthesis, clonal expansion of B cells, and complement activation (reviewed in [28]). 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

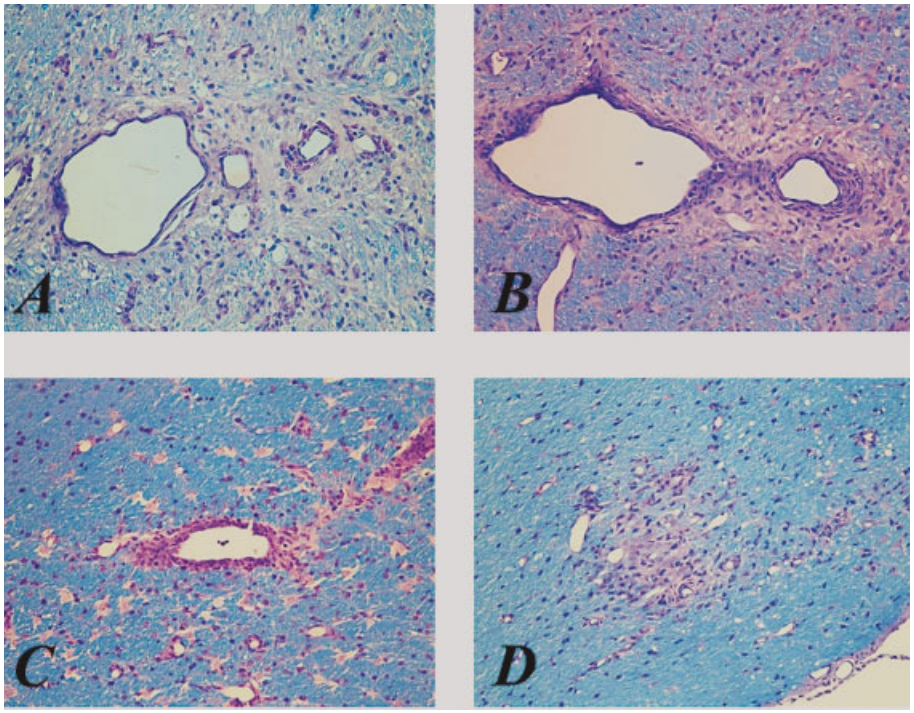

Fig. 8. Neuropathology of MBP-immunized animals after passive transfer of anti-MOG antibodies. Representative CNS lesions in animals that received anti-MOG antibodies reactive against both conformational and linear epitopes [anti-MOG-P/C; (A) 437–99, (B) U015–99], and in animals receiving MOG peptide-specific anti-MOG antibodies [anti-MOG-P; (C) U026–00, (D) U029–00]. All antibody fractions were derived from $rMOG_{1–125}$-immune serum. LFB stains, magnification 200×.

remains largely unknown. The concomitant presence in MS serum of pathogenic and non-pathogenic antibodies that cannot easily be distinguished one from another may be responsible for the lack of clear correlation between antibody presence and disease phenotype that has so far been reported. The current studies systematically investigated functional properties of anti-MOG antibodies according to epitope recognition in outbred marmosets, an EAE model where the diversity of humoral responses parallels that encountered in MS, and include determinants of MOG commonly found in humans [4, 6]. We show that marmosets develop radically different forms of EAE after immunization with either MOG peptides or $rMOG_{1–125}$, and that the EAE phenotype correlates with the presence or absence of a conformation dependent anti-MOG antibody response.

It is important to note that the differences between $rMOG_{1–125}$- and MOG peptide-induced EAE predominantly involved patterns of disease dissemination and demyelination, and not severity of EAE. Some animals in both groups developed either severe, rapidly progressive disease or mild to moderate forms, as can be expected in this outbred species. However, MOG peptide-immunized animals showed reduced, albeit significant demyelination compared to $rMOG_{1–125}$-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 $rMOG_{1–125}$-immunization [1]. This pattern of pathology was consistent in all MOG peptide-induced EAE cases regardless of the choice of immunizing peptide within the sequence of 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 disease and prominent demyelination.

A major finding of the current study is the clear demonstration of a link between antibody determinant recognition and density and distribution of CNS lesions. The presence of 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. The subpial localization of demyelinating infiltrates in MOG peptide-immunized marmosets is also strikingly similar to CNS pathology observed in C57BL/6 mice immunized with MOG aa 35–55 [16, 29]. Possible biological explanations for these differences include differential binding affinity or, as discussed below, different effector mechanisms activated by 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.

Our findings in actively induced EAE were fully corroborated by passive antibody transfer experiments in MBP-immunized marmosets, which develop non-demyelinating antibody responses [3]. MBP-immune animals that received only anti-MOG-P did not develop widespread demyelinating disease unlike those that received preparations containing the antibodies reactive against conformational MOG epitopes. We conclude that the latter fraction is responsible for the typical MS-like phenotype induced by immunization with $rMOG_{1–125}$, in partial agreement with studies of mice lacking B cells which fail to develop severe EAE after immunization with $rMOG_{1–120}$, but do so after passive transfer of whole serum from $rMOG_{1–120}$-immune wild-type mice [30].

However, the marmoset experiments demonstrate that anti-MOG-P antibodies may also be pathogenic, because MBP-immune animals that received these antibodies showed significantly more pathology than animals that received naive marmoset Igκ. Indeed, certain similarities were observed between MOG peptide- and $rMOG_{1–125}$-induced EAE. The lesion pattern observed in the former includes evidence of myelin vacuolation, which is also present at the edge of lesions in $rMOG_{1–125}$-induced EAE [2, 17]. 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 [31], and could also be an effect of MOG-specific antibodies. Antibodies specific for MOG aa 21–40 have been detected in close association with disintegrating myelin membranes in lesions of $rMOG_{1–125}$-induced marmoset EAE, and in those of human MS [2]. 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, as could be the case when naturally occurring MOG dimers are being disrupted [24].

The recognition of structural epitopes of MOG could not only influence antibody binding *in vivo* but also result in different effector mechanisms for antibody pathogenicity. While it was noteworthy that macrophage infiltration and activation was present to a similar extent in both MOG peptide- and $rMOG_{1–125}$-induced EAE, consistent deposition of IgG and the C9 component of the complement lytic complex was only detected in $rMOG_{1–125}$-immunized animals, and was absent from lesions of MOG peptide-induced EAE. This suggests that anti-

bodies 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. Studies of EAE [18, 32] and MS [33–36] 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 in primates is thus of essential importance.

In summary, we present here a comprehensive analysis of functional heterogeneity of the MOG-specific antibody repertoire in an outbred species that shares complexity and similar structural epitopes with humans. We formally demonstrate for the first time that antibody responses against a single target antigen of myelin can be sharply dichotomized in terms of pathogenic and functional properties, and that antibody epitope recognition is a critical factor for phenotypic expression of CNS autoimmunity. 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.

4 Materials and methods

4.1 Antigens

A recombinant protein corresponding to the sequence of the extracellular domain of rat MOG ($rMOG_{1–125}$) was expressed and purified to homogeneity as fusion protein with a His_6-Tag in *E. coli* following published procedures [37]. Eleven synthetic overlapping linear 20mer peptides corresponding to the sequence of rat MOG aa 1–120, and the C-terminal peptide of rMOG1–125 (WINPGRSRSHHHHHH) were synthesized using standard solid phase chemistry (Research Genetics, Huntsville, AL) and purified >95% by HPLC. Purity was confirmed by mass spectrometry. Human MBP was purified from brain as previously described [38].

4.2 Animals, immunization and characterization of EAE

C. jacchus marmosets used in this study were cared for in accordance with all guidelines of the Institutional Animal Care and Usage Committee. Marmosets were actively immunized with either 50 μg of $rMOG_{1–125}$ (group I) or 100 μg of MOG-derived 20mer peptides (group II, individual peptides or combinations, please also refer to Table 1) dissolved in phosphate-buffered saline (PBS) and emulsified with complete Freund's adjuvant (CFA) as previously described [3]. The peptides or combinations of peptides were selected according to previous mapping studies that have characterized the immunodominant T cell and antibody epitopes of $rMOG_{1–125}$ in marmosets [4, 39].

EAE was assessed by daily clinical examination and animals were observed for a total of 12–140 days (marmoset expanded scale, score 0 to 45 [40]). At the end of the observation period, euthanasia was performed under deep pentobarbital anesthesia by intracardial perfusion with 4% paraformaldehyde, and the entire neuraxis obtained and examined in serial consecutive sections (2 mm each). Five-micrometer, paraffin-embedded sections were stained with luxol fast blue /periodic acid-Schiff (LFB/PAS) or used for immunohistochemical analysis. Pathological findings were graded according to separate inflammation and demyelination scores. Inflammation score: 0, no inflammation present; +, rare (1–3) inflammatory infiltrates/average whole section; ++, moderate numbers (3–10) 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) demyelination; +++, extensive demyelination with large confluent lesions.

4.3 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 MOG aa 1–120 (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 buffer (pH 8.0) and extensively dialyzed against PBS. Thus, in these experiments antibody reactivity found in follow-through fractions (if present) could not represent any epitope of MOG directed against a linear feature, and was considered to represent anti-MOG-C.

The binding characteristics of all eluted and follow-through fractions were analyzed by ELISA. Anti-MOG-C, if present, were further affinity-purified by passing pepMOG column follow-through fractions over Sepharose columns containing covalently linked $rMOG_{1–125,}$ 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 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.

4.4 Epitope specificity

Epitope specificities of whole unfractionated sera, fractionated sera or affinity-purified antibodies were determined by ELISA. Plastic wells (maleic anhydride plates; Pierce) were coated with $rMOG_{1-125}$ or MOG-derived 20mer peptides. Control wells contained no antigen, the recombinant GST from *E. coli*, and the $(His)_6$ C-terminal peptide of $rMOG_{1-125}$. Wells were blocked with PBS containing 0.05% Tween-20 (PBS-T) and 3% bovine serum albumin, and the following samples were added in blocking buffer and incubated for 1 h at 37°C: (1) whole immune serum, 1:200; (2) 3 μg/ml of affinity-purified anti-MOG-P antibodies; (3) group I ($rMOG_{1-125}$-), 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, St. Louis, MO) was added, and after incubation for 1 h, wells were developed with tetramethylbenzidine (Pierce) and read at 450 nm.

4.5 Immunohistochemistry

Sections of *C. jacchus* brain were deparaffinized, hydrated and treated with a citrate-based antigen-unmasking solution (Vector Labs, Burlingame, CA) at high temperature for 20 min. Endogenous peroxidase activity was blocked by incubation of sections in 0.3% H_2O_2 in methanol for 30 min. Sections were blocked with 5% normal goat serum (Sigma) in PBS-T for 1 h 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; (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 h at 37°C and washes with PBS-T, the appropriate biotinylated secondary antibodies were applied and incubated for another hour at 37°C [rabbit anti-mouse 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 (Vector). All slides were counterstained with hematoxylin and permanently mounted. Biotinylated anti-MOG-P (from $rMOG_{1-125}$- and MOG peptide-immune animals, 7 μg/ml and 20 μg/ml, respectively) and anti-MOG-C (10 μg/ml) were used to measure their ability to bind to native, full-length MOG expressed *in situ* by oligodendrocytes in marmoset CNS.

4.6 T cell proliferative responses

Antigen-specific T cell proliferative responses were measured in PBMC obtained at euthanasia by centrifugation, using a standard 3-day [^{3}H]thymidine incorporation assay with 10 μg/ml of $rMOG_{1-125}$ or MOG-derived peptides. Stimulation indices were calculated as the ratio of counts in stimulated/control (no antigen) wells.

4.7 Passive antibody transfers

A total of six animals were immunized with 2 mg MBP emulsified in CFA as described previously [3], and after the development of MBP-specific antibody responses (37–43 days post-immunization, determined by ELISA) received by i.v. injection the following antibody preparations: (1) the whole rMOG-reactive antibody fraction from rMOG-immune *C. jacchus* sera obtained by immuno-affinity chromatography over rMOG columns as described above (anti-rMOG-P/C, 250 μg/animal, *n*=2); (2) the peptide-reactive fraction from rMOG-immune sera obtained by affinity purification over MOG peptide columns (anti-MOG-P, 125 μg/animal, *n*=2); (3) Igκ from naive marmoset sera obtained by affinity purification over protein L columns (protein L binds to κ light chains of all antibody isotypes) (naive antibodies, 153–180 μg/animal, *n*=2). All animals were euthanized 2–7 days after the passive antibody transfer and their tissue processed for histology as described above. Persistence of anti-MOG antibodies in peripheral blood was confirmed by ELISA (data not shown).

4.8 Statistics

The following 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 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, > ten stained cells/lesion; IgG (cellular distribution, B cells), > two 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, unpaired Student's *t*-test.

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References

1 **Genain, C. and Hauser, S.,** Experimental allergic encephalomyelitis in the common marmoset C. jacchus. *Immunol. Rev.* 2001. **183:** 159–172.

2 **Genain, C., Cannella, B., Hauser, S. and Raine, C.,** Identification of autoantibodies associated with myelin damage in multiple sclerosis. *Nat. Med.* 1999. **5:** 170–175.

3 **Genain, C. P., Nguyen, M. H., Letvin, N. L., Pearl, R., Davis, R. L., Adelman, M., Lees, M. B., Linington, C. and Hauser, S. L.,** Antibody facilitation of multiple sclerosis-like lesions in a non human primate. *J. Clin. Invest.* 1995. **96:** 2966–2974.

4 **von Büdingen, H.-C., Tanuma, N., Villoslada, P., Ouallet, J.-C., Hauser, S. and Genain, C.,** Immune responses against the myelin/oligodendrocyte glycoprotein in experimental autoimmune demyelination. *J. Clin. Immunol.* 2001. **21:** 155–170.

5 **Haase, C. G., Guggenmos, J., Brehm, U., Andersson, M., Olsson, T., Reindl, M., Schneidewind, J. M., Zettl, U. K., Heidenreich, F., Berger, T., Wekerle, H., Hohlfeld, R. and Linington, C.,** The fine specificity of the myelin oligodendrocyte glycoprotein autoantibody response in patients with multiple sclerosis and normal healthy controls. *J. Neuroimmunol.* 2001. **114:** 220–225.

6 **von Büdingen, H.-C., Hauser, S., Fuhrmann, A., Nabavi, C., Lee, J. and Genain, C.,** Molecular characterization of antibody specificities against myelin oligodendrocyte glycoprotein in autoimmune demyelination. *Proc. Natl. Acad. Sci. USA* 2002. **99:** 8207–8212.

7 **Sun, J., Link, H., Olsson, T., Xiao, B. G., Andersson, G., Ekre, H.-P., Linington, C. and Diener, P.,** T and B cell responses to myelin oligodendrocyte glycoprotein in multiple sclerosis. *J. Immunol.* 1991. **146:** 1490–1495.

8 **Xiao, B. G., Linington, C. and Link, H.,** Antibodies to myelin oligodendrocyte glycoprotein in cerebrospinal fluid from patients and controls. *J. Neuroimmunol.* 1991. **31:** 91–96.

9 **Lindert, R., Haase, C., Brehm, U., Linington, C., Wekerle, H. and Hohlfeld, R.,** Multiple sclerosis: B- and T-cell responses to the extracellular domain of the myelin oligodendrocyte glycoprotein. *Brain* 1999. **122:** 2089–2100.

10 **Reindl, M., Linington, C., Brehm, U., Egg, R., Dilitz, E., Deisenhammer, F., Poewe, W. and Berger, T.,** Antibodies against the myelin oligodendrocyte glycoprotein and the myelin basic protein in multiple sclerosis and other neurological diseases: a comparative study. *Brain* 1999. **122:** 2047–2056.

11 **Karni, A., Bakimer-Kleiner, R., Abramsky, O. and Ben-Nun, A.,** Elevated levels of antibody to myelin oligodendrocyte glycoprotein is not specific for patients with multiple sclerosis. *Arch. Neurol.* 1999. **56:** 311–315.

12 **Vojdani, A., Vojdani, E. and Cooper, E.,** Antibodies to myelin basic protein, myelin oligodendrocytes peptides, alpha-beta-crystallin, lymphocyte activation and cytokine production in patients with multiple sclerosis. *J. Intern. Med.* 2003. **254:** 363–374.

13 **Berger, T., Rubner, P., Schautzer, F., Egg, R., Ulmer, H., Mayringer, I., Dilitz, E., Deisenhammer, F. and Reindl, M.,** Antimyelin antibodies as a predictor of clinically definite multiple sclerosis after a first demyelinating event. *N. Engl. J. Med.* 2003. **349:** 139–145.

14 **Mantegazza, R., Cristaldini, P., Bernasconi, P., Baggi, F., Pedotti, R., Piccini, I., Mascoli, N., Mantia, L. L., Antozzi, C., Simoncini, O., Cornelio, F. and Milanese, C.,** Anti-MOG autoantibodies in Italian multiple sclerosis patients: specificity, sensitivity and clinical association. *Int. Immunol.* 2004. **16:** 559–565.

15 **Hjelmstrom, P., Juedes, A., Fjell, J. and Ruddle, N.,** Cutting edge: B cell deficient mice develop experimental allergic encephalomyelitis with demyelination after myelin oligodendrocyte glycoprotein sensitization. *J. Immunol.* 1998. **161:** 4480–4483.

16 **Lyons, J., San, M., Happ, M. and Cross, A.,** B cells are critical to induction of experimental allergic encephalomyelitis by protein but not by a short encephalitogenic peptide. *Eur. J. Immunol.* 1999. **29:** 3432–3439.

17 **Raine, C., Cannella, B., Hauser, S. and Genain, C.,** Demyelination in non-human primate autoimmune encephalomyelitis and acute multiple sclerosis lesions: a case for antigen-specific antibody mediation. *Ann. Neurol.* 1999. **46:** 144–160.

18 **Brehm, U., Piddlesden, S., Gardinier, M. and Linington, C.,** Epitope specificity of demyelinating monoclonal autoantibodies directed against the human myelin oligodendrocyte glycoprotein (MOG). *J. Neuroimmunol.* 1999. **97:** 9–15.

19 **Bourquin, C., Iglesias, A., Berger, T., Wekerle, H. and Linington, C.,** Myelin oligodendrocyte glycoprotein-DNA vaccination induces antibody-mediated autoaggression in experimental autoimmune encephalomyelitis. *Eur. J. Immunol.* 2000. **30:** 3663–3671.

20 **Bourquin, C., Schubart, A., Tobollik, S., Mather, I., Ogg, S., Liblau, R. and Linington, C.,** Selective unresponsiveness to conformational B cell epitopes of the myelin oligodendrocyte glycoprotein in H-2b mice. *J. Immunol.* 2003. **171:** 455–461.

21 **Albouz-Abo, S., Wilson, J. C., Bernard, C. C. and von Itzstein, M.,** A conformational study of the human and rat encephalitogenic myelin oligodendrocyte glycoprotein peptides 35–55. *Eur. J. Biochem.* 1997. **246:** 59–70.

22 **Oliver, A. R., Lyon, G. M. and Ruddle, N. H.,** Rat and human myelin oligodendrocyte glycoproteins induce experimental autoimmune encephalomyelitis by different mechanisms in C57BL/6 mice. *J. Immunol.* 2003. **171:** 462–468.

23 **Breithaupt, C., Schubart, A., Zander, H., Skerra, A., Huber, R., Linington, C. and Jacob, U.,** Structural insights into the antigenicity of myelin oligodendrocyte glycoprotein. *Proc. Natl. Acad. Sci. USA* 2003. **100:** 9446–9451.

24 **Clements, C. S., Reid, H. H., Beddoe, T., Tynan, F. E., Perugini, M. A., Johns, T. G., Bernard, C. C. and Rossjohn, J.,** The crystal structure of myelin oligodendrocyte glycoprotein, a key autoantigen in multiple sclerosis. *Proc. Natl. Acad. Sci. USA* 2003. **100:** 11059–11064.

25 **Ichikawa, M., Johns, T., Adelmann, M. and Bernard, C.,** Antibody response in Lewis rats injected with myelin oligodendrocyte glycoprotein derived peptides. *Int. Immunol.* 1996. **8:** 1167–1674.

26 **Adelmann, M., Wood, J., Benzel, I., Fiori, P., Lassmann, H., Matthieu, J., Gardinier, M., Dornmair, K. and Linington, C.,** The N-terminal domain of the myelin oligodendrocyte glycoprotein (MOG) induces acute demyelinating experimental autoimmune encephalomyelitis in the Lewis rat. *J. Neuroimmunol.* 1995. **63:** 17–27.

27 **Bernard, C., Johns, T., Slavin, A., Ichikawa, M., Ewing, C., Liu, J. and Bettadapura, J.,** Myelin oligodendrocyte glycoprotein: a novel candidate autoantigen in multiple sclerosis. *J. Mol. Med.* 1997. **75:** 77–88.

28 **Cross, A., Trotter, J. and Lyons, J.-A.,** B cells and antibodies in CNS demyelinating disease. *J. Neuroimmunol.* 2001. **112:** 1–14.

29 **Svensson, L., Abdul-Majid, K. B., Bauer, J., Lassmann, H., Harris, R. A. and Holmdahl, R.,** A comparative analysis of B cell-mediated myelin oligodendrocyte glycoprotein-experimental autoimmune encephalomyelitis pathogenesis in B cell-deficient mice reveals an effect on demyelination. *Eur. J. Immunol.* 2002. **32:** 1939–1946.

30 **Lyons, J. A., Ramsbottom, M. J. and Cross, A. H.,** Critical role of antigen-specific antibody in experimental autoimmune encephalomyelitis induced by recombinant myelin oligodendrocyte glycoprotein. *Eur. J. Immunol.* 2002. **32:** 1905–1913.

31 **Selmaj, K. and Raine, C. S.,** Tumor necrosis factor mediates myelin damage in organotypic cultures of nervous tissue. *Ann. N.Y. Acad. Sci.* 1988. **540:** 568–570.

32 **Piddlesden, S. J., Lassmann, H., Zimprich, F., Morgan, B. P. and Linington, C.,** The demyelinating potential of antibodies to myelin oligodendrocyte glycoprotein is related to their ability to fix complement. *Am. J. Pathol.* 1993. **143:** 555–564.

33 **Compston, D. A. S., Morgan, B. P., Campbell, A. K., Wilkins, P., Cole, G., Thomas, N. D. and Jasani, B.,** Immunocytochemical localization of the terminal complement complex in multiple sclerosis. *Neuropathol. Appl. Neurobiol.* 1989. **15:** 307–316.

34 **Scolding, N., Frith, S., Linington, C., Morgan, B., Campbell, A. and Compston, D.,** Myelin-oligodendrocyte glycoprotein (MOG) is a surface marker for oligodendrocyte maturation. *J. Neuroimmunol.* 1989. **22:** 169–176.

35 **Storch, M. K., Piddlesden, S., Haltia, M., Iivanainen, M., Morgan, P. and Lassmann, H.,** Multiple sclerosis: in situ evidence for antibody- and complement-mediated demyelination. *Ann. Neurol.* 1998. **43:** 465–471.

36 **Lucchinetti, C., Brück, W., Parisi, J., Scheithauer, B., Rodriguez, M. and Lassmann, H.,** Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination. *Ann. Neurol.* 2000. **47:** 707–717.

37 **Amor, S., Groome, N., Linington, C., Morris, M. M., Dornmair, K., Gardinier, M. V., Matthieu, J. M. and Baker, D.,** Identification of epitopes of myelin oligodendrocyte glycoprotein for the induction of experimental allergic encephalomyelitis in SJL and Biozzi AB/H mice. *J. Immunol.* 1994. **153:** 4349–4356.

38 **Deibler, G. E., Martenson, R. E. and Kies, M. W.,** Large scale preparation of myelin basic protein in central nervous tissue of several mammalian species. *Prep. Biochem.* 1972. **2:** 139–165.

39 **Brok, H., Uccelli, A., Kerlero De Rosbo, N., Bontrop, R., Roccatagliata, L., de Groot, N., Capello, E., Laman, J., Nicolay, K., Mancardi, G., Ben-Nun, A. and 't Hart, B.,** Myelin/oligodendrocyte glycoprotein-induced autoimmune encephalomyelitis in common marmosets: the encephalitogenic T cell epitope pMOG24–36 is presented by a monomorphic MHC class II molecule. *J. Immunol.* 2000. **165:** 1093–1101.

40 **Villoslada, P., Hauser, S., Bartke, I., Unger, J., Heald, N., Rosenberg, D., Cheung, S., Mobley, W., Fisher, S. and Genain, C.,** Human nerve growth factor protects common marmosets against autoimmune encephalomyelitis by switching the balance of T helper type 1 and 2 cytokines within the central nervous system. *J. Exp. Med.* 2000. **191:** 1799–1806.

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Relevant antibody subsets against MOG recognize conformational epitopes exclusively exposed in solid-phase ELISA

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A pathogenic role for circulating anti-myelin antibodies is difficult to establish in multiple sclerosis (MS). Here, we unravel a broad heterogeneity within the anti-myelin oligodendrocyte glycoprotein (MOG) antibodies in humans and non-human primates, and demonstrate that detection of important epitopes of MOG within the pathogenic repertoire is exclusively dependent on presentation on a solid-phase MOG conformer. Results of ELISA and those of a liquid-phase assay were compared using a MOG protein with identical sequence but different conformations. We tested sera from 50 human subjects, plasma of *Callithrix jacchus* marmosets known to contain antibodies reactive to either conformational or linearized MOG, and monoclonal, conformation-dependent anti-MOG antibodies. We have found no antibody reactivity against the soluble MOG conformer in human serum, and show that this lack of detection is not due to technical artifacts. Rather, dominant epitopes of MOG are not displayed in soluble phase, as shown by a complete lack of binding of conformation-dependent mAb. In MP4-immune marmosets that exhibit demyelinating pathology due to spreading of antibody determinants to myelin-embedded MOG, only ELISA can detect pathogenic circulating anti-MOG antibodies. Thus, the accurate detection of important subsets of pathogenic anti-MOG antibodies requires methods in which MOG is displayed similarly to its natural conformation in myelin.

Key words: Antigens/peptides/epitopes · Autoantibodies · Autoimmunity · Human · Neuroimmunology

Introduction

Anti-myelin antibodies play an important role in the pathogenesis of demyelinating diseases, in particular those directed against myelin oligodendrocyte glycoprotein (MOG), which is an exposed antigen mostly expressed in CNS myelin [1]. In experimental allergic encephalomyelitis (EAE), passive transfer of anti-MOG antibody produces demyelination, a hallmark of the multiple sclerosis (MS) plaque [2–4]. A causal involvement of circulating anti-MOG antibodies in MS has been difficult to assess because anti-MOG responses have been detected at similar levels and incidence in sera of MS patients, healthy subjects, and other diseases

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Abbreviations: **HC:** healthy controls · **hCA:** human carbonic anhydrase · **HM:** horse myoglobin · **HWM:** human white matter · **LiPhELIA:** liquid-phase based enzyme-linked immune assay · **MBP:** myelin basic protein · **MOG:** myelin oligodendrocyte glycoprotein · **pepMOG:** linear MOG peptides · **PLP:** proteolipid protein · **TT:** tetanus toxoid

(reviewed in [5], see also [6–8]). Recent advances in the field suggest that several subsets of anti-MOG antibodies, separated on the basis of the tertiary structure of MOG (conformationally folded *vs*. linearized) may occur in both EAE and MS. These distinct subsets of anti-MOG antibodies have radically different pathogenic properties, *i.e*., they influence disease phenotype in terms of CNS plaque dissemination, and magnitude of demyelination [9–12]. The controversy surrounding the pathogenic role of anti-MOG (and other anti-myelin) antibodies in humans is due to many factors. To name a few, commonly used assays do not discriminate subsets of antibodies that bind to the different epitopes of MOG, regardless of whether the protein is conformationally folded or linearized; additionally, published studies have used different forms and preparations of MOG that are variable in length and post-translational modifications, as well as different assay systems, *i.e*., ELISA, Western blots, or cell-based assays [5–8, 10, 13, 14]. Clearly, there is a need to better define anti-MOG antibody reactivity at the molecular, single epitope level, to understand the nature of the information provided by each technique and the potential value of these paraclinical measurements.

Here, we further explore the basis for heterogeneity of structural epitopes of anti-MOG antibodies. We provide rational and conformation-based explanations for the apparent differential reactivity of serum anti-MOG antibodies, IgG antibody preparations, and monoclonal IgG and Fab fragments. This study makes use of a single antigen with identical sequence corresponding to the extracellular domain of recombinant human MOG (termed $rhMOG_{125}$) that adopts different conformations (conformers) in either solid or liquid phase. We have compared antibody reactivities by ELISA (solid phase) with a liquid phase-based enzyme-linked immune assay (LiPhELIA) that requires only antigen biotinylation. We have confirmed the lack of specific anti-MOG binding by MS sera in LiPhELIA, as previously reported. Using a marmoset MS model (where pathogenic and non-pathogenic antibodies can be separated according to conformation and epitope binding), and monoclonal IgG and Fab directed against epitopes displayed only by the solid-phase conformer (ELISA), we can demonstrate that important, dominant epitopes that are targeted *in vivo* by the pathogenic responses are not accessible on the solubilized MOG conformer (LiPhELIA). These results show that some of the disease-relevant anti-MOG antibodies can only be detected by solid-phase (or cell-based) assays, where intact MOG adopts a conformation, which simulates its presentation to the immune system in the lipid-rich, membrane-bound environment of CNS myelin.

Results

Differences between LiPhELIA and ELISA for anti-$rhMOG_{125}$ reactivity in human samples

Human serum samples ($n=50$) were chosen randomly from a larger cohort to sample the full range of anti-$rhMOG_{125}$ reactivity as defined by binding ratios of sample/background from low (binding ratio <2) to high (binding ratio >15) (Fig. 1). Anti-tetanus toxoid (TT) binding ratios exhibited a similar distribution in this cohort, with no difference between ELISA and LiPhELIA ($p=0.1$), and a highly significant between-assays correlation (ELISA *vs*. LiPhELIA, $r=0.7$, $p<0.001$). Thus, there was no apparent bias for the purpose of this study in this sample cohort. By contrast, in LiPhELIA none of the human serum samples bound to solution-phase $rhMOG_{125}$ (Fig. 1; $p<0.0001$ compared to ELISA). The LiPhELIA was 27 times more sensitive than the ELISA for anti-TT detection: OD at 50% maximal binding (OD_{50}) required dilutions of 1:600 for ELISA *vs*. 1:16 200 for LiPhELIA, and serum dilutions for the threshold of detection were 1:5400 and 1:145 800, respectively (data not shown).

Several technical factors potentially accounting for the lack of anti-$rhMOG_{125}$ reactivity in LiPhELIA could be eliminated, such as degradation of antigen by protease-activity in human sera (addition of protease inhibitors), and additional nonspecific serum factors (protein G-purified human IgG bound to $rhMOG_{125}$ in ELISA, but not in LiPhELIA). Faulty readings due to low antibody titers were ruled out by diluting sera with high titers of anti-TT IgG in anti-TT-negative serum or in 1%

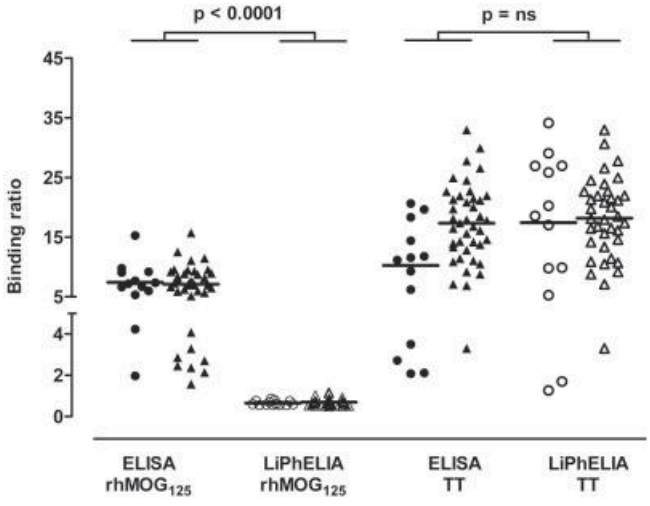

Figure 1. IgG reactivity against $rhMOG_{125}$ and TT in HC (●, ○) and MS patients (▲, △), as assessed by solid-phase ELISA (solid symbols; ▲, ●) and LiPhELIA (open symbols; ○, △). Results expressed as binding ratios. Statistical analysis by unpaired, two-tailed *t*-test.

BSA-PBS-T; TT reactivity in LiPhELIA was reduced, but still remained detectable in the diluted sera; reactivity was not affected at all in ELISA (data not shown).

Differences between ELISA and LiPhELIA for anti-rhMOG$_{125}$ reactivity in marmoset plasma in relation to antibody subsets

Previous studies have established that marmoset monkeys immunized with whole human white matter (HWM, containing native, membrane-embedded MOG), or recombinant rat and human MOG$_{125}$ develop an anti-MOG humoral response that includes demyelinating antibodies [4, 9, 15]. Antibodies generated by immunization with one or more 20-mer linear peptides of MOG (together and subsequently designated as pepMOG) in this species are by contrast restricted to linear determinants and are associated with limited demyelination and limited dissemination of plaques within the CNS [9]. Immuno-affinity purification of IgG from MOG$_{125}$-immunized animals confirmed that only the fraction containing anti-MOG antibodies directed against conformational determinants was associated with extensive demyelination [9].

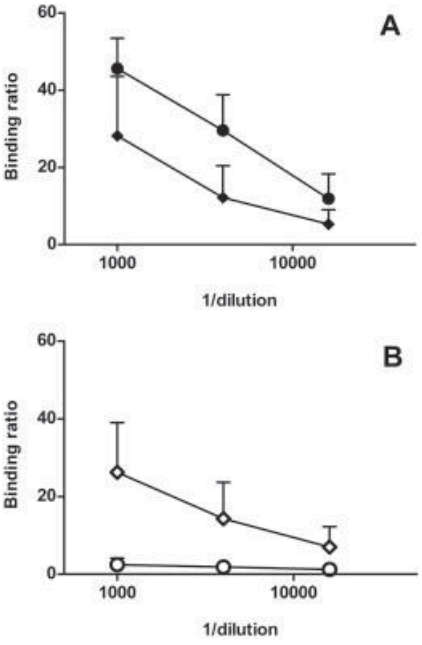

Figure 2. Antibody reactivity in plasma of *C. jacchus* marmosets immunized with HWM (-◆-, -◇-) or pepMOG (-●-, -○-) in ELISA and LiPhELIA. (A) ELISA reactivity, solid symbols (◆, ●). (B) LiPhELIA reactivity, open symbols (◇, ○); 1:4 serial serum dilutions, starting at 1:1000. Results are expressed as binding ratios. Mean ± SD, five animals per group. Reactivity of pepMOG-immune plasma is significantly less in LiPhELIA compared to HWM-immune plasma, while this difference was not observed for ELISA ($p<0.05$ and p=n.s., respectively; unpaired, two-tailed *t*-test).

Fig. 2 shows the binding ratios for serial dilutions of plasma from HWM-immunized, and pepMOG-immunized animals. The ELISA measured similar anti-rhMOG$_{125}$ reactivity regardless of the immunizing antigen, *i.e.*, HWM *vs*. pepMOG (p=ns, Fig. 2A). In contrast, while LiPhELIA detected anti-MOG antibodies in the HWM-immunized monkeys, there was no reactivity at any plasma dilution in pepMOG-immunized animals ($p<0.05$, ELISA *vs*. LiPhELIA; unpaired two-tailed *t*-test for all dilutions, Fig. 2B). Pre-immune naive plasma did not react in either assay system, when assessed at 1:100 dilutions (data not shown).

Differences between ELISA and LiPhELIA for affinity-purified anti-MOG IgG

It could be argued that the lack of reactivity of the human samples in LiPhELIA might be due to the presence of antibodies exclusively specific for linear MOG epitopes, as demonstrated for pepMOG-immunized marmoset plasma (Fig. 2B). To address this question, two sets of experiments were designed. First, IgG from pepMOG-immunized marmoset plasma were purified on pepMOG affinity columns [9]. These purified IgG bound only minimally in LiPhELIA: at saturating IgG concentrations binding was 11.7 times less than by ELISA (Fig. 3A). Second, anti-MOG IgG from human MS patients were purified on an rhMOG$_{125}$ column, and, to segregate antibodies specific for conformational rhMOG$_{125}$ epitopes, the pepMOG-specific antibody subsets were removed by immuno-affinity. These human conformational antibodies also reacted poorly in LiPhELIA (binding ratio of 1.4 at 50 μg/mL, approximately 12.6 times less than ELISA, Fig. 3B). These experiments confirm that the anti-MOG reactivity measured by ELISA in humans is directed against epitopes other than those exposed on the soluble MOG conformer, and cannot simply be accounted for by an exclusive specificity for linear epitopes. Epitope considerations set aside, our results also indicate that none of the human antibodies in serum binds to the soluble conformer of MOG.

Definition of fine epitope specificity on rhMOG$_{125}$ with mAb

ELISA and LiPhELIA reactivities and affinity constants were determined for the murine mAb 8.18c5 [16] that is known to recognize rhMOG$_{125}$ and ratMOG (aa 1–125), and three marmoset-derived Fab that had been selected for their reactivity against recombinant ratMOG$_{1\text{-}125}$. None of these monoclonal reagents binds to linear MOG epitopes [10, 17, 18]. Each of the marmoset Fab define a different conformational epitope on the exposed surface of ratMOG$_{1–125}$ in solid-phase (ELISA conformer) [10,

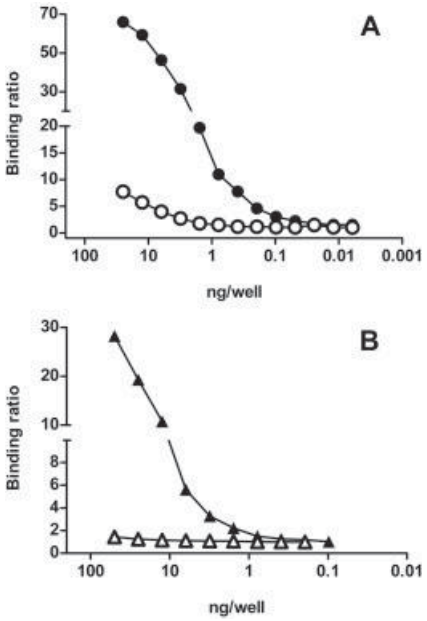

Figure 3. Reactivities of immuno-affinity purified anti-MOG marmoset IgG directed against linear MOG epitopes from a pool of pepMOG (-●-, -○-) -immune plasma, and human IgG directed against $rhMOG_{125}$ (-▲-, -△-). (A) Reactivity of marmoset IgG; these IgG fractions are strictly depleted of any antibody reacting to conformational MOG [9]. (B) Reactivity of human IgG from a pool of four MS patients with high anti-$rhMOG_{125}$ reactivity in ELISA. Solid symbols (-▲-, -●-) represent ELISA reactivity; open symbols (-△-, -○-) represent LiPhELIA reactivity. Twofold serial dilutions starting at 50 ng/well. Results expressed as binding ratios.

11, 18]. In contrast to 8.18c5 that bound equally well to liquid-phase and solid-phase $rhMOG_{125}$ (Fig. 4A), none of the marmoset Fab showed significant binding to liquid-phase $rhMOG_{125}$, exhibiting only low reactivity at saturated concentrations (Fig. 4B). The Fab designated M3–24, despite its lack of reactivity in liquid phase, has an affinity constant similar to that of 8.18c5 ($K_D = 1.1 \times 10^{-8}$ M and 1.0×10^{-8} M, respectively; Fig. 4). These findings suggest that immunodominant and disease-relevant epitopes of $rhMOG_{125}$ are not exposed in an aqueous environment. Very importantly, a MOG epitope defined in marmosets by the monoclonal Fab M3-24 is shared by the circulating anti-MOG antibody repertoire of human MS [18]. This implies that at least, certain disease-relevant and/or pathogenic antibodies present in humans may be missed in liquid-phase assays.

Differences between ELISA and LiPhELIA for anti-$rhMOG_{125}$ reactivity in MP4-immunized marmosets

From the observations in marmosets, it appears that immunization with the correctly folded MOG conformer (contained in white matter and lipid soluble) is a prerequisite for detecting reactivity in LiPhELIA (Fig. 2). This is the case in humans for immunization with TT, an antigen that is more water soluble than $rhMOG_{125}$.

To consolidate this interpretation of the current data, we measured the reactivities of plasma of marmosets immunized with MP4, a recombinant protein construct that encompasses solely myelin basic protein (MBP) and proteolipid protein (PLP) amino acid sequences [19]. Three of these animals had developed antibody responses that included inter-molecular epitope spreading with antibodies against MOG as well as MBP and PLP, even though they had not encountered MOG through peripheral immunization [19]. Reactivity against $rhMOG_{125}$ was readily detected by ELISA in these three animals, whereas no binding to liquid-phase $rhMOG_{125}$ was measurable in LiPhELIA (binding ratio $\sim$1) (Fig. 5). Demyelination with IgG deposition was present in the marmosets that were anti-MOG positive, and not in the anti-MOG negative ones (all by ELISA) [19]. These observations bolster our contention that the absence of liquid-phase reactivity in plasma does not exclude presence of disease-relevant and pathogenic antibodies against MOG. Additionally, they indicate that antibody responses against MOG generated during the course of disease by inter-molecular spreading (*e.g.*,

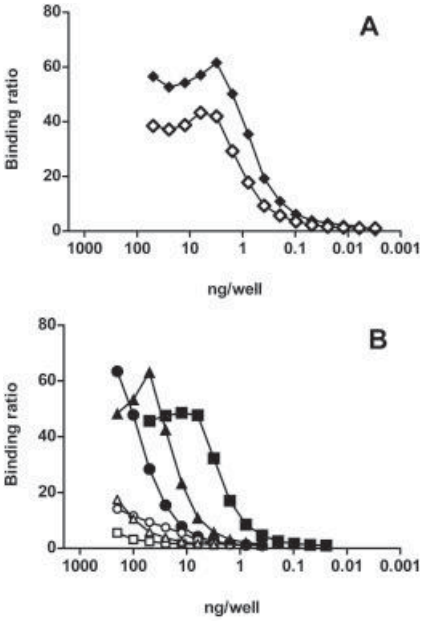

Figure 4. Anti-$rhMOG_{125}$ reactivity of 8.18c5 and *C. jacchus*-derived Fab in ELISA (solid symbols) and LiPhELIA (open symbols). (A) 8.18c5 (-◆-, -◇-), $K_D = 0.1 \pm 0.1 \times 10^{-7}$ M.
(B) M3–24 (-●-, -○-), $K_D = 0.11 \pm 0.006 \times 10^{-7}$ M;
M26 (-■-, -□-), $K_D = 0.81 \pm 0.37 \times 10^{-7}$ M;
M3–31 (-▲-, -△-), $K_D = 1.63 \pm 1.56 \times 10^{-7}$ M.
Serial dilutions starting at 200 ng/well (Fab) and 50 ng/well (8.18c5). Results expressed as binding ratios. Affinity constants K_D ($\pm$ SD) measured as described [46, 47].

directed against native, myelin-embedded MOG) are not detected by liquid-phase assays.

Reactivity of multimerized rhMOG$_{125}$ in native PAGE

It has been reported that recombinant mouse MOG (aa 1–117) forms dimers in solution that may block a major T cell epitope, thus preventing an immune response and formation of specific autoantibodies [20]. Although in the current studies we used a different protein (human, extended by 8 aa), we investigated whether multimerization of MOG could be a factor explaining the lack of reactivity in the soluble-phase system. Under mild SDS-PAGE denaturing conditions by addition of SDS to the running buffer only, rhMOG$_{125}$ appears mainly as a monomer at 15 kDa, and in a minor fraction as a dimer at about 30 kDa in (Fig. 6A). Horse myoglobin (HM) and human carbonic anhydrase (hCA) were used as markers for monomeric (HM, lane 1) and dimerized rhMOG$_{125}$ (hCA, lane 2) due to their biological properties similar to MOG, *i.e.*, molecular size and isoelectric point (p*I*) (Fig. 6A). When rhMOG$_{125}$ was analyzed in native PAGE, only multimers (dimer and trimer) and no monomer forms existed (Fig. 6B); the majority of the protein was shown to migrate well above the 29.5-kDa marker (hCA, lane 2) compatible with MOG trimers and a smaller portion just below it (comprising MOG dimer), but none at, or in close vicinity of the monomer marker (HM, lane 1). Polymerization did not adversely affect antibody binding to MOG epitopes; LiPhELIA-negative antibodies, such as the marmoset Fab, the IgG contained in pepMOG-immune marmoset sera and those contained human sera, exhibited binding to rhMOG$_{125}$ trimers and dimers (Fig. 6C). Thus, it is not the formation of MOG polymers that is responsible for lack of binding in LiPhELIA.

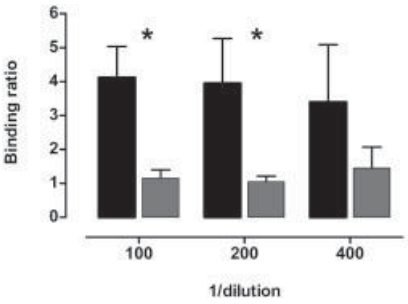

Figure 5. Antibody reactivity in plasma of *C. jacchus* marmosets after induction of demyelinating EAE with MP4 [19] in ELISA and LiPhELIA. Serial plasma dilutions, starting at 1:100. Results are expressed as binding ratios. Mean ± SD (n=3). Black bars represent ELISA reactivity; gray bars represent LiPhELIA reactivity. Stars (*) indicate significant differences of binding ratios (p<0.05, unpaired, two-tailed t-test).

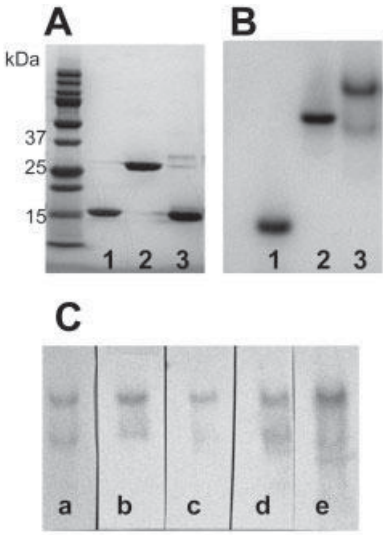

Figure 6. Assessment of dimer/multimer formation of soluble rhMOG$_{125}$ and the resulting antigenicity in immunoblot. (A) Denaturing SDS-PAGE (10–20% gradient gel). Lane 1, HM; lane 2, hCA; lane 3, rhMOG$_{125}$. Biosafe Coomassie protein stain, 2 μg protein/lane. (B) Native continuous PAGE (6% gel). Lane 1, HM; lane 2, hCA; lane 3, rhMOG$_{125}$. Biosafe Coomassie protein stain, 2 μg protein/lane. (C) Native Western blot of rhMOG$_{125}$ after native PAGE. Blot strips were probed as follows: lane a, total protein stain with GelCode (Pierce) after transfer; lane b, M26, 5 μg; lane c, M3–24, 5 μg; lane d, 8.18c5, 0.25 μg; lane e, pepMOG-immune serum pool, 1:1000. Substrate Opti-4CN.

Discussion

Liquid-phase assays have proven to be more sensitive and of greater clinical value than ELISA in diabetes mellitus [21]. We investigated whether this concept could be applied to circulating anti-MOG antibodies found in MS. The assay presented here uses soluble, biotinylated antigen and is technically able to detect antibodies in human serum with high sensitivity, as demonstrated for anti-TT IgG (Fig. 1). It failed however, to detect antibodies against soluble rhMOG$_{125}$ in any of 50 samples studied [MS patients or healthy controls (HC)], while some of these samples were highly positive in solid-phase ELISA (Fig. 1) and were also positive in a cell-based assay using native, membrane-embedded MOG [22]. The liquid-phase assay findings are consistent with results reported that used a the full-length human MOG protein (aa 1–218) [23], glycosylated and dimerized rhMOG (aa 1–121) [24], or tetrameric MOG in solution [25]. The current study differs from the previous ones in that it is the only one to have employed the same recombinant MOG protein in a comparative study of liquid- *vs*. solid-phase systems, and makes use of recombinant antibodies that have well-defined binding characteristics at the single conformational epitope level. Our results further unravel the complex hetero-

geneity of structure-function relationships for circulating anti-MOG antibodies.

The lack of detection in human samples by the non-radioactive LiPhELIA is not attributable to technical shortfalls of the assay. First, antibodies against the soluble $rhMOG_{125}$ conformer are readily detectable in marmoset EAE induced with native, HWM-embedded MOG; the efficiency of a comparable assay has recently been reported [26]. Second, in LiPhELIA soluble MOG is capable of binding to the murine mAb 8.18c5, an exclusively conformational antibody [10]. Thirdly, the lack of soluble MOG reactivity in humans is not due to formation of dimers or multimers after aqueous solubilization of $rhMOG_{125}$ (Fig. 6). Fourthly, we have ruled out the possibility that low antibody affinity is a limiting factor for binding to the $rhMOG_{125}$ conformer (Fig. 4). Fifth, we are confident that biotinylation does not alter the conformation of $rhMOG_{125}$; (i) when biotinylated $rhMOG_{125}$ was coated in ELISA wells, reactivity to 8.18c5 and the Fab can be elicited; (ii) terminating the biotinylation reaction prematurely and thus attaching less than 1 mol biotin/mol $rhMOG_{125}$ did not result in reactivity to human sera, while binding to 8.18c5 was retained; (iii) reactivity to TT was not affected by biotinylation; and (iv) solution-phase assays using biotinlylated antigens have been used extensively in the past without adverse affects to antibody binding due to conformational changes [26, 27].

In general, it is a known phenomenon that proteins may undergo conformational changes dependent on the physico-chemical properties of the assay used, thus limiting immunodominant epitope exposure [28–30]. The restriction in epitope presentation on the soluble MOG conformers probably reflects the fact that an aqueous environment is not physiological for MOG, a membrane-bound protein expressed in lipid-rich CNS myelin; alternatively, the increased reactivity in ELISA may be due to partial unfolding/denaturation of the protein, which exposes cryptic epitopes upon coating to polystyrene [28, 31]. However, polystyrene binding is not sufficient to cause complete denaturation creating linear polypeptides [28] as confirmed here by the strong binding of the marmoset Fab to $rhMOG_{125}$ in ELISA.

The monoclonal Fab are high affinity (similar to 8.18c5), and yet do not bind to biotinylated soluble $rhMOG_{125}$, making it highly unlikely that differences in affinity account for the lack of reactivity for other anti-MOG antibodies in liquid phase. Achenbach *et al.* [32] have reported that low-affinity and low-titer antibodies directed against insulin are capable of binding their target proteins in radioimmunoassays (RIA) (10^{-5} M, almost 100-fold lower than M3-31); Kennel *et al.* [33] reported similar results for anti-fibrinogen mAb (4.7×10^{-6} M, 10-fold less than M3-31). ELISA may promote the binding of low-affinity antibodies, whereas solution-phase assays detect antibodies of higher affinity in an antibody concentration-dependent fashion [34]; however, there is evidence that human serum contains anti-MOG antibodies of high affinity, such as the ones cross-reacting with the M3–24 Fab. In this study, sera with high binding ratios can be diluted beyond dilutions of 1:8000 retaining ELISA reactivity, an observation corroborated by previous reports [6, 7, 13].

We formally demonstrate here that linear epitopes are not exposed on soluble $rhMOG_{125}$. Hence, LiPhELIA is not appropriate for detection of antibodies directed against linear epitopes of MOG (Fig. 2 and 3). In contrast, using ELISA, antibodies against linear and/or conformational epitopes are detected together and indiscriminately [9]. The human antibody response comprises antibodies against linear and conformational epitopes, or a combination of both (T. Menge, H. C. von Büdingen and C. P. Genain; unpublished observation). However, even immuno-affinity-purified human anti-MOG antibodies or concentrated marmoset IgG directed against linear peptides of MOG (pepMOG) fail to react in LiPhELIA (Fig. 3). These observations underscore the fact that only a limited number of epitopes are exposed on water-soluble $rhMOG_{125}$.

To summarize, the exposure of the major determinants of $rhMOG_{125}$ on either the water-soluble or polystyrene-coated conformers, and how it correlates with antibody detection by ELISA or LiPhELIA, differ. We conclude that only one subset of the various anti-MOG antibodies studied, namely the ones induced in marmosets on immunization with HWM, are detectable by LiPhELIA. LiPhELIA also detects the epitope defined by 8.18c5, a murine antibody directed against native rat cerebellar MOG, but does not detect any of the mAb binding to conformationally folded MOG that are present in the circulation of immune marmosets, or some of those in humans. Finally, LiPhELIA fails to detect antibodies reactive to linearized MOG epitopes. These observations underscore the fact that an exceedingly restricted number of relevant epitopes are displayed on the water-soluble MOG conformer. By contrast, ELISA captures most of the antibody reactivity, including the subsets of antibodies directed against conformational and linear epitopes. When put in perspective to our recently published results on the immunological differences of solid-phase *vs*. membrane-embedded MOG [22], MOG epitopes are exposed differently in these three assays, and certain antibodies do not cross-react between assays. Only a minor fraction of antibodies are specific for epitopes exposed in all three assays, *e.g.*, 8.18c5 and IgG from HWM-immune marmosets, while the Fab are either reactive to solid-phase and membrane-bound MOG (M26, M3-31) or exclusively to an epitope exposed on solid-phase $rhMOG_{125}$ (M3-24) [22]. Importantly, all Fab bind to

Table 1. Summary of anti-MOG responses in marmosets immunized with different encephalitogenic antigens

Immunizing antigen/antibody[a)]	Epitope class specificity[b)]	Demyelination	Anti-rhMOG$_{125}$ reactivity in ELISA	Anti-rhMOG$_{125}$ reactivity in LiPHELIA	Reference
pepMOG	L	-	+	-	9
HWM	C	+	+	+	45
MP4	n.d.	+ [c)]	+ [c)]	-	19
8.18c5	C	+ [d)]	+	+	16
Fab fragments	C	+ [e)]	+	-	18

a) pepMOG: linear 20-mer MOG peptides; HWM: human white matter homogenate; MP4: recombinant fusion protein of MBP and PLP [19].
b) L: linear, reactivity to linear 20-mer MOG peptides only; C: conformational, reactivity to conformational MOG only; n.d.: not determined.
c) Only those animals were included that were positive for anti-ratMOG$_{125}$ antibodies by ELISA [19].
d) Demyelinating properties demonstrated in passive transfer experiments [2, 3].
e) Direct demyelinating properties not accessible due to lack of complement binding Fc-fragment; however, the Fab were derived from demyelinating marmoset EAE [18].

MOG *in situ*, as demonstrated by immunohistochemistry [18].

The most significant findings of this study were the relationships between epitope recognition, detection in LiPhELIA *vs*. ELISA, and functional (*e.g*., pathogenic) properties amongst antibodies that all and individually, recognize MOG. The detailed studies using marmosets immunized with antigenic preparations that contained MOG sequences in different conformations, and the correlations to neuropathology provided a framework to understand these relationships (Table 1). It can be seen that: (i) antibody reactivity to soluble MOG has no relationship to the occurrence of pathogenic demyelinating antibodies; (ii) certain pathogenic immunodominant epitopes of MOG relevant to disease are not exposed on the soluble conformer; and (iii) the anti-MOG antibodies present in MP4-immunized animals [19], or those generated from MS-like demyelinating marmoset EAE (Fab) [9], were reactive only to solid-phase rhMOG$_{125}$ (ELISA), but did not react against the soluble conformer (LiPhELIA) (Fig. 4 and 5). These antibodies are of critical importance, because they represent a signature of the spreading of antibody responses, implying that the subject's own native MOG protein has been exposed, degraded, or both. As such they may be pathogenic, and/or serve as disease markers. It is reasonable to state that LiPhELIA or similar assays employing soluble conformers of MOG do not detect antibodies against certain conformational epitopes of this protein that are relevant to pathogenesis of demyelination in EAE. Further, such antibodies can be found in human MS serum [18], and likely could be missed by liquid-phase assays.

Our data suggest that for MOG (and perhaps all myelin autoantigens), the conformational antigen containing properly folded binding sites has to be encountered by the peripheral immune system. This is apparent from a strong LiPhELIA antibody detection in marmosets immunized with HWM as opposed to those animals immunized with linear MOG peptides or MP4 (Fig. 2 and 5). Possible analogies to human situations include patients that were involuntarily immunized with MBP during rabies vaccination (Semple strain) who not only developed acute demyelinating encephalomyelitis (ADEM) and antibodies against solid-phase MBP [35], but also an anti-MBP response in RIA [36]. We recognize that spreading to restricted epitopes may occur locally in the target organs as demonstrated by O'Connor *et al*. [24] for an anti-MOG response. The MBP example, which to date has no MOG counterpart, suggests that circumstantial exposure to the target brain autoantigen must happen under relatively "physiological" conditions, which for proteins like MOG and other transmembranous myelin proteins exclude an aqueous environment, and hence liquid-phase based assays are inappropriate [36, 37]. In contrast, the usefulness of such assays is proven for antibodies against water-soluble proteins that are physiologically present in an aqueous environment, such as insulin or IFN-β [21, 38].

The preparation of MOG used in the current study was expressed in *E. coli* and does not display post-translational modifications of the protein, *e.g*., glycosylation. This, however, does not invalidate our results. The sugar moieties attached to MOG are readily available for antibody binding, as suggested in the crystallization studies of ratMOG$_{125}$ [17]; and glycosylated MOG$_{35–55}$ peptide yields higher antibody titers in MS than its non-glycoslylated counterpart [39]. However, it has also been demonstrated that increased binding is due to a sugar epitope not specific for MOG, but for a conformationally imbedded glycosylated asparagine [40]; glycosylation does not alter the

secondary structure of MOG peptide or ratMOG$_{125}$ [24, 41–43]. Neither the pathogenic mAb 8.18c5 (against glycosylated rat cerebellum) nor sera of patients with ADEM require glycosylation of a soluble MOG tetramer for antibody detection [25]. From our own experience, marmoset IgG generated after immunization with glycosylated HWM bind equally to glycosylated human MOG [22] and to non-glycosylated rhMOG$_{125}$ (Fig. 2). While we cannot rule out the possibility that MOG *in vivo* may be altered post-translationally within an inflammatory environment, this question remains speculative and would require further studies that are beyond the scope of this work. It would also be ideal to compare structurally different MOG preparations, soluble, coated and membrane-embedded MOG. While soluble recombinant ratMOG$_{125}$ has been assessed by crystallography and circular dichroism [17, 43], to date there are no methods available for a side-by-side analysis. Further studies in our laboratory are underway to further understand the differential exposure of disease-relevant MOG epitopes.

In conclusion, this study is the first to make in-depth attempts to dissect heterogeneity within the MOG antibody repertoires with practical assays in higher mammals. Although the precise molecular structure of conformational epitopes of human MOG is awaiting to be discovered, we have demonstrated that the lack of reactivity in LiPhELIA (soluble MOG conformer) is due to restricted exposure of epitopes on rhMOG$_{125}$ that prevents serum antibody detection, and not to technical flaws, a view shared by others [5]. Liquid-phase assays do not appear to be the proper systems to conduct anti-MOG serology studies in humans. Solid phase-based methods (insoluble MOG conformer), are superior in measuring anti-MOG antibodies; however, they lack the discriminative power to isolate the disease-relevant antibodies from epiphenomenal ones. Novel methodologies, such as cell-based assays that measure reactivity against native membrane-embedded MOG, detect a different subset of antibodies that appear to be stage specific and disease relevant [14, 22].

Material and methods

Antigens and antibodies

Water-soluble rhMOG was expressed to create an MOG antigen with identical amino acid sequence that could adopt separate conformations in ELISA and LiPhELIA. The cDNA encoding for the extracellular domain of rhMOG spanning aa 1–125 (termed rhMOG$_{125}$) was amplified using pfu-Polymerase (Promega, Madison, WI) 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′-CGGGGACCATGGGGCAGTTCAGAGTGATAGGACCAAGAC-ATAGGACCAAGACA-3′ and 5′-atccatgagatctaggatcttctactttc-aattccattgctgcc-3′. After digestion with Nco I and Bgl II (New England Biolabs, Ipswich, MA) 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 of the rhMOG$_{125}$ target gene ascertained. M15 cells (Qiagen) conditioned for chemical transformation according to the manufacturer's protocol were transformed for protein expression, expanded in LB medium supplemented with carbenicillin and kanamycin and induced by IPTG according to the manufacturer's protocol.

rhMOG$_{125}$ 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 Biotechnology, Rockford, IL). Purification was carried out under nondenaturing conditions in 20 mM sodium phosphate, 500 mM NaCl, 10% glycerol and 0.05% sodium deoxycholate, pH 8.0 (MOG buffer) using a Ni-NTA FPLC (HiTrap, Amersham, Piscataway, NJ), through the pQE60′s c-terminal His-tag. Protein was eluted by linear gradient of 250 mM imidazole, 20 mM sodium phosphate, 1 M NaCl, 25% glycerol, 0.05% sodium deoxycholate, pH 8.0. The eluted protein was dialyzed into MOG buffer, containing 15% glycerol, purity confirmed to be >95% by SDS-PAGE on 10–20% gels. Bacterial endotoxin contaminations were below detection limits of 0.06 EU/mL as determined by the Pyrogent Plus kit (Cambrex, East Rutherford, NJ).

The 20-mer linear MOG-peptides with 10-amino acid overlaps were purified as described previously [9]. TT was obtained from Wyeth-Ayerst and dialyzed against PBS.

The murine mAb anti-rat MOG IgG 8.18C5 against native form in cerebellar glycoproteins was a gift of Dr. Chris Linington [16]. The marmoset Fab designated M26, M3-24, and M3-31 were derived from a ratMOG-immunized animal that had developed a mature demyelinating antibody response and were generated in our laboratory as described previously [18]. These Fab define distinct epitopes on the ratMOG on a solid support, do not recognize linear MOG epitopes (as verified by 20mer, and 15mer peptides with overlap of 1), and do not overlap with the 8.18C5-defined epitope as shown by competition experiments (von Büdingen, unpublished observation) [18].

Human subjects

Thirteen HC sera and 37 sera of MS patients seen in our MS center that had not been treated with corticosteroids within 3 months or on immunosuppressive therapy within 6 months of phlebotomy, were included in this study. All patients met criteria for clinically definite MS [44]: 17 patients had a relapsing-remitting and 10 patients a secondary-progressive disease course; 10 patients had primary-progressive MS (25 patients were female). The median age of the patients was 45 years, and that of the HC 55 (age ranges 25-67 for MS and 33-73 for HC, respectively); gender and age distributions between MS and HC were not significantly different (χ^2 test and two-tailed *t*-test, respectively). The median extended disability status scale (EDSS) was 3.0 (range 1.0–8.5) and the median disease duration 117 months (range 4–348). Blood was drawn by venipuncture and clotted serum stored at –40°C.

Informed consent was obtained from all subjects, and the study was conducted in accordance with Institutional Review Board approval.

Animals

Callithrix jacchus marmosets were cared for in accordance with the guidelines of the Institutional Animal Care and Usage Committee.

Three protocols were used for EAE induction in marmosets. Animals were immunized with the following respective antigens emulsified in complete Freund's adjuvant supplemented with 3 mg/mL H37/Ra, and receive killed *B. pertussis* on the day of immunization and 48 h later [45]: immunized with: (i) 100 mg HWM containing conformational, glycosylated, native MOG ($n=5$) [45]; (ii) 100 μg synthetic 20-mer linearized MOG peptides purified to >95% purity (together designated as pepMOG; aa 1–40, 21–40, 51–70), or combination of 20-mer peptides spanning the extracellular portion of MOG resulting in non-demyelinating EAE ($n=5$); see [9]; (iii) 0.8–1.0 mg MP4, a recombinant fusion protein of MBP and PLP, lacking any MOG sequence ($n=3$) [19]. These three animals were part of a larger study of MP4-induced EAE, and were the only ones that developed prominent demyelination in conjunction with an antibody response against ratMOG in ELISA [21]. Plasma samples drawn 4–9 weeks after immunization were stored at –20°C until use.

Solid-phase ELISA

$rhMOG_{125}$, diluted to 5 μg/mL in PBS, and TT were coated in duplicates at 100 μL/well in 96-well microtiter plates (Maxisorb, Nunc, Rochester, NY). After incubation at 4°C overnight plates were washed in PBS and ddH_2O followed by 2 h blocking at room temperature with 1% BSA (A7030, Sigma, St. Louis, MO) in PBS, supplemented with 0.05% Tween-20 (PBS-T) (ELISA buffer). After washing with PBS-T and ddH_2O, 100 μL/well serum or purified antibodies diluted in ELISA buffer were added and plates incubated for 90 min at room temperature. Human sera were diluted 1:200 for $rhMOG_{125}$ and 1:600 for TT; for marmoset sera and monoclonal agents, see *Results*. Duplicate wells without antigens were used as individual background controls. After washing as above, bound human serum antibodies were detected by alkaline phosphatase-labeled anti-human IgG, marmoset serum antibodies by HRP-conjugated anti-monkey Ig, 8.18c5 by HRP-conjugated anti-mouse IgG (all Sigma), and Fab by HRP-conjugated Protein L (Pierce), all diluted in ELISA buffer. After incubation for 1 h at room temperature plates were washed as above. For human sera, binding was detected by reading absorbance (A) at 405 nm in a microplate reader (SpectraMax, Molecular Devices, Sunnyvale, CA) after incubation with *p*-nitrophenyl phosphate (Moss, Pasadena, MD) for 30 min in the dark at room temperature. The marmoset sera, Fab and 8.18c5 were developed with 3,3′,5,5′-tetramethylbenzidine (Pierce) for 15 min at room temperature and the A read at 450 nm. A signal-to-background binding ratio was calculated as the ratio of A (signal) over A (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.

Affinity binding constants

Competition assays were performed to determine antibody affinity, according to the protocol of Friguet *et al.* [46] with modifications. After determining their antibody concentrations yielding an OD_{50}, 8.18c5 and Fab were incubated in solution with $rhMOG_{125}$ in concentrations ranging from 1.0 nM to 1000 nM overnight at 4°C. K_D were calculated as described [46] and corrected for monovalent binding where applicable [47].

LiPhELIA

TT and $rhMOG_{125}$ were biotinylated at their primary amines *via N*-hydroxysuccinimide ($NHS\text{-}PEO_4$ biotin, Pierce) according to the manufacturer's protocol in a 20:1 ratio for 30 min at room temperature. Unreacted biotin was eliminated by extensive dialysis against PBS (TT) or MOG-buffer ($rhMOG_{125}$), respectively. A specific activity >10 biotin/mol was confirmed by the 2-hydroxyazobenzen-4′-carboxylic acid/avidin method (HABA/Avidin, Pierce). 12.5 ng/well biotinylated proteins were diluted in 0.1% BSA-PBS-T and mixed with sera, Fab or 8.18c5 in dilutions as used in ELISA in inert polypropylene 96-well plates. In the case of sera, the diluent was supplemented with a protease inhibitor cocktail (Roche, Indianapolis, IN) and 1 mM PMSF (Sigma). 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 ddH_2O and blocked with Superblock (Pierce) according to the manufacturer's recommendations. After incubation for 1 h at 4°C on a rocker, plates were washed with PBS-T and ddH_2O and bound immunocomplexed biotinylated protein detected by addition of 50 μL/well HRP-conjugated streptavidin (50 ng/mL, Pierce), diluted in 1% BSA-PBS-T. Plates were incubated for 1 h at 4°C and washed as above. Substrate reactions and result expression were similar to ELISA.

Immuno-affinity antibody purification

For human anti-MOG antibody purification, 25 mL serum were pooled from four MS patients with high anti-MOG reactivity in ELISA. Total IgG was purified over a protein G column (HP, Amersham) according to the manufacturer's protocol, diafiltrated into MOG buffer and loaded onto a NHS-activated Sepharose (Amersham) to which $rhMOG_{125}$ was covalently coupled according to the manufacturer's protocol. Specifically bound IgG was eluted with 0.1 M glycine, 25% glycerol, 125 mM NaCl, pH 2.7 and dialyzed into PBS.

Marmoset IgG reactive to linear MOG epitopes were purified as described before [9].

Continuous native PAGE and immunoblot

Proteins were separated by continuous PAGE in custom-made 6% gels at pH 9.4 in 60 mM Tris/40 mM CAPS (T/C buffer) omitting any denaturing agents as described before [48]. For comparison, standard SDS-PAGE was run in parallel on 10–20% precast gels (ReadyGel, Bio-Rad, Hercules, CA) in 25 mM Tris, 192 mM glycine running buffer with 0.1% SDS at pH 8.3. After electrophoresis at 200 V for 1 h, the gels were either stained with Biosafe Coomassie (Bio-Rad) or subjected to immunoblotting. Since pre-formulated molecular weight markers are not available for T/C buffer-based continuous PAGE, the following proteins were used to match the molecular mass and p*I* of rhMOG$_{125}$ (15 kDa and 7.2–7.5, respectively): HM (M-0630, Sigma), 17.6 kDa, p*I* ~7.3; hCA (C-6165, Sigma), 29.5 kDa, p*I* ~ 7.4.

For immunoblotting the proteins were transferred onto nitrocellulose in a Mini Trans-Blot cell (Bio-Rad) using T/C buffer supplemented with 20% methanol for 1 h at 350 mA on ice. After washing in PBS-T, membranes were blocked for 1 h in 3% BSA-PBS-T and incubated with primary antibody in 1% BSA-PBS-T overnight at 4°C (see *Results* for dilutions). After washing, membranes were incubated with the respective HRP-labeled secondary antibody diluted in 1% BSA-PBS-T for 1 h at room temperature; for human sera: goat-anti-human IgG (A0170, Sigma); for marmoset sera, 8.18c5 and Fab see ELISA protocol. After washing, blots were developed with the Opti-4CN system (Bio-Rad) for 15 min at room temperature.

Statistical analysis

Statistics were performed using GraphPad Prism 4.0 software. Binding ratios were shown to be normally distributed for both ELISA and LiPhELIA. Groups were compared by unpaired two-tailed *t*-test, multiple comparisons were analyzed with one-way ANOVA followed by Student-Newman-Keuls *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.

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References

1 **Raine, C. S., Cannella, B., Hauser, S. L. and Genain, C. P.,** Demyelination in primate autoimmune encephalomyelitis and acute multiple sclerosis lesions: a case for antigen-specific antibody mediation. *Ann. Neurol.* 1999. **46:** 144–160.

2 **Schluesener, H. J., Sobel, R. A., Linington, C. and Weiner, H. L.,** A monoclonal antibody against a myelin oligodendrocyte glycoprotein induces relapses and demyelination in central nervous system autoimmune disease. *J. Immunol.* 1987. **139:** 4016–4021.

3 **Linington, C., Bradl, M., Lassmann, H., Brunner, C. and Vass, K.,** Augmentation of demyelination in rat acute allergic encephalomyelitis by circulating mouse monoclonal antibodies directed against a myelin/oligodendrocyte glycoprotein. *Am. J. Pathol.* 1988. **130:** 443–454.

4 **Genain, C. P., Nguyen, M. H., Letvin, N. L., Pearl, R., Davis, R. L., Adelman, M., Lees, M. B. *et al.*,** Antibody facilitation of multiple sclerosis-like lesions in a nonhuman primate. *J. Clin. Invest* 1995. **96:** 2966–2974.

5 **Reindl, M., Khalil, M. and Berger, T.,** Antibodies as biological markers for pathophysiological processes in MS. *J. Neuroimmunol.* 2006. **180:** 50–62.

6 **Reindl, M., Linington, C., Brehm, U., Egg, R., Dilitz, E., Deisenhammer, F., Poewe, W. and Berger, T.,** Antibodies against the myelin oligodendrocyte glycoprotein and the myelin basic protein in multiple sclerosis and other neurological diseases: a comparative study. *Brain* 1999. **122:** 2047–2056.

7 **Mantegazza, R., Cristaldini, P., Bernasconi, P., Baggi, F., Pedotti, R., Piccini, I., Mascoli, N. *et al.*,** Anti-MOG autoantibodies in Italian multiple sclerosis patients: specificity, sensitivity and clinical association. *Int. Immunol.* 2004. **16:** 559–565.

8 **Gaertner, S., de Graaf, K. L., Greve, B. and Weissert, R.,** Antibodies against glycosylated native MOG are elevated in patients with multiple sclerosis. *Neurology* 2004. **63:** 2381–2383.

9 **von Budingen, H. C., Hauser, S. L., Ouallet, J. C., Tanuma, N., Menge, T. and Genain, C. P.,** Frontline: Epitope recognition on the myelin/oligodendrocyte glycoprotein differentially influences disease phenotype and antibody effector functions in autoimmune demyelination. *Eur. J. Immunol.* 2004. **34:** 2072–2083.

10 **Brehm, U., Piddlesden, S. J., Gardinier, M. V. and Linington, C.,** Epitope specificity of demyelinating monoclonal autoantibodies directed against the human myelin oligodendrocyte glycoprotein (MOG). *J. Neuroimmunol.* 1999. **97:** 9–15.

11 **Mathey, E., Breithaupt, C., Schubart, A. S. and Linington, C.,** Commentary: Sorting the wheat from the chaff: Identifying demyelinating components of the myelin oligodendrocyte glycoprotein (MOG)-specific autoantibody repertoire. *Eur. J. Immunol.* 2004. **34:** 2065–2071.

12 **Marta, C. B., Oliver, A. R., Sweet, R. A., Pfeiffer, S. E. and Ruddle, N. H.,** Pathogenic myelin oligodendrocyte glycoprotein antibodies recognize glycosylated epitopes and perturb oligodendrocyte physiology. *Proc. Natl. Acad. Sci. USA* 2005. **102:** 13992–13997.

13 **Lindert, R. B., Haase, C. G., Brehm, U., Linington, C., Wekerle, H. and Hohlfeld, R.,** Multiple sclerosis: B- and T-cell responses to the extracellular domain of the myelin oligodendrocyte glycoprotein. *Brain* 1999. **122:** 2089–2100.

14 **Zhou, D., Srivastava, R., Nessler, S., Grummel, V., Sommer, N., Bruck, W., Hartung, H. P. *et al.*,** Identification of a pathogenic antibody response to native myelin oligodendrocyte glycoprotein in multiple sclerosis. *Proc. Natl. Acad. Sci. USA* 2006. **103:** 19057–19062.

15 **Brok, H. P., Uccelli, A., Kerlero De Robso, N., Bontrop, R. E., Roccatagliata, L., de Groot, N. G., Capello, E. *et al.*,** Myelin/oligodendrocyte glycoprotein-induced autoimmune encephalomyelitis in common marmosets: The encephalitogenic T cell epitope pMOG24–36 is presented by a monomorphic MHC class II molecule. *J. Immunol.* 2000. **165:** 1093–1101.

16 **Linnington, C., Webb, M. and Woodhams, P. L.,** A novel myelin-associated glycoprotein defined by a mouse monoclonal antibody. *J. Neuroimmunol.* 1984. **6 :** 387–396.

17 **Breithaupt, C., Schubart, A., Zander, H., Skerra, A., Huber, R., Linington, C. and Jacob, U.,** Structural insights into the antigenicity of myelin oligodendrocyte glycoprotein. *Proc. Natl. Acad. Sci. USA* 2003. **100:** 9446–9451.

18 **von Budingen, H. C., Hauser, S. L., Fuhrmann, A., Nabavi, C. B., Lee, J. I. and Genain, C. P.,** Molecular characterization of antibody specificities against myelin/oligodendrocyte glycoprotein in autoimmune demyelination. *Proc. Natl. Acad. Sci. USA* 2002. **99:** 8207–8212.

19 **McFarland, H. I., Lobito, A. A., Johnson, M. M., Nyswaner, J. T., Frank, J. A., Palardy, G. R., Tresser, N. *et al.*,** Determinant spreading associated with demyelination in a nonhuman primate model of multiple sclerosis. *J. Immunol.* 1999. **162:** 2384–2390.

20 **Clements, C. S., Reid, H. H., Beddoe, T., Tynan, F. E., Perugini, M. A., Johns, T. G., Bernard, C. C. and Rossjohn, J.,** The crystal structure of myelin oligodendrocyte glycoprotein, a key autoantigen in multiple sclerosis. *Proc. Natl. Acad. Sci. USA* 2003. **100:** 11059–11064.

21 **Verge, C. F., Stenger, D., Bonifacio, E., Colman, P. G., Pilcher, C., Bingley, P. J. and Eisenbarth, G. S.,** Combined use of autoantibodies (IA-2 autoantibody, GAD autoantibody, insulin autoantibody, cytoplasmic islet cell antibodies) in type 1 diabetes: Combinatorial Islet Autoantibody Workshop. *Diabetes* 1998. **47:** 1857–1866.

22 **Lalive, P. H., Menge, T., Delarasse, C., Della, G. B., Pham-Dinh, D., Villoslada, P., von Budingen, H. C. and Genain, C. P.,** Antibodies to native myelin oligodendrocyte glycoprotein are serologic markers of early inflammation in multiple sclerosis. *Proc. Natl. Acad. Sci. USA* 2006. **103:** 2280–2285.

23 **Lampasona, V., Franciotta, D., Furlan, R., Zanaboni, S., Fazio, R., Bonifacio, E., Comi, G. and Martino, G.,** Similar low frequency of anti-MOG IgG and IgM in MS patients and healthy subjects. *Neurology* 2004. **62:** 2092–2094.

24 **O'Connor, K. C., Appel, H., Bregoli, L., Call, M. E., Catz, I., Chan, J. A., Moore, N. H. *et al.*,** Antibodies from inflamed central nervous system tissue recognize myelin oligodendrocyte glycoprotein. *J. Immunol.* 2005. **175:** 1974–1982.

25 **O'Connor, K. C., McLaughlin, K. A., De Jager, P. L., Chitnis, T., Bettelli, E., Xu, C., Robinson, W. H. *et al.*,** Self-antigen tetramers discriminate between myelin autoantibodies to native or denatured protein. *Nat. Med.* 2007. **13:** 211–217.

26 **Ngai, P. K., Ackermann, F., Wendt, H., Savoca, R. and Bosshard, H. R.,** Protein A antibdy-capture ELISA (PACE): An ELISA format to avoid denaturation of surface-adsorbed antigens. *J. Immunol. Methods* 1993. **158:** 267–276.

27 **Brickelmaier, M., Hochman, P. S., Baciu, R., Chao, B., Cuervo, J. H. and Whitty, A.,** ELISA methods for the analysis of antibody responses induced in multiple sclerosis patients treated with recombinant interferon-beta. *J. Immunol. Methods* 1999. **227:** 121–135.

28 **Friguet, B., Djavadi-Ohaniance, L. and Goldberg, M. E.,** Some monoclonal antibodies raised with a native protein bind preferentially to the denatured antigen. *Mol. Immunol.* 1984. **21:** 673–677.

29 **Vaidya, H. C., Dietzler, D. N. and Ladenson, J. H.,** Inadequacy of traditional ELISA for screening hybridoma supernatants for murine monoclonal antibodies. *Hybridoma* 1985. **4:** 271–276.

30 **Dekker, E. L., Porta, C. and Van Regenmortel, M. H.,** Limitations of different ELISA procedures for localizing epitopes in viral coat protein subunits. *Arch. Virol.* 1989. **105:** 269–286.

31 **Sodoyez-Goffaux, F., Koch, M., Dozio, N., Brandenburg, D. and Sodoyez, J. C.,** Advantages and pitfalls of radioimmune and enzyme linked immunosorbent assays of insulin antibodies. *Diabetologia* 1988. **31:** 694–702.

32 **Achenbach, P., Koczwara, K., Knopff, A., Naserke, H., Ziegler, A. G. and Bonifacio, E.,** Mature high-affinity immune responses to (pro)insulin anticipate the autoimmune cascade that leads to type 1 diabetes. *J. Clin. Invest.* 2004. **114:** 589–597.

33 **Kennel, D. M., De Bouwerie, M., Kolopp-Sarda, M. N., Faure, G. C., Bene, M. C. and Bernard, C. C.,** Anti-myelin oligodendrocyte glycoprotein B-cell responses in multiple sclerosis. *J. Neuroimmunol.* 2003. **135:** 117–125.

34 **Sodoyez, J. C., Koch, M., Lemaire, I., Sodoyez-Goffaux, F., Rapaille, A., Francois-Gerard, C. and Sondag, D.,** Influence of affinity of antibodies upon their detection by liquid phase radiobinding assay and solid phase enzyme linked immunosorbent assay. Demonstration using monoclonal antibodies raised against rDNA human proinsulin. *Diabetologia* 1991. **34:** 463–468.

35 **Hemachudha, T., Griffin, D. E., Giffels, J. J., Johnson, R. T., Moser, A. B. and Phanuphak, P.,** Myelin basic protein as an encephalitogen in encephalomyelitis and polyneuritis following rabies vaccination. *N. Engl. J. Med.* 1987. **316:** 369–374.

36 **O'Connor, K. C., Chitnis, T., Griffin, D. E., Piyasirisilp, S., Bar-Or, A., Khoury, S., Wucherpfennig, K. W. and Hafler, D. A.,** Myelin basic protein-reactive autoantibodies in the serum and cerebrospinal fluid of multiple sclerosis patients are characterized by low-affinity interactions. *J. Neuroimmunol.* 2003. **136:** 140–148.

37 **Moller, J. R., Johnson, D., Brady, R. O., Tourtellotte, W. W. and Quarles, R. H.,** Antibodies to myelin-associated glycoprotein (MAG) in the cerebrospinal fluid of multiple sclerosis patients. *J. Neuroimmunol.* 1989. **22:** 55–61.

38 **Pachner, A. R., Oger, J. and Palace, J.,** The measurement of antibodies binding to IFNbeta in MS patients treated with IFNbeta. *Neurology* 2003. **61:** S18–S20.

39 **Mazzucco, S., Mata, S., Vergelli, M., Fioresi, R., Nardi, E., Mazzanti, B., Chelli, M. *et al.*,** A synthetic glycopeptide of human myelin oligodendrocyte glycoprotein to detect antibody responses in multiple sclerosis and other neurological diseases. *Bioorg. Med. Chem. Lett.* 1999. **9:** 167–172.

40 **Lolli, F., Liuzzi, G. M., Vergelli, M., Massacesi, L., Ballerini, C., Amaducci, L. and Riccio, P.,** Antibodies specific for the lipid-bound form of myelin basic protein during experimental autoimmune encephalomyelitis. *J. Neuroimmunol.* 1993. **44:** 69–75.

41 **Carotenuto, A., D'Ursi, A. M., Nardi, E., Papini, A. M. and Rovero, P.,** Conformational analysis of a glycosylated human myelin oligodendrocyte glycoprotein peptide epitope able to detect antibody response in multiple sclerosis. *J. Med. Chem.* 2001. **44:** 2378–2381.

42 **Albouz-Abo, S., Wilson, J. C., Bernard, C. C. and von Itzstein, M.,** A conformational study of the human and rat encephalitogenic myelin oligodendrocyte glycoprotein peptides 35–55. *Eur. J. Biochem.* 1997. **246:** 59–70.

43 **Ngu-Schwemlein, M., Corzett, M., Thornton, K. H., Balhorn, R. and Cosman, M.,** Extracellular domain of myelin oligodendrocyte glycoprotein (MOG) exhibits solvent-dependent conformational transitions. *Protein Pept. Lett.* 2003. **10:** 483–490.

44 **Poser, C. M., Paty, D. W., Scheinberg, L., McDonald, W. I., Davis, F. A., Ebers, G. C., Johnson, K. P. *et al.*,** New diagnostic criteria for multiple sclerosis: Guidelines for research protocols. *Ann. Neurol.* 1983. **13:** 227–231.

45 **Genain, C. P. and Hauser, S. L.,** Experimental allergic encephalomyelitis in the New World monkey *Callithrix jacchus*. *Immunol. Rev.* 2001. **183:** 159–172.

46 **Friguet, B., Chaffotte, A. F., Djavadi-Ohaniance, L. and Goldberg, M. E.,** Measurements of the true affinity constant in solution of antigen-antibody complexes by enzyme-linked immunosorbent assay. *J. Immunol. Methods* 1985. **77:** 305–319.

47 **Stevens, F. J.,** Modification of an ELISA-based procedure for affinity determination: Correction necessary for use with bivalent antibody. *Mol. Immunol.* 1987. **24:** 1055–1060.

48 **McLellan, T.,** Electrophoresis buffers for polyacrylamide gels at various pH. *Anal. Biochem.* 1982. **126:** 94–99.

Antibodies to native myelin oligodendrocyte glycoprotein are serologic markers of early inflammation in multiple sclerosis

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Myelin oligodendrocyte glycoprotein (MOG) is an integral membrane protein expressed in CNS oligodendrocytes and outermost myelin lamellae. Anti-MOG Abs cause myelin destruction (demyelination) in animal models of multiple sclerosis (MS); however, such pathogenic Abs have not yet been characterized in humans. Here, a method that specifically detects IgG binding to human MOG in its native, membrane-embedded conformation on MOG-transfected mammalian cells was used to evaluate the significance of these auto Abs. Compared with healthy controls, native MOG-specific IgGs were most frequently found in serum of clinically isolated syndromes ($P < 0.001$) and relapsing-remitting MS ($P < 0.01$), only marginally in secondary progressive MS ($P < 0.05$), and not at all in primary progressive MS. We demonstrate that epitopes exposed in this cell-based assay are different from those exposed on the refolded, extracellular domain of human recombinant MOG tested by solid-phase ELISA. In marmoset monkeys induced to develop MS-like CNS inflammatory demyelination, IgG reactivity against the native membrane-bound MOG is always detected before clinical onset of disease ($P < 0.0001$), unlike that against other myelin constituents. We conclude that (*i*) epitopes displayed on native, glycosylated MOG expressed *in vivo* are early targets for pathogenic Abs; (*ii*) these Abs, which are not detected in solid-phase assays, might be the ones to play a pathogenic role in early MS with predominant inflammatory activity; and (*iii*) the cell-based assay provides a practical serologic marker for early detection of CNS autoimmune demyelination including its preclinical stage at least in the primate MS model.

allergic encephalomyelitis | autoimmunity | clinically isolated syndrome | demyelination

Multiple sclerosis (MS) is an immune-mediated, chronic demyelinating disorder of CNS white matter mediated by $CD4^+$ myelin-reactive T helper 1 cells (1). Humoral immunity may play a role in MS pathogenesis, as suggested by intrathecal Ab synthesis and oligoclonal B cell expansion, observations of Ab deposition associated with myelin vacuolization in MS plaques (2), and involvement of IgG and complement deposition (3). Myelin oligodendrocyte glycoprotein (MOG) is a target myelin antigen for both humoral and cellular CNS-directed immune responses. The full-length protein contains 218 aa and two predicted transmembrane domains, is posttranscriptionally processed as suggested by apparent electrophoretic mobility, and is a potential site for glycosylation, phosphorylation, isoprenylation, and myristoylation (4). The encephalitogenic properties of MOG are believed to result from the extracellular location of its IgV-like domain on the outermost myelin lamellae, which makes it an exposed target accessible to initial autoimmune attack on compact myelinated axons (5). In both rodent and primate MS models of experimental allergic encephalomyelitis (EAE), Abs against MOG directly induce demyelination (6, 7). Moreover, certain Abs against MOG have been proposed as predictors of early conversion to clinically definite MS in the context of a first demyelinating event (8).

A common problem encountered in human studies of humoral immunity against MOG is that accurate detection of Ab depends highly on the conformation of the antigens used for detection. Previous studies, either in MS or EAE, have not fully characterized the structural 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 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 may fail to detect reactivity against epitopes displayed by native MOG expressed *in situ* on myelin sheaths, as it would be upon an initial immune response.

Here, we describe a cell-based assay that specifically measures Abs directed against conformationally folded, cell membrane-expressed human MOG (designated $hMOG_{cme}$), and evaluate the relative incidence of these antinative MOG Abs in serum of humans and *Callithrix jacchus* marmosets with MS-like EAE. We find that $hMOG_{cme}$ Abs predominate during clinically isolated syndrome (CIS) and relapsing-remitting MS (RRMS) and can be detected during the preclinical stage of EAE. These findings underscore the potential value of antinative MOG Abs as a practical candidate biomarker for detecting MS at its early, inflammatory stage.

Results

Chinese Hamster Ovary (CHO)-MOG Assay (MOG_{cme}) Validation. Fig. 1 shows high levels of MOG_{cme} expression, as demonstrated by staining of MOG-transfected CHO cells with the monoclonal anti-MOG Ab 8-18C5. Detection of $hMOG_{cme}$-specific Abs with this cell-based assay was sensitive because a concentration of <1 ng/ml of 8-18C5 produced a binding ratio (BR) >1.5 (data not shown). Staining with a positive control serum (patient 1158) is shown in Fig. 1*C*. This control was used in each assay to

Conflict of interest statement: P.H.L., T.M., H.-C.v.B., and C.P.G. are coinventors of an international patent application that contains claims to methods of diagnostics and prognosis of multiple sclerosis. Such methods may be ultimately commercialized.

Abbreviations: MOG, myelin oligodendrocyte glycoprotein; $rMOG_{125}$, recombinant rat $MOG_{aa1-125}$; $hMOG_{125}$, recombinant human $MOG_{aa1-125}$; $hMOG_{cme}$, cell-membrane expressed human MOG; CHO, Chinese hamster ovary; ntCHO, nontransfected CHO; EAE, experimental allergic encephalomyelitis; CIS, clinically isolated syndrome; MS, multiple sclerosis; RRMS, relapsing-remitting MS; SPMS, secondary progressive MS; PPMS, primary progressive MS; HC, healthy control; BR, binding ratio; Gmean, geometric mean intensity.

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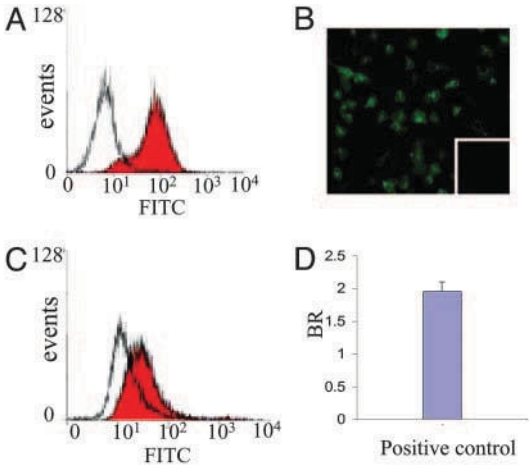

Fig. 1. Cell-based ($hMOG_{cme}$) assay. (*A* and *B*) Staining of MOG-transfected CHO cells with anti-MOG Ab 8-18C5 (0.5 μg/ml) and detection by FACS (*A*) and immunofluorescence (*B*). (*B Inset*) Negative control omitting primary Ab. (*C*) Positive control patient serum (RRMS 1158, 1:10) displaying a clear shift for MOG-transfected CHO cells (filled trace) when compared with ntCHO cells (open trace). (*D*) Mean BR (BR ± SEM) calculated as the Gmean from nine independent assays.

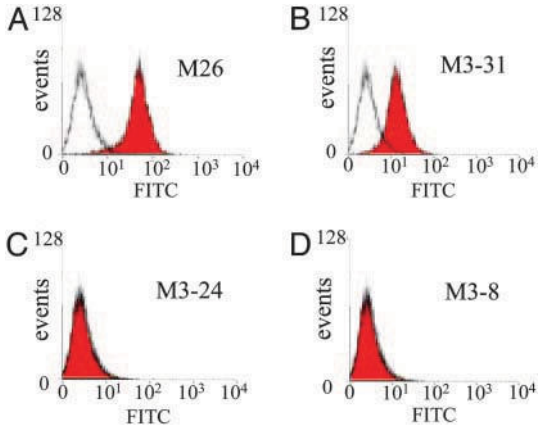

Fig. 2. Binding characteristics of MOG-specific marmoset recombinant Fab fragments. Binding of monoclonal Fabs specific for $rMOG_{125}$ (0.5 μg/ml) by FACS against $hMOG_{cme}$. Background is represented by the binding to the ntCHO cells (open traces) and compared with the binding to MOG-transfected CHO cells (filled traces). Both Fabs M26 (*A*) and M3-31 (*B*) strongly recognize an epitope displayed on $hMOG_{cme}$, in contrast to Fabs M3-24 (*C*) and M3-8 (*D*).

normalize for interassay variability and minimize experimental errors such as variation in surface expression of MOG. The mean (± SEM) BR to $hMOG_{cme}$ of this control from nine independent experiments was 1.96 ± 0.145 (Fig. 1*D*), 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 $hMOG_{cme}$. We analyzed the binding properties of monoclonal, recombinant marmoset Fab Ab fragments produced against the nonglycosylated extracellular domain of recombinant rat $MOG_{aa1–125}$ ($rMOG_{125}$). Four Fabs, designated M3-8, M26, M3-24, and M3-31, were selected by their ability to recognize $rMOG_{125}$ 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. 2 *A* and *B*). On the contrary, no binding was observed for the two other Fabs, M3-24 and M3-8 (Fig. 2 *C* and *D*). 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 $rMOG_{125}$ on solid ELISA support. The observation that only three of five monoclonal reagents tested bind to the transfected cells also renders a nonspecific binding effect unlikely.

IgG Reactivity in Human Serum. Compared with age-matched healthy controls (HCs), the titers of IgG directed against membrane-bound $hMOG_{cme}$ were most significantly increased in CIS ($P < 0.001$). Increased titers were also present in RRMS and secondary progressive MS (SPMS) subtypes, compared with HC ($P < 0.01$ and $P < 0.05$, respectively) (Fig. 3). The differences were also significant when comparing primary progressive MS (PPMS) with all other subtypes [$P < 0.001$ (CIS), $P < 0.01$ (RRMS), and $P < 0.05$ (SPMS)]. No statistical difference was found between PPMS and HC (*P* not significant) or between the CIS, RRMS, or SPMS subtypes when paired comparisons were made. No treatment-related difference was found.

IgG Reactivity in Marmoset EAE. Eleven *C. jacchus* marmosets immunized with human white matter were tested for plasma reactivity against $hMOG_{cme}$ 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 $hMOG_{cme}$ was consistently detected in the earliest blood sample obtained after immunization (mean ± SD = 14 ± 2 days; range = 13–18 days) (Table 1) and was clearly present in each animal before the appearance of any clinical sign (mean ± SD = 21 ± 9 days; range = 16–43 days). The difference between time of appearance of serum IgG reactivity to $hMOG_{cme}$ and appear-

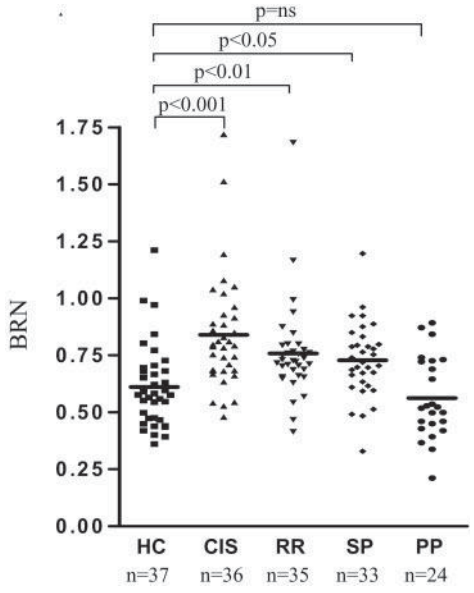

Fig. 3. Analysis of human serum IgG reactivity against $hMOG_{cme}$. BR normalized (BRN) is the Gmean value of IgG binding to MOG-transfected CHO cells divided by that of IgG binding to ntCHO cells, normalized to the value of the positive control tested in each plate. Horizontal bars = median. See text for details.

Table 1. Time course of *C. jacchus* marmoset EAE and serum IgG binding against $hMOG_{cme}$

Animal no.	Clinical onset dpi	Death dpi	Maximal clinical score (dpi)	α-$hMOG_{cme}$ 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 ± 9	52 ± 28	2 ± 1 (49 ± 22)	14 ± 2

dpi, day postimmunization.

ance of clinical signs was highly significant ($P < 0.0001$) (Fig. 4). Reactivity was not detected in preimmune plasma.

Comparison of Human IgG Binding on $hMOG_{125}$ and $hMOG_{cme}$. The serum binding characteristics of the CIS cohort ($n = 36$) were tested by ELISA using recombinant human $MOG_{aa1–125}$ ($hMOG_{125}$) and compared with $hMOG_{cme}$ reactivity by FACS on the MOG-transfected CHO cells. Although some CIS patients displayed high reactivity against $hMOG_{125}$, unlike for $hMOG_{cme}$ reactivity, there was no statistical significant difference between CIS and HCs (data not shown). 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. 5*A*).

Specificity of MOG and Differential MOG-Epitope Binding in Human Serum. To discriminate the epitopes displayed by $hMOG_{125}$ from those displayed on $hMOG_{cme}$ 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

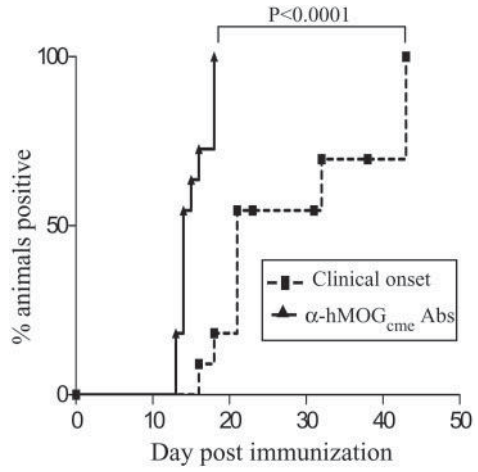

Fig. 4. Time course of serum IgG directed against $hMOG_{cme}$ in marmoset EAE. Results are from 11 EAE *C. jacchus* marmosets immunized with human white matter, 3 of which were killed before onset of clinical disease. First occurrence of serum IgG directed against $hMOG_{cme}$ is compared with time of clinical onset of EAE in a Kaplan–Meier survival plot. Please see text and Table 1 for details.

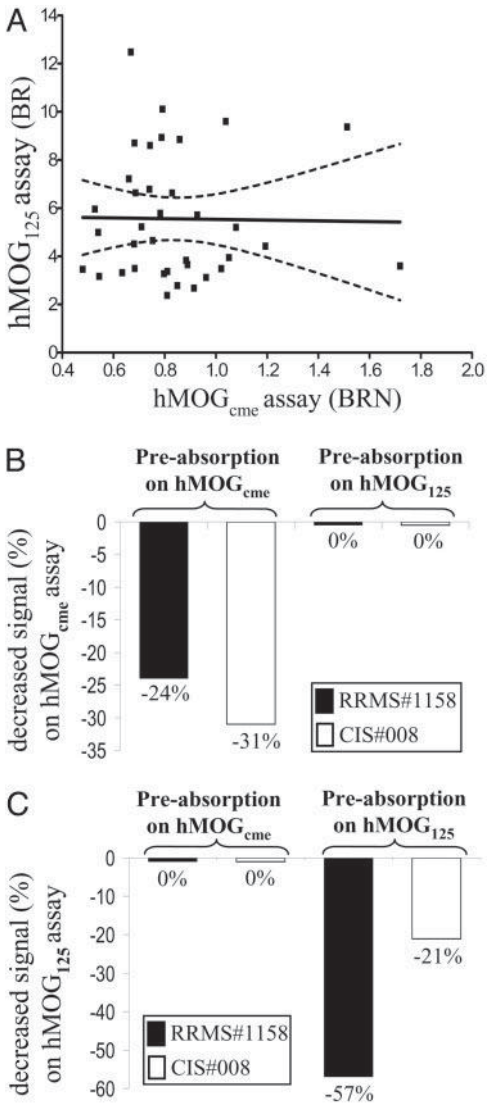

Fig. 5. Selective epitope presentation on $hMOG_{cme}$. (*A*) Binding to $hMOG_{125}$ by ELISA compared with binding to $hMOG_{cme}$ by FACS in the CIS cohort ($n = 36$). By linear regression analysis, there is no correlation between the results of these two methods (P not significant, $r^2 = 0.00023$, Spearman r; straight line is the linear regression curve; dotted line indicates 95% confidence interval) even when clear serum reactivity is present in both assays. BRN, BR normalized. (*B* and *C*) Preabsorption of serum on either $hMOG_{cme}$ (*Left*) or $hMOG_{125}$ (*Right*), followed by testing by FACS (*B*, $hMOG_{cme}$) or ELISA (*C*, $hMOG_{125}$). Preabsorption on $hMOG_{125}$ or $hMOG_{cme}$ only altered the reactivity in the corresponding system of detection. See *Materials and Methods* for additional details.

$hMOG_{cme}$ and $hMOG_{125}$ (CIS 008). Preabsorption against ntCHO cells served as control in the $hMOG_{cme}$ assay (FACS of MOG-transfected CHO cells), and preabsorption against 1% BSA served as a control for the $hMOG_{125}$ ELISA assay. Compared with these controls, preabsorption of either sample on $hMOG_{125}$ did not affect $hMOG_{cme}$ reactivity. Preabsorption on $hMOG_{cme}$ resulted in a decrease in BR (31% for CIS 008 and 24% for RRMS 1158), when tested with $hMOG_{cme}$ expressed on the MOG-transfected cells (Fig. 5*B*). Similarly, when tested on $hMOG_{125}$, no change in reactivity occurred when samples were preabsorbed on the MOG-transfected cells ($hMOG_{cme}$). On the contrary, the samples preabsorbed on $hMOG_{125}$ displayed a decrease in BR, by 21% and 57%, respectively, when tested on $hMOG_{125}$ (Fig. 5*C*). These experiments unequivocally demon-

strate that $hMOG_{cme}$ and $hMOG_{125}$ display separate epitopes of the MOG protein.

Discussion

Methods to study Ab reactivity to MOG (ELISA, Western blot, liquid-phase assay) commonly use linear peptides, polypeptide, 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 $hMOG_{cme}$ 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 $hMOG_{125}$ (solid-phase ELISA) and $hMOG_{cme}$ (MOG-transfected CHO cells), and preabsorption demonstrates that there is no cross-reactivity between epitopes of MOG displayed in these two different assays (Fig. 5). This finding also argues, along with the significant signal quenching in the cell-based assay achieved only by preabsorption on $hMOG_{cme}$ (MOG-transfected cells), and not by preabsorption on either $hMOG_{125}$ or ntCHO cells, against a nonspecific "sticky" effect of MS serum. The $hMOG_{cme}$ 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 $hMOG_{cme}$ 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. There is therefore a humoral immune response specifically directed against intact MOG expressed on myelin oligodendrocytes in those groups of patients. The predominance of $hMOG_{cme}$-specific Abs in CIS suggests that these Abs represent early stages of the immune response against intact (as opposed to degraded) myelin, and thus may represent a marker of inflammatory phases of disease related to blood-brain-barrier opening and/or molecular mimicry. Our results are in partial agreement with a recent report showing an increase of Ig response directed against *ex vivo* glycosylated-native-MOG in first demyelinating events (14). RRMS is the most common MS subtype that includes $\approx$85% of the patients at initial presentation. The secondary progressive pattern is known to follow RRMS in $\approx$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 $hMOG_{cme}$-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 $hMOG_{cme}$ 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 $hMOG_{cme}$ in PPMS is in contrast with the increased IgG reactivity against recombinant $rMOG_{125}$ and against neurons in this disease subtype (unpublished data and ref. 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 $hMOG_{cme}$-reactive Abs in CIS, i.e., 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 $hMOG_{cme}$-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 $hMOG_{cme}$ 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 $hMOG_{cme}$-reactive Abs may be useful to help diagnose MS at its earliest stages. To consolidate this contention, the $hMOG_{cme}$ assay was used to study the time course of the Ab response against $hMOG_{cme}$ in marmoset EAE induced by immunization with human white matter. In these animals, serum reactivity against $hMOG_{cme}$ 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 $hMOG_{cme}$, these findings imply that the $hMOG_{cme}$-reactive Abs are the ones that initiate and/or first result from active demyelination. Regardless of whether they are causative or not, $hMOG_{cme}$-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 $rMOG_{125}$ and had an established anti-MOG Ab response, are the only ones among those tested that recognize $hMOG_{cme}$. 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 patients with a CIS does compete with Fabs M3-31 and M26, these data suggest 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 strongly suggest that analogous to certain serologic markers that are predictive of type I diabetes (23) anti-$hMOG_{cme}$ Abs could be used in humans as a biomarker to diagnose MS or MS risk. Further studies are needed to validate this biomarker and understand the benefits and information that in combination with other Ab profiling techniques (8, 21) it could provide to scientists, treating neurologists, and individuals with suspected or established MS.

Materials and Methods

Patients. Ninety-two patients with clinically definite MS (Poser criteria) (24), 36 patients with CIS, and 37 HCs were recruited from the University of California, San Francisco MS Center, and

Table 2. Clinical characteristics of patients and HCs

Patients	No. of cases	Female/male 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 (0–4)
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 (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)	—	—

EDSS, expanded disability status scale (25).

the MS Center of Pamplona, Spain (CIS). All investigations were conducted according to the Declaration of Helsinki. Blood was obtained by venipuncture after informed consent in full compliance with the Institutional Review Board, and clotted serum was stored at −40°C until use. Patients were classified as RRMS ($n = 35$), SPMS ($n = 33$), and PPMS ($n = 24$) MS by clinical history (24). A CIS was defined by a first clinical event indicative of demyelination with no history of previous neurological symptom. Age, gender, disease duration, and disability state [Expanded Disability Status Score (EDSS) (25)] 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 (Table 2). All RRMS and 19/33 SPMS patients were treated with IFN-β. Two of the PPMS patients were treated with mitoxantrone and monthly pulsed steroids.

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. CHO cells were transfected with a full-length construct corresponding to the major α-1 form of human MOG as described (28). CHO cells were cultured in T-225 flasks (Costar), in RPMI medium 1640 supplemented with 10% FCS, 1× Glutamax, 1 mM sodium pyruvate, and 50 μg/ml gentamycine. G418 (500 μg/ml, GIBCO) was added to the medium of transfected cells. Stable surface expression of MOG was verified by immunofluorescence and FACS on transfected cells after multiple passes and washes; cells were used for flow cytometry (FACS) assay when a confluence of 80–90% was reached. To check for surface expression of $hMOG_{cme}$, 3×10^4 MOG-transfected CHO cells were deposited on a slide and fixed with 100% methanol for 5 min at −20°C. After blocking with PBS containing 2% BSA and 2% FCS for 30 min, cells were incubated 1 h at 37°C with the mouse monoclonal anti-MOG Ab 8-18C5 (5 μg/ml, gift of C. Linington, University of Aberdeen, Aberdeen, Scotland). Fluorescence was revealed after 1-h incubation at 37°C by a goat anti-mouse IgG FITC Ab (Sigma) and examined under a fluorescence microscope (Nikon Eclipse E600). Negative controls were done with secondary Ab alone.

Serum IgG Reactivity. Cells were trypsinized, diluted in FACS buffer (PBS, 0.1% Na azide, and 2% FCS), 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-h incubation at 4°C. After washing, cells were incubated with a goat anti-human IgG FITC (Caltag, South San Francisco, CA) at the recommended concentration for 30 min at 4°C. After a final wash, cells were resuspended in FACS buffer containing propidium iodide (Molecular Probes) at 2 μg/ml and gently shaken. Samples were kept on ice and analyzed by gating the selected live cell population (10^4 cells) within 1 h of harvesting by trypsinization. Each FACS experiment included an internal positive control consisting of the monoclonal anti-MOG 8-18C5 Ab (0.5 μg/ml) and rabbit anti-mouse FITC (DAKO) as secondary Ab. For each sample, the geometric mean intensity (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.

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 $hMOG_{cme}$ was considered positive when the BR (Gmean preimmunization/Gmean time point tested) was >1.5.

Differential Reactivity of Monoclonal Fab Fragments. Recombinant Fabs were derived from a *C. jacchus* marmoset immunized with $rMOG_{1–125}$ produced in *Escherichia coli* ($rMOG_{125}$) (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) diluted at 1:100 was used as secondary Ab for 30 min at 4°C. FACS was performed as described above.

ELISA Assay. $hMOG_{125}$ 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 + 0.05% Tween (BSA-PBS-T) for 2 h at room temperature, sera (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, i.e., signal over BSA background.

Preabsorption of Sera on $hMOG_{cme}$ and $hMOG_{125}$. 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 $hMOG_{cme}$ 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 900 × *g* 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 3,600 $\times$ *g* for 5 min and collected for subsequent experiments.

For $hMOG_{125}$ preabsorption, ELISA plates were coated with either 1 μg BSA or 0.5 μg $hMOG_{125}$ 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 $hMOG_{125}$. After the final preabsorption step, supernatants were collected as above.

Data Analysis. Statistical analyses were performed by using Kruskal–Wallis with Dunn's post hoc test for multiple comparisons for interpatient group differences. Interassay correlation was analyzed with Spearman *r* correlation. Survival analysis for time-dependent variables was assessed by Kaplan–Meyer analysis and the Cox proportional hazard model (PRISM 3.0).

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1. Steinman, L. (1996) *Cell* **85,** 299–302.
2. Genain, C. P., Cannella, B., Hauser, S. L. & Raine, C. S. (1999) *Nat. Med.* **5,** 170–175.
3. Lucchinetti, C., Bruck, W., Parisi, J., Scheithauer, B., Rodriguez, M. & Lassmann, H. (2000) *Ann. Neurol.* **47,** 707–717.
4. Mesleh, M. F., Belmar, N., Lu, C.W., Krishnan, V. V., Maxwell, R. S., Genain, C. P. & Cosman, M. (2002) *Neurobiol. Dis.* **9,** 160–172.
5. von Budingen, H.-C., Tanuma, N., Villoslada, P., Ouallet, J. C., Hauser, S. L. & Genain, C. P. (2001) *J. Clin. Immunol.* **21,** 155–170.
6. Schluesener, H. J., Sobel, R. A., Linington, C. & Weiner, H. L. (1987) *J. Immunol.* **139,** 4016–4021.
7. Genain, C. P., Nguyen, M. H., Letvin, N. L., Pearl, R., Davis, R. L., Adelman, M., Lees, M. B., Linington, C. & Hauser, S. L. (1995) *J. Clin. Invest.* **96,** 2966–2974.
8. Berger, T., Rubner, P., Schautzer, F., Egg, R., Ulmer, H., Mayringer, I., Dilitz, E., Deisenhammer, F. & Reindl, M. (2003) *N. Engl. J. Med.* **349,** 139–145.
9. Haase, C. G., Guggenmos, J., Brehm, U., Andersson, M., Olsson, T., Reindl, M., Schneidewind, J. M., Zettl, U. K., Heidenreich, F., Berger, T., *et al.* (2001) *J. Neuroimmunol.* **114,** 220–225.
10. Carotenuto, A., D'Ursi, A. M., Nardi, E., Papini, A. M. & Rovero, P. (2001) *J. Med. Chem.* **44,** 2378–2381.
11. Ichikawa, M., Johns, T. G., Adelmann, M. & Bernard, C. C. (1996) *Int. Immunol.* **8,** 1667–1674.
12. von Budingen, H. C., Hauser, S. L., Fuhrmann, A., Nabavi, C. B., Lee, J. I. & Genain, C. P. (2002) *Proc. Natl. Acad. Sci. USA* **99,** 8207–8212.
13. Brehm, U., Piddlesden, S. J., Gardinier, M. V. & Linington, C. (1999) *J. Neuroimmunol.* **97,** 9–15.
14. Gaertner, S., de Graaf, K. L., Greve, B. & Weissert, R. (2004) *Neurology* **63,** 2381–2383.
15. Lampasona, V., Franciotta, D., Furlan, R., Zanaboni, S., Fazio, R., Bonifacio, E., Comi, G. & Martino, G. (2004) *Neurology* **62,** 2092–2094.
16. Kerlero de Rosbo, N., Hoffman, M., Mendel, I., Yust, I., Kaye, J., Bakimer, R., Flechter, S., Abramsky, O., Milo, R., Karni, A., *et al.* (1997) *Eur. J. Immunol.* **27,** 3059–3069.
17. Hu, Y., Doudevski, I., Wood, D., Moscarello, M., Husted, C., Genain, C., Zasadzinski, J. A. & Israelachvili, J. (2004) *Proc. Natl. Acad. Sci. USA* **101,** 13466–13471.
18. Weinshenker, B. G., Bass, B., Rice, G. P., Noseworthy, J., Carriere, W., Baskerville, J. & Ebers, G. C. (1989) *Brain* **112,** 1419–1428.
19. Runmarker, B. & Andersen, O. (1993) *Brain* **116,** 117–134.
20. Silber, E., Semra, Y. K., Gregson, N. A. & Sharief, M. K. (2002) *Neurology* **58,** 1372–1381.
21. Menge, T., Lalive, P. H., von Büdingen, H.-C., Cree, B., Hauser, S. L. & Genain, C. P. (2005) *J. Allergy Clin. Immunol.* **116,** 453–460.
22. Marta, C. B., Montano, M. B., Taylor, C. M., Taylor, A. L., Bansal, R. & Pfeiffer, S. E. (2005) *J. Biol. Chem.* **280,** 8985–8993.
23. Verge, C. F., Gianani, R., Kawasaki, E., Yu, L., Pietropaolo, M., Jackson, R. A., Chase, H. P. & Eisenbarth, G. S. (1996) *Diabetes* **45,** 926–933.
24. Poser, C. M., Paty, D. W., Scheinberg, L., McDonald, W. I., Davis, F. A., Ebers, G. C., Johnson, K. P., Sibley, W. A., Silberberg, D. H. & Tourtellotte, W. W. (1983) *Ann. Neurol.* **13,** 227–231.
25. Kurtzke, J. F. (1983) *Neurology* **33,** 1444–1452.
26. Genain, C. P. & Hauser, S. L. (2001) *Immunol. Rev.* **183,** 159–172.
27. Massacesi, L., Genain, C. P., Lee-Parritz, D., Letvin, N. L., Canfield, D. & Hauser, S. L. (1995) *Ann. Neurol.* **37,** 519–530.
28. della Gaspera, B., Pham-Dinh, D., Roussel, G., Nussbaum, J. L. & Dautigny, A. (1998) *Eur. J. Biochem.* **258,** 478–484.

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Highly reactive anti-myelin oligodendrocyte glycoprotein antibodies differentiate demyelinating diseases from viral encephalitis in children

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Abstract

Background: Myelin oligodendrocyte glycoprotein (MOG) may be implicated in the immunopathogenesis of multiple sclerosis (MS) inducing demyelination in the animal model of MS. In adults reported anti-MOG antibody frequencies have been variable across a number of studies and can also be detected in controls.

Objective: To measure antibodies against MOG in paediatric patients with demyelinating disorders of the central nervous system and in controls.

Methods: Serum antibodies against MOG and myelin basic protein were measured by ELISA, flow cytometry (FACS) and in the liquid phase in 11 children with acute disseminated encephalomyelitis (ADEM), 22 children with MS, seven children with acute viral encephalitis and 13 healthy controls. The serostatus of Epstein–Barr virus (EBV) infections were assessed.

Results: Anti-MOG antibodies, measured either by ELISA or FACS were exclusively detected in children with demyelination. In ADEM these antibodies were highly reactive. Anti-MBP reactivity was detectable equally in all groups. The presence of either autoantibodies did not associate with EBV serostatus, age, gender or disease course.

Conclusions: This study independently corroborates recently published results of seroprevalence and specificity of the assay. Due to their low sensitivity anti-MOG antibodies will not serve as disease-specific biomarkers, but could help to support the diagnosis of ADEM in difficult cases.

Keywords

acute disseminated encephalomyelitis, antibody assays, autoantibodies, Epstein–Barr virus, myelin/oligodendrocyte glycoprotein, paediatric multiple sclerosis

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Introduction

Formation of autoantibodies directed against myelin antigens may be induced when the immune system encounters infectious pathogens by virtue of molecular mimicry.[1] Specifically, autoantibodies reactive against myelin oligodendrocyte glycoprotein (MOG) have been

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implicated in multiple sclerosis (MS) and acute disseminated encephalomyelitis (ADEM), because they exhibit demyelinating properties in experimental settings.[2] Anti-MOG antibodies can be detected in both adult patients with MS and controls with varying frequencies and low specificity.[3] Recently, reactivity against native MOG epitopes was assessed in children affected with MS and ADEM.[4–7]

Several lines of evidence render the Epstein–Barr virus (EBV) a candidate environmental factor contributing to the pathogenesis of MS; anti-EBV antibodies are significantly more prevalent in patients with MS, and appear to be associated with paraclinical disease activity as measured by magnetic resonance imaging (MRI).[8,9] Additionally, cross-reactivity between EBV-specific T cells and myelin antigens, namely myelin basic protein (MBP) has been reported.[10,11]

We aimed to compare the anti-MOG IgG status of paediatric patients with different inflammatory central nervous system (CNS) diseases, including MS, ADEM and viral encephalitis, with a control cohort of healthy children. Paediatric patients provide an invaluable opportunity to study antibody responses against autoantigens and viral antigens very early in the disease course when only limited myelin damage has occurred. Technical issues, such as MOG preparations and detection assays used, have been suspected to be at least partially the reason for conflicting inter-laboratory results.[3] We therefore employed an ELISA and a liquid-phase immunoassay using recombinant human MOG,[12] a cell-based cytometric assay for the detection of antibody reactivities against native, membrane-embedded MOG[13] as well as an ELISA against purified MBP. These antibody frequencies were correlated to the EBV serostatus given the association of EBV with MS and the cross-reactivity between EBV and MBP.[8,10,11]

Methods

Patients

The patient sera were derived from different sources. They were either sent for routine anti-MOG diagnostics in the course of acute inflammatory CNS diseases and were included retrospectively to perform extended anti-MOG testing. The majority of sera had been obtained in the course of prospective studies on inflammatory CNS disease in childhood after written parental consent had been obtained, in accordance with the local ethics committees' regulations. Eleven paediatric patients with ADEM, recruited in France, in the course of the KIDSEP study of the French Neuropediatric Society, and by the Department of Pediatrics of RWTH Aachen University Hospital, Germany, and 22 children with clinically definite MS (i.e. documented dissemination in time and space) were included. Diagnosis of ADEM was made as previously described.[14] Serum was stored at -20°C until assayed. Seven children with serologically proven acute viral encephalitis and 13 healthy children (HC) served as controls. HC sera had been collected in the course of a prospective study approved by the Ethics Committee of RWTH Aachen University Hospital focusing on inflammatory CNS diseases in infants and children. The patients' demographics are compiled in Table 1.

Immunoassays against myelin oligodendrocyte glycoprotein and myelin basic protein

Serum IgG antibodies against human recombinant MOG spanning its extracellular domain ($rhMOG_{125}$) were detected by established assay methodology using an ELISA and a liquid-phase assay (LiPhELIA)

Table 1. Demographics and sero-status to Epstein–Barr virus antigens

Parameter	ADEM	MS	Encephalitis	Controls
Number of patients	11	22	7	13
Age (years; mean ± SD)	7.1 ± 4.1	14.9 ± 2.5[a]	7.1 ± 6.2	11.1 ± 3.4
Sex[b] (% female)	28	82	100	15
EDSS[c] (median; IQR)	3.5 (2.0–5.0)	2.0 (1.0–3.0)	NA	NA
OCB (positive/negative)	1/5	22/0	NA	NA
Follow-up (months; median; IQR)	59 (15–85)	NA	NA	NA
EA-IgG (% positive)	0.0	9.1	0.0	15.4
EBNA-IgG (% positive)	27.3	100	85.7	76.9
VCA-IgG (% positive)	27.3	100	85.7	69.2

[a]Older than ADEM and encephalitis ($p < 0.05$; ANOVA).
[b]Sex matching was not deliberately performed.
[c]EDSS of ADEM and pediatric MS not statistically different ($p > 0.05$; Mann–Whitney test).
ADEM: acute disseminated encephalomyelitis; EA: early antigen; EBNA: Epstein–Barr nuclear antigen; EDSS: Expanded Disability Status Scale; IgG: immunoglobulin G; IQR: interquartile range; MS: multiple sclerosis; OCB: oligoclonal bands; NA: not applicable; VCA: virus capsid antigen.

as described.[12,13] Sera of marmosets immunized with MOG served as positive controls.[12] ELISA plates were also coated with purified human MBP (AbD Serotec, Oxford, UK) at 0.5 μg/well. Results were expressed as binding ratio (BR) (i.e. optical density against $rhMOG_{125}$/MBP divided by optical density against background).[12]

Antibodies against native, membrane-embedded MOG were detected by flow cytometry using MOG-transfected Chinese hamster ovary (CHO) cells as previously described, termed the CHO-MOG assay.[13] The BR was defined as the mean fluorescence intensity (MFI) obtained from the MOG-transfected CHO cells divided by the MFI of non-transfected cells.

Anti-Epstein–Barr virus humoral responses

IgG reactivity against the EBV antigens early antigen (EA), virus capsid antigen (VCA) and Epstein–Barr nuclear antigen (EBNA) was determined using commercial ELISA kits, following the manufacturer's instructions (Institut Virion\Serion, Würzburg, Germany). Results were expressed quantitatively (U/ml). Seropositivity was defined by the lot specific cut-off values provided by the manufacturer.

Data analysis and statistics

Samples were deemed positive if the individual BR was greater than a cut-off BR defined from the HC samples; that is, the mean OD + 2 standard deviations. Positive samples were considered *highly reactive* if the sample's BR was (1) above the mean BR of HC plus 5 × SD, and also (2) 5-fold above its individual background (i.e. BR > 5). Data were analysed using GraphPad Prism 4.0 for Windows (La Jolla, CA, USA) by the Kruskal–Wallis test and Dunn's post hoc test for multiple comparison given the small samples sizes. Categorical datasets were compared by the chi-square test.

Results

Of the 53 children recruited for this study, 33 were affected by demyelinating CNS disorders, ADEM or MS. Patients with MS were significantly older than patients with ADEM and encephalitis (Table 1). In addition, owing to the inherent difficulties of recruiting healthy paediatric controls, the cohorts were not sex-matched (Table 1).

Children with demyelinating disorders, ADEM or MS, were significantly more frequently antibody positive against $rhMOG_{125}$ as measured by ELISA than controls or patients with encephalitis (27.3% vs. 5.0%; $p = 0.045$, χ^2, Figure 1). The majority of these samples (77.8%) were highly reactive; that is, they had strong antibody reactivities as defined. Antibodies against native MOG in the CHO-MOG assay were also significantly more frequent in the demyelinating disorders cohort (12.1% vs. 0.0%; $p = 0.049$; χ^2, Figure 1), and were considered highly reactive in all ADEM samples (mean ratio 10.4). In contrast, anti-MBP reactivity was not exclusively associated with demyelination (18.2% seropositivity in ADEM/MS vs. 10% in encephalitis/controls; $p = 0.42$; χ^2). Overall, anti-MOG reactivity could be detected in 30.3% of demyelinating samples with either assay compared to 5% in the non-demyelinating samples ($p = 0.0049$; χ^2). Reactivity against native MOG (CHO-MOG assay) associated with anti-$rhMOG_{125}$ reactivity as measured by ELISA in 75% of cases with demyelination.

Autoantibody status was not associated with age, gender, presence of oligoclonal bands or clinical disability as measured by the Expanded Disability Status Scale (EDSS) in any of the groups (data not shown).

Importantly, reactivity against soluble $rhMOG_{125}$ as measured by LiPhELIA could not be detected in any of the patients or controls (data not shown).

Samples were also analysed for the presence of IgG antibodies against EBNA and VCA indicative of an EBV infection in the past, and against EA to identify recently infected patients. While all patients with MS, 85.7% of patients with encephalitis, and 76.9% of controls were EBNA-/VCA-IgG positive, only 27.3% of the ADEM were EBNA-/VCA-IgG positive, a significant difference ($p < 0.001$; χ^2; Table 1). It has to be noted, however, that, overall EBNA-IgG negative patients were significantly younger than those positive for EBNA-IgG (mean age 7.5 ± 4.2 years vs. 12.5 ± 4.6 years; $p = 0.0015$, unpaired T-test). Titres of EBNA-IgG were not different between groups (data not shown). Neither the serostatus nor the titre of EBNA-IgG were associated with the presence of autoantibodies against MOG or MBP (data not shown).

Discussion

The major finding of this study was that the anti-MOG humoral immune response is exclusively confined to paediatric demyelinating CNS disorders, whereas CNS inflammation alone, as apparent in seven patients with viral encephalitis, did not induce such a response. Secondly, anti-MOG reactivity could not be detected in paediatric controls and is thus specific for CNS demyelination. This is in contrast to anti-MOG IgG reactivity in adults; for example, anti-MOG IgG positivity in 32.9% of healthy adults in a recent study.[3,15] These findings are corroborated by the observation that reactivity against MBP is equally detectable in patients with MS/ADEM and in patients with encephalitis. Notably, while MOG is known to induce

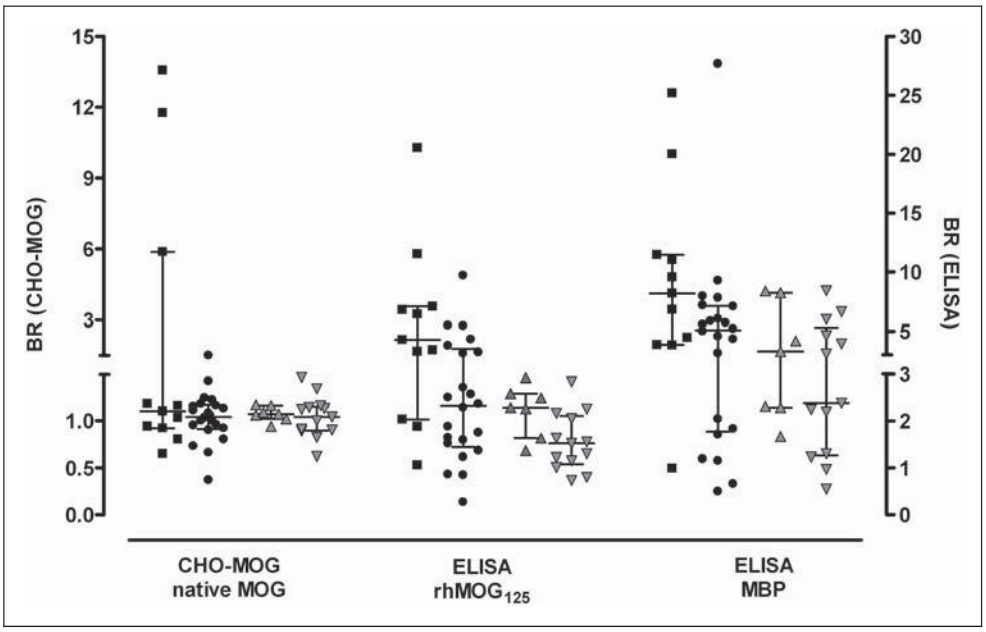

Figure 1. IgG reactivity against cell-expressed native MOG (CHO-MOG), rhMOG$_{125}$, and MBP in paediatric patients with ADEM (black squares), MS (black circles), viral encephalitis (grey triangles) and healthy controls (grey reverse triangles). Results were expressed as binding ratios (BRs). Horizontal bars represent medians and interquartile ranges. ELISA: enzyme-linked immunosorbent assay; ADEM: acute disseminated encephalomyelitis; MBP: myelin basic protein; MOG: myelin oligodendrocyte glycoprotein; MS: multiple sclerosis.

Table 2. Summary of studies testing for anti-MOG IgG antibodies in pediatric cohorts

Study	Assay	CNS demyelinating disease (*n*)	% positive	Control group (*n*)	% positive
This study	FACS	ADEM (11)/pMS (22)	27.3/4.5	Healthy controls (13)	0
	ELISA		54.5/13.6		0
	LiPhELIA		0/0		0
O'Connor et al.[4]	RIA (tetramer)	ADEM (56)/pMS (19)	18/5		
	FACS		9/5		
McLaughlin et al.[5]	FACS	Paediatric MS (131)	21.3	OND (34)	6
				NND (37)	0
				Juvenile diabetes (28)	0
Brilot et al.[7]	FACS	ADEM/CIS (47)	47	Healthy controls (30)	0
				OND (29)	7

ADEM: acute disseminated encephalomyelitis; ELISA: enzyme-linked immunosorbent assay; FACS: fluorescence activated cell sorting; LiPhELIA: liquid phase enzyme-linked immunoassay;[12] MS: multiple sclerosis; OND: other neurological diseases; NND: non-neurological diseases; pMS: paediatric MS; RIA: radioimmunoassay.

demyelination experimentally, MBP causes inflammation but no demyelination in animal models of MS.[2]

Despite the restriction of anti-MOG reactivity to patients with ADEM/MS, this was not very sensitive, being present in 30.3% of the patients only. This is in line with several recently published studies on paediatric patients with demyelinating CNS disorders (Table 2), reporting anti-MOG reactivity in 18–47% of the patients. The reasons for these discrepant anti-MOG prevalences are most likely the different assays systems used, different cell lines and transfection techniques, different detection methods applied and cut-off margins for seropositivity individually defined by each study.[4,5,7] The specificity of anti-MOG antibodies for ADEM/MS in contrast, appears to be high and was calculated at 95% in our study, which is consistent

with the previous studies.[4,5,7] In line with these studies our data confirm that antibodies against native MOG are highly reactive;[4,5,7] the mean CHO-MOG ratio was at least five-fold higher than the highest samples of our previously published adult MS cohort (10.4 vs. 1.72, CIS sample).[13] It is intriguing to speculate about the pathogenetic mechanism behind this phenomenon; however larger numbers of ADEM patients are needed to robustly detect possible associations, for instance the amount of myelin damage as measured by MRI. Thus, our study confirms that anti-MOG antibodies do not serve as diagnostic biomarkers due to lack of sensitivity. Yet, it may be helpful to measure MOG reactivity in uncertain cases; the presence of highly reactive anti-MOG IgG would be indicative of ADEM. It is, however, important to note that one case of ADEM tested positive for anti-MOG, was later re-diagnosed as MS; this was due to a second relapse that occurred 7 months after the initial presentation leading to the diagnosis of ADEM. The median follow-up of the ADEM cases was 59 months (Table 1), and it is thus quite unlikely that further cases of ADEM will have relapses and will have to be reclassified as MS given that most relapses occur with in first 12 months after ADEM diagnosis.[16]

Comparable with the data by Selter et al., anti-MOG IgG serostatus in our study did not allow to distinguish ADEM from relapsing cases and were not associated with EBNA-IgG.[6] However, in our ADEM cohort the proportion of EBV-infected children was considerably lower (27.3% vs. 42.1%), but the EBNA-IgG prevalences of controls higher (91% vs. 50%) due to the younger age of the patients with ADEM (Table 1).[6]

Interestingly, despite highly reactive samples in both the ELISA and the CHO-MOG assay none of the samples was reactive to soluble MOG corroborating our previous conclusion that soluble MOG does not expose epitopes relevant to demyelination.[12] It is intriguing to speculate about the discrepant specificities of anti-MOG observed paediatric cohorts (this study, Table 2) versus adult cohorts.[3,15] While it was shown that molecular mimicry to EBV infection may not be the only infectious agent triggering demyelinating CNS disease, other infectious pathogens or non-infectious proteins are possible candidates for cross-reactivity with CNS antigens, that children may not have yet encountered at blood sampling.[17,18] It is worth noting that the missing association between anti-MOG antibodies and EBV clearly does not rule out the possible involvement of EBV in the pathogenesis of MS.

In summary, this report provides evidence that the humoral immune response against MOG in children is specific for demyelinating inflammatory CNS disorders. Our results confirm independently, by different assay methodologies and patient cohorts, previous reports of low sensitivity of anti-MOG antibodies in children with MS and ADEM. This is particular noteworthy as in adults large numbers of studies yielded widely divergent results. Thus in children suspected to be affected by ADEM, the measurement of highly reactive anti-MOG IgG may supplement the diagnostic tools and may help to differentiate from acute encephalitis.

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Conflict of interest statement

Dr. Wiendl has received funding for travel and speaker honoraria from Bayer Schering Pharma, Biogen Idec/Elan Corporation, Sanofi-Aventis, Merck Serono, and Teva Pharmaceutical Industries Ltd; he has served/serves as a consultant for Merck Serono, Medac, Inc., Sanofi-Aventis/Teva Pharmaceutical Industries Ltd, Biogen Idec, Bayer Schering Pharma, Novartis, and Novo Nordisk; and he receives research support from Bayer Schering Pharma, Biogen Idec/Elan Corporation, Sanofi-Aventis, Merck Serono, and Novo Nordisk. Dr. Schroeter has received honoraria from Astellas Pharma, Bayer Healthcare, Biogen Idec, GlaxoSmithKline, Janssen Cilag, Merck Serono, Pfizer, Roche, Sanofi Aventis, Talecris, Teva. Dr. Kieseier has received honoraria for lecturing, travel expenses for attending meetings, and financial support for research from Baxter, BayerSchering, Biogen Idec, Medac, Merck Serono, Novartis, Roche, SanofiAventis, Talecris, and TEVA Neurosciences. Dr. Menge has received honoraria for lecturing, for travel expenses and for consulting from Bayer Schering, Biogen Idec, Merck Serono and Novartis.

References

1. Sospedra M and Martin R. Immunology of multiple sclerosis. *Annu Rev Immunol* 2005; 23: 683–747.
2. von Büdingen HC, Tanuma N, Villoslada P, Ouallet JC, Hauser SL and Genain CP. Immune responses against the myelin/oligodendrocyte glycoprotein in experimental autoimmune demyelination. *J Clin Immunol* 2001; 21: 155–170.
3. Reindl M, Khalil M and Berger T. Antibodies as biological markers for pathophysiological processes in MS. *J Neuroimmunol* 2006; 180: 50–62.
4. O'Connor KC, McLaughlin KA, De Jager PL, Chitnis T, Bettelli E, Xu C, et al. Self-antigen tetramers discriminate between myelin autoantibodies to native or denatured protein. *Nat Med* 2007; 13: 211–217.
5. McLaughlin KA, Chitnis T, Newcombe J, Franz B, Kennedy J, McArdel S, et al. Age-dependent B cell autoimmunity to a myelin surface antigen in pediatric multiple sclerosis. *J Immunol* 2009; 183: 4067–4076.
6. Selter RC, Brilot F, Grummel V, Kraus V, Cepok S, Dale RC, et al. Antibody responses to EBV and native MOG in pediatric inflammatory demyelinating CNS diseases. *Neurology* 2010; 74: 1711–1715.

7. Brilot F, Dale RC, Selter RC, Grummel V, Kalluri SR, Aslam M, et al. Antibodies to native myelin oligodendrocyte glycoprotein in children with inflammatory demyelinating central nervous system disease. *Ann Neurol* 2009; 66: 833–842.
8. Ascherio A and Munger KL. Environmental risk factors for multiple sclerosis. Part I: The role of infection. *Ann Neurol* 2007; 61: 288–299.
9. Farrell RA, Antony D, Wall GR, Clark DA, Fisniku L, Swanton J, et al. Humoral immune response to EBV in multiple sclerosis is associated with disease activity on MRI. *Neurology* 2009; 73: 32–38.
10. Wucherpfennig KW and Strominger JL. Molecular mimicry in T cell-mediated autoimmunity: viral peptides activate human T cell clones specific for myelin basic protein. *Cell* 1995; 80: 695–705.
11. Lunemann JD, Jelcic I, Roberts S, Lutterotti A, Tackenberg B, Martin R, et al. EBNA1-specific T cells from patients with multiple sclerosis cross react with myelin antigens and co-produce IFN-gamma and IL-2. *J Exp Med* 2008; 205: 1763–1773.
12. Menge T, von Büdingen HC, Lalive PH and Genain CP. Relevant antibody subsets against MOG recognize conformational epitopes exclusively exposed in solid-phase ELISA. *Eur J Immunol* 2007; 37: 3229–3239.
13. Lalive PH, Menge T, Delarasse C, Della GB, Pham-Dinh D, Villoslada P, et al. Antibodies to native myelin oligodendrocyte glycoprotein are serologic markers of early inflammation in multiple sclerosis. *Proc Natl Acad Sci U S A* 2006; 103: 2280–2285.
14. Mikaeloff Y, Caridade G, Husson B, Suissa S and Tardieu M. Acute disseminated encephalomyelitis cohort study: prognostic factors for relapse. *Eur J Paediatr Neurol* 2007; 11: 90–95.
15. Wang H, Munger KL, Reindl M, O'Reilly EJ, Levin LI, Berger T, et al. Myelin oligodendrocyte glycoprotein antibodies and multiple sclerosis in healthy young adults. *Neurology* 2008; 71: 1142–1146.
16. Menge T, Hemmer B, Nessler S, Wiendl H, Neuhaus O, Hartung HP, et al. Acute disseminated encephalomyelitis: an update. *Arch Neurol* 2005; 62: 1673–1680.
17. Guggenmos J, Schubart AS, Ogg S, Andersson M, Olsson T, Mather IH, et al. Antibody cross-reactivity between myelin oligodendrocyte glycoprotein and the milk protein butyrophilin in multiple sclerosis. *J Immunol* 2004; 172: 661–668.
18. Hughes LE, Smith PA, Bonell S, Natt RS, Wilson C, Rashid T, et al. Cross-reactivity between related sequences found in Acinetobacter sp., *Pseudomonas aeruginosa*, myelin basic protein and myelin oligodendrocyte glycoprotein in multiple sclerosis. *J Neuroimmunol* 2003; 144: 105–115.

Antibody responses against galactocerebroside are potential stage-specific biomarkers in multiple sclerosis

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Background: Galactocerebroside, the major glycolipid of central nervous system myelin, is a known target for pathogenic demyelinating antibody responses in experimental allergic encephalomyelitis (EAE), the animal model of multiple sclerosis (MS).

Objective: To address the importance of anti-galactocerebroside (α-GalC) antibodies in MS and to evaluate them as biomarkers of disease.

Methods: α-GalC IgGs were quantified from sera of patients with MS and in marmoset EAE by a new immunosorbent assay.

Results: We report a significant difference in serum α-GalC IgG titers between patients with relapsing-remitting (RR)–MS and healthy controls (HCs; $P < .001$). The frequencies of α-GalC antibody-positive subjects (α-GalC titers ≥ mean HC titers + 3 SD) are also significantly elevated in RR-MS compared with HC (40% vs 0%; $P = .0033$). Immunoaffinity purified α-GalC IgGs from human serum bind to cultured human oligodendrocytes, indicating that the ELISA detects a biologically relevant epitope. Corroborating these findings, α-GalC antibody responses in marmoset EAE were similarly found to be specifically associated with the RR forms and not the peracute or progressive forms, in contrast with other anti-myelin antibodies ($P = .0256$).

Conclusion: (1) α-GalC antibodies appear MS-specific and are not found in healthy subjects, unlike antibodies against myelin proteins; (2) when present, α-GalC antibodies identify mostly RR-MS and may 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.

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Key words: *Galactocerebroside, myelin antigens, autoantibody, multiple sclerosis, experimental allergic encephalomyelitis*

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 anti-galactocerebroside (α-GalC) and α-MOG antibodies.[10-13] Antibody responses against these myelin targets are thus factors that potentially regulate

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Abbreviations used
AM: Acute monophasic
CIS: Clinically isolated syndrome
CNS: Central nervous system
EAE: Experimental allergic encephalomyelitis
GalC: Galactocerebroside
α-GalC: Anti-galactocerebroside
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

disease phenotype expression in the context of established CNS inflammation.

The pathogenic involvement of anti-myelin antibodies in human MS is less well established, because antibody titers against the myelin proteins do not unequivocally differ between control populations and patients with MS.[14-19] However, regardless of pathogenicity, anti-myelin 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 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.

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 secondary-progressive (SP)–MS, and 16 primary-progressive (PP)–MS (Table I). 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. 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.

Animals

Callithrix 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 −40°C. The animals were scored every other day for the development of clinical signs and disability using a previously published scale.[22]

α-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 ug/mL, with 100 uL 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 double-distilled H_2O and blocked with 1% BSA (A7030; Sigma, St Louis, Mo) in PBS (ELISA buffer) for 2 hours at RT. After washing with PBS and ddH_2O, 100 uL 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-anti-human IgG (A9544; Sigma) or by a horseradish peroxidase–conjugated rabbit-anti-monkey 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-anti-bovine galactocerebroside antiserum (G9152; Sigma) was used and antibody binding detected by a horseradish peroxidase–labeled goat-anti-rabbit 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 ug/mL and further diluted in ELISA buffer to a final concentration of 2 ug/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 ug 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-anti-monkey 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 + 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

TABLE I. Characteristics of patients with MS and HCs

Variable	HC	CIS	RR-MS	SP-MS	PP-MS
N	20	14	20	15	16
Sex					
Female	10	9	15	9	10
Male	10	5	5	6	6
Age (y)					
Median	51.0	36.0*	43.0	45.0	51.0
Range	28-71	23-50	25-61	37-60	40-65
Disease duration (mo)					
Median	NA	NA	120	120	48
Range	NA	NA	9-266	24-384	9-216
Expanded Disability Status Scale					
Median	NA	1.5†	2.0‡	5.5	4.5
SD	NA	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).
†$P < .001$ and ‡$P < .01$ if compared with SP-MS or PP-MS, respectively (Kruskal-Wallis test with Dunn post hoc test for multiple comparisons).

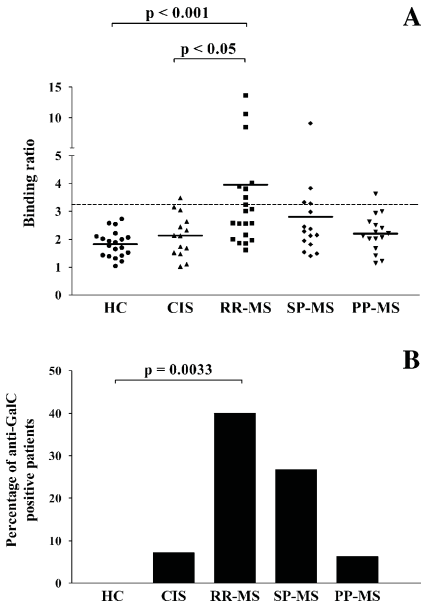

FIG 1. Binding ratios and frequencies of α-GalC IgG responses in human MS and HCs. **A,** α-GalC IgG binding ratios for each disease subgroup. *Solid lines (—)* denote mean binding ratios; *dashed line (--)* denotes threshold of detection (mean binding ratio of HC + 3 SD (see *B*). **B,** Frequencies of α-GalC IgG seropositivity in human sera.

analysis was conducted by using STATA 7.0 (StataCorp LP, College Station, Tex) and GraphPad Prism 3.0 (GraphPad Software, San Diego, Calif). 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 ice-cold 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 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 galactocerebroside solubilized in ELISA buffer (maximal solubility concentration, 2 ug/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 detection was 6.25 ug IgG per well. The interplate and intraplate coefficients of variation were 15% and 4%, respectively.

Detection of α-GalC IgG in patients with MS

Quantitatively, significant differences in α-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; Fig 1, *A*). There was a trend suggesting a difference for the antibody titers between SP-MS and HC ($P = .092$). In contrast, there were no significant differences for α-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 compared with the HC group remained highly significant ($P < .01$).

The threshold for positivity was 3.23 and is indicated in Fig 1, *A* (*dashed line;* see Methods). The frequencies of patients with RR-MS identified as α-GalC antibody–positive by this analysis were significantly higher compared with HC (40% vs 0%; $P = .0033$; Fisher exact test

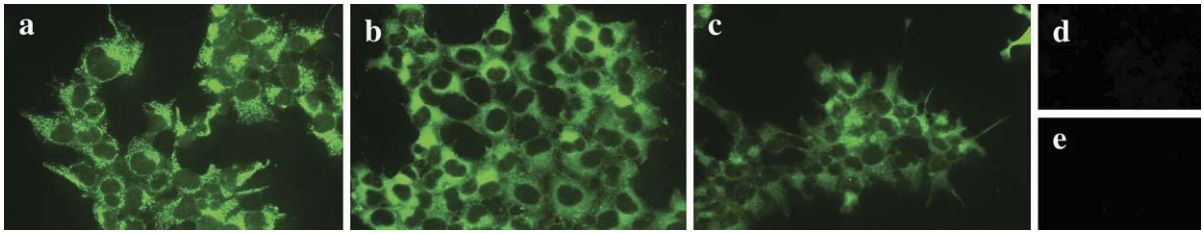

FIG 2. Immunostaining of HOG cells with affinity-purified human α-GalC IgG. **A,** Affinity purified α-GalC IgG (1006-GalC) at 30 ug/mL. **B,** Positive control (rabbit α-GalC antiserum) at 1:50 dilution. **C,** Staining with serum of 1006-GalC at dilution 1:50. **D and E,** Negative controls: fluorescein isothiocyanate–labeled anti-human and anti-rabbit IgG.

with Bonferroni correction for multiple independent comparisons). Again, there was a trend observed for α-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 (Fig 1, *B*).

Immunoaffinity purification of α-GalC IgG and immunohistochemistry

To assess the specificity and biological relevance of the ELISA assay, serum of 1 patient demonstrating a high α-GalC response (#1006) was subjected to immunoaffinity purification of α-GalC IgG. From 50 mL serum, 190 ug 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 ug/mL), and the signal could be quenched by soluble galactocerebroside (45% signal reduction). These galactocerebroside-purified IgGs showed staining of the human oligodendrocytoma cell line HOG identical to the control rabbit α-GalC specific antiserum (Fig 2, *A 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.

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 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 II.

Antibodies against rMOG and MBP were detected in all but 1 of the animals regardless of their disease course, including the preclinical animals (Table II). In contrast, α-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 II). However, this could have resulted from the overall shorter observation period for these animals (median, 28 and 60 days postimmunization vs 70 days postimmunization for RR-EAE; Table II).

The α-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, $P = .001$; Fig 3), indicating that α-GalC antibodies occurred most distant from immunizations and thus onset of EAE in these animals.

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

TABLE II. Characteristics of *C jacchus* marmoset EAE and antibody status

Animal	Disease course	Clinical onset (day PI)	Sacrifice (day PI)†	Maximal clinical score (day PI)†	α-GalC IgG	First day detected (PI)‡	α-rMOG IgG	First day detected (PI)‡	α-MBP IgG	First day detected (PI)‡
326-91	RR	14	120	4 (120)	+	23	+	63	+	23
106-90	RR	16	112	3 (96)	+	80	+	53	+	25
191-92	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	+	15
U057-02	RR	32	82	1.5 (73)	+	62	+	62	+	18
U050-00	RR	21	78	3 (38)	+	78	+	29	+	29
Median ± SD	RR	21 ± 11	98 ± 16	2.0 ± 0.8 (97 ± 16)		70 ± 25		42 ± 17		29 ± 12
127-93	CP	16	68	4 (56)	−	−	+	54*	+	26*
U052-01	CP	21	52	3 (40)	−	−	+	36	+	18
U025-00	AM	21	61	2 (57)	−	−	+	42	+	28
U023-00	AM	16	31	2 (28)	−	−	+	28	+	28
273-93	AM	12	28	3.5 (24)	−	−	+	28*	+	28*
274-93	AM	16	28	2 (20)	−	−	+	30*	+	30*
U021-99	AM	18	23	1.5 (23)	−	−	+	20	+	20
346-92	AM	17	22	3 (22)	−	−	+	23	+	23
Median ± SD	AM	16 ± 3	28 ± 15	2.0 ± 0.8 (23 ± 14)				28 ± 8		28 ± 4
U053-01	Preclinical	NA	38	0	−	−	+	35	+	35
U061-02	Preclinical	NA	31	0	−	−	−	−	+	31
U030-00	Preclinical	NA	23	0	−	−	+	13	+	20

NA, Not applicable; *PI,* postimmunization.
*Monthly blood draw only.
†$P < .001$ 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 Fig 3.

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 RR-MS,[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

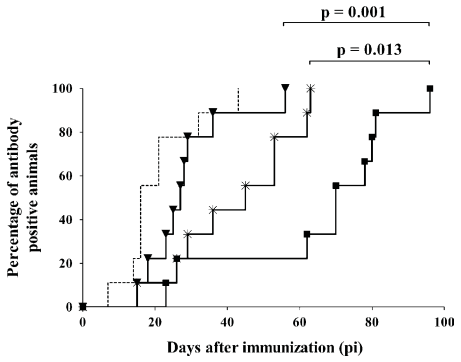

FIG 3. Time course of α-GalC and α-myelin protein IgG responses in immunized *C jacchus*. Serum dilutions, 1:100. *(…)* denotes onset of clinical signs; *(-▼-)* denotes α-MBP positivity; *(-*-)* denotes α-rMOG positivity; *(-■-)* denotes α-GalC positivity. Significant levels for median onset postimmunization (pi) of antibody positivity were determined by a Cox proportional hazard model.

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 anti-myelin protein antibodies, on the other hand, have recently been described as potential predictors of early conversion in patients with CIS.[20]

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 II; Fig 3). This was in contrast to the α-rMOG and α-MBP responses that 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 delayed antibody response to galactocerebroside in MS and EAE is not known, several mechanisms may be postulated. First, glycolipids are not classic, MHC-restricted T-cell antigens but may elicit a T_H1 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 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 considerably lower in human beings compared with titers of antibodies against myelin proteins (Dr Menge, personal observation, ref 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 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 will be used in the near future for MS diagnostics, staging, and prognosis.

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REFERENCES

1. Steinman L. Multiple sclerosis: a two-stage disease. Nat Immunol 2001; 2:762-4.
2. von Büdingen HC, Villoslada P, Ouallet JC, Hauser SL, Genain CP. Immune responses against the myelin/oligodendrocyte glycoprotein in experimental autoimmune demyelination. J Clin Immunol 2001;21: 155-70.
3. Moore GR, Traugott U, Farooq M, Norton WT, Raine CS. Experimental autoimmune encephalomyelitis: augmentation of demyelination by different myelin lipids. Lab Invest 1984;51:416-24.
4. Raine CS, Traugott U, Farooq M, Bornstein MB, Norton WT. Augmentation of immune-mediated demyelination by lipid haptens. Lab Invest 1981;45:174-82.
5. Genain CP, Nguyen MH, Letvin NL, Pearl R, Davis RL, Adelman M, et al. Antibody facilitation of multiple sclerosis-like lesions in a nonhuman primate. J Clin Invest 1995;96:2966-74.
6. Fierz W, Heininger K, Schaefer B, Toyka KV, Linington C, Lassmann H. Synergism in the pathogenesis of EAE induced by an MBP-specific T-cell line and monoclonal antibodies to galactocerebroside or a myelin oligodendroglial glycoprotein. Ann N Y Acad Sci 1988;540:360-3.
7. Schluesener HJ, Sobel RA, Linington C, Weiner HL. A monoclonal antibody against a myelin oligodendrocyte glycoprotein induces relapses and demyelination in central nervous system autoimmune disease. J Immunol 1987;139:4016-21.
8. Linington C, Bradl M, Lassmann H, Brunner C, Vass K. Augmentation of demyelination in rat acute allergic encephalomyelitis by circulating mouse monoclonal antibodies directed against a myelin/oligodendrocyte glycoprotein. Am J Pathol 1988;130:443-54.
9. Morris-Downes MM, Smith PA, Rundle JL, Piddlesden SJ, Baker D, Pham-Dinh D, et al. Pathological and regulatory effects of anti-myelin antibodies in experimental allergic encephalomyelitis in mice. J Neuroimmunol 2002;125:114-24.
10. Fry JM, Weissbarth S, Lehrer GM, Bornstein MB. Cerebroside antibody inhibits sulfatide synthesis and myelination and demyelinates in cord tissue cultures. Science 1974;183:540-2.
11. Raine CS, Johnson AB, Marcus DM, Suzuki A, Bornstein MB. Demyelination in vitro: absorption studies demonstrate that galactocerebroside is a major target. J Neurol Sci 1981;52:117-31.
12. Saida T, Saida K, Silberberg DH. Demyelination produced by experimental allergic neuritis serum and anti-galactocerebroside antiserum in CNS cultures: an ultrastructural study. Acta Neuropathol (Berl) 1979;48:19-25.
13. Menon KK, Piddlesden SJ, Bernard CC. Demyelinating antibodies to myelin oligodendrocyte glycoprotein and galactocerebroside induce degradation of myelin basic protein in isolated human myelin. J Neurochem 1997;69:214-22.
14. Lindert RB, Haase CG, Brehm U, Linington C, Wekerle H, Hohlfeld R. Multiple sclerosis: B- and T-cell responses to the extracellular domain of the myelin oligodendrocyte glycoprotein. Brain 1999;122:2089-100.
15. Reindl M, Linington C, Brehm U, Egg R, Dilitz E, Deisenhammer F, et al. Antibodies against the myelin oligodendrocyte glycoprotein and the myelin basic protein in multiple sclerosis and other neurological diseases: a comparative study. Brain 1999;122:2047-56.
16. Lampasona V, Franciotta D, Furlan R, Zanaboni S, Fazio R, Bonifacio E, et al. Similar low frequency of anti-MOG IgG and IgM in MS patients and healthy subjects. Neurology 2004;62:2092-4.

17. Mantegazza R, Cristaldini P, Bernasconi P, Baggi F, Pedotti R, Piccini I, et al. Anti-MOG autoantibodies in Italian multiple sclerosis patients: specificity, sensitivity and clinical association. Int Immunol 2004;16: 559-65.
18. Rostami AM, Burns JB, Eccleston PA, Manning MC, Lisak RP, Silberberg DH. Search for antibodies to galactocerebroside in the serum and cerebrospinal fluid in human demyelinating disorders. Ann Neurol 1987;22:381-3.
19. Kasai N, Pachner AR, Yu RK. Anti-glycolipid antibodies and their immune complexes in multiple sclerosis. J Neurol Sci 1986;75:33-42.
20. Berger T, Rubner P, Schautzer F, Egg R, Ulmer H, Mayringer I, et al. Antimyelin antibodies as a predictor of clinically definite multiple sclerosis after a first demyelinating event. N Engl J Med 2003;349:139-45.
21. Poser CM, Paty DW, Scheinberg L, McDonald WI, Davis FA, Ebers GC, et al. New diagnostic criteria for multiple sclerosis: guidelines for research protocols. Ann Neurol 1983;13:227-31.
22. Massacesi L, Genain CP, Lee-Parritz D, Letvin NL, Canfield D, Hauser SL. Active and passively induced experimental autoimmune encephalomyelitis in common marmosets: a new model for multiple sclerosis. Ann Neurol 1995;37:519-30.
23. Amor S, Groome N, Linington C, Morris MM, Dornmair K, Gardinier MV, et al. Identification of epitopes of myelin oligodendrocyte glycoprotein for the induction of experimental allergic encephalomyelitis in SJL and Biozzi AB/H mice. J Immunol 1994;153:4349-56.
24. Nakajima H, Katagiri YU, Kiyokawa N, Taguchi T, Suzuki T, Sekino T, et al. Single-step method for purification of Shiga toxin-1 B subunit using receptor-mediated affinity chromatography by globotriaosylceramide-conjugated octyl sepharose CL-4B. Protein Expr Purif 2001;22:267-75.
25. Lily O, Palace J, Vincent A. Serum autoantibodies to cell surface determinants in multiple sclerosis: a flow cytometric study. Brain 2004; 127:269-79.
26. Ichioka T, Uobe K, Stoskopf M, Kishimoto Y, Tennekoon G, Tourtellotte WW. Anti-galactocerebroside antibodies in human cerebrospinal fluids determined by enzyme-linked immunosorbent assay (ELISA). Neurochem Res 1988;13:203-7.
27. Frohman EM, Goodin DS, Calabresi PA, Corboy JR, Coyle PK, Filippi M, et al. The utility of MRI in suspected MS: report of the Therapeutics and Technology Assessment Subcommittee of the American Academy of Neurology. Neurology 2003;61:602-11.
28. Brex PA, Ciccarelli O, O'Riordan JI, Sailer M, Thompson AJ, Miller DH. A longitudinal study of abnormalities on MRI and disability from multiple sclerosis. N Engl J Med 2002;346:158-64.
29. Tabira T, Endoh M. Humoral immune responses to myelin basic protein, cerebroside and ganglioside in chronic relapsing experimental allergic encephalomyelitis of the guinea pig. J Neurol Sci 1985;67: 201-12.
30. Lolli F, Liuzzi GM, Vergelli M, Massacesi L, Ballerini C, Amaducci L, et al. Antibodies specific for the lipid-bound form of myelin basic protein during experimental autoimmune encephalomyelitis. J Neuroimmunol 1993;44:69-75.
31. Beckman EM, Porcelli SA, Morita CT, Behar SM, Furlong ST, Brenner MB. Recognition of a lipid antigen by CD1-restricted alpha beta+ T cells. Nature 1994;372:691-4.
32. Shamshiev A, Donda A, Carena I, Mori L, Kappos L, De Libero G. Self glycolipids as T-cell autoantigens. Eur J Immunol 1999;29: 1667-75.
33. Battistini L, Fischer FR, Raine CS, Brosnan CF. CD1b is expressed in multiple sclerosis lesions. J Neuroimmunol 1996;67:145-51.
34. Lumsden CE. The neuropathology of multiple sclerosis. In: Vinken PI, Bruyn GW, editors. Handbook of clinical neurology. New York: Elsevier; 1970. p. 217-309.
35. Bernard CC, Johns TG, Slavin A, Ichikawa M, Ewing C, Liu J, et al. Myelin oligodendrocyte glycoprotein: a novel candidate autoantigen in multiple sclerosis. J Mol Med 1997;75:77-88.
36. Rosenbluth J, Schiff R, Liang WL, Dou WK, Moon D. Antibody-mediated CNS demyelination: focal spinal cord lesions induced by implantation of an IgM anti-galactocerebroside-secreting hybridoma. J Neurocytol 1999;28:397-416.
37. Robinson WH, Steinman L, Utz PJ. Protein arrays for autoantibody profiling and fine-specificity mapping. Proteomics 2003;3:2077-84.