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Journal of Neuroimmunology 101 (1999) 61–67

Journal of Neuroimmunology

www.elsevier.com/locate/jneuroim

The role of anti-myelin (auto)-antibodies in the phagocytosis of myelin by macrophages

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Received 19 May 1999; received in revised form 28 June 1999; accepted 30 June 1999

Abstract

Plasma cells secreting antibodies directed to myelin components are present in CNS of MS patients and although the pathogenic role of such antibodies has yet to be established it is apparent from animal studies that anti-myelin antibodies are involved in myelin damage. In this study, we have investigated the effect of disease-promoting anti-myelin mAb on the phagocytosis of myelin by macrophages. Monoclonal antibodies directed to myelin basic protein (MBP) — clones 1, 12, 17, 22, 26, proteolipid protein (PLP), galactocerebroside (GalC) and myelin oligodendrocyte glycoprotein (MOG) — clones Y1, Y4, Y6, Y7, Y9, Y10, Y11 and Z12 were incubated with purified murine myelin labeled with DiI. The degree of phagocytosis of antibody-treated myelin by murine macrophages in vitro was determined using a quantitative flow cytometric assay. In comparison to untreated myelin pretreatment with myelin-specific mAb modified the degree of phagocytosis. The degree of opsonization of myelin was dependent on the isotype of antibody and the epitope recognized in addition to the ability of the mAb to fix complement. The greatest degree of opsonization of myelin was observed with the monoclonal antibody MOG Z12 that has previously been shown to enhance EAE and augment demyelination. These findings suggest a major role for anti-myelin antibodies, in particular antibodies directed to MOG, for the phagocytosis of myelin by macrophages in vitro. This may have relevance to the pathogenesis of myelin damage in vivo and provide a helpful tool for the classification of heterogeneous diseases such as MS.

Keywords: Phagocytosis; Macrophages; Antibodies; Myelin; Demyelination

1. Introduction

Intrathecal secretion of oligoclonal IgG antibodies (Ab) are present in more than 90% of multiple sclerosis (MS) patients and is a laboratory marker supporting the diagnosis of this disease. The intrathecal IgG fractions contain many different specific antibodies including myelin-specific Ab. Reports on anti-myelin Ab in CSF and sera are controversial (Bernard et al., 1981; Wajgt and Górny, 1983; Ryberg and Jacque, 1986; Cruz et al., 1987; Möller et al., 1989; Henneberg et al., 1991; Xiao et al., 1991). While several reports describe the presence of anti-myelin Ab in serum and CSF, others find anti-myelin Ab only in the CSF, and yet others describe that anti-myelin Ab are absent in MS patients. However, B lymphocytes, and plasma cells secreting Ab to MBP, MAG, MOG and PLP are consistently detected in the CNS of MS patients (Link et al., 1990; Baig et al., 1991; Sun et al., 1991a,b). Such cells are even measured in the CSF while anti-myelin Ab could not be detected in the same CSF, suggesting that antibodies rapidly bind to target structures such as corresponding auto-antigen or to FcR, and therefore become undetectable (Sun et al., 1991a,b).

While a significance of anti-myelin antibodies in MS has not been established, several in vitro studies and studies in experimental animals suggest that the auto-antibodies directed to myelin components may play a critical pathogenic role in the disease by augmenting demyelination. In organotypic, myelinated cultures of central nervous system (CNS) tissue sera of MS patients promoted myelin breakdown (Raine et al., 1973). Such myelin damage is also observed with anti-GalC sera and anti-whole myelin sera (Seil, 1968; Raine et al., 1981). In the experimental

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0165-5728/99/$ - see front matter
PII: S0165-5728(99)00133-2

autoimmune model of MS, experimental allergic encephalomyelitis (EAE) increased levels of antibody directed to PLP, GalC and MOG correlate with the chronic relapsing demyelinating phase (Linington and Lassmann, 1987; Morris et al., 1998). Although autoaggressive T cells initiate EAE many studies demonstrate that antibody responses are crucial for the development of demyelination. Injection of sera from animals with chronic EAE into the CSF of normal animals induces demyelination (Lassmann et al., 1981). Furthermore, sera of animals with EAE only cause myelin damage in vitro following immunization with whole myelin and not with MBP alone (Raine et al., 1981). Likewise, demyelination is only observed in animals with EAE following immunization with both MBP and GALC and not with MBP alone (Moore et al., 1984).

Taken together, these data suggest that a variety of myelin antigens, particularly those present on the surface of the myelin sheath, may be important targets in antibody-mediated demyelination and in the disease process. This may explain why an antibody directed to MOG, which is exclusively expressed in the CNS on the surface of myelin, augment the severity and duration of clinical signs and induces the formation of large demyelinated plaques within the CNS (Schluesner et al., 1987; Linington et al., 1988). Complement depletion by cobra venom factor (CVF) or treatment with soluble complement receptor 1 suppresses acute EAE and the MOG-augmented EAE suggesting a role for complement in myelin damage (Piddlesden et al., 1991; Piddlesden et al., 1994).

Further investigations on Ab mediated demyelination and the relationship between the ability of anti-myelin mAb to fix complement and their pathogenicity in EAE were performed. It was shown that the anti-MOG mAb Z12 induced the most severe demyelination in rats (Piddlesden et al., 1993) and the most severe clinical signs and demyelination in mice (Morris et al., 1998). MOGZ12 a mouse IgG2a mAb had the best complement fixing ability (Piddlesden et al., 1993) and treatment of mice with CVF could partly abolish the MOGZ12 enhancement of the clinical signs (Morris et al., 1998). Heat-inactivation of sera can decrease the degree of myelin phagocytosis by macrophages (Van der Laan et al., 1996), which means that the complement cascade is activated during the phagocytosis of myelin. One mechanism that may lead to complement activation within the CNS is the formation of antigen:antibody complexes such as myelin:myelin-specific antibody complexes, interacting with FcR on, for example, macrophages and microglia.

In summary, previous studies have shown that anti-myelin antibodies can induce demyelination in vitro and do augment experimental clinical disease, but they do so in a variable extent. Understanding how such antibodies exert their effects and which properties determine their disease producing effects will be relevant to understanding the development of demyelination in MS. For this reason, we investigated in this study the properties of a panel of anti-myelin antibodies with respect to myelin phagocytosis. Our data reveal that monoclonal antibodies that are most effective in promoting EAE show the highest rate of binding to myelin and the greatest enhancement of myelin phagocytosis. Such enhanced-phagocytosis was most effective in the presence of normal, but not heat inactivated serum suggesting a role for complement in antibody-mediated phagocytosis.

2. Materials and methods

2.1. Antibodies

Ab (Table 1) directed to MBP and PLP as an ascitic fluid were kind gifts from Professor N. Groome, Oxford Brookes University, UK. Hybridomas secreting mAb directed to MOG (clone Y10 and Z12) and GalC were used

Table 1
Characteristics of anti-myelin antibodies

Antigen	Clone number	Isotype	Effect in EAE in mice
MBP	1	Mouse IgG2a	No effect
MBP	12	Rat IgG2a	No effect
MBP	17	Rat IgG1	No effect
MBP	22	Mouse IgG2b	↓ clinical signs
MBP	26	Mouse IgG1	↑ clinical signs
GALC	–	Mouse IgG3	↑ inflammation
PLP	–	Rat IgG2a	No effect
MOG	Y1	Mouse IgG1	n.d.
MOG	Y4	Mouse IgG1	n.d.
MOG	Y6	Mouse IgG1	n.d.
MOG	Y7	Mouse IgG1	n.d.
MOG	Y9	Mouse IgG1	n.d.
MOG	Y10	Mouse IgG1	No effect
MOG	Y11	Mouse IgG1	n.d.
MOG	Z12	Mouse IgG2a	↑ clinical signs, ↑ inflammation, ↑ demyelination

to produce ascitis fluid as described before (Ranscht et al., 1982; Piddlesden et al., 1993). The mAb were purified from ascitic fluid by chromatography. The other antibodies directed against MOG were a kind gift from S. Piddlesden, University of Wales, UK. Monoclonal antibodies against CD4 (mouse IgG1) (Diaclone, Besançon, France), CD10 (mouse IgG2a)(Diaclone, Besançon, France), GL113 (rat IgG1) and R7D4 (rat IgG2a) were used as controls.

2.2. Preparation and labeling of myelin

Myelin was prepared from brain tissue of mice and rats as previous described by Norton and Poduslo (1973), using density-gradient centrifugation. To determine the protein concentration in the isolated myelin, a sample of the myelin was compared to a BSA standard curve. The standard was prepared by diluting a stock of 2 mg/ml BSA. 50 μl of each standard and sample was added to 1 ml Bicinchoninic acid (BCA; Pierce, IL, USA) reagent and incubated for 30 min at 37°C. Absorbency was measured at 562 nm. Myelin (6.4 mg protein/ml) was labeled with the lipophilic fluorescent dye 1.1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbo-cyanine perchlorate (DiI; Molecular Probes, Eugene, USA; /ml) by incubation at a final concentration of 12.5 μg DiI/mg myelin protein for 30 min at 37°C. Excess of DiI was removed by washing with PBS the labeled myelin was stored in aliquots at −20°C.

2.3. Flow cytometry analysis of myelin opsonization

Mouse myelin (0.2 mg/ml) was incubated with 75 μg/ml rat or mice mAb against myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), proteolipid glycoprotein (PLP) or galactocerebroside (GalC) in 100 μl PBS in a 96-well plate. After an incubation of 30 min at 4°C the myelin was washed 3 times with PBS. Binding of the antibody to myelin was detected using a second step with fluorescent labeled conjugates donkey-anti-rat IgG (H + L)/RPE (Jackson) or rat-anti-mouse IgG (Fab_2)/RPE (DAKO) in PBS/0.1% BSA for 30 min at 4°C. In the wells with a rat mAb as first step, 0.5% normal mouse serum was added with the conjugate to prevent non-specific binding. Detection of the binding was done by using FACScan flow cytometry (Beckon Dickinson, San Jose, CA, USA) and analyzed employing Cell Quest™ Software.

2.4. Flow cytometry analysis of myelin phagocytosis

Fresh normal mouse serum (NMS) was prepared by centrifugation of clotted peripheral blood of FUB mice.

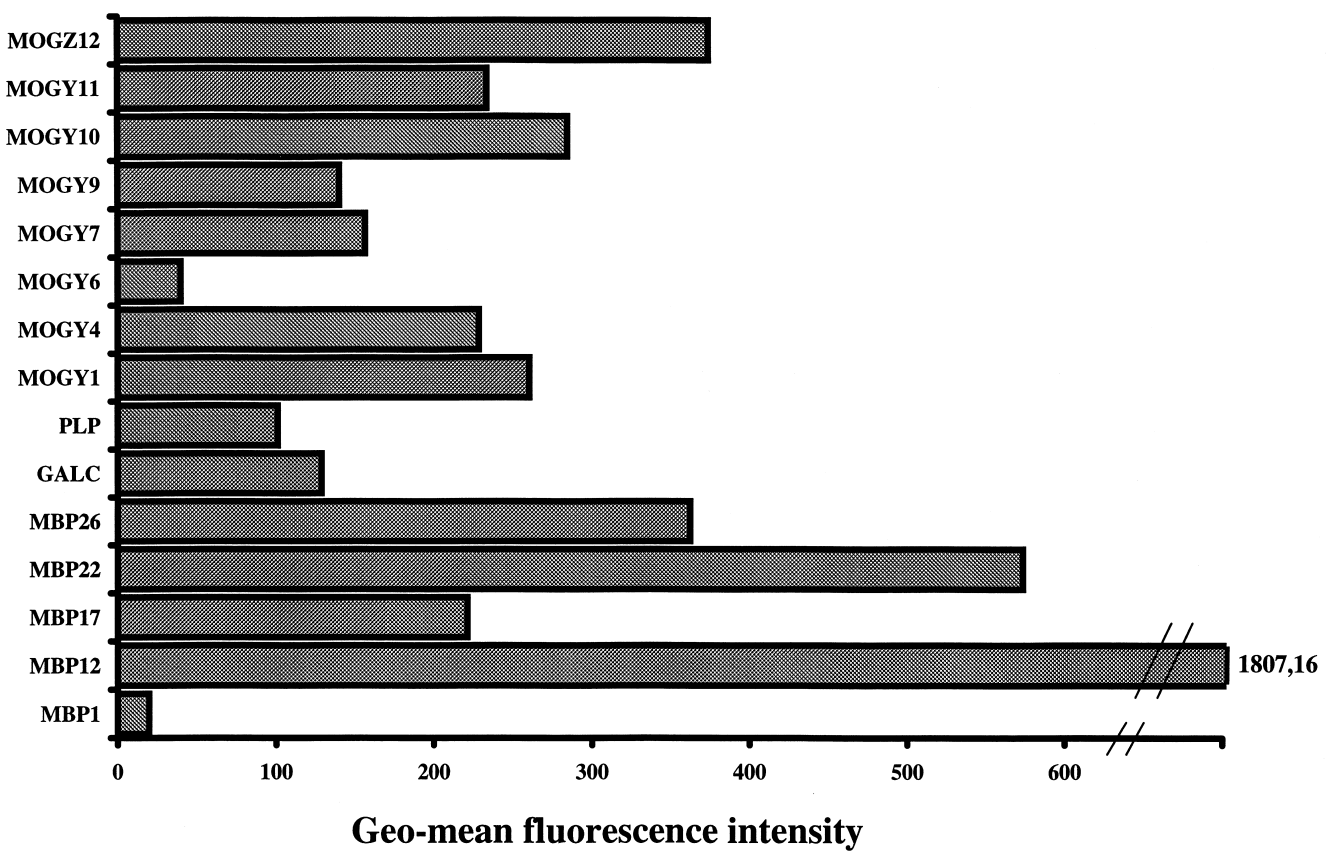

Fig. 1. Incubation of mouse myelin with mAb against myelin components. The binding is visualized by incubating the mAb-myelin with fluorescent labeled conjugates. The fluorescence intensity (FL2) of the myelin was measured using FACScan flow cytometry. The data are presented as the geo-mean of fluorescence of one representative experiment ($n = 4$).

Heat inactivated mouse serum (HiMS) was prepared by incubating serum for 30 min at 56°C to inactivate the complement system. The mouse macrophage cell-line J774.2 (ECACC), was suspended in culture medium (DMEM, Gibco) containing 5% fetal calf serum (FCS, Integro), penicillin (100 IU/ml), streptomycin (50 mg/ml) and 1 mM glutamin) at a concentration of 1.10^6 cells/ml. 0.5 ml cell suspension was added to a well of 24-wells plate and was incubated overnight at 37°C/5% CO_2. The following day, non-adherent cells were removed by washing the wells twice with DMEM, the cells were incubated in 0.5 ml DMEM either with 5% NMS or 5% HiMS. DiI labeled myelin was pre-incubated with antibodies as described above and 20 μg myelin protein was added to each well. After an incubation of 1.5 h, 37°C at 5% CO_2, non-ingested or non-bound myelin was removed by washing the plates 3 times with DMEM. For flow cytometric analysis, macrophages were detached by incubation of the cells with PBS/5 mM EDTA and collected. Fluorescence intensity (FL2 channel) was determined using a FACScan. The geographic mean fluorescence intensity was used as a measurement for binding and uptake of the DiI labeled myelin and was expressed as a percentage of opsonization. Significance was determined using the Student's *t*-test.

3. Results

3.1. Binding of mouse myelin with mAb against myelin components

In order to measure the degree of binding of the panel of anti-myelin mAb, a myelin staining was performed. Accordingly mouse myelin was incubated with the mAb, subsequently with a fluorescent-labeled conjugate. As seen in Fig. 1, the mAb showed varying binding pattern. The maximum binding was observed with the mAb MBP12 and MBP22, which showed a geo-mean fluorescence intensity of 1807.16 and 573.47, respectively. While the mAb MBP26 and MOGZ12 were equally well in binding myelin the mAb MBP17, MOG Y1, MOGY4, MOGY10 and MOGY11 bound to mouse myelin to a lesser extent. In contrast GALC, PLP, MOGY7 and MOGY9 showed only weak binding while MBP1 and MOGY6 were not able to bind myelin. These results suggest that the differential binding is not related to the isotype of the antibody.

3.2. Phagocytosis of pre-incubated mouse myelin by J774.2 cells NMS and HiMS

In a preliminary study, we compared the phagocytosis of mouse myelin by J774.2 cells and primary peritoneal

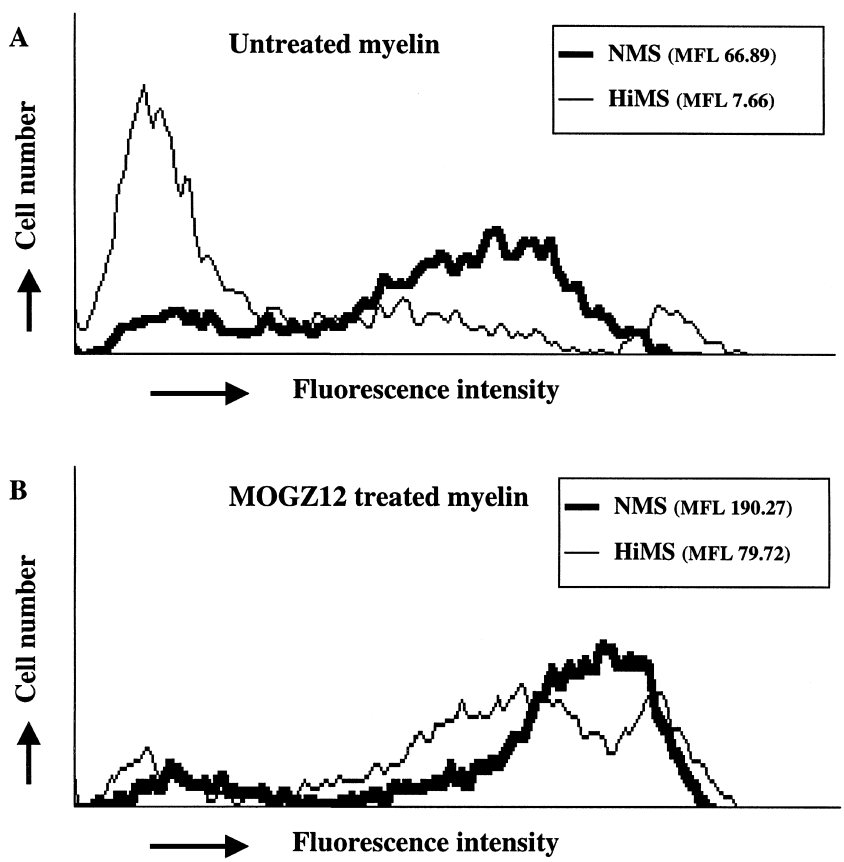

Fig. 2. Phagocytosis of mouse myelin, which was pre-incubated with mAb MOGZ12, by J774.2 cells. The degree of phagocytosis and binding of DiI-labeled myelin is given as the Geo-mean fluorescence of one representative experiment ($n = 5$). (A) Phagocytosis of myelin alone in NMS (thick line) and HiMS (dotted line). (B) Phagocytosis of myelin pre-incubated with MOGZ12 in NMS (thick line) and HiMS (dotted line).

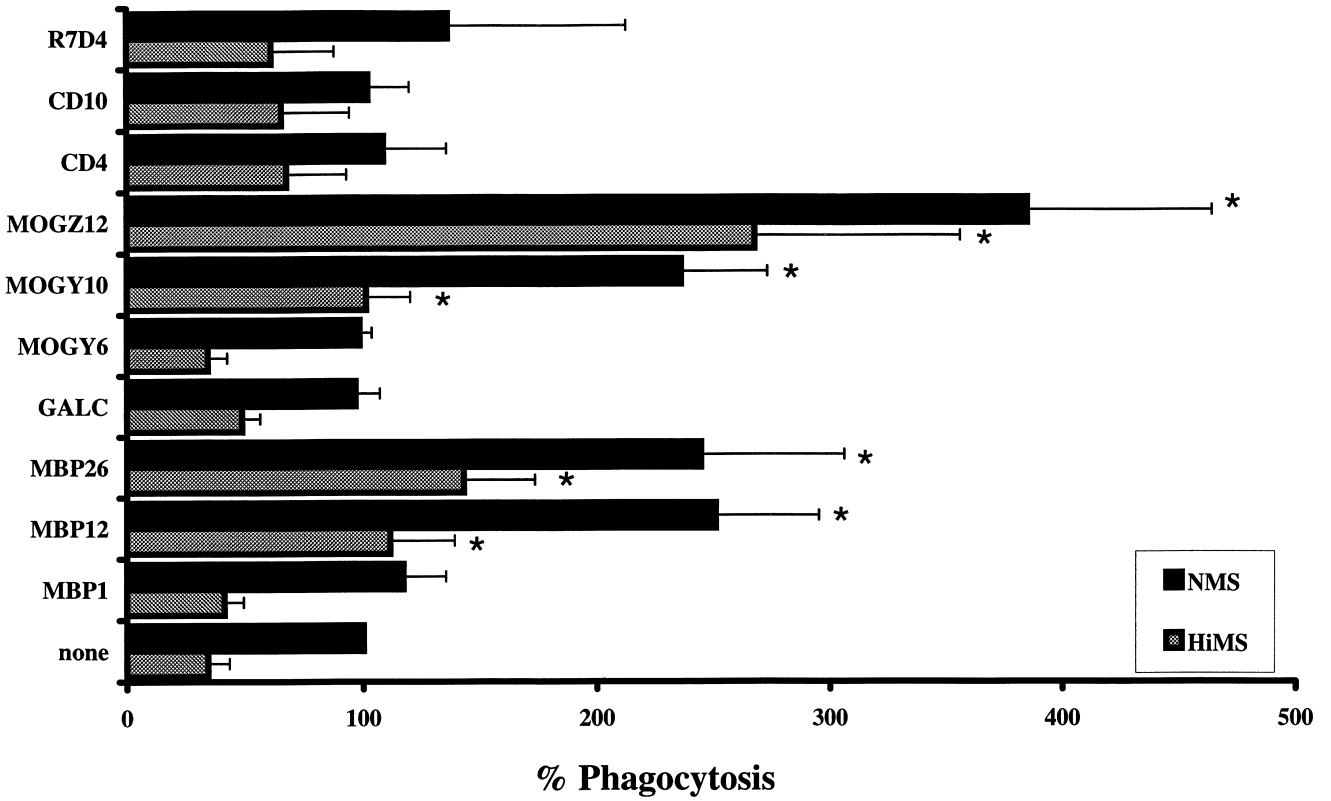

Fig. 3. Phagocytosis of mouse myelin, which was pre-incubated with anti-myelin mAb, by J774.2 cells. Data are presented as the mean percentage of binding and uptake of the fluorescent myelin ± S.E.M. of five experiments. $^{*}P < 0.05$.

macrophages obtained from Balb/c mice following thioglycolate treatment. Due the relatively large numbers of cells required we restricted this preliminary study to MOGZ12 and MBP12. It was observed that not only did the J774.2 cells phagocytose myelin to the same degree as the primary macrophages but that the increase in phagocytosis following incubation of myelin with the myelin-specific mAb was also similar with each cell type (data not shown). Thus, to screen all the mAbs, the macrophage cell line J774.2 was selected for the subsequent studies reported here.

To investigate the effect of anti-myelin mAb on the phagocytosis of myelin by J774.2 cells antibodies representing all degrees of staining were selected. Additionally to investigate factors such as complement, phagocytosis of myelin was performed in the presence of NMS or HiMS (Figs. 2 and 3). Fig. 2 reveals that the degree of phagocytosis of myelin in the presence of HiMS is dramatically reduced compared to NMS 11.5% and 100% (Fig. 2A). Likewise, phagocytosis of myelin, preincubated with MOGZ12, in the presence of HiMS is also reduced 284.5% and 119.2% (Fig. 2B). A similar effect of HiMS was observed for every antibody tested ($p < 0.05$; Fig. 3).

The mAb, which showed weak or no binding to myelin (MBP1, GalC and MOGY6) showed no enhancement of the phagocytosis. Those antibodies which bound more strongly to myelin (MBP12, MBP26, MOGY10 and MOGZ12) showed a significantly ($p < 0.05$) increased phagocytosis 250.7%, 244.5%, 236.1% and 384.6% respectively compared to no Ab treatment in NMS 100% (Fig. 3). After heat-inactivation of the serum, the same Abs showed a significantly ($p < 0.05$) increased phagocytosis 111.5%, 143.1%, 101.2% and 267.4%, compared to no Ab treatment in HiMS 34.1% (Fig. 3).

Table 2
Rat myelin binding and phagocytosis for MBP12 and MBP17 in NRS
$^{*}p < 0.05$.
In the second column, data are represented of the binding of rat mAbs to rat myelin of one representative experiment ($n = 3$). The third column shows the mean percentage ± S.E.M. of binding and uptake of rat myelin, which is pre-incubated with rat mAbs ($n = 3$). MBP12 showed the highest binding of rat myelin measured with fluorescent labeled conjugate. MBP12 also showed the highest increase of the phagocytosis. While MBP17 and PLP bound poorly to myelin they also showed no significant increased phagocytosis.

Antibody	Geo-mean of fluorescence	% Phagocytosis (± S.E.M.)
None	2.21	100
MBP12	594	384.6 ± 29.1*
MBP17	230	149.4 ± 8.1
PLP	99	334.6 ± 45.2

3.3. Rat myelin binding and phagocytosis for MBP12 and MBP17 in NRS

As seen in Table 2, the mAb we used are from different species (rat and mouse). In this study, we examine the possible promoting effects of autoantibodies on phagocytosis of myelin. For this reason, we used three rat mAb MBP12, MBP17 and PLP in a phagocytosis assay with rat peritoneal macrophages and with addition of rat serum. MBP12 showed the best binding to myelin and also the highest enhancement of phagocytosis. Although PLP was less effective as MBP 12 in binding, it gave an enhancement of the myelin phagocytosis. Probably the isotype IgG2a is very important for the enhancement of phagocytosis in the rat system. This could also been seen with MBP17, which is an IgG1 mAb. MBP17 gave a good binding but was less effective in enhancing myelin phagocytosis as compared to MBP12 and PLP both IgG2a mAb.

4. Discussion

In this study, we have examined the effect of myelin-specific antibodies directed to myelin basic protein (MBP) — clones 1, 12, 17, 22, 26, proteolipid protein (PLP), galactocerebroside (GalC) and myelin oligodendrocyte glycoprotein (MOG) — clones Y1, Y4, Y6, Y7, Y9, Y10, Y11 and Z12 on the phagocytosis of myelin. Opsonized DiI labeled myelin was added to the macrophages in the presence of normal or heat inactivated mouse serum to examine the role of complement. In comparison to untreated myelin, pretreatment with myelin-specific Ab modified the degree of phagocytosis. The degree of opsonization of myelin was dependent on the isotype of antibody and the epitope recognized in addition to the ability of the Ab to fix complement.

Of great significance is the finding that the antibody, MOG Z12 that is the most able to augment binding and phagocytosis of mouse myelin also augments clinical disease and demyelination in the mouse EAE model (Morris et al., 1998). This suggests that at least in the case of MOG Z12 there is a correlation between the ability to augment phagocytosis and the capacity to induce demyelination. The enhancement of the phagocytosis by MOGZ12 was dependent on the Fc tail of the mAb, because MOGZ12 Fab_2 fragments could not enhance the phagocytosis (data not shown).

In contrast to MOGZ12, an IgG2a mAb, the opsonizing effect of MOGY10 (IgG1) was less. This is correlated to the observation that MOG Y10 could not enhance clinical disease nor demyelination in the mouse EAE model. This difference may be dependent on the binding capacities of the mAb to myelin and the ability of these monoclonal antibodies to fix complement. Indeed Piddlesden et al., 1993 demonstrated that while all the anti-MOG mAb are capable off fixating complement MOGZ12 is able to fix complement much more strongly than the IgG1 mAb.

Macrophages recognize antibody via many recognized receptors including Fc receptors (FcR) and the type 3 complement receptor (CR3). CR3 is involved in the recruitment of monocytic cells to sites of inflammation and in the stimulation of phagocytosis. The importance of CR3 in EAE is demonstrated by the ability of monoclonal antibodies directed to CR3 to suppress acute EAE (Huitinga et al., 1992) and inhibit the phagocytosis of myelin by macrophages (Van der Laan et al., 1996). It is probable that the enhancement of the phagocytosis by MOG Z12 is dependent on engagement of the FcR. Further studies using FcR and CR3 knockout mice both for in vivo and in vitro studies may help clarify this issue.

We further showed that in the presence of heat-inactive serum enhancement of the phagocytosis of myelin by MOG Z12 was only partially reduced. Suggesting that the opsonizing effect of MOG Z12 is not completely dependent on complement activation. Morris et al. (1998) also described that the clinical disease of EAE could greatly be abolished when the mice were treated with CVF, but that CVF treatment could only partially reduce the clinical signs in MOG Z12 enhanced EAE.

The complement dependent enhancement of antibody-mediated phagocytosis could be due to extracellular degradation of myelin. In the literature it is shown that serum of EAE animals following immunization with whole myelin produce demyelination in organotypic CNS tissue cultures, while serum that has been heat-inactivated does not demyelinate cultures (Grundke-Iqbal et al., 1981). Further it is known that, in the presence of specific antibodies, liposomes can fix complement, which in turn may result in the lysis of the liposomes by activated complement (Rongen et al., 1997). A possible mechanism is that the mAb binds to a specific protein in the myelin, subsequently complement is activated by binding to the Fc tail of the mAb. This results in degradation of big myelin particles into smaller myelin particles, which are easier to ingest for the macrophages.

In summary, previous studies have shown that anti-myelin antibodies induce demyelination in vitro and augment experimental clinical disease, but they do so in a variable extent. Understanding the mechanisms by which anti-myelin antibodies exert their effects and which properties determine their pathogenic effects will be relevant to understanding the development of demyelination in MS. Furthermore, the use of this assay to investigate phagocytosis of myelin in vitro may be adapted for the analysis of the opsonizing capacities of antibodies in CSF or serum of MS patients. Such studies may, as indicated by the results of the present study, give an indication as to the extent of antibody-mediated demyelination in patients and thus provide a helpful tool to classify subtypes of MS and be relevant to the development of specific therapeutic strategies for this otherwise heterogeneous disease.

Acknowledgements

This work was supported financially by the Dutch foundation ''Vrienden MS Research'', project MS 95-202 and the Multiple Sclerosis Society of Great Britain and Northern Ireland.

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