PART III

BODY FLUID BIOMARKERS
EPITOPE SPECIFICITY OF SERUM ANTIBODIES DIRECTED AGAINST THE EXTRACELLULAR DOMAIN OF MYELIN OLIGODENDROCYTE GLYCOPROTEIN: INFLUENCE OF RELAPSES AND IMMUNOMODULATORY TREATMENTS

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ABSTRACT

Only few reports are available on the epitope specificity of anti-myelin oligodendrocyte glycoprotein (MOG) antibodies in multiple sclerosis (MS). In the present study we provide a precise characterization of the epitope specificity of serum antibodies directed against the extracellular domain of MOG, including IgG, IgM and IgA immunoglobulin isotypes in 28 relapsing remitting MS patients and report that linear epitopes amino-acid (aa) 37–48 and aa42–53 are immunodominant. Recently experienced relapses intensified the anti-MOG peptide antibody response. Immunomodulatory treatment with interferon-beta or glatiramer-acetate had no major impact on the anti-MOG peptide immunoreactivity after 1 year of therapy.
INTRODUCTION

However, the pathological significance of anti-MOG antibodies recognizing linear MOG peptides in MS-patients remains to be identified. In order to better characterize the human anti-MOG antibody response we analyzed the epitope specificity of serum anti-MOG antibodies in 28 relapsing remitting MS (RRMS) patients. Because many RRMS patients receive disease modifying treatments we further investigated whether immunomodulatory drugs influenced the epitope specificity after 1 year of treatment.

PATIENTS, MATERIAL AND METHODS
Patients and blood samples
MS patients were diagnosed according to the criteria of Poser (Poser, et al. 1983), had a relapsing remitting disease course and gave informed consent for participation in this prospective study. Serum samples were obtained between 2002 and 2004 and stored at -20 °C until use. Epitope
specificity was investigated before and after 1 year of treatment in 8 MS-patients receiving glatiramer-acetate, 10 MS-patients receiving interferon-beta and 10 MS-patients without any immunomodulatory therapy. Treatment groups were matched for age, sex, disease duration, EDSS (Kurtzke, 1983) and relapse rate before study entry. None of the patients treated with interferon-beta had developed neutralizing antibodies against interferon-beta (Deisenhammer, et al. 1999) during the study period. None of the patients received any immunomodulatory drug prior to study entry. None of the patients had a relapse or received any immunosuppressive treatment within 1 month before blood samples were taken. Neurological examinations were performed every 3 months for 1 year and all relapses had to be confirmed. Serum samples from 20 healthy blood donors (HC) served as controls.

Antigens

Human recombinant MOG immunoglobulin domain aa1–125 was prepared as described before (Reindl, et al. 1999). For detection of the anti-MOG epitope specificity we used a panel of 25 synthetic biotinylated 12 aa long linear peptides with 7 aa overlaps corresponding to the sequence of human MOG aa1–128 (Mimotopes, Australia). Additionally, serum antibody responses were determined against MOG peptide aa38–60 (Peptides and Elephants, Germany).

Purification of serum anti-MOG aa1–125 antibodies

For the immunoaffinity purification of human serum anti-MOG aa1-125 antibodies recombinant MOG aa1-125 was covalently coupled to tosylactivated Dynabeads M-280 (Dynal). For all washing and incubation steps, a magnetic Dynal MPC device (Dynal) and a rotation sampler was used according to the manufactures instructions. Briefly, 2 x 108 dynabeads were washed several times in 0.1 M sodium phosphate pH 7.4 and then incubated with 60 µg recombinant human MOG in 0.2 M sodium acetate pH 4.0 at a concentration of 1 x 109 beads/ml for 16 h at 37 °C. Beads were then washed twice with phosphate-buffered-saline (PBS) -0.1% bovine serum albumin (BSA) and then incubated in 0.2 M Tris pH 8.5, 0.1% BSA for 24 h at 25 °C to block free tosyl-groups. Finally, beads were washed once and stored in PBS-0.1% BSA at 4 °C. For the extraction of antibodies, coated beads were washed once in PBS-0.1% BSA and 500 µl of diluted serum (in 1 ml PBS) were added and incubated overnight at 4 °C. The next day, beads were washed three times in PBS-0.1% BSA and transferred to a fresh tube. Bound antibodies were eluted with 450 µl 0.2 M glycine-HCl pH 2.5 for 10 min at 4 °C. Immediately after this step the supernatant containing the eluted human antibodies was mixed with 50 µl 10 x PBS and 30 µl 1 M NaOH. Purified antibodies were then stored at –20 °C until use.

Immunoassays for anti-MOG peptide antibodies

Epitope specificity of purified anti-MOG aa1-125 antibodies was analyzed by enzyme linked immunosorbent assay (ELISA). 96-well Nunc-Immuno Maxisorp microtitre plates (Nunc, Denmark) were coated with 100 µl of a 5 µg/ml Streptavidin (Sigma, USA) solution in distilled water for one day and one night at 37 °C. The plates were then blocked with 3% BSA (Sigma, USA) in PBS for two hours at room temperature. After washing the plates 4 times with PBS containing 0.05 % Tween 20 (PBS-T), 100 µl of synthetic biotinylated peptides (Mimotopes, Australia) (1:1000 in PBS containing 0.1 % sodium azide and 0.1 % Tween 20) were added and incubated for one hour at room temperature. After washing 100 µl of purified serum anti-MOG antibodies were
then added (1:15 in PBS containing 0.1 % sodium azide and 0.1 % Tween 20) and incubated for one hour at room temperature. After washing, 100 µl of diluted alkaline-phosphatase-conjugated goat anti-human IgG+IgM+IgA (JCH-055064, Jackson, USA) (1:5000 in 0.15% BSA in PBS) were added and plates were incubated for one hour at room temperature. Reaction products were visualized with p-nitrophenylphosphate (Sigma, USA), and the optical density (OD) determined at 405 nm. The streptavidin coated control wells were coated with a negative control peptide aa MASLSRPSLPSCGSGS, which is part of the signal peptide of MOG and incubated with purified anti-MOG antibodies. All data were corrected by subtraction of these background values.

Serum anti-MOG aa38-60 antibodies were detected by ELISA using 96-well Nunc-Immuno Maxisorp microtitre plates (Nunc, Denmark) coated with 100 µl of a 5 µg/ml MOG aa38-60 (MOG 38GWYRPPFSR/VHLYRNGKDQGD60) solution in PBS overnight at 4 °C. At the next day coating solution was removed and the plates were blocked with 10% fetal bovine serum (FBS) in PBS for 1 h at room temperature. After washing the plates 4 times with PBS-T, 100 µl of serum diluted 1:100 in 10% FBS in PBS were added and incubated for 1 h at room temperature. After washing, 100 µl of either diluted horse-radish-peroxidase (HRP)-rabbit-anti-human IgG (1:5000) (Dako P0214, Denmark), HRP-rabbit-anti-human IgM (1:5000) (Dako P0215, Denmark) or HRP-rabbit-anti-human IgA (1:2000) (Dako P0216, Denmark), all diluted in 10% FBS in PBS, were added and incubated for 1 h at room temperature. Reaction products were visualized with 3,3´,5,5´-tetramethyl-benzidine (TMB) liquid substrate, supersensitive (Sigma) and the optical density determined at 450 nm. Control wells were coated with MOG aa38-60 and incubated with either diluted HRP-rabbit-anti-human IgG (1:5000) (Dako P0214, Denmark), HRP-rabbit-anti-human IgM (1:5000) (Dako P0215, Denmark) or HRP-rabbit-anti-human IgA (1:2000) (Dako P0216, Denmark) and all data were corrected by subtraction of these background values.

Statistical evaluation
Statistical evaluations were performed using SPSS, version 12.0.1. and Prism 4, version 4.0b. Comparisons between groups of OD-values and the number of positive reactivities against MOG peptides were done using Mann Whitney U-test. Bonferroni’s correction was applied to adjust p-values for multiple comparisons. Sensitivity and specificity were determined using ROC analyses. McNemar test was used for comparison of the percentage of patients reacting against MOG peptides at baseline and month 12.

RESULTS
Epitope specificity of serum anti-MOG antibodies in MS patients and healthy controls
A panel of 25 synthetic linear MOG peptides with 12 aa length and 7 aa overlaps (Table 1) were used to determine the epitope specificity of purified serum anti-MOG IgG+IgM+IgA antibodies in 28 RRMS patients and 20 HC (Table 2). At baseline (month 0), before patients received a disease modifying treatment, we found significantly increased antibody responses against two overlapping epitopes aa37-48 and aa42-53 in MS-patients compared to HC. Up to 54% of MS-patients had positive immunoreactivities against these immunodominant epitopes when using a cut-off of 95% specificity for MS for each peptide to determine positivity in this
assay (Figure 1). At a specificity of 95% we found a sensitivity of 50% for antibody responses against epitope aa37-48 and a sensitivity of 54% for antibody responses against epitope aa42-53. To confirm our results we additionally analyzed non-purified serum antibody responses from the same blood samples directed against a longer MOG peptide aa38-60 containing both immunodominant epitopes found in the present epitope mapping assay of immunopurified serum anti-MOG antibodies.

### Table 1. Amino acid sequence of synthetic biotinylated MOG peptides

<table>
<thead>
<tr>
<th>Amino acid range</th>
<th>Amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-8</td>
<td>SCGSSSYACQFRVIGP</td>
</tr>
<tr>
<td>2-13</td>
<td>SCGQFRVIGPRHPR</td>
</tr>
<tr>
<td>7-18</td>
<td>SCGGPRHIPRAVLCDD</td>
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<tr>
<td>12-23</td>
<td>SCSGIRALVGDEVELP</td>
</tr>
<tr>
<td>17-28</td>
<td>SCSGDEVLEPCRISP</td>
</tr>
<tr>
<td>22-33</td>
<td>SCSGLPRISPGKSNAT</td>
</tr>
<tr>
<td>27-38</td>
<td>SCSGSPGKNTAGMEVG</td>
</tr>
<tr>
<td>32-43</td>
<td>SCSGATIMECGVRYP</td>
</tr>
<tr>
<td>37-48</td>
<td>SCSGVWYRPVPPSRV</td>
</tr>
<tr>
<td>42-53</td>
<td>SCSGPFRSVVHLVRN</td>
</tr>
<tr>
<td>47-58</td>
<td>SCSGVVHLYRNKDQDD</td>
</tr>
<tr>
<td>52-63</td>
<td>SCSGRNKDDQCDQAP</td>
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<tr>
<td>57-68</td>
<td>SCSGDQCDQAPEYRCR</td>
</tr>
<tr>
<td>62-73</td>
<td>SCSGAFEYRGTELLK</td>
</tr>
<tr>
<td>67-78</td>
<td>SCSGTEGRTEKDAIGE</td>
</tr>
<tr>
<td>72-83</td>
<td>SCSGKLDAIECGKVT</td>
</tr>
<tr>
<td>77-88</td>
<td>SCSGGEKVTLRIRNR</td>
</tr>
<tr>
<td>82-93</td>
<td>SCSGTRLRRNVRFSDE</td>
</tr>
<tr>
<td>87-98</td>
<td>SCSGNVFSDECGFTC</td>
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<tr>
<td>92-103</td>
<td>SCSGDECGFTCCFRDH</td>
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<tr>
<td>97-108</td>
<td>SCSGTCFRRDSYQEE</td>
</tr>
<tr>
<td>102-113</td>
<td>SCSGDDHYQEAAAMEL</td>
</tr>
<tr>
<td>107-118</td>
<td>SCSGEEAAAMELKVEDP</td>
</tr>
<tr>
<td>112-123</td>
<td>SCSGELKVEPFYYWVS</td>
</tr>
<tr>
<td>117-128</td>
<td>SCSGDFFYWVSPGVLV</td>
</tr>
</tbody>
</table>

SGSG, spacer tetrapeptide between the biotin group and the MOG amino acids.

### Table 2. Clinical and demographic data

<table>
<thead>
<tr>
<th></th>
<th>MS all patients</th>
<th>MS-patients treated with</th>
<th>MS-patients treated with</th>
<th>HC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>N (f/m)</strong></td>
<td>28 (14/14)</td>
<td>8 (3/5)</td>
<td>10 (3/7)</td>
<td>10 (8/2)</td>
</tr>
<tr>
<td><em><em>Age</em> (y)</em>*</td>
<td>35.4 (22.1–48.5)</td>
<td>31.5 (26.6–43.4)</td>
<td>36.0 (22.1–43.6)</td>
<td>34.6 (21.3–48.5)</td>
</tr>
<tr>
<td><em><em>Disease duration</em> (y)</em>*</td>
<td>5.4 (1.0–21.2)</td>
<td>5.8 (1.0–19.1)</td>
<td>3.6 (1.2–21.2)</td>
<td>7.2 (1.0–13.5)</td>
</tr>
<tr>
<td><strong>Relapse rate</strong>*</td>
<td>0.7 (0.2–2.1)</td>
<td>0.6 (0.3–2.1)</td>
<td>0.8 (0.4–1.7)</td>
<td>0.4 (0.2–1.9)</td>
</tr>
<tr>
<td><strong>EDSS</strong>*</td>
<td>1.5 (0.0–2.5)</td>
<td>1.8 (1.0–2.0)</td>
<td>1.5 (1.0–2.5)</td>
<td>1.0 (0.0–1.5)</td>
</tr>
</tbody>
</table>

*median values (min–max); y, years; MS, multiple sclerosis; HC, healthy controls; GA, glatiramer-acetate; INF-β, interferon-beta; NOTh, no therapy; EDSS, expanded disability status scale (Kurtzke, 1983).
Anti-MOG aa38-60 IgG (p=0.05) and anti-MOG aa38-60 IgA (p=0.004) antibody responses were significantly increased in MS-patients but not anti-MOG aa38-60 IgM antibodies (p>0.1) compared to healthy controls (Figure 2a-c). Comparison of both peptide-ELISA assays revealed a significant correlation of purified serum anti-MOG aa42-53 IgG+IgM+IgA antibodies with non-purified serum anti-MOG aa38-60 IgG antibodies (p<0.001) and a weak correlation with non-purified serum anti-MOG aa38-60 IgA (p=0.05) antibodies.

**Influence of immunomodulatory therapies on purified serum anti-MOG peptide antibodies**

After the first blood sampling all patients were followed for one year. During this time period 8 patients were treated with glatiramer-acetate, 10 patients were treated with interferon-beta and 10 patients did not receive any immunomodulatory therapy. Figure 3a-c shows the percentage of patients with positive immunoreactivities against each peptide for each treatment group. No significant change of the peptide immunoreactivities occurred after one year of treatment neither with glatiramer-acetate nor with interferon-beta nor in patients without any immunomodulatory therapy. Analyzing the number of positive immunoreactivities from each patient at baseline and month 12 revealed no significant change comparing both time points in each treatment group (data not shown).

**Clinical and demographic data and anti-MOG peptide antibody response**

The percentage of patients with positive immunoreactivities against MOG peptides is not equal in the different treatment groups with the most prominent anti-MOG peptide antibody response in the interferon-beta treatment group and the weakest antibody response in the group of patients...
without any disease modifying treatment (Figure 3a-c). Addressing this issue, we compared the MOG peptide antibody response with clinical data. If the last relapse before study entry occurred within 1 and 6 months, we found that patients had a significantly raised number of positive immunoreactivities against MOG peptides (Figure 4a) and the percentage of patients with positive reactivities against each MOG peptide was clearly higher (Figure 4b) compared to the group of patients in whom the time interval between the last relapse and the time point of study entry was greater than 6 months. Four out of eight patients in the glatiramer-acetate treatment group and all patients in the interferon-beta treatment group experienced the last relapse within 1 and 6 months before study entry. In contrast the time interval between the last relapse and the date of study entry was greater than 6 months in all ten patients in the non-treatment group.

Three patients had a relapse during the observation period of this study, 2 patients in the glatiramer-acetate treatment group and 1 patient in the interferon-beta treatment group. All 3 relapses occurred in the first 6 months of therapy. Comparing the number of positive immunoreactivities against MOG peptides before and after one year revealed a decrease in two patients and an increase in one patient.

We did not find any other correlation between anti-MOG peptide immunoreactivities and other clinical and demographic data (e.g. EDSS, relapse rate, disease duration, progression index, age) in the group of patients analyzed in this study.
Figure 3a-c. Percentage of patients with positive immunoreactivities of immunopurified serum anti-MOG antibodies against peptides corresponding to the extracellular domain of MOG before and one year after initiation of treatment. Antibody responses include IgG+IgM+IgA immunoglobulin isotypes. A cut-off of 95% specificity for MS for each peptide was used to determine positivity. No significant changes of immunoreactivities after one year were observed in patients treated with glatiramer-acetate (Figure 3a), in patients treated with interferon-beta (Figure 3b) and in patients without any immunomodulatory treatment (Figure 3c).
Figure 4a-b. Number of positive immunoreactivities against MOG peptides of each patient (4a) and percentage of patients with positive immunoreactivities against MOG peptides (4b) separately plotted for those patients who experienced the last relapse within 1 to 6 months before study entry (n=14) compared to patients whose last relapse before study entry dated more than 6 months back (n=14). The number of positive immunoreactivities against MOG peptides is significantly raised in patients who experienced the last relapse within 1 to 6 months before study entry (p=0.024) (4a). The percentage of patients with positive immunoreactivities against MOG peptides is increased in patients who experienced the last relapse within 1 to 6 months before study entry. Medians are indicated by horizontal bars (4a).
DISCUSSION


For this purpose we analyzed anti-MOG antibodies purified from serum samples from a group of patients with relapsing remitting MS. Epitope mapping of immunopurified serum IgG+IgM+IgA antibodies analyzing MS-patients before treatment with immunomodulatory drugs and healthy controls revealed a heterogeneous antibody responses. However, antibody responses against two overlapping epitopes aa37–48 and aa42–53 were significantly increased in MS-patients and were therefore immunodominant. Using a cut-off of 95% specificity for MS to determine positivity revealed that epitopes aa37–48 and aa42–53 were recognized by up to 54% of MS patients. These results are consistent with crystallization studies of MOG showing that residues aa41–46 are surface exposed and thus available to bind antibodies (Breithaupt, et al. 2003, Clements, et al. 2003). A higher immunogenicity of this surface exposed epitope and the permanent ability to be recognized by an autoimmune dysregulated immune system might explain the overrepresentation of antibody responses directed against MOG aa37–53 in MS-patients. The fact that anti-MOG aa37–53 antibody responses were extremely low in healthy controls implicates that this region might be a target for pathogenic antibodies in MS. The overrepresentation of the region aa37–53 in MS-patients might be masked when analyzing antibodies directed against the complete extracellular domain of MOG. This could be the reason why the differences between healthy controls and MS-patients were less distinctive in studies using the extracellular domain or full length MOG as an antigen (Karni, et al. 1999, Lampasona, et al. 2004, Lindert, et al. 1999, Mantegazza, et al. 2004, Reindl, et al. 1999).

Former studies on the epitope specificity of anti-MOG antibodies in MS-patients also suggested a heterogeneous antibody response with two frequently recognized epitopes aa1–26 and aa63–87, but the region aa37–53 was not overrepresented (Guggenmos, et al. 2004, Haase, et al. 2001). However, this discrepancy might be due to methodical differences. In these studies analyses of anti-MOG antibodies were performed using longer peptides and only IgG antibodies were detected. Some patients included in their studies underwent plasmapheresis because of a more severe clinical course probably reflecting a more unusual MS patients collective. In the present study epitope mapping of anti-MOG antibodies was performed using short peptides of 12 aa lengths and 7 aa overlaps. In addition, we detected the full spectrum of anti-MOG antibodies using a secondary antibody recognizing human IgG, IgM and IgA antibodies analyzed in MS-patients with a relapsing remitting disease course. The presence of IgG, IgM and IgA antibodies directed against MOG (Egg, et al. 2001, Kennel De March, et al. 2003) and MOG peptides (Kennel De March, et al. 2003, Vojdani, et al. 2003) in MS-patients has recently been demonstrated. To confirm our results from the present study we additionally analyzed non-purified serum antibodies from the same blood samples for each Ig-isotype directed against a
longer MOG peptide aa38–60. This MOG amino acid sequence has been used in two other studies (Guggenmos, et al. 2004, Haase, et al. 2001) and contains both immunodominant epitopes found in our epitope mapping study. We found raised anti-MOG aa38–60IgG and anti-MOG aa38–60IgA antibodies in MS-patients compared to healthy controls. These results indicate that the antibody response directed against the immunodominant epitopes found in this study is dominated by IgG and IgA antibodies. Increased anti-MOG peptide IgA antibody responses favor the hypothesis that molecular mimicry of mucosal associated immune responses with CNS antigens could be involved in the immunopathogenesis of MS (Kennel De March, et al. 2003).


Three interferon-beta preparations and glatiramer-acetate are approved for the treatment of relapsing remitting MS (Jacobs, et al. 1996, Johnson, et al. 1995, PRISMS-Study-Group. 1998, The-IFNB-Multiple-Sclerosis-Study-Group. 1993). Thus, most MS-patients with a relapsing remitting disease course are currently treated with immunomodulatory drugs. Interferon-beta and glatiramer-acetate exert their therapeutic effects by fundamentally different mechanisms (Neuhaus, et al. 2003, Yong. 2002). However, most studies on the mode of action of immunomodulatory drugs investigated the effects on T-cells and there is scant information regarding effects of immunomodulatory drugs on B-cells. Our previous data suggested a partial influence of immunomodulatory treatments on the anti-MOG antibody response measured at a single time point in a large cohort of 261 MS-patients (Egg, et al. 2001).

To investigate, whether the epitope specificity of anti-MOG antibodies is influenced by immunomodulatory drugs, we analyzed 28 RRMS patients receiving either glatiramaer-acetate, interferon-beta or no immunomodulatory drug before and after 1 year of treatment. Although immunoreactivities against MOG peptides were not completely identical before and after 1 year in each treatment group, no significant changes were found in the antibody reactivity against MOG peptides comparing both time points in each patient and in each treatment group, indicating that diverse antibody responses against MOG peptides are rather interindividual. Our findings are in line with recently published studies demonstrating that 1 year of treatment with interferon-beta did not induce any significant changes of anti-MOG antibody levels (Angelucci, et al. 2005, Bitsch, et al. 2004).

The anti-MOG peptide antibody response differed between treatment groups at baseline. Further analyses revealed that anti-MOG peptide immunoreactivities were more prominent in patients who experienced the last relapse within 1–6 months before study entry compared to those patients whose last relapse before study entry dated more than 6 months back. Intramolecular epitope spreading (Robinson, et al. 2003, Vanderlugt and Miller. 2002) might be the underlying mechanism for these findings. Our results indicate that disease activity leads to a time related diversified antibody immune response against MOG peptides in MS-patients. Three patients suffered from a relapse during the study period. Due to the small number of patients experiencing a relapse during the study one cannot deduce a causal relation between diversity of anti-MOG peptide B cell immune responses and subsequent disease activity (Bischof, et al. 2004, Robinson, et al. 2003, Smith, et al. 2005).
In summary, we present a precise analysis of the B-cell epitope specificity of serum anti-MOG antibodies including the isotypes IgG, IgM and IgA before and 1 year after initiation of treatment with immunomodulatory drugs. We demonstrate that two overlapping epitopes aa37–48 and aa42–53 are frequently recognized by up to 54% of MS patients. Additionally, we demonstrate that short-term treatment with immunomodulatory drugs does not significantly alter the antibody response against MOG peptides. Furthermore, this study indicates that recently experienced relapses intensify the anti-MOG peptide antibody reactivity. Our observations provide important implications to further investigate the pathological significance of antibodies directed against MOG peptides encompassing aa37–53 in MS-patients.

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