A Structurally Available Encephalitogenic Epitope of Myelin Oligodendrocyte Glycoprotein Specifically Induces a Diversified Pathogenic Autoimmune Response

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*J Immunol* 2004; 173:600-606; doi: 10.4049/jimmunol.173.1.600

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Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the CNS. MS is characterized by immune reactivity against various myelin proteins, including myelin basic protein (MBP), proteolipid protein (PLP), and myelin oligodendrocyte glycoprotein (MOG) (1). MOG is a type I transmembrane protein of unknown function that comprises only 0.01–0.05% of total myelin protein (2). Several lines of evidence indicate that MOG plays a prominent role in the autoimmune response in MS and its animal model experimental autoimmune encephalomyelitis (EAE). In contrast to other CNS proteins, MOG is located at the outermost surface of the myelin sheath, where it is directly accessible to autoreactive Abs (3). Active MS lesions contain large accumulations of MOG-specific Abs (4) and share a high degree of histopathological similarity with MOG-induced EAE lesions (5, 6). MOG is expressed late in development and exclusively in the CNS, where it is possibly sequestered from the places of immunological tolerance induction in the thymus and in the immune periphery. The epitope MOG92–106 induces EAE in SJL mice (7). Its human homologue is the major T cell epitope of MOG in HLA DR4-positive individuals (8). Determination of the crystal structure of MOG has revealed that this epitope is located at the outer surface of the extracellular Ig-like domain of MOG and a direct target for demyelinating autoantibodies (9, 10).

In this study, we show that the autoimmune response induced by immunization with MOG92–106 differs qualitatively from immune responses elicited by other CNS epitopes derived from MBP and PLP. Immunization of SJL mice with MOG92–106 induced strong EAE, but only poor peripheral T cell activation, indicating that the severity of the disease is not solely determined by autoreactive T cells. Mice with MOG92–106-, but not with MBP84–96- or PLP139–151-induced EAE developed Abs against encephalitogenic epitopes from MBP and PLP and against a broad range of peptide epitopes spanning the complete MBP sequence. The secondary Abs were of the isotypes IgG1 and IgG2b, indicating that self-reactive B cells receive help from activated T cells. In sharp contrast, B cell reactivity in MBP84–96- and PLP139–151-induced experimental autoimmune encephalomyelitis was directed against the disease-inducing Ag only. These data provide direct evidence that the nature of the endogenously acquired immune reactivity during organ-specific autoimmunity critically depends on the disease-inducing Ag. They further demonstrate that the epitope MOG92–106 has the specific capacity to induce a widespread autoimmune response. The Journal of Immunology, 2004, 173: 600–606.
MBP61–80 (RTTHYGLPQKSHQGRQDQE), MBP71–90 (KSQHGRQTDENPVHFKNI), MBP81–100 (NPVVHFFKNIVTPTPPPSQ), MBP91–110 (VTPRTTPPSQKGRGLSLSR), MBP101–120 (GKGRLSLSRF SWGGDRSRS), MBP111–127 (FSWGGDRSRSCSPMAR), MBP84–104 (VVFHFKNIVTPTPPPSQKGR), MBP84–96 (VVFHFKNIVTPTTP), MBP87–98 (FKNIVTPTPPPSQ), MBP75–70 (YELYNIVHAFQYV), PLP104–117 (KTTICCGKGLSATVT), PLP139–151 (HCLGKWLGHFPDKF), PLP178–191 (NTTWCTQ51SAF5PSK), and MOG92–106 (DEGGYCTFDRHDSHYQ) were synthesized using standard d-fluorenylmethoxycarbonyl chemistry and purified by HPLC. Chicken albumin (Sigma-Aldrich, Zwijndrecht, The Netherlands) was used as control Ag in ELISA. (MOG protein (aa 1–120) was kindly provided by T. Ziemssen (University of Dresden, Dresden, Germany) (11).

Immunizations and induction of EAE

Immunizations and EAE induction were performed essentially as described (12), with the following modifications. For immunizations, the indicated amount of peptide Ag in PBS was mixed with an equal amount of CFA containing 4 mg/ml Mycobacterium tuberculosis H37RA (Difco/BD, Alphen aan den Rijn, The Netherlands). Mice were immunized s.c. with 100 μg of the final emulsion.

EAE was induced by s.c. injection of 100 μg of an Ag/CFA emulsion that was distributed over four sites at the flanks. The emulsion contained the indicated amounts of Ag and 200 μg of M. tuberculosis H37RA (Difco). When EAE was induced with peptide Ags, mice were immunized on days 0 and 7 and received at days 0 and 2 in addition 200 ng of pertussis toxin (List Biological Laboratories, Campbell, CA) in PBS i.v. When EAE was induced with M. tuberculosis peptide Ags, mice were immunized on day 0 with 100 μg of rMOG in CFA containing 200 μg of M. tuberculosis H37RA and received a single injection of 200 ng of pertussis toxin. Clinical signs of EAE were assessed using the following score: 0, no apparent abnormalities; 1, tail or hind limb weakness; 2, limp tail and hind limb weakness; 3, severe hind limb paresis; 4, complete hind limb paralysis and front limb weakness; 5, dead (dead mice were scored 5 points when they had previously shown signs of progressive disease).

T cell proliferation assays

Lymph nodes were removed at day 10 after immunization, and single cell suspensions were prepared. Cells of four mice per group were pooled. To determine the specific T cell reactivity in the spleen, single cell suspensions from individual spleens were prepared, and erythrocytes were lysed by incubation in erythrocyte lysis buffer (0.15 M NH₄Cl, 1 mM KHCO₃, 0.1 mM Na₂EDTA, pH 7.5) for 5 min. The prepared single cell suspensions were cultured in 96-well plates containing 10⁵ cells/well in 200 μl of HL-1 medium (BioWhittaker, Walkersville, MD) containing 50 μM 2-ME, 100 μM penicillin, and 100 μg/ml streptomycin (all from BioWhittaker) and the indicated concentration of Ag. After 96 h of culture, 1 μCi/well [³H]thymidine was added, and the cells were harvested after an additional 12 h. Mean incorporation of thymidine was measured in triplicate wells by liquid scintillation counting.

ELISA

Blood was obtained from the tail vein and clotted at 4°C overnight. Cellular components were removed by centrifugation, and serum was stored with 0.1% NaN₃ at 4°C.

To determine Ag-specific Abs in serum samples, polystyrene ELISA plates (Costar, Cambridge, MA) were coated overnight with 10 μg/ml indicated Ag in PBS; washed with PBS, 0.05% Tween 20 (Sigma-Aldrich); and blocked with PBS, 0.05% Tween 20, and 1% milk powder for 1 h. After washing, the plates were incubated for 4 h with triplicates of serum samples diluted 1/50 in blocking buffer. Ag-specific Abs were detected with a 1/1000 dilution of HRP-conjugated secondary Abs specific for IgM, IgG1, IgG2a, or IgG2b (all Zymed Laboratories, Uden, The Netherlands). After thorough washing, the plates were developed with ABTS (Sigma-Aldrich), and OD₄₅₀ was measured in an ELISA reader (Bio-Tek Instruments, Winooski, VT). For IgM, IgG1, IgG2a, or IgG2b (all BD Biosciences, Alphen aan den Rijn, The Netherlands) blocked; incubated with HRP-conjugated secondary Abs; and developed with ABTS. OD₄₅₀ was determined, as above.

Results

Severe EAE and weak T cell responses in MOG92–106-induced EAE

First, we directly compared the capacity of the epitopes MBP84–96, PLP139–151, and MOG92–106 to induce EAE in immunized mice. Even at very high Ag doses, MBP84–96 induced only moderate EAE-symptoms, whereas PLP139–151 and MOG92–106 induced severe disease (Fig. 1). We then assessed whether the ability of these three epitopes to induce autoimmunity correlates to their T cell-stimulating capacity. Primary T cell cultures of mice immunized with MBP84–96, PLP139–151, or MOG92–106 were prepared from draining lymph nodes 10 days after immunization, and T cell proliferation against the immunizing Ag as well as the control Ag purified protein derivative was determined. MBP84–96 and MOG92–106 induced only weak T cell responses (stimulation indices of 4.5 and 5.8, respectively), whereas immunization with PLP139–151 induced very strong T cell proliferation (stimulation index >300; Fig. 2). Thus, despite the fact that MOG92–106 induces only weak T cell responses, MOG-induced autoimmunity is very severe.

FIGURE 1. Comparison of EAE induced with MBP84–96, PLP139–151, and MOG92–106 in SJL mice. Ten mice were immunized on days 0 and 7 with the indicated amount of the respective Ag in CFA containing 200 μg of M. tuberculosis. On days 0 and 2, mice received in addition 200 ng of pertussis toxin in PBS i.v. Clinical signs of EAE were assessed, as described in Materials and Methods, and the mean disease score (MDS) was plotted against the day postimmunization. The incidence rate was 10/10. The shown result is representative for more than five independent experiments.
immunized s.c. with 200 μg of MBP84–96, PLP139–151, or MOG92–106, respectively. At day 10 postimmunization, single cell suspensions of draining lymph nodes were prepared, and proliferation in response to the immunizing Ag was determined by [3H]thymidine incorporation. Background incorporation was <300 cpm, and the stimulation index of the control Ag purified protein derivative, which is a component of the adjuvant, was >20 in all cases (data not shown). Error bars represent SEM. The result is representative for three individual experiments.

**T cell responses to secondary epitopes in MOG-induced EAE**

It has previously been established that during PLP139–151-induced EAE in SJL mice, T cell reactivity against other myelin Ags develops and contributes to the perpetuation of the disease (13–15). It is thus conceivable that T cell reactivity against secondary myelin Ags contributes also to the disease severity in MOG-induced EAE. We assessed mice with MOG92–106-induced EAE for T cell responses against the disease-inducing Ag as well as the encephalitogenic epitopes MBP84–96 and PLP139–151. T cell reactivity against MOG92–106 was present in the spleen at day 21 and to a lesser degree at day 28 after disease induction (Fig. 3). In addition, T cell responses against the weakly encephalitogenic epitope MBP84–96 became apparent at day 21, peaked at day 28, and were still present at day 42 after immunization. There was at no time point any detectable reactivity against PLP139–151.

**B cell reactivity in MBP-, PLP-, and MOG-induced EAE**

We examined mice with MBP-, PLP-, and MOG-induced EAE in weekly intervals for B cell reactivity against a range of well-characterized encephalitogenic epitopes and overlapping dodecamer peptides spanning the complete 14-kDa isofrom of murine MBP.

B cell reactivity in MBP84–96-induced EAE was directed only against the disease-inducing Ag and the partially overlapping epitope MBP87–98 (Fig. 4A). Abs were detected above background levels from day 28 after disease induction until the end of the observation period at day 84. There was no significant B cell reactivity against any of the other epitopes tested.

Similarly, in PLP139–151-induced EAE, B cell reactivity was directed against the disease-inducing Ag only and not against any of the other epitopes (Fig. 4B). Abs against PLP139–151 were first detectable at day 21 after disease induction, and thus developed faster in PLP- as compared with MBP-induced EAE.

In mice with MOG92–106-induced EAE, Abs against the primary Ag were present from day 28 after disease induction until the end of the observation period (Fig. 4C). In striking contrast to MBP- and PLP-induced EAE, mice with MOG-EAE developed massive B cell reactivity against encephalitogenic MBP and PLP epitopes as well as peptide Ags spanning the complete sequence of murine MBP. Abs against these epitopes were not present in CFA-immunized mice, and there was no B cell reactivity against the control Ag albumin. This endogenously acquired self-reactivity appeared at days 35 and 42, 1–2 wk later than Abs against the primary Ag. In striking contrast to what has been observed for T cell epitope spreading (14, 15), the diversification of the B cell reactivity toward secondary self epitopes did not follow a sequential cascade, but rather represented a simultaneous spread toward a broad range of myelin Ags.

**Self-reactive Abs in MOG92–106-induced EAE are of the isotypes IgG1 and IgG2b, but not IgG2a**

In all assays, the detected Abs in MOG-EAE were of the IgG isotype, indicating that B cells that produce these Abs had undergone isotype switching. Isotype switching in B cells requires soluble factors and contact-mediated signals provided mainly by T cells (16). In general, Th1 cells typically produce IFN-γ (17), which induces B cells to secrete Abs of the isotype IgG2a (18), whereas Th2 cells secrete IL-4 (17), which induces isotype switching to IgG1 (19). To further characterize the nature of the B cell responses in MOG-induced EAE, serum samples of individual mice were examined for CNS-specific Abs of the isotypes IgG1, IgG2a, and IgG2b. The Abs against the disease-inducing Ag MOG92–106 as well as Abs against the secondary epitopes PLP139–151 and MBP84–96 were of the isotypes IgG1 and IgG2b, but not IgG2a (Fig. 5A). Sensitivity and specificity of the ELISA were confirmed with isotype control Abs (Fig. 5B).

**No detection of Abs against secondary peptide Ags in MOG protein-induced EAE**

Several studies have shown that the Ab reactivity against MOG protein differs from the reactivity against linear peptide epitopes (20). To assess whether a diversified Ab response is also induced by MOG protein, we established a protocol to induced EAE in SJL mice with rat rMOG protein, aa 1–120 (11) (Fig. 6). SJL mice that received a single s.c. immunization with 200 μg of MOG protein and a single i.v. injection of 200 ng of pertussis toxin developed severe and very consistent disease symptoms starting at day 10 after disease induction. In contrast to MOG peptide-induced EAE, EAE induced by MOG protein did not follow a relapsing remitting course. Serum samples were collected from these mice in weekly intervals and assessed for Abs against peptide epitopes derived from PLP, MOG, and MBP by ELISA. Despite severe clinical
EAE, no Ab reactivity against any of these linear peptide epitopes could be detected (Fig. 7).

**Abs against PLP139–151 enhance MOG92–106-induced EAE**

We next asked whether the Abs against secondary epitopes contribute to disease severity of MOG92–106-induced EAE. To this end, mice were immunized with 200 μg of MOG92–106 in CFA containing 200 μg of *M. tuberculosis* and received i.v. injections of 200 ng of pertussis toxin on days 0 and 2 after immunization. This protocol induced only weak EAE symptoms (Fig. 8). Adoptive transfer of serum obtained from mice immunized with PLP139–151 that contained large amounts of PLP139–151-specific Abs, but not from CFA-immunized
mice, markedly enhanced disease severity (Fig. 8), demonstrating that PLP139–151-specific Abs are pathogenic.

Discussion

In this study, we demonstrate that immunization with the epitope MOG92–106, but not with MBP84–96 or PLP139–151, induces a diversified autoimmune response against a broad range of CNS Ags. Why is this epitope able to induce such a global response, while other clearly immunogenic and encephalitogenic epitopes do not? Because these other epitopes elicit very strong T and B cell responses, the property of MOG92–106 to induce a diversification of the immune reactivity does not seem to be simply a consequence of the magnitude of the response, but rather seems to be due to qualitative properties of the specific epitope and its interaction with the host immune system.

FIGURE 5. Abs against MBP84–96, PLP139–151, and MOG92–106 generated in mice with MOG92–106-induced EAE are of the isotypes IgG1 and IgG2b, and not IgG2a. A. Serum from individual mice with MOG92–106-induced EAE was examined for Abs against MBP84–96, PLP139–151, and MOG92–106 using secondary Abs specific for the isotypes IgG1, IgG2a, and IgG2b. The data are depicted as absorbance at 405 nm. Shown is the result of a single mouse at day 42 after disease induction, which is representative of the results obtained from four individual mice at days 35, 42, 49, and 84 postimmunization. At each time point, control mice immunized with CFA alone were included in the assay. Error bars indicate SEM of triplicate wells. B. Specificity of the secondary Abs was determined by ELISA. Wells were coated with isotype control Abs or albumin and detected with anti-IgG1, anti-IgG2a, and anti-IgG2b, as indicated. Error bars represent SEM.

FIGURE 6. Course of MOG protein-induced EAE in SJL mice. EAE was induced by a single s.c. immunization with 200 μg of rMOG in CFA and i.v. injection of 200 ng of pertussis toxin. The incidence rate was 6/6. The shown result is representative for two independent experiments. Error bars represent SEM.

FIGURE 7. Absence of B cell reactivity against peptide Ags in MOG protein-induced EAE. Serum was obtained from mice with MOG protein-induced EAE at the indicated time points after disease induction. No Abs against the indicated peptide Ags were detected in pooled serum by ELISA using an IgG-specific secondary Ab. A as a positive control (pos. contr.), serum of mice with PLP139–151-induced EAE was tested against PLP139–151 peptide on each ELISA plate. Error bars indicate SEM.

FIGURE 8. Serum containing PLP139–151-specific Abs enhances MOG92–106-induced EAE. EAE was induced by a single immunization with 200 μg of MOG92–106 in CFA containing 200 μg of M. tuberculosis plus i.v. injection of 200 ng of pertussis toxin on days 0 and 2. On days 1, 4, 7, and 11, mice received in addition i.v. injections of 70 μl of serum from PLP139–151- or CFA-primed mice, as indicated. The presence of high titers of PLP139–151-specific Abs in serum from PLP139–151-primed mice was confirmed by ELISA (data not shown).
Previously, several lines of evidence have pointed toward a prominent role of MOG92–106 in CNS-directed autoimmunity. MOG92–106 is encephalitogenic in SJL mice, DA rats (21), and rhesus monkeys (22). Its human homologue is the major T cell epitope in HLA DR4-positive individuals (8). In addition, MOG101–108, in contrast to other parts of the MOG protein, does not display significant homology to potentially tolerogenic protein sequences outside the CNS, and the immune system may thus fail to establish self-tolerance toward this particular epitope (10). Determination of the crystal structure of MOG has revealed that MOG residues 101–108 are located at the outer surface of the MOG protein and MOG is expressed at the outer layer of the multilayered myelin sheath in the CNS (10). In intact myelin, the epitope is thus easily accessible for specific autoantibodies, and such Abs have been shown to induce myelin degradation in vitro and in vivo (9). In contrast, the lack of the spreading of the immune response after MBP84–96 and PLP139–151 immunization may be due to the cryptic nature of these epitopes, which are expressed in the cytoplasm of oligodendrocytes.

That the potency to induce a diversified autoimmune response is a specific property of a self Ag and associated with its accessibility for autoantibodies has recently been observed in a rabbit model for systemic lupus erythematosus (23). In this model, rabbits immunized with the A6 epitope of PLP178–191 was Ag specific, as rabbits immunized with the A6 epitope of nAb specific for secondary epitopes appeared between weeks 4 and 7 after immunization. The property to induce such a global response was Ag specific, as rabbits immunized with the A6 epitope of nuclear riboprotein A or other control epitopes that are not expressed at the surface of the protein and thus not accessible for autoantibodies developed B cell reactivity against the peptide of immunization only.

What may be the mechanism of the diversification of the immune reactivity? Following the initial attack, B cells that are specific for the primary Ag may act as APCs, take up their target Ag, and present it on MHC class II molecules together with other Ags that become available during myelin degradation. This will cause activation of MOG92–106-specific T cells and T cells specific for secondary epitopes. These cells will in turn provide signals to autoreactive B cells that have been triggered by the newly available myelin Ags. That B cells that produce Abs against secondary epitopes indeed receive the T cell help is indicated by the fact that these Abs are of the IgG isotype. The B cells had thus undergone isotype switching, a process that requires soluble factors and contact-mediated signals provided mainly by T cells (16). The Abs against secondary epitopes are pathogenic. Mice with MOG92–106-induced EAE produce PLP139–151-specific Abs, and the adoptive transfer of such Abs enhances MOG92–106-induced EAE. This is in line with previous experiments that demonstrated that adoptive transfer of CNS-specific Abs is disease enhancing in several EAE models (9, 24).

It was previously shown that the Abs responses in MOG protein-induced EAE are at least in part conformation dependent (25, 26). The epitopes recognized by these Abs are determined by the three-dimensional structure of the MOG protein and are not present in linear peptide stretches (4, 10, 27). Consistent with this, no Abs against any of the linear peptide epitopes could be detected in mice with MOG protein-induced EAE. This further demonstrates that the potency to induce a diversified autoimmune response is a specific property of the epitope MOG92–106. The secondary Abs detected by ELISA in mice with MOG92–106-induced EAE were directed against linear peptide epitopes. These Abs have been generated in vivo, and thus recognize endogenously expressed self Ags. Similar observations have been made in individuals with MS.

MS patients generate large amounts of Abs against linear peptide epitopes of several CNS proteins, and such Abs accumulate in MS plaques (4). The Abs destroy their target Ag via complement-mediated lysis or through generation of hydrogen peroxide (28). In addition, the B cells that produce these secondary Abs may again act as APCs. Because B cells are highly efficient APCs for their target Ag (29), these cells may in turn facilitate the activation of CNS-specific T cells. This assumption is perfectly in line with an earlier hypothesis of Mamula and Janeway (30), who suggested that during an autoimmune response, the spread of the reactivity toward secondary epitopes may be driven by Ag-specific B cells that are 10,000-fold more efficient than other APCs in presenting their target Ag to autoreactive T cells.

The observed global autoimmune response in MOG92–106-induced EAE may explain why the disease is severe, while peripheral T cell activation by this epitope is only moderate. This discrepancy between the poor capacity to activate autoreactive T cells and the strong potency to induce an autoimmune disease has also been observed for the homologous epitope MOG91–114 in RT1b rats (31). The severity of the disease induced by this Ag is thus not solely determined by autoreactive T cells.

The observed diversification of the B cell reactivity differs markedly from T cell epitope spreading in EAE (15). T cell epitope spreading in EAE occurs after myelin damage and contributes to relapses and progression of the disease (13, 32). Relapsing remitting EAE induced with PLP139–151 in SJL mice is accompanied by a predictable sequential cascade of newly emerging T cell epitopes (14). The order of the arising T cell epitopes (PLP139–151 > PLP178–191 > MBP84–104) correlates to their relative potential to induce EAE in immunized mice, and it was suggested that this correlation may be a general principle for epitope spreading in EAE (15). The observed diversification of the B cell reactivity observed in this study does not follow a sequential cascade, but rather represents a simultaneous spread toward a broad range of antigenic epitopes. T cell epitope spreading and B cell epitope spreading thus follow different rules and are likely to be mechanistically distinct processes. They contribute to varying degrees to the diversification of the immune response in organ-specific autoimmunity and critically depend on the disease-inducing Ag.

Acknowledgments

We thank Pauline Weder for technical assistance, and Dr. Tjalf Ziemssen, University of Dresden, for rMOG1–120 protein.

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