MOG79-90 PEPTIDE IN COMPLEX WITH RECOMBINANT MHC CLASS II MOLECULES AMELIORATES EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

T. Batsalova¹, M. Vestberg², R. Holmdahl³ and B. Dzhambazov¹
¹Plovdiv University, Biological Faculty, Department of Developmental Biology, Plovdiv, Bulgaria
²University of Oslo, Norwegian Transgenic Center, Oslo, Norway
³Karolinska Institute, Division of Medical Inflammation Research, Stockholm, Sweden

Correspondence to: Balik Dzhambazov
E-mail: balik@uni-plovdiv.bg

ABSTRACT
The recent advancement in the field of biomedical technologies has opened up new possibilities in the treatment of autoimmune disorders such as multiple sclerosis (MS). Here, we report the production of soluble complexes between the myelin oligodendrocytic glycoprotein (MOG) 79-90 peptide and a genetically engineered murine MHC class II molecule Aβ. Using mouse model of MS, we demonstrate that the generated complexes are functional and able to ameliorate the clinical signs and reduce the incidence of experimental autoimmune encephalomyelitis (EAE). Our findings offer a new possibility for the treatment of chronically active autoimmune inflammation such as multiple sclerosis.

Keywords: multiple sclerosis, experimental autoimmune encephalomyelitis, vaccination, MHC II/peptide complex, myelin oligodendrocytic glycoprotein, T cells

Introduction
Multiple sclerosis (MS) is the most common chronic autoimmune disease affecting the central nervous system (CNS) of young adults. Pathologically, the disease manifestations include development of multiple demyelinating plaques (infiltrated mainly with activated T lymphocytes, macrophages and B cells), astrocitic gliosis, loss of oligodendrocytes and axonal destruction within the brain and the spinal cord (8, 22). MS patients exhibit variable neurological symptoms (impaired vision, paresis and paralysis, sensations of burning or pricking, ataxia, fatigue and incontinence, considerable cognitive impairment, difficulties with memory, concentration and other mental skills), which lead to significant physical disability (8). Despite intensive research, the disease aetiology is still uncertain. It is believed that a combination of genetic and environmental factors cause MS development. Like other autoimmune disorders, the strongest known genetic factor involved in MS pathogenesis is the major histocompatibility complex class II (MHC II) region, particularly certain HLA-DR2 alleles (16). The autoaggressive T and B cell responses causing multiple sclerosis could be directed towards different myelin proteins (myelin basic protein (MBP), proteilipid protein (PLP), astroglial S-100 beta protein, myelin-associated glycoprotein (MAG), glial fibrillary acidic protein) (13, 22). Recently, the myelin oligodendrocytic glycoprotein (MOG) gained a notable interest as a candidate autoantigen in MS. MOG is a minor component of the CNS comprising ~0.05% of the myelin sheath outermost surface (11). The extracellular part of this protein could be a potential target for pathogenic antibodies and immune cells. Indeed, MOG-specific autoantibodies have been detected in actively demyelinating lesions and cerebrospinal fluid (CSF) of MS patients (9, 14, 20). In addition, high frequencies of MOG-reactive T cells in blood and CSF have also been reported in MS (6, 10, 13). Immunization of rodents and primates with MOG or MOG-derived peptides leads to experimental autoimmune encephalomyelitis (EAЕ) – a widely accepted animal model resembling human MS in both clinical and pathological signs (1, 12). Thus, manipulating MOG-directed autoimmunity could give raise to a new and more effective
therapy for MS.

In the present article we report the bio- and immunochemical characterization of murine recombinant MHC class II molecules (A\(^8\)) and the subsequent production of A\(^8\)/MOG79-90 peptide complexes. We show that the generated MHC II/peptide complexes are functional in vivo and able to ameliorate active MOG-induced EAE following either intravenous or intranasal administration. Our results represent a new vaccination approach targeting pathogenic T cells in EAE and possibly in MS.

Materials and Methods

A\(^8\) constructs and transfection of SL2 Drosophila melanogaster cell line

The cDNAs for Aα\(^8\) and Aβ\(^8\) were previously produced and described by Dzhambazov et al. (7) The constructs were cloned separately into pMTAL (Invitrogen, La Jolla, CA) or pRMHa-3 (4) vector to allow for heavy metal-induced expression in insect cells. PMTAL contains the resistance gene for hygromycin, while pRMha-3 carried a Copia promoter-driven hygromycin gene as a selection marker.

Following vector linearization the constructs for A\(^8\) α- and β-chain were co-transfected at equimolar ratios into Drosophila melanogaster SL2 cells (American Type Culture Collection, CRL-1963) using calcium phosphate. Stably transfected cell line was generated after 10 days of selection in Schneider’s Drosophila medium (Invitrogen Life Technologies) containing 100 g/ml Hygromycin B (Sigma-Aldrich). Expression and purification of soluble A\(^8\) molecules

Expression and purification procedures were performed as previously reported (7). In brief, for large-scale expression of recombinant protein, the transfected SL2 cells were grown in serum-free Insect express medium (PAA Laboratories) using Fernbach bottles. After induction with 0.7 mM CuSO\(_4\) for 4 days the cell medium was collected and cleared by centrifugation and filtration. Soluble A\(^8\) molecules were purified from the obtained supernatant using Ni-NTA (Qiagen GmbH, Hilden, Germany) affinity chromatography according to the manufacturer’s recommendations. After dialysis against PBS (Gibco\(^\text{TM}\), Paisley, Scotland, UK), positive fractions were pooled, concentrated 5- to 10-fold by Amicon\(^\text{®}\) centrifugal filter devices (MILLIPORE Co, Billerica, MA) and loaded with a peptide to form MHC-peptide complexes. Protein concentration was determined using a Dc protein assay (Bio-Rad Laboratories, Hercules, CA).

ELISA, SDS-PAGE and Western blot analyses

A\(^8\) heterodimers were detected by sandwich ELISA, as previously described (7), using a capturing Y-3P monoclonal antibody (mAb), specific for the native α-chain, and a biotinylated 7-16.17 detection mAb (BD PharMingen, Los Angeles, CA), specific for the β-chain of A\(^8\). 96-well flat-bottom plates (Nunc, Danmark) were coated with 5μg/ml Y-3P antibody for 2h at room temperature. The plates were then washed with PBS and unspecific binding was blocked by incubation with 1% BSA (Sigma-Aldrich) for 1h. After washing 50μl of the protein fractions were plated in triplicates and incubated for 2h at room temperature. Then, the plates were washed, 1μg/ml 7-16.17 antibody was added to each well and incubated for 1h. Following washing with PBS/Tween buffer, the positive signal was revealed by Eu\(^3+\)-labeled streptavidin using dissociation-enhanced time-resolved fluoroimmunoassay (DELFIA) and a multi-label counter (Wallac, Turku, Finland).

The purity of positive protein fractions was evaluated by SDS-PAGE. Samples were electrophoresed in 4-20 % polyacrylamide gradient ready mini-gels (Bio-Rad Laboratories, Hercules, CA) under denaturing and non-reducing conditions. The gels were silver stained according to the manufacturer’s recommendations.

For Western blot analyses, unstained SDS-PAGE gels were electrotransferred onto nitrocellulose membranes. The membranes were blocked in 5 % non-fat dry milk in PBS for 1 h and blotted with different MHC II specific antibodies (M5/114, 7-16.17, 7-23.1, PCQ.6, 34-5-3, Y-3P) at 4°C overnight. After extensive washing, blots were incubated with peroxidase-conjugated goat anti-mouse IgG or goat anti-rat IgG (for M5/114) antibodies (Jackson ImmunoResearch) for 1 h. Immunoblots were developed using diaminobenzidine (Vector Laboratories Inc., Burlingame, CA).

Assembling and purification of peptide/A\(^8\) complexes

Empty soluble A\(^8\) molecules were loaded with 5 to 10-fold molar excess of MOG79-90 peptides at 4°C for 72 h. The formed MHC-peptide complexes were purified by anion-
exchange and gel filtration HPLC (7), concentrated by Amicon® centrifugal filter devices (MILLIPORE Co, Billerica, MA) and stored at −20°C until used.

Animals
Male B10.Q, 8-10 weeks of age, were used in the EAE experiments. The mice were bred at the animal facility of Medical Inflammation Research, Lund University and kept under standardized conditions. All in vivo experiments were approved by Lund-Malmö ethical committee.

Antigens
Mouse myelin oligodendrocytic glycoprotein MOG79-90 peptide (GKVTLRIQNVRF) was purchased from Schafer-N (Copenhagen, Denmark), dissolved in PBS and kept at 4°C until used. Recombinant rat MOG (rMOG) incorporating N-terminal amino acid residues 1-125 from the native protein was dissolved in sodium acetate buffer (pH 3.0) and stored at -20°C.

Induction and clinical evaluation of EAE
For induction of EAE, mice were immunized subcutaneously into the back with 50 µg of rMOG emulsified in adjuvant mixture of 100 µg Mycobacterium tuberculosis H37RA (Difco, Detroit, MI) in IFA (Difco, Detroit, MI). On the same day and day 2, mice were given an intraperitoneal (i.p.) injection of 400 ng Bordetella pertussis toxin (PT) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Animals were monitored daily for disease symptoms and were scored for disease severity according to the following scale: normal appearance (0); tail weakness (1); tail paralysis (2); tail paralysis and/or mild waddle (3); tail paralysis and/or severe waddle (4); tail paralysis and/or paralysis of one limb (5); tail paralysis and/or of both limbs (6); tetraparesis (7); death (8).

Peptide/Aβ complex treatment protocols
Animals were treated by either intravenous (i.v.) injection of 100µg or intranasal (i.n.) administration of 10µg purified MOG/Aβ complexes on days 4 and 8 after immunization.

Statistics
In order to compare nonparametric data for statistical significance, we applied the Mann-Whitney U and Kruskal-Wallis test on all clinical results using the StatView™ programme (SAS, Institute Inc., USA).

Results and Discussion
The constructs for Aβ α- and β-chains were designed from the extracellular part of the native molecule. The transmembrane and intracellular domains were replaced by leucine zipper motifs from Fos and Jun to the C-terminal of the MHC α and β sequences, respectively, which facilitated correct dimerization and assembling of the heterodimer (19). In addition, the α-chain carried C-terminal tag of 6 histidine residues, which enables purification by NiNTA method. The β-chain construct contained C-terminal biotin site that allows biotination of the molecule and subsequent tetramer formation by binding to fluorochrome-labeled streptavidin. The genes for both Aβ chains were put under the control of metallothionein promoter, which enables their transcription and translation only in presence of heavy metal ions in the culture medium. It was previously shown that copper ions are the least toxic for insect cell cultures and do not induce typical heat-shock protein response (4). Thus, we chose CuSO₄ as a chemical agent for induction of recombinant MHC II expression.

After establishment of the new transfected insect cell line that stably incorporated the DNA constructs for Aβ α- and β-chains and optimization of the culture conditions we started large-scale expansion of the cells in order to achieve a significant yield of recombinant proteins. Following 4 days of induction with CuSO₄, transfected SL2 cells produced 2-3 mg of recombinant protein per liter of culture. Aβ molecules were extracted from cell-free culture supernatant by NiNTA affinity chromatography. Non-reducing SDS-PAGE analysis of NiNTA purified Aβ showed two bands (Fig. 1A) with molecular weights of 29 and 33 kDa (approximately corresponding to the predicted sizes of the α- and β-chains). This result demonstrates that the expressed proteins form heterodimers consisting of α- and β-chains.

Soluble Aβ molecules from culture media of transfected SL2 cells were also characterized for proper folding by sandwich ELISA, using specific monoclonal antibodies for the native α- (Y-3P) and the β-chain (7-16.17). As it is clearly shown in Fig. 1B, only induced cells expressed significant amount of the recombinant protein, while the non-induced transfected SL2 cells secreted soluble Aβ at a basal level, detectable only compared to non-transfected cells. The data from the ELISA assay undoubtedly proved that Aβ proteins were correctly folded since the conformation-
The Y-3P, 7-16.17 and 7-23.1 antibodies bound specifically to Aβ heterodimers whereas M5/114 stained both single β-chain and heterodimers. Antibodies 34-5-3 and PCQ.6 did not demonstrate good staining.

The in vivo effects of peptide/Aβ complexes were assessed using the EAE model. Our experiments were based on previous findings where mouse strains expressing the Aβ MHC II haplotype have been shown to display high susceptibility to MOG-induced EAE. In this model, the T cell immunodominant epitope from MOG has been determined and shown to include amino acid residues from position 79 to 96 (1). Interestingly, this epitope partially overlaps with an immunodominant peptide identified in MS patients – MOG63-87 (5, 23). These facts together strongly support the convenience of Aβ-expressing mouse strains in studying disease mechanisms and evaluating new therapies with potential application for treatment of MS. In our experiments we used slightly shorter synthetic MOG peptide (i.e. MOG79-90). Such reduction in the amino acid sequence improves the solubility of the antigenic fragment – an important quality for vaccine production, administration and effectiveness. At the same time the excision of 6 amino acid residues does not affect the immunodominant properties of the peptide (1).

The vaccination experiments included three groups of MOG-immunized B10.Q mice: 1) injected intravenously with MOG79-90/Aβ complexes, 2) treated intranasally with MOG79-90/Aβ, 3) a control group, injected with PBS. All animals were treated simultaneously on days 4 and 8 post immunization. These particular time points were chosen because we aimed to examine the therapeutic effect of MOG79-90/MHC II treatment on already established disease when pathogenic T cell populations have already expanded giving rise to inflammatory process in the CNS. As shown by data presented in Fig. 2, both i.v. and i.n. administration with MOG/Aβ complexes significantly reduced the incidence and clinical score (severity) of EAE. Intravenous injection of peptide/Aβ complexes offered a better protection against EAE, although the intranasal administration was enough to significantly ameliorate the clinical signs of disease. These results demonstrate that treatment with peptide/Aβ complexes affect specifically T cell responses, which led to downregulation of disease symptoms.

In similarity with our results, it has earlier been reported that MOG35-55 peptide covalently bound to recombinant α1-

---

**Table 1**

<table>
<thead>
<tr>
<th>mAbs</th>
<th>single α-chain</th>
<th>single β-chain</th>
<th>α-β dimers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y-3P</td>
<td>0</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>M5/114</td>
<td>-</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>7-16.17</td>
<td>-</td>
<td>0</td>
<td>++</td>
</tr>
<tr>
<td>7-23.1</td>
<td>-</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>34-5-3</td>
<td>-</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PCQ.6</td>
<td>-</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

(0) - no binding, (+) - weak binding, (+++) - medium binding, (++++) - strong binding, (-) - absence of cross-reactivity.
and β1-domains of the human DR2 molecule could induce efficient tolerance in DR2 transgenic mice (21).

Administration of soluble MHC II/peptide complexes represents an effective approach for selective blockage of pathogenic T cells. MHC II/peptide molecules are recognized by antigen-specific T cells – an interaction that provides the first essential signal for T lymphocyte activation. However, in the absence of costimulatory stimulus (B7-1, B7-2, CD40, normally expressed on the surface of antigen-presenting cells (APC)) abrogates the initial activation process and leads to T cell anergy – a state of unresponsiveness to subsequent antigen presentation, proliferation failure and inability for production of pro-inflammatory cytokines (3). Recently, it has been shown that T cells from peptide/MHC II treated mice could transfer the vaccination effect (7). These findings clearly suggest that mechanisms of active tolerance induction like regulatory T cell activation or/and pro/anti-inflammatory cytokine switch are also triggered following MHC II/peptide treatment. Certainly, the precise mechanisms leading to disease inhibition remain to be solved. Most importantly, further research on a possible bystander effect of the MOG79-90/MHC II therapy is needed in order to validate it as an effective vaccination approach applicable in clinical trials with multiple sclerosis patients.

Conclusions
In summary, the data presented in this paper demonstrate:

a) A convenient and productive methodology for generation of functional peptide/recombinant MHC II complexes;

b) Correct folding and binding properties of the recombinant Aα protein;

c) Effective suppression of MOG-induced EAE following therapeutic treatment with MOG79-90/Aα complexes.

Acknowledgment
This work was supported by funds from the King Gustaf V’s 80-Year Foundation, the Anna-Greta Craaford Foundation, the Swedish Strategic Foundation, and the Swedish Science Research Council.
REFERENCES


