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Association of New Putative Epitopes of Myelin Proteolipid Protein (58-74) with Pathogenesis of Multiple Sclerosis

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ABSTRACT

Multiple sclerosis (MS) is an autoimmune disease in which auto-reactive T cells react with self-antigens expressed in the central nervous system (CNS). The main cause of MS is unknown. Nonetheless, the most probable theory is based on molecular mimicry, which suggests that some infections can activate T cells against brain auto-antigens like myelin proteolipid protein (PLP) and initiate the disease cascade. This study is conducted to evaluate the activatory effects of PLP_{58-74} on T lymphocytes and humoral immunity.

PLP_{58-74} was considered as an immunodominant epitope candidate of PLP using bioinformatics tools. Patients and healthy individuals' peripheral blood mononuclear cells (PBMCs) were treated with PLP_{58-74} and its proliferative effects were evaluated through assessing proliferating cell nuclear antigen (PCNA) gene expression changes by real time PCR and immunocytochemistry assay. Finally, the rate of $CD4^+$ and $CD8^+$ T cells were assessed by flowcytometry. ELISA was also performed to measure anti PLP_{58-74} antibody in patients' serum.

PLP_{58-74} induced proliferation in patients' PBMCs while it did not influence PBMCs of healthy individuals. $CD4^+$ T cells were the main activated cells in reaction to PLP_{58-74} which increased from 22% to 39.91%. In addition, immune assay showed threefold increase in specific anti PLP_{58-74} IgG in patients compared to healthy controls.

Results showed that PLP_{58-74} can stimulate $CD4^+$ T cells and humoral immunity. Therefore it seems that the epitopes of some microorganisms mimicking PLP such as PLP_{58-74} might have a potential role in the initiation of MS.

Keywords: Autoimmune disease; Experimental autoimmune encephalomyelitis; Myelin proteolipid protein; Molecular mimicry; Multiple sclerosis

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INTRODUCTION

Multiple sclerosis (MS) is the most common autoimmune disease of the central nerves system (CNS),

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affecting more than 2.5 million people all over the world. Moreover, incidences of MS in females is twice the number of males.[1-3] Disease onset is mostly common in young adults so that it could affect the productivity of their lives.[4] The most applied treatment is based on the suppression of the immune system although there is no effective drug to cure or prevent the disease.[5,6]

Rivers et al. observed that injection of brain extract to healthy monkeys could lead to MS-like disease, thus they revealed that MS is an auto-immune disease.[7] Immune cells including $CD4^+$ and $CD8^+$ T cells, B cells, APCs such as dendritic cells (DCs) and macrophages trigger an immune response against the myelin in the CNS, destroying the myelin and the axons which is typically known as MS.[8]

The etiology of MS is not well understood yet, but there is a significant association between environmental factors such as infections and the disease.[7] The most probable theory for commencing MS is based on molecular mimicry, through T cell activation by infectious agents.[1] Homologies between pathogens and self-antigens could lead to the incidence of cross-reactive T cells.[9] T cell reactivity to brain auto-antigens, including myelin basic protein (MBP), proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG), plays an important role in the initiation of MS.[10,11]

PLP which is the most abundant protein in the CNS myelin, plays an important role in the stability of the structure and function of myelin.[12] PLP's highly conserved sequence consists of 277 amino acid residues and four highly hydrophobic trans-membrane domains. The PLP amino acid sequences of bovine, rat, mouse, dog and human are 99% identical, suggesting that PLP has imperative roles in the CNS.[13,14] The X-linked PLP gene encodes two proteins: PLP and its alternative splice isoform, DM20. Dm20 mRNA lacks 105 bps of exon 3 of PLP mRNA therefore 35 amino acids of its cytosolic loop are lost.[15,16]

The acylation of PLP makes it an effective auto-antigen which can induce experimental autoimmune encephalomyelitis (EAE) in rodents and non-human primates.[12,17] Due to the encephalitogenic potentials of PLP, several continuous peptide were previously selected to induce EAE[18]. Studies showed that residues 43–64, 139–151, 178–191, 104-117, 57-70 of PLP have encephalitogenic potential in PL/J and SJL mice.[19-23] Previous studies have shown that T cells are able to respond to $PLP_{40–60}$,[24] $PLP_{89–106}$,[25] $PLP_{30–49,}$ $PLP_{180–199}$,[26] and $PLP_{184–209}$.[27]

Mimicry hypothesis could be proven regarding the incidence of MS by finding the best epitopes of PLP and significant similarity between these epitopes and some microbial epitopes leading to development of early MS diagnosis which might be useful to prevent or even cure the disease in future. This study is aimed at providing new insights into the etiology of multiple sclerosis.

Based on our previous finding[28] we hypothesized that predicted PLP_{58-74} plays an important role in cell mediated and humoral immunity of MS patients. To investigate this hypothesis, we examined T cell proliferation and IgG assays in MS patients in comparison with healthy individuals. We also looked for homologous sequences between PLP_{58-74} and pathogens in protein databases.

MATERIALS AND METHODS

Epitope Prediction and Peptide Synthesis

PLP peptide 58-74 (YEYLINVIHAFQYVIYG) was synthesized by TAG Copenhagen (Denmark) according to the human sequence of PLP and proved to be >95% pure. PLP peptide 58-74 is an immunodominant epitope according to the predictions of bioinformatics databases.[28] This peptide is highly capable of forming different bindings with a variety of MHC class I and II molecules, including those most commonly found in MS patients population (HLA-A24, DRB1*15, DRB1*04,
DQA1*01:02/DQB1*0602,
DQA1*04:01/DQB1*0402,
DQA1*05:01/DQB1*0201,
A1*05:01/DQB1*0301).[29,30]

Sample Preparation

Blood samples were collected from 30 MS patients who referred to a referral MS clinic (Nabavi Clinic) in Tehran, Iran, with the average age of 26 (ranging from 19-56 years). Clinical evaluations and MRI diagnosed all 30 individual as definite MS patients according to the criteria defined by McDonald.[31] Patients were of relapsing-remitting form of MS (RR-MS), 2:3 female and 1:3 male. The control group consisted of 30 healthy individuals with the average age of 28 (ranging from 20-40 years). Patients had not received corticosteroid or other immunomodulatory therapies at all.

Ethical Aspects

The research project was approved by the Ethics Committee of National Institute of Genetic engineering and Biotechnology (No IR.NIGEB.EC.1395.1.24.) According to ethical guidelines, all samples were obtained from patients who had signed informed consent forms.

PBMC Isolation

Peripheral blood samples (5 ml) were obtained from the cubital vein and were collected in cell preparation tubes containing Heparin. First, blood samples were centrifuged at 300 g for 5 min and the plasma of samples was preserved for ELISA assay. Then, blood samples were diluted with an equal volume of phosphate buffered saline (PBS). Peripheral blood mononuclear cells (PBMCs) were isolated from 5 ml of each blood sample through Ficoll-Hypaque (GE Healthcare, Sweden) density centrifugation.

Cell Culture

PBMCs were washed twice with PBS and treated with PLP_{58-74} in seven concentrations including 0 (untreated control), 25, 50, 75, 100, 125 and 150 μg/ml at a cell density of 10^6 cells/ml for two days in DMEM medium supplemented with 10% heat inactivated fetal bovine serum (FBS), 4 mM L-glutamax, 1% non-essential amino acids and 1% penicillin–streptomycin (Gibco, 1540). Then the 100 μg/ml concentration was used as the best peptide dosage for the rest of the experiments.

Proliferation Assay

The T-cell proliferation assay was performed on PBMCs in triplicate. Two days after induction with PLP, PBMCs were used for RNA extraction using High Pure RNA Isolation kit (Roche, Germany). Concentrations of extracted RNA samples were read by Nanodrop to synchronize all samples. Based on manufacturer protocols, the RNA (0.5 μg) from each sample was used to synthesize first-strand cDNA using a cDNA synthesis kit (Fermentase, Germany).

Assessment of PCNA gene expression using real time PCR was performed to evaluate T cell proliferation. The validity of this test is equivalent to 5-bromo-2'-deoxyuridine test (BrdU.[32] Primers were designed using Oligo 5 online software (www.oligo.net) for proliferating cell nuclear antigen (PCNA) and β-actin genes as housekeeping genes (Table 1).

Real time PCR was performed using AvaGreen florigenic nucleotide to monitor cDNA amplification (Metabion kit, Germany) by measuring the increase in fluorescence intensity and using primer specific for PCNA mRNA and β-actin as the internal control in a real time PCR instrument (Corbett, Germany). Analysis of performed melting curve by real time PCR instrument showed only one peak for each reaction and this was also confirmed by electrophoresis of PCR products that showed only one band of the expected size.

Immunocytochemistry Assay

Immunocytochemistry (ICC) was performed to analyze PCNA expression at protein level. Cells were fixed using paraformaldehyde 4% six days after induction. A solution containing 0.25% Triton X100 was used before incubation with mouse anti-human PCNA anti-body (Milipore, Germany) and followed by washing and incubating with fluoresceinated secondary antibody for 60 minutes (Alexa fluor 594 donkey anti mouse IgG (H+L) invitrogen). Finally, cells were washed and DAPI was added. Preparations were examined and photographed by a Nikon fluorescence microscope (Nikon, Elipse TE 2000U, Japan).

Flowcytometry

To evaluate the quantity of the $CD4^+$ and $CD8^+$ T cells in primary PBMCs and after induction with the peptide, cells were subjected to flowcytometry using anti $CD4^+$ and $CD8^+$ anti-bodies. Treated and untreated PBMCs were centrifuged. Then, the pellet

Table 1. Primer sequences used in RT-PCR and Real time-PCR

Locus	Primers	Accession number	Size
β-actin-R	5'- AGACGCAGGATGGCATGGG-3'	NM_001101.3	161bp
β-actin-F	5'-GAGACCTTCAACACCCCAGCC-3'		
PCNA-F	5'-AGCACCAAACCAGGAGAAAGT-3'	NM_002592.2	191bp
PCNA-R	5'-TCACTCCGTCTTTTGCACAG-3'		

was resuspended in cold PBS to acquire 10^6 cells/ml density. Cells were incubated with mouse anti-human CD4-FITC (Dako, Denmark) or mouse anti-human CD8-PE antibodies (Dako, Denmark). Treated cells were incubated at 4°C for 30 min, and the complex was analyzed by flowcytometer (Partech, Germany). Results were analyzed by Flomax V 2.4.

IgG ELISA

ELISA assay was used for the assessment of specific IgG against PLP_{58-74} in the plasma of patients and healthy individuals. 96 well plates (Nunc Immunoplate MaxiSorp) were pre-coated with bicarbonate buffer and then coated using 20µg/ml peptide diluted in phosphate buffered saline (PBS) overnight at 4°C. The plates were blocked with BSA 1% for 2 hours at room temperature. Diluted serum samples in phosphate buffered saline tween 20 (PBST) containing BSA 1% were added to the plates and incubated overnight at 4°C. HRP conjugated goat Anti Human IgG (SIGMA, USA) diluted 1:1000 was incubated for 2h at room temperature. Subsequently, plates were washed with PBS and 2, 2-Azino-bis3-ethylbenzoThiazoline-6-sulfonic Acid substrate (ABTS), (SIGMA, USA) was added. Finally, after 20 minutes plates were read by ELISA reader (EPSON LQ-100, UK). All tests were assayed in triplicate.

Statistical Analyses

Statistical evaluation was performed using IBM SPSS statistic software v 21. One way ANOVA was carried out to determine the most effective concentrations of peptide on the proliferation of PBMCs. Evaluation of PCNA expression ratio was performed using relative expression software tool REST RG version 3 (REST 2005) to analyze the expression data obtained from real-time PCR. Student T-test was carried out to determine the significant variation in ELISA results. Statistical significance was defined as $p<0.05$.

RESULTS

Peptide

PLP_{58-74} was selected to study MS patients' T cells responses, in order to investigate whether or not patients' T cells are autoreactive to self PLP. This peptide was selected as the most immunodominant epitope of PLP based on meta prediction method hypothesized in our previous study[28] using bioinformatics databases. Bioinformatics results showed a similarity between this epitope and a peptide in bacteria (mainly in the members of *Clostridium* and *Mycobacterium*) and spike protein of Alphacoronavirus1, canine coronavirus and feline coronavirus.

Proliferation Assay using Real Time PCR

Analysis of real time-PCR revealed that there were significant differences in PCNA gene expression rates between treated and untreated patient T cells. PCNA gene expression in patient's T cells treated with PLP_{58-74} increased up to 6.33 folds. However, PCNA gene expression did not show any change in healthy control T cells (Table 2).

While ICC assay showed continuous expression of PCNA protein in the PBMCs of patients, no expression was observed in the PBMCs of healthy individuals (Figure 1).

Flowcytometry

$CD4^+$ T cell population increased in treated patients up to 39.91% although such increase was not observed in the $CD4^+$ T cells of untreated patients, treated healthy and untreated healthy individuals. Even though, $CD8^+$ T cell population in treated patients was

Table 2. Comparison of Tcells proliferation in patients and healthy individuals in response to PLP_{58-74}

Comparison between different groups	*p* value	Rate of change	Standard error
T- individual healthy /U-individual healthy	0.489	0.7^{ns}	±0.4
T- patients /U-patients	0.002	6.33^{**}	±0.42
T-patients/ T-healthy individuals	0.017	2.81^{*}	±0.51
U-patient/U-healthy individuals	0.618	0.97^{ns+-}	±0.8

U; untreated, T; treated

ns: no significant increasing at *p*-value<0.05, *: significant increasing at *p*-value<0.05 **: significant increasing at *p*-value<0.01.

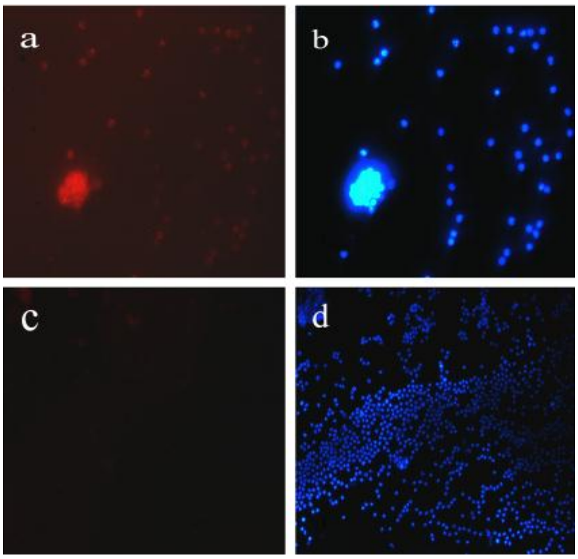

Figure 1. Staining of peripheral blood mononuclear cells (PBMCs) via DAPI and anti-human PCNA in ICC assay. a: treated patient PBMCs staining by anti-PCNA, b: treated patients' PBMCs staining by DAPI, c: untreated patient PBMCs staining by anti-PCNA, d: PBMCs of untreated patients staining by DAPI

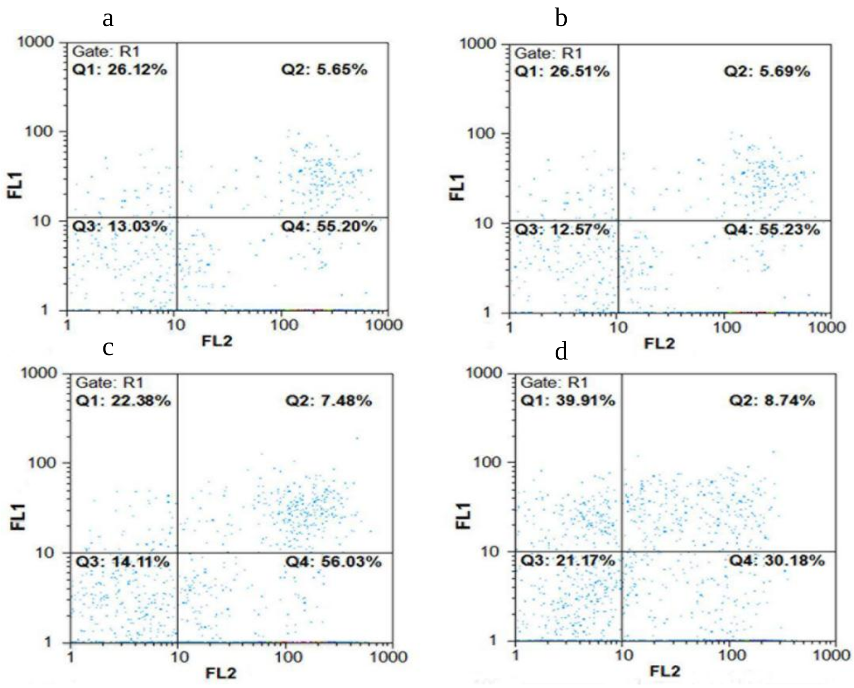

Figure 2. Shows the rate of CD4+ and CD8+ changes in peripheral blood mononuclear cells (PBMCs) after treatment with $PLP_{58\text{-}74}$ analyzed by FACS. a: the rate of CD4+ and CD8+ in the PBMCs of healthy individuals without treatment. b: the rate of CD4+ and CD8+ in healthy individual PBMCs after treatment. c: the rate of CD4+ and CD8+ in patient PBMCs without treatment. d: the rate of CD4+ and CD8+ in patient PBMCs after treatment.

Q1: CD4+ T cells, Q2: the T cells expressed both CD4+, CD8+, Q3: others PBMCs, Q4: T CD8+cells, FL1: CD4-FITC, FL2: CD8-PE

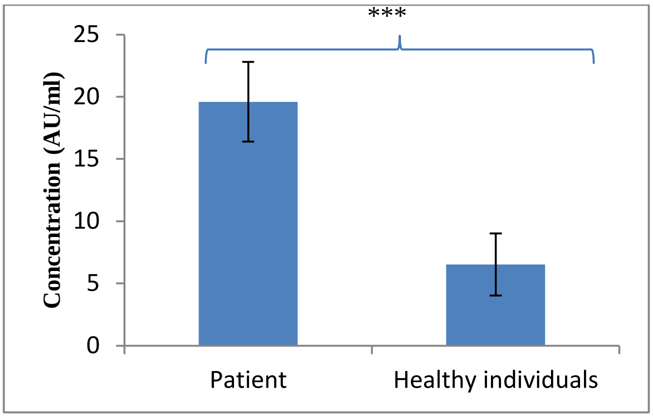

Figure 3: shows the rate of IgG antibody against PLP$_{58-74}$ in patients plasma compared to healthy individuals. *: significant at *p* value≤0.001**

decreased to 30%, $CD8^+$ T cell population was constant in untreated patients, treated healthy and untreated healthy individuals (Figure 2). However, the results associated with flowcytometry revealed the elevating rate of $CD4^+$ T cells compared to $CD8^+$ T cells in PLP$_{58-74}$ treated patient cells.

ELISA

Variations in the rate of IgG antibody against PLP$_{58-74}$ in patient plasma and that of healthy individuals were assessed by ELISA. The results showed that patient plasma IgG antibody was threefold higher compared to healthy individuals (Figure 3).

DISCUSSION

MS is the most common auto-immune disease with unknown etiology in CNS. However, molecular mimicry or cross reactivity between self-antigens and immunodominant epitopes of the pathogens can be the main cause of many auto-immune disorders, particularly MS.

Significant cross reaction was observed between some parvovirus B19 antigens and self-antigens in several auto-immune disorders including rheumatoid arthritis, systemic lupus, anti-phospholipid syndrome and systemic sclerosis.[33] Schloot et al. showed that Coxsackie virus protein P2C mimics the glutamic acid decarboxylase65 (GAD65) in type 1 diabetes patients and isolated T cells from patients were reactive to both P2C and GAD65.[34] Poole et al. remarked that EBV infection can lead to production of cross-reactive antibodies in systemic lupus erythematous (SLE).[35]

We selected PLP$_{58-74}$ as an immunodominant epitope based on Meta prediction method. Bioinformatics results also showed a similarity between this epitope and a peptide in *Clostridium, Mycobacterium* and spike protein of Alphacoronavirus1, canine coronavirus and feline coronavirus. Several studies showed that some pathogenic antigens including *Mycobacterium, Clostridium*, Corona virus, *Haemophilus influenza* have cross reaction with brain proteins.[36,37] They result in activation and proliferation of immune cells, especially T cells, as well as antibodies production against such antigens in MS. Greer et al. showed that the rate of proliferation in patient PBMCs increased in response to 184-199 and 290-209 fragments of PLP.[21] The results obtained in the current study also showed significantly increased PCNA gene expresion in patient cells treated with PLP$_{58-74}$, but not in the cells of healthy individuals. PCNA gene expression at protein level was demonstrated by taking advantage of ICC assay.

Furthermore, T cell reactivity to myelin auto-antigens plays a crucial role in initiating MS.[10,38] Pelfrey et al. decleared that T cells could respond to PLP (40-60) fragment.[24] Massilamany et al. revealed a cross reaction between PLP$_{139-151}$ and *Acanthamoeba castellanii* (ACA)$_{83-95.}$ They showed that these two epitopes could stimulate proliferation of T cell derived from these induced EAE lymph nodes.[9] The cross reactivity potential between some pathogens and PLP was evaluated in the current investigation. Our results accomplished by flowcytometery revealed that the percentage of $CD4^+$ T cells from patients increased in reaction to PLP$_{58-74}$.

Previous studies have focused mostly on cellular immunity of MS. Data have showed that humoral

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immunity plays a crucial role in pathogenesis of MS. It seems that B cells could have a role in the pathogenesis of MS, and ELISA assay results related to this study may support this idea. There was a threefold increase in anti-PLP_{58-74} IgG of patients compared to healthy individuals which can be attributed to the humoral immune activation. This result was in agreement with Munger et al. which revealed that increasing anti-EBNA IgG could lead to MS incidence and progression.[39]

Results suggest that the cross reaction of the immune system to PLP may have originated from bacterial or viral infections. Thus, it can be concluded that PLP_{58-74} can be a target for autoimmune activation processes and it may induce these processes via stimulation of $CD4^+$ T cells and humoral immune activation.

Another study conducted by Wegmann et al. revealed that EAE in mice induced by $PLP_{139-151}$ can be treated by modified ACA_{83-95} and stated that ACA_{83-95} prevents the development of EAE.[40] Moreover, Badawi A. H. and Siahan T. J. in 2013 showed that MOG_{38-50} and $PLP_{139-151}$ induced EAE, which can be suppressed by multivalent bi-functional peptide inhibitors ($MVB_{MOG/PLP}$).[41] Kasarello et al. demonstrated that oral administration of engineered *Lactococcus lactis* expressing three main myelin proteins including PLP, MBP and MOG could improve clinical symptoms of EAE in rats.[42] Because of similarities between human PLP and animal model PLP, conducting such studies in vivo with candidate's epitope seems to be necessary and if the results were acceptable, this peptide might be applicable for MS treatment in the future.

Molecular mimicry has been proposed as one of the phenomena for the occurrence of MS.

It may be possible that in genetically susceptible individuals who are immune-compromised, exposure to microorganisms which have homology with PLP can trigger CNS autoimmunity by molecular mimicry. This study can contribute to a better understanding and clarification of disease mechanism. These results could help us prevent disease initiation or cure MS in the future.

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