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Cross-reactivity between human cytomegalovirus peptide 981-1003 and myelin oligodendroglia glycoprotein peptide 35-55 in experimental autoimmune encephalomyelitis in Lewis rats

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ABSTRACT

Multiple sclerosis (MS) has been documented to have various clinical and pathological presentations. However the underlying mechanisms remain unknown. Viral infections may play a certain role in the etiopathogenesis of MS. This study was designed to explore whether different phospholipid peptides and viral mimic peptides induce antigen specific lesion in experimental autoimmune encephalomyelitis (EAE), an MS animal model. In the present study, Lewis rats immunized with myelin basic protein (MBP) 82-99 or MBP68-86 exhibited clinical signs of EAE and inflammatory infiltrates throughout CNS. Immunization with myelin oligodendroglia glycoprotein (MOG) 35-55 also induced inflammatory infiltrates in spinal cords. Although cytomegalovirus (CMV) 981-1003 failed to induce clinical signs of EAE and inflammatory infiltrates, immunological examination revealed that CMV981-1003 cross-reacted with serum from rats immunized with MOG35-55, and vice versa. Further, MOG35-55 triggered CMV981-1003 specific lymphocytes recruitment in spleen. Together these, this study provides certain evidences for various pathological manifestations of EAE and the linkage of viral mimic peptides with phospholipid peptides. Molecular mimicry may be an explanation the pathogenesis of MS.

1. Introduction

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS), characterized by perivascular inflammatory infiltrates, demyelination and axon injury [1]. As the clinical manifestations and pathological features of MS vary among patients [2], the mechanisms underlying the diversity of pathological features are far to be elucidated. Recent studies have suggested that the symptoms and prognosis of MS are correlated with inflammatory response in the specific regions of the CNS [3,4]. Experimental autoimmune encephalomyelitis (EAE) is the most available animal model of human MS so far. In this model, animals are immunized with myelin components, including myelin basic protein (MBP) and myelin oligodendroglia glycoprotein (MOG) to develop inflammation, and at times demyelination and axon damage [5]. It has been demonstrated without doubt that different genetic backgrounds produce different distribution of lesions and clinical symptoms/courses [6]. Some studies demonstrated that the properties of antigen specific-T cells determined disease sites, clinical presentations, and cellular pathology in mice [7].

The etiology of MS has been always in disputed. Autoantibodies and autoreactive T cells, were detected in MS patients [8]. Although to date no virus has been recognized as a causative factor of MS, viral infections may play an important role in the autoimmunity of the disease [9,10]. Molecular mimicry was proposed to explain how viruses might trigger such autoreactive immune responses in MS [11], which involved de novo activation of autoreactive T cells, due to the cross-reactivity between self epitopes and viral epitopes during virus infection [12]. EBV DNA polymerase 627-641 (EBV627-641) shares similar amino acid sequence with MBP82-99, while human cytomegalovirus major capsid protein UL86 981-1003 (CMV981-1003) shares with rat MOG 35-55. There also exists sequence similarity between core B-cell epitope (MBP86-95) and EBV627-641, and rat T-cell epitope (MOG44-55) and CMV981-1003. These findings trigger a question whether immunization with EBV627-641 and CMV981-1003 induces antigen specific lesion topology through molecular mimicry.

Thus, in the present study, we immunized Lewis rats with different myelin antigens and viral mimic peptides to explore whether they induced different pathological features, and whether there exists cross-reactivity between the myelin antigens and viral mimic peptides.

Abbreviations: EBV627-641, EB virus DNA polymerase 627-641; CMV981-1003, cytomegalovirus major capsid protein UL86 981-1003.

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2. Materials and methods

2.1. Animals

Female Lewis rats (6–8 weeks, 160–180 g, specific pathogen free) were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China), and housed individually in room with specific pathogen free standards at a constant humidity and temperature, with food and water available *ad libitum*. The animal room was on a 12/12 h light/dark cycle. This study was strictly in accordance with institutional animal care procedures of Capital Medical University. All operations were performed under sodium pentobarbital anesthesia.

2.2. Preparation of phospholipid antigens and viral peptides

MBP82-99 (DENPVVHFFKNIVTPRTP), MBP68-86 (YGSLPQKSQRSQDENPV), MOG35-55 (MEVGWYRSPFSRVVHLYRNGK), EBV627-641(TGGVYHFVKKHVHES), and CMV 981-1003 (HEYNWLRSPFSRYSATCPNVLH) were synthesized by Ji'er Biochemical Company (Shanghai, China). The amino acid sequences were analyzed and the peptides were purified with high pressure/performance liquid chromatography. The purity was $\geqslant$98%.

2.3. Induction and clinical assessment of EAE

Lewis rats were divided randomly into seven groups (group A–G, 10–20 rats/group). Group A served as normal control. Rats in group B were subject to bilateral subcutaneous injection of 100 μl Freund's complete adjuvant (CFA; Sigma, St. Louis, MO, USA) containing 4 mg/ml *Mycobacterium tuberculosis* (BD Difco, Detroit, MI, USA) and 100 μl PBS (pH 7.4) at the base of the tails. Rats in group C–G were immunized with MBP82-99 (100 μg), MBP68-86 (100 μg), EBV627-641 (250 μg), MOG35-55 (200 μg), or CMV981-1003 (200 μg), in addition of 100 μl CFA and 100 μl PBS. Neurological signs were evaluated and body weights were measured daily in a blinded fashion by two investigators. Clinical scores of EAE were graded according to the following criteria: 0, healthy; 1, limp tail; 2, mild to moderate hind limb paraparesis; 3, hind limb paraplegia; 4, quadriplegia; and 5, moribund or dead [13].

2.4. Histology and immunohistochemistry

At the 14th day post immunization, rats were sacrificed and perfused intracardially with saline and 4% paraformaldehyde. Cerebrums, brain stems, cerebellums, and spinal cords were removed and immersed in 4% paraformaldehyde for 4 h at 4 °C [14]. These tissues were then fixed in paraffin and cut into 4-μm-thick sections.

Ten equidistant sections from cerebrum, brain stem, cerebellum, and spinal cord of each rat were stained with hematoxylin and eosin (H&E). The number of perivascular inflammatory infiltrates with 20 or more aggregated cells was evaluated blindly at 200× magnification, and the number of perivascular inflammatory infiltrates per square centimeter (cm2) was calculated. Ten additional sections containing specific brain region from each animal were selected for Luxol Fast Blue staining.

Neuronal axons were evaluated with immunohistochemistry according to the manufacturer's instructions. Briefly, the sections were treated with 3% hydrogen peroxide for 10 min to quench endogenous peroxidase. The sections were incubated with primary antibody (anti-neurofilament-200, 1:50, Abcam) at 4 °C overnight. The sections were then incubated with horseradish peroxidase(HRP)-conjugated secondary antibody for 30 min at room temperature. Immunohistochemical reaction was revealed by using 0.05% 3,30-diaminobenzidine and 0.03% hydrogen peroxide as chromogen. After each incubation, the sections were thoroughly washed with PBS. Control sections were incubated with secondary antibody alone [15]. Digital images were taken using Adobe Photoshop (Adobe Systems), and analyzed by a blinded observer.

2.5. Real-time RT-PCR

Real-time RT-PCR was performed to examine mRNA expression of CCL-7, vascular cell adhesion molecule-1 (VCAM-1) and neurofilament medium (NEFM) according to previous study [16]. At the 10th day post immunization, cerebrums, brain stems, cerebellums, and spinal cords were removed and total RNA was extracted and reversely transcribed into cDNA respectively. The resultant cDNA was amplified by real-time PCR with designed primers. CCL7 primers were sense: 5′GGGACCAATTCATCCACTTGC3′ and antisense: 5′TCAGCACAGACTTCCATGCC3′. VCAM-1 primers were sense: 5′GGAAATGCCACCCTCACCTT3′ and antisense: 5′CAC CTGA GATCCAGGGGAGA3′. NEFM primers were sense: 5′TCTGTACACAC ACCGACAGC3′ and antisense: 5′CTGTGAGGGCGTCTTCCATT3′. Melting curve, which was measured immediately after amplification, showed single peak, indicating good product specificity. Results were presented as the levels of expression following normalization to housekeeping gene glyceraldehyde-3-phosphate dehydrogenase using the ΔΔCt method.

2.6. Determination of antibody cross-reactivity

Antibody cross-reactivity between phospholipid peptides and viral mimic peptides was tested with indirect enzyme linked immunosorbent assay (ELISA) according to previous study [17]. Briefly, microtiter plates were coated with 4 μg/ml of MBP68-86, MBP82-99, EBV627-641 or CMV981-1003 overnight at 4 °C. A total of 100 μl serum sample from group A, B, C, D, E, F, G diluted in bovine serum albumin (1:100) was added in wells in duplicate for 2 h. After wash, HRP-conjugated anti-rat IgG was added for 1 h, followed by 3,3′5,5′-tetramethyl benzidine dihydrochloride (TMB). Optical density (OD) was measured at 450 nm using ELISA reader. Absorbance values were corrected by subtracting the OD value obtained in wells without antigen. Anti-MOG35-55 antibody in serum was detected with anti-MOG35-55 IgG quantitative ELISA kit (Anaspec, Fremont, CA, USA) following the manufacturer's instructions. The results were expressed as ng/ml.

2.7. Preparation of spleen mononuclear cells (MNCs) and enzyme linked immunospot assay (ELISpot)

Spleens were dissected under aseptic conditions, and spleen MNCs were enriched with a 1.083 g/ml Percoll gradient centrifuge (Pharmacia, Piscataway, NJ, USA) for 20 min at 450 g. Vital cells were counted by means of trypan blue dye exclusion staining.

An ELISpot was performed to examine the cross-reactivity in MNCs between phospholipid peptides and viral mimic peptides, according to the manufacturer's instructions. In brief, the Multi-Screen$_{HTS}$ filter plates were coated with IFN-γ antibody and blocked with RPMI 1640 medium containing 10% fetal calf serum for 4 h. The MNCs suspension (4 × 105 cells/well) containing different myelin peptide or viral peptide (10 μg/ml) was added. The plates were then incubated for 20 h in a humidified incubator with 5% CO_2 at 37 °C. TMB was added in the plates until the distinct spots emerged. The spots were inspected and counted with an ELISpot reader. Data were presented as mean spot-forming units per 0.4 × 106 cells. Concanavalin A (5 μg/ml; Sigma, St. Louis, MO, USA) was used as positive control.

2.8. Statistics

Data were presented as mean ± SD. SPSS16.0 software was used in statistical analysis. Statistical differences for continuous data were performed by One-way analysis of variance and Student's *T*-test. Clinical scores were analyzed using non-parametric Mann–Whitney *U* test. The correlation analysis was performed with Pearson correlation or partial correlation. $P < 0.05$ was considered statistically different.

3. Results

3.1. Clinical features of EAE

Clinical signs and marked loss of body weight were observed in the rats immunized with MBP82-99 or MBP68-86 (Fig. 1A and B). The clinical signs were more severe in MBP68-86-immunized rats, compared to those in MBP82-99-immunized rats (Fig. 1A). In contrast, the rats immunized with CFA or other peptides did not exhibit any signs. The mean onset time of clinical signs in MBP82-99 and MBP68-86-immunized rats were 11.3 ± 2.07 and 10.8 ± 0.84 days, respectively.

3.2. Pathological manifestations

The primary features of CNS lesion topology in EAE were summarized in Table 1, and the representative sections exemplifying CNS lesions were presented in Fig. 2A.

Infiltrating cells were found in the meninges and perivascular spaces of the parenchymal tissues in rats immunized with MBP82-99, MBP68-86, or MOG35-55, but not in the other rats. The number of inflammatory infiltrates per cm2 was shown in Fig. 2B. The number of infiltrates in spinal cords in the three groups was higher than that in the cerebrums, brain stems and cerebellums. In spinal cords, there was no statistical difference of infiltrates between MBP82-99 and MBP68-86 groups; however, the both groups had more infiltrates than MOG35-55 group. Moreover, MBP68-86 group exhibited more severe inflammation in the brain stem, compared to the MOG35-55 group ($P < 0.05$). Correlation analysis revealed that the clinical score was positively correlated with the number of infiltrates in the spinal cord parenchyma in MBP82-99-immunized rats ($R = 0.494$). No demyelination and axon damage were seen in all groups.

3.3. mRNA expression of CCL7, VCAM-1 and NEFM

The mRNA levels were presented as percentage of that in the CFA group. More than 2.0 or less than 0.5 was considered to be upregulated or downregulated, respectively. The mRNA expression of CCL7 and VCAM-1 was upregulated in the cerebrum, cerebellum, stem and spinal cord in MBP82-99 and MBP68-86 groups (Fig. 3A and B). The number of infiltrates revealed positive correlation with mRNA levels of CCL7 and VCAM-1 ($R = 0.52$ and 0.17, respectively). mRNA level of NEFM was decreased in the spinal cord and brain stems in the MBP82-99 and MBP68-86 groups (Fig. 3C), and there was a negative correlation between its mRNA expression and the number of infiltrates ($R = -0.61$).

Table 1
CNS inflammation topology in rats immunized with MBP82-99, MBP68-86, MOG35-55.[a]

Location		MBP82-99	MBP68-86	MOG35-55
Cerebrum	Meninges	0/6[b]	2/5	0/6
	Parenchyma	2/6	2/5	0/6
Brain stem	Meninges	1/6	1/5	0/6
	Parenchyma	5/6	4/5	0/6
Cerebellum	Meninges	0/6	0/5	0/6
	Parenchyma	2/6	3/5	0/6
Spinal cord	Meninges	6/6	5/5	6/6
	Parenchyma	6/6	5/5	1/6

[a] CNS tissues were obtained at the 14th day post immunization. Presence of infiltrates was assessed with H&E staining. Infiltrate was defined if the aggregations contained 20 or more infiltrating cells.
[b] The number of rats exhibiting infiltrates compared to the total number of rats used.

3.4. Antibody cross-reactivity between phospholipids and viral peptides

Antibody reactivity was tested with ELISA using serum samples collected at different weeks after immunization. Detailedly, the serum in all groups were collected at the 2nd week. The serum in group A, B, F and G were also collected at the 8th week.

3.4.1. Antibody cross-reactivity between MOG35-55 and CMV

The concentration of antibodies in the serum from rats immunized with CMV981-1003 was detected using anti-rat MOG35-55 IgG Quantitative ELISA Kit. The results showed that the concentration was significantly increased, compared to CFA group (Fig. 4A). This suggests that the antibodies against CMV981-1003 cross-reacted with MOG35-55. Likewise, we also found that antibody to MOG35-55 cross-reacted with CMV981-1003 (Fig. 4B).

3.4.2. Antibody cross-reactivity between MBP peptides and EBV

Rats immunized with MBP68-86 exhibited only positive antibody titers against the corresponding antigen that was used to immunize animals, and there was no obvious antibody

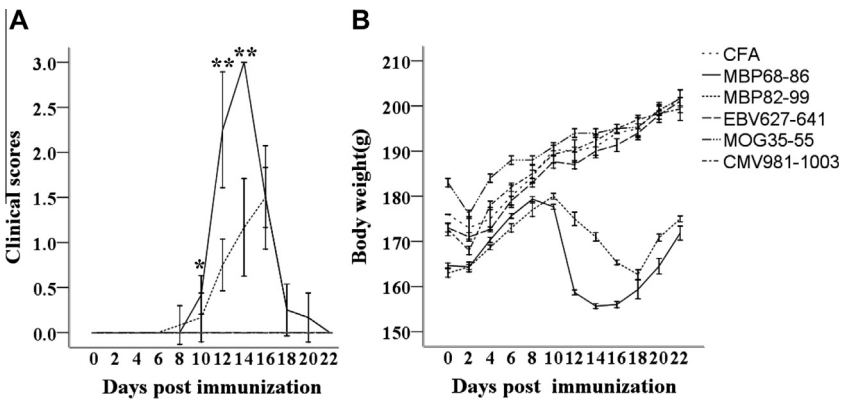

Fig. 1. Clinical features of EAE animals. Lewis rats were immunized with CFA alone, or CFA plus MBP82-99, MBP68-86, EBV627-641, MOG35-55 or CMV981-1003. Clinical scores (A) and body weights (B) were shown. Results were presented as mean ± SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ *vs* the MBP 82-99 group.

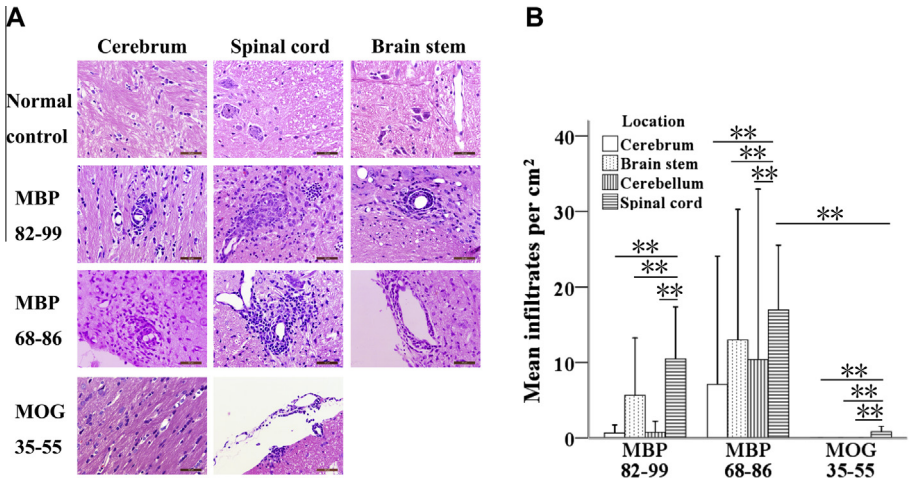

Fig. 2. Inflammatory infiltrates in the CNS of rats at the 14th day post immunization. Cerebrums, brain stems, cerebellums and spinal cords were removed and H&E staining was performed. Representative images (400×, A) and the number of infiltrates per cm2 (B) in each type of tissue were shown. Results were presented as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$.

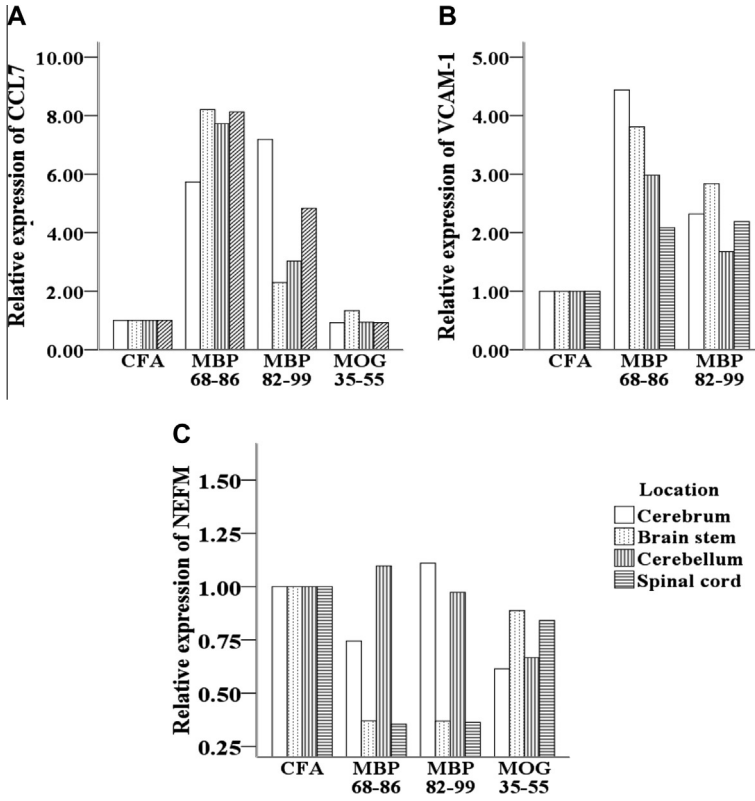

Fig. 3. Relative mRNA expression of CCL7, VCAM-1 and NEFM. Cerebrums, brain stems, cerebellums and spinal cords were extracted to measure mRNA expression of CCL7, VCAM-1 and NEFM with real time PCR. The results are presented as percentages of the CFA group. Results were presented as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$.

cross-reactivity against other antigens. However, rats immunized with MBP82-99 exhibited a positive antibody titer against both EBV627-641 and MBP82-99 (Fig. 4C), indicating that antibody against MBP82-99 cross-reacted with EBV627-641.

3.5. MOG35-55 triggered CMV981-1003 specific lymphocytes

MNCs from rats immunized with CMV981-1003 were cultured for 20 h with MOG35-55 in MultiScreen HTS filter plates for ELISpot assay. MOG35-55 was found to trigger CMV981-1003-specific lymphocytes (Fig. 4D), while no specific spots were generated when lymphocytes from the group MOG35-55 were cultured with CMV981-1003.

4. Discussion

Clinical and histological features of MS vary among patients [2]. The symptoms and prognosis of MS have been identified to be correlated with inflammation of specific regions in the CNS, but the mechanisms remain uncertain. In the present study, we established an EAE model by immunizing Lewis rats with different

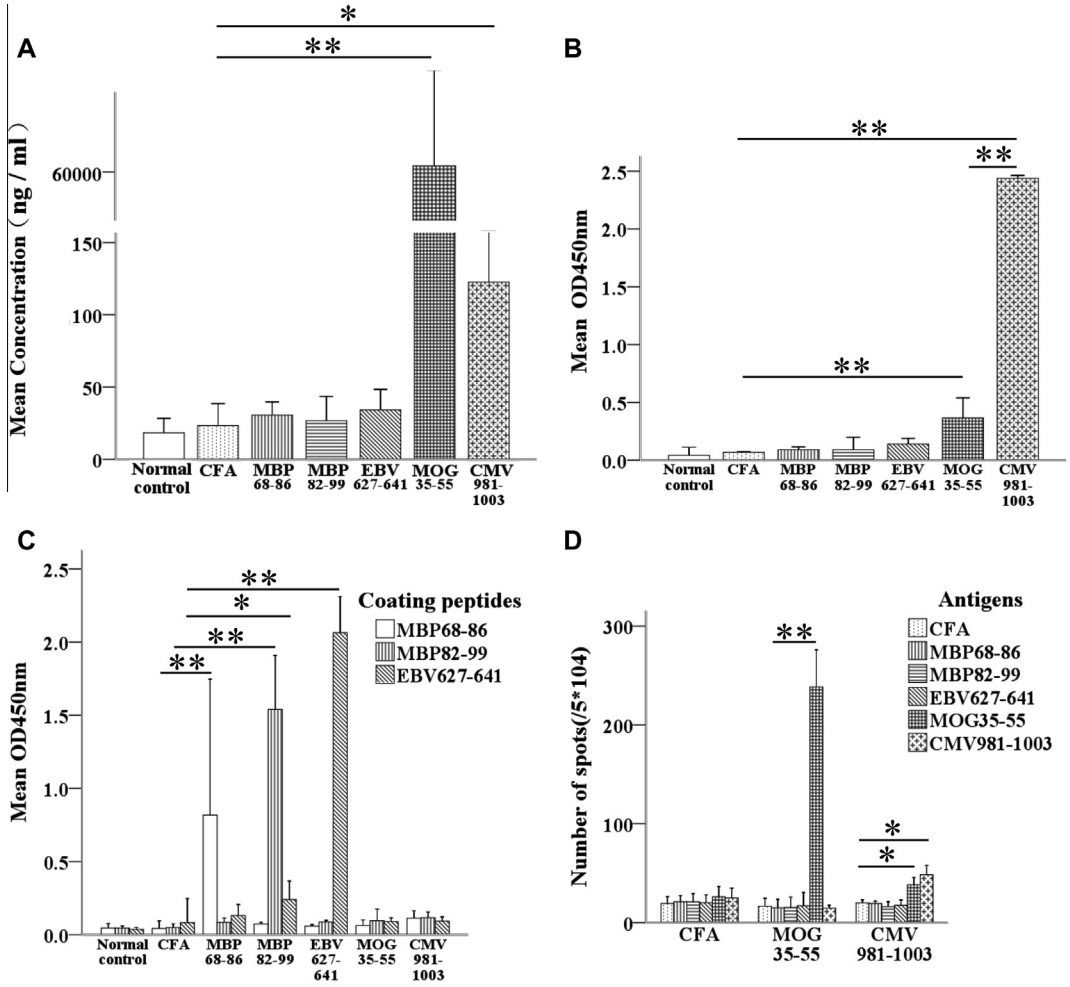

Fig. 4. Cross-reactivity between phospholipid peptides and viral peptides. ELISA was performed to monitor antibody cross-reactivity between phospholipid peptides and viral peptides. Microtiter plates were coated with different peptide (4 μg/ml) overnight at 4 °C. Serum samples from rats immunized with CFA alone, or CFA plus MBP82-99, MBP68-86, EBV627-641, MOG35-55 or CMV981-1003 were added in corresponding wells. OD value was measured at 450 nm. Cross-reactivity between MOG35-55 and other peptides specific antibody (A), CMV981-1003 and other peptides specific antibody (B), and MBP peptide and other peptides (C) were shown. ELIspot was performed to evaluate the cross-reactivity between MOG35-55 and CMV981-1003 in spleen MNCs (D). Results were presented as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$.

myelin antigens or viral mimic peptides. Using this model, we found that different peptides induced different pathological features, and there existed cross-reactivity between myelin antigens and viral mimic peptides.

EAE in Lewis rats is a reliably reproducible monophasic disease model, and frequently different strains and different antigens were studied. Previous studies showed the genetic and antigenic differences in EAE models rendered the histological studies difficult work. In the present study, the results were obtained by comparing Lewis rats from the identical colony, which almost completely eliminates genetic variance.

MBP68-86 and MBP82-99 are dominant encephalitogenic peptides, and the former is frequently used to induce EAE in Lewis rats [18]. A previous study revealed that MOG35-55 was another encephalitogenic peptide and induced strong T and B cell responses in Lewis rats [19]. The present study showed that MBP peptides and MOG35-55 induced different inflammatory infiltrate topology in Lewis rats. Inflammatory infiltrates were found in the spinal cords, cerebrums, stems, cerebellums of rats immunized with MBP82-99, MBP68-86, but only in the spinal cord of rats immunized with MOG35-55. The exact reasons remain unclear why the targets of the autoimmune attack differed between these stimulations. It has been documented that genetic background could contribute to the different disease manifestations. However, in the present study, the differences were still observed in the same colony of rats, thereby eliminating genetic variance. Different solubility and bioavailability of the antigens is one of the possible explanation for the different encephalitogenic activities. A study from Lassmann lab showed that the major differences in lesion topography in a passive EAE model induced by transfer of different neuroantigens specific T cells in Lewis rats depending on the neuroantigens used for the priming of the transferred T cells [20]. Therefore, the antigen specificity of T cells, rather than the biophysical properties of the antigens, may be responsible for the different EAE manifestations.

Axon damage, when assessed with immunohistochemistry using anti-neurofilament-200 antibody, was not observed in the present study. However, mRNA expression of NEFM, which reflects axon size at some extent, was decreased in the spinal cord and brain stems of rats immunized with MBP82-99 or MBP68-86. Further, the mRNA expression of NEFM exhibited a negative correlation with the number of infiltrates in these rats. The results indicated that axon injury may be present during the early stages of EAE.

CCL7 and VCAM-1 are important in the development of EAE. CCL7 is a chemokine that attracts several types of leukocytes including monocytes and lymphocytes, and is upregulated in the CNS in several disorders with increased inflammatory infiltrates [21]. VCAM-1 is an adhesion molecule that can be expressed in vascular endothelial cells as well as astrocytes in the CNS

parenchyma [22]. In the present study, the mRNA expression of CCL7 and VCAM-1 was upregulated in CNS regions with increased inflammatory infiltrates of rats immunized by MBP82-99 and MBP68-86, further was positively correlated with the number of infiltrates.

In the present study, no clinical signs of EAE or inflammation induction were observed in the rats immunized with EBV627-641 and CMV981-1003 peptides. Until now, there are few investigations by using viral peptide to successfully induce EAE, although a lot of data have revealed some relationship exists between viral infections and MS. It is of note that the exploration in this field is significant for the possible role that viruses play in the pathogenesis of MS. In the subsequent immunological studies in the present study, EBV627-641 cross reacted with antibody against MBP82-99. MOG35-55-specific antibody cross reacted with CMV peptide, and vice versa. Moreover MOG35-55 triggered CMV981-1003 specific lymphocytes recruitment to spleen, possibly via molecular mimicry. Indeed, several studies have demonstrated the possibility that molecular mimicry plays a role in the induction of autoimmunity [9,10].

If viral mimic peptides are important for the initiation of autoimmunity, they have to be capable of inducing potent T-cells activation, which in turn results in proliferation of autoreactive T cells. In this study, *in vitro* stimulation of MOG35-55 caused CMV-reactive T-cell clonal expansion, demonstrating T-cell cross-activation. MOG is a unique myelin autoantigen, and is capable of triggering both encephalitogenic T cell responses and demyelinating autoantibody responses [23]. The immunodominant T cell epitope is contained in MOG44-55 (FSRVVHLYRNGK) [24], which shares three same amino acids with CMV983-992 (YNWLRSPFSR). This may provide a possible explanation for the T-cell cross-reactivation. Previous study showed that MOG37-46 (VGWYRSPFSR) strongly reacted with the antibodies from MOG35-55-immunized Lewis rats [19], implying that MOG37-46 is a core B cell epitope. In addition, CMV983-992 (YNWLRSPFSR) had seven amino acids identical to rat MOG37-46 (VGWYRSPFSR), suggesting the explanation for cross-reactivity between the MOG35-55 and CMV981-1003.

The autoantibody binding site in MBP residues is located in MBP86-95 (VVHFFKNIVT), which also contains MHC/T cell receptor contact residues of T cell epitope [25]. An important difference between the autoantibody and T cell epitope is that the residues 89-91 of MBP86-95 has to be absolutely conserved for autoantibody binding [25]. The binding of viral peptides to autoantibodies requires the presence of at least four or five same amino acids in the core of the epitope. Our results showed that serum from rats immunized with MBP82-99 peptide (DENPVVHFFKNIVTPRTP) reacted with EBV627-641 (TGGVYHFVKKHVHES). The five same amino acids between them may contribute to the cross-reactivity.

Together, this present study provides certain evidence for the understanding of the diversity of pathological manifestations of EAE in Lewis rats. Molecular mimicry may be an important factor in the pathogenesis of EAE. It is a little farfetched to bring molecular mimicry hypothesis to MS pathogenesis due to the limited satisfactory results in EAE model. Undoubtedly, many differences exist between animal models and disease entity of human being. Nevertheless, it can be inferred that viral molecular mimicry may play a role in MS pathogenesis.

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