Evaluation of a Rat Model of Experimental Autoimmune Encephalomyelitis with Human MBP as Antigen

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Experimental autoimmune encephalomyelitis (EAE) is a good model for human multiple sclerosis (MS) research. However, there are some defects in the traditional models. Here, we improved the model by using the human myelin basic protein (MBP) as antigen. EAE was induced by immunization of female Wistar rats with human MBP. Compared with the traditional models, the new model was evaluated by clinical signs to pathological changes. The immune state of the model was assessed by the lymphocyte infiltrative response and levels of TNF-α, IFN-γ, IL-10. It was found that most of rats exhibited tail tone loss and hind-limb paralysis, also there were demyelination, infiltrative lymphocyte foci, "Neuronophagia" in the cortex of cerebra and the white matter of spinal cords. PBMC and spleen lymphocytes were strongly response to the stimulation of MBP and PHA. The levels of TNF-α, IFN-γ were altered with the severity of EAE. In the remitting phase, IL-10 was increased significantly. This study demonstrate that the animal model of EAE induced by human MBP bears resemblance to the features of human multiple sclerosis and promises to be a better model than ever before for the study of MS. *Cellular & Molecular Immunology*. 2004;1(5):387-391.

Key Words: EAE, model, MS, MBP

Introduction

Experimental autoimmune encephalomyelitis (EAE) is a demylinating, inflammatory disease in the nervous system and induced by immunization of susceptible strains of rats or mice with myelin antigens [mainly consisted of myelin basic protein (MBP)] combined with adjuvant. EAE has been served as an animal model of human multiple sclerosis (MS) for several decades.

The disease course of EAE and MS has been well characterized by previous studies. In the pathologic process of MS and EAE, the immune balance *in vivo* is skewed to Th1-mediated immune response (1). MBP-specific, auto-immune reactive Th1 cell can be found in the blood, immune organs and central nervous system of EAE and MS. They secrete a number of immune molecules (such as proinflammatory cytokines, adhesion molecules and chemokines) to destroy the brain-blood barriers, to induce the apoptosis of oligodendrocytes and cause the loss of sheaves around the neuron axon. TNF-α and IFN-γ are important cytokines secreted by Th1 cells. They increased in MS and EAE serum and play a pivotal role in the activation of T cells and demyelation. Moreover, adoptively transfer of these T cells into naïve mice also induces clinical signs in the receipt. All these findings demonstrate that EAE/MS is a Th1 cell mediated autoimmune disease and MBP is the major antigen.

Although we have made great progress of MS by the research of EAE animal model, there are differences between EAE and MS. In human MS, the axon and neuron damages exist in the brain and spinal cord (2). The lesion has never been found in animal models. And the natural process of MS is remitting and relapsing. In the EAE animal, it is difficult to induce relapsing after the remitting.

What's the reason for these differences? In traditional models, MBP, a pivotal component as the encephalitogen in the whole procedure, was obtained from the rodent or bovine animals. However, there are great differences in the amino acid component, biochemical structure and physical

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Abbreviations: EAE, experimental autoimmune encephalomyelitis; MS, multiple sclerosis; MBP, myelin basic protein; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; IL-10, interleukin-10; GP, guinea pig.

properties between human MBP and animal MBPs. The similarity of amino acid component between rat MBP and human is only 62%; the main epitopes, which existed in the peptide 82-102 and 143-168, are completely different between them. The MBPs difference not only caused the discrepancy between EAE and human MS, but also hinder the further therapeutic research (such as human MBP antibodies). So, we suppose that using human MBP, as an alternative of animal MBPs, to induce EAE model maybe mostly mimics the characters of human MS, and this model may be used in the following MS and EAE research. To test our hypothesis, we immunized the rats with human MBP, and observed the clinical, pathological and immunological features. Our results indicated that the model of EAE induced by human MBP is a better model than ever before for the study of MS.

Materials and Methods

Animals and reagents

Female Wistar rats with 6-8 weeks of age (200 ± 20 g) were purchased from the Experimental Animal Center of Henan province (Grade: II; Number 4100117), housed in the animal center of Jiangsu province and used according to the institutional guidelines. ELISA kits for TNF-α and IFN-γ were purchased from the Bender MedSystems Inc., USA. Rat IL-10 ELISA kit was purchased from the BioSource International, USA.

Induction of EAE

Human MBP was extracted and purified from the brains of the unclaimed cadavers dying of incidents, which were supplied by the Centre of Medicolegal Expertise, Nanjing Police Bureau, and identified by SDS-PAGE eletrophoresis and western blotting. The extracting and identifying procedure was in accord with the standard procedure established in earlier work (3). EAE animal models were induced by immunization with human MBP combined with adjuvant (named as hu-group). Briefly, followed the procedure for the inducement of EAE by Guinea Pig (GP) MBP (4), human MBP saline solution (4 mg/ml) was homogenized and emulsified with the same volume of complete Freud's adjuvant. The final emulsion contained 16.7 mg of *Mycobacterium tuberculosis* H37Ra per ml. The animals designed as experimental groups were immunized by subcutaneous injection in the footpads with 0.2 ml emulsion as encephalitogen. Seven days after injection, the animals were administered the same volume of the emulsion by injection again. Part of the experimental rats received the same treatment on 27 day. Besides, seven rats were randomly selected and immunized with GP MBP to replicate the traditional EAE model (named as GP-group). For the control group, the identical procedure was performed except that the emulsion contained no MBP.

Scoring of EAE

The animals were housed in plastic cages in a room and given commercial food and water *ad libitum*. The clinical signs of EAE include loss of body weight, loss of tail tone, limb paralysis and moribund (or death). The animals that appear one of the clinical signs were considered as EAE positive.

Individual rats were examined daily for clinical signs of neurological deficits scored on a 0-6 scale as follows: grade 0, no abnormality; grade 1, limp tail; grade 2, hind limps weakness (waddling gait); grade 3, partial hind limp paralysis; grade 4, complete hind limb paralysis; grade 5, moribund; grade 6, death. The data are reported as the mean daily clinical score in each group.

Histopathology and transmission electron microscope examination

For histopathological examinations, the animals were anesthetized (< 15 sec) with halothane immediately after removal from their home cages at the peak phase of EAE. The brains and spinal cords were harvested were fixed in 4% paraformaldehyde in PBS for 18 h, dehydrated in a graded ethanol series, and embedded in paraffin. Five μM thick sections were prepared and then stained with hematoxylin and eosin or with luxol fast blue. The remains of the central nervous tissue were fixed in 5% glutaradehyde solution and examined by transmission electron microscope.

Quantification of cytokines

After the animals were anesthetized, blood was immediately collected from the heart of each animal into ice-chilled EDTA-coated tubes containing 140 μg aprotinin followed by centrifugation at 2,500 g, 4 °C for 10 min. The plasma were harvested and stored at -20 °C. Plasma TNF-α, IFN-γ and IL-10 levels were determined by commercial ELISA kits according to the protocol recommended by the manufactures. The results were expressed as the quantity per ml plasma.

In vitro proliferation assay

Peripheral blood monocytes and spleen lymphocytes were isolated. Cells were cultured in 96-well flat-bottom microculture plates at a density of 5×10^5 cells/well in 200 μl RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM 2-ME and antibiotics. The state of cell growth was observed with light microscope. For assessment of proliferation response, cultures were pulsed with [^{3}H]TdR (0.5 μci/well) for the last 16 h of the 96 h culture and harvested on a micro-96 harvester. Incorporated radioactivity was measured on a microplate beta counter. The results from each group were expressed as stimulating index (SI).

Statistical analysis

Data were presented as $x \pm s$. Group differences were analyzed with one-way ANOVA, using SNK test for multiple comparisons, and $p < 0.05$ was considered as significant.

Results

The clinical signs and scoring of EAE

Following the second injection of human MBP emulsion, 85.71% rats in the hu-group exhibited loss of body weight, loss of tail tone and hind-limb paralysis. The initial phase of the hu-group was at the 8th day and the peak phase (the clinical score > 1) was from 14th to 19th day. Rats began to rehabilitate after the peak phase of 2-3 days, only the tails

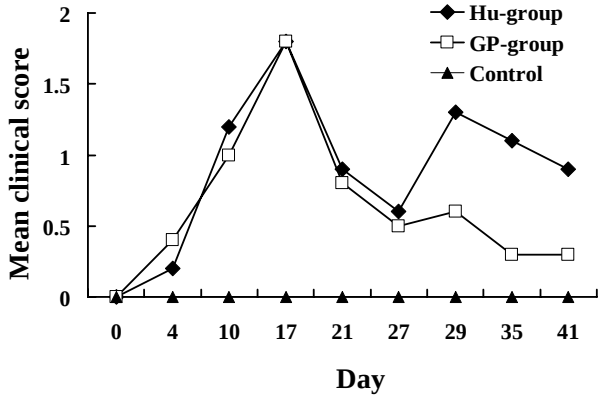

Figure 1. The mean clinical scores of the hu-group, GP-group and control group.

keeping flaccid. Some (5/8) relapsed after they received the third immunization on 27th day. Compared to the GP-group, the incidence of this EAE model is higher, the process longer and easy to relapse (Figure 1).

The pathological characteristics of EAE models

Demyelination, infiltrative lymphocyte foci, damage of axon and neurons are characterized in human MS patients. Histopathological examination showed that all these changes occurred at the summit of EAE rats in the hu-group. There were a number of glial nodules and perivascular cuffs in the cortex of cerebra and the white matter of spinal cords (Figure 2A, 2B). Demyelination was observed in the above place and more severe than the GP-group (Figure 2C, 2D). "Neuronophagia" also was noticed in the brain of improved models, meaning that there was axon and neuron damage in the brains of the hu-group rats (Figure 2E). TEM examination confirmed the above characteristics in the hu-group (Figure 2F).

The change of plasma TNF-α, IFN-γ and IL-10 in the course of EAE

To further understand the immune state in the hu-group rats, we detected the levels of plasma TNF-α, IFN-γ and IL-10, which are predominant cytokines in the development of EAE. In our experiment, the change of plasma TNF-α was related with the severity of EAE (Table 1). The level of TNF-α began to increase after first immunization of human MBP and continued to grow until the clinical scores of EAE culminated. At the peak phase of EAE, the level was the highest. From then, the level began to decrease, but still was higher in the remitting phase than that in the beginning time. The tendency of IFN-γ was similar to that of TNF-α, only showing a sharp increase after the first immunization, higher than the second immunization.

Contrary to the tendency of TNF-α and IFN-γ, the plasma IL-10 level was maintained at the low level until the EAE rats remitted. In the remitting phase, the content of IL-10 was increased significantly compared to the control group ($p < 0.05$).

PBMC and spleen lymphocytes were response to the stimulation of MBP and PHA

To closely determine the contribution of T lymphocytes to the development of EAE models, we assessed the proliferation response of lymphocytes to the stimulation of MBP and PHA at the summit of the hu-group rats by [^{3}H]TdR uptake (Table 2). It was found that after the stimulation of PHA or MBP, the lymphocytes became larger and crowded in cluster. The effect of PHA was greater than that of MBP. The lymphocytes in the control group showed weak response. The SI in the hu-group was

Table 1. *The levels of plasma TNF-α, IFN-γ and IL-10 in the course of EAE (x ± s).*

	n	TNF-α	IFN-γ	IL-10
Control	7	22.34 ± 5.93	34.64 ± 6.19	undetectable
Day 4	8	24.67 ± 6.09*	247.91 ± 40.66*	0.24 ± 0.13
Day 10	8	35.52 ± 20.31*	105.20 ± 40.33*	0.37 ± 0.15
Day 17	8	90.37 ± 53.85*$^{\Delta}$	184.62 ± 60.19*	0.56 ± 0.37
Day 27	8	93.26 ± 49.16*$^{\Delta}$	240.48 ± 90.16*	3.92 ± 2.41$^{\Delta}$

*$p < 0.05$ compared with the control group; $^{\Delta}p < 0.05$, compared with Day 4.

Table 2. *The SI of PMBC and spleen lymphocytes to the stimulation of MBP and PHA (x ± s).*

	n	PBMC		Spleen lymphocytes	
		PHA	MBP	PHA	MBP
Hu-group	8	214.84 ± 55.78*	4.18 ± 2.22*	104.75 ± 29.11*	4.18 ± 2.84*
Control group	7	29.48 ± 22.32	0.78 ± 0.17	14.89 ± 14.17	0.14 ± 0.10

*$p < 0.05$, compared with the control group.

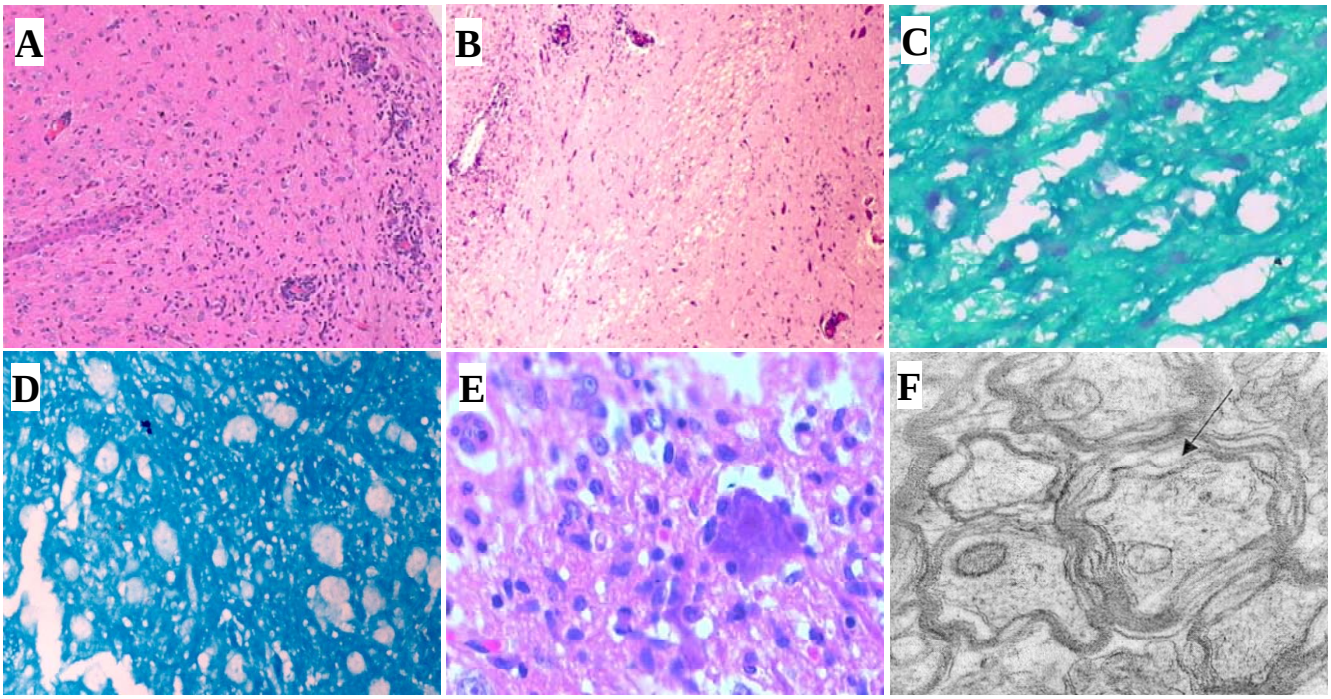

Figure 2. The pathologic characteristics in the hu-group and the GP-group. (A) A number of perivascular cuffs in the cortex of cerebrum in the hu-group (HE × 100); (B) Inflammatory foci were shorter in the cortex of cerebrum in the GP-group (HE × 100); (C) Deymelination in the white matter of the spinal cord in the hu-group (LFB × 400); (D) Deymelination was not obvious in the white matte of the spinal cord in the GP-group (LFB × 400); (E) "Neuronophagia" in the cortex of cerebrum in the hu-group (HE × 400); (F) Deymelination in the white matte of the spinal cord in the hu-group (TEM × 8,000).

significantly higher than that in the control group ($p < 0.05$).

Discussion

The disease course of remitting-relapsing, demyelination, infiltrative lymphocyte foci, damage of axon and neurons in the central system are characterized in the human MS patients. In the traditional EAE models, there are some defects: difficulty to induce relapsing, scare foci of infiltrative lymphocyte, little demyelanation and no neuron damage in the central nervous system. However, EAE rats in the hu-group showed all these characteristics. Compared to the GP-group, the hu-group rats also showed the following features: the higher incidence, the longer disease process (6 d) and the relapsing tendency. Moreover, "Neuronophagia" was also noticed that there was axon and neuron damage in the brains of EAE rats in the hu-group. It was suggested that the EAE model induced by human MBP closely mimics human MS and is superior to other models before.

To further confirm our conclusion and study the immune state of the hu-group rats, we evaluated the tendency of important cytokines *in vivo*, such as TNF-α, IFN-γ and IL-10. TNF-α and IFN-γ are proinflammatory cyto- kines, predominately secreted by auto-immune T cells. They directly destroyed the brain-blood barrier, induced the apoptosis of oligodendrocyte, caused the demyelination (5). The plasma levels are correlated with the severity of EAE and MS and reflect the immune state (6). In hu-group, the levels of plasma TNF-α and IFN-γ were changed with the process of EAE. After the first immunization of antigen, IFN-γ showed great marked increase, which indicated that IFN-γ plays an important role in the T cell activation. Then, the levels of plasma TNF-α, IFN-γ were increased with the development of EAE. At the summit of EAE, the cytokines also culminated. But after the relapsing of EAE, the cytokines didn't restore to the normal level. This means the skewed immune tendency remains in the animal bodies and may be the foundation of the relapsing of EAE after the third immunization on 27 day.

By contrast, IL-10, a cytokine produced by Th2 and regulatory T cells, plays an inhibitory role in the pathologic process of EAE and MS by down-regulating the expression of proinflammatory cytokines, such as IL-1, IL-8, IL-6 and TNF-α (7). In hu-group, plasma IL-10 persisted at the low level until the rats began to rehabilitate, meaning that the Th2 cell-immune reaction is very weak in the initiation and development of EAE. In the remitting stage, IL-10 level was at high level and promoted the rehabilitation of rats. After EAE had remitted, the immune balance was restored partially.

Autoimmune and antigen-specific T lymphocytes existed in the body, which is the foundation of EAE (8, 9). To directly evaluate the state of lymphocytes in EAE rats, we assayed the proliferation response of peripheral blood monocytes and spleen lymphocytes. Our experiment indicated that both of blood monocytes and spleen lymphocytes were response to the stimulation of PHA and MBP and the proliferation ability to the simulation of PHA was superior to that of MBP. Perhaps, MBP is a specific

antigen and only stimulates antigen-specific clone, while PHA is a non-specific stimulator, all T cells are response to the stimulation of PHA. This suggested that in the hu-group there were human MBP specific T cells in the rats body, and these autoimmune T cells were involved in the causing mechanism of EAE and MS.

In summary, the animal model of EAE induced by human MBP bears a resemblance to the features of MS, which promises to be a better model than ever before for the study of MS.

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