Analysis of the direct injury effector of oligodendroglia cells or myelin sheath in an experimental allergic encephalomyelitis model induced by the MOG35-55 peptide

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Received May 27, 2014; Accepted July 28, 2015

DOI: 10.3892/mmr.2015.4358

Abstract. The aim of the present study was to investigate the possible role of cytotoxic T lymphocytes (CTL) and mononuclear macrophages in the pathogenic processes of experimental animals. To construct a chronic experimental allergic encephalomyelitis (EAE) model, an artificially synthesized myelin oligodendrocyte glycoprotein (MOG)35-55 peptide was used to induce C57BL/6 mice. Subsequently, the experimental animals were investigated at the level of their nervous function, histopathological, immunohistochemical and fluorescence immunohistochemical experiments were performed at different time points following immunization. The expression of immune molecules and cytokines associated with the activation of the mononuclear macrophages and CTL during the different stages was assessed by western blotting and reverse transcription-quantitative polymerase chain reaction. As a result, the MOG35-55 peptide was identified as being successful at inducing C57BL/6 mice for the development of the EAE model. A modest level of mononuclear macrophage and lymphocyte infiltration was observed in the central nervous system (CNS), although no infiltration of neutrophils was observed. A sporadic flaky depletion of the myelin sheath was also identified. The activation and proliferation of mononuclear macrophages, including microglia, was clearly demonstrated. Furthermore, the expression levels of major histocompatibility complex class I and II molecules and interleukin-12 in the brain, which is associated with the activation and proliferation of mononuclear macrophages, increased over the duration of the experiment compared with less pronounced changes in the expression levels of interferon (IFN)-γ, Fas and perforin in the CNS, which are associated with the function of CTL. The secretion of IFN-γ in the spleen increased during the morbidity peak, however, any noticeable activation and proliferation of CD8+ T cells was absent. These results demonstrated that the induced immune response mediated by mononuclear macrophages made a more important contribution compared with CTL towards the pathological process of myelin sheath injury. Mononuclear macrophages are therefore, identified as being one of the most significant effector cell types to directly injure the myelin sheath in the CNS.

Introduction

Multiple sclerosis (MS) is an inflammatory autoimmune disease characterized by sporadic or multifocal demyelination in the white matter of the central nervous system (CNS), resulting in possible injury of the axons to differing degrees. Experimental allergic encephalomyelitis (EAE) is an inflammatory allergic disease affecting the CNS in a variety of sensitive experimental animals, and this is often used as an animal model of MS (1,2). Myelin oligodendrocyte glycoprotein (MOG) has been widely used in previous studies as the inducing antigen/immunogen for the EAE model. MOG is a transmembrane glycopeptide of 26-28 kDa, which is expressed in the myelin sheath membrane and on the outer surface of oligodendroglia cells (3,4). Three identifiable epitopes exist in the extracellular region, and MOG35-55 peptide is one of the epitopes which causes encephalitis. MOG has been demonstrated to be the sole autoantigen, not only to induce the T cell response, but also to elicit the production of myelin sheath antibodies among the constitutive proteins of the myelin sheath (5), results which revealed the advantage of using MOG as the inducer for the establishment of the EAE model.

Key words: myelin oligodendrocyte glycoprotein, oligodendroglia cells, cytotoxic T lymphocyte, experimental allergic encephalomyelitis
model and its pathological characteristics (6). The EAE model of C57BL/6 mice induced by MOG approximates closely to human MS, with respect to the pathological and clinical manifestations (7-9), and therefore it is an ideal model to investigate the pathogenesis and therapeutic strategies of MS (10,11).

The pathogenesis of MS remains to be fully elucidated, however, the best-supported hypothesis is that MS is a type of autoimmune disease of the myelin sheath in the CNS, in which autoreactive CD4+Th1 lymphocytes, and the cytokines they release, are the most important factors associated with humoral immunity. The impairment in function of the CNS is characterized by deletion of the myelin sheath, which is predominantly mediated by interferon (IFN)-γ from activated T cells. This induces differentiation of the Th1 cells and the immune response targeted to the myelin sheath, leading to the development of the disease. An imbalance in the Th1/Th2 cell response, and either an inhibition of differentiation of the regulatory T cells (Treg cells) or an impairment of their function, are also likely to contribute towards the disease, even though autoreactive CD4+Th1 lymphocytes and their released cytokines are thought to be primarily responsible as the causative agents (12-14). However, a number of biological phenomena, which are encountered during studies of MS/EAE, may not be explained by the predominant differentiation of the Th1 cells and the inhibition of differentiation or functional impairment of Treg cells under pathological conditions.

CD4+Th1 and CD8+T cells are unable to directly attack the target cells, and they contribute to inflammation and tissue injury predominantly by secreting cytokines, which promote the activation and proliferation of cytotoxic T lymphocytes (CTL), natural killer (NK) cells and phagocytes, and mediate the immune response of the effector cells. Th2 cells are also unable to cause direct injury of the target cells, and these mediate the humoral immune response predominantly by stimulating B lymphocytes to produce antibodies. Activated CD8+T lymphocytes differentiate into CD8+ effector T cells and CTL. It is possible that oligodendroglia cells are attacked by activated CTL and macrophages, since the expression levels of the major histocompatibility complex (MHC) class I molecules are upregulated in oligodendroglia cells during inflammation. The immunocytes, which elicit direct myelin sheath injury of the CNS, are possibly those among the CTL or macrophage systems. The present study focused on an examination of the immune response changes of CTL and mononuclear macrophages using an EAE model, and aimed to ascertain which effector cells led to direct myelin sheath injury of the CNS by comparing the roles of CTL and mononuclear macrophages during the progression of EAE.

Materials and methods

Immunogens. Murine recombinant MOG35-55 peptide was artificially synthesized (Sigma-Aldrich, St. Louis, MO, USA; purity, >98%). The amino acid sequence of the peptide was MEVGWYRSPFSRVVHLYRNGK, with a molecular mass of 2,582.0 Daltons.

Experimental animals. A total of 80 healthy female wild-type C57BL/6 mice, aged 8-10 weeks and weighing 18-22 g, were used in the present study. The mice were provided by the Experimental Animal Center (Wuhan University, Wuhan, China) and fed on nutritional foodstuff in individual cages. The feeding environment was at room temperature (18-25°C), with a relative humidity of 50-60%, and 12 h day/night cycle lighting. The mice were numbered and grouped following adaptive feeding for 1-2 weeks. The study was approved by the Ethics Committee of Huazhong University of Science and Technology (Wuhan, China).

Animal grouping and establishment of the EAE model. The 80 C57BL/6 mice were divided randomly into a blank control group and the EAE group, with 40 mice in each group. The control group animals were injected with 2 mg Complete™ Freund's adjuvant (Sigma-Aldrich, St. Louis, MO, USA) subcutaneously at two points of the inguinal groove for each mouse and 200 ng pertussis toxin (Sigma-Aldrich) was subsequently injected into the abdominal cavity on the day of immunization and 48 h afterwards. Administration of the OG35-55 peptide and immunological adjuvant were used to establish the chronic EAE animal model in the EAE group: 0.2 ml mixed emulsion was injected subcutaneously at two points of the inguinal groove for each mouse, which contained 250 µg artificially synthesized MOG35-55 peptide and 2 mg Complete™ Freund's adjuvant (volume ratio, 1:1). The mice were subsequently injected with 200 ng pertussis toxin into the abdominal cavity on the day of immunization and 48 h afterwards. The behavior and activities of the animals were assessed daily prior to the completion of the experiment, on day 30, with the day of immunization being set as day 0. The severity of clinical nervous symptoms of the EAE group mice were assessed according to the Benson scoring standard (15). A total of eight mice were sacrificed in the control and EAE groups on days 0, 7, 14, 21 and 30, and brain and spleen tissues were extracted for further examination. The mice were anesthetized with 3 ml/kg chloral hydrate (Sigma-Aldrich), following which the thoracic cavity was opened in order to expose the heart. A needle was inserted from the apex cordis to the aorta, and the right auricle was sectioned. A total of 0.9% normal saline (NS; 300 ml) was perfused until the perfusate was transparent, followed by perfusion of 50 ml 4% paraformaldehyde (Servicebio Technology Co., Ltd, Wuhan, China).

The scoring standard was applied as follows: 0 points, no symptoms; 1 point, tail inertia or postcrisis crouch gait accompanied by powerful tail; 2 points, jump crouch gait accompanied by tail inertia (ataxia); 2.5 points, ataxia accompanied by partial paralysis of a single limb; 3 points, complete paralysis of a single limb; 3.5 points, complete paralysis of a single limb accompanied by partial paralysis of another limb; 4 points, complete paralysis of a couple of limbs; 4.5 points, paralysis of four limbs; 5 points, death.

Histological examination. The abdominal skin of the experimental mice was cut from the upper part along a curved line to each side following anesthetization by injection of 10% chloral hydrate (3 ml/kg) into the abdominal cavity. The peritoneum and costal bone on each side was carefully incised to completely expose the thoracic cavity. A perfusion needle was fixed, and inserted from the cardiac apex up to the aorta. The right auricular appendix was cut and perfused with
Western blotting. The rat brains were harvested and homogenized at 4°C using a Teflon glass homogenizer in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10 mM NaF, 1 mM NaVO₃, 10 mM β-mercaptoethanol, 5 mM EDTA, 2 mM benzamidine, 1.0 mM phenylmethylsulfonyl fluoride, 5 mg/ml leupeptin, 5 mg/ml aprotinin and 2 mg/ml pepstatin (Sigma-Aldrich). Three volumes of the homogenized samples were subsequently added to one volume of the extracting buffer (200 mM Tris-HCl, pH 7.6, 8% SDS, 40% glycerol; Sigma-Aldrich). Protein concentrations were determined using a bicinchoninic acid kit (Pierce Biotechnology, Inc., Rockford, IL, USA), and between 9 and 11 mg/ml was used. A final concentration of 10% mercaptoethanol and 0.05% bromophenol blue (Sigma-Aldrich) were then added, and the samples were boiled in a water bath for 10 min. The boiled samples were separated by 10% SDS-PAGE (Sigma-Aldrich) and the separated proteins were transferred onto nitrocellulose membranes (GE Healthcare Life Sciences, Little Chalfont, UK). The membranes were subsequently blocked with 5% nonfat milk dissolved in Tris-buffered saline (TBS)-Tween-20 (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.2% Tween-20; Sigma-Aldrich) for 0.5 h (16), followed by an incubation with primary antibodies (1:200) at 25°C for 0.5 h. The primary antibodies were as follows: Mouse anti-mouse monoclonal IgG GAPDH (cat. no. ab9482), rabbit anti-mouse polyclonal IgG MHC-I (cat. no. ab93364), rat anti-mouse monoclonal IgG MHC-II (cat. no. ab139365) and rat anti-mouse monoclonal IgG L-12 (cat. no. ab80682) (Abcam). The membranes were washed with TBS-Tween-20, and then incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies (1:15,000) for 1 h at room temperature. After a final wash with TBS-Tween-20, the grey scale of the blots was analyzed using an Odyssey Infrared Imaging system (cat. no. 9120; LI-COR Biosciences, Lincoln, NE, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). For RT-qPCR, mice tissue during all the experimental stages was selected and the total RNA was extracted using TRIzol® reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. The cDNA (5 µg) was reverse-transcribed and synthesized using a Prime Script First Strand cDNA Synthesis kit (cat. no. D6110A; Takara Bio, Inc., Beijing, China) under the conditions of 37°C for 15 min and 85°C for 5 sec. The gene products were subsequently amplified by RT-qPCR (CFX Connect™ Optics Module; Bio-Rad Laboratories, Inc.), according to the following method. The reaction system comprised 5 µl SYBR Green mix (Sigma-Aldrich), 0.3 µl the upstream and downstream primers, 1 µl cDNA and 3.4 µl RNase-free water. A total of 40 cycles were used in the program (95°C for 3 min, 95°C for 10 sec, 60°C for 30 sec), and the dissolving curve was examined at 65-90°C. The sequences of the primers used were as follows: β-actin, upstream: 5'-CTGAGAGGGGAAATCGTGCCT-3' and downstream: 5'-CCACAGGATTCCATACCCAAGA-3'; Fas, upstream: 5'-ACCCCTGACCCAGAATACCAAG-3'; and downstream: 5'-AGGCGATTTCTGGGACCTTTGT-3'; perforin, upstream: 5'-CACGCGATCTGCTCTTTCG-3' and downstream: 5'-CGCTTCCGGGTTCGTGTCTTC-3'.

Statistical analysis. SPSS 18.0 software (IBM, SPSS, Chicago, IL, USA) was used for statistical analysis of the experimental data. The data are expressed as the mean ± standard error of the mean. Student's t-test was used to compare the differences between pairs of groups, and one-way analysis of variance was used to compare several samples. P<0.05 was considered to indicate a statistically significant difference.

Figure 1. Clinical function scores of the experimental mice. The data are expressed as the mean ± standard error of the mean. EAE, experimental allergic encephalomyelitis.
Establishment of the animal model. The EAE model of C57BL/6J mice was successfully induced by injection of the MOG35-55 peptide and immunological adjuvant. As shown in Fig. 1, the experiment was terminated on day 30, and all mice in the control group were free of the disease. The disease affected all mice in the EAE model group, with the exception of those which were sacrificed during the early stage. The mice in the EAE group were free of the disease up to 11 days following immunization, at which time certain mice were affected by the disease in rapid succession, between days 12 and 14, with

Results

Establishment of the animal model. The EAE model of C57BL/6J mice was successfully induced by injection of the MOG35-55 peptide and immunological adjuvant. As shown in Fig. 1, the experiment was terminated on day 30, and all mice in the control group were free of the disease. The disease affected all mice in the EAE model group, with the exception of those which were sacrificed during the early stage. The mice in the EAE group were free of the disease up to 11 days following immunization, at which time certain mice were affected by the disease in rapid succession, between days 12 and 14, with
Figure 4. Double fluorescence IHC of CD8 and Ki-67 in the spleen of the experimental mice (magnification, x200). The merged images are presented in the panels on the right. DAPI, 4',6-diamidine-2'-phenylindole dihydrochloride.

Figure 5. Examination of the levels of (A and B) MHC I, (C and D) MHC II and (E and F) IL-12 in the brain of the experimental mice during all stages. The panels on the left demonstrate the western blot analyses. The quantified data are revealed in the panels on the right (*P<0.05, compared with the control group). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; MHC, major histocompatibility complex.
clinical scores of 1-2. The morbidity reached its peak during days 20-24, with scores of 2.5-4.5. The clinical symptoms of certain mice were alleviated following survival for 3-4 days, and their scores declined. However, the symptoms persisted and the disease was able to enter the chronic stage.

**Histological changes in the brain of the experimental mice.** The pathological condition of the mice was monitored throughout the 30 day course of the experiment. The brain tissue in each group appeared normal to the naked eye, however, a noticeable level of congestion and edema were observed in the spleen of the diseased mice in the EAE group. HE staining and LFB staining of the mice brain in the control group revealed no abnormal change. In the EAE group, a modest level of monocyte and lymphocyte infiltration in the brain was observed, as the disease progressed. LFB staining revealed a loosening of the white matter, discontinuity, breaking and vacuole changes in the myelin sheath, and a white and flaky deletion of the myelin sheath in certain regions, conforming to the features of the chronic EAE mode (i.e. prominent myelin sheath destruction and little apparent inflammatory cell infiltration).

Fig. 2 shows the results of the fluorescence IHC experiment of IFN-γ in the spleen and brain of the experimental mice. A marginal quantity of green IFN-γ fluorescence was observed by fluorescence IHC of IFN-γ in the spleen of the control group, as revealed in Figs. 2-4. In the EAE group, green fluorescence was observed in the spleen on day 21 due to an abundant production of IFN-γ by lymphocytes, indicating that the T lymphocytes were activated. The noticeable fluorescence coloration remained visible until day 30. However, no positive identification of IFN-γ was measured by fluorescence IHC in the brain tissue, with the exception of a very slight quantity of green IFN-γ fluorescence around the ventricle on day 21 (data not shown).

Double fluorescence IHC of Iba1 and ki-67 in the brain of the experimental mice was subsequently performed (Fig. 3). No indication of proliferation of the Iba1-positive cells in the control group was evident, and a slight double fluorescence was visible on day 7 after immunization, which remained apparent up to day 14. On conclusion of the experiment at day 30, the proliferation reaction of Iba1-positive cells remained visible. Subsequently, a double fluorescence IPC experiment was performed with the anti-CD8 and anti-ki-67 antibodies in the spleen of the experimental mice. As revealed in Fig. 4, a slight and sporadic proliferation reaction of the CD8+ T lymphocytes was visible on days 14 and 21 after immunization, whereas no proliferation was observed during other stages, indicating that the majority of the activated and proliferating lymphocytes were not CD8+ T lymphocytes.

**EAE altered the expression of the associated proteins.** As revealed in Fig. 5, the expression level of MHC I molecules in the brain tissue of mice in the EAE group increased significantly on day 14, and the higher levels of MHC I persisted until day 30 (P<0.05, compared with the control group). The results indicated that the expression of MHC I molecules increased with the onset of the disease, and the increased levels were maintained at the higher level without a decline. The expression levels of the MHC II molecules in mice in the EAE group increased significantly by day 7 following the onset of the disease, reached a peak on day 14, exhibited a decline on day 21 and subsequently rose again on day 30, (P<0.05, compared with the control group). The secretion of IL-12 in the brain started to increase on day 7 and achieved its maximum level by day 21, maintaining a higher level up until day 30. The increases compared with the control group were significant (P<0.05); however, the level of IL-12 during the chronic stage of the disease was lower compared with that during the peak stage (P<0.05).

**EAE did not affect the mRNA expression levels of Fas and perforin.** Changes in the mRNA expression levels of Fas and perforin were examined in the brain of the mice in the EAE and control groups during the different stages of the experiment. The differences identified were not significant (P>0.05; Fig. 6).

**Discussion**

It would appear that almost all the known immunocytes are involved in processes associated with inflammation and demyelination in the CNS, although the exact mechanisms of various immunocytes and their secreted cytokines in MS development remain to be fully elucidated (17-20). Antigens themselves are not the direct causative agents of disease during the immune response: Immunocytes, and molecules that are activated by the antigen directly or indirectly, are the direct cause. It is generally accepted that injury of the CNS, which
is characterized predominantly by demyelination, is mediated by IFN-γ, a strong proinflammatory cytokine, which elicits the immune response of myelin sheath by inducing differentiation of the Th1 cells. However, a study investigating IFN-γ and its receptor with gene-knockout animals revealed that EAE still occurred, and that the gene-knockout animals were more adversely affected by the disease (21). This demonstrates that MS/EAE may be caused by several types of pathological mechanisms working in concert, and innate immunity, the specific immune response and other regulatory mechanisms of the immune response may be involved.

Cellular functions become diversified following lymphocyte activation during the effector stage. Tγγγγ cells differentiate into CD4+ and CD8+ T lymphocytes and Treg cells. Each type of T lymphocyte then further differentiates into the terminal immune effector cells. CTL can attack the host cells directly, however, Th cells, Treg cells and CD8+ T effector lymphocytes (together with B lymphocytes, another type of humoral immunocyte) only regulate the direction and extent of the immune response by secreting cytokines or antibodies, which act further on macrophages and CTL, without being able to attack the host cells directly. At present, macrophages and CTL are known to cause direct injury of oligodendroglia cells and the myelin sheath.

The macrophages, which are predominately featured in the MS/EAE immune response are mononuclear macrophages and neutrophils. In the present study, no infiltration and aggregation of neutrophils was identified in the CNS, indicating that the major pathogenic macrophages may be oligodendroglia cells in the CNS and/or peripheral mononuclear macrophages. MHC molecules are not constitutively expressed by oligodendroglia cells, although MHC I molecules can be upregulated under pathological conditions, including inflammation, whereas the expression of MHC II molecules by oligodendroglia cells does not occur under any conditions. This further demonstrated that CD4+ Th lymphocytes and Treg cells do not directly attack oligodendroglia cells.

CTL are restricted by MHC I molecules when killing the target cells, and sensitized CTL cells are able to continuously act on other target cells, which carry an identical antigen following killing of the target cells, demonstrating that the effects of CTL are self-propagating. A number of previous studies revealed that the role of CTL in the occurrence and development of MS/EAE is not negligible, however, it is controversial (22-25). Additionally, it was revealed that CD8+ Treg cells express specificity for their target organs, which may lead to the direct killing of the activated immune response cells, or produce cytokines, including TGF-β and IL-10, under the action of IFN-γ, fulfilling the role of immune suppression (26). CTL elicit their effects predominantly via the perforin and Fas/Fas L route. In the present study, changes identified for Fas, perforin and IFN-γ during the occurrence and development of chronic EAE were not significant, and since they are associated with CTL function in the brain, no appreciable changes were observed in the levels of these effector molecules of CTL, which are capable of killing the target cells directly. Nevertheless, the expression of the MHC I and MHC II molecules, which are associated with the antigen-presenting function of mononuclear macrophages, were upregulated, indicating that there is also an increase in the expression levels of MHC I molecules in the CNS, despite the evidence that T lymphocyte activation is marked by IFN-γ production in spleen. These results fail to demonstrate that CTL enhance the immune effect of MHC I positive cells in the CNS via the perforin and Fas/Fas L route.

Mononuclear macrophages are a type of antigen presenting cell (APC), which may also cause injury of the myelin sheath in CNS by phagocytosis, dissolution, and even possibly direct attack, in addition to the induction of the immune response by presenting antigens. IL-12 is a type of cytokine specifically produced by APC cells, including mononuclear macrophages and dendritic cells. Activated microglia cells are one of the predominant sources of IL-12 in the CNS. IL-12 may activate NK cells in innate immunity, however, its major function, identical to IFN-γ, is to promote the differentiation of Th0 cells into Th1 cells. In the present study, the expression levels of IL-12 changed markedly in animals in the EAE group throughout the course of the experiment, whereas the immunofluorescence studies revealed an absence of any appreciable increase in IFN-γ secretion in the brain, indicating that the degree of T lymphocyte activation was not high. Given that the effects of IL-12 secreted by mononuclear macrophages on the CNS are mediated earlier than those of IFN-γ, and the effect of IL-12 in promoting Th cell differentiation and regulating antibody production of B lymphocytes is more marked compared with IFN-γ, mononuclear macrophages exert a more important role compared with CTL in the progression of EAE.

In conclusion, the effector cells causing direct injury to the myelin sheath in the CNS may be mononuclear macrophages other than CTL, including microglia cells, although it was not determined whether the Iba 1-positive cells are mononuclear macrophages migrating from peripheral tissues, or microglia cells residing in the brain. A noticeable activation and proliferation of mononuclear macrophages containing microglia cells during the course of EAE, and the induced immune response fulfills a more important role compared with CTL during the pathological process of myelin sheath injury. Therefore, mononuclear macrophages are one of the most important effector cells causing direct injury of the myelin sheath in the CNS.

Acknowledgements

This study was supported by the National Program on Key Basic Research Project of China (973 Program; no. 2012CB722401), and the National Natural Science Foundation of China (nos. 81030051 and 21177046).

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