Intracerebral injection of myelin basic protein (MBP) induces inflammation in brain and causes paraplegia in MBP-sensitized B6 mice

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SUMMARY
Brain inflammation and paraplegia can be induced by an additional intraperitoneal (i.p.) and intracerebral (i.c.) restimulation in B6 mice after standard immunization with MBP in Freund’s complete adjuvant (FCA) and Bordetella pertussis coadjuvant. Only the combination of i.p. MBP/FCA and i.c. MBP injection could induce clinical paraplegia; either one alone was not effective. Clinical symptoms would develop 2 days after the i.c. injection. The induction of paraplegia was MBP-specific, as irrelevant bovine serum albumin with the same protocol could not induce it. The i.p. restimulation was requisite and needed the MBP in FCA, as MBP in PBS was ineffective. Histopathological observation manifested cellular infiltration by leucocytes in perivascular spaces and cerebral cortex. Neutrophils were prominent at 12 h after i.c. injection, then were replaced by mononuclear cells 24 h later. There were dynamic changes in cell number and immunophenotype of VLA-4\(^+\) expression in cervical lymph node cells after i.c. injection. The cells derived from cervical lymph nodes had higher MBP-stimulated proliferation than that of distal lymph nodes. This additional i.p. and i.c. stimulation provides a new manipulation to study brain inflammation.

Keywords intracerebral stimulation cervical lymphatics experimental allergic encephalomyelitis

INTRODUCTION
Experimental allergic encephalomyelitis (EAE) is a demyelinating disease induced by immunization with MBP in adjuvant and is characterized by perivascular inflammatory lesions in the central nervous system [1–4]. This T cell-mediated autoimmune disease has been used as an animal model for the study of multiple sclerosis. Murine EAE can be induced by an active immunization with myelin antigens in Freund’s complete adjuvant (FCA) and Bordetella pertussis coadjuvant or by a passive transfer of primed and in vitro activated cells into naive recipients [5,6]. Susceptible animals will develop EAE after MBP/FCA sensitization. The induction of MBP-specific EAE is also regulated by genetic factors. Both susceptible and resistant rodent strains have been identified [7–11]. Three commonly used susceptible strains are SJLJ (H-2\(^b\)), PLJ (H-2\(^b\)), and B10.PL (H-2\(^b\)), while resistant strains are C57Bl/10 (H-2\(^b\)), AKR (H-2\(^b\)), and BALB/c (H-2\(^b\)). Shaw et al. [12] reported that reputed resistant mouse strains can indeed be induced to develop EAE by a combination of adoptive transfer and subsequent antigenic challenge.

The brain has been immunologically characterized as an immune privileged site, and the blood–brain barrier by virtue of its selective permeability plays an important role in the regulation of immunoregulatory cells in the brain cell microenvironment. Although the brain lacks draining lymph nodes, it is now known that there is a connection between the brain and the draining deep cervical lymph nodes [13]. Opening of the blood–brain barrier appears to be a common feature and represents ongoing disease activity in patients with mild, relapsing-remitting multiple sclerosis [14,15]. Harling-Berg et al. [16] reported that MBP, infused into cerebrospinal fluid, suppressed EAE. However, in this study we demonstrate that additional i.c. MBP injection followed after i.p. MBP/FCA restimulation in MBP-sensitized B6 mice could induce the so-called resistant strain to develop EAE. The i.c. challenge would recruit the MBP-reactive T cells from the cervical lymph node into the brain and caused the EAE.

MATERIALS AND METHODS
Animals
Breeder mice of B6 strain were purchased from The Jackson Laboratory (Bar Harbor, ME). They were maintained on standard laboratory chow and water ad libitum in the animal facility of the Medical College (National Cheng Kung University, Tainan, Taiwan, Republic of China).
Groups of six B6 mice were immunized subcutaneously with 200 μg of MBP/Freund’s complete adjuvant (FCA) and Bordetella pertussis as described in Materials and Methods. Eighteen days after sensitization, mice were injected intraperitoneally with 200 μg MBP/FCA or bovine serum albumin (BSA)/FCA. On day 20, 100 μg of MBP or BSA in PBS were injected intracerebrally. The mice were examined daily for clinical signs of experimental allergic encephalomyelitis (EAE). The mean of clinical score was expressed.

**MBP preparation**

Crude MBP was prepared from guinea pig central nervous system (CNS) tissue and extracted, homogenized, and delipidated in chloroform:methanol (2:1) as previously described [17,18]. The residue was washed with acetone and distilled water, then re-extracted with Tris–HCl pH 6.8 containing 2.5% SDS, 5% 2-mercaptoethanol (2-ME), and 10% glycerol. Bromophenol blue was added as a tracking dye. Separation gel with 12% acrylamide, 0.1% SDS, and 0.375% Tris–HCl pH 8.8 and stacking gel of 4% acrylamide, 0.1% SDS and 0.125% Tris–HCl pH 6.8 were prepared. Electrophoresis was performed at 20 mA/gel until the bromophenol blue line reached the bottom of the gel. After electrophoresis, gels were stained with coomassie blue.

**Immunization and induction of EAE**

Groups of four to six mice were injected subcutaneously at the base tail and flank with 200 μl of an emulsion containing 200 μg of antigen in FCA containing H37Ra (Difco Labs, Detroit, MI) [19]. The co-adjuvant Bordetella pertussis was injected intravenously at a dose of 1010 and 109 on days 1 and 2, respectively. Formaldehyde-inactivated Bordetella pertussis was provided by Dr C.-H. Lu (National Institute of Preventive Medicine, Taipei, Taiwan). Eighteen days later, mice were restimulated with 200 μg MBP/FCA intraperitoneally. Two days later, 100 μg of MBP in PBS was injected intracerebrally. The mice were examined daily for clinical signs of disease and graded according to the following scale: 0, no abnormality; 1, loss of weight and tail tone, and ruffled coat; 2, flaccid tail and mild hind limb weakness; 3, hind limb paresis, the animal walked with its chest close to the ground; 4, total paresis of both hind limbs, the animal could not lift its chest; 5, premoribund state; 6, death [12].

**Immunofluorescence analysis**

Lymphocytes (50 μl; 2 × 107/ml) were suspended in Hanks’ balanced salt solution (HBSS) containing 2% fetal calf serum (FCS) and 0.1% NaN3. Cells were then incubated with various fluorescence-labelled MoAbs for flow cytometric analysis. The antibodies used included FITC-labelled anti-TCR αβ and anti-CD49d MoAb (PharMingen, San Diego, CA). After incubation for 30–45 min on ice, the mixture was washed twice with ice-cold HBSS, and the cells were resuspended and adjusted in HBSS containing 2% FCS and 0.1% NaN3 to approximately 1 × 106 cells/ml. Stained lymphocytes were analysed by flow cytometry (FACScan; Becton Dickinson, Mountain View, CA) with excitation set at 488 nm.

**Histopathology**

Mice were killed by perfusion via cardiac puncture with PBS. Brains and spinal cords were removed and fixed in 10% buffered formalin solution pH 7.2 for at least 3 days. Representative sections were cut and embedded in paraffin. Sections (4 μm) were made and stained with haematoxylin and eosin. Histological examination was evaluated by two independent observers without knowledge of antecedent treatment.

In vitro T cell proliferation

Lymph node cells (4 × 105) from cervical lymph nodes (superficial/deep cervical and anterior/posterior) or distal lymph nodes (inguinal and axillary) were collected and counted. The cells were cultured in a 96-well flat-bottomed microtitre plate (Falcon; Becton Dickinson Labware, Oxnard, CA) with 0.2 ml RPMI 1640 medium containing 5 × 10−5 M 2-ME and 10% fetal bovine serum (FBS). Cultures were stimulated with the optimal dose of MBP/FCA, MBP/PBS, or BSA/FCA. On day 20, 100 μg of MBP or BSA in PBS were injected intracerebrally. Clinical EAE symptoms developed on day 22. Clinical score was expressed by mean ± s.d. (n = number of mice).

**Table 1. Induction of experimental allergic encephalomyelitis (EAE) by additional i.p. and i.c. restimulation in MBP-sensitized B6 mice**

<table>
<thead>
<tr>
<th>Group</th>
<th>s.c.</th>
<th>i.p.</th>
<th>i.c.</th>
<th>Clinical score†</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>MBP/FCA</td>
<td>MBP/FCA</td>
<td>MBP</td>
<td>2.2 ± 0.5</td>
<td>10</td>
</tr>
<tr>
<td>B</td>
<td>MBP/FCA</td>
<td></td>
<td>MBP</td>
<td>0.0 ± 0.0</td>
<td>4</td>
</tr>
<tr>
<td>C</td>
<td>MBP/FCA</td>
<td>MBP/PBS</td>
<td>MBP</td>
<td>0.0 ± 0.0</td>
<td>3</td>
</tr>
<tr>
<td>D</td>
<td>BSA/FCA</td>
<td>BSA/FCA</td>
<td>BSA</td>
<td>0.0 ± 0.0</td>
<td>4</td>
</tr>
</tbody>
</table>

*Groups of mice were immunized subcutaneously with 200 μg MBP/Freund’s complete adjuvant (FCA) or bovine serum albumin (BSA)/FCA and Bordetella pertussis as described in Materials and Methods. Eighteen days after sensitization, mice were injected intraperitoneally with 200 μg MBP/FCA, MBP/PBS, or BSA/FCA. On day 20, 100 μg of MBP or BSA in PBS were injected intracerebrally. Clinical EAE symptoms developed on day 22. Clinical score was expressed by mean ± s.d. (n = number of mice).
MBP (20 μg/ml), incubated in a humidified atmosphere of 5% CO₂:95% air for 120 h, and labelled with 1 μCi ³H-thymidine (6-7 Ci/mm; ICN Radiochemicals, Irvine, CA) for the final 24 h. Cells were harvested and radioactivity was determined with direct β-counter (Matrix 9600; Packard Instrument Co., Inc., Meriden, CT). Cultures were performed in triplicate and the results were expressed as net ct/min ((mean of ct/min of MBP-containing culture) – (mean ct/min of MBP-free culture)).

RESULTS

Induction of paraplegia by i.p. injection in MBP-sensitized B6 mice

Resistant strains of mice such as B6 mice can not develop clinical EAE symptoms (limp tail, hindleg weakness, paraplegia) after a standard immunization of 200 μg MBP/FCA containing H37Ra and B. pertussis. However, we found that additional i.p. MBP/FCA and i.c. MBP injections induced clinical paraplegia in MBP/FCA-
sensitized B6 mice (Fig. 1). The i.p. restimulation manifested no clinical symptom except weight loss. However, the i.c. challenge induced weight loss, ruffled fur, limp tail, hindleg weakness, and paraplegia 2 days after injection. The symptoms were observed.

**Fig. 2.** Histopathological changes in the brains after i.c. injection in MBP-sensitized B6 mice. Mice of B6 strain were immunized with 200 μg of MBP/Freund’s complete adjuvant (FCA) and Bordetella pertussis. Eighteen days after sensitization, mice were injected intraperitoneally with 200 μg MBP/FCA. On day 20, 100 μg of MBP in PBS were injected intracerebrally. The mice were killed at various times after i.c. challenge. Polymorphonuclear neutrophils appeared in the meninges at 6 h (b, ×400) and 12 h (c, ×400). Mononuclear cells predominated in the meninges at 24 h (d, ×400). Heavy meningeal infiltrate spilled into the parenchyma at 48 h (e, ×400). Mononuclear infiltration gradually decreased after 72 h (e, ×400). Control brain from MBP-sensitized mice is shown in (a) (×400).

**Fig. 3.** Cell number changes in cervical or distal lymph nodes after i.c. injection in MBP-sensitized mice. Groups of four B6 mice were immunized subcutaneously with 200 μg MBP/Freund’s complete adjuvant (FCA) and Bordetella pertussis. Eighteen days after sensitization, mice were injected intraperitoneally with 200 μg MBP/FCA. On day 20, 100 μg of MBP in PBS were injected intracerebrally. The mice were killed at various times after i.c. challenge. The cells of cervical (□) or distal lymph nodes (■) were pooled and counted. The cell number (mean ± s.d., n = 3) per mouse at each time point was expressed. Sensitized and i.p. denote days 18 and 20 after sensitization, respectively.

only in the group that was treated by the combination of i.p. MBP/FCA followed by i.c. MBP injection. Neither i.p. nor i.c. injection alone could induce paraplegia. The induction of paraplegia was MBP-specific, as irrelevant BSA with the same protocol could not induce EAE (group D versus group A, Table 1). Furthermore, the i.p. restimulation was requisite and needed the MBP in FCA, as MBP in PBS was ineffective (groups B and C, Table 1). The antigen used was prepared from acid extract of guinea pig CNS tissues and contained primarily 14-kD and 22-kD MBP on SDS–PAGE (data not shown).

**Histopathological changes in brain after i.c. injection in MBP-sensitized B6 mice**

The histopathological changes in the brain after i.p./i.c. induction of EAE are shown in Fig. 2. There were no cellular infiltrations in

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**Fig. 4. Enhanced T cell proliferation to MBP in cervical lymph nodes.** Groups of four B6 mice were immunized subcutaneously with 200 μg MBP/Freund’s complete adjuvant (FCA) and Bordetella pertussis. Eighteen days after sensitization, mice were injected intraperitoneally with 200 μg MBP/FCA. On day 20, 100 μg of MBP in PBS were injected intracerebrally. The mice were killed at various times after i.c. challenge. The cells of cervical (■) or distal lymph nodes (□) were pooled and MBP-stimulated T cell proliferation was determined as described in Materials and Methods. The net c/min was expressed. The background c/min in various groups was between 100 and 200. Sensitized and i.p. denote days 18 and 20 after sensitization, respectively.

**Fig. 5.** Kinetic changes of VLA-4+ expression in cervical or distal lymph nodes after i.c. injection in MBP-sensitized B6 mice. Groups of four B6 mice were immunized subcutaneously with 200 μg MBP/Freund’s complete adjuvant (FCA) and Bordetella pertussis. Eighteen days after sensitization, mice were injected intraperitoneally with 200 μg MBP/FCA. On day 20, 100 μg of MBP in PBS were injected intracerebrally. The mice were killed at various times after i.c. challenge. The cells of cervical (cLN) or distal lymph nodes (dLN) were pooled and VLA-4+ expression was stained with FITC-anti-VLA-4+ as described in Materials and Methods. Cells from naive mice or mice treated intraperitoneally or intracerebrally alone were included for comparison. The percentage of VLA-4high cells is indicated on the right of the histogram.
the brain of mice challenged intraperitoneally or intracerebrally alone. Only those challenged intraperitoneally followed by intracerebrally manifested cellular infiltration in perivascular spaces and cerebral cortex. The kinetic studies after i.c. injection showed that cells, primarily neutrophils, infiltrated the meninges and periventricular space 12 h after i.c. injection. After 24 h, most infiltrated cells became mononuclear cells (Fig. 2b–e).

Changes of cervical lymphatics after i.c. injection in MBP-sensitized B6 mice

Proteins injected into the brain are primarily drained into the cervical lymphatics. Cervical lymphatics and the blood–brain barrier provide a continuous and highly regulated communication link between the brain and the immune system [13]. The kinetic changes in cervical lymph node cells were monitored after i.c. challenge. Cervical lymph node cells were collected from superficial/deep cervical and anterior/posterior lymph nodes, while distal lymph node cells were collected frominguinal and axillary lymph nodes. There was a transient increase in cell number at 6 h, followed by a drop at 24 h and 48 h after an i.c. injection in cervical lymph node cells (Fig. 3). The decrease of cell number paralleled the abundant cell accumulation in the perivascular area of the subarachnoid space at 24 h or 48 h, suggesting that cells might have been recruited into the brain. The cell number increased again at 7 days after i.c. challenge. In the distal lymph nodes, cell number also increased temporarily at 12 h, but decreased after 24 h and thereafter. Cells were tested for their MBP reactivity. The i.p. stimulation reactivated MBP-reactive cells because higher MBP-stimulated cell proliferation was noted (Fig. 4). Cells derived from cervical lymph nodes also had higher MBP-stimulated cell proliferation than those of distal lymph nodes. Interestingly, there was also a decrease of MBP activity at 24 h or 48 h after i.c. injection. It seemed that the i.c. injection affected not only the number of cells, but also their MBP reactivity, especially in cervical lymph nodes.

VLA-4 was reported to be involved in the migration of CD4+ T cells into the brain to cause EAE [20]. We monitored the kinetic changes of VLA-4 expression on cervical and distal lymph node cells after i.c. injection. VLA-4high cells were depleted at 12 h or 24 h after i.c. challenge in cervical lymph nodes, but increased again at 120 h (Fig. 5). The i.p or i.c. challenge alone did not manifest changes in VLA-4high expression. In the distal lymph node, a transient increase in VLA-4high cells was observed at 12 h. The expression of VLA-4high cells also increased at 120 h. These dynamic changes of VLA-4high expression in lymph node cells indicated that they were activated by i.p./i.c. MBP stimulation. Since the cells, especially MBP-reactive or VLA-4high cells, were depleted at 12–50 h in the cervical lymph node and during the same period many inflammatory cells were found in the meninges and perivascular spaces, it was speculated that the infiltrated cells might be the MBP-reactive and VLA-4high cells derived from the cervical lymph node.

DISCUSSION

Murine EAE can be induced by active immunization with myelin antigens in FCA and B. pertussis coadjuvant in susceptible mouse strains such as SJL/J (H-2b), PL/J (H-2d), and B10.PL (H-2d). In contrast, C57Bl/6 (H-2b) is a resistant strain, because they cannot develop EAE after MBP sensitization. We report in this study that an additional i.p. MBP/FCA stimulation would reactivate the autoreactive T cells, whereas the i.c. challenge recruited these autoreactive T cells into the brain through the cervical lymphatics. The combinative treatment induced EAE in B6 mice.

EAE in Lewis rats developed a single episode of paralysis after MBP induction. The recovered animals were resistant to reinduction of the disease. However, Lindsey et al. [21] reported that SJL and PL/J mice not only remain susceptible to the disease after recovery, but also have an accelerated autoimmune response and recurrent paralysis when rechallenged with the same antigen. They reasoned that the autoreactive T cells generated by the initial stimulation would migrate from the peripheral lymph node to the CNS and cause encephalomyelitis and paralysis. Clinical recovery could be due to either the limited period of T cell activity which occurs after a single stimulation, or an active down-regulatory mechanism. The autoreactive T cells remain present and can be reactivated outside the CNS by a second stimulation. After reactivation, they can again migrate to the CNS and cause a second episode of EAE. Shaw et al. [12] reported that combination of adoptive transfer and antigenic challenge could induce EAE in the B6 resistant strain of mice. Mason [22] also proposed that genetic variation in the stress response determined the susceptibility to EAE in rats. The release of stress hormone corticosterone regulates the outcome of EAE disease. We found that B6 mice could indeed be induced to mount vigorous inflammatory responses in brain, resulting in development of EAE via additional i.p. and i.c. restimulation.

The immune response to the albumin administered into the cerebrospinal fluid has been reported [23,24]. Outflow of MBP and other antigens from CNS to peripheral lymphoid organs can occur by at least two pathways as a consequence of the turnover of CSF [25,26]. First, antigen can drain into cervical lymph nodes via CSF outflow along certain cranial nerves and into the cervical lymph node. Second, antigen in CSF can reach the spleen by moving across the arachnoid villi and into the blood. Harling-Berg et al. [16] reported anti-MBP antibody production after a cerebrospinal fluid infusion of MBP. However, it would suppress the development of clinical symptoms in response to an EAE-inducing challenge. Nevertheless, we found that i.c. injection of MBP induced the development of EAE. The discrepancy was probably in the timing of MBP injection. Harling-Berg gave the MBP injection before the sensitization of MBP/FCA, while we injected MBP 20 days after sensitization. We previously reported an antigen-specific hypersensitivity which does not fit into the traditional classification of hypersensitivity [27]. It is elicited within 1 h following antigen challenge, distinguishing it from either the 5-h immune complex-type or 24-h delayed-type hypersensitivity. This hypersensitivity is referred to as early-type hypersensitivity (ETH). Most importantly, the ETH is manifest by increased vasopermeability, capillary congestion, leakage of plasma proteins, and causation of oedema without cell infiltration. The CNS of mammals is considered to be an immunologically privileged site because it lacks an area of lymphatic drainage and is separated from the blood compartment by the blood–brain barrier. The i.c. injection of MBP in our system might have activated the local brain environment and opened the tight junctions of CNS endothelial cells to allow the entry of activated autoreactive T cells. Matyszak & Perry also show that bacille Calmette–Guérin (BCG) sequestrated behind the blood–brain barrier after intracranial injection would provoke a delayed-type hypersensitivity in the brain after subsequent peripheral sensitization of the immune system with BCG in FCA [28]. We believe that these two systems have common characteristics in the immune effector mechanism. CSF normally contains not more than
5 ng/ml of MBP; however, some neuropathological conditions such as trauma and infection are associated with a marked increase in the release of MBP into the CSF [29–31]. It has been proposed that elevated concentrations of MBP in CSF may provide a stimulus for eliciting autoimmune reactions against brain tissue and play a role in the etiology of multiple sclerosis in genetically susceptible individuals [32,33].

EAE can be produced in susceptible animals by the transfer of MBP-reactive T cells. However, the mechanism responsible for the entry of autoreactive T cells into the CNS and the development of disease is not known yet. It has been postulated that an interaction between the luminal surface of endothelial cells in the CNS and sensitized lymphocytes constitutes an early step in the pathogenesis of EAE [34]. Increased expression of intercellular adhesion molecule-1 (ICAM-1) or vascular cell adhesion molecule-1 (VCAM-1), which are natural ligands of lymphocyte function-associated antigen-1 (LFA-1) or very late activation antigen-4 (VLA-4), respectively, has been observed on brain endothelial cells of EAE animals [35,36]. Expression of these molecules may facilitate lymphocyte migration and extravasation across the blood–brain barrier. The transient decrease in MBP-reactive or facilitate lymphocyte migration and extravasation across the cells of EAE animals [35,36]. Expression of these molecules may facilitate lymphocyte migration and extravasation across the blood–brain barrier. The transient decrease in MBP-reactive or VLA-4+ cells at 24 h (1 day before clinical symptoms) followed by an increase thereafter is compatible with the hypothesis that activated T cells were recruited into the brain from the cervical lymph node. Baron et al. [20] demonstrated that surface expression of VLA-4 was important for CD4+ T cell entry into brain parenchyma. The VLA-4 integrins may be crucial in allowing activated effector T cells to leave the blood and enter the brain. Our unpublished data also found ICAM-1 and VCAM-1 expression in the brain after i.p. and i.c. restimulation.

Histopathological observation of kinetic changes revealed an early neutrophil infiltration at 12 h after i.c. injection. The neutrophil accumulation might be a non-specific inflammatory response caused by the trauma of i.c. stimulation or the anti-MBP antibody immune complex-associated inflammation, since anti-MBP antibodies were produced 20 days after immunization. However, 24 h after i.c. challenge, the infiltrated cells became mononuclear cells. The immunological reactions underlying the pathogenesis of EAE are primarily T cell-mediated, and the disease represents a specific case of DTH. The pattern of inflammatory response in resistant B6 mice in this study is different from that of classical EAE in susceptible SJL and PL/J mice [37]. It is hypothesized that EAE induced in resistant B6 mice is due to the i.c. induction of inflammatory responses that connect to the draining cervical lymph nodes. Therefore, this approach has several features which should make it a useful model. The reactivation of a previously established autoimmune response is more equivalent to multiple sclerosis patients. The predictable time of onset will allow kinetic studies of activated cells. Further manipulation in the brain should provide more information about the recruitment of autoreactive cells into the CNS and the immunoregulation within it.

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