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Infiltrated Cells in Experimental Allergic Encephalomyelitis by Additional Intracerebral Injection in Myelin-Basic-Protein-Sensitized B6 Mice

Key Words
Experimental allergic encephalomyelitis
Adhesion molecule
$CD4^-CD8^-CD3^+$ cells
Intracerebral stimulation
Cervical lymphatics

Abstract
We previously reported that murine experimental allergic encephalomyelitis can be induced by an additional intraperitoneal and intracerebral (i.c.) restimulation in resistant B6 mice after standard immunization with myelin antigens in complete Freund's adjuvant and *Bordetella pertussis* coadjuvant. Neutrophils infiltrated into perivascular spaces at 12 h, followed by mononuclear cells 24 h after i.c. injection. In this study, we report that the i.c. injection induced the expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). The kinetic expression of ICAM-1 or VCAM-1 on brain endothelial cells paralleled the infiltration of neutrophils and mononuclear cells, respectively. The infiltrated lymphocytes also expressed very late antigen-4 (VLA-4) molecules. The microvascular endothelial cells were positive for VCAM-1, whereas the surrounding mononuclear cells were VLA-4 positive. Furthermore, we found a unique subpopulation of cells with characteristics of $CD4^-CD8^-V_\beta8^+$ markers. The kinetic studies of this population showed that these cells were transiently depleted from 12 to 24 h after i.c. challenge (before the development of clinical symptoms) in cervical lymph nodes. These $CD4^-CD8^-V_\beta8^+$ cells can be expanded by in vitro culture with myelin basic protein or IL-2. No significant changes of $CD4^+/CD8^+$ cells were noted. $CD4^+CD8^-CD3^+$ cells were also found in brain by double histochemical stains and were the major infiltrating cells at 24 or 48 h after i.c. challenge.

Experimental allergic encephalomyelitis (EAE) is a demyelinating disease and causes inflammatory lesions in the central nervous system (CNS) [13, 22–24]. The illness is characterized by acute onset of paralysis, perivascular and parenchymal infiltration of the brain and spinal cord by mononuclear cells and inflammation of the lesions similar to a delayed-type hypersensitivity reaction. 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

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myelin basic protein (MBP) in complete Freund's adjuvant (CFA) and *Bordetella pertussis* coadjuvant or by a passive transfer of $CD4^+$ T cells into naive recipients [33, 35]. Among T cell clones specific for MBP, some were encephalitogenic and induced EAE, while others were nonencephalitogenic or even inhibited subsequent EAE induction [1, 2, 10]. The mechanisms responsible for the migration of lymphocytes into the CNS and the development of disease remain unclear. The brain has been physiologically regarded as an immune-privileged site. The blood-brain barrier, by virtue of its selective permeability, plays an important role in the regulation of immunoregulatory cells in the brain 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 [9]. 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 [7, 15]. Increased expression of intercellular adhesion-molecule-1 (ICAM-1) or vascular cell adhesion molecule-1 (VCAM-1) has been observed on brain endothelial cells of EAE animals [18, 29, 38]. The appearance of very late antigen-4 (VLA-4) on $CD4^+$ T cells allowed the activated effector T cells to enter the brain [2]. We have previously established a new protocol to induce EAE in resistant B6 mice by additional intraperitoneal (i.p.) and intracerebral (i.c.) manipulation [34]. The autoreactive T cells were recruited into the brain by i.c. challenge. In this study, we report the cell infiltration induced by i.c. stimulation in MBP-sensitized B6 mice.

Materials and Methods

Animals

Breeder mice of the B6 strain were purchased from the Jackson Laboratory, Bar Harbor, Me., USA, or Charles River Japan (Atsugi, Japan). 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. The animals were raised and cared for following the guidelines set up by the National Science Council of the Republic of China. Six- to twelve-week-old mice were used in all experiments.

MBP Preparation

Crude MBP was prepared from guinea pig CNS tissue. The protein was extracted, homogenized and delipidated in chloroform:methanol (2:1) as previously described [6, 30]. The residue was washed with acetone and distilled water and then reextracted with Tris-HCl (pH 3.0). The acid extract was centrifuged (48,000 *g*, 4°C, 10 min), and the supernatant was dialyzed against distilled water at 4°C for 24 h. The acidic dialysate was lyophilized and stored at 4°C as crude MBP.

Immunization and Induction of EAE

Groups of 4–6 mice were injected subcutaneously at the base tail and flank with 200 µl of an emulsion containing 200 µg of antigen in CFA containing H37Ra (Difco Laboratories, Detroit, Mich., USA) [5]. The coadjuvant *B. pertussis* was injected intravenously at a dose of 10^{10} and 10^9 cells on days 1 and 2, respectively. Formaldehyde-inactivated *B. pertussis* was provided by Dr. C.-H. Lu (National Institute of Preventive Medicine, Taipei, Taiwan). Eighteen days later, the mice were restimulated with an i.p. challenge of 200 µg MBP/CFA. Two days later, 100 µg of MBP in PBS was injected i.c. 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, and 6, death [28].

Immunofluorescence Analysis

Fifty microliters of lymphocytes (2×10^7/ml) were suspended in HBSS containing 2% FCS and 0.1% NaN_3. Cells were then incubated with various fluorescence-labeled monoclonal antibodies (mAb) for flow cytometric analysis [37]. The antibodies used included FITC-labeled anti-Vβ8 and PE-labeled anti-CD4 anti-CD8 mAb (PharMingen, San Diego, Calif., USA). 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% NaN_3 to approximately 1×10^6 cells/ml. Stained lymphocytes were analyzed by flow cytometry (FACScan; Becton-Dickinson, Mountain View, Calif., USA), with the excitation set at 488 nm. In some experiments, cells (4×10^5) 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 96-well flat-bottom microtiter plates (Falcon; Becton-Dickinson Labware, Oxnard, Calif., USA) with 0.2 ml of RPMI-1640 medium containing 5×10^{-5} *M* 2-mercaptoethanol and 10% fetal bovine serum. Cultures were stimulated with MBP (15 µg/ml) or IL-2 (10 U/ml) and incubated in a humidified atmosphere of 5% CO_2:95% air for 24 or 48 h. Then, the cells were harvested and stained with FITC-labeled anti-Vβ8 and PE-labeled anti-CD4 anti-CD8 mAb.

Immunohistochemistry

Mice were sacrificed by perfusion with PBS via cardiac puncture. Brains and spinal cords were removed and embedded in OCT compound and then frozen in liquid nitrogen. Five-micron cryosections were made and stained with rat primary mAb. Peroxide-conjugated sheep anti-rat IgG was used as secondary antibody. A peroxide stain (reddish-brown coloring) was developed by the aminoethyl carbazole substrate kit (ZYMED Laboratories, San Francisco, Calif., USA). In double-staining experiments, peroxide-conjugated goat anti-hamster IgG and alkaline-phosphatase-conjugated sheep anti-rat IgG were used. Alkaline phosphatase (dark-blue coloring) was developed with nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate substrate (NBT/BCIP). Positive double staining appeared as purple coloring. The antibodies used included rat anti-CD4 anti-CD8 anti-CD49d anti-CD106 (VCAM-1) mAb and hamster anti-CD54 (ICAM-1) anti-CD3 mAb (PharMingen).

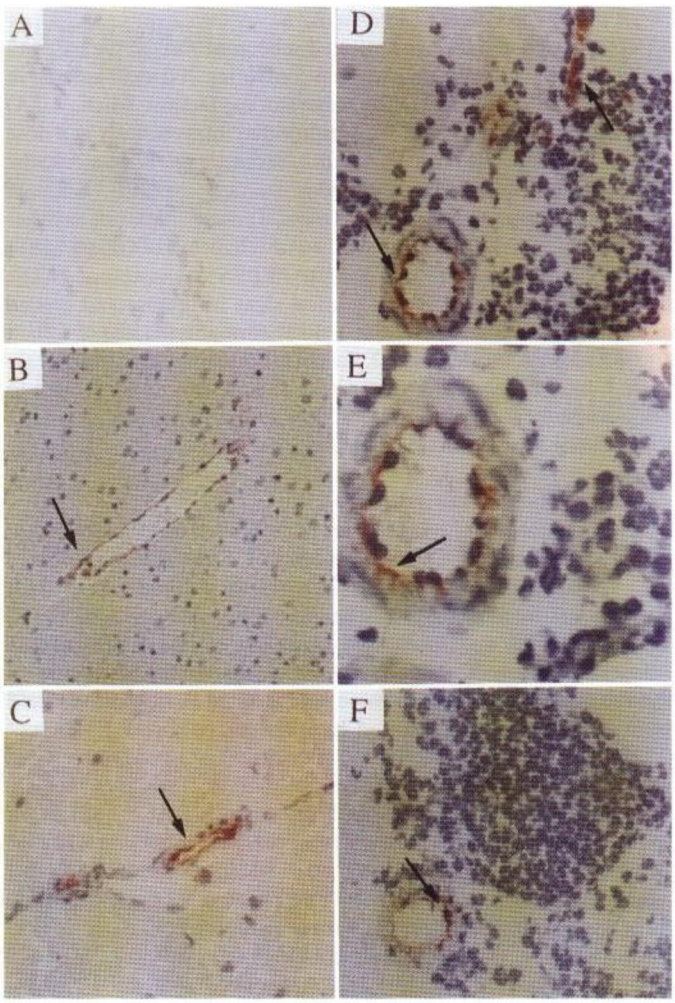

Fig. 1. Expression of ICAM-1 on cerebral endothelial cells in EAE mice. Mice of the B6 strain were immunized subcutaneously with 200 μg of MBP/CFA and *B. pertussis*. Eighteen days after sensitization, the mice were injected i.p. with 200 μg of MBP/CFA. On day 20, 100 μg of MBP in PBS was injected i.c. The mice were sacrificed at various times after i.c. challenge. Naive mice were injected i.c. with PBS alone. Five-micron cryosections were made and stained with rat anti-ICAM-1 (CD54) mAb. Peroxidase-conjugated sheep anti-rat IgG was used as secondary antibody. The peroxidase signal developed with aminoethyl carbazole substrate is indicated by the arrow. **A** naive, i.c. with PBS. × 100. **B** i.p./i.c., 1 h. × 100. **C** i.p./i.c., 3 h. × 200. **D** i.p./i.c., 6 h. × 200. **E** i.p./i.c., 6 h. × 400. **F** i.p./i.c., 12 h. × 200.

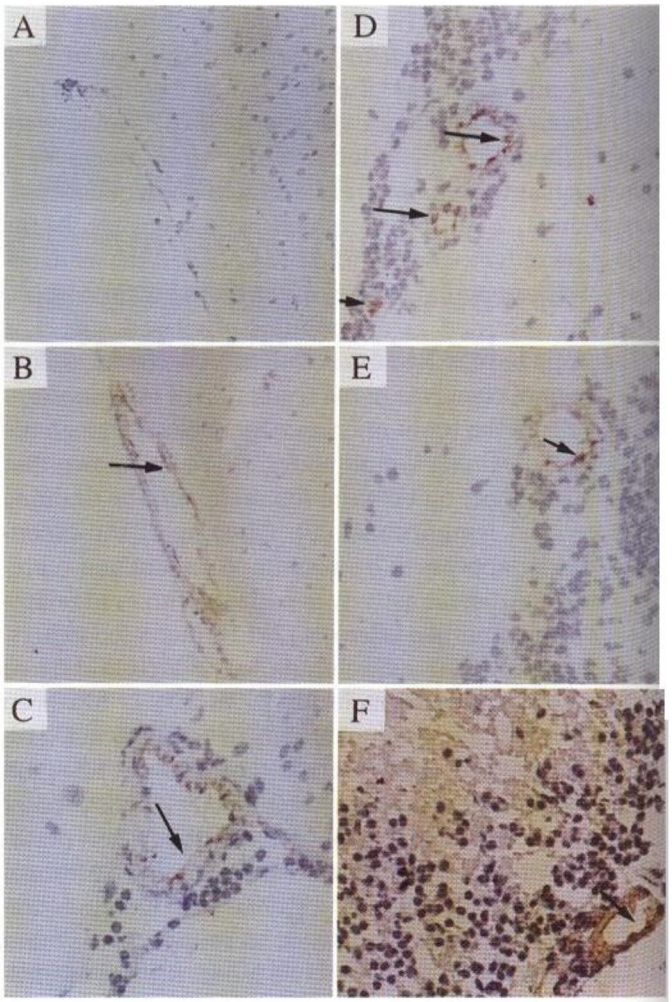

Fig. 2. Expression of VCAM-1 on cerebral endothelial cells in EAE mice. Mice of the B6 strain were immunized subcutaneously with 200 μg of MBP/CFA and *B. pertussis*. Eighteen days after sensitization, the mice were injected i.p. with 200 μg of MBP/CFA. On day 20, 100 μg of MBP in PBS was injected i.c. The mice were sacrificed at various times after i.c. challenge. Naive mice were injected i.c. with PBS alone. Five-micro cryosections were made and stained with rat anti-VCAM-1 (CD106) mAb. Peroxidase-conjugated sheep anti-rat IgG was used as secondary antibody. The peroxidase signal developed with aminoethyl carbazole substrate is indicated by the arrow. **A** naive, i.c. with PBS, 1 h. × 200. **B** i.p./i.c., 1 h. × 200. **C** i.p./i.c., 3 h. × 200. **D** i.p./i.c., 6 h. × 200. **E** i.p./i.c., 12 h. × 200. **F** i.p./i.c., 48 h × 200.

Results

Expression of Adhesion Molecules in the Brains of EAE Mice

The inflammatory cells migrated into the brain via interaction with ICAM-1/VCAM-1 on brain endothelial cells. No ICAM-1 were detected on brains from naive mice injected i.c. with PBS. However, the i.c. injection of MBP induced the expression of ICAM-1. Strong staining was found at 3 h, reached the maximum at 6 h and decreased 12 h after i.c. injection (fig. 1). VCAM-1 were expressed slower and lasted longer than ICAM-1. No VCAM-1 were noted on the brains of naive mice injected i.c. with PBS. The expression of VCAM-1 was

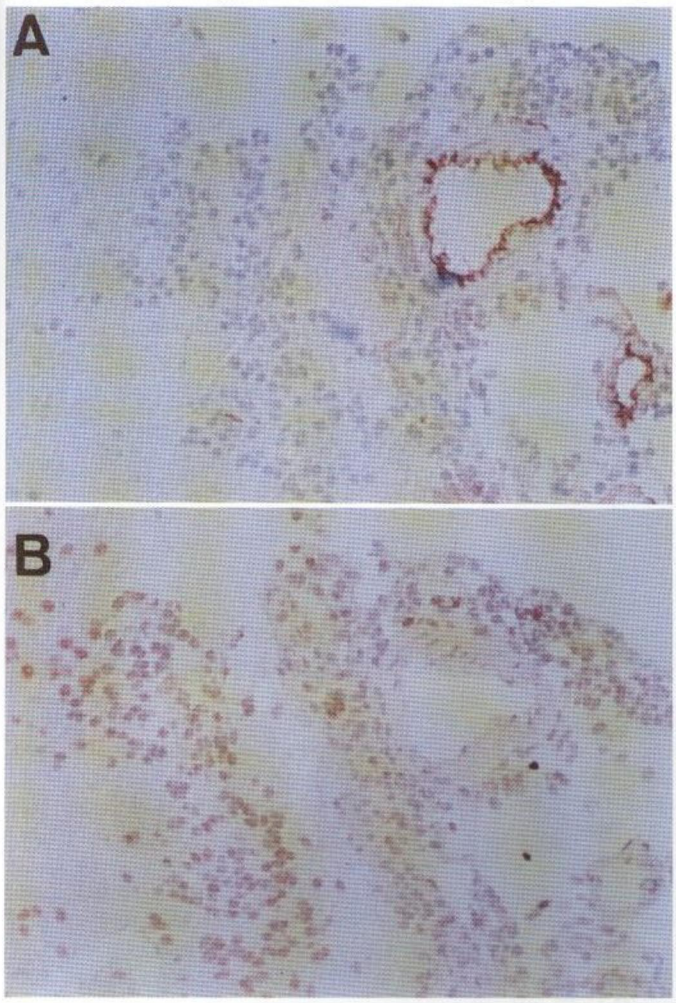

Fig. 3. Expression of VCAM-1 on cerebral endothelial cells and VLA-4 on surrounding mononuclear cells in EAE mice. Mice of the B6 strain were immunized subcutaneously with 200 μg of MBP/CFA and *B. pertussis*. Eighteen days after sensitization, the mice were injected i.p. with 200 μg of MBP/CFA. On day 20, 100 μg of MBP in PBS was injected i.c. The mice were sacrificed 48 h after i.c. challenge. Serial cryosections were made and stained with either rat anti-VCAM-1 (CD106) mAb (**A**) or rat anti-VLA-4 (CD49d) mAb (**B**). Peroxidase-conjugated sheep anti-rat IgG was used as secondary antibody.

induced, gradually increased and persisted till 48 h after the MBP i.c. challenge (fig. 2). The pattern of adhesion molecules was consistent with that of cell infiltration in EAE mice. Neutrophils were prominent at 12 h and were replaced by mononuclear cells 24 h after i.c. injection. The interactions between VCAM-1 on brain microvascular endothelial cells and VLA-4 molecules on surrounding infiltrated mononuclear cells are shown in figure 3. The microvascular endothelial cells were positive for VCAM-1, whereas the surrounding mononuclear cells were VLA-4 positive.

$CD4^-CD8^-V_\beta8^+$ Cells in Cervical Lymph Nodes

Cervical lymphatics drain antigens from the brain and can be modulated by i.c. injection. We have reported a dynamic change of $VLA\text{-}4^+$ cells in cervical lymph nodes after i.c. injection [34]. The kinetic pattern of $CD4^+$ or $CD8^+$ cells are shown in figure 4; the percentage of $CD4^+/CD8^+$ cells was not changed greatly among the cervical and distal lymph nodes after i.c. challenge. However, a unique subpopulation of $CD4^-CD8^-V_\beta8^+$ cells was found in cervical and distal lymph nodes. They constituted 10–15% of total lymph node cells in naive or sensitized mice and increased to 30% 6 h after the i.c. challenge. Then, these cells were gradually depleted to 5% from 12 to 24 h, but increased again at 120 h after i.c. challenge. The changes in the distal lymph nodes were not as obvious as those in the cervical lymph nodes. This population could be expanded in vitro. When these lymph node cells were cultured in vitro with MBP or IL-2 for 24 or 48 h, the percentage of $CD4^-CD8^-V_\beta8^+$ cells increased from 10 to 30% (fig. 5).

Immunohistochemical Stains of $CD4^-CD8^-CD3^+$ Cells in Brain

The transient depletion of $CD4^-CD8^-CD3^+$ cells in cervical lymph nodes suggests that they migrated into the brain after i.c. injection. The cells infiltrated into the brain of EAE mice were stained with primary rat anti-CD4, anti-CD8 and hamster anti-CD3 mAb, followed by sheep anti-rat IgG alkaline phosphatase and goat anti-hamster peroxidase, respectively. Most of the infiltrated mononuclear cells were stained with anti-CD3, but not anti-CD4 or anti-CD8, as shown by the reddish-brown color 24 h after i.c. injection. This indicates that they bore the $CD4^-CD8^-CD3^+$ marker. Very few cells were double stained (purple coloring). These cells were either $CD4^+CD3^+$ or $CD8^+CD3^+$. At 48 h, the number of double-stained $CD4^+CD3^+/CD8^+CD3^+$ lymphocytes increased, but still represented a small percentage of the total infiltrated cells. Ninety-six hours after i.c. challenge, they became the majority of the infiltrated cells (fig. 6). Taken together, these data suggest that the peripheral blood cells were recruited into the brain by VLA-4 and VCAM-1 interaction after i.c. challenge. The early infiltrated lymphocytes were $CD4^-CD8^-CD3^+$.

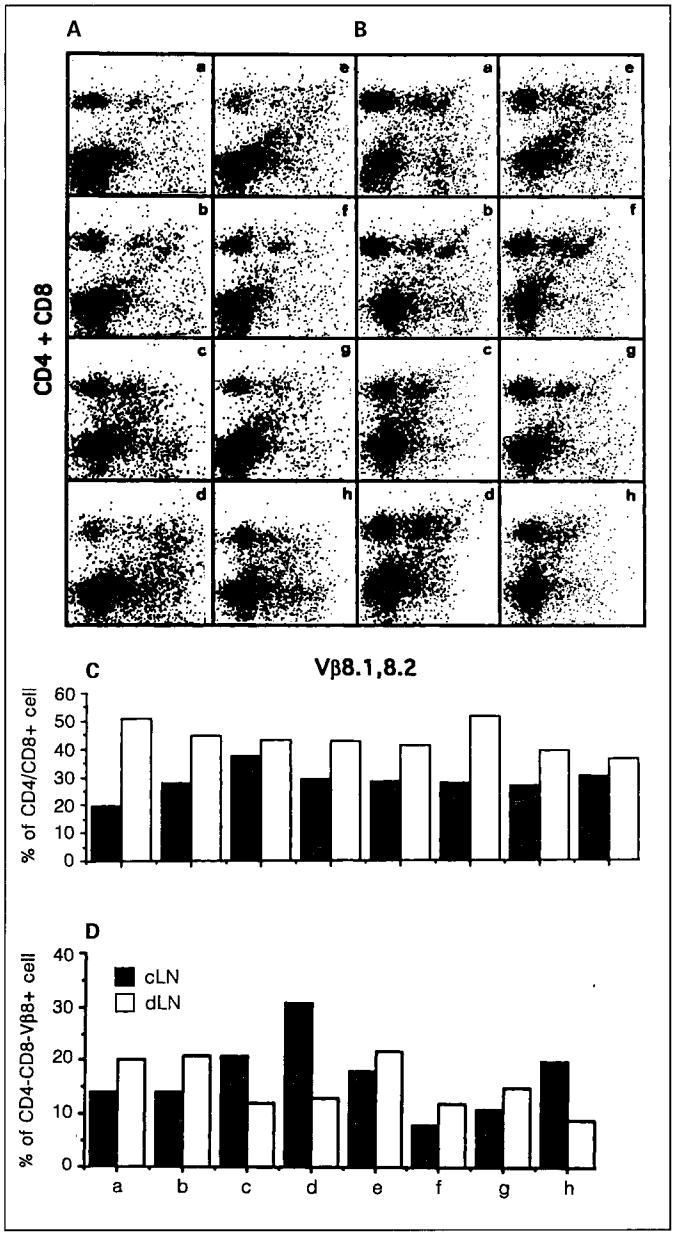

Fig. 4. Kinetic changes of CD4CD8/Vβ8 cells in cervical or distal lymph nodes after EAE induction. Groups of 4 B6 mice were immunized subcutaneously with 200 μg of MBP/CFA and *B. pertussis*. Eighteen days after sensitization, the mice were injected i.p. with 200 μg of MBP/CFA. On day 20, 100 μg of MBP in PBS was injected i.c. The mice were sacrificed at various times after i.c. challenge. The cells of cervical (**A**, ■) or distal lymph nodes (**B**, □) were pooled and stained for CD4CD8/Vβ8 expression with FITC-anti-Vβ8 and PE-anti-CD4/PE-anti-CD8 antibodies as described in 'Materials and Methods'. **a** Naive. **b** Sensitized. **c** i.p. **d** i.p./i.c., 6 h. **e** i.p./i.c., 12 h, **f** i.p./i.c., 24 h. **g** i.p./i.c., 48 h. **h** i.p./i.c., 120 h. **C** Percentages of $CD4^+/CD8^+$ cells. **D** Percentages of $CD4^-CD8^-V_\beta8^+$ cells.

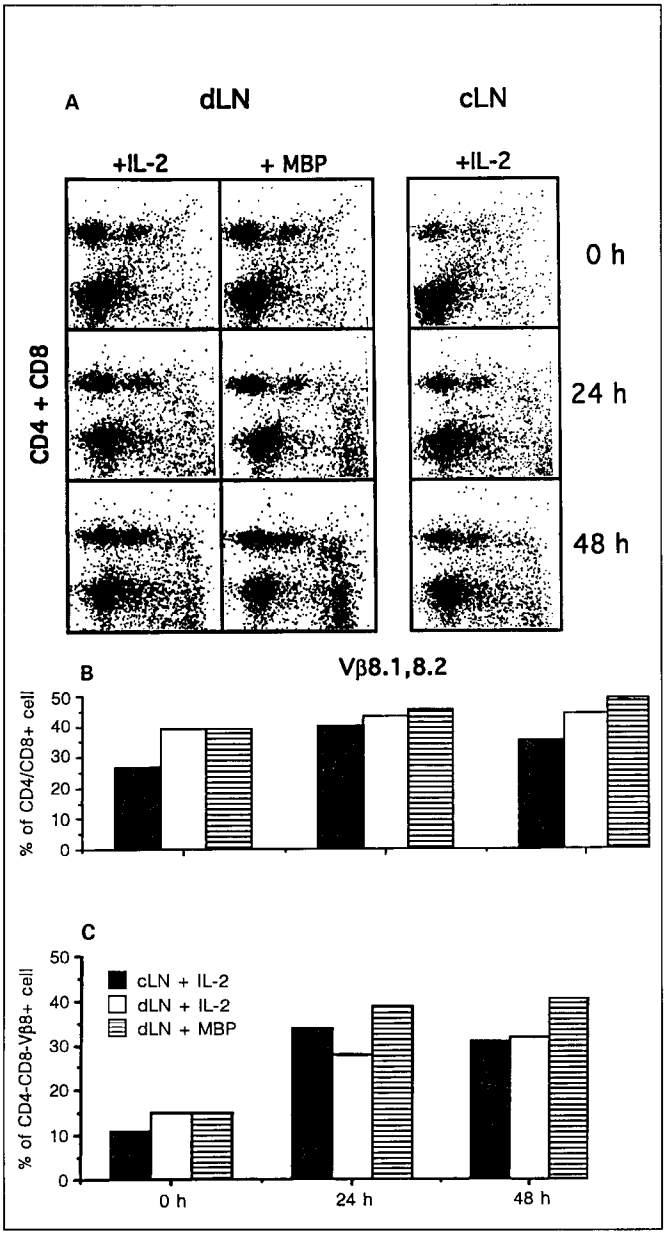

Fig. 5. Expansion of $CD4^-CD8^-V\beta8^+$ cells after in vitro culture. Groups of 4 B6 mice were immunized subcutaneously with 200 μg of MBP/CFA and *B. pertussis*. Eighteen days after sensitization, the mice were injected i.p. with 200 μg of MBP/CFA. On day 20, 100 μg of MBP in PBS was injected i.c. The mice were sacrificed 48 h after i.c. challenge. **A–C** The cells of cervical (cLN, ■) or distal lymph nodes (dLN, □ or ▤) were pooled and cultured with MBP (15 μg/ml) or IL-2 (10 U/ml) for 24 or 48 h. The cells were then stained with FITC-anti-Vβ8 and PE-anti-CD4/PE-anti-CD8 antibodies as described in 'Materials and Methods'. The percentages of $CD4^+/CD8^+$ cells and $CD4^-CD8^-V_\beta8^+$ cells are shown in **B** and **C**, respectively.

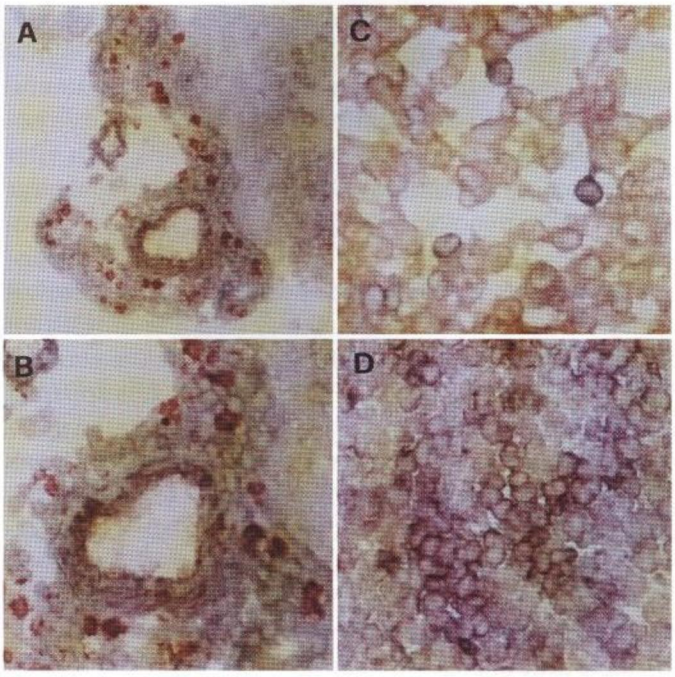

Fig. 6. Infiltration of $CD4^-CD8^-CD3^+$ T cells in brains of EAE mice. Mice of the B6 strain were immunized subcutaneously with 200 μg of MBP/CFA and *B. pertussis*. Eighteen days after sensitization, the mice were injected i.p. with 200 μg of MBP/CFA. On day 20, 100 μg of MBP in PBS was injected i.c. The mice were sacrificed at 24, 48, or 96 h after i.c. challenge. Five-micron cryosections were made and stained with a mixture of hamster anti-CD3, rat anti-CD4 and rat anti-CD8 mAb. Peroxidase-conjugated goat anti-hamster IgG and alkaline-phosphatase-conjugated sheep anti-rat IgG were used as secondary antibodies. The peroxidase signal was developed with aminoethyl carbazole substrate (reddish-brown). The alkaline phosphatase signal was developed with NBT/BCIP substrate (dark blue). Double staining will appear as purple coloring. **A** i.p./i.c., 24 h. × 100. **B** i.p./i.c., 24 h. × 400. **C** i.p./i.c., 48 h. × 400. **D** i.p./i.c., 96 h. × 400.

Discussion

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 are not known yet. It has been postulated that an interaction between the luminal surface of endothelial cells in the CNS and sensitized lymphocytes participates in an early step of the pathogenesis of EAE [19]. Increased expression of ICAM-1 or VCAM-1, which are natural ligands of lymphocyte-function-associated antigen-1 or VLA-4, respectively, has been observed on brain endothelial cells of EAE animals [29, 38]. The expression of these molecules may facilitate lymphocyte migration and extravasation across the blood-brain barrier. Baron et al. [2] reported that surface expression of VLA-4 was important for $CD4^+$ T cell entry into the brain parenchyma. The VLA-4 integrins may be crucial in allowing activated effector T cells to leave the blood and enter the brain. Our staining of ICAM-1 and VCAM-1 on brain endothelial cells and $VLA\text{-}4^+$ molecules on infiltrated lymphocytes in EAE mice confirmed the importance of adhesion molecules in the development of inflammation in EAE mice. The expression of adhesion molecules such as ICAM-1 and VCAM-1 was not due to the trauma caused by the i.c. injection, but rather induced by MBP-associated inflammation and cytokine production [unpubl. obs.]. At the beginning, we used Vβ8 as the marker to characterize T cells in the lymph nodes. However, the mouse mAb we used cannot stain the brain-infiltrated T cells immunohistochemically; we have to switch to the hamster mAb anti-CD3 antibody. The lymphocyte subpopulation in lymph nodes showed the same pattern, no matter whether we used anti-CD3 or anti-TCRαβ antibodies (data not shown). The $CD4^-CD8^-V_\beta8^+$ cells in the lymph nodes are the $CD4^-CD8^-CD3^+$ cells in the brain. The kinetic association between the transient deletion of $CD4^-CD8^-V_\beta8^+$ cells in the cervical lymph nodes and the infiltrated $CD4^-CD8^-CD3^+$ cells in the brain suggests that the infiltrating cells are attracted to the brain from the cervical lymph nodes.

The $TCR_{\alpha\beta}{}^+CD4^-CD8^-$ double-negative T cells, a novel subset of T cells, were reported to be present in normal tissues or in autoimmune diseases of man and mouse [8, 25]. The TCR repertoire of these double-negative T cells was restricted. T cells of this phenotype were immunocompetent and could be activated via the CD3 pathway. In murine splenic and pulmonary $CD4^-CD8^-$ cells, TCR-$V\beta8^+$ genes were preferentially expressed. Some cloned murine splenic $TCR_{\alpha\beta}{}^+CD4^-CD8^-$ T cell lines recognized syngeneic and allogeneic dendritic cells in a major-histocompatibility-complex-unrestricted way [27]. Purified murine splenic $CD4^-CD8^-$ T cells proliferated specifically in response to syngeneic stimulator cells. Splenic $TCR_{\alpha\beta}{}^+CD4^-CD8^-$ T cells from normal mice showed major histocompatibility complex class-II-restricted autoreactivity [26, 31]. Erard et al. [11] reported the switching of $CD8^+$ T cells to noncytolytic $CD4^-CD8^-$ cells and their synthesis of Th2 cytokines. The $TCR_{\alpha\beta}{}^+CD4^-CD8^-$ T cells may represent a unique developmental pathway of a separate lymphoid lineage [3]. Alternatively, they may result from immunoregulatory mechanisms which are particularly prominent in autoimmune conditions.

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 immune response to albumin administered to the cerebrospinal fluid (CSF) has been reported [12, 14]. Outflow of MBP and other antigens from the CNS to peripheral lymphoid organs can occur by at least two pathways as a consequence of the turnover of CSF [4, 39]. First, the antigens can drain into cervical lymph nodes via CSF outflow along certain cranial nerves and into the cervical lymph. Second, the antigens in the CSF can reach the spleen by moving across the arachnoid villi and into the blood. 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. 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 [16, 20, 21]. It has been proposed that elevated concentrations of MBP in the 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 [17, 36].

Histopathological observation of kinetic changes revealed an early neutrophil infiltration 12 h after i.c. injection. The neutrophil accumulation might be a antibody-mediated inflammatory response caused by the i.c. stimulation. Since the injection was given 20 days after immunization, the anti-MBP-antibody immune-complex-associated inflammation developed. Nevertheless, 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 delayed-type hypersensitivity. 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 [32]. It is apparent that EAE induced in resistant B6 mice is due to the intracerebral induction of inflammatory responses that break the blood-brain barrier and 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 on the regulation of activated autoimmune cells.

Acknowledgments

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