

## Tumor Necrosis Factor- $\alpha$ and Transforming Growth Factor- $\beta$ Production by Isolated Mononuclear Cells from the Spinal Cords of Lewis Rats with Experimental Autoimmune Encephalomyelitis

TERUAKI IWAHASHI, CHANG-SUNG KOH, ATSUSHI INOUE  
and NOBUO YANAGISAWA

*Department of Medicine (Neurology), Shinshu University  
School of Medicine, Matsumoto 390*

IWAHASHI, T., KOH, C.-S., INOUE, A. and YANAGISAWA, N. *Tumor Necrosis Factor- $\alpha$  and Transforming Growth Factor- $\beta$  Production by Isolated Mononuclear Cells from the Spinal Cords of Lewis Rats with Experimental Autoimmune Encephalomyelitis.* Tohoku J. Exp. Med., 1997, 183 (2), 123-133 — We demonstrated time course of the number of mononuclear cells (MNCs) isolated from spinal cords (SCs) correlates with the degree of experimental autoimmune encephalomyelitis (EAE) of Lewis rats, and analyzed their tumor necrosis factor (TNF)- $\alpha$  and transforming growth factor (TGF)- $\beta$  production by MNCs, using enzyme-linked immunosorbent assay and enzyme-linked immunospot (ELISPOT) assay. The number of MNCs varied from 5 to  $620 \times 10^4$  per SC of normal Lewis rat and Lewis rat with EAE. MNCs increased and reached a peak on day 2 post clinical onset (Day 2), and subsequently declined through the clinical course. The increase of infiltrating MNCs in SCs paralleled the severity of the disease development. TGF- $\beta$ 1 in plasma of rats with EAE significantly increased on Day 1 and reached the peak on Day 3. TNF- $\alpha$  levels in culture supernatants of MNCs from SCs increased on Day 1, and it decreased from Day 2, and declined on Day 4 when animals began to recover. TGF- $\beta$ 1 was not detected in culture supernatant during the whole clinical course. The number of TNF- $\alpha$  and TGF- $\beta$ 1 producing cells that were detected by ELISPOT assay increased on Day 0, and decreased rapidly after the onset of neurological symptoms. Thus, increase of TNF- $\alpha$  appeared in the early phase of the disease and then promptly decreased. In contrast, TGF- $\beta$ 1 was activated during the later recovering phase of the disease. We consider that TNF- $\alpha$  may play an important role in the pathogenesis of EAE and TGF- $\beta$  may inhibit the development of EAE. ——— experimental autoimmune encephalomyelitis (EAE); multiple sclerosis (MS); tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ); transforming growth factor- $\beta$  (TGF- $\beta$ ); enzyme-linked immunospot (ELISPOT) assay © 1997 Tohoku University Medical Press

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Address for reprints: Chang-Sung Koh, M.D., Ph. D., Department of Medicine (Neurology), Shinshu University School of Medicine, 3-3-1 Asahi, Matsumoto 390, Japan.  
e-mail: kshosei@gipac.shinshu-u.ac.jp

Experimental autoimmune encephalomyelitis (EAE) is an autoimmune disease of the central nervous system (CNS) that can be experimentally induced in susceptible rodents either by sensitization to neural antigens such as myelin basic protein (MBP) or transfer of effector cells, CD4+Th1 cells, sensitized by MBP (Paterson 1978; Ando et al. 1989). EAE in Lewis rats is an acute monophasic paralytic disease, clinical signs appear 10-14 days following immunization with MBP and complete Freund's adjuvant (CFA), and spontaneously recover by day 18 (Paterson 1980; Alvord et al. 1984). The pathology of EAE is characterized by perivascular and meningeal infiltrates of mononuclear cells (MNCs) in brain and spinal cord. EAE provides a model of neuroautoimmune animal disease with clinical and immunohistopathologic similarities to human demyelinating disorders, e.g., multiple sclerosis (MS) (Swanborg et al. 1974). Although clearly EAE is a T cell mediated autoimmune disease, the mechanism through which these T cells injure the CNS has not been established, and the mechanism of recovery from EAE is also yet unresolved. It is important to analyze CNS inflammatory cells quantitatively through the course of EAE.

During the development of EAE, there are increasing evidences that proinflammatory cytokines, such as tumor necrosis factor (TNF)- $\alpha$ , are involved in the early phase of inflammation (Ruddle et al. 1990; Selmaj et al. 1991a; Selmaj and Raine 1995). On the other hand, there are evidences that immunoregulatory cytokines, such as transforming growth factor (TGF)- $\beta$ , relate to the recovery from EAE (Karpus and Swanborg 1991). Because TNF- $\alpha$  and TGF- $\beta$  demonstrate several antagonistic functions in vitro (Ranges et al. 1987; Espevik et al. 1988), TNF- $\alpha$  and TGF- $\beta$  may play important roles in the advance and natural recovery of EAE. But the precise time course of TNF- $\alpha$  and TGF- $\beta$  production in EAE was not clear. We isolated inflammatory MNCs that were infiltrating into SC of rat with EAE, and demonstrated time course of the number of these cells, and also showed these cytokine productivities, especially TNF- $\alpha$  and TGF- $\beta$ , cytokines which set each other off.

## MATERIALS AND METHODS

### *Animals*

Young adult male Lewis rats (200-250 g), obtained from Charles River Co. (Kanagawa), were used in all experiments. The animals were kept in plastic cages containing pine chips, and were given food and water ad libitum.

### *Antigen and induction of EAE*

Guinea pig myelin basic protein (GPMBP) was prepared from guinea pig spinal cords by the method of Swanborg et al. (1974). GPMBP was suspended in phosphate-buffered saline (PBS, pH 7.2) and emulsified in an equal volume of CFA. Each Lewis rat was sensitized in the hind footpads with 25  $\mu$ g of GPMBP emulsified in CFA containing 200  $\mu$ g of *Mycobacterium tuberculosis* (MTB Difco,

Detroit, MI, USA) H37Rv, Jamaican strain.

### *Assessment of clinical severity of EAE*

Rats were observed every 24 hours for neurological signs, which were recorded on a scale of 0 to 3 by using the following grading system: flaccid tonicity of the distal tail, 0.25; lack of tonicity in the entire tail, 0.5; ataxia of gait, 1.0; hind leg paresis, 2.0; and bilateral paralysis of the hind legs, 3.0 as previously described (Koh and Paterson 1987).

### *Designation of study period*

Day 0 was arbitrarily designated as the day when clinical signs were first observed after sensitization. Rats killed 1 day before control recipients first showed clinical signs were designated as Day-1 animals. This designation is workable in Lewis rats because the disease is highly reproducible in this species. Rats were sacrificed on Day-1, Day 0, Day 1, Day 2, Day 3, Day 4 and Day 5. We isolated MNCs from spinal cords as follows.

### *Isolation of inflammatory MNCs from spinal cords of rats with EAE*

Rats were sacrificed under anesthesia. MNCs in the spinal cords were isolated as follows (Irani and Griffin 1991). Each perfused spinal cord was minced gently through a fine mesh screen using a syringe plunger and collected into 10 ml of Hanks' balanced salt solution (HBSS) containing 0.05% collagenase D (Boehringer Mannheim Biochemicals, Indianapolis, IN, USA), 0.1  $\mu$ g/ml of the trypsin inhibitor TLCK (Sigma Chemical Co., St. Louis, MO, USA), 10  $\mu$ g/ml DNAase I (Sigma), and 10 mM Hepes buffer, pH 7.4. The resulting tissue slurry was mixed at room temperature for 60 minutes, and then allowed to settle at unit gravity for 30 minutes. The supernatant was collected, pelleted at  $200 \times g$  for 5 minutes, and resuspended in 10 ml  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HBSS. Five ml of this suspension was carefully layered onto 10 ml a 75% Ficoll-Paque (Pharmacia, Uppsala, Sweden) in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY, USA) in a centrifuge tube. Gradient was centrifuged at  $500 \times g$  for 30 minutes and the overlying media and interface of tissue debris were removed. The entire 10 ml of gradient medium was diluted ten-fold with HBSS and centrifuged at  $300 \times g$  for 10 minutes to pellet the cells. These cells were counted and subsequently used for experiments.

### *In vitro cell culture*

MNCs from spinal cord were adjusted to a concentration of  $1 \times 10^6/\text{ml}$  in a medium and cultured in 96-well flat-bottom microculture plates (Nunc, Roskilde, Denmark) for 48 hours at  $37^\circ\text{C}$  in a humidified 5%  $\text{CO}_2$ . Tissue culture supernatants were collected to determine the presence of secreted TNF- $\alpha$  and TGF- $\beta$ 1 by enzyme-linked immunosorbent assay (ELISA).

*Enzyme-linked immunosorbent assay determination for TNF- $\alpha$  and TGF- $\beta$ 1*

TNF- $\alpha$  and TGF- $\beta$ 1 production by cultured MNCs from spinal cord on Day-1, Day 0, Day 1, Day 2, Day 3 and Day 4 were assayed by ELISA. TNF- $\alpha$  and TGF- $\beta$ 1 in plasma were also measured by ELISA. We used mouse TNF- $\alpha$  ELISA kit, Factor Test mTNF- $\alpha$  (Genzyme Corp., Boston, MA, USA) and TGF- $\beta$ 1 ELISA system (Amarsham, Buckinghamshire, England), as described by the manufacturers. Each system is reacted to rat TNF- $\alpha$  and TGF- $\beta$ 1. Results were read at 492 nm with an ELISA reader (Corona, Hitachi).

*Enzyme-linked immunospot assay for TNF- $\alpha$  and TGF- $\beta$ 1 production*

The original reverse enzyme-linked immunospot (ELISPOT) assay (Czerkinsky et al. 1988; Skidmore et al. 1989) was modified by using nitrocellulose membranes. MNCs collected from spinal cords on Day-1, Day 0, Day 2 and Day 4 were used. Each well of 96-well microculture plates backed with nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) was filled with hamster anti-murine TNF- $\alpha$  mAb (Genzyme) at a concentration of 10  $\mu$ g/ml in 0.5% BSA in PBS or 1:100 diluted chicken anti-human TGF- $\beta$ 1 mAb (King Brewing Co. Ltd., Central Institute [K.B.I, Kakogawa]) at 4°C for overnight. Unabsorbed antibodies were removed and wells were washed with PBS. The plates were then blocked with 1% Blotto (non fat dry milk; Carnation, Los Angeles, CA, USA) for 2 hours at 37°C. The outer surface of the nitrocellulose membrane was carefully dried.  $1 \times 10^5$ /well of MNCs from spinal cords were dispensed in an individual well (100  $\mu$ l/well). Plates were then incubated for 48 hours at 37°C in a humidified 5% CO<sub>2</sub> and washed with PBS-Tween. To each well, 50  $\mu$ l of a 1:250 diluted rabbit anti-mouse TNF- $\alpha$  antibody (Genzyme) or rabbit anti-human TGF- $\beta$  antibody (K.B.I) was added, followed by incubation for 2 hours at 37°C. Plates were washed again and treated with 50  $\mu$ l of 1  $\mu$ g/ml alkaline phosphatase-conjugated goat anti-rabbit IgG (Kirkegaard & Perry Laboratories Inc. [KPL], Gaithersburg, MD, USA) for 2 hours at 37°C. After the plates were washed, each cytokine secreted by single cells was visualized by adding a mixture of nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolphosphate (BCIP) (Gibco). The color reaction of the enzyme was halted after 30 minutes by washing with water and spots were enumerated under  $\times 40$  magnification.

*Statistical analysis*

Statistical analysis was performed for each experiment on day compared to the other days according to Student's *t*-test.

## RESULTS

*Clinical course of EAE*

Clinical neurological signs regularly appeared in rats (Fig. 1). Beginning

with mild signs on Day 0 ( $0.33 \pm 0.12$ ), clinical neurological abnormalities progressively increased to a maximum on Day 3 ( $2.83 \pm 0.18$ ). Thereafter signs abated with rats becoming clinically well.

*Enumeration of mononuclear cells infiltrating spinal cord*

Fig. 2 shows the number of infiltrating MNCs isolated from spinal cord at different time points over the course of EAE, and normal control rats. The number of MNCs varied from 5 to  $620 \times 10^4$  per spinal cord. We could isolate very little number of cells ( $9.4 \pm 3.4 \times 10^4$ ) from spinal cord of normal Lewis rat. A small number of cells ( $38.1 \pm 17.3 \times 10^4$ ) were harvested on Day-1. MNCs increased on the day of clinical onset ( $70.0 \pm 50 \times 10^4$ ) and reached a peak on Day

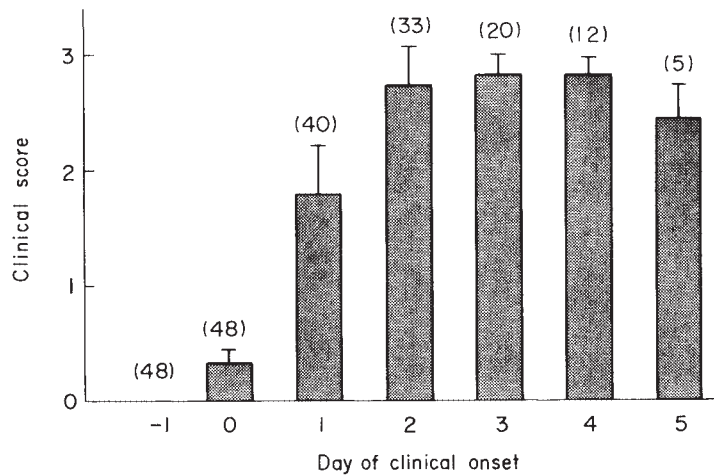


Fig. 1. Time course of severity of neurological signs in Lewis rats with EAE. The number of rats used is shown in the parentheses.

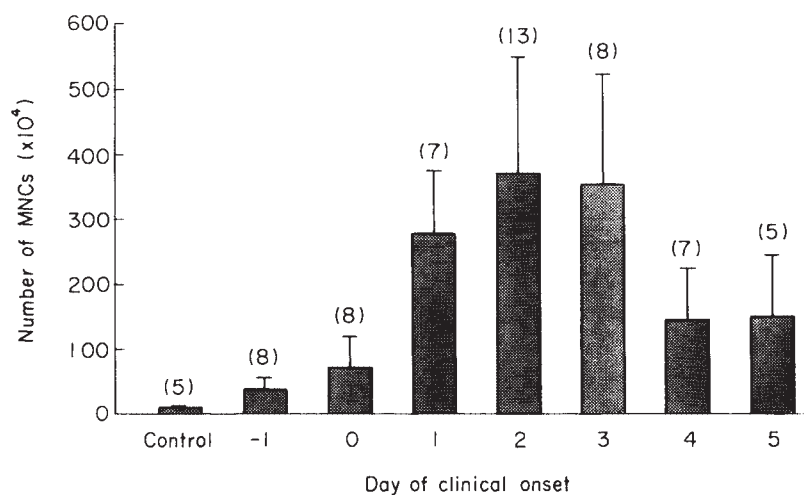


Fig. 2. Time course of the number of MNCs taken from spinal cords of normal Lewis rats and Lewis rats with EAE. Numerals in parentheses represent the number of rats used for determination. Only small numbers of MNCs were detected in normal Lewis rats. The number of MNCs significantly increased in rats with EAE compared with normal control rats. The number of MNCs reached a peak on Day 2, subsequently declined with clinical course.

$2 (370.9 \pm 178.1 \times 10^4)$ , subsequently declined with clinical course to  $149.2 \pm 97.4 \times 10^4$  on Day 5 when animals began to recover.

#### *Assessment of TNF- $\alpha$ and TGF- $\beta$ 1 production during the course of EAE*

TNF- $\alpha$  could not be detected in the plasma throughout the course of EAE (data are not shown). However, TGF- $\beta$ 1 was significantly increased on Day 1 ( $2.58 \pm 1.02$  ng/ml), and reached the peak on Day 3 ( $3.27 \pm 0.45$  ng/ml) (Fig. 3). The time course of TNF- $\alpha$  levels in the supernatants from cultures of MNCs is shown in Fig. 4. TNF- $\alpha$  increased on Day 1 ( $382 \pm 97$  pg/ml), and it decreased on Day 2 ( $255 \pm 85$  pg/ml), and declined to  $14 \pm 9$  pg/ml on Day 4 when animals began to recover. TGF- $\beta$ 1 was not detected during all clinical course (data are not shown).

The number of TNF- $\alpha$  and TGF- $\beta$ 1 producing cells that were detected by

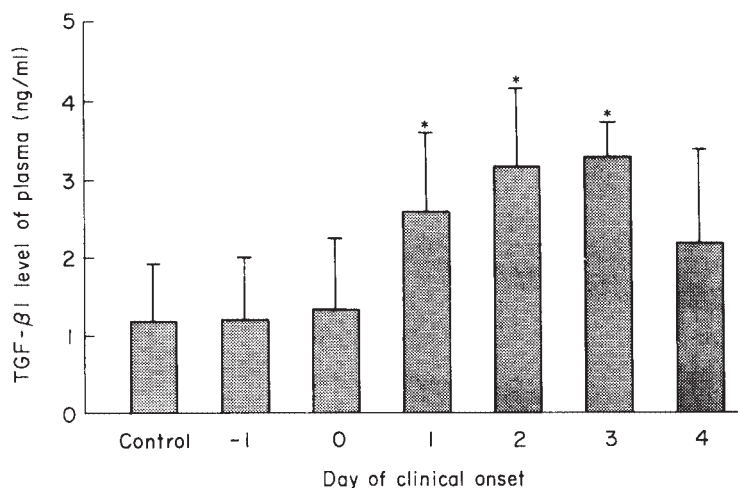


Fig. 3. The plasma levels of TGF- $\beta$ 1 measured by ELISA. TGF- $\beta$ 1 was present in the plasma of Lewis rats with EAE at the height of disease and following recovery (\* $p < 0.05$ ).

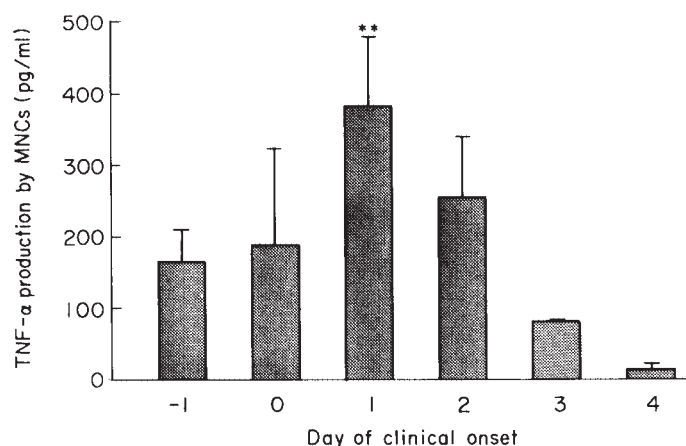


Fig. 4. The levels of TNF- $\alpha$  in the culture supernatants of MNCs from spinal cords, measured by ELISA. The levels of TNF- $\alpha$  significantly increased on Day 1 compared with on Day-1 (\*\* $p < 0.01$ ), and subsequently went down rapidly.

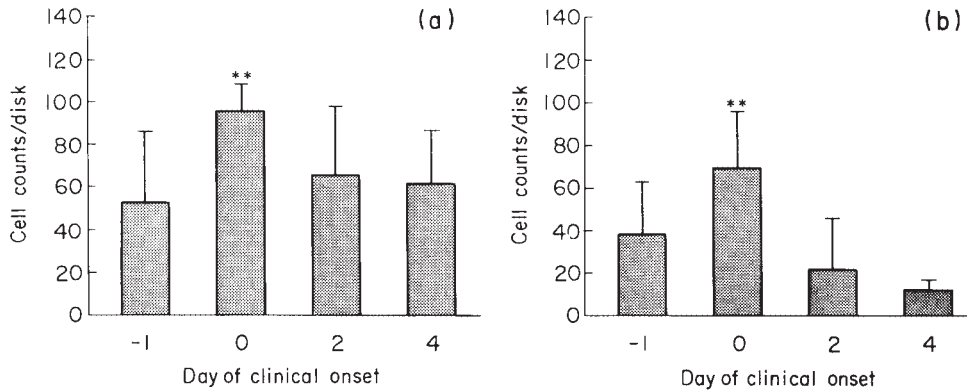


Fig. 5. The levels of TNF- $\alpha$  (a) and TGF- $\beta$ 1 (b) producing cells examined by ELISPOT assay. It was significantly higher in Lewis rats with EAE on Day 0 compared with other days (\*\* $p < 0.01$ ).

ELISPOT assay increased on Day 0, and went down rapidly after the onset of neurological symptoms (Fig. 5).

#### DISCUSSION

EAE in the Lewis rat is characterized morphologically by an infiltration of MNCs around blood vessels in white matter of spinal cord. There is, however, a significant difference between the degree of severity of the disease and histopathology. We showed that the increase of infiltrating cells in the spinal cord of rat with EAE paralleled the severity of the disease development.

We have demonstrated the increase of TNF- $\alpha$  producing cells in the spinal cords of rats with EAE, especially in the early phase of inflammation in EAE. TNF- $\alpha$  is a major mediator of inflammation with a broad spectrum of activity (Durum and Oppenheim 1993), produced largely by activated macrophages as well as certain resident CNS cells such as astrocytes and microglia (Lieberman et al. 1989; Sawada et al. 1989), and it is capable of inducing a delayed-onset lysis of myelin and oligodendrocyte necrosis (Robbins et al. 1987; Selmaj and Raine 1988). Recently, there is an increasing evidence that TNF- $\alpha$  plays an important role for the progression of MS (Selmaj et al. 1991b; Sharief and Hentges 1991). In Theiler's murine encephalomyelitis virus-induced demyelinating disease (TMEV-IDD), another animal model of MS, the level of TNF- $\alpha$  producing cells appears to correlate well with the degree of demyelination, and anti-TNF- $\alpha$  antibody suppresses the development of TMEV-IDD (Inoue et al. 1996). In EAE, TNF- $\alpha$  may play a pivotal role in the autoimmune process and anti-TNF- $\alpha$  antibody treatment abrogates disease progression (Ruddle et al. 1990; Selmaj et al. 1991a; Selmaj and Raine 1995). These findings suggest that TNF- $\alpha$  may play an important role in pathogenicity of CNS inflammation and demyelination. In our study, the number of TNF- $\alpha$  producing cells and TNF- $\alpha$  levels in culture supernatant MNCs in the spinal cords were increased, but TNF- $\alpha$  levels in plasma did not increase through the whole time course of EAE. This suggests that the

increased local production of TNF- $\alpha$  by MNCs plays an important role in the pathogenesis of EAE, such as extravasation of inflammatory cells into CNS across the blood-brain barrier (BBB) and progression of demyelination during the disease. The peak of TNF- $\alpha$  production is on Day 1 by ELISA, but it is on Day 0 by ELISPOT assay. The reason for this difference is not clear, but this difference may be because ELISPOT assay can detect only frequency of cytokine producing cells, and cannot quantity of cytokine production.

We showed that TGF- $\beta$ 1 was present in the plasma of Lewis rats with EAE at the height of disease and following recovery. These data suggest that endogenous TGF- $\beta$  plays a critical role in the natural recovery of EAE. TGF- $\beta$  is a potent immunosuppressive cytokine that suppresses cell mediated immunity as well as humoral immunity, and inhibits the production of most lymphokines and monokines such as IL-1, IL-6 and TNF (Espevik et al. 1987; Chantry et al. 1989). TGF- $\beta$  has been shown to reduce the enhanced binding of lymphocytes to capillary endothelium in the presence of proinflammatory cytokines (Gamble and Vadas 1988, 1991; McCarron et al. 1993), and can thereby reduce the trafficking of lymphocytes into the brain parenchyma. It has been found in the parenchyma, cytoplasm of endothelial cells, and astrocytes (Johns et al. 1992; Racke et al. 1992). In our result, TGF- $\beta$ 1 producing cells were already increased in early phase of EAE, but increase of plasma levels of TGF- $\beta$ 1 was behind. It was not clear why this time lag was observed. One hypothesis is as follows; TGF- $\beta$  is ordinarily secreted by almost all cells as an inactive high molecular mass complex that is not bound to the TGF- $\beta$  receptor (Miyazono et al. 1988; Wakefield et al. 1989). This latent TGF- $\beta$  has been shown to contain the 25 kDa TGF- $\beta$  homodimers in noncovalent association with the amino-terminal region of the TGF- $\beta$  precursor (Wahl et al. 1989). Recent studies suggest that proteases, specifically plasmin, act by cleaving within the amino-terminal region of the TGF- $\beta$  precursor, thereby destabilizing the latent complex and releasing active TGF- $\beta$  (Lyons et al. 1990; Sato et al. 1990). ELISA system we used can detect total TGF- $\beta$ 1 including latent form of TGF- $\beta$ 1 by treatment with acid. But ELISPOT can only detect active TGF- $\beta$ 1 because there was no treatment that change latent form to active form. It was known that some activated cells can also produce active TGF- $\beta$  directly. Our results of ELISPOT and ELISA showed that the number of active TGF- $\beta$  producing cells was increased in the early phase of EAE, but the amount was as little. Most of TGF- $\beta$  was ordinarily secreted as latent TGF- $\beta$ . Latent TGF- $\beta$ , that was secreted by not only infiltrating MNCs but also endothelial cells and astrocytes, may slowly change to active TGF- $\beta$  in inflammatory lesion, by some mechanisms such as local pH change or local plasmin level elevation associated with inflammation (Koh et al. 1990; Sato et al. 1990). These findings suggested TGF- $\beta$  at the time of CNS inflammation contributes to subsequent remission seen in EAE. Increase of active TGF- $\beta$  in late phase of EAE may reduce the binding of EAE effector cells,

down-regulate TNF- $\alpha$  production, thus reduce BBB permeability changes and cause remission.

In conclusion, TNF- $\alpha$  may play an important role in the pathogenesis of EAE and TGF- $\beta$  may inhibit the development of T cells that mediate EAE, at least in part, by inhibiting TNF- $\alpha$  production (Ranges et al. 1987), and we consider that the approach we employed offered more sophisticated and quantitative analysis of CNS inflammatory cells which is unobtainable by tissue section staining, and that it is effective measures to make clear the pathogenesis of EAE.

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