The level of tumor necrosis factor-α producing cells in the spinal cord correlates with the degree of Theiler’s murine encephalomyelitis virus-induced demyelinating disease

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Abstract
The levels of tumor necrosis factor (TNF)-α producing cells were analyzed in mice with Theiler’s murine encephalomyelitis virus-induced demyelinating disease (TMEV-IDD). Using an ELISPOT assay, we demonstrate an increase in TNF-α producing cells in the spinal cords of TMEV-infected SJL/J mice, especially at an active disease stage. The numbers of TNF-α producing cells were extremely high in susceptible SJL/J mice compared with the numbers in resistant BALB/c and C57BL/6 mice. TNF-α producing cells were also immunohistochemically identified in active lesions of TMEV-IDD at acute as well as chronic stages. The percentage of TNF-α producing cells compared with the total number of cells isolated from spinal cords was higher in TMEV-infected SJL/J mice than resistant BALB/c and C57BL/6 mice. Correspondingly, the level of TNF-α was much higher in the culture supernatants of both infiltrating cells in the spinal cords and spleen cells from clinically affected animals than that from similarly treated resistant mice. Treatment of virus-infected mice with a mAb specific for TNF-α at the beginning of the onset of disease suppressed the development of the demyelinating disease. These findings suggest that TNF-α may play an important role in the pathogenicity of TMEV-IDD.

Introduction
Theiler’s murine encephalomyelitis virus (TMEV) is a naturally occurring enteric pathogen of mice and belongs to the picornavirus family (1,2). Either the BeAn 8386 or DA strain of TMEV induces chronic, immune-mediated demyelination when intracerebrally (i.c.) inoculated into susceptible mouse strains (3–5). The clinical signs of TMEV-induced demyelination include a spastic waddling gait, extensor spasms and incontinence (6,7). The histopathology of the virally induced demyelination consists of mononuclear cell infiltration and myelin sheath damage limited to the white matter (3,6,8). An immune response has been demonstrated to be the cause of TMEV-induced demyelination (9–11). In addition, many immunological and genetic parameters associated with susceptibility to this disease parallel those of human multiple sclerosis (MS) (4,5,9,12–17) and thus this system is considered to be one of the best infectious animal models for MS.
Tumor necrosis factor (TNF-α) is one of the major pro-inflammatory cytokines produced mainly from activated macrophages as well as certain resident CNS cells such as astrocytes and microglia (18,19). This cytokine has a broad spectrum of biological activities on many different target cells (20). Several immunologically important molecules are up-regulated by TNF: expression of MHC class I as well as class II antigens is enhanced on various cell types including endothelial cells (21,22). In addition, TNF-α induces intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and endothelial leukocyte adhesion molecule-1 (ELAM-1) which are involved in binding T cells to endothelial cells and antigen-presenting cells (23,24).

Recently, there is increasing evidence that TNF-α plays an important role in the progression of MS (25). TNF-α is identified in acute and chronic MS lesions (26). TNF-α may have a direct cytotoxic effect on oligodendrocytes in culture and induce a delayed onset degradation of myelin in organotypic cultures of nervous system (27). In experimental autoimmune encephalomyelitis, another animal model of MS, TNF-α may play a pivotal role in the autoimmune process and anti-TNF-α antibody treatment abrogates autoimmune demyelination (28-30). Thus, this cytokine may play an important role in the pathogenesis of demyelination. Therefore, in this study, we have focused on the role of TNF-α in the pathogenesis of TMEV-induced myelinating disease (TMEV-IDD). We demonstrate that the level of TNF-α producing cells appears to correlate well with the degree of demyelination during disease progression. Furthermore, suppression of the development of TMEV-IDD by anti-TNF-α antibody suggests the importance of TNF-α in the pathogenesis of viral demyelination.

Methods

Animals

Female SJL/J pathogen-free mice from Jackson Laboratories (Bar Harbor, ME) were maintained and bred at Gokita Breeding Co. (Tokyo, Japan). Inbred mouse strains (C57BL/6, BALB/c and SJL/J) were purchased from either Japan SLC (Shizuoka, Japan) or Gokita Breeding Co. Mice were housed and cared for in a federally approved facility in accordance with National Institutes of Health guidelines.

Virus

The BeAn 8386 strain of TMEV was propagated in BHK (Baby hamster kidney)-21 cells grown in DMEM supplemented with 7.5% donor calf serum and purified by isopycnic centrifugation on Cs2SO4 gradients as previously described (31).

Injection of mice with TMEV

The 6- to 8-week-old SJL/J, BALB/c and C57BL/6 mice (18 mice respectively) were anesthetized with methoxyflurane and inoculated in the right cerebral hemisphere with 1.3×10^6 p.i.u. of virus in 30 μl. This inoculum consistently induced neurological signs in susceptible mouse strains (32). Age-matched SJL/J, BALB/c, and C57BL/6 (18 mice respectively) were used as controls; they received 30 μl DMEM and were subjected to essentially identical manipulation. Mice were divided into three groups and killed on day 40, 90 or 120 after i.c. infection.

Assessment of demyelinating disease

Clinical symptoms of demyelination in TMEV-infected mice were examined daily by observation. Mice were allowed to walk on a polyethylene (Dynalab) walking board and observed for symptoms which included a waddling gait, extensor spasms, paralysis, loss of the righting reflex, incontinence and/or hunched posture.

Immunohistochemical study

Mice were sacrificed under anesthesia on day 40, 90 or 120 after i.c. inoculation. Animals were bled by retroorbital puncture and sera were obtained by centrifugation after clotting. To evaluate the localization of TNF-α producing cells within spinal cord tissue, a qualitative detection of endogenous TNF-α was carried out on cryostat sections using immunohistochemical staining. Fresh spinal cord tissues were embedded in Tissue-Tek OCT compound (Lab-Tek Products Division, Miles Laboratories, Naperville IL), snap-frozen in cold isopentane and stored at −85°C until used. Frozen spinal cord sections were cut longitudinally into 4 μm sections using a cryostat and fixed in cold acetone. Immunohistochemical staining was carried out by streptavidin–biotin system using the Zymed Histostain-DS kit (Zymed, San Francisco, CA). The primary antibody was 1:250 diluted rabbit anti-mouse TNF-α antibody (Genzyme). The procedures were then followed according to the manufacturer’s protocol. A specimen from normal mouse spinal cord was used for negative control.

Isolation of spinal cord infiltrating mononuclear cells

Mononuclear cells in the spinal cords after virus infection were isolated as follows. In order to reduce the possibility of contamination by peripheral blood lymphocytes, mice were anesthetized with methoxyflurane and perfused with 30–50 ml PBS. Spinal cords were minced gently through a fine mesh screen using a syringe plunger and collected into 1 ml of Hank’s balanced salt solution (HBSS) containing 0.05% collagenase D (Boehringer Mannheim, Indianapolis, IN), 0.1 μl/ml of the trypsin inhibitor TLCK (Sigma, St Louis, MO), 10 μl/ml DNase I (Sigma) and 10 mM HEPES buffer, pH 7.4. The resulting tissue slurry was mixed at room temperature for 60 min and then allowed to settle at unit gravity for 30 min. The supernatant was collected, pelleted at 200 g for 5 min and resuspended in 10 ml Ca2+ /Mg2+-free HBSS. Then, 5 ml of this suspension was carefully layered onto 10 ml of 75% Ficoll-Paque (Pharmacia, Sweden) in RPMI 1640 medium ( Gibco, Grand Island, NY) in a centrifuge tube. The gradient was centrifuged at 500 g for 30 min and the overlying media and interface of tissue debris were removed. The entire 10 ml of gradient medium was diluted 10-fold with HBSS and centrifuged at 300 g for 10 min to pellet the cells. These cells were counted and subsequently used for experiments (33).
brane (Bio-Rad) were filled (50 μl/well) with hamster anti-murine TNF-α mAb (Genzyme) at a concentration of 10 μg/ml in 0.5% BSA in PBS by overnight at 4°C. Unabsorbed antibodies were removed and wells were washed with PBS. The plates were then blocked with 1% BLOTTO (non-fat dry milk) for 2 h at 37°C. The outer surface of the nitrocellulose membrane was carefully dried. Mononuclear cells (1×10⁶ well) from spleen or spinal cord in the same culture medium were dispensed in individual wells (100 μl/well). Plates were then incubated for 24 h at 37°C in a humidified 5% CO₂ atmosphere and washed with PBS-Tween. To each well, 50 μl of a 1:250 diluted rabbit anti-mouse TNF-α antibody (Genzyme) was added, followed by incubation for 2 h at 37°C. Plates were washed again and treated with 50 μl of 1 mg/ml alkaline phosphatase-conjugated goat anti-rabbit IgG (KPL) for 2 h at 37°C. After the plates were washed, the TNF-α secreted by single cells was visualized by adding a mixture of nitro-blue tetrazolium and 5-bromo-4-chloro-3-indol phosphate (Gibco). The color reaction of the enzyme was halted after 30 min by washing with water and spots were enumerated under ×40 magnification.

Treatment of mice with anti-TNF-α antibody

A neutralizing anti-mouse TNF-α mAb (MP6-XT22) was obtained from PharMingen (San Diego, CA). MP6-XT22 is a rat IgG1 antibody specific for mouse TNF-α. MP6-XT22 were injected i.p. into mice (10 mice) on days 14, 19, 21, 23, 27 and 29 at a dosage of 100 μg/mouse each time after i.c. infection of TMEV. Control animals (10 mice) received rat IgG1 antibody with unknown specificity (Zymed) i.p. at similar time intervals and were subjected to essentially identical manipulation. At 55 days post-inoculation, three mice randomly selected from each experiment were anesthetized and killed by perfusion through the left ventricle with PBS (pH 7.3), followed by chilled 3% glutaraldehyde in phosphate buffer (pH 7.3). Spinal cords were dissected from the vertebral canal, sectioned at 1 mm intervals, post-fixed in 1% osmic acid and processed for Epon embedding. Ten 1 μm thick sections from each spinal cord were stained with Toluidine blue and examined by light microscopy.

Statistical analysis

The significance of the differences between experimental animal groups with various treatments was analyzed based on the paired t-tests to calculate the two-tailed P value by using the Program (Software, San Diego, CA). The significance of the differences between the experimental (marked with asterisks) and control groups was determined by using the Fisher's exact 2×2 test.

Results

Clinical course of disease and histology

All susceptible SJL/J mice and 10% of BALB/c mice displayed clinical signs of demyelinating disease following viral inoculation. The mean day of onset of the disease was 40 days after i.c. injection with TMEV, which is typical in susceptible SJL/J mice (29). Either C57BL/6 mice inoculated with TMEV or control mice similarly injected with DMEM showed no clinical signs. Representative TMEV-infected mice (both with and without clinical signs) were selected from each group for histological examination; all SJL/J mice and BALB/c mice which exhibited clinical signs of demyelination showed the characteristic mononuclear cell infiltration and demyelination, ranging from mild to severe. Pronounced leptomeningeal and perivascular infiltrates were noted in the spinal cords of TMEV-infected SJL/J mice. The infiltrates were composed of mononuclear cells. Extensive areas of demyelination were present in the vicinity of inflammatory cell infiltrates. The degree of inflammatory demyelination was less in BALB/c mice with clinical disease. All C57BL/6 mice and BALB/c mice with no clinical signs of the disease displayed no histological changes. Control mice which were i.c. injected with only DMEM remained clinically well with normal histology (data not shown).

Enumeration of CNS-infiltrating mononuclear cells

The numbers of mononuclear cells taken from spinal cords of susceptible SJL/J mice and resistant C57BL/6 as well as BALB/c mice were assessed at 40, 90 and 120 days after viral infection (Fig. 1). The cell number was the largest in SJL/J mice at 40 days which showed an active demyelinating disease ("P < 0.001). The number was then decreased after 90 and 120 days, when mice were in the late stages of the disease. Only small numbers of mononuclear cells were detected in resistant BALB/c and C57BL/6 mice throughout the time period.
Assessments of TNF-α producing cells during the course of TMEV-IDD

The levels of TNF-α producing cells in the spinal cords in SJL/J, BALB/c and C57BL/6 mice following TMEV infection were examined using the ELISPOT method. The number of TNF-α producing cells was significantly higher in SJL/J mice 40 days post viral infection (*P < 0.01) than in resistant C57BL/6 and BALB/c mice. In BALB/c and C57BL/6 mice the number of cells taken from spinal cord were so small that we could not describe the exact differences between cell types.

Abrogation of TMEV-IDD by administration of anti-TNF-α antibody

In order to assess the potential role of TNF in the pathogenesis of demyelination induced by TMEV infection, mAb to TNF-α were administered multiple times (2-5 days intervals for six times) into susceptible SJL/J mice infected with virus. Administration of anti-TNF-α antibody suppressed the disease, while a characteristic TMEV-IDD course was found in the control mice that similarly received non-specific rat IgG1 (Fig. 4). The frequency of prevention was 90%. Spinal cord sections from non-specific rat IgG1-treated mice with full clinical signs showed leptomeningeal and parenchymal spinal cord mononuclear cell infiltrates surrounded by numerous demyelinated axons (Fig. 5A). Spinal cord sections from TNF-α-treated mice without clinical signs were typically devoid of inflammatory lesions and demyelination (Fig. 5B).

Discussion

We have demonstrated the increase of TNF-α producing cells in the spinal cords of TMEV-infected SJL/J mice, especially in the active stage of TMEV-IDD. Using an ELISPOT assay, we could identify and enumerate the cells producing TNF-α. The number of TNF-α producing cells was extremely high in spinal cords of SJL/J mice 40 days after viral infection, 80% were monocytes/macrophages. In BALB/c and C57BL/6 mice the number of cells taken from spinal cord were so small that we could not describe the exact differences between cell types.
Fig. 3. Immunohistochemical study. The TNF-α producing cells were identified in the spinal cord section using the streptavidin-biotin system. (A) TNF-α producing cells (arrow) are identified among the mononuclear cell infiltration around the small vessel (V) in the spinal cord of the TMEV-infected SJL/J mice. (B) No TNF-α producing cells were detected in the spinal cord of the resistant C57BL/6 mice. ×800. We could not detect any TNF-α producing cells in the spinal cord of resistant BALB/c mice (Data not shown).

Fig. 4. Abrogation of TMEV-IDD by administration with anti-TNF-α antibody. A neutralizing anti-mouse TNF-α mAb (MP6-XT22) was injected i.p. into mice (10 mice) on days 14, 19, 21, 23, 27, and 29 at a dosage of 100 mg/mouse each time after i.c. infection of TMEV. Control animals (10 mice) received rat IgG1 antibody with unknown specificity (Zymed) i.p. at similar time intervals and were subjected to essentially identical manipulation. In this experiment, 90% of mice that received anti-TNF-α antibody were free of disease, whereas the control mice that received non-specific rat IgG1 showed a characteristic course of the disease. These results showed that the administration of anti-TNF-α antibody suppressed TMEV-IDD.

Response that resistant mice cleared TMEV after infection immediately in the periphery and no inflammation occurred in CNS, although susceptible mice were immunologically activated due to TMEV-IDD both in spleen and CNS for a long time. Taken together, the number of TNF-α producing cells in the spinal cord appears to be correlated with the clinical signs.

Although the precise mechanism of demyelination is not clear in TMEV-IDD, there is much evidence that immune mechanisms play an important role in the pathogenesis. The delayed-type hypersensitivity (DTH) reaction against TMEV antigen correlates with the clinical severity (10). In addition, antibody reaction against the viral capsid protein of TMEV may also be very important in the disease course (36). Histologically, mononuclear cell infiltration has been observed in and around the spinal cord vessel, and the myelin lamellae appear to be stripped by activated macrophages in the active stage of TMEV-IDD (3,8). We have confirmed the findings described by Lindsley et al. (37) that an increased number of spinal cord infiltrating cells correlated with clinical severity of TMEV-IDD.

After i.c. inoculation with TMEV, an initial viremia is followed by a persistent, low-level CNS infection for the life time of the susceptible animal (38). TMEV infects neurons and glial cells in the spinal cords. Neurons sustain high levels of RNA synthesis and are infected only during early disease. Through
out the infection, viral RNA is present in small amounts in glial cells of the white matter of the spinal cord (39). Infected macrophages can be detected at any time during the persistent phase of the infection (40). Antigen presentation may be mediated by either macrophages or glial cells, which contain TMEV antigens. As a consequence of the immunological process, MHC class II-restricted TMEV-specific DTH T cells may be activated, which in turn participate in immune-mediated inflammatory responses. It has been proposed that virus-specific DTH results in the recruitment and accumulation of macrophages in the CNS, producing demyelination via a non-specific 'bystander' mechanism (10) TNF-α is produced
by activated macrophages and by many other cell types including T cells after stimulation with various exogenous or endogenous agents (41). These monocytes/macrophages that entered from circulation to the CNS through the blood-brain barrier may secrete TNF-α and injure the oligodendrocyte. Thus, monocytes/macrophages are likely to play an important role in the viral pathogenesis and produce the majority of TNF-α, although several investigators have reported that significant levels of TNF-α are produced by stimulated astrocytes (42). TNF-α has a broad spectrum of biological actions on many target cells. It involves multiple stimulatory activities on T cells, including enhancement of proliferative response to antigen, increase in IL-2 receptor expression and induction of IFN-γ production (43,44). TNF induces MHC class I antigens on endothelial cells and synergizes with IFN-γ to induce MHC class II (23,44). It also induces ICAM-1 (25), VCAM-1 and ELAM-1 (26) on endothelial cells. These cell adhesion molecules are involved in binding T cells to endothelial cells and antigen-presenting cells. Furthermore, TNF-α is capable of inducing selective pathological changes in myelinated fibers and oligodendrocytes in vitro (27).

The administration of anti-TNF-α antibody might block these various activities of TNF-α. Our results, that anti-TNF-α antibody suppressed TMEV-IDD, suggest that TNF-α could also play a significant role in the pathogenesis of TMEV-IDD. Our present study showed the increased number of TNF-α producing cells in the spinal cord of active TMEV-IDD mice. The correlation of the TNF-α producing cell number and clinical severity suggests an important role of TNF-α in TMEV-IDD. However, the precise mechanism of myelin damage by TNF-α is not clear, although TNF has demonstrated a toxic effect on oligodendrocytes (27). It is of interest that TNF-α has a protective effect on TMEV-IDD when given prior to viral infection (46). Thus, TNF-α at different stages of inflammation may function differently, i.e. regulatory effect prior to inflammatory response and exacerbating effect after establishment of an initial inflammatory response. This concept is consistent with the previous observation (47) that whether administration of TNF-α inhibits or accelerates viral meningitis depends on the severity of inflammation at the time of administration. However, the level of TNF-α is significantly enhanced during the course of TMEV-IDD as shown in our present study. Our data suggest that this cytokine is most likely involved in the pathogenesis rather than protection of virally induced inflammatory demyelinating disease. Furthermore, the administration of anti-TNF-α antibody even after an initial viral infection and the development of virus-specific immunity can exert a profound protection from the development of virus-induced demyelinating disease. These results strongly suggest that the increased production of TNF-α in the CNS plays an important role in TMEV-IDD. These results are in contrast to those obtained with anti-IFN-γ antibody treatment which results in acceleration of TMEV-IDD (48). Therefore, our present study indicates that control of TNF-α levels may provide an effective means to treat patients with similar CNS inflammatory disease.

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