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# Endogenous gamma interferon produced in central nervous system by systemic infection with Theiler's virus in mice

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## Summary

Theiler's virus GD VII strain causes acute encephalomyelitis by intracerebral inoculation. We established acute encephalomyelitis in mice by the intravenous (i.v.) inoculation of Theiler's virus GD VII strain. Replication of Theiler's virus injected i.v. could be observed in both the brain and spinal cord of mice, and interferon (IFN)-γ could be detected in the extracts of brain and spinal cord in parallel with viral replication. Furthermore, by the injection of anti-IFN-γ monoclonal antibody (mAb) on Day 1 post-infection (p.i.), mortality and virus titres in the spinal cord increased compared with the control mice treated with normal rat globulin. The histological exacerbation of inflammation was observed in spinal cord of anti-IFN-γ mAb-treated mice. These results indicate that endogenous IFN-γ, produced locally in the brain and spinal cord of mice through both antiviral action and anti-inflammatory action of IFN-γ in central nervous system, plays an important role in Theiler's virus infection.

## Introduction

Theiler's virus is a cardiovirus which causes enteric and neurological diseases in mice (Theiler and Gard, 1940; Pevear et al., 1987). There is a close relationship between Theiler's virus and human enteroviruses, in terms of structural genetic relatedness (Robart et al., 1988) and comparable biological behavior (Theiler and Gard, 1940). The target organ for Theiler's virus is the central nervous system (CNS), similar to the human enteroviruses (Theiler and Gard, 1940).

There are many strains of Theiler's virus isolated from mice. They are divided into two subgroups on the basis of differences in biological behavior (Lipton, 1975; Lorch et al., 1981), antigenicity (Nitayaphan et al., 1985), and RNase T oligonucleotide two-dimensional maps (Lorch et al., 1981). Strains belonging to the subgroup GD VII (GD VII and FA strains) produce an acute neural infection in mice with neither demyelination nor virus persistence (Lipton, 1980). Strains in subgroup TO (TO and DA strains), produce a biphasic disease consisting of poliomyelitis occurring within a few days post-infection (p.i.), followed by persistent infection with chronic paralysis and demyelination manifestation after a latency of several weeks (Friedman and Lorch, 1985). The acute phase of infection caused by both subgroups is characterized by the replication of the virus in CNS gray matter, causing disease resembling poliomyelitis (Lipton, 1980). In the acute neural infection by Theiler's virus, cellular immune mechanisms might be involved in the protection of mice. Administration of cyclophosphamide, anti-L3T4 serum, and other inhibitors of immune function (e.g. silica quartz dust, protease inhibitors) to Theiler's virus TO strain-infected mice resulted in severe acute encephalomyelitis and high mortality (Lipton and Dal Canto, 1977; Rodriguez and Quddus, 1986; Welsh et al., 1987; Rodriguez and Sriram, 1988; Paya et al., 1989). Gamma interferon (IFN-γ) has been postulated to be an important cytokine which modulates immune effector cells during CNS inflammation. For example, endogenous IFN-γ was detected in the cerebrospinal

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fluid of experimental viral inflammation (Frei et al., 1988), and acute aseptic meningitis (Abbott et al., 1987). Therefore, it is possible that IFN-γ might play an important role in the protection against Theiler's virus infection.

The aim of this study was to detect endogenous IFN-γ produced locally in the infected tissues of mice during Theiler's virus infection and to demonstrate the significance of endogenous IFN-γ in host defence against acute encephalomyelitis by administration of anti-IFN-γ monoclonal antibody (mAb).

## Material and methods

*Mice*

Female ddY mice (4 weeks old) were obtained from SLC (Hamamatsu, Shizuoka, Japan).

*Cells and virus*

Baby hamster kidney cells (BHK-21-P1436) were grown in RPMI 1640 medium containing 5% fetal calf serum (FCS, Gibco Laboratories, Grand Island, NY) at 37°C in a 5% $CO_2$ atmosphere. Theiler's virus GD VII strain was multiplied on BHK-21 cell monolayers in a serum-free RPMI 1640 medium. The whole cultures were frozen and thawed twice, and then centrifuged at $500 \times g$ for 30 min to obtain the supernatant fluids. The supernatant fluids were sterilized by filtration through a 0.2-mm pore size membrane filter (Gelman Science, Inc., Ann Arbor, MI). The filtrate containing $5 \times 10^8$ PFU/ml of Theiler's virus was dispensed and stored at −70°C as the virus stock. BHK-21 cells and Theiler's virus GD VII strain were provided by Dr. Hiroshi Sato of the Institute for Animal Experiment, Nagasaki University School of Medicine, Nagasaki, Japan.

*Plaque assay*

Theiler's virus was quantitated by plaque assay on BHK-21 cells. The organs of Theiler's virus-infected mice were removed aseptically and homogenized in RPMI 1640 medium with a Dounce tissue grinder. The homogenate of the brain was 30% (w/v) of the brain tissue, and the homogenate of the spinal cord was 10% (w/v) of the spinal cord tissue. The resulting tissue homogenates were frozen and thawed twice and clarified by centrifugation at $500 \times g$ for 30 min. 0.3 ml of diluted tissue homogenates was added to the monolayer of BHK-21 cells in each 60-mm plastic dish (Becton Dickinson, Oxnard, CA) and allowed to adsorb BHK-cells for 1 h at 37°C. Subsequently, the cells were overlayed with 1% agar noble (Difco Laboratories, Detroit, MI) in an RPMI 1640 medium containing 5% FCS. After incubation of these dishes for 4 days at 37°C, 0.04% Neutral red was added to the nutrient agar, and after 4 h plaques were enumerated.

*Antibodies*

Rat $IgG_1$ (mAb) against the purified preparation of mouse natural IFN-γ, which had been prepared from the ascites fluid in Pristane-primed CD-1 nude mice injected with hybridoma R4-6A2 (Nakane et al., 1989), was purified by $(NH_4)_2SO_4$ precipitation. The characteristics of the mAb were described previously (Spintalny and Havell, 1984). Normal rat globulin (NRG), prepared and purified by $(NH_4)_2SO_4$ precipitation from the pooled sera of Wistar rats (SLC), was injected as a control.

*Viral infection and mAb treatment*

Mice were inoculated i.v. with $10^8$ PFU per mouse of Theiler's virus strain GD VII. 1 mg of anti-IFN-γ mAb or NRG was given i.v. by a single injection on Day 1 p.i.

*IFN-γ assay*

IFN-γ assay of the tissue homogenate was carried out by a double-sandwich enzyme-linked immunosorbent assay (ELISA) as previously reported (Nakane et al., 1990). Rat anti-mouse IFN-γ mAb (R4-6A2) purified by DEAE Affi-Gel Blue column chromatography (Nakane et al., 1989) was used as a capture antibody. Rabbit anti-mouse IFN-γ serum was obtained from a rabbit hyperimmunized with purified recombinant mouse IFN-γ as described previously (Nakane et al., 1989). All ELISAs were run with recombinant mouse IFN-γ that was produced and purified by Genentech, Inc. (San Francisco, CA). The titre of standard IFN-γ used in ELISA was already titrated by antiviral assays.

*Histological study*

Mice were killed on Day 6 and Day 9 p.i. The brains and spinal cords were harvested after perfusion with 10% phosphate-buffered formalin and embedded in paraffin and stained with hematoxylin and eosin (H&E). To evaluate the localization of IFN-γ-expressing cells within CNS tissue infected with Theiler's virus, a qualitative detection of endogenous IFN-γ was carried out on cryostat sections using immunohistochemical staining. Fresh brain tissues were embedded in OCT compound (Ames Co, Div. of Miles Laboratory, Inc, Elkhart, IN), snap-frozen in liquid nitrogen and ethanol, and stored at −85°C until use. 4-μm cryostat sections were mounted on poly-L-lysine (Sigma Chemical Co., St. Louis, MO)-coated glass slides and were fixed in cold acetone. Immunohistochemical staining was carried out by the avidin-biotin-peroxidase complex (ABC) (Vector Laboratories, Burlingame, CA) method (Hsu et al., 1981). The first antibody to murine IFN-γ was R4-6A2. The second antibody to rat IgG

was biotynyl rabbit anti-rat IgG (Vector Laboratories, Burlingame, CA). The sections were stained with $H_2O_2$ and 3,3′-diaminobenzidine and by Methyl green nuclear counter staining. Normal rat IgG (Jackson Immunoresearch Laboratories, Inc., Avondal, PA) was used as a control.

*Statistical analysis*

The Wilcoxon test for two samples was used to evaluate the statistical significance of differences in virus titres and mortality between groups of mice that received mAb and NRG control.

## Results

*Theiler's virus replication and IFN-γ production in brain and spinal cord*

Mice were injected i.v. with $10^8$ PFU of Theiler's virus and killed at 6, 12, 24 h, and thereafter at 24-h intervals until Day 17 p.i. The viruses replicated and the peak titres were $10^5$ PFU/g from Day 4 to Day 6 p.i. in the brain and $10^5$ PFU/g on Day 10 to Day 14 p.i. in the spinal cord (Fig. 1B). The virus was not detected in the brain and spinal cord on day 57 p.i. (data not shown)

IFN-γ was detected in the CNS of the Theiler's virus-infected mice. IFN-γ in the brain and spinal cord was detected on Day 5–17 Day p.i. associated with viral replication. From Day 5 to Day 9 p.i., 0.5–20 IU/g of IFN-γ was detected, and the production peaked on Day 6 p.i. in brain (Fig. 1A). In the spinal cord, 5–25 IU/g of IFN-γ was detected from Day 6 to Day 17 p.i. (Fig. 1A). IFN-γ could not detected in serum, liver, spleen, heart, brain, and spinal cord of normal mice and in those of mice on Day 3 p.i. (data not shown). On cryostat sections using immunohistochemical staining, IFN-γ-expressing (IFN-$\gamma^+$) cells in the CNS infected with Theiler's virus were detected on Day 6 p.i. IFN-$\gamma^+$ cells were located in inflammatory lesions of the brainstem on Day 6 p.i. (Fig. 2B, C). Anti-IFN-γ mAb preabsorbed with recombinant mouse IFN-γ did not react with the tissues. The sections treated with control rat IgG were unstained (Fig. 2A). Endogenous IFN-γ in the brain stem was not detected from Day 0 to Day 4 p.i. (data not shown).

*Effect of anti-IFN-γ mAb treatment on the course of infection*

To assess the contribution of endogenous IFN-γ to the host defence against Theiler's virus infection, neutralization of endogenous IFN-γ by the administration of anti-IFN-γ mAb was investigated. Mice were infected i.v. and treated with 1 mg of the mAb on Day 1 p.i. The control group was treated with NRG. The mortality was significantly higher in the anti-IFN-γ mAb-treated group as compared with the NRG-treated group ($P < 0.0001$). All anti-IFN-γ mAb-treated mice were dead within 2 weeks; however, half the controls survived beyond 3 weeks p.i. (Fig. 3).

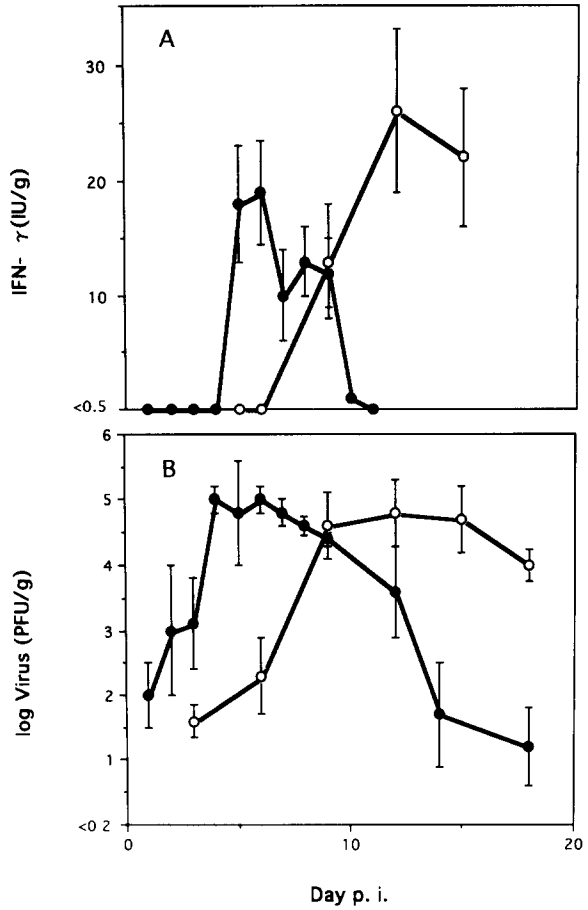


Fig. 1. IFN-γ (**A**) and virus titres (**B**) in brain (closed circles), and spinal cord (open circles). Animals were inoculated i.v. with $1 \times 10^8$ PFU of Theiler's virus. At various times, each group of five mice were killed, tissues were obtained, and IFN-γ and virus were titrated. Titres of IFN-γ and virus were expressed as the average of five different determinations.

*Effect of anti-IFN-γ mAb treatment on viral replication*

To determine the effect of anti-IFN-γ mAb on viral replication, the homogenized CNS tissues (whole brain and spinal cord) from each mouse were plaqued on BHK-21 cells for detection of the infectious viruses. Although there was no significant difference of virus titres between the anti-IFN-γ mAb- and the NRG-treated mice in brain (Fig. 4B), the virus titres in spinal cord of anti-IFN-γ mAb-treated mice were significantly higher than those of NRG-treated mice on Day 9 and Day 11 p.i. ($P < 0.05$) (Fig. 4A). On Day 6 and Day 9 p.i., no viral replication in spleen, liver, heart of anti-IFN-γ mAb and NRG-treated mice was observed (data not shown).

*Histological changes by administration of anti-IFN-γ mAb*

The neuropathology of Theiler's virus-infected mice at the acute phase of infection was characterized by neuronolysis, appearance of microglia, and perivascular inflammatory cell infiltration. These lesions were observed in the spinal cord and brainstem. Inflammatory cell infiltration and microglial nodules were more prominent in the brainstem than those in the spinal cord from Day 6 p.i. to Day 9 p.i. (Figs. 5A, C, 6A, C). In the spinal cord, the appearance of microglia was only observed on Day 6 p.i. (Fig. 5A). Mice treated

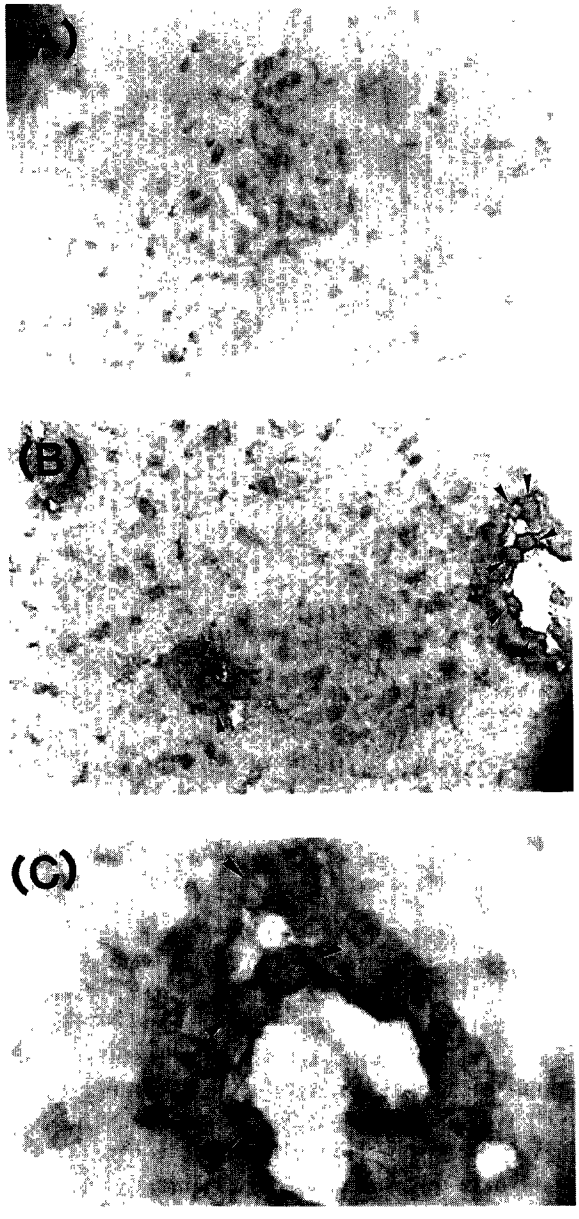


Fig. 2. Identification of cells positive in brain on Day 6 p.i. for IFN-γ by immunohistochemical method (ABC staining). IFN-γ positive cells were indicated by arrows. **(A)** Control (1st antibody was NRG), magnification ×400; **(B)** magnification ×400; **(C)** magnification ×1000.

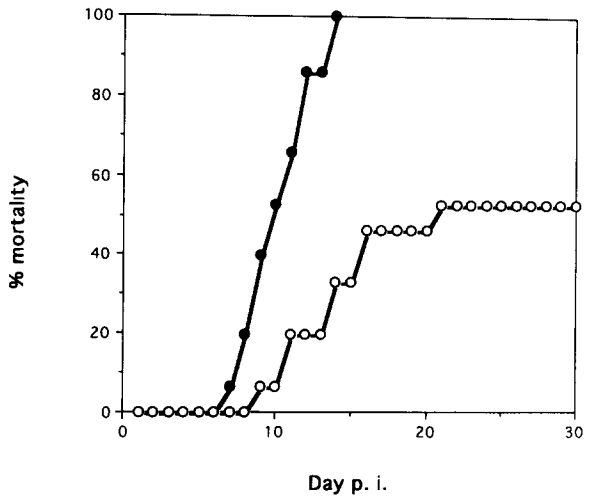


Fig. 3. Mortality rate during the course of Theiler's virus infection in mice treated with anti-IFN-γ mAb on Day 1 p.i. Each group comprised 20 mice. Closed circles, anti-IFN-γ mAb-treated group. Open circles, NRG-treated group. The mortality rates of both groups were significantly different at $P < 0.0001$.

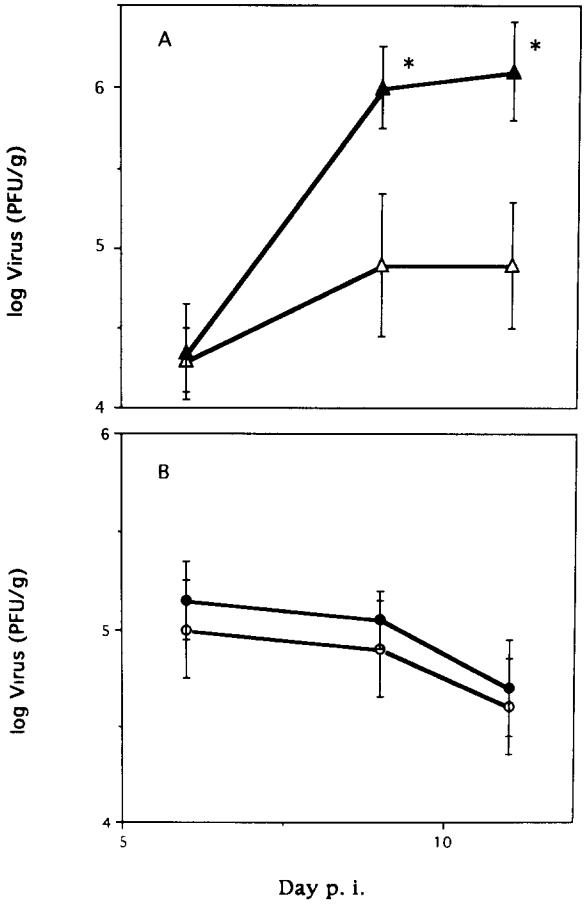


Fig. 4. Theiler's virus titres in spinal cord **(A)** and brain **(B)** of mice which had received anti-IFN-γ mAb (closed symbols) or NRG (open symbols) on Day 6, Day 9, Day 11 p.i. Each value was expressed as the average of eight different determinations. *Significant difference from the value for the anti-IFN-γ mAb-treated group at $P < 0.05$.

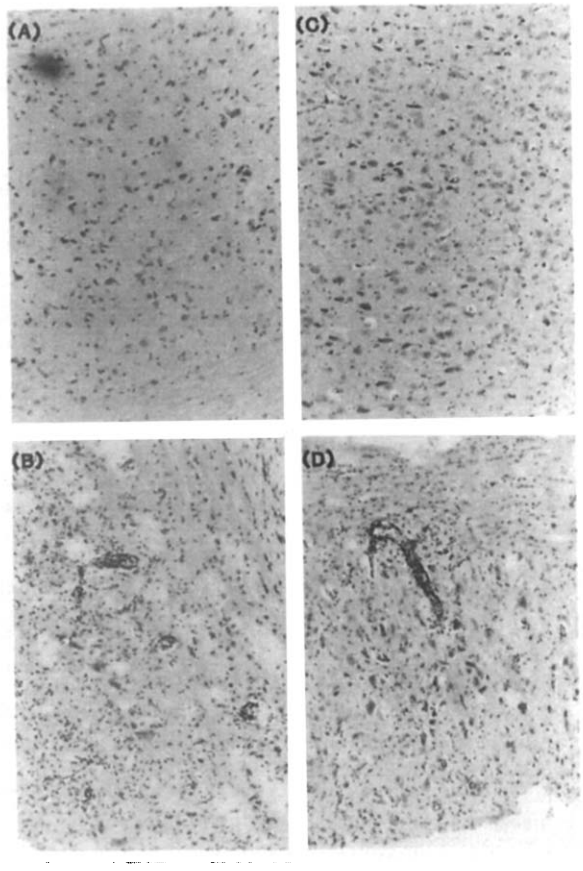


Fig. 5. Histopathology of the spinal cord of mice treated with NRG on Day 6 p.i. (**A**); anti-IFN-γ on Day 6 p.i. (**B**); NRG on day 9 p.i. (**C**); and anti-IFN-γ on Day 9 p.i. (**D**) (H&E; magnification ×200).

with anti-IFN-γ mAb showed severe inflammatory reactions in the spinal cord compared with NRG-treated mice (Fig. 5B). Furthermore, on Day 9 p.i., inflammatory cell reactions were also observed in the spinal cord of NRG-treated mice (Fig. 5C); however, these reactions were less than those of mice treated with anti-IFN-γ mAb (Fig. 5D). In brain, there was no change between anti-IFN-γ mAb-treated mice and NRG-treated mice on Day 6 and Day 9 p.i. (Fig. 6).

## Discussion

Our present study has shown that endogenous IFN-γ was detected in the CNS of mice infected with Theiler's virus. We further demonstrated that the elimination of endogenous IFN-γ by the treatment of mice with a mAb directed against IFN-γ suppressed the protection of mice from Theiler's virus infection in the CNS and that inflammatory cell infiltration was more remarkable in the spinal cord of anti-IFN-γ mAb-treated mice than those of NRG-treated mice.

In our experiments, the production of endogenous IFN-γ in CNS tissue was observed from Day 5 to Day 17 p.i. (Fig. 1A). Endogenous IFN-γ production in CNS tissue corresponded to the proliferation of Theiler's virus (Fig. 1). The localization of endogenous IFN-γ was also evident in the CNS by immunohistochemistry (Fig. 2). Cells positive for endogenous IFN-γ infiltrated vessels and the CNS parenchyma.

We carried out in vivo neutralization of endogenous IFN-γ by the administration of anti-IFN-γ mAb to study the role of this cytokine in the CNS of mice infected with Theiler's virus. The administration of anti-IFN-γ mAb on Day 1 p.i. increased viral replication in the spinal cord and the mortality in comparison to NRG-treated controls (Figs. 3 and 4). These data indicate that endogenous IFN-γ produced in CNS tissue is important in the protective mechanism against Theiler's virus.

The importance of endogenous IFN-γ in various infectious diseases including *Listeria monocytogenes*, *Toxoplasma gondii*, lymphocytic choriomeningitis virus, and mouse hepatitis virus, was verified by the fact that neutralization of endogenous IFN-γ with anti-IFN-γ mAb reduced host resistance (Buchmeier and

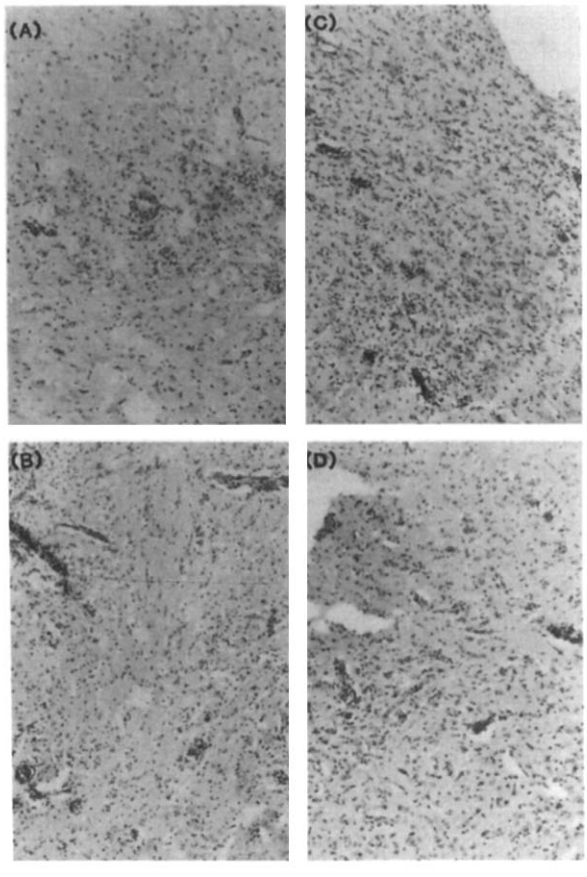


Fig. 6. Histopathology of the brain of mice treated with NRG on day 6 p.i. (**A**); anti-IFN-γ mAb on Day 6 p.i. (**B**); NRG on Day 9 p.i. (**C**); and anti-IFN-γ mAb on Day 9 p.i. (**D**) (H&E; magnification ×200).

Schreiber, 1985; Suzuki et al., 1988; Leist et al., 1989; Nakane et al., 1989; Smith et al., 1991). There are only a few reports on the kinetics of endogenous IFN-$\gamma$ production during infections. In listerial infection, endogenous IFN-$\gamma$ production coincided with bacterial growth (Nakane et al., 1990). Our present data also indicate that endogenous IFN-$\gamma$ production coincides with the viral replication (Fig. 1). We speculate that endogenous IFN-$\gamma$ is produced by cells which are stimulated with the virus. Therefore, it is reasonable that the peak of viral replication coincides with that of endogenous IFN-$\gamma$ production. IFN-$\gamma$ in infectious diseases has been reported to induce activated macrophages (Buchmeier and Schreiber, 1985; Suzuki et al., 1988) and other immune effector cells (Biron et al., 1983; Habu et al., 1984; Spintalny and Havell, 1984; Issekutz et al., 1988). In viral infections, IFN-$\gamma$ may be effective in both the activation of immune effector cells and the induction of antiviral states. In our present study, the enhancement of viral replication was observed after histological changes in the spinal cord (Figs. 4A and 5). If endogenous IFN-$\gamma$ acted on only on the induction of the antiviral state in the host, an increase of viral replication by treatment with anti-IFN-$\gamma$ mAb might precede the histological changes. Alternatively, the inhibition or the elimination of the virus by immune effector cells through anti-IFN-$\gamma$ mAb might cause an histological exacerbation. Nevertheless, in the brain, enhancement of viral replication and histological exacerbation were not observed. In Theiler's virus infection, inflammatory cell infiltration consisted mainly of macrophages in the spinal cord until 7–10 days p.i.; and T cells and macrophages were present in the brain from 4–5 days p.i. Treatment with anti-T cell receptor-$\alpha\beta$ augmented viral replication in brain, but not in spinal cord (unpublished data). Therefore, we speculate that endogenous IFN-$\gamma$ produced in Theiler's virus infection might act as a macrophage activating factor and activated macrophages might be important in protecting the spinal cord. On the other hand, the protective mechanism in brain might be dependent on T cells.

The regulation of a putative mechanism by IFN-$\gamma$ in Theiler's virus infection is difficult to evaluate because of its pleiotropic functions. Duong et al. (1992) showed that anti-IFN-$\gamma$ mAb treatment exacerbated experimental allergic encephalomyelitis (EAE). They speculated that a feed back regulatory loop existed between transforming growth factor (TGF)-$\beta$ and IFN-$\gamma$, and neutralization of IFN-$\gamma$ by the mAb may prevent TGF-$\beta$ release (Twardzik et al., 1990). TGF-$\beta$ has been shown to abrogate the transfer of EAE by encephalitogenic T cell lines (Kuruvilla et al., 1991). It is possible that an anti-inflammatory effect of endogenous IFN-$\gamma$ might act as a protector against the destruction of CNS tissue by Theiler's infection.

Ongoing work is examining the actions of endogenous IFN-$\gamma$ in Theiler's virus infection and differences in the mechanism of viral replication and host defence between spinal cord and brain.

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