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Theiler's virus is eliminated by a gamma-interferon-independent mechanism in the brain

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Abstract

The intravenous infection of Theiler's virus GD VII strain causes acute encephalomyelitis in infected mice. To determine the cellular mechanism of resistance and interferon (IFN)-γ-producing cell populations, mononuclear cells isolated from tissues of the brain were analyzed by the flow cytometry method. Antibodies specific for CD3, CD4, CD8, T cell receptor (TCR)-$\alpha\beta$, and Asialo GM1 were used to deplete the corresponding cell populations in Theiler's virus-infected mice. $CD4^+$ lymphocytes and $CD8^+$ lymphocytes infiltrated in the brains of infected mice from 5 days postinfection (p.i.). The number of $CD3^+/TCR\text{-}\gamma\delta^+$ lymphocytes increased in the brains on Day 6 p.i. The elimination of $CD3^+$ lymphocytes or $CD4^+$ lymphocytes augmented viral replication and suppressed the production of IFN-γ. The suppression of IFN-γ production by anti-CD3 monoclonal antibody (mAb) persisted, although the suppression by anti-CD4 mAb was observed only on Day 6 p.i. The depletion of $CD8^+$ lymphocytes as well as TCR-$\alpha\beta^+$ lymphocytes also augmented the viral replication; however, it did not alter the production of IFN-γ. Anti-Asialo GM1 antibody had no effect on viral replication and IFN-γ production. These results indicate that T lymphocytes are important for eliminating Theiler's virus from the brain, $CD3^+/CD4^+/CD8^-$ lymphocytes and $CD3^+/TCR\alpha\beta^-/CD4^-/CD8^-$ lymphocytes would produce IFN-γ in brain. However, from the result on the experiment of the depletion of TCR-$\alpha\beta^+$ lymphocytes, the defence mechanisms by T lymphocytes against Theiler's virus would be independent of endogenous IFN-γ production.

Key words: Theiler's virus; T lymphocytes; Endogenous interferon-γ; Acute encephalomyelitis

1. Introduction

Theiler's virus is a cardiovirus (Pevear et al., 1987), and is divided into two subgroups (GD VII strain and TO strain) on the basis of biological behavior (Lorch et al., 1981). Strains of GD VII and FA are characterized by the replication of the virus in the central nervous system (CNS) gray matter, causing a disease resembling poliomyelitis (Lipton, 1980). For a 50% lethal dose of GD VII strain injected intravenously (i.v.) and having a count of 1.4×10^8 PFU/mouse, the infected mice show symptoms of wasting and paralysis, and the elimination of the virus from CNS tissues was observed (Kohanawa et al., 1993). The mechanisms on the elimination of GD VII strain virus from CNS tissues have not been elucidated. In viral infections, the elimination of viruses from infected tissues is performed by a T lymphocyte-dependent mechanism (Nash et al., 1987; Perry and Lodmell, 1991). T lymphocytes play important roles, i.e. helping immune reaction, cytotoxicity to infected cells, and secretion of cytokines (Ertl et al., 1989; Lindsley et al., 1991; Nakane et al., 1991). $CD4^+$ T lymphocytes secrete cytokines (Nakane et al., 1991) and show helper activities (Ertl et al., 1989). $CD8^+$ lymphocytes act as cytotoxic T lymphocytes (CTL) and destroy virus-infected cells (Lindsley et al., 1991).

In our observation on Theiler's virus GD VII strain, endogenous interferon gamma (IFN-γ) was produced in the brains and spinal cords of the infected mice (Kohanawa et al., 1993). IFN-γ which is secreted by natural killer (NK) cells and T lymphocytes (Klavinskis et al., 1989; Taylor et al., 1989; Nakane et al., 1991) is an important cytokine in viral infections (Klavinskis et al., 1989; Leist et al., 1989; Smith et al., 1991; Lucin et

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al., 1992). In lymphocytic choriomeningitis virus infection, $CD8^+$ lymphocytes and $CD4^+$ lymphocytes which are protective against this virus produce endogenous IFN-γ (Gessner et al., 1989). In Theiler's virus infection, endogenous IFN-γ is important in the defence mechanisms. We confirmed that endogenous IFN-γ is important for defending the spinal cord tissues from destruction by Theiler's virus infection, although the administration of anti-IFN-γ mAb was not effective in augmenting viral replication and exacerbating disease lesion histologically in the brain (Kohanawa et al., 1993). This observation indicates that the defence mechanisms in the brain may be different from those in the spinal cord and independent of endogenous IFN-γ.

In our study, we show that T lymphocytes in the brain eliminate Theiler's virus and produce endogenous IFN-γ, although this elimination is independent of endogenous IFN-γ produced.

2. Materials and methods

2.1. Mice and viral infection

Female ddY mice (4 weeks old) were obtained from SLC (Hamamatsu, Shizuoka, Japan). Theiler's virus GD VII strain was multiplied on BHK-21 cell monolayers in a serum-free RPMI 1640 medium. Mice were inoculated i.v. with 10^8 PFU per mouse.

2.2. Plaque assay

The brains 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. The resulting tissue homogenates were frozen and thawed twice and clarified by centrifugation at $500 \times g$ for 30 min. The virus in the supernatant of the tissue homogenate was quantitated by plaque assay on BHK-21 cells (Kohanawa et al., 1993).

2.3. Antibodies

Hybridoma cells secreting monoclonal antibodies (mAbs) directed against CD4 (L3T4) (GK1.5, rat immunoglobulin G2b) (Dialynas et al., 1983), CD8 (Lyt-2) (53–6.72, rat immunoglobulin G2a) (Ledbetter and Herzenberg, 1979), CD3 (145-2C11, hamster immunoglobulin G) (Leo et al., 1987), and T cell receptor (TCR)-$\alpha\beta$ (H37-597, hamster immunoglobulin G) (Kubo et al., 1989) were used. 145-2C11 cells were kindly provided by Dr. H. Yagita and Dr. K. Okumura, Department of Immunology, Juntendo University School of Medicine, Tokyo, Japan. H37-597 cells were kindly donated by Dr. E. Nakayama, Department of Parasitology, Okayama University, Medical School, Okayama, Japan.

These mAbs were prepared from ascites fluid in Pristane-primed CD-1 *nu/nu* mice. Partial purification by 50% $(NH_4)_2S0_4$ precipitation was followed by exhaustive dialysis against phosphate-buffered saline (PBS, pH 7.4). Rabbit anti-asialo GM1 (ASGM1) antibody was prepared and partially purified by 50% $(NH_4)_2SO_4$ precipitation as described previously (Nakane and Minagawa, 1984). The antibodies were aliquoted and stored at −70°C.

2.4. In vivo depletion of $CD3^+$ cells, $CD4^+$ cells, $CD8^+$ cells, TCR-$\alpha\beta^+$ cells, and $ASGM1^+$ cells

We gave each mouse a single 400-μg i.v. injection of anti-CD4 mAb, or Anti-CD8 mAb (in 0.2 ml of pyrogen-free saline) on Day −1 of the infection. Normal rat globulin was injection as a control.

200 μg of anti-CD3 mAb or anti-TCR-$\alpha\beta$ mAb was injected intravenously on Day −3 of the infection. Normal hamster globulin was injected as a control.

400 μg of anti-ASGM1 antibody was given i.v. in a volume of 0.2 ml on Day −1 of the infection. Normal rabbit globulin was injected as a control.

Evaluation of in vivo cell depletion has been shown previously (Nakane and Minagawa, 1984; Nakane et al., 1991; Hiromatsu et al., 1992).

2.5. Isolation of brain mononuclear cells (MNC) and flowcytometry analysis

The method of isolation of the brain's MNC was shown previously (Clatch et al., 1990). After the infected mice were killed by perfusion with PBS through the hearts, the brains were removed. The brain tissues of the 5–8 mice were dissociated by passage through 100-mesh stainless steel screens and resuspended in RPMI 1640 medium. Dissociated brain tissues were centrifuged for 10 min at $200 \times g$ and resuspended in 4 ml of 70% Percoll (Pharmacia LKB Biotechnology Inc. Piscataway, NJ) in PBS at 24°C. 4 ml of 30% Percoll was carefully overlaid over the 70% Percoll layer containing the dissociated tissues, and the gradients were centrifuged for 15 min at $500 \times g$ at 24°C. Fractions from the gradients were collected by puncturing a hole in the bottom of the tube.

For flowcytometry analysis (FACScan, Becton Dickinson & Co., Mountain View, CA) of the distribution of the $CD3^+$, TCR-$\gamma\delta^+$, Mac-1^+, $CD4^+$ and $CD8^+$ cells, MNC were stained by phycoerythrin (PE)-conjugated anti-L3T4 mAb (Becton Dickinson), anti-TCR-$\gamma\delta$ mAb (Pharmingen, San Diego, CA), fluorescein isothio-

cyanate (FITC)-conjugated anti-Lyt-2 mAb (Becton Dickinson), FITC-anti-CD3 mAb, biotin-conjugated anti-Mac-1 mAb (Caltag Laboratories, South San Francisco, CA) and FITC-conjugated avidin (Becton Dickinson). FITC-conjugated anti-CD3 mAb was prepared in our laboratory.

2.6. IFN-γ assay

IFN-γ assay in 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 (R46A2) (Spintalny and Havell, 1984) purified by DEAE Affi-Gel Blue column chromatography 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 that described previously (Nakane et al., 1990). 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 had already been

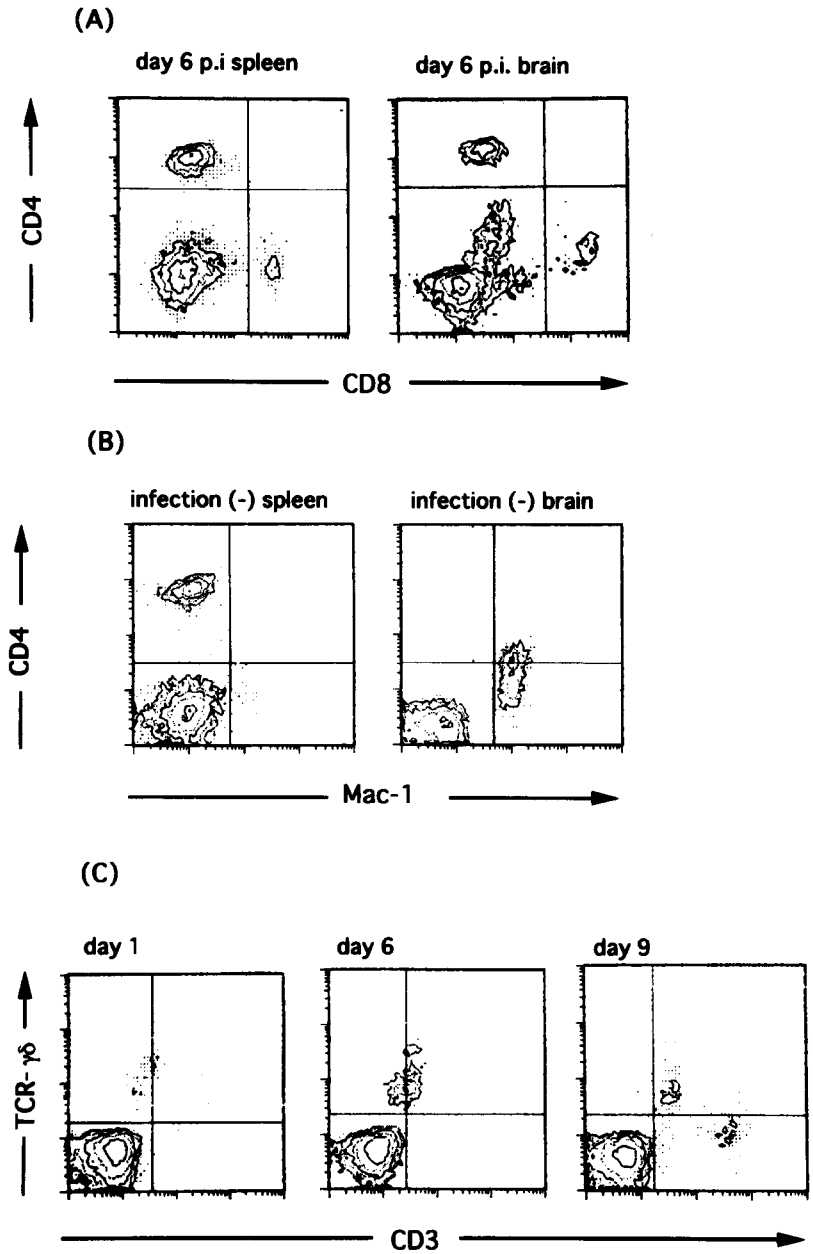

Fig. 1. FACS analysis of the mononuclear cells infiltrated in the brains and spleen. These cells were stained with PE-anti-CD4(GK 1.5), FITC-anti-CD8(Lyt-2), FITC-anti-Mac-1, anti-CD3 (145-2C11)-FITC, and anti-TCR-γδ (UC7-13D5)-PE. The cells separated from the brains of about 5–7 mice were analyzed in each analysis. (A) On day 6 p.i. (B) Uninfected. (C) On day 1, day 6 and day 9 p.i.

Table 1
Surface makers and numbers of mononuclear cells isolated from the brains

	Day post inoculation				
	1	3	5	7	9
$CD4^+$ (%)	0.0	0.2	0.5	10.2	2.9
$CD8^+$ (%)	0.2	0.1	5.0	6.0	2.5
Number of isolated MNC [a]	0.34	0.62	0.50	1.34	0.77

[a] $\times 10^5$ cells per mouse.

titred by antiviral assays. We were able to detect 0.1 international unit (IU)/ml of IFN-γ with the ELISA.

2.7. Statistical analysis

The Wilcoxon test for two samples was used to evaluate the statistical significant differences in virus titres between groups of mice that received mAb and normal globulin.

3. Results

3.1. Analysis of surface CD3, CD4, CD8, TCR-γδ, and Mac-1 molecule expression on the MNC from the brains

Mice were injected i.v. with 10^8 PFU of Theiler's virus and killed on Day 1, Day 3, Day 5, Day 7, and Day 9 p.i. $CD4^+$ lymphocytes in the brains which had an equal intensity of fluorescence to $CD4^+$ lymphocytes in the spleen were detected (Fig. 1A). In the brains of uninfected mice, the low CD4-positive cells existed and these cells were Mac-1-positive (Fig. 1B). The peak infiltration of $CD4^+$ lymphocytes and $CD8^+$ lymphocytes was 10.2% and 6.0% on Day 7 p.i., respectively (Table 1). We analyzed the existence of $CD3^+/TCR\text{-}\gamma\delta^+$ lymphocytes in CNS tissues on Day 1, Day 6, and Day 9 p.i. (Fig. 1C). $CD3^+/TCR\text{-}\gamma\delta^+$ lymphocytes increased on Day 6 p.i. and the proportion was 10.8%.

3.2. Effect of depletion of $CD4^+$ lymphocytes and $CD8^+$ lymphocytes in viral replication and endogenous IFN-γ production

400 μg of anti-CD4 mAb or anti-CD8 mAb was injected intravenously on Day −1 of the infection. On Day 6 p.i., the treatment of anti-CD4 mAb and anti-CD8 mAb did not alter the viral replication (Fig. 2A). The virus titres in the anti-CD4 mAb-treated mice and anti-CD8 mAb-treated mice were significantly higher than those of the control mice on Day 9 p.i. at $P < 0.01$ and $P < 0.05$, respectively (Fig. 2A).

Anti-CD4 mAb-treatment suppressed endogenous IFN-γ production only on Day 6 p.i., and not on Day 7, Day 8, Day 9 p.i. (Fig. 2B). Anti-CD8 mAb-treatment showed no effect on endogenous IFN-γ production (Fig. 2B).

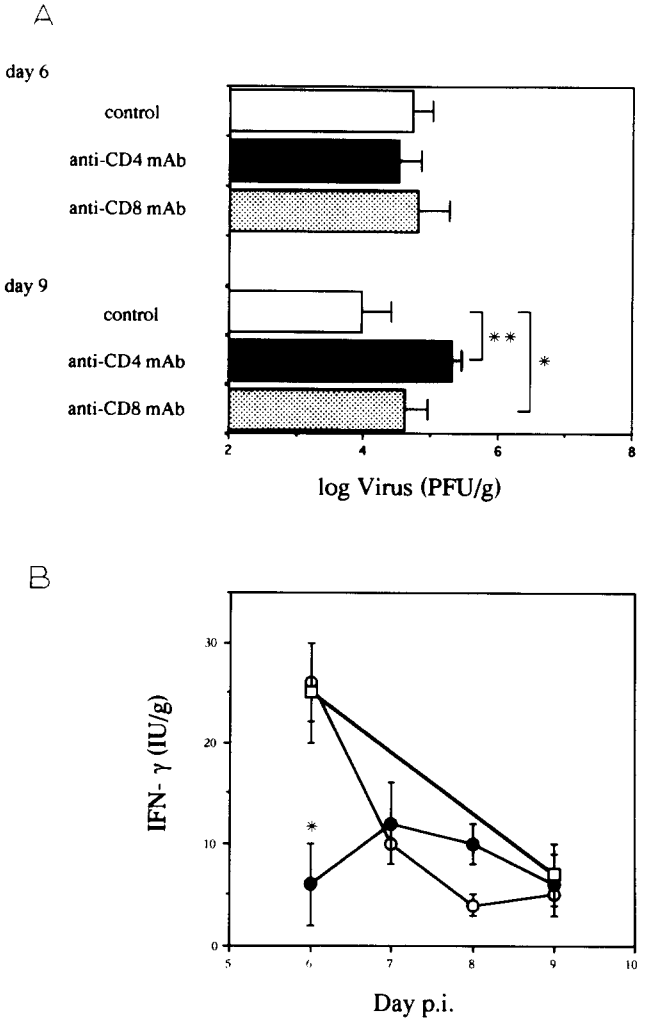

Fig. 2. Theiler's virus titres (**A**) and endogenous IFN-γ (**B**) in mice which had received normal rat globulin (control, **A**: open bars, **B**: open circles), anti-CD4 mAb (**A**: black bars, **B**: closed circles), or anti-CD8 mAb (**A**: gray bars, **B**: open squares). Each value was expressed as the average of 10 different determinations. Symbol: significant difference at * $P < 0.05$, ** $P < 0.01$.

3.3. Effect of depletion of $CD3^+$ lymphocytes and TCR-$\alpha\beta^+$ lymphocytes in viral replication and endogenous IFN-γ production

200 μg of anti-CD3 mAb or anti-TCR-αβ mAb was injected intravenously on Day-3 of the infection. On Day 6 p.i., the treatment of anti-CD3 mAb or anti-TCR-αβ mAb did not alter the viral replication (Fig. 3A). The virus titres in the anti-TCR-αβ mAb-treated mice as well as anti-CD3 mAb-treated mice were significantly ($P < 0.01$) higher than those of the control mice on Day 9 p.i. (Fig. 3A).

The production of endogenous IFN-γ was significantly suppressed ($P < 0.05$) in the anti-CD3 mAb-

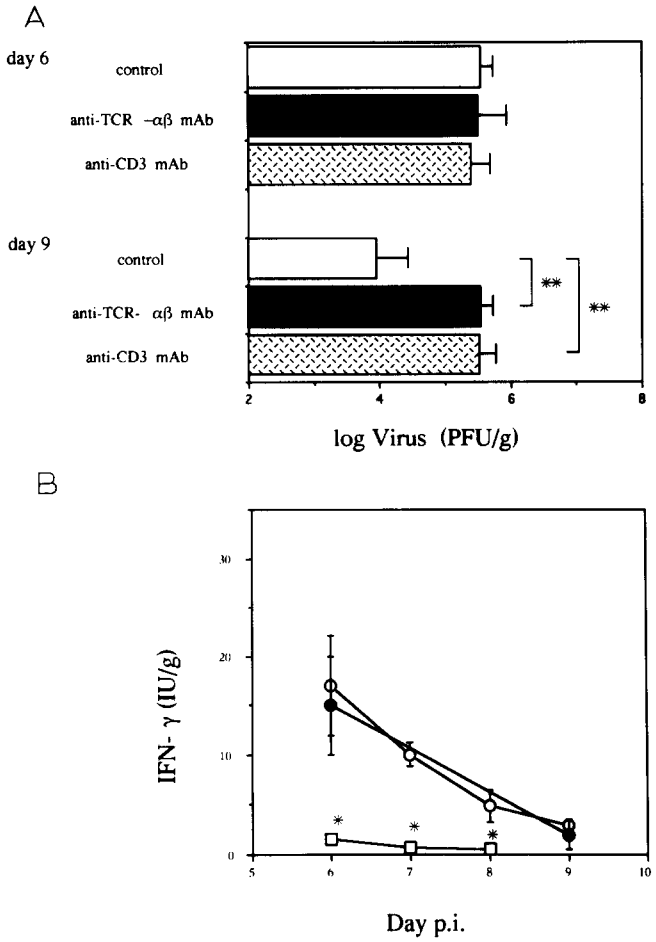

Fig. 3. Theiler's virus titres (**A**) and endogenous IFN-γ (**B**) in mice which had received normal hamster globulin (control, **A**: open bars, **B**: open circles), anti-TCR-αβ mAb (**A**: black bars, **B**: closed circles), and anti-CD3 mAb (**A**: hatched bars, **B**: open squares). Each value was expressed as the average of 10 different determinations. Symbol: significant difference at * $P < 0.05$, ** $P < 0.01$.

treated mice on Day 6, Day 7, and Day 8 p.i. (Fig. 3B). Anti-TCR-αβ mAb showed no effect on the endogenous IFN-γ production (Fig. 3B).

3.4. Effect of depletion of ASGM1$^+$ cells in viral replication and endogenous IFN-γ production

The administration of anti-ASGM1 Ab showed no effect on the viral replication and endogenous IFN-γ production (data not shown).

3.5. Effect of depletion of TCR-αβ$^+$ cells in infiltration of TCR-γδ$^+$ cells in the brains

On Day 9 p.i., TCR-γδ$^+$ cells in the mice treated with anti-TCR-αβ mAb increased as compared with those in the control mice (Fig. 4).

4. Discussion

Our present study shows that T lymphocytes are important for eliminating Theiler's virus from the brains of infected mice. The following is a discussion on the findings of these results.

First, IFN-γ-producing cells in the brains of infected mice are divided into two phenotypes. One of the phenotype endogenous IFN-γ producing cells might be CD3$^+$/CD4$^+$/CD8$^-$, and another CD3$^+$/CD4$^-$/CD8$^-$. In the brains of infected mice, CD3$^+$/TCR-γδ$^+$ lymphocytes existed in the brains (Fig. 1C). It has been reported that γδ$^+$ T lymphocytes can produce IFN-γ (O'Brien et al., 1989), and the phenotype of these cells is mostly CD3$^+$/CD4$^-$/CD8$^-$ or CD3$^+$/CD4$^-$/CD8$^+$. In T lymphocytes, a CD3$^+$/TCR-αβ$^+$/CD4$^-$/CD8$^-$ population also exists. However, anti-TCR-αβ mAb showed no effect on the endogenous

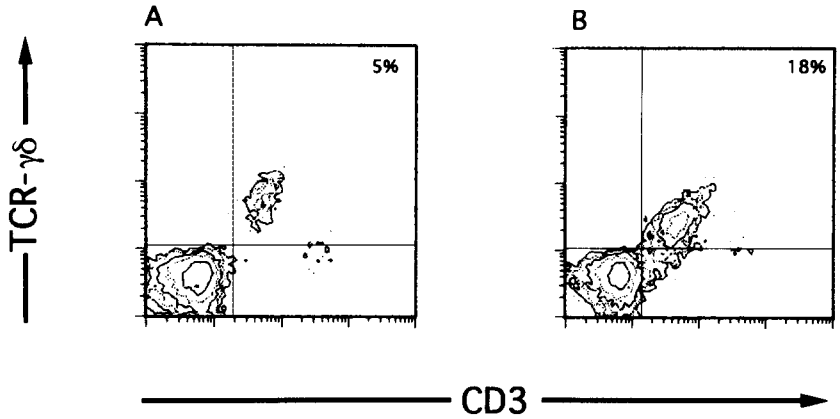

Fig. 4. FACS analysis of the CD3$^+$/TCR-γδ$^+$ cells infiltrated on Day 9 p.i. in the brains of the mice which were treated with anti-TCR-αβ mAb. These cells were stained with PE-anti-TCR-γδ$^+$ and FITC-anti-CD3. (**A**) normal hamster globulin-treated mice. (**B**) anti-TCR-αβ mAb-treated mice.

IFN-γ production (Fig. 3B). Therefore, we presume that the TCR-γδ$^+$ lymphocytes might produce IFN-γ in the CNS tissues. γδ$^+$ T lymphocytes exist mostly in epidermis (Koning et al., 1987), intestinal intraepithelium (Goodman and Lefrancoise, 1988), and reproductive organs (Lafaille et al., 1989). In CNS tissues, it has been reported that γδ$^+$ T lymphocytes exist on multiple sclerosis (Selmaj et al., 1991a), experimental allergic encephalitis (Sobel and Kuchroo, 1992) and Sindbis virus infection (Griffin et al., 1992). γδ$^+$ T lymphocytes have the ability to be cytotoxic to cells infected with viruses (Maccario et al., 1993) as well as to produce cytokines (O'Brien et al., 1989). It has been reported that γδ$^+$ T lymphocytes have a few repertoires in the V region (Garman et al., 1986) and would react with only some proteins (involving heat shock protein) through their TCR (O'Brien et al., 1989). On this property, we consider that γδ$^+$ T lymphocytes in the brain might act as cytokine-producing cells rather than as cytotoxic cells in Theiler's virus infection. However, it cannot be denied that γδ$^+$ T lymphocytes have cytotoxicity to infected cells with Theiler's virus because it has been reported that TCR-γδ$^+$ lymphocyte clone was raised by immunization of herpes simplex virus glycoprotein I (Johnson et al., 1992), and TCR-γδ$^+$ lymphocytes induces myocarditis by Coxsackievirus B3 (Huber et al., 1992).

Second, T lymphocytes infiltrated in the brains and were essential in eliminating Theiler's virus from the brains (Table 1 and Fig. 2). In the infection by Theiler's virus GD VII strain, the viral titres decreased from Day 5 to 6 p.i. until Day 18 p.i. (Kohanawa et al., 1993), and the virus was completely cleared on day 30 p.i. in ddY mice (unpublished data). The beginning of the elimination of the virus from the brain coincides with the beginning of the infiltration of T lymphocytes into the brain (Table 1). Moreover, the depletion of CD4$^+$ cells or CD8$^+$ cells by administration of the mAbs augmented the viral titres in the brain. Therefore, T lymphocytes might be essential in eliminating the virus from the brain. The immune reactions by T lymphocytes involve cytotoxicity and cytokine production. CD8$^+$ lymphocytes would eliminate Theiler's virus from the brain through cytotoxic mechanisms, because CD8$^+$ lymphocytes are well recognized as cytotoxic T lymphocytes (Lindsley et al., 1991). Cytokine production by T lymphocytes might also be important in eliminating Theiler's virus from the brain. It is possible that CD4$^+$ lymphocytes would act on the brain through endogenous IFN-γ and other cytokine production. However, from our present study, it is reasonable to assume that CD4$^+$ lymphocytes might eliminate Theiler's virus from brain through IFN-γ independent fashion, because, firstly, the elimination of CD4$^+$ lymphocytes were able to suppress the production of IFN-γ only temporarily (Fig. 2B). Secondly, the elimination of TCR-αβ$^+$ lymphocytes could augment viral replication as well as anti-CD4 mAb-treatment, in spite of the failure to suppress endogenous IFN-γ production (Figs. 2 and 3). In the brain, Theiler's virus was eliminated under conditions of lack of endogenous IFN-γ (Kohanawa et al., 1993), and the elimination of T lymphocytes augmented the viral replication in the presence of endogenous IFN-γ. These data indicate that the defence mechanisms by CD4$^+$ lymphocytes against Theiler's virus might not be dependent on endogenous IFN-γ production. CD4$^+$ T lymphocytes might be important for producing other cytokines or class II-restricted cytotoxic cells in the brain. For example, CD4$^+$ T lymphocytes produce tumor necrosis factor (TNF) (Nakane et al., 1992) and kill target cells by induction of apoptosis (Selmaj et al., 1991b; Rahelu et al., 1993). In the infection of Theiler's virus DA strain, TNF inhibits demyelination (Paya et al., 1990). Any other cytokines would be important in the protective mechanisms by T lymphocytes.

The CD4 low positive cells existed in the brains of infected mice (Fig. 1A). In humans and rats, microglias and macrophages are CD4$^+$ cells (Crocker et al., 1987; Perry and Gordon, 1987). In mice, peritoneal macrophages, splenic macrophages, and Kupffer cells are CD4$^-$ (Crocker et al., 1987). However, the microglias of mice are Mac-1$^+$ and CD4$^+$ (Akiyama and McGeer, 1990; Hassan et al., 1991). In our present study, spleen cells included CD4$^+$/Mac-1$^-$ or CD4$^-$/Mac1$^+$ (Fig. 1B). In the brains of normal mice, CD4 low positive cells were Mac-1$^+$ (Fig. 1B). Therefore, we presume that CD4 low positive cells in the CNS tissues might be a member of the macrophage lineage cells, microglia.

In our present experiment, the treatment of anti-TCR-αβ mAb did not alter the production of endogenous IFN-γ (Fig. 3). TCR-αβ$^+$ lymphocytes consist of CD4$^+$/CD8$^-$, CD4$^-$/CD8$^+$, CD4$^-$/CD8$^-$, and CD4$^+$/CD8$^+$ lymphocytes. The treatment of anti-TCR-αβ mAb-depleted CD4$^+$ lymphocytes and CD8$^+$ lymphocytes (Hiromatsu et al., 1992), and augmented the number of TCR-γδ$^+$ cells in the brains of the infected mice (Fig. 4). Also, in the peritoneal infection of *Listeria monocytogenes*, a significantly increased number of TCR-γδ$^+$ cells was detected in the peritoneal cavity of the TCR-αβ-suppressed rats (Hasegawa et al., 1992). Therefore, anti-TCR-αβ mAb would have little effect on the suppression of endogenous IFN-γ production because of the compensative production of IFN-γ by TCR-γδ$^+$ lymphocytes.

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