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Protection of mice from a lethal coronavirus infection in the central nervous system by adoptive transfer of virus-specific T cell clones

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Summary

The protective effect of a mouse hepatitis virus type-4 (MHV-4)-specific CD8^{+} cytotoxic T cell clone and a CD4^{+} helper T cell clone was examined by the adoptive transfer into brains of mice lethally infected with MHV-4. Mice survived acute encephalitis if more than 5×10^5 cells of either type of the virus-specific T cell clones had been transferred into H-2-matched recipients by 1 day post-infection. Although the adoptive transfer of both types of the T cell clones suppressed viral growth and viral antigen-positive cells in the brains, a significant inhibition of virus replication by the cytotoxic T cell clone was detected prior to that induced by the helper T cell clone. Histologically, cell destruction was prominent in the brains of mice which received the cytotoxic T cell clone. These results demonstrate that both the CD8^{+} cytotoxic T cell and the CD4^{+} helper T cell can protect mice from a lethal MHV-4 infection in the central nervous system.

Introduction

Mouse hepatitis virus type-4 (MHV-4) infection in mice is one of the best examples of virus-in-

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duced central nervous system (CNS) disease in an animal model (Wege et al., 1982; Kyuwa and Stohlman, 1990). Intracerebral (i.c.) infection with MHV-4 induces an acute fatal encephalomyelitis in almost all the laboratory strains of naive mice (Stohlman and Frelinger, 1978; Knobler et al., 1981). Survivors show evidence of chronic demyelination and occasionally suffer severe hind leg paralysis. Various factors including virus strain, route of infection, genetic background and immune status of the host are known to modify the infection. Recently, some reports have focused on immunological therapy of MHV-4 infection in the

CNS and demonstrated that in vivo treatment with antiviral monoclonal antibodies protected mice from a lethal MHV-4 infection (Buchmeier et al., 1984; Fleming et al., 1989). In addition, adoptive transfer of CD4^{+} helper T cell (Th) clones which induce a delayed-type hypersensitivity (DTH) response also protects mice (Stohlman et al., 1986). However, viral replication in the brains of these mice is scarcely affected by these therapeutic treatments.

Since T cells, particularly cytotoxic T lymphocytes (CTL), are thought to play an important role in viral infections (Blanden and Gardner, 1976; Yap et al., 1978; Byrne and Oldstone, 1984; Oldstone et al., 1986), we established MHV-4-specific CD8^{+} CTL clones and CD4^{+} Th clones from infected BALB/c mice to better understand the role of these cells in MHV-4-induced diseases (Kyuwa et al., 1987; Yamaguchi et al., 1988). In this study we transferred MHV-4-specific T cell clones into infected mice and examined whether these T cell clones could protect mice from acute fatal encephalitis. Both types of T cell clones protected H-2-matched mice, accompanied by a significant inhibition of virus replication and clearance of viral antigen-positive cells in the CNS; however, the CTL clone was more active in terms of viral clearance. Thus, not only CD4^{+} Th but also CD8^{+} CTL can protect mice from a lethal MHV-4 infection in the CNS.

Materials and methods

Mice

BALB/c mice were obtained from colonies in this institute. B10.D2, B10.A and B10.BR mice were purchased from Shizuoka Laboratory Animal Center, Hamamatsu, Japan. Female, 5- to 8-week-old mice were used throughout the experiments. Breeding colonies were routinely screened serologically for the absence of MHV.

Virus

MHV-4 was propagated and assayed for plaque-forming units (PFU) on DBT cells as described (Hirano et al., 1978). Virus was stored at $-70\,^{\circ}$C until use.

T cell clones

CD8^{+} CTL clones P11D, P1C5, P4A3, P16A and 2.55 and CD4^{+} DTH-inducer Th clone P12B were established from the spleens of MHV-4-infected mice (Kyuwa et al., 1987; Yamaguchi et al., 1988). Briefly, BALB/c mice were inoculated intraperitoneally with 10^4 PFU of MHV-4. One week later the spleens were removed, and 10^8 splenocytes were co-cultured with 2×10^7 BALB/c spleen cells, which had been gamma irradiated (2000 rad) and infected with MHV-4 (multiplicity of infection 0.05), in 40 ml of RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 5×10^{-5} M 2-mercaptoethanol and 20 μg/ml of gentamicin in 75 cm^2 flasks at 37°C. One to 2 weeks later limiting dilution was performed in the presence of 10% rat concanavalin A (ConA)-conditioned medium and 10^6 irradiated, MHV-4-infected BALB/c spleen cells in 96-well flat-bottom plates. After expansion of growing cells, cells were recloned by the same procedure. T cell clones were maintained in Iscove's modified Dulbecco's medium supplemented with 10% FCS, 10% rat ConA-conditioned medium, 5×10^{-5} M 2-mercaptoethanol, 25 mM Hepes and 20 μg/ml of gentamicin and stimulated with MHV-4-infected BALB/c spleen cells weekly. The Sendai virus-specific T cell line SV-1 was kindly provided by Dr. Yamamoto, Institute of Public Health, Tokyo (Yamamoto et al., 1988).

Virus inoculation and transfer of T cell clones

Mice were inoculated i.c. in the right cerebral hemisphere with 100 PFU of MHV-4 in 20 μl of Eagle's minimum essential medium (MEM). The i.c. LD$_{50}$ of MHV-4 in young adult BALB/c mice is approximately 2 PFU. Five or 6 days after stimulation with MHV-4-infected spleen cells, T cell clones were centrifuged on Ficoll-Conray gradients to exclude dead cells. One million T cell clones were injected i.c. into the right cerebral hemisphere of recipients in a volume of 20 μl on day 1 post-infection (p.i.) except for the time course study. Control mice having received either T cell clone or RPMI 1640 medium without infection did not show any clinical symptoms.

Virus titration

Mice were sacrificed on days 2, 5 and 8 p.i. The brains, spleens and livers were removed aseptically and infectious virus titer was determined by plaque assay on DBT cells as described above. Significance was determined using the Student t-test.

Histopathology

Groups of BALB/c mice were sacrificed and the brains were removed on days 3 and 5 p.i. The brains were fixed in 10% formalin in phosphate-buffered saline (PBS) and then embedded in paraffin. Sections (4 μm thick) were stained with hematoxylin and eosin (HE).

Detection of MHV-4 antigen-positive cells

Viral antigen was detected by immunoperoxidase staining. Deparaffinized sections were rinsed in PBS and treated with 1% normal swine serum in PBS for 30 min at room temperature. The sections were then incubated for 1 h at 37°C with rabbit anti-MHV-4 serum, purified by Sepharose-Protein A column chromatography. After washing for 30 min in PBS the sections were exposed to peroxidase-conjugated anti-rabbit IgG swine serum (Dakopatts, Denmark) diluted 1/100 in PBS for 45 min. Negative controls included sections of MHV-4-infected brains incubated with normal rabbit serum and sections of uninfected brains incubated with the anti-MHV-4 serum. The peroxidase reaction was performed according to the methods of Kaplow (1975). Sections were counterstained with Mayer's hematoxylin. The number of MHV-4 antigen-positive cells was determined as follows. Two coronal sections of brains approximately 4 and 8 mm from the tip of the brain were prepared from each mouse. The total number of viral antigen-positive cells per two sections from each mouse was counted microscopically. Neurons and glial cells were classified according to the morphology.

Results

Effect of adoptive transfer of MHV-4-specific T cell clones

We have established virus-specific CD8$^+$ CTL clones and CD4$^+$ Th clones from MHV-4-infected

TABLE 1

PROTECTION OF MICE FROM LETHAL MHV-4 INFECTION BY ADOPTIVE TRANSFER OF MHV-4-SPECIFIC T CELL CLONES [a]

T cell clone			Survivor/ total	Survival time (mean day ± SD)
	Specificity	Type		
None	–	–	0/16	6.4 ± 1.1
SV-1	Sendai virus	Th	0/10	6.2 ± 1.0
P11D	MHV-4	CTL	12/12	> 30
P1C5	MHV-4	CTL	3/3	> 21
P4A3	MHV-4	CTL	3/3	> 21
P16A	MHV-4	CTL	3/3	> 21
2.55	MHV-4	CTL	3/3	> 21
P12B	MHV-4	Th	12/12	> 30

[a] BALB/c mice which had been infected i.c. with 100 PFU of MHV-4 received 1×10^6 T cell clones on day 1 p.i., and were observed for more than 3 weeks.

BALB/c mice (Kyuwa et al., 1987; Yamaguchi et al., 1988). Adoptive transfers were used to examine the effect of these T cell clones on an acute fatal MHV-4 infection. Groups of BALB/c mice were infected i.c. with 100 PFU of MHV-4. The next day 1×10^6 cells of each T cell clone were transferred into the brains. Subsequently, mice were observed for more than 3 weeks. As controls, the Sendai virus-specific CD4$^+$ T cell line SV-1, also established from BALB/c mice, was transferred into the brains of MHV-4-infected mice. Table 1 shows that the MHV-4-specific CTL clones P11D, P1C5, P4A3, P16A and 2.55 and the Th clone P12B protected recipients from a lethal MHV-4 infection and that the recipients survived for more than 3 weeks. Survivors showed no clinical symptoms of chronic disease. In contrast, both the mice which received the Sendai virus-specific T cell line SV-1 and the infected untreated mice had symptoms of hyperirritability, rough coat and loss of body weight within 4 days p.i. and died between 5 and 8 days p.i. The protective effect of the CTL clone P11D and the Th clone P12B was studied further. Although the sera of each group of mice were examined on days 2, 5, 9 and 14 p.i., if possible, neutralizing antibodies were detected neither in MHV-4-infected mice without T cell clones nor in mice having received the T cell clone P11D or P12B (less than 1 : 10). When transferred 1 day before, on the same day or 1 day after virus

TABLE 2

PROTECTIVE EFFECT BY THE TIMING OF TRANSFER OF T CELL CLONES

T cell clone		Time of transfer [a] (days p.i.)	Survivor/ total	Survival time (mean day ± SD)
	Type			
None	–	–	0/3	7.7 ± 1.0
P11D	CTL	−1	2/3	> 30 (8) [b]
		0	3/3	> 30
		+1	3/3	> 30
		+2	0/3	8.3 ± 0.9
		+4	0/3	6.7 ± 0.5
P12B	Th	−1	3/3	> 30
		0	2/3	> 30 (7) [c]
		+1	3/3	> 30
		+2	0/3	7.7 ± 0.9
		+4	0/3	8.3 ± 0.5

[a] One million cells of each T cell clone were adoptively transferred into lethally infected BALB/c mice 1 day before, on the same day, or 1, 2 or 4 days after infection.
[b] One mouse died on day 8 p.i.
[c] One mouse died on day 7 p.i.

TABLE 3

DOSE–RESPONSE OF T CELL CLONE-MEDIATED PROTECTION OF MICE FROM LETHAL MHV-4 INFECTION [a]

T cell clone		No. of cells transferred	Survivor/ total	Survival time (mean day ± SD)
	Type			
None	–	–	0/6	7.5 ± 2.6
P11D	CTL	1×10^6	4/4	> 30
		5×10^5	4/4	> 30
		1×10^5	2/4	> 30 (5, 6) [b]
		5×10^4	0/4	4.5 ± 0.5
P12B	Th	1×10^6	4/4	> 30
		5×10^5	4/4	> 30
		1×10^5	0/4	7.5 ± 1.7
		5×10^4	0/4	8.3 ± 0.4

[a] BALB/c mice which had been infected i.c. with 100 PFU of MHV-4 received various numbers of T cell clones on day 1 p.i., and were observed for more than 1 month.
[b] Two mice died on days 5 and 6 p.i.

inoculation, both the T clones protected mice from a lethal MHV-4 infection. However, adoptive transfer carried out on day 2 or 4 p.i. was not effective (Table 2). The protective effect of both T cell clones was dose dependent and required more than 5×10^5 cells to protect a mouse (Table 3).

Genetic restriction of the protective effects of T cell clones in vivo

The T cell clones P11D and P12B were derived from BALB/c (H-2^d). Since the proliferation re- sponse of the T cell clones P11D and P12B is H-2-restricted (Kyuwa et al., 1987), H-2 restriction of the protective effect of these T cell clones was examined using H-2-congeneic strains derived from C57BL/10 mice, B10.D2 (H-2^d), B10.A (H-2^a) and B10.BR (H-2^k). Table 4 shows that adoptive transfer of CTL clone P11D prevented a lethal infection in B10.D2 mice; however, neither B10.A nor B10.BR mice were protected. Similarly, adoptive transfer of the Th clone P12B protected B10.D2 mice but failed in B10.A and B10.BR mice. Thus, the T cell clones P11D and P12B were

TABLE 4

GENETIC RESTRICTION OF T CELL CLONE-MEDIATED PROTECTION OF MICE FROM LETHAL MHV-4 INFECTION [a]

T cell clone		Recipient mouse	H-2 KID	Survivor/total	Survival time (mean day ± SD)
	Type				
None	–	B10.D2	ddd	0/5	7.4 ± 0.5
		B10.A	kkd	0/5	7.4 ± 0.5
		B10.BR	kkk	0/5	7.6 ± 0.5
P11D	CTL	B10.D2	ddd	5/5	> 16
		B10.A	kkd	0/5	8.0 ± 2.3
		B10.BR	kkk	0/5	7.4 ± 1.0
P12B	Th	B10.D2	ddd	5/5	> 16
		B10.A	kkd	0/5	7.6 ± 0.5
		B10.BR	kkk	0/5	8.8 ± 0.4

[a] Mice which had been infected i.c. with 100 PFU of MHV-4 received 1×10^6 T cell clones on day 1 p.i., and were observed for more than 16 days. No mice having received either T cell clone died without MHV-4 challenge.

able to protect mice from a lethal MHV-4 infection only in the context of the appropriate genetic environment, presumably involving recognition of viral antigen in the context of the products of H-2 genes.

Alteration of virus titers in the brains by adoptive transfer of MHV-4-specific T cell clones

To examine the effect of CTL and Th clones on virus replication, titers of MHV-4 in the brains were examined. The brains of MHV-4-infected mice, which had received the MHV-4-specific CTL clone P11D, the Th clone P12B or the Sendai virus-specific T cell line SV-1, were assayed for virus on days 2, 5 and 8 p.i. A 90% drop in virus titers in the CNS was observed as early as 1 day after adoptive transfer in mice which had received the CTL clone P11D (Table 5). However, complete virus clearance was not observed by 8 days p.i. In contrast, only a slight reduction in virus titer was observed 1 day following adoptive transfer of the Th clone P12B. Significant decreases in virus titer were detected by 4 days after adoptive transfer in mice having received the Th clone P12B. The adoptive transfer of the Sendai virus-specific T cell line SV-1 did not restrict MHV-4 replication.

Histopathology and viral antigen-positive cells in the brains

On days 3 and 5 p.i. histological changes in the brains were compared among mice having received the CTL clone P11D or the Th clone P12B and infected untreated mice. Basically, there was little difference between the mice having received
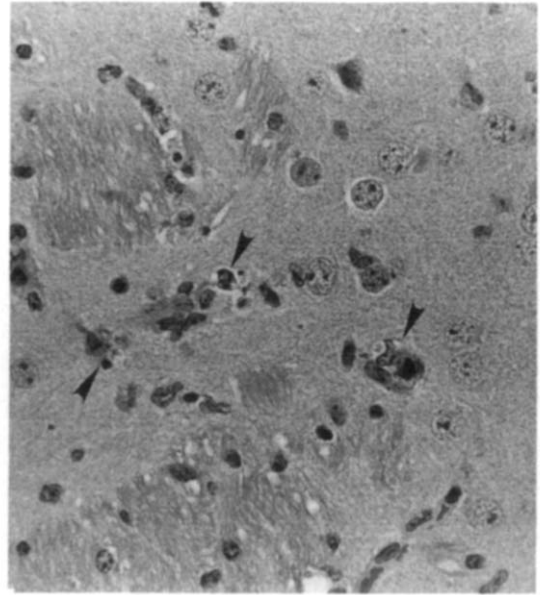

Fig. 1. Cell destruction (arrowhead) associated with mononuclear cell infiltration in the brain of mice having received CTL clone P11D. Caudate nucleus. Day 5 p.i. ×180 (HE).

the CTL clone and the infected untreated mice; the degree of cellular infiltration was at a minimum in the brains of mice in these groups. However, cell destruction looked prominent in the brains having received the CTL clone P11D on day 3 p.i. (Fig. 1). By contrast, typical changes following transfer of the Th clone P12B were moderate infiltration of mononuclear cells and meningitis (Fig. 2).

The numbers of MHV-4 antigen-positive neurons and glial cells were determined in mice which

TABLE 5

INHIBITION OF VIRUS REPLICATION IN BRAINS BY ADOPTIVE TRANSFER OF T CELL CLONES [a]

T cell clone			Viral titer ($\log_{10}$ PFU/g)		
	Specificity	Type	Day 2 p.i.	Day 5 p.i.	Day 8 p.i.
None	–	–	5.7 ± 0.2 [b]	6.6 ± 0.1	NT [c]
SV-1	Sendai virus	Th	5.6 ± 0.3	6.4 ± 0.3	NT
P11D	MHV-4	CTL	$\underline{4.7 \pm 0.1}$ [d]	$\underline{4.9 \pm 0.1}$	3.3 ± 0.1
P12B	MHV-4	Th	5.1 ± 0.3	$\underline{5.4 \pm 0.1}$	3.2 ± 0.1

[a] BALB/c mice which had been infected i.c. with 100 PFU of MHV-4 received 1×10^6 T cell clones on day 1 p.i.
[b] Mean viral titer ± SD from three mice.
[c] Viral titer was not tested due to death of mice.
[d] Viral titer significantly different ($p < 0.01$) from the titer of the brain without T cell clone transfer is underlined.
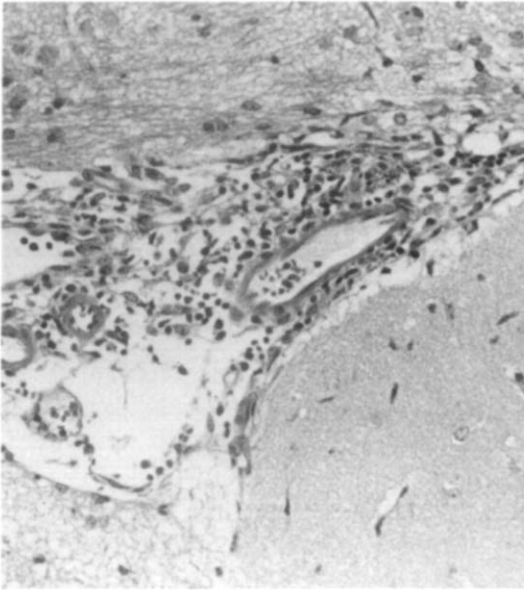
Fig. 2. Moderate mononuclear cell infiltration adjacent to the lateral ventricle of the brain of mice having received Th clone P12B. Days 5 p.i. ×830 (HE).

had received the MHV-4-specific CTL clone P11D or the Th clone P12B and compared to infected untreated mice (Fig. 3). The adoptive transfer of the CTL clone P11D drastically reduced the num-
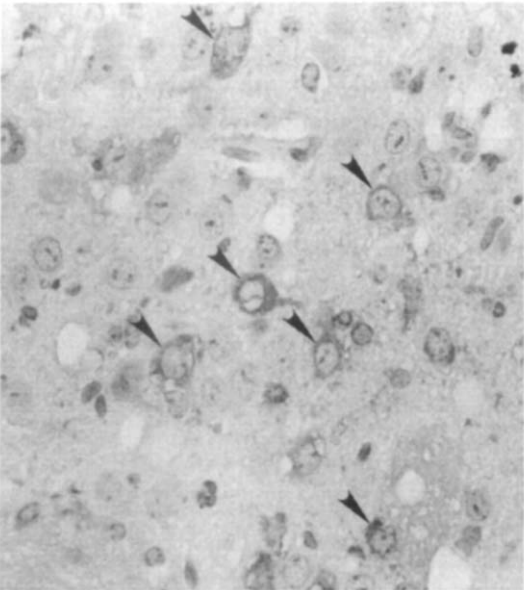

Fig. 3. Detection of MHV-4 antigens (arrowhead) in the brain by immunoperoxidase staining. Mammary body. Day 5 p.i. ×830.

TABLE 6

VIRAL ANTIGEN-POSITIVE CELLS IN THE BRAINS OF MICE HAVING RECEIVED T CELL CLONES [a]

T cell clone	Type	No. of mice tested	No. of positive cells [b] (mean ± SD)	
			Neuron	Glial cells
None	–	4	38 ± 17	523 ± 129
P11D	CTL	4	2 ± 2	31 ± 25
P12B	Th	4	1 ± 1	41 ± 19

[a] BALB/c mice which had been infected i.c. with 100 PFU of MHV-4 received 1×10^6 T cell clones. On day 5 p.i. brains were removed and viral antigen was detected as described in Materials and Methods.
[b] Viral antigen-positive cells on two coronal sections of brain from each mouse were counted microscopically.

bers of viral antigen-positive cells in the brains (95% reduction in neurons and 94% reduction in glial cells). Similarly, a 93% reduction in antigen-positive cells was also observed following the adoptive transfer of the Th clone P12B (Table 6). These data indicated that both T cell types can effectively limit the number of antigen-positive cells in the CNS of infected mice.

Discussion

The immune response is an important factor which determines the fate of MHV-4 infection in the CNS of mice. The adoptive transfer of anti-viral monoclonal antibodies specific for three structural proteins (S, M and N) can protect mice from a lethal infection (Buchmeier et al., 1984; Fleming et al., 1989; S.A. Stohlman, personal communication). In addition, the adoptive transfer of CD4+ DTH-inducer T cell clones also results in protection of mice from the infection (Stohlman et al., 1986). Interestingly, protection mediated by adoptive transfer into C57BL/6 mice was not associated with a decrease in viral replication within the CNS but rather with neuronal protection. In the present study we demonstrate that the adoptive transfer of virus-specific CD8+ CTL clones also protects mice from a lethal MHV-4 infection. In this case, however, protection is associated not only with decreased neuronal infection but also with a significant inhibition

of viral growth. Thus, protection of mice from a lethal MHV-4 infection is always accompanied by inhibition of neuronal infection, irrespective of the therapeutic treatment. These findings support the hypothesis that neuronal infection is a major fatal factor in acute fatal encephalomyelitis induced by MHV-4 (Knobler et al., 1981, 1982).

The adoptive transfer of either CTL or Th clones at day 2 p.i. or later failed to protect the mice. This may suggest that approximately 50 LD_{50} virus given i.c. had produced an overwhelming infection which would not be restrained by the action of the T cell clones. Recently, we have made an interesting finding in this regard. Expression of class I molecules on J774.1 cells decreased after high multiplicity of MHV-4 infection in vitro (S. Kyuwa, unpublished data). For example, this down-regulation of class I molecules, which is indispensable for recognition by $CD8^+$ CTL, may provide an escape mechanism for MHV-4 from CTL-mediated protection as described below.

$CD4^+$ Th cell-mediated protection was first reported by Stohlman et al. (1986). In this study we have confirmed that the adoptive transfer of virus-specific $CD4^+$ Th cells protects mice from a MHV-4 lethal infection. While viral replication in the CNS was not inhibited in their study, viral replication was slightly suppressed following transfer of the $CD4^+$ Th clone P12B. The absence of detectable antibody in the recipients suggests that this was probably not due to provisional help for the secretion of neutralizing antibody. Although both $CD4^+$ Th clones (4B10 and P12B) induce a virus-specific DTH response, no other functions have been identified, which may be important for virus clearance. For example, it is not known whether these $CD4^+$ Th clones provide help for virus-specific CTL, secreting a variety of lymphokines in vivo. In addition, differences in virus and mouse strain may account, at least potentially, for this discrepancy.

Although CTL clone P11D and Th clone P12B required almost the same number of cells and transfer timing for the protection, there was a slight difference in time when a significant drop in virus titer began. Virus titers in mice which received the CTL clone P11D decreased significantly 1 day after transfer. In contrast, a significant drop in virus titer in recipients of the Th clone P12B was detected on and after 4 days following the transfer and the inhibition was less than that induced by the CTL clone P11D. These findings suggest that $CD8^+$ T cells are indeed responsible for the reduction in virus titer and fit with the hypothesis that $CD4^+$ T cells may function as helper T cells for the generation of virus-specific $CD8^+$ CTL (Sussman et al., 1989); however, we cannot rule out the possibility that the difference is due to the antigen specificity of T cell clones rather than the type of T cell clones. In addition, this hypothesis is supported by recent findings that the in vivo treatment with not only anti-CD8 but also anti-CD4 monoclonal antibodies inhibits virus clearance from the CNS (Williamson and Stohlman, 1990).

The mechanism(s) of protection conferred by virus-specific CTL is a most interesting point. Generally, $CD8^+$ T cells recognize a small fragment of endogenously synthesized protein bound to major histocompatibility complex (MHC) class I molecules on target cells prior to lysis of the targets (Sweetser et al., 1989; Townsend and Bodmer, 1989; Whitton and Oldstone, 1989). Therefore, two components are necessary for target cell lysis. First, potential targets must be infected with MHV-4. In this regard MHV-4 can infect almost all of the major cell types in the CNS: neurons, oligodendrocytes and astrocytes. Second, the targets must express MHC class I molecules. Although there is little expression of class I molecules on cells in the CNS, recent reports indicate that some cytokines including interferon-γ (IFN-γ), IFN-α/β and tumor necrosis factor increase expression of MHC class I molecules on cells in the CNS (Wong et al., 1984, 1985; Lavi et al., 1988). In addition, elevation of MHC class I antigen expression on glial cells following murine coronavirus infection has been described (Suzumura et al., 1986). Taken together, these results suggest that glial cells represent the most probable targets of MHV-4-specific CTL. In fact, the adoptive transfer of the $CD8^+$ CTL clone P11D induced cell destruction and decreased the number of viral antigen-positive cells. To our knowledge, this finding is notable because there is little direct evidence that CTL can kill target cells in vivo as they do in vitro (Mullbacher and Ada, 1987). Complete clearance of infectious virus from

the brains was not observed by 8 days p.i. This phenomenon is similar to incomplete clearance of lymphocytic choriomeningitis virus from the CNS following adoptive transfer of CD8^{+} T cells (Oldstone et al., 1986). These findings may be due to the limited expression of MHC antigens in the CNS (Vitteta and Capra, 1978; Williams et al., 1980)

In addition to lysis of virus-infected cells, CD8^{+} CTL can secrete a variety of lymphokines including IFN-γ after target recognition. In fact, we have detected IFN activity in the culture supernatant of CTL clone P11D after stimulation with MHV-4-infected J774.1 cells, which we used as the targets in the CTL assays (unpublished data). Although direct lysis rather than secretion of IFN-γ was suggested as the antiviral mechanism of CTL (Lukacher et al., 1984; Mullbacher and Ada, 1987), it might be still worthy of consideration, especially in the CNS, an immunologically unique site. Furthermore, IFN-γ secreted by CTL would also facilitate in target recognition by CTL as a result of the up-regulation of MHC class I molecules on cells in the CNS.

A recent study has demonstrated that CD8^{+} T cells play an important role not only in acute virus clearance, but also in the development of MHV-4-induced demyelination (J.O. Fleming, personal communication). Although adoptive transfer of virus-specific monoclonal antibodies or CD4^{+} T cells protects mice from a lethal infection, these therapeutic treatments cannot protect mice from demyelination. Their data suggest that MHV-4-induced demyelination is immune-mediated and that CD8^{+} T cells are responsible for the development of demyelination. Although we did not examine the effect of our CD8^{+} CTL clones on virus-induced demyelination in this study, future experiments may reveal the interaction between MHV-4, CD8^{+} T cells and cells in the CNS, providing important information on the mechanism of MHV-4-induced demyelination.

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