CHARACTERIZATION OF MOUSE HEPATITIS VIRUS-REACTIVE T CELL CLONES

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INTRODUCTION

Mouse hepatitis virus(MHV) is a member of the family Coronaviridae and known to induce various types of disease, hepatitis, enteritis and encephalitis in mice depending upon the host strain, age, and immune status[1]. Using nude or immunomodulated mice, T cell-mediated immunity in MHV infections was suggested to play an important role[2,3]. By the adoptive transfer of spleen cells from immune euthymic mice the growth of MHV in the recipient nude mice was inhibited, and the transfer effect was abolished by treating immune spleen cells with anti-BAT serum and complement[3].

In various virus infections in mice, $L3T4^+$, Lyt 2^- T cell population was shown to have helper activity and to mediate DTH response in association with class II MHC product[4,5]. Although DTH response was demonstrated in MHV infections using polyclonal[6] or cloned[4,7] T cells, the role of DTH effector T cells on MHV infection remains obscure. On the other hand, $L3T4^-$, Lyt 2^+ T cells were shown to have cytotoxic activity against virus-infected cells and to be restricted by class I MHC product[8,9]. However, cytotoxic T lymphocytes(CTL) activity in MHV infections has not been demonstrated. Recently, several laboratories have successfully established and maintained T cell clones of various subsets and of several virus specificities[5,7,8]. In addition, using these homogenous populations several important properties and roles of T cells have been revealed[4,9].

To investigate the mechanisms of T cell-mediated immunity in MHV infection, we attempted to establish MHV-reactive T cell clones. This paper deals with the establishment and characterization of T cell clones reactive for MHV.

MATERIALS AND METHODS

Animals. Female BALB/c, C57BL/6, C3H/He, DBA/2, SWR/J and SJL/J mice were obtained from this Institute. A/J mice were purchased from Shizuoka Laboratory Animal Center, Hamamatsu. Congenitally athymic nude mice having a background of BALB/c and C3H/He were bred in this Institute. All the mice were used at the age of 4 to 8 weeks. Female Fischer rats were kindly supplied from Charles River Japan, Atsugi. The mouse and rat breeding colonies had been routinely checked serologically for the absence of MHV[10].

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Virus. An MHV strain JHM(MHV-JHM) was propagated and assayed for plaque forming units(PFU) on DBT cells as described elsewhere[11] and stored at -70°C.

IL 2. The IL 2 sample was prepared according to the methods of Gillis et al.[12] Briefly, spleens of Fischer rats were taken aseptically and pooled. Single cell suspension at a concentration of 4×10^6/ml was prepared in RPMI 1640(GIBCO, Chagrin Falls, Ohio) supplemented with 5% fetal calf serum(FCS; M.A. Bioproducts, Walkersville, MD), 5 μg/ml of concanavalin A(Con A), 5×10^{-5} M 2-mercaptoethanol(2-ME; Nakarai, Kyoto), 25 mM HEPES(Sigma, Kenilworth, NJ) and cultured for 24 hr at 37°C in humidified 5% CO_2 in air. The supernatant was harvested, added 20 mg/ml of α-methyl-D-mannoside to neutralize Con A, filtered through 0.22 μm membrane filter(Millipore, Bedford, MA), and kept at -20°C until use.

T cell clones. BALB/c mice were inoculated intraperitoneally(i.p.) with 10^4 PFU of MHV-JHM. Seven to 8 days later spleens were taken and 10^8 cells were co-cultured with 2×10^7 spleen cells("stimulator cells"), which had been gamma-irradiated(2,000 rads) and MHV-infected(multiplicity of infection 0.05), in 40 ml of RPMI 1640 supplemented with 10% FCS, 5×10^{-5} 2-ME and 20 μg/ml of gentamycin in 75 cm^2 tissue culture flasks at 37°C in an atomosphere of 5% CO_2 in air. For clonig 1 to 4,000 viable cells at 8 to 14 days of co-culture were cultured together with 10^6 "stimulator cells" in 0.2 ml of Iscove's modified Delbecco's medium supplemented with 10% FCS, 10% IL 2, 5×10^{-5} M 2-ME, 25 mM HEPES and 20 μg/ml of gentamycin(IMDM medium) using 96 flat-bottom multiwell plates. After incubation for 7 to 14 days each well was checked for cell growth, and from wells showing positive growth at cell dilution level giving less 33% positive wells, cultures were expanded into 24 multiwell plates in the presence of 5×10^6 "stimulator cells" and fed 2 ml of IMDM medium. The cell lines were passaged in 5 ml of IMDM medium every 3 or 4 days co-culturing with 2×10^7"stimulator cells" every one passage. One to 2 months later 0.3 to 10 cells were subjected to recloning by the same procedure as described above. Four clones, D4C, P9A, P11D and P12B, thus obtained were subcultured every 3 to 4 days using fresh IMDM medium, and weekly they were co-cultured with "stimulator cells".

Detection of surface markers on T cell clones. The T cell clones were examined for surface markers by membrane immunofluorescence. One million cells of each clone were incubated with 1 μg of biotin-conjugated anti-Thy 1.2, anti-Lyt 1 or anti-Lyt 2 monoclonal antibodies(Becton Dickinson, Moutain View, CA), or with rat anti-L3T4 monoclonal antibody(GK 1.5) at 4°C for 45 min. After incubation cells were washed three times with phenol red-free Eagle minimal essential medium(Nissui, Tokyo) supplemented with 0.02 M NaN_3. Subsequently, cells were incubated at 4°C for 45 min with 1 μg of fluorescein isothiocyanate(FITC)-conjugated avidin(Tago, Burlingame, CA) or with FITC-labeled rabbit anti-rat IgG serum. Cells were washed three times and analysed by a flow cytometry(Spectrum III, Ortho, Ratitan, NJ).

Proliferation assay. The cloned T cells(2×10^4), which had been co-cultured with "stimulator cells" 7 days before, were washed three times and co-cultured at 37°C for 40 hr with 10^6 "stimulator cells" in the presence or absence of 10% IL 2 in 0.2 ml of IMDM medium in 96 flat-bottom multiwell plates. As control, the cloned T cells were cultured with uninfected, gamma-irradiated spleen cells or medium only, in the presence or absence of IL 2. The cultures were then pulsed with 1 μCi of [^{3}H]-thymidine for 8 hr and then [^{3}H]-thymidine uptake was measured by a liquid scintillation counter(LS 9000, Beckman, Fullerton, CA).

Production of lymphokines. The clones T cells(6×10^5) were co-cultured for 24 hr with 2×10^7"stimulator cells" in 5 ml of IL 2-free IMDM medium. Then, supernatants were harvested, exposed to UV lamp, and stored at -20°C.

Assays for IL 2, IL 3 and IFN. IL 2 and IL 3 activities were measured as [^{3}H] -thymidine incorporation by IL 2-dependent CTLL-2[12] and by IL 3-dependent FDC-P2[13] respectively. IFN activity was determined by inhibition of plaque formation induced by vesicular stomatitis virus on L929 cells.[14]

DTH response. DTH response was measured by footpad swelling as previously described[6] with a minor modification. Naive mice received 10^6 cloned T cells and 30 µl of MHV-infected DBT cell lysate at the right hind footpad and the same numbers of the cloned T cells and 30 µl of uninfected DBT cell lysate at the left hind footpad, respectively. Twenty-four, 48 or 72 hr later the footpad thickness was measured and percent increase was calculated by the following formula. Previous report showed that MHV-infected DBT cell lysate without T cell clones did not induce footpad swelling in naive mice.[6]

$$\frac{[\text{Thickness of the left footpad}]-[\text{Thickness of the right footpad}]}{[\text{Thickness of the right footpad}]} \times 100$$

RESULTS

Established T cell clones were examined for surface markers by immunofluorescence and analysed by a flow cytometry. Clone D4C expressed Lyt 1

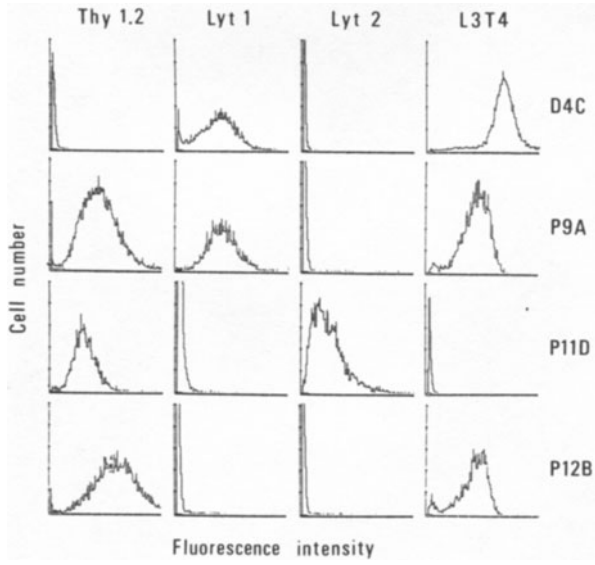

Fig. 1. Surface antigens of MHV-reactive T cell clones by flow cytometry analysis.

Table 1. Proliferation of MHV-reactive T cell clones in response to antigen

Clone	IL 2	[^{3}H]-thymidine incorporation (cpm)		
		Medium	Naive SPC	MHV-infected SPC
D4C	+	34,400	20,900	38,800
	-	500	500	40,100
P9A	+	2,200	2,500	8,800
	-	800	1,200	5,300
P11D	+	2,000	4,000	16,000
	-	1,500	1,200	2,100
P12B	+	3,400	4,400	8,700
	-	1,000	500	9,000

and lacking Thy 1.2 and Lyt 2, while the P9A did Thy 1.2, Lyt 1 and L3T4 lacking Lyt 2. Clone P11D expressed Thy 1.2 and Lyt 2 lacking Lyt 1 and L3T4, and the P12B expressed Thy 1.2 and L3T4 lacking Lyt 1 and Lyt 2. No surface immunoglobulin was detected in all the clones(Fig. 1).

The T cell clones were examined for reactivity for MHV in a proliferation assay. Seven days after co-culturing with "stimulator cells", the T cell clones were co-cultured with MHV-infected and gamma-irradiated spleen cells or those uninfected and gamma-irradiated in either presence or absence of IL 2. In the presence of MHV-infected spleen cells without IL 2, clones D4C, P9A and P12B showed marked proliferation whereas the P11D clone required both MHV-infected spleen cells and exogenous IL 2 for its optimal proliferation(Table 1).

Table 2. H-2 restriction of proliferative response of MHV-reactive T cell clones

Stimulator cell	H-2 haplotype	Infection of stimulator cells	[^{3}H]-thymidine incorporation (cpm)			
			D4C	P9A	P11D	P12B
BALB/c	d	-	300	300	600	200
		+	77,000	63,500	4,800	60,200
BALB/c (nu/nu)	d	-	500	500	200	300
		+	20,400	19,300	4,100	15,100
DBA/2	d	-	400	36,100	300	400
		+	70,500	23,800	1,700	66,700
C57BL/6	b	-	500	900	500	100
		+	600	400	700	500
C3H/He	k	-	300	300	900	7,700
		+	700	500	600	4,600
SJL/J	s	-	800	200	400	200
		+	1,000	300	1,000	300
SWR/J	r	-	600	500	700	200
		+	3,100	700	1,000	500
A/J	a	-	400	500	1,300	700
		+	400	300	2,000	800

Table 3. IL 2, IL 3 and IFN production by MHV-reactive T cell clones

Clone	IL 2	IL 3	IFN
D4C	Yes	Yes	Yes
P9A	Yes	Yes	Yes
P11D	No	No	No
P12B	Yes	Yes	Yes

Next the T cell clones were examined for H-2 restriction in proliferative response. Each clone was co-cultured in the absence of IL 2 with MHV-infected and gamma-irradiated spleen cells or those uninfected and gamma-irradiated from different strains of mice. When co-cultured with MHV-infected spleen cells of BALB/c($H\text{-}2^d$), BALB/c-nu/nu($H\text{-}2^d$) or DBA/2 ($H\text{-}2^d$), the D4C, P9A and P12B clones showed marked proliferation. The response of clone P11D was very weak even in the presence of H-2 compatible MHV-infected cells. However, the P9A clone obviously responded when co-cultured with spleen cells of DBA/2 regardless of MHV infection(Table 2).

For testing lymphokine production by the T cell clones after exposure to MHV antigen, the T cell clones were co-cultured with MHV-infected and gamma-irradiated BALB/c spleen cells in the absence of IL 2. Twenty-four hour later the culture supernatants were sampled and tested for their IL 2, IL 3 and IFN activities. Clone D4C, P9A and P12B were found to produce detectable levels of IL 2, IL 3 and IFN (Table 3).

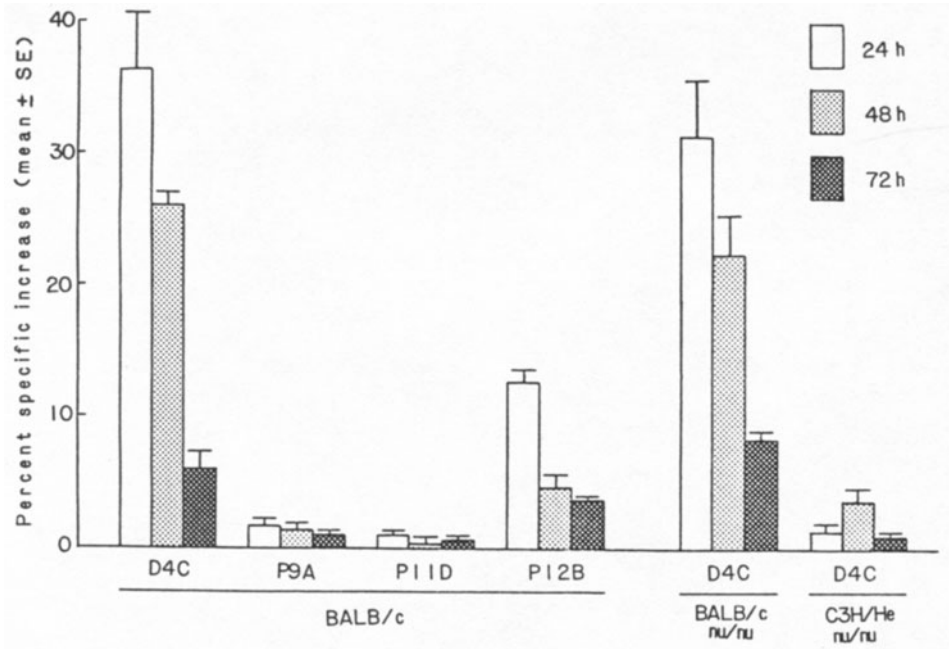

Fig. 2. DTH response induced by MHV-reactive T cell clones.

For testing DTH responses induced by the T cell clones, naive BALB/c mice were injected with the T cell clones and MHV antigen at the hind footpad, and increase of footpad thickness was measured 24, 48 and 72 hr later. The D4C and P12B clones induced a significant DTH response in BALB/c mice, but the P9A and P11D clones did not. Moreover, induction of DTH response by clone D4C was observed in syngeneic BALB/c nude mice but not in H-2 incompatible C3H/He nude mice(Fig. 2).

DISCUSSION

Four MHV-reactive T cell clones established in this study were designated as D4C, P9A, P11D and P12B. These clones were from MHV-infected BALB/c mice and maintained by co-culturing with "stimulator cells", which were MHV-infected and gamma-irradiated syngeneic spleen cells, in the presence of exogenous IL 2 for more than 10 months.

The P9A clone expressed Thy 1.2, Lyt 1 and L3T4 lacking Lyt 2, and was suggested to be of helper/inducer T cells. In fact, this clone release lymphokines such as IL 2, IL 3 and IFN.

Clones D4C having Thy 1.2^-, Lyt 1^+, Lyt 2^- and L3T4$^+$ as well as P12B having Thy 1.2^+, Lyt 1^-, Lyt 2^- and L3T4$^+$ did not have typical helper/inducer T cell markers, while they might be of helper/inducer T cells expressing L3T4 antigen, which may correlate with reactivity to class II MHC product[15]. They mediated DTH response *in vivo* and/or produced IL 2, IL 3 and IFN showing no cytotoxic activity. The present study as well as previous studies describing helper and DTH effector T cell clones secreting lymphokines and lacking Lyt 1 and Lyt 2[5,16] indicated that the absence of Lyt 1 surface antigen was not correlated with disfunction of this type of T cells. Similarly, the lack of expression of Thy 1.2 antigen might not be correlated with disfunction of T cells.

The P11D clone expressing both Thy 1.2 and Lyt 2 lacking Lyt 1 and L3T4 antigens was suggested to be of suppressor/cytotoxic T cells. It did not mediate DTH response nor secrete IL 2, IL 3 and IFN while having cytotoxic acitvity against MHV-infected J774.1 and P388D1 cells in a preliminary experiment(data not shown).

The D4C, P9A and P12B clones were proliferated when co-cultured with MHV-infected and gamma-irradiated BALB/c spleen cells but not when co-cultured with uninfected and gamma-irradiated spleen cells in the absence of exogenous IL 2. The P11D, however, showed a weak response in the absence of exogenous IL 2 when co-cultured with "stimulator cells". These results indicated that the proliferative response might be correlated with IL 2 production by antigen stimulation, supporting a hypothesis that the proliferation of T cell clones may require IL 2 secreted by antigen-stimulated T cells(autocrine mechanism). In addition, the T cell clones were proliferated only when co-cultured with H-2 compatible "stimulator cells", suggesting that T cells were activated after recognition of not only MHV antigen but also MHC-encoded products.

Some of the presently established clones possessed a potential to secrete IL 2, IL 3 and IFN after exposure to MHV antigen. IL 2 and IL 3 are known to be a T cell growth factor[12,17,18] and growth and differentiation factor of multiple stem cells[19], respectively. IFN was shown to have multiple biological activities in immune responses[14]. By these lymphokines produced by MHV-reactive T cells either MHV-specific or non-specific antiviral activity may be augmented.

In other virus infections DTH response has been proposed to manifest

antiviral activity by attracting and activating mononuclear cells[20,21]. The establishment of DTH response in MHV infection was reported using polyclonal and cloned T cells[4,5]. We also indicated that the D4C and P12B induced DTH response in vivo. Local adoptive transfer of DTH effector T cell clones protected mice from lethal MHV infection[4], while the mechanism remains unclear.

Earlier studies showed that T cell-mediated immunity played an important role in protection against MHV infection in mice by adoptive transfer of T cells from immune mice[3]. Subsets of effective T cells in the protection has not been understood clearly. These T cell clones established and characterized in this study may provide useful tools to clarify the role of T cells in MHV infections in mice.

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