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Mutation of neutralizing/antibody-dependent enhancing epitope on spike protein and 7b gene of feline infectious peritonitis virus: Influences of viral replication in monocytes/macrophages and virulence in cats

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ABSTRACT

We previously prepared neutralizing monoclonal antibody (MAb)-resistant (*mar*) mutant viruses using a laboratory strain feline infectious peritonitis virus (FIPV) 79-1146 (Kida et al., 1999). *Mar* mutant viruses are mutated several amino acids of the neutralizing epitope of Spike protein, compared with the parent strain, FIPV 79-1146. We clarified that MAb used to prepare *mar* mutant viruses also lost its activity to enhance homologous *mar* mutant viruses, strongly suggesting that neutralizing and antibody-dependent enhancing epitopes are present in the same region in the strain FIPV 79-1146. We also discovered that amino acid mutation in the neutralizing epitope reduced viral replication in monocytes/macrophages. We also demonstrated that the mutation or deletion of two nucleotides in 7b gene abrogate the virulence of strain FIPV 79-1146.

1. Introduction

Feline infectious peritonitis virus (FIP virus: FIPV), a feline coronavirus (FCoV) of the family *Coronaviridae*, causes a fatal disease called FIP in wild and domestic cat species. Cats that developed FIP were affected in several organs, including the liver, lungs, spleen, and central nervous system, forming lesions accompanied by necrosis and pyogenic granulomatous inflammation (Pedersen, 2009). In some cats, pleural effusion and ascitic fluid accumulated.

FCoV is mainly composed of nucleocapsid (N) protein, envelope (E) protein, membrane (M) protein, and peplomer spike (S) protein (Pedersen, 2009). FCoV is classified into serotypes I and II according to the amino acid sequence of its S protein (Motokawa et al., 1995, 1996). Both serotypes consist of two biotypes: FIPV and feline enteric coronavirus (FECV). FECV infection is asymptomatic in cats. In contrast, FIPV infection causes FIP. It has been proposed that FIPV arises from FECV by mutation (Poland et al., 1996; Vennema et al., 1998), but the exact mutation and inducing factors have not yet been clarified. Stoddart and Scott (1989) reported that the proliferation in peritoneal macrophages was associated with the virulence of FIPV because FECV exhibited a lower proliferation in peritoneal macrophages than FIPV.

FIPV targets monocytes/macrophages, and monocytes/macrophages infection is enhanced in the presence of antibodies (antibody-dependent enhancement, ADE). ADE activity in FIPV infection is induced when anti-FIPV-S antibody-bound viruses infect monocytes/macrophages by binding to the Fc portion of Fc receptors on the cell surface (Hohdatsu et al., 1991; Corapi et al., 1992; Olsen et al., 1992).

The S protein of coronavirus plays an important role in infecting target cells. Coronavirus S protein is divided into S1 and S2 regions (Bosch et al., 2003). The S1 region binds to the cell surface virus receptor, and the S2 region is involved in viral-cell fusion. The presence of neutralizing and antibody-dependent enhancing epitopes in the S1 region of FCoV has also been reported (Corapi et al., 1995; Kida et al., 1999).

We previously prepared a neutralizing monoclonal antibody (MAb)-resistant (*mar*) mutant virus using a virulent type II laboratory strain, FIPV 79-1146 (Kida et al., 1999). *Mar* mutant viruses are those with several amino acids of the neutralizing epitope in the S1 region mutated as compared with the parent strain, FIPV 79-1146. Here, strain FIPV 79-1146, *mar* mutant viruses, and type II laboratory strain FECV 79-1683 were used to compare the differences in proliferation in monocytes/macrophages, and virulence in cats.

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Furthermore, it was investigated whether biological properties of *mar* mutant viruses were related to mutations in the genome.

2. Materials and methods

2.1. Cell cultures and viruses

Felis catus whole fetus-4 (Fcwf-4) cells were grown in Eagles' minimum essential medium containing 50% L-15 medium, 5% fetal calf serum (FCS), and antibiotics. Feline monocytes and alveolar macrophages were cultured in RPMI 1640 medium containing 10% FCS and antibiotics. Strain FIPV 79-1146, strain FECV 79-1683, and all *mar* mutant viruses were grown in Fcwf-4 cells at 37 °C. Strain FIPV 79-1146 was supplied by Dr. M.C. Horzinek of State University Utrecht, The Netherlands. Stain FECV 79-1683 was supplied by Dr. A.J. McKeirnan of Washington State University, Pullman. *Mar* mutant viruses were derived from strain FIPV 79-1146, as reported previously (Kida et al., 1999). Briefly, *mar* mutant viruses were prepared using monoclonal antibody (MAb) 5-7-2, MAb 6-4-2, and MAb 7-4-1. For example, single *mar* mutant virus 5-7-2.C2 was prepared using MAb 5-7-2. Double *mar* mutant viruses were derived from the single *mar* mutant virus. *Mar* 5-7-2.7-4-1 was derived from *mar* 5-7-2.C2, and *mar* 6-4-2.7-4-1 was derived from *mar* 7-4-1.C2.

2.2. Recovery of feline monocytes and alveolar macrophages

Feline monocytes were isolated from specific pathogen-free (SPF) cats as previously described by Dewerchin et al. (2005). Feline alveolar macrophages were obtained by broncho-alveolar lavage with Hanks's balanced salt solution (HBSS) from anti-FCoV antibody-negative SPF cats, as previously described by Hohdatsu et al. (1991).

2.3. ADE and neutralizing activities of MAbs against mar mutant viruses

ADE activity (enhancing titer index) of each MAb was measured employing the method below: virus suspension (1×10^4 $TCID_{50}$) and MAb 5-7-2, MAb 6-4-2, or MAb 7-4-1 solution were mixed at an equivalent volume ratio and reacted at 4 °C for 1 h, and 0.1 ml of this reaction solution was used to inoculate feline alveolar macrophages (2×10^6 cells) cultured in each well of 24-well multi-plates. As control, medium alone was added. After virus adsorption at 37 °C for 1 h, the cells were washed with HBSS and 1 ml of maintenance medium. The culture supernatant was collected after 72 h. The culture supernatant was used for plaque assay. Enhancing titer index ($\log\% = \log[100 \times (A - B)/A]$. A is the number of plaques after reaction with MAb (culture fluid from the hybridoma cell line) and B is the number of plaques after reaction with medium.

Neutralizing activities (neutralizing titer index) of MAb 5-7-2, MAb 6-4-2, and MAb 7-4-1 were measured referring to the method reported by Kida et al. (1999). Neutralization titer index ($\% = 100 \times (C - D)/C$. C is the number of plaques after reaction with medium, and D is the number of plaques after reaction with MAb (culture fluid from the hybridoma cell line).

2.4. Plaque assay

Confluent Fcwf-4 cell monolayers in 24-well multi-plates were inoculated with 100 μl of the sample dilutions. After virus adsorption at 37 °C the cells were washed with HBSS and 1 ml of growth medium containing 1.5% carboxymethyl cellulose was added to each well. The cultures were incubated at 37 °C for 2 days, fixed in 10% buffered formalin, and stained with 1% crystal violet.

2.5. Inoculation of Fcwf-4 cells, feline monocytes, and alveolar macrophages with virus

Confluent Fcwf-4 cells in 24-well multi-plates were inoculated with strain FIPV 79-1146, strain FECV 79-1683, and *mar* mutant viruses (each at 1×10^4 $TCID_{50}$) at 37 °C for 1 h. After washing, the cells were cultured in media, and the culture supernatants were collected every 6–12 h. Feline alveolar macrophages (2×10^6 cells) or feline monocytes (2×10^5 cells) in 24-well multi-plates were inoculated with strain FIPV 79-1146, strain FECV 79-1683, and *mar* mutant viruses (each at 1×10^4 $TCID_{50}$) at 37 °C for 1 h. After washing, the cells were cultured in the media, and the culture supernatants were collected every 24 h.

2.6. Experimental animals

Strain FIPV 79-1146, strain FECV 79-1683, and *mar* mutant viruses (each at 10^4 $TCID_{50}$/ml) were administered orally to 46 SPF cats (6- to 8-month-old). These cats were checked daily for clinical signs, and we measured their body temperature and weight. Cats were euthanized when reaching a previously determined the humane endpoint or 60 days after challenge. We set the humane endpoint as meeting two of the following points: lack of appetite (more than 5 days), extreme anemia due to dyspnea (prolongation of capillary refill time), fever of 40 °C or higher followed by body temperature reduction (below 38 °C), and jaundice of visible mucosa. Cats showing neurological symptoms were immediately euthanized. FIP diagnoses were confirmed upon postmortem examination, revealing peritoneal and pleural effusions, and pyogranuloma in major organs. All experiments were performed in accordance with the Guidelines for Animal Experiments of Kitasato University.

2.7. Virus genomic RNA isolation and cDNA preparation

Virus genomic RNA was extracted from virus-infected Fcwf-4 cells using a High Pure RNA Isolation Kit (Roche Diagnostics, Switzerland) according to the instructions of the manufacturer. RNA was dissolved in elution buffer. Using virus genomic RNA as a template, cDNA was synthesized using Ready-to-Go RT-PCR beads (GE Healthcare Life Sciences, U.S.A.). Reverse transcription was performed in a 50-μl final volume containing 0.5 μg $oligo(dT)_{12-18}$ primers. The resulting solution was incubated at 42 °C for 1 h to synthesize cDNA.

2.8. Sequence analysis of virus genomes

cDNA was amplified by PCR. The primer sequences and target regions are shown in Fig. 6 and Table 2. PCR was performed in a total volume of 50 μl. One microliter of sample cDNA was mixed with 10 μl of 5-fold PrimeSTAR Buffer (TaKaRa, Japan), 4 μl of dNTP Mixture (TaKaRa, Japan) containing 2.5 mM of each dNTP, 1 μl of 20 μM primer mix, 0.5 μl of PrimeSTAR HS DNA Polymerase (2.5 U/ml; TaKaRa, Japan), and 33.5 μl of distilled water. Using a PCR Thermal Cycler Dice (TaKaRa, Japan), the DNA was amplified at 98 °C for 1 min, followed by 30 cycles of denaturation at 98 °C for 10 s, primer annealing at 55 °C for 15 s, and synthesis at 72 °C for 1 min, with a final extension at 72 °C for 5 min.

PCR products (21 μl) were electrophoresed with DNA markers on 1.5% agarose gel. Singlet bands were excised and transferred to microtubes, and DNA was purified using a QIAquick Gel Extraction Kit (QIAGEN GmbH, Germany). The purified DNA was sent to Sigma Aldrich (Japan) for sequencing. The sequences of virus genomes were determined and analyzed employing the GENETYX computer program (Software Development, Japan).

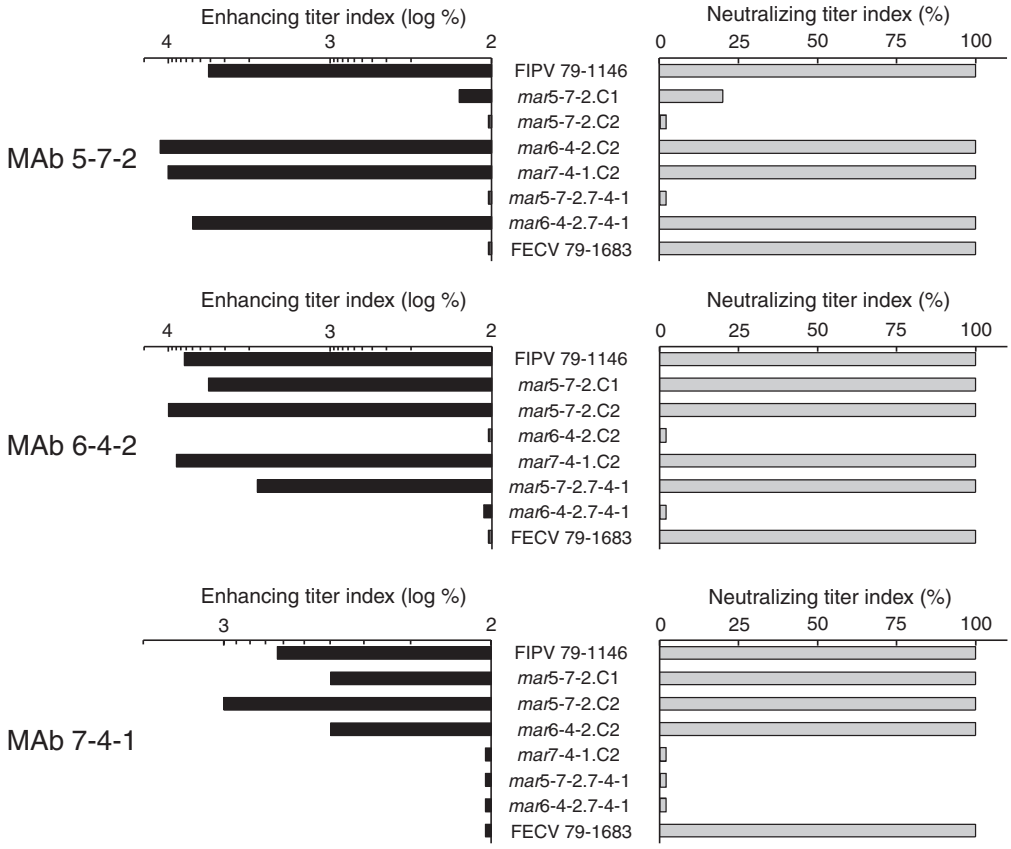

Fig. 1. Comparison of neutralizing and ADE activities for each virus among MAb 5-7-2, MAb 6-4-2, and MAb 7-4-1. The left graph (black bar) shows the enhancing titer index (%) of MAb for each virus ($n = 3$). The right graph (gray bar) shows the neutralization titer index (%) of MAb against each virus ($n = 3$). Enhancing titer index (log %) = log $[100 \times (A - B)/A]$. A is the number of plaques after reaction with MAb (culture fluid from the hybridoma cell line) and B is the number of plaques after reaction with medium. Neutralization titer index (%) = $100 \times (C - D)/C$. C is the number of plaques after reaction with medium, and D is the number of plaques after reaction with MAb (culture fluid from the hybridoma cell line).

2.9. Statistical analysis

The data was analyzed using the one-way ANOVA test. The data in Fig. 5 was analyzed employing the Mann–Whitney test. p-Values <0.05 were considered to indicate a significant difference between compared groups.

3. Results

3.1. ADE and neutralizing activities of MAb 5-7-2, MAb 6-4-2, and MAb 7-4-1 against mar mutant viruses

ADE and neutralizing activities of MAb 5-7-2, MAb 6-4-2, and MAb 7-4-1 against mar mutant viruses were analyzed (Fig. 1). All these MAbs exhibited potent neutralizing and ADE activities for the parent strain, FIPV 79-1146. On the analysis of *mar* mutant virus reactivity with each MAb, both neutralizing and ADE activities of MAb 5-7-2 were reduced for *mar* 5-7-2.C1, *mar* 5-7-2.C2, and *mar* 5-7-2.7-4-1. Reductions of both neutralizing and ADE activities of MAb 6-4-2 for *mar* 6-4-2.C2 and *mar* 6-4-2.7-4-1 and MAb 7-4-1 against *mar* 7-4-1.C2, *mar* 5-7-2.7-4-1, and *mar* 6-4-2.7-4-1 were also observed. Interestingly, the three MAbs exhibited potent neutralizing activity but no ADE activity for the FECV 79-1683 strain.

3.2. Virus replication in Fcwf-4 cells, feline monocytes, and alveolar macrophages

Replication of the *mar* mutant virus in Fcwf-4 cells, feline monocytes, and alveolar macrophages was investigated (Fig. 2). No significant difference was noted in virus production among Fcwf-4 cells inoculated with strain FIPV 79-1146, *mar* mutant viruses, and strain FECV 79-1683. In contrast, virus production was significantly decreased in feline monocytes and alveolar macrophages inoculated with *mar* mutant virus *mar* 5-7-2.C2, *mar* 5-7-2.7-4-1, *mar* 6-4-2.7-4-1, and FECV 79-1683, compared with strain FIPV 79-1146. In particular, viral production of *mar* 5-7-2.C2, *mar* 5-7-2.7-4-1, and FECV 79-1683 was markedly decreased in feline alveolar macrophages.

3.3. Virulence of mar mutant viruses for SPF cats

The virulence of *mar* mutant viruses were investigated using SPF cats. Changes in the body weight and temperature after mar mutant virus inoculation were measured. In all cats, the body weight did not markedly change, but tended to decrease 1–2 weeks before FIP development in cats developing FIP (Fig. 3). The body temperature rose 2–4 days after virus inoculation in all cats (Fig. 4). The body temperature markedly changed in cats inoculated with FIPV 79-1146, whereas the variation was small in cats inoculated with FECV 79-1683. In cats inoculated with *mar* 6-4-2.C2, *mar* 7-4-1.C2, and *mar* 6-4-2.7-4-1, body temperature changes were similar to those in FIPV 79-1146-inoculated cats, and the changes in cats inoculated with *mar* 5-7-2.C1, *mar* 5-7-2.C2, and *mar* 5-7-2.7-4-1 were similar to those in FECV 79-1683.

Fig. 5 shows the survival rate of cats inoculated with strain FIPV 79-1146 ($n = 13$), strain FECV 79-1683 ($n = 3$), and *mar* mutant viruses (each, $n = 5$). The survival rate of cats inoculated with strain FIPV 79-1146 was 23%. The survival rates in cats inoculated with

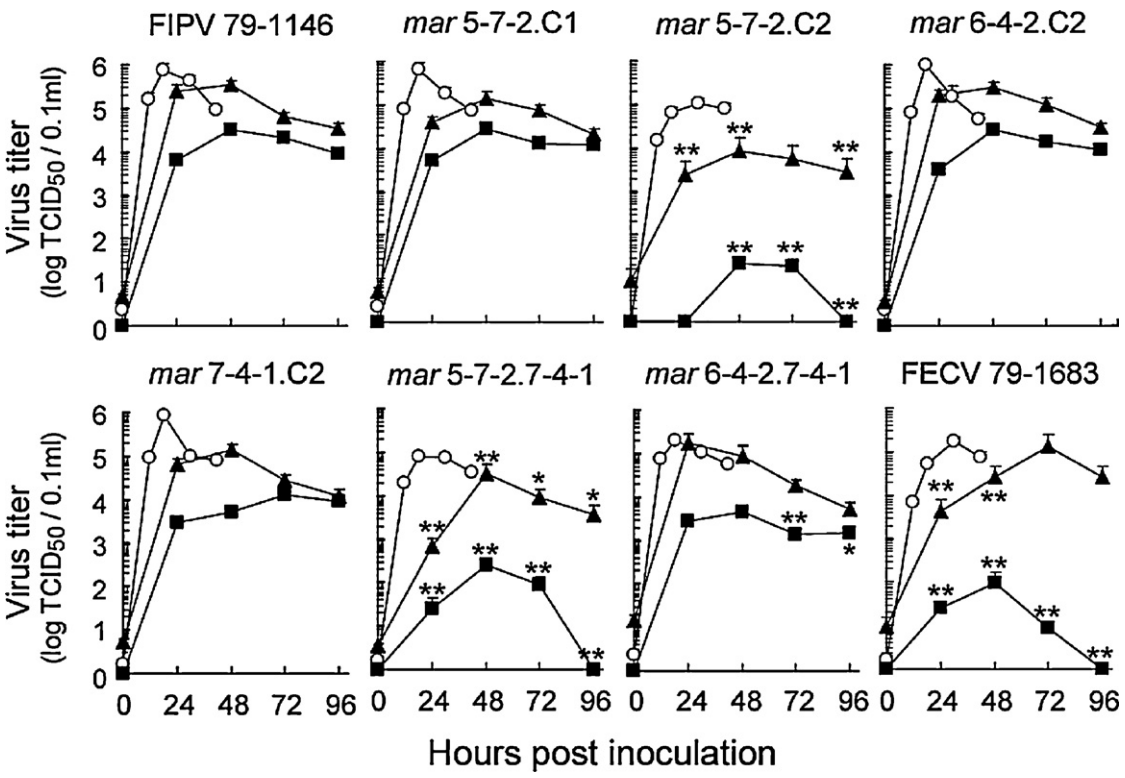

Fig. 2. Growth kinetics of *mar* mutant viruses in Fcwf-4 cells, monocytes, and alveolar macrophages. Cells were infected with strain FIPV 79-1146, strain FECV 79-1683, and *mar* mutant viruses, and incubated at 37 °C. Virus titers in the culture supernatants at different times after inoculation were measured. Circle: Fcwf-4 cells ($n = 3$), triangle: monocytes ($n = 9$), square: alveolar macrophages ($n = 5$). $^{**}p < 0.01$ vs. strain FIPV 79-1146, $^{*}p < 0.05$ vs. strain FIPV 79-1146.

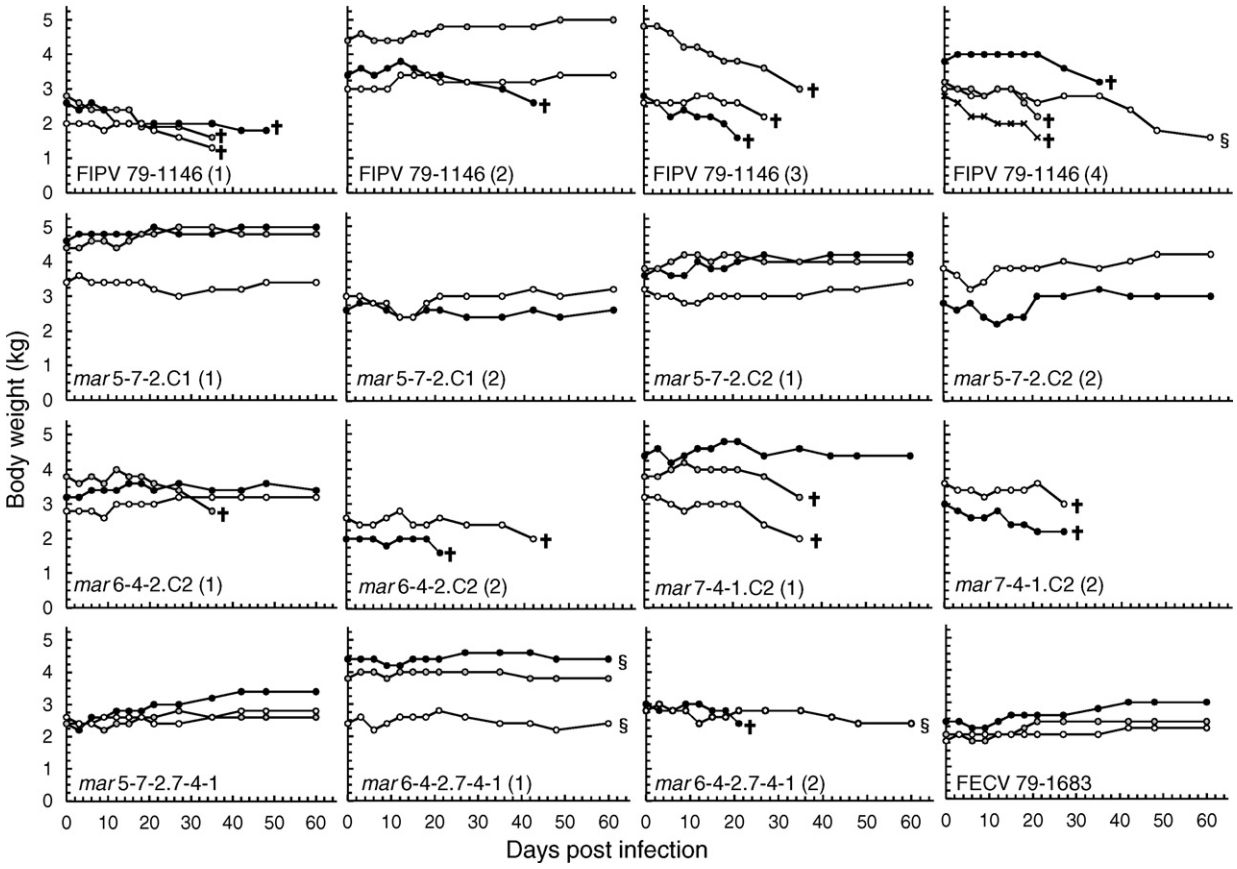

Fig. 3. Body weight after *mar* mutant virus infection in cats. (§) FIP development was confirmed at completion of the experiment (60 days after virus inoculation). (+) Animal was euthanized because its clinical condition reached the humane endpoint.

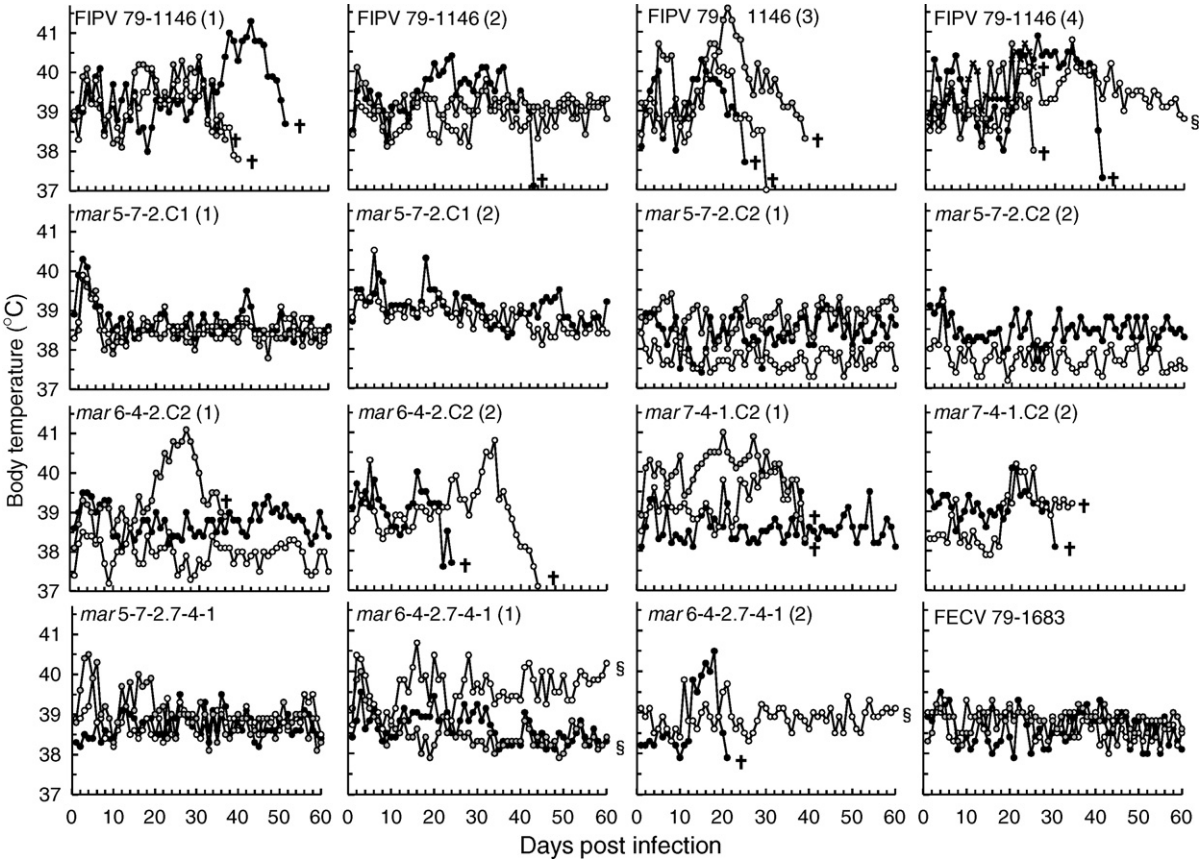

Fig. 4. Body temperature after *mar* mutant virus infection in cats. (§) FIP development was confirmed at completion of the experiment (60 days after virus inoculation). (+) Animal was euthanized because its clinical condition reached the humane endpoint.

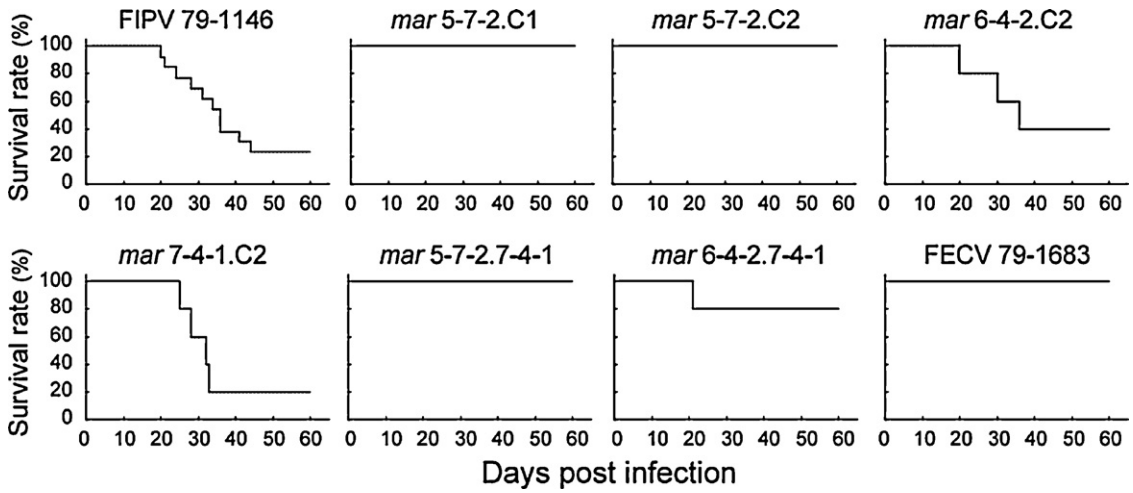

Fig. 5. Survival rate after *mar* mutant virus infection in cats.

mar 6-4-2.C2 and *mar* 7-4-1.C2 were 40% and 20%, respectively, showing no significant difference compared with that in cats inoculated with strain FIPV 79-1146. In contrast, *mar* 5-7-2.C1, *mar* 5-7-2.C2, *mar* 5-7-2.7-4-1, and *mar* 6-4-2.7-4-1 showed a significant decrease ($p < 0.01$) in virulence in SPF cats compared to strain FIPV 79-1146. Cats euthanized 60 days after virus inoculation were autopsied. In a cat inoculated with FIPV 79-1146, granulomatous lesions were present in the omentum and intestine. In three cats inoculated with *mar* 6-4-2.7-4-1, pleural effusions and granulomatous lesions were observed in the lung. No lesion was observed in any other cat.

3.4. Sequence analysis of the genome of mar mutant viruses

The nucleotide sequences of the genome of *mar* mutant viruses were determined, and compared with strain FIPV 79-1146. We referred to the report by Kida et al. (1999) for the nucleotide sequences of the ORF 2 (S) of *mar* mutant viruses. In *mar* mutant virus, the nucleotide sequence in the S1 region of the S gene was mutated (Table 1). In the ORF7b genes of *mar* 5-7-2.C1, *mar* 5-7-2.C2, and *mar* 5-7-2.7-4-1, nucleotides 531 and 532 of the ORF7b gene were deleted, and a nonsense mutation was found. In these viruses, 29 amino acids at the N-terminal of the 7b protein were

Table 1
Differences in the predicted amino acid sequences, virulence in cats, and cell proliferation between strain FIPV 79-1146 and *mar* mutant viruses.

Virus	ORF 1ab	ORF 2 (S)		ORF 3abc	ORF 4 (E)	ORF 5 (M)	ORF 6 (N)	ORF 7		Pathogenicity in cats	Proliferation of cells	
		S1	S2					a	b		Monocyte	Macrophage
FIPV 79-1146	*	*	*	*	*	*	*	*	*	High	High	High
mar 6-4-2.C2	*	T → K (554)[a,b]	*	*	*	*	*	*	*	High	High	High
mar 7-4-1.C2	*	A → V (480)[a] R → C (647)[a,b]	*	*	*	*	*	*	*	High	High	High
mar 6-4-2.7-4-1	*	A → V (480)[a] T → K (554)[a,b] R → K (647)[a,b]	*	*	*	*	*	*	*	Moderate to low	Moderate to high	Moderate to low
mar 5-7-2.C1	*	L → S (589)[a,b]	*	*	*	*	*	*	Deleted (N-terminal 29aa)	Low	High	High
mar 5-7-2.C2	*	M → T (542)[a,b] L → S (589)[a,b]	*	*	*	*	*	*	Deleted (N-terminal 29aa)	Low	Low	Low
mar 5-7-2.7-4-1	*	M → T (542)[a,b] L → S (589)[a,b] R → L (647)[a,b]	*	*	*	*	*	*	Deleted (N-terminal 29aa)	Low	Low	Low

The predicted amino acid sequence conserved between strain FIPV 79-1146 and FIPV *mar* mutant virus indicated by asterisks.
[a] These information were referred to the article by Kida et al. (1999).
[b] Amino acids present in the neutralizing epitope (A1 and A2 sites) of FIPV 79-1146 (Corapi et al., 1995; Kida et al., 1999).

deleted (Table 1). No mutation was found in the ORF 1a/b, ORF 3abc, ORF 4 (E), ORF 5 (M), ORF 6 (N), and ORF 7a of *mar* mutant viruses.

4. Discussion

Mar mutants of MHV, Ross River virus, rabies virus, and Venezuelan equine encephalitis virus became less virulent than their parent strains (Coulon et al., 1998; Johnson et al., 1990; Vrati et al., 1996; Wang et al., 1992). However, the virulence of *mar* mutant virus of FIPV in cats remains unexamined. Cats inoculated with strain FIPV 79-1146 frequently develop FIP. Specifically, the *mar* mutant virus of strain FIPV 79-1146, used to inoculate cats, clearly demonstrates changes in virulence. Furthermore, strain FIPV 79-1146 is suitable for examining the correlation between the proliferation of FIPV in monocytes/macrophages and its virulence in cats because of the high proliferation of strain FIPV 79-1146 in monocytes/macrophages.

Corapi et al. (1995) and Hohdatsu et al. (1993) reported that FIPV 79-1146-neutralizing MAb exhibited potent ADE activity in macrophages. Their report suggested that neutralizing and antibody-dependent enhancing epitopes are present in the same region in FIPV 79-1146, but this hypothesis has not been verified. We revealed that MAbs which lost neutralizing activity against mar mutant viruses also lost ADE activity, showing that neutralizing and antibody-dependent enhancing epitopes are present in the same region of the S protein in FIPV 79-1146, as hypothesized. On the other hand, MAb which neutralized FECV 79-1683 on fcwf-4 cells did not exhibit ADE activity on macrophages. The reason for the absence of ADE activity for FECV 79-1683 is unclear. FECV 79-1683

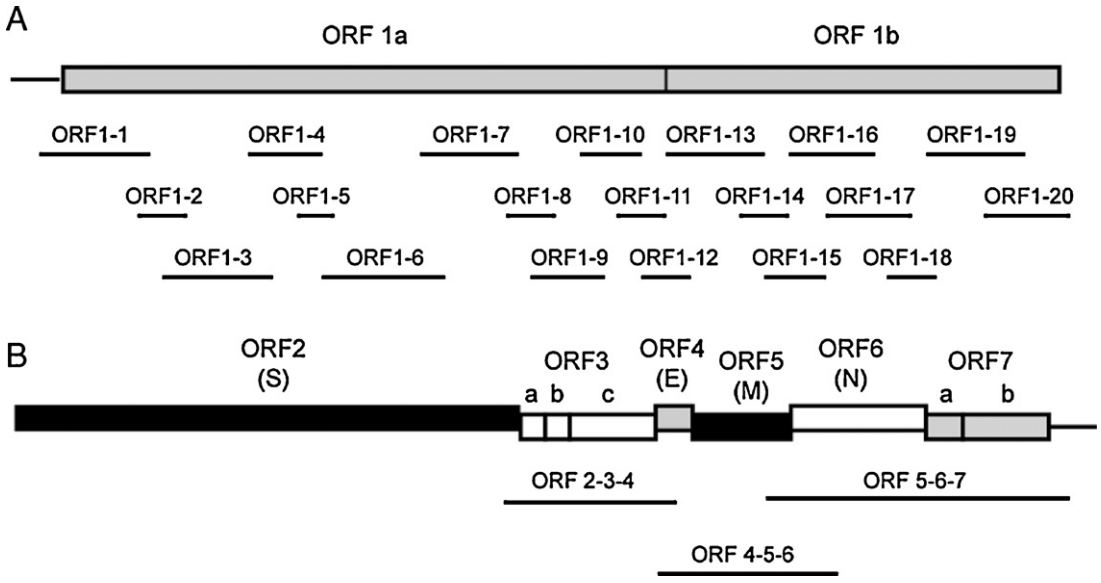

Fig. 6. Genome of feline coronavirus and PCR products. (A) ORF 1ab. (B) ORF 2, ORF 3abc, ORF 4, ORF 5, ORF 6, and ORF 7ab. Bold lines indicate the region which are shown in Table 2.

Table 2
Sequences of primers.

Region	Primer	Orientation	Nucleotide sequence (5′–3′)	Length (bp)	Reference
ORF1-1	orf1-1f	Forward	ACCAGTTTGGCAATCACTCC	2022	GenBank Accession No. AY994055
	orf1-1r	Reverse	AGCAATAGCAGCATCGAGGT		
ORF1-2	orf1-2f	Forward	TGCTGATGCTTGGAAAGTTG	968	
	orf1-2r	Reverse	TGTACCAGATGGCACCAAAA		
ORF1-3	orf1-3f	Forward	ACCTCGATGCTGCTATTGCT	2281	
	orf1-3r	Reverse	GCCACCCACGTGTCTAAGAT		
ORF1-4	orf1-4f	Forward	CGTGGAAGGTTCTGGTGTTT	1621	
	orf1-4r	Reverse	TAGGCCTTATCGGCATCAAC		
ORF1-5	orf1-5f	Forward	TCTTTTGGTTTGTGGCACTG	662	
	orf1-5r	Reverse	ACAGGGCTGGTTTGTTCAAT		
ORF1-6	orf1-6f	Forward	AGGTCCTGTAGTGGGTGACG	2325	
	orf1-6r	Reverse	GGGCAGGACTTGTCAAATGT		
ORF1-7	orf1-7f	Forward	ACCTCGACGTGACATTCTCC	1940	
	orf1-7r	Reverse	TTGCCATAAGCCACCCTTAC		
ORF1-8	orf1-8f	Forward	TACTGGTTCGATGGGAGAGG	1007	
	orf1-8r	Reverse	CAGTAGGCGTGAATTCGTCA		
ORF1-9	orf1-9f	Forward	GCTGGTACCTGTGGGTCAGT	1686	
	orf1-9r	Reverse	GCACGAGCTTTTTCATAGGC		
ORF1-10	orf1-10f	Forward	TGTAATGCAGCCAGCTTTTG	1202	
	orf1-10r	Reverse	TACCATAAGCATCGCCATCA		
ORF1-11	orf1-11f	Forward	CTTGACAAAATGGCAGAGCA	1048	
	orf1-11r	Reverse	CGGTTGGTACTTGGACGAAT		
ORF1-12	orf1-12f	Forward	TGATGGCGATGCTTATGGTA	915	
	orf1-12r	Reverse	CTCATGGTCCATAACGCTCTT		
ORF1-13	orf1-13f	Forward	CTGCAATTGATGGATTGTGC	1952	
	orf1-13r	Reverse	TAAGCATGTTGTCCCAACCA		
ORF1-14	orf1-14f	Forward	GCGTGGATTCTTTGAAGAGG	865	
	orf1-14r	Reverse	GTTTGAATCAACGCCCAAAA		
ORF1-15	orf1-15f	Forward	ATTGCACAGGCGGTTTTTAC	1261	
	orf1-15r	Reverse	TTCCTTAGGGCCGATAACCT		
ORF1-16	orf1-16f	Forward	TTAGCGTTGGACCACATGAA	1893	
	orf1-16r	Reverse	TGTGTGCTAAAAACGCCTTG		
ORF1-17	orf1-17f	Forward	AGGTTATCGGCCCTAAGGAA	1806	
	orf1-17r	Reverse	TCTGCACTATACGGCGTCTG		
ORF1-18	orf1-18f	Forward	TGTTGCCATTACGAGAGCAA	1105	
	orf1-18r	Reverse	ACCCACATCATGAATTGCAG		
ORF1-19	orf1-19f	Forward	CGGGATCTTTGAGCATGAAT	2085	
	orf1-19r	Reverse	GCATCGTCTGGTAACCACCT		
ORF1-20	orf1-20f	Forward	GCTGTGAAAGGGCTTAGTGC	1791	
	orf1-20r	Reverse	TGCTATTTTCCATGGCTTCC		
ORF2-3-4	Primer 1	Forward	GCCATTCTCATTGATAAC	1491	Haijema et al. (2004)
	Primer 9	Reverse	CAGGAGCCAGAAGAAGACGCTAA		GenBank Accession No. AY994055
ORF4-5-6	Mpr1	Forward	TGAATGACCTCACGTTGCAT	1532	GenBank Accession No. AY994055
	Mpr2	Reverse	TTCCTTACGCTGGCCTTTTA		
ORF5-6-7	Primer 11	Forward	GGTGATTACTCAACAGAAGC	2155	Haijema et al. (2004)
	Primer 12	Reverse	GACCAGTTTTAGACATCG		GenBank Accession No. AY994055

exhibits 90% or higher amino acid sequence homologies with structural proteins (S, E, M, and N) and ORF 7a of the FIPV 79-1146 strain, but homology with non-structural proteins other than ORF 7b was less than 65%. Moreover, non-structural protein-encoding ORF 1ab of FECV 79-1683 has not been analyzed. To investigate the difference in ADE activity between FECV 79-1683 and FIPV 79-1146, the investigation of differences in the replication mode including the cellular invasion process in monocytes/macrophages is necessary.

Corapi et al. (1995) and Kida et al. (1999) investigated the neutralizing epitope in FIPV 79-1146 using mar mutant viruses. They divided the neutralizing epitope in the S1 region of S protein into A1 (residues 542–591) and A2 (residues 643–656) sites, but it was unclear whether the A1 and A2 sites are involved in the replicating ability of FIPV 79-1146 in monocytes/macrophages. We confirmed that the viral replicating ability in monocytes/macrophages was reduced in viruses with two or more amino acid mutations in the A1 and A2 sites (*mar* 5-7-2.C2, *mar* 5-7-2.7-4-1, and *mar* 6-4-2.7-4-1), suggesting that the A1 and A2 sites of FIPV 79-1146 are important for viral replication in monocytes/macrophages.

In *mar* 5-7-2.C1, *mar* 5-7-2.C2, and *mar* 5-7-2.7-4-1, nucleotides 531 and 532 of the ORF7b gene were deleted, and nucleotides 534–536 were mutated into a stop codon (TGA). It has been proposed that FIPV becomes less virulent when the 7b gene is deleted (Herrewegh et al., 1995). In FIPV, 7b gene is easily deleted after viral passage in cells. However, no deletion was found in the 7b gene of *mar* 6-4-2.C2 and *mar* 7-4-1.C2, in spite of undergoing the same number of passages as *mar* 5-7-2.C1 and *mar* 5-7-2.C2. The reason why the 7b gene is deleted after the passage of FIPV is unknown. Further studies are needed in the future.

Mar 5-7-2.C1 containing two nucleotide deletions in the 7b gene replicated well in monocytes/macrophages despite the virulence for cats being lost, showing that the reduced virulence of FIPV with 7b gene mutation is not related to the replicating ability in monocytes/macrophages. The function of protein encoded by the 7b gene (7b protein) in FIPV-infected cats is unclear. A non-structural protein of FCoV similar to 7b protein, 3c protein, has been suggested to be involved in viral transfer from intestinal epithelial cells to macrophages (Chang et al., 2010), but the essential functions of 3c and 7b proteins have not been analyzed. It is necessary to prepare recombinant proteins of these and investigate their functions.

In every round of replication multiple mutations might be expected, due to the infidelity of RNA-dependent RNA polymerase of coronaviruses (Woo et al., 2009). In FCoV, multiple mutations induced by several passages have been observed in FIPV strain

Black (FIPV Black: type I FCoV) (Tekes et al., 2008). However, a few mutations were found in genome despite the fact that *mar* mutant viruses were generated from FIPV 79-1146 (type II FCoV) during several passages, independently of each other in different cultures in the presence of the neutralizing monoclonal antibodies. Although the reason for this contradiction is unclear, a difference in the fidelity of RNA-dependent RNA polymerase between types I and II FCoV may have been involved. Vignuzzi et al. (2006) suggested that genomic diversity decreases in viruses with high-fidelity RNA-dependent RNA polymerase. Actually, many gene mutations were observed among type I FCoV strains, but no mutation was detected in type II FCoV. Further investigation is necessary concerning the relationship between FCoV polymerase fidelity and genomic mutation.

The proliferation of *mar* mutant viruses in monocytes/macrophages tended to be correlated with the virulence in cats. However, *mar* 5-7-2.C1 becomes less virulent in cats, although it proliferated well in monocytes/macrophages *in vitro*. This result is inconsistent with a theory that the proliferation of FCoV in monocytes/macrophages correlates with virulence. Pedersen (2009) reported that oral inoculation using strain FIPV 79-1146 caused FIP in 92.6% of cats, and intraperitoneal inoculation, 100%. In contrast, oral inoculation using strain FIPV UCD4 caused FIP in 0% of cats, and intraperitoneal inoculation, 37.5%. Thus, *mar* 5-7-2.C1 may also become virulent via other inoculation routes. In the future, the association among the proliferation in monocytes/macrophages, onset of FIP, and the developmental mechanism via a parenteral transmission route should be examined using strain *mar* 5-7-2.C1 and other *mar* mutant viruses. In addition, it is necessary to prepare MAb 5-7-2-resistant mutant virus without the mutation of regions other than the neutralizing epitope and perform a similar experiment.

It has been proposed that FIPV arises from FECV by mutation (Poland et al., 1996; Vennema et al., 1998). However, recently, Brown et al. (2009) theoretically demonstrated that FIP develops via the horizontal infection of FIPV. On the basis of their theory, FIPV and FECV are prevalent among cats. As mentioned above, some FIPV may cause few clinical symptoms via some transmission routes. As indicated in our results, the virulence of FIPV alters based on a few amino acid changes. From these results, FCoV, including FIPV, is regarded as a group of various virulent viruses. In the future, changes in virulence caused by gene mutations and the manifestation of virulence due to transmission routes should be examined for FCoV to comprehensively elucidate the virulence of FIPV.

Involvement of FIPV replication in monocytes/macrophages in the aggravation of FIP has been suggested. If an agent which inhibits FIPV infection of monocytes/macrophages is developed, it may be used to treat FIP. For example, neutralizing epitope-binding peptidomimetics may inhibit FIPV infection of monocytes/macrophages without exhibiting ADE activity. However, the neutralizing or antibody-dependent enhancing epitope of type I FIPV, accounting for about 80% of field FIPV, has not been identified, unlike type II FIPV. To prepare a therapeutic drug targeting the neutralizing epitope, identification of the neutralizing epitope in type I FIPV is necessary. Recently, a method for preparing type I FCoV employing a reverse genetics system has also been established (Tekes et al., 2008). In the future, it will be desirable to examine the neutralizing/antibody-dependent enhancing epitope of type I FCoV *in vitro* and *in vivo*.

Our results indicated that the virulence of FIPV is based on a few amino acid changes. Indeed, a few amino acid changes were demonstrated in the mar mutant viruses but the viruses with reduced virulence also had the deletion in the 7b gene. ORF 7b gene deletions are probably readily acquired during passage of FCoV *in vitro* and have been suggested to correlate with loss of virulence (Herrewegh et al., 1995). We speculated that important virulence mutations actually related to deletions in 7b gene in this study. We suggested that mutations in the neutralizing epitope of type II FIPV and deletions in ORF 7b gene are related to loss of virulence also in cats. In descried above, the FCoV-infected cats developing FIP are not type II FIPV in fields, and it is mostly type I FIPV (Hohdatsu et al., 1992; Kummrow et al., 2005; Pedersen, 2009). It is necessary to perform a similar study concerning type I FIPV.

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