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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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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 of FCoV 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 mutared 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.

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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.
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 MAb against mar mutant viruses

ADE activity (enhancing titer index) of each MAb was measured employing the method below: virus suspension (1 × 10^6 TCID50) 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 × (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 × (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 × 10^5 TCID50) 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 × 10^5 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 TCID50) 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 TCID50/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 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 post-mortem 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).
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 5-7-2.7-4-1, 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

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**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 × (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 × (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).
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         |           |           |       |                       |                        |
| FIPV 79-1146  | *       | *         | *        | *         | *         | *         | *     | High                  | High                   |
| mar 6-4-2.C2  | T → K (554)*,b | * | * | * | * | * | * | High | High |
| mar 7-4-1.C2  | A → V (480)* | * | * | * | * | * | * | High | High |
| mar 6-4-2.7-4-1| T → K (554)*,b | A → V (480)* | * | * | * | * | * | Moderate to low | Moderate to high |
| mar 5-7-2.C1  | T → K (554)*,b | L → S (589)*,b | * | * | * | * | * | Deleted (N-terminal 29aa) | Low |
| mar 5-7-2.C2  | M → T (542)*,b | L → S (589)*,b | * | * | * | * | * | Deleted (N-terminal 29aa) | Low |
| mar 5-7-2.7-4-1| M → T (542)*,b | L → S (589)*,b | * | * | * | * | * | Deleted (N-terminal 29aa) | Low |

The predicted amino acid sequence conserved between strain FIPV 79-1146 and FIPV mar mutant virus indicated by asterisks.

* 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 mar mutant virus of 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 MAbs 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, MAbs 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...
exhibits 90% or higher amino acid sequence homologies with struc-
tural 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
exhibited by several passages have been observed in FIPV strain
induced by several passages have been observed in FIPV strain
replicated well in monocytes/macrophages despite the virulence
replication ability in monocytes/macrophages was
confirmed that the viral replicating ability in monocytes/macrophages was
unclear whether the A1 and A2 sites are involved in the replicating
A1 (residues 542–591) and A2 (residues 643–656) sites, but it was
divided the neutralizing epitope in the S1 region of S protein into
neutering epitope in FIPV 79-1146 using mar mutant viruses. They
investigation of differences in the replication mode including the
ence in ADE activity between FECV 79-1683 and FIPV 79-1146, the
FECV 79-1683 has not been analyzed. To investigate the differ-
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 mono-
protein encoded by the 7b gene
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
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to be involved in viral transfer from intestinal epithelial cells to
macrophases.

Table 2 Sequences of primers.

| Region | Primer | Orientation | Nucleotide sequence (5’–3’) | Length (bp) | Reference |
|--------|--------|-------------|-----------------------------|-------------|-----------|
| ORF1-1 | orf1-1f | Forward     | ACCACCTTTGGCAGAATCCTCC      | 2022        | GenBank Accession No. AY994055 |
| ORF1-2 | orf1-2f | Reverse     | GCAATAACGACCATGAGGT          | 968         |           |
| ORF1-3 | orf1-3f | Forward     | TTGCTGATGCTGAGGAATTTG        | 2281        |           |
| ORF1-4 | orf1-4f | Reverse     | TTGCTGATGCTGAGGAATTTG        | 1621        |           |
| ORF1-5 | orf1-5f | Reverse     | TTGCTGATGCTGAGGAATTTG        | 2325        |           |
| ORF1-6 | orf1-6f | Forward     | TTGCTGATGCTGAGGAATTTG        | 662         |           |
| ORF1-7 | orf1-7f | Forward     | TTGCTGATGCTGAGGAATTTG        | 1940        |           |
| ORF1-8 | orf1-8f | Forward     | TTGCTGATGCTGAGGAATTTG        | 1007        |           |
| ORF1-9 | orf1-9f | Forward     | TTGCTGATGCTGAGGAATTTG        | 1893        |           |
| ORF1-10| orf1-10f| Forward     | TTGCTGATGCTGAGGAATTTG        | 915         |           |
| ORF1-11| orf1-11f| Forward     | TTGCTGATGCTGAGGAATTTG        | 1202        |           |
| ORF1-12| orf1-12f| Forward     | TTGCTGATGCTGAGGAATTTG        | 1048        |           |
| ORF1-13| orf1-13f| Forward     | TTGCTGATGCTGAGGAATTTG        | 915         |           |
| ORF1-14| orf1-14f| Forward     | TTGCTGATGCTGAGGAATTTG        | 1952        |           |
| ORF1-15| orf1-15f| Forward     | TTGCTGATGCTGAGGAATTTG        | 865         |           |
| ORF1-16| orf1-16f| Forward     | TTGCTGATGCTGAGGAATTTG        | 1261        |           |
| ORF1-17| orf1-17f| Forward     | TTGCTGATGCTGAGGAATTTG        | 1893        |           |
| ORF1-18| orf1-18f| Forward     | TTGCTGATGCTGAGGAATTTG        | 915         |           |
| ORF1-19| orf1-19f| Forward     | TTGCTGATGCTGAGGAATTTG        | 1105        |           |
| ORF1-20| orf1-20f| Forward     | TTGCTGATGCTGAGGAATTTG        | 2085        |           |
| ORF2-3-4| Primer 1| Forward     | GCCACCTTTCATGTTGAC            | 1491        | GenBank Accession No. AY994055 |
| ORF4-5-6| Mpr1    | Forward     | GCTTGATGCTGAGGAATTTG        | 1532        | GenBank Accession No. AY994055 |
| ORF5-6-7| Primer 11| Forward   | GCTTGATGCTGAGGAATTTG        | 2155        | GenBank Accession No. AY994055 |
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 pepitidometimics 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 described 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.

References

Brown, M.A., Troyer, J.L., Pecon-Starrhey, J., Roeke, M.E., O’Brien, S.J., 2009. Genetics and pathogenesis of feline infectious peritonitis virus. Emerg. Infect. Dis. 15, 1445–1452.

Bosch, B.J., van der Zee, R., de Haan, C.A., Rottier, P.J., 2003. The coronavirus spike protein is a class I virus fusion protein: structural and functional characterization of the fusion core complex. J. Virol. 77, 8801–8811.

Chang, Y.H., de Groot, R.J., Egberink, H.F., Rottier, P.J., 2010. Feline infectious peritonitis: insights into feline coronavirus pathobiography and epidemiology based on genetic analysis of the viral 3c gene. J. Gen. Virol. 91, 415–420.

Corapi, W.V., Darrel, R.J., Audonnet, J.C., Chappuis, G.E., 1995. Localization of antigenic sites of the S glycoprotein of feline infectious peritonitis virus involved in neutralization and antibody-dependent enhancement. J. Virol. 69, 2858–2862.

Corapi, W.V., Olsen, C.W., Scott, F.W., 1992. Monoclonal antibody analysis of neutralization and antibody-dependent enhancement of feline infectious peritonitis virus. J. Virol. 66, 6695–6705.

Coulon, P., Ternaux, J.P., Flamand, A., Tuffereau, C., 1998. An avirulent mutant of rabies virus is unable to infect motoneurons in vivo and in vitro. J. Virol. 72, 2772–2778.

Dewerchin, H.L., Cornelissen, E., Nauwyczyn, J., 2005. Replication of feline coronaviruses in peripheral blood monocytes. Arch. Virol. 150, 2483–2500.

Hajema, B.J., Volders, H., Rottier, P.J., 2004. Live attenuated coronavirus vaccines through the directed deletion of group-specific genes provide protection against feline infectious peritonitis. J. Virol. 78, 3863–3871.

Herrewegh, A.A., Vennema, H., Horzinek, M.C., Rottier, P.J., de Groot, R.J., 1995. The molecular genetics of feline coronaviruses: comparative sequence analysis of the ORF7a/7b transcripton unit of different biotypes. Virology 212, 622–631.

Hohdatsu, T., Okada, S., Ishizuka, Y., Yamada, H., Koyama, H., 1992. The prevalence of type I and II feline coronavirus infections in cats. J. Vet. Med. Sci. 54, 557–562.

Hohdatsu, T., Nakamura, M., Ishizuka, Y., Yamada, H., Koyama, H., 1991. A study on the mechanism of antibody-dependent enhancement of feline infectious peritonitis virus infection in feline macrophages by monoclonal antibodies. Arch. Virol. 120, 207–217.

Hohdatsu, T., Yamada, H., Ishizuka, Y., Koyama, H., 1993. Enhancement and neutralization of feline infectious peritonitis virus infection in feline macrophages by neutralizing monoclonal antibodies recognizing different epitopes. Microbiol. Immunol. 37, 499–504.

Johnson, R.J., Brubaker, J.R., Ehrlich, J.T., Trent, D.W., 1996. Variants of Japanese equine encephalitis virus that resist neuralization define a domain of the E2 glycoprotein. Virology 177, 676–683.

Kida, K., Hohdatsu, T., Fuji, K., Koyama, H., 1999. Selection of antigenic variants of the S glycoprotein of feline infectious peritonitis virus and analysis of antigenic sites involved in neutralization. J. Vet. Med. Sci. 61, 935–938.

Kummrow, M., Melli, M.L., Haessler, M., Goenzi, E., Poland, A., Pedersen, N.C., Hofmann-Lehmann, R., 2005. Feline coronavirus serotypes 1 and 2: seroprevalence and association with disease in Switzerland. Clin. Diag. Lab. Immunol. 12, 1209–1215.

Motokawa, K., Hohdatsu, T., Aizawa, C., Koyama, H., Hashimoto, H., 1995. Molecular cloning and sequence determination of the peplomer protein gene of feline infectious peritonitis virus type I. Arch. Virol. 140, 469–480.

Motokawa, K., Hohdatsu, T., Hashimoto, H., Koyama, H., 1996. Comparison of the amino acid sequence and phylogenetic analysis of the peplomer, integral membrane and nucleocapsid proteins of feline, canine and porcine coronaviruses. Microbiol. Immunol. 40, 425–433.

Olsen, C.W., Corapi, W.V., Ngchabe, C.K., Baines, J.D., Scott, F.W., 1992. Monoclonal antibodies to the spike protein of feline infectious peritonitis virus mediate antibody-dependent enhancement of infection of feline macrophages. J. Virol. 66, 956–965.

Pedersen, N.C., 2009. A review of feline infectious peritonitis virus infection: 1963–2008. J. Feline Med. Surg. 11, 225–258.

Pedersen, N.C., 2009. A review of feline infectious peritonitis virus infection: 1963–2008. J. Feline Med. Surg. 11, 225–258.

Poland, A.M., Vennema, H., Foley, J.E., Pedersen, N.C., 1996. Two related strains of feline infectious peritonitis virus isolated from immunocompromised cats infected with a feline enteric coronavirus. J. Clin. Microbiol. 34, 3180–3184.

Stoddard, C.A., Scott, F.W., 1989. Intrinsic resistance of feline peritoneal macrophages to coronavirus infection correlates with in vivo virulence. J. Virol. 63, 436–440.

Tekes, G., Hofmann-Lehmann, R., Stallkamp, I., Thiel, V., Thiel, H.J., 2008. Genome organization and reverse genetic analysis of a type I feline coronavirus. J. Virol. 82, 1851–1859.

Vennema, H., Poland, A., Foley, J.E., Pedersen, N.C., 1998. Feline infectious peritonitis virus arise by mutation from endemic feline enteric coronaviruses. Virology 243, 150–157.
Vignuzzi, M., Stone, J.K., Arnold, J.J., Cameron, C.E., Andino, R., 2006. Quasispecies diversity determines pathogenesis through cooperative interactions in a viral population. Nature 439, 344–348.

Vrati, S., Kerr, P.J., Weir, R.C., Dalgar, L., 1996. Entry kinetics and mouse virulence of Ross River virus mutants altered in neutralization epitopes. J. Virol. 70, 1745–1750.

Wang, F.I., Fleming, J.O., Lai, M.M., 1992. Sequence analysis of the spike protein gene of murine coronavirus variants: study of genetic sites affecting neuropathogenicity. Virology 186, 742–749.

Woo, P.C., Lau, S.K., Huang, Y., Yuen, K.Y., 2009. Coronavirus diversity, phylogeny and interspecies jumping. Exp. Biol. Med. 234, 1117–1127.