Feline infectious peritonitis virus (FIPV) is classified as a feline coronavirus (FCoV). FCoVs have a single-stranded, positive-sense, linear RNA genome and are mainly composed of nucleocapsid (N), transmembrane and spike (S) proteins (Olsen, 1993). FCoVs are classified into types I and II according to their S protein properties (Hohdatsu et al., 1991a; Motokawa et al., 1995, 1996). FCoVs are also classified into two biotypes based on differences in pathogenicity: feline enteric coronavirus (FECV) and FIPV. FECV is asymptomatic in cats, but FIPV infection induces feline infectious peritonitis (FIP). FIP is a fatal disease in wild and domestic cat species, mainly leading to the development of immune complex vasculitis. There are types I and II FECV and FIPV in FCoV. Type II FCoVs are known to utilize feline aminopeptidase N (fAPN) as virus receptor (Hohdatsu et al., 1998).

FIPV targets the monocyte/macrophage lineage, infection of which is enhanced in the presence of antibodies (antibody-dependent enhancement or ADE). ADE activity in FIPV infection is induced when anti-FIPV-S antibody-bound viruses infect cells of the monocyte/macrophage lineage by binding to the Fc portion of Fc receptors on the cell surface (Corapi et al., 1992; Hohdatsu et al., 1991b; Olsen et al., 1992). When anti-FIPV-S antibodies are absent, FIPV infects the monocyte/macrophage lineage via fAPN (Rottier et al., 2005). However, whether the virus receptor, fAPN, is involved in ADE of FIPV infection is not clear.

It has been reported that mouse hepatitis virus type 2 (MHV-2) and severe acute respiratory syndrome coronavirus (SARS-CoV) enter cells through a low pH-dependent, endosomal pathway (Qiu et al., 2006; Simmons et al., 2005). Van Hamme et al. (2007) studied the kinetics of binding and internalization, demonstrating clearly that FIPV enters the cell via endocytosis. However, it is unclear whether acidification of the endosome is necessary for cellular invasion by FIPV, regardless of the presence or absence of antibodies. In this study, we investigated the involvement of the virus receptor (fAPN) in ADE of FIPV infection and the necessity of acidification of the endosome for cellular invasion by viruses.

Feline alveolar macrophages were isolated from anti-FCoV antibody-negative specific pathogen-free (SPF) cats as described previously (Hohdatsu et al., 1991b). Feline monocytes were isolated from SPF cats as described by Dewerchin et al. (2005). mAb 6-4-2 (IgG2a), used in the present study, recognizes the S protein of type II FIPV, as demonstrated by immunoblotting. It has been reported that mAb 6-4-2 exhibits a neutralizing activity in Felis catus whole fetus (Fcwf)-4 and Crandell feline kidney (CrFK) cells, but an enhancing activity in feline macrophages, depending on the reaction conditions (Hohdatsu et al., 1993). As a mAb to recognize fAPN, R-G-4 (IgG1), prepared by our laboratory (Hohdatsu et al., 1998), was used. Mouse IgG1 mAb recognizing feline gamma interferon, as control for
R-G-4, was prepared by our laboratory. RNA was isolated from cells by a method reported previously (Takano et al., 2007). To synthesize cDNA from FIPV negative-strand RNA, 1 μl RNA extract and 0.02 mol sense primer for the FIPV N gene (positions 876–895, 5'-CAACTGGGAGATGAACCTT-3') were added to Ready-to-Go RT-PCR beads (GE Healthcare Life Sciences) and the volume was adjusted to 50 μl with water. The resulting solution was incubated at 42 °C for 1 h to synthesize cDNA. cDNA was amplified by PCR using primers specific for the FCoV N gene (sense primer, positions 1644–1663, 5'-GGTAGCATTTGGCAGCGTTA-3') and antisense primer, positions 592–571, 5'-CAACTGGGAGATGAACCTT-3'). PCR was performed as reported previously (Takano et al., 2007). Data were analysed by Student's t-test. P values of <0.01 and <0.05 are used to indicate significant differences between compared groups.

The influence of the presence of anti-fAPN antibodies on ADE of FIPV infection of the feline monocyte/macrophage lineage was investigated. When feline macrophages and monocytes were treated with a mixture of type II FIPV strain 79-1146 (kindly provided by Dr M. C. Horzinek, Utrecht University, the Netherlands) and mAb 6-4-2, which enhances FIPV infection, virus production was increased significantly (P<0.01) compared with that after inoculation with virus alone, showing that infection was enhanced (Fig. 1; Methods A and C). When feline alveolar macrophages and monocytes were incubated with mAb R-G-4 at 4 or 37 °C beforehand and then incubated with FIPV strain 79-1146, infection was inhibited completely (Fig. 1; Method B). However, when feline alveolar macrophages and monocytes were incubated with mAb R-G-4 at 4 or 37 °C beforehand and then incubated with a mixture of FIPV and mAb 6-4-2, infection was not inhibited, but enhanced, as in cells without mAb R-G-4 treatment (Fig. 1; Method D).

Whether acidification of the endosome is necessary for cellular invasion by FIPV, regardless of the presence or absence of antibodies, was investigated. Cells were treated with lysosomotropic agents and the influence on viral replication after FIPV infection was investigated. A lysosomotropic agent, ammonium chloride (Wako Pure Chemical), chloroquine (Wako Pure Chemical) or bafilomycin A1 (Sigma-Aldrich), was added to the medium. Feline alveolar macrophages (2×10⁶ cells) and monocytes (2×10⁶ cells), cultured in the presence of lysosomotropic agents, were incubated with a mixture of FIPV strain 79-1146 (1×10⁵ TCID₅₀) and mAb 6-4-2, and intracellular expression of FIPV negative-strand RNA was measured. None of the added lysosomotropic agents showed any toxic effect on cell viability, as detected by using a WST-8 Cell Proliferation Assay kit (Kishida Chemical). The intracellular expression level of FIPV negative-strand RNA decreased with an increase in lysosomotropic-agent concentration (Fig. 2). Virus production in the culture supernatant also decreased in a manner dependent on lysosomotropic-agent concentration (Fig. 2).

Experiments using the anti-fAPN antibody R-G-4 suggested that the virus receptor, fAPN, was not involved in ADE of FIPV infection. Thus, we confirmed that acidification of the endosome is necessary for the route of Fc receptor-mediated cellular invasion by FIPV by using U937 cells, which have been reported to express no fAPN. The absence of fAPN on the surface of the U937 cells used in this study was confirmed by flow cytometry using mAb R-G-4 (culture fluid) at 4 or 37 °C for 1 h. After washing, FIPV strain 79-1146 (1×10⁶ TCID₅₀) was added to the culture and adsorbed to the cells at 37 °C for 1 h. After washing, medium was added and the cells were cultured at 37 °C. Method B: feline alveolar macrophages or monocytes were treated with anti-fAPN mAb R-G-4 (culture fluid) at 4 or 37 °C for 1 h. After washing, FIPV strain 79-1146 was added to the culture and adsorbed to the cells at 37 °C for 1 h. After washing, medium was added and the cells were cultured at 37 °C. Method C: feline alveolar macrophages or monocytes were treated with mouse IgG1 mAb control (culture fluid) at 4 or 37 °C for 1 h. After washing, FIPV strain 79-1146 reacted with mAb 6-4-2 at 4 °C for 1 h was added to the culture and adsorbed to the cells at 37 °C for 1 h. After washing, medium was added and the cells were cultured at 37 °C. Method D: feline alveolar macrophages or monocytes were treated with anti-fAPN mAb R-G-4 (culture fluid) at 4 or 37 °C for 1 h. After washing, FIPV strain 79-1146 reacted with mAb 6-4-2 at 4 °C for 1 h was added to the culture and adsorbed to the cells at 37 °C for 1 h. After washing, medium was added and the cells were cultured at 37 °C. In all methods, the culture supernatant was collected after 40 h (for monocytes) or 48 h (for alveolar macrophages), and the virus titre in the supernatant was determined (n=3). ND, Not detected.
cells reacted with mAb R-G-4, but U937 cells did not. In addition to ADE of FIPV infection, we investigated the effects of lysosomotropic agents: U937 cells \((2 \times 10^5)\) were cultured in medium containing the lysosomotropic agents at \(37^\circ C\) for 1 h. After washing, FIPV strain 79-1146 \((1 \times 10^6 \text{ TCID}_{50})\) reacted with mAb 6-4-2 or HBSS at \(4^\circ C\) for 1 h was added to the culture and adsorbed to the cells at \(37^\circ C\) for 3 h in the presence of the lysosomotropic agents. The cells were washed and cultured at \(37^\circ C\) for 48 h, and supernatants and cells were collected. When U937 cells were inoculated with a mixture of FIPV and mAb 6-4-2, the virus apparently replicated (Fig. 3). However, when cells were inoculated with the mixture in the presence of the lysosomotropic agents, intracellular expression of FIPV negative-strand RNA and virus production in the culture supernatant decreased in a manner dependent on the lysosomotropic-agent concentration (Fig. 3). When cells were inoculated with the virus alone without mAb 6-4-2 treatment, no viral replication was noted, regardless of the presence or absence of the lysosomotropic agents.

In addition to FIPV, there have been several reports concerning ADE of other virus infections (Halstead, 2003; Sullivan, 2001; Tirado & Yoon, 2003). For human immunodeficiency virus (HIV) infection, the necessity of the HIV receptor, CD4, for ADE induction, in addition to Fc receptors, has been reported (Takeda et al., 1990). In contrast, in foot-and-mouth disease virus infection, virus receptor-independent ADE activity was detected in an experiment using a mutant virus lacking virus receptor-binding ability (Mason et al., 1994). The involvement of Fc receptors in ADE of FIPV infection has been reported, but the necessity of the virus receptor, fAPN, was unclear. First, we investigated whether anti-fAPN mAb blocks ADE of FIPV infection in feline alveolar macrophages and monocytes. When anti-fAPN mAb was incubated with cells at \(37^\circ C\), ADE of FIPV infection was not blocked. However, it was considered possible that capping could have occurred on the cell surface at \(37^\circ C\), resulting in incorporation of anti-fAPN mAb into the cytoplasm, which avoided blocking of FIPV binding to the virus receptor. Thus, we reduced the anti-fAPN mAb reaction temperature to \(4^\circ C\) and repeated the experiment, but ADE of FIPV infection was not blocked, as in the experiment at \(37^\circ C\). We also clarified that FIPV alone could not establish infection in fAPN-negative, Fc receptor-positive U937 cells, but the virus apparently replicated when a mixture of FIPV and an anti-FIPV-S antibody, mAb 6-4-2, was used for
inoculation, suggesting strongly that the virus receptors are not necessary for the induction of ADE of FIPV infection.

We demonstrated that (i) cellular invasion by FIPV via the virus receptors was inhibited by lysosomotropic agents in a concentration-dependent manner, and (ii) cellular invasion via Fc receptors (ADE of FIPV infection) was also inhibited by lysosomotropic agents in a concentration-dependent manner, revealing that acidification of the endosome is necessary for cellular invasion by FIPV, regardless of the presence or absence of anti-FIPV-S antibodies. FIPV that entered cells of the monocyte/macrophage lineage may somehow escape digestion in late endosomes and lysosomes, and release the viral genome into the cytoplasm. Gollins & Porterfield (1986) reported that the viral core transfers to the cytosol in pre-lysosomal endosomes (late endosomes) 1–3 min after antibody-mediated entrance of West Nile virus into the cell. However, this process is limited to viruses that use virus receptors in ADE. It is not clear whether this phenomenon occurs when the virus receptors are not used, as in ADE of FIPV infection. Detailed investigation of the following by electron-microscopic and fluorescence-microscopic analyses is expected: (i) the mechanism by which FIPV that has entered cells via Fc receptors escapes digestion in late endosomes and lysosomes, and (ii) whether endosomal protease is involved in cellular invasion by FIPV, as in that by SARS-CoV and MHV-2 (Qiu et al., 2006; Simmons et al., 2005).

Addie et al. (1995) reported that FCoV reinfection of anti-FCoV antibody-positive domestic cats might not result in the development of ADE. Although the details are unclear, differences in the immunological condition of FCoV-infected cats may be the reason that the phenomenon noted in this experiment does not occur in domestic cats. It is assumed that ADE does not occur in cats that have acquired strong cellular immunity, even if they possess anti-FCoV antibodies, escaping from FIP development. It may also be possible that anti-FCoV antibodies prevent FIPV infection, avoiding ADE, when the anti-FCoV neutralizing-antibody titre is high.

We clarified that FAPN is not involved in ADE of FIPV infection and that acidification of the endosome is necessary for cellular invasion by FIPV, regardless of the presence or absence of antibodies. We reported previously that ADE activity in FIPV infection of feline alveolar macrophages caused overproduction of tumour necrosis factor alpha, which may lead to serious FIP symptoms (Takano et al., 2007). ADE-associated aggravation of the pathology of FIP in cats is being elucidated, but further studies are necessary to understand the aggravation mechanism. Clarification of ADE of FIPV infection is also important for understanding ADE of other virus infections.
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References


