Feline infectious peritonitis virus (FIPV) may cause a highly lethal infection in cats, in spite of a usually strong humoral immune response. Antibodies seem unable to identify infected cells and mediate antibody-dependent cell lysis. In this study, the effect of antibodies on *Feline coronavirus* (FCoV)-infected monocytes was investigated. Upon addition of FCoV-specific antibodies, surface-expressed viral proteins were internalized through a highly efficient process, resulting in cells without visually detectable viral proteins on their plasma membrane. The internalization was also induced by mAbs against the Spike and Membrane proteins, suggesting that both proteins play a role in the process. The internalization did not occur spontaneously, as it was not observed in cells incubated with medium or non-specific antibodies. Further, the internalization could not be reproduced in feline cell lines, indicating its cell-type specificity. This study sheds new light on the immune-evasive nature of FIPV infections.

Feline infectious peritonitis virus-infected monocytes internalize viral membrane-bound proteins upon antibody addition

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Feline infectious peritonitis virus (FIPV) and feline enteric coronavirus (FECV) are two coronaviruses described in cats. These feline coronaviruses are spread worldwide and infect all members of the family Felidae. Very little is known about the interactions of FIPV with the host immune system. Cats with clinical feline infectious peritonitis (FIP) often have very high titres of FIPV-specific antibodies; however, these antibodies are not able to block infection. This suggests that, for unknown reasons, antibodies and antibody-driven immune effectors are not able to clear the body of virus and/or virus-infected cells efficiently. There are indications that the immune system even plays an adverse role in the development of FIP. It has been reported in experimental infections that cats that have obtained FIPV-specific antibodies actively or passively develop FIP faster and more severely than naive cats (Pedersen & Boyle, 1980). This accelerated FIP has been the reason for the failure of most vaccination attempts (Woods & Pedersen, 1979; Pedersen & Black, 1983; Barlough et al., 1984, 1985; Vennema et al., 1990; McArdle et al., 1992). A mechanism was proposed that could explain this accelerated development of FIP in the presence of antibodies: antibody-dependent enhancement of infectivity (ADEI) (Hohdatsu et al., 1991; Corapi et al., 1992; Olsen et al., 1992). ADEI suggests that antibodies might help spread the virus in an infected cat by facilitating the virus uptake through the formation of virus–antibody complexes that are taken up by uninfected monocytes/macrophages via the Fc receptor. ADEI may explain why a larger number of cells can be infected in the presence of antibodies, but it cannot explain why these infected cells are not eliminated by the immune system. It is believed that the only effective defence against FIP is cell-mediated immunity (Pedersen, 1987).

The role of antibodies in the pathogenesis of naturally occurring FIP and, more specifically, how antibodies interact with infected cells is unknown. In the present study, we investigated the effect of FCoV-specific antibodies on FCoV-infected monocytes to clarify why antibodies seem to be unable to identify infected cells and/or mark them for antibody-dependent cell lysis. *Feline coronavirus* (FCoV)-, *Feline leukemia virus* (FeLV)- and *Feline immunodeficiency virus* (FIV)-negative cats were used as blood donors. Monocytes were isolated as described previously (Dewerchin et al., 2005) and seeded on glass coverslips, which allowed mounting on microscope slides using glycerin/DABCO (Janssen Chimica). The adherent cells consisted of 86 ± 7 % monocytes (assessed with monocyte marker DH59B; Veterinary Medical Research and Development). At 36 h post-seeding, monocytes were inoculated at an m.o.i. of 5 with third passages of FIPV 79-1146 and FECV 79-1683 on Crandell feline kidney (CrFK) cells (McKeirnan et al., 1981). FIPV 79-1146 was obtained from the ATCC and FECV 79-1683 was kindly provided by Dr Egberink (Utrecht University, The Netherlands). Twelve hours after inoculation, monocytes were incubated with FCoV-specific antibodies, which were obtained in the form of mAbs against the Spike and Membrane proteins, which might be involved in the process. The antibodies were purified with Protein G-Sepharose beads (Amersham). The mAbs were tested by FCoV-specific neutralization (Hohdatsu et al., 1992; McArdle et al., 1992). The mAbs were used at a concentration of 10 μg/ml, which resulted in a 90-95 % reduction of viral replication. The role of antibodies in the pathogenesis of naturally occurring FIP and, more specifically, how antibodies interact with infected cells is unknown. In the present study, we investigated the effect of FCoV-specific antibodies on FCoV-infected monocytes to clarify why antibodies seem to be unable to identify infected cells and/or mark them for antibody-dependent cell lysis. *Feline coronavirus* (FCoV)-, *Feline leukemia virus* (FeLV)- and *Feline immunodeficiency virus* (FIV)-negative cats were used as blood donors. Monocytes were isolated as described previously (Dewerchin et al., 2005) and seeded on glass coverslips, which allowed mounting on microscope slides using glycerin/DABCO (Janssen Chimica). The adherent cells consisted of 86 ± 7 % monocytes (assessed with monocyte marker DH59B; Veterinary Medical Research and Development). At 36 h post-seeding, monocytes were inoculated at an m.o.i. of 5 with third passages of FIPV 79-1146 and FECV 79-1683 on Crandell feline kidney (CrFK) cells (McKeirnan et al., 1981). FIPV 79-1146 was obtained from the ATCC and FECV 79-1683 was kindly provided by Dr Egberink (Utrecht University, The Netherlands). Twelve hours after inoculation, monocytes were incubated with FCoV-specific antibodies, which were obtained in the form of mAbs against the Spike and Membrane proteins, which might be involved in the process. The antibodies were purified with Protein G-Sepharose beads (Amersham). The mAbs were tested by FCoV-specific neutralization (Hohdatsu et al., 1992; McArdle et al., 1992). The mAbs were used at a concentration of 10 μg/ml, which resulted in a 90-95 % reduction of viral replication.
isothiocyanate (FITC) (Molecular Probes). In order to find the infected cells in the population easily, the cells were incubated with a mixture of mAbs 7-4-1, F19-1 and E22-2, recognizing respectively the Spike (S), Membrane (M) and Nucleocapsid (N) proteins (kindly provided by Dr Hohdatsu, Kitasato University, Japan), and visualized with goat anti-mouse–Texas red (Molecular Probes) (not shown). For the controls, cells were incubated with non-specific polyclonal antibodies that were obtained from specific-pathogen-free cats vaccinated with Nobivac Tricat (Intervet). After fixation of the cells, surface expression of viral proteins was visualized by a subsequent incubation with a mixture of anti-S and anti-M mAbs and goat anti-mouse–Texas red. Next, the cells were permeabilized and incubated with goat anti-cat–FITC (Sigma-Aldrich) to visualize possible internalization caused by the non-specific polyclonal antibodies (not shown). In Fig. 1(a), confocal images illustrate that, after FCoV-specific antibody addition, the surface-expressed viral proteins moved from the plasma membrane into the cytoplasm. In contrast, after addition of non-specific antibodies, the surface-expressed viral proteins remained in the plasma membrane. Fig. 1(c) shows that internalization of the viral glycoproteins was initiated very shortly after antibody addition and was completed rapidly.

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**Fig. 1.** Internalization assays with polyclonal antibodies. (a) Images show the localization of surface-expressed viral proteins in FCoV-infected monocytes at different times after addition of α-FCoV or non-specific polyclonal antibodies. (b) Spontaneous-internalization assay. The distribution of surface-expressed proteins was visualized at 0 or 30 min incubation with RPMI medium, α-FCoV or non-specific polyclonal antibodies. All images are a single section through the cell. Bar, 5 μm. (c) Internalization of surface-expressed viral proteins in FIPV- or FECV-infected monocytes after addition of polyclonal antibodies. ●, FIPV; ○, FECV; ▽, non-specific polyclonal antibody. Data represent means ± SD of triplicate assays.
In the graph, the internalization was represented as the percentage of cells that were internalizing viral proteins and not as number of internalized antibody–antigen complexes per cell, because the amount of viral proteins that is expressed in the plasma membrane varies strongly between cells (Dewerchin et al., 2005). The curves indicate that 89 ± 9 and 84 ± 4 % of respectively FIPV- and FECV-infected monocytes showed internalization of the plasma membrane-bound viral proteins after 3 min incubation. At 30 min, almost 100 % of the infected monocytes internalized their membrane-bound proteins (98 ± 3 and 97 ± 4 % for FIPV and FECV infection, respectively). Considering that an immune-evasive nature is attributed only to FIPV, it was remarkable to find that FIPV and FECV show almost-identical internalization kinetics. These identical kinetics imply that the difference between FIPV and FECV pathogenesis cannot be explained by the ability to internalize viral proteins. Fig. 1(c) also shows that internalization was not observed after addition of non-specific antibodies. This indicates that specific Fab–antigen interactions are needed and that the internalization is not mediated by the Fc-binding capacity that has been described for the S protein of several coronaviruses (Oleszak et al., 1993). To confirm that the membrane-bound structures were single proteins and not virus particles, infected cells were fixed and membrane expression was visualized. Then, the cells (and virus membrane) were permeabilized and nucleocapsid proteins were stained. No co-localization was found between the nucleocapsid and membrane-bound proteins, which shows that the structures were single proteins (data not shown).

Next, a spontaneous-internalization assay was performed. In this assay, it was tested whether internalization could occur spontaneously and whether it could be induced by non-specific cat antibodies. For this assay, all surface-expressed proteins were labelled with biotin. Monocytes, at 12 h after inoculation, were placed on ice, washed twice with ice-cold PBS solution and incubated with 2 mM of the biotinylation reagent EZ-Link sulfo-NHS-LC-Biotin (Pierce). After 30 min, the biotin was removed and replaced by cold medium supplemented with 10 mM glycine for 10 min. Then, the monocytes were washed twice with cold, supplemented medium and twice with cold medium without fetal bovine serum or heparin. Cells were shifted to 37°C and incubated with anti-FIPV polyclonal antibodies, non-specific antibodies or RPMI medium for 30 min. Control cells were fixed before the temperature shift. To visualize internalized, biotinylated proteins, cells were fixed, permeabilized and incubated with streptavidin–Texas red (Molecular Probes). Afterwards, cells were incubated with polyclonal feline anti-FIPV–FITC (VMRD) to enable identification of infected cells (not shown). Fig. 1(b) shows that a temperature shift by itself did not lead to internalization; nor did incubation with non-specific antibodies. Only monocytes that were incubated with anti-FoCV antibodies showed internalized proteins. These results indicate that spontaneous internalization did not occur.

To further specify which membrane-bound viral proteins are of importance for the internalization process, the redistribution of proteins induced by mAbs directed against the S or M protein was studied. At 12 h after inoculation, monocytes were incubated with anti-S (7-4-1, subisotype IgG2b), anti-M (F19-1, subisotype IgG1) or a combination of both antibodies. At different times post-antibody addition, cells were fixed, permeabilized and incubated with goat anti-mouse–Texas red to visualize the distribution of the antigen–antibody complexes. Next, the cells were incubated with FITC-labelled anti-FIPV antibodies to allow easy recognition of infected cells (not shown). The confocal images in Fig. 2(a) illustrate that both anti-S and anti-M antibodies were able to induce internalization. Fig. 2(b) shows that 82 ± 9 and 66 ± 4 % of the infected cells showed internalization at 10 min after addition of anti-S or anti-M antibodies, respectively. These percentages further increased and, after 1 h incubation with anti-S or anti-M antibodies, respectively 85 ± 4 and 81 ± 4 % of the cells showed internalization. The results demonstrate that internalization induced by mAbs occurred less efficiently than internalization induced by polyclonal anti-FIPV antibodies. Incubation with both anti-S and anti-M antibodies led to internalization in 100 % of infected monocytes; thus, the same efficiency was reached as with polyclonal antibodies. In cells where all antigen–antibody complexes were internalized with one monoclonal antibody against S or M protein, no residual expression could be found in the plasma membrane by using a polyclonal antibody (data not shown). In cells where not all complexes were internalized with one mAb against the S or M protein, the complexes that were in the plasma membrane could also be stained for the other protein (data not shown). These results indicate that S and M proteins reside in the plasma membrane as complexes. Interactions between the S and M proteins have already been described in mouse hepatitis virus infection, during which the M and S proteins form heteromultimeric complexes (Opstelten et al., 1995). Taken together, these findings suggest that S and M proteins operate together to mediate the internalization process. Internalization could not be induced by using anti-N antibodies (data not shown). As the mAbs against S and M protein are of mouse origin, non-specific mouse monoclonals of the same isotype were tested as a control in order to exclude isotype-specific interactions. Inoculated monocytes were incubated for different time periods with a mixture of non-specific, isotype-matched mAbs: 41D3 (isotype IgG1), recognizing porcine sialoadhesin, and Mil2 (isotype IgG2b), recognizing porcine CD14 (Duan et al., 1998; Thacker et al., 2001; Vanderheijden et al., 2003). After fixation of the cells, surface expression of viral proteins was visualized by incubation with biotinylated anti-FoCV polyclonal antibodies and then streptavidin–Texas red (Molecular Probes). Next, the cells were permeabilized and incubated with goat anti-mouse–FITC (Sigma-Aldrich) to visualize possible internalization caused by the non-specific mAbs. No internalized, non-specific antibodies were found (not shown). Fig. 2(a) shows that addition of non-specific antibodies did not lead to internalization,
confirming that the internalization process requires FCoV-specific antibodies and is not mediated by isotype-specific interactions.

Commonly used feline cell lines, CrFK cells and the macrophage-like ‘Felis catus whole fetus’ (fcwf) cells, were tested for their ability to internalize surface-expressed viral proteins. For this, cells at 12 h post-inoculation were incubated with biotinylated anti-FCoV polyclonal antibodies. At 30 min post-antibody addition, cells were fixed, permeabilized and incubated with streptavidin–Texas red. Then, cytoplasmic expression of antigens was visualized with anti-FIPV–FITC (not shown). Fig. 3 shows that the cell lines expressed viral proteins on their cell surface and that, after addition of antibodies, the antigens were somewhat more clustered than in non-treated cells. However, internalization was not observed. These findings suggest that the internalization process requires cellular machinery that is not, or not completely, present in cell lines. It also means that a more thorough study of this internalization pathway using

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**Fig. 2.** Internalization assays with mAbs. (a) Images show the localization of surface-expressed viral proteins after addition of monoclonal anti-S (IgG2b) or anti-M (IgG1) antibodies, a combination of both or non-specific, isotype-matched mAbs 41D3 (IgG1) and Mil2 (IgG2b). The images show a section through the cells. Bar, 5 μm. (b) Internalization of surface-expressed viral proteins in FIPV-infected monocytes after addition of monoclonal antibodies. ●, Anti-S + anti-M mAb; △, anti-S mAb; ▼, anti-M mAb; ○, non-specific mAbs. Data represent means ± SD of triplicate assays.

**Fig. 3.** The distribution of surface-expressed viral proteins upon antibody addition in CrFK and fcwf cells. The images show a section through the middle of the cells. Bar, 5 μm.
dominant-negative mutants will be hampered by the lack of adequate cell lines.

In this study, a mechanism is presented that might aid in explaining why the humoral immune system is not effective against an FIPV infection: internalization of viral plasma membrane-bound proteins induced by antibodies. This immune-evasion mechanism was described for the plasma membrane-bound proteins induced by antibodies. This immune-evasion mechanism was described for the first time by Favoreel et al. (1999). They found that viral plasma membrane-bound proteins in Pseudorabies virus (PrV)-infected pig monocytes were internalized upon antibody addition. This internalization process is clathrin-mediated and dependent on a YXXΨ motif (Y stands for tyrosine, X for any amino acid and Ψ for a bulky hydrophobic amino acid) in the cytoplasmic tail of the gB protein (Van de Walle et al., 2001; Favoreel et al., 2002). The viral plasma membrane-bound proteins in FIPV-infected cells (S and M) contain putative internalization motifs in their cytoplasmic tails. The S protein contains a dileucine motif and a YXXΨ motif and the M protein contains two of each. The presence of these putative internalization motifs is another indication that both viral proteins are of importance in antibody-mediated internalization. The role of these motifs will be investigated in the future.

In previous work, we reported that only half of FIPV-infected monocytes express viral proteins on their plasma membrane (Dewerchin et al., 2005). Here, we report that cells that do express viral proteins internalize these proteins upon antibody addition. With these findings, the following hypothetical model may aid in explaining FIP pathogenesis. In an FIPV-infected cat, a proportion of the FIPV-infected monocytes may remain immune-masked because no viral antigens are expressed at the plasma membrane and a proportion of the cells may express viral proteins. When antibodies bind to these membrane-bound proteins to mark the infected cells for cell lysis, internalization may be triggered. The plasma membrane is cleared of viral proteins and the infected cell remains invisible to the humoral immune system. In this way, the cell may be able to continue the production of progeny virus without being eliminated or it may enter a quiescent-infection state, as is seen in PrV-infected monocytes that were cultured in the presence of specific antibodies (Favoreel et al., 2003). This quiescent infection state would be an excellent cover for a carrier cell and might explain the sometimes long incubation period of an FIPV infection. For PrV-infected pig monocytes, it has also been shown that cells with internalized viral glycoproteins are protected against antibody-dependent, complement-mediated cell lysis (Van de Walle et al., 2003). Whether this is also true for an FIPV-infected monocyte will be investigated in the near future.

In conclusion, it can be stated that surface-expressed viral proteins in FIPV- and FECV-infected monocytes are internalized upon FCoV-specific antibody addition in a very efficient manner. This internalization does not occur spontaneously; nor can it be induced by non-specific antibodies. These findings might lead to new insights in strategies for immune evasion developed by feline coronaviruses.

Acknowledgements
We are grateful to Dr Hohdatsu and Dr Egberink for supplying antibodies. H. L. D. and E. C. were supported by the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT-Vlaanderen).

References


