A study on the mechanism of antibody-dependent enhancement of feline infectious peritonitis virus infection in feline macrophages by monoclonal antibodies

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Summary. Enhancement of feline infectious peritonitis virus (FIPV) infection of feline macrophages was studied using monoclonal antibodies (MAbs) to the FIPV strain 79-1146. Adherent cells recovered from the feline lung and peritoneal cavity phagocytosed fixed red blood cells, and formed Fc-mediated rosettes. Enhancement of virus infection by MAb was investigated by inoculating alveolar macrophages with a mixtures of viral suspension and MAb, and examining the cells for intracellular viral antigen by the immunofluorescence assay and the amount of infectious virus in the supernatant fluid after incubation. The replication of FIPV in macrophages was enhanced by non-neutralizing MAbs recognizing peplomer protein (S) and transmembrane protein (M) of the virus. Even among the MAbs having the ability to neutralize FIPV strain 79-1146, some reversely enhanced virus infection when they were diluted. The enhancement was suppressed by pretreatment of the MAb with protein A. The enhancement was reduced by the use of $F(ab')_2$ fragment of MAb. These results demonstrated antibody-dependent enhancement (ADE) of FIPV infection in macrophage. The replication of FIPV 79-1146 strain in macrophages from FIPV antibody-positive cats was more enhanced than in those from antibody-negative cats.

Introduction

Feline infectious peritonitis virus (FIPV), family *Coronaviridae,* genus *Coronavirus,* produces a chronic, progressive, immunologically-mediated disease in domestic and exotic cats. At the present time, no practical method of immunization has been reported. Cats immunized with virulence-attenuated live or inactivated FIPV are not protected against virulent FIPV challenge-exposure [15, 20]. Cats immunized with closely related coronaviruses, such as feline

enteric coronavirus (FECV), canine coronavirus (CCV), and transmissible gas-
troenteritis virus (TGEV), are also sensitive to exposure to virulent FIPV [2,
16, 18, 22, 29]. Infection of FIPV and the onset of the disease are accelerated
in cats with FIPV antibodies in comparison with those without FIPV antibody
[17, 18, 27]. When cats passively immunized with FIPV antibodies were infected
with virulent FIPV, severe symptoms were observed and some of them died
soon after the infection [17, 27]. These phenomena are considered to have been
caused by a mechanism similar to that of the dengue shock syndrome. Antibody-
dependent enhancement (ADE) of viral infection, which occurs with sub-neu-
tralizing antiviral antibodies and results from the formation of virus-antibody
complexes, attachment of complexes to cell Fc receptors, and internalization
by mononuclear phagocytes, has been demonstrated with some viral infections
such as dengue virus infection [1, 4–6, 12–14, 25]. The feline macrophage is
known to be one of the target cells for FIPV [7, 19, 28]. The in vitro growth
of FIPV in feline macrophages has been reported by Stoddart and Scott [23,
24], but the details of ADE of FIPV infection are unknown. This paper describes
in vitro ADE of FIPV infection in feline macrophages.

Materials and methods

Virus

The 79-1146 strain of FIPV was obtained from Dr. M.C. Horzinek of the State University
Utrecht, the Netherlands, and the 79-1683 strain of FECV was obtained from Dr. A. J.
McKeirnan of the Washington State University, Pullman. These strains were passaged 2
or 3 times in feline fetal cell cultures (fcwf-4), and stored at −80 °C until use.

Monoclonal antibodies (MAbs)

These were prepared by fusion of spleen cells from BALB/c mice immunized with the FIPV
79-1146 strain and mouse myeloma cells (P-3/x-63-Ag8-6, 5, 3). The MAbs used in the
present study were F19-1, F18-2, and F30-1 recognizing protein M of the virus and F80-
1, F25-1, 5-6-2, 6-4-2, and 7-4-1 recognizing protein S of the virus. Of these MAbs, 5-6-2,
6-4-2, and 7-4-1 have the ability to neutralize FIPV strain 79-1146, and recognize different
epitopes of S protein of the virus. Other MAbs (F19-1, F18-2, F30-1, F80-1, and F25-1)
have no neutralizing ability. These MAbs were characterized by the authors [8, 9]. The
culture fluid of a hybridoma was used for the experiment.

Collection of macrophages

Macrophages were collected from coronavirus antibody-negative adult cats. Alveolar mac-
rophages were obtained from alveolar washing with Hanks's balanced salt solution (HBSS).
The recovered cells were spun down, washed 3 times with HBSS and a 2×10^6 cells/ml
suspension was made in a growth medium (Eagle's minimum essential medium containing
50% Leibovitz L-15 medium, 10% fetal calf serum, 100 units of penicillin per ml, and
100 μg of streptomycin per ml). The suspension was transferred in 1 ml volumes to each
well of 24-well multiplates each of which contained a cover glass. The cells were incubated
at 37 °C for 2 h, the supernatant fluid was removed, and the cell layers were washed with
HBSS to remove non-adherent cells. After addition of growth medium the cells were

incubated at 37 °C for 2 h, and washed with HBSS. The cells thus obtained were used as macrophages in the experiments.

Peritoneal macrophages were recovered from cats by lavaging the peritoneal cavity with HBSS 4 or 5 days after intraperitoneal administration of 100 ml/kg of liquid paraffin. The recovered cells were treated in the same manner as alveolar macrophages, and used for the experiments.

Identification of macrophages

Macrophages were identified on the basis of phagocytosis of fixed red blood cells and Fc-mediated rosetting. Phagocytosis was carried out by adding 0.5% sheep red blood cells (SRBC) fixed in 2.5% glutaraldehyde solution to cultured macrophages and incubating at 37 °C for 2 h. After incubation, the cells were washed with phosphate buffered saline (PBS), fixed in methanol, and stained with 10% Giemsa solution. Fc-mediated rosetting was induced by adding SRBC coated with rabbit anti-SRBC antibodies to the cultured macrophages and incubating the mixture at 37 °C for 1 h. After incubation, the specimens were gently washed with PBS, and observed under a microscope.

Indirect fluorescent antibody assay (IFA)

A mixture of MAbs that recognize peplomer protein (S), transmembrane protein (M) and nucleocapsid protein (N) of FIPV strain 79-1146 was used as the primary antibody. The MAbs were added to acetone-fixed cells and allowed to stand at 37 °C for 30 min. After the specimens were washed with PBS three times, they were stained with rabbit antimouse-IgG, IgA and IgM serum conjugated with fluorescein isothiocyanate (Miles Laboratories, Naperville, U.S.A.). After left at 37 °C for 30 min, they were washed with PBS, mounted in 50% glycerol buffer and observed under a fluorescence microscope.

Plaque assay

Confluent fcwf-4 cell monolayers in 60-mm plastic petri dishes were inoculated with virus dilution in 0.1 ml amounts. After virus adsorption at 37 °C for 60 min, the inoculated cultures were covered with 5 ml of agar overlay medium which consisted of 1% Bacto agar in MEM. The cultures were incubated in a CO_2 incubator at 37 °C for 2 days, and stained by incubating at 37 °C for 6 h under a second overlay medium containing 0.01% neutral red. The infectious titer was expressed in plaque-forming units (PFU).

ADE assay of viral infection

Equal amounts of MAbs and viral suspension were allowed to react at 37 °C for 1 h. The mixtures were inoculated in 0.1 ml volumes into wells of multiplate cultures of macrophages incubated for 3 days. After adsorption at 37 °C for 1 h, 1 ml of growth medium was added, and incubated at 37 °C for 36 h in a CO_2 incubator. The culture supernatant was determined for PFU, and the recovered cover slips were examined by IFA. The percentage of ADE was obtained by the following formula:

$$\text{percent of ADE} = A - B/B \times 100$$

where A is the rate of positivity of IFA or PFU in the presence of the antibodies and B is the rate of positivity of IFA or PFU in the absence of the antibodies.

Pepsin digestion of MAb

IgG was purified from mouse ascitic fluid by affinity chromatography using protein A sepharose 4B (Pharmacia, Sweden). The purified IgG was dialyzed against 0.2 M sodium
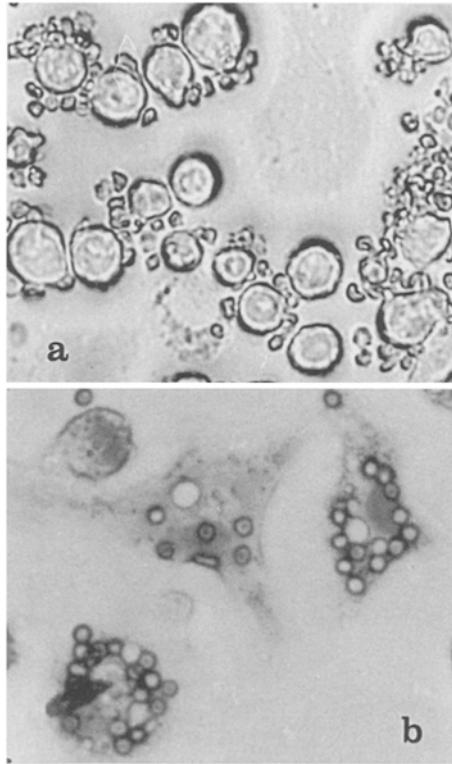
Fig. 1. Fc-mediated rosetting of the alveolus macrophage (a), and phagocytosis of fixed red blood cells by alveolus macrophage (b)

acetate buffer (pH 4.5), and 1 mg of pepsin per 100 mg of IgG was added to the specimen for digestion at 37 °C for 20 h. Subsequently, 1 N NaOH was added to adjust the pH to 8.0, and the reaction was terminated. The supernatant obtained after centrifugation at 2,000 × g for 30 min was loaded onto a protein A sepharose 4B column, and the flow-through fraction was used as the F $(ab')_2$ fragment. This fraction retained the IFA ability to detect FIPV antigen.

Feline coronavirus antibody-positive cats

Two feline coronavirus antibody-negative cats were orally inoculated with 10^6 tissue culture infectious doses $(TCID_{50})$ of the FECV strain 79-1683. After the antibodies to feline coronavirus were confirmed to be present by IFA technique and neutralization test 30 days after the inoculation, the cats were used to recover macrophages.

Results

Adherent cells recovered from the alveolus and peritoneal cavity of cats demonstrated the Fc-mediated rosetting and phagocytosis of fixed red cells (Fig. 1). Furthermore Giemsa staining showed more than 90% of the cells to have the morphology of macrophages.

Using MAbs recognizing protein M or S, ADE of feline macrophage infection with the FIPV 79-1146 strain was studied. Table 1 summarizes the results of MAbs without neutralizing ability. These MAbs were shown to enhance

Table 1. Antibody-dependent enhancement (ADE) by monoclonal antibody (MAb) without virus-neutralizing activity of feline macrophage infection with FIPV 79-1146 strain

MAb no.	Indirect fluorescent assay		Plaque assay	
	positive (%)	enhancement (%)	PFU/ml	enhancement (%)
F 19-1	3.8	171	7.9×10^5	229
F 18-2	4.6	229	7.0×10^5	192
F 30-1	3.3	136	9.0×10^5	275
F 80-1	2.3	64	6.5×10^5	171
F 25-1	3.8	171	5.4×10^5	125
Without MAb	1.4	–	2.4×10^5	–

infection by both the indirect fluorescence assay (IFA) and the plaque assay. The enhancement demonstrated by IFA decreased with the antibody dilution (Fig. 2). Figure 3 shows the results of enhancement detected by IFA for neutralizing MAbs 5-6-2 [neutralization (NT) titer; 1:128], 6-4-2 (NT titer; 1:256) and 7-4-1 (NT titer; 1:64). Virus infection was completely inhibited by MAb 5-6-2 at a dilution of 1:100 or less. At a dilution of more than 1:100, no enhancement was observed. Undiluted MAbs 6-4-2 and 7-4-1 inhibited virus infection, but at a dilution of 1:10 infection was reversely enhanced. MAb 6-4-2 induced a particularly strong enhancement.

Effects of blockade of the Fc region of MAbs on ADE of viral infection were studied. Protein A (Pharmacia, Sweden) was diluted to 0.05–500 µg/ml, and added to F18-2 MAb. The mixture was allowed to stand at 4 °C for 1 h. Viral suspension was added to the mixture, incubated at 37 °C for 1 h, and
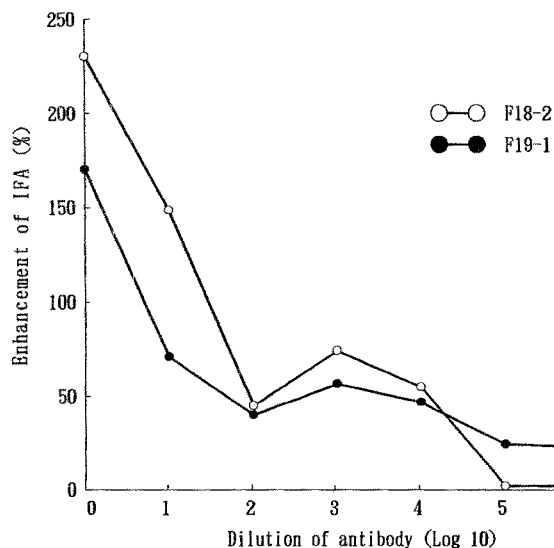
Fig. 2. Relationship between the dilution of antibody and the enhancement of IFA
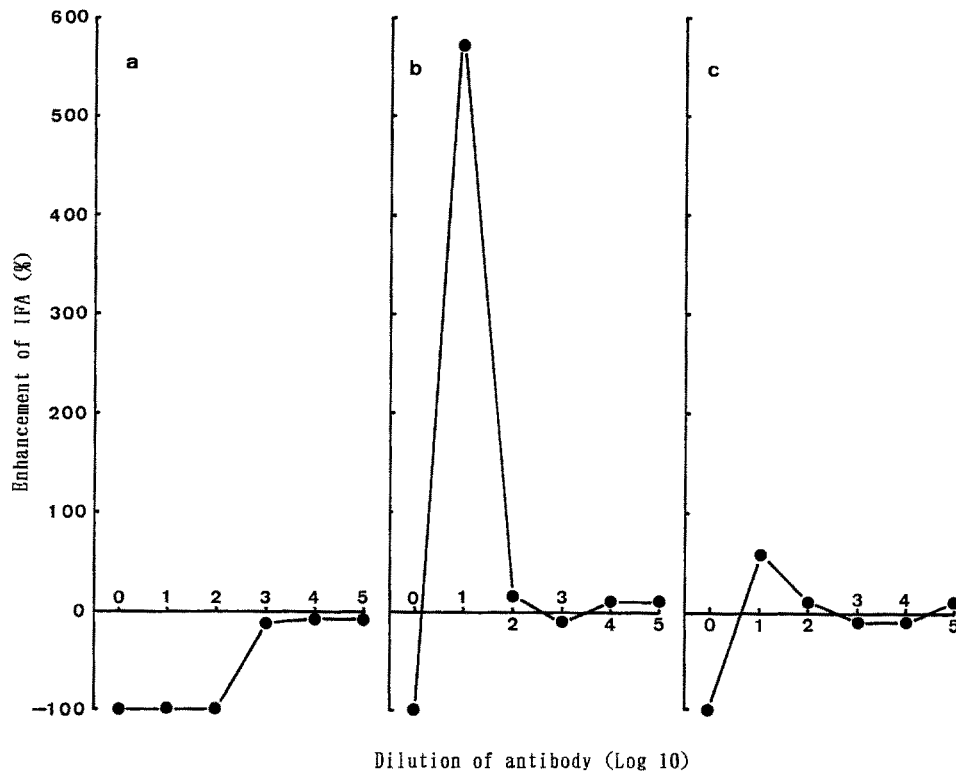
Fig. 3. Antibody-dependent enhancement (ADE) by monoclonal antibody (MAb) with virus-neutralizing activity of feline macrophage infection with FIPV 79-1146 strain. MAbs: **a** 5-6-2, **b** 6-4-2, **c** 7-4-1

inoculated onto alveolar macrophages. ADE was suppressed with increasing concentration of protein A (Fig. 4). Effects on ADE of IgG purified from mouse ascitic fluid and $F(ab')_2$ fragment in which the Fc fragment was removed from IgG by digestion with pepsin were studied in alveolar macrophages. The percent enhancement was determined by IFA. Regardless of the concentration of antibody, ADE was more suppressed by $F(ab')_2$ fragment than by IgG (Fig. 5).

Macrophages were recovered from the alveolus and peritoneal cavity of 2 cats experimentally infected with the FECV 79-1683 strain and of 2 coronavirus antibody negative cats. Alveolar and peritoneal macrophages, incubated for 3 days, were inoculated with the FIPV 79-1146 strain. Thirty-six hours later the virus replication in these cells was compared. Table 2 shows the results. Virus replication was distinctly better in the peritoneal and alveolar macrophages recovered from the antibody-positive cats than those from the antibody-negative cats. When no virus was inoculated onto the macrophages recovered from the antibody-positive cats, of course no viral antigen was detected by IFA, and no infectious virus was found even in the culture supernatant. When the peritoneal and alveolar macrophages were compared, viral replication tended to be better
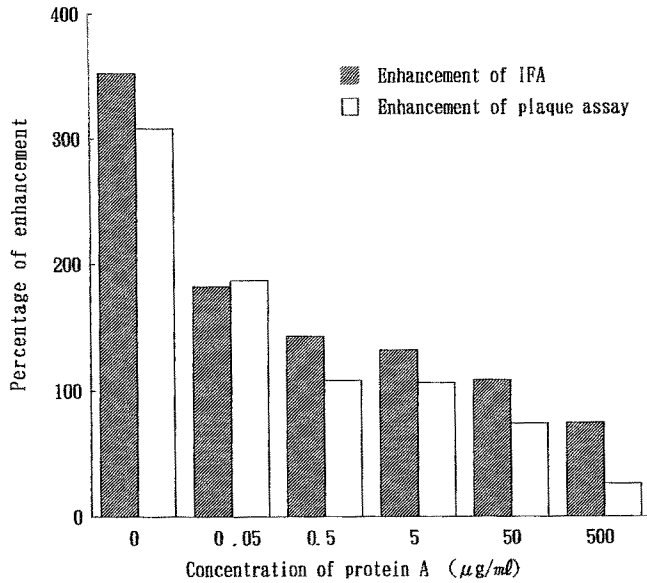
Fig. 4. Effect of protein A on antibody-dependent enhancement of FIPV strain 79-1146 infection in feline alveolar macrophages
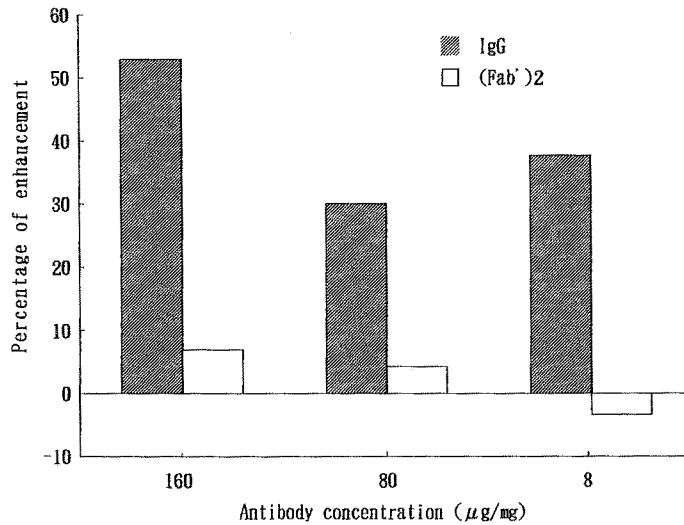
Fig. 5. Effect of pepsin-digested IgG on antibody-dependent enhancement of FIPV strain 79-1146 infection in feline alveolar macrophages

in the peritoneal macrophages from both the antibody-negative and -positive cats.

Discussion

The in vitro growth of FIPV was first demonstrated in cultures of cells from the peritoneal exudate of an experimentally infected kitten in 1976 [19]. Macrophages in inflammatory lesions of the infected cat were shown to contain virus by the electron microscopic observation, suggesting the macrophage to

Table 2. Replication of FIPV strain 79-1146 in macrophages from feline coronavirus antibody-negative and -positive cats

Cell	Assay	Antibody-negative cats (mean)	Antibody-positive cats (mean)
Peritoneal macrophages	IFA[a]	3.83%	15.94%
	Plaque[b]	2.3×10^5	34.5×10^5
Alveolar macrophages	IFA	2.11%	10.14%
	Plaque	1.9×10^5	20.5×10^5

[a] Percentage of IFA positive cells
[b] PFU/ml

be one of the target cells for FIPV [19]. Stoddart and Scott have recently reported the growth of FIPV in macrophages recovered from the peritoneal cavity [23, 24]. In the present study adherent cells recovered from the alveolus and peritoneal cavity phagocytosed fixed red blood cells and were able to form Fc-mediated rosettes. These phenomena indicate the adherent cells to have properties of macrophages. When the adherent cells were inoculated with the FIPV 79-1146 strain, about 1–2% of the cells were revealed to be virus antigen-positive by the indirect fluorescence assay, and the supernatant fluid contained 2×10^5 PFU/ml of virus. However, no cytopathic effect was observed, suggesting the infection to be persistent and lead to a virus-carrier state.

Antibody-bound virus invades cells via Fc receptors and infection is enhanced. This phenomenon has been observed in macrophages and macrophage-like cell lines with various viruses [1, 4–6, 12–14, 25]. The present study disclosed that viral infection of alveolar macrophages is enhanced by intervention with MAbs without virus neutralizing activity (Table 1). The enhancement was suppressed by blockade of the Fc region of antibody (Figs. 4 and 5). Even of the MAbs having the ability to neutralize FIPV strain 79-1146, some reversely enhanced the infection after they were diluted (Fig. 3b and c). Each of the neutralizing MAbs used in this study recognizes the different epitopes of protein S of the virus. MAb 5-6-2 showed no enhancement of infection regardless of dilution (Fig. 3a). The inhibition of virus proliferation by the MAb may be exerted after adsorption of viruses to cells. Detailed mechanisms of neutralization and enhancement of FIPV infection by these neutralizing MAbs should be further studied. A difference in viral type is considered to be closely related to the ADE of infection and to cause dengue shock syndrome in dengue virus infection [6, 14]. We discovered the existence of at least 2 sero types of FIPV by using MAbs with ability to neutralize FIPV strain 79-1146 [8]. Effects of this type difference on ADE of FIPV infection should be studied in future.

Vennema et al. combined protein S gene of FIPV with vaccinia virus [26]. Cats immunized with the recombinant virus demonstrated no protection against

challenge with virulent FIPV. The onset of disease was accelerated by the immunization. This observation is further supported by our results indicating the MAb to protein S to show ADE. Furthermore, the present study showed that the MAb to protein M also has the enhancing activity.

FECV is known to be antigenically cross-reactive with FIPV. Cats previously inoculated with FECV showed an accelerated onset of the disease upon infection with virulent FIPV [16–18]. The replication of FIPV was distinctly better in macrophages recovered from FECV antibody-positive cats than in those from antibody-negative cats. It also remains unsolved whether this interesting phenomenon is due to ADE by cytophilic antibody [21] or not. Some studies have shown that viral growth is better in activated macrophages [3, 10]. We also obtained better replication of FIPV in peritoneal macrophages induced by stimulation with liquid paraffin than in alveolar macrophages (Table 2). ADE of FIPV infection should also be studied by taking into consideration a difference in the activation of macrophages. The possibility of using fcwf-4 cells, which are believed to have macrophage properties [11], for the determination of ADE of FIPV infection is under investigation, taking cell homogeneity into consideration.

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