Arch Virol (2000) 145: 1–12

Archives of Virology
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Printed in Austria

Neutralization of feline infectious peritonitis virus: preparation of monoclonal antibody that shows cell tropism in neutralizing activity after viral absorption into the cells

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Accepted September 9, 1999

Summary. Feline infectious peritonitis virus (FIPV) infection of feline macrophages is enhanced by mouse anti-FIPV monoclonal antibody (MAb). This antibody-dependent enhancement (ADE) of FIPV infection is dependent on mouse MAb subclass, and MAb of IgG2a subclass has a strong ADE activity. Furthermore, MAb showing strong neutralizing activity in Felis catus whole fetus (fcwf-4) cells and Crandell feline kidney (CrFK) cells shows strong enhancing activity in feline macrophages, indicating that the neutralizing epitope and the enhancing epitope are closely related. In this study, we prepared MAb FK50-4 that showed a strong neutralizing activity in feline macrophages, despite the fact that the MAb belonged to the IgG2a subclass. However, MAb FK50-4 did not exhibit neutralizing activity in CrFK cells or fcwf-4 cells, thus showing a very unusual property. MAb FK50-4 recognized FIPV small integral membrane glycoprotein (M protein). Even when feline macrophages were pretreated with MAb FK50-4 prior to FIPV inoculation, this antibody prevented FIPV infection. This reaction disappeared after treatment of FK50-4 with protein A. The neutralizing activity of FK50-4 was also effective on feline macrophages after the cells were inoculated with FIPV. These findings indicated that the FIPV replication mechanism differs between feline macrophages and CrFK/fcwf-4 cells and that a neutralizing epitope that can prevent FIPV infection of feline macrophages after viral absorption is present on M protein.

Introduction

Feline infectious peritonitis (FIP) is a virus-induced disease that is chronically progressive and usually fatal disease in domestic and wild Felidae. The causative agent of this disease is FIP virus (FIPV), which belongs to the family

Coronaviridae, genus *Coronavirus*. In the field, feline enteric coronavirus (FECV) is also prevalent. FECV is antigenically similar to FIPV, and infection with FECV induces mild enteritis in cats. It has been noted that based on genetic and antigenic comparison, FIPV and FECV belong to one feline coronavirus (FCoV) group with a broad range of pathogenicity, rather than considering each virus as independent entity. It is considered that due to reduced immunity in the host (cats), mutation from FECV to FIPV occurs in the host, and the mutated virus induces FIP [23, 29].

Generally, macrophages play an important role in non-specific defense against viral infections. However, it is also known that some viruses bound to antibodies invade macrophages via the Fc region of the antibody and the Fc gamma receptor (FcγR) of the macrophage, and eventually, the antibody leads to the enhancement of infection [1, 4–7, 14, 22, 24, 25]. Dengue hemorrhagic fever/dengue shock syndrome is caused by this infection mechanism. This phenomenon is called antibody dependent enhancement (ADE) of virus infection. The antibody against FIPV is also known to enhance the FIPV infection and accelerate the disease onset in cats [19–21]. Following experimental FIPV infection, cats with FIPV-neutralizing antibody frequently develop FIP more rapidly and more severe than do sero-negative cats [19, 30].

The FIPV virions consist of 3 main structural proteins: nucleocapsid protein (N), small integral membrane glycoprotein (M) and large spike glycoprotein (S) [3, 17]. We previously reported that in vitro FIPV infection of feline alveolar macrophages is enhanced by murine MAbs to the S protein of FIPV [9, 12]. This ADE activity increased with the MAb that showed neutralizing activity with feline kidney cells (CrFK), suggesting that there was a distinct correlation between ADE activity and the neutralizing activity [2, 12, 18]. The ADE activity was dependent on the mouse MAb Ig subclass, and this dependence was closely correlated with the ability of the antibody to bind to feline macrophage FcγR. Even among the MAbs that have been shown to recognize the same antigenic site, IgG2a MAbs strongly enhanced FIPV infection, whereas IgG1 MAbs did not [11]. The close association between enhancing and neutralizing epitopes is an obstacle to developing a vaccine containing only neutralizing epitopes without enhancing epitopes, and detailed analysis is expected.

In this study, we prepared a very unusual MAb that showed cell tropism in neutralizing activity on FIPV infection. This MAb showed a strong neutralizing activity in feline macrophages without ADE activity, despite belonging to the IgG2a subclass. However, this antibody did not exhibit any neutralizing activity for feline fibroblastoid cell lines such as CrFK cells.

Materials and methods

Virus and cell cultures

FIPV strain 79–1146 was obtained from Dr. M. C. Horzinek (The State University, Utrecht, The Netherlands). CrFK cells, Felis catus whole fetus (fcwf-4) cells and feline alveolar macrophages were cultured in Eagle's minimum essential medium (MEM) containing 50%

Leibovitz's L-15 medium, 10% fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin. The maintenance medium was MEM containing 50% L-15 and antibiotics as above. Feline alveolar macrophages were collected from adult cats negative for anti-coronavirus antibody as described previously [9].

Preparation of the virus antigen

The virus antigen was prepared with the FIPV 79–1146 strain grownin fcwf-4 cell cultures. Infectious culture fluid concentrated nearly tenfold by ammonium sulfate precipitation was layered onto a discontinuous sucrose density gradient (20 and 60%) in an RPS 28 roter (Hitachi Koki Co., Ltd., Japan) and centrifuged at 27,000 r.p.m. for 2 h. The virus bands formed were collected, diluted in NTE buffer, (0.1 M NaCl, 0.01 M Tris-HCl, pH 7.4, 0.001 M EDTA) and centrifuged at 80,000 **g** for 1 h. The virus-containing pellet was suspended in a 1/500 volume of NTE buffer and treated with 0.1% Triton X-100 at 4 °C for 1 h.

Monoclonal antibodies (MAb)

MAbs 5-6-2 and 6-4-2 were used as MAbs recognizing S protein [12]. MAb F18-2 and MAb E22-2 were used as MAbs recognizing M protein and N protein, respectively [10]. Among these MAbs, MAbs 5-6-2 and 6-4-2 had neutralizing activity in CrFK and fcwf-4 cells. Furthermore, MAb 5-6-2 is a mouse IgG1 antibody that exhibits only neutralizing activity in feline macrophages. However, MAb 6-4-2 shows a strong ADE activity in feline macrophages because it belongs to the IgG2a subclass.

Production of MAb FK50-4 secreting hybridomas

For preparation of MAbs against FIPV 79–1146 strain, BALB/c mice of about 5 weeks of age, were inoculated intraperitoneally with a mixture of 50 μg of the virus antigen prepared as described above and 10^9 cells of pertussis adjuvant. Four to 6 weeks later, the mice received an intravenous booster dose of 50 μg of viral antigen, and spleen cells were obtained for fusion 3 days later. The fusion was carried out by essentially the same method described by Köhler and Milstein [16]. Polyethyleneglycol-4,000 (Merck, Federal Republic of Germany) was used as a fusing agent, and the ratio of mouse spleen cells and mouse myeloma cells (P-3/x-63-Ag8-6,5, 3) was 10:1. The selective medium contained hypoxanthine (10^{-4} M), aminopterin (4×10^{-7} M) and thymidine (1.6×10^{-5} M). The fused cells, at a concentration of 3.5×10^6 spleen cells per ml, were dispended in 100 μl volumes into 96-well flat-bottomed microplates (Corning Glass Works, Corning, NY) and incubated at 37 °C in a humid atmosphere containing 5% CO_2. After incubation for 2 weeks, the wells were examined, and those that contained hybridoma cultures were tested for feline coronavirus specific antibody by indirect fluorescent antibody (IFA) assay (see below). The colonies in antibody-positive wells were passaged in 24-well multiplates (Corning Glass Works, Corning, NY) and incubated in a medium containing hypoxanthine (10^{-4} M) and thymidine (1.6×10^{-5} M). The cells were then cloned by the soft agar method.

Determination of antibody class and subclass

The supernatant fluids of antibody-secreting hybridoma cultures were concentrated ten-fold by 50% saturation with ammonium sulfate and used for the determination of antibody class and subclass by double diffusion in 1% agar gel containing 0.1% NaN_3. Rabbit antisera against mouse immunogloblins, IgG1, IgG2a, IgG2b, IgG3, IgM and IgA were placed in center wells, and test samples were added to adjacent wells. The plates were incubated overnight at room temperature in a humidified chamber.

Purification of polypeptides recognized by MAb FK50-4 by affinity chromatography

Polypeptides recognized by MAb FK50-4 were purified from the virus antigen prepared as described above by affinity chromatography, using HiTrap NHS-Activated Affinity Columns (Pharmacia, Biotech, Sweden), according to the manufacturer's protocol. As the ligand, IgG purified from the mouse ascitic fluid of MAb FK50-4 with protein A-sepharose CL-4B (Pharmacia Biotech, Sweden) was used. The molecular weight of the purified polypeptides was determined by Western immunoblotting.

Western immunoblotting

Viral antigen separated in polyacrylamide gel by sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) was transferred to nitrocellulose sheets of 0.45 μg pore size. The transfer was carried out electrophoretically by a method adapted from that described by Towbin et al. [26] in a Transfer-Blot cell apparatus at 120 mA and 10 V for 14 h, using a transfer buffer consisting of 3 g/l Tris (pH 8.3), 20% methanol and 43.2 g/l glycine. The nitrocellulose sheets were then cut into strips and incubated at 37 °C for 2 h in PBS containing 10% fetal-calf serum. The supernatant fluid of antibody-secreting hybridoma cultures was added in 1 ml volumes to individual strips and incubated at 37 °C for 2 h. The strips were then washed 3 times with PBS containing 0.05% Tween-20 and incubated at 37 °C for 2 h with horseradish peroxidase-conjugated rabbit antibody against mouse IgG, A, M (Miles Lab., U.S.A.) diluted 1:300 with PBS containing 10% fetal calf serum. The strips were then washed and treated with substrate solution containing 0.05 g diaminobenzidine and 50 μl of 30% H_2O_2 in 100 ml of 0.05 M Tris-HCl, pH 7.2. When distinct bands appeared about 10 min later, the reaction was stopped by pouring off the substrate solution and rinsing with distilled water.

Indirect fluorescent antibody (IFA) assay

Hybridoma culture supernatant fluid was added to acetone-fixed FIPV-infected cells, incubated for 30 min at 37 °C, washed 3 times with phosphate buffered saline solution (PBS) and then stained with rabbit anti-mouse-IgG, A, M serum conjugated with fluorescein isothiocyanate (FITC) (Miles Lab., U.S.A.). After a further 30 min incubation at 37 °C, slides were washed in PBS. Stained cells were mounted in buffered glycerol and examined using a fluorescence microscope.

Neutralization (NT) test

Neutralization tests were performed using fcwf-4 cells, CrFK cells and feline macrophages. Viral solutions of FIPV strain 79–1 146 adjusted to 200 $TCID_{50}$ were used for fcwf-4 cells and CrFK cells, and a viral solution adjusted to 2 000 $TCID_{50}$ was used for feline macrophages. Equal volumes of virus and MAb were mixed and reacted with fcwf-4 cells or CrFK cells at 37 °C, and with feline macrophages at 4 °C for 60 min. Then the reaction mixture was added to fcwf-4 cells, CrFK cells or feline macrophages for viral absorption at 37 °C for 60 min. After the reaction solution was removed by aspiration, maintenance medium was added and the cells were incubated at 37 °C for 3 days. For fcwf-4 cells and CrFK cells, the neutralizing antibody titer was expressed as the reciprocal of the highest dilution of MAb that completely inhibited cytopathic effect in the test. For feline macrophages, viral protein-specific fluorescence was measured by the IFA method to estimate the infection rate. The infection rate was obtained by the following formula: infection rate = $(A - B) / B \times 100$, where A is the IFA positivity rate in the presence of the antibody and B is the IFA positivity rate in the absence of the antibody. An infection rate of 0% or lower indicates neutralizing activity, and an infection rate higher than 0% indicates enhancing activity.

Results

Preparation and characterization of MAb FK50-4

Cell fusion was performed using spleen cells from a mouse immunized with viral antigen. Hybridoma cells that produced IgG2a antibody and were IFA-positive for FIPV were selected. The neutralizing activity of these hybridoma cultures on strain 79–1 146 was examined using fcwf-4 cells, CrFK cells or feline macrophages. MAb FK50-4 showing neutralizing activity only in feline macrophages was obtained. NT test results in feline macrophages are shown in Fig. 1, and the results in fcwf-4 cells and CrFK cells are shown in Table 1. Neither MAb FK50-4 in the culture supernatant nor the MAb FK50-4 mouse ascites showed neutralizing activity in fcwf-4 cells or CrFK cells. In contrast, MAb FK50-4 showed a strong neutralizing activity but did not show enhancing activity in feline macrophages. MAb 5-6-2 used as a control showed neutralizing activity in all cell types. MAb 6-4-2 showed neutralizing activity in fcwf-4 cells and CrFK cells, while it showed a strong enhancing activity in feline macrophages.

Polypeptide specificity of MAb FK50-4 against FIPV strain 79–1146

The specificity of MAb FK50-4 to FIPV strain 79–1 146 polypeptide was investigated by Western immunoblotting. MAb FK50-4 did not specifically react with any S, M or N viral structural protein (data not shown). Therefore, we attempted to purify the polypeptide recognized by MAb FK50- 4 by affinity chromatography. MAb FK50-4 was immobilized with a HiTrap NHS-activated affinity column (Pharmacia Biotech, Sweden). Viral antigens were loaded on the column, and the bound polypeptide was eluted. MAb FK50-4-affinity purified polypeptide was investigated by Western immunoblotting. MAbs 6-4-2, F18-2 and E22-2,

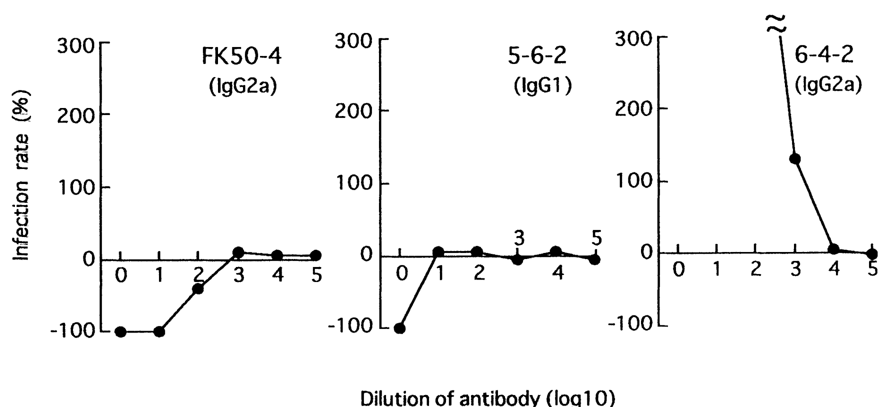

Fig. 1. Neutralization of FIPV infection in feline macrophages by MAb FK50-4. The neutralizing activity of MAb FK 50-4 on FIPV strain 79–1 146 was studied in feline macrophages. MAbs 5-6-2 and 6-4-2 were used as a control. Each MAb and FIPV were allowed to react at 4 °C for 1 h, then the mixture was inoculated into feline alveolar macrophages. The cells were examined for the viral antigen by IFA 36 h later to determine proliferation of the virus. Infection rate was calculated as described in Materials and methods

Table 1. Neutralization titer of FIPV infection in fcwf-4 and CrFK cells

MAb	Culture fluids of hybridoma		Mouse ascitic fluid[b]		Ig isotype
	fcwf-4	CRFK	fcwf-4	CRFK	
5-6-2	40[a]	40	40960	40960	IgG1
6-4-2	80	80	40960	40960	IgG2a
FK50-4	<1	<1	<1	<1	IgG2a

[a]Neutralization titer

[b]BALB/c were injected intraperitoneally with 0.5 ml of pristane 14 days before intraperitoneal inoculation of 2×10^6 hybridoma cells. Ten days later, the ascitic fluids were collected, clarified by centrifugation and stored at −80 °C until use

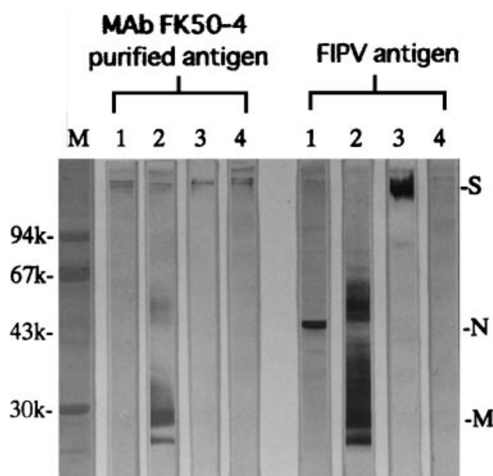

Fig. 2. Polypeptide specificity of MAb FK50-4 against FIPV strain 79– 1 146. Polypeptide recognized by MAb FK50-4 was purified from FIPV antigens by affinity chromatography and used in Western immunoblotting. MAbs E22-2 (*1*), F18-2 (*2*) and 6-4-2 (*3*), recognizing proteins N, M and S, respectively, were used to detect the polypeptide recognized by MAb FK50-4. HBSS was used as a negative control (*4*). *M* represents molecular weight markers

which recognized FIPV S, M and N protein, respectively, were used to detect the polypeptide recognized by MAb FK50-4. The polypeptide recognized by MAb FK50-4 specifically reacted only with MAb F18-2, which recognized M protein (Fig. 2). This finding strongly suggested that the viral structural protein recognized by MAb FK50-4 is M protein.

Effect of pretreatment of feline macrophages with MAb FK50-4 prior to FIPV inoculation on neutralizing activity

Although the Ig subclass was IgG2a, MAb FK50-4 prevented the FIPV strain 79–1 146 from infecting feline macrophages. Therefore, feline macrophages were pretreated with MAb FK50-4 prior to FIPV inoculation, and then the effect of the neutralizing activity on FIPV infection was examined. As a result, when feline macrophages were pretreated with MAb 5-6-2, which belonged to the IgG1 subclass, used as a control, the neutralizing activity disappeared. In contrast, MAb FK50-4 neutralized FIPV as it did when a mixture of the virus and the antibody

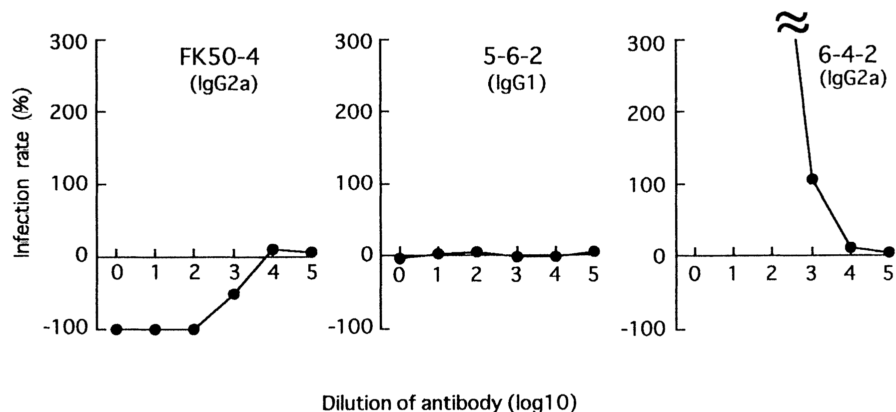

Fig. 3. Neutralization of FIPV infection determined by virus inoculation into MAb FK50-4 pretreated feline alveolar macrophages. Each MAb was added to macrophages and allowed to react at 37 °C for 1 h. After washing 3 times with HBSS, the cells were inoculated with FIPV. The inoculated macrophages were examined for the viral antigen by IFA after 36 h of infection, and the infection rate was calculated as described in Materials and methods

was inoculated into feline macrophages (Fig. 3). Furthermore, MAb 6-4-2, which also belonged to the same IgG2a subclass as MAb FK50-4, showed a strong enhancing activity, as it did when the virus was premixed with this antibody and inoculated.

The neutralizing activity of MAb FK50-4 was confirmed to act via the antibody Fc region and the macrophage Fc*g*R. Protein A-treated MAb FK50-4 was added to feline macrophages and incubated at 37 °C for 1 h. After washing with HBSS, the cells were inoculated with the virus. As a result, protein A-treated FK-50-4 completely lost its neutralizing activity (Fig. 4).

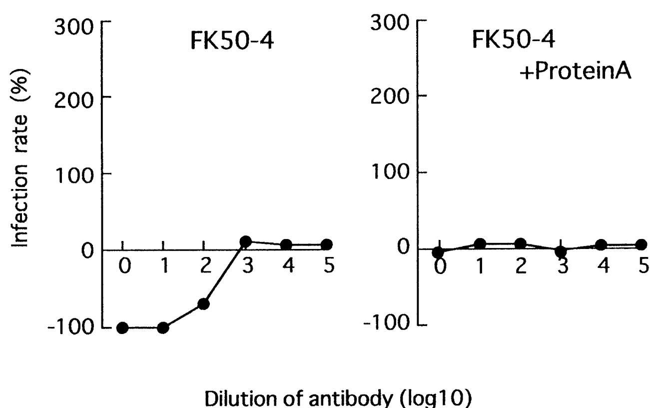

Fig. 4. Neutralizing activity of protein A-treated MAb FK50-4. MAb FK50- 4 was treated with 1000 μg/ml of protein A at 37 °C for 1 h and added to feline alveolar macrophages. After 3 washings with HBSS, the cells were inoculated with FIPV. The inoculated macrophages were examined for the viral antigen by IFA after 36 h of infection, and the infection rate was calculated as described in Materials and methods

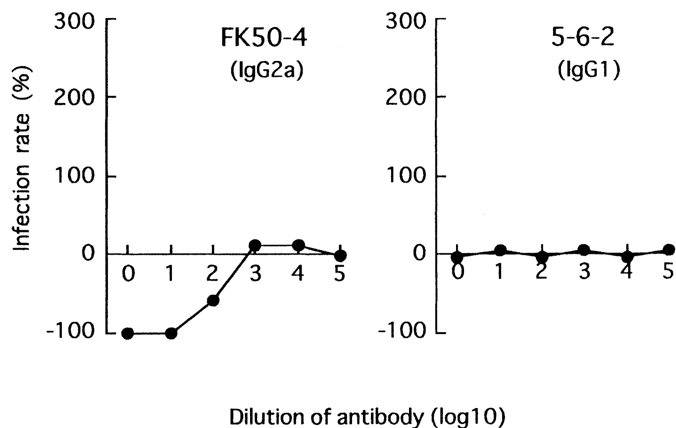

Fig. 5. Neutralizing activity of MAb FK50-4 on FIPV-inoculated feline macrophages. After FIPV was inoculated into macrophages and absorbed at 4 °C for 2 h, the cells were washed with HBSS 3 times. Then MAb FK50-4 or 5-6-2 was added and incubated at 37 °C for 1 h. The cells were examined for the viral antigen by IFA 36 h later to determine proliferation of the virus. Infection rate was calculated as described in Materials and methods

Effect on neutralizing activity of FK-50-4 after FIPV absorption into feline macrophages

After FIPV was absorbed in feline macrophages at 4 °C for 2 h, the cells were reacted with MAb FK-50-4, and the effect on the neutralizing activity was investigated. MAb 5-6-2 showing a strong neutralizing activity in feline macrophages was used as a control. As a result, MAb FK-50-4 prevented FIPV infection, but MAb 5-6-2 did not (Fig. 5).

Discussion

In this study, we succeeded in obtaining a MAb FK50-4 that prevented FIPV strain 79–1 146 infection of feline macrophages despite its belonging to the IgG2a subclass. However, MAb FK50-4 did not neutralize FIPV infection of fcwf-4 cells or CrFK cells, showing a very unusual property. Since MAb FK50-4 specifically reacted only with FIPV-infected cells on IFA assay, it was obvious that MAb FK50-4 recognizes FIPV antigen (data not shown). However, a specific reaction was not detected on Western immunoblotting using purified FIPV antigen. Probably, MAb FK50-4 recognizes the region that cannot be recognized when the tertiary structure is changed after the FIPV antigen was denatured by SDS. Therefore, the polypeptide recognized by MAb FK50-4 was purified by affinity chromatography. Then the polypeptide specificity was examined by Western immunoblotting with known MAb recognizing S, M or N protein. FK50-4-affinity purified polypeptide specifically reacted only with MAb F18-2, which recognized M protein. This finding strongly suggested that the polypeptide recognized by FK50-4 is contained in M protein.

It is known that when feline macrophages are pretreated with IgG2a MAb with neutralizing activity in fcwf-4 cells and CrFK cells, then inoculated with FIPV

after washing, the antibody shows a strong enhancing activity [11]. However, MAb FK50-4 showed only neutralizing activity even when feline macrophages pretreated with MAb FK50-4 were inoculated with FIPV. MAb 5-6-2, which was IgG1, used as a control, did not show any neutralizing activity in this method, although it showed a strong neutralizing activity in the method in which the virus-antibody mixture was inoculated into feline macrophages. We concluded that IgG1 MAb could not bind to feline macrophage Fc*g*R, and then the MAb was removed by washing. In contrast, MAb FK50-4, which was IgG2a, may not have been removed by washing due to its strong binding to feline macrophage Fc*g*R, expressing neutralizing activity. When protein A-treated MAb FK50-4 was examined by the same experiments, the neutralizing activity was lost. It was considered that protein A was bound to the MAb FK50-4 Fc region, blocking its binding to feline macrophage FC*g*R. Furthermore, MAb FK50-4 showed neutralizing activity even when the antibody was added to feline macrophages inoculated with FIPV. These findings suggested that MAb FK50-4 acts on cells after FIPV absorption into the cells and prevents the infection. Antibodies that exhibit neutralizing activity after viral absorption into cells were also reported for herpes simplex virus and adenovirus [8, 31]. Anti-herpes simplex virus glycoprotein-D MAb exhibited neutralizing activity after viral absorption in the cells, similarly to MAb FK50-4. Neutralizing activity was also detected in anti-adenovirus penton antiserum by the same method. Although the neutralization mechanism has not been clarified, FK50-4 may express neutralizing activity via the same mechanism as these antibodies. Furthermore, it was confirmed in this study that MAb FK50-4 recognizes M protein. Coronavirus M protein is thought to play an important role in the viral budding from cells [15]. Viral budding occurs after viral structural protein synthesis. When FIPV reacted with MAb FK50-4 was inoculated into feline macrophages, FIPV antigens were not detected in the cells on IFA assay, indicating that FIPV neutralized by MAb FK50-4 cannot synthesize viral proteins. Therefore, we concluded that the neutralization by FK50-4 is exerted by blocking a certain step in the FIPV replication cycle between fusion of cellular and viral membranes and translation. Further analysis of the mechanism is necessary. Since MAb FK50-4 showed cell tropism in neutralizing activity, it is possible that the FIPV replication manner differs between fcwf-4/CrFK cells and feline macrophages, and this also requires further investigation.

Preparation of anti-M protein MAb that neutralizes FIPV infection in fcwf-4 cells and CrFK cells has not yet been reported. However, Horzinek et al. [13] suggested that an anti-M protein antibody has complement- requiring neutralizing activity. We previously reported that certain M protein-recognizing MAb have enhancing activity. However, Vennema et al. [27,28] found that when they injected FIPV to cats vaccinated with recombinant vaccinia vaccine expressing S or M protein, FIP onset was advanced in cats immunized with recombinant vaccinia virus expressing S protein, while three of eight cats immunized with recombinant vaccinia virus expressing M protein survived and the disease onset was not advanced. The enhancing activity of MAb on M protein that we reported

was very weak compared to that of MAb recognizing S protein. There are many unclear points in the role of M protein in host immunity against FIPV infection, and further investigation is necessary. All reported IgG2a MAbs showing FIPV-neutralizing activity induced enhancing activity in feline macrophages, indicating that neutralizing epitopes and enhancing epitopes of FIPV are located at close sites. However, MAb FK50-4 showed only a neutralizing activity in feline macrophages despite its belonging to the IgG2a subclass. This finding strongly suggested the presence of a new epitope that can prevent FIPV infection of feline macrophages. FIPV infection of feline macrophages is thought to be important in the development of FIP. Identification of the epitope recognized by FK50-4 may allow the development of a vaccine for the prevention of disease development.

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Received May 26, 1999