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Characterization of monoclonal antibodies against feline infectious peritonitis virus type II and antigenic relationship between feline, porcine, and canine coronaviruses

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Summary. Seven monoclonal antibodies (MAbs) with neutralizing activity against feline infectious peritonitis virus (FIPV) strain 79-1149 (type II) were prepared. When the polypeptide specificity recognized by these monoclonal antibodies (MAbs) was investigated by Western immunoblotting, all of the MAbs reacted with peplomer glycoprotein (S) of the virus. By competitive binding assay these MAbs were found to recognize at least 3 different epitopes. The reactivity of these MAbs with 6 viruses classified as FIPV type I (UCD-1, UCD-2, UCD-3, UCD-4, NW-1, and Black), feline enteric coronavirus (FECV) type II strain 79-1683, canine coronavirus (CCV) strain 1-71, and transmissible gastroenteritis virus (TGEV) strains TO-163 and SH was examined by neutralization tests. All MAbs neutralized FECV strain 79-1683, CCV strain 1-71, and TGEV strains TO-163 and SH, while they did not neutralize the 6 FIPV type I viruses. Moreover, the MAb against TGEV strain TO-163, which has strong neutralizing activity against 7 TGEV viruses, neutralized CCV strain 1-71, FECV strain 79-1683, and FIPV strain 79-1146, but did not neutralize the 6 FIPV type I viruses.

These results demonstrated that there are at least 3 epitopes involved in the neutralization of FIPV type II strain 79-1146, and that these epitopes are not present in FIPV type I viruses but are present in FECV strain 79-1683 which does not induce feline infectious peritonitis, TGEV strains TO-163 and SH, and CCV strain 1-71. These results suggest the presence of 2 serotypes of FIPV which can be clearly distinguished by the neutralization test using MAbs.

Introduction

The mammalian members of the family *Coronaviridae* are divided into 2 distinct antigenic groups on the basis of serologic tests [23, 25]. One antigenic group

contains mouse hepatitis virus, neonatal calf diarrhea coronavirus, human coronavirus HCV-OC43, hemagglutinating encephalomyelitis virus of swine, and rat coronavirus. The second antigenic group consists of human respiratory coronavirus HCV-229E, transmissible gastroenteritis virus (TGEV) of swine, canine coronavirus (CCV), and feline infectious peritonitis virus (FIPV) [8, 22]. In addition to these members, a feline enteric coronavirus (FECV) has been isolated, which is very closely related to FIPV, TGEV, and CCV [19, 20].

Feline coronaviruses are divided into FIPV types I and II, and FECV types I and II, on the basis of the disease types, that is, whether it causes feline infectious peritonitis (FIP) or not, the ability of the viruses to proliferate in cell cultures, and the antigenic relationship to TGEV and CCV [17]. Among these types, FIPV type II and FECV type II are reported to be antigenically closer to TGEV and CCV. Between TGEV Purdue strain and FIPV 79-1146 strain a high degree of homology in the primary structure of the peplomer protein (S, 210k) has been described (39% for amino acid residues 1 to 274 and 93% for residues 275 to 1447) [9]. The authors prepared MAbs which react only with the feline coronaviruses FIPV type II strain 79-1146 and FECV type II strain 79-1683. Some of these MAbs reacted with either TGEV or CCV in indirect fluorescent antibody assays. Also, some differences in antigenically between FIPV type I virus strains were noted in an analysis using MAbs (unpubl. obs.).

In this study, we prepared MAbs with neutralizing activity against FIPV strain 79-1146, and investigated the differences in the epitopes recognized by these MAbs, using a competitive binding assay. Using these MAbs and MAbs with neutralizing activity against TGEV strain TO-163, we examined the neutralizing epitopes for differences between feline coronaviruses, and TGEV and CCV viruses.

Materials and methods

Cell cultures

Feline whole fetus cells (fcwf-4), Crandell feline kidney cells (CrFK), and swine kidney cells (CPK) were grown in Eagle's minimum essential medium (MEM) containing 20% L-15 medium, 10% fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin. The maintenance medium was MEM containing 20% L-15 and antibiotics as above. The cells were maintained in a humidified 5% CO_2 incubator at 37 °C.

Viruses

The coronavirus isolates used in this study and their sources are shown in Table 1. Among the FIPV strains used in the study, strains UCD-1, NW-1, UCD-2, UCD-3, UCD-4, and Black show cell-associated growth, and are therefore regarded as type I virus strains in the classification of Pedersen et al. [17].

FIPV and FECV, TGEV, and CCV were passaged 2 or 3 times in fcwf-4 cells, CPK cells, and CrFK cells, respectively, and were used for the study.

Table 1. Source of coronavirus isolates

Virus strain		Source	Reference
FIPV	79-1146	M. C. Horzinek, State University Utrecht	[12, 20]
	UCD-1	N. C. Pedersen, University of California, Davis	[18, 21]
	NW-1	J. K. Yamamoto, University of California, Davis	[14, 18]
	UCD-2	J. K. Yamamoto, University of California, Davis	[16]
	UCD-3	J. K. Yamamoto, University of California, Davis	[16]
	UCD-4	J. K. Yamamoto, University of California, Davis	[16]
	Black	J. K. Yamamoto, University of California, Davis	[2, 14]
FECV	79-1683	A. J. McKeirnan, Washington State University, Pullman	[12, 20]
TGEV	TO-163	National Institute of Animal Health of Japan	[4]
	SH	National Institute of Animal Health of Japan	[6]
CCV	1-71	E. Takahashi, University of Tokyo	[1]

Preparation of virus antigen

The antigen was prepared with the FIPV 79-1146 strain grown in fcwf-4 cell cultures. Infectious culture fluid concentrated about tenfold by ammonium sulfate precipitation was layered onto a discontinuous sucrose density gradient (20 and 60%) in a RPS 28 rotor (Hitachi Koki Co., Ltd, Japan) and centrifuged at 27,000 r.p.m. for 2 h. The virus bands formed at the interface of 20% and 60% sucrose layers 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.

Production of antibody-secreting hybridomas

For preparation of neutralizing MAbs against FIPV 79-1146 strain, BALB/c mice, about 5 weeks of age, were inoculated intraperitoneally with a mixture of 50 μg of the viral antigen prepared as above and 10^9 cells of pertussis adjuvant. Four or 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 [11]. Polyethyleneglycol-4,000 (Merck, Federal Republic of Ger-

many) 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 wells of 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 which contained hybridoma cultures were tested for feline coronavirus specific antibody by a neutralization (NT) test (see below). The colonies in antibody positive wells were passaged in 24-well multiplates (Corning Glass Works, Corning, NY) and incubated in medium containing hypoxanthine (10^{-4}M) and thymidine (1.6×10^{-5}M). The cells were then cloned by the soft agar method.

The neutralizing MAbs against TGEV TO-163 strain used were previously reported by Hohdatsu et al. [7].

Determination of antibody class and subclass

The supernatant fluid of antibody-secreting hybridoma cultures were concentrated tenfold by 50% saturation of ammonium sulfate and used for determination of antibody class and subclass by double diffusion in 1% agar gel containing 0.1% NaN_3. Rabbit antisera against mouse immunoglobulins, IgG 1, IgG 2 a, IgG 2 b, IgG 3, IgM and IgA, and κ and λ chains (Miles Laboratories, Naperville, U.S.A.) were placed in center wells and test samples were added to peripheral wells. The plates were incubated overnight at room temperature in a humidified chamber.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed in slab gels under a nonreducing condition. The separation gel contained 10% polyacrylamide and the stacking gel contained 3% polyacrylamide. The sample buffer contained final concentration of 2% (w/v) SDS, 10% (v/v) glycerol, 0.1% (w/v) bromphenol blue and 100 mM Tris-HCl (pH 6.8). The electrode buffer (pH 8.8) contained 6.1 g/l Tris, 28.8g/l glycine and 0.1% SDS. Samples were mixed with sample buffer and kept at room temperature for 12 h without boiling. Electrophoresis was performed at room temperature for 6 h at a constant current of 20 mA.

Western immunoblotting

Viral antigen separated in polyacrylamide gel by SDS-PAGE were transferred to nitrocellulose sheets of 0.45 µm pore size. The transfer was carried out electrophoretically by the method adapted from Towbin et al. [24] in a Transfer-Blot cell apparatus (Bio-Rad Laboratories, Richmond, CA) at 120 mA and 10 V for 14 h using 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 were 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, IgA, and IgM (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, 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.

Competitive binding assay

This was carried out by a modified enzyme-linked immunosorbent assay (ELISA) [10]. Serial tenfold dilutions in 100 µl volumes of each competing MAb from ascitic fluid were added to wells of a flat-bottomed Microelisa plate coated with virus antigen (see above), and incubated at 37 °C for 60 min. After 3 washings with washing solution (0.85% NaCl solution containing 0.02% Tween-20), 100 µl of biotin-labeled MAb diluted so as to show an optical density (OD) of 1.0 was added, and incubated at 37 °C for 60 min. After 3 washings with washing solution, peroxidase conjugated avidin (Cappel, Cooper Biomedical, Inc. Malvern, PA) were diluted to the optimal concentration with PBS containing 10% fetal calf serum and 0.05% Tween-20 and 100 µl of the dilution was added to each well of the plates. After incubation at 37 °C for 30 min, each well received 100 µl of substrate solution and incubated at 25 °C for 20 min in a dark room. After completion of the incubation, the reaction was stopped with 3 N H_2SO_4 solution and the OD at 492 nm was determined. The amount of competitive binding was estimated from the OD in the presence or absence of unlabeled competing antibodies. The percentage of competition was determined by the formula, 100 (A − n)/(A − B), where A is the OD in the absence of competing antibody, B is the OD in the presence of homologous antibody (10^4 ELISA units), and n is the OD in the presence of a competitor.

Neutralization (NT) test

Serial twofold dilutions of the MAb were mixed with an equal volume of a virus suspension diluted so as to contain approximately 200 $TCID_{50}$/0.1 ml. The mixtures were incubated at 37 °C for 60 min. Each mixture was then inoculated into cell cultures in flat-bottomed microplates (Corning Glass Works, Corning, NY), and incubated in an atmosphere of 5% CO_2 in air at 37 °C for 6 days. Two wells were employed for each antibody dilution. The anitbody titer was expressed as the reciprocal of the highest dilution of MAb that completely inhibited cytopathic effect in the test.

Results

Isolation and characterization of monoclonal antibodies

Using the spleen cells of mice immunized with FIPV strain 79-1146, cell fusion was conducted 6 times. As a result, 7 MAbs (5-6-2, 5-7-2, 6-1-1, 6-4-2, 7-1-1, 7-3-1, and 7-4-1) with neutralizing activity against FIPV strain 79-1146 were obtained. The immunoglobulin isotype and polypeptide specificity of these MAbs are shown in Table 2. Polypeptide specificity was determined by Western immunoblotting. All Mabs were found to recognize the S protein of the viruses. Figure 1 shows an example of the reaction.

Results of competitive binding assay

To determine the differences in the epitope specificity of the 7 neutralizing MAbs, competitive binding assay was performed, and the results are summarized in Table 3. Binding of the biotin-labeled MAbs 5-6-2, 5-7-2, 7-1-1, and 7-3-1 to virus antigen was completely blocked by all of these unlabeled MAbs. The biotin-labeled MAb 7-4-1 was completely blocked only by the homologous MAb. Biotin-labeled MAbs 6-4-2 and 6-1-1 were completely blocked by all unlabeled MAbs. Unlabeled MAbs 6-4-2 and 6-1-1 blocked biotin-labeled MAbs

Table 2. Immunoglobulin isotype and polypeptide specificity of monoclonal antibodies

MAb no.	Isotype		Polypeptide specificity
	heavy chain	light chain	
5-6-2	IgG 1	κ	S
5-7-2	IgG 2 a	κ	S
6-1-1	IgG 1	κ	S
6-4-2	IgG 2 a	κ	S
7-1-1	IgG 2 a	κ	S
7-3-1	IgG 1	κ	S
7-4-1	IgG 2 b	κ	S

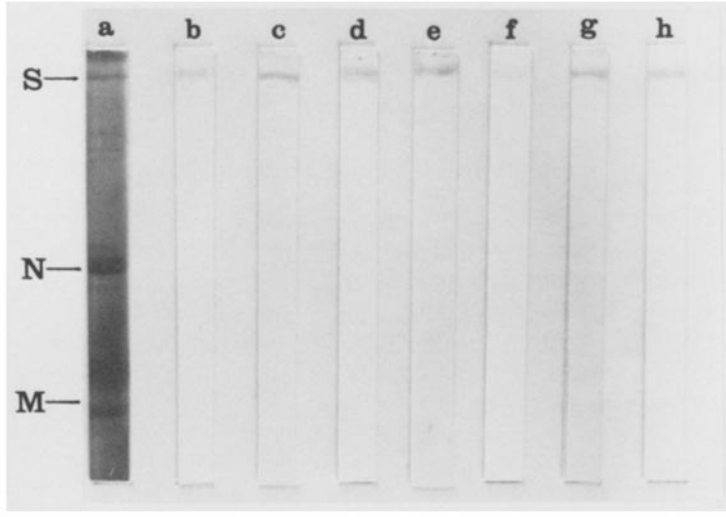

Fig. 1. Polypeptide specificity of neutralizing MAbs against FIPV 79-1146 strain in Western immunoblotting. *a* Anti-FIPV mouse serum, *b* 5-6-2, *c* 5-7-2, *d* 7-1-1, *e* 7-3-1, *f* 7-4-1, *g* 6-1-1, *h* 6-4-2

Table 3. Results of competitive binding assay

Biotin labelled MAb	Unlabelled MAb							Epitope specificity
	5-6-2	5-7-2	7-1-1	7-3-1	7-4-1	6-4-2	6-1-1	
5-6-2	+	+	+	+	−	49.8	34.1	I
5-7-2	+	+	+	+	−	65.1	57.6	I
7-1-1	+	+	+	+	−	67.3	67.9	I
7-3-1	+	+	+	+	−	50.2	38.0	I
7-4-1	−	−	−	−	+	76.4	75.4	II
6-4-2	+	+	+	+	+	+	+	III
6-1-1	+	+	+	+	+	+	+	III

\+ Represents ⩾80% inhibition of binding
− Represents ⩽20% inhibition of binding

Table 4. Reactivity of the neutralizing monoclonal antibodies against FIP strain 79-1146 to feline, porcine, and canine coronaviruses

MAb[a] no.	FIPV							FECV	CCV	TGEV		Epitope specificity
	type I						type II	79-1683	1-71	SH	TO-163	
	UCD-1	UCD-2	UCD-3	UCD-4	NW-1	Black	79-1146					
5-6-2	<2[b]	<2	<2	<2	<2	<2	40,960	20,480	10,240	5,120	1,280	I
5-7-2	<2	<2	<2	<2	<2	<2	40,960	20,480	1,280	10,240	5,120	I
7-1-1	<2	<2	<2	<2	<2	<2	20,480	10,240	1,280	40,960	5,120	I
7-3-1	<2	<2	<2	<2	<2	<2	40,960	6,400	100	800	1,600	I
7-4-1	<2	<2	<2	<2	<2	<2	20,480	40,960	800	25,600	1,600	II
6-1-1	<2	<2	<2	<2	<2	<2	40,960	40,960	40,960	40,960	20,480	III
6-4-2	<2	<2	<2	<2	<2	<2	40,960	40,960	40,960	40,960	40,960	III

[a] Mouse ascitic fluid
[b] Neutralization titer

Table 5. Reactivity of the neutralizing monoclonal antibodies against TGEV strain TO-163 to feline, porcine, and canine coronaviruses

MAb[a] no.	FIPV							FEV	CCV	TGEV		Epitope specificity
	type I						type II	79-1683	1-71	SH	TO-163	
	UCD-1	UCD-2	UCD-3	UCD-4	NW-1	Black	79-1146					
55-2	<10[b]	<10	<10	<10	<10	<10	128	5,120	20,480	20,480	40,960	A
38-A	<10	<10	<10	<10	<10	<10	<10	<10	<10	32	640	B
66-A	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	204,800	C

[a] Mouse ascitic fluid
[b] Neutralization titer

5-6-2, 5-7-2, 7-1-1, 7-3-1, and 7-4-1 by 34.1～76.4%. On the basis of these results, epitopes recognized by these MAbs were named I, II, and III.

Reactivity of the neutralizing MAbs against FIPV strain 79-1146 with feline, porcine, and canine coronaviruses

Reactivity of the MAbs which recognize the 3 neutralizing epitopes in FIPV strain 79-1146, with feline, porcine, and canine coronaviruses was investigated with the neutralization (NT) test. As shown in Table 4, these MAbs did not neutralize the 6 FIPV type I strains, but neutralized FECV strain 79-1683, as well as the porcine and canine coronaviruses. In particular, MAbs which recognize epitope III (6-1-1 and 6-4-2) also showed a high neutralizing activity against these viruses, as against FIPV strain 79-1146.

Reactivity of the neutralizing MAbs against TGEV strain TO-163 with feline, porcine, and canine coronaviruses

The reactivity of the 3 neutralizing MAbs (55-2, 38-1, and 66-A) [7] prepared by using TGEV strain TO-163 as immunogen, with feline, canine, and porcine coronaviruses was examined with the NT test. All these 3 MAbs recognize different epitopes of the S protein of TGEV. Among these MAbs, MAb 55-2 possesses a strong neutralizing activity 1:12,800～1:40,960) against 7 TGEV strains (TO-163, h-5, Kanagawa, Purdue, Ukiha, SH, Miller strains), and similarly, MAb 38-A has a weak neutralizing activity (1:8～1:640) against the 7 TGEV strains. MAb 66-A possesses a neutralizing activity only against the TO-163, h-5 and Kanagawa strains of TGEV.

The results are shown in Table 5. While MAb 55-2 neutralized the FIPV type II strain 79-1146, FECV type II strain 79-1683, and CCV strain 1-71, it did not neutralize FIPV type I viruses. MAbs 38-A and 66-A did not neutralize any coronaviruses other than TGEV.

Discussion

In this study, we prepared 7 MAbs with neutralizing activity against FIPV strain 79-1146, and used them to investigate the serological relationships between feline, porcine, and canine coronaviruses.

It is generally accepted that epitopes associated with neutralization are present on the S protein of viruses. Fiscus and Teramoto have reported this phenomenon in feline coronaviruses [3]. All 7 MAbs with neutralizing activity were observed to recognize S protein. This finding confirms the presence of a neutralizing epitope on the S protein. However, there have been no reports on the characteristics of this epitope. The results of the competitive binding assay using the 7 MAbs revealed the existence of at least 3 different neutralizing epitopes (I, II, and III) in FIPV strain 79-1146 (Table 3). We believe that the topographical relationship between epitopes I, II, and III is as follows. Since

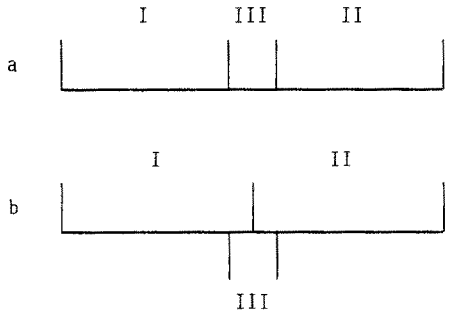

Fig. 2. Model for the location of the epitopes recognized by our panel of neutralizing monoclonal antibodies against FIPV 79-1146 strain

MAbs which recognize epitopes I and II did not inhibit the binding of each other's biotin-labeled MAb to the virus antigen, they were considered to exist independently. In contrast, 2 MAbs which recognize epitope III were inhibited by all unlabeled MAb. Moreover, the unlabeled MAb of epitope III inhibited the labeled MAbs of epitopes I and II by 34.1~76.4%. Consequently, it was postulated that epitope III exists at an intermediate site between epitopes I and II, very close to both epitopes (Fig. 2 a) resulting in blocking by steric hindrance, or that epitope III overlaps part of epitopes I and II (Fig. 2 b).

Differences in the epitopes recognized by the 7 MAbs were determined. The NT activity of these 7 MAbs against several feline, porcine, and canine coronaviruses was examined. These MAbs did not neutralize the 6 FIPV type I viruses, but neutralized FECV strain 79-1683, TGEV strains TO-163 and SH, and CCV strain 1-71, which do not cause feline infectious peritonitis (Table 4). Moreover, a MAb against TGEV strain TO-163, which neutralized the 7 TGEV strains, neutralized FIPV strain 79-1146, FECV strain 79-1683, and CCV strain 1-71 (Table 5). These results support the classification by Pedersen et al. [17]. When the 5 virus groups (FIPV type I, FIPV type II, FECV type II, TGEV, and CCV) are serologically compared, FIPV type II, FECV type II, TGEV, and CCV are found to form a group. FIPV type I virus seems to be a virus of a distinct serotype. In other words, FIPV inducing feline infectious peritonitis seem to consist of viruses of at least 2 serotypes. Pedersen et al. [17] have indicated that many cases of feline infectious peritonitis in cats are induced by type I virus infection. We also found that, out of 24 cats with feline infectious peritonitis-like symptoms and antibody-positive by indirect fluorescent antibody assay, only 6 were positive for neutralizing antibody against FIPV strain 79-1146 (FIPV type II) (unpubl. data). It is probable that FIPV type I is more prevalent in a natural environment. In the future, examination of the effects of different virus types on the anitbody-mediated enhancement of FIPV infection [15, 26], as seen in dengue virus infection [5, 13], would be valuable. At present, we are investigating the effects of the neutralizing MAb prepared in this study against in vivo FIPV infection. Differences between TGEV strain Purdue and FIPV type II strain 79-1146 at the gene level of S protein have been reported [9]. Analysis of FIPV type I at the gene level is also desired.

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