ネコ汎白血球減少症ウイルスに対するモノクローナル抗体を用いたネコパルボウイルス亜種ウイルス株の比較

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Comparison of Feline Parvovirus Subspecific Strains Using Monoclonal Antibodies Against a Feline Panleukopenia Virus

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ABSTRACT. Four monoclonal antibodies (mAb) against a feline panleukopenia virus (FPLV) TU 1 strain, one of the host range variants of feline parvovirus (FPV), were produced and applied for antigenic analysis of FPLV, canine parvovirus (CPV) and mink enteritis virus (MEV). All mAbs were considered to be directed at epitopes on the virus capsid surface because they neutralized the infectivity and inhibited the hemagglutination (HA) of the homologous virus as well as other FPV strains. They were of the mouse IgG1 type. High antigenic homogeneity among FPLV strains was confirmed by HA-inhibition (HI) test with the mAbs and polyclonal immune sera against FPLV or CPV. But the TU 11 strain of FPLV was antigenically distinguished from the remaining 14 FPLV strains by both the HI test and the micro-neutralization test with one of the mAbs produced. MEV Abashiri strain was found to be antigenically indistinguishable from FPLV. Most of the CPV strains isolated after 1981 were considered to be antigenically different from earlier CPV isolates when some mAbs were applied in the serological tests, confirming the replacement of CPV by an antigenic variant in Japan. However, antigenically different CPVs were detected at the end of 1984 from unrelated epizootics occurred a month apart in the same area.—KEY WORDS: cat, dog, mink, monoclonal antibody, parvovirus.

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Feline panleukopenia (FPL) is an acute contagious disease of probably all members of the family *Felidae*. The disease is characterized by severe enteritis and lymphopenia with high mortality for especially non-immune kittens. The etiological agent, FPL virus (FPLV) is one of the host range variants (subspecies) of feline parvovirus (FPV) to which mink enteritis virus (MEV) and canine parvovirus (CPV) also belong as subspecies viruses [32]. Some members of *Mustelidae, Procyonidae* and *Viverridae* are susceptible to FPLV as well [28]. CPV was the most recently emerged, assumptively from FPLV or MEV by mutation, as a new canine pathogen which caused enteritis and myocarditis [2, 7] and its infection was panzootic by 1980 including Japan [3].

There is a great deal of homology between the subspecies of FPV on the virus proteins [6, 17] as well as on the DNA sequences [5, 29, 30], which imply very few nucleotide or amino acid changes differentiate the antigenic and host range specificity of FPV subspecies viruses [23, 29]. Although it has been nearly impossible to find subtle antigenic heterogeneity between the subspecies or among virus strains of one species by using conventional (polyclonal) immune sera [3, 8, 10, 14, 15, 21, 24, 29], Parrish *et al.* [22, 24] were able to define antigenic diversity between the viruses by using monoclonal antibodies (mAb) against CPV and FPLV. In this paper we describe some properties of mAbs formed against FPLV TU 1 strain, results of the antigenic distinction of FPLV, MEV and CPV strains collected for the past decade in Japan using the mAbs, and an FPLV strain distinguished from other FPLV stocks by serologic tests

with a certain mAb.

MATERIALS AND METHODS

Viruses and cell cultures: Fourteen strains of FPLV and Cp 49 strain of CPV isolated either from diseased animals or as a contaminant in cat cell cultures in Tokyo area during the period between 1973 and 1980, Phillips-Roxane (Ph. R.) strain of FPLV [13], and 19 strains of CPV isolated from diseased animals in western part of Japan during 1980 to 1987 were applied in the present study. Some strains of FPLV and CPV had been characterized [1, 3, 14]. The Abashiri strain of MEV [10] was also used as a representative of MEV isolates in Japan. Year of isolation and cell culture passage histories are listed in Table 1. Virus was produced by the method described by Johnson *et al.* [12] by using Crandell feline kidney (CRFK) cells. CRFK cells were cultured in Eagle's minimum essential medium (Eagle's MEM) containing 10% NU-SERUM (NUS: Collaborative Res., Inc.), 10% tryptose phosphate broth and antibiotics (100 U of penicillin G, 100 μg of streptomycin and 5 μg amphotericin B/m*l*) (Eagle's MEM/10NUS). Murine myeloma (NS-1) cells were obtained from Dept. of the Second Biochem., Faculty of Medicine, Kagoshima University. The NS-1 cells were cultivated in RPMI 1640 medium (Flow Lab.) with 15% NUS and the antibiotics (NS-1 medium).

Polyclonal immune sera: Antisera against FPLV TU 1 strain, CPV Cp 49 strain and CPV 29F strain were prepared by using rabbits and their specificities had been confirmed elsewhere [3, 14, 19].

Monoclonal antibody production: Monoclonal antibodies were raised against FPLV TU 1 strain. The virus was partially purified from the infected CRFK cell culture fluid by the method described previously [20] and it was emulsified with an equal amount of Freund's complete adjuvant. Specified-pathogen-free Balb/c mice were initially inoculated subcutaneously with about 100 μl of the emulsion. After a month, serum anti-FPLV antibody titer of each mouse was tested by hemagglutination-inhibition (HI) test and two mice with the highest HI titers were each inoculated intraperitoneally with the purified virus in 100 μl of phosphate buffered saline (PBS). The spleens were harvested 3 days later for fusion with the NS-1 cells by the polyethylene glycol method of Galfrè and Milstein [9]. After the fusion with polyethylene glycol (PEG 1500: Boehringer Mannheim GmbH), the cells were suspended at 3×10^6/m*l* of spleen cell density in the conditioned medium (a mixture of equal parts of freshly prepared NS-1 medium and once-used medium for cultivation of NS-1 cells) that contained hypoxanthine, aminopterine and thymidine (×50 HAT concentrate: Boehringer Mannheim GmbH). Then 100 μl of the cell suspension was distributed into each well of four 96-well microculture plates (Corning Glass Works). After 14 days incubation, cell culture fluid of the wells containing hybridoma colonies were determined for virus-specific antibody using HI test. Cells from the antibody-positive wells were serially cloned 2–3 times by the limiting dilution technique using the 96-well microculture plates and mouse thymocytes suspended at 1×10^7/m*l* of cell density in the conditioned medium. The medium from cloned hybridoma cell culture was collected and utilized as mAbs for further experiments. Ascites and sera containing certain of the mouse mAb were also prepared by pristane-primed Balb/c mice transplanted with 10^7 hybridoma cells.

Monoclonal antibody Ig classes and subclasses were determined by the immunodiffusion system of mouse monoclonal typing kit (MMT 01K: Serotec).

Hemagglutination-inhibition test: The test

was performed by the microtiter system with the formalin-fixed pig erythrocytes [16] and 1/15 M PBS (pH 6.05–6.30) supplemented with 0.15 M NaCl and 0.3% bovine serum albumin (HI buffer). Hybridoma culture fluid, and mouse serum and ascites were pretreated by adsorption of the fixed erythrocytes and kaolin, and heat-inactivation (56°C for 30 min). Then two-fold dilution of the samples were made in the HI buffer using 25 μl amounts and equal amounts of virus antigen containing 4 hemagglutinating (HA) units were added to the dilution. After the mixture was left at room temperature for 1 hr, 50 μl portions of 0.75% erythrocytes suspension were added to each well and the plate was kept at 4°C overnight. The HI titer (HIU) was expressed as a reciprocal of the highest dilution that completely inhibited viral HA.

Micro-neutralization test: Micro-virus titration and micro-neutralization test (MNT) were performed in the microculture plates. After serial ten-fold virus dilutions were made with the Eagle's MEM/10NUS, 50 μl of aliquot was transferred to 4 wells per dilution. Then 50 μl of CRFK cell suspension at 2×10^5/ml of cell density in the Eagle's MEM/10NUS was added per well. The plate was gently tapped for mixing and incubated at 37°C for 5 days in a humidified chamber containing 5% CO_2. The end point was determined by the HAin production of each well and the titer was expressed as median tissue culture infective dose per 50 μl ($TCID_{50}$/50 μl). For MNT, 25 μl of hybridoma culture fluid was serially diluted two-fold with the Eagle's MEM/10NUS in the plate. Each well in the serum titration row was mixed with an equal volume of a virus suspension containing 100 $TCID_{50}$/50 μl. The plate was gently agitated for mixing and it was incubated at 37°C for 1 hr in the chamber. Then 50 μl of the CRFK cell suspension was added to each well, then the plate was incubated for 5 days. The MNT titer was defined as a reciprocal of the highest serum dilution at which the HAin production had been suppressed completely.

Immunoblotting analysis: The immunoblotting assay was used to examine the reactivity of mAbs with electrophoretically separated viral proteins. The TU 1 strain of FPLV was applied in the experiment. The methods of virus concentration, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretic transfer were described previously [17, 18, 20]. The immunoblotting analysis was performed with the ascites of mice inoculated with the hybridoma cells, biotinylated goat anti-mouse IgG (diluted 1:2,000; TAGO, Inc.) and Avidin-Biotin peroxidase complex (VECTASTAIN ABC Kit: Vector Lab., Inc.).

RESULTS

Four hybrid cell lines secreting anti-FPLV mAb were successfully established and cloned by the serial limiting dilution technique in 4 microculture plates of the fusion experiment. They were designated as 2C7, 2D9, 3G5 and 4G1, and all were of the mouse IgG1 type. After the second cloning, however, the hybrid cells of 2C7 stopped growing, and a large amount of the hybrid cells could not be achieved. The ascitic HI titers of the Balb/c mice inoculated with the hybridoma cells of 2D9, 3G5 and 4G1 at 4 weeks postinoculation were found to be $1{:}10^6$ HIU against the homologous virus strain, respectively, but the hybridoma cells of 2C7 did not grow in the abdominal cavity of the mice, of which serum HI titer was less than 1:40 HIU.

None of the mAbs 2D9, 3G5 and 4G1 reacted with the electroblotted FPLV proteins.

Results of the HI tests employing various FPV strains, the hybridoma culture fluids

Table 1. Hemagglutination-inhibition (HI) titers of monoclonal antibodies and polyclonal immune sera against feline parvoviruses

	Virus	Year of isolation	PN[a]	Monoclonal antibodies against FPLV TU 1 strain				Polyclonal immune sera against		
				2C7	2D9	3G5	4G1	FPLV TU 1	CPV Cp49	CPV 29F
FPLV[b]	TU 1	1973–	36	128[c]	1024	2048	128	2560	2560	2560
	TU 2	1975	14	128	1024	1024	128	1280	1280	640
	TU 3		14	128	1024	1024	128	2560	1280	1280
	TU 4		13	128	1024	1024	128	2560	1280	1280
	TU 5		16	128	1024	1024	128	1280	1280	1280
	TU 6		13	128	1024	1024	128	2560	1280	1280
	TU 7		17	128	1024	1024	128	1280	1280	1280
	TU 8	1976–	12	128	1024	1024	128	1280	1280	1280
	TU 9	1977	13	128	1024	1024	128	1280	640	1280
	TU 10		16	128	1024	1024	128	1280	1280	2560
	TU 11		17	<2	1024	1024	128	1280	1280	1280
	TU 12	1978–	13	128	1024	1024	128	1280	2560	1280
	TU 14	1980	11	128	1024	1024	128	1280	2560	1280
	TU 15		11	128	1024	1024	128	1280	1280	1280
	Ph.R.[d]	v.s.[e]	10	64	1024	1024	128	1280	1280	1280
MEV	Abashiri	1978	10	64	1024	1024	128	1280	1280	1280
CPV	Cp 49	1979	17	64	512	1024	64	640	1280	2560
	29F	1980	3	64	512	512	64	640	1280	1280
	817-F		4	32	128	256	16	1280	1280	1280
	KY-1		4	32	128	256	16	1280	1280	640
	BCP-1		4	32	256	512	16	1280	1280	1280
	BCP-3	1981	5	64	64	256	4	1280	640	640
	BCP-4		2	64	64	256	4	1280	640	640
	BCP-5		2	32	64	256	8	1280	1280	1280
	BCP-8	1982	3	32	64	256	8	1280	1280	1280
	KS5701		5	32	32	128	2	640	640	640
	KS5702		5	32	32	128	2	640	640	640
	KS5703		5	32	32	128	2	640	640	640
	KS5802	1983	5	32	32	128	2	640	640	640
	CPK-2	1984	4	64	64	256	4	640	640	640
	CPK-5		4	64	64	256	4	640	640	640
	CPK-7		4	128	512	1024	64	1280	1280	1280
	CPK-61	1986	2	128	64	256	8	640	640	640
	CPK-8	1987	1	128	64	256	8	640	640	640
	CPK-9		2	128	64	256	4	640	640	640
	CPK-12		2	64	64	256	4	640	640	640

a) PN: Passage number *in vitro*.
b) FPLV: Feline panleukopenia virus, MEV: Mink enteritis virus, CPV: Canine parvovirus.
c) HI titer is expressed as a reciprocal of the highest dilution that completely inhibited viral HA.
d) Ph.R.: Philips-Roxane strain.
e) v.s.: Vaccine strain, see the text.

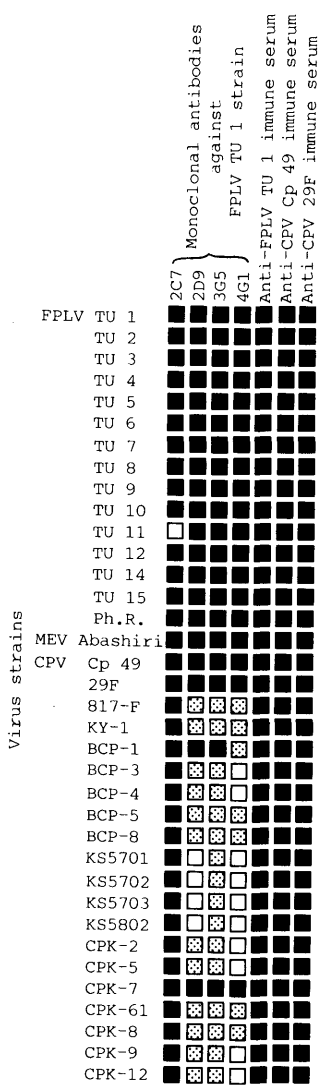

Fig. 1. The diagram of hemagglutination-inhibition titers of monoclonal antibodies and polyclonal immune sera against feline parvoviruses presented in Table 1.
■: ± 4 × the titer with the homologous virus.
▩: < 1/4 but ≧ 1/20 the titer with the homologous virus.
□: < 1/20 the titer with the homologous virus.

containing mAbs against FPLV TU 1 strain and the polyclonal immune sera against FPLV or CPVs are shown in Table 1. Figure 1 schematizes the antibody titers presented in Table 1 according to the diagram adopted in the report of Parrish and Carmichael [22]. Antigenic homogeneity was found among 15 FPLV strains with an exception. TU 11 strain of FPLV, which had been isolated from blood of the diseased cat, did not react with the mAb 2C7 at all. No antigenic difference was obtained between the Abashiri strain of MEV and the FPLV strains.

However, some antigenic heterogeneity was recorded among CPV strains when examined by using the mAbs 4G1 and 2D9. Canine parvovirus strains Cp 49, 29F and CPK-7 isolated in 1979, 1980 and 1984, respectively, were found to show the same antigenicity as the FPLV strains. Almost none or considerably low HI titers were obtained for the remaining CPV strains isolated during 1981 to 1987. Some CPV strains isolated in 1980 moderately reacted with the mAbs 2D9 and 4G1. When the mAbs, 2C7 and 3G5, or the polyclonal immune sera were used in the HI test no obvious antigenic differences were found among the CPV strains.

Table 2 shows the results of MNT between the mAbs and the polyclonal immune sera, and some virus strains of interest. All mAbs neutralized not only the homologous FPLV TU 1 strain but other viruses. However, the TU 11 strain of FPLV was not neutralized by the mAb 2C7, lower MNT titers were obtained for the CPV strains when the mAb 4G1 was used, and the mAb 2D9 did not differentiate CPVs from FPLVs by the MNT.

DISCUSSION

The four mAbs against the FPLV TU 1 strain in the present study are probably directed at epitopes on virus capsid surface because they have efficiently neutralized the infectivity and inhibited the HA of the viruses. These neutralizing mAbs are unusual in other viruses, canine adenoviruses,

Table 2. Micro-neutralization titers of monoclonal antibodies and polyclonal immune sera against different feline parvoviruses

Virus		Monoclonal antibodies against FPLV TU 1 strain				Polyclonal immune sera against		
		2C7	2D9	3G5	4G1	FPLV TU 1	CPV Cp49	CPV 29F
FPLV[a)]	TU 1	512[b)]	1024	512	64	1280	1280	1280
	TU 11	<2	4096	2048	128	2560	5120	2560
MEV	Abashiri	128	4096	2048	128	2560	2560	2560
CPV	Cp 49	256	512	1024	16	1280	2560	5120
	KS5701	256	1024	2048	2	2560	2560	5120
	KY-1	256	1024	1024	8	2560	2560	5120

a) FPLV: Feline panleukopenia virus, MEV: Mink enteritis virus, CPV: Canine parvovirus.

b) Micro-neutralization titer is expressed as a reciprocal of the highest serum dilution at which the HAin production of 100 $TCID_{50}$ of virus had been suppressed completely.

for instance [34]. A simple viral morphology peculiar to parvovirus, namely, repeated and identical capsomeres composed of a few viral structural polypeptides [32], as discussed by Parrish and Carmichael as well [22], may be one of the reasons why many mAbs against FPV possess neutralizing activity [4, 22, 24, 33].

The ascites containing the mAb against FPLV TU 1 strain did not recognize any polypeptide bands electrotransferred to nitrocellulose membrane. The most probable cause is that the epitopes have denatured after the SDS-PAGE as discussed by others [22, 33]. More than 60% (31/41) of the rat mAbs screened in the study of Surleraux *et al.* [33] did not react with the electroblotted CPV polypeptides presumably attributed to the irreversible denaturation of viral antigens during SDS-PAGE.

The two mAbs, 2C7 and 3G5, may be reacting with common epitopes of FPV subspecies virus. It was, however, an unexpected finding that the mAb 2C7 did not neutralize infectivity nor inhibit HA of the TU 11 strain of FPLV. The strain showed similar antigenicity to other FPLV strains by other mAbs or the polyclonal immune sera as presented in Tables 1 and 2. Although no conspicuous antigenic difference among FPLV strains has been reported [11, 14], Parrish *et al.* [24] demonstrated antigenic differences between the vaccine strain and the natural field strain of FPLV in immunodiffusion tests using anti-CPV dog serum. This antigenic difference was not resolved by their panel of 13 mAbs against CPV and 8 mAbs against FPLV [22]. Because only the TU 11 strain out of the present 15 strains showed the antigenic variation, this may result very exceptionally from a mutation causing large amino acid substitution of one of the common epitopes of FPV subspecies viruses. Parrish and Carmichael [22] concluded that surfaces of FPV appear to be composed of several different, but overlapping antigenic sites, each consisting of many slightly different epitopes. Another possible explanation is that there may be some antigenic variants in nature detectable only by mAbs just similar to the three antigenic variations in MEV isolates [25]. At any rate, the TU 11 strain of FPLV is antigenically distinct from other FPV subspecies viruses when examined by the HI test as well as the serum neutralization test with the mAb 2C7

produced in this study. Additional studies on biological characteristics of the TU 11 strain relating to its unique antigenicity are in progress.

As compared with high antigenic homogeneity among FPLV strains, considerable antigenic variations were found among CPV strains when the mAbs 2D9 and 4G1, but not the polyclonal immune sera, were applied in the HI test. Only a few CPV strains of interest were examined by the MNT, but the significant differences of MNT titers were not obtained when the mAb 2D9 but not 4G1 was used, differing from the results obtained by the HI test as shown in Table 1. Although the reason for this discrepancy is not known at present, there may be some qualitative difference between the mAbs 2D9 and 4G1, which appear to recognize distinct epitopes in probably different antigenic sites for neutralization. Antigenicities of CPV strains isolated after 1981 were particularly different from those of FPLV strains, the MEV strain and some CPV strains isolated in 1979 and 1980. Parrish *et al.* [27] discovered that CPV isolates in the U.S.A. after 1980 were antigenically different from earlier isolates by the HI test with mAbs against CPV and FPLV. While the earlier isolates during 1980 is called the 'old' CPV, this antigenic variant is termed the 'new' CPV. The Cp 49, 29F and CPK-7 strains isolated in 1979, 1980 and 1984, respectively, are considered to be the 'old' type, and other CPV strains isolated during 1981 to 1987 in the present study are probably what they call the 'new' CPV from the results obtained by using the mAbs 2D9 and 4G1. As a matter of course, mAbs specific for the 'new' CPV are necessary for positive identification. While the manuscript for this article was being prepared, Senda *et al.* [31] applied CPV strains from various parts of Japan collected during 1979 to 1985 and the mAbs made by Parrish *et al.* [22, 24] in the experiment, and they suggested the possibility that hemagglutinability and antigenicity of CPVs have altered during passage in dogs in nature. They applied our 10 CPV isolates, which were examined in the present study as well, by the HI test with the mAbs including an mAb specific for the 'new' CPV and the results were very similar to those obtained in this report. Quite recently Parrish *et al.* [26] reported the natural global replacement of CPV by the 'new' antigenic type over a period of 2 to 3 years between 1979 and 1982. They postulated from the results obtained that both CPV types had diverged from a common ancestor, and the 'new' CPV had been derived from one source, and subsequently had spread around the world within a period similar to that of the original spread of the 'old' type. The CPK-7 strain of CPV isolated from feces of a dog in November 1984 in Kyoto area still showed the serologic characteristics of the 'old' type, whereas the CPK-5 strain which was considered to be the 'new' type had been isolated from another dog in the same area a month before the CPK-7 isolation. Thus there may be the 'old' type CPV in nature as ever, but mostly the variant 'new' CPVs predominantly cause the disease in dogs in Japan at present.

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要　約

ネコ汎白血球減少症ウイルスに対するモノクローナル抗体を用いたネコパルボウイルス亜種ウイルス株の比較：望月雅美・小西信一郎[1]・安食政幸[2]・赤星隆雄（鹿児島大学農学部家畜微生物学講座，[1]東京大学農学部家畜微生物学教室，[2]㈱微生物化学研究所）――ネコパルボウイルス（FPV）の宿主変異亜種ウイルスの一つであるネコ汎白血球減少症ウイルス（FPLV）TU 1 株に対するモノクローナル抗体（mAb）を作出し，FPV の別の亜種ウイルスであるミンク腸炎ウイルス（MEV）およびイヌパルボウイルス（CPV）と FPLV 間の抗原性を比較した．マウス IgG 1 タイプの mAb を産生する 4 種クローンが作出され，総ての mAb がウイルス中和能と血球凝集阻止能を有することから，ウイルスカプシド表在のエピトープを認識するものと考えられた．血球凝集阻止（HI）試験に FPLV あるいは CPV に対するポリクロナール免疫血清を用いて，FPV 亜種ウイルス36株の抗原性を検討したが，有意差は認められなかった．mAb を用いても FPLV 15株間の抗原の異質性は検出されなかったが，例外的に 1 分離株（TU11株）は FPV に共通のエピトープを認識する mAb の一つと全く反応しない変異株であった．また，MEV Abashiri 株と，1980年以前および1984年11月に分離された CPV3株は，作出した mAb を用いた HI 試験で FPLV と同じ抗原性を示した．しかし，1981年以降に分離された CPV17株のこれら mAb に対する反応性は弱く，抗原性の変化が認められることから，わが国でも，最近では CPV 抗原変異株がイヌ間に優勢に広まっているものと推察された．