PLAQUE ASSAY, POLYPEPTIDE COMPOSITION AND IMMUNOCHEMISTRY OF FELINE INFECTIOUS PERITONITIS VIRUS AND FELINE ENTERIC CORONAVIRUS ISOLATES

John F. Boyle[1], Niels C. Pedersen[1], James F. Evermann[2], Alison J. McKeirnan[2], Richard L. Ott[2] and John W. Black[3]

[1]Department of Medicine, School of Veterinary Medicine University of California, Davis, CA 95616

[2]Department of Veterinary Clinical Medicine and Surgery School of Veterinary Medicine, Washington State Univ. Pullman, WA 99164

[3]Specialized Assays, P.O. Box 25110 Nashville, TN 37202

INTRODUCTION

The coronaviral nature of feline infectious peritonitis virus (FIPV) and feline enteric coronavirus (FECV) has been well documented by morphological, physicochemical and antigenic studies[1-10]. However, biochemical and detailed immunochemical analyses of FIPV and FECV have been difficult due to the inability to prepare sufficient quantities of viral material. Recently, we have been able to propagate FIPV and FECV in continuous cell culture of feline origin[8,11-13].

The purpose of this report is to describe the purification of feline coronaviruses from infected cell culture and to compare five strains with respect to: 1) plaque characteristics, 2) viral structural polypeptide composition and 3) serologic reactivity of experimentally infected cats against the structural polypeptides of homologous and heterologous strains.

MATERIALS AND METHODS

Cells and virus

Fetal cat whole fetus (fcwf-4) cells were used to propagate all feline coronavirus strains. Cells were cultured with Eagle's

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minimum essential medium supplemented with 20% Leibovitz's L-15 medium, L-glutamine, antibiotics and 10% fetal bovine serum (FBS). The isolation and pathogenic characterization of FIPV-UCD1 (UCD1), FIPV Black High passage (BHP), FIPV-Black Low Passage (BLP), FIPV-79-1146 and FECV-79-1683 have been previously described[8,9,11-15].

Titration of virus infectivity

Feline coronaviruses were titered by either dilution to endpoint of infectivity or by plaque assay. For dilution to endpoint titrations, a standard tissue culture infectious dose assay was used. Tissue culture infectious doses for 50% of the cultures (TCID50) were calculated by the method of Reed and Muench[16].

Plaque assays were performed in 6 or 12 well Costar cluster plates (Costar, Cambridge, MA). Monolayers of fcwf-4 cells were prepared in the plates. The culture medium was aspirated from the wells, and serial five or ten-fold dilutions of virus in Hank's balanced salt solution were inoculated onto the cultures. The inocula were adsorbed for 45 minutes at 37 degrees C, with occasional rocking. At the end of the adsorption period, the cultures were overlaid with medium containing 0.5% Noble agar and incubated at 37 degrees C for 1 to 5 days. Overlay medium was then removed, the monolayer rinsed with water, and stained with crystal violet.

Strains UCD1, BLP, BHP, 79-1146 and 79-1683 of feline coronavirus were cultured and plaque picked three times under 0.5% agar. After the third pick, the virus was inoculated onto monolayers of fcwf-4 cells, allowed to replicate until CPE became apparent and then freeze-thawed at -70 degrees C twice. Clarified culture supernatants were aliquoted and stored at -70 degrees C for later use as stock virus.

Enzyme-linked immunosorbent assay (ELISA)

An indirect method of ELISA was used for the detection of coronavirus antigen at various stages in the purification. This assay was performed essentially as previously described by Osterhaus et al[17] and Horzinek et al[10].

Protein determinations

The Coomassie Blue dye binding method of Bradford[18] was adapted for use in 96 well round bottom microplates.

Metabolic radiolabeling of virus

Confluent monolayers of fcwf-4 cells were prepared in

plastic cell culture flasks (Costar, Cambridge, MA). The medium was removed and the cultures were inoculated with a 1:5 dilution of the appropriate stock virus. The virus was adsorbed at 37 degrees C for 45 minutes with occasional rocking. The inocula were then removed, and Eagle's MEM containing 25% normal concentration of amino acids and 2% dialyzed FBS was added (10 ml/75 cm^2). The cultures were incubated for 4 hours at 37 degrees C, after which time 2 uCi/ml of [3H] amino acids was added to the medium. The cultures were harvested soon after CPE was apparent.

Virus purification

Virus was purified by polyethylene glycol precipitation, a discontinuous sucrose gradient centrifugation step and isopycnic centrifugation in a 20-50 percent (wt/wt) linear sucrose gradient essentially as described by Sturman et al[19].

SDS - polyacrylamide gel electrophoresis (SDS-PAGE)

Polyacrylamide gradient slab gels (8-16%), with 3% stacking gels, were run using the discontinuous system of Laemmli[20]. The acrylamide gradient was stablized during pouring with glycerol. Samples were disrupted in treatment buffer for 15 minutes at 25 degrees C. The final concentration of the components of the sample treatment buffer were 62.5 mM Tris, 2% SDS, 2% 2-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue, pH 6.8. The gels were electrophoresed at 15 mA per gel. Molecular weights were determined using a series of eight molecular weight standards (200kd - 14.4kd, Bio Rad, Richmond, CA).

Radioactivity profiles were generated by electrophoresing the proteins of metabolically labeled purified virus in a 1.5 mm thick 25 cm long SDS-polyacrylamide gel. The appropriate lane of the slab gel was cut from the gel and sliced horizontally to generate 1.5 cm x .15 cm x .4 cm pieces. The gel fractions were digested at 37 degrees C for 2 hours with 0.4 ml of NCS tissue solubilizer (9 parts NCS: 1 part water, Amersham Corp., Arlington Heights, IL). Ten milliliters of 2 parts PCS: 1 part xylene was used as scintillation fluid. Disintegrations per minute were calculated from counts per minute using the external standard channels ratio method.

Immunoblots

The electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose paper and the immunologic detection of the blotted proteins (immunoblotting) was performed similar to the method of Towbin et al[21]. Serum

samples were diluted 1:50 in dilution buffer consisting of 0.15 M NaCl, 0.05 M Tris, pH 7.4, 0.005 M EDTA, 0.1% bovine serum albumin and 0.05% tween 20. ^{125}I labeled rabbit anti-feline IgG was prepared by the chloramine T method[22] and used at a final concentration of 1 - 5 x 10^5 cpm/ml in dilution buffer.

RESULTS AND DISCUSSION

Plaque assay of feline coronaviruses

Plaques produced by 79-1146 and 79-1683 could readily be observed 2 days post infection, while UCD1, BLP and BHP usually took up to 5 days to become large enough to be viewed without the microscope. Plaque size varied considerably between strains but was consistent for each strain. UCD1 and BLP produced small plaques, about 1-2 mm 5 days post-infection. FIPV-BHP which originated from FIPV-BLP stocks, but was passaged in cell culture more than 50 times, has the ability to form slightly larger plaques (3mm in 5 days). FIPV-79-1146 and FECV-79-1683 produced much larger plaques, > 3mm 2 days post infection (Figure 1). Early CPE produced by the five strains consisted of syncytia formation, while later CPE was characterized by rounding of polykaryons and detachment from the plastic substrate (Figure 2). Titers of the stock virus also varied between strains: UCD1 and BLP having low titers ($<10^4$ pfu/ml), BHP and 79-1146 having moderate titers (about 10^5 pfu/ml) and 79-1683 having the highest titer ($>10^6$ pfu/ml).

The results of the plaque assay of the five strains of feline coronavirus we have investigated indicate a great difference in their plaque forming characteristics. Two strains, FIPV-UCD1 and FIPV-BLP, form small plaques and do not replicate to high titer in the culture system used. FIPV-BHP forms a slightly larger plaque and produces moderate titers of virus. The continuous passage of BHP has also caused it to attenuate to the extent that it is no longer pathogenic to cats[14]. Strains 79-1146 and 79-1683, on the other hand, form large plaques and replicate to high titers. In culture without agar overlay, these strains show extensive CPE 24 hours after infection. This is unlike the other feline coronavirus isolates grown in monolayers without agar overlay. These other isolates do not show CPE until at least 48 hours post infection. The CPE is then limited to small foci of infection which does not appear to spread to other areas of the monolayer, unless the infected cell cultures are passed. This may be due to the low multiplicities of infection (MOI) used, since it is difficult to obtain MOIs greater than 0.002 for these low titer virus strains. Another possible explanation is that FIPV-UCD1 and the Black isolates are highly cell associated, and that transmission to adjacent normal cells

occurs primarily via fusion with infected cells. On the other hand, FIPV-79-1146 and FECV-79-1683 progeny virions may be more freely released into the medium, thereby infecting cells in more distant areas of the monolayer.

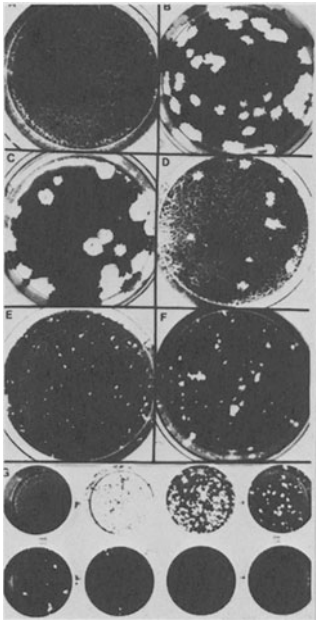

Figure 1. Plaques produced by feline coronaviruses: uninfected monolayer of fcwf cells (A), two days post-infection FIPV-79-1146 (B) and FECV-79-1683 (C), five days post-infection FIPV-BHP (D), FIPV-BLP (E) and FIPV-UCD1 (F). Plaque assay of FIPV-UCD2, first well was sham inoculated control, subsequent wells inoculated with serial five-fold dilutions (G).

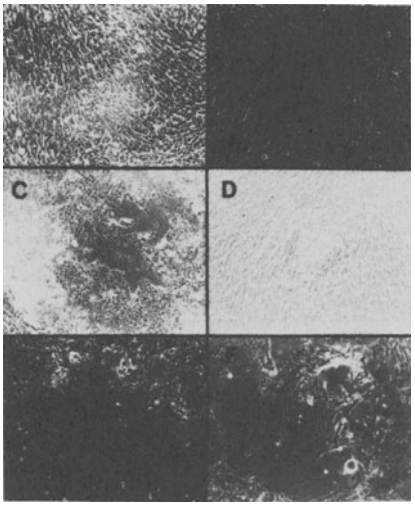

Figure 2. Cytopathic effect of feline coronaviruses: uninfected monolayer of fcwf cells (A), FECV-79-1683; 18 hours post infection (B) and 48 hours post-infection (C), FIPV-UCD1; 48 hours post-infection (D) and 5 days post-inoculation (E), FIPV-BHP; five days post-inoculation (F).

Purification of feline coronavirus from infected cell cultures

The procedure of Sturman et al 19 was used to purify feline coronavirus from infected cell cultures of feline origin. The virus routinely banded at 1.18 g/ml in isopycnic sucrose density gradients (Figure 3), which has been observed for other members of the Coronaviridae 23,24.

The recovery of infectious virus from a representative purification is summarized in Table 1. Despite the low percentage of infectious virus recovered from the band in the final centrifugation step (usually about 2%), the amount of infectious virus per milligram of protein was increased 30 fold. Using methods similar to those applied here, other investigators have reported a much higher percentage recovery of infectious particles after purification than the 2% observed for FIPV-BHP, i.e., 25% for TGEV[25]; 32% for hemagglutinating encephalomyelitis virus (HEV) of swine[26], and 66% for mouse hepatitis virus A59 (MHV-A59)[19]. This may be due the innate lability of the virion. Indeed, negative stain electron microscopy of FIPV-BHP and TGEV purified identically shows that many of the virions in the FIPV preparations are broken open, whereas virions in the TGEV preparations are uniformly intact[27].

Feline coronavirus polypeptides

The structural proteins that compose the virion of various coronaviruses have been analyzed by several laboratories[28]. In general, the coronavirus particle appears to have 4 to 7 proteins. A simple model for the structure of the coronavirion has been proposed by Sturman et al[19], who suggest that the major viral protein (N; apparent molecular weight (Mr)=50kd) is associated with the RNA. The nucleocapsid is surrounded by a lipid envelope containing a transmembrane integral glycoprotein (E1; Mr=23kd) which associates with the nucleocapsid in the interior of the virion while simultaneously exposing a portion of its structure to the external environment. The third and fourth major polypeptides are glycoproteins (E2; Mr=90kd and 180kd). The 180kd molecule is a dimer of the 90kd polypeptide, and together they comprise the characteristic peplomers of the coronavirion[29].

Radiolabeled polypeptides of UCD1 and BHP were analyzed by SDS-PAGE. Figures 4 and 5 showed that these two strains of feline coronavirus shared the 3 major polypeptides found in many members of the coronaviridae. The major polypeptide, with an apparent molecular weight of 45,000 daltons (45kd) is likely to be the nucleocapsid protein (N). The small polypeptide, which migrated as a broad band in the range of 33,000 to 27,000

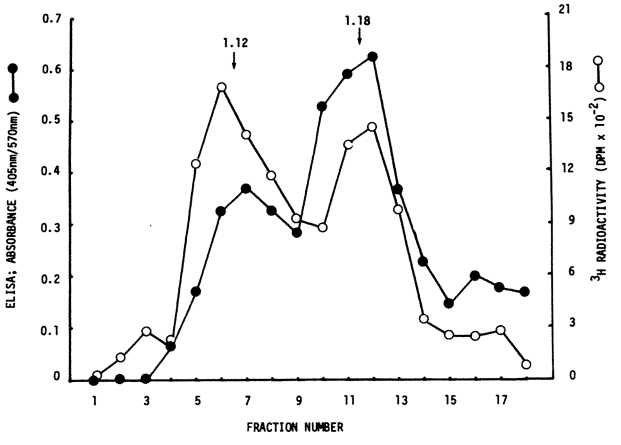

Figure 3. Enzyme-linked immunosorbent assay reactivities and ^{3}H radioactivities of fractions from a representative 20-50% (w/w) linear sucrose gradient used to purify metabolically radiolabeled FIPV-BHP.

daltons is probably the envelope protein (E1), while the large polypeptide, Mr of 210,000 daltons, is most likely the peplomer protein. At the top of the gel, a very large protein was sometimes observed. The molecular weight of this component was difficult to determine precisely, as the highest molecular weight standard used was 200kd, but it is estimated to be at least 400kd. A minor protein, Mr 42kd, was also reproducibly seen.

TABLE 1 - FIP virus purification and recovery of infectious virus.

Virus sample	Infectivity ($TCID_{50}$ x 10^5/ml)	Volume (ml)	% Recovery of infectivity	Total protein (mg)	$TCID_{50}$ x 10^5 mg of protein
Clarified infected cell culture supernatant	2.3	160	100	325	1.1
Polyethylene glycol precipitate	26	8	57	19.6	11
Interphase from 20/50% discontinuous sucrose gradient	18	3	15	1.87	29
Band from 20-50% continuous sucrose gradient (1.175 - 1.19 g/cm^3)	3.5	2	2	0.23	30

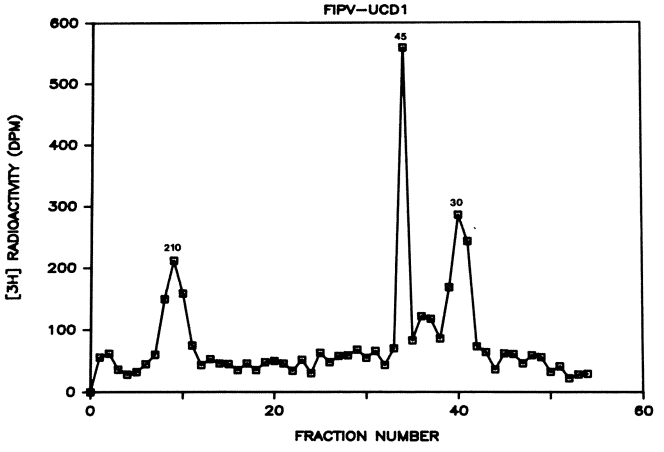

Figure 4. SDS-Polyacrylamide gel electrophoresis profile of ^{3}H amino acid labeled FIPV-UCD1. Numbers above the individual peaks represent molecular weight in kilodaltons.

The polypeptide structure of the feline coronaviruses was confirmed by coupling SDS-PAGE with the ability to detect resolved viral proteins immunologically once they were transferred to nitrocellulose paper (immunoblots). Cell culture material which had been sham inoculated (no virus) and prepared

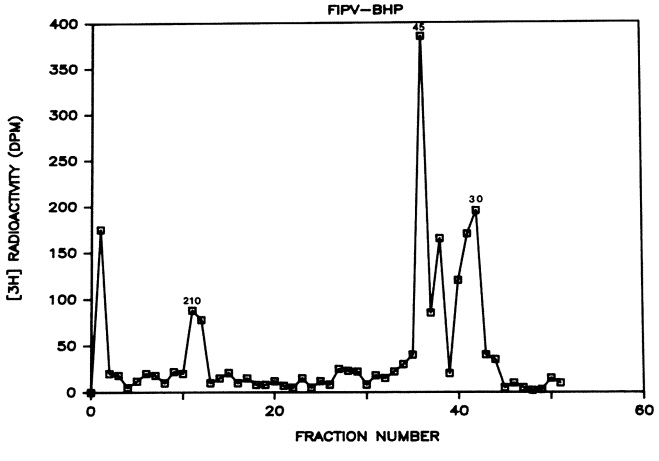

Figure 5. SDS-Polyacrylamide gel electrophoresis profile of ^{3}H amino acid labeled FIPV-BHP. Numbers above the individual peaks represent molecular weight in kilodaltons.

identically to feline coronavirus preparations was used as a virus negative control. Figure 6 shows the results of an immunoblot in which isopycnically banded FIPV-BHP was detected with various feline anti-coronavirus serum samples. Figures 7 through 9 represent the results of experiments in which five strains of feline coronavirus and sham inoculated cell preparations were electrophoresced in preparative gels, the separated proteins transferred to nitrocellulose and the polypeptides detected with serum samples from various cats. The viral antigen used to generate the blots in figures 7 through 9 was from the 20/50% interface of a discontinuous sucrose gradient. Table 2 summarizes the apparent molecular weights of the proteins for each strain. The nomenclature of Sturman et al[11] has been used to classify the polypeptides. The most prominent protein (N; Mr 45kd) was recognized by all serum samples used. The broad 30kd band (E1) seen in radioactivity profiles was resolved as a broad triplet. The precise nature of the relationship between the individual components of the E1 complex has not been investigated, but it is likely that differences in the glycosylation in the amino-terminal domain contributes to the polymorphism, as has been demonstrated by Stern et al[30] for the infectious bronchitis virus (IBV) E1 family.

The large 210kd peplomer protein was detected only when certain serum samples were used. Although the E2 polypeptide was easily seen in [^{3}H] SDS-PAGE profiles of metabolically labeled virus, it was barely detectable in the immunoblots. Three possibilities could contribute to this: (1) inefficient transfer to nitrocellulose paper during blotting, (2) lack of humoral response to this polypeptide by cats infected with feline coronaviruses or (3) loss of critical antigenic determinants during SDS-PAGE or electrophorectic transfer. The first possibility seems least likely, however, since Coomassie Blue staining of gels and amido black staining of nitrocellulose paper after blotting shows that the majority of even the larger molecular weight proteins have left the gel and bound to the nitrocellulose. Also of interest is the fact that no protein was seen which would serve as a likely candidate for a monomer E2 polypeptide as has been demonstrated in the murine coronavirus system[29].

Other proteins were also readily detected by this technique; Mr: 400kd, 190kd, 162kd, 76kd, 66kd, 57kd, 15kd, 11kd and 8kd. However, these are not likely to be viral polypeptides, because: 1) blots using sham inoculated culture material as antigen also contain bands of 190, 162, 76, 66 and 75kd (Figure 7), 2) infected animals' antibody responses to the 15, 11 and 8kd proteins do not increase through the course of infection (Figure 9) and (3) these proteins are not seen in metabolically labeled virus preparations (Figures 4 and 5).

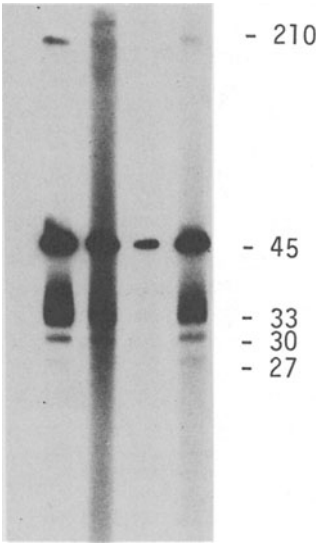

Figure 6. Immunoblot of highly purified FIPV-BHP from the 1.18 g/ml fraction of an isopyknic sucrose gradient, reacted with various feline serum samples. Values are apparent molecular weights in kilodaltons.

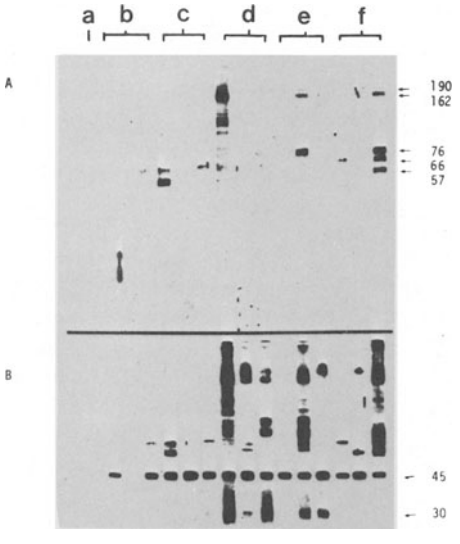

Figure 7. Immunoblots of sham inoculated fcwf cell culture material (panel A) and FIPV-UCD1 inoculated culture material (panel B), reacted with serum samples from cats with the following natural histories: pre-infection (a lane), died FIP; 79-1146 (b lanes), convalescent FECV; 79-1683 (c lanes), convalescent FIP; UCD1 (d lanes), died FIP; UCD1 (e lanes) and convalescent FECV; UCD1 (f lanes). Values are apparent molecular weights in kilodaltons.

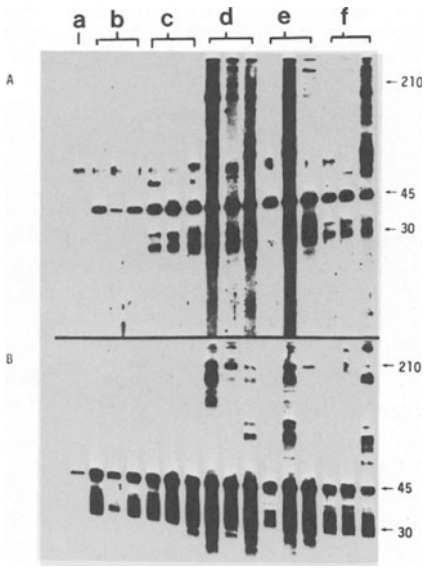

Figure 8. Immunoblots of FIPV-BLP (panel A) and FIPV-BHP (panel B) inoculated cell culture material, reacted with serum samples from cats with the following natural histories: pre-infection (a lane), died FIP: 79-1146 (b lanes), convalescent FECV; 79-1683 (c lanes), convalescent FIP; UCD1 (d lanes), died FIP; UCD1 (e lanes) and convalescent FECV; UCD1 (f lanes). Values are apparent molecular weights in kilodaltons.

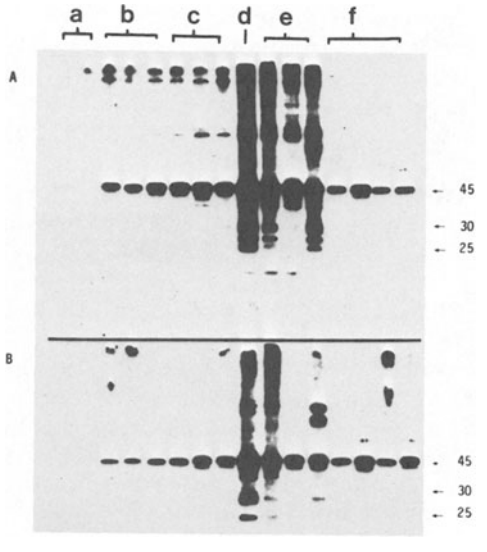

Figure 9. Immunoblots of FIPV-79-1146 (panel A) and FECV-79-1683 (panel B) inoculated cell culture material, reacted with serum samples from cats with the following natural histories: pre-infection (a lanes), died FIP; 79-1146 (b lanes), convalescent FECV; 79-1683 (c lanes), convalescent FIP; UCD1 (d lane), died FIP; UCD1 (e lanes) and convalescent FECV; UCD1 (f lanes). Values are apparent molecular weights in kilodaltons.

It therefore appears that the protein structure of the feline coronaviruses is similar to other members of the Coronaviridae[28].

Comparison of feline serological response to various feline coronaviruses

Serum samples were collected from cats which had been inoculated pronasally with various strains of feline coronavirus[9,13,14]. Figures 7 through 9 show the results of immunoblots using convalescent or pre-mortem serum samples from cats infected with the FIPV UCD1, FECV UCD, 79-1146 and 79-1683 to detect homologous and heterologous antigen. Table 3 summarizes the cross-reactivity between heterologous and homologous feline sera and viral antigen preparations. The humoral response of cats to the individual polypeptides of homologous and heterologous virus preparations, as determined by immunoblotting, appeared to be fairly consistent. Animals infected with FIPV-UCD1, FECV-UCD, FIPV-79-1146 and FECV-79-1683 all had serum antibodies which recognized the N and E1 proteins of all coronavirus strains used, i.e., FIPV-UCD1, FIPV-BLP, FIPV-BHP, 79-1146 and 79-1683. However, the anti-FIPV-UCD1 and anti-FECV-UCD1 samples tended to react more strongly with all of the virus strains. This is most likely due to the fact that these samples had higher titers to coronaviral antigen, as determined by indirect fluorescent and ELISA antibody tests (data not shown).

On the other hand, most animals had very low or undetectable levels of antibodies to the E2 peplomer protein. The only animals that responded to this polypeptide were those infected with FIPV-UCD1 and FECV-UCD1, and these antibodies reacted only with FIPV-UCD1, FIPV-BLP and FIPV-BHP, not with 79-1146 or 79-1683. This does not necessarily indicate more distant relationship of these strains, because animals infected with 79-1146 and 79-1683 also did not appear to recognize the homologous E2 antigen. This may be due to the low titers of these serum samples or to loss of critical antigenic determinants during SDS disruption of antigen.

Of particular interest is the fact that animals which had died of feline infectious peritonitis and those we considered to be immune to FIP showed comparable humoral reactivity to the individual polypeptides of the virion (Table 3). This evidence, along with the observation that high neutralizing antibody titers to FIPV are not protective, supports the hypothesis that cell mediated immunity plays a more important role in the recovery of a cat from infection with FIPV[14,15].

Table 2. Apparent molecular weights of FIPV, FECV and TGEV polypeptides. Values represent kilodaltons, NS = not seen.

	POLYPEPTIDE		
STRAIN	N	E1	E2
FIPV-UCD1	45	32	210
		30	
FIPV-BLP	45	33	210
		30	
		27	
FIPV-BHP	45	33	210
		30	
		27	
79-1146	45	30	NS
		27	
		25	
79-1683	45	30	NS
		27	
		25	
TGEV (Miller)	48	31	200
		27	

Table 3. Summary of cross-reactivity of serum samples from cats infected with various strains of feline coronavirus to heterologous and homologous viral antigen preparations. Convalescent samples were from cats having recovered from experimental infection. Pre-mortem samples were taken from cats in the terminal stages of FIP (experimentally induced) just prior to euthanasia. Cross-reactivity was broken down into 6 categories, by density of the individual bands on the autoradiographs: ++++ = greatest reactivity, 0 = no reactivity.

	Natural history of serum sample				
Viral Antigen	Died 79-1146	Conval. 79-1683	Conval. FIPV-UCD1	Died FIPV-UCD1	Conval. FECV-UCD1
79-1146					
N	++	++	++++	+++	++
E1	+	++	+++	+++	+
E2	0	0	0	0	0
79-1683					
N	+	++	+++	+++	+
E1	0	+	++	++	+
E2	0	0	0	0	0
FIPV-UCD1					
N	+++	+++	+++	+++	+++
E1	+	++	++	++	+
E2	0	0	+	+/-	0
FIPV-BHP					
N	+++	+++	+++	+++	+++
E1	++	++	+++	++	++
E2	0	0	++	++	+
FIPV-BLP					
N	++	+++	+++	+++	+++
E1	+	++	+++	+++	++
E2	0	0	+	+	+

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