Virion polypeptide specificity of immune complexes and antibodies in cats inoculated with feline infectious peritonitis virus

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SUMMARY

Immune complexes purified from sera and ascitic fluids of cats after inoculation with feline infectious peritonitis (FIP) virus contained proteins and proteolytic fragments of the peplomer, nucleocapsid, and envelope polypeptides; in addition, host proteins were demonstrated in the immune complexes. Free (uncomplexed) antibodies against the 3 classes of virion polypeptides were detected and quantitated; the weakest and latest response was directed against the peplomer protein.

Immunofluorescence titers showed the best correlation with the antibody response directed against the envelope polypeptides. Differences in reactivity were not found between sera and ascitic fluids from the same animals and between seropositive healthy cats and cats which had died of FIP. Humoral antibody and hypergammaglobulinemia showed a linear correlation, but the wide variation in antiviral titers at a given concentration of γ-globulin indicated that additional (autoimmune) reactions occur during the pathogenesis of FIP.

Feline infectious peritonitis (FIP) is a progressively debilitating, usually fatal condition affecting domestic and wild *Felidae*. The most prominent features in the dead animals are diffuse fibrinous polyserositis, mesothelial hyperplasia, and focal necrosis in the parenchymal organs. Feline infectious peritonitis is caused or triggered by coronaviruses; affected animals have high antibody titers which are presently used for diagnostic purposes. It has been shown, however, that other coronaviruses which are antigenically indistinguishable from FIP virus occur in cats, but cause only mild-to-moderate enteric infections; they may also lead to seroconversion.[1-4]

An immune pathogenesis has been postulated for FIP, and experimental evidence in favor of this hypothesis has been presented[5-8]; the evidence includes demonstration of sessile[9] and circulating[10] immune complexes (ICX) in naturally occurring and experimentally induced FIP. Since the antigen component in the ICX is unknown, identification experiments were done in the present study. Differences between feline coronaviral strains are not reflected by variations in serologic reactivity toward individual virion polypeptides, and comparable patterns are encountered in immune and fatally infected animals.[11] The present quantitative studies confirm this conclusion; in addition, the time of the appearance of antibody directed against the 3 virion proteins was studied. Finally, the virus specificity of the hypergammaglobulinemia was investigated, since it is commonly considered to have diagnostic value in FIP.

Materials and Methods

Virus—The Dahlberg strain of FIP virus (FIPV) was propagated in suckling mouse brain as described.[12] The UCD1 strain was grown in the fcwf line of feline macrophages.[13,a] The NOR-15 strain of FIPV, as well as the NLFK line of feline kidney cells used for its propagation, was obtained from Norden Laboratories.[b]

Transmissible gastroenteritis virus of swine (TGEV), strain Purdue, was used to inoculate porcine kidney cells of the PD5 line.[c] The system used for heterotypic antibody determination by immunofluorescence was described.[14]

Sera and ascitic fluids—The materials used in the present study were obtained from cats used in earlier infection experiments,[9,10] from cats with clinical infections of FIP, or from healthy animals included in a previous seroepizootiologic study.[15]

Sera devoid of coronaviral antibody were obtained from a specific pathogen-free cattery.[d] All materials were stored at −20 C. Immunoglobulin (Ig) concentrations in cat sera and ascitic fluids were determined by nephelometry,[e] using a standard preparation of purified IgG. The data are presented as relative light-scattering (RLS) values.

Virus labeling and purification—Monolayers of NLFK cells in 25-cm² Costar flasks were inoculated with the NOR-15 strain of FIPV at a multiplicity of about 10; the cells had been pre-

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[a] Both the UCD1 strain of feline infectious peritonitis virus and the fcwf line were provided by Dr. N. C. Pedersen, School of Veterinary Medicine, University of California, Davis.

[b] Norden Laboratories, Lincoln, Neb.

[c] The PD5 cell line has been provided by Duphar, Weesp, The Netherlands.

[d] CPB Zeist, The Netherlands.

[e] Hyland Laser-Nephelometer PDQ, Costa Mesa, Calif.

washed with phosphate-buffered saline solution (PBSS) containing 50 μg of diethyl aminoethyl dextran[f]/ml. After an adsorption period of 1 hour at 37 C, the inoculum was removed, and 5 ml of Dulbecco's modification of Eagle's minimum essential medium containing 2% to 5% bovine fetal serum (BFS) was added. Four hours later, the medium was removed, the monolayers were rinsed once, and 2 ml of methionine-free Dulbecco's modification of Eagle's minimum essential medium supplemented with 2% dialyzed BFS was added; this labeling medium contained 220 μCi of ^{35}S methionine (sp act, 1,440 Ci/m*M*).[g] Thirteen hours after inoculation, the medium was harvested and clarified by low-speed centrifugation.

After 200 μl of BFS was added to the harvest, the first virus purification step consisted of ammonium sulphate precipitation (50% saturation) for a minimum of 3 hours at 4 C. The precipitate was obtained by low-speed centrifugation, resuspended in 1 ml of a 150 m*M* Tris-HCl buffer (pH 8.6), and layered on top of linear 20% to 50% (w/v) sucrose gradients in TES buffer (20 m*M* Tris-HCl, pH 7.4, 1 m*M* EDTA, 100 m*M* NaCl). After centrifugation for 16 hours at 85,000 × g in a Spinco SW 27.1 rotor, the gradient was fractionated, and the radioactive peak was localized by liquid scintillation counting.

Gel electrophoresis-derived enzyme-linked immunosorbent assay (GED ELISA)—Sodium dodecyl sulfate (SDS)-polyacrylamide-gel electrophoresis (PAGE) was done as described[14]; also, the experimental details of the GED ELISA procedure have been published.[16] In the present study, FIPV-inoculated suckling mouse brain material was extracted with an equal volume of a 2% (w/v) solution of Tween 20[h] in distilled water and incubated at 37 C for 1 hour. The clarified supernatant was adjusted to a protein concentration of 5 mg/ml in 1% Tween 20/PBSS and used immediately or after it had been extracted 3 times with diethyl ether.[17] After electrophoresis in a 12.5% polyacrylamide gel, the slab containing the array of polypeptides was cut into 5-mm slices perpendicular to the direction of separation. The slices were then soaked 3 times for 5 minutes in water to remove excess SDS and homogenized in a constant volume of distilled water containing 0.2% (w/v) sodium azide. Elution of the proteins was allowed to proceed for at least 24 hours at 4 C with occasional shaking of the gel slurries.

The data are presented either as absorbance values at 405 nm or, for comparative purposes, as absorbance ratios per gel fraction; in the latter case, the ELISA readings obtained with the positive serum were divided by those of a standard negative serum (P/N ratio). This eliminated the necessity for standardization of the enzyme-substrate reaction time and facilitated comparing the data. Quantitation of the reactivity of a serum with respect to individual virus proteins was achieved by comparing the integrals under the P/N ratio curve after localization of the polypeptides, using a molecular weight marker set.

Electroblotting—The procedure was similar to that described,[16] with the following modifications: Transfer of electrophoretically separated proteins from polyacrylamide gel slabs was made to nitrocellulose filter sheets[i] (BA85, pore diameter 0.45 μm) at 4 C for 90 minutes at 0.5 A (6 to 10 V); the same gel could be blotted at least twice. Transfer buffer consisted of 0.375*M* Tris-HCl (pH 8.8) in 80% water/20% methanol containing 0.015% SDS. After transfer, the filters were soaked overnight at 4 C in a 0.01*M* Tris-HCl buffer (pH 7.4) containing 0.9% NaCl (rinsing buffer) supplemented with 3% bovine serum albumin and stored at −20 C until use.

An ELISA procedure was chosen for the demonstration of virus-specific antigens in protein blots. The nitrocellulose sheets were incubated for 2 hours at room temperature with a horse radish peroxidase conjugate,[18] using purified IgG from an ascitic fluid of a cat with FIP; purification included ammonium sulfate precipitation and chromatography on protein A Sepharose.[f] The conjugate was diluted 1:100 in rinsing buffer supplemented with 3% bovine serum albumin, 1% newborn calf serum, 0.2% Triton X-100, 0.1% sodium deoxycholate, and 0.02% SDS. After 4 cycles of washing (3 times in rinsing buffer supplemented with 1% Triton X-100 and once in rinsing buffer alone), the substrate solution was added which, in 100 ml of rinsing buffer, contained 250 μl of a 1% solution of orthodianisidine[j] in methanol and 33 μl of 30% H_2O_2. The reaction was stopped after 30 to 60 minutes by rinsing of the filter strips in running tap water. The patterns of bands became more distinct when the strips were left in water overnight or longer at 4 C.

Radioimmunoprecipitation (RIP)—Purified radioactively labeled FIPV (15 μl, containing 200 to 400 cpm/μl) was diluted 6-fold in lysis buffer (TES buffer, pH 7.4, containing 0.5% Triton X-100, 0.5% [w/v] 1,5 naphthalene disulphonate disodium salt, and 2 m*M* phenyl methyl sulphonyl fluoride) and mixed with 10 to 15 μl of antiserum or ascites. After overnight incubation at 4 C, KCl was added to a final concentration of 0.5*M*, and the ICX were adsorbed to formaldehyde-fixed *Staphylococcus aureus* cells for 45 minutes at room temperature. After repeated washes in TES buffer containing 0.1% Triton X-100, the immune precipitates were dissolved in Laemmli buffer and analyzed by PAGE and fluorography. Quantitation of the polypeptide-specific response was done by liquid scintillation counting of the respective bands which had been excised from the dried gel after localization by fluorography.

Preparation of ICX—From clarified serum or ascitic fluid (10,000 × g, 10 minutes), ICX were precipitated by the addition of 1/5 volume of a mixture containing 30% polyethylene glycol 6000 (PEG)[k] and 0.2*M* EDTA·Na_2 in PBSS, resulting in a final PEG concentration of 3.5%. After overnight incubation at 4 C, the precipitate was obtained, and the pellet was washed twice, using the PEG/EDTA mixture at a 1:6 dilution. Further purification was achieved by mixing the pellet material dissolved in PBSS with a 9-fold volume of a suspension of protein A Sepharose[f] (final concentration 5%). The suspension was stirred gently for 30 minutes at room temperature and centrifuged at low speed, and the pellet was washed twice in PBSS. Elution of the ICX was done by incubation of the sediment for 15 minutes in a glycine/NaCl solution (0.1*M*, pH 2.8). Supernatant material was withdrawn, and the pH was adjusted to physiologic values, using 1.0*M* Tris, and analysed by PAGE and electroblotting.

Results

Analysis of ICX in sera and ascitic fluids—The electroblot-ELISA procedure was used for the demonstration of antigen components in circulating ICX. For the purpose of comparison and protein identification, the mouse brain-adapted Dahlberg strain, the UCD1 strain of FIPV, and the Purdue strain of TGEV were included (Fig 1). For the most prominent nucleocapsid (N) protein, a value of about 43,000 was determined; with frequencies varying per antigen preparation, accompanying structures of lower molecular weight (mol wt) were observed. Three major envelope (E) proteins were detected in the blotting experiments, which will be referred to as p32, p30, and p27; additional smaller E structures were encountered in antigen preparations from FIPV-inoculated mouse brain. The data are summarized in Table 1.

As shown in Figure 1, FIPV and TGEV differ in the mo-

[f] Pharmacia, Uppsala, Sweden.
[g] All isotopes were from Amersham International, England.
[h] Merck, Darmstadt, West Germany.
[i] Schleicher & Schuell GmbH, Dassel, West Germany.
[j] Fluka, Buchs, Switzerland.
[k] BDH Chemicals Ltd, Poole, England.

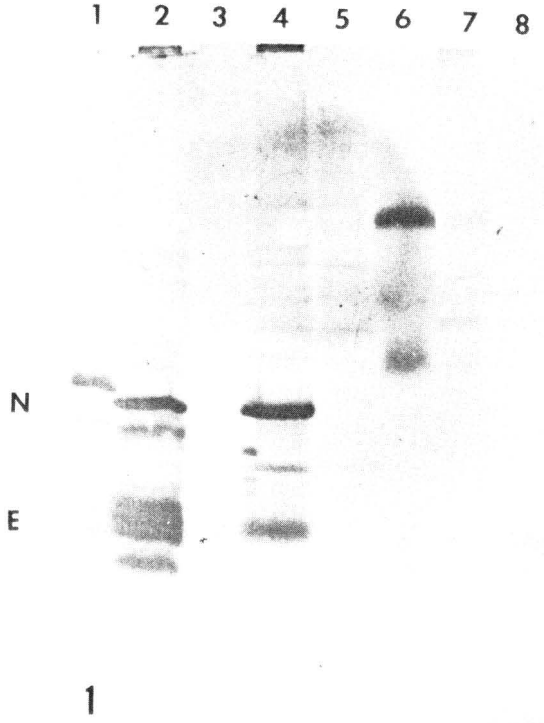

Fig 1—Electroblot-ELISA for the demonstration of antigen in semipurified transmissible gastroenteritis virus (lane 1), feline infectious peritonitis virus (FIPV)-inoculated and noninoculated suckling mouse brain material (lanes 2 and 3, respectively), and FIPV-inoculated and noninoculated lysates from fcwf cells (lanes 4 and 5, respectively). The remaining lanes show antigens detected in ascitic fluid of field instances of FIP (lane 6) and in a polyethylene glycol precipitate from this material, analyzed either directly (lane 7) or after purification on protein A Sepharose (lane 8). The positions of the nucleocapsid protein (N) and the envelope proteins (E) are indicated. Note host-specific reactions in the higher molecular weight range detected by the peroxidase conjugate (FIP ascitic fluid).

TABLE 1—Molecular weight values of feline infectious peritonitis virus structural polypeptides and their proteolytic fragments, as determined by electroblotting/ELISA

Protein	No. of blotting experiments	Molecular weight
PEPLOMER		
p83	3	83,000 ± 1,700
NUCLEOCAPSID		
p43	10	42,600 ± 1,300
p41	7	40,800 ± 2,000
p40	4	40,000 ± 2,200
p37	4	36,600 ± 1,500
ENVELOPE		
p32	7	31,500 ± 1,000
p30	7	29,500 ± 800
p27	7	27,200 ± 400
p26	3	26,000 ± 900
p24	5	23,500 ± 1,100
p21	4	21,100 ± 300
p19	4	18,500 ± 1,000

Data are expressed as mean ± SD

lecular weight of the N proteins and their (proteolytic) split products; the TGEV proteins corresponding to p43 and p40 showed an apparent molecular weight of 45,000 and 41,000. In contrast, the E proteins of TGEV appeared at the same position as did those of FIPV. Conspicuous differences appeared in the patterns of blotted proteins from mouse brain inoculated with the Dahlberg strain (Fig 1, lane 2) and fcwf cells inoculated with the UCD1 strain (lane 4) of FIPV; in the latter, the main N protein (p43) and 1 minor fragment (p37) and only the p32 E protein were demonstrated. The specificity of the ELISA used for antigen detection is evident from the empty lanes 3 and 5 containing noninoculated mouse brain and fcwf cell extracts, respectively.

In ascitic fluid from a randomly selected cat that had died of FIP, the E proteins p32 and p27 were detected immediately (Fig 1, lane 6), after PEG precipitation (lane 7), and in the eluate from protein A Sepharose (lane 8).

Analysis of the ICX in sera and ascitic fluids of 7 experimental instances of FIP demonstrated the polypeptides of all 3 structural constituents of FIPV, although to varying degrees (Fig 2; Table 2). For optimal demonstration of all viral antigens, 2nd blots of the same gel were used. As shown in a Coomassie blue-stained gel after 2 blots (Fig 2), various amounts of host proteins are retained in the higher molecular weight region of the gel.

Correlation of total IgG with antiviral antibody—Hypergammaglobulinemia is considered characteristic of FIP; in view of the various arrays of nonviral proteins encountered in ICX of inoculated animals (Fig 2) and antibody production against host components inferred from this observation, the relationship between IgG levels and anti-FIPV titers was determined; total antibody activity was determined by ELISA. The results obtained with 40 ascitic fluids from confirmed instances of FIP are depicted in Figure 3. In 20 additional samples, both the IgG levels and the antibody titers were low (not shown). For 31 selected values (disregarding 9 measurements giving extreme RLS and ELISA readings), a correlation coefficient of $r = 0.8223$ was calculated which, at the given df, indicated that a linear correlation existed ($P < 0.001$). Also, the border values of highest RLS readings in the presence of lowest antiviral titer showed a linear correlation ($r = 0.9993$; $n = 5$; $P < 0.001$). In 4 instances, extremely high ELISA titers were recorded in ascitic fluids with low RLS readings (Fig 3, empty symbols).

Quantitation of antibody reactivity to virion polypeptides—Since the analysis of ICX had demonstrated the presence of all 3 classes of virion polypeptides, the corresponding noncomplexed antibodies were expected to occur during infection. Experiments, using different extraction procedures of FIPV-inoculated suckling mouse brain, were done to determine the optimal method of antigen preparation for GED ELISA. Although ether extraction resulted in preferential loss of antigen from the E region (Fig 4),

TABLE 2—Viral proteins identified in immune complexes purified from the ascitic fluids (A) and sera (S) of 7 cats after infection with feline infectious peritonitis virus (see Fig 2)

Cat No.	Protein		
	P	N	E
1(A)	. . .	. . .	p32, p30, p27, p19
2(A)	p83	p43	p32, p30, p27, p19
(S)	p83	p43	p32, p30, p27
3(A)	p83	p40	p32, p30, p27, p21
(S)	p83	p43	p32, p30, p27
4(A)	p83	. . .	p32, p30, p27, p21
5(A)	. . .	. . .	p32, p30, p27
(S)	. . .	. . .	p32, p30, p27
6(A)	p83	. . .	p32, p30, p27
(S)	p83	. . .	p32, p30, p27
7(A)	p83	p43	p32, p30, p27

P = peplomer protein. N = nucleocapsid protein. E = envelope protein.

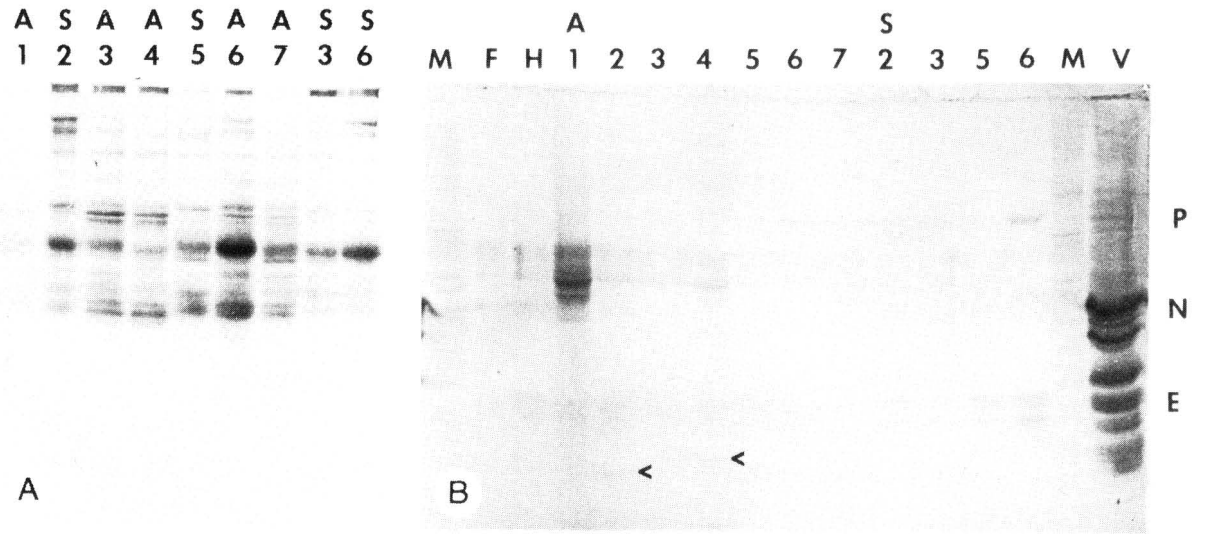

Fig 2—Electroblot-ELISA for the demonstration of antigen in immune complexes purified from ascitic fluids (A) and sera (S) of 7 inoculated cats. Nonviral signals were prominent in the high molecular weight region of the polyacrylamide gel after staining with Coomassie blue (a), whereas specific reactions appeared in the second electroblot (b). The positions of the nucleocapsid protein (N), the envelope proteins (E), and the peplomer protein p83 (P) are indicated. Arrowheads point to the p19 (A2) and the p21 (A4) proteolytic fragments of the E protein.

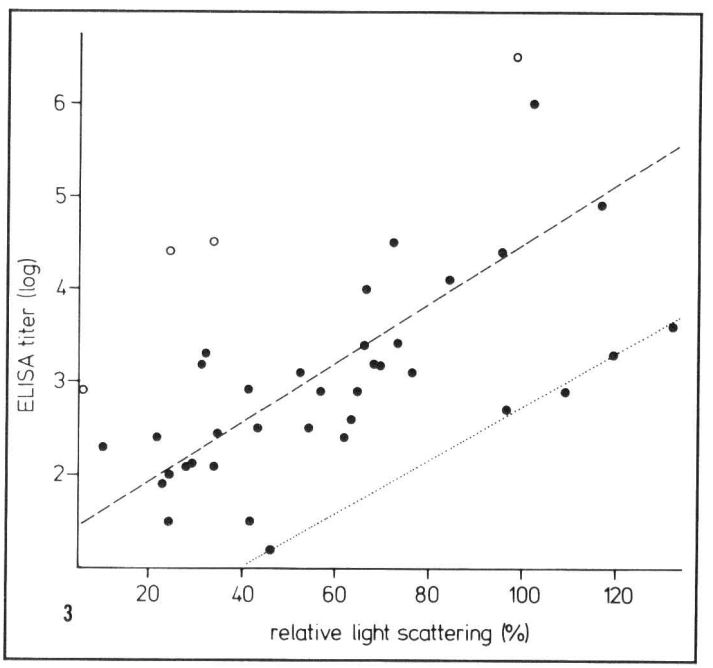

Fig 3—Correlation between antibody titers (as measured by ELISA) and immunoglobulin G contents (as measured by laser beat nephelometry) in 40 ascitic fluids from field instances of feline infectious peritonitis. A linear regression (dotted line, $r = 0.993$, $n = 5$, $P < 0.001$) was obtained for the lowest titer values with corresponding highest relative light-scattering readings. Also, the remaining values, with the exception of 4 extremely high-titer preparations (open symbols), showed a linear correlation (dashed line, $r = 0.8223$, $n = 31$, $P < 0.001$).

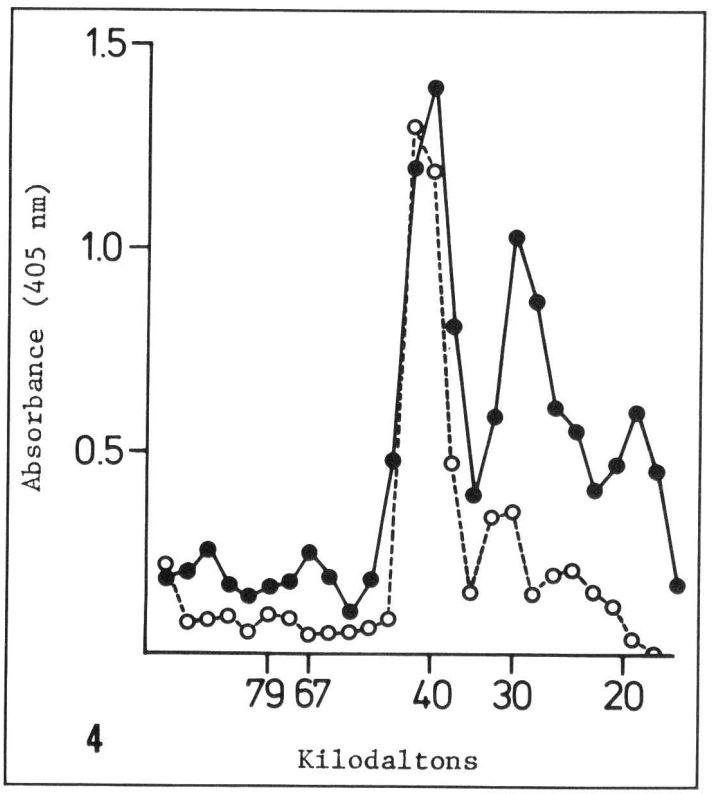

Fig 4—Gel electrophoresis-derived ELISA profiles obtained with the same antibody, using feline infectious peritonitis virus mouse brain antigen extracted with Tween 20/diethylether (open symbols) and Tween 20 alone (closed circles). Note decrease of activity in the envelope protein region after ether extraction.

it proved indispensable for proper separation of the viral proteins. All subsequent experiments were therefore done, using brain homogenates extracted with Tween 20/ ether.

The profiles of representative experiments are given in Figure 5. Demonstration of the peplomer (P) peak was poor in most gels, and the various E protein fragments were hardly ever resolved. The profiles obtained with serum and ascitic fluid of the same animal were virtually identical (Fig 5a). Although most body fluids contained clearly demonstrable antibodies directed both against the N and the E proteins, some preparations were lacking one of these (Fig 5b and c). A comparison of 8 sera from cats with clinical FIP and 8 sera from healthy seropositive cats showed no significant differences (Fig 5d). The day-to-day reproducibility of the ELISA P/N ratios was good.

The areas under the P, N, and E curve peaks were measured, and the percentages were calculated per serum. When comparing sera from cats with clinical FIP with

TABLE 3—Comparison of antibody activities directed against feline infectious peritonitis virus structural proteins (gel electrophoresis-derived ELISA measurements) between clinical field instances and healthy cats

Serum	Anti-P*	(%)	Anti-N	(%)	Anti-E	(%)	Total
CATS WITH FIP							
4329	1.36	14	2.88	30	5.47	56	9.71
4391	3.82	33	7.00	60	0.93	8	11.75
4330	11.16	40	7.77	28	8.96	32	27.89
4411	13.42	45	7.21	24	8.96	30	29.58
1346	2.14	6	12.60	36	19.81	57	34.55
4394	17.78	32	11.62	25	19.60	43	46.00
4361	27.84	52	13.07	24	12.80	24	53.71
4413	14.64	24	9.87	16	35.84	59	60.35
Mean	11.28	33	9.09	26	14.12	41	34.49
HEALTHY SEROPOSITIVE CATS							
1039	0.94	12	2.94	37	4.06	51	7.94
1042	1.36	16	4.98	60	1.94	23	8.28
1333	6.32	38	2.74	17	7.43	45	16.49
1153	3.22	18	5.07	28	9.76	54	18.05
1405	5.98	26	6.68	29	10.53	45	23.19
1406	7.06	25	12.52	44	8.96	31	28.56
1056	7.60	18	11.26	27	23.58	56	42.44
1160	12.02	27	13.75	31	18.23	41	44.00
Mean	4.42	20	6.11	27	11.93	53	22.46

* The poorly resolved P peak (*see* Fig 6) was entered into the calculations, using the triangular approximation with a peak value at an apparent molecular weight of 195,000.

P = peplomer protein. N = nucleocapsid protein. E = envelope protein.

those from clinically healthy, seropositive cats, a wide variation was noted, but the overall reaction pattern was similar (Table 3).

Time course of the protein-specific humoral response—The RIP experiments were done, using sera obtained from cats at different times after inoculation. As shown in Figure 6, the earliest immune response was directed against the N protein (only p43 recognized in this test, using the Norden strain radiolabeled in NLFK cells). Antibodies to the E proteins appeared later; the unresolved p32/p30 and the p27 species were reproducibly immune precipitated. The increase in serum titers, as measured by immunofluorescence, correlated with the E signal rather than with that of the N protein. Latest and weakest in appearance was antibody directed against the P protein; only the 195,000 species was detected by RIP. It must be noted that antibody recognition is different from the stochiometric occurrence of structural proteins in the virion as evidenced by the incorporation of ^{35}S methionine. Thus, more N protein and far less of the P protein was immune precipitated than was present in the virion equivalent (Table 4).

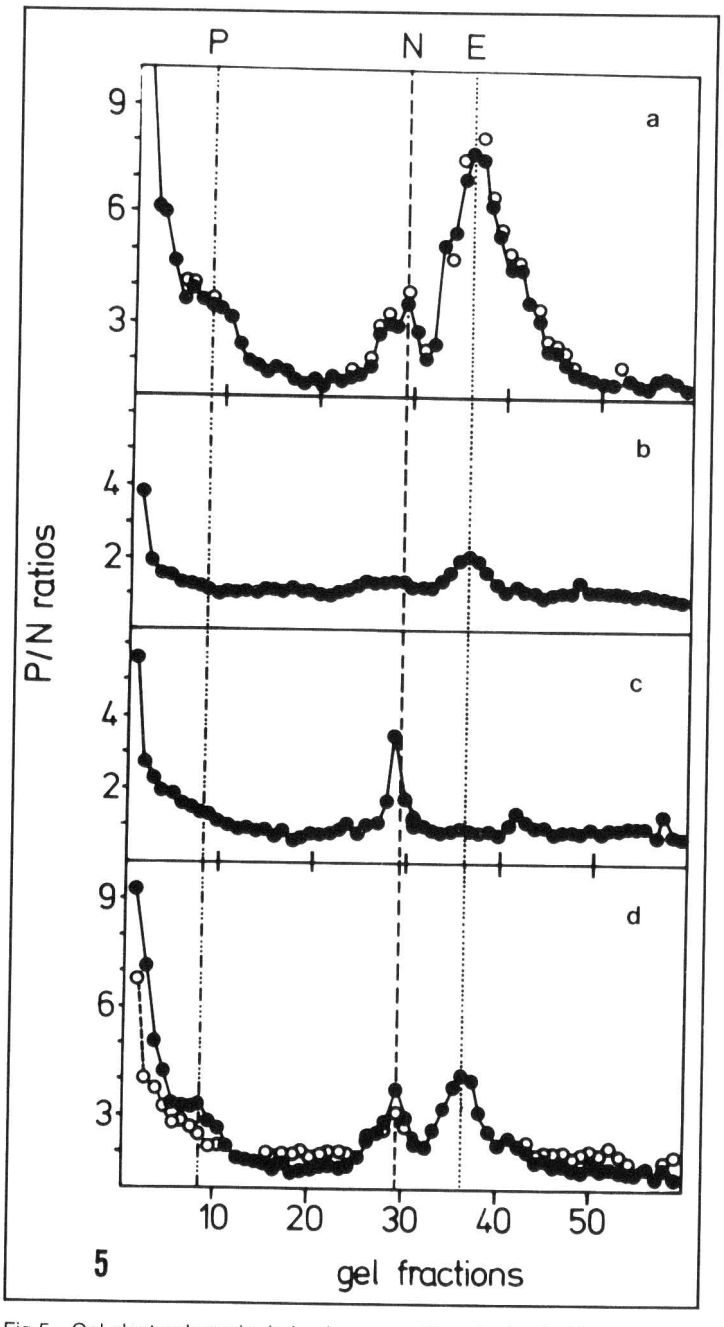

Fig 5—Gel electrophoresis-derived ELISA profiles obtained with ascitic fluid (open circles) and serum (closed circles) from a field instance of feline infectious peritonitis (a) and with sera recognizing the envelope (E) protein (b) or the nucleocapsid (N) protein (c) only. Panel d shows average activities determined in 8 sera from cats which had died of feline infectious peritonitis (closed circles) and from seropositive healthy cats (open circles). P = peplomer protein.

Discussion

Immune complexes present in the body fluids of cats with FIP were shown to contain all 3 classes of virion polypeptides. From the various relative abundance of the virion polypeptides noted between different sera and ascitic fluids, it may be assumed that the majority of ICX does not contain intact virions, but rather virion components and/or their proteolytic split products. It has been shown that the N protein of mouse hepatitis virus, another coronavirus, is readily degraded into distinct fragments by ubiquitous proteases.[19] Cell destruction with proteolysis also occurs during FIP pathogenesis, and fragments carring antigenic determinants (which may not be exposed in the intact molecule) are expected to induce an antibody response. Therefore, mouse brain extracts of the Dahlberg strain of FIPV,[12] rather than purified virus were used as controls in the present ICX blotting experiments and in the analysis of antibody specificities. As demonstrated in Figure 1, arrays of virus-specific proteolytic fragments were reproducibly formed in the tissue, some of which were also demonstrable in the ICX (Fig 2; Table 2). These may have contained various amounts of host cellular material (Fig 1 and 2), an observation indicative of autoimmune phenomena.

The high molecular weight P protein of 195,000 was

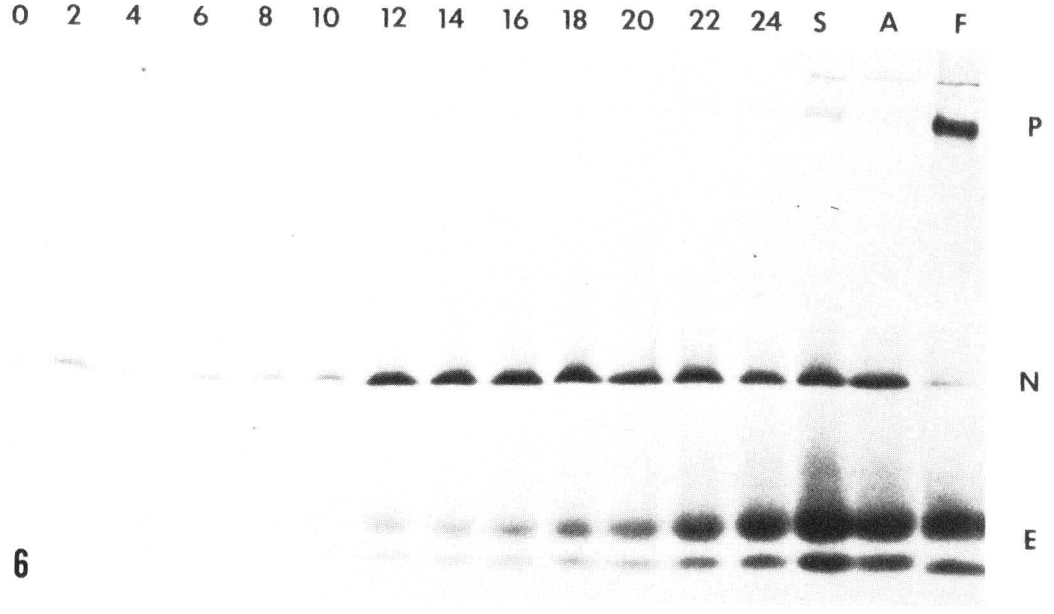

Fig 6—Radioimmune precipitation and sodium dodecyl sulfate polyacrylamide gel electrophoresis of ^{35}S methionine-labeled feline infectious peritonitis virus, using cat serum samples obtained at different times after inoculation. Lane numbers indicate days after inoculation; reactions, using the serum (S) and ascitic fluid (A) of the succumbed animal (28 days after inoculation), are presented as is the radiolabeled virus preparation used in this experiment (F). The positions of the nucleocapsid protein (N), the envelope proteins (E), and the peplomer protein p195 (P) are indicated. A quantitative evaluation of the reactivities is given in Table 4. Note nonspecific adsorption of the N and E proteins to the *Staphylococcus aureus* cells (lanes 0-10).

TABLE 4—Quantitation of the polypeptide-specific serologic response at different times after inoculation (from the radioimmunoprecipitation experiment depicted in Fig 6)

	Days after infection					
Item	10	14	18	22	28	FIPV
PROTEINS						
P	90*	95	69	91	113	200
N	95	227	320	197	156	104
E	243	336	348	422	1079	689
RATIOS						
E/P	2.7	3.5	5.0	4.6	9.6	3.5
E/N	2.6	1.5	1.1	2.1	6.9	6.6
IFT titer	< 5	10	80	320	640	...

* The values are cpm determined in the respective band areas of the dried polyacrylamide gel after localization by fluorography.

P = peplomer protein. N = nucleocapsid protein. E = envelope protein. IFT = antibody titer as determined by indirect immunofluorescence assay. FIPV = values encountered in purified preparations of feline infectious peritonitis virus.

found only on one occasion in extracts of inoculated cells and never in ICX after blotting (Fig 1 and 2). Since this protein, as well as its homologous antibody, was readily detected in metabolically labeled FIPV and after RIP (in the presence of protease inhibitors, Fig 6), it was assumed that the P-specific 83,000 structure detected in ICX (Fig 2) was the result of proteolysis after cytopathologic damage or that it constituted the intracellular precursor of the high mol wt P protein, as has been shown for mouse hepatitis virus.[19]

Not unexpectedly, free antibody was also found to be directed against the 3 classes of FIPV proteins (Fig 6). In sera taken at different intervals after inoculation, the earliest and most pronounced humoral response concerned the N protein, which was taken as an additional indication for virus disintegration and antigenaemia. The gradual increase of the anti-E response correlated with the serum titers determined in immunofluorescence tests; a similar conclusion was reached by Fiscus et al,[20] using a monoclonal antibody competition assay. The P protein invariably gave a weak signal—contrasting with its relative abundance in the virion, for which different technical reasons have been discussed.[11,l] When using RIP instead of immunoblotting from SDS gels (to avoid possible loss of conformational [discontinuous] determinants), identical results were obtained (Fig 6) which may reflect poor antigenicity of this structure or the preferential induction of low-affinity antibodies.

The characteristically high levels of γ-globulin[21,22] and the increased immunofluorescence titers[15,23] in the body fluids of preterminal instances of FIP are of diagnostic value; they invite the conclusion that hypergammaglobulinaemia is due to the antiviral immune response. A statistical correlation of linearity was found (Fig 3). However, in view of a more than 1,000-fold difference between the minimal vs the maximal titers (empty symbols) at the same concentration of IgG, it is unlikely that viral antigen alone triggered the hypergammaglobulinemia. The antiviral titers probably reflected the extent of virus replication, and the concomitant cell damage in turn may have stimulated an autoimmune reaction. Anticellular activities have been noted in PAGE of ICX (Fig 2), immunoblotting (Fig 1), ELISA,[l] and GED ELISA.[m] These observations do not necessarily imply that the autoimmune reaction is pathogenetically relevant; it may rather be a consequence than the cause of the disease process.

Using GED ELISA, we quantitated the humoral reaction to FIPV proteins in seropositive healthy cats and in fatal instances of FIP. This experimental approach was chosen to obtain serologic clues of prognostic value. Our working hypothesis was based on the following precedent: with

[l] Ingersoll JD. *Feline coronaviruses: development of homologous serological assays and determination of antigenic relatedness*. MSc Thesis, Department of Biological Sciences, Graduate College, University of Nebraska, Lincoln, 1985.

[m] Horzinek MC, Institute of Virology, Veterinary Faculty, State University, The Netherlands: Unpublished data, 1984.

morbilliviruses, differences in the immune reaction to the matrix (M) protein were found between patients recovered from acute measles and those with atypical measles[24] and subacute sclerosing panencephalitis.[25-27] In patients with atypical measles, a pronounced antibody response to the M antigen is part of a generally accentuated immune response; in subacute sclerosing panencephalitis, no or only low titers against the M protein are seen in spite of increased antibody titers to most measles virus components. Similar observations were made in dogs persistently infected with canine distemper virus.[28,29] The E transmembrane protein of coronaviruses fulfills criteria of a M protein; its hydrophobicity, a reflection of its lipid association within the viral membrane,[30] is demonstrated by its preferential removal during ether extraction (Fig 4).

It should be observed that the activities recorded in Figure 5 are without dimension and represent the P/N ratios. In the same animal, a difference in antibody specificities was not observed between serum and ascitic fluid, indicating that the latter was transudate in nature. Quantitation of free (uncomplexed) antibody directed against individual FIP viral polypeptides did not reveal differences between healthy cats and animals which had died of the disease (Fig 5d; Table 3). A missing reactivity against the N (Fig 5b) or E protein (Fig 5c) in some animals has been observed before[n] and may be due to exhaustive ICX formation, rather than to a lack of immune reaction to these antigens. The body fluids were tested at a constant and relatively low dilution (1:100) at which probably only high-affinity antibodies are detected[31]; the question remains unanswered whether low-affinity antibody does have a part in FIP. Also, the test system did not distinguish between subclasses of feline IgG[32,33] which may differ in their biologic activity (binding to Fc receptors; activation of complement) and relevance to the immune phenomena encountered in FIP.

The similarity in the humoral immune response to individual polypeptides between seropositive healthy animals and fatally infected cats along with the observation that high neutralizing antibody titers are not protective have been taken as indications that cell-mediated reactions have an important part in the immunity to FIP.[34,35] On the other hand, the serologic analyses done so far are probably still too coarse to reveal differences in response to FIPV and its nonfatal relative, feline enteric coronavirus; this problem is presently being approached at the epitope level. However, virulence in feline coronaviruses may not possess a virion structural covariant at all.

[n] Fiscus SA, Syngene Products and Research, Fort Collins, Colo: Personal communication, 1984.

References

1. McKeirnan AJ, Evermann JF, Hargis A, et al. Isolation of feline coronaviruses from two cats with diverse disease manifestations. *Feline Pract* 1980;11(3):16–20.

2. Pedersen NC, Boyle JF, Floyd K, et al. An enteric coronavirus infection in cats and its relationship with feline infectious peritonitis. *Am J Vet Res* 1981;42:368–377.

3. Hayashi T, Watabe Y, Nakayama H, et al. Enteritis due to feline infectious peritonitis virus. *Jpn J Vet Sci* 1982;44:97–106.

4. Pedersen NC, Black JW, Boyle JF, et al. Pathogenic differences between various feline coronavirus isolates. *Adv Exp Med Biol* 1984;173:365–380.

5. Horzinek MC, Osterhaus ADME. The virology and pathogenesis of feline infectious peritonitis. *Arch Virol* 1979;59:1–15.

6. Horzinek MC, Daha M, van Dam RH, et al. Arguments in favour of an immune pathogenesis of feline infectious peritonitis (FIP). Fourth WHO Symposium on Microbiology, Munich, 1979.

7. Pedersen NC, Boyle JF. Immunologic phenomena in the effusive form of feline infectious peritonitis. *Am J Vet Res* 1980;41:868–876.

8. Weiss RC, Scott FW. Antibody-mediated enhancement of disease in feline infectious peritonitis: comparison with dengue hemorrhagic fever. *Comp Immunol Microbiol Infect Dis* 1981;4:175–189.

9. Jacobse-Geels HEL, Daha MR, Horzinek MC. Isolation and characterization of feline C_3 and evidence for the immune complex pathogenesis of feline infectious peritonitis. *J Immunol* 1980;125:1606–1610.

10. Jacobse-Geels HEL, Daha MR, Horzinek MC. Antibody, immune complexes, and complement activity fluctuations in kittens with experimentally induced feline infectious peritonitis. *Am J Vet Res* 1982;43:666–670.

11. Boyle JF, Pedersen NC, Evermann JF et al. Plaque assay, polypeptide composition, and immunochemistry of feline infectious peritonitis virus and feline enteric coronavirus. *Adv Exp Med Biol* 1984;173:133–147.

12. Osterhaus ADME, Horzinek MC, Wirahadiredja RMS. Feline infectious peritonitis virus. II. Propagation in suckling mouse brain. *Zentralbl Veterinarmed [B]* 1978;25:301–307.

13. Jacobse-Geels HEL, Horzinek MC. Expression of feline infectious peritonitis coronavirus antigens on the surface of feline macrophage-like cells. *J Gen Virol* 1983;64:1859–1866.

14. Osterhaus ADME, Horzinek MC, Reynolds DJ. Seroepidemiology of feline infectious peritonitis virus infections using transmissible gastroenteritis virus as antigen. *Zentralbl Veterinarmed [B]* 1977;24:835–841.

15. Horzinek MC, Osterhaus ADME. Feline infectious peritonitis: a worldwide serosurvey. *Am J Vet Res* 1979;40:1487–1492.

16. Horzinek MC, Lutz H, Pedersen NC. Antigenic relationships among homologous structural polypeptides of porcine, feline, and canine coronaviruses. *Infect Immun* 1982;37:1148–1155.

17. Mussgay M, Rott R. Studies on the structure of a hemagglutinating component of a group A arbovirus (Sindbis). *Virology* 1964;23:573–581.

18. Nakane PK, Kawoi A. Peroxidase-labelled antibody: a new method for conjugation. *J Histochem Cytochem* 1974;14:929–936.

19. Rottier PJM, Spaan WJM, Horzinek MC, et al. Translation of three mouse hepatitis virus strain A59 subgenomic RNAs in *Xenopus laevis* oocytes. *J Virol* 1982;38:20–28.

20. Fiscus SA, Teramoto YA, Mildbrand MM, et al. Competitive enzyme immunoassays for the rapid detection of antibodies to feline infectious peritonitis virus polypeptides. *J Clin Microbiol* 1985;22:395–401.

21. Gouffaux M, Pastoret PP, Henroteaux M, et al. Feline infectious peritonitis, proteins of plasma and ascitic fluid. *Vet Pathol* 1975;12:335–348.

22. Potkay S, Bacher JD, Pitts TW. Feline infectious peritonitis in a closed breeding colony. *Lab Anim Sci* 1974;24:279–289.

23. Pedersen NC. Serologic studies of naturally occurring feline infectious peritonitis. *Am J Vet Res* 1976;37:1449–1453.

24. Norrby E, Orvell C, Vandvik B, et al. Antibodies against measles virus polypeptides in different disease conditions. *Infect Immun* 1981;34:718–724.

25. Hall WW, Choppin PW. Evidence for lack of synthesis of the M polypeptide of measles virus in brain cells in subacute sclerosing panencephalitis. *Virology* 1979;99:443–447.

26. Hall WW, Lamb RA, Choppin PW. Measles and subacute sclerosing panencephalitis virus proteins: lack of antibodies to the M protein in patients with subacute sclerosing panencephalitis. *Proc Natl Acad Sci USA* 1979;76:2047–2051.

27. Wechsler SL, Weiner HL, Fields BN. Immune response in subacute sclerosing panencephalitis: reduced antibody response to the matrix protein of measles virus. *J Immunol* 1979;123:884–889.

28. Krakowka S, Olsen R, Confer A, et al. Serologic response to canine distemper viral antigens in gnotobiotic dogs infected with canine distemper virus. *J Infect Dis* 1975;132:384–392.

29. Miele JA, Krakowka S. Antibody responses to virion polypeptides in gnotobiotic dogs infected with canine distemper virus. *Infect Immun* 1983;41:869–871.

30. Rottier P, Brandenburg D, Armstrong J, et al. Assembly in vitro of a spanning membrane protein of the endoplasmic reticulum. The El glycoprotein of coronavirus MHV-A59. *Proc Natl Acad Sci USA* 1984;81:1421–1425.

31. Lehtonen OP, Eerola E: The effect of different antibody affinities on ELISA absorbance and titer. *J Immunol Methods* 1981;54:233–240.

32. Schultz RD, Scott FW, Duncan JR, et al. Feline immunoglobulins. *Infect Immun* 1974;9:391–393.

33. Tizard I. *An introduction to veterinary immunology*. Philadelphia: WB Saunders Co, 1982.

34. Pedersen NC, Black JW. Attempted immunization of cats against feline infectious peritonitis, using avirulent live virus or sublethal amounts of virulent virus. *Am J Vet Res* 1983;44:229–234.

35. Pedersen NC, Black JW, Boyle JF, et al. Pathogenic differences between various feline coronavirus isolates. *Adv Exp Med Biol* 1984;173:365–380.