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Antibody and cytokine responses in kittens during the development of feline infectious peritonitis (FIP)

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Abstract

Two recombinant FIPV spike proteins were assessed for their immunogenic properties in 8-week-old kittens, which were then challenged intranasally with FIPV 79-1146. Humoral responses were assessed by ELISA and serum neutralisation test. Changes in PBMC cytokine mRNA levels were detected by a reverse transcription, semiquantitative polymerase chain reaction assay (RT-sqPCR), assessing IL-2, IL-4, IL-6, IL-10, IL-12 and IFN γ . All of the kittens developed clinical signs typical of FIP, which were confirmed on gross post mortem examination. The recombinant proteins induced little or no specific antibody response prior to challenge, and failed to alter the course of disease compared to controls. One week after virus challenge, the stimulated PBMCs showed small increases in the expression of IL-6 and IFN γ mRNA, which correlated with a transient pyrexia. After this time expression of IL-6 mRNA remained unaltered but, as FIP developed, mRNA levels of IL-2, IL-4, IL-10, IL-12 and IFN γ became markedly depressed. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

Feline infectious peritonitis (FIP) is an immune-mediated, usually fatal disease of domestic and exotic felids. The disease presents as a fibrinous serositis, with accumulations of highly proteinaceous fluid within body cavities, disseminated pyogranulomata formation, hypergammaglobulinaemia, and the formation of immune complexes (reviewed by Pedersen, 1995). It is caused by infection with certain variants of

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feline coronavirus (Pedersen, 1987; Vennema et al., 1995; Herrewegh et al., 1995). The pathogenesis of the disease is complex, with cell mediated immunity (CMI) playing a protective role, while humoral responses are generally detrimental, aiding viral dissemination by opsonising its uptake by macrophages, and resulting in immune complex (IC) formation (Weiss and Scott, 1981a; Pedersen, 1987). The ICs are deposited in small blood vessels, where they fix and activate complement (C3) and become phagocytosed by macrophages (Weiss and Scott, 1981b). Infected macrophages then promote immune-mediated tissue reactions via the interaction of their altered cell membranes with complement-fixing antibody or cytotoxic T-cells. Degeneration of infected macrophages leads to the release of new virus and complement components (Jacobse-Geels et al., 1980; Weiss and Scott, 1981a; Jacobse-Geels and Horzinek, 1983). A vicious cycle of complement-mediated vascular damage ensues, generating chemotactic complement components and attracting neutrophils, which then release proteolytic enzymes, leading to further tissue damage. These reactions can be seen as degenerative and proliferative changes in blood vessel walls, particularly in the endothelial and medial layers of small veins and arteries in the peritoneal and pleural serosa, and in the interstitial connective tissue of parenchymatous organs (Hayashi et al., 1977; Jacobse-Geels et al., 1980; Weiss and Scott, 1981a, b).

Cytokines are intimately involved in the control of immune reactions (Mosmann and Coffman, 1986; Romagnani, 1997). While it is not yet known which cytokines are of particular importance in the development of FIP, it has been postulated that, as the disease progresses, increased production of IL-6 and possibly IL-4 (typical Th2 cytokines) and reduced production of IL-2 and/or interferon γ (IFN γ) (typical Th1 cytokines) may result in decreased Th1-associated protective CMI, and increased Th2-associated non-protective antibody responses. Evidence in support of this comes from studies showing that clinical FIP is associated with markedly increased expression of IL-10 within lymphoid tissues (Dean et al., 1997), IL-10 usually being classified as a Th2 cytokine. FIP is also associated with high levels of IL-6 and IL-1 in both sera and ascitic fluid (Goitsuka et al., 1990), the cytokines being secreted by FCoV-infected macrophages within the granulomata and peritoneal exudate (Goitsuka et al., 1987b, 1988, 1991a, b; Hasegawa and Hasegawa, 1991). IL-6 is also produced, along with IL-1, by vascular endothelium within areas of vascular damage (Carvalho and Savage, 1997). The presence of IL-6 can polarise naive Th cells to effector Th2 cells, inducing the initial production of IL-4 by CD4⁺ T-cells (Rincon et al., 1997). Acting in synergy, IL-6, IL-1 and IL-4 can then stimulate B cell activation and induce the non-protective hypergammaglobulinaemia that is seen commonly in clinical FIP (Maggi et al., 1987; Sparkes et al., 1991; Clerici and Shearer, 1993). Increased levels of plasma leukotriene B4 (LTB4) and prostaglandin E2 (PGE2) have also been seen associated with clinical FIP (Weiss et al., 1988). PGE2 favours the development of a Th2 response, augmenting the production of IL-6 and IL-10 (Berger et al., 1996), and inhibiting the production of Th1 cytokines (IL-12 from dendritic cells and IFN γ from T-cells) (Van der Pouw Kraan et al., 1995; Hilkens et al., 1996). While, taken together, these findings support the hypothesis that FIP is a Th2 disease, a number of results are in disagreement with this conclusion, including mild elevations in IL-12 within the spleen, reduced levels of IL-4 within the thymus and minimal changes in TNF α (Dean et al., 1997).

2. Materials and methods

2.1. Experimental design

Twelve, 8–9-week old specific pathogen-free (SPF) kittens were obtained from a commercial supplier and housed in pathogen-free quarters. The kittens were divided into three groups, one of which was left as controls (Control), one was immunised with recombinant FIPV 79-1146 spike protein (S) and the third was immunised with a recombinant modified spike protein from which three major enhancing epitopes had been deleted (SDAD). The recombinant proteins were obtained after co-transfection of sf9 cells with BacPAC 6 Baculovirus linear DNA and the Baculovirus transfer plasmid pBacPAC 8 containing the FIPV coding sequences. The proteins were administered subcutaneously with 0.1% Carbopol 941 as adjuvant, being given on three separate occasions, 3-weeks apart. Kittens were challenged oronasally with 10^5 CCID₅₀FIPV79-1146 3 weeks after the final immunisation.

Daily health checks were performed from the day of challenge. These included taking rectal temperatures and assessing the kittens for evidence of inappetence, depression, dyspnoea, ascites, jaundice, lymphadenopathy, diarrhoea, ocular or nasal discharge, oral or nasal ulceration, hypersalivation, conjunctivitis, sneezing or neurological signs. Serial blood samples were collected for the assessment of serological responses and monitoring of stimulated peripheral blood mononuclear cells (PBMC) cytokine mRNA levels.

2.2. FCoV virus neutralisation test

Virus neutralisation tests were performed in 96 well flat bottom microtiter™ plates (Costar, USA) using doubling dilutions of serum. Each serum sample was assessed in quadruplet wells. Each sample was mixed with an equal volume of cell growth medium (Eagle's minimum essential medium (MEM) (Sigma) supplemented with 5% (v/v) foetal bovine serum (Sigma), 0.23% (w/v) sodium bicarbonate, 1% (v/v) non-essential amino acids (GibcoBRL), 0.05% (v/v) lactalbumin hydrolysate (GibcoBRL), 2 mM L-glutamine and 50 µg/ml gentamicin) containing 100 CCID₅₀ of FIPV 79-1146, then incubated at 35°C for 30 min. 5×10^3 whole feline embryo (WFE) cells (Harbour et al., 1991) were then added and the cultures were incubated for four days at 37°C. Titres were expressed as the reciprocal of the dilution that completely neutralised the FIPV 79–1146.

2.3. Serum anti-FCoV IgG ELISA

Serum FCoV-specific IgG was detected using an ELISA method. Ninety-six well microtiter™ plates (Dynatech Laboratories, USA) were coated overnight at 5°C with 100 µl of sucrose gradient-purified FIPV 79–1146 in carbonate/bicarbonate coating buffer (Voller et al., 1982). The plates were then washed five times in PBS/0.1% (v/v) Tween 20. A doubling dilution series of each serum sample was added and the plates were incubated for 2 h at room temperature, then washed as before. Mouse anti-cat IgG monoclonal antibody (K62.2G4, a gift from Dr K. Haverson, University of Bristol, UK) was added at the optimum dilution in PBS/0.1% (v/v) Tween 20 and the plates incubated

for a further 30 min at room temperature. After incubation, washing was repeated and goat anti-mouse/alkaline phosphatase-conjugated monoclonal antibody (Sigma) added at a 1:1000 dilution. The plates were incubated for 30 min at room temperature. After further washing, paranitrophenol phosphate (1 mg/ml) was added in carbonate/bicarbonate coating buffer. The reaction was allowed to progress for 30 min, then stopped by adding 50 μ l of 0.02% (v/v) sulphuric acid. Titres were expressed as the reciprocal of the highest dilution of serum to give an optical density (OD) of at least twice that of the standard negative serum.

2.4. PBMC isolation and culture

Blood samples were centrifuged at $2000\times g$ for 10 min. Plasma was removed and stored at -20°C prior to assessment of antibody response to FCoV (see above). Buffy coat cells were diluted to 5 ml in sPBS, overlaid onto 5 mls of Ficoll-Paque (Pharmacia Biotech) in 15 ml conical tubes and centrifuged for 20 min at $750\times g$, room temperature. The cells which collected at the interface were transferred to a fresh tube, washed twice with sPBS, then re-suspended at 5×10^6 cells per ml in lymphocyte growth medium (RPMI 1640 (Sigma) supplemented with 5% (v/v) foetal bovine serum (Sigma), 1% (v/v) HEPES, 0.1 μ g/ml glutamine, 50 μ g/ml gentamicin) and Con A at 10 μ g/ml. Separated cells consisted of greater than 90% lymphocytes, with over 90% viability as determined by trypan blue exclusion. The cells were cultured in 2 ml aliquots in 12-well tissue culture plates (Costar, USA) for 4 h at 37°C . After culture both the adherent and non-adherent cells were harvested. The non-adherent cells were transferred directly to fresh tubes, washed twice with sPBS, then pelleted. 1 ml of TRI REAGENT (Sigma) was added to each culture well to lyse the adherent cells, pipetted repeatedly, then transferred to the tube containing the corresponding cell pellet. Repeated gentle pipetting ensured complete cell lysis prior to storage at -70°C .

2.5. RT-PCR for the detection of cytokine mRNA

RNA was extracted using 1 ml of TRI REAGENT per 5×10^6 cells. The cells were lysed by repeated pipetting until the lysate changed from a viscous to a watery consistency. Each lysate was then vortexed for 30 s prior to adding 200 μ l of HPLC grade chloroform (Sigma). Each sample was vortexed for a further 30 s, then centrifuged for 15 min at $13\,000\times g$, at 4°C (Hettich Zentrifugen EBA12R). The upper aqueous layer was transferred to a fresh 1.5 ml microcentrifuge tube and an equivalent volume (~ 0.5 ml) of cold isopropanol (Sigma) was added. The sample was vortexed, then chilled at -20°C for at least 30 min (preferably greater than 2 h). After chilling the sample was centrifuged for 15 min at $13\,000\times g$, 4°C . The supernatant was discarded and the RNA pellet washed by adding 1 ml of 75% ethanol (molecular grade – Hayman), vortexed, then centrifuged for ten minutes at $8000\times g$, 4°C . The supernatant was again discarded and the RNA was dried briefly in air, prior to being re-suspended in diethylpyrocarbonate (DEPC)-treated water (~ 20 μ l per 5×10^6 cell equivalents). The amount of RNA extracted varied from between 10–30 μ g per 5×10^6 cells, with A_{260} : A_{280} ratio between 1.7–2.0.

The RNA was DNase treated. Each aliquot of RNA solution was heated to 94°C for 5 min, then chilled quickly on ice. The reaction mixture was adjusted to contain 0.1 µg/µl total RNA, 20 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, 0.1 U/µl DNase I (molecular biology grade, Gibco BRL) and DEPC-treated water to a final volume of 50 µl. The mixture was incubated for 15 min at room temperature. To terminate the reaction 2.5 mM EDTA was added and the mixture incubated at 65°C for 15 min.

To perform reverse transcription the RNA solution (containing 2–5 µg of total RNA) was incubated with 0.5 µg oligodeoxythymidine triphosphate (dT)12-18 primers for 10 min at 70°C, then chilled quickly on ice for at least 1 min. Each sample was adjusted with additional reagents to the following conditions: 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM MgCl₂, 0.5 mM deoxynucleic acid triphosphate (dNTP) mix, 10 mM DTT and 10 U/µl SuperScript II RT reverse transcriptase (GibcoBRL), in a total volume of 20 µl. The samples were incubated at 45°C for 55 min, heat inactivated at 70°C for 15 min, then chilled on ice. Each sample was then treated with 0.1 U/µl of *E.coli* RNase H and incubated for 20 min at 37°C. The cDNA was either used directly in the PCR reaction, or stored at –20°C, prior to further processing.

Primers were designed to the feline housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (G3PDH) after sequencing it. The cytokine primer sequences used initially were those designed by Rottman et al. (1995), based on published sequences (IL-2 – Cozzi et al., 1993; IL-6 – Bradley et al., 1993; Ohashi et al., 1993). A number of primers were redesigned based on new published data (IL-4 forward (F) and reverse (R); IL-10 (R); IFN γ (R); shown on Table 1 (IL-4 – Schijns et al., 1995b; IFN γ – Argyle et al., 1995; Schijns et al., 1995a). At the start of the study the nucleic acid sequence for feline IL-12 was not available. To synthesise primers for the more highly conserved IL-12 p35

Table 1
Details of primers

Primer	Size (bp)	TTm (°C)	G+C (%)	Priming site	Prod. size (bp)	Sequence 5' → 3'
IL-2 F	21	62	48	102→122	387	GCA CCT ACT TCA AGC TCT ACA
IL-2 R	22	62	41	467←488		GAT GCT TTG ACA AAA GGT AAT C
IL4 F	22	68.5	55	51→72	334	GCA TTT ACC AGC ACC TTC GTC C
IL-4 F	23	68.4	48	362←384		GCA TGA TCG CTT TTA GCC TTT CC
IL-6 F	21	62	48	2→22	695	CTA TGA ACT CCC TCT CCA CAA
IL-6 R	20	62	55	677←696		TGC CCA GTG GAC AGG TTT CT
IL-10 F	20	60	50	229→248	421	TAC TTG GGT TGC CAA GCC TT
IL-10 R	22	67.9	55	628←649		CCA AGG AGC TGT TTG GTT CTC C
IL-12 F	21	68.5	57	159→179 ^a	470 ^a	TCC TGC ACC ACC TCA GTT TGG
IL-12 R	23	65.9	48	597←629 ^a		CAG CTA TTG ATG AGC TGA TGG AG
IFN- γ F	21	54	29	202→223	355	ATT TGA AGA ATT GGA AAG AAG
IFN- γ R	24	68.9	46	533←556		GGC AGG ACA ACC ATT ATT TCG ATG
G3PDH-F	22	62	43	167→189	399	CCT TCA TTG ACC TCA ACT ACA T
G3PDH-R	21	62	48	546←566		CCA AAG TTG TAC TGG ATG ACC

^aBased on the Macaque sequence.

TTm – Theoretical annealing temperature based on 100% identity.

F – forward primer.

R – reverse primer.

subunit, consensus sequences for man, macaque, mouse, pig and cow were determined using the Genbank database. Primer sequences for IL-12 (F) and (R) are shown on Table 1. Potential sites for PCR primers were identified and consensus sequences aligned using computer assistance (MacVectorTM 5.0 – Kodak). Primers were made by Genosys.

The components for the first round of the PCR reaction were combined together into a master mix adjusted to give reaction conditions: 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.0 mM MgCl₂, 200 μM dNTP mix, 0.1 U/μl *Taq* DNA polymerase (AmpliTaq GoldTM – Perkin Elmer), 2 μl (5% [v/v]) of the cDNA solution and 0.3 μM primers. DEPC-treated water was added to make a final volume of 20 μl. In order to study large numbers of samples, the PCR was performed using a 96-well polycarbonate plate format, on a temperature cycler with a heated lid (Touchdown – Hybaid). The optimised PCR protocol for detecting message for IL-2, IL-4, IL-10, IL-12 and G3PDH consisted of an initial incubation of 94°C (10 min), followed by 30 cycles of melting 94°C (1 min), annealing 58°C (1.5 min) and extension 72°C (1 min), then a single extension of 72°C (10 min). For IL-6 and IFN γ the optimised protocol consisted of an initial incubation of 94°C (10 min), followed by 35 cycles of 94°C (1 min), 50°C (1.5 min) and 72°C (1 min), then a single extension of 72°C (10 min).

Controls were incorporated with each set of PCR reactions. Positive controls included amplifying cDNA generated from kit supplied RNA, using the kit supplied PCR primers, and later, using experimental cDNA samples known to give strong positive results. Negative controls were established for each cytokine and G3PDH estimation, and included ‘no RT’, ‘no primer’ and ‘no cDNA’ controls.

2.6. Detection of RT-PCR products

Once it had been demonstrated that the probes detected only single and specific bands, the PCR products were applied directly to nylon membranes (HybondTM-N Amersham), prior to detection using the enhanced chemiluminescence detection system (ECLTM, Amersham Life Science). The PCR products were denatured using a thermal cycler (Touchdown – Hybaid) at 100°C for 10 min, then chilled quickly on ice. Three microlitres of the denatured PCR product were then applied directly to the nylon membrane and air dried, then fixed by UV cross linking for 45–60 s on a UV transilluminator (UVP, Cambridge, UK).

Oligonucleotide probes were designed using computer assistance (MacVectorTM 5.0 – Kodak) according to published sequences for IL-2, IL-4, IL-6, IL-10, and IFN- γ , and the feline G3PDH sequence (Helps, unpublished), and on consensus sequences from man, macaque, mouse, pig and cow for IL-12 (Table 2). Probes were made by Genosys.

Labelling and detection of the oligonucleotide probes was performed using ECL 3'-oligolabelling and detection systems. All procedures were performed following the manufacturer's recommendations. Oligonucleotide probes were labelled with fluorescein-11-dUTP to give a final probe concentration of 0.6 μM. Blots were pre-hybridised for two to eight hours at 42°C. Labelled probe was added at 5 ng/ml of hybridisation buffer (5 \times SSC, with 0.1% (w/v) kit-supplied hybridisation buffer component, 0.02% (w/v) SDS, and 20-fold dilution of kit-supplied liquid block). Hybridisation was

Table 2
Details of oligonucleotide probes

Probe	Size (bp)	Tm (°C)	Binding site	Sequence 5'→3'
IL-2	22	57.8	300→321	GAG CTA AAT TTA GCA CTT CCT C
IL-4	24	62.8	180→203	CCC CTA AGA ACA CAA GTG ACA AGG
IL-6	20	61.0	253→272	AAC AAC CTG AAT CTT CCG AA
IL-10	20	66.1	378→397	CAC AGG GCA GAA ATC GAT GA
IL-12	26	62.8	314→339 ^a	GAA GAG ATC GAT CAT GAA GAT ATC AC
IFN- γ	21	60.8	244→264	AAG GAG AAC AAT TTG GCT CTG
G3PDH	20	65	286→305	ATC TTC CAG GAG CGA GAT CC

^a Based on Macaque sequence.

Tm – Theoretical annealing temperature based on 100% identity.

performed for 2 h at 42°C. Membranes were washed as directed and unbound probe removed by incubating twice in an excess of stringency buffer (1×SSC with 0.1% (w/v) SDS) for 15 min at 42°C. Membranes were rinsed, blocked and conjugated, excess conjugate was removed and the signal generated as per the manufacturer's recommendations. The luminescent image was detected on autoradiography film (HyperfilmTM-ECL- Amersham), the film being exposed for 5 min prior to development (GBX developer and replenisher – Kodak) and fixation (GBX fixer and replenisher – Kodak).

2.7. Semi-quantification of RT-PCR product

To allow comparison between samples it was necessary to relate the amount of starting material to the detected product. To accomplish this the protocol was standardised and measurements were made at as many steps as possible. PBMCs were incubated at fixed concentrations and the concentration of the extracted RNA was determined by spectrophotometry (Ultraspec II – LKB Biochrom); one unit of optical density at 260 nm being equivalent to approximately 40 mg of single stranded RNA (Ausubel et al., 1995).

$$\text{Total RNA isolated (mg)} = 40 \times \text{optical density} \times \text{dilution} \times \text{volume (ml)}$$

To control for variation in the RT-PCR, G3PDH, a 'housekeeper' gene involved in glycolysis, was selected as an internal control and its mRNA assessed at the same time as that for the cytokines.

Following amplification, RT-PCR products were dotted onto membranes in duplicate. To control for variation in the labelling procedure all samples for a particular cytokine were analysed simultaneously. The autoradiograph images were quantified by densitometry using a Model GS-700 Imaging Densitometer (Bio-Rad Laboratories, UK) and molecular analysis software version 2.1 (Macintosh/Bio-Rad Laboratories, UK). The calculated mean relative optical density (OD) corresponds to the volume of the dot multiplied by its density, after background density had been subtracted. Duplicate dot

values were averaged. The G3PDH signals were used to control for variation in the quantity of RNA between samples and the efficiency of the RT-PCR.

Using cDNA known to give strong signals, dilution series were performed to determine the relationship between product and template. The relative densities were then plotted and linear regression correlation performed.

2.8. Analysis of results

Results were expressed as the log₁₀ of the cytokine mRNA/G3PDH mRNA ratio. Each cDNA sample represented a single time point for an individual cat. Where cats were considered in groups, these values were averaged and the standard deviation calculated. When graphed the log₁₀ of the cytokine mRNA/G3PDH mRNA ratio was adjusted to zero at the start of the experiment, and all later values expressed as changes relative to that time point. Statistical analysis was performed using ANOVA with Bonferroni's post test. The relationship between OD and the concentration of the PCR product was analysed using Pearson's correlation coefficient.

3. Results

3.1. Clinical progress

The proteins were well tolerated, and no abnormal signs developed in any of the kittens during the period prior to virus challenge. In the week after the challenge all of the kittens developed a transient pyrexia (defined as >39°C). The temperatures then returned to normal, before pyrexia reappeared as clinical disease became apparent (Fig. 1).

Within 4 weeks of the challenge all of the kittens developed clinical signs typical of FIP (Fig. 2); characterised by ascites and dyspnoea, with occasional other signs including sneezing, hypersalivation and diarrhoea. None of the groups showed accelerated development of disease. Kittens were euthanased when they became anorexic and depressed. All were subject to gross post mortem examination and showed typical lesions consisting of granulomata in the visceral organs; many also had peritoneal or pleural exudates.

3.2. Serological responses

All of the kittens were seronegative for FCoV at the start of the study. No neutralising antibodies were detected before challenge. Three weeks following challenge, all of the kittens had developed low to moderate neutralising antibody titres, which rose further until euthanasia (Fig. 3). There was no indication of an anamnestic response in the immunised groups compared to the unimmunised control group.

No serum anti-FCoV IgG antibody was detected after the first immunisation, but all the kittens developed low levels of antibody after the second dose of protein had been administered. These titres altered little by the third immunisation, but rose following

Temperature °C

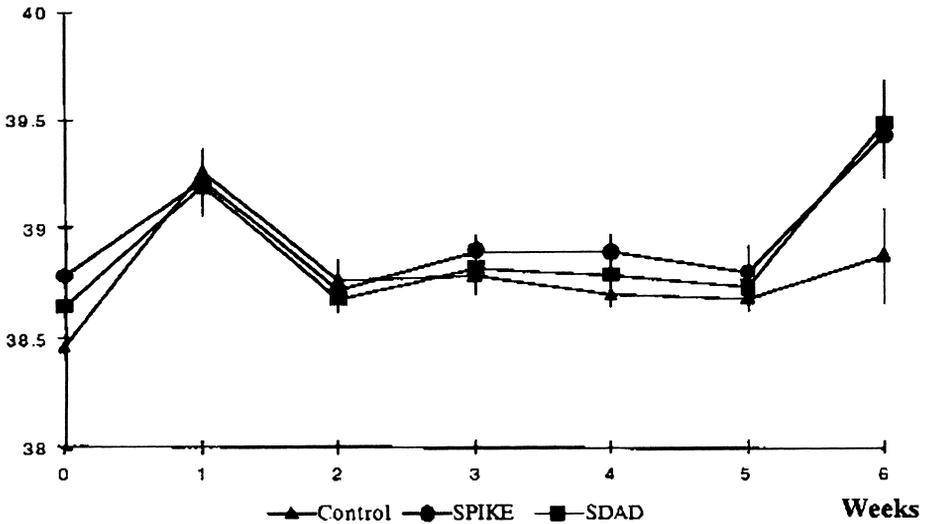


Fig. 1. Rectal temperatures from the day of challenge. Rectal temperatures were taken daily, then averaged for each group of kittens and displayed as weekly values with standard deviation bars. Time zero represents the day of challenge.

Clinical Score

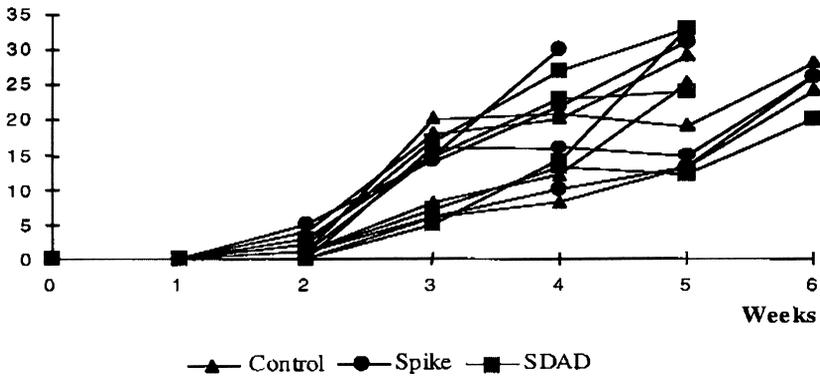


Fig. 2. Clinical scores from the day of challenge. Clinical scores were assessed daily, one or two points being given for each of the clinical signs demonstrated, depending of the severity of the sign. The results for each cat were then pooled and displayed as weekly values. Each line represents a single cat. Time zero represents the day of challenge.

challenge and continued to increase until euthanasia (Fig. 4). There was no indication of an anamnestic response in the immunised groups compared to the unimmunised control group.

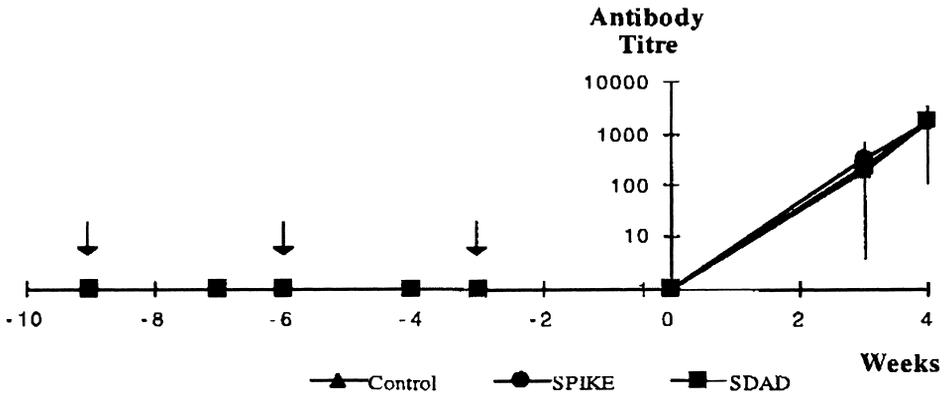


Fig. 3. FCoV neutralisation tests. Serum neutralisation of FCoV was assessed from 1-week prior to the first immunisation. The times of immunisation are indicated by arrows. Time zero represents the day of challenge. Titres are expressed as the reciprocal of the serum dilution that completely neutralised 100 CCID₅₀ of FIPV 79–1146. Titres for each vaccine group were averaged and displayed with standard deviation bars.

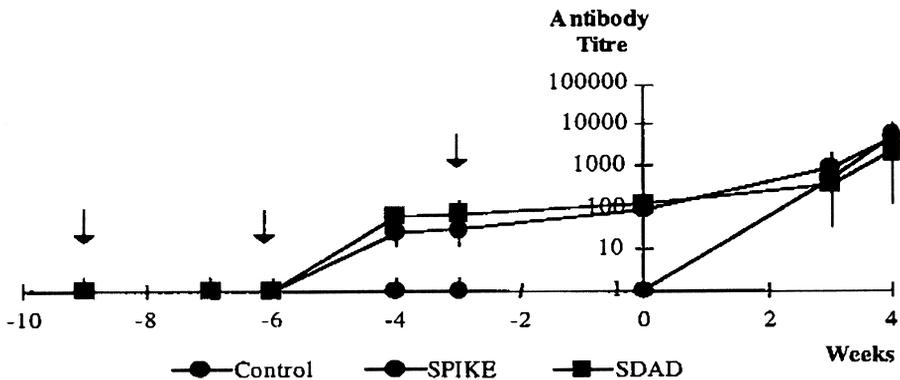


Fig. 4. Serum anti-FCoV IgG detected by ELISA. Serum IgG response to FCoV was assessed by ELISA from 1-week prior to the first immunisation. The times of the immunisations are indicated by arrows. Time zero represents the day of challenge. Titres are expressed as the reciprocal of the highest dilution of serum to give an OD of twice that of the background value, using sucrose gradient-purified FIPV 79–1146 as antigen. Titres for each vaccine group were averaged and displayed with standard deviation bars.

3.3. Optimisation of PCR methodology

The PCR protocol was optimised to ensure that, for each cytokine assessed, the product generated a single band on an agarose gel and ran at the expected molecular weight (Fig. 5). Thirty to thirty-five PCR cycles were shown to be within the linear phase of amplification, as determined by assessing replicate tubes run for multiples of five cycles. Titration of the cDNA template prior to amplification revealed a linear relationship between the amount of template added and the intensity of the resulting autoradiograph

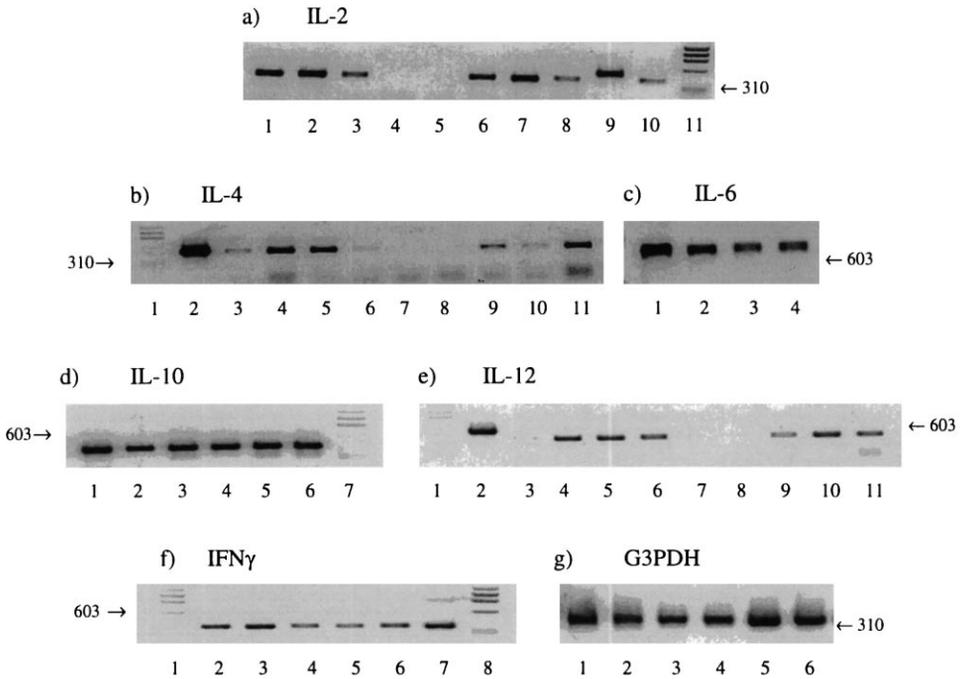


Fig. 5. RT-PCR products run on ethidium bromide stained gels. (a) IL-2 RT-PCR products. Lanes 1–8 and 10; products generated from PBMCs from separate SPF cats. Lane 9; product from the kit-supplied RNA control. Lane 11; markers. (b) IL-4 RT-PCR products. Lane 1; markers. Lanes 2–11; products from the same nine SPF cats. (c) IL-6 RT-PCR products. Lanes 1–4; products from four SPF cats. (d) IL-10 RT-PCR products. Lanes 1–6; products from six SPF cats. Lane 7; markers. (e) IL-12 RT-PCR products. Lane 1; markers. Lane 2; product from the kit-supplied RNA control. Lanes 3–11; products from the nine SPF cats. (f) IFN γ RT-PCR products. Lanes 1 and 8; markers. Lanes 2–7; products from the six SPF cats. (g) G3PDH RT-PCR products. Lane 1–6; products from the six SPF cats.

image. The intensity of the product was shown to be proportional to the logarithm of the template concentration (Fig. 6).

The cytokine assays were found not to be compromised by DNA contamination. This was confirmed by running 'no RT'-PCR controls and by removing the DNase step. The 'no RT'-PCR controls failed to generate product bands and removing the DNase step had no effect on the product. For the cytokines the RT-PCR reactions produced single bands of the expected molecular weight, (albeit rather weak in the case of IL-12), while the 'no RT'-PCR reactions failed to generate visible product. The G3PDH 'no-RT'-PCR reaction produced a very faint band of the same molecular weight as that seen for the corresponding RT-PCR. Southern blotting and ECL labelling confirmed the specificity of the bands in the RT-PCR reactions and revealed no bands in the cytokine 'no RT'-PCR controls. It also confirmed the specificity of the G3PDH band in the 'no RT'-PCR control. The expression of this band did not appear to be due to contaminating genomic DNA as it

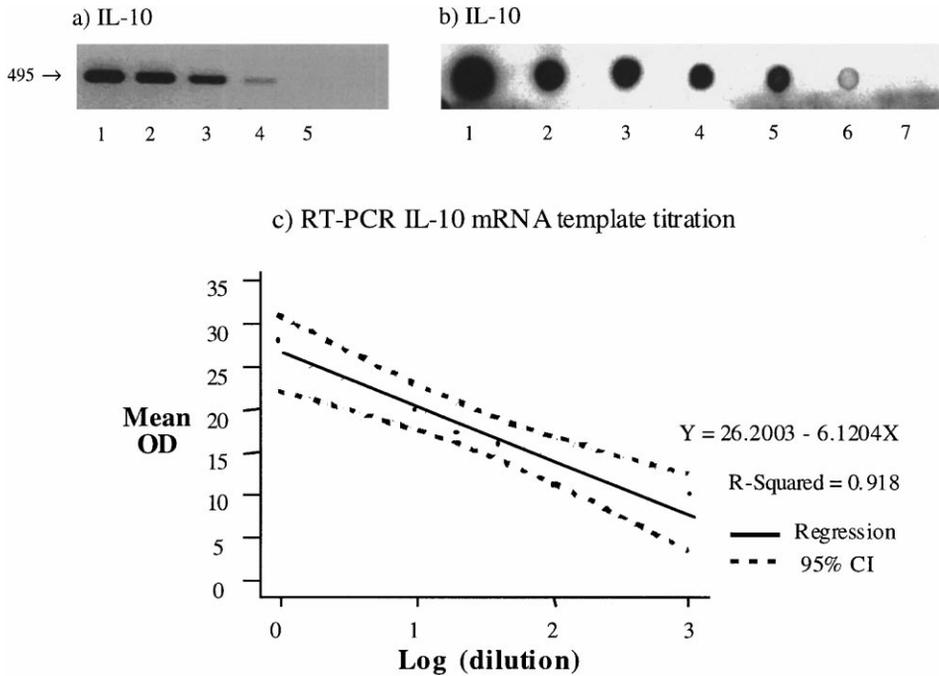


Fig. 6. Ethidium bromide-stained gel, autoradiograph and logarithm plot showing the relationship between the template concentration and the intensity of the RT-PCR product. The RNA was extracted from PBMCs of an SPF cat. The PCR was run using IL-10 primers. (a) Ethidium bromide-stained gel loaded with RT-PCR products. Lane 1; product gained from undiluted template. Lanes 2–5; from 10-fold serially-diluted template. (b) Autoradiograph of blotted RT-PCR products. Dot 1; from undiluted template. Dots 2–3; from two-fold serially-diluted template. Dots 4–7; from 10-fold serially-diluted template. (c) Logarithm plot of the relative OD of the autoradiograph shown in (b), analysed using Pearson's correlation coefficient.

was unaffected by DNase treatment. Since it was shown that DNase treatment failed to alter the product generated, the step was not incorporated in later studies.

3.4. Changes in cytokine mRNA levels

Due to the kittens' small size it was not possible to collect sufficient blood to assess PBMC cytokine mRNA at the start of the experiment. The first cytokine assessment therefore occurred 5-weeks into the trial, that is, 1-week after the second immunisation, 5-weeks before viral challenge. No cDNA was generated from the RNA extracted from blood taken on the day of challenge from kittens 2 and 10. A number of individual PCR reactions also failed to generate product. These included the 1-week post challenge reactions for IL-2 (kittens 7 and 9), and IL-12 (kittens 1, 2 and 3), and the day of euthanasia sample for IL-6 (kitten 9).

After PCR amplification and denaturation of the PCR products, all samples pertaining to an individual cytokine were dotted onto a single membrane and labelled en masse by

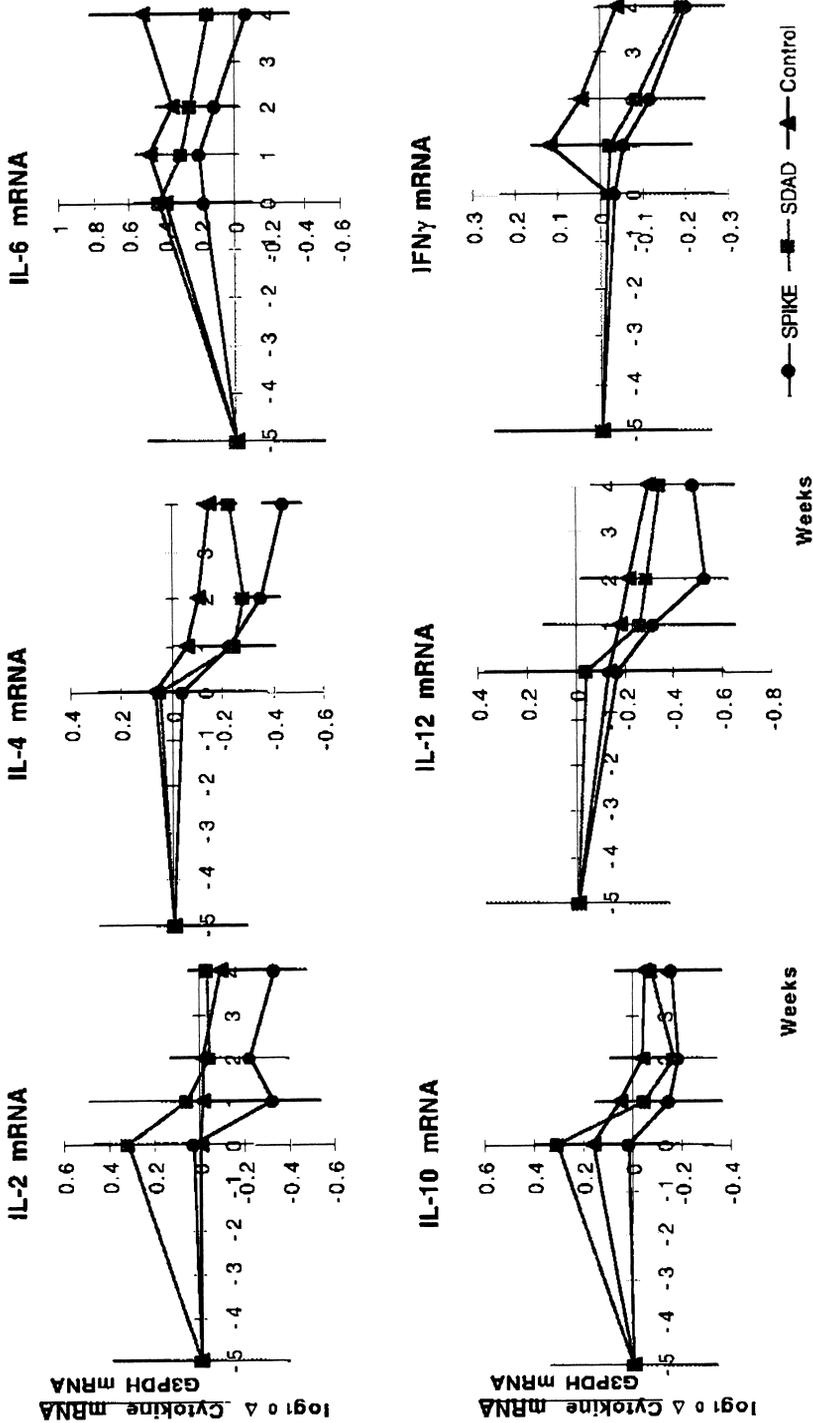


Fig. 7. Change in cytokine mRNA expression by stimulated PBMC. Results are expressed as the \log_{10} of the changes in the cytokine mRNA / GAPDH mRNA ratio, adjusting the value of the ratio when first assessed (five weeks pre-challenge) to zero, then graphing the changes from that point. For each time point the results for each vaccine group were averaged and displayed with standard deviation bars.

ECL. The results are graphed in Fig. 7. Between 5-weeks before challenge and on the day of the challenge, the expression of IL-2, IL-6 and IL-10 mRNA increased slightly. This trend was also seen with IL-4 mRNA, while the expression of IL-12 mRNA was slightly reduced. From the day of challenge to euthanasia mRNA levels for IL-2, IL-4, IL-10 and IL-12 were significantly reduced. This downward trend was also followed by IFN γ , but with this cytokine the fall was preceded by a small rise 1-week after challenge, most obviously in the Control group. Overall, the IL-6 mRNA levels remained unchanged as the disease progressed.

While the cytokine mRNA levels showed quite marked variation between individual kittens, there was little evidence that the immunisations had any effect. Statistically there was no significant difference between the three treatment groups; all showed suppression of IL-2, IL-4, IL-10, IL-12 and IFN γ mRNA levels after challenge. When looking at the IL-6 levels in individual kittens, two basic patterns emerged; the majority showed elevated IL-6 expression, at least initially, while in three of the kittens (Nos. 4, 6 and 12) there was no elevation in IL-6 (Fig. 8). Interestingly, these were three of the earliest kittens to show evidence of clinical disease.

4. Discussion

Numerous attempts have been made to develop a suitable vaccine against FIP. Unfortunately most have failed, and a number have actively enhanced disease (Woods and Pedersen, 1979; Pedersen and Black, 1983; Barlough, 1984; Pedersen et al., 1984; Pedersen and Floyd, 1985; Barlough and Stoddart, 1990; Gerber et al., 1990; Scott et al., 1992, 1995a, b). When enhancement occurs the earlier onset of clinical signs and higher mortality results from the presence of antibody to FCoV prior to virulent challenge (Weiss and Scott, 1981a, c; Pedersen, 1987). This 'antibody dependent enhancement' (ADE) probably results from antibody-mediated opsonisation of infection, with improved phagocytosis of immune complexes by macrophages, and accelerated lysis of virus-infected cells (Weiss and Scott, 1981a).

It is generally the spike (S) protein that stimulates the production of both neutralising and enhancing antibodies (Spaan et al., 1988 and references therein; Vennema et al., 1990; Hohdatsu et al., 1991; Olsen et al., 1992, 1993; Corapi et al., 1992, 1995), and although the exact relationship between enhancing and neutralising epitopes remains to be determined, a number of attempts have been made to develop suitable subunit vaccines. While some success has been seen with the murine MHV system (Flory et al., 1993, 1995; Brown et al., 1994), in cats recombinant vaccinia vectors expressing FCoV, CCV or TGEV S proteins have generally caused enhancement of disease rather than protection (Vennema et al., 1990; Chalmers et al., 1993). More recent work has tried to define the exact locations of both the neutralising and enhancing epitopes (Hohdatsu et al., 1991; Corapi et al., 1992, 1995; Olsen et al., 1992, 1993).

The kittens in each of the groups in this study experienced a clinical time course similar to that seen in previous experiments when using seronegative kittens and virulent FCoV challenge (Hayashi et al., 1982; Pedersen et al., 1984; Stoddart et al., 1988; Vennema et al., 1990). The mean rectal temperature curves were also similar to those

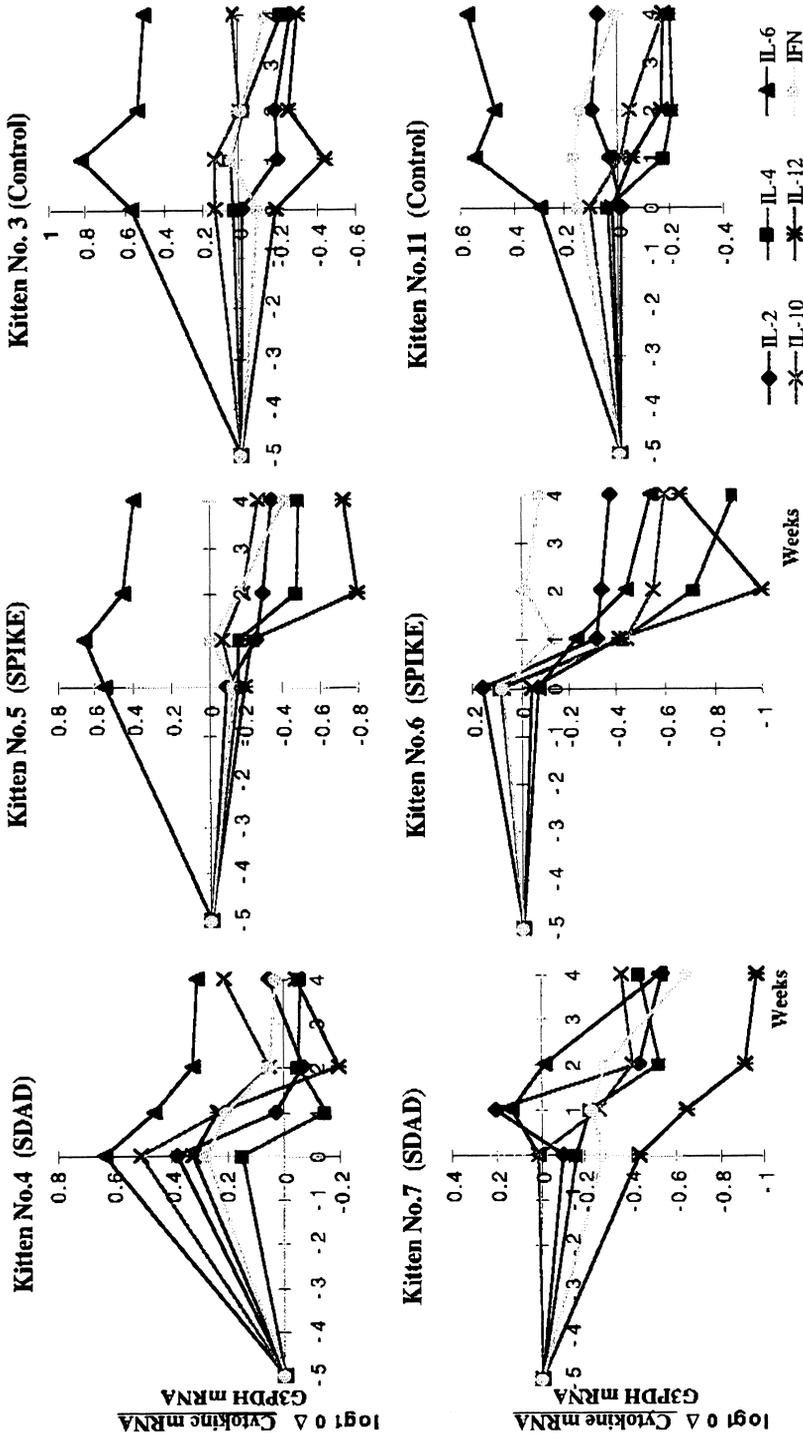


Fig. 8. Change in cytokine mRNA expression in stimulated PBMCs from individual kittens. Results are expressed as the \log_{10} of the change in the cytokine mRNA / G3PDH mRNA ratio. The ratio when first assessed (five weeks pre-challenge) was adjusted to zero, and the rest of the results graphed from that point. The Figure shows results from six kittens, two from each vaccine group. Each graph shows the cytokine changes for a single kitten.

seen previously, with a fever spike in the week following challenge, after which time the temperatures returned to normal, prior to more persistent pyrexia developing alongside the development of clinical signs (Weiss and Scott, 1981c; Vennema et al., 1990).

Why the vaccines used in this study failed to generate an immune response remains unclear. Previously the baculovirus system has successfully generated biologically active feline proteins, including IFN γ (Sakurai et al., 1992; Argyle et al., 1996), and has produced feline viral proteins that are immunogenic in cats (ENV and GAG proteins of FIV) (Lutz et al., 1995; Berlinski et al., 1996), and mice (feline herpesvirus hemagglutinin) (Maeda et al., 1996). The carbopol adjuvant has been successfully used in a number of vaccine studies (Gualandi et al., 1988; Mumford et al., 1994). Since the protein in the current study induced low levels of IgG which were not neutralising, it may have been presented inappropriately, perhaps being incorrectly folded.

Previous studies into feline cytokine transcription have shown that the peak expression of most feline cytokines occurs between 2 and 4 h after stimulation with Con A (Goitsuka et al., 1986; Rottman et al., 1995, 1996). Con A, a putative T-cell mitogen, tends to be used since feline lymphocytes respond poorly to PHA and LPS, but respond well to Con A (Schultz and Adams, 1978; Rottman et al., 1996). Feline macrophages respond to stimulation by LPS (Lin and Bowman, 1991). The optimal dose of Con A has been determined to be $\sim 10 \mu\text{g/ml}$ (Goitsuka et al., 1986; Rottman et al., 1995). Prior to stimulation, feline IL-2 mRNA is not readily detected (Cozzi et al., 1993), while IL-6, IL-10, IL-12 and TNF α appear to be expressed constitutively at low levels (Rottman et al., 1995). However, since these latter cytokines are synthesised by monocytes and macrophages this low level expression may result from cell activation during processing (Rottman et al., 1995). After stimulation with Con A, feline PBMCs readily produce mRNA for IL-2, IL-4, IL-6, IL-10, IL-12, IFN γ and TNF α (Cozzi et al., 1993; Rottman et al., 1996). Our study confirmed this finding for each of these cytokines, except TNF α , which was not assessed.

In a study analysing unfractionated human PBMC, TNF α , IL-6 and IL-10 were found to be the most abundant cytokines, being secreted by CD14 $^{+}$ cells, which include circulating monocytes and granulocytes (Hagiwara et al., 1995). Less abundant cytokines included IFN γ , IL-2 and IL-4. These came mainly from T-cells, with CD8 $^{+}$ cells being the major source of IFN γ in most individuals, although in others it appeared to be being secreted by either CD4 $^{+}$ or CD14 $^{+}$ cells (Zhou et al., 1994; Hagiwara et al., 1995). In disagreement with these findings, IL-6 and TNF α are normally difficult to detect in unstimulated PBMC cultures (Al-Janadi et al., 1993). The reason for this discrepancy may relate to inadvertent activation of the monocytes during the isolation process, since the separation of PBMC on a hyperosmolar solution, such as Ficoll-Hypaque, and their culture *ex vivo* may modify PBMC function (De Groote et al., 1992). Stimulation with PHA failed to alter the production of IL-6, IL-10 or TNF α , but caused a significant increase in IL-2, IL-4 and IFN γ . This probably arose because the T-cell mitogen had a positive effect on the CD4 $^{+}$ cells, which are the primary source of IL-2 and IL-4, along with the CD8 $^{+}$ cells that also secrete IFN γ . However, a T-cell mitogen is unable to affect the CD14 $^{+}$ monocytes that are the main source of IL-6 or IL-10 production in humans (Hagiwara et al., 1995). This is unlike the situation in mice, where IL-6 and IL-10 come from CD4 $^{+}$ cells.

Our results show that RT-sqPCR can be used successfully to detect cytokine mRNA expression in stimulated PBMC, and can monitor relative changes in cytokine expression over time. While the expression of cytokine mRNA does not necessarily correlate precisely with the production of functional protein, the mRNA expression and protein levels do usually follow a similar trend (Rottman et al., 1995).

The current study demonstrated that the cytokine mRNA levels expressed by stimulated PBMCs changed once the kittens had been challenged. Small increases in IL-6 and IFN γ expression were seen in the week following the challenge, after which IL-6 expression stayed fairly constant, while the expression of the other cytokines became significantly reduced. The initial increase in IL-6 corresponded with the initial fever spike, and since IL-6 is pyrogenic, it is possible that the two events are linked (Helle et al., 1988), with the source of IL-6, and possibly IFN γ , being virus-infected monocytes/macrophages. While clinical signs of illness did not arise until more than 2-weeks after the viral challenge, the general depression of cytokine expression was seen very early, then progressed as clinical signs developed. This is consistent with findings seen in the lymphoid tissues of cats with FIP, where changes in cytokine production occur rapidly after a virulent challenge (Dean et al., 1997).

Interestingly, the changes seen in this study do not fully agree with other studies, or the overall theory that FIP is a Th2 disease. IL-6 apart, this study demonstrates overall immunosuppression, with reduced expression of cytokines from both monocytes/macrophages (IL-10, IL-12), and T-cells (IL-2, IL-4, IL-10, IFN γ). FIP has previously been shown to suppress blastogenic responses to Con A by both PBMC and lymphocytes from affected lymph nodes (Haagmans et al., 1996), and reduced IL-2 expression has been seen in other feline viral diseases, including FeLV infection (Goitsuka et al., 1987a). Lymphocyte apoptosis and lymphopenia may be responsible for the lack of T-cell cytokine expression from the PBMCs, while the reduced expression of monocyte/macrophage cytokines may perhaps relate to their exhaustion or dysfunction. Dean et al. (1997) demonstrated increases in IL-10 within lymphoid tissue, with small increases in IL-12 within spleen, and reduced levels of IL-4 within thymus. However, it is difficult to correlate these findings with those of the present study since tissue samples rather than PBMC were assessed.

The IL-6 result requires further consideration. While IL-6 activity is not seen in the plasma of healthy cats (Goitsuka et al., 1990), it is markedly increased in the plasma and ascitic fluid of cats with FIP (Goitsuka et al., 1990). While it may have been expected that circulating monocytes would be expressing this cytokine at high levels (Jirik et al., 1989; Sironi et al., 1989), from this study this appears not to be the case. That said, since the expression of the other monocyte-derived cytokines were depressed in this study, while the overall level of IL-6 remained constant, the overall expression of IL-6 may be enhanced to some degree. Evidence for IL-6 being generated mainly outside the blood stream comes from studies showing that IL-6 is secreted by FCoV-infected macrophages within granulomata and peritoneal exudates, from where it then diffuses into the circulation (Goitsuka et al., 1987b, 1988, 1990, 1991a, b; Hasegawa and Hasegawa, 1991). The case of kittens 4, 6 and 12, is of particular interest. These were three of the earliest kittens to show evidence of clinical disease, and in each case they failed to show an elevation in their IL-6 expression at any point after challenge. The significance of this finding remains unclear.

Cats with FIP show an overall lymphopenia, with apoptosis and T-cell depletion in their lymphoid organs, with the spleen and mesenteric lymph nodes being most severely affected. Apoptosis is seen in both T- and B-cell areas of the lymphoid organs, plus the omentum, pyogranulomata, and thymus. Although, it is not yet proven that the apoptotic cells are T-cells, the virtual removal of all T-cells from affected tissues, together with thymus atrophy, argue that it is more likely to be the T-cells that are dying, rather than the B-cells (Stoddart, 1985; Pedersen, 1987; Haagmans et al., 1996), particularly given that the apoptosis is occurring in the face of hypergammaglobulinaemia and immune complex formation (Jacobse-Geels et al., 1980; Sparkes et al., 1991; Haagmans et al., 1996). Apoptosis occurs as a result of the release of soluble mediators from virus-infected monocytes/macrophages and neutrophils, rather than from viral infection of the lymphocytes per se. The exact nature of the soluble factors has still to be determined, but it is known that it does not involve TNF α or transforming growth factor β (TGF β) (Haagmans et al., 1996). Our results showing profound reductions in PBMC cytokine expression is further evidence of the immunosuppressive nature of this disease.

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