Production of IFN-γ in feline whole blood after incubation with potential T-cell epitopes of the nucleocapsid protein of feline coronavirus

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ABSTRACT

Interferon gamma (IFN-γ) plays an important role in cell mediated responses against mutated feline coronavirus strains (FCoV) involved in the pathogenesis of feline infectious peritonitis (FIP). The aim of this study was to establish a combined *in silico* and *in vitro* approach to assess feline leukocyte production of IFN-γ in response to selected peptides of the nucleocapside protein (N) of FCoVs.

To this aim, we designed, through a bioinformatic approach, 8 potentially immunogenic peptides from the protein N corresponding to sequences of residues 14, 182, 198 detected only in FCoVs from FIP cats (virulent strains), only in FCoVs from healthy cats (avirulent strains) and both in FIP and in healthy cats (mixed strains).

The peptides or a sham solution were incubated with whole blood from 16 cats (7 healthy and 9 with chronic diseases other than FIP) and IFN-γ concentration was measured on plasma using an ELISA system. RT-PCR expression of IFN-γ mRNA was also evaluated after incubation of the peptides or a sham solution with whole blood from 4 clinically healthy cats.

The mean plasma concentration of IFN-γ in samples incubated with peptides decreased and the expression of IFN-γmRNA did not change compared with the sham solution, except for some cats with chronic diseases (which probably have a "pre-activated" immune response). These cats responded to "avirulent" or "mixed" peptides by increasing the concentration of IFN-γ and the expression of IFN-γ mRNA.

The combined approach employed in this study allowed us to identify potentially immunogenic peptides of FCoV N protein that can modulate the production of IFN-γ especially in cats with a "pre-activated" cell mediated response.

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1. Introduction

Feline infectious peritonitis (FIP) is a fatal immune-mediated disease triggered by infection with feline coronavirus (FCoV). Feline infectious peritonitis virus (FIPV) is a virulent biotype of feline enteric coronavirus (FECV), a low pathogenic FCoV that induces mild enteritis in kittens. The analysis of FIPV and FECV genomes suggests that FIPV may arise from FECV by a series of genomic mutations that provide the virus with the ability to replicate within cells of the monocytic lineage and thus to disseminate throughout the body (Vennema et al., 1998; Pedersen et al., 2009; Chang et al., 2010).

The pathogenesis of FIP is not completely understood, but the development of viral quasispecies and feline immune response to infection are considered to be key pathogenic events. Usually a predominantly humoral immunity response is considered ineffective in virus neutralization since the presence of antibodies could enhance the uptake of FCoVs by macrophages (Pedersen,

2009). In this type of immune response the deposition of macrophages and virus-antibody complexes around small venules leads to a pyogranulomatous vasculitis that is responsible for the formation of effusions typical of wet FIP.

If a strong cell-mediated immune response develops in the early stage of the infection, virus replication could be lowered and infection can be limited or successfully cleared with no evidence of clinical signs. Conversely, if the cell-mediated response is weak and unable to reduce virus replication an intermediate response can also occur with a continuous release of virokines and chemokines that recruit neutrophils and macrophages into infection sites. This leads to the formation of pyogranulomas, typical of dry FIP. Dry FIP may also become effusive in the terminal stage of the disease when the immune system collapses. Thus, the development of FIP depends on the balance between humoral and cellular immune response (Pedersen, 2009). The cytokine interferon-γ (IFN-γ) is one of the main modulators of cell mediated immunity (Berg et al., 2005). IFN-γ shifts the immune response from T-helper 2 to T-helper 1 with a consequent enhancement of immune cytotoxic activity due to the activation of macrophages and CD8+ T cells. This type of immunity can protect the host against the development of the disease or even more against the infection (Pedersen, 2009).

All coronaviruses contain at least three main structural proteins: spike (S), membrane (M) and nucleocapsid (N). The nucleocapsid (N) protein is one of the most produced viral proteins during viral infections and may exert several functions. It is primarily involved in the encapsidation and packaging of viral genomic RNA; in addition to this structural role, it also participates in viral RNA transcription, replication and translation (Lai and Canavagh, 2007), and in modulating the metabolism of host cells (Eleouet et al., 2000; Surijt et al., 2006).

Several studies have identified antigenic determinants in N protein of human and animal coronaviruses and have demonstrated that N protein is one of the immunodominant antigens in the CoV family (Collisson et al., 2000; Liu et al., 2001; Zhao et al., 2007).

The N protein may play an important role in the pathogenesis of FIP. Different recombinant vaccines based on N protein have already been tested and in some cases they proved to be effective in preventing the progression of FIP. This suggests that a cell mediated immunity against the FIPV antigen was induced by the vaccine (Wasmoen et al., 1995; Hohdatsu et al., 2003) and that therefore N protein could be a suitable vaccine candidate.

The precise identification of T-cell-stimulating epitopes represents a difficult task but it is an important pre-requisite for accurate epitope mapping and for formulation of vaccines and immunotherapies. A systematic approach based upon the synthesis and testing of large sets of overlapping peptides is very expensive. In contrast, bioinformatic prediction is extremely cheap and can be useful for selecting potentially immunogenic peptide regions, reducing the number of peptides to be screened. The strategy that combines the predictive power of these theoretical approaches with experimental in vitro or in vivo approaches allows a reliable and rapid identification of T cell stimulating epitopes (Schirle et al., 2001). Many web-based databases and prediction algorithms are now available and have been applied successfully to predict T-cell stimulating epitopes in infectious disease, cancer, autoimmunity and allergy.

In this study, the possible cell mediated immune response induced by N protein was investigated using both a computational prediction method and an in vitro assay to assess the T-helper 1 responses. Specifically, 8 potential immunogenic peptides from N protein were designed through a bioinformatic approach and the production of IFN-γ was assessed after incubation of feline leucocytes with these peptides.

2. Materials and methods

2.1. Animals and samples

The method employed to assess IFN-γ production was based on incubation of whole blood with different protein N peptides. The cell viability provided by this method was already evaluated in a previous study where it appeared greater than 95% after 1-h of incubation (Gelain et al., 2006).

Blood samples (0.8–3 ml) were collected from the jugular vein of 20 privately owned cats, after informed consent was obtained by the owners, and put into EDTA-coated tubes.

Specifically, the blood from 16 of the 20 cats (cats #1 to #16) was used to assess IFN-γ production by an ELISA system (see the details below). At the time of sampling, these cats were diagnosed as clinically healthy ($N = 7$) or showing symptoms of diseases other than FIP ($N = 9$), such as hepatic lipidosis ($N = 2$), neoplasia ($N = 2$), chronic gastroenteritis ($N = 2$), cardiopathy ($N = 1$), bone fractures ($N = 1$) and trauma ($N = 1$).

The blood from additional 4 cats (cats A–D) was used to assess the expression of mRNA coding for feline IFN-γ (see the details below). When sampled, all these 4 cats were diagnosed as clinically healthy.

For each sample a complete blood cell count (CBC) was obtained using an automated haematology analyser (Sysmex XT-2000iV) together with a manual differential leukocyte count performed on May-Grunwald Giemsa stained smears. An aliquot of each sample (200 μl) was then centrifuged (8 min at $450 \times g$) and plasma was harvested and transferred to Eppendorf plain tubes for FCoV serology and, in 10 cases, for the assessment of the baseline (pre-incubation) value of IFN-γ. Specifically, the presence of anti-FCoV antibody titres was investigated using an indirect immunofluorescence test performed on multiwell slides produced at the University of Zurich according to Osterhaus et al. (1977) and using the procedure routinely performed in our laboratory (Paltrinieri et al., 2008). The concentration of IFN-γ was measured with the method described below.

To assess the possible non specific influence of peptides on IFN-γ measurement by the ELISA technique described below, a pool of fresh feline plasma from blood samples routinely submitted to our Institution was also included in the study in addition to the blood samples described above, as negative control.

2.2. Peptide design and synthesis

Putative Cytotoxic T Lymphocytes (CTLs) epitopes, were predicted combining different prediction algorithms: binding matrices, Artificial Neural Networks (ANN) and Support Vector Machines (SVM). The following programs were used: CTLPred (http://www.imtech.res.in/raghava/ctlpred/) (Bhasin and Raghava, 2004) and RANKPEP (http://bio.dfci.harvard.edu/RANKPEP/) (Reche et al., 2002).

CTLPred has the ability to predict CTL epitopes from a protein sequence on the basis of ANN and SVM; in our case a consensus prediction was performed to enhance the specificity of the results compared to individual ANN and SVM approaches.

RANKPEP predicts peptide binders to MHC-I molecules from a protein sequence using position-specific scoring matrices (PSSMs). In addition, it predicts those MHC-I ligands whose C-terminal end is likely to be the consequence of a proteasomal cleavage.

The amino acid sequences of N protein from 36 FCoVs strains (NCBI accession no. GU017091–GU17126) detected in faeces from healthy cats (N = 31) and in extra-intestinal organs obtained during necropsy from cats with lesions histologically consistent with FIP (N = 5) were individually tested using these algorithms. A previous analysis has already evidenced the presence of several sites under positive selection in these protein N sequences, which might represent immunological domains (Battilani et al., 2010).

The criteria used to select the candidate epitopes were: having an higher binding score and occurring in prediction results more than three times or at least not less than two.

Finally the order-disorder profile of N protein was used to restrict the panel of peptides to be synthesized. All the coronavirus N proteins share the same modular organization, with the presence of three intrinsically disordered regions located in the central domain and at the N and C-terminus. Since disordered regions are often involved in biomolecular interactions and disordered regions of coronavirus N proteins have already been reported to be probable interaction sites with functional implications (Tusell et al., 2007), we have limited the choice of predicted peptides to these regions.

A total of 8 peptides (P1–P8), consisting of 9–12 amino acids, were designed from the viral sequences centered on residues 14, 182 and 198. Specifically, two sequences from viruses detected during necropsy in extra intestinal organs of cats with lesions consistent histologically of FIP and classified as "virulent strains" or "FIPV-like"; three sequences detected in viruses from faeces of clinically healthy cats and classified as "avirulent strains" or "FECV-like" and three sequences detected both in viruses from tissues of FIP cats and in viruses from faeces of clinically healthy cats and classified as "mixed" ("FECV/FIPV"). Peptides were synthesized by 9-fluorenylmethoxy carbonil-based solid-phase chemistry (Thermo Electron Corporation, Germany); the purity of peptides was >90% as determined by reversed-phase high-performance liquid chromatography and mass spectrometry. Details of peptides are summarized in Table 1. Stock solutions were made at 1 mg/mL in water, except for peptides 6 and 7, which, based on their amino acid composition and on the

Table 1

Sequences of the peptides designed on the basis of RNA sequences detected in feline coronaviruses from faeces of healthy cats (FECV-like or "non virulent strains"), from tissues of cats with feline infectious peritonitis (FIPV-like or "virulent strains") or both (FECV/FIPV-like or "mixed strains").

Peptide	Sequence	Position	Biotype
P1	PSKRRGSN	14–22	FECV/FIPV
P2	PSKSGRSNS	14–22	FECV
P3	SNNQNNVEDTIV	182–193	FECV/FIPV
P4	PNNQNNNVDTIV	182–193	FIPV
P5	FNNQNNNVEDITIV	182–194	FECV
P6	KLGVTDKLRS	198–207	FIPV
P7	RLGVTSDNKQ	198–207	FECV
P8	KLGVTDKQRS	198–207	FECV/FIPV

results of preliminary tests with small amounts of peptides, were less soluble in water than others and were thus dissolved in 10% dimethylsulfoxide (DMSO)/water mixture. All the peptide aliquots were stored at −80 °C.

2.3. Stimulation of whole blood with peptides

After performing the CBC and separating a part of the blood for FCoV serology as previously described, each whole blood sample was subdivided in aliquots of 250 μl. When sample volume was enough 9 aliquots were prepared, in order to test the effect of all the 8 peptides and of a sham solution (see below). When the sample volume was low, 2–9 aliquots were prepared, in order to test at least one peptide and the sham solution.

Each aliquot was assayed in a 96-well ELISA plate and each well was then added with the peptides or with the sham solution (negative control or P0: RNase free distilled water). Specifically, the final concentration of peptide was 5 μg/1 × 10^6 leukocytes/μl in each well.

The plate was incubated for 1 h at 37 °C in a moist chamber with 5% of CO_2. After incubation, each aliquot was re-suspended and transferred in an Eppendorf tube.

Aliquots from the 16 samples dedicated to the ELISA test (see below) were centrifuged (450 × g for 8 min) to obtain plasma that was then stored at −20 °C until IFN-γ evaluation was performed.

In order to assess the possibility of non specific influence of peptides on IFN-γ measurement, pooled fresh plasma was added with all the 8 peptides just before the ELISA testing.

2.4. ELISA test to measure IFN-γ production

Plasma IFN-γ concentration was determined using a specific ELISA for feline IFN-γ (DuoSet ELISA, R&D System Europe, Ltd) according to a protocol already performed in our laboratory (Giordano and Paltrinieri, 2009). All IFN-γ measurements were performed in duplicate, and the mean values of the two readings were used to statistically analyse the results.

2.5. RT-PCR

RNA was extracted using a commercially available kit (QIAamp RNA Blood Mini Kit, Qiagen, Milan, Italy) in

accordance with the manufacturer's instructions. To avoid contamination by genomic DNA (gDNA), a DNase treatment was performed on-column (RNase-Free DNase, Qiagen). Prior to cDNA synthesis, 1 μl of RNA from each sample was subjected to PCR analysis for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Samples that showed a PCR product for GAPDH were considered to have genomic contamination and were subjected to a second DNase treatment (DNA-FREE, Ambion, Cambridgeshire, United Kingdom) and re-checked for DNA contamination. All the samples were negative for gDNA after the second DNase treatment. The synthesis of the first-strand cDNA templates with oligodT (200 ng/μl) was carried out with a commercial kit (Ready-To-Go™ You-Prime First-Strand Beads, Amersham Biosciences, Piscataway, NJ, USA) in a volume of 33 μl at 42 °C for 50 min followed by inactivation of reverse transcriptase at 70 °C for 15 min. Samples were stored at −80 °C.

PCR was performed using the cytokine primer sequence for feline IFN γ (Forward: TGGTGGGTCGCTTTTCGTAG; Reverse GAAGGAGACAATTTGGCTTTGAA) designed in a previous study (Gelain et al., 2006). The optimized PCR protocol was the following: denaturation at 94 °C (45 s), annealing for 50 s at 59 °C for 38 cycles and extension at 72 °C (80 s) with a final extension step at 72 °C of 10 min. Ten microlitre of reaction product was visualized on a 1.5% agarose gel containing ethidium bromide and an image of the gel was digitally photographed on an ultraviolet transilluminator. Expected PCR products (85 bp) were identified using a 100 bp standard marker (Gene Ruler 100 bp DNA Ladder plus, Fermentas UAB, Vilnius, LIT).

Results were subjectively evaluated by two different observers and expressed as negative (−) when no bands were visible, weak (+/−) when bands were visible but with lower intensity compared with the bp ladder, and positive (+) when bands of the same intensity compared with the bp ladder were present.

Results obtained from wells incubated with the peptides were then compared with those obtained in P0 and expressed as unchanged compared with P0 (e.g. both negative, weak or positive), increased (e.g. weak or positive compared with a negative P0; positive compared with a weak P0) or decreased (e.g. negative or weak compared with a positive P0; negative compared with a weak P0).

2.6. Statistical analysis

Statistical analyses were performed with an Excel (Microsoft Corp, Washington, US) and the Analyse-it set of macroinstructions (Analyse-it Software, Leeds, UK).

Descriptive statistics and a Kolgomorov–Smirnov normality tests were used to examine the distribution of data regarding the concentration of IFN-γ recorded in plasma of the whole study group, in plasma of cats grouped according to the disease status or to the anti-FCoV serology and in wells incubated with sham solutions or with peptides. The concentration of IFN-γ recorded in plasma of cats with or without diseases and with or without anti-FCoV antibodies was compared to each other using a Mann–Witney U-test. Results recorded in native plasma and in plasma incubated with sham solutions were

compared to each other using a Wilcoxon test. This latter test was also employed to compare the concentration recorded in paired samples on which each single peptide and the sham solution were tested. The possible presence of correlation between the concentration of IFN-γ in plasma and in P0 or in P0 and in each test well was assessed using a Spearmann correlation test.

Results were considered statistically significant when $p < 0.05$.

Due to the low number of observation and to the semiquantitative method empoloyed, data from the RT-PCR testing were not statistically analyzed. Results were expressed as number of increased or decreased band intensity compared with P0.

3. Results

3.1. Laboratory observation in sampled cats

Eleven of the 16 cats included in this study had antibody titres lower than or equal to 1:100, that represents the cut-off limit currently used in the Author's laboratory to discriminate between infected and not infected cats. Some of these cats showed hematological changes as a slight neutrophilia (5 cases, with neutrophil counts ranging from 13.5 to 17×10^6 cells/l: upper reference limit: 13×10^6 cells/l) or a moderate lymphopenia (2 cases, with a lymphocyte count of 0.6 and 0.8×10^6 cells/l respectively; lower reference limit: 1.0×10^6 cells/l) or a slight lymphocytosis (1 case with a lymphocyte count of 8.9 cells/l; upper reference limit 7.0×10^6 cells/l).

Of the remaining cats, 3 cats had an antibody titre of 1:400 and no hematological changes and in 2 cases the antibody titre was not evaluated.

3.2. IFN-γ concentration in plasma and in wells incubated without peptides

The comparison of the results obtained from native pooled fresh plasma and from fresh pooled plasma added with the peptides (Table 2) did not show evident changes in the concentration of IFN-γ.

The IFN-γ concentration recorded in plasma before incubation with the peptides was variable (Table 3): in 6 out of 10 cats in which native IFN-γ concentration was tested, the IFN-γ concentration was within reference interval for healthy cats (Giordano and Paltrinieri, 2009)

Table 2
Results obtained in native plasma and in plasma added with peptides P1–P8.

	IFN-γ (ng/mL)
Native plasma	3.21
Plasma + P1	3.18
Plasma + P2	3.21
Plasma + P3	3.18
Plasma + P4	3.26
Plasma + P5	3.23
Plasma + P6	3.15
Plasma + P7	3.14
Plasma + P8	3.14

Table 3
Individual results recorded in the study: for each cat the concentration of IFN-γ (ng/mL) recorded in the different wells after incubation with sham solution (P0) or with the different peptides (P1–P8) is reported. When available, also the concentration of IFN-γ recorded in plasma before incubation is reported.

Cat	Status	Plasma	P0	P1 R14M	P2 R14A	P3 R182M	P4 R182V	P5 R182A	P6 R198V	P7 R198A	P8 R198M
1	S	**8.98**	4.12	2.49		1.41	0.66	0.20			0.15
2	S	0.29	6.81				4.29				
3	H	**1.73**	1.67	0.76	0.33	0.22	0.18	8.55	2.69	1.47	1.75
4	H	0.89	0.26	0.2							
5	H		7.77	4.68		3.09	1.65	0.90			0.25
6	S	0.44	0.14			6.34					
7	S		4.12	1.65	1.52	0.35	0.16	0.19	0.12	2.62	
8	H		1.63	1.16	0.98	0.53	0.26	0.25			0.17
9	S		5.04	4.00		3.06	1.98	0.98			0.48
10	H	0.41	0.17	6.02	3.14	2.13	2.2	0.78	0.36	0.49	0.17
11	S	**6.9**	5.96	1.92	1.94	0.7	0.3	0.37	0.15	10.72	6.97
12	S		4.22	2.05	0.47	0.19		0.2	0.15	10.43	
13	S+	**4.19**	3.07		1.48				2.25		
14	S	1.04	0.64		0.62				0.44		
15	H+	1.39	0.57		0.47				0.6		
16	H+		3.34		1.15				0.66		

R14, R182, R198: residues 14, 182 and 198, respectively. A: avirulent (FECV biotype); V: virulent (FIPV biotype); M: mixed (FECV/FIPV biotype); S: sick; H: healthy; +: anti-FCoV titre > 1:100; in bold: values higher than the reference interval.

whereas in 4 cats it was higher than the reference interval. These last cats belonged to both the group of diseased cats (3 out of 6 cats tested) and clinically healthy cats (1 out of 4 cats tested) and none of them showed any hematological abnormality.

No significant difference in IFN-γ concentration ($p = 0.522$) was however noted between the diseased cats (mean ± D.S. = 3.64 ± 3.67 ng/mL; median: 2.61 ng/mL; min-max range = 0.29–8.98 ng/mL) and the clinically healthy cats (1.10 ± 0.57 ng/mL; 1.14 ng/mL; 0.41–1.73 ng/mL) in spite of evident differences in mean and median values. The concentration of IFN-γ was higher than the reference interval in one of the FCoV positive cats tested.

The difference between IFN-γ concentration in plasma recorded before incubation (2.62 ± 3.06 ng/ml) and after incubation with the sham solution (P0, 2.34 ± 2.52 ng/ml) was not statistically significant ($p = 0.08$). These results were however affected by the markedly increased IFN-γ concentration after incubation noted in one sample (cat #2). In the absence of this sample, the concentration of IFN-γ recorded after incubation with the sham solution (1.85 ± 2.09 ng/mL) was significantly lower ($p = 0.004$) than the one observed in plasma (2.88 ± 3.12 ng/ml). No significant correlation

between the amount of IFN-γ in plasma and after incubation with sham solution was observed ($p = 0.760$). Also in P0, the difference between the concentration of IFN-γ recorded in diseased cats (mean ± SD = 3.79 ± 2.22 ng/mL; median = 4.12 ng/mL; reference interval = 0.14–6.81 ng/mL), was not significantly different ($p = 0.210$) from that of clinically healthy cats (2.20 ± 2.69 ng/mL; 1.63 ng/mL; 0.17–7.77 ng/mL). Similarly, no significant differences ($p = 0.611$) were found between FCoV-positive ($N = 3$; .2.32 ± 1.52 ng/mL; 3.07 ng/mL; 0.57–3.34 ng/mL) and FCoV-negative cats ($N = 13$; 3.27 ± 2.67 ng/mL; 4.12 ng/mL; 0.14–7.77 ng/mL).

3.3. IFN-γ concentration after incubation with FCoV peptides

The comparison of the results obtained from each cat after incubation of the blood with the different FCoV peptides is reported in Table 3. Mean and median results are summarized in Table 4 and show the results obtained in paired samples on which both P0 and each peptide were determined. Compared with P0, the mean values of IFN-γ concentration recorded in the presence of peptides decreased with all the peptides except P7. However, these

Table 4
Summary of the results of ELISA testing recorded after incubation with sham solution or with peptides (mean ± D.S. and, between parenthesis, median values) and results of statistical analysis.

	IFN-γ (ng/mL)		Wilcoxon test
	Sham	Peptide	
P1 – R14M ($n = 10$)	3.50 ± 2.50 (4.12)	2.49 ± 1.85 (1.97)	$p = 0.084$
P2 – R14A ($n = 10$)	2.54 ± 1.90 (2.37)	1.21 ± 0.87 (1.07)	$p = 0.048$
P3 – R182M ($n = 10$)	3.48 ± 2.52 (4.12)	1.80 ± 1.95 (1.06)	$p = 0.160$
P4 – R182V ($n = 9$)	4.14 ± 2.56 (4.12)	1.30 ± 1.39 (0.67)	$p = 0.019$
P5 – R182A ($n = 9$)	3.86 ± 2.36 (4.12)	1.38 ± 2.71 (0.37)	$p = 0.164$
P6 – R198V ($n = 9$)	2.64 ± 1.99 (3.07)	0.83 ± 0.96 (0.44)	$p = 0.097$
P7 – R198A ($n = 5$)	3.23 ± 2.29 (4.12)	5.15 ± 5.01 (2.61)	$p = 0.437$
P8 – R198M ($n = 7$)	3.77 ± 2.72 (4.12)	1.41 ± 2.51 (0.25)	$p = 0.218$

P1–P8: number of the peptide; R14, R182, R198: residues 14, 182 and 198, respectively. A: avirulent (FECV biotype); V: virulent (FIPV biotype); M: mixed (FECV/FIPV biotype);
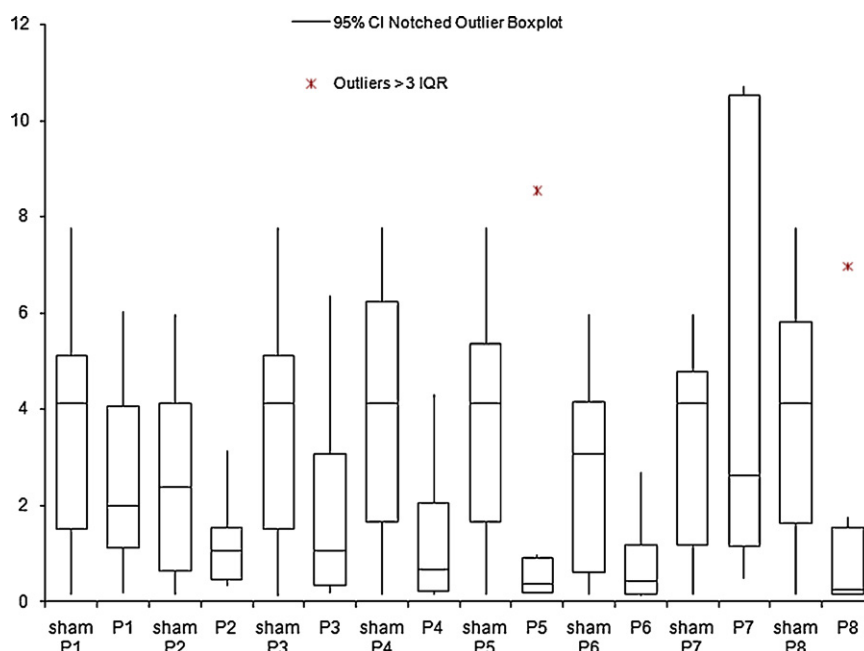
Fig. 1. Distribution of individual results after incubation with sham solution or with peptides.

differences were statistically significant only with P2 with P4 and close to statistical significance with P1 with P6.

The analysis of data distribution reported in Fig. 1, however, reveals that the median values were lower than P0 with all the peptides but with some of them the individual variability was extremely high and characterized by the presence of outliers (e.g. peptides P5 and P8) or of a very wide distribution range. For example, it is interesting to note that, contrarily to that recorded for the other peptides, the distribution range observed with P7 was wider than in samples treated with the sham solution. This can be explained by the fact that in 3/5 cats IFN-γ concentration was higher than the one observed in the control wells (see also Table 3). The analysis of individual data shows that values higher than P0 were also recorded in all the samples from cat #10 and, occasionally in other cats, mostly in the case of incubation with peptides from "mixed" or "avirulent" strains (e.g. P3, P5, and P8).

3.4. Expression of mRNA coding for feline IFN-γ

The results of RT-PCR are reported in Table 5. Three out of 4 cats did not show any visible band in cells treated with P0. In all these cats, however, weak or positive bands were found in aliquots of blood incubated with some peptides. Specifically, compared with P0, peptides from the residue 14 did not change the intensity of the bands, except for a decreased intensity in one aliquot incubated with P2. Using the peptides designed around residue 182 an increased intensity was found once, while a decreased band intensity was found for one aliquot incubated with P4 and one with P5; as regards peptides designed around residue 198 a decreased intensity was found only in one aliquot incubated with P6, while P7 and P8 increased the intensity of the bands in 2 and 1 case, respectively.

Table 5
Summary of the RT-PCR results recorded after incubation with sham solution or with peptides.

	Cat A		Cat B		Cat C		Cat D		D	U	I
	AI	RI	AI	RI	AI	RI	AI	RI			
P0 – Sham	−	n/a	−	n/a	−	n/a	+	n/a	n/a	n/a	n/a
P1 – R14M	−	U	−	U	−	U	+	U	0/4	4/4	0/4
P2 – R14A	−	U	−	U	−	U	+/−	D	1/4	3/4	0/4
P3 – R182M	+/−	I	−	U	−	U	+	U	0/4	3/4	1/4
P4 – R182V	+/−	I	−	U	−	U	+/−	D	1/4	2/4	1/4
P5 – R182A	+/−	I	+	I	−	D	+/−	U	1/4	2/4	1/4
P6 – R198V	−	U	−	U	−	U	+/−	D	1/4	3/4	0/4
P7 – R198A	−	U	+	I	+	I	+	U	0/4	2/4	2/4
P8 – R198M	+/−	I	−	U	−	U	+	U	0/4	3/4	1/4

AI: absolute intensity (i.e. intensity recorded in each lane). RI: relative intensity (i.e. intensity compared with P0). P1–P8: number of the peptide; R14, R182, R198: residues 14, 182 and 198, respectively. A: avirulent (FECV biotype); V: virulent (FIPV biotype); M: mixed (FECV/FIPV biotype); n/a: not applicable; U: unchanged compared with P0; I: increased compared with P0; D: decreased compared with P0

4. Discussion

It has been hypothesized that virulent FCoV strains arise by a genomic mutation, possibly within the 3c gene (Pedersen, 2009; Pedersen et al., 2009; Chang et al., 2010), but the mutation potentially involved in this pathogenic shift has never been identified (Kennedy et al., 2001; Rottier et al., 2005; Dye and Siddell, 2007; Lin et al., 2009). Experimental studies reported a low morbidity rate in cats inoculated with "virulent" FCoVs formerly classified as FIPV (Pedersen, 1987; Kipar et al., 2001; Dean et al., 2003; Kiss et al., 2004). All these findings support the hypothesis that the development of FIP depends on a specific FCoV–host interaction, mostly based on cell-mediated immunity (Pedersen, 1987, 2009). In turn, the strength of the immune response could be influenced by viral epitopes, since immune-modulating sequences have been found in the FCoV genome (Zhao et al., 2007). The resistance/susceptibility to FCoV infection could thus depend on a balance between individual factors referred to both the virus (type of immunomodulating peptides) and the host (cell response to specific epitopes).

To verify this hypothesis we assessed the rate of IFN-γ production by feline blood cells incubated with peptides designed on the sequence of viral strains identified from the faeces of clinically healthy cats ("non-virulent" strains), or from tissues of cats with FIP ("virulent" strains). Some sequences were found both in cats with FIP and in faeces from clinically healthy cats and we thus designed these additional peptides that have been classified as "mixed". Specifically, peptides corresponding to the residues 14, 182, 198 of protein N of FCoVs were selected using a bioinformatic approach. Immunomic tools have been largely applied in several species (De Groot et al., 2003; Díaz et al., 2009), but not in cats. Thus, in this study, epitope mapping algorithms designed for human and mouse were used since the sequence analysis of feline MHC class I reveals a remarkable conservation of both nucleotide sequence and functional organization with human and mouse MHC loci (Yuhki et al., 1989). Obviously, a more classical approach using overlapping peptides from the N protein would have provided a more exhaustive determination of T-cell epitopes. However, the bioinformatic approach allowed us to save time and resources by selecting only those peptides with a high probability to be immunologically relevant.

The effect of these peptides on IFN-γ production was assessed on blood randomly collected during routine activities. This random selection allowed us to reproduce a field situation, where stimuli not necessarily associated with FCoV infection influence IFN-γ responses. Thus, our caseload included cats with chronic diseases potentially characterized by a persistent stimulation of the immune system (e.g. neoplasia and chronic inflammation). Similarly, both FCoV-negative and FCoV-positive cats were sampled, since, based on our knowledge on host–FCoV interaction, it is very likely that, when sampled, they were respectively non-infected and infected by FCoVs (Addie et al., 2003).

Results from native plasma suggest that the concentration of IFN-γ in blood is influenced by the presence of chronic diseases rather than by the serological status of cats. This allowed us to assess whether the response to immunogenic peptides of FCoVs could be influenced by a previous priming of IFN-γ responses. Moreover, the moderate hematological changes found in both healthy and sick cats can depend on stress (neutrophilia and lymphopenia) or fear (neutrophilia and lymphocytosis) (Stockham and Scott, 2008), rather than on a stimulation of the immune system.

The incubation with the sham solution decreased the concentration of IFN-γ in almost all the cats on which IFN-γ concentration was assessed both in plasma and in P0. It is unlikely that this depends on analytical interferences of the peptides, since the presence of peptides in the pooled fresh plasma did no alter the concentration of IFN-γ. Only one cat (cat #2), had a higher concentration of IFN-γ in P0 than in plasma in spite of the lack of evident hematological changes or of a high anti-FCoV titre. Unfortunately we do not have information about the IFN-γ concentration in the plasma of 6 cats, 5 of which had high level of IFN-γ in P0. Thus, we cannot definitely conclude whether this high level in P0 was due to an even higher concentration of IFN-γ in plasma (suggesting that cat #2 was hyper-responsive) or if their plasma values were normal (suggesting that in the majority of cats, including cat #2, the simple incubation activates IFN-γ production). The first hypothesis, however, is more likely, based on the results from cats in which both plasma and P0 values were analyzed and on RT-PCR results, that were negative in 3 out of 4 P0 samples consistently with that reported in a previous study (Gelain et al., 2006) on incubation of feline whole blood.

In turn, the decreased IFN-γ concentration in P0 can be due to the degradation of pre-existing IFN-γ coupled with the lack of significant production of IFN-γ. Also this hypothesis is supported by PCR results of P0 samples. Thus, the protocol used in this study maintains an acceptable level of IFN-γ on resting cells without stimulating IFN-γ production, thus allowing for the investigation of IFN-γ responses to FCoV peptides.

This comparison, however, was biased by the low number of observations and by the individual variability of IFN-γ responses, which did not allow for the detection of significant changes despite evident differences in mean values. It is thus not possible to draw definitive conclusions on the effect of potentially immunogenic peptides. Nevertheless in most cases the incubation with peptides further decreased the concentration of IFN-γ compared with P0, especially after incubation with the peptides designed around residues 14 and 182 and with the peptide designed on the "virulent" sequence around residue 198. Moreover, in this case, the decreased IFN-γ concentration could be due to the degradation of pre-existing IFN-γ (possibly induced by the peptides itself) not associated with an equivalent IFN-γ production since RT-PCR did not suggest an increased mRNA expression compared with P0 after incubation with most of these peptides. Conversely, both the concentration of IFN-γ and mRNA expression increased in individual cats, especially after incubation with peptides designed on the basis of "avirulent" or "mixed" sequences of the residue 198 peptide. As regards to the ELISA testing, however, the possible presence of a previous priming of the cells is very likely, since "hyper-responsive" cats were

mostly found among cats with chronic diseases potentially stimulating the immune system.

Finally, based on our data, it is not possible to state whether the different responses to the peptides were associated with the presence/absence of anti-FCoV antibodies, specific diseases or hematological changes. Some of the cats that showed an increased IFN-γ production in the presence of a given peptide did not respond in the same way to other peptides, suggesting that independently on the possible priming, this responsiveness is possibly "peptide-specific".

5. Conclusion

In conclusion, this is the first time that an *in silico* prediction and *in vitro* confirmation has been used for evaluating the IFN-γ production in cats and in particular for studying FCoV–host interaction. The proposed selection of peptides and the "*in vitro*" assay employed in this study can thus be used for future studies. The preliminary results about the responses to FCoV peptides are encouraging but further investigations on the possible role of protein N immunostimulating epitopes. Specifically, although biased by the low number of observations and by the high individual variability, these results suggest that some sequences of FCoV protein N from "avirulent" or "mixed" strains can stimulate cell-mediated immunity, especially in cats in which cell-mediated immunity is primed by chronic diseases. Future larger studies are required to confirm the possible role of FCoV itself in stimulating the production of IFN-γ, the key cytokine of cell-mediated immunity involved in the response against FCoVs and consequently responsible of viral persistence (Gunn-Moore et al., 1998; Dean et al., 2003; Foley et al., 2003; Kiss et al., 2004; Berg et al., 2005; Gelain et al., 2006; Giordano and Paltrinieri, 2009).

Conflict of interest statement

Acknowledgments

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