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Cellular composition and interferon-γ expression of the local inflammatory response in feline infectious peritonitis (FIP)

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Abstract

Feline infectious peritonitis (FIP) is one of the most important viral diseases of cats. International studies estimate that approximately 80% of all purebred cats are infected with the causative agent, feline coronavirus (FCoV). Out of these, 5–12% develop clinical symptoms of FIP. The pathogenesis of the disease is complex with many unresolved issues relating to the role of the immune system. The aim of the present study was to determine the proportions of various inflammatory cell types in FIP lesions by using a panel of cat specific, thoroughly validated, monoclonal antibodies. In addition, the expression of interferon-γ within the inflammatory lesions was examined by RT-PCR. Our results confirm the mixed nature of the inflammatory reaction in FIP, involving B cells and plasma cells as well as CD4+ and CD8+ T cells. However, one cell type stands out as being the key element in both the "wet" and "dry" forms of FIP: the macrophage. Upregulation of IFN-γ expression within the inflammatory lesions suggests a local activation of macrophages, which might result in increased viral replication.
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Keywords: FIP; Inflammatory response; Interferon; Macrophages

1. Introduction

Feline infectious peritonitis (FIP) is one of the most important viral diseases of cats. International studies estimate that approximately 80% of all purebred cats are infected with the causative agent, feline corona-virus (FCoV). Out of these, 5–12% develop the classical symptoms of effusive/wet FIP, the non-effusive/dry form of FIP or a combination of both. The outcome of clinical FIP is almost always fatal (de Groot and Horzinek, 1995).

The pathogenesis of the disease is complex with many unresolved issues relating to the role of the immune system. Humoral immunity is not protective and conventional vaccines seem to accelerate the

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disease progress rather than being protective (Vem-mema et al., 1990). This has been attributed to antibodies that facilitate the uptake of virus into macrophages (Hayashi et al., 1983; Pedersen, 1995; Olsen et al., 1992, 1993; Hohdatsu et al., 1994). The histopathological lesions in the wet form of FIP (mainly vasculitis) are suggestive of a type III hypersensitivity reaction. On the other hand, the histopathological picture in the dry form of FIP (mainly granuloma formations) point in the direction of a type IV immune reaction. Based on these and other observations it has been hypothesized that animals with a weak cell-mediated immunity (CMI) in combination with a strong humoral immune response are likely to develop wet FIP. In contrast, cats with a moderately strong CMI would develop the dry form of the disease. Finally, cats with a strong CMI may not develop the disease at all (Pedersen, 1995). The viral influence on the type of immune response mounted against the viral infection is not known, but is assumed to be acquired, via mutation from the harmless feline enteric coronavirus (FECV) into the lethal feline infectious peritonitis virus (FIPV) (Vennema et al., 1998).

Considering the importance of the immune system in the pathogenesis of FIP, comparatively few studies have been aimed at investigating the local inflammatory response. The aim of the present study was to determine the proportions of various inflammatory cell types in FIP lesions, using a panel of cat specific, thoroughly validated, monoclonal antibodies. In addition, the expression of IFN-γ within the inflammatory lesions was examined by RT-PCR. Our results confirm the mixed nature of the inflammatory reaction in FIP, involving B cells and plasma cells as well as CD4+ and CD8+ T cells. However, one cell type stands out as being the key element in both forms of FIP: the macrophage.

2. Materials and methods

2.1. Animals

Six cats naturally infected with FCoV and clinically diagnosed with FIP were used in this study. All cats were euthanized by pentobarbital overdose. Details about the cats are given in Table 1.

Table 1
Cats included in the study

Cat no.	Age/sex[a]	Breed	Form of FIP	Duration of illness
1	0.4/M	Devon rex	Wet	2 Weeks
2	1.5/M	Persian	Wet	4 Months
3	4/F	Domestic shorthair	Wet	2 Months
4	1/M	Domestic shorthair	Dry	2 Weeks
5	1/M	Birman	Wet	Not known
6	1.5/F	Birman	Wet/dry	Not known

[a] Age in years; M, male; F, female.

2.2. Necropsy and histopathology

In all cases, necropsy was performed within a few hours of death. Tissue samples were taken from macroscopically visible changes (granuloma, serosal surfaces with fibrinous coating) and fixed in 10% buffered formalin. After paraffin embedment, sections were cut 4 μm thick and stained with haematoxylin and eosin (HE) for histopathological evaluation. Parallel tissue samples were embedded in OCT medium, snap frozen in liquid nitrogen and stored at −70 °C for immunohistochemistry and RT-PCR.

2.3. Immunohistochemistry

Frozen tissue samples were cut at 4 μm thickness, dried for 30 min at room temperature, fixed for 10 min in acetone and dried again for 30 min at room temperature. In order to identify various cell types within the inflammatory lesions, a panel of cat specific monoclonal antibodies was applied to the sections (Table 2). These antibodies had previously been tested on feline lymphoid tissue and peripheral blood mononuclear cells, verifying their reactivity and specificity (Lundgren et al., 1995; Berg et al., 1999). To identify macrophages, a biotinylated Griffonia Simplicifolia lectin (Vector Laboratories) was used. The monoclonal antibodies were applied overnight, after blocking steps with avidin, biotin and normal horse serum. On day 2, a biotinylated horse anti-mouse antibody (Vector Laboratories) was applied to the sections for 30 min at a dilution of 1:200. Following quenching of endogenous peroxidase activity with 3% H_2O_2 for 15 min, an avidin–biotin complex (ABC) (Vector Laboratories) was added to the sections for 30 min. Bound antibodies were visualized with AEC. Washing with

Table 2
Panel of monoclonal antibodies

Antibody	Dilution used	Source	Specificity	Reference
CF54A	1:800	VMRD, Pullman, WA, USA	CD3 (pan T)	Lundgren et al. (1995), Berg et al. (1999)
CAT30A	1:100	VMRD	CD4 (T helper)	Tompkins et al. (1990), Lundgren et al. (1995)
CAT82A	1:200	VMRD	MHC II	Lundgren et al. (1995), Berg et al. (1999)
F46A	1:800	VMRD	CD21 (B cells)	Lundgren et al. (1995)
FT2	1:50	Southern Biotechnology, Birmingham, AL, USA	CD8 (cytotoxic T cells)	Klotz and Cooper (1986), Lundgren et al. (1995), Berg et al. (1999)
FIPV3-70	1:100	Customs Monoclonals International, W. Sacramento, CA, USA	FCoV	Kipar et al. (1998)

TRIS-buffered saline (TBS) was performed between every incubation step. The biotinylated lectin was applied to paraffin sections for 60 min at room temperature. Blocking of unspecific background staining, washing steps and visualization followed essentially the protocol used for frozen tissue sections. After the completion of immunohistochemistry, all sections were counterstained with haematoxylin.

2.4. Quantification of antibody-labelled cells

In each section, microscopical granuloma or areas of more diffusely demarcated inflammation were defined. In total, 28 inflammatory lesions were identified. Nineteen of the investigated inflammatory lesions were located in the intestinal serosa, four in the kidney, one in the mesenteric lymph node and two in the liver and lungs, respectively. The number of antibody- or lectin-labelled cells within each of these lesions was scored in a semi-quantitative fashion as follows: 0, none; 1, occasional; 2, scattered; 3, moderate and 4, predominant (>50% of the total cell infiltrate). Plasma cells were identified based on their morphological appearance in HE-stained sections and scored according to the same system.

2.5. Statistical analysis

Mean score ± S.D. was calculated for each antibody/cell type (see above under quantification of antibody-labelled cells). Inflammatory lesions were divided into two basic morphological types A and B (see below under Section 3). Differences in antibody labelling scores between types A and B lesions were evaluated using the Mann–Whitney U-test, two-tailed distribution. A P-value < 0.05 was considered to be statistically significant.

2.6. RNA extraction

The following frozen tissue samples were used for RNA extraction: kidney, liver and spleen from cat no. 4; liver, jejunum and spleen from cat no. 5; liver and lung from cat no. 6 (Table 4). Total RNA was extracted from frozen tissue samples using the Trizol reagent (Life Technologies). The RNA was dissolved in nuclease-free water and stored at −70 °C. RNA concentration was determined by spectrophotometry at 260 nm.

2.7. ConA stimulation of feline PMBCs

Feline blood was collected in heparine tubes and PBMC purified via a standard Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden) centrifugation. Cells were seeded onto plastic wells and ConA was added to a final concentration 10 μg/ml. The cells were collected 6 h thereafter and RNA prepared as described above.

2.8. RT-PCR assay

The reverse transcription (RT) reaction was performed in a final volume of 50 μl containing 1 μg of total RNA, 2.5 μl cDNA primer (530 μg/ml), 0.5 mM dNTPs, 50 mM Tris–HCl (pH 8.3), 40 mM

KCl, 6 mM MgCl$_2$, 5 mM dithiothreitol (DTT), 2.5 μl RNAse inhibitor and 400 units SuperScript II (Gibco BRL) at 42 °C for 50 min, followed by 75 °C for 15 min. 10 μl was taken aside before addition of Superscript to be used as negative control in the interferon RT-PCR assays.

The cDNA primer has a unique sequence at the 5′ end (CCT GAC CCA ACCAGT AGA CCA TTT TTT TTT TTT TTT TTT TN) that was used in a multiplex first step PCR. The last base was either an A, C or a G.

PCR was performed in a total volume of 25 μl and containing the following concentrations of the ingredients (1 × PCR buffer, 2.0 mM MgCl$_2$, 0.25 mM dNTP and 1 unit of Taq polymerase) as recommended by the manufacturer (MBI Fermentas, Lithuania) and 0.4 μM of the primers IFN-γ forward (ACT GGA AAG AGG AGA GTG ATA) and back (TGA CCC AAC CAG TAG ACC A). One microlitre of the RT reaction was used for the PCR.

The nested PCR was performed using 1 μl of the first PCR, using the same conditions as described above and 0.4 μM of the primers IFN-γ 2 and IFN-γ 3 (AGC CAA ATT GTC TCC TTC TAC and GGA CAA CCA TTA TTT CGA TGC). The number of cycles in the nested PCR was variable from 20 to 35 cycles as a mean for relative quantification. PCR with primers for β-actin was performed in parallel to check for RNA quality. The PCR products were analyses by agarose-gel electrophoresis. The primers for feline INF-γ were designed based on the sequences from GenBank (NM001009873). The primers were designed to be on different exons based on the human interferon-γ gene. Feline β-actin primers were designed based on the partial coding sequence in GenBank (AB051104).

3. Results

3.1. Gross pathology

The cats with wet FIP had peritoneal and/or pleural effusion of yellow colour, containing fibrinous flocks. Fibrinous serositis, peritonitis and/or pleuritis were present in all cats with wet FIP, as were multiple granulomas in the intestinal serosa, mesenteric lymph nodes and kidneys. The cat with dry FIP (no. 4) showed enlarged retropharyngeal and popliteal lymph nodes, and multiple granulomas in the kidneys. The cat with mixed wet/dry FIP had pleural effusion, fibrinous pleuritis, granulomas in the lungs, liver and portal lymph nodes.

3.2. Histopathology

Two basic types of lesions were observed: (A) a diffuse serosal inflammation with extensive fibrinous exsudation, necrosis and perivasculitis/vasculitis and (B) demarcated or confluent granulomas, with or without a necrotic centre (Fig. 1a and b). In both types of lesions, the composition of inflammatory cells was mixed, although type A lesions tended to be less cellular than type B lesions. Neutrophilic granulocytes occurred in both types of lesions, mainly in connection with necrosis. Both types of lesions occurred in the cats with wet, or mixed wet/dry, FIP. In the only case classified as dry FIP (Table 1, no. 4), no type A lesions were observed (Table 4). However, vasculitis was present within the type B lesions in the kidney of this cat. In total, 8 FIP lesions were classified as type A and 20 as type B. A further subdivision of type B lesions was made with granulomas containing a necrotic centre ($n = 15$) and granulomas without necrosis ($n = 5$) as separate groups.

An attempt was made to separate cases of chronic duration (Table 1, nos. 2 and 3) from those in the subacute stage of the disease (Table 1, nos. 1 and 4). There was no apparent morphological distinction between these two groups. Type B lesions of both subtypes occurred in the subacute as well as in the chronic cases.

In addition to the distinct types A and B lesions, other more diffuse inflammatory changes were observed. These included periportal and perivascular mononuclear cell infiltrates in the liver, histiocytosis and plasmacytosis in the red pulp of the spleen, and lymphoid hyperplasia or atrophy. The immunohistochemical investigation was confined to types A and B lesions.

3.3. Composition and proportions of inflammatory cells

The two subgroups of type B lesions, granulomas with and without necrosis, did not differ significantly in antibody labelling pattern. Accordingly, comparisons with type A lesions were done based on data
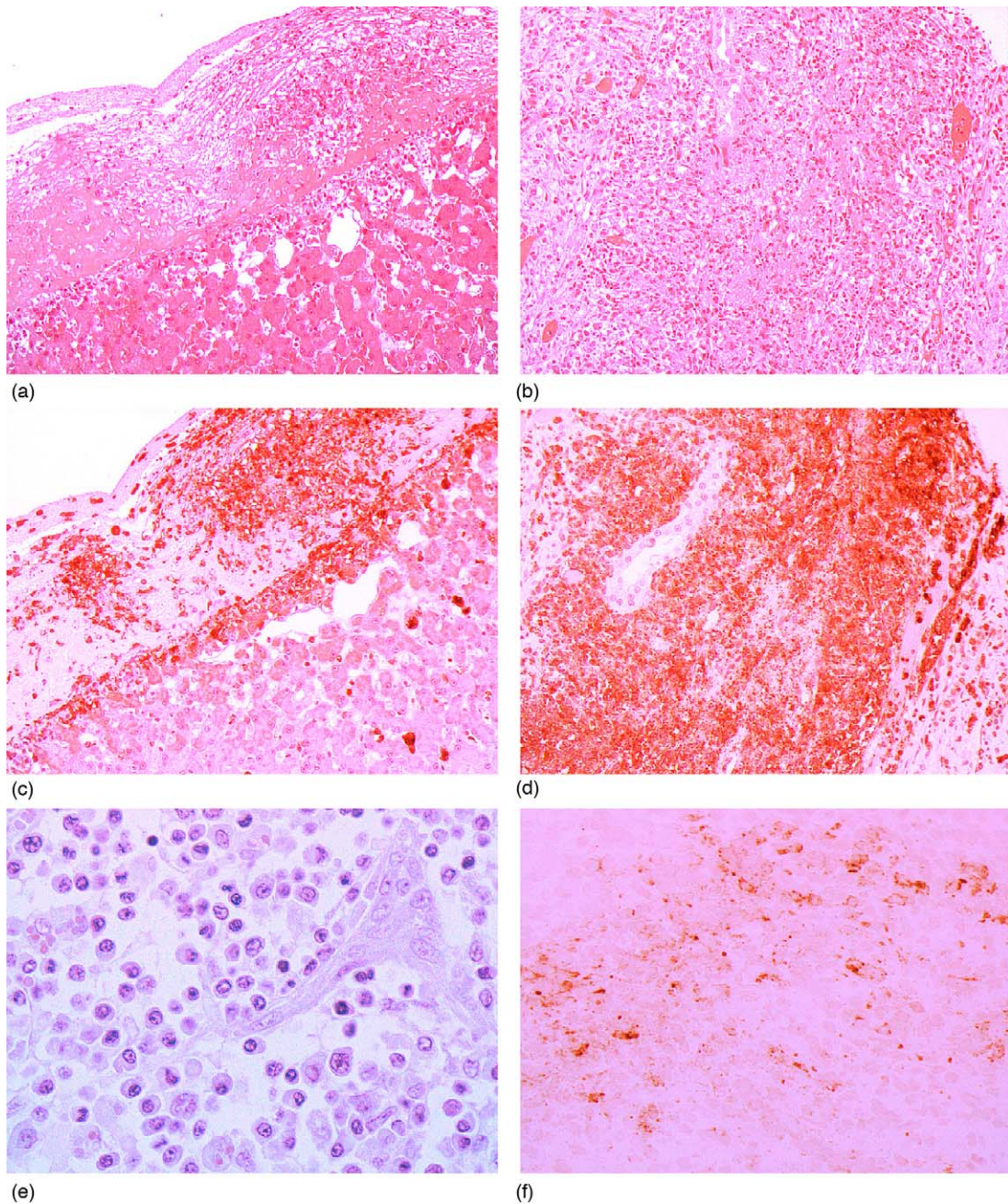
Fig. 1. Histopathological and immunohistochemical characterization of FIP lesions. (a) Diffuse serosal inflammation and fibrinous exsudation (type A lesion) in the liver (cat no. 5). (b) Granuloma formation (type B lesion) in the kidney (cat no. 4). (c) Numerous monocytes/macrophages (red cells) within the same inflammatory area as shown in (a). (d) Numerous monocytes/macrophages (red cells) within the same granuloma as shown in (b). (e) Plasma cells and a multinucleated giant cell (upper right corner) within a type A lesion in the liver (cat no. 6). (f) Inflammatory cells (presumably macrophages) containing FCoV antigen (red cells) within an intestinal granuloma (cat no. 2). FCoV-positive granules are also present extracellularly. Stainings: HE (a, b and e), lectin immunohistochemistry (c and d) and FCoV immunohistochemistry (f). Magnifications: ×100 (a–d), ×200 (f) and ×400 (e).
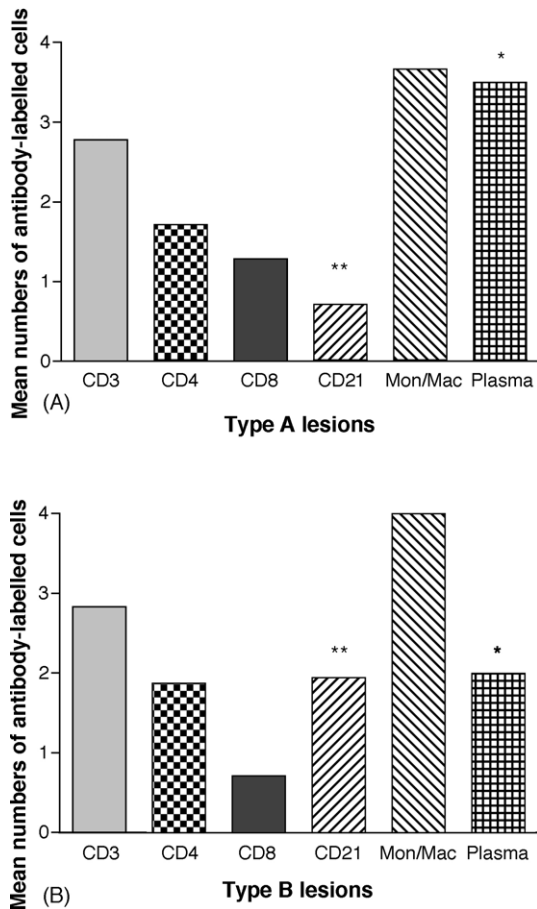
(A) Type A lesions

(B) Type B lesions

Fig. 2. Mean number of cells expressing different types of CD markers in type A lesions (a) and type B lesions (b). $^*p < 0.05$, $^{**}p < 0.005$ Mann–Whitney *U*-test.

from all type B lesions. Regardless of type of lesion, the vast majority of inflammatory cells were MHC class II positive. Monocytes/macrophages, identified by positive labelling with lectin, were by far the most predominant cells within type B granulomas as well as in the more diffuse type A lesions (Figs. 1c and d and 2a and b). Multinucleated giant cells were occasionally observed in type B granulomas (Fig. 1e).

T cells appeared in moderate numbers in both type A and B lesions. The proportion of CD4+ versus CD8+ T cells was higher in type B lesions; this difference, however, was not statistically significant (Fig. 2a and b). The CD21 antibody, a marker for B lymphocytes but not for mature plasma cells, labelled scattered to moderate numbers of cells in both types of lesions. The number of CD21+ cells was significantly higher in type B lesions. In contrast, plasma cells were significantly more frequent in type A lesions (Figs. 1e and 2a and b).

Scattered cells infected with FCoV occurred in the majority of type A as well as type B lesions. A proportion of antibody-labelled viral antigen appeared to be extracellularly located (Fig. 1f). Considerable numbers of neutrophilic granulocytes were present within the necrotic areas of type A and B lesions, but were not otherwise prominent. No further quantification of this cell type was performed.

A comparison between chronic cases (Table 1, nos. 2 and 3) and subacute cases (Table 1, nos. 1 and 4) revealed no significant differences in composition and proportions of inflammatory cells. The results are summarized in Fig. 2 and Table 3.

3.4. IFN-γ profiles

IFN-γ mRNA levels were increased in the majority of tissues with inflammatory lesions in all three examined cats (Fig. 3, Table 4). Type B lesions tended to have the strongest expression of IFN-γ, although considerable levels were present in some type A lesions as well (Table 4).

Table 3
Proportions of inflammatory cell populations within FIP types A and B lesions

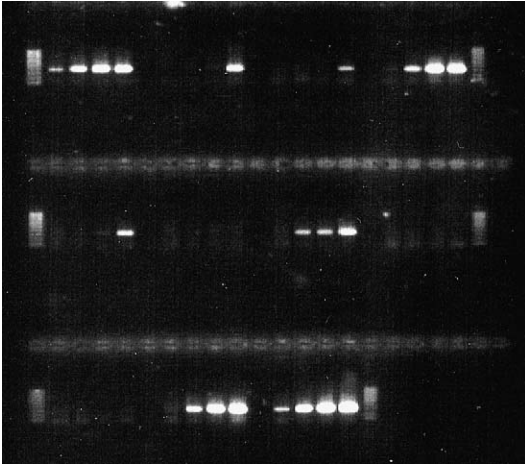|	CD3 (pan T)		CD4		CD8		CD21 (B cells)		Plasma cells		Monocytes/macrophages	
	A	B	A	B	A	B	A	B	A	B	A	B
Mean (range)	2.8 (1–4)	2.8 (1–4)	1.7 (1–3)	1.9 (0–4)	1.3 (0–2)	0.7 (0–2)	0.7 (0–1)	1.9 (1–3)	3.5 (3–4)	2.0 (1–4)	3.7 (3–4)	4.0 (4–4)
Median	3.0	3.0	1.0	2.0	1.0	1.0	1.0	2.0	3.5	2.0	4.0	4.0
P-value[a]	NS		NS		NS		0.0019		0.0107		NS	

[a] NS, non-significant.

Fig. 3. Agarose-gel electrophoresis of interferon-γ RT-PCR products. All PCR reactions were run for 20, 25, 30 and 35 cycles for estimation of relative expression levels. Well number 1, molecular weight marker; 2–5, ConAstimulated feline PBMC; 6, empty; 7–10, feline PBMC without stimulation; 11, empty; 12–15, FIP cat (167/98) liver; 16, empty; 17–20, FIP cat (167/98) lung; 21, Mwmarker; 22, empty; 23, marker; 24–27, FIP cat (125/98) lymph node; 28, empty; 29–32, FIP cat (125/98) spleen; 33, empty; 34–37, FIP cat (125/98) intestine; 38, empty; 39–42, FIP cat (125/98) liver; 43, marker; 44, empty; 45, marker; 46–49, FIP cat (94/97) liver; 50, empty; 51–54, FIP cat (94/97) spleen; 55, empty; 56–59, FIP cat (94/97) kidney; 60, marker; 61–66, empty.

4. Discussion

Our findings confirm the heterogeneous nature of FIP lesions, previously described by others (Kipar et al., 1998; Paltrinieri et al., 2001). Indeed, the results of the present study suggest that the commonly used

Table 4
Expression of IFN-γ mRNA in types A and B lesions

Cat no.	Tissue	Type of lesion	Expression of IFN-γ[a]
4	Kidney	B	++++
	Liver	None	−
	Spleen	Histiocytosis, plasmacytosis	+++
5	Liver	A	−
	Spleen	A	−
	Jejunum	A (severe)	+++
	Lymph node	A (severe)	++
6	Liver	B	+
	Lung	A and B	+++

[a] Expression of IFN-γ: −, no detectable expression; +, minimal; ++, slight; +++, moderate; ++++, strong expression.

subdivision of the disease into "wet" and "dry" forms is based more on clinical presentation than on any morphological criteria. We could not identify a distinct histopathological picture distinguishing wet from dry cases, nor acute/subacute from chronic ones. Although two major types of lesions were observed, one basically necrotizing/fibrinous (type A) and the other one basically granulomatous (type B), there was considerable overlap between the two categories. Vasculitis was for instance present within granulomas in the kidney of a "dry" case and more or less demarcated granulomas were found in parallel with diffuse serosal inflammation in "wet cases".

The composition and proportion of inflammatory cells within types A and B lesions was also strikingly similar with a strong predominance of macrophages and moderate numbers of T cells as common features. Here, our results differ somewhat from Kipar et al. (1998), who reported that T cells only occurred in low numbers in all types of lesions. This discrepancy in results might be due to different specificities of the antibodies used for immunohistochemistry: non-cat specific (Kipar et al.) versus cat specific. CD4+ cells tended to be more numerous than CD8+ cells in type B lesions, providing some support for the notion of a type IV hypersensitivity reaction in the pathogenesis of FIP (Paltrinieri et al., 1998b). The same predominance of CD4+ cells over CD8+ cells in FIP granulomas was reported by Paltrinieri et al. (1998a), who used a different panel of monoclonal antibodies. A further indication of a type IV hypersensitivity reaction is the presence of multinucleated giant cells in type B lesions. This cell type, not commonly reported as a feature of FIP, usually occurs during conditions of chronic stimulation of macrophages (e.g. tuberculosis) (Tizard, 1987).

However, the only significant difference between types A and B lesions was a higher proportion of mature plasma cells, and a lower proportion of B lymphocytes, in type A lesions. Accumulation and maturation of B lymphocytes into FCoV antibody-producing plasma cells is supposed to lead to deposition of immune complexes within vessel walls and complement-mediated vascular injury (Olsen, 1993; de Groot and Horzinek, 1995; Pedersen, 1995). It can thus be assumed that since type A lesions are closely associated with vasculitis and peritoneal/pleural effusion, our findings of numerous plasma cells in type A lesions support this theory.

A somewhat unexpected finding was the increased expression of IFN-γ within inflammatory lesions of both types, but in particular type B. In contrast, previous studies on experimentally infected cats have indicated that IFN-levels are low in these animals, accounting for a poor CMI, and thus development of disease (Gunn-Moore et al., 1998; Kiss et al., 2004). However, those results were obtained using peripheral blood mononuclear cells only. It seems likely that cytokine profiles run on samples from tissues with relevant inflammatory lesions would reflect the local cytokine response more adequately. In a recent study, Dean et al. (2003) found a non-significant tendency of elevated IFN-γ mRNA expression in lymphoid tissues in FIP-infected cats as compared to controls. Also, in a study focused on the neurological form of FIP, Foley et al. (2003) showed that cats with generalized FIP (but not cats with purely neurological affection) had upregulation of IFN-γ as well as several other Th1-associated cytokines in the brain. However, the variation between cats was high and no consistent Th1- or Th2-pattern could be distinguished.

In the heterogeneous and sometimes confusing histopathological picture of FIP one distinct morphological feature stands out: the granulomatous nature of the inflammatory reaction. Macrophages are, without question, the most predominant inflammatory cells in FIP lesions. Cytokines secreted by local T cells will attract more macrophages to the lesions, thereby stimulating and preserving the granulomatous inflammation. Of particular interest in this context is the role of IFN-γ. A strong local IFN-γ expression would be expected to result in activation of macrophages, including an increase in the number of Fc-receptors on the macrophage cell membrane. Thus, viral uptake in macrophages is enhanced and viral replication increased. On the other hand, a strong IFN-γ response is considered to be vital in the defense against viral agents, stimulating the differentiation of cytotoxic T cells.

Although the exact role of IFN-γ in the pathogenesis of FIP cannot presently be determined, it is obvious that the infection is not controlled by the inflammatory response. The immune response against a virus is a complex interplay between different cells and the molecules they produce, cytokines, chemokines and their receptors. In addition, many molecules are redundant, having many different effects and acting on different cells. To further elucidate the intriguing immunopathogenesis of FIP it is necessary to perform complete cytokine profiles on relevant inflammatory lesions from a larger number of cats with the three major forms of the disease, as well as to determine the timing of cytokines and inflammatory cells in experimentally infected cats.

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