Measurement of IL-12 (p40, p35), IL-23p19, and IFN-γ mRNA in Duodenal Biopsies of Cats with Inflammatory Enteropathy

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Background: Dietary hypersensitivity and inflammatory bowel disease (IBD) are important causes of chronic vomiting and diarrhea in cats. IL-23 has been recently found to be a key factor in the immunopathogenesis of IBD in humans but the involvement in IBD has not been investigated in cats.

Hypothesis/Objectives: Expression of genes encoding Il-12p35 and p40, IL-23p19, and IFN-γ may be up-regulated in duodenal biopsy specimens taken from cats with histologic evidence of inflammation.

Animals and Methods: Duodenal biopsy specimens were collected from control cats (n = 21) and cats with inflammatory enteropathy (n = 13). Routine histopathology, immunohistochemistry (IHC), and qRT-PCR were used to assess expression of MHC class II and to measure gene transcripts encoding the p35, p40, and p19 subunits of the IL-12 family of cytokines and IFN-γ.

Results: There were significant differences in expression of mRNA encoding IL-12p35 and IL-23p19 between healthy cats and cats with inflammatory enteropathy. IL-12p35 mRNA was lower in the duodenal mucosa of cats with inflammatory enteropathy compared with the mucosa of healthy cats ($P = .001$). In contrast, IL-23p19 mRNA expression was higher in duodenal biopsy specimens from cats with inflammatory enteropathy than in those from healthy controls ($P = .001$). There was no difference in expression of IL-12p40 and IFN-γ mRNA ($P > .05$). The majority of cats with inflammatory enteropathy had histologic evidence of moderate to severe colitis (score 2).

Conclusions and Clinical Importance: The results of this preliminary study suggest that IL-23 plays a role in the pathogenesis of feline inflammatory enteropathy.

Key words: Dietary hypersensitivity; Food-responsive diarrhea; GALT; Inflammatory bowel disease.

I nflammatory bowel disease (IBD) is a common and important disorder of the cat.[1] The underlying etiology and pathogenesis of IBD remain unclear, but may involve immune dysregulation leading to inappropriate immunologic responsiveness to commensal flora and dietary antigens.[1] Characterization of mucosal cellular infiltrates using immunohistochemical techniques in canine IBD[2,3] and in healthy cats and cats with IBD[4,5] has provided new insights into the cellular changes that may accompany the disease process. Recent reports have shown alterations in cytokine gene expression profiles in the small intestine of cats with IBD.[6,7] One of the key cytokines with a role in the immunopathogenesis of IBD is interleukin-12 (IL-12) which is a heterodimeric molecule composed of p35 and p40 subunits and which promotes development of IFN-γ-producing Th1 CD4$^+$ cells.[8]

Interleukin-23 (IL-23) is a member of the IL-12 family of cytokines and is composed of p19 and p40 subunits, the latter of which is shared with IL-12.[9] The role of IL-12 in the pathogenesis of IBD is being

Abbreviations:

GALT	gut-associated lymphoid tissue
HE	hematoxylin and eosin
IBD	inflammatory bowel disease
IHC	immunohistochemistry
IL	interleukin
MHC-II	major histocompatibility complex class II
qRT PCR	quantitative reverse transcription polymerase chain reaction

re-evaluated in light of recent reports suggesting that IL-23 may have greater relevance to the pathogenesis of various autoimmune and inflammatory diseases.[10] Significant differences have been reported in the levels of expression of mRNA encoding IL-12p40, but not IL-12p35 between cats with and without histologic evidence of IBD.[6] In light of the new findings related to IL-23, up-regulation of p40 mRNA may be associated with parallel up-regulation of IL-23p19 gene expression. IL-23p19 has not been studied previously in cats with gastrointestinal (GI) disease. Therefore, the aim of this study was to compare IL-12p35, IL-12p40, IL-23p19, and IFN-γ mRNA expression in duodenal biopsy specimens taken from control cats and cats with inflammatory enteropathy.

Materials and Methods

Study Protocol

Duodenal biopsy specimens were collected from control cats (n = 21) and cats with IBD or dietary hypersensitivity (n = 13), collectively referred to as 'inflammatory enteropathy'. Control cats were specific pathogen-free (SPF) animals 2–4 years of age with no history or clinical signs of GI disease. These animals were part of an experimental study, and the samples included in

this study were taken before the start of the experiment. Duodenal biopsy specimens were collected on 2 occasions from each cat and results represent an average of these 2 time points. The cats were kept under conditions approved by the UK Home Office and Institutional Ethical Committee.

Cats with inflammatory enteropathy had evidence of vomiting, diarrhea, weight loss, or some combination of these signs for a minimum of 2 weeks. Each of these cats was subjected to a complete clinical examination and routine laboratory testing (hematology, serum biochemistry, retroviral screening, and T4 concentration for cats >7 years of age) including fecal parasitologic examination and bacterial culture, and diagnostic imaging. All cats received a course of fenbendazole to eliminate possible *Giardia* infection. These animals therefore were considered to have either IBD or dietary hypersensitivity. A restriction diet trial was not administered in order to distinguish between these possibilities and thus the term 'inflammatory enteropathy' was applied.

Collection of Endoscopic Biopsy Specimens

Samples of duodenal and colonic mucosa (colonic mucosa was only collected from cats in the clinical group) were collected by endoscopy as previously described.[4] In brief, 6 biopsy specimens were fixed in 10% neutral buffered formalin, embedded in paraffin wax, sectioned at 5 μm and stained with hematoxylin and eosin (HE). Another set of 6 biopsy specimens was snap-frozen and stored at −80°C until used for real time reverse transcriptase-polymerase chain reaction (RT-PCR). Whenever possible, both inflamed and normal tissues were biopsied.

Histopathology and Immunohistochemistry

Biopsy specimens from all cats were scored for pathologic changes based on the architecture of the section and cellular infiltration of lamina propria in both villus and crypt areas. The study predated availability of the WSAVA Gastrointestinal Standardization Group monograph[11] but utilized a previously published scoring system.[4] All slides were examined by a board-certified veterinary pathologist (MJD) who was blinded to the animal's identity.

Labeling for MHC class II expression was performed using formalin-fixed tissues and evaluated as previously described.[4,5] Briefly, for scoring epithelial expression of MHC class II, areas in both crypt and villus epithelium were scored according to the intensity of MHC class II labeling of the epithelial cells (grades 0–4). An overall score then was calculated by adding together the grades of all areas examined.[5] For each group, the average and standard deviation of score in all cats then were calculated. The density of class II$^+$ cells in the lamina

propria was scored from 1 to 4 using the following grading system:

1. Normal density (grade 1).
2. Mild increase in cellularity (grade 2).
3. Increased cellularity (grade 3).
4. Lamina propria densely populated with differentiated dendritic cells (grade 4).

Statistics

All data were assessed for normal distribution using the normal probability plot before applying parametric or nonparametric analysis where appropriate. For detection of differences between groups, general linear model (GLM) procedures were applied. When F-tests were significant, means were compared using Scheffe's test. Student's t-tests were used to determine whether there were significant differences between the 2 groups for expression of mRNA encoding cytokines and the presence of immune cells. Pearson's correlation coefficient test was used to check for positive or negative correlations between variables.

All statistical analysis procedures were performed using statistical package for social scientists software.[a] The significance level was set at $P < .05$.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

RNA Isolation. Isolation and DNase digestion of total RNA from 2 to 4 endoscopic biopsy specimens (8–18 mg) were performed as described previously.[6] The quantity and quality of the resulting total RNA were assessed by automated gel electrophoresis.[b]

Primer and Probe Design. IL-12p35, IL-12p40, IFN-γ, and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) primers and probes were the kind gift of Dr Nghia Van Ngyun (Table 1).[6] No feline-specific IL-12p19 sequence was available from GenBank for the design of primers and probes for this target. Therefore, a consensus sequence was obtained by aligning the human (GenBank accession number: NM_016584), murine (NM_031252) and canine (XM_538231) sequences. Consensus primers were designed using this consensus sequence (Forward: AGAGCCAGCCAGATYTGAGAAG Reverse: CTGCTCCRTG GGCAAAGA), which amplified a 580 base pair product from canine cDNA. These primers were used to amplify products from feline intestine and lymph node cDNA which were agarose gel-purified and sequenced.[c] The sequence data generated from these experiments were submitted to GenBank (accession number: DQ195102). Primers and probes were designed against the IL-23 p19 sequence using Primer 3 (www–genome.wi.mit.edu/cgi–bin/

Table 1. Primer and probe sequences used in this study.

Cytokine	Forward Primer 5′→3′	Reverse Primer 3′→5′	Probe 5′→3′	Optimal Annealing Temperature (°C)	Efficiency (%)
G3PDH	GCTGCCCAGA ACATCATCC	GTCAGATCCACG ACGGACAC	TCACTGGCATG GCCTTCCGT	64	93.2
IL-12p19	GAGAACAGGG AGATGACGAGAC	TGAGTCCTTGGG GATCACAG	AACCAGCCATG GCAACGCCTCC	60	98.0
IL-12p35	AATGTTCCA GTGCCTCAACC	CTAGAGTTTGT CTGGCCTTCTG	CTGCGAGCCAT CAGCAACCG	64	99.1
IL-12p40	GCCTACCCATT GAAGTCGTG	GGTTTGATGAT GTCCCTCATG	CTGCGAGCCA TCAGCAACACG	58	94.8
IFN-γ	TGCAAGTAA TCCAGATGTAGCAG	GTTTTATCACT CTCCTCTTTCCAG	CAAAATGTCTAC GAAAAGCGACCCACC*	58	94.4

primer/primer3_www.cgi) and M-Fold as described previously (Table 1).[12] Primers and probes were synthesized by Eurogentec Ltd[d] and reconstituted in EB buffer (10 mM Tris–HCl pH–8.4[e]) before use.

Quantitative RT-PCR. qRT-PCR for IL-12 p35, IL-12p40, and IFN-γ was carried out as previously described.[6]

The absence of genomic contamination of all RNA samples was confirmed before the RT reactions by performing qPCR on the purified RNA without reverse transcription with the G3PDH assay as previously described.[6] None of the samples showed evidence of amplifiable genomic DNA with this assay. A negative control of nuclease-free water and a positive control sample with a known Ct value were included with all sample runs to control for run-to-run Ct variation. A PCR reaction was run for each RT repeat resulting in 2 Ct values for each RNA sample. A mean Ct value was calculated for each sample using these values. G3PDH also was used as a 'housekeeper gene' to normalize all of the threshold cycle (Ct) values of other cytokine products as previously described.[6]

In brief, a G3PDH Ct value of 20 was chosen as a normalization value. The correction value for G3PDH of each sample was calculated as follows:

$$\text{G3PDH correction value} = \text{mean sample Ct G3PDH value} - 20.$$

$$\text{Corrected cytokine Ct value} = \text{mean cytokine Ct} - \text{G3PDH correction value.}$$

The relative number of cDNA copies in the sample was calculated using the following equation:

$$\text{Relative cytokine copy number} = 2^{(42 - \text{corrected cytokine Ct value})}$$

IL-23p19 qPCR Assay Optimization. Conventional PCR to obtain the amplicon for direct sequencing was carried out using the HotStarTaq[d] Master Mix as described previously[6] but a thermocycling protocol of 95°C for 15 minutes and 45 cycles of 95°C for 15 seconds, 60°C for 20 seconds, and 72°C for 30 seconds before a final incubation at 72°C for 5 minutes was used in a PTC–200 DNA engine.[f]

Results

Signalment

Cats with inflammatory enteropathy were presented with chronic history of diarrhea (n = 13), vomiting (n = 3), weight loss (n = 13), and all of these mentioned signs (n = 3). The age of cats with inflammatory enteropathy (n = 13) ranged from 5 to 132 months (mean, 51.43; median, 27). Breeds of cats in this group were Domestic Shorthair (DSH), Ragdoll, Siamese, British Blue, Burmese, Bengal, and Norwegian Forest. There was no sex predisposition and cats were male (n = 4), female (n = 3), neutered male (n = 4), and neutered females (n = 2). All the control group cats were DSH. Both males (n = 13) and females (n = 8) were represented. Details of age, sex, and breed distribution of both groups are summarized in Table 2.

Histopathology and Immunohistochemistry

Histopathologic scores of healthy cats ranged from normal (score = 0) to moderate cellular infiltration (score = 2) (mean, 0.8, median, 1). In cats with inflammatory enteropathy, duodenal biopsy specimens had an average score of 0.9 (median, 1), whereas colonic biopsy specimens had a consistent score of 2 (moderate colitis) even when no signs of colitis were reported by the owners (mean, 1.63, median, 2). Both the density of cells expressing MHC class II within the duodenal lamina propria and the epithelial expression of this molecule by enterocytes were slightly higher in cats with inflammatory enteropathy than in healthy cats,

Table 2. Signalment of cats with inflammatory enteropathy and healthy controls.

Group	Sex		Age (months)				Breed		
	MN (M)	FN (F)	Min	Max	Mean	Median	DSH	Ragdoll	Other
Inflammatory enteropathy	4 (4)	3 (2)	5	132	51.43	27	5	2	6
Healthy	12 (1)	4 (4)	17	36	27	26	21	0	0

MN, neutered male; M, male; FN, neutered female; F, female; Min, minimum; Max, maximum age in the study group; DSH, domestic shorthair.

Two groups were included in this study: cats with inflammatory enteropathy and healthy SPF cats.
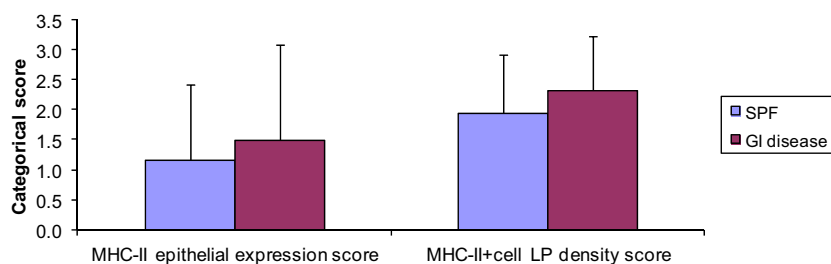

Fig 1. MHC class II[+] epithelial expression and lamina propria cell density scoring in duodenal biopsies of healthy controls and in cats with inflammatory enteropathy.

but the difference was not significant ($P > .05$). IHC results are summarized in Figure 1.

qRT-PCR Results

IL-12p35 gene expression was significantly lower in duodenal biopsies of cats with inflammatory enteropathy than in healthy cats ($P = .0001$), whereas p40 gene expression was not significantly different between the 2 groups ($P = .079$). IL-23p19 mRNA was significantly higher in duodenal biopsies of cats with inflammatory enteropathy than in healthy cats ($P = .001$), but IFN-γ gene expression was not significantly different between the 2 groups ($P = .993$). Cytokine mRNA expression is summarized in Figures 2 and 3.

There was a positive correlation between all measured cytokines for the control group ($P < .001$; Table 3). There was a highly significant positive correlation between IL-12p40 and IL-12p35 gene expression ($P = .001$) in duodenal biopsies of cats with inflammatory enteropathy (Table 4). IL-23p19 gene expression was positively correlated with that for IL-12p35 ($P = .04$), IL-12p40 ($P = .002$), and IFN-γ ($P = .015$) in the duodenal biopsies of cats with inflammatory enteropathy. IFN-γ mRNA was not significantly correlated with that encoding IL-12p40 ($P > .05$).

Discussion

This study characterized IL-23 p19 gene expression in the duodenal mucosa of healthy cats and cats with chronic idiopathic inflammatory enteropathy. Such analysis has not been performed previously in cats or dogs with IBD.

As reported in the literature, cats referred with chronic signs of inflammatory enteropathy were middle- to older-aged animals.[13] There was no breed predilection, although there were relatively more DSH and Ragdoll cats (n = 5 and 2, respectively), but the study sample was relatively small. Both male and female cats were represented equally in the study sample.

Significant differences in the levels of expression of mRNA encoding IL-12p40, but not IL-12p35 have been reported between the healthy cats and the cats with histologic evidence of intestinal inflammation.[6] Since that study, another cytokine has been characterized which shares the p40 subunit with IL-12 and is now referred to as IL-23.[9] In light of these new findings, the observed increase in expression of the IL-12 p40 gene reported in cats could indicate translation into IL-23, rather than IL-12, protein.

All cats included in the clinical group of this study had histopathologic changes suggestive of IBD, but some cats in the control group also had mild inflammatory infiltration of the duodenal mucosa. We have noted the presence of such inflammation in clinically normal cats of this experimentally housed colony in previous investigations.[6] Evaluation of cellular infiltration in HE-stained endoscopic biopsy specimens can be difficult, but evaluation has now been simplified by availability of the standards published by the World Small Animal Veterinary Association (WSAVA) Gastrointestinal Standardization Group.[11] In this
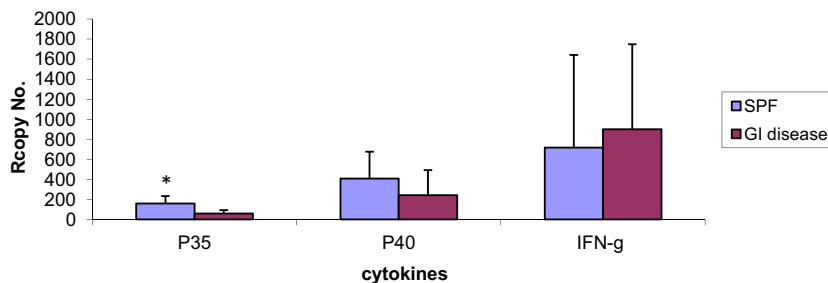

Fig 2. IL-12 p35, p40, and IFN-g mRNA expression in duodenal biopsies of control cats and cats with inflammatory enteropathy.
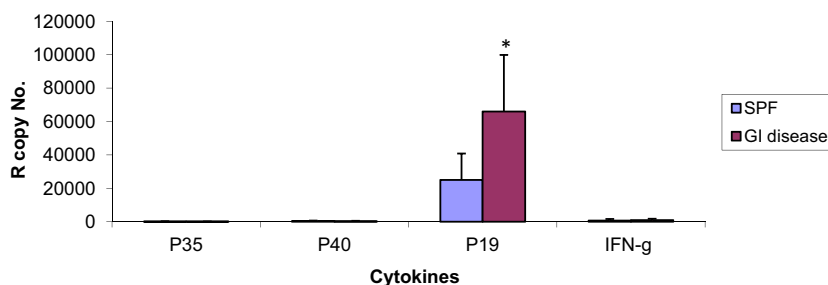

Fig 3. IL-23p19 mRNA expression levels in duodenal biopsies of healthy cats and cats with inflammatory enteropathy.

Table 3. Cytokine mRNA correlation in duodenal biopsies of control cats.

Control Group	P35	P40	P19	IFN-γ
P35	1	0.606[a]	0.617[a]	0.701[a]
P40		1	0.798[a]	0.806[a]
P19			1	0.854[a]
IFN-γ				1

The values presented are the Pearson correlation coefficient.
[a]Correlation is significant at the .01 level (2-tailed).

Table 4. Cytokine mRNA correlations in duodenal biopsies of cats with inflammatory enteropathy.

GI Disease Group	P35	P40	P19	IFN-γ
P35	1	0.796[b]	0.57[a]	0.654[a]
P40		1	0.776[b]	0.517
P19			1	0.581[a]
IFN-γ				1

The values presented are the Pearson correlation coefficient.
[a]Correlation is significant at the .05 level (2-tailed).
[b]Correlation is significant at the .01 level (2-tailed).

study, there was no interobserver variation because all biopsies were evaluated on 1 occasion by a single pathologist.

SPF-derived cats may differ from healthy pet cats, but ethical and legal constraints prevented sampling the intestinal mucosa of clinically normal pet animals. Therefore, comparisons with these controls should be made with caution. Dilemmas in the choice of a control group also apply to clinical studies of GI disease in humans and dogs where similar ethical constraints related to the availability of patient tissue arise.

MHC class II expression by epithelial cells was higher in cats with inflammatory enteropathy, although the increased level of expression was not significant. We have previously shown that MHC class II expression was significantly up-regulated in cats with IBD as compared to cats with GI disease or healthy controls.[4] In this study, the number of cases included was smaller than in the previous study. We were not able to subgroup the cases according to severity of histopathologic changes. This may explain the lack of significant difference despite the higher expression score obtained for the cats with inflammatory enteropathy.

IL-12p40 and IFN-γ gene expression was not significantly different between the 2 groups, whereas IL-12p35 mRNA was significantly lower in cats with inflammatory enteropathy. IL-12p40 mRNA was highly significantly correlated with p19 mRNA in cats with inflammatory enteropathy. Unlike in the previous report[6], IL-12p35 mRNA was significantly lower in cats with inflammatory enteropathy. IL-23p19 gene expression was significantly higher in cats with inflammatory enteropathy. Significant variation in the level of expression of mRNA encoding IL-23p19 after the introduction of novel diets to clinically healthy cats has been observed (N.E. Waly et al, unpublished data). Taken

together, these results support the hypothesis that IL-23 is implicated in the immunopathogenesis of feline inflammatory enteropathy. The significant positive correlation between IFN-γ, IL-23p19, and IL-12p35 suggest that all of these cytokines may still have a role in the pathogenesis of GI inflammation in cats.

The 2 major forms of IBD in humans are Crohn's disease (CD) and ulcerative colitis (UC). CD has been associated with a Th1-dominated cytokine profile, whereas a Th2 cytokine response has been linked to UC.[14] IL-23 is now known to play an important role in the pathogenesis of CD in humans.[14,15] An IL-12-driven Th1 response may dominate during the early stages of CD, implying that susceptibility to IL-12-mediated modulation is strongly dependent on the stage of the disease,[16] although activities that have been attributed to IL-12 in the past may have been mediated by IL-23.[17] Several studies have now demonstrated an important role for Th17 cells in intestinal inflammation.[18,19]

GI inflammation in cats also has a complicated immunopathogenesis which involves both proinflammatory and immunoregulatory cytokines.[6,7] The results of this study add to this complexity by showing for the first time that there is significant up-regulation of IL-23 gene expression in the duodenal mucosa of cats with inflammatory enteropathy. This suggests that in feline chronic inflammatory enteropathy, a Th17-type response also may occur.

Footnotes

[a] SPSS 14.0 for Windows; SPSS Inc 2005, Chicago, IL
[b] Experion; Bio-Rad Laboratories, Hemel Hempstead, UK
[c] University of Dundee, Dundee, UK
[d] Eurogentec Ltd, Southampton, UK
[e] Qiagen Ltd, Manchester, UK
[f] iCycler; Bio-Rad Laboratories

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