Cytokine mRNA abundance in intestinal biopsies from dogs with chronic diarrhea

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ABSTRACT: The differentiation between inflammatory bowel disease (IBD) and food responsive diarrhea (FRD) is difficult and no objective markers are available. We postulated that patterns of selected key cytokines would help to objectively differentiate between the two subcategories of chronic enteropathies in dogs. We studied mRNA patterns of selected cytokines in dogs with chronic enteropathies. Ten dogs with FRD (= group FRDbef) and seven dogs with IBD (= group IBDbef) were presented for endoscopy at the Small Animal Clinic, University of Bern. A control endoscopy was performed in both groups after treatment with an elimination diet for four weeks (FRDaft) or with an elimination diet combined with prednisolone for 10 weeks (IBDaft). Intestinal control samples of gastrointestinally healthy dogs from an independent study were additionally available. Dogs were clinically examined and scored using the canine IBD activity index (CIBDAI). mRNA abundance of interleukin (IL)-5, -10, -12p40, and -13, tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β1, and interferon (IFN)-γ were analyzed in intestinal samples by reverse transcription and real time polymerase chain reaction. Median CIBDAI decreased in FRDaft ($P < 0.01$) and IBDaft ($P = 0.07$) during treatment. In duodenum, IL-12p40 mRNA levels tended to be lower in FRDbef than in IBDbef ($P = 0.07$). The abundance of TNF-α mRNA was higher in IBDbef than in control dogs ($P < 0.05$). IL-5 mRNA levels decreased in FRD dogs during treatment ($P = 0.06$), and IL-10 mRNA levels decreased in IBD dogs ($P < 0.05$). In colon, IL-5 and IL-12p40 mRNA levels were lower in FRDbef than in IBDbef ($P < 0.05$) and control dogs ($P < 0.01$). IL-13 mRNA abundance was lower in FRDbef than in control dogs ($P < 0.05$) and IFN-γ mRNA abundance was lower in FRD and IBD dogs than in control dogs ($P < 0.01$). Feeding the elimination diet additionally reduced IFN-γ mRNA levels ($P < 0.01$), but increased TNF-α mRNA levels ($P < 0.05$) in FRD dogs. In conclusion, mRNA levels of the selected cytokines before treatment did not show clear differences between FRD and IBD dogs.

Keywords: chronic enteropathy; duodenum; colon; cytokines; canine

Chronic enteropathies (CE) in dogs are characterized by changed attitude and appetite, increased defecation frequency, reduced feces consistency, and vomitus (Jergens et al., 2003). The small and (or) large intestine can both be affected. Histopathological analyses of intestinal samples report increased infiltration of the *lamina propria* of the intestinal mucosa with eosinophils, lymphocytes, plasma cells and (or) neutrophils (German et al., 2001; Craven et al., 2004). However, histopathological reports are variable and do not necessarily correlate with the clinical presentation of the animals (Willard et al., 2002). Based on the combined results of clinical examination, histopathology, responsiveness to treatment and contingent relapses, two main differential diagnoses of CE – food responsive diarrhea (FRD) or inflammatory bowel disease (IBD) – can potentially be distinguished (Willard et al., 2002). However, difficulties in diagnosing FRD or IBD remain and the usefulness for follow-up and prognosis in dogs suffering from CE is questionable. Examination of nuclear receptors and nuclear receptor target genes already revealed differences in the mRNA levels of the different factors between IBD dogs, FRD dogs and healthy control dogs (Greger et al., 2006). Furthermore it has been shown in mice and in humans that nuclear receptors are involved in the development of IBD (Panwala et al., 1998; Langmann et al., 2004; Rousseaux et al., 2005). Differences in

cytokine mRNA patterns might be helpful to further differentiate these two disease entities and for follow-up examinations and prognosis.

Cytokines are mediators of inflammatory processes and play an important role in the development of CE as demonstrated in several animal models, including transgenic or knockout mice (Pizarro et al., 2003), and humans (Desreumaux et al., 1997; Melgar et al., 2003).

In dogs suffering from CE, cytokine profiles are variable (Cave, 2003). German and others (German et al., 2000) evaluated the mRNA expression pattern of selected cytokines in duodenal samples of German shepherd dogs with small intestinal enteropathies. Levels of interferon-γ (IFN-γ), interleukin-2 (IL-2), IL-12p40, IL-5, tumor-nekrosis factor-α (TNF-α), and transforming growth factor-β (TGF-β) were significantly increased in these dogs when compared with healthy control dogs. Ridyard et al. (2002) examined the mRNA pattern in colonic samples in a mixed population of dogs suffering from lymphoplasmacytic colitis by means of semiquantitative polymerase chain reaction (PCR). They found significantly increased mRNA levels of IL-2 and TNF-α in these dogs when compared with healthy control dogs. Jergens and others (Jergens et al., 2003) investigated the mRNA pattern in IBD dogs suffering from small intestinal symptoms or from large intestinal symptoms. In dogs with small intestinal IBD, mRNA levels of IL-1α, IL-1β, IL-2, IL-10, TNF-α, and IFN-γ were decreased when compared with healthy dogs, whereas IL-12 mRNA levels were increased. In dogs with large intestinal IBD, mRNA levels of IL-2 and TGF-β were decreased, whereas IL-4 levels were increased when compared with healthy dogs. Recently, Peters et al. (2005a, b) studied cytokine mRNA abundance in the healthy intestine of dogs using real-time reverse transcription (RT) – PCR and in diseased dogs. They failed to detect significant differences in the cytokine mRNA pattern in dogs suffering from chronic enteropathies when compared with gastrointestinally healthy dogs. Yet, to our knowledge, there are no data available using real-time PCR on cytokine mRNA patterns in duodenal and colonic samples before and after treatment in a mixed population of dogs affected by FRD or IBD.

Based on these premises we postulated that differences in intestinal cytokine mRNA patterns could help to differentiate further and in an objective way between FRD and IBD. Samples of gastrointestinally healthy control dogs were included for comparisons. Furthermore, we postulated that changes in cytokine mRNA levels during treatment could be helpful for follow-up examinations and prognosis in dogs suffering from FRD or IBD. Therefore, we investigated the mRNA abundance of IL-5, IL-10, IL-12p40, IL-13, IFN-γ, TNF-α, and TGF-β1 in duodenal and colonic tissue samples of a mixed population of dogs suffering from CE before and after treatment.

MATERIAL AND METHODS

Animals and experimental procedures. All experimental procedures were approved by the Committee overseeing Animal Experimentation in the canton of Bern (Number 72/02) and by the Ethical Committee of the Veterinary Faculty, University of Bern (2002).

In the frame of a study on CE in dogs, a mixed population of dogs (n = 17) was referred by private veterinarians to the Small Animal Clinic at the Veterinary Faculty in Bern for diagnostic gastro-, duodeno-, and colonoscopy. Details on recruitment procedure have been described elsewhere (Allenspach et al., 2004; Sauter et al., 2005, 2006; Spichiger et al., 2005). Animals were clinically examined and scored using the Canine Inflammatory Bowel Disease Activity Index (CIBDAI) (Jergens et al., 2003b) to define the severity of disease. A scoring from 0 to 3 indicates clinically insignificant disease, from 4 to 5 a mild degree of clinical symptoms, from 6–8 a moderate degree of clinical symptoms, and above 9 the scoring indicates a severe degree of clinical symptoms. Results of clinical examination, blood analyses, histopathological analyses of samples obtained during first endoscopy, and responsiveness to treatment with an elimination diet for one week (as described below) were used to stratify dogs into the groups FRDbef and IBDbef.

The FRD group consisted of seven males and three females. The mean age was 28 ± 4 months (range 7 to 42 months). Dogs were of the following breeds (n = 1 each): German Shepherd, Labrador Retriever, Bernese Mountain dog, Leonberger, Great Dane, Landseer, Border Collie, Border Terrier, Shi Tzu, and mixed breed. The dogs had suffered for 8 ± 2 months from gastrointestinal symptoms. Symptoms included reduced appetite (1/10), intermittent vomitus (5/10), diarrhea (10/10), increased frequency of defecation (6/10), haematochezia (5/10), excess fecal mucus (4/10), tenesmus (3/10), and weight loss (5/10).

The IBD group consisted of four males and three females: The mean age was 62 ± 15 months (range 6 to 128 months). Dogs were of the following breeds (n = 1 each): German Shepherd, Boxer, Dachshound, Rottweiler, Shar Pei, Mastiff, and mixed breed. They had suffered for 25 ± 11 months from gastrointestinal symptoms. Symptoms included reduced appetite (4/7), intermittent vomitus (3/7), diarrhea (7/7), increased defecation frequency (6/7), haematochezia (1/7), excess fecal mucus (2/7), tenesmus (2/7), and weight loss (6/7).

The (control) group consisted of four males and seven females. The mean age was 32 ± 7 months (range 13 to 82 months). They were all Beagles. They showed no gastrointestinal diseases.

Owners agreed by written consent to perform an endoscopy and to present their dogs for a control endoscopy. Dogs were hospitalized and fasted for 72 hours. One day prior to the endoscopy, they were given an oral electrolyte solution produced by a local pharmacy (Sauter et al., 2006; Spichiger et al., 2006). All dogs were sent home and were exclusively fed an elimination diet based on novel protein sources (salmon and trout), canola meal and rice [Purina Canine LA® (Limited Antigen) Diet, St. Louis, MO].

Dogs with FRD showed a significant improvement of clinical signs already after one week of treatment with the elimination diet and they were presented for control endoscopy after four weeks (FRDaft). Dogs with IBD did not show sufficient improvement of clinical signs after one week of elimination diet. They needed additional oral prednisolone treatment [1 mg/kg body weight (BW) for 10 d twice daily, then 0.5 mg/kg BW for 10 days twice daily, then 0.5 mg/kg BW for 10 days once daily, then 0.5 mg/kg BW for 10 days every other day] besides above mentioned elimination diet. They were presented for control endoscopy after 10 weeks (IBDaft).

Tissue sampling, histopathology, laboratory analyses. Details on tissue sampling in diseased and healthy dogs have been described in earlier studies (Sauter et al., 2005, 2006; Spichiger et al., 2005). Treatment and storage of samples until analysis were the same as described before (Sauter et al., 2005, 2006; Spichiger et al., 2005). Extraction of total RNA, reverse transcription as well as real-time PCR reaction were described in detail in previously (Sauter et al., 2005, 2006).

Primers and real-time polymerase chain reaction (PCR). Sequences of canine β-actin, glyceraldehyde phosphate-dehydrogenase (GAPDH), IL-5, IL-10, IL-12p40, IL-13, IFN-γ, TGF-β1, and TNF-α were available on NCBI gene bank (www.ncbi.nlm.nih.gov/Genbank/GenbankSearch.html). Details on primers have also published published earlier (Sauter et al., 2005, 2006).

Statistical analyses. Data were analyzed using NCSS 2001 software (Kaysville, UT, USA). Data on age and duration of symptoms were expressed as means ± SEM with the corresponding range. Data on CIBDAI scoring were expressed as median with corresponding range. Data of PCR results $(2^{(-\Delta\Delta CP)}) \times 100$ were expressed as median with the 25^{th} and 75^{th} percentile.

Normality of distribution of all measured parameters was tested using NCSS 2001. The level of significance was set at $P < 0.05$, and the level of trend at $P < 0.1$. All tested effects on cytokine patterns were analyzed separately within duodenal and colonic samples. In order to achieve normality in the respective distributions, mRNA values for IL-5, IL-12p40, IL-13, IFN-γ, TNF-α, IL-10, and TGF-β1 were $\log_{10}$ transformed, and group comparisons were performed on the log-transformed data.

Differences in mRNA levels of cytokines among control dogs, FRDbef and IBDbef were localized by one-way analysis of variance (ANOVA). Results were corrected by Bonferroni for multiple comparisons. Effects of sex were tested separately in the different groups by using the Two Sample Test. Differences between FRDbef and FRDaft, and between IBDbef and IBDaft in cytokine values were evaluated by paired t-test. Differences in CIBDAI scoring before and after treatment were analyzed by Wilcoxon Signed Rank Test.

The sum of values of log cytokine values of duodenum and colon were correlated with CIBDAI scores and results were expressed as Spearman correlation coefficients. Cytokine values of control dogs, having a CIBDAI score of 0, were included into the calculation.

RESULTS

CIBDAI, blood parameters and histopathology. Median CIBDAI score in FRDbef dogs was six (range 2 to 8) and decreased during treatment to score one (range 0 to 3) ($P < 0.001$). The median score was nine in IBDbef (range 6 to 9) and tended to decrease during treatment to score five (range 0 to 10) ($P = 0.07$). Results of blood analyses of control dogs, FRD, and IBD dogs were all within the range of healthy dogs and did not reveal group differences (data not shown).

Histopathology revealed no pathological changes in the control dogs. In groups FRD and IBD, there were no pathological changes or up to severe infiltration of the intestinal mucosa by eosinophils, lymphocytes and (or) plasma cells. Details are listed in Table 1.

Abundance of mRNA of cytokines in duodenal samples (Figures 1a–7a). The IL-12p40 mRNA abundance was lower in FRDbef than in IBDbef ($P = 0.07$). TNF-α mRNA levels were significantly higher in IBDbef than in control dogs ($P < 0.05$). IL-5 mRNA levels decreased in FRD dogs during treatment ($P = 0.06$) and IL-10 mRNA expression levels significantly decreased in IBD dogs during treatment with the elimination diet and prednisolone ($P < 0.05$). There were no significant group or treatment effects on IL-13, IFN-γ and TGF-β1 mRNA levels in duodenum, but gender influenced IL-13 and IFN-γ mRNA levels in FRD and IBD dogs: mRNA expression levels were

Table 1. Histopathological diagnoses of duodenal and colonic samples before and after treatment of dogs suffering from food-responsive diarrhea (FRD) or inflammatory bowel disease (IBD)

Group	Histopathological diagnoses of first endoscopy		Histopathological diagnoses of second endoscopy	
	duodenum	colon	duodenum	colon
FRD	moderate plasmacytic	NPC	moderate plasmacytic	mild lymphoplasmacytic
	moderate lymphoplasmacytic	severe lymphocytic	moderate lymphoplasmacytic	mild neutrophilic
	mild lymphoplasmacytic and eosinophilic	mild lymphoplasmacytic and eosinophilic	moderate eosinophilic	moderate eosinophilic
	moderate eosinophilic and plasmacytic	mild plasmacytic	moderate plasmacytic	NPC
	mild lymphoplasmacytic and eosinophilic	NPC	mild lymphoplasmacytic	NPC
	mild eosinophilic	moderate lymphoplasmacytic and eosinophilic	NPC	NPC
	mild eosinophilic	mild eosinophilic	mild eosinophilic	mild neutrophilic
	mild eosinophilic	NPC	moderate lymphoplasmacytic	NPC
	moderate lymphoplasmacytic	NPC	NPC	NPC
	NPC	NPC	mild lymphoplasmacytic	NPC
IBD	mild lymphoplasmacytic and eosinophilic	NPC	moderate to severe lymphoplasmacytic and eosinophilic	moderate to severe lymphoplasmacytic and eosinophilic
	moderate eosinophilic	mild to moderate lymphoplasmacytic	moderate eosinophilic	moderate eosinophilic
	mild eosinophilic	mild to moderate eosinophilic and lymphoplasmacytic	moderate lymphoplasmacytic	NPC
	moderate plasmacytic	NPC	mild to moderate lymphoplasmacytic	NPC
	moderate eosinophilic and lymphoplasmacytic	moderate to severe eosinophilic and lymphoplasmacytic	moderate eosinophilic	moderate eosinophilic
	moderate eosinophilic, lymphangiectasia	NPC	NPC	mild neutrophilic and eosinophilic
	NPC	NPC	moderate lymphoplasmacytic	moderate lymphoplasmacytic

NPC = no pathological changes

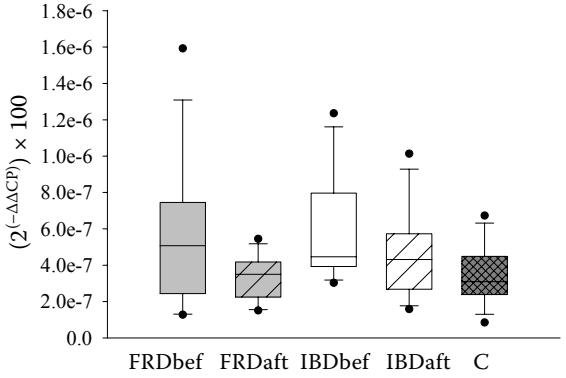

Figure 1. (**a**) IL-5 mRNA abundance in duodenal biopsies of FRDbef (dogs with food responsive diarrhea before treatment), FRDaft (dogs with food responsive diarrhea after treatment), IBDbef (dogs with inflammatory bowel disease before treatment), IBDaft (dogs with inflammatory bowel disease after treatment), and of C (gastrointestinally healthy dogs). Non $\log_{10}$ transformed data were used for generation of the graphs. Data are presented as median with 25th and 75th quartile in each box plot. The whiskers indicate 1.5 times the length of the quartiles. Points outside this range indicate outliers

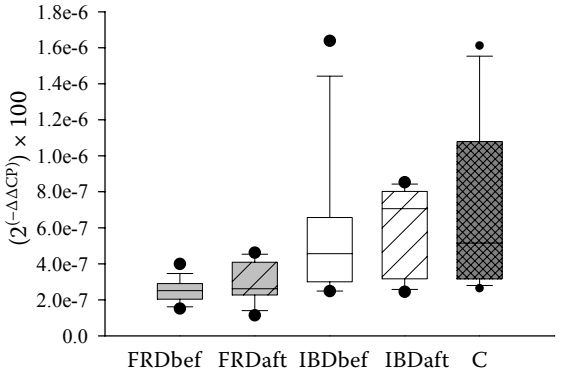

Figure 1. (**b**) IL-5 mRNA abundance in colonic biopsies of FRDbef (dogs with food responsive diarrhea before treatment), FRDaft (dogs with food responsive diarrhea after treatment), IBDbef (dogs with inflammatory bowel disease before treatment), IBDaft (dogs with inflammatory bowel disease after treatment), and of C (gastrointestinally healthy dogs). Non $\log_{10}$ transformed data were used for generation of the graphs. Data are presented as median with 25th and 75th quartile in each box plot. The whiskers indicate 1.5 times the length of the quartiles. Points outside this range indicate outliers. IL-5 mRNA levels were lower in FRDbef than in IBDbef and C ($P < 0.05$)

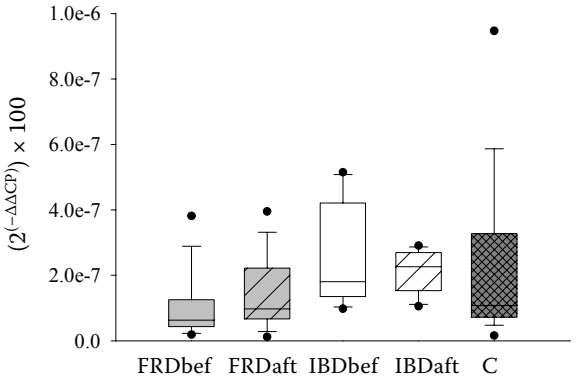

Figure 2. (**a**) IL-12p40 mRNA abundance in duodenal biopsies of FRDbef (dogs with food responsive diarrhea before treatment), FRDaft (dogs with food responsive diarrhea after treatment), IBDbef (dogs with inflammatory bowel disease before treatment), IBDaft (dogs with inflammatory bowel disease after treatment), and of C (gastrointestinally healthy dogs). Non $\log_{10}$ transformed data were used for generation of the graphs. Data are presented as median with 25th and 75th quartile in each box plot. The whiskers indicate 1.5 times the length of the quartiles. Points outside this range indicate outliers

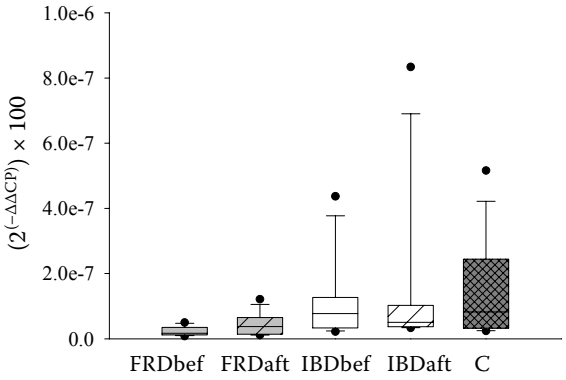

Figure 2. (**b**) IL-12p40 mRNA abundance in colonic biopsies of FRDbef (dogs with food responsive diarrhea before treatment), FRDaft (dogs with food responsive diarrhea after treatment), IBDbef (dogs with inflammatory bowel disease before treatment), IBDaft (dogs with inflammatory bowel disease after treatment), and of C (gastrointestinally healthy dogs). Non $\log_{10}$ transformed data were used for generation of the graphs. Data are presented as median with 25th and 75th quartile in each box plot. The whiskers indicate 1.5 times the length of the quartiles. Points outside this range indicate outliers. IL-12p40 mRNA levels were lower in FRDbef than in IBDbef and C ($P < 0.05$)

lower in female than in male dogs for IFN-γ mRNA levels in FRDbef ($P < 0.05$) and for IL-13 mRNA levels in IBDbef ($P = 0.07$).

Abundance of mRNA of cytokines in colonic samples (Figures 1b–7b). The mRNA levels of IL-5 and IL-12p40 mRNA were significantly lower in

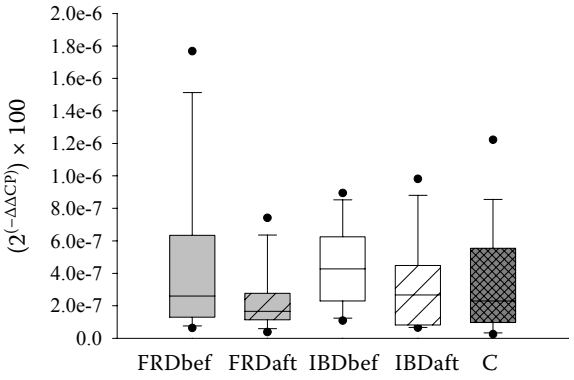

Figure 3. (**a**) IL-13 mRNA abundance in duodenal biopsies of FRDbef (dogs with food responsive diarrhea before treatment), FRDaft (dogs with food responsive diarrhea after treatment), IBDbef (dogs with inflammatory bowel disease before treatment), IBDaft (dogs with inflammatory bowel disease after treatment), and of C (gastrointestinally healthy dogs). Non $\log_{10}$ transformed data were used for generation of the graphs. Data are presented as median with 25th and 75th quartile in each box plot. The whiskers indicate 1.5 times the length of the quartiles. Points outside this range indicate outliers

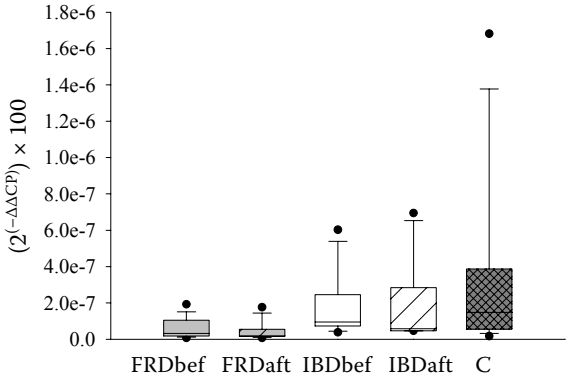

Figure 3. (**b**) IL-13 mRNA abundance in colonic biopsies of FRDbef (dogs with food responsive diarrhea before treatment), FRDaft (dogs with food responsive diarrhea after treatment), IBDbef (dogs with inflammatory bowel disease before treatment), IBDaft (dogs with inflammatory bowel disease after treatment), and of C (gastrointestinally healthy dogs). Non $\log_{10}$ transformed data were used for generation of the graphs. Data are presented as median with 25th and 75th quartile in each box plot. The whiskers indicate 1.5 times the length of the quartiles. Points outside this range indicate outliers. IL-13 mRNA levels were lower in FRDbef than in C ($P < 0.05$)

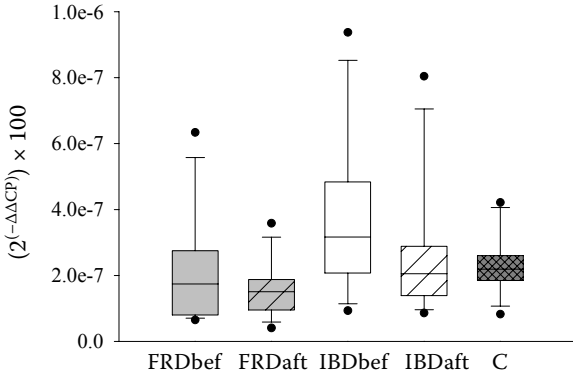

Figure 4. (**a**) IFN-γ mRNA abundance in duodenal biopsies of FRDbef (dogs with food responsive diarrhea before treatment), FRDaft (dogs with food responsive diarrhea after treatment), IBDbef (dogs with inflammatory bowel disease before treatment), IBDaft (dogs with inflammatory bowel disease after treatment), and of C (gastrointestinally healthy dogs). Non $\log_{10}$ transformed data were used for generation of the graphs. Data are presented as median with 25th and 75th quartile in each box plot. The whiskers indicate 1.5 times the length of the quartiles. Points outside this range indicate outliers

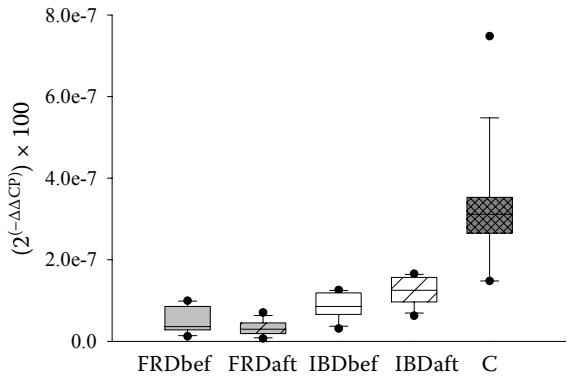

Figure 4. (**b**) IFN-γ mRNA abundance in colonic biopsies of FRDbef (dogs with food responsive diarrhea before treatment), FRDaft (dogs with food responsive diarrhea after treatment), IBDbef (dogs with inflammatory bowel disease before treatment), IBDaft (dogs with inflammatory bowel disease after treatment), and of C (gastrointestinally healthy dogs). Non $\log_{10}$ transformed data were used for generation of the graphs. Data are presented as median with 25th and 75th quartile in each box plot. The whiskers indicate 1.5 times the length of the quartiles. Points outside this range indicate outliers. IFN-γ mRNA levels were lower in FRDbef and IBDbef than in C ($P < 0.01$). Levels decreased from FRDbef to FRDaft ($P < 0.05$)

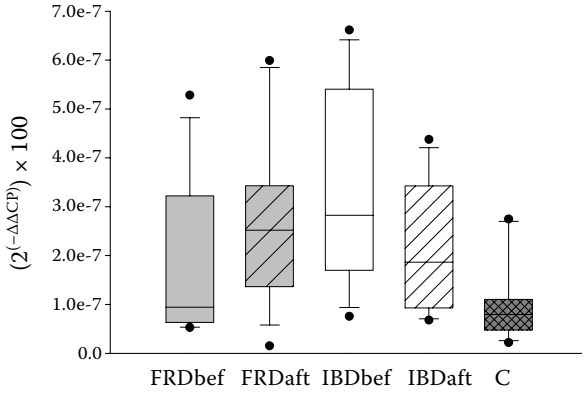

Figure 5. (**a**) TNF-α mRNA abundance in duodenal biopsies of FRDbef (dogs with food responsive diarrhea before treatment), FRDaft (dogs with food responsive diarrhea after treatment), IBDbef (dogs with inflammatory bowel disease before treatment), IBDaft (dogs with inflammatory bowel disease after treatment), and of C (gastrointestinally healthy dogs). Non $\log_{10}$ transformed data were used for generation of the graphs. Data are presented as median with 25th and 75th quartile in each box plot. The whiskers indicate 1.5 times the length of the quartiles. Points outside this range indicate outliers. TNF-α mRNA levels were higher in IBDbef than in C ($P < 0.05$)

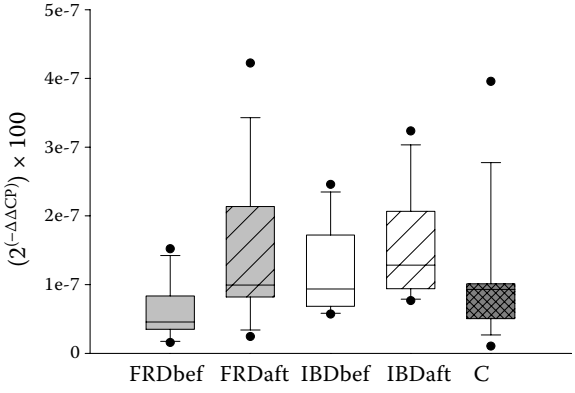

Figure 5. (**b**) TNF-α mRNA abundance in colonic biopsies of FRDbef (dogs with food responsive diarrhea before treatment), FRDaft (dogs with food responsive diarrhea after treatment), IBDbef (dogs with inflammatory bowel disease before treatment), IBDaft (dogs with inflammatory bowel disease after treatment), and of C (gastrointestinally healthy dogs). Non $\log_{10}$ transformed data were used for generation of the graphs. Data are presented as median with 25th and 75th quartile in each box plot. The whiskers indicate 1.5 times the length of the quartiles. Points outside this range indicate outliers. Levels increased from FRDbef to FRDaft ($P < 0.05$)

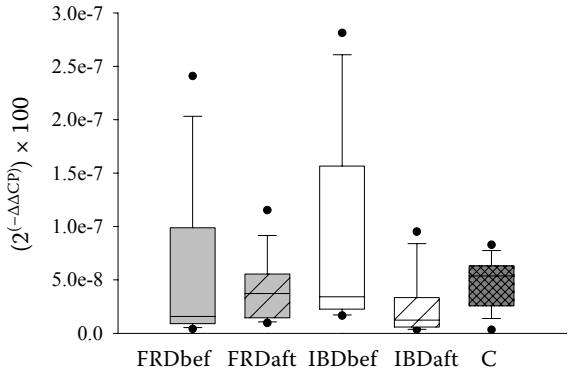

Figure 6. (**a**) IL-10 mRNA abundance in duodenal biopsies of FRDbef (dogs with food responsive diarrhea before treatment), FRDaft (dogs with food responsive diarrhea after treatment), IBDbef (dogs with inflammatory bowel disease before treatment), IBDaft (dogs with inflammatory bowel disease after treatment), and of C (gastrointestinally healthy dogs). Non $\log_{10}$ transformed data were used for generation of the graphs. Data are presented as median with 25th and 75th quartile in each box plot. The whiskers indicate 1.5 times the length of the quartiles. Points outside this range indicate outliers. IL-10 mRNA levels were lower IBDaft than in IBDbef ($P < 0.05$)

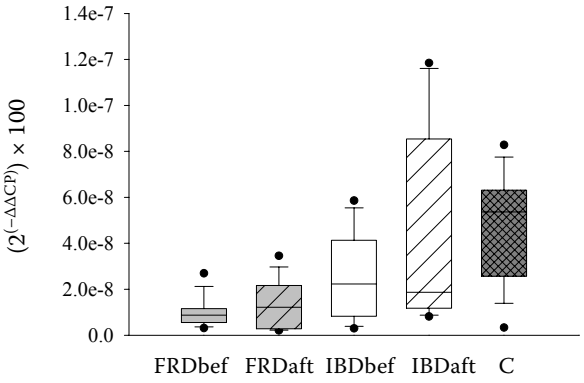

Figure 6. (**b**) IL-10 mRNA abundance in colonic biopsies of FRDbef (dogs with food responsive diarrhea before treatment), FRDaft (dogs with food responsive diarrhea after treatment), IBDbef (dogs with inflammatory bowel disease before treatment), IBDaft (dogs with inflammatory bowel disease after treatment), and of C (gastrointestinally healthy dogs). Non $\log_{10}$ transformed data were used for generation of the graphs. Data are presented as median with 25th and 75th quartile in each box plot. The whiskers indicate 1.5 times the length of the quartiles. Points outside this range indicate outliers

FRDbef than in IBDbef ($P < 0.05$) and in control dogs ($P < 0.01$). The mRNA levels of IL-13 were significantly lower in FRDbef than in control dogs ($P < 0.05$). The IFN-γ mRNA levels were significantly decreased in FRDbef and IBDbef when compared with control dogs ($P < 0.001$). Treatment with

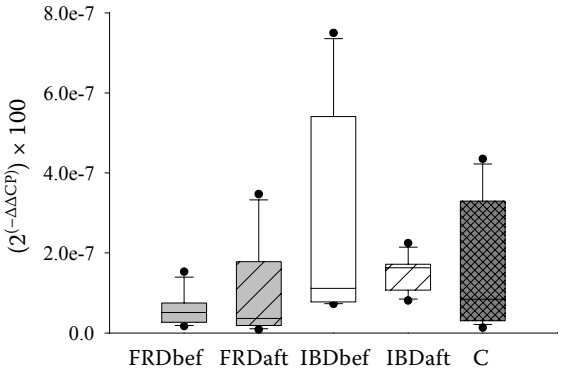

Figure 7. (**a**) TGF-β1 mRNA abundance in duodenal biopsies FRDbef (dogs with food responsive diarrhea before treatment), FRDaft (dogs with food responsive diarrhea after treatment), IBDbef (dogs with inflammatory bowel disease before treatment), IBDaft (dogs with inflammatory bowel disease after treatment), and of C (gastrointestinally healthy dogs). Non $\log_{10}$ transformed data were used for generation of the graphs. Data are presented as median with 25th and 75th quartile in each box plot. The whiskers indicate 1.5 times the length of the quartiles. Points outside this range indicate outliers

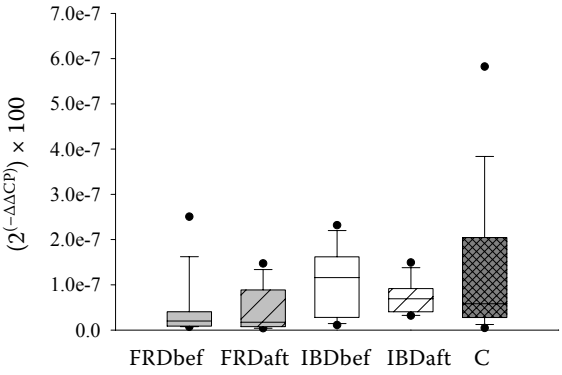

Figure 7. (**b**) TGF-β1 mRNA abundance in colonic biopsies FRDbef (dogs with food responsive diarrhea before treatment), FRDaft (dogs with food responsive diarrhea after treatment), IBDbef (dogs with inflammatory bowel disease before treatment), IBDaft (dogs with inflammatory bowel disease after treatment), and of C (gastrointestinally healthy dogs). Non $\log_{10}$ transformed data were used for generation of the graphs. Data are presented as median with 25th and 75th quartile in each box plot. The whiskers indicate 1.5 times the length of the quartiles. Points outside this range indicate outliers

the elimination diet further decreased IFN-γ mRNA levels ($P < 0.01$) in FRD dogs, whereas abundance of TNF-α mRNA significantly increased ($P < 0.05$). There were no significant effects on IL-10 and TGF-β1 mRNA levels in colon.

Correlation CIBDAI scores and cytokine values. CIBDAI scores only correlated significantly with mean values of TNF-α before treatment r_{sp} = 0.86, $R^2 = 0.56$, $P < 0.05$).

DISCUSSION

Health status and histopathology. The CIBDAI score was higher in IBD dogs than in FRD dogs. This reflected the more severe degree of disease in IBD than FRD dogs. Clinical symptoms greatly improved in FRD dogs after the treatment with an alternative protein source such as fish as indicated by the strongly reduced CIBDAI scoring. As reviewed by Allenspach and Gaschen (2003), feeding an alternative protein source can be sufficient to treat FRD. In IBD dogs, there was also an improvement of clinical signs, but the median CIBDAI score still indicated a mild degree of gastrointestinal symptoms, in accordance with other studies Jergens et al. (2003b). These dogs needed prednisolone treatment in addition to dietary treatment. Values of blood analyses were all within the range of healthy animals (Kraft and Dürr, 1999).

Histopathological results were very variable. There were no associations between CIBDAI scores and histopathological reports except in two animals of the IBD group: the CIBDAI scoring indicated a moderate degree of gastrointestinal signs, whereas histopathology reported moderate to severe infiltration of the gastrointestinal mucosa with immune competent cells. In contrast to studies in cats with IBD (Goldstein et al., 2003), no architectural changes of the GIT structure were reported in the dogs that were included into the study.

Cytokines. There was a great individual variability in mRNA expression levels of the different cytokines in dogs with FRD, IBD and even in control dogs. Differences in age, growth, sex and (or) nutritional status probably also accounted for the variability in cytokine mRNA profiles, in agreement with studies in humans (Moxley et al., 2002; Grimble, 2003; Huang et al., 2005). However, there were significant gender influences on INF-γ mRNA levels of FRD dogs and on IL-13 mRNA levels in IBD dogs in duodenal samples. Because there were no gender effects on cytokine levels in the control group, gender seems to affect the expression of these cytokines in sick dogs. The variations in number and type of immune-competent cells that infiltrated the

intestinal mucosa were most likely responsible at least for some of the individual variability in cytokine expression patterns. Because we have used a pool of total extracted RNA from about 10 biopsy specimens, which were randomly collected in both localizations, the cytokine pattern was likely also representative for much of the changes within the intestinal mucosa, at least as it concerns the upper part of the small intestine and the entire colon of each individual dog. Effects of fasting on cytokine expression patterns can be excluded because all dogs (even control dogs) underwent a fasting period of 24 h to 72 h. Effects of glucocorticoids on cytokine levels in IBD dogs could also be neglected because treatment was stopped 2–4 weeks prior to the first and second endoscopy. It can be expected that effects of prednisolone were abolished by then. However, because mRNA expression levels of cytokines can change within hours, as demonstrated in challenge studies of humans with allergies (Ferreira, 2003), it cannot be excluded that at the time point of the second endoscopy possible changes in cytokine expression were missed. This could be an additional reason for failing treatment effects. Furthermore, differences in study populations, treatment procedures, in the clinical presentation of the dogs, and in methodologies for the determination of mRNA levels of selected cytokines between our study and earlier studies (German et al., 2000; Fujiwara et al., 2002; Ridyard et al., 2002; Jergens et al., 2003a; Peters et al., 2005a) possibly contributed to different results of cytokine patterns.

The IL-5 stimulates and activates eosinophils (Lopez et al., 1988). Eosinophilic accumulation is a common feature of gastrointestinal disorders in human medicine (Rothenberg, 2004) and some categories of canine IBD (Allenspach and Gaschen, 2003), although eosinophilic infiltration can also be found in the normal gastrointestinal tract (Kato et al., 1998). Eosinophils have pleiotropic functions such as the release of preformed secondary granula, cytokines, chemokines, neuropeptides or lipid mediators. Furthermore, they can be involved in antigen presentations to T-cells (Rothenberg, 2004).

Decreased IL-5 levels in duodenal samples of FRD dogs after treatment may indicate a reduced activation of eosinophils in the intestinal layer, probably going along with a reduced degree of inflammation after feeding the elimination diet as indicated by the reduced CIBDAI scores. Similarly, IL-5 mRNA levels decreased during treatment in a study of German et al. (2000). On the other hand, reduced colonic IL-5 mRNA levels in FRD dogs when compared with IBD and control dogs were surprising.

The IL-12 is a pro-inflammatory cytokine, which is characteristic for Th1 immune responses and is mainly produced by activated macrophages and dendritic cells (Abbas et al., 1996). It is a heterodimeric protein compromised of a p35 and a p40 subunit, of which the p40 subunit seems to be the more important one (Holscher, 2004). Reduced IL-12p40 mRNA levels in duodenum and colon of FRDbef dogs when compared to IBDbef dogs may (1) indicate a tendency towards a Th2 immune response in FRD dogs and (or) (2) reflect a lesser degree of gastrointestinal inflammation in FRD dogs than in IBD dogs as mirrored by the reduced CIBDAI scoring. However, we failed to find a significant association between the CIBDAI score and IL-12p40 mRNA levels. In experimental models of human IBD, Simpson et al. (1998) demonstrated that the disease could develop without enhanced IL-12 expression. In contrast to these findings, earlier studies found increased IL-12p40 mRNA levels in dogs with IBD as compared with healthy dogs (German et al., 2000; Ridyard et al., 2002; Jergens et al., 2003a) and in cats with IBD (Goldstein et al., 2003). Differences in age, degree and duration of disease, and differences in methodology probably are responsible for diverging results.

Th2-cells mainly produce IL-13, a known inhibitor of the production of pro-inflammatory cytokines (Wynn, 2003). Reduced colonic mRNA levels of IL-13 in FRD dogs when compared with control dogs are possibly related to reduced IL-5 mRNA levels. Results suggest an increased responsiveness of the duodenum to allergens and concomitantly a subordinate role of the colon in FRD dogs before treatment with the elimination diet. However, comparative data on mRNA levels of IL-5 and IL-13 in colonic samples of dogs with IBD or FRD are missing. In accordance with our data, Vainer et al. (2000) found diminished IL-13 abundance at the protein and mRNA level in human IBD patients when compared to healthy individuals.

The IFN-γ is a pro-inflammatory cytokine that is predominantly produced by Th1 cells and natural killer cells upon activation by IL-12 (Abbas et al., 1996). Significantly reduced IFN-γ mRNA levels in colonic samples in FRD and IBD dogs when compared with control dogs were surprising. These findings were in contrast to studies of German et al. (2000), who found increased IFN-γ mRNA levels in diseased dogs when compared to their control dogs. They also differed

from Ridyard et al. (2002) and Peters et al. (2005b) who found no significant effects. However, in line with our results, Fujiwara et al. (2002) and Jergens et al. (2003) found significantly decreased IFN-γ levels in IBD dogs when compared to healthy dogs. On the one hand, our results indicate a subordinate role of IFN-γ in disease development. As shown by Simpson et al. (1998), experimental colitis can develop even in the absence of enhanced IFN-γ production and this may also be the case in dogs. On the other hand, there might be an association between enhanced IFN-γ mRNA abundance in our control group and relatively high intestinal bacterial cell counts. Garden et al. (1999) found increased levels of IFN-γ mRNA, measured by *in situ* hybridization, in jejunal samples of Beagle dogs when compared with healthy or gluten-sensitive Irish setter dogs. Unfortunately, we were not able to sample duodenal juice of all dogs, especially of the control dogs, in order to evaluate bacterial cell numbers.

The TNF-α is a potent pro-inflammatory cytokine (Vassalli, 1992) and its role in human IBD has widely been demonstrated by successful anti-TNF-α treatments in human IBD patients (Sandborn, 2005). Increased TNF-α mRNA levels in duodenum in IBDbef dogs when compared with control dogs was in accordance with previous studies (German et al., 2000; Ridyard et al., 2002). TNF-α mRNA levels probably reflected the more severe degree of inflammation in these dogs as indicated by a significant correlation to the CIBDAI scores and elevated plasma haptoglobin levels (Spichiger et al., 2006). Increased TNF-α mRNA levels in FRD dogs after treatment are difficult to explain. Increased TNF-α mRNA levels might mirror an activated immune status in these dogs, which was needed to resolve the gastrointestinal inflammation as indicated by the significantly reduced CIBDAI scoring. On the other side, German et al. (2000) found significantly reduced TNF-α levels after treatment in his study population.

The IL-10 is a key regulatory cytokine and immunesuppressive. IL-10 is mainly produced by regulatory T-cells and helps to down-regulate the production of pro-inflammatory cytokines (Moore et al., 2001). The importance of IL-10 in controlling the intestinal inflammation was shown by IL-10 deficient mice which eventually developed enterocolitis (Kuhn et al., 1993) or by beneficial therapeutic effects of IL-10 in human IBD and in experimental colitis (Li and He, 2004; Lindsay et al., 2004). However, there were no significant differences in IL-10 mRNA abundance in duodenum and colon between FRD, IBD and control groups, in accordance with earlier studies (German et al., 2000; Ridyard et al., 2002). However, IL-10 mRNA levels in duodenum decreased in IBD dogs during treatment. It is worth mentioning that Melgar et al. (2003) found increased IL-10 levels in patients with active ulcerative colitis, which correlated with disease activity. Similarly, Goldstein et al. (2003) found increased levels of IL-10 mRNA in cats with IBD, which correlated with the clinical severity of the disease. One might speculate that a decrease of the immune-suppressive IL-10 mRNA levels is necessary in order to stimulate the gastrointestinal immune system.

In contrast to German et al. (2000), but in accordance with Ridyard et al. (2002), there was no up-regulation of TGF-β1 in FRD and IBD dogs when compared with healthy dogs. Earlier studies (Fujiwara et al., 2002; Jergens et al., 2003a) even found decreased TGF-β1 mRNA expression in diseased dogs when compared with healthy dogs. Nevertheless, recent studies showed that normal or even increased expression levels of TGF-β1 in human IBD patients do not necessarily correlate with the clinical status of patients and that TGF-β1 is not able to fully down-regulate the inflammatory processes in an inflamed intestine (Monteleone et al., 2004). Additionally it is known that TGF-β1 undergoes major post-translational modifications (Letterio and Roberts, 1998) and that mRNA abundance of TGF-β1 does not necessarily reflect the TGF-β protein levels.

In summary, due to the high variability in cytokine mRNA levels, differences in patterns between FRD and (or) IBD dogs of IL-5, IFN-γ or TNF-α mRNA levels were often difficult to explain. These results underline the complexity of the system and the difficulties to understand fully the pathogenesis and pathophysiology of FRD and IBD in dogs. In line with our initial hypothesis, TNF-α abundance may be a potential factor to differentiate between FRD and IBD.

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