Remission of histiocytic ulcerative colitis in Boxer dogs correlates with eradication of invasive intramucosal Escherichia coli

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REMISSION OF HISTIOCYTIC ULCERATIVE COLITIS IN BOXER DOGS CORRELATES WITH ERADICATION OF INVASIVE INTRAMUCOSAL *ESCHERICHIA COLI*

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Running head: *E coli* in Boxer colitis

Abstract

Background: Historically, histiocytic ulcerative (or granulomatous) colitis (HUC) of Boxer dogs was considered an idiopathic immune-mediated disease with a poor prognosis. Recent reports of dramatic responses to enrofloxacin and the discovery of invasive *E. coli* within the colonic mucosa of affected Boxer dogs support an infectious etiology.

Hypothesis: Invasive *E. coli* is associated with colonic inflammation in Boxer dogs with HUC, and eradication of intramucosal *E. coli* correlates with clinical and histologic remission.

Animals: 7 Boxer dogs with HUC.

Methods: Colonic biopsies were obtained at initial evaluation in 7 dogs, and in 5 dogs after treatment with enrofloxacin. Biopsies were evaluated by standardized histopathology, and fluorescence in situ hybridization (FISH) with probes to eubacteria and *E. coli*.

Results: Intramucosal *E. coli* was present in colonic biopsies of 7/7 Boxers with HUC. Clinical response was noted in 7/7 dogs within 2 wks of enrofloxacin given at a mean dose of 7 mg/kg/d for a mean duration of 9.5 weeks, and was sustained in 6 dogs (mean disease-free interval to date of 45 months). Post-enrofloxacin FISH was negative for *E. coli* in 4/5 dogs. *E. coli* resistant to enrofloxacin were present in the FISH positive dog that relapsed clinically.

Conclusions and clinical relevance: The correlation between clinical remission and the eradication of mucosally invasive *E. coli* during treatment with enrofloxacin supports the causal involvement of *E. coli* in the development of HUC in susceptible Boxer dogs. A poor response to enrofloxacin treatment may be due to colonization with enrofloxacin-resistant *E. coli.*

Key words: colitis, canine, enrofloxacin, Crohn’s disease, 16S rDNA, FISH, adherent and invasive *E. coli*

Granulomatous colitis (GC) of Boxer dogs was first described by Van Kruiningen in a kennel of Boxer dogs in 1965.[1] Some subsequent reports of this disease use the term Histiocytic Ulcerative Colitis (HUC),[2-5] and this term is more familiar to many veterinarians. The clinical hallmarks of the disease are severe large bowel diarrhea that is often accompanied by profound weight loss, anemia and hypoalbuminemia.[1-5] HUC, although rare, occurs world-wide with reported cases originating from Australia, Japan, North America and Europe.[1-6] Boxer dogs are most commonly affected although other breeds such as Mastiff, Alaskan Malamute, Doberman Pinscher, and French Bulldogs, are sporadically affected.[6-8]

The pathognomonic lesion of HUC in Boxer dogs is mucosal infiltration with large numbers of macrophages staining positively with period-acid-Schiff (PAS), and is usually accompanied by mucosal ulceration and loss of goblet cells.[1] However, both the distribution and severity of lesions within the colon are variable, and in some cases they are focally distributed, and macrophages can be scarce and ulceration absent.[9,10] Early reports describe a response of 6/9 dogs to chloramphenicol and the presence of intracellular bacteria within macrophages.[1,11] However, these findings were poorly reproducible in subsequent studies[9,10] and experimental attempts to reproduce HUC, by infecting Boxer dogs with *Mycoplasma spp.* isolated from colonic mucosa were unsuccessful.[12] In-depth pathological studies describe a loss of goblet cells and an increase in IgG_3 and IgG_4 plasma cells, CD_3-T cells, and L_1 (macrophages and polymorphoneulcear granulocytes) and MHCII positive cells, similar to ulcerative colitis in people.[13] Thus HUC has until recently been regarded as an idiopathic immune-mediated disease that typically responds poorly to treatment with empirical therapies such as dietary change, antibiotics, and immunosuppressive agents.[4,5]

The poor response of HUC to immunosuppression led to a re-appraisal of antibiotic therapy, and there are now three independent studies that describe dramatic improvements in clinical signs and histological lesions of Boxer dogs treated with antibiotic protocols containing enrofloxacin.[14-16] These observations along with the discovery of a bacterial cause for the intestinal infiltration of PAS positive macrophages in people with Whipple's disease[17] initiated the search for an infectious agent using contemporary culture-independent methodologies. Colonic biopsies from affected Boxer dogs (*n*=13) and controls (*n*=38) were examined by fluorescent in situ hybridization (FISH) with a eubacterial16S rDNA probe. Culture, 16S ribosomal DNA

sequencing, and histochemistry were used to guide subsequent FISH. Intramucosal Gram-negative coccobacilli were present in 100% of HUC samples from Boxer dogs but not controls, and invasive bacteria hybridized with FISH probes to *E. coli*.[16] Independent support for these findings is provided by the immunolocalization of *E. coli* to macrophages within the colons of 10/10 Boxer dogs with HUC.[18] *E. coli* strains isolated from affected Boxer dogs are novel in phylogeny, and have an adherent and invasive phenotype (AIEC) similar to strains isolated from people with Crohn's disease.[16,19,20] These findings suggest that *E. coli* invasion may play a critical role in the initiation and/or progression of HUC in Boxer dogs, likely facilitated by a breed-specific susceptibility.

It is against this background that we sought to determine the temporal association of clinical and histological response to the presence or absence of intramucosal *E. coli* in Boxer dogs with HUC before and after treatment with enrofloxacin.

Materials and Methods

Seven Boxers with signs of colitis referred to MUVTH (dogs 1-5) and Cornell University Hospital for Animals (dogs 6, 7) between 2002 and 2006 were enrolled in this study. Diagnostic evaluation included hematology, serum biochemistry, urinalysis, zinc sulphate fecal flotation, and fecal culture for enteric pathogens, including *Campylobacter* and *Salmonella*. Colonic biopsies were obtained at the time of diagnostic evaluation in all seven dogs (endoscopic biopsies) and at variable intervals after enrofloxacin[a] treatment in 5 dogs (endoscopic in dogs 1,3,4 and 5; full thickness at post-mortem in dog 7). For endoscopic biopsy a minimum of seven to twelve colonic biopsies were collected after standard colonoscopic preparation with an oral electrolyte solution[b] and pre-endoscopic enema with warm water. Enrofloxacin[a] was prescribed at dose rates ranging from 4.8 to 12.8 mg/kg/day for a period of 4-16 weeks, equating to a mean daily dose of 7 mg/kg/day, for a mean duration of 9.5 weeks. Two of these dogs (Dogs 1 and 2) have been reported in a previous publication.[14]

Histopathology

In all dogs, haematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) stained paraffin-embedded formalin fixed sections of colon were retrospectively evaluated by a veterinary pathologist (SPM) in a blinded manner to confirm the original histological diagnosis, and to assess the severity of colitis

Fluorescence in situ hybridization

Colonic sections were examined by fluorescence in situ hybridization (FISH) in a blinded manner. Eubacterial and *E coli*-specific probes targeting 16S ribosomal rDNA were employed, using established methodologies.[16]

Colonic mucosal culture

Colonoscopic biopsies obtained from dogs 3, 4 and 5 prior to enrofloxacin were stored in *Brucella* broth with 30% glycerol at -70°C prior to culture for *Shigella*, *E. coli*, *Salmonella, Mycobacterium bovis*, *Mycobacterium* spp, *Chlamydia* and *Mycosplasma*. Colonic biopsies obtained from dog 7 after clinical relapse were cultured for *E. coli* using methods described previously.[20] Antimicrobial sensitivity of *E. coli* was determined by disk diffusion**.** [21]

Results

Initial Evaluation:

Seven Boxer dogs with a definitive diagnosis of HUC were included in this study (3 neutered females, 2 entire female, 1 neutered male, 1 entire male). The age of onset of clinical signs ranged from 1.5 to 20 months, with a mean of 8.8 months. The age at presentation ranged from 10-24 months, with a mean of 17.4 months. At the time of diagnosis, colitis was graded as severe in 4/7 dogs and moderate in 3/7, typically with large numbers of PAS+ macrophages (Figures 1 and 2). Commonly observed histologic changes were neutrophilic infiltration (6 dogs; marked in dog 6, moderate in dogs 1,3,7 and mild in dogs 4 and 5), epithelial erosion/ulceration (6 dogs;1, 3-7), crypt hyperplasia and crypt distortion (6 dogs;1-6). Decreased numbers of goblet cells were readily apparent in PAS stained sections from 6/7 dogs (4 severe, dogs 1,3,4,6; 2 moderate, dogs 5 and 7). Only one dog (dog 2) had a normal distribution and number of goblet cells.

FISH analysis was positive for intramucosal *E. coli* in each of the 7 affected dogs.

Routine fecal cultures and parasitology were negative for known pathogens. Culture of colonic biopsies from dogs 3, 4 and 5 was positive for *E. coli*, but negative for *Shigella*, *Salmonella, Mycobacterium bovis*, *Mycobacterium* spp, *Chlamydia* and *Mycosplasma*. *E. coli* isolates were sensitive to a wide range of antibiotics including enrofloxacin, gentamicin, amoxicillin-clavulanate and cephalexin.

Clinical response:

Clinical response was evaluated in 7 Boxer dogs with HUC after treatment with enrofloxacin[a]. Five of these dogs of these dogs had not responded to previous treatment with sulfasalazine[c] (4), corticosteroids (3), erythromycin[d] (1) metronidazole[e] (2), amoxicillin-clavulanate[f] (1), and azathioprine[g] (1). Two dogs had received no medications prior to enrofloxacin. A positive clinical response (cessation of tenesmus, mucoid feces and hematochezia) was noted in 7/7 dogs within 2 wks of enrofloxacin (mean 7 mg/kg/d), and was sustained in 6 dogs, with a mean disease-free interval to date of 45 months (17-62 months). Dog 7 initially responded well to intermittent treatment with enrofloxacin[a] (3 courses of approximately 5.0 mg/kg/day for 2-4 weeks) over a 7 month period, but became unresponsive to enrofloxacin. Culture of colonic biopsies obtained after clinical relapse yielded two different strains of *E. coli*, both of which were resistant to enrofloxacin and gentamicin, and sensitive to amoxicillin-clavulanate, cefoxitin, ceftriaxone, amikacin and kanamycin. Further treatment with

amoxicillin-clavulanate[f] failed to elicit clinical improvement and this dog was euthanatized approximately 4 weeks later due to the continued severity of clinical signs.

Histopathological response:

Blinded evaluation of biopsies obtained before and after enrofloxacin[a] revealed a marked reduction in the severity of inflammation in 4 of the 5 dogs evaluated (dogs 1,3,4 and 5) (Figures 1 and 2). Histologic remission lagged behind clinical remission in each of the responders. In dog 1 (Figure 1) histologic evaluation of colonoscopic biopsies 2 weeks after diagnosis showed marked improvement of the initially severe, erosive HUC, but a mild infiltration of PAS+ macrophages persisted. Colonoscopic biopsies collected from this dog on a third occasion, 7 months after the initial diagnosis and were histologically within normal limits though rare macrophages and crypt distortion persisted (Figure 1). An impact of HUC on goblet cells was readily appreciated in PAS stained sections from this dog, with marked increases in goblet cells and mucus at 7 months (Figure 1). In dog 3, colonic histopathology 10 weeks after diagnosis had changed from severe HUC with extensive epithelial erosions to mild, lymphoplasmacytic colitis, though small to moderate numbers of PAS positive macrophages were observed within the lamina propria and submucosa respectively (Figure 2). In dog 4, the initially moderate HUC with focal epithelial erosions had regressed to mild HUC with rare PAS+ macrophages (predominantly in the submucosa) at repeat histological evaluation of colonic biopsies by 8 weeks after the initial diagnosis (Figure 2). The moderate HUC initially documented in dog 5 had also improved, though mild, focal PAS positive macrophages persisted on re-evaluation of colonoscopic biopsies 8 weeks later (Figure 2). In dog 7, which relapsed after an initial response to enrofloxacin[a], severe granulomatous inflammation and large numbers of PAS+ macrophages was present in biopsies obtained 7 months after initial evaluation. Partial resolution of epithelial erosion, a more focal distribution of inflammation, and a decrease in neutrophils were observed after four weeks of amoxicillin-clavulanate[f] but this did not translate into clinical improvement.

Fluorescence in situ hybridization (FISH) post-treatment:

Post-treatment FISH analysis was negative for intramucosal *E. coli* in 4/5 dogs (1,3,4,5) at all time points evaluated after treatment with enrofloxacin[a] (Figures 1 and 3). Intramucosal *E. coli* was detected in colonic

mucosal biospies from the dog (dog 7) that had become clinically unresponsive to treatment with enrofloxacin. Re-evaluation of colonic biopsies following treatment with amoxicillin-clavulanate[f], showed persistence of intramucosal bacteria, though they did appear reduced in number.

Discussion

The clinical response of Boxer dogs with HUC to antibiotic regimens containing enrofloxacin[a] strongly suggests bacterial involvement in the inflammatory process.[14-16] This possibility is significantly strengthened by the recent discovery of invasive *E. coli* within the colonic mucosa and macrophages of affected Boxer dogs, but not dogs with other forms of colitis.[16] Our observations that colonic biopsies obtained during the initial diagnostic evaluation of 7 HUC affected Boxers contained intramucosal *E. coli* provides independent confirmation of these previous findings. The inclusion of dogs from Australia and the USA in the present study, and dogs from the UK and USA in the previous study[16] demonstrates that intramucosal colonization of *E. coli* in HUC is not a consequence of a geographically restricted infectious agent. In agreement with previous reports,[4,5,14,15] we observed a poor clinical response in all dogs treated with various combinations of prednisolone,[c] sulfasalazine,[d] and non-enrofloxacin[a] antimicrobials. We found that enrofloxacin[a] (mean 7 mg/kg/d) for a mean duration of 9.5 weeks resulted in a positive clinical response in all dogs within two weeks of administration, and this was sustained in 6/7 dogs, resulting in mean disease-free interval to date of 45 months (17-62 mo.). Sustained response correlated with the eradication of invasive intramucosal *E. coli*. The dog that relapsed was positive for intramucosal *E. coli* that was resistant to enrofloxacin[a]. The correlation between clinical remission and the eradication of mucosally invasive *E. coli* during treatment with enrofloxacin[a], supports the causal involvement of *E. coli* in the development of HUC in susceptible Boxer dogs

The association of *E. coli* with granulomatous intestinal inflammation in Boxer dogs parallels a growing number of studies in people with Crohn's disease that report the presence of *E. coli* antigens and DNA within affected mucosa,[20,22,25] the isolation of higher numbers of invasive *E. coli* from Crohn's mucosa,[20,24-26] and increased circulating antibodies against *E. coli* OmpC.[27] It is noteworthy that the *E. coli* strains isolated from Boxer dogs are strikingly similar to those implicated in Crohn's ileitis,[16-20,24] in that they are able to invade, persist and replicate in epithelial cells and macrophages and evoke the production of pro-inflammatory cytokines such as

TNF-α, IFN-γ and IL8.[19,20,28] These characteristics are generally associated with pathogenic bacteria, and it seems likely that this emergent group of adherent and invasive *E. coli* (AIEC) are opportunistic pathogens able to exploit a susceptible individual rather than harmless commensals.[20,25,28] This possibility is consistent with the prevailing view that inflammatory bowel disease is the result of an over-exuberant inflammatory response to a subset of resident enteric bacteria in a genetically susceptible individual.[29,30] Genetic polymorphisms associated with granulomatous intestinal inflammation in people involve defective intracellular processing of bacterial components such as polymorphisms in the intracytosolic muramyl dipeptide receptor NOD2, and autophagy genes such as ATG16L1, and an overactive mucosal inflammatory response (e.g. IL-23r promoting loss of oral tolerance).[31] Since HUC in dogs is remarkably breed-specific it may be due to a heritable anomaly in Boxer dogs that confers susceptibility to invasion and persistence of AIEC within the colonic mucosa. As Boxer dogs are also prone to other granulomatous diseases such as intestinal and systemic protothecosis and cutaneous intracellular mycobacteria (canine leproid granuloma syndrome),[32,33] it is possible that Boxers have a genetic defect in how they respond to granuloma-inducing microorganisms in general, e.g. defective bacterial killing. Further study is needed to identify the host and bacterial factors related to invasion and persistence of *E. coli* in HUC susceptible Boxers.

Our finding that clinical remission in response to enrofloxacin[a] correlates with the eradication of invasive *E.coli* and precedes complete histologic resolution of inflammation highlights the importance of eliminating *E. coli* infection in Boxers with HUC. Serial evaluation of dog 1, where the ongoing inflammation observed at 2 weeks had almost completely resolved at 7 months suggests the lag between bacterial eradication and normalization of colonic histology reflects the time taken to heal and remodel the severely damaged mucosa. These observations are similar to findings in people with Whipple's disease where regression of PAS positivity lags behind eradication of *Trophyrema whipplei*.[34]

In the present study, resistance to enrofloxacin[a] was demonstrated in two *E. coli* strains isolated from the colonic mucosa of a dog whose clinical signs recurred after an initial response to enrofloxacin. The development of resistance to enrofloxacin[a] has been extensively demonstrated in dogs with *E. coli* and other

Enterobacteriaceae infections[35-38] and often involves acquisition of resistance plasmids.[38] Interestingly, the dog that relapsed had received several short courses of treatment with enrofloxacin (2-4 weeks) and it is possible that repeated withdrawal of enrofloxacin[a] prior to complete eradication of infection facilitated recrudescence and subsequently development of enrofloxacin[a] resistance. Short-term treatment of this dog with amoxicillin-clavulanate[g], based on the results of antimicrobial susceptibility testing, resulted in a reduction in intramucosal *E. coli* and partial histologic improvement, but this did not translate to clinical improvement. While it is possible that a longer duration of treatment may have been effective it is important to consider that differences in the ability of antibiotics to penetrate infected macrophages may also affect outcome. Evaluation of the ability of antibiotics (at peak serum concentrations) to kill *E. coli* isolated from colonic Crohn's disease within J774-A1 macrophages indicates greatest efficacy of ciprofloxacin, with decreasing impact of rifampicin, tetracycline, trimethoprim, clarithromycin and azithromycin.[39] This study also found the combination of ciprofloxacin, tetracycline and trimethoprim was more effective than ciprofloxacin alone, and that ampicillin was not effective against intra-cellular CD associated *E. coli.* Hence, the ability to effectively penetrate and kill *E. coli* within macrophages may explain the positive response of HUC of Boxers to enrofloxacin, and also to chloramphenicol,[1] and the negative response of the dog in the present study to amoxicillin-clavulanate[g]. To optimize the treatment of HUC of Boxers in the future it would seem prudent that FISH and colonic mucosal culture are performed as part of the diagnostic evaluation of Boxer dogs suspected of HUC. This would enable antimicrobial selection based on sensitivity testing of mucosal *E. coli* isolated from FISH positive individuals and their ability to penetrate tissues and macrophages. Based on the results of the present and previous studies,[14-16] Boxer dogs with enrofloxacin sensitive HUC should be treated for at least 6 to 8 weeks at doses of 5-10mg/kg body weight /day.

Previous reports have documented resolution or improvement of clinical signs in some affected Boxers managed with dietary therapy and sulfasalazine.[4,5] Mesalamine, but not hydrocortisone, at therapeutic concentrations has recently been shown to inhibit MAPK-dependent IL-8 release in response to adherent and invasive IBD and colon cancer *E. coli* isolates *in vitro*, and it may have some therapeutic benefit *in vivo*.[40] However, as the dogs in this present study that received sulfasalazine[d] failed to respond, a meaningful effect of

sulfasalazine[d] in dogs with severe HUC seems unlikely. Whether sulfasalazine[d] has a role in treating Boxers with mild HUC or as an adjunct to enrofloxacin[a] remains to be determined

In conclusion, this case series serves to corroborate the association of mucosally invasive *E. coli* with HUC in Boxer dogs and the responsiveness of this disease to enrofloxacin[a]. The correlation between clinical remission and eradication of mucosally invasive *E. coli* supports the potential causal involvement of *E. coli* in the development of HUC in susceptible Boxer dogs. As clinical relapse was associated with enrofloxacin[a] resistance, we suggest that sensitivity profiles of mucosal *E. coli* and the ability of antibiotics to penetrate macrophages are used to guide antimicrobial selection.

Footnotes

a. Baytril®, Bayer
b. Colonlytely®, Dendy Pharmaceuticals
c. Salazopyrin®, Pharmacia
d. Erythrocin Stearate®, Abbott
e. Flagyl®, Aventis
f. Clavulox®, Pfizer
g. Imuran®, Faro Pharmaceuticals

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Legends for Figures:

Figure 1. Colonic histopathology and fluorescence in situ hybridization (FISH) before and after treatment of HUC with enrofloxacin

Colonic biopsies from dog 1 at initial diagnosis were dominated by PAS positive macrophages (a) and invasive intramucosal bacteria that hybridized with a Cy-3 labeled probe to *E. coli* (b: bacteria are orange, DAPI stained nuclei are blue). After 2 wks of enrofloxacin mucosal infiltration with PAS positive macrophages persists (c) but no invasive bacteria are visualized by FISH (d). 7 months after diagnosis, the infiltration of PAS positive macrophages had resolved (though occasional PAS positive macrophages remained), goblet cells containing abundant PAS staining mucus were apparent (e), and FISH (f) remained negative.

Figure 2. Colonic histopathology before and after treatment of HUC with enrofloxacin

H&E and PAS stained sections of endoscopic colonic biopsies from dogs 2,3 and 4 before (a, c, e) and eight to ten weeks after treatment with enrofloxacin (b, d, f). In untreated HUC (a, c, e) epithelial erosion, loss of glandular structure and marked cellular infiltration with PAS positive macrophages (insets) were readily apparent. After treatment with enrofloxacin (b, d, f) histological abnormalities had markedly improved in all three dogs, though small numbers of PAS positive macrophages and infiltrates of lymphocytes and plasma cells were still present. An increase in PAS positive goblet cells was observed in all three dogs post treatment (insets, b,d,f).

Figure 3: Fluorescence in situ hybridization (FISH) before and after treatment of HUC with enrofloxacin

Fluorescence in situ hybridization (FISH) of colonic biopsies from dogs 2, 3 and 4 prior to enrofloxacin (a, c, e) revealed multifocal intramucosal bacteria that hybridized with a Cy-3 labeled probe to *E. coli* (bacteria are orange, DAPI stained nuclei are blue). Many of the *E. coli* appeared to be within

macrophages (insets a, c, e). After treatment with enrofloxacin sections from all 3 dogs (b, d, f), were FISH negative.