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Identification of Histamine Receptors in the Canine Gastrointestinal Tract

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Identification of histamine receptors in the canine gastrointestinal tract

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The role of histamine in chronic gastrointestinal diseases has been increasingly recognized in humans, but the role of histamine in the canine gastrointestinal tract has not been thoroughly investigated. The presence and distribution of all 4 histamine receptors (H1, H2, H3, and H4) in the stomach, duodenum, ileum, jejunum, and colon of healthy dogs were evaluated with a commonly employed immunohistochemistry technique using antibodies predicted to cross react with canine histamine receptors. All 4 histamine receptors were identified in the canine gastrointestinal tract, and differed in location and density within sections of the canine gastrointestinal tract. Antibody specificity was evaluated with Western blot. With the establishment of a method to study histamine receptors in the canine gastrointestinal tract, additional research to evaluate histamine receptors in dogs is warranted to further understand the pathophysiology and treatment of chronic canine enteropathies.

DEDICATION

I would like to dedicate this research to my parents, who have loved me and supported me in every way possible. I will never be able to thank them enough. I would also like to dedicate this to my precious husband, Hayden, who graciously allowed me to chase my dreams and cheered me on the whole way. And lastly, I dedicate this to our beautiful daughter, Riley, who brings me endless joy with her quick wit and joyful spirit.

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CHAPTER I

INTRODUCTION

Histamine Discovery and Biology

In 1910, Henry Dale isolated and identified 2- (4-imidazole)-ethylamine, now known as histamine, from an extract of ergot.[1] Since its discovery, histamine has been linked to well over 20 different physiological responses.[1,2] Initial histamine research led to groundbreaking discoveries in the realm of human and veterinary medicine, including the development of specific therapies for anaphylaxis, asthma, gastric ulcers, and allergic reactions. More recently, the roles of histamine and its receptors in inflammatory, immune-mediated, and neoplastic diseases have been intensely investigated in human medicine.[3] Human research using new histamine antagonists has advanced our understanding of these complex conditions and offers exciting therapeutic potentials, particularly in regards to the treatment of complicated inflammatory conditions.

Histamine is a primary amine synthesized intracellularly through oxidative decarboxylation of the amino acid histidine and stored in granules within mast cells and basophils.[2] The enzyme L-histadine decarboxylase catalyzes and regulates histamine production, and the gene for this enzyme is expressed intracellularly in many cell types.[4] Mast cells, which are located throughout the body but are especially concentrated in the skin, lungs, and gastrointestinal tract, are the main sites of histamine synthesis.[5] Enterochromaffin-like cells of the gastric mucosa, endothelial cells, platelets, basophils,

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dendritic cells, T cells, and histaminergic neurons also synthesize and secrete histamine.[2,4,6,7] Histamine is present in varying concentrations in most organs, particularly within the stomach, lymph nodes, and thymus.[7] Histamine is released following immunological and non-immunological stimulation of histamine-producing cells, and exerts its diverse biological actions through binding of four different G-protein coupled histamine receptors (H1, H2, H3, and H4) located throughout the body.[3] Overall, only 2-3% of histamine is excreted unchanged in the urine.[3] The majority of histamine (50-80%) is metabolized to its primary metabolite, N-methylhistamine, by the cytosolic enzyme N-methyltransferase, which degrades intracellular histamine but spares stored histamine.[2,7,8] The remainder of histamine is metabolized by the enzyme diamine oxidase.[4,8]

Histamine Receptors

Investigation of histamine receptors has to date been limited in the veterinary literature, but these receptors have been studied in human medicine for over 75 years.[1] Histamine exerts its actions via binding of four different G-protein coupled receptors, named H1, H2, H3, and H4, which are members of the largest family of membrane proteins in the human genome.[5] Upon histamine binding, these receptors transmit the extracellular signal to intracellular second messengers that trigger a cascade of molecular events that direct a particular physiological response, which, in turn, depends on the subtype and location of the engaged histamine receptor.[2] Each receptor varies in its affinity for histamine and its level of expression throughout the body.[9] Although there are many similarities in receptor distribution across species, differences between species in receptor location and function have been documented.[1,2] In the following section,

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location of histamine receptors is based on research in humans and laboratory animals primarily, unless otherwise noted.

H1 Receptor

The H1 histamine receptor was the first of the four histamine receptors to be discovered.[5] It was characterized in 1937, and the bovine H1 receptor was cloned in 1991.[10] The receptor was later cloned in several other species, including the dog, guinea pig, and human.[1] Histamine binding of the H1 receptor stimulates the inositol phosphate signaling pathway, which leads to increased intracellular calcium.[4] Stimulation of the H1 receptor also induces synthesis of prostacyclin and platelet-activating factor, activation of NF-κB transcription factor, and release of von Willebrand factor and nitrous oxide.[4]

The H1 receptor is ubiquitously expressed in vascular endothelium, vascular and airway smooth muscle, hepatocytes, chondrocytes, epithelial cells, neurons, glial cells and immune cells, including dendritic cells, monocytes, neutrophils, T cells and B cells.[3,4] It has also been identified in the gastrointestinal tract, genitourinary tract, heart, skin and adrenal medulla.[11] Biological actions mediated by the H1 receptor include the well-known Type 1 hypersensitivity reaction, including smooth muscle contraction in the intestines responsible for abdominal cramping, pruritus, bronchoconstriction, potent vasodilation, anaphylactic shock, increased vascular permeability and edema, and release of catecholamines from the adrenal medulla.[2,4] H1 receptor-mediated excitation in the central nervous system is the major mechanism for cortical activation and wakefulness in the wake-sleep cycle.[5]

Common pathological processes mediated by the H1 receptor include allergic rhinitis, conjunctivitis, asthma, urticaria, atopic or allergic dermatitis, and anaphylaxis.[2,4]

The well-known antihistamines chlorpheniramine, diphenhydramine, and clemastine are H1 receptor antagonists that have been used for over 70 years for the treatment of allergic conditions and anaphylactic shock.[12] These anti-allergy medications inhibit the effects of histamine on H1 receptors and stabilize mast cells.[9] Because of the sedating effects of these first-generation H1 antagonists, non-sedating second-generation antagonists such as cetirizine, lorantadine, and desrolorantadine have been developed.[12,13]

H2 Receptor

In 1972, the H2 receptor was identified after researchers noticed that several effects of histamine, such as the stimulatory effect of histamine on gastric acid secretion, were not inhibited by the H1 antagonist mepyramine.[5] The H2 receptor was cloned from the dog in 1991, and was subsequently cloned from the rat, mouse, guinea pig, and human.[13,14] The human H2 receptor is coupled to both adenylate cyclase and phosphoinositide second messenger systems.[4] Binding of histamine to the receptor may produce either a stimulatory or inhibitory action   on H2 receptor-expressing cells.[15].

Like the H1 receptor, the H2 receptor is widespread throughout the body. It is abundant on the parietal cells of the gastric mucosa and on the endothelial cells, astrocytes, and neurons in the brain.[1] It is also located in smooth muscle cells, myocytes, enteric neurons, neutrophils, eosinophils, monocytes, macrophages, dendritic cells, and T and B lymphocytes.[2,11,15]

The H2 receptor is best known for its role in controlling gastric acid secretion. Gastrin secreted by the gastric antrum, acetylcholine released from the vagal nerve, and histamine released by gastric enterochromaffin-like (ECL) cells stimulate hydrochloric acid release from parietal cells.[16] Gastrin initiates the process through stimulation of ECL

cells, which synthesize and secrete histamine that binds to the H2 receptor on parietal cells.[16] This binding then activates the H/K-ATPase pump on the parietal cell.[16] This pump is the common target for all stimulatory mechanisms that increase gastric acid production.[16] Without the central role of histamine and its corresponding H2 receptor on parietal cells, gastric acid production is significantly hampered.[17]

In addition to its role in gastric acid secretion, the H2 receptor also mediates vasodilation, inotropy and chronotropy, and smooth muscle relaxation in the uterus, vasculature, and airways.[4,18] Unlike the H1 receptor, which has a pro-inflammatory role, the H2 receptor has an anti-inflammatory role through the inhibition of cytokine production and chemotaxis and dampening of T cell responses.[2,19] Therefore H2 antagonism may predispose to or exacerbate allergy, inflammation, or infection, particularly in the gastrointestinal (GI) tract.[19,20]

Cimetidine was the first H2 antagonist developed in 1977, and it revolutionized the understanding and treatment of peptic ulcers, dyspepsia, and gastrointestinal reflux.[16] More potent H2 antagonists such as ranitidine and famotidine were later developed, and all are considered safe, with very little affinity for H1 receptors.[5] Other potential uses of H2 antagonists include cardioprotection in ischemic conditions and improvement of cognitive function.[18,21]

H3 Receptor

The third histamine receptor was characterized in 1983, and cloned in humans in 1999.[22] It was later cloned in the rat, guinea pig, mouse, and monkey, and has been found to be over 90% conserved between species.[1,5] This receptor shows low sequence homology with the H1 and H2 receptors, and different isoforms of the receptor have been

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identified.[22,23] The H3 receptor was discovered as a presynaptic receptor on histaminergic nerve terminals in the central nervous system, where it functions to control the synthesis and release of histamine.[12] When the H3 receptor is bound to its G-protein coupled receptor, adenylate cyclase activity is inhibited along with calcium channels at certain nerve endings, thereby controlling neurotransmitter release.[2]

Primary locations of the H3 receptor in the central nervous system (CNS) include the hypothalamus, thalamus, frontal cortex, hippocampus, caudate nucleus, and basal ganglia.[2,3] This receptor is also located in the peripheral nervous system, particularly in the enteric nervous system.[3] The H3 receptor has also been found in other non-neurological tissues, including the respiratory epithelium, gastric and intestinal mucosa, skin, vascular endothelium, thymus, heart, and kidney of the rat.[5,24] The H3 receptor is involved primarily in negative feedback-mediated inhibition of the synthesis and release of not only histamine but also other neurotransmitters such as dopamine, serotonin, glutamate, acetylcholine, and norepinephrine.[1,2] Neurogenic inflammation may also be controlled in part through the interaction of histamine on mast cell H3 receptors through a neuron-mast cell feedback loop.[15,25] In addition to its control of histamine and neurotransmitter synthesis and release, the H3 receptor is also involved in the sleep-wake cycle, memory, appetite, and cognition.[4,26] Gastrointestinal motility is altered by the H3 receptor due to influence on the release various neurotransmitters.[2,27] The H3 receptor also appears to have a protective role against the formation of gastrointestinal ulcers.[28,29]

Several H3 agonists and antagonists have been developed, and all are in the preclinical phases of study.[5] Thioperamide is a particularly useful, potent H3 antagonist that has had a pivotal role in investigation of the H3 receptor.[12] Treatment of several brain

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disorders with H3 receptor antagonists have been investigated, including narcolepsy, Alzheimer's, and attention disorders, , and the results support their use in these complex disorders.[15] Treatment of pain with H3 antagonists has yielded conflicting results, but in a recent study an experimental H3 antagonist was safe and efficacious for neuropathic or osteoarthritic pain.[1,30]

H4 Receptor

In 2000, the H4 receptor was discovered, and it has since been cloned in the rat, mouse, dog, guinea pig, monkey, and pig.[5, 31] Sequence homology between the canine and human H4 receptor is 71%.[31] Histamine has a high affinity for the H4 receptor, but species differences in ligand binding and downstream signaling are evident.[5] There is an approximately 40% sequence homology between the H3 and H4 receptor, and less homology between the H1 and H2 receptors.[26] Similar to the H3 receptor, the H4 receptor inhibits cAMP formation after it is bound by histamine.[5,15]

Expression of H4 receptors is highest in the bone marrow and peripheral hematopoietic cells.[4] The receptor is found on neutrophils, eosinophils, mast cells, dendritic cells, basophils, T cells and B cells.[1,2,4] H4 receptors have been identified in the spleen, thymus, brain, liver, lung, heart, epidermis, peripheral nerves, enteric nerves, and the large and small intestines.[1,4] In 2011, H4 receptor mRNA was first identified in healthy canine skin, colon, liver, spleen and kidney, and showed similar tissue distribution to that of humans and rodent models.[32] New sites of H4 receptor expression are emerging as research with this receptor continues at a rapid pace, but the dynamic expression levels of the receptor, particularly in inflammation, have led to inconsistent findings.[5,33]

The primary function of the H4 receptor is mediation of inflammation through induction of cytokine release and chemotaxis of inflammatory cells, especially eosinophils and mast cells.[3,5] Overall, the H4 receptor appears to exert a pro-inflammatory state, and evidence for its role in chronic inflammatory diseases and autoimmunity is abundant.[1,3,4] The H4 receptor mediates increased pruritus and promotes allergic inflammation, along with H1 receptor.[2,34,35] Immune cell proliferation and differentiation is also affected by the H4 receptor.[5] H4 receptor dysregulation has also been linked to certain neoplasia, such as breast and colon cancer.[1,4]

H4 receptor antagonists have been developed for research and most are in phase I preclinical trials.[5,22] However, the antagonist JNJ-39758979 has recently been advanced into clinical trials, and has shown efficacy in treating allergic conditions that have previously not responded fully to H1 antagonism.[36] This antagonist along with another well-studied H4 antagonist, JNJ 7777120, has also been effective in various animal models of inflammation such as asthma, dermatitis, and colitis.[22,36,37] H4 antagonism improved pruritus in a mouse model of atopic dermatitis, and in dogs with inflammatory skin disease.[5,37] Efficacy of H4 antagonists in chronic pain has recently been demonstrated as well.[5]

Emerging Roles of Histamine and Histamine Receptors

With the development of new histamine receptor agonists and antagonists over the last two decades, especially in the case of the H3 and H4 receptors, histamine's role in numerous biological processes has proven to be far more diverse than ever imagined.[38] Newly discovered effects of histamine on the immune response are complex, and they offer exciting new therapeutic opportunities in chronic inflammatory disease and

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immune-mediated disorders.[4] Furthermore, involvement of different histamine receptors in certain gastrointestinal disorders, allergic diseases, and cancers is being unraveled.[1-5]

Histamine in Inflammation

Histamine is intimately involved in many functions of the immune system, and directly affects the activity of individual immune cells.[15] Even wound healing, cellular proliferation and differentiation, and tissue regeneration involve histamine.[4] Histamine not only mediates several aspects of acute inflammation, including cytokine release, chemotaxis, and the function of individual immune cells, but also modulates chronic inflammatory events.[3,4,39,40] Consequently, our understanding of several complex and debilitating inflammatory conditions has been enhanced through histamine research.

Effects of Histamine on Immune Cells

Mast cells and basophils are the primary source of histamine storage in normal tissues.[2] Histamine is released from these cells after immunological activation of their immunoglobulin E receptor, which binds antibody or antigen and incites histamine release.[2] Non-immunological means of histamine release include cytotoxic and non-cytotoxic stimulation by cytokines, free radicals, and products of the complement system.[38] Endothelial cells express histamine receptors, and histamine incites upregulation of adhesion molecule expression and regulates epithelial barrier function and permeability, mostly via the H1 receptor.[4] Mast cells, basophils, monocytes, and lymphocytes express H1, H2, and H4 receptors, and their cellular functions are affected by histamine.[2,41] Furthermore, histamine modulates cytokine production.[2,3,41] Notably, histamine increases IL-1 and IL-6 production and induces IL-10 synthesis.[41,42,43]

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Perhaps one of the most consequential effects of histamine on the immune system is its role in chemotaxis and accumulation of granulocytes at target sites.[5] The primary location of the H4 receptor on virtually every immune cell highlights the importance of this particular receptor in inflammation. Overall, the H1 and H4 receptors mediate a pro-inflammatory role, and the H2 receptor mediates downregulation of the immune system.[4,19] The H3 receptor primarily mediates control of histamine release, therefore indirectly influences the immune response.[5] Both the H1 and H4 receptors enhance eosinophil chemotaxis, while the H2 receptor is inhibitory.[5,19,40] Eosinophil adhesion to the endothelium, which is a pivotal step in eosinophil migration, is mediated by the H4 receptor.[44] Similarly, mast cell migration is induced by the H4 receptor.[45,46] Histamine-mediated accumulation of eosinophils and mast cells promotes chronic inflammation and tissue damage, and the use of H4 antagonists with or without concurrent H1 antagonists may improve outcome in chronic inflammatory conditions. On the other hand, the H2 receptor suppresses neutrophil chemotaxis and function, and H2 antagonism may permit further inflammation.[5,19]

Antigen-presenting dendritic cells express all 4 histamine receptors, and are differentially influenced by each receptor.[15] Both the H1 and H4 receptor mediate chemotaxis of natural killer cells and dendritic cells, and promote increased antigen-presenting capacity and pro-inflammatory cytokine release.[15,47,48] Histamine also provides a link between the innate and adaptive immune response through its influence on the dendritic cell/T cell interaction and subsequent polarization into Th1 and Th2 cells.[41] Specifically, the H1 receptor increases cellular immunity via Th1 cells, and the H4 receptor promotes Th2 polarization through release of the key Th2 cytokine, IL-4, as well

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as IL-10 release from dendritic cells.[40,41,42,49] H4 antagonism reduces production of the Th2 cytokines IL4, 5, and 13, as well as production of the pro-inflammatory IL-6 and IL-17, emphasizing the powerful role of histamine in T cell function.[41] Th2 cells favor IgE production, further increasing histamine secretion by mast cells. Hence, histamine's role in T cell mediated diseases such as rheumatoid arthritis, asthma, and multiple sclerosis can be explained by histamine's influence on innate and adaptive immunity, and is supported by the reduction of inflammation by H4 antagonists in experimental models of the diseases.[40]

Pro-inflammatory effects of histamine are counterbalanced by the H2 receptor, which enhances humoral immunity and IL-10 production, and dampens both Th1 and Th2 cell-associated cytokines and responses.[15,42,49] The anti-inflammatory effects of H2 receptor are consistent with its proposed role in peripheral tolerance and/or suppression of the inflammatory response.[15,50] In fact, H2 antagonists have benefited immune-suppressed patients battling secondary infections.[51]

Histamine in Asthma and Allergic Inflammation

Despite clear evidence that histamine is involved in asthma, efficacy of H1 antagonists in the treatment of asthma has been poor.[4] However, H4 antagonists may offer new hope. Because the H4 receptor mediates CD4+ T cell activation and induces eosinophil chemotaxis, the receptor mediates allergic responses.[41] H4 receptor deficient mice and mice treated with H4 antagonists display lower concentrations of eosinophils and lymphocytes in their lungs and have decreased Th2 responses.[41] In further support of the role of H4 receptors in asthma, in vivo studies have shown that the highly selective

H4 receptor antagonist JNJ7777120 blocks histamine-mediated mast cell accumulation in respiratory tissues.[52]

Historically, the H1 receptor was identified as the histamine receptor responsible for allergic dermatitis and pruritus. However, involvement of the H3 and H4 receptor in the itch sensation was demonstrated in 2011, creating new therapeutic options for pruritus.[35] Use of H4 antagonists has relieved chronic pruritus in mouse models of atopic dermatitis, and the H4 receptor now appears to be more involved in pruritus than the H1 receptor.[34] Similarly, clinical data with the H4 receptor antagonist JNJ 39758979 support its efficacy in reducing pruritus in humans.[42]

Histamine in Gastrointestinal Inflammation

Histamine's contribution to gastrointestinal pathology has been a productive focus of research. In addition to its central role in gastric acid secretion, histamine has an important role in many functions of the gastrointestinal tract, including neurotransmission, visceral nociception, and mucosal immunity.[2,53] Because of histamine's far-reaching effects on numerous physiological processes, an increase in histamine or a dysregulation of its receptors has a significant impact on normal organ structure and function.

Hyperhistaminemia is a well-established risk factor for gastroduodenal ulceration and perforation.[2] Ion transport across the intestinal epithelium is also increased by histamine/H1 receptor interactions, contributing to diarrhea.[54,55] The influence of histamine on the enteric nervous system via all 4 histamine receptors may contribute to increased motility and cramping.[54,55] Levels of histamine in the gut lumen of human patients with inflammatory bowel disease are elevated, likely because gastrointestinal

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lesions in these patients have dense accumulations of histamine-producing mast cells.[26] Intestinal tissue samples and secretions of patients with chronic enteropathies such as Crohn's disease have elevated histamine levels, and the histamine levels correlate with disease severity.[56,57,58,59] Gastrointestinal mucosal IgE and mast cells, as well as urinary N-methylhistamine levels, are elevated some human and canine inflammatory bowel disease (IBD) patients.[42,56,60,61]

In 2006, the H1, H2, and H4 receptors were demonstrated, through PCR and immunohistochemistry techniques, in healthy and diseased human GI tract biopsies.[11] H3 receptor expression was only detected in 2 patients in this study.[11] One year later, Breunig, et al discovered that all 4 histamine receptors were involved in excitation of the human enteric nervous system.[27] The H1 receptor is involved in intestinal smooth muscle contraction, mucosal ion transport, and nociception.[62] A recent study showed that visceral hypersensitivity in irritable bowel patients is mediated by the H1 receptor, and that administration of the H1 receptor antagonist, ebastine, substantially reduces abdominal pain by blocking a common nociceptor involved in visceral hypersensitivity.[62]

The H2 receptor is widely dispersed in the gastrointestinal tract.[1,2] In addition to its previously described role in gastric acid secretion, it downregulates the immune response.[19] It promotes an anti-inflammatory cytokine profile and therefore is protective in the realm of gastrointestinal immunity.[19] Indeed, antagonism of the H2 receptor has led to exacerbations of clinical signs of Crohn's disease, has been linked to worsening of intestinal bacterial inflammation, and has been associated with susceptibility to immune-mediated inflammation.[19,20]

Regulation of histamine and intestinal neurotransmitter release is mediated in part by the H3 receptors, which are densely concentrated in the enteric nervous system.[63] Additionally, the H3 receptor may be involved in gastroprotection, but current data is somewhat conflicting.[28,63] New H3 antagonists are, however, showing good results for relief of chronic pain, and may have a future role in treatment of visceral hypersensitivity.[1,30]

Many of histamine's effects on GI mucosal immunity are mediated by the H4 receptor. For example, the H4 receptor is a strong mediator of eosinophil chemotaxis, and eosinophilic enteritis is a frequent finding in gastrointestinal tissue of both human and canine IBD patients.[64] Toll-like receptors (TLRs), which are implicated in the pathogenesis of inflammatory bowel disease, interact with the H4 receptor to influence cytokine and chemokine production by dendritic cells [26,41,65] Treatment of experimentally induced peritonitis with an H4 antagonist blocks TLR-mediated inflammation.[26] The trinitrobenze sulphonic acid model of colonic inflammation, which mimics Crohn's disease, involves TLR, T cell and dendritic cell pathology that is effectively reduced by H4 antagonism.[26] H4 antagonists also inhibit TNF-α and IL-6, cytokines involved in the pathogenesis colonic inflammation.[53] Similar to the H3 receptor, the H4 receptor may have a role in controlling gastric acid secretion.[28,29] Research suggests it might promote healing of gastrointestinal ulcers alone or in combination with the H3 receptor.[28,29,53] These developments in histamine research are exciting for the future of human and veterinary gastroenterology, particularly in regards to the potential of H4 antagonists.

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Histamine in Neoplasia

Histamine's effects on cell proliferation and differentiation led to investigation of histamine receptors in certain malignancies. Indeed, histamine receptors H2 and H4 have been identified on a variety of malignant cells.[1,5] H2 receptors have been documented in breast cancer cells and significantly altered populations of H1, H2, and H4 receptors have been detected in colorectal cancers.[66] Decreased expression of the H4 receptor has been noted in colorectal carcinoma.[67] H2 antagonism has been shown to decrease growth of some tumor cell lines.[68] Due to the immune-suppressive effects of the H2 receptor, treatment with H2 antagonists in order to stimulate a more robust immune attack on tumor cells has been explored.[69] Also, the influence of H4 receptor-mediated inhibition of proliferation has been demonstrated in melanoma and breast cancer cells.[70]

Importance of the Research

Recently, H4 receptor mRNA was identified in the canine colon for the first time.[11] The presence of all four histamine receptors in the canine GI tract has not been documented to our knowledge. Histamine receptor research in humans has led to the development of new histamine receptor antagonists that show promise in treating chronic intestinal inflammation. Further documentation of histamine receptors in the canine gastrointestinal tract will provide additional research opportunities to further our understanding of the pathophysiology of the canine counterparts of chronic enteropathies, particularly the role of histamine receptors in these diseases d to potentially explore new histamine antagonist treatment strategies for these common yet highly variable diseases.

Canine Chronic Enteropathies

Inflammatory bowel disease is one of the most common causes of chronic gastrointestinal signs in dogs. The predisposition of certain breeds to IBD supports a possible genetic cause in some patients.[71] The Yorkshire terrier, German shepherd, Rottweiler, Basenji, Norwegian Lundehund, soft-coated Wheaten terrier, Irish setter and boxer dog are common breeds that are predisposed to IBD. In two separate studies of intestinal biopsies from dogs with chronic GI signs, nearly 25% had some form of IBD.[72,73] While the exact etiopathogenesis of IBD is unknown, a breakdown of immunological self-tolerance to dietary and bacterial antigens in the GI lumen is widely accepted to play a central role in the pathogenesis of the condition.[74] Clinical signs and severity in dogs varies significantly in affected patients, and different sections of the GI tract are often variably affected.

Highly variable patient responsiveness to current therapy highlights the need for a greater understanding of the pathogenesis of canine IBD, in order that better treatment options can be explored. Exploration of the role of GI histamine receptors in the canine gastrointestinal tract is an important step in this process. Canine IBD shares many similarities with Crohn's disease and ulcerative colitis in humans. Both diseases involve immune-mediated inflammation in the gut wall that responds most consistently, but not always, to medications that suppress the immune system and dietary changes. Histamine certainly appears to have a role in contributing to the progression of IBD in dogs.[61,74] Dogs with IBD have been shown to have increased intestinal mast cell density, and some also have high levels of fecal and urinary N-methlyhistamine.[61,74] The large body of evidence supporting the role of histamine and its receptors in intestinal inflammation in

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humans suggests a potential therapeutic role for histamine receptor antagonists, particularly H4 antagonists, in chronic canine intestinal diseases.

Histamine Research Techniques

Histamine receptors are dispersed throughout the body, with each different receptor mediating unique responses in the body. Documentation of histamine receptors is limited in the veterinary literature, but is abundant in human medicine. The majority of work has been performed in humans, mice, rats, monkeys, and guinea pigs.[1,3,4,5] The function of each receptor has been uncovered through the use of ligands, specifically histamine receptor agonists and antagonists.[12] Radiolabelled agonists have also been used to study the location of various receptors in tissues.[12] Successful cloning of all 4 receptors opened the door for researchers to use the polymerase chain reaction techniques to search tissues containing histamine receptor cDNA.[1,3,4,5]

Assessment of histamine and/or N-methylhistamine levels is a non-invasive means of indirectly studying histamine.[75] Recently, a method for quantitating plasma histamine in dogs, mice, rats, and humans was validated.[76] Histamine has a short half-life in circulation, buts it major metabolite, N-methylhistamine (NMH), is stable and measureable.[75] A method for NMH measurement in human plasma was described in 1997, and this was modified by Ruaux, et al to develop a chromatography-mass spectrometry assay for measurement of fecal and urinary N-methylhistamine levels in dogs.[75.77] Despite the correlation of urinary NMH with disease severity in Crohn's disease, NMH levels appear to be inconsistently elevated in dogs with chronic enteropathies and do not predict mast cell activation or clinical signs.[61,78]

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Arguably, immunohistochemistry and/or immunofluorescence techniques are some of the most clinically useful means for determining the location and density of histamine receptors in target tissues.[79] These methods depend on detection of receptor expression, versus molecular methods such as PCR that detect mRNA of the receptors. The presence of mRNA does not necessarily equate to the level or location of protein expression in a certain disease or inflammatory state. In order to investigate the location and density of histamine receptors in canine tissues, development of a straightforward, affordable histopathologic immunohistochemical technique is ideal. Then, the same tissue samples obtained for routine histopathology could be submitted for evaluation of histamine receptors via immunohistochemistry. Although there are currently no commercially available canine histamine receptor antibodies, cross reactivity amongst species is supported based on sequence homologies.[22,32] Establishment of an immunohistochemical technique can be used to not only study canine intestinal tissues, but also other tissues such as skin and lung in order to evaluate the role of histamine receptors in dermatological and respiratory diseases, respectively.

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CHAPTER II

IDENTIFICATION OF HISTAMINE RECEPTORS IN THE CANINE

GASTROINTESTINAL TRACT

Introduction

The role of histamine in allergic responses, anaphylaxis, and gastric acid secretion has been well described in both human and veterinary medicine. New roles for histamine and its receptors, however, have been elucidated over the last two decades, and novel histamine receptor antagonists have also recently been developed.[1,2] Histamine has been shown to have an important role in many functions of the gastrointestinal (GI) tract, including neurotransmission, visceral nociception, and mucosal immunity.[3,4] Histamine is a primary amine that is synthesized via decarboxylation of the amino acid histidine in mast cells, platelets, basophils, histaminergic neurons, and enterochromaffin cells, and then stored within intracellular vesicles.[3] Mast cells, which are abundant throughout the body, but especially in the skin and GI tract, are the predominant storage site of histamine.[5] Immunological and non-immunological stimulation of histamine-producing cells triggers release of histamine. Histamine then exerts its actions via binding to four different G-protein coupled receptors (H1, H2, H3, and H4) throughout the body.[6] Histamine mediates many different physiologic responses, of which the best-known is the Type I hypersensitivity reaction. In the GI tract, histamine is involved in regulation of gastric acid production, smooth muscle motility, and mucosal ion transport.[7] Histamine

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also has a role in mucosal defense and neurotransmission.[3] Importantly, histamine is a potent mediator of inflammatory responses, inciting cytokine and chemokine release and inducing chemotaxis and adhesion molecule production.[5,8,9]

Histamine receptors are dispersed throughout the body, with each individual receptor mediating unique responses. Investigation of histamine receptors has to date been limited in the veterinary literature, but these receptors have been more thoroughly explored in human medicine. In humans, histamine receptors have been demonstrated with polymerase chain reaction (PCR), Western blotting, and immunohistochemistry techniques.[7] Approximately 50 years ago, it was first recognized that not all histamine receptors were alike, and that various cells expressed different histamine receptors.[2]

The first two receptors to be identified and categorized in humans were the H1 and H2 receptors which are both widely distributed throughout the body.[2] H1 receptors mediate smooth muscle contraction, particularly in the airways and GI tract, and are heavily involved in hypersensitivity reactions such as asthma.[3] H1 receptors are also involved in the pathogenesis of some of the classic features of the inflammatory reaction, such as vasodilation and increased vascular permeability.[5] The best-known function of the H2 receptor is regulation of gastric acid secretion. The H2 receptor also influences intestinal secretion and neurotransmission, and has been shown to mediate both pro-inflammatory and anti-inflammatory processes in the immune response.[1,8]

The presence of the H3 receptor in humans was confirmed approximately 25 years ago, and the receptor was successfully cloned in 1999.[2] The H3 receptor is unique, as it controls feedback of histamine release, particularly in the central nervous system, where it modulates neurotransmitter release and acts at the presynaptic histaminergic

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neurons, inhibiting release and synthesis of histamine.[3] H3 receptors are also involved in mucosal defense and inhibition of neurotransmitter release within the GI tract in humans.[10,11]

The newest histamine receptor to be discovered is the H4 receptor, which was identified in humans and cloned in 2000.[2] Genetically, the H4 receptor is closely related to the H3 receptor, but is restricted to cells within the spleen, intestines and thymus, and to immune cells such as T cells, mast cells, neutrophils and eosinophils.[2] In humans, the H4 receptor plays a significant role in regulation of inflammatory responses by inducing inflammatory cell chemotaxis and cytokine release from inflammatory cells.[9,12]

Histamine and histamine receptors have recently become a focus of intense interest in the area of chronic GI disease in people. Many different cell types store and secrete histamine in the GI tract, especially mast cells. Mucosal mast cell accumulation and increased mucosal and luminal concentrations of histamine have been documented in humans with chronic GI diseases such as Crohn's disease, ulcerative colitis, irritable bowel syndrome and allergic enteropathy.[1,2,8] In the first study to document expression of all histamine receptors in the human GI tract, mRNA associated with H1, H2, and H4 receptors was distributed equally in the mucosa, submucosa and muscular layers, and immunostaining patterns correlated with mRNA expression.[7] Later studies also confirmed the presence of the H3 receptor in the human GI tract.[10] Human patients with irritable bowel syndrome and food allergy have significantly higher expression of H1 and H2 mRNA within the GI tract compared to unaffected individuals.[7]

Rodent models have also been used to study GI tract histamine receptors. Studies using a commonly employed rodent model, in which the oxidizing hapten TNBS

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(trinitrobenzenesulfonic acid) induces GI inflammation that mimics Crohn's disease, have demonstrated increased expression of the H4 receptor in the GI tract (and correlation of colitis progression with receptor density.[11,13]

The role of histamine in canine inflammatory bowel disease (IBD) has not been thoroughly investigated, but dogs with IBD have been shown to have increased GI tract mast cell density, and some have high levels of urine N-methylhistamine, a metabolite of histamine.[14] In 2008, the canine H4 receptor was cloned, and PCR techniques were used to identify H4 receptor expression in the canine small intestine.[15] More recently, H4 receptor mRNA was identified in the canine colon.[16] To the authors' knowledge, the specific locations and distribution of all 4 histamine receptors in the entire canine GI tract have never been documented. The purpose of this study was to identify the presence and location of all four histamine receptors in the normal canine GI tract using immunohistochemical techniques. This information will provide additional research opportunities into the pathophysiology and treatment of canine chronic enteropathies.

Materials and Methods

Tissue Collection and Evaluation

Multiple biopsies were obtained from the GI tract of 6 adult, clinically healthy purpose-bred, colony-kept, research dogs immediately after humane euthanasia. These dogs were deemed healthy based on physical examination and history. A packed cell volume and total protein were performed prior to anesthesia in all dogs and were in normal range. All dogs were fed a standardized diet for at least 6 weeks before being euthanized as an endpoint of an unrelated project approved by the university's Institutional Animal Care and Use Committee (IACUC).[1] Specifically, for each dog, 2

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full-thickness wedge biopsies and 1 full-thickness 8 mm punch biopsy of the stomach, duodenum, ileum, jejunum, and colon were obtained immediately after humane euthanasia. All tissue sections were fixed in 10% neutral buffered formalin, routinely processed, embedded in paraffin and sectioned at 5μm for either staining with hematoxylin and eosin or for immunohistochemistry. Similarly, formalin-fixed paraffin-embedded tissue sections from a high grade malignancy (Grade III) canine cutaneous mast cell tumor were used as a positive control for H1, H2, and H4 receptor identification.[17]Peripheral nervous system tissue (intestinal ganglia) obtained from the full-thickness GI biopsies served as the positive control for H3 receptor identification.[2]

Evaluation of Gastrointestinal Inflammation

Gastric, duodenal, and colonic tissues from each of the 6 control dogs were evaluated for inflammation according to World Small Animal Veterinary Association (WSAVA) guidelines.[18]Tissue samples were sectioned at 5 μm and stained with hematoxylin and eosin and evaluated with light microscopy. Mucosal pathology and inflammatory cell infiltrates of the lamina propria were described according to WSAVA standards as normal, mild, moderate, or marked.[18]

Immunohistochemistry

A standard avidin-biotin-peroxidase complex technique was applied according to manufacturer recommendations (Dako LSAB2 System; Agilent Technologies, CA, USA) for immunostaining of histamine receptors. Briefly, all tissue sections were deparaffinized and rehydrated prior to antigen retrieval with heat and citrate buffer. Slides were rinsed in Tris-buffered saline (TBS) and endogenous peroxidase was

quenched with 3% H_2O_2 for 10 minutes at room temperature. Sections were rinsed with TBS and blocked with Dako protein block serum-free solution (Dako; Agilent Technologies, CA, USA) for 1 hour before incubation with primary polyclonal goat or rabbit histamine receptor antibodies.

Optimal primary antibody incubation times and concentrations were determined for the H1, H2, H3, and H4 receptor antibody. Sections were incubated at room temperature for 8 hours with the H1 (goat) and H2 (rabbit) primary antibodies (Acris Antibodies; CA, USA) at a dilution of 10 μg/ml. Sections were incubated at room temperature for 4 hours with the H3 (rabbit) primary antibody (Acris Antibodies; CA, USA) at a dilution of 5 μg/ml, and with the H4 (rabbit) primary antibody (Santa Cruz Biotechnology; CA, USA) for 2 hours at a dilution of 3 μg/ml. Negative controls were established for immunohistochemical evaluation by omission of the primary antibody for all experiments with the 4 histamine receptors.

After incubation with primary antibodies, sections were washed with TBS and incubated with a biotinylated goat anti-rabbit and goat anti-mouse secondary antibody for 30 minutes at room temperature, according to manufacturer recommendations (Dako; LSAB2 System-HRP; Agilent Technologies, CA, USA). After another rinse with TBS, the antigen-antibody complex was visualized with 3, 3' diaminobenzidine (DAB) chromogen solution (Dako; Agilent Technologies, CA, USA). Tissues were counterstained with hematoxylin and dehydrated with a series of ethanol and xylene before being mounted with cover slips. Tissue sections were examined under an optical microscope.

Histamine Receptor Scoring

Immunostaining intensity of H1, H2, H3, and H4 receptors were subjectively scored by a board-certified pathologist as follows: 0, negative; 1+, weak; 2+, moderate; and 3+, strong (Figure 2.1). Layers and tissues within the stomach, duodenum, jejunum, ileum, and colon tissue sections of each dog were evaluated and scored individually. Specifically, the superficial and deep mucosal layers and submucosa, smooth muscle, submucosal ganglia, and myenteric ganglia were scored in each section of the GI tract in all 6 dogs. Staining that was suspected to be non-specific was not scored.
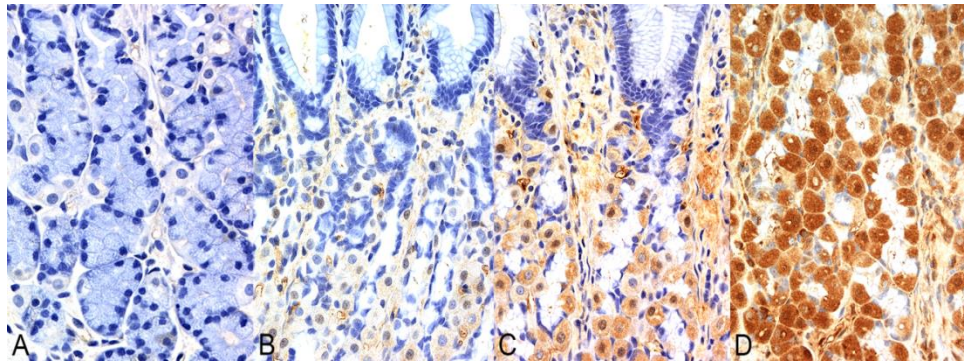
Figure 2.1   Histamine receptor scoring in the canine gastrointestinal tract

Immunostaining of histamine receptors in the canine gastrointestinal tract was scored as 0; negative (A), 1; mild staining (B), 2; moderate staining (C), or 3; strong staining (D).

Western Blot Technique

Histamine receptor expression was evaluated using Western blotting as previously described.[19]Gastric, small intestine, and colonic tissue samples were lysed with radioimmunoprecipitation assay (RIPA) buffer (3 ml RIPA per 1 gram of tissue) (Santa Cruz Biotechnology; CA, USA). For each ml of RIPA buffer, 10 μLs each of phenylmethylsulfonyl fluoride (PMSF) solution, sodium orthovanadate solution, and

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protease inhibitor cocktail solution (Santa Cruz Biotechnology; CA, USA) were added. Protein quantification of the lysates was achieved using the bicinchoninic acid (BCA) assay according to the manufacturer's instructions (Pierce Biotechnology; IL USA). Tissue lysates were resolved on a 10% SDS-PAGE and proteins transferred to nitrocellulose membranes. The membranes were blocked overnight and incubated for one hour at room temperature with the primary H1, H2, and H3 receptor antibodies at concentrations of 2 μg/mL and overnight at 4°C with the primary H4 receptor antibody at a concentration of 1 μg/mL. Membranes were also incubated overnight with commercially available smooth muscle actin monoclonal mouse antibody according to manufacturer recommendations at 1 μg/mL at 4°C (Dako; Agilent Technologies, CA, USA). This antibody is widely used in immunohistochemistry and served as a positive control for gastrointestinal tissue lysates.[20]Membranes were washed and then incubated with the appropriate alkaline phosphatase-conjugated secondary antibody (Sigma-Aldrich; MO, USA) for one hour and then washed. Immunoreactivities were revealed with the 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT) substrate (Sigma-Aldrich; MO, USA).

Statistical Analysis

To determine if there were differences in histamine receptor immunostaining scores among the gastrointestinal wall layers/tissues within the different sections of the gastrointestinal tract (stomach, duodenum, jejunum, ileum, and colon), a method similar to the non-parametric Friedman's test was conducted. Separate analyses were conducted for each layer/tissue. For each histamine receptor scored (H1, H2, H3, and H4) within a section of the gastrointestinal tract, the data was first ranked within each dog. An

analysis of variance using PROC MIXED in SAS for Windows v9.4 (SAS Institute Inc.; Cary, NC) was then conducted on the ranked data with dog and layer/tissue as fixed effects. To prevent type I errors due to multiple comparisons, differences in least squares means with Bonferroni adjustment were determined for histamine receptor scores.

Results

WSAVA Assessment of Gastrointestinal Inflammation

Based on WSAVA guidelines, gastric tissue was normal in all dogs except Dog 5, which had mild lymphocytic plasmacytic gastritis, mild mucosal atrophy, and mild hyperplasia of submucosal Peyer's patch lymphoid follicles. All dogs had mild lymphocytic plasmacytic duodenal inflammation, and Dog 4 had mild eosinophilic inflammation as well. Duodenal mucosal morphology was normal in all dogs. Mild eosinophilic colitis was present in Dog 4 and Dog 6. Colonic tissue in the remaining dogs was normal.

Western Blot Validation of Histamine Receptor Antibodies

Expression of the histamine receptor protein in gastric, small intestine and colonic lysates was examined by Western blotting (Figures 2.2A-2.2D). Expression of the smooth muscle actin protein at 50 kDa in all lysates was confirmed. The H1 receptor and H3 receptor antibodies yielded bands at the expected molecular weight of approximately 50 kDa. However, additional bands were present, suggesting non-specific binding of the H1 and H3 receptor antibody. Successful utilization of the H2 receptor antibody for Western blotting was not possible with any of the lysates, despite the known presence of H2 receptors in the canine stomach. For the H4 receptor antibody, a band was present at the

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expected molecular weight of approximately 50 kDa in the gastric, small intestinal, and colonic tissue lysates (Figure 2.2 D). This finding was repeatable, as was the absence of this band using the control anti-rabbit antibody, indicating excellent specificity of the H4 receptor antibody.
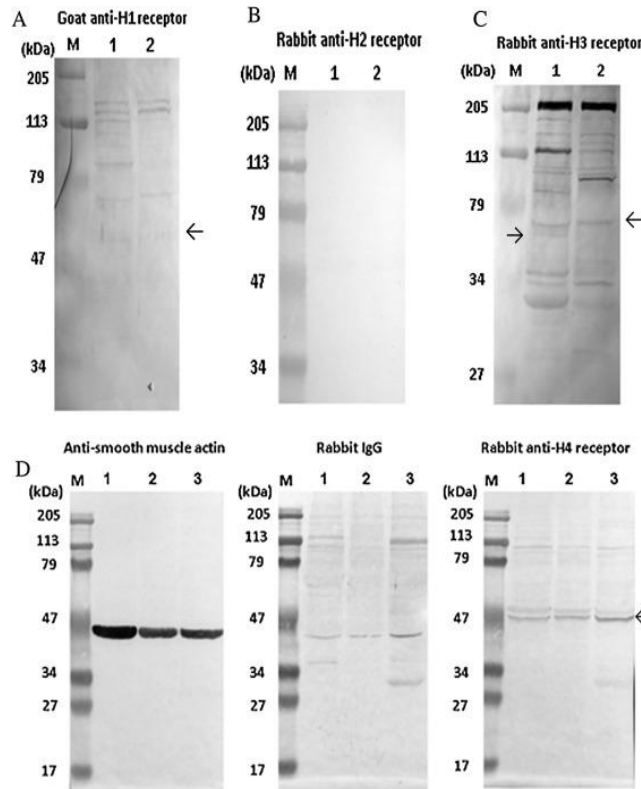
Figure 2.2   Western blots for histamine receptor antibodies

A A band (see arrow) at the expected molecular weight (approximately 55 kDa) of the H1 receptor was present in lysates of the stomach (Lane 1) and the duodenum (Lane 2). Additional bands are also present, consistent with non-specific staining noted with immunohistochemistry.
B A band at the expected molecular weight (approximately 50 kDa) of the H2 receptor was not obtained in lysates of the stomach (Lane 1) or the duodenum (Lane 2), despite excellent immunohistochemistry results.
C A band (see arrows) at the expected molecular weight (approximately 70 kDa) of the H3 receptor was present in lysates of the stomach (Lane 1) and the duodenum (Lane 2). Additional bands are also present, consistent with non-specific staining noted with immunohistochemistry.
D A band (see arrow) at the expected molecular weight (47-50 kDa) of the H4 receptor was present in lysates of the stomach (Lane 1), duodenum (Lane 2), and the colon (Lane 3) using the H4 receptor antibody but not with the rabbit control antibody. A strong band at the expected molecular weight of smooth muscle actin (42 kDa) in all tissue lysates was present.

Histamine Receptor Immunohistochemistry Descriptive Findings

Moderate to strong immunostaining of H1, H2, and H3 receptors and weaker staining of H4 receptors in mast cells was identified in the control mast cell tumor tissue sections. Moderate to strong endothelial staining of H1, H2, and H4 receptors was identified in the mast cell tumor sections. Strong immunostaining of the H3 receptor was present in control intestinal ganglia sections.

The presence and distribution of H1, H2, H3, and H4 receptors varied among layers and tissues within the canine GI tract. When present in a specific layer or tissue, the receptors were typically found consistently in all 6 dogs with minimal variation in location and staining intensity amongst dogs. No submucosal staining of histamine receptors was identified in any dog. Overall, the distribution of H1, H2, and H3 receptors was greater than that of H4 receptors throughout the entire GI tract.

The H1 receptor was located in all sections of the GI tract. Superficial and/or deep mucosal staining was only present in the stomach except in Dog 2, which had mild staining in the superficial colonic mucosa. Staining in the stomach was intermittent and included staining of parietal cell, chief cells and superficial epithelial cells (Figure 2.3A). Deep gastric mucosal staining was strongest and more uniform in the dog with mild gastritis (Dog 5) (Figure 2.3B). Lymphoid follicles in the ileum and colon were positive for the H1 receptor, with the strongest staining in lymphoblasts and germinal centers (Figure 2.3C). The H1 receptor was identified in all smooth muscle and ganglia. Non-specific staining of the H1 receptor antibody was identified in collagen and in enterocyte nuclei.

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Distribution of the H2 receptor in the GI tract was widespread in the mucosa of the stomach, duodenum, jejunum, ileum, and colon (Figure 2.3D-2.3F). Gastric mucosal staining was strong, especially in the parietal and chief cells (Figure 2.3D). Moderate to strong staining of lymphoid tissue was present in the duodenum, jejunum, ileum and colon. H2 receptors were not identified in smooth muscle but were present in all ganglia. Subjectively, the specificity of the H2 immunostaining was excellent, with consistently specific staining of parietal cells, chief cells and enterocytes in the small intestine and colon. No background or non-specific immunostaining was suspected.

The H3 receptor was most frequently located in the gastric mucosa and all ganglia (Figure 2.3G). Mucosal staining was present in the stomach and colon, but not in the small intestine. Gastric mucosal staining was typically greater in the superficial mucosal enterocytes, but the dog with mild gastritis (Dog 5) had strong staining in both the superficial and deep mucosa (Figure 2.3H). Lymphoid follicles of the ileum and colon were positive for the H3 receptor, and strong staining of a gastric lymphoid follicle was also present in the dog with gastritis (Figure 2.3I). The H3 receptor was identified in all smooth muscle and ganglia. Non-specific nuclear and collagen staining was present with the H3 receptor antibody, similar to that seen with the H1 receptor antibody.

Immunostaining for the H4 receptor was identified in gastric and colonic mucosa, but not in the small intestinal mucosa (Figure 2.3J and 2.3K). The strongest gastric staining was in the deep mucosa of the dog with mild gastritis (Dog 5). Lymphoid follicles in the ileum were weakly positive in Dog 1 and Dog 2 (Figure 2.3L). Smooth muscle and ganglia staining for the H4 receptor was mild to absent throughout the GI

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tract. Occasional non-specific collagen staining occurred with the H4 receptor antibody, but less so than with the H1 and H3 receptor antibody.
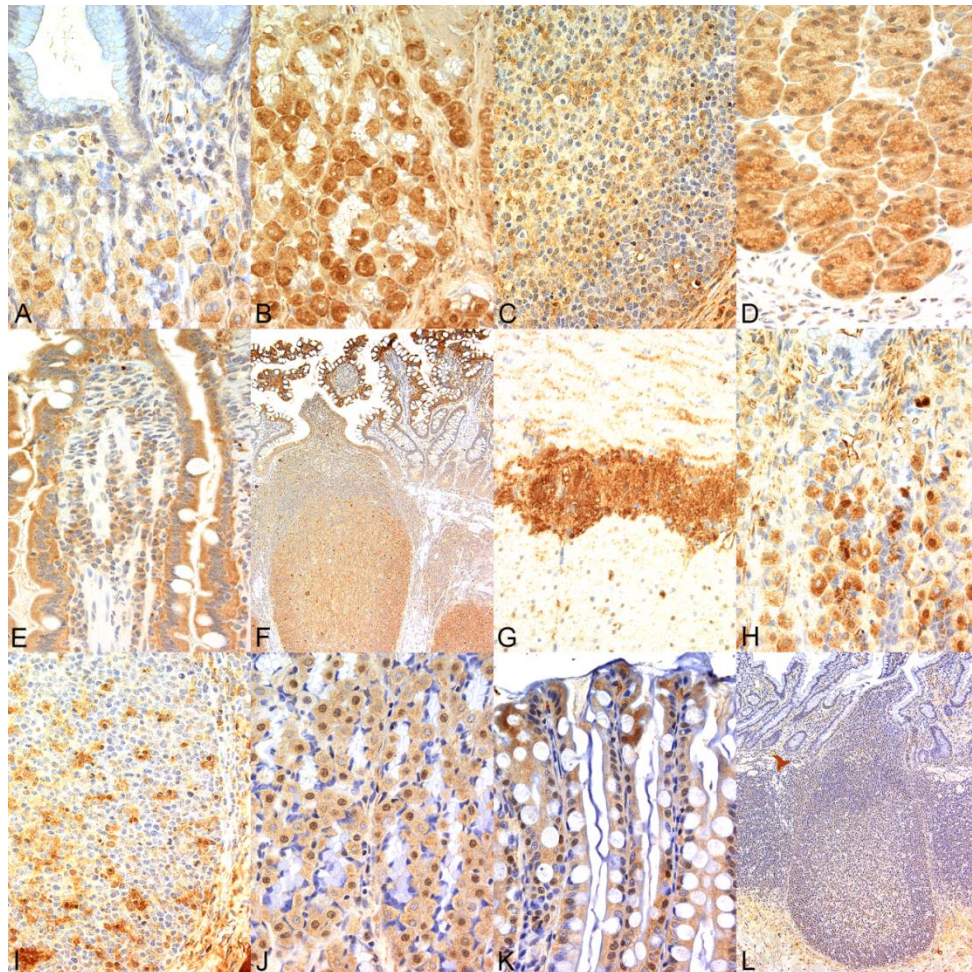
Figure 2.3   Histamine receptor staining patterns in the canine gastrointestinal tract.

Immunostaining of H1 receptor in the gastric mucosal parietal and chief cells was moderate (A) in most dogs but strong in the dog with gastritis (B). Lymphoblasts in the germinal center of a lymphoid follicle in the ileum stained positively for the H1 receptor (C). Moderate to strong staining of the H2 receptor in gastric mucosal parietal and chief cells (D), superficial duodenal enterocytes (E), and an ileal lymphoid follicle (F) is illustrated. All ganglia stained strongly for the H3 receptor (G) and moderate staining of gastric mucosal parietal cells (H) and a gastric lymphoid follicle (I) was identified. Staining for H4 receptor was moderate in the gastric mucosal parietal and chief cells (J) and superficial colonic enterocytes (K). Mild staining of the H1 receptor in an ileal lymphoid follicle is illustrated (L).

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Histamine Receptor Immunohistochemistry Statistical Analysis

For all 4 histamine receptors, there were multiple significant differences (p value < 0.05) among layers (superficial and deep mucosal layers) and tissues within each section of the gastrointestinal tract (Figure 2.4).

A — H1 Receptor

	Stomach	Duodenum	Jejunum	Ileum	Colon
Superficial Mucosa	2	0	0	0	0
Deep Mucosa	1	0	0	0	0
Lymphoid Tissue	0	0	0	1.5	0.5
Smooth Muscle	2	2	2	2	1
Submucosal Ganglia	2.5	3	3	3	2.5
Myenteric Ganglia	3	3	3	3	3

B — H2 Receptor

	Stomach	Duodenum	Jejunum	Ileum	Colon
Superficial Mucosa	0	3	3	3	3
Deep Mucosa	3	2	2.5	1.5	3
Lymphoid Tissue	0	3	3	3	3
Smooth Muscle	0	0	0	0	0
Submucosal Ganglia	3	2	2	2	2
Myenteric Ganglia	3	2	2	2	2

C — H3 Receptor

	Stomach	Duodenum	Jejunum	Ileum	Colon
Superficial Mucosa	2	0	0	0	2
Deep Mucosa	0.5	0	0	0	0
Lymphoid Tissue	0	0	0	2	2
Smooth Muscle	1	1	1	1	1
Submucosal Ganglia	3	3	3	3	3
Myenteric Ganglia	3	3	3	3	3

D — H4 Receptor

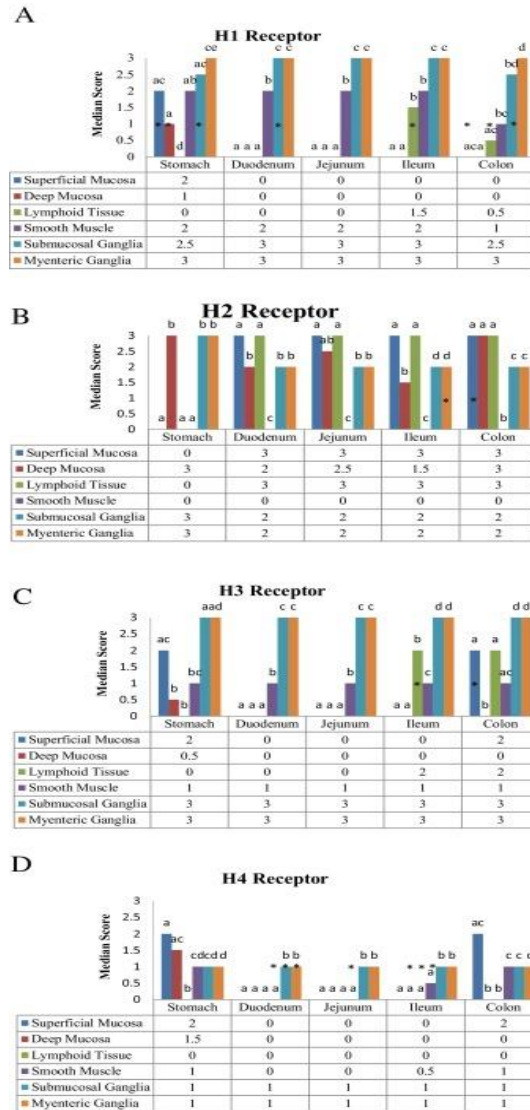|	Stomach	Duodenum	Jejunum	Ileum	Colon
Superficial Mucosa	2	0	0	0	2
Deep Mucosa	1.5	0	0	0	0
Lymphoid Tissue	0	0	0	0	0
Smooth Muscle	1	0	0	0.5	1
Submucosal Ganglia	1	1	1	1	1
Myenteric Ganglia	1	1	1	1	1

Figure 2.4   Distribution of histamine receptors in the canine gastrointestinal tract

Presence and intensity of staining for H1 (A), H2 (B), H3 (C), and H4 (D) is illustrated. The interquartile range (IR) for each median score is denoted by an asterisk (*) unless IR=0.

Differences in Histamine Receptor Distribution

In the stomach, there were significantly less H1 receptors in lymphoid tissue compared to all other targets. In the small intestine, significant differences in H1 receptor locations were comparable within the duodenum and jejunum. In contrast to the duodenum and jejunum, ileal lymphoid tissue had significantly more H1 receptors than the superficial and deep mucosa.

Gastric deep mucosa and ganglia had significantly more H2 receptors than other gastric layers/tissues. In the small intestine, there were significantly more H2 receptors in the duodenal superficial mucosa and lymphoid tissue than all other duodenal layers/tissues. Significant differences in H2 receptors were similar in the jejunum and ileum. H2 receptors in the colon were significantly greater in the superficial and deep mucosa and lymphoid tissue than in the smooth muscle and ganglia.

In the stomach, there were significantly more H3 receptors in the superficial mucosa than the deep mucosa. In the duodenum, jejunum, ileum, and colon, H3 receptors were significantly greater in the ganglia than other sites.

H4 receptors in the gastric superficial mucosa were significantly greater than all other sites except the deep mucosa. The lymphoid tissue had significantly less H4 receptors than all other layers/tissues. In the colon, H4 receptors in the superficial mucosa were significantly greater than the deep mucosa.

The authors refer the reader to Figures 3A-3D, which demonstrate additional statistically significant differences (p value < 0.05) in histamine receptor distribution among the different layers/tissues.

Discussion

Functions of histamine and histamine receptors have been studied for many years, and H1 and H2 receptor antagonists such as diphenhydramine and famotidine are used commonly in both human and veterinary medicine to treat a variety of conditions, including anaphylaxis, allergies, pruritus and GI ulcers. With the discovery of the H3 and H4 receptors, new roles for histamine have been elucidated, and new drug therapies are being explored.[3] An understanding of histamine receptor location and function is necessary for the development of novel histamine receptor antagonists and agonists.

The current study identified the presence and location of all four histamine receptors in the GI tract of 6 healthy adult dogs. Although no clinical signs of GI disease were noted, mild inflammation was noted in at least one section of the GI tract in all dogs based on WSAVA guidelines. Interpretation of GI inflammation in asymptomatic dogs is challenging for several reasons. A lack of agreement in regards to normal histology exists, despite development of the WSAVA guidelines.[21] Significant variation in intraepithelial lymphocyte numbers occurs between normal dogs as well.[18] Furthermore, in the absence of clinical signs or other clinical criteria for inflammatory bowel disease, the current veterinary literature does not explain the significance of histopathological abnormalities in the GI tract of healthy dogs.[18]

Distribution of histamine receptors in the intestines varies among species, but our findings are similar to those previously reported in humans and guinea pigs.[2,9] H1 receptors were located mainly in the gastric mucosa, smooth muscle, and lymphoid tissue, corresponding to the known function of this receptor in smooth muscle contraction and humoral immunity.[5] H2 receptors were most abundant in the gastric mucosa,

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supporting the major role of this receptor in controlling gastric acid secretion. The H3 receptor predominantly mediates neurotransmission, and findings of intense staining of the H3 receptor antibody in all ganglia of the canine GI tract are consistent with this function.[10] Similar to other species, the H4 receptor was not as widely distributed in the canine GI tract as the other histamine receptors.[11] H4 receptors were, however, located in the gastric and colonic mucosa, as well as in lymphoid tissue. In humans, the H4 receptor appears to have a major influence on many aspects of the immune response, including chemotaxis of neutrophils and eosinophils, and T cell responses.[4] Therefore, presence of the H4 receptor in lymphoid tissue was expected.

To date, canine-specific histamine receptor antibodies are not commercially available. In our study, we used antibodies predicted by the manufacturers to cross react with canine histamine receptors. The specificity of the H4 receptor antibody was confirmed with Western blotting, which was consistent with the limited amount of non-specific staining observed on immunohistochemistry. Non-specific staining of nuclei and collagen was noted with the H1 and H3 receptor antibodies on immunohistochemistry and, although Western blotting demonstrated bands at the expected molecular weights of these histamine receptors, additional bands were also present. In addition to non-specific binding of collagen and nuclear material, the additional bands could be due to the presence of isoforms of the H1 and H3 antibodies.[8] Despite some degree of non-specific staining, immunohistochemistry results with the H1 and H3 antibodies were typically consistent and repeatable among all dogs, and were also consistent with previously published findings in other species, including humans.

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The H2 receptor antibody yielded excellent immunohistochemical staining with no non-specific background staining, and results were consistent and repeatable. Immunohistochemical findings in the dogs in our study were also very comparable with previously documented H2 receptor distribution in other species.[2] Despite multiple attempts, a band at the correct molecular weight of the H2 receptor could not be obtained with Western blotting. The authors suspect that the rabbit H2 receptor antibody used in our study recognizes a native conformational protein instead of a linear sequence produced as a result of necessary reducing conditions for Western blot, and is, therefore, not compatible with Western blot methodology. To confirm the specificity of the H2 antibody, an additional method, such as immunoprecipation followed by mass spectrophotometry, would need to be performed.

Whether histamine receptor distribution is altered in canine GI diseases remains unknown, but human research has documented altered histamine receptor density in certain GI diseases.[4,7] Additionally, a novel H4 receptor antagonist, JNJ7777120, has been shown to reduce histological inflammation and clinical signs in experimentally-induced colitis.[22] Interestingly, the dog in our study with histologic evidence of mild gastritis had stronger mucosal staining for the H1 receptor and stronger lymphoid tissue staining for the H3 receptor compared to the dogs with normal gastric biopsies.

One limitation of our study was that all control dogs had histologic evidence of mild GI inflammation according to established WSAVA guidelines despite being apparently healthy purpose-bred research dogs housed in a controlled environment with no history of vomiting, diarrhea or other signs of GI disease, and despite having normal physical examinations and negative fecal flotations.[18] These findings are similar to

previous reports of mild histopathology changes in asymptomatic healthy, control dogs.[23,24] Histology of the canine GI tract is affected by many factors, including age, intestinal parasites, diet, intestinal flora, medications, and environmental allergens.[18,24] All dogs received monthly topical parasiticide[2] and deworming every 3 months;[3] therefore intestinal parasitism is an unlikely explanation for the mild GI inflammation noted. The authors agree with the observation by Junginger et al, that mild histopathological abnormalities may simply represent background variation in the GI tract of normal dogs.[23] We, therefore, believe that, with the possible exception of the dog with mild gastritis, the histologic findings in the dogs in our study are consistent with a healthy GI tract, and that the histamine receptor distribution reported in our study reflects the distribution in clinically normal dogs.

Our study has established immunohistochemical techniques for the staining of H1, H2, H3, and H4 receptors in canine GI tissue, and described the distribution of these receptors in normal dogs. The results of our study provide the foundation for similar work in dogs with GI disease. Future research in the role of histamine receptors in canine IBD, for example, may provide evidence to suggest additional treatment options for a common, yet sometimes refractory, disease.

Conclusion

All four histamine receptors were located throughout the canine GI tract using immunohistochemistry. Differences in their distribution patterns were appreciated amongst the different segments of the canine GI tract, as well as in the different histologic layers and tissues. The presence of each receptor was typically consistent among and within dogs. Based on our findings, H1, H3, and H4 receptor density may be

increased with gastritis, but further investigation is necessary, as only one of the dogs in our study had mild gastritis. The results of our study will provide valuable baseline information in healthy dogs as a basis for additional histamine receptor research. Histamine blockade with novel antagonists may have therapeutic implications for GI inflammation in the dog, and further investigation of the distribution of histamine receptors in dogs with GI diseases such as IBD is, therefore, warranted.

Footnotes

1. Institutional Animal Care and Use Committee (IACUC) #12-065, approval granted on 8-21-2012 and expired on 8-20-2015 for the 4th year veterinary student elective course, Advanced Small Animal Surgery CVM 5754

2. Advantage Multi®; Bayer, Kansas, USA.

3. Drontal® Plus; Bayer, Kansas, USA

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CHAPTER III

CONCLUSION

In this study, the presence and distribution of all four histamine receptors (H1, H2, H3 and H4) were documented in the dog for the first time. An immunohistochemical technique to study canine histamine receptors was established using commercial goat and/or rabbit antibodies predicted to cross react with the canine histamine receptors. Despite some non-specific staining with the H1 and H3 receptor antibodies, interpretation of staining patterns was straightforward, and results were repeatable with all 4 receptor antibodies. The location and distribution of histamine receptors in 6 clinically normal adult dogs were very similar to findings in humans.[1,2] Furthermore, findings were consistent with the mRNA distribution of the histamine receptors in previous canine research.[1,3]

Additional research opportunities may now be explored utilizing the immunohistochemical technique established in normal dogs. Of particular interest is the possible role of histamine receptors in canine inflammatory bowel disease, and the potential to explore the use of new histamine receptor antagonists in the dog. In collaboration with the Texas A&M University Gastroenterology Laboratory, the correlation of canine urinary and/or fecal N-methylhistamine levels with gastrointestinal histamine receptor expression in canine enteropathies may now be investigated. Similar research in cats has not been conducted in veterinary medicine, but the current study

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provides a foundation for future work in this species. In addition to advancing research in veterinary gastroenterology, histamine receptor research also opens the door for advancing scientific knowledge in small animal veterinary dermatology, oncology, and respiratory medicine.

Use of validated histamine receptor antibodies for additional research is important for producing reliable and repeatable results. Initial validation methods include verification of antibody specificity with western blotting to confirm a band at the expected molecular weight of the antibody's target protein. Use of positive and negative controls with the test antibodies also provides valuable information about antibody specificityl. Staining patterns with the antibodies should be consistent with known biological functions. Furthermore, an important criterion for antibody validation is reproducibility of staining patterns with different lots of antibodies and between multiple runs.

To date, commercial canine-specific histamine receptor antibodies are not available. The H1 and H3 histamine receptor antibodies used in the current study are derived from the goat and rabbit; however, based on shared genetic sequences, these antibodies should cross react with the canine species. Staining was present in positive control tissues, was repeatable between antibody lots and runs with these antibodies, and was consistent with previously established functions of the H1 and H3 receptors. However, since there was a degree of non-specific staining using current antibodies, exploration of other available H1 and H3 receptor antibodies for clinical use in further canine research would be ideal. Current literature documents particular challenges with the H4 receptor antibodies with regards to poor specificity and cross-reactivity across

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species.[1,4,5] However, western blot results for the H4 receptor antibody used in the current study were encouraging, indicating good specificity in canine intestinal tissue. Immunostaining with the H2 receptor antibody was superior to all other antibodies, despite failure to yield a band on western blot after undergoing standard denaturing processing. Therefore, additional validation methods will be sought for this antibody, which appears very specific for canine histamine receptors. Both the H2 and H4 receptor antibodies demonstrated excellent and consistent staining between antibody lots and runs, and staining correlated with published biological functions and tissue distribution, including in positive control tissues. In the author's opinion, these antibodies are worthy of continued use in clinical canine histamine research using the described immunohistochemistry technique.

Results of the study provide valuable information necessary to move forward with further research in the canine gastrointestinal tract. Of particular interest is the role of these receptors in canine inflammatory bowel disease, which shares similarities with Crohn's disease and ulcerative colitis. Inflammatory bowel disease (IBD) is a common diagnosis in young adult and middle-aged dogs displaying mild to severe gastrointestinal (GI) signs. A definitive diagnosis is based on a combination of clinical signs and histologic analysis of biopsy samples obtained from the GI tract. There is considerable variation in clinical signs and histologic disease severity amongst canine IBD patients. Treatment for canine IBD typically involves specific dietary changes and modulation of the immune system. Unfortunately, not all patients respond to standard treatment modalities. Individual-to-individual differences in disease severity and treatment responsiveness in our canine IBD patients highlight the pressing need to better

understand the pathogenesis of this important condition. Clinically, patients who have comparable histological findings on GI biopsy can respond very differently to similar treatment regimens: some dogs have minimal response to aggressive therapy, while other respond well. Commonly used canine IBD protocols have been shown to lead to only partial or no treatment responses in up to 50% of patients.[6] In one large retrospective study of canine IBD, 13% of affected dogs had intractable disease and were euthanized, and a further 50% still had intermittent clinical signs despite treatment with a variety of immunosuppressants and antibiotics.[7] In another large prospective study in dogs with chronic enteropathies, 18% of dogs were euthanized due to refractory disease.[8] This lack of predictability in response to therapy serves to underscore our limited understanding of certain aspects of IBD in dogs, including the role of histamine receptors.

Histamine receptor distribution within the human GI tract has been extensively studied over the past decade, and alterations in histamine levels and histamine receptor expression have been described in human patients with chronic enteropathies such as ulcerative colitis and Crohn's.[1,9] Human patients with irritable bowel syndrome and food allergy have higher expression of H1 and H2 mRNA within the GI tract compared to unaffected individuals.[2] Rodent models of inflammation have also been used to study GI tract histamine receptors. Studies using a common mouse model of GI inflammation that mimics Crohn's disease in humans, for example, have demonstrated increased expression of the H4 receptor in the GI tract, and a correlation of colitis progression with receptor density.[9,10]

Application of the current technique to evaluate histamine receptor distribution in canine IBD is an important next step in this research. Findings may not only yield

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valuable knowledge in the realm of veterinary gastroenterology but also in human gastroenterology, since canine and human chronic enteropathies appear to share similar etiologies. A naturally-occurring canine model of inflammatory bowel disease could prove more valuable, for instance, than an experimental rodent model. The current immunohistochemical technique can readily be applied to full-thickness biopsies obtained routinely in dogs with chronic enteropathies. Application of the technique to gastrointestinal endoscopic biopsies will help determine the feasibility of using mucosal pinch biopsy tissue samples in future research. A retrospective study using archived tissue samples, as well as prospective studies, can be conducted using the current staining technique. Immunostaining patterns in affected dogs can be compared to those of the six clinically normal dogs in the current study, in order to determine if significant differences in histamine receptor distribution and density occur in dogs with chronic enteropathies such as IBD. In a prospective study, concurrent evaluation of urinary and fecal N-methylhistamine levels would further aid our understanding of the pathophysiological role of histamine in canine IBD, and help determine the usefulness of this metabolite as a potential biomarker of gastrointestinal disease in dogs.

The large body of evidence supporting the role of histamine and histamine receptors in intestinal inflammation in human patients and rodent models suggests a potential therapeutic use for histamine receptor antagonists in chronic intestinal diseases.[11,12] The histamine receptors vary in location, histamine binding ability, signaling, and functions; therefore antagonism of these receptors has a variety of therapeutic potentials.[13] In particular, the recently developed histamine H4 receptor antagonists have gained increasing interest as a novel therapeutic approach to a wide

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range of inflammatory conditions. This is due to the unique location of the H4 receptors on the immune cells that play an integral role in the pathogenesis of inflammatory conditions such Crohn's disease.[14] H4 receptor ligands have been used in multiple studies over the last decade to demonstrate the role of the H4 receptors in gastric acid secretion, intestinal motility, mucosal defense, abdominal pain, intestinal immunity, and carcinogenesis.[9] In experimentally-induced GI inflammation in a rodent model, a beneficial effect of H4 antagonists was seen in reducing intestinal damage, inflammatory cell infiltration, and release of IL-6 and TNFα, two cytokines known to play an important role in the pathogenesis of human IBD.[10] Additionally, in a mouse model of peritonitis, H1 and H4 receptor antagonists both decreased influx of inflammatory cells.[11] The H1 receptor antagonist pyrilamine has been shown to inhibit histamine release and ion transport in patients with inflammatory bowel disease, suggesting a potential role for antihistamines in treating the clinical signs associated with IBD.[15] The H1 receptor antagonists loratadine and ketotifen reportedly decrease clinical signs of IBD such as abdominal pain and bloody stool in people with active IBD (loratadine) and children with ulcerative colitis (ketotifen).[16]

If continued research into histamine receptors in canine chronic enteropathies suggests involvement of/or alterrations in gastrointestinal histamine receptors, then evaluation of new histamine antagonists in dogs will be warranted. The anti-inflammatory properties of H4 receptor antagonists, in particular, are intriguing, and may be relevant to the treatment of canine inflammatory bowel disease. Pharmacokinetic studies determining efficacy and safety of the H4 antagonists have not been conducted in veterinary medicine. This work could prove to be revolutionary in the treatment of

chronic canine enteropathies, given the promising results associated with H4 antagonists in experimentally-induced gastrointestinal inflammation in rodent models and in pre-clinical human trials.

Certainly, histamine receptors have a significant role in human gastrointestinal immunology, and scientific evidence supports their role in gastrointestinal pathology. There is a pressing need to better understand the pathogenesis of chronic intestinal diseases in the dog, and to develop new treatment strategies, particularly for inflammatory bowel disease. The newly established immunohistochemistry technique and results of the current study will now serve as the basis for future histamine receptor research in veterinary medicine.

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