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The Role of Mucosal Mast Cells in Injury and Defence in the Gastrointestinal Tract

by

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ABSTRACT

THE ROLE OF MUCOSAL MAST CELLS IN INJURY AND DEFENCE IN THE GASTROINTESTINAL TRACT

Mast cells are found in considerable numbers in the gastrointestinal mucosa and contain mediators which have potent effects on other cells. Knowledge of the physiological and pathological effects of mast cells in the gastrointestinal tract is incomplete. The broad objective of the research described in this dissertation was to examine the role of mast cells in injurious reactions in the gastrointestinal tract. Central to this work were techniques for modifying mast cell numbers and assessing mast cell activation *in vivo*.

Studies were undertaken to determine how mast cells modulate mucosal integrity. Although increasing or decreasing the number of mast cells was without effect, acute antigenic activation of mast cells significantly increased susceptibility of the stomach to injury induced by ethanol. Separate studies described and characterized an interesting phenomenon whereby chronic oral antigen challenge of sensitized rats caused mucosal mast cells to become unresponsive to subsequent antigen-induced degranulation.

Experiments were performed to explore the contribution of mast cells to gastric injury induced by NSAIDs. Mast cell degranulation did not occur after NSAID administration, and the extent of injury was not affected by changes in gastric mucosal mast cell numbers. Moreover, mast cells did not appear to be the targets of

prostaglandins used clinically to reduce NSAID-induced injury. Therefore, mast cells do not play a significant role in the pathogenesis of experimental NSAID-gastropathy.

Studies were conducted to examine whether changes in hepatic mast cell numbers and mediator content were associated with fibrotic changes in experimental biliary cirrhosis. Hepatic mast cell hyperplasia and degranulation occurred during prolonged cholestasis in the rat. Although these changes did not correlate with the onset of hepatic fibrosis, they did occur when significant deposition of collagen was taking place. Hepatic mast cells, by releasing profibrogenic mediators, may contribute to fibrotic changes in biliary cirrhosis.

Overall, this work shows that mast cell activation does not underlie the deleterious effects of common ulcerogens in the gastrointestinal tract. However, specific activation of mast cells does compromise mucosal defence. These studies also highlight the dynamic nature of mucosal mast cells in terms of number and mediator release.

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List of Abbreviations and Symbols

©	copyright
×	magnification
°C	degree(s) Celsius
~	approximately
′	minute
ANOVA	analysis of variance
anti-IgE	antibody directed against immunoglobulin E
BDR	bile duct resection
CTMC	connective tissue mast cell
ELISA	enzyme-linked immunosorbent assay
g	acceleration due to gravity
HCl	hydrochloric acid
IgE	immunoglobulin E
IP	intraperitoneal
IV	intravenous
KCl	potassium chloride
LTC_4	leukotriene C_4
mm Hg	millimetres of mercury
MMC	mucosal mast cell
mmol/L	millimolar
N	normal, concentration of ionizable groups
NS	not significantly different
NSAIDs	nonsteroidal anti-inflammatory drugs
OA	chicken ovalbumin
P	probability
PAF	platelet-activating factor
PBS	phosphate buffered saline, pH 7.4
PCA	passive cutaneous anaphylaxis
RMCP-II	rat mast cell protease II
SC	subcutaneous
SEM	standard error of the mean

All measurements are expressed using Système International units and prefixes.

When I'm running each day, I take one day at a time. When I'm running, I take one mile at a time and I take one corner at a time. Every signpost, I'm looking at it and reaching out for it. Every corner, I'm reaching out...

- Terry Fox (1958-1981)

CHAPTER 1

GENERAL INTRODUCTION

The first documented observation of mast cells occurred in 1863 when von Recklinghausen published excellent illustrations of granular cells in unstained frog mesentery.[1] However, the unique nature of these cells was not appreciated until Paul Ehrlich observed that they stained violet or red when treated with the basic aniline dye, dahlia. He was so impressed by the abundance of metachromatic granules contained by these cells that he referred to them as "mastzellen," which in German means "well-fed cell." Having refined the techniques for tissue fixation and histochemical staining to best visualize mast cells, Ehrlich then described their tissue distribution in various animal species. He also gained insight into the potential physiological and pathological significance of mast cells when he noted that they were closely associated with blood vessels, nerves and epithelial cells, and were increased in number in neoplastic and inflammatory lesions. Ehrlich's descriptive and experimental studies of mast cells were compiled in his doctoral thesis which he presented in 1878.[2] Ehrlich also described a circulating leukocyte which contained cytoplasmic granules with staining properties similar to those of mast cells.[3] This blood-borne cell would eventually be known as a basophil.

In the century subsequent to the discovery of mast cells, intensive scientific study has been aimed at better understanding their cellular workings, immunological functions,

and role in human disease. This has certainly been a fruitful area of work for many investigators. The discovery of histamine, for example, and the relationship between mast cells and immunoglobulin E certainly represent landmark achievements in medicine. Progress in this area has proceeded at such a rapid pace that much is now known about the origin and ontogeny of mast cells, the signals and second messengers involved in their activation, and pharmacological agents which modify the actions of mast cells.

In this chapter, the immunology and cellular biology of mast cells will be reviewed. This will, by no means, be an exhaustive treatment of these subjects. Instead, emphasis will be placed on existing knowledge about mast cells in the gastrointestinal tract. In addition, some new or emerging concepts about mast cells will be highlighted. This chapter ends with a statement of the objectives which guided the work presented in this dissertation. Additional introductory information will be provided in chapters 3 to 6 to aid understanding of the methods and results described therein.

1.1 Origin, Growth, and Differentiation

Much insight about the origin and ontogeny of mast cells has been gained from the development of techniques to culture mature mast cells *in vitro*[4] and studies of genetically mast cell-deficient mice.[5] As will be described in this section, mast cells are derived from multipotent hematopoietic precursors which originate in the bone marrow. These cells circulate throughout the body via blood and migrate to various sites (*e.g.* peritoneal fluid, skin, mucosal surfaces) where they complete their differentiation to

mature mast cells. The phenotype of mature mast cells is determined by soluble factors and physical interactions with cells in the local micro-environment.

Discovery of mast cell progenitors. Mast cells were first cultured *in vitro* by Ginsberg from dispersed mouse thymus in the presence of serum and fibroblasts, suggesting that mast cell precursors were lymphoid in origin.[6] It was later shown that large, pure cultures of mast cells could also be obtained from mouse bone marrow cells when grown in the presence of accessory factors from mitogen-stimulated T-cells.[7] Definitive proof that mast cells are derived from a bone marrow stem cell has come from elegant studies performed by Kitamura using genetically mast cell-deficient *W/W^v* mice.[8] In these studies, allogeneic transplantation of bone marrow from normal +/+ mice to mast cell-deficient *W/W^v* mice restored the full complement of mast cells in the recipient mice.

As will be described in greater detail in the next section, mast cells display considerable phenotypic heterogeneity. The diversity of mast cell phenotype relates not to the presence of multiple distinct mast cell precursors, but rather to effects of local micro-environment on phenotypic expression. This was demonstrated by studies in which cloned mast cell precursors were transferred by micro-injection into skin or gastric mucosa of mast cell-deficient *W/W^v* mice.[9] The common precursor gave rise to mature mast cells with distinct phenotypes at each site.

Although basophils are very similar to mast cells in terms of mediator content and expression of the high affinity IgE receptor, it is important to note that it has never been proven that they originate from a common progenitor cell.[4]

Factors involved in mast cell differentiation. Compared to mature mast cells, mast cell progenitors are large, rapidly dividing, and agranular.[4] Acquisition of high affinity receptors for IgE ($Fc_{\varepsilon}RI$) is an early event which indicates committed differentiation.[10] Further marking the process of maturation is the accumulation of cytoplasmic granules containing histamine and proteoglycan. As previously stated, factors derived from activated T-lymphocytes are vital to the successful growth and differentiation of murine mast cells *in vitro*. This essential T-cell-derived factor has been shown to be interleukin-3.[11] The ability of IL-3 to promote mast cell growth and differentiation have, more importantly, been demonstrated *in vivo*. For example, IL-3 is a critical mediator of the mucosal mast cell (MMC) proliferative response observed in nematode infection in rodents,[12] and repeated administration of purified IL-3 to athymic mice causes a significant increase in intestinal MMC.[13]

Interleukin-4 is also an important mast cell growth factor. Although IL-4 alone does not promote differentiation of rodent mast cells from cultured bone marrow cells, it potently enhances the effects of IL-3.[14] Considering the ability of IL-4 to promote B-cell isotype switching to produce IgE antibodies, it is believed that concurrent production of IL-3 and IL-4 during nematode infection co-ordinates mast cell hyperplasia with the IgE response.[15]

Stem cell factor (SCF) is a fibroblast-derived growth factor which is essential to mast cell development. The critical role of SCF in mast cell development is best illustrated by the fact that mice that do not produce SCF due to a genetic mutation are devoid of mature mast cells.[16, 17]

Genetically mast cell-deficient rodents. Mice which have mutant alleles at the dominant spotting (*W*) locus on chromosome 5 have varying degrees of mast cell deficiency. For example, mice with two mutant alleles, designated W/W^v, have less than 1% of the normal number of mast cells in skin and are completely lacking mast cells at other sites such as the stomach.[8] In addition, W/W^v mice are anaemic, infertile, and lack fur pigmentation.

The precise nature of the genetic mutations in W/W^v mice is known (*see* Nocka *et al.*[18]). The *W* locus contains the *c-kit* proto-oncogene which encodes a transmembrane receptor whose signalling capacity is dependent upon the tyrosine kinase activity of its intracellular region. The mutation designated W^v results in the substitution of a single amino acid in a region of the tyrosine kinase domain which is critical to its catalytic activity. The *W* mutation refers to a deletion of the membrane-spanning region of *c-kit* which abrogates its surface expression.

Mast cell deficiency has also been observed in mice that have two mutant alleles at the Steel (*Sl*) locus on chromosome 10. The Steel locus contains the gene for stem cell factor (SCF) which is a ligand for the *c-kit* receptor.[17] In the *Sl* mutant allele, all of the coding sequence for SCF has been deleted, whereas in Sl^d mutants, this sequence has been truncated. In mice with the genotype Sl/Sl^d, the absence of functional SCF causes anaemia and profound mast cell deficiency which are corrected when exogenous SCF is administered.[17] However, in contrast to W/W^v mice, mast cell deficiency in Sl/Sl^d mice is not corrected by bone marrow transplantation.

Therefore, essentially the same mechanisms underlie mast cell deficiency in *W/W^v* and *Sl/Sl^d* mice. In the case of *Sl/Sl^d* mice, there is a deficiency in SCF which promotes mast cell proliferation and maturation. In *W/W^v* mice, SCF is present, but mast cell progenitors are unable to respond to it due to a defective SCF receptor.

Genetically mast cell-deficient rats have also been developed. *Ws/Ws* rats are homozygous mutants at the "white spotting" locus and, as a result, are devoid of both MMC and CTMC.[19] The *Ws* mutation has been identified as a 12-base deletion in the gene encoding the *c-kit* receptor which dramatically reduces the tyrosine kinase activity of the functional protein.[20] Like *W/W^v* mice, *Ws/Ws* rats lack coat pigmentation and are severely anaemic at birth.[19] However, *Ws/Ws* rats are able to reproduce and the anaemia becomes less severe in adulthood. Since mast cells in the rat gastrointestinal tract are better characterized than in any other animal, *Ws/Ws* rats will be particularly useful for studying the biological effects of mast cells in organs of the digestive system. There is also a definite practical advantage to the larger size of the rats compared to mice. However, the use of *Ws/Ws* for scientific experimentation has been limited by the fact that they are not widely available.

Genetically mast cell-deficient rodents are valuable tools for studying the origin and ontogeny of mast cells, factors controlling phenotypic expression, as well as probing the role of mast cells in various physiological and pathological responses. Galli and Kitamura have been great proponents of the use of mast cell-deficient mice and have placed them at the centre of a systematic approach to study the physiological/pathological effects of mast cells.[16] However, it should be noted that the use of mast cell-deficient

rodents is not without disadvantage. For example, the severe and persistent anaemia that occurs in genetically mast cell-deficient rodents certainly complicates interpretation of experiments involving such animals. Another consideration when using mast cell-deficient rodents is that there are generally always redundant mechanisms of physiological control and therefore a deficiency of mast cells may be compensated for by other cells. These adaptations would tend to confound the interpretation of information derived in this manner.

Depletion of MMC by corticosteroids. In rats, corticosteroids have been shown to cause rapid and near-complete depletion of MMC, without significantly affecting the CTMC population.[21] This is not simply due to degranulation of mucosal mast cells or phenotypic changes rendering them histochemically undetectable. Rather, depletion of MMC by acute corticosteroid treatment is due to their engulfment by macrophages and subsequent phagocytosis.[22] This process occurs without significant release of mast cell-derived mediators and is therefore not associated with inflammation.

The growth and differentiation of mast cell progenitors, and the continued functional integrity of mature mast cells are intimately dependent upon lymphocyte-derived cytokines and growth factors, most notably IL-3.[11-13] However, inhibited production of such factors by corticosteroids does not appear to underlie their ability to deplete mucosal mast cells, as in congenitally athymic *rnu/rnu* rats, the virtual absence of functional T cells does not preclude depletion of MMC by dexamethasone.[23] Instead, corticosteroids may act directly on mast cells to induce apoptosis. The process of programmed cell death may then signal the actions of macrophages.

Corticosteroids are widely used in the treatment of allergic diseases such as asthma, hay fever, and urticaria (reviewed by Schleimer[24]). Their effectiveness in these conditions is linked to their ability to suppress antigen-induced histamine release *in vitro*. Depletion of mucosal mast cells may also underlie this effect *in vivo*. In addition, it has been speculated that the beneficial effects of glucocorticoids on chronic inflammatory diseases may be due, in part, to their effects on mast cells. In an experimental setting, dexamethasone-induced depletion of gastrointestinal MMC serves as a useful tool for studying the involvement of mucosal mast cells in physiological and pathological processes.

Targeting of mast cells to the GI tract. The following paragraph summarizes studies performed by Guy-Grand *et al.*[12] which have shed light on how the gut becomes populated by MMC and what mechanisms underlie the MMC proliferative response in intestinal nematode infection in rodents. These investigators have shown that precursors of mast cells are present in large numbers in the intestinal mucosa of mice. Since the essential source of these precursors is the bone marrow, there must be mechanisms in place which allow mast cell progenitors to migrate to the gut. The scarcity of mast cell progenitors in various lymphoid organs or thoracic duct lymph suggests that mucosal mast cell precursors migrate directly from blood to the gut mucosa. The studies of Guy Grand *et al.*[12] of morphology and rate of unstimulated proliferation suggested that mast cell progenitors in the gut are more differentiated precursors than those found in bone marrow. During *N. brasiliensis* infection, profound intestinal MMC hyperplasia occurs due to local release of IL-3 from T cells which promotes division and differentiation of

MMC precursors resident in the gut. MMC hyperplasia occurs at sites in the gastrointestinal tract that are distant to the focus of infection, in part, because mast cell precursors, stimulated by local IL-3 release, migrate in efferent lymph. The work of Guy-Grand *et al.*[12] suggests that the increase in mast cell numbers in *N. brasiliensis* infection occurs as a result of the combined effects of division and development of resident and recruited mast cell progenitors.

1.2 Phenotypic Heterogeneity of Mast Cells

Detailed histochemical and morphological studies performed by Enerback have shown that at least two distinct phenotypes of mast cells exist in rats.[25] This classification scheme also generally applies to other animals, including humans (reviewed by Irani and Schwartz[26]). Other phenotypic differences among mast cells have been recognized and include the types and amounts of mediators produced, sensitivity to secretagogues, and sensitivity to agents which inhibit mediator release.

Histochemical staining. Mast cells were originally divided into two separate classes according to histochemical properties. In 1966 Enerback showed that mast cells in the rat small intestine required special fixation techniques in order to be stained by the cationic dyes such as alcian blue.[25] Enerback demonstrated that a non-aldehyde fixative such as Carnoy's solution preserved the dye-binding capacity of intestinal MMC, whereas mast cells from intestinal serosa, skin, and peritoneum could be stained after conventional fixation in neutral buffered formalin. Although originally distinguished on the basis of

their sensitivity to formalin fixation, the two basic types of rodent mast cells have been named according to their general anatomical location: mucosal mast cells (MMC) and connective tissue mast cells (CTMC).

When rat intestine is fixed with Carnoy's solution and stained with alcian blue, both MMC and CTMC will bind the blue dye, but when safranine O is applied to the same preparation as a counterstain, only CTMC will stain red.[25] This serves as a useful technique for distinguishing MMC and CTMC in the same histological section.

Chemical mediators. Proteoglycans are large complexes of polysaccharide and protein which form a major component of mast cell granules where they serve as a substrate for other mediators such as cationic proteases and histamine.[26] The different affinity of rat CTMC and MMC for cationic dyes is due to differences in granule proteoglycans. Chondroitin sulfate proteoglycans predominate in MMC granules, whereas the more highly sulfated heparin is the major component of CTMC proteoglycan.

MMC cells isolated from rat small intestine contain approximately one-tenth the amount of histamine found in mast cells isolated from the rat peritoneum (1.3 pg/cell compared with 15 pg/cell).[27] However, due to differences in the rigors of handling during preparation of MMC and CTMC for such measurements or differences in state of maturity or activation of these mast cell populations, this may not accurately reflect levels of histamine found *in vivo*.

Neutral serine proteases are present in substantial quantities in the exocytotic granules of rodent mast cells. In rats, two distinct proteases have been described. Rat

mast cell protease I (RMCP-I) is found almost exclusively in CTMC whereas rat mast cell protease II (RMCP-II) is found in MMC.[28] As described below, the release of RMCP-II serves as a very useful marker of mucosal mast cell activation.

Eicosanoids are lipid mediators which are synthesized *de novo* when mast cells are activated by antigen. In rat mucosal mast cells, the major eicosanoid produced is leukotriene C_4 (~30 pg/10^6 cells), whereas PGD_2 is the major lipid mediator derived from activated rat CTMC (~10 pg/10^6 cells).[26]

Sensitivity to secretagogues. Both MMC and CTMC degranulate when surface-bound IgE molecules are crosslinked by exposure to the sensitizing antigen. The neuropeptide substance P is also a physiologically relevant activating stimulus for both CTMC and MMC.[33] However, differences in responses to stimuli which are not immunologically relevant further highlight phenotypic differences between rodent mast cells. For example, the polycationic compound 48/80 is a very effective stimulus for degranulation of CTMC, but not MMC.[26]

Sensitivity to agents which inhibit mediator release. Many pharmacological agents have been developed which effectively inhibit activation and release of mediators from mast cells.[26] It is clear, however, that these agents are not equally effective on MMC and CTMC. For example, disodium cromoglycate and theophylline effectively inhibit antigen-induced histamine release from CTMC but not from rat intestinal MMC. In contrast, quercetin and doxantrazole (both structurally related to sodium cromoglycate) inhibit antigenic activation of MMC as well as CTMC. It is remains unclear how these agents inhibit mediator release and why some have different effects on MMC and CTMC.

Although Enerback's original system of classifying mast cells as either MMC or CTMC[25] is convenient, recent evidence suggests that this may be a gross oversimplification. Tainsh and Pearce have shown that within a group of mast cells which are typically classified as CTMC (*i.e.* those isolated from rat skin, peritoneum, mesentery and lung), there is considerable difference in terms of histochemical staining.[29] This is believed to reflect a fundamental biochemical difference, that is, the relative proportion of chondroitin sulfate and heparin proteoglycans contained by the exocytotic granules. Also noted were differences in sensitivity of these "CTMC" cells to mast cell secretagogues. These authors emphasized that classification of mast cells as CTMC or MMC is an artificial dichotomy. It is to be appreciated that, *in vivo*, there is likely a continuum of mast cell phenotypes, the extremes of which are represented by CTMC and MMC.

Much knowledge about mast cells comes from studies of rodent mast cells. The relative ease of obtaining CTMC from peritoneal lavage fluid compared to the rigorous enzymatic dispersion procedure for isolating MMC has meant that most studies of mast cells have been performed on CTMC.[27] Much remains to be learned about the unique behaviours of mucosal-type mast cells. Moreover, it is difficult for data derived from studies of rodent mast cells to be related to humans because it is becoming increasingly clear that human mast cells behave quite differently.[26]

1.3 Mast Cell Activation

Antigenic activation. In general, sensitization occurs when the humoral immune response to a foreign antigen is dominated by immunoglubulin E (IgE) production. Antigen-specific IgE molecules bind to high affinity IgE receptors ($Fc_{\varepsilon}RI$) on the surface of mast cells. The extremely low dissociation constant of $Fc_{\varepsilon}RI$ (10^{-10}M) means that IgE binding is practically irreversible.[30] Exposure of sensitized mast cells to antigen causes crosslinkage of $Fc_{\varepsilon}RI$ which initiates a program of biochemical and ultrastructural changes ending in exocytotic release of cytoplasmic granules (reviewed by Landry[31]). IgE-dependent activation causes release of both granule-associated mediators as well as lipid-derived and cytokine mediators which are produced *de novo*.

Animal models of allergy have been developed to aid in the study of the mechanisms and manifestations of mast cell activation. Early work by Ellen Jarret has identified several factors which determine the degree to which experimental animals can be actively sensitized:[32] (1) *Genetically-defined factors.* The subtle interplay of genes which control immune responses may skew the humoral response to production of IgE. Genes which ultimately affect production of IL-4, a key cytokine which promotes IgE isotype switching in B cells, are likely candidates. (2) *Age at sensitization.* It is well known that young animals are more amenable to sensitization than are adult animals. (3) *Route and dose of sensitizing antigen.* Parenteral administration of very low doses of antigen are likely to produce a better primary IgE response. (4) *Concurrent administration of adjuvants.* Adjuvants such as pertussis toxin or aluminum hydroxide

aid induction of a primary IgE response by stimulating helper T cells, macrophages and B cells.

Activation by neuropeptides. Mast cells in the intestinal mucosa can be found in close proximity to nerve terminals.[33] In rodents, both MMC and CTMC can be activated by the neuropeptide substance P *in vitro*, and mast cells are a source of neuro-active mediators such as histamine and serotonin. Together, this suggests the existence of bidirectional interactions between mast cells and nerves. Indeed, in the gastric mucosa, mast cells may be part of a neurally-mediated reflex which signals for increased mucosal blood flow in the face of a luminal irritant.[44]

Endogenous modulators of mast cell reactivity. The release of mediators from activated mast cells can be modified by native factors of the immune system. Nitric oxide, for example, has been shown to attenuate mast cell histamine release, and it appears that mast cells themselves constitutively release NO which acts in an autoregulatory manner to modulate its reactivity. [106] Mast cell degranulation has been observed as a consequence of inhibition of nitric oxide production.[107] Hogaboam *et al.* have shown that IL-1β inhibits release of pro-inflammatory mediators from ionophore-activated mast cells *in vitro*.[64] Interestingly, this effect of IL-1β appears to be dependent upon its ability to stimulate NO release from mast cells. Finally, prostaglandins have been shown to potently inhibit release of TNF-α from ionophore-activated rat MMC and CTMC.[65] The responses of mast cells to activating stimuli appear to subject to self-regulation and influence by soluble factors in the micro-environment. Inflammatory responses may greatly affect the profile and amounts of mediators released by mast cells.

1.4 Mast Cell-Derived Mediators

Mast cells are a source of a variety of mediators contained by exocytotic granules (*e.g.* histamine, proteglycans) and mediators which are synthesized *de novo* upon mast cell activation. This section will focus on two classes of mast cell-derived mediators, the neutral serine proteases and cytokines.

Proteases. Rat mast cell protease II is a 25 kDa serine protease which is specifically associated with the proteoglycan matrix of exocytotic granules in rat mucosal mast cells.[34] When mucosal mast cells degranulate, RMCP-II is released into the interstitium and, being highly soluble, is rapidly cleared and appears in blood.[28] A significant rise of the concentration of RMCP-II in blood reflects activation of mucosal mast cells. Tissue levels of RMCP-II reflect the number of mucosal mast cells in that location.[35]

Proteases comprise a major component of mucosal mast cell granules. For example, during *N. brasiliensis* infection, it has been estimated that the concentration of RMCP-II in rat intestinal mucosa is 4-6 mg/g wet weight.[36] However, the precise function of RMCP-II remains unknown. It has been suggested that mast cell-derived proteases increase vascular permeability or degrade extracellular matrix substrates and thereby enhance the pro-inflammatory actions of other mast cell mediators such as histamine and cytokines.[34]

Elegant studies recently performed by Scudamore *et al.* have shed light on the potential substrates of RMCP-II and its role in anaphylactic reactions.[36] In *N. brasiliensis*

infected rats, systemic infusion of worm antigen caused significant increases in levels of RMCP-II in blood and intestinal lumen. Degranulation of MMC was accompanied by a rapid and striking increase in intestinal epithelial permeability. This effect of antigen challenge was mimicked by close arterial infusion of purified RMCP-II. Interestingly, altered permeability was not accompanied by gross epithelial injury as assessed by histology. Since it is unlikely that RMCP-II could have such rapid effects by acting on type IV collagen of the basement membrane, the authors suggested that the target of RMCP-II may instead be a protein of the intestinal epithelial tight junction complex.

Cytokines. Recent literature shows that mast cells have the capacity to synthesize practically every major cytokine.[37] Many of investigations of mast cell-derived cytokines could be classified as purely descriptive studies of *in vitro* phenomenon. The greater challenge will be to determine the significance of these findings in the context of the immune system *in vivo*. The capacity of mast cells to produce cytokines expands the potential roles of the mast cell to include participation in acute and chronic inflammatory reactions. It has long been recognized that chronic or "late phase" inflammatory reactions accompanied allergic disorders, especially in the airways. But little regard has been given to the possibility that chronic late-phase inflammatory reactions accompany acute allergic reactions in the gut.

1.5 Physiological and Pathological Roles of Mast Cells in the Gastrointestinal Tract

Mast cells are granulated immunocytes which reside in abundance in the gastrointestinal tract. As shown in Figure 1.1, mast cells typically segregate in the superficial aspect of the lamina propria of both stomach and intestines. Mast cells are also found in considerable numbers in the liver where they are closely associated with the portal tracts (*see* Figure 6.1). Very little information is known about the precise biological function of mast cells resident in the liver, but it has been suggested that hepatic mast cells may regulate local blood flow and participate in local immunological defenses.[134]

In some animal species including humans, mast cells are the predominant source of histamine in the glandular mucosa (reviewed by Håkanson[38]). Enterochromaffin-like cells are another source of histamine in the gastric mucosa and are the major source of this mediator in mice and rats. It remains a subject of considerable debate as to whether mast cell-derived histamine is involved in the physiological regulation of gastric acid secretion.[38] There is no clear evidence that mast cells are capable of releasing histamine in response to acid secretagogues such as gastrin or acetylcholine[39] and, in general, mast cells are distributed in a uniform fashion throught the gastric mucosa without a particular association with parietal cells.

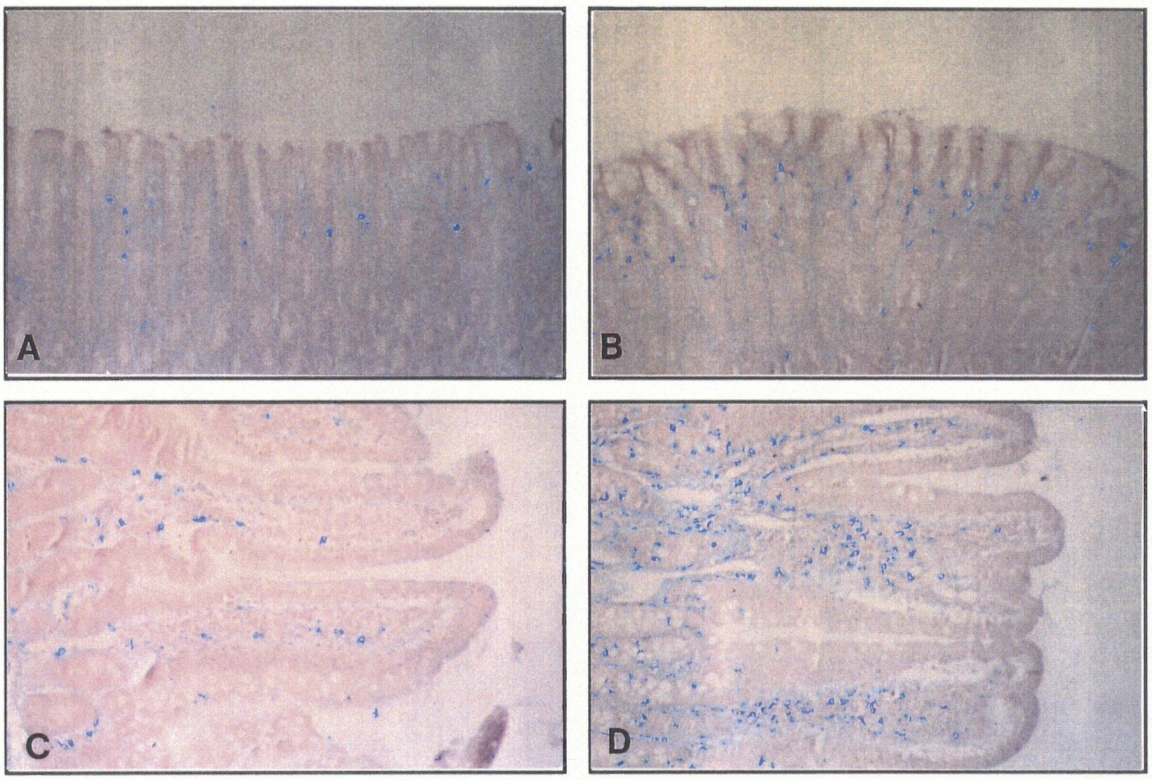

Figure 1.1. **Mucosal mast cells in the rat stomach and intestine.** Sections of rat stomach and small intestine were fixed in Carnoy's Solution, stained with alcian blue and safranine, and visualized by light microscopy. *(A)* In the stomach, mast cells segregate in the superficial aspect of the mucosa and are closely associated with the gastric pits. *(C)* Mast cells are found in even greater numbers in the small intestine, predominantly along the villus-crypt axis. Infection of rats with the nematode parasite *Nippostrongylus brasiliensis* induces an increase in the number of mucosal mast cells, which is most profound in *(D)* the small intestine, but occurs also in *(B)* the stomach. This response is an important component of the mechanism by which the infection is cleared.

In the intestines, mast cells play a key role in immunological responses which defend against parasitic infection. Infection of rats with the nematode parasite *Nippostrongylus brasiliensis* is associated with profound increase in the number of mucosal mast cells in the gastrointestinal tract.[40] The most significant MMC hyperplasia occurs in the proximal small bowel which is the preferred site of worm infection, although MMC hyperplasia occurs to a lesser degree at other sites in the GI tract. The MMC hyperplasia does not occur in athymic animals suggesting that T-cell derived factors are essential mediators of this response. [41] Mast cells are actively involved in eradicating the infection, the so-called "self-cure." Infection is also associated with an acute inflammatory response and changes in enteric nerve and smooth muscle function.

The ability of mast cells to promote injury in the gastrointestinal tract relates to the fact that they are a source of a variety of mediators which have been shown to cause or promote injury at mucosal surfaces. However, it would not be correct to presuppose that mast cells can only have detrimental effects on mucosal integrity. In fact, mast cells are equally a source of mediators which may prevent injury or promote repair of injured tissue.

1.6 Challenges of Defining the Role of Mast Cells in Physiological or Pathological Events in the Gastrointestinal Tract

Several general strategies have been devised for defining and characterizing the role of mast cells in biological responses (reviewed by Wershil and Galli[42]). Although

these are definitely useful approaches, they do have shortcomings which often make it difficult or complicated to precisely define the contributions of mast cells to a given physiological or pathological effect. To be sure, however, when these approaches are used in combination with one another or along with other tools such as genetically mast cell-deficient mice, a confident assessment of the functional importance mast cells can be made.

Mast cell number. Mast cells have been implicated in biological responses due simply to the observation that their numbers are either increased or decreased during the course of the response in question. As degranulation depletes mast cells of proteoglycans which are the target of histochemical stains, a decrease in the number of histochemically detectable mast cells points to mast cell degranulation. However, in the event that a decrease in the number of mast cells is associated with a pathological effect, it is not entirely clear whether this is a result of mast cell activation and degranulation or simply the result of mast cell lysis. Mast cell hyperplasia has been described in a wide variety of diseases, but it often remains unclear whether this has any pathological significance.

Mast cell mediator release. Another approach which has been employed to examine the relationship between mast cells and a given biological response is to directly assess whether mast cell-derived mediators are released during the response or to see if mast cell-derived mediators mimic the response when administered exogenously in pure form. The major drawback to this approach is that many other types of cells can produce mediators traditionally believed to be derived from mast cells (*e.g.* in addition to mast cells, basophils and neurons are a source of histamine). Moreover, it is interesting to

demonstrate that mast cell mediator release occurs during a biological response, but often the greater issue is to prove whether such mediator release is essential to the response.

Effect of pharmacological agents which affect mast cells. Drugs which suppress mast cell activation (*e.g.* sodium cromoglycate) or antagonize the effects of mast cell-derived mediators (*e.g.* antihistamines) have been used to demonstrate the role of mast cells in biological responses. However, it is to be appreciated that drugs which purportedly have specific effects on mast cells or their mediators may also have effects on other cells which account for the observed outcome.

Endogenous factors which may influence mast cells. During the course of a biological response, certain factors may be present which affect mast cells, for example, by promoting their activation or proliferation. Although it is often presumed that mast cells are the only cells involved in IgE-mediated reactions, other cells such as basophils and monocytes may be activated by IgE-dependent mechanisms. Furthermore, agents which activate mast cells in an IgE-independent manner are also very likely to have effects on other cell types.

1.7 Objectives

Four series of studies have been compiled to form the body of this dissertation. These studies are presented individually in chapters 3 to 6. The broad objectives of this research project were as follows:

(1) To examine the effects of increasing or decreasing the number of mast cells in the stomach on the susceptibility of the gastric mucosa to injury induced by common ulcerogenic agents.

(2) To examine the effect of mast cell activation on the susceptibility of the gastric mucosa to injury induced by a topical irritant.

(3) To describe and characterize the effect of chronic activation of mast cells in the gastrointestinal tract on responsiveness of mucosal mast cells to repeated activation.

(4) To study associations between mast cells and fibrotic reactions.

CHAPTER 2

GENERAL MATERIALS AND METHODS

Three fundamental approaches were employed to study the role of mast cells in mucosal injury and defence in the gastrointestinal tract. One approach which helped to highlight the role of mast cells in physiological and pathological events involved techniques for specifically increasing or decreasing the number of mast cells in the gastrointestinal mucosa. A second approach aimed at studying the effects of mast cell-derived mediators on mucosal integrity employed techniques for activating mast cells *in vivo* in an immunologically relevant manner. Finally, a strategy for studying the potential involvement of mast cells in pathological processes in the gastrointestinal tract made use of techniques for detecting whether mast cells had been activated to release mediators. In this chapter these key methods will be described in detail. Ancillary techniques will be described in subsequent chapters where such information is pertinent.

2.1 Animals

Male Wistar and Sprague Dawley rats were obtained from Charles River Breeding Farms, (Montréal, PQ). Male and female Hooded Lister rats were obtained from the Department of Biological Sciences at the University of Calgary. Mast cell-deficient mice ($WBB6F_1/J$-*W/W*^{v}) and congenic normal littermates ($WBB6F_1/J$-+/+) were obtained from Jackson Laboratories (Bar Harbour, ME). All animals were housed in transparent plastic

cages at 22°C in a light-controlled room with a 12-hour day/night cycle, and were allowed free access to standard laboratory chow and tap water. All experimental procedures were performed in accordance with the guidelines established by the Canadian Council on Animal Care.

2.2 Mucosal Mast Cell Hyperplasia Induced by Nippostrongylus brasiliensis

***Laboratory culture of* Nippostrongylus brasiliensis.** *Nippostrongylus brasiliensis* cultures were maintained by serial passage in Sprague Dawley rats according to a procedure described by Befus *et al.*[43] Seven days after *N. brasiliensis* infection (see below), faeces were collected from 3-4 infected rats over a twenty-four hour period. The faeces, a source of *N. brasiliensis* eggs, were placed in a plastic beaker and soaked in an equal volume of tap water for one hour. Approximately equal volumes of activated charcoal and finely minced vermiculite were then added to achieve the consistency of loose soil. The mixture was transferred to a 500 mL plastic container and gently pressed into place. To complete the process, vermiculite chips were pressed into the surface of the culture medium and the container was covered with a mesh lid for aeration. After 4-7 days at room temperature, the *N. brasiliensis* eggs hatch and the larvae migrate to the vermiculite chips. To harvest stage-three larvae, the chips were suspended in warm water in a glass funnel lined with cheesecloth. To hold the water in the mouth of the funnel, a transparent flexible tube was attached to the spout and clamped shut. Within one hour, the larvae settled to the bottom of the tube and were collected into a 50 mL polypropylene

tube. The larvae were washed 3 times with 25 mL of 0.9% saline and resuspended in 5 mL saline. The concentration of worms was determined by counting the number of worms in a 25 μL sample under a dissecting microscope. The mixture was diluted with saline to give a final concentration of 6000 worms/mL.

Infection. Male Sprague Dawley rats (175-200 g body weight) were infected with *Nippostrongylus brasiliensis* by injecting 3000 stage-three larvae subcutaneously at the scruff of the neck. The infective larvae were delivered in a volume of 0.5 mL of saline using a syringe fitted with a 21 gauge needle. Rats were used in experiments thirty-five to forty-two days later, by which time the infection and associated acute inflammatory changes had resolved, but mastocytosis and sensitization persisted. Control rats received an equivalent volume of saline administered in the same manner and were age- and weight-matched to the experimental group.

Antigen Preparation. Eight to ten days after infection with *N. brasiliensis*, rats were sacrificed and the entire small bowel was excised, cut open longitudinally, and placed on a large gauze pad. The corners of the gauze pad were gathered and tied, and the entire assembly was suspended in a beaker of phosphate buffered saline (PBS, pH 7.4) at 37°C for one hour. The worms were collected from the bottom of the beaker, washed three times with PBS, and resuspended to give a concentration of 1500 worms/mL. The suspension of worms was sonicated for five minutes at room temperature to release soluble worm antigens. The insoluble debris was sedimented by centrifugation (2000*g*, 5 minutes, 4°C) and the supernatant was collected. This solution

of soluble worm antigen was diluted to a final concentration of 100 worm equivalents/mL and stored at -20°C.

2.3 Depletion of Mucosal Mast Cells by Dexamethasone

Dexamethasone has been shown to deplete mucosal mast cells in the intestine by causing their engulfment by macrophages.[22] This effect has also been shown in the rat stomach.[44] Dexamethasone (Sabex, Boucherville, PQ) was administered to rats by intraperitoneal injection at a dosage of 1.0 mg/kg once daily for two consecutive days. Control rats received sterile 0.9% saline administered in the same manner. Rats were used in experiments 24 hours after the second injection.

2.4 Sensitization to Ovalbumin

Preparation of sensitizing antigen. Rats were sensitized to chicken ovalbumin (OA, Grade V, Sigma Chemical Co., St. Louis, MO) using aluminum hydroxide as both immunological adjuvant and vehicle for delivery of OA.[45] Stock solutions of OA (150 μg/mL) and aluminum potassium sulfate (alum, $AlK(SO_4)_2 \cdot 12H_2O$, 100 mg/mL, Fisher Scientific, Fair Lawn, NJ) were prepared in 0.9% sodium chloride. One millilitre of OA solution was mixed with 1.0 mL of alum solution. Two drops of 0.02% phenol red were added as a pH indicator for the subsequent alkaline precipitation reaction. Sodium hydroxide (1.0 M) was added dropwise until the solution turned pink. The aluminum

hydroxide precipitate containing antigen was sedimented by centrifugation (2000*g*, 10 minutes, 4°C) and the supernatant was discarded. The pellet was resuspended in 10 mL of normal saline and gently mixed to disperse the precipitate. After centrifugation, the precipitate was resuspended in 10 mL of saline to give a solution which was used in the sensitization procedure.

Sensitization. Male Hooded Lister rats (75-100 g body weight) were sensitized to ovalbumin by intraperitoneal injection of 1.0 mL of the OA-aluminum hydroxide suspension. Control rats received 1.0 mL of aluminum hydroxide suspension prepared in the absence of ovalbumin.

Confirmation of sensitization. The passive cutaneous anaphylaxis (PCA) reaction was used to measure serum levels of reaginic antibodies directed against ovalbumin.[46] Under halothane-induced anaesthesia (Halocarbon Laboratories, River Edge, NJ), ~500 μL of blood was withdrawn from each control and sensitized rat by cardiac puncture using a 25 gauge needle. Sera obtained from each blood sample were stored at -20°C. In preparation for the PCA reaction, serum samples to be tested were thawed and diluted 1:8, 1:16, 1:32, or 1:64 with 0.9% saline.

Under halothane-induced anaesthesia, 300 μL of each dilution was injected intradermally on the shaved backs of naive Sprague Dawley or Hooded Lister rats (300-400 g body weight) using a 30 gauge needle fitted to a 1.0 mL syringe. Forty-eight hours later, the PCA reaction was developed by intracardiac injection of 2.0 mL of a solution containing 1.0% w/v Evans Blue Dye and 0.5% w/v OA prepared in 0.9% saline. After 30 minutes, the shaved back of each rat was inspected. A distinct, circular blue

disk measuring at least 5 mm at the injection site indicated a positive reaction. Rats were considered sensitized if sera diluted ≥1:32 produced a positive PCA reaction. This degree of sensitization was chosen because antigen challenge produced large and reproducible increases in serum RMCP-II in rats sensitized to this level, and a reasonable rate of successful sensitization could be achieved using the above methods.

2.5 Histochemical Staining of Mucosal Mast Cells

Tissue collection and fixation. Immediately after rats were sacrificed, tissues were excised from stomach (corpus region), small intestine (15-20 cm distal to pylorus), or liver (margin of right lobe). The tissues were fixed in Carnoy's solution (ethanol:chloroform:glacial acetic acid, 6:3:1) for 24-48 hours and then transferred to 50% ethanol. This fixation procedure preserves the proteoglycans of mucosal mast cell exocytotic granules which are the targets of histochemical stains.

Paraffin embedding. Tissues were further processed by routine histological techniques. Automated equipment was used for serial passage of the tissues through agents for dehydration (ethanol: 50% ⇨ 70% ⇨ 90% ⇨ 95% ⇨ 100%), clearing (AmeriClear®, Baxter Diagnostics Inc., Deerfield, IL) and infiltration (SurigPath®, Oxford Labware, St. Louis, MO). To complete the wax infiltration process, the tissues were transferred to a beaker of infiltration wax at 50°C and left for 10 minutes under vacuum. The tissues were oriented in stainless steel histology moulds and embedded in paraffin wax (ParaPlast+®, Oxford Labware, St. Louis, MO).

Sectioning and staining. Sections measuring 6 μm in thickness were cut, mounted on glass microscope slides, and heat-fixed at 50°C overnight. The mounted sections were deparaffinized by immersion in xylenes and then rehydrated by serial passage through graded alcohols and finally tap water. Alcian Blue 8GX (Sigma Chemical Co., St. Louis, MO) and Safranine O (ICN Biochemicals, Cleveland, OH) were each prepared at a concentration of 1.0% in 0.7 N hydrochloric acid and filtered by gravity (Grade 202 Reeve Angel filter paper, Clifton, NJ). Immediately after rehydration, the sections were stained by immersion for 2.5 hours in Alcian Blue 8GX, rinsed for 30 seconds in 0.7N HCl, and immersed in Safranine O for 1 hour.[47] The microscope slides were rinsed with distilled water, dried at room temperature overnight, and coverslips applied using Entellan® (Merck, Darmstadt, Germany).

Identification and enumeration of mucosal mast cells. Mast cells were visualized by light microscopy. In stomach and small intestine, all distinctly stained mast cells were counted in five randomly selected microscope fields (magnification 200×) and results expressed as mucosal mast cell number "per high power field" or "per villus-crypt unit," respectively. In liver, the area of each section was measured by computerized planimetry and every mast cell within that section was counted. The number of hepatic mast cells was therefore expressed "per square millimetre." In all instances, sections were coded so that the identity of the specimen was unknown at the time the mast cells were counted.

2.6 Rat Mast Cell Protease II Assay

Serum collection. Under halothane-induced anaesthesia, ~500 μL of blood was collected by cardiac puncture using a 25 gauge needle fitted to a 1.0 mL syringe. Serum obtained from each blood sample was stored at -20°C until assay.

Tissue collection. Tissue samples (100-300 mg wet weight) were excised from stomach (corpus region), small intestine (15-20 cm distal to pylorus), or liver (margin of right lobe). Using the method of Woodbury and Miller,[35] tissues were placed in 1.0 mL of ice-cold 0.15 mol/L potassium chloride (KCl). The samples remained on ice throughout all subsequent procedures. The tissues were minced with scissors and homogenized for 45 seconds (Polytron Homogenizer PT10/35, PTA-7 probe, Brinkmann, Rexdale, ON). After centrifugation (36,000*g*, 30 minutes, 4°C), the supernatants were collected and stored at -20°C until assay.

RMCP-II ELISA. Rat mast cell protease II was measured in serum and tissue by enzyme-linked immunosorbent assay (ELISA). RMCP-II ELISA kits were obtained from Moredun Animal Health Ltd. (Midlothian, United Kingdom). RMCP-II ELISA kits were used according to the instructions of the manufacturer. ELISA plates (96-well NUNC-Maxisorp® plates, Intermed, Denmark) were coated with "capture" antibody (monoclonal mouse anti-RMCP-II, stock 1.0 mg/mL) diluted 1:1000 in 0.1 mmol/L carbonate buffer pH 9.6. Serum samples were diluted 1:50, 1:100, 1:200, and 1:400 with PBS/0.5% v/v Tween20/4% w/v bovine serum albumin (PBS/Tween20/BSA). Tissue samples were diluted *1:500, 1:1000, 1:2000,* and *1:4000* (stomach), or *1:5000, 1:10000, 1:20000,* and

1:40000 (small intestine), or *undiluted, 1:10, 1:100, 1:1000* (liver) with PBS/Tween20/BSA. Plates were washed 6 times (200 µL per well) with PBS/Tween20 using an automated microtitre plate washer and then incubated with PBS/Tween20/BSA for 30 minutes. RMCP-II standards (0.25 ng/mL to 12 ng/mL) were prepared in PBS/Tween20/BSA. Each plate was washed once with PBS/Tween20 and then incubated with samples and standards for 1.5 hours at room temperature. After washing 6 times with PBS/Tween20, the secondary antibody (sheep anti-RMCP-II conjugated to horse radish peroxidase, diluted 1:400 with PBS/Tween20/BSA) was applied for 1 hour. After a final wash, the substrate for the colour reaction (10 mg ortho-phenylene diamine and 10 µL 30% hydrogen peroxide prepared in 25 mL citrate/phosphate buffer pH 5.0) was added to each well. The colour reaction took 10-15 minutes to develop and was stopped by adding 2.5 mmol/L sulphuric acid. The optical density (492 nm) was determined by spectrophotometer. The concentrations of RMCP-II in samples were interpolated from the standard curve of absorbance values plotted as a function of known concentrations of purified RMCP-II.

2.7 Statistical Analyses

All data are expressed as mean ± SEM. Statistical comparison of two means was made using Student's *t* test. For comparison of more than two means, analysis of variance was employed, with the Student-Newman-Keuls post-hoc test. Groups of data were considered significantly different if $P < 0.05$.

CHAPTER 3

ANTIGENIC ACTIVATION OF MUCOSAL MAST CELLS PREDISPOSES THE GASTRIC MUCOSA TO INJURY INDUCED BY ETHANOL

3.1 Introduction

The role of mast cells in allergic and anaphylactic responses is well established, as is the contribution of the mast cell to the functional disturbances associated with such responses in the intestine.[48-51] For many years, the mast cell has also been suspected of playing an important role in the pathogenesis of gastric ulceration, including recent studies implicating the mast cell in gastric mucosal injury associated with duodenogastric reflux[52] and *Helicobacter pylori* infection.[53] Studies utilizing various experimental models of gastric mucosal injury have provided ample evidence consistent with such a role. This evidence includes the following observations: (1) agents that can cause mast cell degranulation (*e.g.* compound 48/80) can induce gastric mucosal injury,[54] (2) agents which have been described as "mast cell stabilizers" are capable of reducing the severity of gastric injury in various models,[55-58] (3) mast cell numbers in the gastric mucosa, determined histologically, are reduced after administration of an ulcerogenic agent or subjecting the animal to stress,[55,59-62] (4) mast cell-deficient mice are significantly more resistant to gastric injury induced by ethanol than normal littermates,[63] and (5) agents capable of protecting the stomach from injury (*e.g.* prostaglandins, IL-1) can inhibit the release of pro-ulcerogenic mediators from mast cells.[64,65] While this body of evidence is

considerable, questions could be raised about the conclusions of many of these studies. For example, the agents which cause mast cell degranulation are non-physiological and may have many other actions which could account for the effects reported. The mast cell stabilizers used also exert actions unrelated to effects on mast cells (*e.g.* ketotifen[66]). A decrease in mast cell number following administration of an ulcerogen could be a consequence of the injury induced by the agent, rather than contributing to it. Moreover, the decrease in histologically detectable mast cells could be due to lysis, rather than degranulation.

The mast cell is capable of liberating a wide array of mediators, many of which have been shown to affect gastric mucosal integrity. For example, leukotrienes and platelet-activating factor (PAF) can be produced by mucosal mast cells, and have been shown to have effects on the gastric mucosa consistent with a role in the pathogenesis of ulceration.[67] However, these mediators can also be released from a variety of other types of cells. Studies to directly assess the contribution of mast cell-derived mediators have not been performed. In the present study, rats previously infected with the nematode *Nippostrongylus brasiliensis* were used to determine the effects of elevated mucosal mast cell number on the susceptibility of the gastric mucosa to injury induced by a topical irritant. Moreover, experiments were performed to evaluate the effects of activation of mast cells using a physiologically relevant stimulus (*i.e.* antigen) on susceptibility of the gastric mucosa to injury. Finally, attempts were made to identify the factors liberated in response to antigen administration that account for the observed effects on mucosal integrity.

3.2 Materials and Methods

Sensitization. Sprague Dawley (175-200 g body weight) rats were infected with *Nippostrongylus brasiliensis* (*see* Section 2.2). All experiments were performed 35-42 days after infection, a time by which the nematode had been expelled but mastocytosis persisted. It has previously been demonstrated that this infection results in a 2- to 3-fold increase in the number of mucosal mast cells in the gastric mucosa.[44] Moreover, primary infection with *N. brasiliensis* provides an immunologically relevant means of mast cell activation, that is, by re-exposure to sensitizing worm antigens. The control group consisted of age-matched, sham-infected rats.

Assessment of Gastric Injury. The *ex vivo* gastric chamber apparatus described by Wallace *et al.*[68] was a central technique in this series of experiments. Rats were anaesthetized with sodium pentobarbitol (65 mg/kg, IP) and mid-line laparotomy was performed. The pylorus was ligated and the stomach was opened by an incision along the greater curvature. After pinning the stomach to an overlying plexiglass support, a plexiglass ring was clamped onto the exposed mucosa to form a chamber to which solutions could be added. Each experiment consisted of six 10 minute periods (Figure 3.1). At the beginning of each period the solution bathing the mucosa was changed. The bathing solutions were warmed to 37°C before being added to the chamber. During the first two and final three periods, the mucosa was bathed with 100 mM hydrochloric acid (HCl). During the third period, 20% (v/v) ethanol was topically applied to the mucosa.

Three groups of experiments were performed, with control and sensitized rats used in each ($n \geq 5$ per group). The first set of experiments was performed as described above. In the second set of experiments, 100 μL of antigen (*see* Section 2.2) was administered via the carotid artery at the beginning of the second period (*i.e.* 10 minutes prior to topical application of ethanol). In the third set of experiments, antigen was administered, but the mucosa was not challenged with ethanol (100 mM HCl was applied to the mucosa in the third period). Blood samples were taken at the end of the second, third and fifth periods for determination of serum levels of RMCP-II (*see* Section 2.6), a specific marker of mucosal mast cell degranulation.[35] At the end of each experiment the mucosa was photographed and the extent of macroscopically visible injury was quantified by computerized planimetry. Damage area was expressed as a percentage of the total area of glandular mucosa.

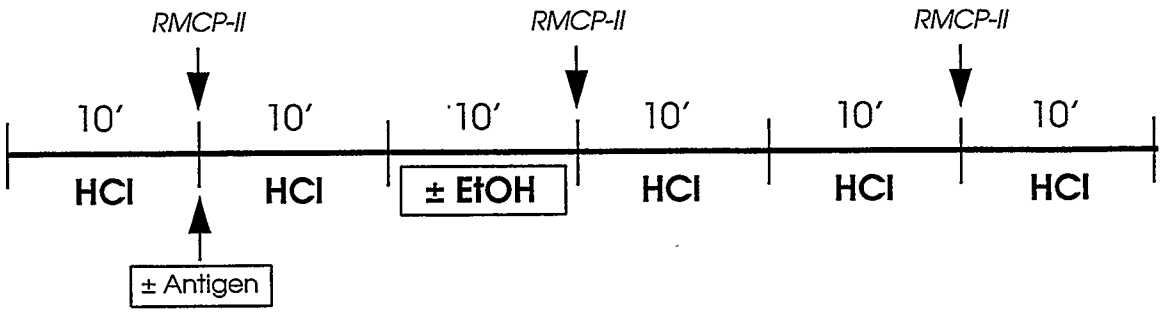

Figure 3.1. **Basic experimental protocol for studying effects of mast cells on gastric mucosal integrity.** Each experiment consisted of six periods of ten minutes each. The gastric mucosa was bathed in 100 mmol/L hydrochloric acid during periods 1, 2, 4, 5 and 6. In the first experiment, 20% v/v ethanol was applied topically to the gastric mucosa during the third period. In the second experiment, antigen was infused systemically ten minutes prior to the application of ethanol. In the third experiment, antigen was infused after period one, but 100 mM HCl was applied in place of ethanol during period three. RMCP-II was measured as an index of mucosal mast cell degranulation both before and after exposure to antigen and/or ethanol. Extent of macroscopically visible gastric mucosal injury was measured by computerized planimetry.

Additional experiments were performed in which a carotid artery was cannulated for continuous measurement of systemic arterial pressure, and gastric perfusion velocity (as an index of mucosal blood flow) was measured by laser-Doppler flowmetry.[68] These experiments were performed to determine the effect of antigen administration to sensitized rats on these parameters.

Mast Cell Staining. Mucosal mast cell numbers were determined histologically (*see* Section 2.5) using tissue excised from control and sensitized rats (n = 6 per group) that were surgically unmanipulated.

Drug Treatments. The role of the mast cell and vaso-active mediators that can be liberated by mast cells in mediating the effects of antigen administration on mucosal susceptibility to injury was further investigated by testing the effects of a number of drugs. Control and sensitized rats were treated with dexamethasone (*see* Section 2.3) prior to setting up the *ex vivo* gastric chamber preparation. Experiments were then performed, as described under *Assessment of Gastric Injury,* in which the rats received intra-arterial antigen followed by topical ethanol. Mucosal mast cell depletion was confirmed by measurement of serum RMCP-II levels and by histochemical identification of mucosal mast cells.

In other experiments, control and sensitized rats were pretreated with a PAF receptor antagonist (WEB-2086; 5.0 mg/kg, IV) or a leukotriene D_4 receptor antagonist (MK-571; 3.0 mg/kg, IV) 20 minutes prior to antigen administration. The doses of these compounds used were selected based on previous work which documented their effectiveness.[68,69] While MK-571 is an LTD_4 receptor antagonist, Wallace *et al.* have

previously demonstrated that it will block the vasoconstrictor effects of LTC_4, LTD_4 and LTE_4 in the rat stomach.[69] The role of prostanoids in the effects of antigen administration were examined by repeating experiments in control and sensitized rats pretreated with indomethacin (5.0 mg/kg, IP) 30 minutes prior to the beginning of the experiment.

Serum Peptido-Leukotriene Levels. Serum samples taken in the various experiments for the determination of RMCP-II levels were also used for measurement of leukotrienes $C_4/D_4/E_4$. The levels of these peptido-leukotrienes were measured using a commercially available ELISA kit (Amersham, Oakville, ON) which utilizes an antibody that does not distinguish between the three peptido-leukotrienes. Unfortunately, peptido-leukotrienes were not detectable in any of the serum samples tested, regardless of the treatments the animals had received. It is believed that this was, in large part, due to interactions of rat immunoglobulins and possibly other serum proteins with anti-rat antibodies used in this ELISA kit.

Materials. WEB-2086 was kindly provided by Dr. H. Heuer of Boehringer Ingelheim (Ingelheim, Germany). MK-571 was kindly provided by Dr. A. Ford-Hutchinson of Merck-Frosst (Montréal, PQ). All other chemicals were obtained from Sigma Chemical Company (St. Louis, MO) or Fisher Scientific (Edmonton, AB).

3.3 Results

Infection with *N. brasiliensis* has previously been shown to cause a 2- to 3-fold increase in the numbers of gastric mucosal mast cells.[44] As shown in Figure 3.2, the

mean basal concentration of RMCP-II in serum from sensitized rats was not significantly different from that of controls. While antigen administration to control rats did not affect serum RMCP-II, antigen administration to sensitized rats resulted in a marked increase.

Topical application of 20% ethanol to control rats produced macroscopically visible damage to approximately 10% of the glandular mucosa (Figure 3.3). Prior administration of antigen did not significantly affect the extent of injury induced by ethanol in control rats. In sensitized rats, the extent of gastric injury induced by topical ethanol did not differ from that observed in controls. However, following antigen administration to sensitized rats, administration of ethanol resulted in a >3-fold increase in the extent of damage ($P < 0.01$). Antigen administration without subsequent topical administration of ethanol did not produce detectable gastric damage in control or sensitized rats (data not shown). Application of 20% ethanol to the gastric mucosa of control or sensitized rats did not, in itself, influence serum RMCP-II levels, regardless of whether or not antigen had been administered previously. For example, in rats not receiving antigen, application of ethanol caused a change in serum RMCP-II of -1.0 ± 4.0% in control rats and +4.1 ± 10.2% in sensitized rats (n = 4; NS).

Antigen administration to sensitized rats resulted in a transient, but significant decrease in systemic arterial blood pressure and gastric mucosal blood flow (Figure 3.4). However, both of these parameters had returned to basal levels within 5 min of antigen administration and remained at basal levels thereafter. Thus, neither blood pressure nor blood flow were compromised at the time of application of ethanol to the gastric mucosa.

Pretreatment with dexamethasone resulted in a marked reduction of histologically detectable mucosal mast cells and a significant reduction in serum RMCP-II levels in both control and sensitized rats (Figure 3.5). However, dexamethasone-induced depletion of mucosal mast cells did not significantly affect the susceptibility of either group of rats to damage induced by topical ethanol (Figure 3.6). In contrast, dexamethasone pretreatment reduced the severity of gastric damage induced by antigen and ethanol in sensitized rats to the levels seen in controls. Thus, depletion of mucosal mast cells abolished the augmentation of gastric injury induced by antigen administration. Further evidence that the effects of antigen on systemic blood pressure did not contribute to the gastric injury seen in sensitized rats was provided by the observation that in dexamethasone-treated rats, antigen still caused transient reductions in arterial blood pressure comparable to those shown in Figure 3.4, but no longer caused the marked increase in gastric damage.

Pretreatment with indomethacin increased the extent of gastric injury induced by antigen and topical ethanol in both control (28.2 ± 4.1, $n = 13$) and sensitized (44.7 ± 5.9, $n = 10$) rats, with the damage in the latter group still being significantly greater than in controls ($P < 0.05$). Pretreatment with WEB-2086, a PAF receptor antagonist, did not significantly affect the extent of damage in sensitized rats given antigen and ethanol compared with untreated controls (Figure 3.7). WEB-2086 did, however, significantly increase damage in nonsensitized control rats. In contrast, pretreatment with MK-571 reduced the extent of damage induced by antigen and ethanol in the sensitized rats to the level observed in untreated controls ($P < 0.01$).

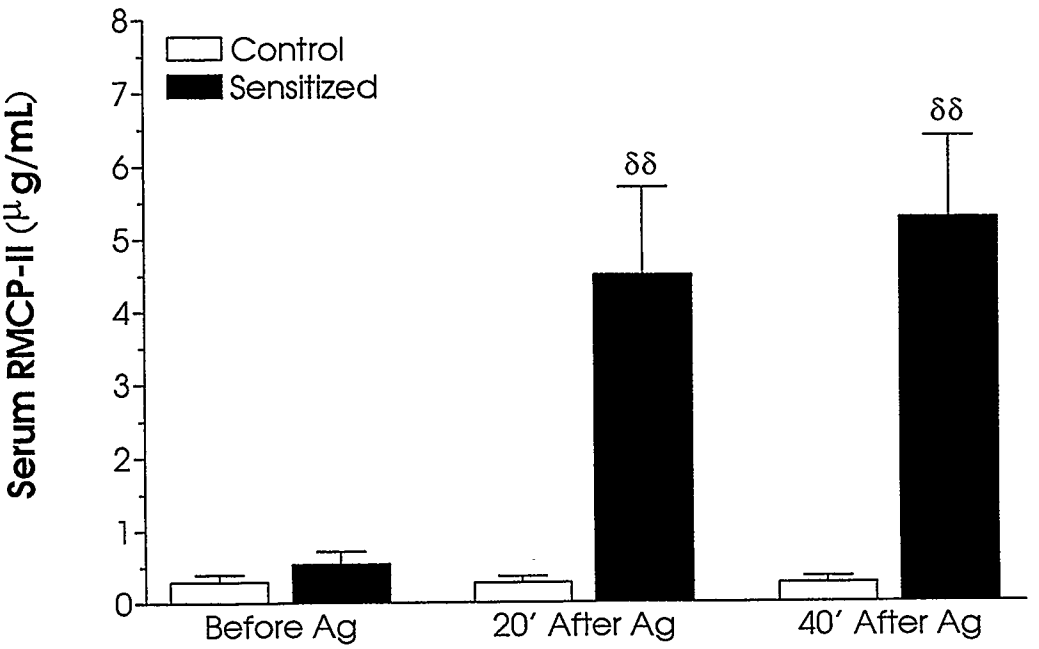

Figure 3.2. **Effect of antigen challenge on serum levels of rat mast cell protease II.** Serum levels of RMCP-II were measured by ELISA. Basal ("Before Ag") serum RMCP-II levels in control and sensitized rats were not significantly different. In rats sensitized to *N. brasiliensis*, intravenous infusion of worm antigen (Ag, 10 worm equivalents) caused a significant increase in RMCP-II within 20 minutes of challenge ($^{\delta\delta}P < 0.001$ compared with corresponding "Before Ag" levels). In control rats, serum levels of RMCP-II were unaffected by antigen. Each bar represents the mean ± SEM of 5 rats per group.

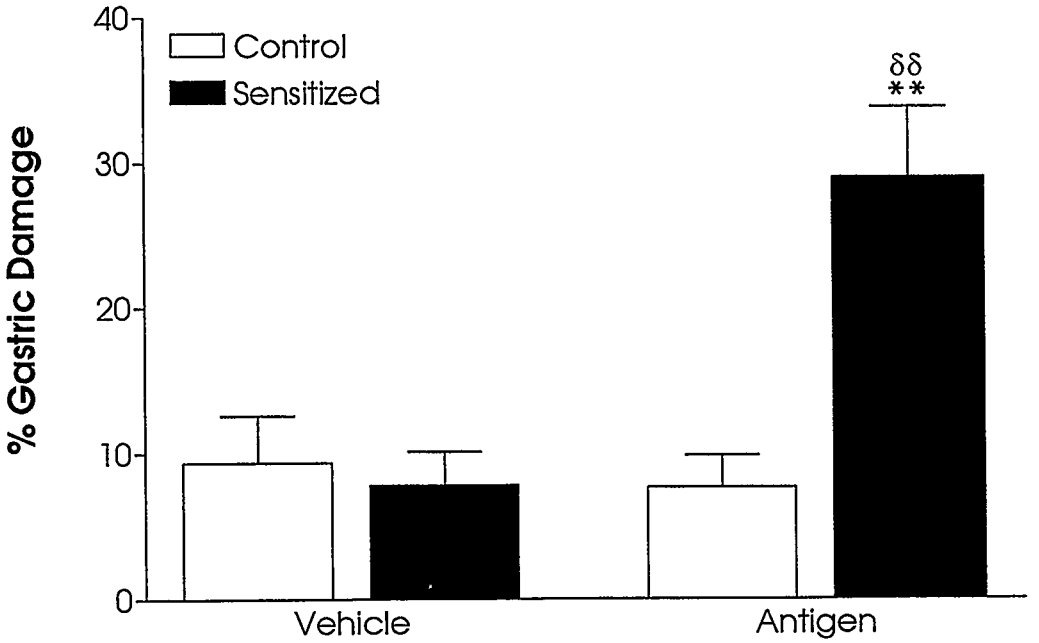

Figure 3.3. **Effect of antigenic activation of mast cells on ethanol-induced gastric mucosal injury.** Antigen administration 10 minutes prior to the topical application of 20% ethanol resulted in a significant increase in the extent of damage in sensitized, but not control rats ($^{**}P < 0.01$ compared with the control group given antigen; $^{\delta\delta}P < 0.01$ compared with the sensitized group given vehicle). Each bar represents the mean ± SEM of at least 5 rats per group.

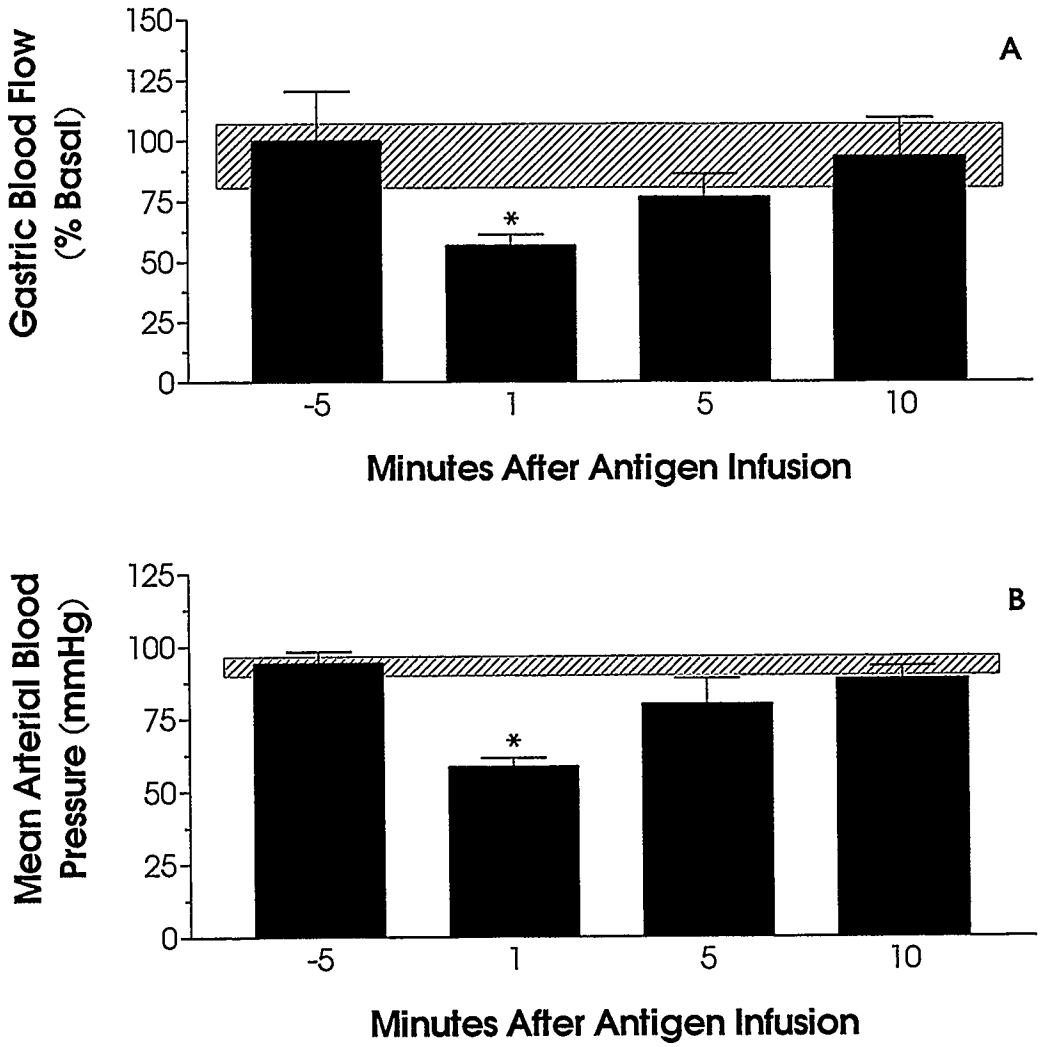

Figure 3.4. **Changes in gastric mucosal blood flow and mean arterial blood pressure after administration of antigen to sensitized rats.** *(A)* Intravenous administration of antigen caused a rapid, but transient decrease in gastric blood flow, which paralleled the decrease in *(B)* systemic arterial blood pressure ($^{*}P < 0.05$). By 5 minutes after antigen administration, both gastric blood flow and arterial pressure had returned to basal levels (mean ± SEM basal levels shown by hatched bar). Data are expressed as mean ± SEM and represent a series of measurements made on 5 rats.

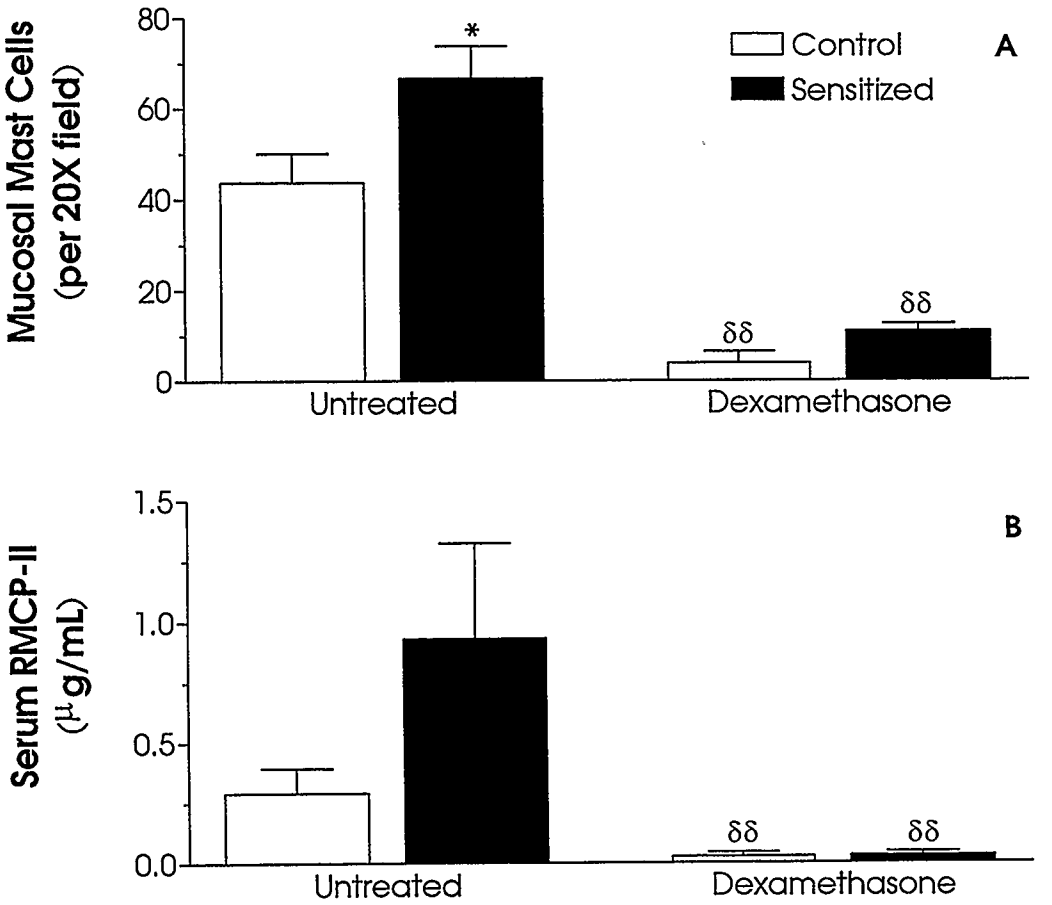

Figure 3.5. **Effect of dexamethasone on number of histologically detectable mast cells in stomach and serum levels of rat mast cell protease II.** Mast cells were identified by light microscopy in sections stained with alcian blue. Pretreatment with dexamethasone significantly reduced *(A)* mast cell numbers and *(B)* serum RMCP-II levels in both control and sensitized rats compared with respective untreated rats ($^{\delta\delta}P < 0.01$). Note also that prior infection of rats with *N. brasiliensis* caused a significant increase in the number of gastric mucosal mast cells ($^{*}P < 0.05$ compared with control). Each bar represents the mean ± SEM of 3 rats in the dexamethasone-treated control group and at least 6 rats in the other groups.

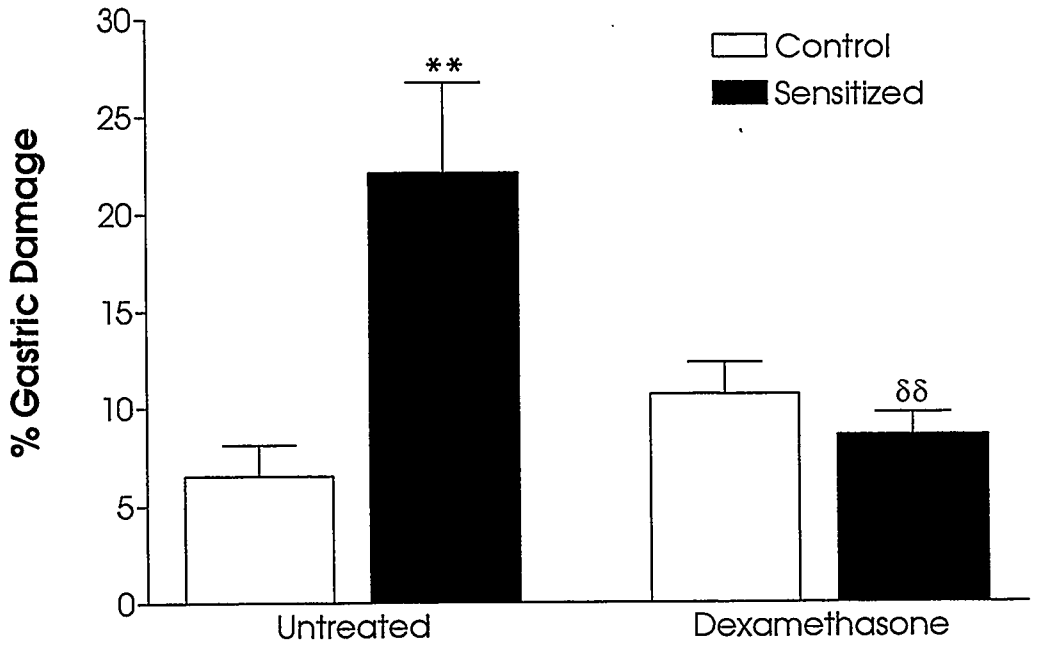

Figure 3.6. **Effect of mucosal mast cell depletion on the extent of ethanol-induced gastric damage.** Antigen was administered intravenously to all rats 10 minutes prior to topical application of ethanol. As shown previously (*see* Figure 3.3), antigen challenge significantly increased ethanol-induced gastric damage in sensitized rats compared with controls ($^{**}P < 0.01$). In sensitized rats depleted of mucosal mast cells by dexamethasone pretreatment, the extent of damage induced by antigen plus ethanol was significantly reduced compared with sensitized rats which did not receive dexamethasone pretreatment ($^{\delta\delta}P < 0.01$). Each bar represents the mean ± SEM of at least 5 rats per group.

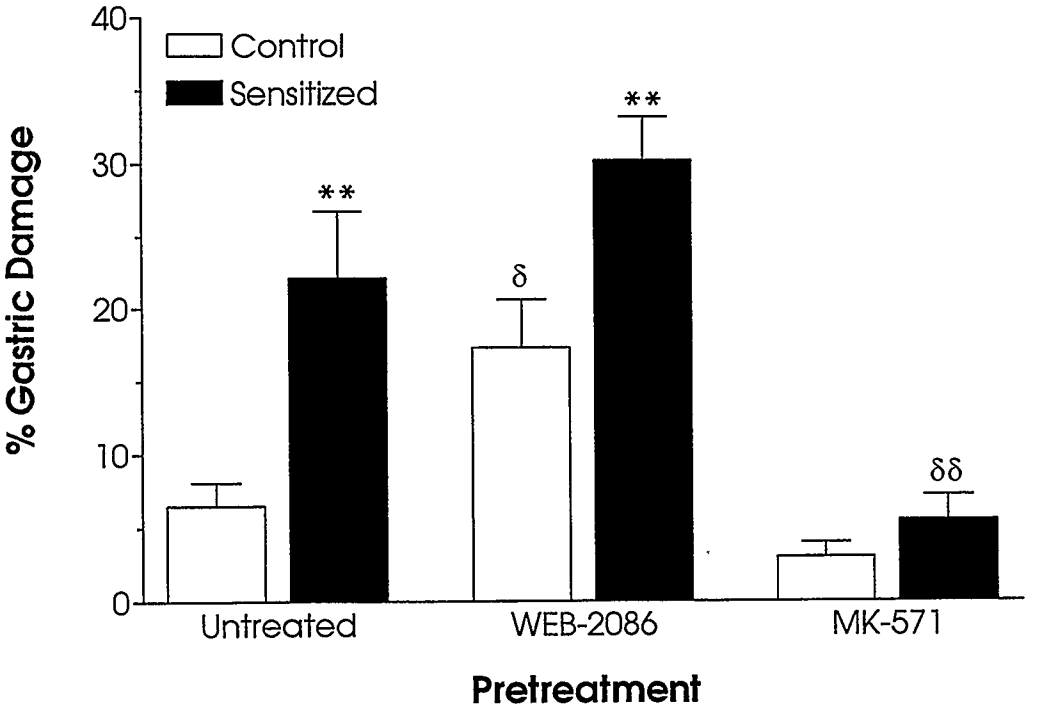

Figure 3.7. **Effects of pretreatment with a platelet-activating factor receptor antagonist (WEB-2086, 5.0 mg/kg) or a leukotriene C_4 receptor antagonist (MK-571, 3.0 mg/kg) on ethanol-induced gastric damage.** All animals were challenged with antigen 10 minutes prior to the application of ethanol. MK-571 significantly reduced the extent of damage in sensitized rats ($^{\delta\delta}P < 0.01$ compared with the untreated, sensitized group). In control rats pretreated with WEB-2086, gastric damage was significantly increased compared with control rats which were not pretreated ($^{\delta}P < 0.05$). $^{**}P < 0.01$ compared with the corresponding control group. Each bar represents the mean ± SEM of at least 5 rats per group.

3.4 Discussion

Mast cells are capable of releasing a wide array of mediators capable of modulating mucosal resistance to injury. These mediators include some with documented protective effects on the gastric mucosa, such as nitric oxide and IL-1, and others which have been shown to increase the susceptibility of the mucosa to injury, such as PAF, endothelin and leukotrienes. Previous studies have suggested that mast cell activation may contribute to the pathogenesis of experimental ulceration. The results of the present study suggest that an increase in mast cell number within the gastric mucosa does not, in itself, influence susceptibility to damage induced by a topical irritant (20% ethanol). However, activation of the mast cells with antigen results in a profound increase in the extent of gastric damage induced by the irritant. A similar conclusion was drawn from an earlier preliminary study.[70] Depletion of the mucosal mast cell population, through pretreatment with dexamethasone, abolished the increase in gastric injury observed in sensitized rats given antigen while having no effect on the controls. The ability of the leukotriene D_4 receptor antagonist, MK-571, to block the increase in gastric injury induced by antigen suggests that peptido-leukotrienes (LTC_4, LTD_4, LTE_4) are responsible for the increased susceptibility to injury. Attempts were made to detect changes in serum levels of the peptido-leukotrienes in this study, but technical problems associated with the assay prevented this from being accomplished. However, there is ample evidence in the literature that mucosal mast cells from the gastrointestinal tract of

the rat are capable of releasing LTC_4 in response to antigen.[71,72] In a previous study it was demonstrated that intravenous administration of any of the three peptido-leukotrienes resulted in a marked increase in the extent of gastric damage induced by topical ethanol and the effects of all three could be blocked by MK-571.[69] Pretreatment with a PAF receptor antagonist did not affect the augmentation of damage caused by antigen administration to sensitized rats although, paradoxically, the antagonist significantly increased the extent of damage in control (nonsensitized) rats. It is difficult to explain this observation, as such an effect has not been documented in previous studies of this compound.[68] While PAF has been shown to exert ulcerogenic effects in various models of experimental ulceration, previous studies suggest that PAF does not play an important role in the gastric damage induced by ethanol.[73]

A role for mast cells in the pathogenesis of ethanol-induced gastric injury has been suggested based on evidence that mast cell stabilizers reduce the severity of injury and histologically detectable mast cell numbers are reduced following administration of ethanol at concentrations that induced substantial mucosal injury. Whether the reduction of mast cell number is due to their degranulation or purely to lysis has not been examined. In the present study, topical application of 20% ethanol to the gastric mucosa did not significantly affect serum levels of the mucosal mast cell marker, RMCP-II. This suggests that mast cell degranulation did not occur in response to ethanol, or that the level of degranulation elicited was not sufficient to cause detectable changes in serum RMCP-II. It is also possible that mast cell activation, leading to the generation of mediators such as PAF and leukotrienes, could occur in the absence of detectable degranulation.

However, the observations that the pretreatment with a leukotriene antagonist or depletion of mucosal mast cells with dexamethasone did not alter the extent of ethanol-induced damage in control rats, suggests that activation of mast cells is not an important factor in the development of mucosal injury with this irritant. Previous studies suggested that mast cell degranulation or lysis occurs following topical administration of higher concentrations of ethanol,[55] while very low concentrations of ethanol can inhibit mast cell activation.[74] A role for mast cell degranulation/lysis in ethanol-induced gastric damage was suggested by Galli *et al.*,[63] who reported that mast cell-deficient mice were more resistant to absolute ethanol-induced gastric injury than normal littermates. Thus, the role of the mast cell in ethanol-induced damage appears to be dependent upon the concentration of ethanol being used: with high concentrations, mast cell degranulation/lysis occurs and contributes to injury, while at lower concentrations mast cell degranulation/lysis does not occur, and may even be inhibited.

It is noteworthy that pretreatment with dexamethasone, while depleting mucosal mast cells from the stomach, did not affect the systemic blood pressure responses to antigen administration. It is possible that these systemic effects were due to release of vaso-active substances (*e.g.* amines) from connective tissue mast cells or other IgE-binding cells. However, since dexamethasone reduced gastric damage in sensitized rats receiving antigen, it would appear that the mediators responsible for the systemic blood pressure responses did not contribute significantly to the effects of antigen on gastric mucosal susceptibility to injury.

To ensure that an equivalent amount of antigen was delivered to each animal an intra-arterial route of administration was used in this study. This is an important consideration since sensitization to some allergens has been shown to alter intestinal permeability, thereby affecting antigen uptake.[75] There is considerable evidence from previous studies that antigen, including the *N. brasiliensis* antigen used in the present study, can be taken up by the gastric mucosa in sufficient quantities to induce mast cell activation and/or functional changes.[76,77] For example, Catto-Smith *et al.*[76] demonstrated that gastric acid secretion could be stimulated and gastric emptying inhibited by intragastric administration of ovalbumin to sensitized but not control rats. More recently, Curtis *et al.*[78] have provided evidence for an active uptake system for luminal antigen in the gastric mucosa. Taken together with the results of the present study, these findings suggest that luminal antigen could trigger mast cell activation in the stomach, leading to the liberation of mediators which alter the susceptibility of the mucosa to injury. Indeed, intramucosal injection of antigen to sensitized rodents has been shown to cause gastric ulcers.[79] Such events may have relevance to the pathogenesis of gastric ulceration in man. Intragastric administration of food allergens to sensitized patients has been shown to cause mucosal swelling, erosions and bleeding, with decreases in tissue mast cell numbers and histamine content.[80] Mast cells have been implicated in the pathogenesis of ulceration associated with duodenogastric reflux[52] and *Helicobacter pylori* infection.[53,81,82] For example, cell wall fragments of *H. pylori* can augment rat mast cell degranulation induced by compound 48/80[53] and chronic gastritis patients have been shown to have basophil-bound and serum IgE directed against *H. pylori*.[81] It is also

interesting that increased mucosal generation of leukotriene C_4, the putative mediator of the enhanced mucosal injury observed in the present study, has been documented in *H. pylori* colonization.[83]

In addition to releasing mediators which can increase the susceptibility to mucosal injury, mast cells may also participate in the regulation of gastric acid secretion,[84,85] and in adaptive mucosal blood flow responses when the mucosa is exposed to an irritant.[69] While the role of mast cells in human peptic ulcer disease remains to be firmly established, the results presented herein support the hypothesis that the mast cell can modulate gastric mucosal defence.

CHAPTER 4

CHRONIC ORAL ANTIGEN CHALLENGE RESULTS IN SUPPRESSION OF MUCOSAL MAST CELL DEGRANULATION

4.1 Introduction

Most studies of allergic reactions in the gastrointestinal tract have focused on the effects of acute antigen challenge. In sensitized rats, for example, acute luminal antigen challenge causes alterations in intestinal permeability and water and electrolyte transport,[50,86] and disrupts co-ordinated patterns of intestinal motility.[87] In the stomach, luminal antigen challenge of sensitized rats evokes acid secretion and delays gastric emptying.[76] By comparison, few studies of animal models of intestinal allergy have examined the effects of chronic antigen challenge on the gut. This situation is probably more clinically relevant, as patients with documented food allergies are likely to be repeatedly exposed to the sensitizing antigen.

There have been some recent attempts to determine the effects of repeated or prolonged exposure to antigen on mucosal mast cell numbers and reactivity. For example, Marshall[88] demonstrated that repeated subcutaneous administration of low doses of antigen to sensitized rats resulted in changes in mucosal mast cell numbers, but the effects were dependent upon the dose of antigen used. At the lower doses of antigen (3 or 30 μg), Marshall noted significant increases in mucosal mast cell number, while at a higher dose (300 μg) there was no effect on mucosal mast cell number. Interestingly,

repeated subcutaneous challenge with the higher dose of antigen appeared to induce a change in mast cell reactivity, in that final challenge with antigen failed to significantly increase serum levels of a mast cell-derived protease.[88] Curtis *et al.*[89] examined the effects of administration of antigen in drinking water over a period of 9 days on mucosal mast cell numbers, and like Marshall[88] observed a marked intestinal mucosal mast cell hyperplasia.

This study examined the effects of chronic oral antigen administration to sensitized rats on the ability of mucosal mast cells to respond to these challenges. In broad overview, these investigations demonstrated that mucosal mast cells become refractory to antigenic stimulation after repeated challenge but remain responsive to activation by anti-IgE. In the face of repeated antigenic activation, this adaptive response of mast cells may serve to limit the potentially harmful effects of mast cell-derived mediators.

4.2 Materials and Methods

Immunization and challenge protocols. Male, Hooded Lister rats (75-100 g body weight) were sensitized by intraperitoneal injection of a chicken ovalbumin (OA) using aluminum hydroxide as an adjuvant (*see* Section 2.4). Control animals received adjuvant alone. Eleven days later, blood was withdrawn for measurement of OA-specific reaginic antibody titres by the passive cutaneous anaphylaxis (PCA) reaction (*see* Section 2.4).

Both sensitized and control animals were then subjected to either acute or chronic oral antigen challenge according to the protocols illustrated in Figure 4.1. Rats were deprived of food, but not water, for 15-18 hours before each oral challenge. In the acute challenge protocols, rats were given a single dose of ovalbumin (10 mg in 1.0 mL saline) by gavage 14 days after sensitization. This dose of antigen was selected because in previous studies by Catto-Smith *et al.*[76] it was found to evoke mast cell degranulation, as well as functional alterations (elevated acid secretion and delayed gastric emptying). In the chronic challenge group, 14 days after sensitization, rats were begun on a series of challenges (10 mg in 1.0 mL saline by gavage), a total of seven over the course of a three week period. In experiments lasting beyond two weeks, serum OA-specific reaginic antibodies were re-evaluated at the end of the protocol to ensure that the rats had remained sensitized.

Some experiments were performed in which rats received 10 mg OA subcutaneously, rather than orally. Antigen was administered every three days over a three week period, as in the experiments described above, and the final challenge with OA was given orally.

In other experiments, rats were given antigen chronically by gavage as described above, but the final (35 days after sensitization) challenge consisted of either (a) OA given intravenously instead of orally, or (b) intravenous infusion of anti-IgE. In these experiments, the rats were anaesthetized with sodium pentobarbital (65 mg/kg, IP) and the carotid artery was cannulated for blood sampling and continuous monitoring of blood pressure. The left jugular vein was cannulated for infusion of either OA or anti-IgE.

After a 15 minute stabilization period, OA (45 mg/kg) or anti-IgE (40 mg/kg, Serotec, Oxford, UK) were given as bolus intravenous infusions. These doses were selected based on preliminary studies in which they were shown to rapidly and effectively activate mucosal mast cells (MMC), but only produce a transient reduction of systemic blood pressure. Blood samples were collected both before and 30 minutes after infusion of OA or anti-IgE.

To examine whether the effects of chronic antigen challenge on mucosal mast cell responsiveness were dose-related, sensitized rats were chronically challenged with one-tenth of dose of antigen used in the previous protocols. In this experiment, 1.0 mg of OA was administered by gavage on three-day intervals for a total of six challenges. For the seventh challenge, 10 mg of OA was administered by gavage

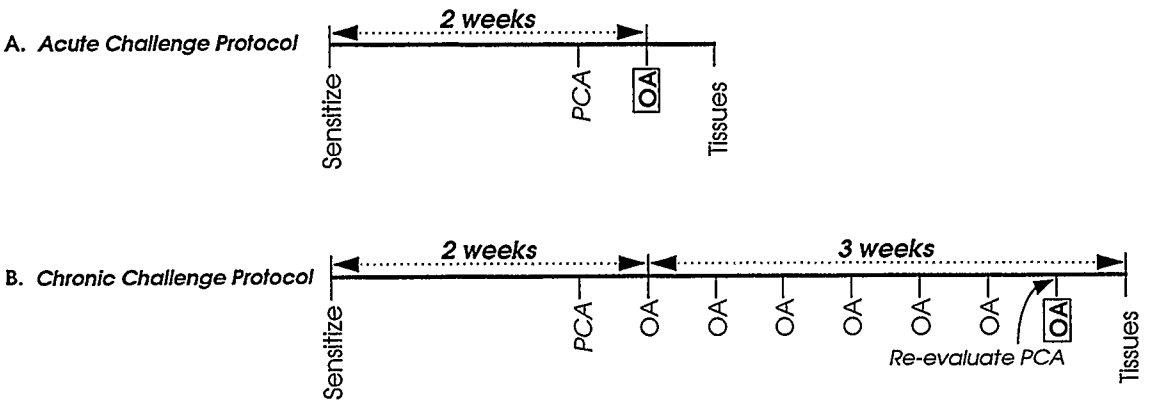

Figure 4.1. **Basic experimental protocols for acute and chronic antigen challenge.** Rats were sensitized by intraperitoneal injection of chicken ovalbumin (OA), using aluminum hydroxide as an adjuvant. Control rats received adjuvant alone. The passive cutaneous anaphylaxis (PCA) reaction was used to measure the degree of sensitization. *(A)* In the acute challenge protocol, rats were challenged with OA (10 mg in 1.0 mL of saline) by gavage. *(B)* In the chronic challenge protocol, rats were challenged repeatedly on three-day intervals for a total of 7 challenges. Blood was collected immediately before and three hours after the challenges for measurement of RMCP-II. At the end of the study period, tissues were excised for measurement of tissue RMCP-II levels and for histological enumeration of mucosal mast cells.

Rat Mast Cell Protease-II. Levels of RMCP-II were measured in blood serum and in tissues by ELISA (*see* Section 2.6). Blood samples were collected by cardiac puncture under halothane-induced anaesthesia both before and three hours after OA challenge. Tissue samples for RMCP-II measurement were excised from gastric corpus and small intestine three days after the final antigen challenge.

Histochemical identification of mucosal mast cells. Samples of gastric corpus and small intestine were excised three days after the final antigen challenge and prepared for mast cell staining (*see* Section 2.5).

Gastric acid secretion. Fasted rats were anaesthetized with 20% urethane (6.0 mL/kg, IP) and following laparotomy, cannulae were inserted via the mouth and duodenum into the stomach. The stomach was continuously perfused with 0.9% saline (37°C) at a rate of 3.0 mL/h through the orogastric cannula, and samples were collected from the duodenogastric cannula. Following a 30 minute stabilization period, perfusates were collected for three subsequent 30 minute periods. Sensitized rats from both acute and chronic challenge groups were given antigen intragastrically (10 mg in 1.0 mL) immediately after the first thirty minute period. In other experiments, pentagastrin (Peptavalon, Ayerst Laboratories, Montréal, PQ) was infused (20 mg/kg intravenous bolus followed by continuous infusion of 20 $mg \cdot kg^{-1} \cdot h^{-1}$) at the end of the first thirty minute period. Perfusates were collected for each thirty minute period and stored at -20°C. Gastric acid output was measured by back-titration of perfusates to pH 7.0 using an automated titration system (Impulsomat 614, Brinkmann Instruments, Rexdale, ON).

4.3 Results

Mucosal mast cell activation. Serum RMCP-II levels were measured as an index of MMC degranulation in sensitized and control rats (Figure 4.2). In control rats, the mean basal level of RMCP-II in serum was approximately 250 ng/mL and this was not significantly changed by oral administration of antigen. However, in sensitized rats, oral antigen challenge resulted in a 30-fold increase in serum RMCP-II levels. In sensitized rats subjected to chronic antigen challenge, there was still a significant increase in serum RMCP-II levels after the final challenge, but the response was significantly reduced from that observed in rats which were acutely challenged. This decrease in MMC degranulation occurred despite the fact that these rats maintained their original degree of sensitization, as shown by PCA.

In separate experiments, serum RMCP-II responses to oral antigen were measured after the first, fifth, and seventh challenges (Figure 4.3). These experiments showed a progressive decrease in MMC response to challenge which was dependent upon the number of times the rats were challenged during the chronic challenge protocol. To examine whether the diminished response to oral challenge observed in the chronic challenge group compared with the acute challenge group was simply due to differences in age at the time of challenge, additional experiments were performed in which a single oral challenge was administered five weeks after rats were sensitized to OA. In this group of rats, the mean serum RMCP-II response to oral challenge was not significantly different from that of an initial challenge at two weeks (Figure 4.3).

Studies were then performed to determine whether this apparent hyporesponsiveness of MMC after repeated administration of antigen simply reflected depletion of the mediator - RMCP-II - which was used as a marker of MMC activation. On the contrary, however, when tissue levels of RMCP-II were measured, they were found to be significantly increased in both the stomach and small intestine of sensitized rats which had been subjected to chronic oral antigen challenge (Figure 4.4). Histological enumeration of MMC showed that there was no significant difference in the number of MMC in the stomach of sensitized rats which were chronically challenged compared with unsensitized controls (*control:* 23.1 ± 5.8; *sensitized:* 30.4 ± 6.2). There was, however, a statistically significant decrease in the number of histologically detectable MMC in the proximal small bowel of chronically challenged sensitized rats (*control:* 18.2 ± 1.8; *sensitized:* 12.8 ± 1.8, $P < 0.05$).

To examine whether repeated parenteral challenge could result in hyporesponsiveness of MMC, antigen was given repeatedly to sensitized rats by the subcutaneous rather than the oral route. After the first subcutaneous challenge, a marked rise in serum RMCP-II occurred in all 5 rats tested (Figure 4.5A). However, after 6 subcutaneous challenges over the course of 18 days, a final oral challenge failed to evoke a response in any of the 5 rats tested (Figure 4.5B) despite having maintained their original degree of sensitization.

An alternative explanation for the apparent hyporesponsiveness of MMC after challenge is decreased uptake of antigen from the gastrointestinal lumen. To address this possibility, experiments were performed to measure the response of MMC to

intravenously administered antigen. In sensitized rats, intravenous infusion of antigen caused a significant increase in serum RMCP-II levels, with an increase of greater than two standard deviations above basal levels observed in 9 out of 9 rats tested (Figure 4.6A). In sensitized rats which had received chronic oral antigen, a final intravenous antigen challenge failed to produce significant changes in serum RMCP-II levels. As shown in Figure 4.6B, 5 out of 8 rats showed no response to antigen, while in the remaining 3 rats the serum levels of RMCP-II increased to a similar extent as seen in animals receiving only a single antigen challenge.

The decreased MMC degranulation observed following chronic antigen administration may have occurred as a result of changes in the mast cell itself. To address this possibility, experiments were performed to determine whether chronic antigen challenge rendered MMC hyporesponsive to activation by other agents. In sensitized rats which had not previously been challenged with antigen, intravenous infusion of anti-IgE produced measurable RMCP-II responses in all 5 rats studied (Figure 4.7A). In sensitized rats which had received 6 oral antigen challenges over the course of 18 days, infusion of anti-IgE on day 21 still resulted in significant increases in serum RMCP-II (Figure 4.7B).

To study whether a lower dose antigen would induce mucosal mast cell hyporesponsiveness, sensitized rats were repeatedly challenged with 1.0 mg OA (one-tenth the dose used in previous experiments) by gavage. This dose of antigen was effective at degranulating MMC as shown by the significant increase in serum RMCP-II after acute challenge (Figure 4.8). After six challenges, MMC no longer responded to a

1.0 mg OA challenge, but a final challenge with a higher dose of antigen (10 mg) caused significant release of RMCP-II.

Gastric acid secretion. In sensitized rats, intragastric administration of antigen resulted in a significant increase in gastric acid secretion (Figure 4.9A). However, in sensitized rats that had been chronically challenged with the antigen by gavage, subsequent intragastric administration of antigen had no effect on gastric acid secretion (Figure 4.9B).

Repeated oral challenge with antigen did not affect the ability of the stomach to secrete acid, as intravenous administration of pentagastrin to sensitized rats which had been chronically challenged resulted in an increase in acid secretion from a basal level equivalent to that observed in control rats (Figure 4.10).

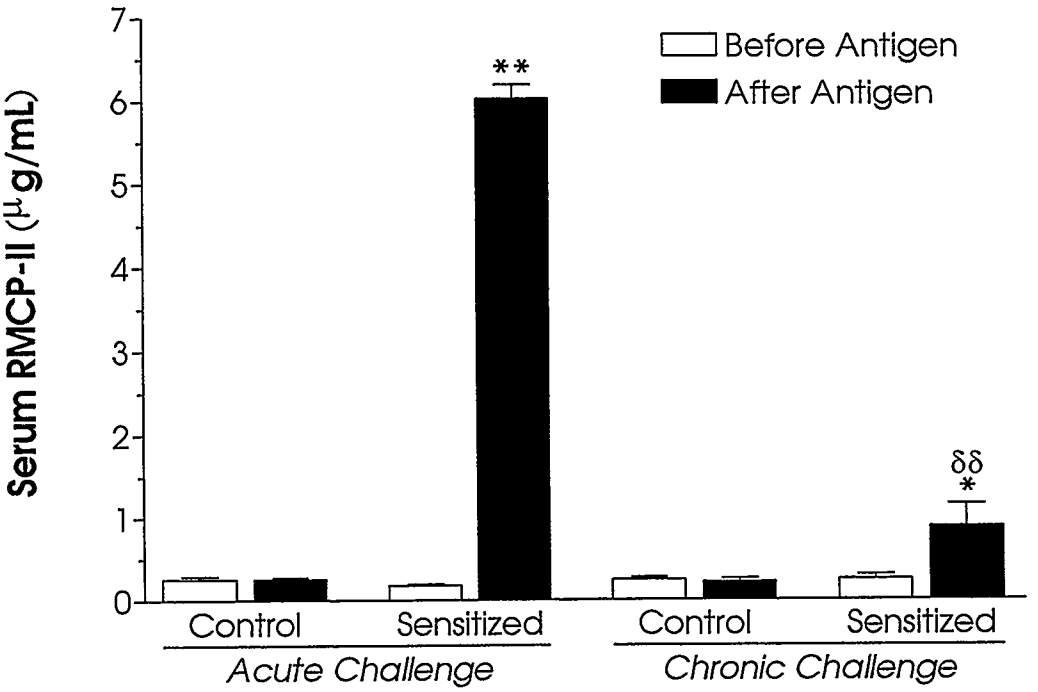

Figure 4.2. **Effects of acute and chronic oral antigen challenge on serum rat mast cell protease II levels in sensitized and control rats.** Following chronic antigen challenge, administration of antigen caused release of RMCP-II into serum that was significantly less than that observed with a single antigen challenge ($^{**}P < 0.001$, $^{*}P < 0.05$, compared with the corresponding "before antigen" group; $^{\delta\delta}P < 0.001$ compared with the corresponding "acute challenge" group). Each bar represents the mean ± SEM of at least 5 rats per group.

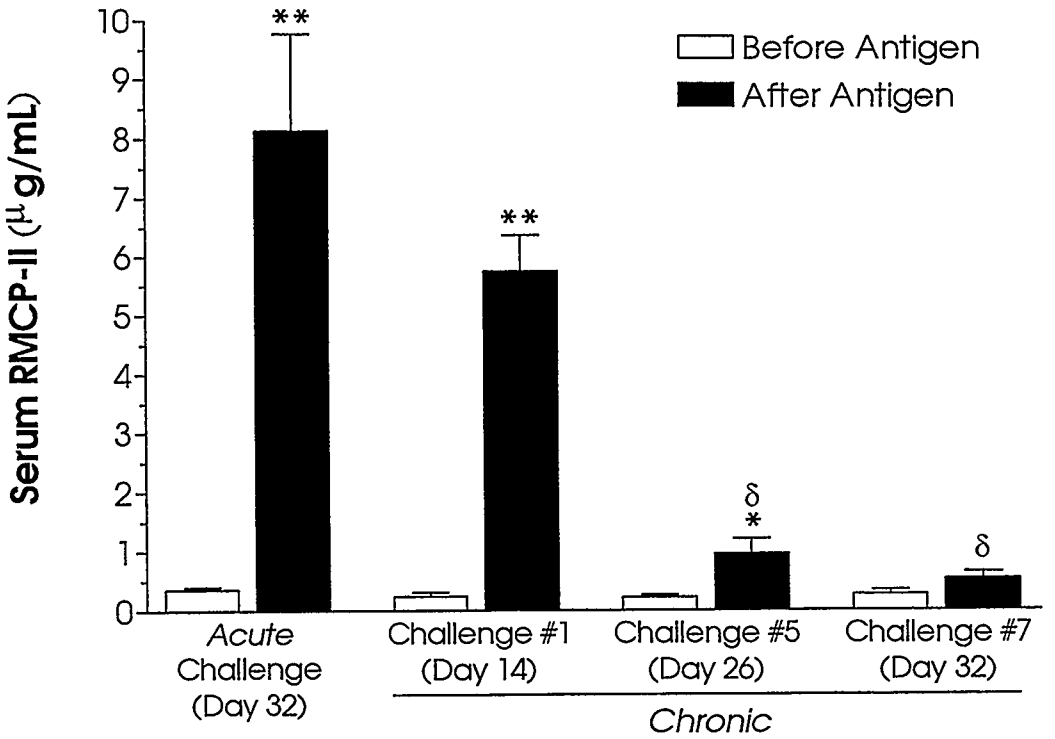

Figure 4.3. **Time-course of diminished responsiveness of mucosal mast cells to activation after repeated oral antigen challenge.** After challenge #1 of the *chronic* challenge protocol, there was a very marked rise in serum RMCP-II in sensitized rats. Although there was still a significant rise in RMCP-II in sensitized rats after challenge #5, this response was significantly decreased from that of challenge #1, and after challenge #7 there was no longer a significant rise in serum RMCP-II. The *acute* challenge group showed that this phenomenon was not simply an effect of age. Note that "days" listed on x-axis refer to the number of days after sensitization. $^{**}P < 0.001$, $^{*}P < 0.05$ compared with the corresponding "before antigen" group; $^{\delta}P < 0.05$ compared with after antigen challenge #1. Each bar represents the mean ± SEM of 6 rats.

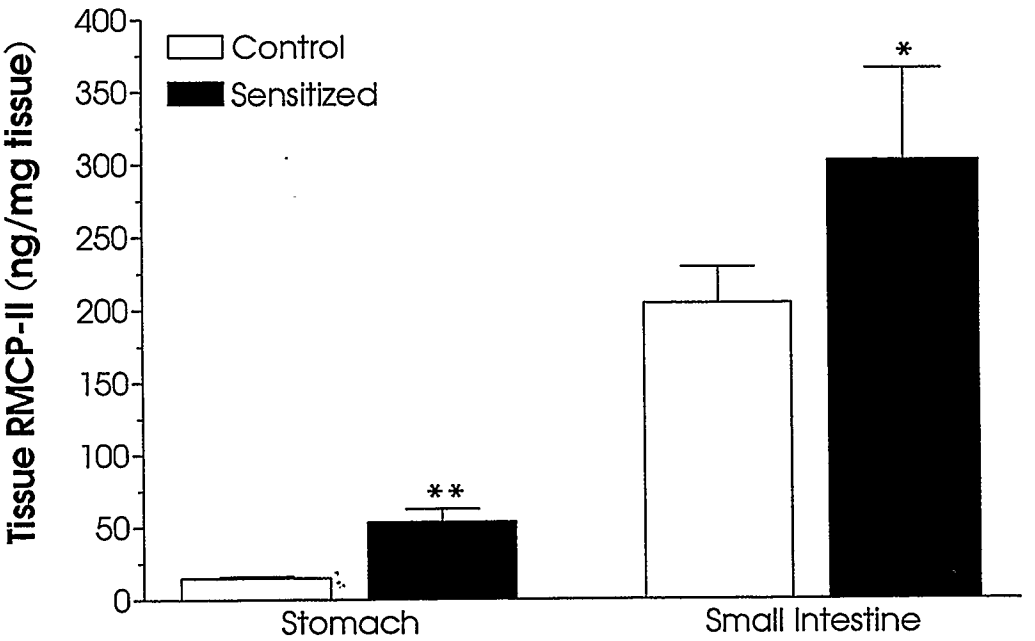

Figure 4.4. **Effects of chronic oral antigen challenge of sensitized rats on tissue levels of rat mast cell protease II in stomach and small intestine.** Tissues were excised three days after the final challenge for measurement of RMCP-II by ELISA. In both stomach and small intestine of sensitized rats there was a significant increase in RMCP-II compared with control rats ($^{*}P < 0.05$, $^{**}P < 0.01$).

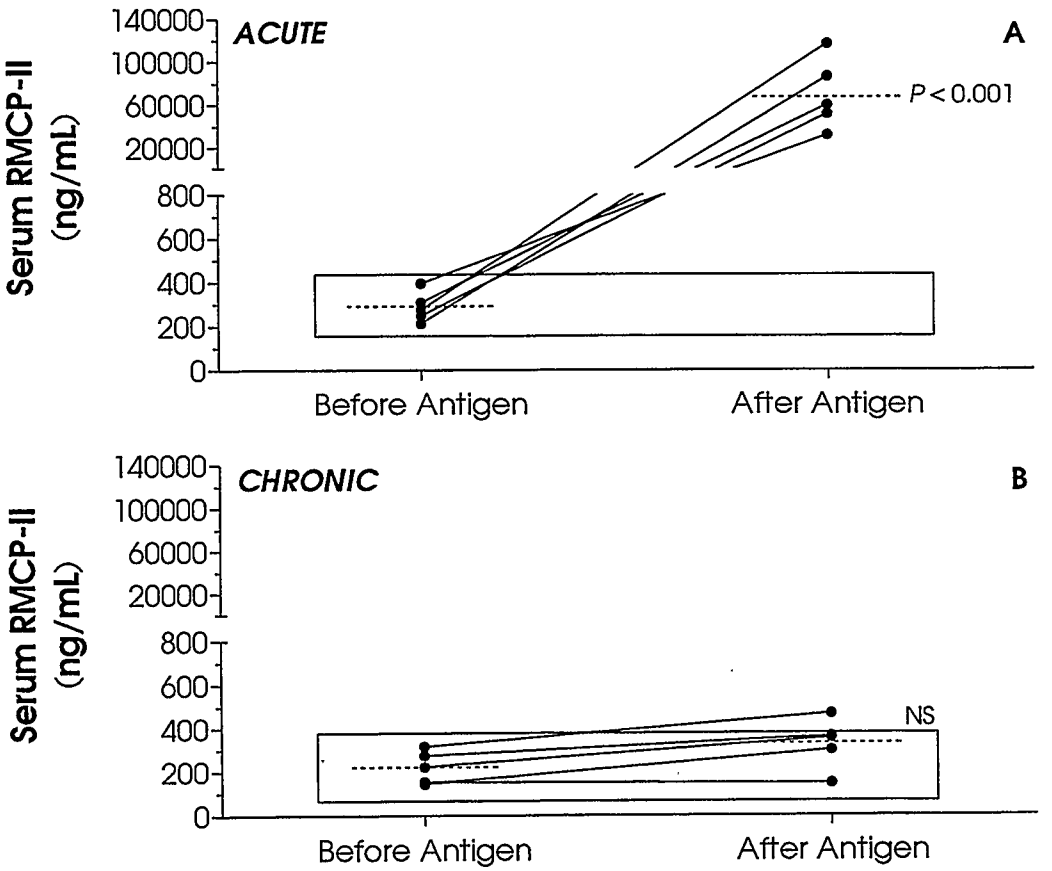

Figure 4.5. **Effect of chronic subcutaneous antigen challenge of sensitized rats on subsequent response of mucosal mast cells to oral antigen challenge.** Serum RMCP-II levels served as a marker of mucosal mast cell degranulation. Each line (•—•) connects the before and after antigen challenge RMCP-II level for a single sensitized rat; the dashed lines (-----) represent the mean values. The boxes show the mean ± 2 standard deviations of the "before antigen" serum RMCP-II level. *(A)* A single subcutaneously administered dose of antigen caused a significant increase in serum RMCP-II. *(B)* After a series of six subcutaneous challenges, rats failed to respond to the final antigen challenge which was administered by the oral route.

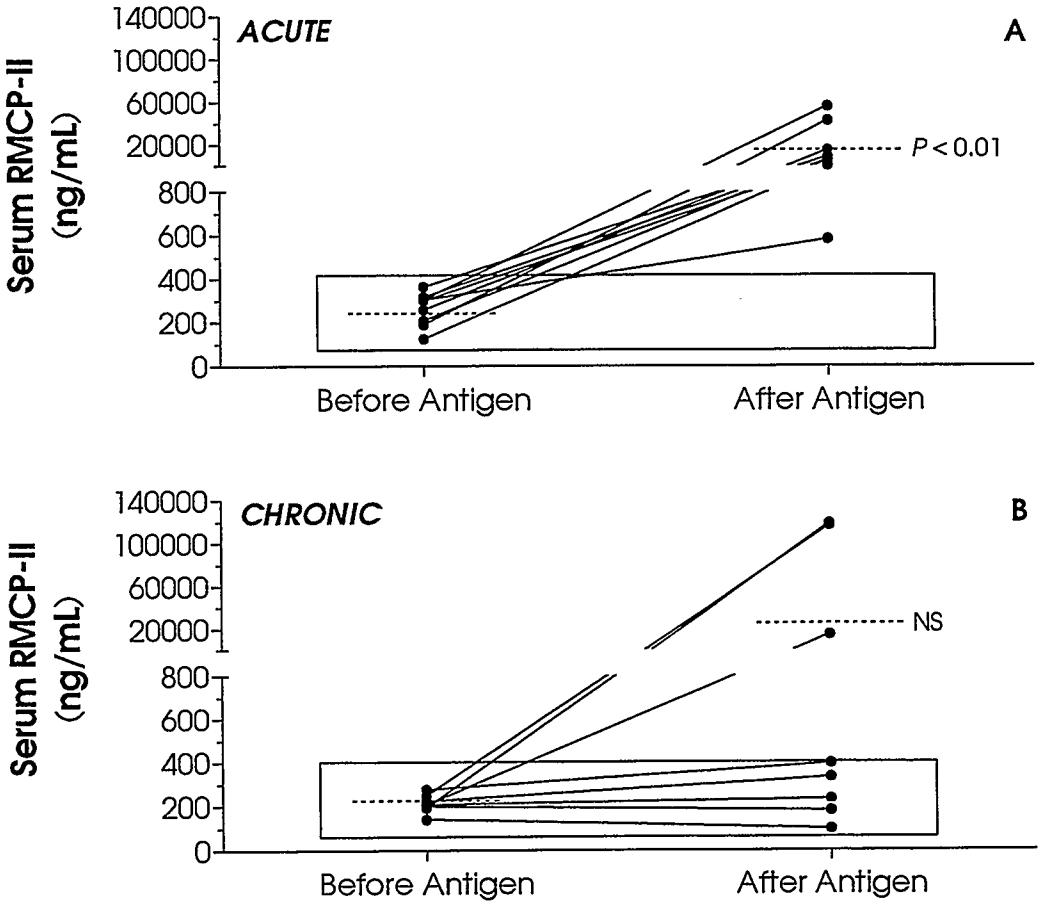

Figure 4.6. **Effect of intravenously administered antigen on mucosal mast cell activation in sensitized rats after acute and chronic oral antigen challenge.** Each line (•—•) connects the before and after antigen challenge RMCP-II level for a single sensitized rat; the dashed lines (-----) represent the mean values. The boxes show the mean ± 2 standard deviations of the "before antigen" serum RMCP-II level. *(A)* A single intravenously administered dose of antigen caused a significant increase in serum RMCP-II. *(B)* After a series of six oral antigen challenges, 5 out of 8 rats failed to respond to the final antigen challenge which was administered by the intravenous route.

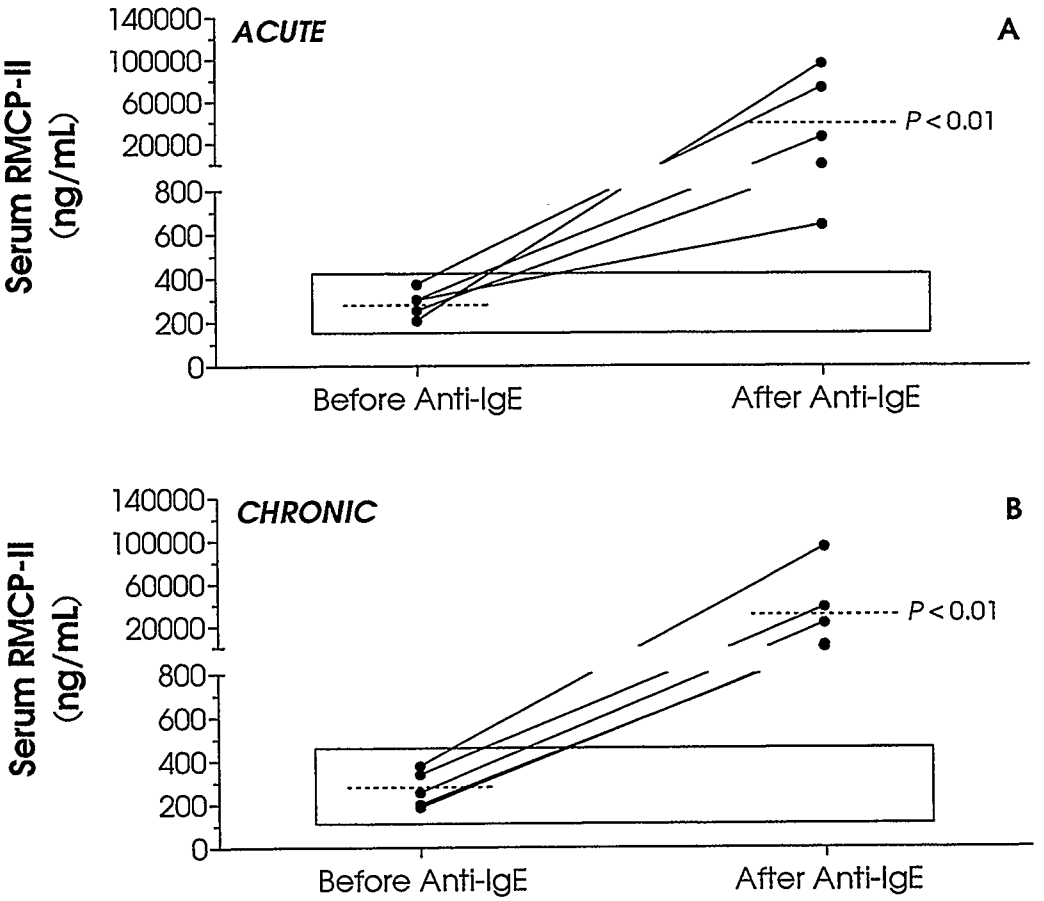

Figure 4.7. **Effect of chronic oral antigen challenge of sensitized rats on subsequent response of mucosal mast cells to intravenously administered anti-IgE.** Each line (•—•) connects the before and after antigen challenge RMCP-II level for a single sensitized rat; the dashed lines (-----) represent the mean values. The boxes show the mean ± 2 standard deviations of the "before antigen" serum RMCP-II level. *(A)* A single intravenously administered dose of anti-IgE caused a significant increase in serum RMCP-II. *(B)* After a series of six oral challenges with ovalbumin, mucosal mast cells still responded significantly to intravenously administered anti-IgE.

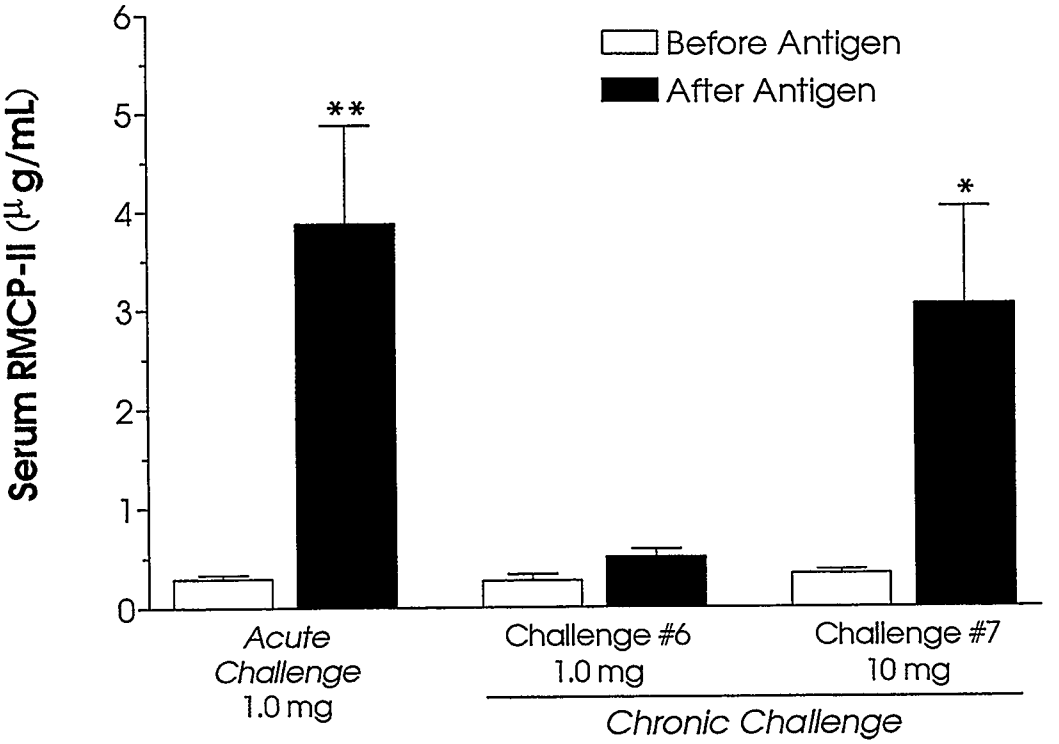

Figure 4.8. **Effect of chronic challenge with 1.0 mg ovalbumin on subsequent response of mucosal mast cells to 10 mg challenge.** Three hours after acute challenge with 1.0 mg ovalbumin (prepared in 1.0 mL saline and administered by gavage), there was a significant increase ($P < 0.01$) in serum RMCP-II levels compared with levels measured "before antigen." However, in sensitized rats that were chronically challenged with 1.0 mg ovalbumin, there was no longer a significant rise in serum RMCP-II by the sixth challenge despite the fact that they had maintained their original degree of sensitization (PCA titre $\geq$ 1:32). A seventh and final challenge with a higher dose of antigen (10 mg) evoked significant release of RMCP-II ($P < 0.05$) demonstrating that mucosal mast cells were still able to respond to antigen. Each group consisted of 6 rats.

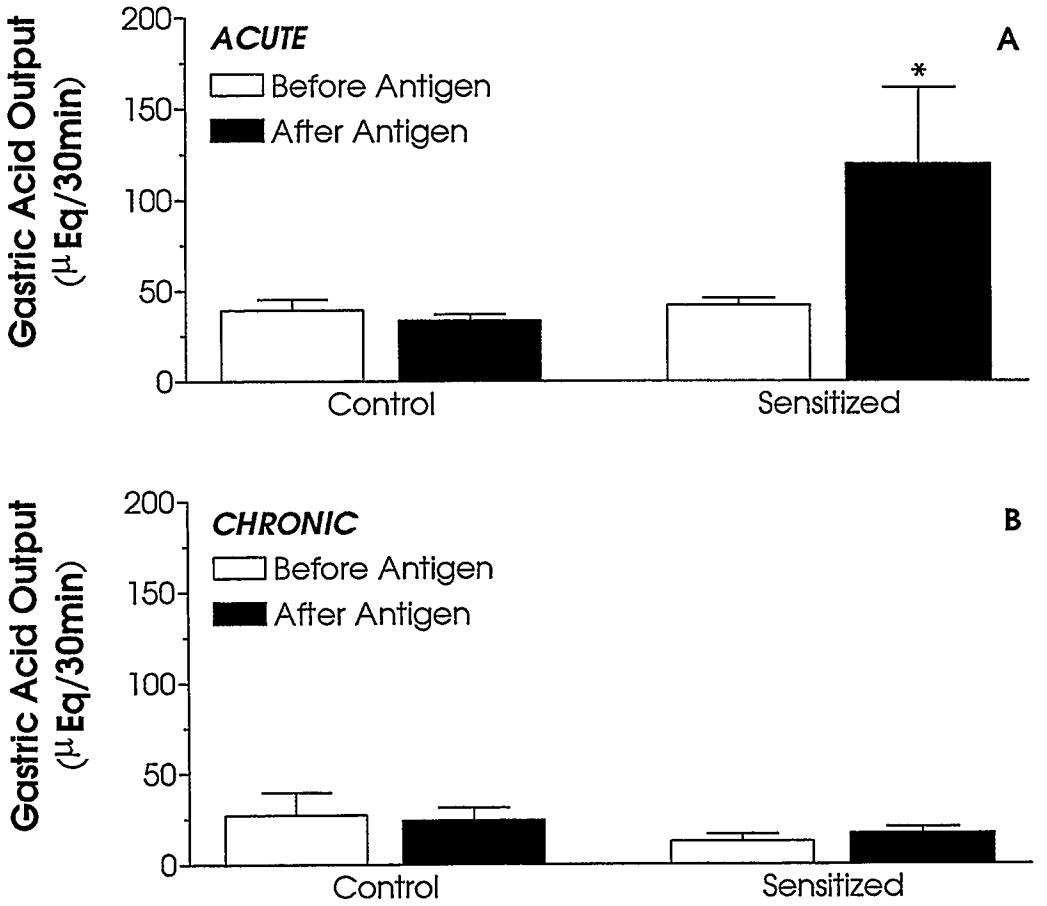

Figure 4.9. **Effect of acute and chronic luminal antigen challenge on gastric acid secretion.** The stomachs of sensitized and control rats were continuously perfused with saline. After a basal period, antigen was added to the perfusate (10 mg/mL). *(A)* In sensitized rats, acute luminal challenge caused a significant increase in gastric acid output ($^{*}P < 0.05$ compared with "before antigen"), whereas antigen had no effect on gastric acid output in control rats. *(B)* After a series of six oral antigen challenges, sensitized rats no longer secreted gastric acid in response to luminal challenge with antigen. Rates of basal secretion did not differ between groups.

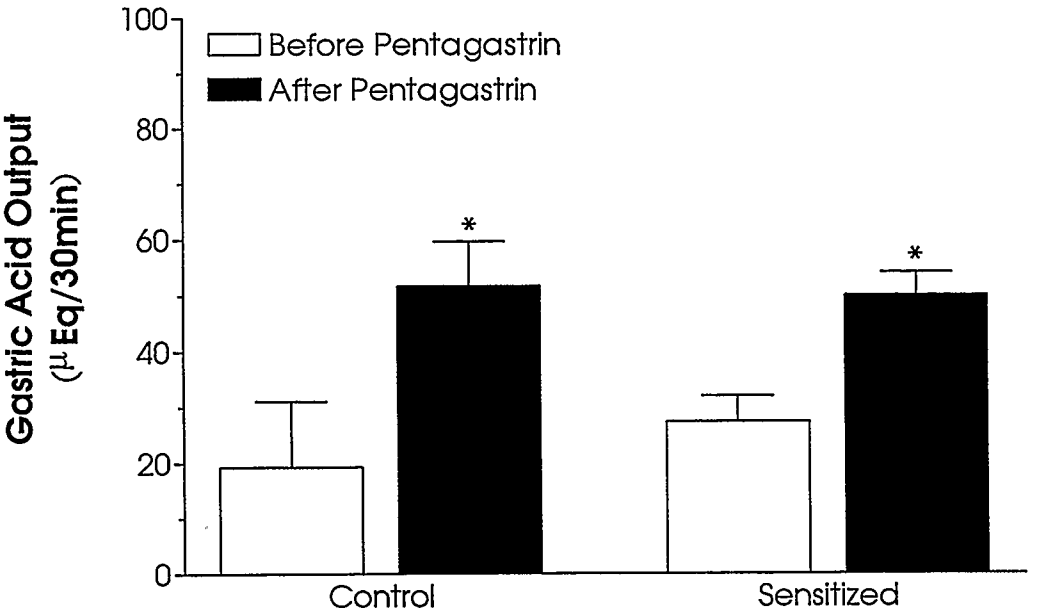

Figure 4.10. **Effect of chronic oral antigen challenge on pentagastrin-induced gastric acid output.** Although refractory to the acid secretory effects of luminal antigen (*see* Figure 4.9), sensitized rats subjected to chronic oral antigen challenge still responded to pentagastrin to the same degree as unsensitized control rats ($^{*}P < 0.05$ compared with corresponding "before pentagastrin."

4.4 Discussion

The results of this study demonstrate a marked and progressive down-regulation of mast cell degranulation during repeated challenge with the sensitizing antigen. This effect was not attributable simply to a depletion of cellular stores of RMCP-II, a protease released when mast cells degranulate, as tissue levels of this enzyme actually increased during chronic antigen administration. The down-regulation of mast cell responsiveness to antigen was also not due to decreased serum anti-OA specific reaginic antibodies, since PCA confirmed that titres were maintained throughout the experiment. In concordance with the observed decrease in mast cell degranulation, as measured through serum RMCP-II levels, a decreased functional correlate of mast cell degranulation was also observed. Antigenic activation of mast cells in the stomach leads to stimulation of gastric acid secretion, presumably attributable to histamine release from these cells.[76] This phenomenon was confirmed in the present study, and it was also demonstrated that following repeated challenge with antigen, an acid-secretory response to oral antigen could no longer be elicited. This was not due to changes in the capacity of the stomach to secrete acid, since secretory responses to pentagastrin were similar in control and chronic antigen-treated rats.

Adaptation to repeated antigen exposure, leading to an apparent down-regulation of mast cell responsiveness could occur through several mechanisms. For example, changes in epithelial uptake of antigen in response to repeated oral antigen challenge have been reported.[90] This raises the possibility that the diminished mast cell degranulation

observed during chronic antigen administration may have been due to decreased delivery of the antigen to the mast cell. However the suppressed mast cell response in the present study was not related to the luminal delivery of the antigen, since similar adaptation was observed when the antigen was given repeatedly via the subcutaneous route. Secondly, when the gastrointestinal epithelium was "by-passed" by delivering the final antigen challenge intravenously, the down-regulated mast cell response was still evident. Overall, a statistically significant increase in serum RMCP-II levels was not observed in this group in response to the intravenous antigen challenge. However, careful examination of the data revealed a separation into two subgroups. In 5 of 8 rats studied, the post-antigen serum RMCP-II levels were within two standard deviations of basal levels, while in the remaining 3 rats there was an increase in serum RMCP-II levels similar to that seen following an acute antigen challenge. By comparison, 9 out of 9 rats receiving the same dose of antigen intravenously but not previously challenged with antigen exhibited significant increases in serum RMCP-II levels.

Whereas mucosal mast cells did not respond to challenge with the antigen, they still responded consistently to anti-IgE. These experiments clearly demonstrated that the mucosal mast cells were still capable of degranulating, but the responsiveness to the sensitizing antigen was greatly down-regulated. It should be noted that there was a major difference in terms of the dose of antigen used in the present study versus that of Marshall.[88] Whereas Marshall used microgram doses of antigen that caused only small increases in serum RMCP-II levels (of ~ 100 ng/mL), in the present study, milligram doses of antigen were used that increased serum RMCP-II levels by over 7000 ng/mL.

The doses of antigen used in this study were selected because in previous studies they were found to cause functional and/or structural alterations in the gastrointestinal tract.[76,86,87]

Whereas mast cells in sensitized rats remained responsive to repeated subcutaneous challenge with low doses antigen (3 or 30 μg), Marshall showed that mast cells were refractory to a final challenge after repeated challenge with a relatively high dose of antigen (300 μg).[88] In the present study, when a lower dose of antigen was used (1.0 mg instead of 10 mg) in the chronic challenge system, mast cells were again rendered unresponsive to activation at the end of the protocol. However, in these animals, a final challenge with a higher dose of antigen (10 mg) was effective at activating MMC. Further studies are required to see if MMC will remain responsive to repeated challenge when even lower doses of antigen are used.

One explanation for these findings is that repeated exposure to antigen results in an increase in the threshold of stimulation required for mast cell degranulation to occur. The fact that intravenous antigen challenge caused mast cell degranulation in a subset of the rats that had previously been repeatedly challenged with antigen are consistent with this hypothesis. When the antigen was delivered intravenously, a higher concentration of antigen would be delivered to the mast cell surface, and therefore there would be a greater likelihood of exceeding the threshold for activation. In further support of this hypothesis, it was noted that intravenously administered anti-IgE was able to activate mucosal mast cells after they had become refractory to the effects of chronic antigen challenge by gavage. In this situation, it is clear that anti-IgE was a more effective stimulus, being

able to exceed what seems to be an altered threshold of activation. Also consistent with this hypothesis was the observation that high dose antigen was able to cause degranulation of mast cells after they had become refractory to stimulation by a lower dose of antigen. While there have been no studies of repeated antigen challenge of mucosal mast cells *in vitro*, Levi-Schaffer *et al.*[91] reported on the effects of repeated or continuous exposure of peritoneal mast cells to antigen *in vitro*. While the peritoneal mast cells remained responsive during continuous exposure to antigen, the percent release of histamine progressively declined. They suggested that this may be due to a down-regulation of IgE receptors on the mast cell.

Both Marshall[88] and Curtis *et al.*[89] observed a significant increase in mucosal mast cell numbers during repeated antigen challenge. In the present study, repeated oral challenge with antigen resulted in a significant increase in tissue RMCP-II levels, consistent with an increase in mucosal mast cell numbers. However, histological enumeration of mast cells showed no change in gastric mucosal mast cell numbers and a slight, but significant decrease in intestinal mucosal mast cell numbers. The differences between tissue RMCP-II and histological enumeration of mast cells could be attributable to higher amounts of RMCP-II per cell. Alternatively, these differences may be attributable to the histological enumeration of cells being a less quantitative method, given that it relies upon counting only a few sections of tissue, versus the RMCP-II measurement in a much larger tissue sample.

Marshall[88] suggested that the up-regulation of mast cell numbers observed with low dose antigen challenge was an adaptive response aimed at increasing the sensitivity

of the individual to the antigen. It is possible that the observations in the present study represent a corollary to this hypothesis; that is, with repeated exposure to a high dose of antigen, there is a down-regulation of mast cell reactivity. As mast cell degranulation has been shown to increase the susceptibility of the gastric mucosa to damage[96] and to be associated with alterations in epithelial function and/or integrity in the small intestine,[50,75,86,92,93] an adaptive down-regulation of mast cell reactivity during repeated exposure to antigen would seem logical. This adaptation appears to occur at the level of the mast cell, perhaps as a consequence of altered threshold of activation. However, further studies are required to fully characterize the mechanism through which mucosal mast cell hyporesponsiveness occurs.

CHAPTER 5

GASTRIC MUCOSAL MAST CELLS: POTENTIAL TARGETS OF NONSTEROIDAL ANTI-INFLAMMATORY DRUGS AND CYTOPROTECTIVE PROSTAGLANDINS

5.1 Introduction

Mast cells are prevalent in the superficial aspect of the gastric mucosa where they may be involved in defensive hyperaemic responses to luminal irritants[44] and, in some species, physiological regulation of gastric acid secretion.[85] There is evidence that mucosal mast cells may contribute to injury in the stomach induced by agents such as stress,[60] ethanol,[63] and *Helicobacter pylori*.[81,82] Little attention, however, has been paid to the possibility that mast cells may contribute to gastric mucosal injury associated with the use of nonsteroidal anti-inflammatory drugs (NSAIDs).

Mast cells are a source of several potent pro-ulcerogenic and pro-inflammatory mediators whose effects on mucosal integrity in the gastrointestinal tract have been studied. For example, mast cell-derived mediators shown to be important in the development of gastrointestinal injury include leukotriene C_4,[69,94-96] platelet-activating factor,[73,95] histamine,[97,98] endothelin,[99] and tumour necrosis factor-α.[100,101,102] In addition, activation of mast cells, either through administration of chemical agonists[59,103] or antigen[96] have been shown to produce or exacerbate mucosal injury, whereas mast cell "stabilizers" have been reported to reduce gastric mucosal injury in various experimental models.[55-58]

The injurious effects of NSAIDs on the gastric mucosa are thought to be largely attributable to the ability of these agents to block production of prostaglandins. Prostaglandins are key regulators of gastric mucosal defence, modulating release of gastric mucus and bicarbonate, regulating mucosal blood flow and increasing the resistance of the gastric mucosa to injury induced by topical irritants.[104,105] An important component of the protective actions of prostaglandins in the stomach may be their ability to inhibit mediator release from mast cells at extremely low concentrations.[65] Therefore, NSAIDs, by reducing prostaglandin synthesis in the gastric mucosa, may remove an endogenous stabilizer of mast cells thus increasing their reactivity and perhaps even leading to unstimulated mediator release. It is possible that this contributes to the pathogenesis of mucosal injury caused by these drugs. This is analogous to the deleterious effect of inhibiting nitric oxide synthesis. Like prostaglandins, nitric oxide is an endogenous modulator of mast cell reactivity. Nitric oxide is constitutively produced by rat mast cells and seems to act in an autoregulatory fashion to suppress histamine and PAF release.[64,106] When nitric oxide synthesis is blocked, mast cell degranulation has been observed[107] and is accompanied by increased intestinal permeability.[108]

The objective of this study was to assess the role of gastric mucosal mast cells in injury to the stomach induced by NSAIDs in the rat and mouse. Specifically, experiments were performed to determine whether mucosal mast cell degranulation occurs after administration of an NSAID and whether the number of mast cells in the gastric mucosa influences susceptibility to NSAID-induced damage.

5.2 Materials and Methods

Animals. Male Wistar and Sprague Dawley rats (200-250 g body weight), and male mast cell-deficient mice (W/W^v) and normal congenic littermates (+/+) (23-27 g body weight) were used in these experiments (*see* Section 2.1)

Indomethacin-induced gastric mucosal injury. Rats were deprived of food, but not water, for 15-18 hours before each experiment. A basal blood sample was collected by cardiac puncture after inducing anaesthesia with halothane. Rats assigned to the experimental group received indomethacin (sodium salt, Sigma Chemical Co., St. Louis, MO) by gavage at a dose of 20 mg/kg, whereas control rats received an equivalent volume (0.1 mL/100 g body weight) of vehicle alone (5% w/v aqueous sodium bicarbonate). Three hours later, rats were anaesthetized with halothane and a second blood sample was collected by cardiac puncture, after which the rats were sacrificed by cervical dislocation. The stomach was excised and cut open along the greater curvature. Macroscopically visible gastric damage was assessed by an observer unaware of the treatments the rats had received. Damage scores were assigned according to an established system which involves measuring the lengths (in mm) of each lesion and summing them for each stomach.[109] In some experiments, tissue samples (~150 mg, wet weight) were cut from the corpus region of the stomach for measurement of prostaglandin E_2 (PGE_2) synthesis by enzyme immunoassay (Cedarlane Laboratories Ltd., Hornby, ON), as reported elsewhere.[110]

Mucosal mast cell degranulation. After macroscopic gastric damage had been assessed, tissue samples were taken from the corpus region for histochemical identification of mast cells (*see* Section 2.5). RMCP-II was measured in full-thickness tissue samples (~150 mg wet weight) excised from the gastric corpus (*see* Section 2.6). Serum RMCP-II levels were also determined, using the blood samples collected at the beginning and end of each experiment.

Manipulation of mucosal mast cell numbers. To induce gastric mucosal mast cell hyperplasia, male Sprague Dawley rats were infected with *Nippostrongylus brasiliensis* (*see* Section 2.2). Dexamethasone was used to deplete the gastric mucosa of mast cells (*see* Section 2.3).

Rat antral ulcer model. The lesions produced in the rat stomach by acute indomethacin administration are superficial and largely confined to the corpus region, whereas clinically significant NSAID-induced ulcers in man occur primarily in the antrum. Therefore, additional experiments were performed to assess the role of mast cells in a model of NSAID-induced antral ulceration in the rat.[111] Rats were fasted for 24 hours, then allowed to feed for 2 hours, after which time saline or prostaglandin E_2 (100 mg/kg), followed by naproxen (80 mg/kg) were administered by gavage. Twenty-four hours later, the stomachs were excised and the number and area of antral ulcers were measured by planimetry. To evaluate the role of mast cells in the formation of these ulcers, one group of rats (n = 14; 8 saline-treated and 6 PGE_2-treated) was pretreated with dexamethasone 48 and 24 hours prior to the administration of naproxen to deplete the

stomach of mucosal mast cells, while the control group (n = 13; 7 saline-treated and 6 PGE_2-treated) received vehicle instead of dexamethasone.

Mast cell-deficient mice. Mast cell-deficient mice (W/W^v)[63] and normal littermates (+/+) were fasted for 24 hours and then given indomethacin (20 mg/kg administered in 5% aqueous sodium bicarbonate by subcutaneous injection). Three hours later, the mice were killed by cervical dislocation and each stomach was excised, opened along the greater curvature, and pinned out on a wax platform. According to the initial experimental design, macroscopically visible damage was to be scored. However, these animals did not develop macroscopically visible lesions as seen in the rat, so instead damage was scored histologically. The stomach was divided in half along the lesser curvature. One-half of the stomach was fixed in Carnoy's solution and the other half in neutral buffered formalin for alcian blue/safranine and hematoxylin/eosin staining, respectively. The samples stained in hematoxylin/eosin were coded and then randomized to prevent observer bias in the evaluation of damage by light microscopy. The severity of damage was scored using the following criteria: 0 = no damage; 1 = superficial damage to the epithelium; 2 = damage extending up to half the depth of the mucosa; 3 = damage involving the full thickness of the mucosa. The samples stained with alcian blue/safranine were examined to confirm the absence of mast cells in the W/W^v mice.

5.3 Results

Oral administration of indomethacin (20 mg/kg) inhibited gastric PGE_2 synthesis by ~80% compared with vehicle-treated control rats (indomethacin: 18.4 ± 4.0 pg/mg n = 6; vehicle: 88.8 ± 29.6 pg/mg n = 5; $P < 0.05$). Indomethacin also caused hemorrhagic erosions in the gastric mucosa. These erosions were generally confined to the crests of the rugal folds in the corpus region and the mean damage score was 28.8 ± 6.3 (n = 6). Histologically, the lesions were found to be confined to the mucosal layer (*i.e.* not penetrating through the muscularis mucosae). No gastric damage was observed in vehicle-treated control rats.

Effects of indomethacin on mast cell degranulation. In samples of tissue excised from the gastric corpus and fixed in Carnoy's solution, combined staining with alcian blue and safranine enabled visualization of mast cells by light microscopy. By this method, mast cells in the rat stomach distinctly stained blue and were found to segregate in the superficial aspect of the mucosa near the bases of gastric pits. On average, approximately 40 mast cells were present per 20× microscope field (Figure 5.1A).

When mast cells degranulate, they become depleted of proteoglycans which form the matrix of mast cell granules. Since proteoglycans are the targets of the histochemical stains used to identify mast cells, degranulation of mast cells renders them undetectable by histological means. Administration of indomethacin did not significantly change the number of histologically detectable mast cells in the gastric mucosa compared with

vehicle-treated controls (Figure 5.1A), suggesting that mast cell degranulation did not occur.

Mast cell degranulation was also assessed by measurement of gastric tissue and serum levels of RMCP-II. Administration of indomethacin did not significantly alter RMCP-II levels in tissue or serum, again suggesting that mast cell degranulation did not occur (Figures 5.1B and 5.1C).

Effect of increasing gastric mucosal mast cell numbers. In rats studied 35-42 days after infection with *N. brasiliensis* there was a 75% increase in the number of mast cells in the gastric mucosa compared with controls ($P < 0.05$) (Figure 5.2A). However, there was no significant change in the susceptibility of the gastric mucosa to indomethacin-induced injury in *Nippostrongylus*-infected rats compared with controls (Figure 5.2B).

Effect of decreasing gastric mucosal mast cell numbers. Pretreatment with dexamethasone 48 and 24 hours prior to indomethacin administration resulted in nearly complete depletion of histologically detectable gastric mucosal mast cells ($P < 0.0001$; Figure 5.3A). The depletion of gastric tissue mast cells was confirmed by the marked reduction of tissue RMCP-II levels ($P < 0.05$; Figure 5.3B). However, the rats depleted of mucosal mast cells did not exhibit any difference in the susceptibility to indomethacin-induced damage (Figure 5.3C).

Rat antral ulcer model. Administration of naproxen according to the "refeeding" protocol caused discrete ulcers in the antrum region of the stomach in all 7 saline-pretreated rats (Figure 5.4). The average ulcer area was 18.9 ± 3.3 mm^2. Pretreatment

with dexamethasone using the protocol that results in depletion of mucosal mast cells (as above) did not significantly affect the incidence (100%) or area (25.8 ± 7.8 mm^2) of antral ulceration. Pretreatment with PGE_2 almost completely abolished antral ulcer formation, both in the group pretreated with saline and the group in which mast cells were depleted by treatment with dexamethasone.

Mast cell-deficient mice. Unlike the rat, mice given indomethacin did not develop macroscopically detectable gastric damage. However, when examined histologically, gastric injury was detectable. This damage consisted primarily of necrosis of the superficial epithelium, although in some animals the damage penetrated to about the top third of the mucosa. Semi-quantitative, blinded scoring of the damage yielded a mean damage score in the five mast cell-deficient (W/W^v) mice of 1.6 ± 0.2. This level of damage was indistinguishable from that observed in the five normal congenic littermates (mean damage score of 1.8 ± 0.2). The staining with alcian blue/safranine confirmed that the W/W^v mice were completely devoid of mucosal mast cells.

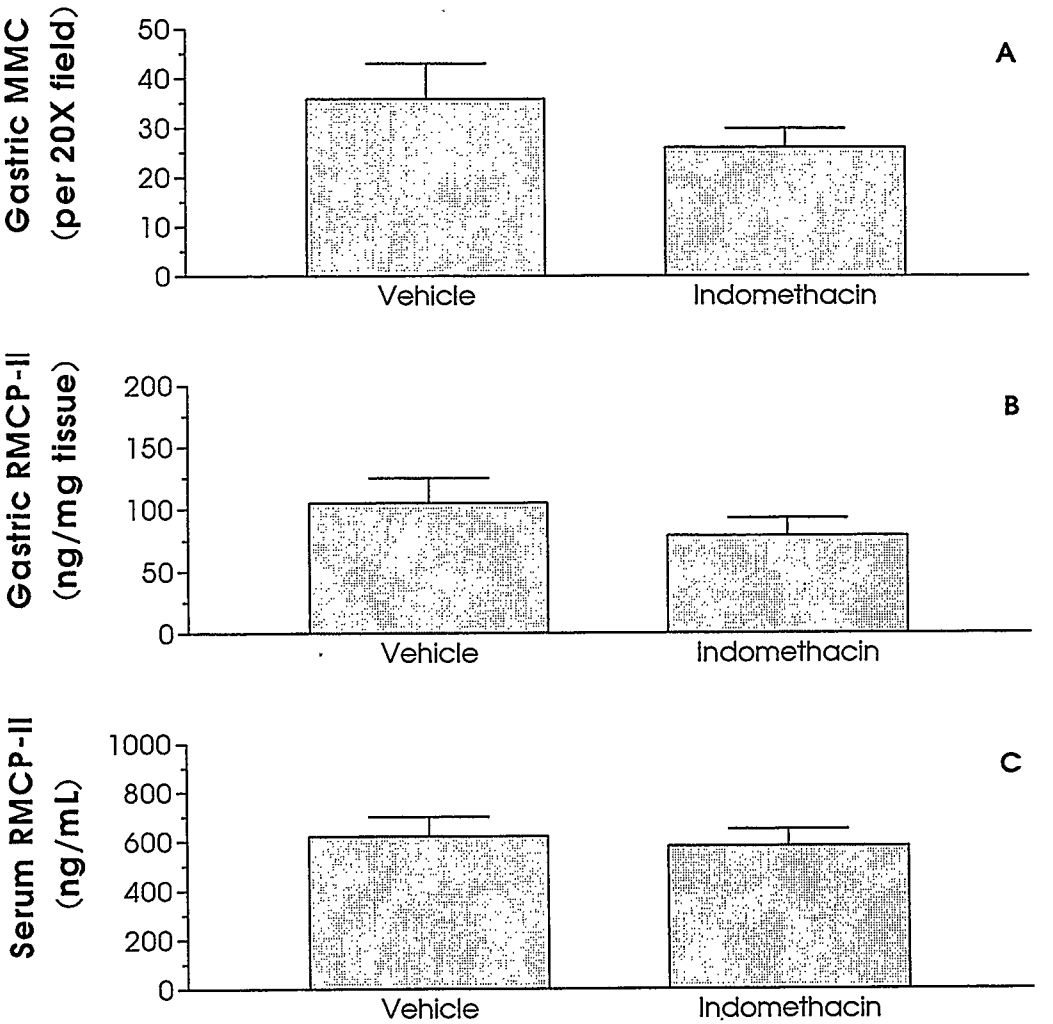

Figure 5.1. **Effect of indomethacin on number of histologically detectable mucosal mast cells in *(A)* stomach, and levels of rat mast cell protease II in *(B)* stomach and *(C)* serum.** Samples of peripheral blood and tissue from gastric corpus were collected 3 hours after administration of indomethacin (20 mg/kg) or vehicle (5% bicarbonate) by gavage. Light microscopy showed that there was no significant change in the number of gastric mucosal mast cells associated with indomethacin administration. In addition, there was no significant effect of indomethacin on the levels of RMCP-II in tissue or serum. Each bar represents mean ± SEM of 6 rats per group.

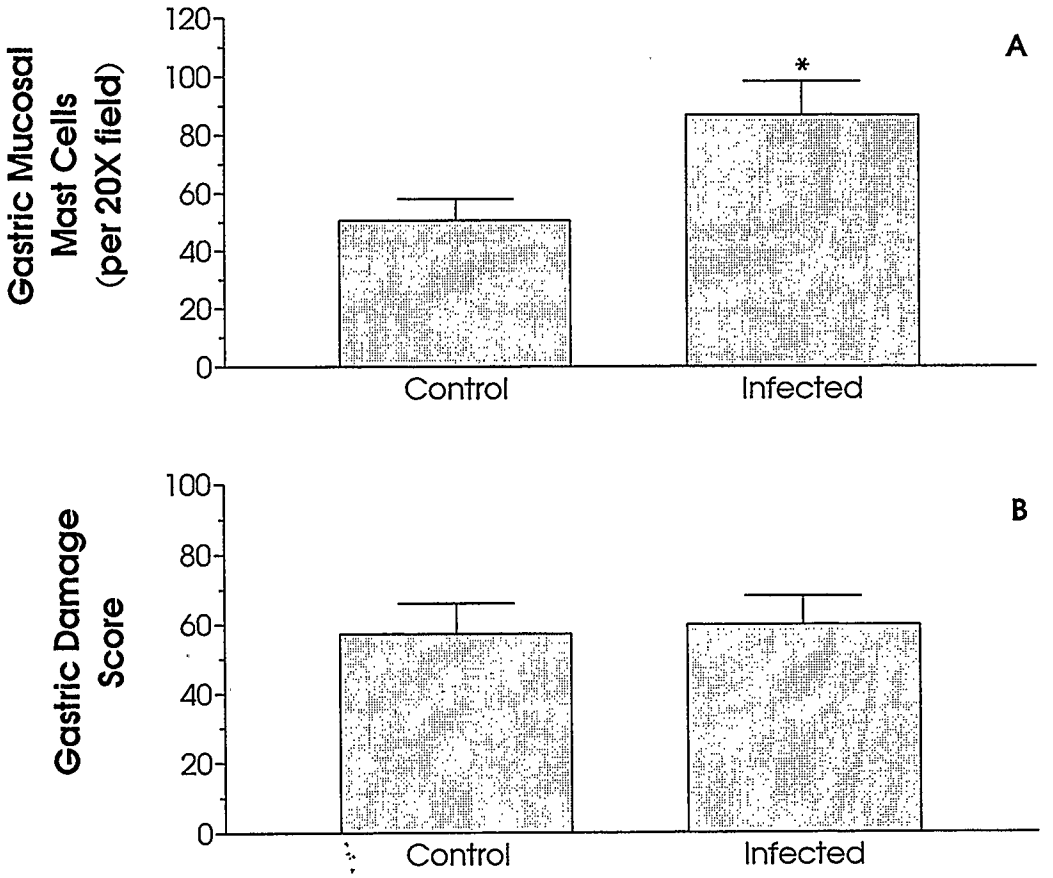

Figure 5.2. **Effect of gastric mucosal mast cell hyperplasia on susceptibility of rat stomach to superficial injury induced by indomethacin.** Rats were studied 35-42 days after infection with *Nippostrongylus brasiliensis*, by which time the infection and associated acute inflammatory changes had resolved but significant mucosal mast cell hyperplasia persisted. *(A)* In rats previously infected with *N. brasiliensis* there was a 75% increase ($^{*}P < 0.05$ compared with naive control rats) in the mean number of gastric mucosal mast cells. *(B)* The rats with mast cell hyperplasia were no more or less susceptible to indomethacin-induced gastric injury than control rats. Each bar represents mean ± SEM of 6-8 rats per group.

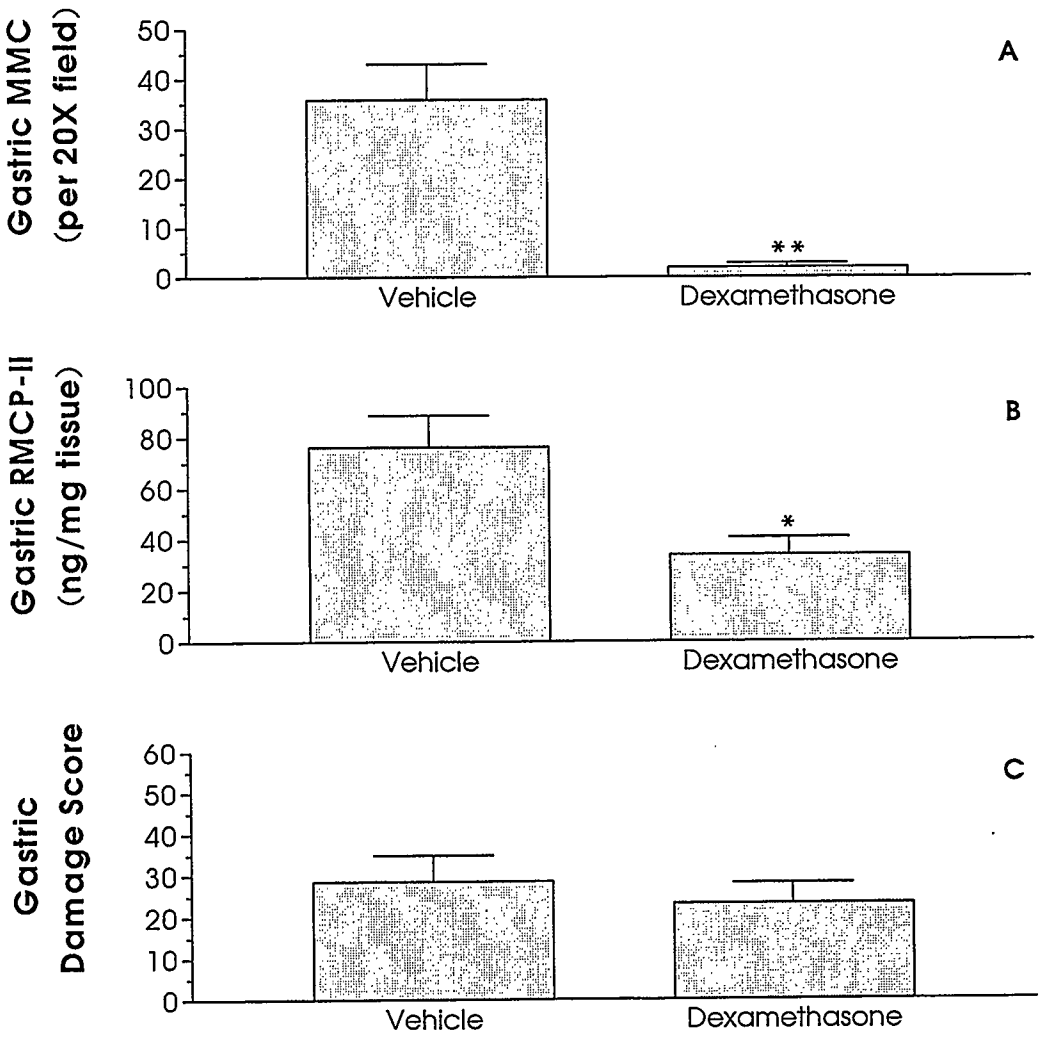

Figure 5.3. **Effect of depletion of gastric mucosal mast cells on susceptibility of rat stomach to injury induced by indomethacin.** Rats were given dexamethasone (1.0 mg/kg, IP) 24 and 48 hours prior to administration of indomethacin (20 mg/kg) by gavage. *(A)* Dexamethasone significantly ($^{**}P < 0.001$) reduced the number of histologically detectable MMC in the stomach and *(B)* the gastric tissue levels of RMCP-II ($^{*}P < 0.05$). Despite depletion of gastric mucosal mast cells, there was no significant effect on *(C)* susceptibility to indomethacin-induced gastric damage. Each bar represents mean ± SEM of 6 rats per group.

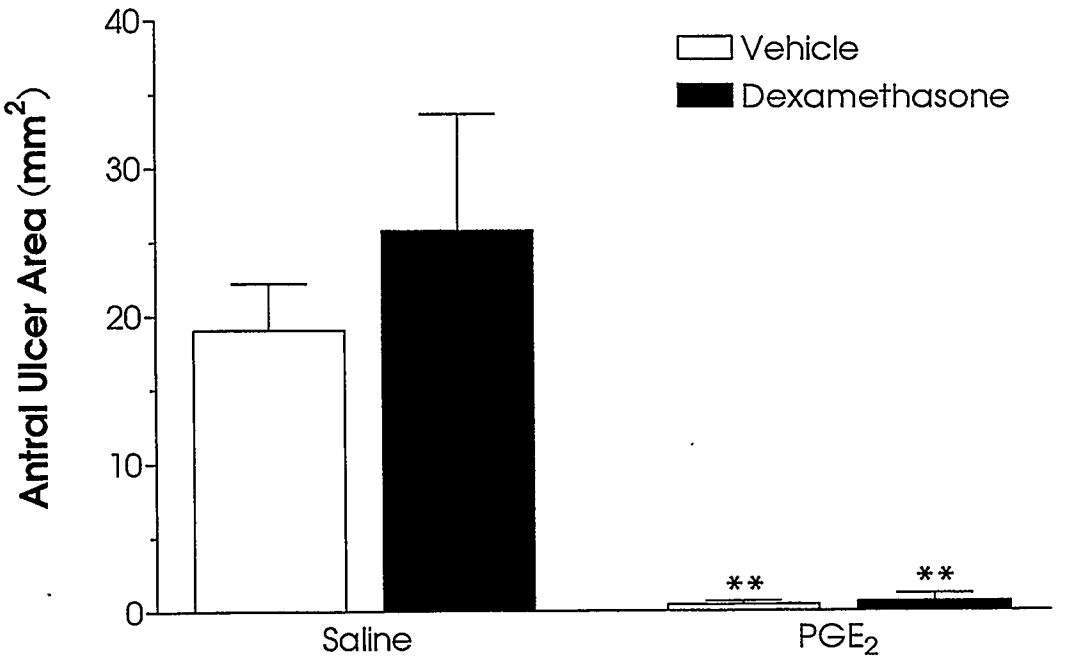

Figure 5.4. **Effect of depletion of mucosal mast cells on susceptibility to naproxen-induced antral ulceration, and on the ability of prostaglandin E_2 to prevent this damage.** Mast cells were depleted by prior treatment with dexamethasone. Rats were given saline or PGE_2 (100 mg/kg) by gavage immediately prior to administration of naproxen (80 mg/kg) by gavage. Antral ulcer area was measured 24 hours later. In rats with the normal complement of mucosal mast cells (vehicle pretreated), PGE_2 significantly reduced the area of antral ulceration induced by naproxen ($^{**}P < 0.01$ compared with corresponding saline-treated group). Depletion of gastric mucosal mast cells by dexamethasone pretreatment did not affect the ability of PGE_2 to protect against naproxen-induced antral ulceration. Bars represent mean ± SEM of 6-8 rats per group.

5.4 Discussion

The results of this study suggest that mast cells do not contribute to the gastric mucosal injury induced by NSAIDs in the rat or mouse. Clearly, the number of mast cells present in the gastric mucosa *per se* does not influence susceptibility to acute NSAID-induced gastric injury. Depleting mucosal mast cells through pretreatment with dexamethasone had no effect on extent of indomethacin-induced gastric damage in rats, while genetically mast cell-deficient mice showed comparable susceptibility to indomethacin-induced damage as their normal congenic littermates. In the "refeeding" model, in which a more chronic-type ulcer is induced in the antral region of the stomach, depletion of mucosal mast cells with dexamethasone had no effects on the severity of antral ulcers induced by naproxen. Elevating the number of mucosal mast cells in the rat stomach through prior infection with *Nippostrongylus brasiliensis* also did not affect susceptibility to indomethacin-induced damage.

The mucosal injury observed following indomethacin administration in the mouse was very superficial and not macroscopically detectable. Others have previously noted this, and found that co-administration of bethanechol resulted in an exacerbation of tissue injury and conversion from microscopic to macroscopic damage.[112] However, the use of bethanechol to augment the detectability of indomethacin-induced erosions would have been of limited value in the present study because mast cell-deficient mice have previously been shown to have a significantly reduced acid secretory response to this agonist.[85] Thus, any difference in susceptibility to damage between mast cell-deficient

and normal mice might be attributable to differences in the acid secretory response to bethanechol rather than to true differences in mucosal susceptibility to injury.

While prostaglandins have been shown to be potent inhibitors of mast cell reactivity,[65] NSAID administration, at doses causing significant mucosal injury and significant suppression of prostaglandin synthesis, did not cause mast cell degranulation (measured histologically and by tissue and serum RMCP-II levels). This is in contrast to effects of topical irritants such as ethanol which, when administered into the lumen, cause a marked reduction in the number of histologically detectable mast cells within the gastric mucosa.[56] It is possible, of course, that agents such as ethanol cause release of granules from mast cells as a result of lysis, rather than through activation of these cells. The absence of an effect of indomethacin on mast cell degranulation suggests that endogenous prostaglandins do not tonically suppress mast cell reactivity in the stomach, although these data do not rule out the possibility that prostaglandins could tonically modulate the reactivity of gastric mucosal mast cells to other agonists.

The ability of prostaglandins to "stabilize" mast cells has also been suggested as the mechanism through which these substances can protect the gastric mucosa from injury induced by topical irritants or NSAIDs.[61] However, the results of the studies utilizing the "re-feeding" model of antral ulceration clearly demonstrated the ability of prostaglandin E_2 to prevent NSAID-induced ulceration in a situation in which mucosal mast cells had been depleted from the gastric tissue. It therefore seems highly unlikely that actions on the mast cell account for the "cytoprotective" effects of prostaglandins, at least in this model.

In summary, the studies described herein suggest that mast cells do not contribute significantly to gastric injury induced in rats or mice by NSAIDs. If mediators such as leukotrienes[94,95,112] or tumour necrosis factor[101,102] contribute to the injury induced in the stomach by NSAIDs, they must be derived from sources other than the mast cell. The ability of prostaglandins to prevent NSAID-induced injury in the stomach does not appear to be related to actions of these substances on the mucosal mast cell.

CHAPTER 6

HEPATIC MUCOSAL MAST CELL HYPERPLASIA IN SECONDARY BILIARY CIRRHOSIS

6.1 Introduction

Mast cells are granulated immunocytes which are believed to play a role in fibrotic reactions in a variety of diseases. Increased numbers of mast cells have been observed in the vicinity of fibrotic lesions in rheumatoid arthritis,[113] inflammatory bowel disease,[114,115] scleroderma,[116] and fibrotic disorders of the lung.[117] Moreover, mast cell-derived mediators are known to promote fibroblast growth[118] and collagen synthesis,[119] and affect the organization and accumulation of connective tissue elements.[120] Recent studies have also demonstrated that mast cells are themselves capable of synthesizing components of the extracellular matrix such as collagen and laminin.[121]

In the normal liver, mast cells are closely associated with the portal tracts, but their precise function is not known. It is reasonable to speculate that, as in other tissues, hepatic mast cells may be involved in local immunological responses and maintenance and repair of connective tissue.[122] Despite active interest in the role of mast cells in many chronic inflammatory and fibrotic disorders, mast cells have received little attention as possible participants in pathological processes in the liver. In one of the few studies of hepatic mast cells that exist, Murata *et al.* examined postmortem liver biopsies taken from patients who had died of liver diseases and showed that mast cell hyperplasia occurred in

cirrhotic livers.[123] Moreover, in this study, the number of mast cells seemed to correlate with the accumulation of collagen and other extracellular matrix elements in the liver.

Gittlen and colleagues have reported that patients with chronic cholestatic liver disease have significantly increased serum histamine levels compared with controls.[124] The mean serum histamine concentration was particularly high in a subset of patients that suffered from pruritis compared with the nonpruritic patients. Histamine, a known pruritogen, may underlie this common and troublesome symptom of cholestatic liver disease. Since mast cells are the major source of histamine, it was speculated that mast cell activation occurs in prolonged cholestasis.[124] Work by Quist *et al.*[125] leads to further speculation that the activating stimulus may be bile acids which accumulate intrahepatically and systemically when biliary outflow is blocked.

In the series of experiments reported here, the broad objective was to study associations between hepatic mast cells and fibrotic reactions in the liver. Specifically, these studies examined the number of mast cells in the liver at several different stages in experimentally-induced hepatic fibrosis, and assessed the possibility that hepatic mast cells release mediators which could contribute to the fibrotic reaction. This was studied in a rat model of biliary cirrhosis in which cholestasis was induced by bile duct resection. In this model, fibrosis is a prominent and consistent finding, whereas only moderate hepatic inflammation and necrosis are present. In overview, it was demonstrated that hepatic mast cell hyperplasia and mediator release occurred at a time when the fibrotic reaction in the cholestatic liver was most active.

6.2 Materials and Methods

Model of cholestasis. Male Sprague Dawley rats (200-250 g body weight) were used in these experiments. Under halothane-induced general anaesthesia, laparotomy was performed and the bile duct was isolated, double ligated, and resected between the ligatures as described by Cameron and Oakley.[126] Sham resection consisted of laparotomy and bile duct identification and manipulation without ligation or resection. Experiments were performed 7, 14, or 21 days after bile duct resection (BDR) or sham resection (sham).

Serum biochemistry. To confirm cholestasis, serum total bilirubin was determined by colorimetric assay using a diagnostic kit obtained from Sigma Chemical Company (St. Louis, MO).

Hepatic collagen deposition. To study inflammatory and fibrotic changes in the liver associated with cholestasis, samples of liver tissue were excised from the margin of the right lobe, and were immersed in formaldehyde, processed in graded alcohols, and embedded in paraffin wax. Sections (5 μm) were mounted on glass slides and stained with hematoxylin and eosin. Inflammation was assessed by light microscopy. As a measure of hepatic fibrosis, collagen was identified in liver using a method adapted from that of López-DeLeón.[127] Briefly, liver sections (3 μm) were mounted on glass slides, deparaffinized, and immersed for 10 seconds in saturated aqueous picric acid containing 0.1% Fast Green FCF (Sigma Chemical Co., St. Louis, MO). After rinsing thoroughly with distilled water, sections were immersed for 15 minutes in 0.1% Sirius Red F3BA

(Polysciences Inc., Warrington, PA) prepared in saturated aqueous picric acid. By this method, collagen stained distinctly red and noncollagenous proteins stained light-green. Coded sections were examined by an observer unaware of the identity of the specimen and a semiquantitative measure of hepatic fibrosis (scored from 1 to 4) was obtained according to the following criteria: 1 = Collagen restricted to portal areas. 2 = Mild expansion of loose periportal collagen into surrounding parenchyma associated with bile ductular proliferation. 3 = Moderate expansion of periportal collagen into surrounding parenchyma with significant organization of fibrous tissue and mild porto-portal bridging. 4 = Extensive porto-portal and porto-central vein bridging of fibrotic bands.

Identification and enumeration of hepatic mast cells. Other liver biopsies, taken for mast cell staining, were placed in Carnoy's fixative solution for 48 hours and then processed by routine histological techniques (*see* Section 2.5). Using light microscopy, mast cells were identified in sections stained with alcian blue/safranine[25] or toluidine blue[128] and counted by a "blinded" observer. The area of each liver section was measured by computerized planimetry and all identifiable mast cells with distinct nuclei within that section were counted. Results were expressed as number of mast cells per square millimetre (#/mm^2). At least five liver sections were counted for each group and the data were averaged.

Immunohistochemical techniques were also employed to identify hepatic mast cells and assess mediator content. Liver samples excised from the right lobe were fixed in freshly prepared 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (4% in PBS, Sigma Chemical Co., St. Louis, MO) for subsequent histamine immunostaining, or Zamboni's

solution for subsequent serotonin immunostaining.[129] Cells containing serotonin or histamine were then identified by indirect immunofluorescence microscopy using commercially available primary antibodies (*serotonin:* Incstar, Stillwater, MN, 1:1000; *histamine:* Incstar, 1:500) and a fluorescently-labelled secondary antibody (sheep anti-rabbit conjugated to CY3, Sigma Chemical Co., St. Louis, MO, 1:200). Mast cells identified in this manner were counted as described above.

Rat Mast Cell Protease-II. Levels of RMCP-II were measured in liver extracts and in serum by ELISA (*see* Section 2.6).

6.3 Results

Confirmation of cholestasis. BDR rats showed clinical evidence of cholestasis with jaundice, dark urine, and icteric serum. A marked increase in serum total bilirubin was observed in BDR rats compared with sham-resected controls studied 7, 14, and 21 days after surgery (Table 1).

Hepatic fibrosis. A semi-quantitative measure of hepatic fibrosis was obtained by examining Fast Green-Sirius Red stained liver sections and judging the amount of collagen present and its degree of organization (Table 2). In sham rats studied 7, 14, and 21 days after surgery, hepatic collagen was limited to the portal areas. In day 7 BDR rats, significant deposition of loose collagen was observed and was accompanied by mild bile ductular proliferation. With increasing duration of cholestasis due to bile duct resection, hepatic fibrosis worsened. In BDR rats studied 14 days after surgery, organization and

expansion of fibrotic areas with associated parenchymal loss were observed. Bridging (porto-central vein) hepatic fibrosis was a consistent feature in rats after 21 days of cholestasis.

Identification and enumeration of hepatic mast cells. When liver sections stained with alcian blue and counterstained with safranine were examined by light microscopy, mast cells were distinctly stained blue. As shown in Figure 6.1, hepatic mast cells were rhomboidal or irregularly flattened in form with compact nuclei, and were found predominantly in the connective tissue of the portal tracts in both sham and BDR rats. To a lesser extent, mast cells were also located in the connective tissue capsule which encases the liver (not shown).

Although pathological changes due to cholestasis (*i.e.* portal zone expansion and bile ductular proliferation) were evident in BDR rats studied 7 days after surgery (Figure 6.1A), there was no apparent difference in the morphology or number of hepatic mast cells compared with day 7 sham rats (Figure 6.1B). There was, however, a clear increase in the number of hepatic mast cells in rats studied 21 days after induction of cholestasis by BDR (Figure 6.1C) compared with that present in sham rats (Figure 6.1D).

To evaluate the magnitude of this change, all distinctly identifiable mast cells in alcian blue/safranine-stained liver sections were counted by a blinded observer and expressed per unit area of that section. As shown in Figure 6.2, equal numbers of hepatic mast cells were found in BDR and sham rats studied both 7 and 14 days post-surgery. However, in day 21 BDR rats, there were twice as many hepatic mucosal mast cells as in the corresponding group of sham rats ($P < 0.001$). In light of these data, subsequent

studies of the effects of cholestasis on hepatic mast cells were focused on day 21 BDR and sham rats.

To confirm that hepatic mast cell numbers were indeed increased in day 21 BDR rats, other staining methods were used to identify mast cells for enumeration. Toluidine blue staining revealed a one- to two-fold increase ($P < 0.05$) in hepatic mast cell numbers in day 21 BDR rats compared with sham rats (Figure 6.3). Moreover, the number of hepatic mast cells identified by toluidine blue staining was very similar to that observed in alcian blue-safranine stained sections of liver from both sham and BDR rats studied 21 days after surgery.

Hepatic mast cells were also identified by immunofluorescence microscopy using antibodies against serotonin or histamine, two constituents of mast cell granules.[130] In day 21 BDR (Figure 6.4A) and sham (Figure 6.4B) rats, serotonin immunoreactivity was discretely localized to cells within the portal zone. Moreover, enumeration of serotonin-containing cells revealed a two-fold increase ($P < 0.05$) in day 21 BDR rats compared with sham-resected controls (Figure 6.3). Qualitatively, there was also a striking increase in the number of histamine-containing cells in day 21 BDR rat livers (Figure 6.4C) compared with sham (Figure 6.4D), but this difference could not be quantified due to the diffuse appearance of histamine immunofluorescence in day 21 BDR rats. The presence of diffuse histamine immunoreactivity surrounding indistinctly stained mast cells in day 21 BDR rats, but not in sham rats, was an interesting finding in itself because it suggested that hepatic mast cell degranulation had occurred in these animals.

Rat Mast Cell Protease-II. To further investigate the possibility that cholestasis results in liver mast cell degranulation, RMCP-II levels were measured in homogenates of liver from BDR and sham rats (Figure 6.5A). Despite conspicuous pathological changes, liver RMCP-II levels in day 7 BDR rats were unchanged compared with day 7 sham rats. Liver RMCP-II content was significantly ($P < 0.01$) decreased in BDR rats studied 14 days post-surgery, and there was a further decrease in BDR rats studied at day 21 such that levels were approximately 50% of those in sham rats. The decrease in liver RMCP-II content in day 21 BDR rats was particularly striking considering that there was also a two-fold increase in the number of hepatic mast cells in this group of animals.

A substantial decrease in tissue RMCP-II due to its rapid release from mast cells is accompanied by an appreciable and persistent increase in serum RMCP-II levels.[131] Despite evidence of depletion of RMCP-II from hepatic mast cells possibly due to degranulation in day 14 and day 21 BDR rats, a corresponding increase in serum RMCP-II levels was not observed. On the contrary, at all time points studied, serum RMCP-II was significantly decreased in BDR compared with sham rats (Figure 6.5B).

Considering that activation of serum proteases has been reported in patients with the cholestatic liver disease, primary biliary cirrhosis,[132] it was possible that the decrease in serum RMCP-II levels in BDR rats was due to proteolytic degradation. To study this, sera from day 21 BDR and sham-resected rats (n = 3 per group) were spiked with 600 ng/mL of purified RMCP-II (Moredun Animal Health Ltd., Midlothian, UK) and incubated at 37°C for one hour. In this system, no measurable degradation of exogenous RMCP-II occurred in sera from BDR rats (data not shown).

Table 6.1. **Serum total bilirubin (mg/dL)**

	Days After Surgery		
Surgical Treatment	7	14	21
Sham	0.463 ± 0.135	0.190 ± 0.146	0.424 ± 0.111
BDR	4.15 ± 0.29 **	3.93 ± 0.75 **	7.26 ± 0.91 ** δδ

Data are expressed as mean ± SEM of at least 5 rats per group.

** $P < 0.001$ compared with corresponding sham

δδ $P < 0.01$ compared with day 7 and day 14 BDR

Table 6.2. **Degree of hepatic collagen staining**

	Days After Surgery		
Surgical Treatment	7	14	21
Sham	1.2 ± 0.2	1.0 ± 0.0	1.4 ± 0.2
BDR	2.2 ± 0.2 **	3.4 ± 0.2 ** δδ	4.0 ± 0.0 ** δ

Data are expressed as mean ± SEM of at least 4 rats per group.

** $P < 0.001$ compared with corresponding sham

δδ $P < 0.01$ compared with day 7 BDR

δ $P < 0.05$ compared with day 14 BDR

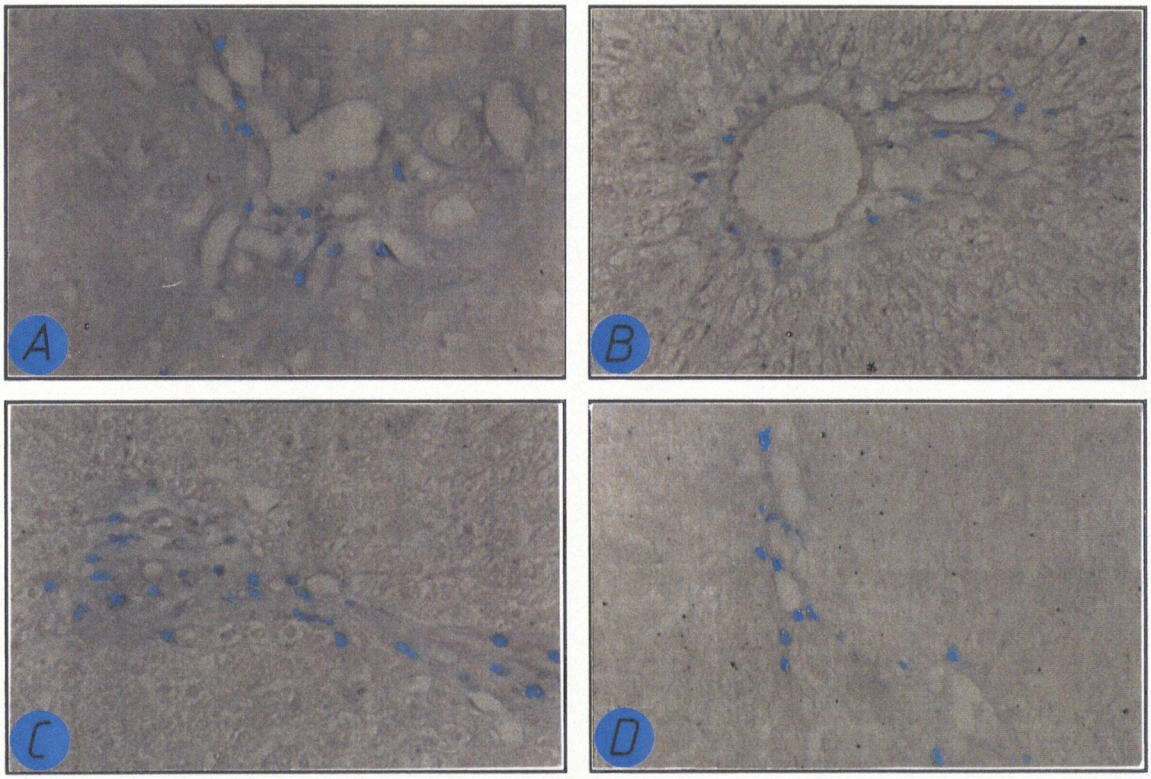

Figure 6.1. **Histochemical identification of hepatic mast cells in BDR and sham rats.** Mast cells in the rat liver were found in the periportal connective tissue and, according to their alcian blue/safranine staining properties, were phenotypically similar to mucosal mast cells. Whereas equal numbers of hepatic mast cells were found in *(A)* day 7 BDR rats compared with *(B)* day 7 sham rats, a marked increase in the number of hepatic mast cells was apparent in *(C)* day 21 BDR rats compared with *(D)* day 21 sham rats. (Original magnification: ×100).

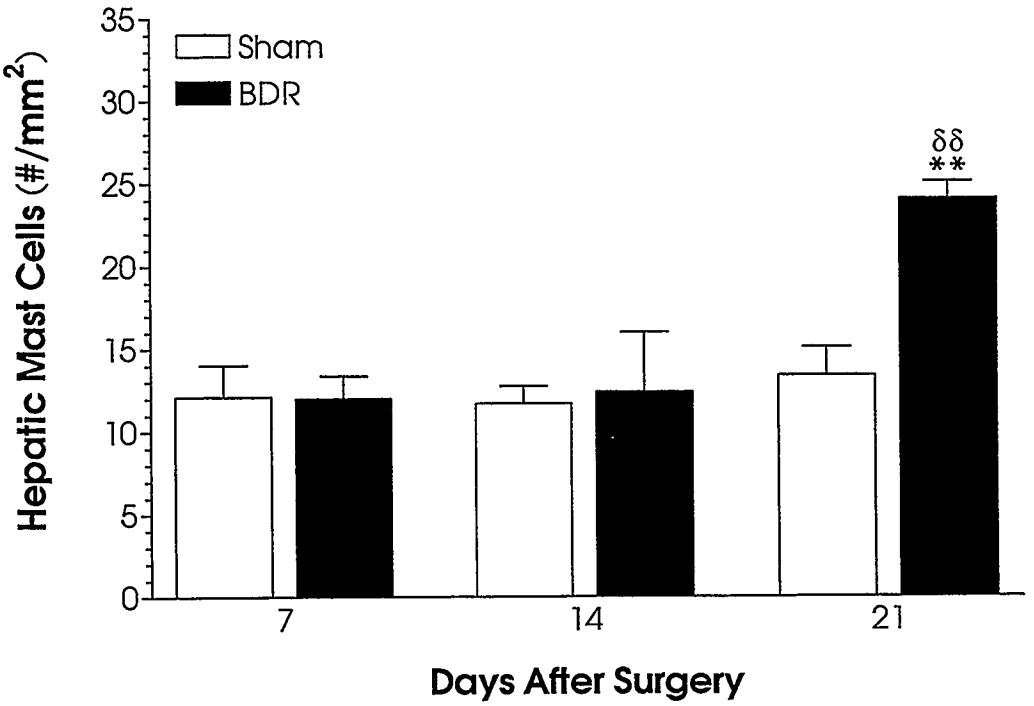

Figure 6.2. **Effect of cholestasis on the number of histologically detectable mast cells in the liver.** Mast cells were identified by light microscopy in sections of liver stained with alcian blue and safranine. Similar numbers of hepatic mast cells were found in BDR and sham rats 7 and 14 days after induction of cholestasis by surgical bile duct resection. At 21 days, there was a significant increase in the number of hepatic mast cells in BDR rats ($^{**}P < 0.001$ compared with corresponding sham; $^{\delta\delta}P < 0.001$ compared with day 7 and day 14 BDR). Each bar represents the mean ± SEM of at least 7 rats per group.

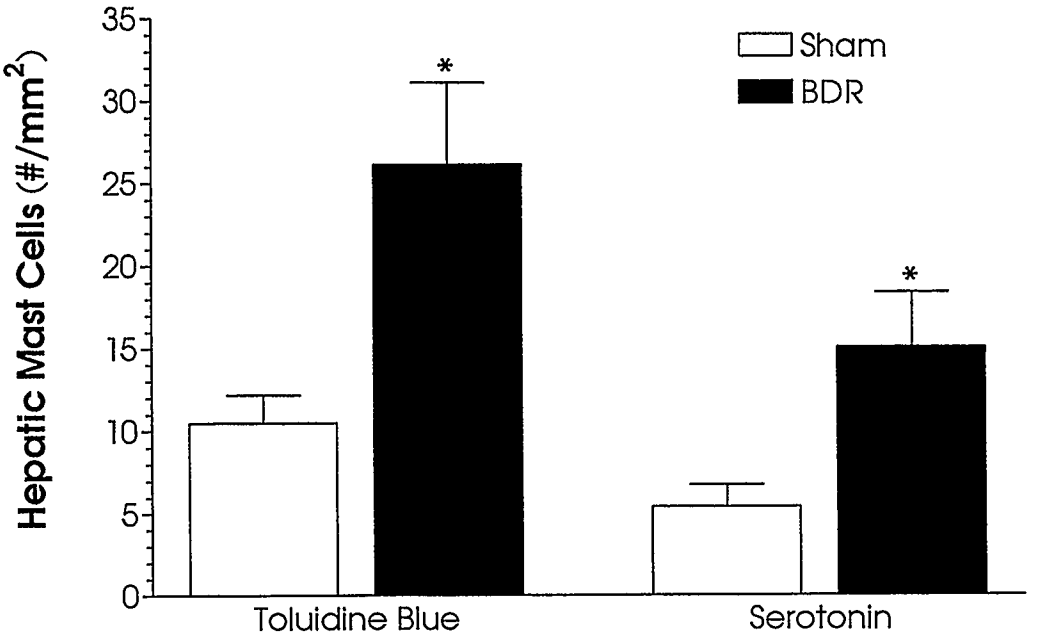

Figure 6.3. **Confirmation of increased mast cell number in day 21 BDR rats.** Hepatic mast cells were identified in day 21 BDR and sham rats by histochemical (toluidine blue) and immunohistochemical (fluorescently-labelled antibody directed against serotonin) stains. Both staining procedures showed that there were approximately twice as many hepatic mast cells in day 21 BDR rats as in sham-operated controls ($^{*}P < 0.05$). These data are consistent with results obtained using alcian blue to identify mast cells in the liver (*see* Figure 6.2). Each bar represents mean ± SEM of at least 6 rats per group.

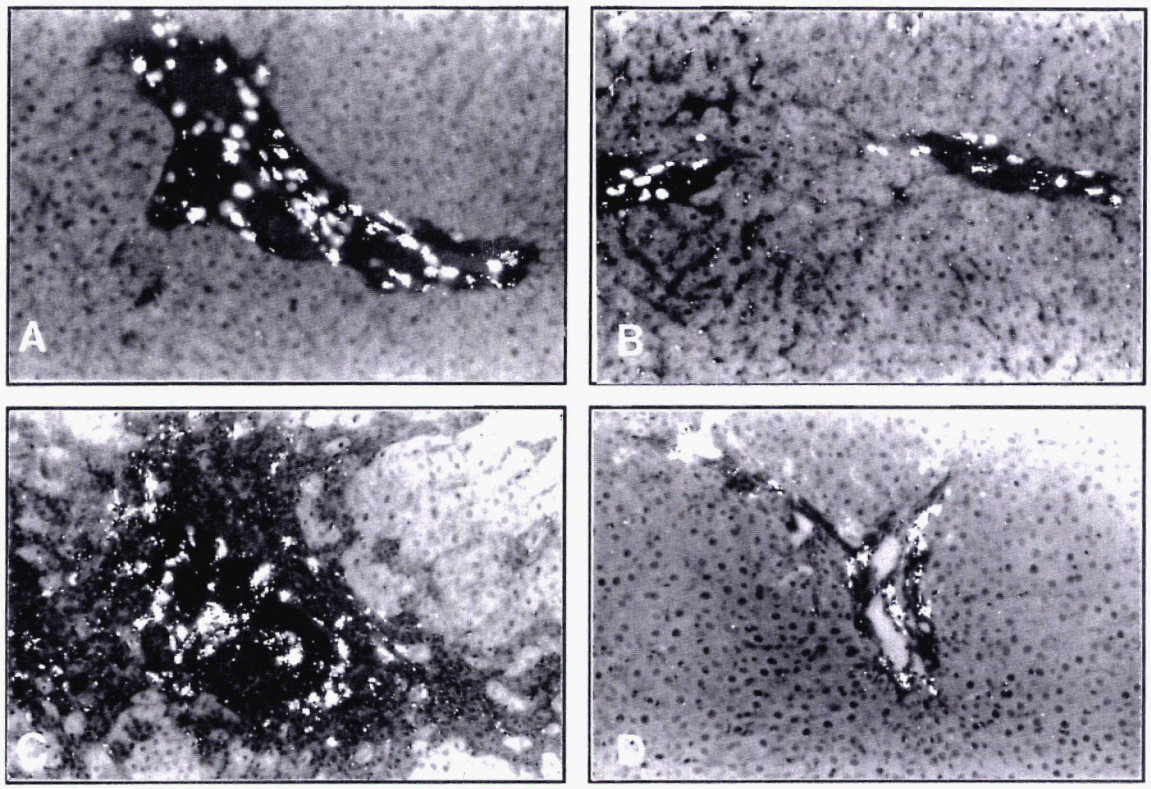

Figure 6.4. **Identification of hepatic mast cells by indirect immunofluorescence microscopy.** Samples of liver from day 21 BDR and sham rats were stained with fluorescently-labelled antibodies directed against serotonin or histamine. Serotonin was distinctly identified in cells within portal tracts of the liver and, qualitatively, there was a striking increase in the number of serotonin-containing cells in *(A)* BDR rats compared with *(B)* sham rats (this difference was quantified - *see* Figure 6.3). There was also an apparent increase in the number of histamine-containing cells in *(C)* BDR rats compared with *(D)* sham rats. This difference could not be quantified due to the diffuse pattern of histamine immunofluorescence in BDR rats, but not sham rats, which is suggestive of mast cell degranulation.

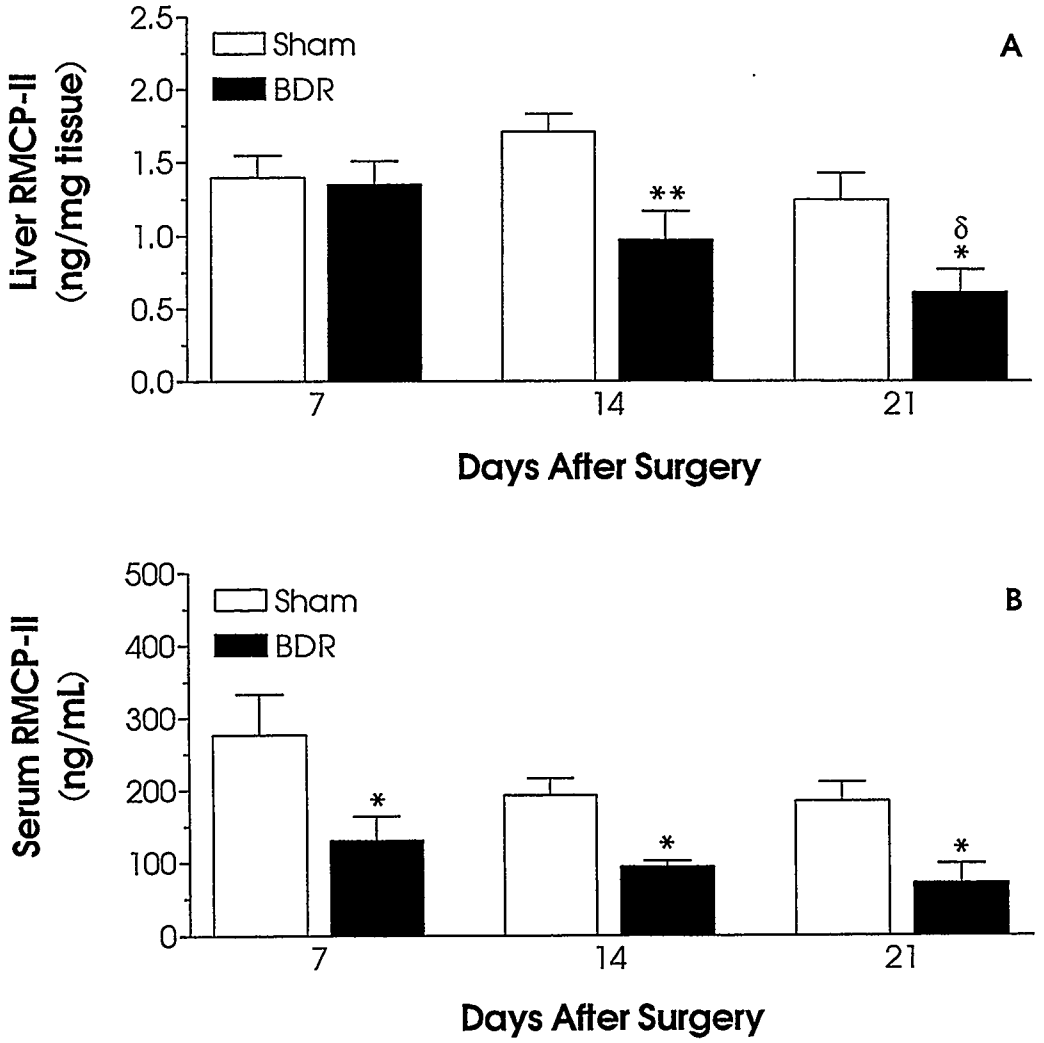

Figure 6.5. **Effect of cholestasis on rat mast cell protease II levels in liver and serum.** RMCP-II was measured by ELISA in homogenates of liver or peripheral blood from BDR and sham rats. *(A)* Hepatic RMCP-II levels were significantly decreased in day 14 and 21 BDR rats ($^{*}P < 0.05$, $^{**}P < 0.01$ compared with corresponding shams; $^{\delta}P < 0.05$ compared with day 7 BDR). *(B)* Serum RMCP-II levels were significantly decreased in BDR rats at all time points studied ($^{*}P < 0.05$ compared with corresponding sham). Each bar represents the mean ± SEM of at least 5 rats.

6.4 Discussion

Rodent mast cells comprise at least two distinct phenotypes - MMC and CTMC - which were initially distinguished by their anatomical location and metachromatic staining properties.[130] Based on differences in binding of cationic dyes by granule proteoglycans, primary staining with alcian blue combined with safranine counterstaining allows distinction of MMC and CTMC histologically.[25] By this method, MMC stain blue and CTMC stain red. In the present study, mast cells in the rat liver distinctly stained blue and, accordingly, can be classified as MMC. In contrast, CTMC could not be detected in the rat liver by this staining procedure.

Mucosal mast cells and CTMC can also be distinguished from one another by identifying certain proteases that are specific for each subtype of mast cell. In rats, MMC contain RMCP-II, whereas CTMC contain a distinct protease designated RMCP-I.[34] In the present study, substantial amounts of RMCP-II were found in homogenates of liver from both sham and BDR rats. Therefore, in addition to sharing the histochemical staining properties of MMC, mast cells in the rat liver are also biochemically analogous to MMC. Previous studies of mast cells in the liver have not made this distinction.[133,134] Recently, however, Hagmann *et al.* showed that hepatic mast cells were the major source of leukotriene production during anaphylactic reactions in the rat liver.[135] Since leukotriene C_4 is the predominant eicosanoid produced by immunologically activated MMC, but not CTMC,[71] the results of Hagmann and colleagues also suggest that hepatic mast cells are MMC.

Elevated serum histamine levels have been documented in patients with primary biliary cirrhosis and primary sclerosing cholangitis, suggesting that prolonged cholestasis causes mast cell degranulation.[124] In the present study, histamine immunostaining of liver sections from day 21 BDR rats produced a diffuse pattern of fluorescence surrounding hepatic mast cells which is consistent with mast cell degranulation. This pattern of immunofluorescence staining was not seen in any of the samples of sham liver that were studied in this manner. Despite evidence of histamine release, there was no evidence of serotonin release from hepatic mast cells in day 21 BDR rats. This suggests the occurrence of differential mediator release, a process by which some preformed, granule-associated mediators and/or *de novo* synthesized mediators are preferentially released from mast cells in response to various stimuli.[136,137]

RMCP-II is a 25 kDa serine protease that is specifically found in the exocytotic granules of rat mucosal mast cells.[34] RMCP-II is highly soluble such that, when released from mast cells upon degranulation, it is rapidly cleared from the tissue interstitium and appears in the blood. Therefore, degranulation of mucosal mast cells results in decreased tissue levels of RMCP-II and, if appreciable amounts of RMCP-II are released, a measurable increase in serum RMCP-II levels may also occur.[131] Significant decreases in liver RMCP-II levels were observed in both day 14 and day 21 BDR rats compared with their respective sham-operated controls. These data, together with immunohistochemical evidence of histamine release from hepatic mast cells, suggest that liver mast cells may release mediators as a result of long-standing cholestasis.

Despite depletion of RMCP-II from hepatic mast cells, a significant increase in levels of RMCP-II in serum was not observed. Contrary to this, serum levels of RMCP-II were significantly decreased in BDR rats as early as 7 days after surgery. *In vitro* studies of the stability of exogenously added RMCP-II in sera from BDR rats eliminated the possibility that degradation of RMCP-II by activated serum proteases accounted for these findings. Instead, it is possible that cholestasis may reduce RMCP-II synthesis in mucosal mast cells and thereby reduce basal levels present in serum. This latter suggestion is supported by a report that dexamethasone suppresses RMCP-II synthesis in a MMC-like cell line.[138] Increased levels of endogenous glucocorticoids have been reported in BDR rats,[139] and could account for the reduced serum RMCP-II levels that were observed in such animals in the present study. Considering these reports, it is not possible to conclude that the significant depletion of liver RMCP-II that was observed in day 14 and 21 BDR rats was due exclusively to its exocytotic release from hepatic mast cells. Although it is possible that suppression of RMCP-II synthesis, and not depletion due to degranulation, may account for the significant decreases in both serum and liver RMCP-II levels in these animals, two points argue against this possibility: (1) Serum RMCP-II levels were significantly reduced as early as 7 days after induction of cholestasis whereas liver RMCP-II levels were not affected until 14 days. If decreased liver and serum RMCP-II levels were due to suppression of RMCP-II synthesis, both effects might be expected to occur at the same time. (2) Evidence of hepatic mast cell degranulation provided by measurements of liver RMCP II is supported by the results of histamine immunofluorescence staining. In addition, using electron microscopy to

directly examine mast cells in the rat liver, preliminary evidence has been obtained showing that the number and density of exocytotic granules of hepatic mast cells are decreased after prolonged cholestasis.

Our studies clearly showed that hepatic mucosal mast cell hyperplasia did not correlate with the onset or early stages of hepatic fibrosis. Although the methods for measuring hepatic collagen deposition were only semiquantitative, results of the present study are supported by those of Aronson *et al.*, who have performed quantitative studies of hepatic collagen deposition in BDR rats.[140] Aronson also showed that the most substantial increases in volume density of collagen in liver occurred between the second and fourth weeks following induction of cholestasis. This corresponds with the approximate time at which a marked increase in the number of hepatic mucosal mast cells was observed in the present study. Together, these data suggest that the occurrence of hepatic mucosal mast cell hyperplasia correlates with the time of most active collagen synthesis in this model of biliary cirrhosis. However, it remains unclear whether mast cells are acting to promote or limit the fibrotic reaction.

Ito cells (fat-storing cells) are known to be the primary source of collagen and other extracellular matrix components which are deposited during hepatic fibrogenesis of diverse etiologies.[141] In experimental biliary cirrhosis in the rat, marked Ito cell proliferation occurs as early as 5 days after induction of cholestasis,[142] whereas the present study demonstrated that significant changes in the number of hepatic MMC occur only after 3 weeks. These observations suggest that the factors which stimulate Ito cell proliferation are different from those which underlie the increase in MMC number.

Although several cytokines and novel Kupffer cell-derived factors have been shown to enhance production of matrix components by Ito cells,[141] the effects of hepatic mast cell-derived mediators such as leukotriene C_4, histamine, and proteoglycans on matrix deposition by Ito cells have not been studied.

Future studies will focus on identifying the factors which may cause hepatic mast cell hyperplasia in biliary cirrhosis. Toward this end, preliminary experiments extending from the present study have demonstrated the presence of cells which produce transforming growth factor beta (TGF-β) in liver from day 21 BDR rats, but not sham rats. This is consistent with other reports of upregulated local TGF-β expression associated with hepatic fibrosis of various causes.[143,144] A recent investigation has shown that TGF-β is a very potent mast cell chemotactic factor.[145] Therefore, a component of the profibrotic effects of TGF-β in the liver may be its ability to promote hepatic mast cell proliferation.

CHAPTER 7

GENERAL DISCUSSION

The research presented in this dissertation was aimed at gaining insight into the role of mast cells in defensive and injurious reactions in the gastrointestinal tract. These studies were quite broadly-based in that several distinct pathological events were examined in different organs of the digestive system, and the injurious reactions were initiated by a variety of different agents. For example, some studies focused on the role of mast cells in gastric mucosal injury caused by common ulcerogens - ethanol or NSAIDs (*see* Chapters 3 and 5). Other experiments were designed to examine the possible association between mast cells and fibrotic reactions in the liver, an accessory organ of the gastrointestinal tract (*see* Chapter 6). Yet other studies were aimed at describing and understanding a phenomenon whereby mast cells in the gastrointestinal mucosa become hyporesponsive to antigenic activation after repeated challenge (*see* Chapter 4). Although these individual studies stand quite well on their own, they certainly do not represent a series of connected works which progress from one to another, building upon accumulated knowledge. Detailed analyses and discussions of each study have been presented in the previous four chapters. In this chapter, an attempt will be made to derive unifying principles from four apparently disjointed studies and highlight interesting parallels between them. In addition, the validity of some key

methods will be discussed. Finally, as the pursuit of answers in science invariably leads to new questions, future avenues of research will be discussed.

Intestinal mucosal mast cell hyperplasia is a prominent feature of intestinal nematode infection.[40] Several experiments presented in this dissertation have employed *Nippostrongylus brasiliensis*-induced gastrointestinal MMC hyperlasia as a tool for studying mast cells in the gastrointestinal tract. When the mucosal mast cell population is selectively increased, it is reasonable to assume that their physiological and pathological effects would be heightened, making them more amenable to observation. Chapters 3 and 5 describe studies which were performed to examine the effect of increasing the number of mucosal mast cells in the stomach on its susceptibility to injury induced by ethanol or NSAIDs. Despite a 2- to 3-fold increase in the number of gastric mucosal mast cells in rats previously infected with *N. brasiliensis*, there was neither an increase nor a decrease in the extent of injury induced by such agents. The physiological or pathological effects of mast cells seem to be dependent upon their activation to release chemicals mediators, not on their absolute numbers. Indeed, neither in the case of topically applied ethanol nor in the case of indomethacin was there detectable mucosal mast cell degranulation. Therefore, mast cells do not appear to mediate injury induced by two distinct pro-ulcerogenic agents.

Unlike the situation in which *N. brasiliensis*-induced MMC hyperplasia was a *pre-existing* condition used as a tool for studying MMC, in two of the studies presented in this dissertation there was evidence of MMC hyperplasia occurring as a *consequence* of the experimental manipulations. In chapter 6, it was shown that a marked increase in the

number of histologically detectable hepatic mucosal mast cells occurred in rats with prolonged cholestasis. Similarly, in chapter 4, sensitized rats that were chronically challenged with antigen showed indirect evidence of mucosal mast cell hyperplasia in that levels of RMCP-II were significantly increased in both stomach and small intestine. The latter is consistent with reports of an increase in the number of histologically detectable intestinal MMC as a consequence of repeated mast cell activation.[88,89]

Together, the studies presented in chapters 4 and 6 suggest that repeated or continuous activation of mast cells promotes proliferation of these cells. Antigen is clearly the stimulus for mast cell activation in the chronic antigen challenge experiments. The mast cell specificity implicit in antigen-IgE reactions indicates that activated mast cells may themselves be the major source of factors promoting proliferation of the mast cell population. But to clarify one point, in conditions of cholestasis, the activating stimulus for mast cell activation may be bile acids, as bile acids have been shown both *in vitro* and *in vivo* to be effective at evoking mast cell degranulation.[125] In cholestasis, due to stagnation of biliary outflow and intrahepatic retention of bile, interstitial levels of bile acids may be increased leading to local activation of mast cells. This hypothesis is consistent with our findings of hepatic mast cell degranulation in cholestatic rats. It would appear that this effect takes time to develop as the first evidence of mast cell degranulation only occurred two weeks after induction of cholestasis. Studies by Gittlen *et al.*[124] also suggest that chronic cholestasis may cause widespread activation of mast cells, as humans with cholestatic liver disease have very markedly increased serum histamine levels.

Looking to the *N. brasiliensis* model, it is likely that expansion of the MMC population represents the combined effects of growth and differentiation of resident MMC precursors and recruitment of new MMC progenitors.[12] Which mast cell-derived factors could potentially mediate mast cell proliferation? There has been much recent interest in mast cells as a source of cytokines,[37] and among the many cytokines shown to be produced by activated mast cells are IL-3 and IL-4 which promote growth and differentiation of mast cell precursors resident in a particular tissue.[12-14] In addition, recruitment of mast cell precursors may also occur when mast cells are repeatedly or continuously activated, as these cells are capable of producing TGF-β, a potent mast cell chemotactic factor.[145] In an analogous fashion, early and continuous activation of MMC in *N. brasiliensis* infection may significantly contribute to the gastrointestinal MMC proliferative response. This possibility has perhaps not been adequately addressed, as most studies of the mechanism underlying this response have focused on the role of T cells in MMC proliferative responses.

Measurement of serum RMCP-II as an index of mucosal mast cell activation was a central technique in each of the four studies presented in this dissertation. In some experiments, RMCP-II was measured to ensure that antigen had effectively activated MMC in primed rats. For example, in *N. brasiliensis*- and ovalbumin-sensitized rats, acute challenge with the sensitizing antigen caused significant increases in serum RMCP-II levels indicating that MMC degranulation had occurred. However, in other experiments, serum RMCP-II levels were measured to see if MMC degranulation was associated with a particular biological response. It is important to point out that a rise in

serum RMCP-II levels reflects the fact that MMC have been activated in such a manner that degranulation occurred. Yet even in the absence of a detectable rise in serum RMCP-II after a particular stimulus, it is possible that mast cells were activated to produce mediators *de novo*. For instance, detectable increases in serum RMCP-II were not observed after NSAID administration, or topical application of ethanol to the gastric mucosa, or a final challenge in a series of chronic challenges. But from these data it is not possible to absolutely rule out the possibility that mast cells were activated to release factors such as PAF, LTC_4, or cytokines. Measurement of RMCP-II is a fast, sensitive, reliable, and stable index of MMC degranulation, but this technique ignores the possibility that mast cell may contribute to biological responses by exclusively releasing newly-synthesized mediators. The use of supplementary techniques such as dexamethasone-induced depletion of MMC helped to eliminate this uncertainty.

At this point, some comments are in order about the use of dexamethasone to deplete mucosal mast cells. By no means is MMC depletion the exclusive effect of dexamethasone when administered in the manner used here (*see* Section 2.3). Dexamethasone has multiple effects on physiological, biochemical, and immune functions which may confound interpretation of its particular effect on MMC.

Focusing on immune function, glucocorticoids are effective anti-inflammatory agents because they inhibit production of a wide variety of chemical mediators of inflammation. Dexamethasone, for example, inhibits production of lipid and cytokine mediators of inflammation by leukocytes,[146] as well as expression of the inducible isoform of nitric oxide synthase.[147] Dexamethasone also inhibits expression of adhesion

molecules on leukocytes and vascular endothelial cells, but this effect may be due to its ability to suppress synthesis of cytokines such as IL-1 rather than direct effects on adhesion molecules.[146] These considerations were particularly important in the experiments in which the effects of dexamethasone-induced MMC depletion on NSAID-induced gastric mucosal injury were examined. If dexamethasone pretreatment, for example, had reduced NSAID-induced gastric injury, it may not have been entirely clear whether this effect was due to depletion of MMC or altered leukocyte-endothelial cell interactions which have been shown to be critical to the development of NSAID gastropathy.[110] Since MMC depletion is a relatively long-lasting effect, administration of dexamethasone as a pretreatment (24 hours before experimentation) would tend to minimize these complicating issues.

Enumeration of MMC using histochemical means of detection of MMC has an inherent problem. Since histochemical stains target proteoglycans present within mast cell granules, mast cell degranulation renders them undetectable.[25] In conditions where both increased mast cell number and mast cell mediator release are apparent, histochemical identification of mast cells tends to underestimate the degree of mast cell hyperplasia. For example, the increase in hepatic MMC number in day 21 BDR rats may have been grossly underestimated because of concurrent degranulation. Further suggestion of the insensitivity of histochemical methods of detecting MMC comes from the chronic challenge studies in which increased tissue RMCP-II levels were not accompanied by similar increases in the number MMC detected histologically.

Several aspects of the work presented in this dissertation represent innovation. The description of mast cell hyporesponsiveness after chronic antigen challenge is certainly a novel and interesting finding. These studies were conceived when it was recognized that most of the studies of immediate hypersensitivity reactions in the gastrointestinal tract examined the phenomena associated with acute antigen challenge, whereas in instances of documented food allergy, repeated exposure to the sensitizing antigen might certainly occur. Also, the associations between mast cells and fibrotic reactions in the liver have never before been studied in a controlled experimental setting. Although purely a descriptive observation, it is interesting that mast cells in the rat liver have the phenotypic characteristics of mucosal mast cells. Considering that so little is known about the normal physiological role of hepatic mast cells, this perhaps suggests that hepatic mast cells have immunological functions which are analogous to those of mast cells in the mucosa of the stomach and intestine.

Future Directions

Neither increasing nor decreasing the number of gastric MMC had a significant effect on injury induced by ethanol or indomethacin. However, prior antigenic activation of mast cells did significantly increase the extent of injury induced by topical ethanol application. As an extension of this finding, it might also be interesting to examine whether prior activation of mast cells (*e.g.* in OA-sensitized rats acutely challenged with

antigen) increases the susceptibility of the gastric mucosa to injury induced by indomethacin given by gavage or subcutaneous injection.

The studies which demonstrated hepatic MMC hyperplasia associated with chronic cholestasis raise the issue of whether an increase in mast cell numbers might also be found at other sites, such as the skin or gastrointestinal tract. In addition, to gain greater insight into the role of hepatic MMC in local fibrotic reactions, it would be useful to perform experiments in which BDR and sham rats are treated with a MMC stabilizer such as doxantrazole. Finally, measurement of the profile and time course of cytokine messenger RNA expression (*e.g.* IL-3, IL-4, and TGF-β) would be useful for identifying locally produced factors which may mediate hepatic MMC hyperplasia.

In particular, the studies which describe and characterize the effects of chronic antigen challenge on MMC responsiveness raise a number of additional questions. For example, do sensitized rats in which MMC were rendered hyporesponsive by chronic antigen challenge eventually regain the ability to respond to antigen at some time after challenge has been stopped? Although chronically challenged rats are no longer responsive to the sensitizing antigen, they do remain responsive to other agents such as anti-IgE, or a higher dose of antigen. It would be interesting to see whether suppressed mast cell responsiveness is an antigen-specific effect. To address this issue, rats could simultaneously be sensitized to two antigens and studies performed to examine whether chronic challenge with one antigen abolishes the response to a final challenge with the second antigen. If an antigen-specific effect were noted, one possible explanation would be that, in the course of chronic oral challenge, other neutralizing antibodies (*e.g.*

circulating IgG or IgM, or secretory IgA) have been raised against the challenge antigen. Finally, additional chronic challenge studies could be performed to address the issue that the observed effects of chronic challenge are due to altered threshold of MMC activation. Such studies of mast cell biology and biochemistry are beyond the present technical means available in this laboratory.

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APPENDIX

THIS DISSERTATION IS COMPILATION OF THE FOLLOWING MANUSCRIPTS:

Rioux KP, Wallace JL. Mast cell activation augments gastric mucosal injury through a leukotriene-dependent mechanism. *Am J Physiol* 266: G863-G869, 1994.

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Rioux KP, Sharkey KA, Wallace JL, Swain MG. Hepatic mucosal mast cell hyperplasia in rats with secondary biliary cirrhosis. *Hepatology* 23: 888-895, 1996.