

Hypersensitivity mechanisms and mucus in the immune
expulsion of Nippostrongylus brasiliensis from the rat.

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Doctor of Philosophy

University of Edinburgh

1986



Statement.

I hereby declare that :

- (i) this thesis has been composed by myself,
- (ii) it has not been accepted in any previous application and,
- (iii) the work described has been carried out by myself or, where jointly, this fact has been acknowledged.

Acknowledgements

I would like to thank those who assisted me in the course of my studies, especially my supervisors Dr H.R.P Miller and Dr A. Ferguson who were always available to give me advice and encouragement.

I would like to acknowledge the expert technical assistance given by Mr G. Newlands who prepared the histological specimens and I am grateful to Mr J. Huntley and Dr S. Gibson for valuable discussion and provision of some reagents.

I wish to thank Miss K. Donald and Mr E. Gray for help with the scanning electron microscopy and Mr B. Easter and Mr A. Inglis for excellent photographic work.

I would also like to thank Dr J. Dawes and Miss K. Reilly for their collaboration and for assaying of glycosaminoglycan.

I am indebted to Dr R. Woodbury without whom much of this thesis would not have been possible for the gift of purified rat mast cell protease II.

I would also like to show appreciation for the members of the Pathology Dept., Moredun Institute for making my stay in Edinburgh a happy and profitable one.

I gratefully acknowledge financial support from the Agricultural and Food Research Council.

Finally I wish to thank my wife and family for continued support and encouragement throughout the preparation of this thesis.

ABSTRACT

Significant numbers of N.brasiliensis were trapped in mucus on days 10 and 11 of primary infection, trapping also occurred on day 7. Trapping was also demonstrated by scanning electron microscopy on day 10 but not on day 6 of infection, worms were embedded in a thick blanket of mucus on day 10.

The secretion of rat mast cell protease II (RMCPII) a specific granule product of mucosal mast cells (MMC) was examined during N.brasiliensis primary infection. Peak levels of enzyme occurred in the sera of rats around day 10 when MMC were either absent or present in very low numbers. MMC counts were similar whether stained for NADC-esterase, for RMCPII by immunoperoxidase or for glycosaminoglycan (GAG) demonstrating the absence of selective release of RMCPII from MMC. There was a time- and dose-dependent release of RMCPII into the serum of rats during systemic anaphylaxis, high levels of protease were also present in the intestinal lumen of shocked rats. The systemic concentrations of RMCPII were highly correlated with the enteric concentrations of Evan's blue, which were significantly increased during anaphylaxis. RMCPII could thus increase mucosal permeability during N.brasiliensis primary infection.

The major source of RMCPII in naive and immune rats was the jejunum, there being depletion of both MMC and RMCPII from this site following anaphylaxis. RMCPII was absent from connective tissue mast cells.

Anaphylactic pathology and permeability changes were abrogated by prior treatment of primed rats with corticosteroids. The number of MMC were significantly reduced following steroid treatment, mast cell changes occurred 4-24 hours after treatment with steroid. The fate of MMC was not established.

The number of MMC stained for NADC-esterase and GAG were always highly correlated and there was a highly significant relationship between the circulating levels of RMCPII and GAG during anaphylaxis, secreted GAG may thus be of MMC origin.

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CHAPTER 1 General Introduction.

Introduction

Despite over 50 years of research on the subject of the expulsion of intestinal nematodes from mammalian hosts no universally accepted mechanism of expulsion has been identified. Most workers agree that the rejection of primary infections with intestinal helminths involves antibodies and lymphocytes and there is evidence to implicate a bone marrow component (rev. Ogilvie & Love, 1974; Wakelin, 1978; Miller, 1984). However, due to the multiplicity of events promoted by lymphocytes and the extensive infiltration of the parasitized mucosa with a variety of cells including mast cells and basophils (Taliaferro & Sarles, 1939; Miller, 1984) it would seem that worm expulsion is likely to be a complex interaction of host responses.

One of the major features of helminthiasis is the development of inflammatory changes in the mucosa (Taliaferro & Sarles, 1939) and a long standing theme is that inflammation may bring about worm expulsion (Larsh & Race, 1975; Wakelin, 1978). Immediate hypersensitivity reactions may render the intestine inhospitable for worm survival (Stewart, 1955), promote changes in mucosal permeability (Murray, 1972), induce mucous release and the infiltration of other cells such as eosinophils to the inflamed mucosa (rev. Wakelin, 1978; Wasserman, 1983). The cells most frequently identified with allergy are mast cells and basophils

and it has become apparent that mast cells at mucosal surfaces are quite different from their connective tissue counterparts. The concept of mast cell heterogeneity will be reviewed later in this introduction.

It has been assumed for the purpose of this review that the lymphocytes have already been primed and the forthcoming discussion will deal only with the efferent arm of the mucosal response to the intestinal nematode Nippostrongylus brasiliensis in the rat and, in particular, the roles of mucosal mast cells (MMC) and of goblet cell mucus.

As much of this thesis is devoted to a study of the mucosal, as opposed to connective tissue mast cell responses to N.brasiliensis, the concept of heterogeneity within mast cells will be discussed at the beginning of this chapter.

Section I Mast cell heterogeneity.

Mast cell heterogeneity in the rat

Enerback (1966) must be credited with the first comprehensive study of mast cell heterogeneity through his studies on fixation and staining properties. However, 60 years earlier Maximow (1906) had drawn attention to a population of metachromatically granulated cells which were found in the intestinal mucosa of a number of species. Maximow (1906) stated that these cells were different from other mast cells both morphologically and in their staining properties. These cells were interpreted to be a special type of mast cell and were termed "atypical mast cells". The early work of Maximow (1906) has been reviewed by Michels (1938).

In 1959 Riley had reported that treatment with compound 48/80 did not affect tissue mast cells or the histamine content of the small intestine (Riley, 1959) an observation later to be confirmed in vitro showing isolated mast cells (atypical-mucosal) to be refractory to the potent secretagogue 48/80 (Befus, Pearce, Gauldie, Horsewood & Bienenstock, 1982b). It has become clear that many of the properties of the familiar rat peritoneal mast cell cannot be readily extrapolated to mucosal mast cells (MMC), (Miller, 1980; Enerback, 1981; Jarrett & Haig, 1984). MMC have been implicated in the expulsion of N.brasiliensis from the rat (Murray, 1972) and it is from this system that much of our present information in terms of MMC ontogeny and pharmacology has

been gained.

Origin

The proliferation of mucosal mast cells (MMC) in response to parasite infection is thymus-dependent because thymectomized or T-cell depleted rats and athymic mice fail to respond to enteric nematodiasis with mucosal mastocytosis (Mayrhofer, 1979; Mayrhofer & Fisher, 1979; Crowle & Reed, 1984; Ruitenbergh & Elgersma, 1976). This defect can be repaired by thymic grafts in mice (Ruitenbergh & Elgersma, 1976) or intravenous injection of normal thymocytes into nude mice (Olsen & Levy, 1976). An accelerated MMC response to N.brasiliensis in the rat can also be transferred by immune thoracic duct lymph cells or the putative T-cell fraction thereof, but not the B-cell fraction (Nawa & Miller, 1979).

Connective tissue mast cells (CTMC) originate in the bone marrow (Kitamura, Go & Hatanaka, 1978; Kitamura, Shimadu, Go, Matsuda, Hatanaka & Seki, 1979) since studies employing mice with genetically determined large mast cell granules demonstrated that CTMC in the skin were derived from bone marrow and were thymus independent (Kitamura et al., 1979)

Using athymic (deficient in MMC) and W/W^V anaemic mice (deficient in both MMC and CTMC), Crowle & Reed (1984) were able to show that the MMC defect in athymic mice could be repaired by thymus grafts, and could be

corrected by grafts of bone marrow in anaemic W/W^V mice. It would seem, therefore, that both CTMC and MMC derive from the bone marrow, MMC needing thymic influences to develop.

In vitro studies on the proliferation of MMC have shown that lymphocytes from N.brasiliensis-infected rats when stimulated with worm antigen will release factors which cause the differentiation of MMC from cultures of normal bone marrow (Haig, McKee, Jarrett, Woodbury & Miller, 1982). Furthermore, MMC will differentiate from cultures of bone marrow when treated with the supernatant from Concanavalin-A stimulated lymphocytes (Haig, McMenamin, Gunneberg, Woodbury & Jarrett, 1983). The cultured cells were classified as MMC because they contained rat mast cell protease II and because of the histochemical properties of the granule glycosaminoglycans (Haig et al., 1983; Haig, Jarrett & Tas, 1984).

Cultured mesenteric lymph node cells provide the best conditioned medium for the growth of MMC, which would indicate that they are one of the major sources of primed T-cells. It has been suggested that an expanded MMC progenitor population leaves the bone marrow and the proliferation and differentiation of these cells in the gut is dependent upon the presence of a lymphokine produced by activated T-cells (Jarrett & Haig, 1984).

Characteristics of MMC

In a series of papers (Enerback, 1966a,b,c,d) attention was focused on a population of mast cells in the lamina propria of the small intestine which could only be demonstrated when special fixation and staining techniques were used (Enerback, 1966a,b). Carnoy's solution and low concentrations of formaldehyde-acetic acid preserved these cells (MMC) well, whilst normal aldehyde fixatives did not (Enerback, 1966a). However, MMC can be demonstrated in normal aldehyde fixed tissue, if they are stained for 5-7 days in Toluidine blue (Wingren & Enerback, 1983). A more recent study showed that brief immersion of tissues in 4% neutral buffered paraformaldehyde (6 hours) allows both MMC and CTMC to be stained using conventional techniques (Newlands et al., 1984).

In appropriately fixed sections MMC and CTMC differ in their staining properties (Enerback, 1981). MMC stain strongly with copper phthalocyanine dyes, whereas CTMC will take up Safranin in the Astra blue/Safranin sequence. This suggests that MMC contain a weakly sulphated glycosaminoglycan (GAG) as opposed to heparin present in CTMC. Furthermore, MMC do not take up berberine sulphate (Wingren & Enerback, 1983) which is also indicative of a low sulphated GAG, and is in agreement with the findings of Tas & Bernsden (1977) who demonstrated the absence of heparin in MMC. MMC are also smaller than CTMC and less

densely granulated (Enerback, 1981).

Mucosal mast cells contain histamine (Enerback & Wingren, 1980) and are the main repository for histamine in the intestinal mucosa. Comparison of the histamine content of isolated MMC and CTMC (Befus et al., 1982b) has shown that MMC contain much less histamine (1.3pg/cell) than CTMC (15 pg/cell). MMC also differ from

CTMC in their content of serine proteinases and in their content of 5-HT (Enerback, 1981). These mediators will be discussed later in this section.

Although both CTMC and MMC have surface IgE another point of difference between them is that MMC in Nippostrongylus-infected rats have intracytoplasmic IgE (Mayrhofer, Bazin & Gowans, 1976). This could provide an enriched concentration of IgE in intestinal secretions by migration and shedding of MMC from the mucosa or by specific degranulation by antigen (Mayrhofer et al., 1976). The precise role of IgE in the cytoplasm remains to be established.

In vivo treatment of rats with agents which cause degranulation of CTMC, such as compound 48/80 and polymyxin B, has no immediate effect on MMC, but is followed by an increase in the number of MMC (Enerback & Lowhagen, 1979; Enerback, Lowhagen, Lowhagen & Wingren, 1981). The cause of the increase in MMC number is unclear, but it has been suggested that products released from degranulating CTMC are involved, however both

histamine and 5-HT fail to induce an increase in MMC when injected in amounts expected to be released from degranulating CTMC (Enerback et al., 1981). There are, therefore, many differences between MMC and CTMC in the rat and these delineate concepts of mast cell heterogeneity; they are summarized in Table 1.

Pharmacological properties

Heterogeneity between rat CTMC and MMC is evident if we consider their responses to a variety of basic secretagogues and anti-allergic compounds. Thus the highly potent CTMC secretagogues compound 48/80 and bee venom peptide 401 are completely without effect on MMC (Pearce, Befus, Gauldie & Bienenstock, 1982) and, similarly, phosphatidyl serine, which enhances histamine secretion from CTMC, does not potentiate histamine release from MMC (Pearce et al., 1982).

Disodium cromoglycate and theophylline which inhibit antigen-induced histamine secretion from CTMC are ineffective against MMC, but both cell types are inhibited by doxantrazole (Befus et al., 1982b; Pearce et al., 1982). Additionally, a series of flavonoid compounds have different orders of anti-allergic activity for MMC when compared with CTMC (Pearce, Befus & Bienenstock, 1984). The pharmacological properties of MMC and CTMC are shown in Table 2.

Table 1 Characteristic features of MMC and CTMC

	MMC	CTMC
Shape/granules	small, variable, sparsely granulated	large, uniform, densely granulated
GAG	Chondroitin sulphate di-B	Heparin
Origin	bone marrow	bone marrow
Life span	short (40 days)	long (6 months)
T-cell dependence	strong	weak
IgE	surface and cytoplasmic	surface
Histamine	1.5 pg/cell	15 pg/cell
5-HT	low	high
Arachidonic acid metabolism	unknown	PGD ₂
Nematode infections	proliferate	not known

Table 2 Pharmacological properties of MMC and CTMC

Agent	MMC	CTMC
Antigen	+	+
Anti-IgE	+	+
48/80	0	++
Peptide 401	0	++
A23187	+	++
Con-A	+	+
Cromoglycate	0	++a
Theophylline	0	++a
Doxantrazole	++a	++a
Phosphatidyl serine	0	++b

0 no effect +/++ weak to strong secretagogue activity

++a inhibition of histamine release

++b potentiation of histamine secretion

Murine bone marrow derived mast cells

Mouse bone marrow cells cultured in the presence of T cell factors differentiate into a relatively pure culture of granulated cells which resemble mast cells in terms of the staining of intracellular granules and overall ultrastructural morphology (Razin, Cordon-Cardo & Good, 1981; Schrader, Lewis, Clark-Lewis & Culvenor, 1981; Sredni, Friedman, Bland & Metcalfe, 1983; Razin, Stevens, Austen, Caulfield, Hein, Liu, Clabby, Nabel, Cantor & Friedman, 1984b). Mouse bone marrow derived mast cells (BMC) contain chondroitin sulphate E proteoglycan in their secretory granules (Razin, Stevens, Akiyama, Schmid, & Austen, 1982) whereas mouse and rat serosal mast cells contain heparin proteoglycan (Razin et al., 1982; Yurt, Leid, Austen & Silbert, 1977; Stevens, Razin, Austen, Hein, Caulfield, Seno, Schmid & Akiyama, 1983).

Upon in vitro activation with A23187 or with antigen after passive sensitization with monoclonal IgE, BMC oxidatively metabolize arachidonic acid through the 5-lipoxygenase pathway to LTC₄ and LTB₄ and generate minimal quantities of PGD₂ (Razin et al., 1982; Razin, Mencia-Huerta, Stevens, Lewis, Liu, Corey & Austen, 1983; Mencia-Huerta, Razin, Ringel, Corey, Hoover, Austen & Lewis, 1983). In contrast, rat CTMC activated immunologically generate PGD₂ and when stimulated with A23187 produce largely PGD₂ with some LTB₄ but no LTC₄ (Lewis, Soter, Diamond, Austen, Oates & Roberts, 1982;

Roberts, Lewis, Austen & Oates, 1979).

BMC are also distinguished from the mouse serosal mast cell by monoclonal antibodies prepared against mouse macrophages. Both subclasses of mast cell react with monoclonal B54.2 but only the serosal mast cell with B1.1 and only the BMC reacts with B23.1 (Katz, LeBlanc & Russell, 1983).

On the basis of the absence of heparin proteoglycan, distinct arachidonic acid metabolism, dependence on T cell factors : interleukin 3 (Razin, Ihle, Seldin, Mencia-Huerta, Katz, LeBlanc, Hein, Caulfield, Austen & Stevens, 1984a) and ultrastructural observations, mouse BMC are thought to represent the murine counterpart of rat mucosal mast cells.

Mast cell-derived mediators

The mast cell can release both preformed and generated (primarily lipid) materials which comprise the mediators of immediate hypersensitivity. The biochemical processes that are involved in the activation of mast cells have been reviewed extensively in recent years (Ishizaka & Ishizaka, 1978; Ishizaka, Conrad, Schulman, Sterk & Ishizaka, 1983; Siraganian, 1983; Kennerly, 1984). Essentially the bridging of cell surface IgE molecules generates a signal which results in membrane lipid metabolism and granule solubilization and discharge.

The mast cell contains, or is capable of elaborating in excess of twenty mediators (rev. Wasserman, 1980; Metcalfe, Kaliner & Donlon, 1981; Wasserman, 1983) and it is not clear whether MMC contain the same profile of mediators as CTMC since the two cell types differ functionally and biochemically (Jarrett & Haig, 1984)

Therefore I have selected those mediators known to be present in rat or putative murine MMC for further discussion and will indicate some of the possible functions of these mediators.

Histamine

The mast cell represents the main depot of tissue histamine and, under physiological conditions, the tissue content of histamine corresponds to the number of mast cells (Riley & West, 1953).

The effects of histamine are mediated by at least two histamine receptors : H1 and H2 receptors on target cells and tissues (Plaut, 1979). Following release of histamine from mast cells a number of local and systemic effects can be observed (rev. Metcalfe et al., 1981; Wasserman, 1983). Release of histamine in the gut would be expected to lead to cramping, acid and pepsin release. (Table 3)

Table 3 The effects of histamine mediated by H1 or H2 receptors

- H1 Increase vascular permeability
 Bronchial/smooth muscle contraction
 Pulmonary vasoconstriction
 Nasal mucus production
 Enhanced chemotaxis
- H2 Augment nasal mucus production
 Increase gastric acid secretion
 Increase vascular permeability
 Increase cAMP levels

Serotonin

Serotonin (5-Hydroxytryptamine : 5-HT) is present in rat MMC (Enerback, 1966d, Wingren et al., 1981, Wingren, Enerback, Ahlman, Allenmark & Dahlstrom, 1983), but by far the greatest amount of 5-HT is stored in the platelets (Vanhoutte & Cohen, 1983).

5-HT is active in inducing vasopermeability responses, and constriction of some smooth muscle and increases gastrointestinal motility (Metcalf et al., 1981).

Oxidative products of arachidonic acid

Arachidonic acid (AA) is liberated from mast cell membrane phospholipids by the action of phospholipase A₂, phospholipase C and diglyceride lipase (Kennerly, Sullivan & Parker, 1979) and, once liberated, AA may be metabolized via two main pathways : cyclooxygenase leading to the production of prostaglandins (PG) or via lipoxygenase to generate leukotrienes (LT). It is unclear whether rat MMC metabolize AA to PG or LT and hence both classes of mediator will be briefly discussed.

(i) Prostaglandins

Rat serosal mast cells preferentially generate large amounts of PGD₂ (Lewis et al., 1982; Roberts et al., 1979). By contrast, murine bone marrow derived mast cells generate minimal quantities of PGD₂ (Razin et al., 1983).

The possible physiological/pathological effects of PG are extensive (Metcalf et al., 1981; Wasserman, 1983) Liberated PGD₂ causes peripheral and central airway constriction in animals, inhibits platelet aggregation, elevates cAMP and causes vasodilation and secretion of mucus from cultured human airways (Wasserman, 1983; Soter, Lewis, Corey, Austen, 1983; Marom, Shelhamer & Kaliner, 1981).

(ii) Leukotrienes

The action of 5-lipoxygenase enzymes (rev. Samuelsson, 1983) on AA leads to the production, via a series of unstable intermediates, to the sulphidopeptide leukotrienes LTC₄, LTD₄ and LTE₄ or the potent chemoattractant LTB₄. The substance once referred to as slow-reacting substance of anaphylaxis (SRS-A) is now known to comprise LTC₄, LTD₄ and LTE₄ (Lewis & Austen, 1984).

Mouse bone marrow-derived mast cells metabolize AA predominantly to LTC₄ (Razin et al., 1983) with the production of some LTB₄ (Mencia-Huerta et al., 1983). In view of the fact that these cells are thought to represent the murine counterpart of rat MMC, it is possible that rat MMC likewise generate leukotrienes. The biological effects of the leukotrienes are listed in Table 4.

Table 4 Pharmacological actions of leukotrienesLTB₄

1. Increase neutrophil granule release and metabolism
2. Chemotactic and chemokinetic for neutrophils and eosinophils
3. Stimulate neutrophil adherence to endothelial cells
4. Increase vascular permeability

LTC₄, LTD₄ and LTE₄

1. Constrict smooth muscle (enhance peristalsis)
2. Increase vascular permeability (oedema formation)
3. Generate prostaglandins
4. Increase airway mucus production
5. Vasoconstriction
6. Constrict small airways

References

Piper (1984), Samuelsson (1983), Lewis & Austen (1984), Malmsted (1984).

Granule-associated neutral proteinases

1. Group-specific proteases

In 1971 new enzymes were purified from rat small intestine and skeletal muscle which reacted with apo-proteins of pyridoxal enzymes (Katunuma, Kominami & Kominami, 1971). These proteases were studied further and shown to have pH optima in the alkaline region, activity with substrates of chymotrypsin (N-acetyl-L-tyrosine ethyl ester) but not trypsin and seryl residues at their active centre (Katunuma, Kominami, Kobayashi, Banno, Suzuki, Chichibu, Hamaguchi & Katsunuma, 1975).

The enzymes separately purified from skeletal muscle and small intestine were shown to be distinct based on the lack of cross reactivity of the protease from muscle with antisera prepared against the intestinal enzyme (Katunuma et al., 1975). It was suggested that these enzymes, called 'group specific proteases' due to the apparent limited and selective activity, initiated the degradation of pyridoxal phosphate enzymes such as ornithine aminotransferase (OAT) (Katunuma et al., 1975).

The enzyme from small intestine was purified by Woodbury & Neurath (1978) and the quantitative relationships of protease to OAT examined. The disproportionate amounts of protease with respect to OAT activity in adult rats and the absence of any correlation in developing rats were inconsistent with the concept

that OAT is a physiological substrate for the protease.

2. Mast cell origin of Group specific proteases

The properties of the protease purified from rat skeletal muscle were found to be identical to that from rat peritoneal mast cells (Woodbury, Everitt, Sanada, Katunuma, Lagunoff & Neurath, 1978a). Polyacrylamide gel electrophoresis demonstrated identical mobilities corresponding to a molecular weight of 26000 and the relative amino acid sequences were found to be identical, furthermore immunodiffusion tests demonstrated that the two proteases were immunologically identical (Woodbury et al., 1978a)

Similarly the protease isolated from small intestine was found to be of mast cell origin (Woodbury, Gruzinski & Lagunoff, 1978b). This enzyme was localized by immunofluorescence to the mucosal mast cells of the lamina propria and intraepithelial cells of the intestine and also beneath the epithelium of the bronchioles (Woodbury et al., 1978b). All cells were found to contain low levels of the protease from skeletal muscle (Woodbury et al., 1978b).

Although both types of mast cell contained both intestinal and muscle proteases these enzymes did not cross-react immunologically (Woodbury et al., 1978b). Recent studies by Gibson & Miller (1986) have shown that using monospecific antisera that the enzyme of skeletal

muscle is exclusively located in CTMC and the protease from intestine is present only in MMC.

3. Terminology

To avoid the complicated and confusing names given to the mast cell proteases it was proposed by Woodbury & Neurath (1978) that the enzyme in skeletal muscle be termed RMCPI, and that in intestine RMCPII. This terminology will be observed throughout this thesis.

4. RMCP

A chymotrypsin like enzyme was localized to the mast cell granule by histochemical techniques over 30 years ago (Gomori, 1953), and its presence was further demonstrated by cleavage of synthetic substrates by whole mast cells (Benditt & Arase, 1959), cell extracts (Lagunoff & Benditt, 1963) and granule preparations (Lagunoff & Pritzl, 1976). This enzyme was termed chymase (Lagunoff & Benditt, 1963) and had a molecular weight of 23000-29000 (Kawiak, Vensel, Komander & Barnard, 1971) and was cationic assessed by its mobility at alkaline pH (Lagunoff, Phillips, Iseri & Benditt, 1964).

RMCP (chymase) has an apparent molecular weight by SDS PAGE of 26000 (Woodbury, Everitt & Neurath, 1981). Amino acid analysis has shown that the enzyme has a relatively high content of basic residues (19%) consistent with a pI of 9.5 (Woodbury et al., 1978a).

The amino acid sequence of the first 51 amino terminal residues reveals approximately 40% homology with bovine chymotrypsin A (Woodbury, Katunuma, Kobayashi, Titani & Neurath, 1978c) and greater than 75% sequence identity with RMCPII (Woodbury et al., 1978c). RMCPI is relatively insoluble requiring high salt concentrations for its isolation from CTMC or muscle (Woodbury et al., 1981).

There is a high degree of similarity with neutrophil cathepsin G (Woodbury & Neurath, 1980) and it has been suggested that RMCPI and cathepsin G are one and the same enzyme (Starkey, 1978). However, reactivity towards 4-nitroanilide peptides clearly indicates that the enzymes are quite different (Yoshida, Everitt, Neurath, Woodbury & Powers, 1980).

The activity of RMCPI is partially masked by its binding to mast cell heparin (Yurt & Austen, 1977) with which it is released after activation of isolated mast cells (Yurt & Austen, 1977). Furthermore, the active site of RMCPI is partially inhibited in vitro by 5-HT; at concentrations comparable to those present in the intact mast cell (Yurt & Austen, 1977).

5. RMCPII

Mucosal mast cells also possess a chymotrypsin like enzyme with esterase activity (Lagunoff & Benditt, 1963; Newlands, Huntley & Miller, 1984). Amino acid sequence

analysis shows RMCPII, like RMCPI, to be a single polypeptide chain of molecular weight 25000 (Woodbury et al., 1978c). Like RMCPI, RMCPII is isolated in a fully active form and seems to lack a zymogen precursor (Woodbury & Neurath, 1980). RMCPII has 15% basic and only 9% acidic residues and is basic at neutral pH, but not as basic as RMCPI (Woodbury et al., 1978c).

In contrast to RMCPI, RMCPII is not inhibited by N-p-tosyl-L-phenylalanine chloromethyl ketone. However, RMCPII is inhibited by extended peptide chloromethyl ketones that contain phenylalanine and hydrophobic residues. The structures of such inhibitors are analogous to the preferred substrates of RMCPII (Woodbury & Neurath, 1980).

The cell specific localization of RMCPII suggests that it is a secretory enzyme (Woodbury & Neurath, 1978) and hence the measurement of extracellular RMCPII should provide a specific marker for the in vivo activation of MMC.

The amount of RMCPII in an adult rat intestine has been estimated to be 3mg (Woodbury & Neurath, 1978) and in rats 6-8 weeks old it represents 1% (by weight) of the total protein of the small intestine (Woodbury & Neurath, 1978). Unquestionably RMCPII represents a major protein product of MMC.

6. RMCP II, MMC and development

Since RMCP II has been shown to be located in MMC (Woodbury et al., 1978b; Gibson & Miller, 1986) then the number of these cells would be expected to reflect the concentration of protease in the intestine. Woodbury & Neurath (1978) examined the levels of RMCP II in the intestines of developing rats and demonstrated a rapid 4 fold rise in the levels of protease 4-6 weeks after birth. The levels then decreased to adult levels (0.5 mg RMCP II/g of tissue). The mast cell response followed a similar pattern increasing 2 fold between weeks 4 and 6, remaining high and then decreasing to adult numbers (Woodbury & Neurath, 1978).

7. RMCP II and parasitism

Infection with N.brasiliensis is associated with a proliferation of MMC in the jejunum, and at the peak of this response the levels of RMCP II are increased almost nine fold in the intestinal mucosa (Woodbury & Miller, 1982). Early in the proliferation of MMC, only a proportion of the cells in the lamina propria stained for RMCP II with immunoperoxidase, none was stained in the epithelium (Woodbury & Miller, 1982), although intraepithelial MMC were detected after staining with basic dyes. Fourteen to twenty days after infection and in rats immunized by previous infection, staining for RMCP II was both stronger and also present in

intraepithelial mast cells (Woodbury & Miller, 1982).

Woodbury et al (1978b) were able to demonstrate RMCPII immunohistochemically in intraepithelial mast cells of normal rats, in contrast to the results of Woodbury & Miller (1982). However, this required fixation by perfusion with paraformaldehyde which resulted in considerable extracellular fluorescence; Carnoy's fixation in agreement with the results of Woodbury & Miller (1982) did not permit the adequate visualization of intraepithelial mast cells (Woodbury et al., 1978b).

When the parasites had been expelled (day 12), MMC numbers were significantly increased, but many of the MMC (detected by Alcian blue) were found to contain little protease assessed by the levels of RMCPII in the jejunum or in mucosal mast cells by immunofluorescence (Woodbury & Miller, 1982). The failure to detect protease in intraepithelial mast cells suggests that it is either not present, present in lower concentrations (Woodbury & Miller, 1982) or is released disproportionately to proteoglycan. Granule discharge has been demonstrated in N.brasiliensis infection (Miller & Walshaw, 1972) and the low levels of RMCPII are compatible with histochemical evidence of granule discharge. Selective release of granule components cannot be ruled out until good immunohistochemical localization and concomitant proteoglycan staining can be achieved.

The finding that RMCPII levels in the gut mucosa were low when the majority of parasites had been expelled (Woodbury & Miller, 1982) could mean that MMC are secreting rather than storing enzyme. This led to a study of the release of this enzyme into the serum of rats following challenge with either parasites or parasite antigens.

Rats primed by infection with N.brasiliensis and challenged with 5 day old parasites respond by the systemic release of RMCPII which is both time- and dose-dependent (Miller, Woodbury, Huntley & Newlands, 1983). Maximal values (3000ng/ml serum) were observed in recipients of 2665 parasites one hour after challenge. Trace amounts of protease were also present in naive and primed rats given saline intraduodenally. Systemic secretion of RMCPII occurred in naive rats challenged with parasites and was slower and less pronounced than in primed rats (Miller et al., 1983). No RMCPII was present in the sera of naive rats challenged with day 4 old parasites supporting the view that the parasite does not produce mast cell degranulator until it is 5-6 days old (Miller, 1979b). Few morphological changes were evident granule exocytosis was not observed in MMC, and the tissue concentration of RMCPII was unaltered following worm challenge (Miller et al., 1983).

Soluble Nippostrongylus antigen had no effect when given either intraduodenally or intravenously to naive

rats, but one hour after intraduodenal challenge of primed rats with antigen a concentration of $10.5\mu\text{g RMCPII/ml}$ was detected in the sera (Miller et al., 1983). Furthermore, after intravenous challenge of primed rats with antigen there was substantial release of RMCPII ($122\mu\text{g/ml}$) and depletion of both MMC and RMCPII from the intestinal mucosa (Miller et al., 1983). Signs of severe anaphylactic shock were evident ie. hyperaemia, mucus secretion, epithelial shedding and the appearance of Evan's blue dye in the gut lumen indicative of increased epithelial permeability (Miller et al., 1983). These experiments demonstrate that MMC are activated in response to parasite antigens and that the measurement of RMCPII can be used to assess the degree of activation and participation of MMC.

The stage was set to analyze the release, if any, of RMCPII into the blood of rats during primary infection, and two nematode infections were investigated (Woodbury, Miller, Huntley, Newlands, Palliser & Wakelin, 1984). Following infection with Trichinella spiralis most of the worm burden was expelled between days 9 and 12, maximal accumulation of MMC occurred on day 12 when peak levels of RMCPII were detected in the sera. Significant enzyme release was detected in blood, however as early as day 6 of infection (Woodbury et al., 1984). In N.brasiliensis infection protease secretion was less pronounced than in T.spiralis infection (1000ng/ml compared

with 4000ng peak levels). Peak levels of enzyme occurred on day 9-10 and coincided with worm expulsion. These results demonstrated for the first time that MMC were active during the immune expulsion of nematode parasites (Woodbury et al., 1984).

8. Functions of RMCPI and RMCPII

Although RMCPI (chymase) has been studied for many years its precise physiological role remains somewhat obscure. There is evidence to suggest that chymase is involved in the degradation of connective tissue proteoglycans (Seppa, Vaananen & Korhonen, 1979), in the selective degradation of basement membrane type IV collagen (Sage, Woodbury & Bornstein, 1979) as well as promoting vascular permeability changes (Seppa, 1978). The enzyme may, however, have some as yet undefined intracellular role, an observation supported by the fact that it is fully active as an esterase and protease even when bound to mast cell heparin (Yurt & Austen, 1977).

The function of RMCPII is likewise obscure. The involvement of MMC in parasite expulsion will be discussed (Section II) and if MMC are part of the host response to parasitism then RMCPII could play a role in the events leading to parasite expulsion.

As RMCPI and RMCPII are antigenically distinct and because RMCPII predominates in MMC, measurement of this enzyme by immunoassay allows the participation

of MMC in parasitism and allergic conditions to be selectively monitored.

Table 5. RMCPII the unique proteinase of rat MMC

Localization

Exclusively to MMC by immunoperoxidase techniques.

Biochemical Properties

single polypeptide chain

224 amino acids

molecular weight 24655

seryl residue at active site

pH optima in alkaline region

active with substrates of chymotrypsin

preferential cleavage of peptide bonds between hydrophobic residues

cleaves native basement membrane type IV collagen

75% sequence identity with RMCPI in the first 51 amino terminal amino acids

soluble, not requiring high salt concentrations for its purification or assay

Section II Allergy, mast cells and parasitism.

Parasitism and Allergy

A characteristic feature commonly associated with helminth infections is the occurrence of hypersensitivity reactions with a proliferation of mast cells and eosinophils together with the biosynthesis of substantial quantities of IgE (rev. Murray, 1972; Sadun, 1972; Askenase, 1980; Jarrett & Miller, 1982; Miller, 1984). Positive Prausnitz-Kustner reactions have been demonstrated in man thus providing evidence of the immediate nature of the hypersensitivity state (Andrews, 1962).

Allergy to helminth antigens is exemplified by the reactions of animals sensitive to Ascaris species, where rapid local and occasionally systemic reactions have been demonstrated in horses challenged with enteric fluid from A. equorum (Weinberg & Julien, 1911). Cutaneous, asthmatic and anaphylactic symptoms have also been described in dogs challenged intradermally with A. lumbricoides and Toxocara canis antigens (Brunner, Altman & Bowman, 1944). Laboratory rodents are also demonstrably hypersensitive to helminths, rats and mice sensitized with N. brasiliensis, T. spiralis, or Nematospiroides dubius and injected with homologous antigen undergo systemic or local anaphylactic shock (Briggs & Degiusti, 1966; Urquhart, Mulligan, Eadie & Jennings, 1965; Panter, 1969; Miller et al., 1983).

It became evident that hypersensitivity reactions expressed as oedema, dermatitis, diarrhoea and even anaphylactic shock were involved in the pathogenesis of

parasitic infections (Sadun, 1972). Such reactions are normally regarded as being harmful to the host but a constant idea has been that immediate hypersensitivity mechanisms may play a part in specific protective responses at mucosal surfaces.

Although the concept of anaphylaxis introduced by Portier & Richet (1902) to describe the apparent decreased resistance of animals to foreign protein on a second exposure and the term 'allergy' introduced by Von Pirquet (1906) bore striking resemblances to the observations with helminth antigens, it was not until the 1950's that the observations of Taliaferro & Sarles (1939) and Hamann (1943) describing mast cell degranulation and an increased level of histamine in tissues of parasitized rats were linked to allergic reactions. This was chiefly achieved by the identification of mast cells as the major source of histamine released by specific anaphylactic degranulation (Mongar & Schild, 1950).

IgE, Allergy and Parasitism

A fundamental immunological component of immediate hypersensitivity is the production of IgE which binds to high-affinity receptors on mast cells and basophils (Ishizaka et al., 1983)

Mast cells and basophils are not the only cells to possess Fc receptors for IgE, macrophages (Dessaint,

Torpier, Capron, Bazin & Capron, 1979), T-lymphocytes (Yodoi & Ishizaka, 1979), monocytes (Ferreri, Howland & Spiegelberg, 1986) and eosinophils (Capron & Capron, 1980) have all been shown to possess such receptors, albeit of a lower affinity than those on mast cells and basophils.

The production of substantial quantities of IgE is one of the most conspicuous events associated with helminth infestations and N.brasiliensis infection of rats has proved a useful model to examine the parameters of IgE production and its role in parasite rejection and allergic conditions (Jarrett & Miller, 1982).

Conventional passive cutaneous anaphylaxis (PCA) assays have failed to show any relationship between the expulsion of N.brasiliensis and the levels of parasite specific IgE (Ogilvie, 1967; Jarrett, Jarrett, Miller & Urquhart, 1967); IgE generally being detected in the sera only after the completion of parasite expulsion (Ogilvie, 1967). However, if other methods are employed IgE-like reactivity can frequently be demonstrated before parasite expulsion is complete. Although in vivo gut sensitivity to intravenous administration of worm antigen generally occurs after parasite rejection, systemic anaphylaxis can be induced as early as 13 to 16 days after infection with N.brasiliensis, the reaction being confined to the small intestine (Keller, 1970). Urquhart et al (1965) have reported susceptibility to

systemic anaphylaxis as early as day 7 of infection. Similarly, peritoneal mast cells are sensitized within 7 to 10 days of infection (Wilson & Bloch, 1967). A small degree of bronchial sensitivity to N.brasiliensis can be demonstrated before IgE is detectable in the sera (Church, 1975) and active cutaneous anaphylaxis can be demonstrated as early as day 10 of infection (Jarrett & Stewart, 1973; Befus, Johnson, Berman & Bienenstock, 1982). It is therefore possible that mast cells are sensitized with IgE before IgE can be shown to be present in the sera (Askenase, 1980).

Immediate hypersensitivity and parasite expulsion

Some of the most notable features occurring during helminth infections are inflammatory changes in the enteric mucosa with extensive accumulation of mast cells and basophils (Taliaferro & Sarles, 1939; Wells, 1962; Murray, Jarrett, Jarrett & Jennings, 1971a; Askenase, 1980; Rothwell & Dineen, 1972) and altered physicochemical conditions in the gut lumen (Castro, Roy & Stockstill, 1974; Castro, Badial- Aceves, Adams, Copeland & Dudrick, 1976). Consequently a theme expressed in many reviews on the mechanisms of helminth immunity is that inflammation may bring about parasite expulsion (Murray, 1972; Larsh & Race, 1975; Wakelin, 1978).

A relationship between local hypersensitivity and worm expulsion was demonstrated by Stewart (1955). It

was shown that adult Haemonchus contortus were frequently expelled after a further intake of infective larvae (Stewart, 1953; Stewart, 1955). Coincident with expulsion there was a rise in whole blood histamine and a local anaphylactic reaction characterized by abomasal oedema with increased peristalsis and segmentation (Stewart, 1953). This expulsion process termed 'self-cure' (Stewart, 1955) was inhibited by antihistamine drug treatment, parenteral administration of histamine or exposure in vitro of the adult worms to histamine did not have any direct effect on the worms themselves (Stewart, 1953). Stewart (1955) concluded that histamine did not act directly on the parasite but the local hypersensitivity reaction altered the environment within the abomasum so that the parasite could no longer survive. Challenge with an unrelated parasite could also cause expulsion indicating that the mechanism of expulsion was immunologically non-specific (Stewart, 1953).

This idea was examined by others who studied infection of rats with N.brasiliensis, a system in which a clearly demarcated and quantifiable self-cure occurs (Ogilvie & Jones, 1971). Expulsion of N.brasiliensis does not require the stimulus of reinfection and starts usually 10-11 days after infection and proceeds over several days (Jarrett, Jarrett & Urquhart, 1968; Ogilvie & Love, 1974).

At the time of worm expulsion rats are demonstrably hypersensitive to N.brasiliensis antigens and show immediate skin reactions on intradermal injection and anaphylactic shock following intravenous injection of antigen (Urquhart et al., 1965). The anaphylactic reaction is most prominently displayed by gross alterations in the small intestine with hyperaemia, increased capillary permeability, mucus secretion, oedema and plasma leakage into the gut lumen (Urquhart et al., 1965). Subsequently a lesion similar to that induced experimentally was shown to be present in the intestines of rats during the expulsion of N.brasiliensis (Barth et al., 1966). The experiments of Urquhart et al. (1965) led them to propose that local anaphylaxis may affect worm expulsion in two ways. Firstly, by physical changes in the intestine as originally postulated by Stewart (1955) or secondly, by promoting the passage of antibody and other serum derived components into the gut lumen as a result of increased mucosal permeability.

The second hypothesis was tested by Barth et al. (1966) who studied the effect of heterologous intestinal anaphylaxis in rats harbouring transplanted N.brasiliensis. These experiments demonstrated that rats given hyperimmune serum plus anaphylactic shock (egg albumin (EA) intravenously into EA sensitized rats) expelled worms sooner than rats given anaphylactic shock or serum alone (Barth et al., 1966) indicating a link between antibody

and permeability changes.

An increased translocation of plasma-derived macromolecules into the gut lumen occurs in N.brasiliensis infected rats (Murray et al., 1971a, Nawa, 1979) and ultrastructural studies have shown gaps between epithelial cells indicative of increased mucosal permeability (Murray et al., 1971a). Anaphylactic degranulation of MMC could promote changes in mucosal permeability but Nawa (1979) was unable to show any relationship between MMC counts and permeability. A different conclusion was reached by Murray et al (1971a) who demonstrated a close agreement between permeability and MMC numbers.

MMC and parasites

Mucosal mast cell (MMC) hyperplasia is a prominent feature following enteric infection with parasite nematodes (rev. Askenase, 1980; Befus & Bienenstock, 1982; Miller, 1984) and the role of MMC in the expulsion of gastrointestinal nematodes remains controversial (Askenase, 1980). Although nematode infections of laboratory animals invariably provokes mucosal mastocytosis (Miller, 1980; Jarrett & Miller, 1982; Miller, 1984), the precise timing of the onset of mastocytosis and its relationship to expulsion varies both between different strains of the same host (Nawa & Miller, 1979; Lee & Wakelin, 1982) and with different levels of infection

(Nawa & Miller, 1979).

Experimental infection of rats with N.brasiliensis is associated with a fluctuation both in number and distribution of MMC (Miller & Jarrett, 1971; Wells, 1962). Shortly after this parasite reaches the intestine there is a marked degranulation of MMC (Miller, 1971) which may reflect the release from the worms of a mast cell degranulating agent (Murray, Miller & Jarrett, 1968). During the exponential rise in MMC there is release of amines, proteoglycan and basic protein from MMC which can be detected by electron microscopy and histochemical techniques (Miller, Murray & Jarrett, 1967; Murray et al., 1968; Miller & Walshaw, 1972). It is at this stage when transformation of MMC into globule leukocytes is most marked (Miller & Jarrett, 1971) the partially discharged MMC migrating into the intestinal epithelium to give rise to GL. If worms are removed by drug treatment this transformation does not occur (Murray, Miller, Sanford & Jarrett, 1971b) whereas the rise in MMC numbers occurs whether the worms have been removed or not.

Discharge of MMC is also indicated by the detection of RMCPII in the sera of N.brasiliensis-infected rats, the levels of this enzyme reach a peak 10-12 days after infection coincident with worm expulsion (Woodbury et al., 1984).

The evidence cited above, of activation of MMC

during self-cure, has led to the suggestion that they are involved in the expulsion of nematodes via anaphylactic triggering and release of mediators when MMC surface IgE interacts with parasite antigens.

Mediators released from degranulating MMC may affect the parasite directly (Rothwell, Prichard & Love, 1974), alter mucosal integrity (Miller *et al.*, 1983) and secretory activity (Castro, 1982) and act on smooth muscle (Castro, 1981).

The arguments for and against a role for MMC in parasite expulsion

Kinetic studies documenting increased numbers of intestinal MMC and the relationship of these increases to parasite expulsion have provided conflicting data as to the role of MMC. Whilst on one hand mastocytosis and expulsion are related temporally during T. spiralis (Alizadeh & Wakelin, 1982), Strongyloides ratti (Olsen & Schiller, 1978; Mimori, Nawa, Korenga & Tada, 1982) and Trichostrongylus columbriformis (Handlinger & Rothwell, 1981) infections of mice, rats and guinea pigs respectively the relationship in S. ratti (Carroll, Mayrhofer, Dawkins & Grove, 1984) or Trichuris muris (Lee & Wakelin, 1982) infections of mice is less clearcut.

The situation is made even more complex in that increased numbers of mast cells have been recorded at or about the time of the expulsion of N. brasiliensis from

rats (Wells, 1962; Jarrett et al., 1967; Miller & Jarrett, 1971) whereas other reports have shown that the rise does not occur until the majority of parasites have been expelled (Keller, 1971; Kelly & Ogilvie, 1972). Furthermore, in some strains of rats the mast cell response does not occur until 1-2 days after the onset of expulsion, but coincides with the final stages of worm expulsion (Nawa & Miller, 1979).

The overall impression from studies involving mast cell counts is that no firm conclusions can be made as to the role of MMC in expulsion. Furthermore it must be questioned as to whether histological evidence alone adequately portrays the situation in the parasitized gut. The latter problem is exemplified by the studies of Woodbury et al. (1984) on the release of RMCPII during infection of rats with N.brasiliensis. It was demonstrated that RMCPII was secreted into the serum and at its peak coincided with parasite expulsion even though MMC were low in number. Therefore on the basis of MMC counts alone, a role for MMC would seem unlikely, but the presence of RMCPII in serum demonstrates that MMC are activated and their role must be reevaluated in the light of these studies.

The role of MMC has been further explored by the use of a variety of drugs which act on or compete with biogenic amines. Whilst on one hand the antihistamine promethazine (Urquhart et al., 1965) or promethazine

plus anti-serotonin drugs (Murray, Smith, Waddell & Jarrett, 1971c) or reserpine (Sharp & Jarrett, 1968) inhibited the expulsion of N.brasiliensis, similar drugs had no effect when tested by other workers (Keller, 1971; Keller & Ogilvie, 1972). Therefore, whilst some experiments are in favour of a mast cell involvement in worm expulsion others reach the opposite conclusions.

However, it is clear that experiments dealing with mediator antagonists are often flawed because the drugs have multiple actions and side effects. For example, although reserpine depletes mast cell amines, treatment of guinea pigs with this drug renders them dehydrated through the action of the drug on the enterochromaffin tissues of the gut (Askenase, 1977).

Of all the drugs tested, the glucocorticosteroids are those invariably shown to inhibit worm expulsion (Ogilvie, 1965; Urquhart et al., 1965). These drugs suppress the mast cell response to N.brasiliensis infection (Jarrett et al., 1967) and also block the rapid expulsion of T.spiralis (Bell, McGregor & Adams, 1982) and N.brasiliensis (Miller & Huntley, 1982b) from primed rats. Again corticosteroids have a wide spectrum of actions and their action against MMC requires further analysis.

The lack of conclusive evidence obtained from drug blocking experiments, has led to the use of animals deficient in MMC in order to clarify the role of MMC in

expulsion. However, even these experiments have failed to produce unequivocal results, with Uber, Roth & Levy, (1980) demonstrating normal expulsion kinetics for N.brasiliensis and Kojima, Kitamura & Takatsu (1980), Crowle & Reed (1981) and Crowle (1983) showing infection was prolonged in mast cell-deficient mice. Reconstitution of W/W^V mice (congenitally deficient in MMC) with bone marrow or spleen cells from $+/+$ littermates repaired the mast cell defect, but insufficient data points were included to comment on the effect of reconstitution on expulsion (Crowle, 1983). Reconstituted or not, such mice were slower at rejecting N.brasiliensis than their normal litter mates (Crowle, 1983). The expulsion of T.spiralis from mast cell-deficient W/W^V mice is delayed (Ha, Reed & Crowle, 1983) and transfer of $+/+$ bone marrow cells to W/W^V mice accelerates parasite expulsion (Ha et al., 1983).

It is important at this point to indicate that the parameters for the identification of murine MMC have not been fully established (Miller, 1980). For example, the granules of intraepithelial lymphocytes exhibit metachromasia similar to mast cell granules when stained with Toluidine blue (Guy-Grand, Griscelli & Vassalli, 1978) and the extent to which these cells may have been misinterpreted as MMC is unclear.

In conclusion, the experimental results, with the exception of those dealing with the measurement of RMCPII,

are highly contradictory with regard to the role of MMC in the expulsion of primary nematode infections. The observations on the release of RMCPII during expulsion are to date the most convincing evidence to suggest that MMC may play some role in expulsion. It is also reasonable to suggest that, in addition to RMCPII other mediators may be generated/released and, since mast cells are capable of producing a wide battery of potent biologically active compounds, the effects of MMC secretion could be of great importance in the expulsion mechanism.

Secondary infections - Rapid expulsion

Rapid expulsion (RE) is a phenomenon directed against infective larvae as they enter the intestine and is ultra rapid, apparently occurring before establishment takes place, the worms being expelled within 24-48 hours of challenge. RE has been described in both rats and mice challenged with T.spiralis (McCoy, 1940; Russell & Castro, 1979; Alizadeh & Wakelin, 1982), in rats challenged with N.brasiliensis (Miller, Huntley & Wallace, 1981b) and in guinea pigs challenged with T.colubriformis (Rothwell et al., 1974)

Secondary challenge of rats with T.spiralis is associated with a response that prevents 80% of an intraduodenal challenge of 7500 larvae from embedding in the intestine (Russell & Castro, 1979). The host response occurs within 15 minutes after contact of the

parasite with the intestinal mucosa, the residual worm burden being expelled 4-8 days later (Russell & Castro, 1979).

Infective larvae are rejected uninjured and are able to establish normally in naive rats (Hessell, Ramaswamy & Castro, 1982; Russell & Castro, 1985). This is in agreement with the early observations of McCoy (1940) who detected a reduced worm burden 12 hours after secondary challenge of T. spiralis in immune rats, the worms being infective for naive rats. Two inductive stimuli are required to promote RE in rats challenged with T. spiralis, one is a consequence of prior infection with pre-adult T. spiralis and the second is a non-specific activation of the intestine, since an unrelated parasite (Heligmosomoides polygyrus) will also promote RE (Bell & McGregor, 1980).

The relative speed at which challenge infections are expelled from immune rats and the occurrence of high levels of parasite-specific IgE and increased numbers of MMC after the expulsion of primary infections (Murray, 1972) raises the distinct possibility that challenge infections are expelled by an anaphylactic reaction (Murray, 1972; Askenase, 1980; Miller, 1984).

Following secondary challenge of rats immune to T. spiralis there is a rapid accumulation of fluid in the gut (Castro, Hessell & Whalen, 1979) which is associated with RE of this parasite (Russell & Castro,

1979). Studies in vitro of these effects have shown there to be immunologically specific alterations in trans-epithelial transport of sodium-hexose and an increase in trans-epithelial electrical potential difference (Russell & Castro, 1985). Anaphylactic reactions on exposure to Trichinella antigens may cause structural/functional changes in the intestinal epithelium and thus confer resistance to the host indirectly by modification of the habitat of the parasite (Castro, 1982; Russell & Castro, 1985). Castro has proposed that vasoactive intestinal peptide released from mast cells may play a role (Castro, 1982) or, alternatively, mediators such as histamine, serotonin or prostaglandins (Russell & Castro, 1985) may mediate these effects.

The RE of N.brasiliensis occurs within 4-5 hours of intraduodenal challenge of primed rats with day 4 old worms. The response is unaffected by challenge dose, but is dependent upon the immune status of the host (Miller et al., 1981b) Evidence that MMC are involved in this response has come from a study of the release of RMCPII during the RE of N.brasiliensis (Miller et al., 1983). The release of RMCPII from MMC was examined following challenge of primed rats with 4 or 5 day old parasites. Primed rats responded in a dose- and time-dependent fashion with the secretion into blood of 1-3 μg RMCPII/ml of serum one hour after challenge (Miller

et al., 1983). These results unequivocally demonstrate MMC secretion during the RE of N.brasiliensis. It would seem reasonable to suggest that RMCPII secretion is accompanied by the release of other mediators which may in concert bring about the rapid expulsion of N.brasiliensis.

In an attempt to assess the role of immediate hypersensitivity in the RE of T.spiralis, Bell et al. (1982) employed various inhibitors of hypersensitivity and examined their effects on RE. However, no consistent inhibitory effects could be seen (Bell et al., 1982). Promethazine, cobra venom factor (anti-complementary) and cortisone caused some inhibition of RE, but a variety of antihistamines and anti-5-HT drugs were without effect on RE (Bell et al., 1982). Furthermore, treatment of rats with aspirin or indomethacin (anti-prostaglandin/inflammation) or carbachol (smooth muscle stimulant) did not prevent RE. It was concluded that immediate hypersensitivity played a minor role or none at all in the RE of T.spiralis (Bell et al., 1982).

In guinea pigs infected with T.colubriformis a role for histamine and 5-HT in parasite expulsion has been suggested (Jones, Rothwell, Dineen & Griffiths, 1974; Rothwell, Dineen & Love, 1971) the rejection of transplanted worms being inhibited by anti-histamine treatment of immune guinea pigs (Rothwell, Love & Evans, 1978).

There are, however, restrictions which apply to experiments dealing with mediator inhibition. Firstly, it is possible that following mediator release local tissue concentrations are so high that the drugs are ineffective. Furthermore, the effects of histamine may be a combination of both H1 and H2 receptor activation such that blockade with one antihistamine is not sufficient. Secondly, it is not only histamine which is important in the pathogenesis of allergy, and other mediators may not be amenable to the pharmacological regimes used. Thirdly, MMC may not be affected by the drugs which are known to inhibit mediator release from CTMC. Any of the above mechanisms may contribute to a lack of success in demonstrating the inhibition of RE by anti-allergic compounds (Bell et al., 1982).

Glucocorticosteroids have proved universally effective in inhibiting worm expulsion, blocking the rapid expulsion of N.brasiliensis (Miller & Huntley, 1982b) and T.spiralis (Bell et al., 1982), and it is clear that their potent anti-anaphylactic properties and effects on MMC in relation to the suppression of rapid expulsion are worthy of further study.

Section III Mucus.

Gastrointestinal mucus

Mucus is usually defined as the viscoelastic fluid that lines the epithelium of the gastrointestinal, respiratory and genito-urinary tracts and is a mixture of large molecular weight glycoproteins (mucins), water, electrolytes, sloughed epithelial cells, DNA, IgA, lysozyme, plasma proteins, bacteria and bacterial products (Forstner, 1978).

Chemical and physical properties

The molecules responsible for the viscous and gel forming properties of mucus are the glycoproteins or mucins (Hollander, 1954), which constitute between 1 and 10% by weight of the gel. The bulk of mucus consists of water, up to 95% by weight, and an electrolyte composition similar to that of plasma (Hollander, 1963). The remainder is non dialyzable glycoproteins, proteins, and nucleic acids (Allen, 1978). Mucus is heterogeneous and contains, in addition to those substances defined above, virtually all other constituents found in gastrointestinal juice (Glass, 1964).

The general features of mucus glycoproteins are shown in Table 6. Mucins are macromolecules in which the peptide core, generally between 10 and 30% of weight, is linked to oligosaccharide side chains by O-glycosidic bonds between serine and/or threonine and N-acetyl-galactosamine residues (Ginsberg & Neufeld, 1969; Spiro,

Table 6 General features of mucin glycoproteins

1. High molecular weight, greater than one million
2. Over 70% carbohydrate by weight
3. Carbohydrate side chains contain : N-acetylglucosamine
N-acetylgalactosamine, galactose, sialic acids and
fucose
4. Do not contain : uronic acids, mannose or glucose
5. O-glycosidic bonds : N-acetylgalactosamine-threonine
(or serine)
6. Carbohydrate side chains : 2-22 sugars in length, often
branched
7. Negatively charged due to sialic acid and ester sulphate
8. Mucins are viscous and form gels



1970). The oligosaccharide composition is variable but consists of those sugars listed in Table 6. In all instances fucose and sialic acids are found at the non-reducing ends. Carbohydrate side chains vary from 2-15 sugars in length and may be branched (Oates, Rosbottom & Schragar, 1974). Mannose, uronic acids and glucose are not present, sulphate is usually present but its linkage point has not been fully characterized.

Various models of mucin structure have been proposed and these include the "windmill" model for porcine gastric mucin (Allen & Snary, 1972), a beaded mucin model (Robinson & Monsey, 1975) and the "flexible thread" configuration for rat goblet cell mucin (Forstner, Jabbal & Forstner, 1973). These structures are stabilized by non-covalent bonds, disulphide bridges, hydrophobic and hydrogen bonds (Forstner, 1978), leading to the formation of a viscous elastic gel typical of mucus (Allen, 1981). The gel has properties intermediate between those of a solid gel and a liquid (Litt, Wolf & Khan, 1977) arising from a balance between polymer-polymer interactions of the glycoproteins themselves and the polymer-solvent interactions of the hydrophilic glycoproteins and the aqueous environment (Morriss & Rees, 1978).

MUCUS glycoproteins not only stick to each other, but to other molecules and cell surfaces (Allen & Minnikin, 1975) ensuring that mucus adheres to the mucosal surface and provides a slimy coat to facilitate the passage of

food and faecal material.

Physicochemical function of mucus

Mucus is a product of secretory epithelia which line internal body cavities where tissue is in contact with air (as in the lung) or solid material (as in the intestine).

One major function of mucus in the gut is to protect the delicate mucosal epithelium from damage by the passage of food and faecal material, and to lubricate the epithelium so as to ease the transit of contents along the intestine (Florey, 1955).

Another important feature of the mucus gel is its ability to retain water (Silverberg, Meyer, Gilboa & Gelman, 1977) so as to provide a perpetual aqueous environment for the mucosal surface. Mucus may also function as a water-proofing agent and hence regulate epithelial cell hydration (Gibson, Matthews, Minihan & Patti, 1971; Gordon, 1974). This function is likely to be related both to the concentration of mucin and the extent of intertangling of the mucin fibres, and in vitro studies have shown that the rate of water permeability through mucus gels is inversely correlated with the concentration of mucin glycoprotein (Lukie, 1977).

There is some circumstantial evidence that mucus may protect against ulceration, hence drugs which inhibit gastric mucus production such as aspirin and corticosteroids aggravate ulceration (Kent & Allen, 1967; Menguy & Masters,

1963; Menguy & Masters, 1965). In contrast, the anti-ulcer agents such as prostaglandins (Bolton, Palmer & Cohen, 1978; Domschke, Domschke, Hornig & Demling, 1978) are reported to increase mucus production.

It is often assumed that the mucus layer protects the gastric mucosa from damage by intraluminal acid. However the mucus gel does not provide an impenetrable barrier to acid since it is permeable to H^+ ions. It has been proposed that mucus protects the mucosa by delaying the diffusion of H^+ from the lumen (Heatley, 1959; Williams & Turnberg, 1980) providing a medium for neutralization of acid by bicarbonate (Williams & Turnberg, 1979).

The mucus gel may protect the underlying epithelium from proteolytic damage by digestive enzymes, however many mucus gel secretions are degraded by proteolysis. For example, pepsin gradually erodes the luminal surface of the gastric mucus gel (Scawen & Allen, 1977). There is likely to be a dynamic equilibrium between erosion of the gel and secretion of mucus, the thick mucus gel restricting the diffusion of digestive enzymes through the gel matrix.

Mucus and bacteria

In 1955, Florey suggested that mucus in the gut could entrap bacteria and parasites so as to facilitate their removal from the intestine by peristalsis (Florey, 1955). This idea gained further support from the work of

Dixon (1960) who concluded that mechanical removal by peristaltic action aided by the secretion of mucus was probably the main fate of viable bacteria which entered the small intestine.

For colonization of mucosal surfaces by bacteria, high affinity interactions of ligands on the organism with receptors on epithelial cells are essential. Such receptors are usually glycolipids or glycoproteins (Jones, 1977) with the properties of blood group determinants and are, therefore, similar to mucins (Watkins, 1966).

Protection against bacteria

Mucus may physically shield the receptors on the epithelial surface and prevent bacterial binding, it may also act as a barrier through which organisms must traverse to reach mucosal receptors. For example, whilst penetrating the mucus gel occasional Vibrio cholerae may become entrapped within the mucus and not reach the epithelial surface or penetrate down into the crypt region of the intestine (Jones, Abrams & Freter, 1976). Such trapping may facilitate clearance of the mucosal surface as the mucus is propelled along the gut.

Mucin inhibits the attachment of oral Streptococci to human buccal epithelial cells and may cause desorption of previously adherent organisms (Williams & Gibbons, 1975). In this instance mucin may be antigenically similar to the

epithelial cell receptor and actively compete for the bacterial ligands. Other evidence of competition for epithelial cell receptors is given by Strombeck & Harrold (1974) who found gastric mucin competed with cholera toxin for binding to small intestinal receptor groups.

Salivary mucin aggregates some pathogenic Streptococci (Kashket & Donaldson, 1972) such aggregates could be swept away by saliva and promote bacterial clearance. It has also been suggested that the epithelial cell receptor is shed and entrapped in the mucus gel which would aid in the retention of bacteria in the mucus gel (Fox, 1979).

Blood group-reactive mucins from human saliva bind and agglutinate Streptococcus mutans and other oral organisms (Gibbons & Qureshi, 1978; Levine, Hertzberg, Levine, Ellison, Stinson, Li & Van Dyke, 1978) and whereas neuraminidase treatment of mucin does not alter binding of S.mutans, binding of S.sanguis is inhibited, showing sialic acid to be an important component for the binding of this organism (Levine et al., 1978).

Component sugars of mucins may also affect binding, for example, adsorption of S.mutans is inhibited by galactose (Gibbons & Qureshi, 1979) and the adhesion of V.cholerae to rabbit brush border membranes is inhibited by fucose (Jones et al., 1976). Since salivary mucin comprises part of the acquired pellicle of teeth, bacteria-mucin interactions may enhance or inhibit bacterial clearance (Levine et al., 1978). If binding is by free

salivary components, agglutinated bacteria may be rapidly cleared by swallowing, whereas if mucin is adherent to teeth, binding and agglutination could increase bacterial colonization.

Far from being protective, mucus may actually enhance the virulence of bacteria. Thus various Gram positive and negative bacteria, if suspended in mucin, initiate systemic infections more readily than if suspended in buffer (Anderson & Oag, 1939). However, iron compounds can replace mucin in the establishment of meningococcal infections (Calvert, Kenny & Lavergne, 1979) and the mucin enhancing effect may be caused by iron atoms bound to the mucin rather than the glycoprotein content (Ford & Hayhoe, 1976)

Caecal mucin inhibits the normal bacteriocidal effects of serum on Escherichia coli (Winses, Midtvedt & Trippestad, 1976) but this may reflect the presence of serum inhibitors in the mucin preparation. As mucin binds avidly to bacteria such as S. mutans (Gibbons & Qureshi, 1978) such binding may bind blood group activity to the bacteria and help to avoid antigenic recognition.

Bacteria, mucus and antibody

Although all classes of immunoglobulin are represented in intestinal secretions, the predominant secretory

immunoglobulin is IgA (Bienenstock & Befus, 1980). Secretory antibodies are retained within the mucus layer of the gut by interactions with cystine residues present in mucins (Walker & Isselbacher, 1977). This stationary location of antibodies allows for a more effective interaction with intestinal antigens coming in contact with the mucosal barrier.

There is decreased adherence of bacteria to epithelial cells after exposure of the bacteria to specific secretory IgA, suggesting that secretory IgA (sIgA) blocks specific binding sites on the bacterial cell wall (Williams & Gibbons, 1972).

Magnusson & Stjernstrom (1982) examined the effects of sIgA on the association of Salmonella typhimurium with the intestine of the rat and with a column of hog gastric mucin. It was found that sensitization with sIgA increased the affinity of the bacteria both for the mucus layer of the intestine and the mucin column, suggesting that sIgA had made the bacteria considerably more mucophilic and thus indicating a link between antibody and mucin.

Mucus and intestinal parasites

The association between increased resistance to nematode parasites and mucus was first reported over 40 years ago by Ackert, Edgar & Frick (1939). It was suggested that the greater amount of mucus present in

older chickens was responsible for heightened resistance to infection with Ascaridia galli (Ackert et al., 1939). Furthermore, duodenal mucus from chickens inhibits the growth of A.galli in vitro (Frick & Ackert, 1941).

Infection with A.galli provokes an increase in the number of goblet cells in the intestinal epithelium (Frick & Ackert, 1948). Increased numbers of goblet cells in the gut mucosa have been reported in sheep infected with Trichostrongylus vitrinus (Jackson, Angus & Coop, 1983) or Oesophagostomum columbianum (Dobson, 1967) and in all instances these observations were found to be associated with increased resistance to infection.

Goblet cell hyperplasia has been shown to occur during infection of laboratory animals with parasitic helminths and is temporally related to the expulsion of N.brasiliensis (Wells, 1963; Miller & Nawa, 1979a,b; Uber, Roth & Levy, 1981) and T.spiralis (Alizadeh & Wakelin, 1982) from rats and mice.

The mechanisms which underlie the increase in goblet cells have not been fully established, but factors which influence goblet cells also control MMC (Miller, 1979). Thus, adoptive immunization with the T-cell enriched fraction from TDL effectively transfers both goblet cell and MMC hyperplasia (Miller et al., 1979), as does passive immunization with hyperimmune serum (Miller & Nawa, 1979a). Treatment of passively immunized rats with corticosteroids which reduces the number of MMC (Jarrett et al., 1967)

or reserpine which depletes mast cell amines (Askenase, 1980) blocks both goblet cell hyperplasia and worm expulsion (Miller & Nawa, 1979a). These findings further suggest that MMC, goblet cells, and worm expulsion could be interlinked.

An increase in the number of goblet cells could signify that the synthesis and turnover of mucus is increased at the time of worm expulsion. Miller, Huntley & Dawson (1981a) have demonstrated that the incorporation of ^{14}C -glucosamine into mucin was maximal about the time of worm rejection. Although there is an increase in the proportion of goblet cells and in the turnover of mucus during worm expulsion in N.brasiliensis infection the exact role, if any, of mucus and goblet cells remains to be elucidated.

Considerably more information has been gained on the protective anti-parasitic role of mucus from studies of the rapid expulsion of T.spiralis and N.brasiliensis from primed rats. During the rapid expulsion of T.spiralis, larvae fail to penetrate the epithelium and many are to be found trapped in mucus (Lee & Ogilvie, 1981); the number of larvae penetrating into the epithelium after intraduodenal challenge of immune rats is approximately one half of that in control rats 90 minutes after challenge (Lee & Ogilvie, 1981). After oral challenge many more worms were found in mucus globules in immune rats than there were in control animals. The number

being lower than after intraduodenal challenge, most probably due to the mucus globules being continually propelled along the intestine (Lee & Ogilvie, 1982).

Lee & Ogilvie (1981) were able to develop an in vitro model of mucus trapping which enabled analysis of the factors which may contribute to trapping. Firstly, as normal intestinal mucus cannot be obtained in practical amounts mustard oil was used to stimulate the intestine (Lee & Ogilvie, 1981). It is unclear whether this mucus is typical of mucus in normal animals consequently experiments dealing with this product may be based on an artifact.

Infective larvae were incubated at 37°C with immune or control serum or bile and then were agitated with mucus for 2 hours. After incubation, the mucus was layered onto sucrose and incubated in which time untrapped larvae separate from the mucus layer.

Control or immune mucus trapped about 40% of the larvae which had been incubated with control serum, this figure dropped to about 5% after heat inactivation (56°C) of the serum, indicating that complement was involved (Lee & Ogilvie, 1982).

Inactivated immune serum trapped 38.5% of larvae with immune mucus, and the number trapped with control mucus was similar. Bile, whether from control or immune donors did not cause trapping (Lee & Ogilvie, 1981).

The in vitro studies suggest that there are two

systems interacting with mucus to promote trapping a) the complement system and b) antibody which can be serum derived and does not appear to be IgA (Lee & Ogilvie, 1981, 1982).

A trapping function has also been suggested during the rapid expulsion (RE) of N.brasiliensis (Miller, Huntley & Wallace, 1981b). Perfusion of the small intestines of naive rats that had been challenged intraduodenally with pre-adult worms showed that 70-80% of the challenge dose was adherent to the gut mucosa, whereas in immune rats only 28-31% of the worms were attached to the mucosa and 3-23% were enveloped in mucus (Miller et al., 1981b). Exclusion of the parasites from the mucosa occurred within 30 minutes of challenge and significant mucus trapping was noted 2 hours after challenge (Miller et al., 1981b). It must be questioned whether mucus trapping is an essential component of RE, since in the case of N.brasiliensis it occurred at a stage when the parasites had already moved into the distal half of the intestine, and it is possible that after failing to establish they are enveloped in mucus (Miller et al., 1981b).

Experiments by Bell, Adams & Ogden (1984) showed that the RE of T.spiralis may occur in the absence of mucus trapping. Under normal conditions, in rats primed by full infection with T.spiralis and challenged orally, 69% of the worms in the gut lumen were trapped in mucus.

However, if rats were immunized by a T.spiralis infection truncated by chemotherapy which prevented deposition of newborn larvae in the tissues, RE occurred but in the absence of significant mucus trapping. Therefore, to facilitate mucus trapping systemic exposure to target antigens is necessary which may happen during the migration of N.brasiliensis parenterally and, for T.spiralis, during the deposition of muscle larvae (Bell et al., 1984). Hessel, Ramaswamy & Castro (1982) found that 75% of T.spiralis rejected by RE were infective for naive recipients, which would indicate that few of them could have really been trapped in mucus. Thus, whilst mucus trapping has been shown to occur the most important functional role of mucus may be in preventing the establishment of the parasite (Miller et al., 1981b). Support for a 'barrier' role has come from experiments in which rats were treated with a mucolytic or mustard oil to disperse or deplete mucus respectively (Miller & Huntley, 1982a). In both instances worm expulsion was blocked, indicating that mucus is important in N.brasiliensis infection. Furthermore, mucus trapping and RE are blocked by pretreatment of rats with corticosteroids (Miller & Huntley, 1982b). A similar steroid abrogation of RE has been demonstrated in T.spiralis infection (Bell, McGregor & Adams, 1982).

The role of mucus in Entamoeba histolytica infection, a parasitic protozoan which is able to breach the

mucosal barrier of the large intestine and cause erosive lesions (Brandt & Tamago, 1970) has been recently studied. Within one hour of inoculation of trophozoites into rat colonic loops considerable numbers of trophozoites were adsorbed by mucus and, by four hours, they appeared within the lumen with aggregated sloughed mucus blanket fragments; as adsorption to mucus occurred, motility was reduced (Leitch, Dickey, Udezulu & Bailey, 1985). It was concluded that mucus provides a significant barrier to trophozoite access to the intestinal epithelial target tissue (Leitch et al., 1985).

Extracts of intestinal mucus from nematode resistant sheep have been shown to be potent inhibitors of in vitro larval migration (Douch, Harrison, Buchanan & Greer, 1983); extracts from normal or susceptible sheep having no such effect. The inhibitory extracts had properties and activities similar to SRS-A (increased skin permeability and inactivation by arylsulphatase). Although some activity could have arisen from the tissues, as the mucus was collected by scraping the mucosa, the results demonstrate that products of immediate hypersensitivity may paralyse nematode larvae either directly, or due to an effect on the quantity or quality of mucus.

Stimulation of mucus release

A number of different substances have been shown to induce the release of mucus from goblet cells (Specian & Neutra, 1982; Neutra, O'Malley & Specian, 1982). These include irritants such as mustard oil and alcohol, neurotransmitters, hormones and bacterial toxins (Forstner, 1978). Furthermore, immune complexes and intestinal anaphylaxis induce release of mucus from rat goblet cells (Walker, Wu & Bloch, 1977; Lake, Bloch, Sinclair & Walker, 1980; Miller et al., 1983).

There is abundant information concerning the influence of mast cell mediators on mucus secretion in the lung (Kaliner, Marom, Patow & Shelhamer, 1984), and although the profile of mediators and their interactions may not be the same in the intestine, it is relevant to note the mediators shown to have effects in lung.

Cultured human bronchi have been widely used to test the effects of mediators on mucus release and in this respect anaphylaxis and histamine (Shelhamer, Marom & Kaliner, 1980), prostaglandins but not of the E_2 series (Marom, Shelhamer & Kaliner, 1981), monoeicosatetraenoic acids (Shelhamer et al., 1980; Marom, Steel, Shelhamer, Goetzl & Kaliner, 1982a) and the leukotrienes C_4 and D_4 (Marom, Shelhamer, Bach, Morton, & Kaliner, 1982b) induce mucus secretion. More recently mucus release by prostaglandin generating factor of anaphylaxis (PGF-A) has been demonstrated, most probably by increasing the

generation of lipoxygenase products (Marom, Shelhamer, Steel, Goetzl & Kaliner, 1984).

The effects of LTC_4 and LTD_4 on mucus release have also been studied on canine trachea in vitro. Using this system LTD_4 was 1000 times more potent than LTC_4 in causing mucus release (Johnson & McNee, 1983). Blocking with diphenylhydramine (H type 1) revealed an as yet undefined role for histamine in the LTC_4 enhanced release of mucus (Johnson, Chinn, Morton, McNee, Miller & Nadel, 1983).

Any or all of these mechanisms, as well as non-immunological stimuli (Forstner, 1978), could be involved in the hypersecretion of mucus during RE, but the prominence of MMC and evidence that histamine (Kowalowski, Pachkowski & Secord, 1976) and serotonin (Forstner, 1978) potentiate mucus release suggests that IgE-mediated intestinal hypersensitivity reactions play a major role. It is also possible that mast cell-derived mediators induce the release of mucus during N.brasiliensis primary infection where MMC migrate into the epithelium to lie in close proximity to goblet cells.

Scope of this thesis

The introduction documents a close interrelationship between mucus, mucosal mast cells and immediate hypersensitivity and the role of these three components in the expulsion of N.brasiliensis will be examined. The questions asked in this thesis are (i) does mucus trap parasites during primary infection and could this be an important mechanism to bring about the rejection of N.brasiliensis, and (ii) are MMC involved in expulsion and if so what is their role in relation to expulsion and mucosal permeability.

CHAPTER 2 Materials and methods.

Experimental Animals

Male and female Wistar rats, 10-40 weeks old, were used for all experiments and for the routine maintenance of Nippostrongylus brasiliensis. For all experimental procedures rats were anaesthetized with ether. Animals were killed by exsanguination from the carotid artery and, for the preparation of worm antigen, or the assay of passive cutaneous anaphylaxis, by cervical dislocation. Both procedures were performed on ether anaesthetized rats.

All the rats were bred within the animal breeding department of the Moredun Research Institute and were allowed free access to food and water. For experiments involving collection of tissues or estimation of worm burdens rats were fasted for 24 hours.

For the collection of faeces and the monitoring of daily faecal egg production, rats were kept in cages with wire mesh floors suspended over trays containing tap water.

Buffers

Carbonate-bicarbonate pH 9.6

1.59g of Na_2CO_3 and 2.93g of NaHCO_3 were dissolved in one litre of distilled water. This buffer was stored at 4°C for not more than two weeks.

Phosphate-citrate pH 5.0

Two stock solutions were prepared consisting of 0.1M citric acid and 0.2M disodium hydrogen orthophosphate. 24.3ml of citrate and 25.7ml of phosphate were combined and the volume made up to 100ml with distilled water.

Phosphate-buffered-saline (PBS) pH 7.4

The following chemicals were dissolved in 5 litres of distilled water; 400g NaCl, 10g KCl, 57.5g Na_2HPO_4 and 10g KH_2PO_4 . This is a ten times concentrate of PBS and an appropriate dilution was made before use.

Tris-maleate pH 7.5

24.2g of Tris (hydroxymethyl) aminomethane and 23.2g of maleic acid were dissolved in one litre of distilled water. 25ml of this solution and 28ml of 0.2M NaOH were made up to 100ml with distilled water.

Hank's balanced salt solution

Sterilized Hank's balanced salt solution was obtained from the Microbiology Department, Moredun Research Institute. One gram of bovine serum albumin (Sigma Chemical Co. Ltd.) was stirred into 100ml of this solution before the pH was adjusted to 7.4 with HEPES buffer (Flow Laboratories)

The pH of each buffer was checked using a pH meter before use.

Fixatives

Carnoy's fluid

100ml of this fixative was prepared with 30ml of chloroform, 60ml of absolute ethanol and 10ml of glacial acetic acid.

4% paraformaldehyde

Four grams of paraformaldehyde were dissolved in 100 ml of PBS by heating and stirring on a hot plate. The fixative was cooled to room temperature before use.

10% aqueous acrolein

The above was prepared by adding 90ml of distilled water to 10ml of acrolein solution (Sigma). This was performed in a well ventilated fume cupboard.

Parasitological Techniques

The parasite

The strain of N.brasiliensis used in these experiments was originally established and maintained in the Department of Experimental Parasitology, University of Glasgow, and is now maintained in our laboratory by passage in Wistar rats.

Culture and routine maintenance of N.brasiliensis

The techniques used were essentially as described by Nawa & Miller (1978). Seven days after infection faeces were collected into water, washed and mixed to form a thick paste. Previously washed granular charcoal (10-18 mesh, BDH Chemical Co.) was added to the faeces, mixed, and the preparation incubated on wet filter paper (Whatman no. 1) in covered Petri dishes in humidified plastic sandwich boxes in an incubator at 28°C.

Seven to 14 days after incubation the infective third stage larvae (L_3) were collected into water at 37°C and then transferred to a steel mesh covered with rice paper (K-dex-8/9, Kleenaroll Ltd, London) through which they migrated. The larvae were collected by sedimentation in a filter funnel and then washed several times with sterile saline. 50µl samples of thoroughly mixed larval suspensions were counted under a dissecting microscope. The concentration of the larval suspension was adjusted to the required dose and 0.5ml inoculated subcutaneously into the flank of anaesthetized rats.

Faecal egg counts

Faecal egg counts were monitored by using a modification of the McMaster slide counting technique as described by Whitlock (1948).

Method for counting worm burdens

The relationship of the parasite to the intestinal mucosa and the extent of mucus trapping was as described by Miller et al (1981b). Anaesthetized rats were bled out from the carotid artery and the entire small intestine removed. This was divided into two equal lengths and each portion flushed with 50ml of saline followed by 10ml of air, lightly adherent mucus and intestinal contents were dislodged by gently drawing the intestine between thumb and forefinger.

The gut washings were emptied into a surgical gauze suspended over a 250ml beaker, and the gauze incubated in saline at 37°C for 1 hour. The number of worms that had migrated or passed straight through the gauze were counted and designated FREE in the lumen.

The gauze contents were examined under a stereo microscope for worms TRAPPED in mucus, using a dissecting needle to distinguish worms trapped from those lying on the mucus gel. In addition, the worms that had passed straight through the gauze were examined for the presence of adherent mucus.

To count the number of worms ADHERENT to the gut mucosa, the intestines were divided into 10 equal lengths and the parasites counted in situ in longitudinally opened gut segments. Both gauze and intestines were examined immediately or stored at -20°C for analysis at a later date.

Preparation of whole worm antigen

Whole worm antigen was prepared as described by Nawa et al (1981). Adult worms were harvested from donor rats 6-7 days after primary infection, by incubating the jejunum, slit longitudinally and enveloped in surgical gauze, in saline for 1 hour at 37°C. The worms were collected and washed several times in sterile saline by decantation and sedimentation and their concentration adjusted to 4000/ml. The worm suspension was homogenized in a teflon/glass homogenizer cooled in ice and the homogenate clarified by centrifugation at 2500 revs/min for 15 minutes (4°C). The supernatant was removed and kept on ice, the pellet resuspended in the original volume of saline was homogenized and centrifuged for a second time. The supernatants were combined and the pooled antigen preparation stored at -20°C as 2000 worm equivalents (we) of antigen per ml.

Histology and cell counts

(a) Fixation

Carnoy's fixation

Segments of jejunum and ileum 2 cm in length (specimens adjacent to those used for RMCPII assay), mesenteric lymph node, thymus, left lobe of lung, trachea and tongue were placed in Carnoy's fluid for 24 hours. Tissues were then processed through a graded ethanol series, cleared in toluene, and embedded in paraffin wax.

Sections were cut at 5 μm , deparaffinized in xylene, rehydrated and stained.

Paraformaldehyde fixation

Portions of jejunum, ileum, stomach, colon and ear pinnae were fixed for 6 hours in 4% paraformaldehyde and then transferred to 70% ethanol and stored overnight at 4°C (Newlands et al., 1984). The tissue samples were dehydrated, processed into wax and sections cut at 5 μm .

(b) Staining

Glycosaminoglycan (GAG)

Carnoy's-fixed sections were stained with 0.1% Alcian blue 8GX in 0.7N HCl pH 0.3 for 30 minutes and were counterstained with Safranin O in 0.125N HCl for 5 minutes (Enerback, 1966a). Sections fixed in paraformaldehyde were stained with Toluidine blue (0.5% in 0.5N HCl, pH 0.5) for 15 minutes (Enerback, 1966a).

Serine esterase

Esterase activity was demonstrated by incubating paraformaldehyde-fixed sections in a synthetic esterase substrate : naphthol AS-D chloroacetate and Fast Garnet GBC (Gomori, 1953; Huntley et al., 1984).

Fifteen milligrams of naphthol AS-D chloroacetate (Sigma) were dissolved in 2ml of dimethylformamide (BDH) to which was added 5ml of ethylene glycol monomethyl

ether (BDH), 10ml of Tris-maleate buffer (pH 7.5) and 8ml of distilled water. This solution was added to 15mg of Fast Garnet GBC salt (Sigma), immediately mixed, and filtered through a 0.22 μ m millipore membrane onto the slides. Staining was for 5 minutes, followed by washing with distilled water. Coverslips were mounted with polyvinyl pyrrolidone (PVP).

Immunoperoxidase staining of RMCPII

Paraformaldehyde fixed sections were first pretreated with periodic acid and sodium borohydride to block endogenous peroxidase activity (Heyderman & Neville, 1977). The sections were rinsed in three 5 minute changes of 100mM Tris/HCl (pH 7.4) and incubated overnight in a humid chamber with a 1/50 dilution of rabbit anti-RMCPII in Tris/HCl containing 10% ovalbumin (Sigma). After a further three washes in Tris/HCl, sections were incubated for 1.5 hours with sheep F(ab')₂ anti-rabbit Fab peroxidase conjugate (25 μ g/ml) (Miller et al., 1981a) diluted with Tris/HCl-ovalbumin. After a further three washes in Tris/HCl, peroxidase activity was revealed with 3-amino-9-ethyl-carbazole (Graham, Lundholm & Karnovsky, 1965). For control purposes a 1/50 dilution of normal rabbit serum replaced anti-RMCPII.

(c) Cell counting techniques

Mucosal mast cells in jejunum and ileum were counted per villus crypt unit (VCU) as defined by Miller & Jarrett (1971), a minimum of 15 VCU was counted for each section.

Mast cells in tongue, MLN, lung, and thymus were not enumerated but cell density was a subjective assessment with(+++) substantial increase, (++) an increase in the density of mast cells when compared with controls in which mast cells were either present (+) or completely absent (-). Mast cells in trachea were counted per 1mm length of tracheal mucosa.

Sections of ear pinnae were examined microscopically and the total number of mast cells enumerated. The areas of tissue in which mast cells were counted, excluding cartilage, was measured by projecting the sections onto equiweight paper using a photographic enlarger and then calculating the area by weight analysis of the tissue outline.

To compare MMC counts in jejunum, ileum, colon and stomach the numbers of MMC/unit area was calculated. This takes into account the smaller mucosal volume of the ileum compared to jejunum and relates MMC numbers to mucosal volume (Pitts & Mayrhofer, 1983). A calibrated grid was aligned along the muscularis mucosae and the total number of MMC counted in the grid area. The area of mucosa was calculated with reference to the calibrated

grid. Mast cell numbers are expressed as MMC/mm² mucosa.

All cell counts were performed using a Leitz Dialux 20EB microscope.

Measurement of IgE antibody

The levels of circulating IgE against N.brasiliensis were determined by passive cutaneous anaphylaxis (PCA) (Ovary, 1964). The preparation of worm antigen has already been described.

Doubling dilutions of test sera were made, and 100 μ l of each were injected intradermally into the shaved backs of recipient rats maintained under ether anaesthesia. Seventy-two hours later rats were injected intravenously with 1000 we of antigen plus 2.5 mg Evan's blue/100g body weight. Reciprocals of the highest dilution of serum giving a blue spot greater than 5mm in diameter were determined by examination of the underside of the skin 30 minutes after antigen challenge.

To determine the effects of corticosteroids on PCA, recipient rats were injected intradermally with immune serum (doubling dilutions) and immediately injected with 25 mg/kg methylprednisolone (Upjohn Ltd) or saline intramuscularly. Steroid or saline treatment was repeated 24 hours later and PCA titres determined after a further 24 hours.

Intestinal permeability

To measure the permeability of the intestinal mucosa Evan's blue dye was used as an indicator (Nawa, 1979). Experimental animals were injected intravenously with Evan's blue and either worm antigen or saline in a dose of 1ml. The dose was calculated so that, in addition to 2.5 mg Evan's blue/100g body weight, rats would receive 50-800 we of antigen.

At the end of each experimental time period, rats were bled out under ether anaesthesia, their entire small intestines removed, and divided into two equal lengths. Each half was perfused with 10ml of saline plus 10ml of air from a 20ml syringe. Intestinal perfusates were centrifuged for 10 minutes at 10000 rev/min in a Beckman J2-21 centrifuge at 4°C. The clear supernatants (SOL) were removed and the pellets (GEL) resuspended in 5ml of saline and homogenized in a teflon/glass homogenizer. The concentrations of Evan's blue were measured spectrophotometrically in both sol and gel after acetone extraction (Nawa, 1979).

Briefly, 1ml of acetone was added to an equal volume of sol or gel, mixed well and centrifuged at 4000 rev/min for 10 minutes. The optical density of the clear supernatants was measured in a Pye-Unicam spectrophotometer at 620nm. The amount of Evan's blue in each sample was then calculated from an Evan's blue standard curve prepared from solutions of Evan's blue (0-10 µg/ml)

assayed as described above.

To determine whether the dye was transuding freely or bound to protein, both sol and gel phases were treated with 10% trichloroacetic acid (TCA). More than 90% of the dye was precipitable with TCA indicating that the dye was predominantly bound to protein. (Table 1).

Table 1 The precipitation of protein-bound Evan's blue : its recovery from intestinal perfusates.

	SOL		GEL	
	A	B	A	B
Immune Control	0.2	0(0)	2.9	0(0)
Immune ^a Challenge	2.9	0(0)	2.5	0.1(4)
Immune ^b Challenge	8.0	0.5(6.3)	18	0.2(1)

a 5 minutes after challenge b 60 minutes after challenge

A concentration of Evan's blue in sample ($\mu\text{g/ml}$)

B concentration after TCA precipitation ($\mu\text{g/ml}$)

values in parentheses refer to the % retained after TCA precipitation

Note values for both sol and gel were corrected for background interference from normal gut contents before and after TCA precipitation.

Scanning electron microscopy (SEM)

Six rats were infected with 5000 N. brasiliensis L₃ and three animals were killed on day 6 and the remaining three on day 10 of infection. The animals were fasted overnight before sacrifice.

Small intestines from 2 rats at each time point were removed and divided into two equal lengths. Each half was perfused with 50ml of saline. The perfusates were passed over surgical gauze suspended over a 250ml beaker and the gauzes containing mucus and possible trapped worms were opened flat and 2 cm² areas trimmed out from the central portion of the gauze. These samples were pinned flat to cork board. The intestines from the remaining rats were opened longitudinally and small segments, 25 cm distal to the pylorus and 1.5 cm in length were pinned flat to cork board and debris gently brushed from their surface.

Specimens were suspended over 10% acrolein in screw topped jars and fixed for 1 hour at room temperature in a well-ventilated fume cupboard (Garland et al., 1982). After fixation the specimens, still attached to cork, were transferred to a solution containing 3% paraformaldehyde-3% glutaraldehyde in PBS for 2 hours, trimmed and then left overnight in fresh paraformaldehyde-glutaraldehyde.

Specimens were then processed according to the revised - tannin method of Murakami (1974). Tissues

were first transferred to a solution containing 2% arginine hydrochloride, 2% glycine, 2% sodium glutamate and 2% sucrose in distilled water and left overnight at room temperature. After washing in three 15 minute changes of distilled water tissues were placed in 2% tannic acid in distilled water (pH 4.0) and left for 24 hours at room temperature. After a further three washes the samples were immersed in 2% aqueous osmium tetroxide for 6 hours and then washed three times with distilled water. The samples were then dehydrated through an ascending acetone series (30%, 50% and 80% 30 minutes at each stage) followed by three 30 minute periods in 100% acetone, after which they were left overnight in 100% acetone. Samples were then critical point dried from CO₂ and sputter coated with 1.5nm of a gold/palladium mixture. Specimens were examined in a JEOL electron microscope.

Rat mast cell protease II (RMCPII)

Source

RMCPII was generously supplied by Dr R.G Woodbury (University of Washington, Seattle) and was prepared from the intestines of Sprague Dawley rats by affinity-adsorption chromatography on potato chymotrypsin inhibitor immobilized on Sepharose 4B (Woodbury & Neurath, 1978). Antisera to RMCPII had previously been prepared in rabbits within the Moredun Institute. Anti-RMCPII antibody had a titre in Ouchterlony gels against antigen of 32. RMCPII antigen was a 1mg/ml solution in PBS and was stored at -20°C until use.

RMCPII was also provided by Dr S Gibson (Moredun Institute) prepared from the intestines of N.brasiliensis infected rats by ion exchange chromatography using a Pharmacia Fast Protein Liquid Chromatograph with Mono-S cation exchange column (Gibson & Miller, 1986). This preparation was, by SDS-PAGE, found to contain a single polypeptide (Figure 1) indistinguishable both in terms of molecular weight and reactivity with anti-RMCPII antibody from that provided by Dr Woodbury.

Specificity of antiserum by gel diffusion

Possible cross-reaction of anti-RMCPII antibody with RMCPI was checked both in ELISA (see later) and by gel diffusion. One gram of agarose (BDH) was dissolved in 100ml of PBS and 3ml was poured onto glass microscope



Figure 1. Discontinuous SDS-PAGE gel stained with Coomassie blue showing in lane I molecular weight standards (12300-77000 da) and in II and III RMCPII (5 μ g) and RMCPI (3 μ g) respectively.

Standards were cytochrome c(12.3k), myoglobin(17.2k), chymotrypsinogen A(25.7k), ovalbumin(45k), albumin(66.25k) and ovotransferrin(77k).

(Photograph kindly supplied by Dr S Gibson)

slides. Six wells were punched in the agarose concentrically around a central well using a gel puncher and template (LKB). Ten microlitres of antiserum were placed in the central well and extracts of peritoneal mast cells ($1-6 \times 10^6$ cells) prepared in 0.15M KCl and 1.5M KCl (see preparation of tissues) placed in the surrounding wells. Both purified RMCPII and jejunal homogenate were included as positive controls.

Precipitin lines were allowed to develop overnight at room temperature. The gel was washed extensively in PBS, pressed and dried. The gel was then stained with Coomassie blue as described for radialimmunodiffusion.

No precipitin lines were observed against extracts of peritoneal mast cells even when prepared in 1.5M KCl. By contrast, strong precipitin lines were observed against both jejunal homogenates and standard RMCPII preparations. (Figure 2).

Collection of samples for RMCPII assay

Serum

Rats were anaesthetized with ether and bled out from the carotid artery. Blood was allowed to clot before sera was separated by centrifugation. Sera was stored at -20°C until assay.

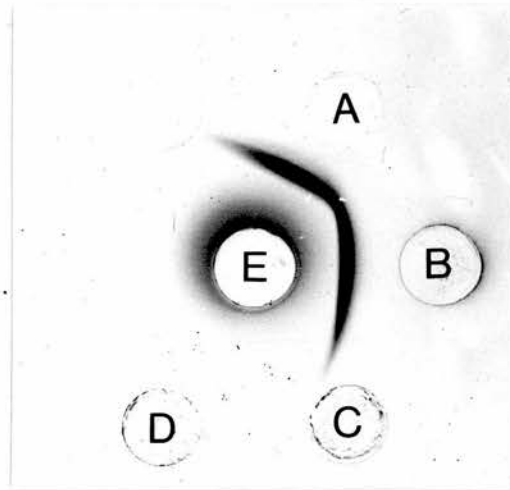


Figure 2. Immunodiffusion demonstrating the specificity of the anti-RMCPII antibody. Samples were 20 μ l of :

A purified RMCPII (104 μ g RMCPII/ml)
B jejunal homogenate (100 μ g RMCPII/ml)
C peritoneal mast cell extract in 0.15M KCl
D peritoneal mast cell extract in 1.5M KCl
E anti-RMCPII antiserum.

Intestinal perfusates

Perfusates were collected as described earlier. Samples (1ml) of sol and gel were removed and stored at -20°C .

Tissue samples

Two centimetre lengths of the following tissues were collected: jejunum (25cm distal to the pylorus), ileum (10cm proximal to the ileocaecal valve) and colon. Stomach, mesenteric lymph node (MLN) and trachea were removed and divided into two portions (one for histology). Right lobe of lung, spleen, liver, kidney, femoral muscle and skin were also collected. Samples were rinsed in saline and all, except skin and muscle, were weighed and homogenized in 3 volumes of 0.15M KCl in a teflon/glass homogenizer. The trachea was prepared in 6 volumes of KCl. Muscle and skin samples were weighed, diced with scissors and homogenized in 3 volumes of 1.5M KCl.

Samples of bone marrow were prepared as follows: bone marrow was obtained by perfusing the femur with saline (1ml) and the perfusate was rapidly freeze-thawed in a mixture of dry ice/isopentane followed by warming in a water bath to 37°C . Cell debris was removed by centrifugation and the supernatant stored.

To recover peritoneal cells, exsanguinated rats were injected intraperitoneally with 40ml of ice-cold saline, the peritoneal washings were aspirated and the

cells recovered by centrifugation. They were washed several times with Hank's balanced salt solution containing 1% BSA and mast cells were counted from an aliquot of cells that had been diluted 1 part to 9 parts of Azure A (1%) in 50% propylene glycol. The remaining cells were pelleted by centrifugation and resuspended in approximately 3 volumes of 0.15M KCl or 1.5M KCl and freeze-thawed rapidly 5-6 times before centrifugation to remove cell debris. The concentrations of RMCPII were analysed in the supernatant fluids.

Measurement of RMCPII

(a) Radial Immunodiffusion

This assay was suitable for the determination of RMCPII at concentrations greater than 5 μ g/ml. The concentrations of RMCPII in sera, intestinal perfusates and tissue homogenates was measured using minor modifications of the assay described by Woodbury & Neurath (1978). An assay gel was prepared from 15ml of 1% agarose in PBS containing a 1/100 dilution of rabbit anti-RMCPII antibody. Samples and standards (5-200 μ g) were applied to the gel and precipitin lines allowed to develop for 48 hours at 4 $^{\circ}$ C (24 hours at room temperature) The gel was then pressed, dried and washed with three 15 minute changes of PBS and stained with 0.5% Coomassie Brilliant Blue R250 in ethanol:acetic acid : water (90 : 20 : 90). The gel was then rinsed with tap water

and destained with ethanol : acetic acid : water to visualize precipitin rings. The diameters of the rings were then measured and the concentrations of RMCPII determined from a standard curve. For skin, peritoneal mast cells and muscle, assay gels were set up both in standard buffer and in 1.5M KCl with RMCPII standards for each buffer system.

(b) Enzyme-linked immunosorbant assay (ELISA)

Principle

1. Antigen is attached to the solid phase microtitre wells which are then washed.

2. Samples are then mixed with antibody and the mixture added to, and incubated in the coated wells which are again washed.

3. Enzyme-labelled anti-globulin (reactive with antibody used in step 2) is then added, incubated and the solid phase washed.

4. Enzyme substrate is then added. This is a competitive inhibition assay with high antigen concentrations resulting in less colour development at the end of the test.

Procedure

The assay is essentially similar to that described by Miller et al (1983) with minor modifications.

1. Plastic, 96 well microtitre plates (Dynatech Laboratories)

were incubated overnight at 4°C with (150µl/well) RMCPII at 1µg/ml in carbonate-bicarbonate buffer, pH 9.6. The protease solution was removed and the plates washed 5 times with PBS containing 0.5% ovalbumin and 0.05% Tween 20 (PBS-OVA-T20).

2. In a separate microtitre plate test sample dilutions were prepared in PBS-OVA-T20 containing 4% normal rat serum. The dilution ranges and layout of the samples and standards is shown in Table 2. Samples and standards were incubated with 180µl of a 1/3000 dilution of rabbit anti-RMCPII prepared in PBS-OVA-T20 for 1 hour at 37°C. Duplicate samples (150µl) were then transferred to the RMCPII coated plate and incubated in the wells for 2 hours at 37°C. The samples were then removed and the plates washed 5 times with PBS-OVA-T20.

3. The wells were then incubated with 150µl of sheep F(ab')₂ anti rabbit Fab peroxidase conjugate (Miller et al., 1981a) diluted 1/1000 with PBS-OVA-T20 for 1 hour at 37°C. The plates were then washed 5 times and blotted dry.

4. Enzyme substrate was then added. This consists of 150µl of freshly prepared o-phenylene diamine (40mg) dissolved in 100ml of phosphate-citrate buffer plus 40µl of hydrogen peroxide (30% w/v). Colour development was stopped by the addition of 50µl of 2.5M sulphuric acid.

Table 2 Dilution ranges and layout of ELISA plate.

Stage 2 :

	<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>	<u>5</u>		<u>12</u>
A	-	+ve	a	e	e	. . .	e
B	-	+ve	b	f	f	. . .	f
C	-	+ve	c	g	g	. . .	g
D	-	+ve	d	h	h	. . .	h

Column 1 - blank

- 2 - positive control (180 μ l) PBS-OVA-T20 + 4% normal rat serum (NRS)
- 3 - standards a 1/2000, b 1/8000, c 1/32000, d 1/128000 dilution of RMCPII in PBS-OVA-T20-NRS
- 4 - samples e 1/25, f 1/100, g 1/400, h 1/1600 dilution of test sera in PBS-OVA-T20-NRS

Stage 3 :

180 μ l of antisera are added to all wells (except column 1) and after incubation, 2 x 150 μ l samples from row A above are added to rows A and B on the sensitized plate. This is repeated for row B above (to row C and D) and for rows C and D.

NRS is added to control for the possible protease-inhibitor complexes in the samples.

Plates were read in a Titertek Multiscan reader at 492nm. The concentration of RMCPII in the unknown samples was calculated from the 50% end point optical density changes for both unknown and standard RMCPII samples. This calculation was performed by microcomputer using specially designed computer software.

Assay of glycosaminoglycan (GAG)

The competitive binding assay as described by Dawes & Pepper (1982) was used in which test material competes with ^{125}I -labelled heparin for binding to protamine-Sepharose. In the current assay system polybrene-Sepharose replaced protamine-Sepharose and tracer and standard were heparan sulphate rather than heparin. GAG was analysed in gut homogenates and perfusates, prepared as described earlier, and in plasma.

Plasma collection

Glass tubes, marked at the 3ml level, and containing 0.3ml of the platelet release inhibitor solution (ETP) which consists of 78mM EDTA, 10mM theophylline, and 0.33 μg /ml prostaglandin E_1 were used. The tubes were immersed in an ice-water mixture so that all the tube below the 3.0ml mark was cooled. Rats were anaesthetized with ether and the carotid artery sectioned. Blood was immediately aspirated using a 5ml syringe and transferred to the cooled sample tube. Following collection the tube was

capped and pushed further down into the ice mixture. Samples were maintained at 0°C until separation which must be within 3 hours of collection. Samples were centrifuged at 4°C after which the plasma was collected. Aliquots of 0.7ml of plasma were removed from the middle one-third of the plasma layer and dispensed into 1.5ml plastic sample tubes. Samples were stored at -20°C.

Assay procedure

The assay mixture contained 50µl of polybrene-Sepharose suspension, 50µl of ¹²⁵I-heparan sulphate, 50µl of heparan sulphate standard or sample and phosphate buffer pH 7.7 containing Tween 20 (1%) to a final volume of 300µl. The concentration of polybrene-Sepharose which bound 50% of the tracer in the absence of unlabelled heparan sulphate was used, which was normally a 1/25 to 1/50 dilution of the solid phase. Standard heparan sulphate was serially diluted to 2ng/ml.

Assay tubes were shaken for 16 hours at room temperature after which the polybrene-Sepharose was washed twice with assay buffer, the solid phase was then counted in a gamma counter. The concentration of GAG in the sample was determined by reference to the standard curve. The less ¹²⁵I-heparan sulphate bound, the greater the concentration of GAG in the unknown sample.

Statistical methods

1. Student's t-test

To compare the means of two samples a Student's t-test was used. A two tailed alternative was used as no prior knowledge of experimental outcome was known. The assumptions made in the test were that the data were normally distributed and that the true variances of the two populations to be compared were the same.

Student's t with $n_1 + n_2 - 2$ degrees of freedom is given by :-

$$s \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}} = t$$

$$\text{where } s^2 = \frac{E_1(x - \bar{x}_1)^2 + E_2(x - \bar{x}_2)^2}{n_1 + n_2 - 2}$$

Data were considered significant if the t value was equal to or exceeded the tabulated value at 5% ($P < 0.05$).

2. Standard error of the mean (SEM)

The standard error of the mean is the error in the estimation of the mean and is

$$\text{SEM} = \frac{\text{standard deviation}}{\sqrt{n}}$$

3. Fisher exact probability test (Chapter 3)

This test is an extremely useful non-parametric technique for analysing data when two independent samples are small in size. It is used when the scores from two samples all fall into one or the other of two mutually exclusive classes. The scores are represented by frequencies in a 2X2 contingency table and for Chapter 3 this was :

	Trapping +ve	Trapping -ve	
Control	A	B	A+B
Infected	C	D	C+D
Total	A+C	B+D	N

A, B, C, and D stand for frequencies and the test determines whether control and infected differ significantly in the proportion of pluses and minuses attributed to them.

$$P = \frac{(A+B)! (C+D)! (A+C)! (B+D)!}{N! A! B! C! D!}$$

The value yielded will be for a one tailed test, and the P value calculated is doubled for a two-tailed test.

4. Mann-Whitney U-test

The U-test may be used to test whether two independent groups have been drawn from the same population. The U test is a most useful non-parametric alternative to the t-test in that it avoids the t-test's assumptions and it is relatively quick and easy to apply. The null hypothesis is that population A and B have the same distribution against the alternative that A is larger than B, or B larger than A.

Let n_1 = the number of observations in A and n_2 the number in B. To apply the test, we combine the scores from both groups and rank these in order of increasing size. Ties are given the average of the ranks they would have had if no ties had occurred.

$$\text{Then } U = \frac{n_1 n_2 + n_1 (n_1 + 1)}{2} - R_1$$

or equivalently substituting n_2 into this formula and replacing R_1 with R_2 .

Where R_1 and R_2 are the sum of the ranks assigned to groups n_1 and n_2 respectively.

These two formulas yield different U values and it is the smaller required. The larger = U' and $U = n_1 n_2 - U'$
Significance of U was obtained by reference to tables.

5. Regression analysis

This statistical operation was carried out using a Tektronix microcomputer. The input data (x,y) is fitted to eight regression equations and the equation which has maximum r^2 value indicated.

CHAPTER 3 A role for mucus in the expulsion of a primary
N.brasiliensis infection in the rat.

Introduction

The mechanism of expulsion of intestinal nematodes is thought to involve the collaboration of immunologically specific and non-specific components (Ogilvie & Love, 1974; Wakelin, 1978; Miller, 1984) and it has been suggested that goblet cell hyperplasia and mucus release are part of the non-specific component of worm expulsion (Miller & Nawa, 1979a; Askenase, 1980; Uber et al., 1980; Levy & Frondoza, 1983).

Increased mucus secretion could make the environment unsuitable for parasite establishment by excluding them from the mucosa (Miller et al., 1981b) or providing a slippery surface to which damaged parasites cannot cling (Askenase, 1980). Alternatively, mucus may physically trap the parasite as observed in the rapid expulsion of T.spiralis (Lee & Ogilvie, 1981; Bell et al. 1984) and N.brasiliensis (Miller et al., 1981b; Miller & Huntley, 1982a).

The present study was undertaken to determine if immune exclusion and mucus trapping occurred during the expulsion of a primary infection with N.brasiliensis. The results show that a small, but significant, number of parasites are trapped in mucus at the time of expulsion, but there was no evidence of immune exclusion.

Experiments and Results

Experiment A.

Eighty-five female rats infected with 5000 N.brasiliensis larvae were used. Groups of 8-10 rats were killed daily and the numbers and distribution of worms counted in the small intestine. The worm burdens are presented in Table 1.

Reduction in total worm burden occurred on day 11 ($P < 0.001$ vs day 7) when significant losses of lumen-dwelling parasites ($P < 0.05$) and of those adherent to the gut mucosa ($P < 0.001$) occurred. There was also a reduction in lumen-dwelling worms on days 9 and 10 ($P < 0.05$ vs day 7). Thirteen days after infection the few remaining worms within the intestine were predominantly adherent (Table 1).

Small numbers of worms were trapped in mucus 7-12 days after infection (0.03-1.22%), trapping was maximal on day 11. Such worms were enveloped in mucus and, using a dissecting needle, could not be liberated from the mucus gel. No trapping occurred on days 4, 6 or 13 (Table 2).

Experiment B.

Forty-two female rats were used and groups of 14 were infected with 1000, 3000 or 6000 N.brasiliensis L₃. Six, ten and thirteen days after infection, 5 rats from each group (4 from those given 6000 L₃) were killed and

Table 1 Worm distribution following an infection of 5000 N.brasiliensis L₃ : the number of worms free in the lumen and attached to the gut mucosa.

Day ^a	Free	Adherent	TWB ^b
4	334.0 ± 117	351.4 ± 106	685.4 ± 211
6	600.3 ± 44	598.4 ± 42	1198.7 ± 47
7	828.7 ± 154	597.6 ± 61	1427.8 ± 212
8	812.2 ± 171	604.3 ± 34	1416.9 ± 194
9	448.6 ± 86 ⁺	497.4 ± 79	946.5 ± 137
10	406.1 ± 61 ⁺	516.6 ± 53	924.3 ± 106
11	224.0 ± 49 ⁺⁺	197.2 ± 52 ⁺⁺⁺	426.4 ± 100 ⁺⁺⁺
12	140.2 ± 29 ⁺⁺⁺	194.1 ± 39 ⁺⁺⁺	334.7 ± 62 ⁺⁺⁺
13	18.1 ± 5 ⁺⁺⁺	31.0 ± 11 ⁺⁺⁺	49.1 ± 16 ⁺⁺⁺

a day after infection

b Total worm burden, including those trapped (see Table 2)

+ P < 0.05, ++ P < 0.005, +++ P < 0.001 when compared with day 7 (Student's t-test)

all data presented as mean ± SEM

Table 2 The number of worms trapped in mucus recovered by intestinal perfusion of rats infected with 5000 Nippostrongylus larvae.

Day ^a	Trapped (mean \pm SEM)
4	0
6	0
7	1.5 \pm 0.3 ⁺⁺⁺
8	0.4 \pm 0.2
9	0.5 \pm 0.3
10	1.6 \pm 0.6 ⁺
11	5.2 \pm 1.2 ⁺⁺
12	0.4 \pm 0.2
13	0

a day after infection

+ P=0.0062

++ P=0.0014

+++ P=0.0007

when compared with day 6 (Fishers exact probability test)

the numbers of worms adherent to the mucosa, free in the gut lumen and trapped in mucus determined (Table 3).

Six days after infection the majority of worms were adherent (72%) in rats infected with 1000 L_3 and with 3000 L_3 (60%). The converse was true for rats infected with 6000 L_3 where 60% were free in the lumen (Table 3). Ten days after infection worm distribution was unaltered in recipients of 3000 L_3 , whereas rats given 1000 L_3 had an increased proportion of worms free in the gut lumen (44%). Worms were distributed equally (50% free, 50% adherent) 10 days after infection with 6000 L_3 .

Significant worm expulsion had occurred in rats that had been infected with the smallest challenge dose, the worm burden decreasing by half on day 10 (Table 3), this being accounted for by a reduction in the number of worms adherent to the gut mucosa. Rats infected with 6000 L_3 lost proportionately more worms from the gut lumen (40% decrease) than from the mucosa.

Thirteen days after infection expulsion was almost complete, there being a small residual worm burden in one rat from each group (Table 3). Trapping of worms in mucus was observed only on the tenth day of infection (Table 3), the numbers of parasites trapped in mucus recovered from rats infected with 3000 L_3 being significantly greater than those from either rats given 1000 L_3 ($P < 0.001$) or 6000 L_3 ($P < 0.05$). Between 1 and 2% of worms were trapped in mucus which is consistent with Experiment A

Table 3 The effect of different levels of infection on worm distribution and mucus trapping.

Day ^a	Free	Adherent	Trapped	TWB ^b
6	122 ± 24	314 ± 29	0	436 ± 47
A 10	101 ± 11	127 ± 18 ⁺⁺	2 ± 0.3 ^{++a}	230 ± 27 ⁺
13	0	0.2 ± 0.2	0	0.2 ± 0.2
6	369 ± 41	571 ± 78	0	940 ± 108
B 10	295 ± 45	461 ± 40	16 ± 2.4 ^{++a}	772 ± 77
13	2 ± 2	26 ± 26	0	28 ± 28
6	739 ± 89	517 ± 57	0	1256 ± 82
C 10	450 ± 83	463 ± 63	9 ± 0.8 ^{++a}	922 ± 123
13	0	3 ± 3	0	3 ± 3

a day after infection with either (A) 1000 l_3 (B) 3000 l_3 or (C) 6000 l_3

b Total worm burden (TWB) all data presented as mean ± SEM
+ $P < 0.005$ ++ $P < 0.001$ when compared with day 6 (Student's t-test)

+a $P = 0.028$ ++a $P = 0.0078$ when compared with day 6 or 13
(Fisher's exact probability test)

(Tables 2 & 3).

Worms were enveloped in clear viscous globules of mucus, occasionally in clumps of 4-5, but mainly as ones and twos. Trapping in clumps was most obvious in rats infected with 3000 L₃.

Scanning Electron Microscopy (SEM).

The data from the previous two experiments suggested that the properties of mucus during parasite expulsion differed from its properties on day 6. Therefore samples of jejunum and mucus (gauze contents) collected on days 6 and 10 were fixed in acrolein vapour and processed for examination by SEM.

The disposition of mucus is shown in Figures 1-6. On day 6 there is little or no mucus present either on the mucosal surface or around the worms (Figures 1 & 2) whereas on day 10 worms are embedded in a thick blanket of mucus, which, in many instances enveloped the worms (Figures 3-6). These findings were consistent in each of the 3 rats examined at each time point.

Figure 1. Jejunum day 6 (x75) note little contact or attachment of mucus to parasites.

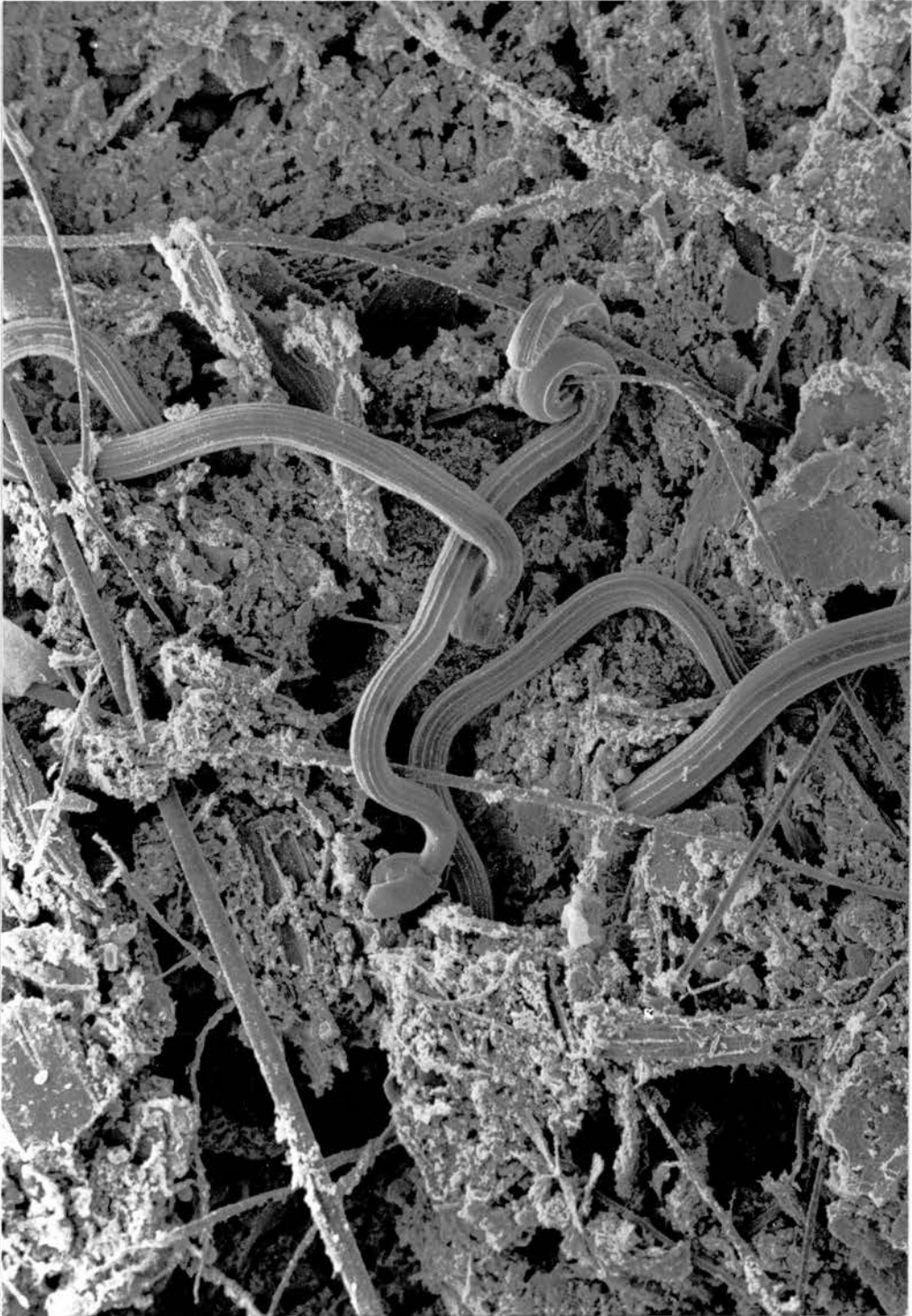


Figure 2. Jejunum day 6 (x75) little mucus present, parasites lying on the surface of the jejunum.

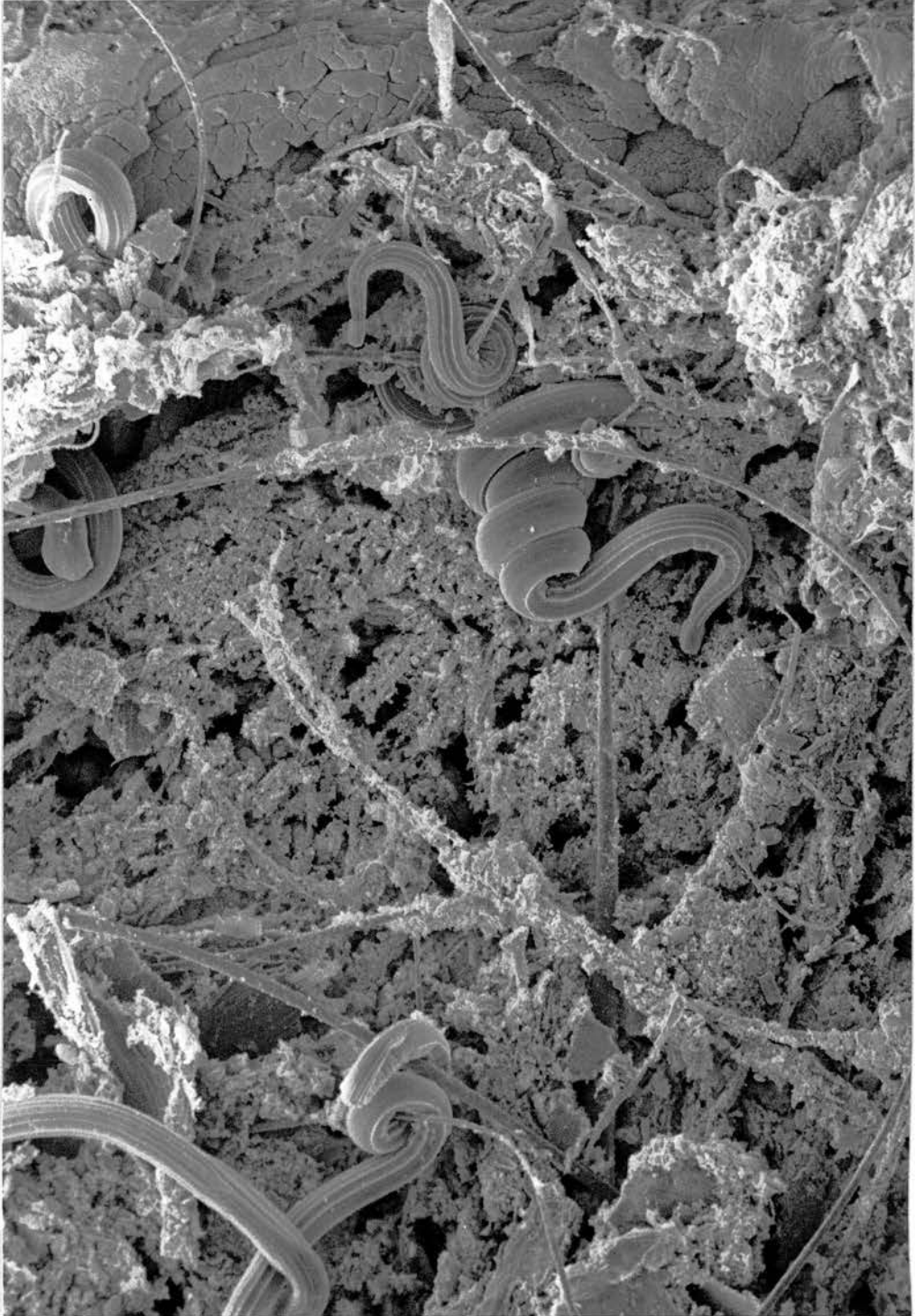


Figure 3. Jejunum day 10 (x300) attachment of mucus to parasite, envelopment of some areas of the parasite in mucus.

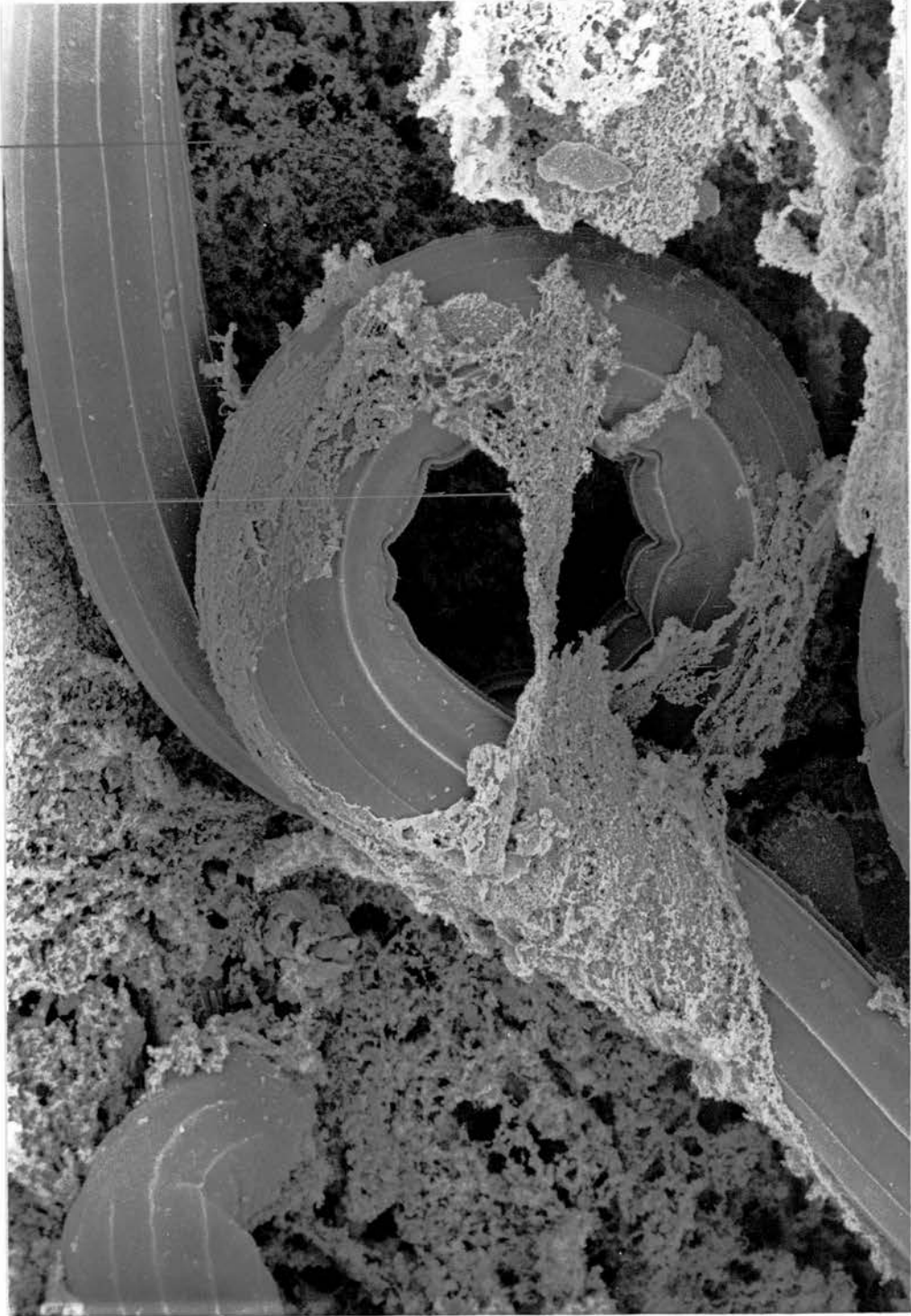


Figure 4. Jejunum day 10 (x150) parasite embedded in mucus. Note greater amount of mucus present compared with day 6.

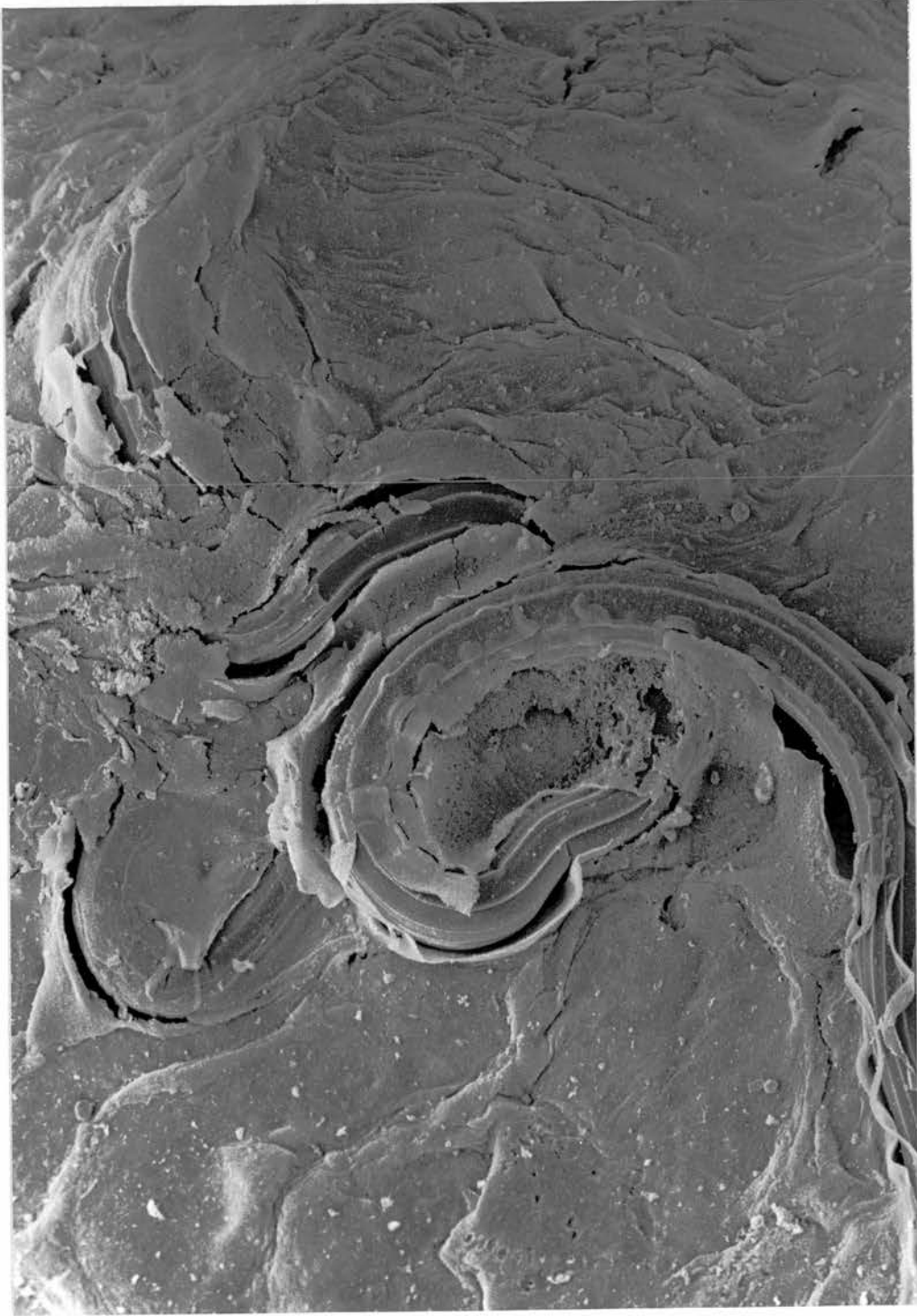


Figure 5. Gauze day 10 (x225) parasite embedded in a thick blanket of mucus.

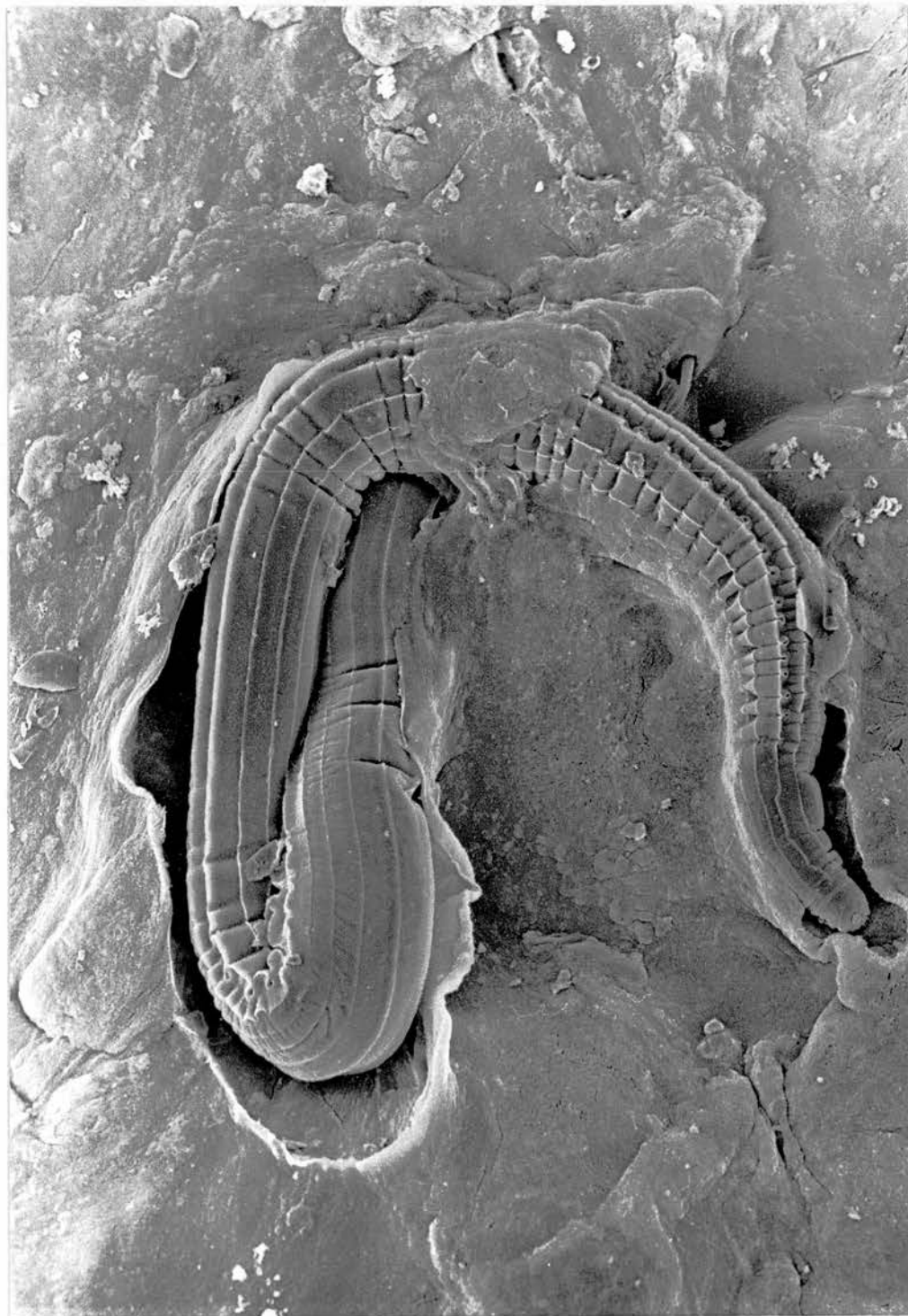
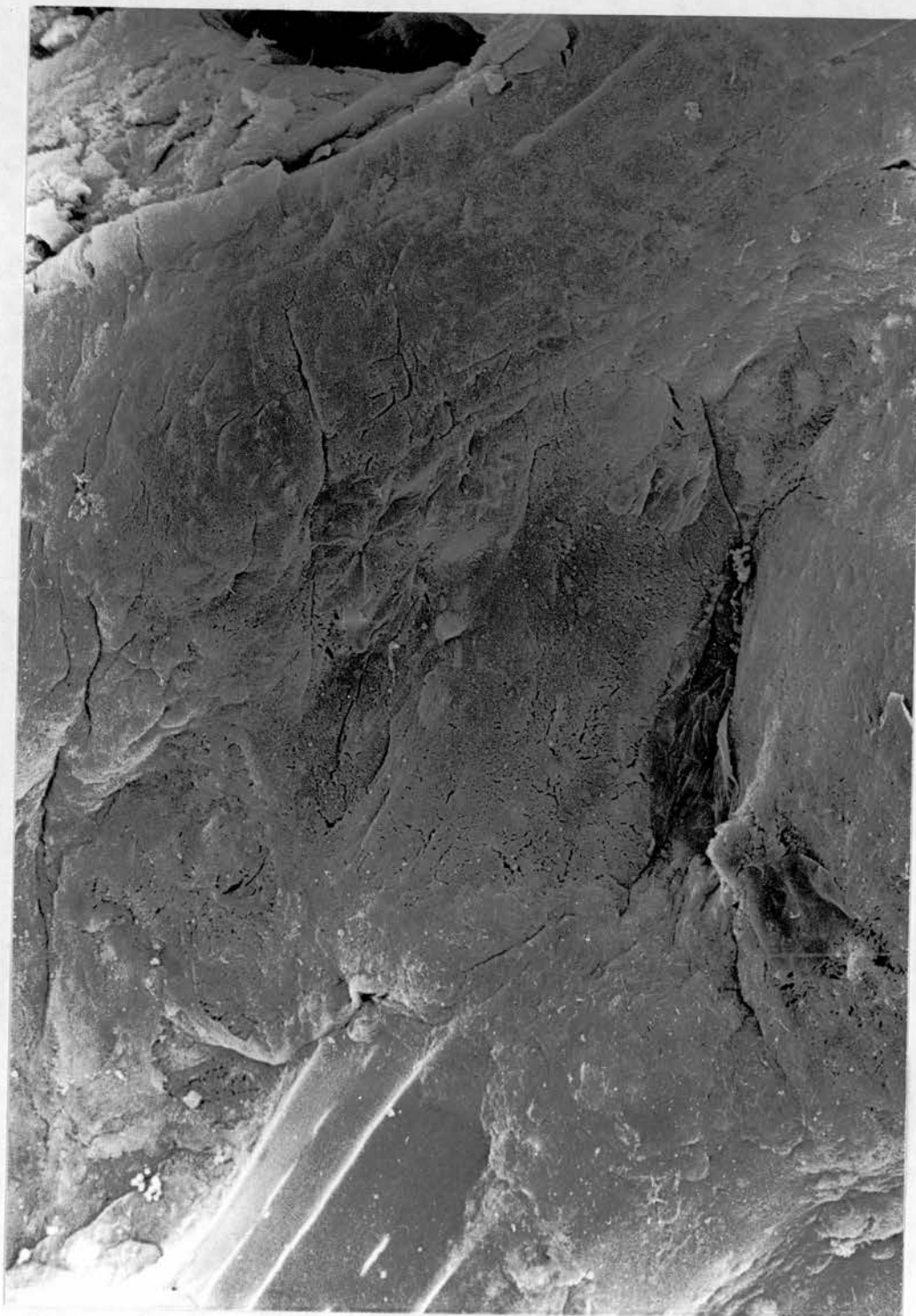


Figure 6. Gauze day 10 (x525) mucus covering a worm which can just be seen (bottom left).



Discussion

In this study the role of mucus in the expulsion of N.brasiliensis from the rat was examined and the results show that (a) small, but significant numbers of worms were trapped in mucus on days 10 and 11 of infection (b) none was trapped on days 6 and 13 and (c) a preliminary examination of the intestines of infected rats by SEM revealed a thick blanket of mucus enveloping worms on day 10 but not on day 6 of infection.

To quantitate the numbers of worms trapped in mucus at each stage of infection the intestinal perfusion technique originally described by Miller et al (1981b) was used. Trapping was maximal on days 10 and 11 (1-2%) but also occurred on day 7 (0.1%). Additional preliminary evidence that mucus trapping does occur during primary infection was obtained by using SEM. Whereas on day 6 very little mucus was evident, by day 10 the parasites were embedded in a thick blanket of mucus (Figures 1-6). Furthermore, SEM of the contents retained in the gauze from perfusates on day 10 also revealed worms to be enveloped in mucus.

Mucus trapping occurs during the rapid expulsion (RE) of T.spiralis (Lee & Ogilvie, 1981; Bell et al., 1984) and N.brasiliensis (Miller et al., 1981b; Miller & Huntley, 1982a) from primed rats. Comparison of the present data with that of Miller et al (1981b) shows that although the percentage of total worm burden trapped in

mucus during primary infection (1-2%) is considerably less than during RE (3-22%), in terms of actual numbers of worms trapped (1-24) on day 10 of primary infection, they are similar to the numbers trapped 30 and 60 minutes after challenge of immune rats (9 and 19 respectively) (Miller et al., 1981b).

A very conspicuous event in RE is the exclusion of the parasite from the intestinal mucosa (Russell & Castro, 1979; Miller et al., 1981b). However in the present experiments exclusion was not observed, in that at no stage of primary infection was there a significantly greater number of worms free in the intestinal lumen.

Could mucus trapping during primary infection account for the loss of up to 900 worms over a 72 hour period? It is important to note that although small numbers of parasites were trapped at each time point examined, no information can be gained as to the number of worms trapped over a 24 hour period (ie. the rate of trapping and loss) between sampling. For example if up to 16 worms are trapped and expelled per hour then mucus trapping could be of great significance, as the loss of only 10 worms per hour is required to expel the worm burden over a 72 hour period.

It must be questioned whether the perfusion technique employed was adequate for the quantitative recovery of all the worms trapped in mucus. Although only a preliminary study, SEM clearly demonstrated that worms were embedded

in mucus which covered the intestinal villi on day 10, it is not yet known if this mucus is dislodged by perfusion. Furthermore, experimental animals were routinely fasted overnight and the effect of this on the trapping of worms is uncertain as it would have probably affected the feeding pattern of the parasites which are known to move into food boluses of fed rats (Croll, 1976).

The absence on day 6, and presence on day 10 of trapped worms may reflect differences in the solubility of mucus at these two stages of infection. The solubility of mucus is strongly influenced by the interactions of mucin glycoproteins with other luminal constituents. For example, the viscosity of mucus increases dramatically after incubation in vitro with albumin (Forstner, 1978). Mucosal inflammation could cause mucus to transform into a more viscous gel.

Infection with N.brasiliensis is associated with an increased synthesis and turnover of mucin (Miller et al., 1981a) and goblet cell hyperplasia (Miller & Nawa, 1979a; Uber et al., 1980) both of which are temporally related to worm expulsion. Alterations in the histochemical properties of goblet cell mucin have been observed during Nippostrongylus-infection; the mucin staining with Alcian blue in normal rats and later a proportion (20-30 %) of goblet cells were PAS positive suggesting that they contained neutral mucin (Miller & Nawa, 1979b). Since the sialic acids and sulphate groups which confer

acidic properties to mucins are terminal on carbohydrate side chains, accelerated goblet cell differentiation may be accompanied by an incomplete synthesis of mucin glycoproteins. Secretion of mucin at an earlier stage of its synthesis could confer considerable differences both in the solubility of the mucus gel and its interaction with other components.

In Experiment A, although there were significant ($P < 0.05$) reductions in the numbers of worms free in the gut lumen on days 9 and 10 when compared with day 7, a significant reduction in total worm burden was not demonstrated until day 11 ($P < 0.001$ vs day 7). The total worm burden on day 11 was significantly reduced when compared with day 10 ($P < 0.005$) this being due to a reduction in adherent parasites ($P < 0.001$). It was over this time period when mucus trapping was maximal, but whether trapping could promote the expulsion of approximately 20 parasites per hour cannot be established from this data. However, gut motility is increased during parasitism (Castro, Hessel & Whalen, 1979; Farmer, 1981) and trapped worms could be rapidly lost from the intestine. Major worm loss occurred from those adherent to the mucosa where parasites will first experience "changes" in mucus.

In conclusion, the data clearly shows that mucus trapping does occur during the expulsion of N. brasiliensis. Trapping was maximal on days 10 and 11 coincident with

major reductions in worm burden. The numbers of worms trapped in mucus were low and without data on the kinetics of trapping over a 24 hour period definite conclusions cannot be made. However, if as many as 10 parasites are trapped and expelled per hour trapping could be of considerable functional significance.

CHAPTER 4 N.brasiliensis primary infection : mucosal
mast cell counts and the levels of RMCPII during
infection.

Introduction

Mucosal mastocytosis within the intestinal lamina propria is a prominent feature following infestation of laboratory animals with parasitic nematodes (Askenase, 1980; Miller, 1980; Miller, 1984). However the temporal relationships between the onset of mastocytosis and worm expulsion has raised doubts as to the involvement of MMC in the rejection mechanism. For example, mastocytosis and expulsion are related in T.spiralis infection (Alizadeh & Wakelin, 1982) whereas mast cell hyperplasia may occur either during (Murray et al., 1971a) or after the expulsion of N.brasiliensis (Keller, 1971; Kelly & Ogilvie, 1972). However, by measuring the concentrations of RMCPII, a MMC specific proteinase, Woodbury et al (1984) were able to demonstrate the secretion of this enzyme into blood during the expulsion of T.spiralis and N.brasiliensis, even when MMC were absent or in very low numbers. This raises the possibility that (a) there is differential release of protease during infection or (b) release is from MMC that are not demonstrable by conventional staining regimes.

The present study was designed to investigate these two possibilities and shows that there is no evidence to suggest differential release or secretion by "hidden" MMC which are undetected by staining for glycosaminoglycan.

Experiments and Results

Groups of 14 rats were infected with 1000, 3000 or 6000 *N. brasiliensis* larvae and 5 from each group (4 from those given 6000 L₃) were bled out 6, 10 and 13 days after infection. An additional group of 5 naive rats was killed for control purposes.

In rats infected with 1000 or 6000 L₃ the immune response, as monitored by faecal egg counts, began on day 9, counts falling to zero 12 days after infection. Egg counts did not fall until 10 days after infection with 3000 L₃ although there were few eggs present on day 12. This is consistent with the majority of worms being expelled between 10 and 13 days after infection.

The intestinal worm burden was significantly ($P < 0.005$) reduced 10 days after infection with 1000 L₃ when compared with that on day 6; the worm counts in rats given the higher doses of larvae were unchanged over this time period (Table 1). Expulsion was all but complete on day 13, there being a small residual worm burden in one rat from each group.

Table 1 Worm burdens (mean \pm SEM) six, ten and thirteen days after infection of rats with 1000, 3000 or 6000 L₃.

Day	1000 L ₃	3000 L ₃	6000 L ₃
6	436 \pm 47	940 \pm 108	1256 \pm 82
10	230 \pm 27 ^a	772 \pm 77	922 \pm 123
13	0.2 \pm 0.2	28 \pm 28	3 \pm 3

a $P < 0.005$ when compared with day 6 (Student's t-test)

The concentration of RMCPII in jejunum.

Six days after infection, concentrations of RMCPII in jejunal homogenates had significantly ($P < 0.001$) fallen when compared with control values (Table 2). There was, in addition, significantly less RMCPII in homogenates prepared from the jejunum of rats infected with 3000 or 6000 L_3 than from rats given 1000 L_3 ($P < 0.05$, Table 2).

Ten days after infection the levels of RMCPII in jejunal homogenates were unchanged (Table 2) whereas on day 13 the concentrations of enzyme had increased significantly ($P < 0.001$) when compared with control, day 6, or day 10 values (Table 2).

Systemic levels of RMCPII (Figure 1).

To monitor the appearance of RMCPII in the sera of infected rats, 5 animals from each group were bled via the tail vein on day 4 and daily thereafter (except day 12), 4-5 rats were exsanguinated on day 6, 10 and 13.

RMCPII was not detected in the sera of uninfected control rats. Trace amounts of protease were present in the sera of rats infected with 1000 L_3 4 and 5 days after challenge after which on day 6, there was a highly significant increase in the circulating concentration of RMCPII ($P < 0.005$ vs day 5). The levels of RMCPII rose steadily to reach a peak on day 13 ($P < 0.05$ vs day 6) when there was 1043 ng RMCPII/ml serum.

Table 2 The concentration of RMCPII in the jejunum of rats infected with N.brasiliensis : the effect of different doses of larvae.

Day	$\mu\text{g RMCPII/g wet wt tissue}^a$		
	1000	3000	6000
0	390 \pm 13	390 \pm 13	390 \pm 13
6	232 \pm 20 ^{+++a}	182 \pm 3 ^{+++a}	180 \pm 0 ^{+++a}
10	208 \pm 13 ^{+++a}	182 \pm 3 ^{+++a}	165 \pm 9 ^{+++a}
13	1327 \pm 186 ^{+++b}	1392 \pm 2 ^{+++b}	1584 \pm 181 ^{+++b}

a the concentration of RMCPII (mean \pm SEM) in homogenates of jejunum prepared from rats infected with 1000, 3000 or 6000 N.brasiliensis l_3 .

+++a $P < 0.001$ significant depletion when compared with controls (day 0)

+++b $P < 0.001$ significant increase when compared with controls or day 6 or 10

On day 6 the concentration of RMCPII (1000 l_3) was greater ($P < 0.05$) than that from 3000 l_3 or 6000 l_3 infected rats.

On day 10 the mucosal concentration of RMCPII in rats given 1000 l_3 was greater ($P < 0.05$) than that from rats infected with 6000 l_3 .

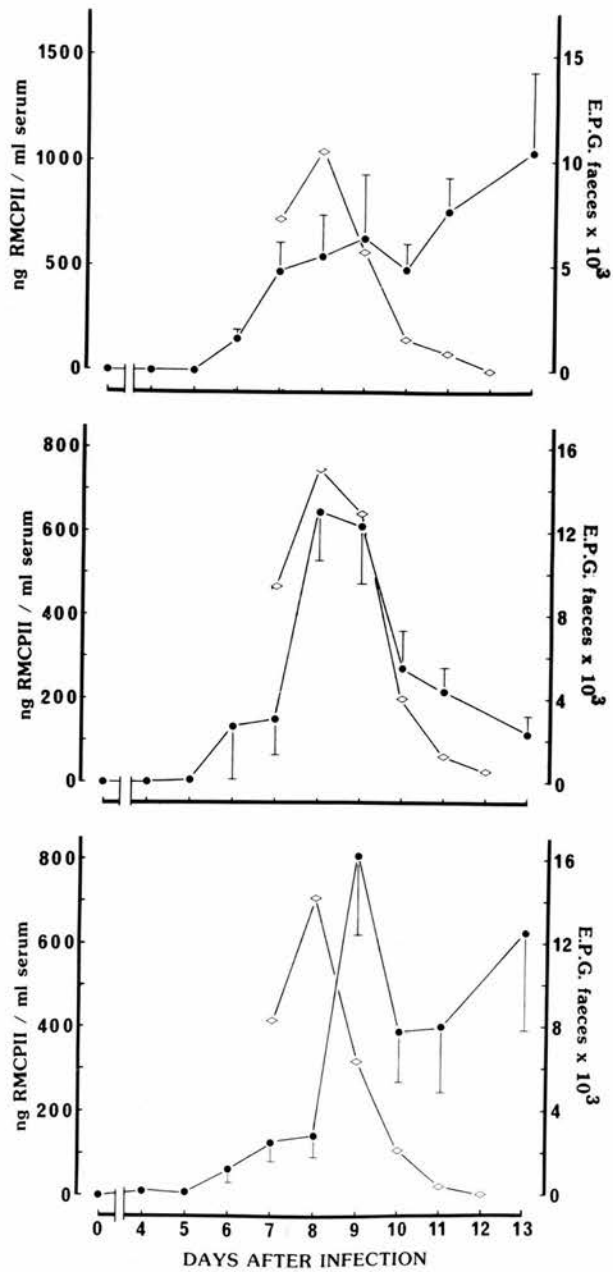


Figure 1. The profiles of release of RMCPII into the serum of rats infected with 1000 L₃ (top), 3000 L₃ (centre) or 6000 L₃ (bottom). The levels of RMCPII (●) ng/ml of serum (mean \pm SEM). Eggs per gram (EPG) faeces also shown as (◇).

Following infection of rats with 3000 L₃, trace amounts of protease were present on day 5, none was present on day 4. In rats that had been infected for 7 days there was significantly ($P < 0.025$) more RMCPII present in the sera when compared with day 5. The levels of protease reached a peak on day 8 (645 ng/ml) and remained high on day 9 after which they declined. The concentration on day 8 was significantly ($P < 0.001$) greater than on day 7 or 13.

A similar response was recorded in rats infected with 6000 L₃, there being 800 ng RMCPII/ml serum on day 9 ($P < 0.001$ vs day 6) the concentration remaining high (625 ng/ml) 13 days after infection.

Comparison of MMC staining methods.

MMC were enumerated both within epithelium and lamina propria after staining with Toluidine blue, naphthol AS-D chloroacetate (NADC), or with anti-RMCPII immunoperoxidase, the cell counts are shown in Table 3. Comparison by regression analysis of the number of MMC detected by each staining method showed a highly significant correlation between methods whether cells were demonstrated by their content of GAG or RMCPII (Table 4, total cell counts). Furthermore, the relationship between mast cells (immunoperoxidase, total) and the mucosal concentration of RMCPII was highly significant ($y = -163 + 42x$, $r=0.88$, $P < 0.001$).

Table 3 The number of MMC in the jejunum of rats detected by staining for esterase, glycosaminoglycan or RMCPII.

Day	MMC/VCU (mean \pm SEM)		
	Esterase	Toluidine blue	RMCPII
6	6.6 \pm 0.6	6.2 \pm 0.4	5.3 \pm 0.3
A) 10	14.7 \pm 0.9	14.2 \pm 0.6	14.5 \pm 1.0
13	33.2 \pm 0.5	33.2 \pm 1.5	33.1 \pm 0.6
6	5.3 \pm 0.3	4.5 \pm 0.2 ⁺	5.9 \pm 0.4
B) 10	9.8 \pm 0.3	10.0 \pm 0.4	10.4 \pm 0.2
13	33.8 \pm 2.2	36.6 \pm 2.0	34.3 \pm 2.2
6	5.3 \pm 0.6	5.3 \pm 0.5	5.6 \pm 0.4
C) 10	18.5 \pm 1.0	16.7 \pm 1.2	17.2 \pm 0.8
13	38.0 \pm 2.4	37.4 \pm 2.8	37.9 \pm 2.4
Control	9.6 \pm 0.8	9.5 \pm 0.7	N.D

A 1000 l_3

B 3000 l_3

C 6000 l_3

+ significantly less when compared with esterase ($P < 0.05$)
or RMCPII ($P < 0.01$) (Student's t-test)

N.D not done

Further analysis of the data showed that on day 6 of infection there was no correlation between cell counts when NADC and immunoperoxidase or Toluidine blue and immunoperoxidase methods were compared (Fig. 2). The relationship between counts of NADC and Toluidine blue positive cells was however significant ($P < 0.05$). The mean cell counts (total) on day 6 did not differ significantly and the poor correlation may reflect a lack in data "spread" as most cell counts were predominantly between 5 and 6 per VCU. Both on day 10 and 13 regression analysis showed cell counts to be significantly correlated when staining methods were compared (Fig. 2, Table 3).

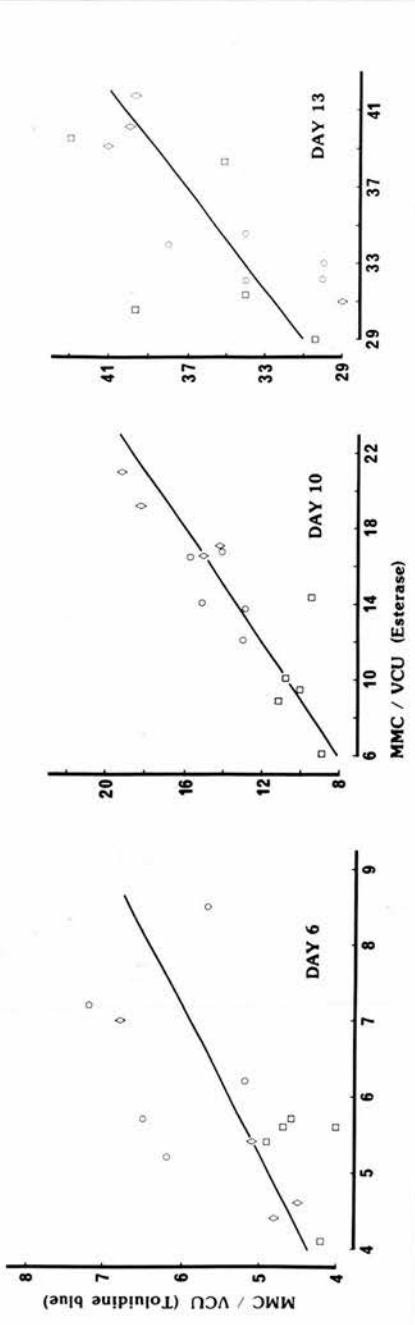
Table 4 The relationships between total cell counts on esterase, Toluidine blue and immunoperoxidase stained sections.

Data	Equation of Best Fit	r	P
E^a vs T^b	$y = 1.02x + -0.5$	0.98	0.001
E^a vs R^c	$y = 0.99x + -0.003$	0.99	0.001
T^b vs R^c	$y = 0.94x + 0.96$	0.98	0.001
R^c vs R^d	$y = 42x + -163$	0.88	0.001

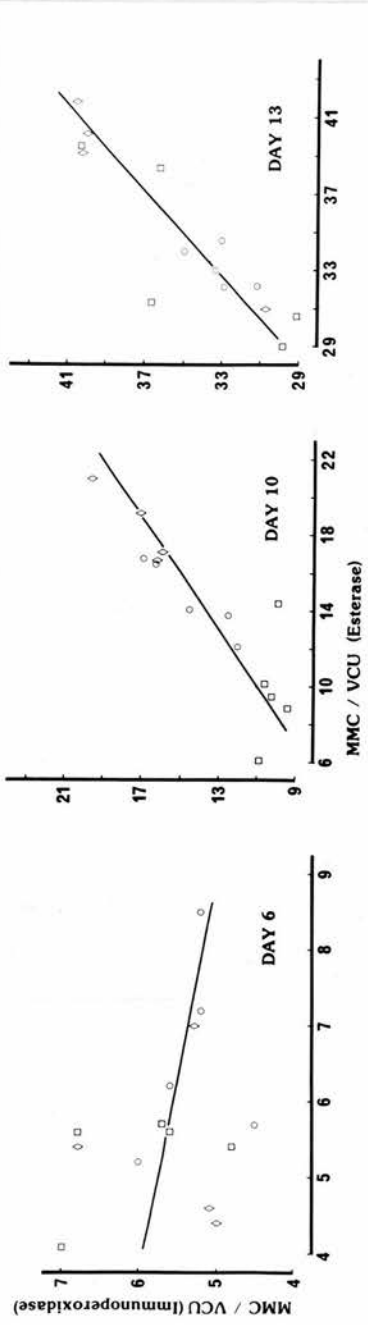
a esterase b Toluidine blue c anti-RMCPII

d mucosal RMCPII

a



b



c

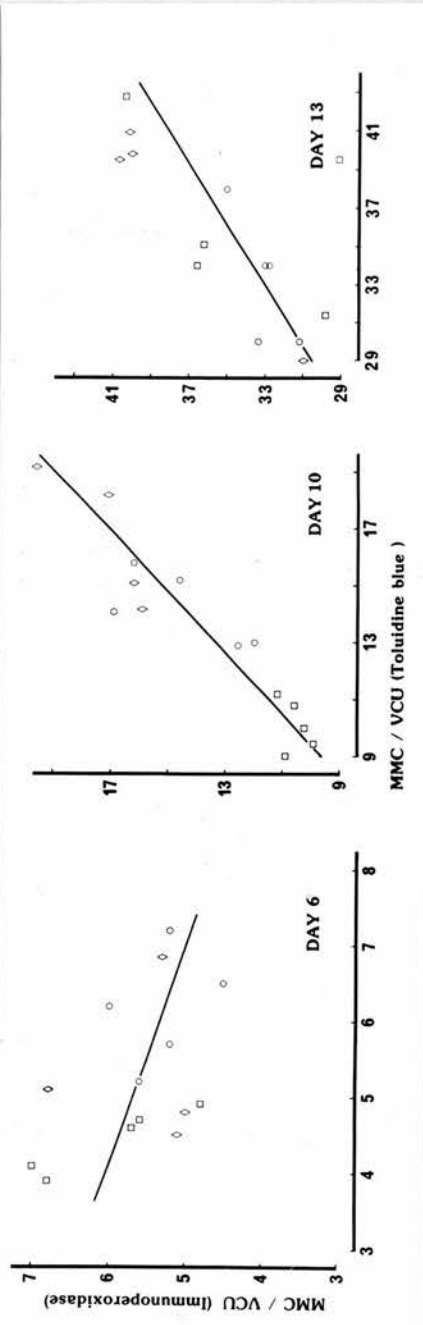


Figure 2. The relationships on day 6, 10 and 13 between MMC counts performed on NADC-esterase, Toluidine blue and immunoperoxidase stained sections.

Symbols for rats infected with 1000L₃ (○), 3000L₃ (□) and 6000L₃ (◇).

(a) Esterase vs Toluidine blue.

day 6 ($y = 0.5x + 2.4$, $r=0.58$)

day 10 ($y = 0.65x + 4.3$, $r=0.87$)

day 13 ($y = 0.75x + 9.3$, $r=0.69$)

(b) Esterase vs Immunoperoxidase.

day 6 ($y = -0.19x + 6.7$, $r=0.29$)

day 10 ($y = 0.67x + 4.3$, $r=0.87$)

day 13 ($y = 0.89x + 3.9$, $r=0.90$)

(c) Toluidine blue vs Immunoperoxidase.

day 6 ($y = -0.36 + 7.5$, $r=0.47$)

day 10 ($y = 0.92x + 1.4$, $r=0.93$)

day 13 ($y = 0.62x + 12.6$, $r=0.68$)

Kinetics of MMC following infection.

Six days after infection, the numbers of MMC were significantly reduced when compared with counts in uninfected controls (Table 3). This change was apparent whether cells were demonstrated by their content of RMCPII (esterase) or GAG, and although there was no further depletion of MMC at the two highest levels of infection after staining for esterase, there was a significantly greater reduction ($P < 0.05$) in the number of MMC demonstrated with Toluidine blue when doses of 3000 and 1000 L_3 were compared.

Ten days after infection there was a highly significant increase in the number of MMC when compared with day 6, and in animals given 1000 or 6000 L_3 the numbers were greater than in uninfected controls. In rats that had been infected with 3000 L_3 , mast cell changes were less pronounced and although the numbers were increased on day 10 when compared with day 6, they had not exceeded the numbers in uninfected controls (Table 3).

On day 13 the numbers of MMC had increased to be significantly ($P < 0.001$) greater than on day 6 or 10, but there was no difference between groups infected with different doses of larvae (Table 3).

Morphology of mast cells during infection.

MMC in normal rats contained numerous small granules

which stained an intense red colour with NADC. Six days after infection MMC were sparse and some villi were devoid of MMC, those present had poor granule definition. The cells on day 10 were predominantly immature having large nuclei and abundant cytoplasm containing few granules which stained strongly with each of the techniques used. Occasionally fully mature cells were present. By day 13, mast cells were mainly mature containing many closely packed granules.

Discussion

The histochemical demonstration of rat mucosal mast cells (MMC) is primarily based upon the staining of mast cell glycosaminoglycan (GAG) with basic dyes such as Alcian blue or Toluidine blue (Enerback, 1981). However, MMC also contain a granule-associated serine proteinase : rat mast cell protease II (RMCPII) which can be demonstrated within MMC by immunocytochemistry (Woodbury et al., 1978b; Woodbury & Miller, 1982) or by its activity on synthetic esterase substrates (Newlands et al., 1984). In the present study, these three methods have been compared to determine if there is selective release of mast cell granule constituents during Nippostrongylus-infection.

Comparable numbers of MMC were detected after staining for GAG, esterase or RMCPII. This is in agreement with previous results (Newlands et al., 1984) and occurred on days 6, 10 and 13 of N.brasiliensis infection (Table 3), indicating no selective loss or gain of granule constituents.

The mast cell responses were similar, irrespective of infective dose on days 6 and 13, but on day 10 there was a significantly greater number of MMC in the jejunum of rats given 6000 L₃ compared to 1000 L₃ ($P < 0.025$) or 3000 L₃ ($P < 0.005$) indicating that the higher doses of larvae may have provided greater antigenic stimulus for the sensitization of T-cells.

A previous study in which MMC, detected by their content of GAG or RMCPII were quantitated (Woodbury & Miller, 1982) demonstrated a close agreement between the two methods on day 7 of infection, but on day 12 approximately twice as many cells were detected by their content of GAG than by staining of RMCPII with immunoperoxidase. The latter observation is not consistent with the present results (Table 3). The major difference in experimental protocol between the present study and that of Woodbury & Miller (1982) is in tissue fixation. Woodbury & Miller (1982) employed Carnoy's fixation, whereas in this study tissues were fixed in four percent paraformaldehyde which optimizes staining with basic dyes, NADC and immunoperoxidase (Newlands et al., 1984). Woodbury & Miller (1982) were unable to detect intra-epithelial mast cells even though they contained GAG and these authors suggested that Carnoy fixation reduces the sensitivity of the immunoperoxidase technique. Consequently intraepithelial mast cells which are partially depleted of protease and other granule constituents are not detected (Miller & Walshaw, 1972; Huntley et al., 1984).

The numbers of MMC were, in agreement, with previous results (Woodbury & Miller, 1982; Woodbury et al., 1984), low on days 6-10 even when significant enzyme was detected in the sera (Figure 1). Nevertheless, the numbers of MMC increased 2-3 fold from day 6 to 10 although the concentration of RMCPII in the jejunal mucosa remained

low on day 10, even in groups of rats where MMC counts were increased. This, as well as the raised systemic levels of RMCPII, support the view that MMC may be secreting rather than storing enzyme (Woodbury & Miller, 1982; Woodbury et al., 1984) and the fact that MMC were predominantly immature and few in number at the time of maximal enzyme release indicates that they may be more functionally active than the fully granulated cell. The concentration of RMCPII and numbers of MMC in the jejunum increased soon after the completion of parasite expulsion.

In conclusion, the results of this study confirm previous observations on the relationship between RMCPII secretion and the onset of worm expulsion (Woodbury et al., 1984). However in these studies (Woodbury et al., 1984) RMCPII was detected in the sera of infected rats when the numbers of MMC (detected by Alcian blue) were very low. The enzyme could, therefore, have been released from MMC which were undetectable by conventional techniques. The present investigation examined this possibility by staining for GAG, RMCPII and esterase and comparing cell counts. The results have shown that even early in infection when RMCPII is detectable in the sera there are few MMC regardless of the technique used to demonstrate them. The role of MMC in parasite expulsion will, thus, need to be reevaluated in the light of these findings.

CHAPTER 5 Anaphylactic release of RMCPII and its relationship to mucosal permeability in Nippostrongylus-primed rats.

Introduction

It has long been thought that an immediate hypersensitivity reaction may play some part in the expulsion of gastrointestinal helminths (rev. Murray, 1972). The role for local anaphylaxis was emphasised by the studies of Urquhart et al (1965) who suggested that anaphylaxis could affect worm expulsion either by changes in the environment of the parasite or by potentiating the translocation of parasite-damaging antibody into the gut lumen as a result of increased mucosal permeability. Increased mucosal permeability was subsequently demonstrated in Nippostrongylus-infection (Murray et al., 1971a; Nawa, 1979).

The causes of increased mucosal permeability may be the presence of worms themselves (Nawa, 1979), the severe inflammatory reaction in response to infection or the increase in both number and functional activity of MMC.

The studies in Chapter 4 show that MMC are secreting RMCPII in response to N.brasiliensis infection even when MMC are very few in number. The systemic levels reached a peak 9-10 days after infection when mucosal permeability is maximal (Nawa, 1979).

The experiments described in this chapter were undertaken to analyse the relationship between RMCPII secretion and gut permeability during systemic anaphylaxis. The results show that RMCPII secretion

is directly related to permeability changes in the gut and indicate that it could, therefore, play some role in causing the increases in mucosal permeability during N.brasiliensis primary infection.

Experiments and Results

Time course of RMCPII release and mucosal permeability

Twenty-five immune and 10 naive rats were used in this experiment. Twenty immune and five naive rats were injected intravenously with 500 we of worm antigen plus Evan's blue. The remaining ten rats were injected with Evan's blue in saline. Challenge control rats and immune and naive rats treated with saline were killed 30 minutes after injection. Immune recipients of antigen were killed 5, 15, 30, and 60 minutes after injection of antigen and Evan's blue.

No RMCPII was detected in the sera of saline-treated immune rats or of naive rats given saline or antigen (Table 1). Immune rats injected with antigen responded by the secretion into the sera of substantial amounts of RMCPII (Table 1). Regression analysis of these data (Fig. 1) shows a highly significant correlation between time after challenge and RMCPII levels in the sera of immune rats ($y = 41 + 11x$, $r=0.87$, $P < 0.001$). RMCPII was detected in the sol and gel phases of intestinal perfusates from immune rats injected with antigen (Fig.2). None was present in the sol or gel phases of perfusates from immune or naive recipients of saline or naive rats given antigen (Table 2). Although the concentrations of RMCPII in the perfusates of immune-challenged rats was not time-dependent it was related both to Evan's blue accumulation in the intestine and serum levels of RMCPII (Table 3).

Table 1 The systemic secretion of RMCPII in primed rats challenged intravenously with worm antigen.

Group ^a	Challenge ^b	Time ^c	µg RMCPII/ml serum
1	Saline	30	0
2	500 we	5	121 ± 27
3	500 we	15	190 ± 43
4	500 we	30	351 ± 95
5	500 we	60	630 ± 132
6	500 we	30	0
7	Saline	30	0

a Groups 1-5 immune, 6&7 naive

b rats were challenged intravenously with 500 we of worm antigen or saline.

c Time after challenge (minutes)

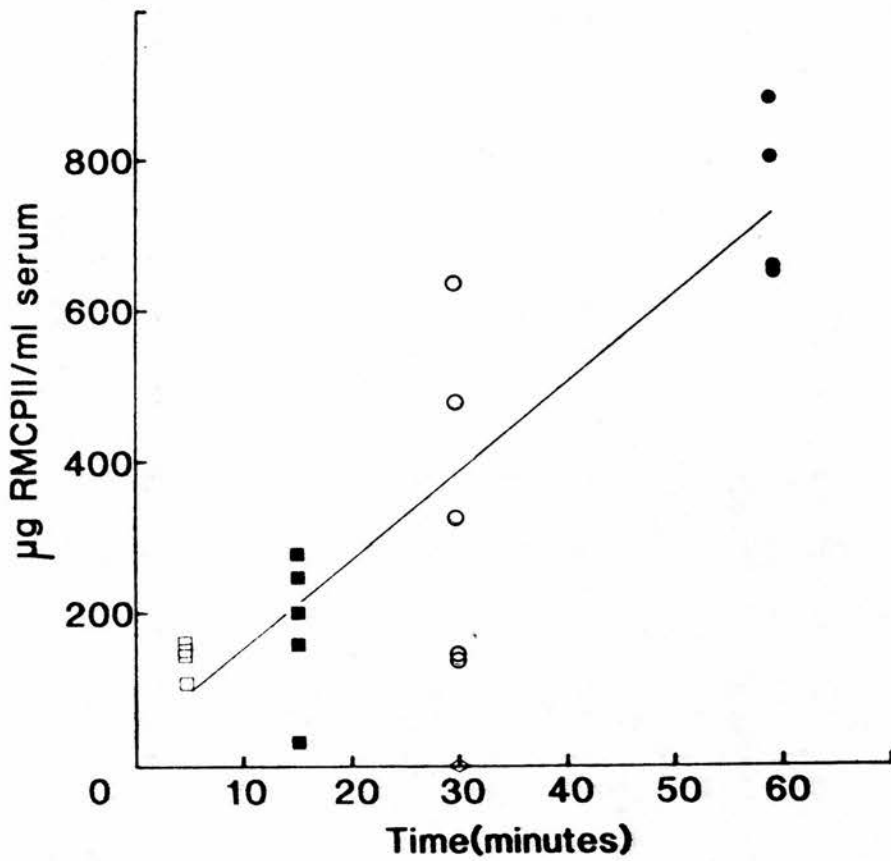


Figure 1. The levels of RMCPII in the sera of primed rats plotted against time after challenge with 500 we of antigen. The mean value for immune rats given saline shown at 30 minutes (\diamond).

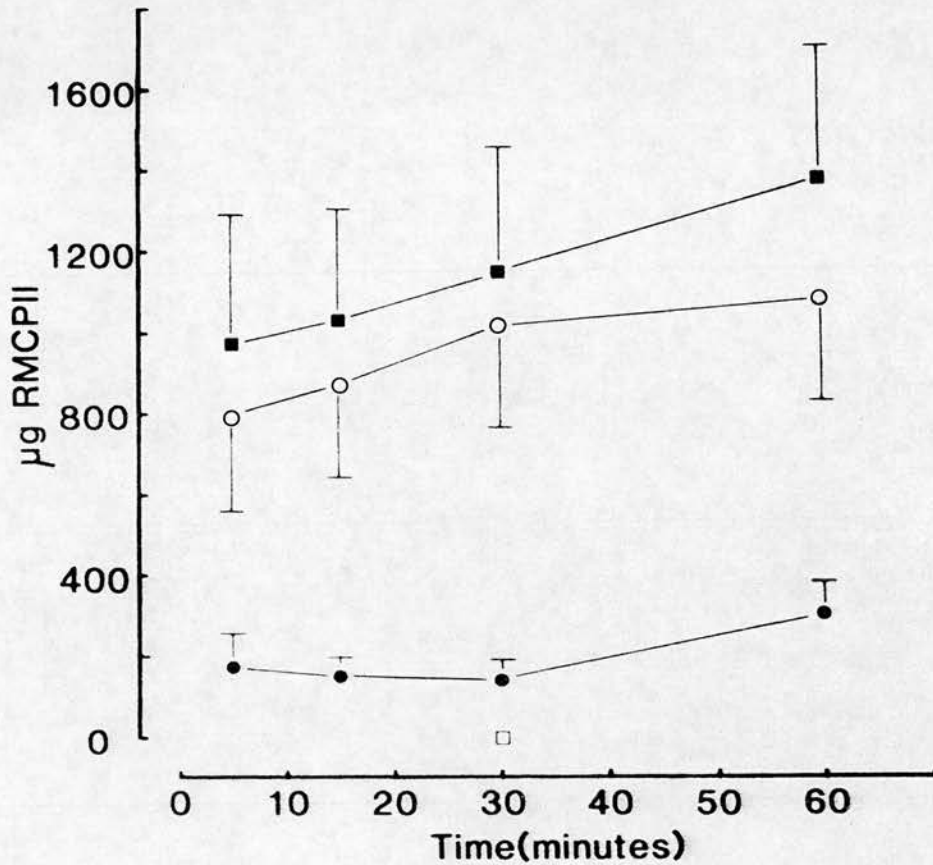


Figure 2. The partitioning of RMCPII (mean \pm SEM) between sol and gel phases of intestinal perfusates. Sol phase (○), gel phase (●) and total RMCPII (■). Immune control at 30 minutes (□).

The total amount of RMCPII (μg) in the sol (○) was calculated by multiplying the concentration of RMCPII in the sol ($\mu\text{g}/\text{ml}$) by the volume of sol. The total amount of RMCPII (μg) in the gel (●) was calculated by multiplying the concentration of RMCPII in the gel ($\mu\text{g}/\text{ml}$) by the volume of rehomogenized gel.

The total amount of RMCPII (■) represents the sum of sol and gel.

Table 2 The partitioning of RMCPII between sol and gel phases of intestinal perfusates following intravenous challenge with worm antigen.

Group ^a	Time ^b	$\mu\text{g RMCPII (mean } \pm \text{ SEM)}$		
		SOL	GEL	TOTAL
1	30	0	0	0
2	5	801 \pm 242	176 \pm 83	977 \pm 322
3	15	879 \pm 236	156 \pm 47	1035 \pm 276
4	30	1020 \pm 253	139 \pm 52	1159 \pm 303
5	60	1085 \pm 248	310 \pm 75	1395 \pm 315
6	30	0	0	0
7	30	0	0	0
Distribution (%) of RMCPII		83 \pm 2	17 \pm 2	

a group status and treatment as described in Table 1

b time after challenge (minutes)

Table 3 Analysis of the relationships between time, systemic and enteric secretion of RMCPII, and enteric accumulation of Evan's blue.

Data	Equation of best fit	r	P
T vs R ^a	$y = 929 + 7.7x$	0.25	NS
R ^b vs R ^a	$y = -1425 + 475\log x$	0.73	(P < 0.001)
R ^a vs EB ^c	$y = -451 + 88\log x$	0.72	(P < 0.001)

R^a RMCPII recovered from sol and gel of perfusate (μg)

R^b RMCPII in serum ($\mu\text{g}/\text{ml}$)

EB^c Total Evan's blue from sol and gel of perfusate (μg)

T Time after challenge (minutes)

NS Not Significant

Immune rats given saline had significantly less Evan's blue intraluminally (34 $\mu\text{g}/\text{perfusate}$) than their challenged counterparts (186 $\mu\text{g}/\text{perfusate}$) at 30 minutes (P < 0.001, Fig. 3). Five minutes after intravenous injection of antigen the levels of Evan's blue in the intestines of primed rats increased two-fold (Table 4, Fig. 4). Perfusates from naive rats given saline contained significantly less (P < 0.001) Evan's blue (24 μg) than those from naive recipients of worm antigen (55 μg) yet no RMCPII was detected in the sera of either

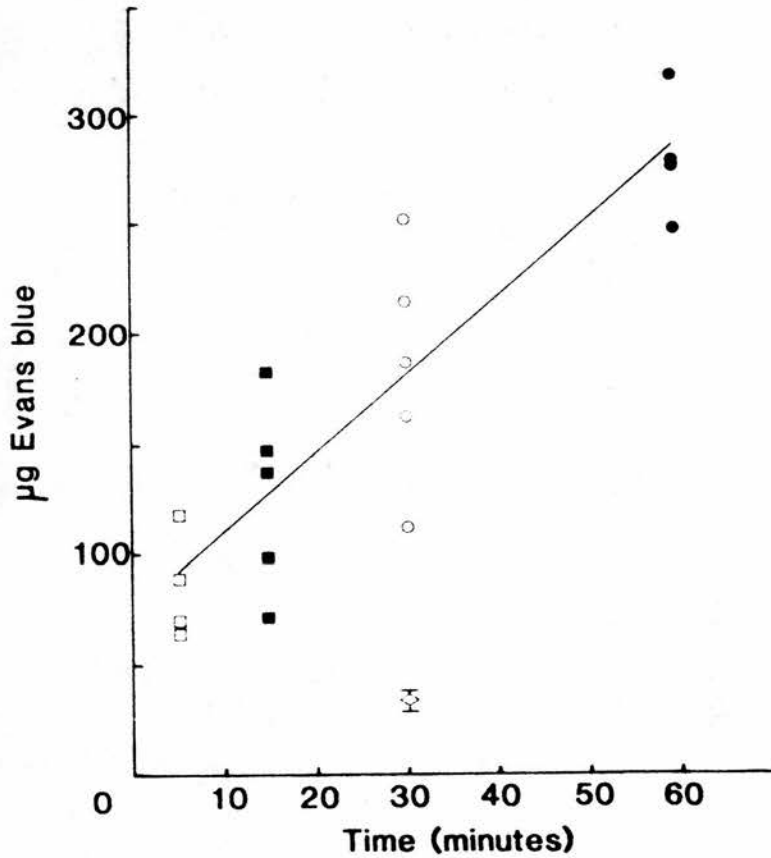


Figure 3. The levels of Evan's blue in the gut lumen plotted against time after challenge with 500 we.

Mean \pm SEM for immune control shown at 30 minutes as (\diamond).

The amount (μg) of Evan's blue represents the total from both sol and gel phases of each perfusate (see pp 144 for details).

Table 4 The distribution of Evan's blue between sol and gel phases of intestinal perfusates.

Group ^a	<u>µg Evan's blue (mean ± SEM)</u>		
	SOL	GEL	TOTAL
1	11 ± 5	23 ± 1	34 ± 6
2	65 ± 11	21 ± 2	86 ± 12
3	99 ± 14	29 ± 6	128 ± 20
4	147 ± 22	39 ± 10	186 ± 24
5	195 ± 11	86 ± 3	281 ± 14
6	39 ± 2	14 ± 1	53 ± 3
7	6.5 ± 3	17.5 ± 3	24 ± 2

a see Table 1 for group status and treatments

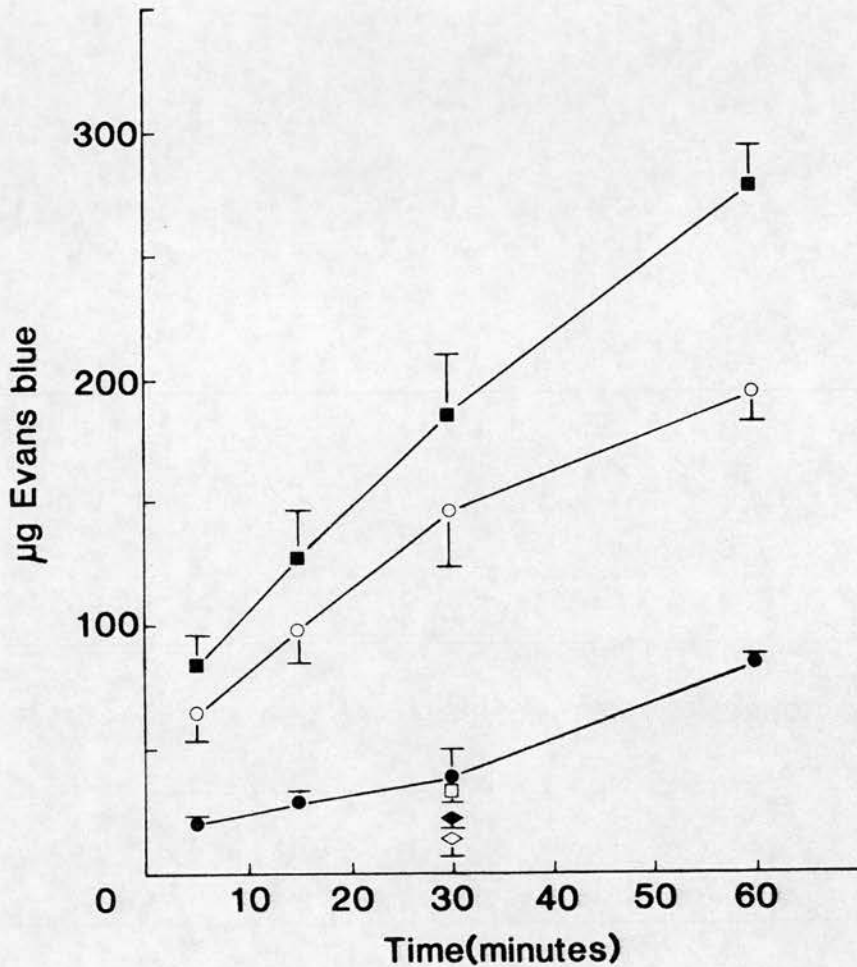


Figure 4. The relative contribution of sol (○) and gel (●) phases to total (■) Evan's blue in the gut lumen plotted against time after challenge. Immune recipients of saline, sol (◇), gel (◆) and total (□) shown at 30 minutes (mean \pm SEM).

The total amount of Evan's blue (μg) in the sol (○) was calculated by multiplying sol volume by the concentration of Evan's blue ($\mu\text{g}/\text{ml}$). The amount of Evan's blue in the gel (μg) was calculated by multiplying the concentration of Evan's blue in the gel ($\mu\text{g}/\text{ml}$) by the volume of rehomogenized gel and is shown as (●)

The total amount of Evan's blue (■) represents the sum of sol and gel.

group (Table 1). Therefore, if RMCPII secretion had occurred, it was below the level of detection. Evan's blue accumulation in the intestines of primed rats was a function of time after challenge (Fig.3 $y = 74 + 3.5x$, $r=0.88$, $P < 0.001$), and there was a highly significant correlation (Fig.5) between the levels of RMCPII in the sera and Evan's blue in the gut lumen of these rats ($y = 76 + 0.26x$, $r=0.87$, $P < 0.001$).

The relationship between RMCPII secretion and permeability in shocked rats was then examined over a short time period. The treatment schedules for this experiment are summarized in Table 5. RMCPII was present in the serum of primed rats within 90 seconds of injection with worm antigen (Table 5). None was detected systemically in primed rats injected intravenously with ovalbumin or in naive rats given antigen. Analysis of the relationship between time and the concentrations of RMCPII in the sera of shocked rats showed that maximal secretion of RMCPII occurred 6 minutes after challenge, the levels 15 minutes after challenge being similar to those at 6 minutes as indicated by the regression equation of RMCPII levels in serum against time ($y = 149 + -123/x$, $r=0.85$, $P < 0.001$).

The accumulation of Evan's blue in the gut lumen (Table 6) was time-dependent, there was a highly significant correlation between the time after challenge and levels of Evan's blue in the gut lumen ($y = 40 + 21x$,

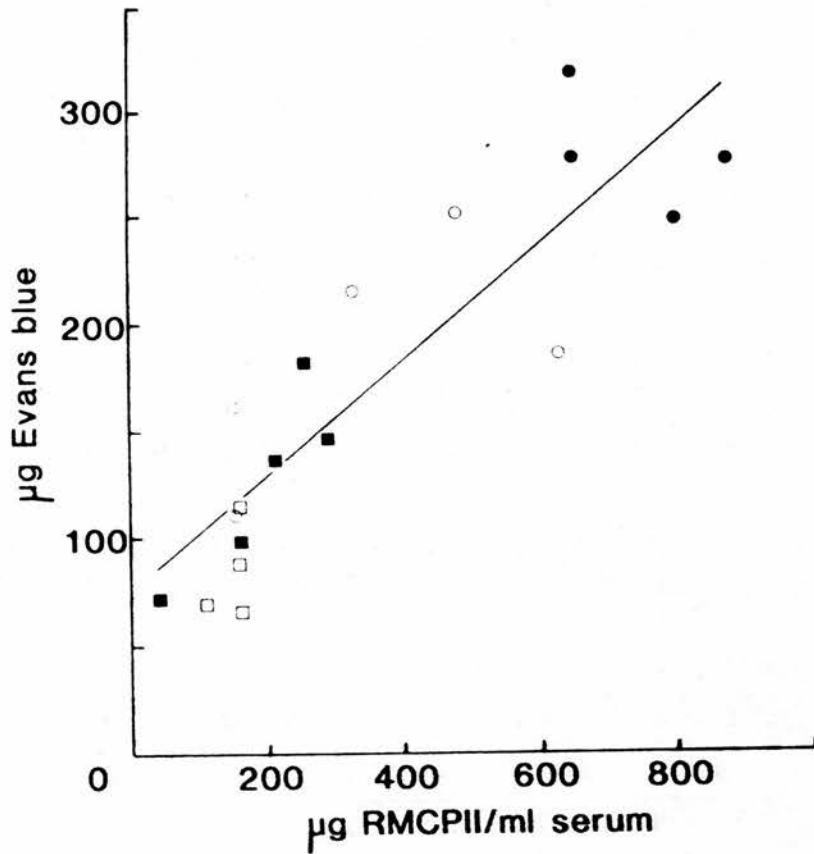


Figure 5. Regression analysis of the relationship between RMCP II secretion and levels of Evan's blue in the gut lumen. 5 (\square), 15 (\blacksquare), 30 (\circ) and 60 (\bullet) minutes after challenge.

The amount (μg) of Evan's blue represents the total from both sol and gel phases of each perfusate (see pp 144 for details).

Table 5 The concentrations of RMCPII in the sera of immune rats challenged intravenously with whole worm antigen.

Time ^a	Challenge ^b	μg RMCPII/ml serum
1.5	500 we	66 \pm 16
3.0	500 we	106 \pm 7
6.0	500 we	139 \pm 3
15.0	500 we	144 \pm 12
15.0	1% OVA	0

a Time (minutes) after challenge

b Rats were challenged intravenously with 1ml of saline containing 500 we of antigen or 1% ovalbumin (OVA) plus 2.5 mg Evan's blue/100g body weight.

Table 6 The distribution of Evan's blue between sol and gel phases of intestinal perfusates from immune rats injected intravenously with antigen or ovalbumin.

Time	μg Evan's blue (mean \pm SEM)		
	SOL	GEL	TOTAL
1.5	60 \pm 14	34 \pm 9	94 \pm 23
3.0	68 \pm 13	26 \pm 3	94 \pm 16
6.0	116 \pm 13	32 \pm 8	148 \pm 21
15.0	259 \pm 66	115 \pm 29	374 \pm 91
15.0	21 \pm 6	13 \pm 2	34 \pm 8

$r=0.80$, $P < 0.001$). There was a significant relationship between RMCPII secretion into the serum and the levels of Evan's blue in the gut perfusates ($y = 29\text{EXP}(0.013x)$), $r=0.64$, $P < 0.01$). Gross examination of the intestines of primed rats injected with worm antigen revealed considerable mucosal damage with shedding of epithelium and accumulation of Evan's blue (Table 7); the intestines were markedly hyperaemic and in some instances there was accumulation of blood within the perfusates. By contrast, the intestines of primed rats injected with ovalbumin appeared normal with little signs of gut blueing or damage.

Table 7 The effect of intravenous administration of worm antigen on the small intestine of primed rats.

Time ^a	Hyperaemia	Epithelial Shedding	Gut blueing	Gel ^b blue
1.5	4/4	3/4	3/4	4/4
3.0	3/4	2/4	4/4	4/4
6.0	3/4	3/4	4/4	4/4
15.0	4/4	4/4	4/4	4/4
15.0 ^c	0/4	0/4	0/4	0/4

a time after challenge with antigen (minutes)

b deeply stained mucus and epithelial cells

c primed rats injected with 1% ovalbumin plus Evan's blue

(the numbers in the table represent the numbers of animals affected out of the total of 4 animals/time point)

Dose-response relationship.

Eleven primed rats were allocated to four groups and rats in each group were injected intravenously with worm antigen plus Evan's blue as summarized in Table 8. All rats were killed one hour after injection of antigen and Evan's blue and the concentrations of RMCPII in serum and intraluminal levels of Evan's blue are shown in Table 8. In contrast to all other experiments, the intestine was perfused with 100ml of saline instead of 20 ml.

Both the intestinal accumulation of Evan's blue and systemic secretion of RMCPII were dose-dependent (for RMCPII $y = -549 + 146 \log x$, $r=0.75$, $P < 0.01$ and for Evan's blue $y = -226 + 56 \log x$, $r=0.76$, $P < 0.01$). There was in addition a highly significant correlation between the concentration of RMCPII in the sera and Evan's blue in the intestinal perfusates, ($y = -11 + 0.36x$, $r=0.97$, $P < 0.001$, Fig. 6).

A second experiment was carried out using twenty - nine immune and five naive rats. These were allocated to groups and treated as described in Table 9. All animals were killed one hour after injection and the concentrations of RMCPII in sera and Evan's blue in intestinal perfusates are shown in Table 9.

No RMCPII was detected in the sera of immune rats given saline or in the sera of naive rats given antigen. Despite the relatively poor response to 50-200 we there was a highly significant dose-dependent release of RMCPII

Table 8 The systemic secretion of RMCPII and the intraluminal accumulation of Evan's blue in the small intestine following challenge of primed rats with whole worm antigen.

Group	Treatment ^a	RMCPII ^b	Evan's blue ^c
1	50	24 ± 5	3 ± 4
2	100	110 ± 67	24 ± 13
3	200	264 ± 171	76 ± 69
4	400	312 ± 115	113 ± 38

a worm antigen (we) plus Evan's blue by intravenous injection.

b µg RMCPII/ml serum (mean ± SEM)

c µg Evan's blue/perfusate (mean ± SEM). Note, the intestine was perfused with 100ml of saline, see Materials and Methods, and Evan's blue was measured in the sol phase only.

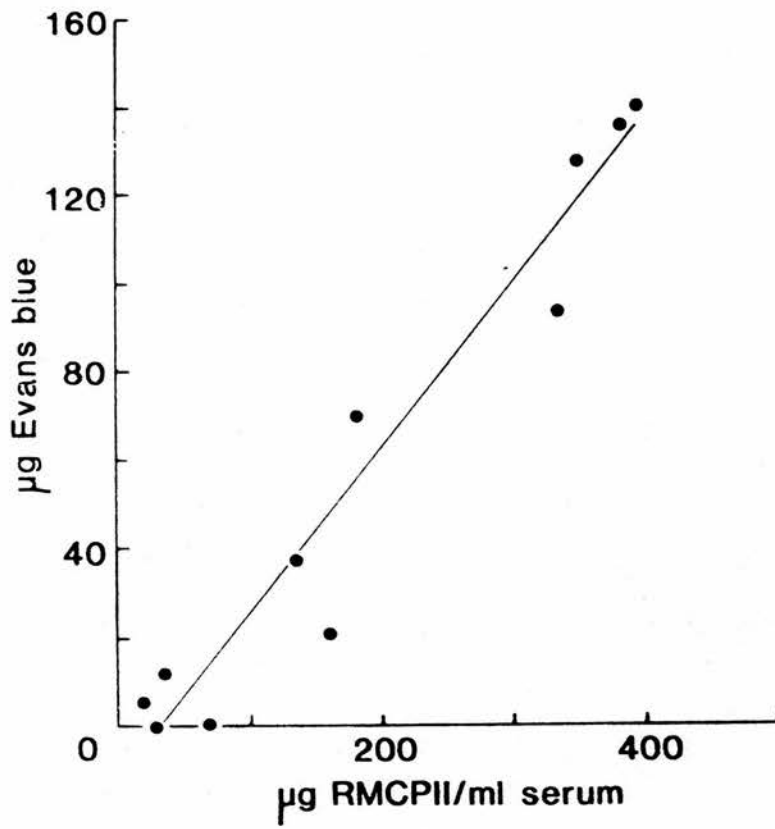


Figure 6. Regression analysis of the levels of RMCPII in the sera of primed rats and the levels of Evan's blue in the gut lumen. (total in sol phase only)

Table 9. The systemic secretion of RMCPII and levels of Evan's blue detected in the gut lumen following intravenous challenge with worm antigen.

Group	Treatment	RMCPII ^a	Evan's blue ^b
I	800 we	498 ± 148	198 ± 29
II	400 we	295 ± 147	138 ± 31
III	200 we	7 ± 2	35 ± 7
IV	100 we	6 ± 2	24 ± 2
V	50 we	11 ± 11	44 ± 11
VI	saline	0	21 ± 5
VII	400 we	0	24 ± 5

a RMCPII (µg/ml serum)

b Evan's blue (µg/perfusate)

all data presented as mean ± SEM

Groups I to VI immune

Group VII naive

in primed rats given worm antigen ($y = -51 + 0.65x$, $r=0.71$, $P < 0.001$).

The amount of Evan's blue detected in the perfusates of immune rats injected with saline and of naive recipients of antigen did not differ significantly (Table 9). Immune rats given worm antigen responded with a dose-dependent increase in the amount of Evan's blue in the gut lumen. The relationship between dose and Evan's blue was highly significant ($y = 13 + 0.23x$, $r=0.84$, $P < 0.001$). On analysis of regression, systemic RMCPII concentrations and the amount of Evan's blue in the perfusates were also significantly correlated ($y = 50 + 0.24x$, $r=0.78$, $P < 0.001$).

Discussion.

Three major findings arise from this study :

(a) RMCPII is secreted systemically in primed rats in a dose and time-dependent manner following intravenous challenge with worm antigen; (b) the enzyme is present in large amounts in the intestinal lumen of these same rats; and (c) systemic release of RMCPII is associated with substantial changes in the permeability of the intestinal mucosa as measured by an intraluminal accumulation of Evan's blue dye.

Earlier studies by Miller et al (1983) showed that the release of RMCPII into the serum was associated with an increase in mucosal permeability because Evan's blue was observed within the gut lumen of shocked rats. By using the perfusion technique of Nawa (1979), this study shows that the accumulation of Evan's blue is both time and dose-dependent and related to the systemic and enteric secretion of RMCPII.

The permeability of the intestinal mucosa was determined by measuring the intraluminal accumulation of Evan's blue dye which combines preferentially with plasma albumin (Rawson, 1943). More than 90% of Evan's blue from gut contents and sera was precipitable with 10% trichloroacetic acid this being in agreement with Nawa's (1979) observations which confirmed that the dye remained bound to protein and was not transuding as free dye (see Materials & Methods). Consequently, the measurement of

Evan's blue in the gut lumen represents the translocation of albumin molecules and reflects combined epithelial and vascular leak of protein.

The method of extraction of Evan's blue (Nawa, 1979) was modified to recover dye from the sol and gel phases of each perfusate. Data from the time course experiments shows that the gel phase contained Evan's blue as early as 90 seconds after antigen challenge, although the contribution of gel to the total was a minor one. Even after homogenization and extraction of the dye into acetone some dye remained in the pellet which indicates that the amount of Evan's blue in the gel is likely to be underestimated.

Relatively large amounts of RMCPII were present in the sol and gel phases of perfusates, even within 90 seconds of challenge of primed rats with worm antigen. Since the translocation of RMCPII into the gut lumen requires only a change in epithelial permeability (Fig.7), and the passage of Evan's blue into the gut lumen is a consequence of both vascular and epithelial permeability changes (Fig. 7), it would follow that RMCPII release and hence epithelial permeability occurred before the development of vascular changes.

The extraction of RMCPII, like that of Evan's blue, is likely to be incomplete from the gel phase, and is probably affected by the interactions of RMCPII and the gel components. The gel phase comprises mucin, epithelial

cells, and DNA from cell breakdown (Ferencz, Orskov, Orskov & Klemm, 1980) as well as considerable amounts of shed epithelium. Because RMCPII is a highly basic protein (Woodbury & Neurath, 1980) it may interact with the acidic groups present within mucin glycoproteins (Woodbury & Miller, 1982). If present in an undegraded form it could play some role in altering the solubility of intestinal mucin.

Although the recoveries of both RMCPII and Evan's blue from the perfusates were correlated, the relationship was not linear and could mean that there is a rate-limiting step ie the development of capillary permeability changes.

The high concentrations of RMCPII in the intestinal lumen are indicative of the extent of MMC secretion from the mucosa and provide the most compelling evidence that this is the major source of enzyme in blood during systemic anaphylaxis (see also Chapter 6). The function of RMCPII in vivo has not been determined. However, it has been shown to have proteolytic activity against basement membrane type IV collagen (Woodbury & Neurath, 1980) and is effective at concentrations as low as 10 ng/ml in causing the release of epithelial cells from gut slices in vitro (Woodbury, pers.commun.). RMCPI, biochemically a similar enzyme to RMCPII (Woodbury et al 1978), is additionally active in the degradation of fibronectin (Vartio, Seppa & Vaheri, 1981). It is,

therefore, possible that RMCPII is responsible for epithelial shedding, although more direct experimentation would be needed to confirm this hypothesis.

Studies by Seppa (1979) have shown that RMCPI from rat CTMC is a mediator of vascular permeability in rat skin and it is possible that RMCPII is likewise involved in the mediation of mucosal vascular permeability. However, histamine, arachidonate metabolites, and other mediators of immediate hypersensitivity (Wasserman, 1983) released from MMC in conjunction with RMCPII are likely to be the most significant mediators of vascular permeability (Fig. 7).

A possible sequence of events occurring in intestinal anaphylaxis is shown in Fig. 7. This illustration summarizes the present results and clearly shows that epithelial permeability precedes vascular changes. By measuring the accumulation of RMCPII and Evan's blue in the gut lumen and RMCPII in serum, it has been possible to dissociate the events of mucosal epithelial permeability and damage from mucosal vascular permeability and damage. However, the precise role of MMC and RMCPII in the pathogenesis of these changes remains to be determined. That RMCPII is released into the serum in Nippostrongylus primary infection (Chapter 4) further supports the view that a mast cell-mediated leak lesion in the gut may play some role in expulsion (Murray, 1972).

Intestinal anaphylaxis in the rat : sequence of events ?

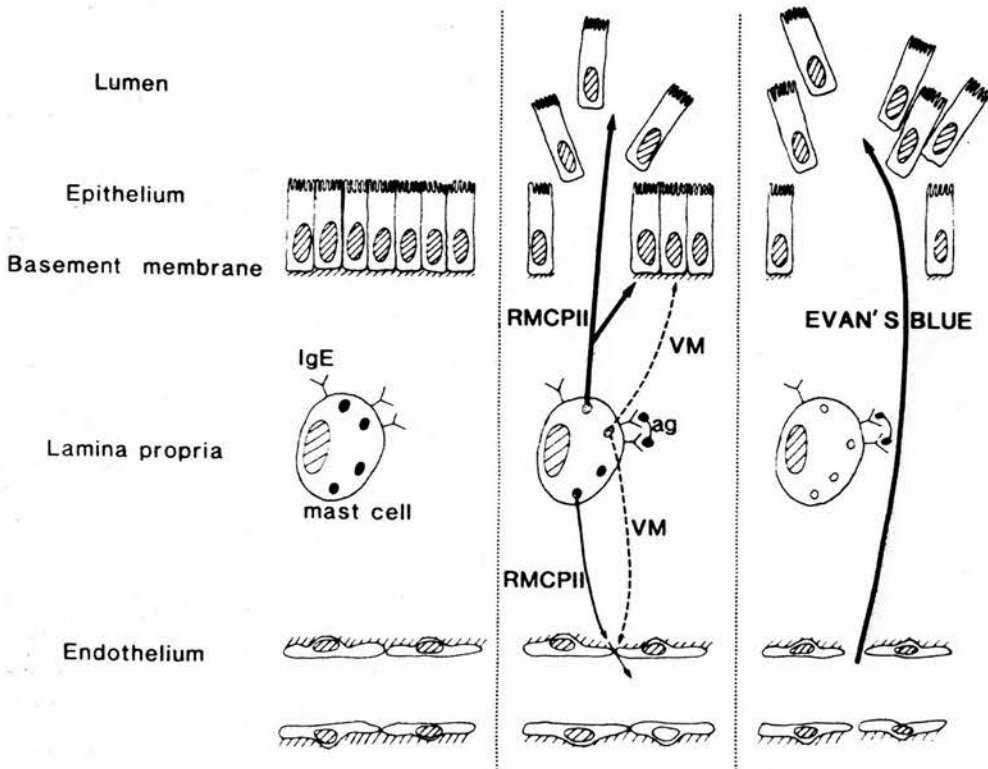


Figure 7. Intestinal anaphylaxis in the rat : sequence of events ?

Left panel. Before challenge with worm antigen. A mucosal mast cell is depicted in the lamina propria. IgE is present on the membrane of the cell. Both the epithelium and endothelium are intact.

Centre panel. Injection of antigen plus Evan's blue. Antigen (ag) cross-links the IgE molecules present on the MMC, leading to transmembrane signals and release of mediators. RMCPII acts on the basement membrane of the epithelium and causes shedding of epithelial cells. RMCPII is then able to pass directly into the gut lumen. RMCPII may also act on the endothelium of the capillary beds : RMCPII passes into the blood circulation. Other mediators (vasoactive mediators; VM) may act on either endothelium or epithelium to facilitate permeability changes and passage of RMCPII.

Right panel. The endothelium has been made more permeable and the epithelium is damaged and there is extensive shedding of epithelial cells. Evan's blue is able to pass directly into the gut lumen.

CHAPTER 6 The major source of secreted RMCPII following systemic anaphylaxis in the rat.

Introduction

Rat mucosal mast cells are characterized by the presence within their granules of rat mast cell protease II (RMCPII) which differs in antigenic properties, substrate specificity and solubility from a similar enzyme (RMCPI) present within connective tissue mast cells (Woodbury & Neurath, 1980; Yoshida et al., 1980).

The relatively high solubility of RMCPII is associated with its release into the blood circulation of Nippostrongylus-primed rats challenged either intravenously with worm antigen (Chapter 5) or intraduodenally with the homologous parasite (Miller et al., 1983). This enzyme is also released systemically during primary infection with N.brasiliensis (Chapter 4; Woodbury et al., 1984).

The major tissue source of RMCPII is not known, but published work would seem to suggest that it is the gut (Miller et al., 1983; Chapter 5). The present study was undertaken to investigate the tissue distribution of RMCPII in both naive and immune rats undergoing anaphylactic shock. The results strongly suggest that gastrointestinal MMC are the major source of RMCPII.

Experiments and Results

Kinetics of RMCPII release into blood and its concentration in enteric tissues.

Thirty rats primed twice by infection 5 weeks and 10 days previously and ten naive rats were used. The treatment schedules are summarized in Table 1; also shown are the concentrations of RMCPII in the jejunal mucosa and the numbers of MMC per villus crypt unit in the jejunum. The time course of appearance of RMCPII in the sera (Chapter 5) is included for information.

Significant decreases occurred both in the numbers of MMC and the concentration of RMCPII in the jejunum (Table 1). Reductions were most substantial 4 hours after challenge (Table 1). Analysis of regression showed the numbers of MMC and concentrations of RMCPII in the jejunum to be significantly correlated ($y = 391 + 82x$ $r=0.84$, $P < 0.001$, Fig.1).

Intestinal MMC as the major source of secreted RMCPII.

The possibility that RMCPII had been released from sources other than the gut was considered and the distribution of RMCPII was examined in other tissues of naive and immune rats. The concentrations of RMCPII in samples of lung, trachea, thymus and MLN are recorded in Table 2.

When compared with naive controls, levels of RMCPII in immune rats were significantly ($P < 0.001$) increased in

Table 1 The levels of RMCPII in jejunum and numbers of mucosal mast cells (MMC) in immune and naive rats.

Group ^a	Challenge/ Time ^b	RMCPII		MMC/VCU
		serum ^c	jejunum ^d	
I	S/30	0	3180 ± 137	33.3 ± 1.0
I	Ag/5	121	2280 ± 239 ⁺	24.3 ± 3.6 ⁺
I	Ag/15	190	2152 ± 472	18.5 ± 3.7 ⁺⁺
I	Ag/30	351	2280 ± 305 ⁺	19.4 ± 2.9 ⁺⁺⁺
I	Ag/60	630	2184 ± 289 ⁺	16.4 ± 4.8 ⁺⁺
I	Ag/240	560	1275 ± 65 ⁺⁺⁺	16.0 ± 2.5 ⁺⁺⁺
N	Ag/30	0	1260 ± 60 ⁺⁺⁺	10.8 ± 2.6 ⁺⁺⁺
N	S/30	0	796 ± 111 ⁺⁺⁺	10.1 ± 0.8 ⁺⁺⁺

a I immune N naive

b Time after challenge (min) with 500 we of antigen (Ag) or saline (S).

c µg RMCPII per ml of serum (mean ± SEM)

d µg RMCPII per g wet weight tissue (mean ± SEM)

+ P < 0.05, ++ P < 0.01, +++ P < 0.001 when compared with immune saline challenge group (Student's t-test).

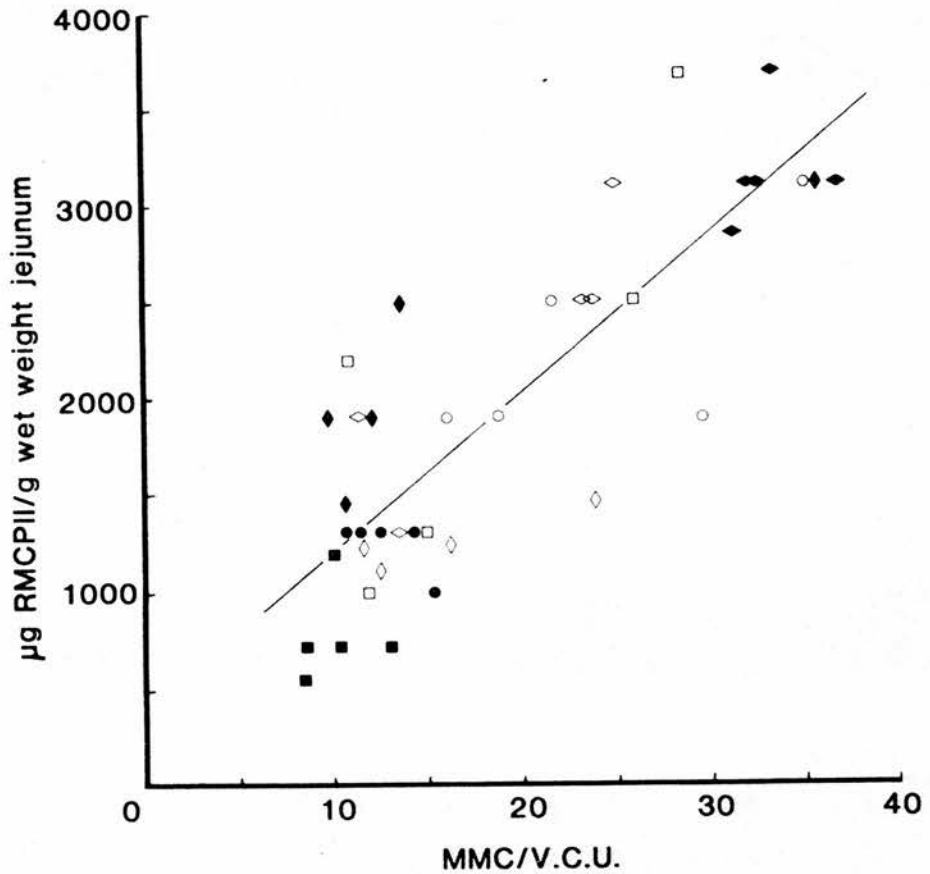


Figure 1. The relationship between the number of MMC and the concentration of RMCPII in the jejunum. Symbols for individual values were as follows :-
 Immune challenge groups (○) 5, (□) 15, (◇) 30, (◆) 60, (◇) 240 minutes after challenge. (◆) immune saline, (●) naive saline and (■) naive challenge.

Table 2 The distribution of RMCPII in tissues, other than jejunum, of naive and Nippostrongylus-primed rats.

Challenge ^a		$\mu\text{g RMCPII/g wet weight (mean } \pm \text{ SEM)}$			
	Time ^b	Lung	Thymus	MLN	Trachea
1	S/30	300 \pm 16	43 \pm 2	90 \pm 10	322 \pm 60
2	Ag/60	280 \pm 25	84 \pm 10 ⁺⁺	179 \pm 5 ⁺⁺⁺	488 \pm 48
3	Ag/240	237 \pm 24	46 \pm 1	97 \pm 8	420 \pm 67
4	S/30	45 \pm 0 ⁺⁺⁺	40 \pm 3	0 \pm 0 ⁺⁺⁺	323 \pm 18

Groups 1, 2, and 3 immune, group 4 naive

a challenge with Ag - 500 w.e. of worm antigen or S-saline

b time (min) after challenge

++ $P < 0.01$ and +++ $P < 0.001$ when compared with immune saline challenge group (Student's t-test)

lung and MLN, although it should be noted that the concentrations in jejunum were ten times those in the lung. Challenge of immune rats with antigen did not alter the concentrations of RMCPII in lung or trachea (Table 2). The amounts of RMCPII in thymus and MLN increased 1 hour after challenge (Table 2) but the levels at 4 hours were similar to those in immune controls given saline.

Histological examination of the tissues revealed that mast cell densities were increased in lung and MLN in primed rats when compared with naive controls (Table 3). Few changes were detected in mast cells in any other organ apart from the intestine (Tables 1 & 3) following challenge, nor, apart from the intestine was there evidence of generalized mast cell disruption (Table 3). Granule exocytosis was observed only in occasional mast cells in the adventitia of the thymus and tongue.

Preparations of peritoneal cells containing $1-6 \times 10^7$ mast cells from both primed and naive rats did not contain any detectable RMCPII. This was true for cells extracted in 0.15M KCL and also 1.5M KCL, and assayed by radialimmunodiffusion.

A second experiment was carried out to further examine the tissue distribution of RMCPII, and to analyse release from sites other than the jejunum. The experimental design and challenge regimes are summarized

Table 3 Assessment of mast cell changes in tissues other than gut.

Group ^a	MMC/mm ^b	mast cell density ^c			
	Trachea	Tongue	Lung	MLN	Thymus
I	46.2 ± 4.2	+	+++	+++	+
II	30.5 ± 6.7	+	+++	+++	+ ^e
III	31.0 ± 10.2	+	++ ^d	++ ^d	+ ^e
IV	34.2 ± 1.2	+	+	-	+

a group status and treatment as in Table 2

b intraepithelial mast cells per 1mm length of tracheal mucosa

c mast cell density by subjective assessment : (+++) substantially increased in immune rats when compared with naive controls in which mast cells were present (+) or absent (-)

d mast cell depletion

e exocytosis of mast cells in adventitia

in Table 4.

The concentrations of RMCPII in jejunum were compared with those in ileum and stomach, and throughout the gastrointestinal tract of immune rats there was a highly significant ($P < 0.001$) increase in RMCPII when compared with naive controls (Table 4). Antigen challenge was without effect on the concentration of RMCPII in the ileum, and only at 30 minutes after challenge did significant depletion from jejunum occur (Table 4). By contrast, there was significant depletion of RMCPII from stomach 10, 30 and 60 minutes after challenge with worm antigen (Table 4) when compared to the concentrations in immune rats given saline.

RMCPII was not detected in liver, kidney, spleen or bone marrow nor in muscle or skin homogenates prepared and assayed in 1.5M KCL.

Histological examination of jejunum and ileum revealed an increase in the numbers of MMC present in immune rats when compared with naive controls (Table 5). This was true whether MMC were detected by their content of serine esterase or proteoglycan (Table 5). Antigen challenge was without effect on the numbers of MMC in jejunum or ileum (Table 5). In agreement with previous results, there were significant correlations between the numbers of MMC in jejunum or ileum and the RMCPII content of each tissue (for jejunum; $y = 384 + 33x$, $r=0.90$, $P < 0.001$, and ileum; $y = 70 + 25x$, $r=0.92$, $P < 0.001$).

Table 4 The concentrations of RMCPII in jejunum, ileum and stomach of naive and Nippostrongylus-primed rats.

Gp ^a	Challenge ^b		$\mu\text{g RMCPII/g wet weight (mean } \pm \text{ SEM)}$		
	Time (min)		Stomach	Jejunum	Ileum
I	Ag/10		132 \pm 10 ⁺⁺⁺	2136 \pm 375	916 \pm 113
II	Ag/30		151 \pm 12 ⁺⁺	2358 \pm 220 ⁺⁺⁺	1002 \pm 120
III	Ag/60		130 \pm 8 ⁺⁺⁺	2406 \pm 253	1122 \pm 95
IV	S/30		189 \pm 5	2988 \pm 137	1104 \pm 65
V	S/30		101 \pm 5 ⁺⁺⁺	822 \pm 72 ⁺⁺⁺	253 \pm 14 ⁺⁺⁺
VI	Ag/30		105 \pm 5 ⁺⁺⁺	792 \pm 85 ⁺⁺⁺	312 \pm 26 ⁺⁺⁺

a Groups I-IV Immune, Groups V and VI naive

b Ag 500we of antigen or S saline intravenously

++ $P < 0.05$, +++ $P < 0.001$ when compared with immune rats given saline (group IV) (Student's t-test)

Table 5 Enumeration of mast cells stained for proteoglycan or esterase in jejunum and ileum of naive and immune rats given saline or worm antigen intravenously.

Group ^a	MMC/VCU (mean \pm SEM)	
	Esterase	Alcian blue
(A) I	52.9 \pm 7.9	52.0 \pm 7.6
II	55.2 \pm 7.8	56.3 \pm 8.1
III	67.0 \pm 3.7	70.0 \pm 11.0
IV	60.1 \pm 3.7	61.2 \pm 2.5
V	14.8 \pm 0.6 ⁺⁺⁺	15.4 \pm 0.6 ⁺⁺⁺
VI	17.5 \pm 0.8 ⁺⁺⁺	18.1 \pm 0.6 ⁺⁺⁺
(B) I	37.7 \pm 5.1	35.3 \pm 5.2
II	33.5 \pm 2.4	34.0 \pm 3.2
III	42.1 \pm 4.1	42.0 \pm 6.0
IV	38.0 \pm 2.8	37.3 \pm 3.7
V	10.7 \pm 0.6 ⁺⁺⁺	10.7 \pm 0.6 ⁺⁺⁺
VI	11.2 \pm 0.6 ⁺⁺⁺	12.0 \pm 1.5 ⁺⁺⁺

a group status and treatments as described in Table 4

A jejunum, B ileum

+++ $P < 0.001$ when compared with group IV (Student's t-test)

Response of CTMC to antigen challenge.

The numbers of CTMC in ear pinnae of immune and naive rats are shown in Table 6. Antigen challenge was without effect on the density of CTMC in immune and naive rats, and the mast cell counts were similar whether demonstrated by the esterase or proteoglycan content of their granules (Table 6).

Further analysis of the effect of anaphylaxis on intestinal RMCPII levels.

The lack of reaction in ileum and the sensitivity of stomach to anaphylactic shock prompted further analysis of the responses by these tissues. In addition, the effect of antigen challenge on the levels of RMCPII in colon was examined 30 minutes after challenge. The levels of RMCPII in these tissues were determined and compared to the concentrations in jejunum (Table 7). The concentrations of RMCPII in stomach, ileum and colon were, as previously, significantly ($P < 0.001$) increased in immune rats when compared with naive controls (Table 7), however the levels in these tissues were much lower when compared with those in jejunum (Table 7). There was no anaphylactic depletion of RMCPII from ileum or colon, whereas the concentrations of RMCPII in jejunum and stomach were reduced 30 minutes after challenge (Table 7) when compared with immune rats given saline.

The numbers of mast cells per unit area of mucosa

Table 6 The numbers of CTMC in the ear pinnae of immune and naive rats challenged with worm antigen or saline.

Group	Challenge ^a	mast cells/mm ² tissue ^b	
		Esterase	Alcian blue
I	Ag	57.6 ± 3.2	57.7 ± 2.5
I	S	68.7 ± 6.9	68.7 ± 3.8
N	Ag	82.0 ± 4.0	81.2 ± 4.8
N	S	73.4 ± 5.5	74.8 ± 3.8

a animals challenged intravenously with antigen (Ag) or saline (S) and killed 30 min after challenge

b the total number of mast cells in sections of ear pinnae were counted and the area of tissue, excluding cartilage, calculated by weight analysis of the tissue outline.

Alcian blue vs Esterase $y=15.5+0.78x$, $r=0.90$, $P < 0.001$
(Simple regression analysis)

Table 7 The concentrations of RMCPII at four sites along the gastrointestinal tract in naive and Nippostrongylus-primed rats.

Group ^a		$\mu\text{g RMCPII/g wet weight tissue (mean } \pm \text{ SEM)}$			
		Stomach	Jejunum	Ileum	Colon
I	I/S	189 \pm 5	2985 \pm 235	962 \pm 79	221 \pm 9
II	I/Ag	155 \pm 10 ⁺	2100 \pm 132 ⁺	930 \pm 18	215 \pm 11
III	N/S	141 \pm 11 ⁺⁺⁺	1042 \pm 43 ⁺⁺⁺	453 \pm 9 ⁺⁺⁺	159 \pm 5 ⁺⁺⁺
IV	N/Ag	131 \pm 6 ⁺⁺⁺	1074 \pm 61 ⁺⁺⁺	430 \pm 18 ⁺⁺⁺	159 \pm 6 ⁺⁺⁺

a I - immune or N - naive rats challenged intravenously with either Ag - 500 w.e. of antigen or (S) Saline
 + $P < 0.01$, ++ $P < 0.001$ when compared with group I (Student's t-test)

Table 8 The number of mast cells per unit area in stomach, jejunum, ileum and colon in naive and primed rats.

		mast cells/mm ² tissue (mean \pm SEM)			
Group ^a		Stomach	Jejunum	Ileum	Colon
I		93 \pm 3	580 \pm 22	489 \pm 19	161 \pm 11
II		66 \pm 7 ⁺⁺	391 \pm 24 ⁺⁺⁺	433 \pm 32	113 \pm 10 ⁺
III		63 \pm 5 ⁺⁺⁺	194 \pm 17 ⁺⁺⁺	176 \pm 7 ⁺⁺⁺	81 \pm 3 ⁺⁺
IV		68 \pm 3 ⁺⁺⁺	192 \pm 6 ⁺⁺⁺	176 \pm 8 ⁺⁺⁺	86 \pm 4 ⁺⁺⁺

a group status and treatment as in Table 7

+ $P < 0.01$, ++ $P < 0.005$, +++ $P < 0.001$ when compared with group I Student's t-test

are shown in Table 8. All four sites in immune rats had increased numbers of MMC when compared with those in naive controls (Table 8, $P < 0.001$). After challenge, there was a reduction in the density of mast cells in stomach ($P < 0.005$), jejunum ($P < 0.001$) and colon ($P < 0.01$) but not in ileum (Table 8). Although the density of mast cells in jejunum and ileum were similar there were 2-6 times as many cells per mm^2 in the intestine as in the colon or stomach (Table 8).

Regression analysis of the relationships between mast cell density and RMCPII concentrations are given in Table 9. At all four sites along the gastrointestinal tract the number of MMC/mm^2 correlated with the RMCPII content of the area (Table 9).

Table 9 Analysis of the relationships between mast cell density and RMCPII content of stomach, jejunum, ileum and colon.

Tissue	Equation of best fit	r	P
Stomach	$y = 67 + 1.2x$	0.72	0.001
Jejunum	$y = 155 + 4.8x$	0.97	0.001
Ileum	$y = 187 + 1.6x$	0.88	0.001
Colon	$y = 280 + -9125x$	0.70	0.001

Discussion.

This study unequivocally demonstrates that the unique serine proteinase RMCPII which is a granule product of MMC (Woodbury et al., 1978b) is most abundant in the gastrointestinal tract of both naive and Nippostrongylus-primed rats. Furthermore, following intravenous challenge of immune rats with worm antigen the concentrations of this enzyme are reduced in gastric and jejunal mucosae. In the absence of significant depletion from any other site, it would seem likely that the gut is the major source of secreted RMCPII following systemic anaphylaxis in the rat.

Analysis of other tissues showed RMCPII to be absent from most sites, and where present, the concentrations were much lower than those in the jejunal and ileal mucosae (Tables 2, 4 & 7). RMCPII was not detected in muscle, skin or peritoneal mast cells, even when assayed in the presence of 1.5M KCL, nor could RMCPII be detected in peritoneal mast cell extracts by ELISA.

Within the gastrointestinal tract itself there is considerable variation in the levels of RMCPII, the highest concentration occurred in the jejunum and corresponded with maximal numbers of MMC. However, although there was a 2-3 fold difference in concentrations of RMCPII in jejunum relative to ileum, there was no such difference in the density of mast cells in these organs. This is in agreement with earlier observations (Pitts &

Mayrhofer, 1983; Saavedra-Delgado, Turpin & Metcalfe, 1984) that when the numbers of MMC are expressed per unit area they are comparable in both jejunum and ileum.

It may be expected that for an equal weight of tissue that there would be more mucosa relative to muscle in jejunum when compared with ileum, thus a 2-3 fold difference in the amount of RMCPII may reflect a difference in the amount of mucosal tissue homogenized. Alternatively, MMC in jejunum may contain more RMCPII than their counterparts in ileum.

The density of mast cells and the concentrations of RMCPII were, by regression analysis, highly correlated in stomach, jejunum, ileum and colon (Table 9). Within each tissue an increase in the concentration of RMCPII was accompanied by a similar increase in MMC density following infection with N.brasiliensis. This increase was maximal in the jejunum of infected rats, there being almost a 3-fold increase in both mast cells and RMCPII concentrations (Table 7).

Increased levels of RMCPII were also recorded in lung and MLN after infection with N.brasiliensis. Mast cell infiltration of MLN has been reported in Nippostrongylus-infected rats (Keller, Cottier & Hess, 1974) and pulmonary mastocytosis has been observed to be associated with larval migration (Wells, 1971). The results suggest that a proportion of the additional mast cells induced by parasite infection are MMC.

Depletion of RMCPII occurred only in jejunum and stomach following anaphylactic shock and was associated with a decrease in the number of mast cells in these tissues. Although there was also significant depletion of MMC from colonic mucosae this was not reflected by RMCPII concentrations which remained unaltered. The reasons for this are not clear, although it is possible that released enzyme is not so rapidly cleared from colonic tissues as from stomach and jejunum. Neither the number of MMC nor the concentration of RMCPII in ileum was altered following anaphylaxis. In all four tissues (Table 7), infection with N.brasiliensis provoked a highly significant increase in the density of MMC when compared with uninfected controls.

One interesting finding was that thymus and MLN had increased levels of RMCPII 60 minutes after challenge with worm antigen. Serum levels of RMCPII were maximal at this time and the lymphatic drainage from the gut to MLN could be a source of increased enzyme concentrations in these tissues. Since haemoconcentration is a characteristic feature of anaphylaxis in the rat (Sanyal & West, 1958), an alternative explanation is that experimental animals were insufficiently exsanguinated; the increased levels in thymus and MLN might reflect the accumulation of plasma-derived RMCPII.

In all instances, the number of MMC detected by their content of esterase or GAG were very similar (Table 5)

indicating that when MMC are depleted from the mucosa both esterase (RMCPII) and proteoglycan are lost from their granules. This histochemical observation is further supported by a study in which the GAG content of plasma and the concentration of RMCPII in the sera of shocked rats were greatly increased, there being a highly significant correlation between the circulating levels of these two products (Chapter 8).

Few changes were recorded amongst the CTMC in the tongue or adventitia of the thymus following anaphylactic shock (Table 3). Furthermore, quantitative studies failed to show any depletion of CTMC from the pinna of the ear (Table 6) although, in this particular experiment, anaphylactic shock was not severe and there was little depletion of MMC from the enteric mucosa (Table 4).

The release of RMCPII and its depletion from the jejunum may be related to the gross mucosal damage associated with systemic anaphylaxis in the rat. The lesions occurring during anaphylactic enteropathy include epithelial shedding, hyperaemia, mucus secretion and increased mucosal permeability (Urquhart *et al.*, 1965; Miller *et al.*, 1983; Chapter 5). A study of the distribution of secreted RMCPII showed that 1-2 mg of RMCPII was secreted into the intestinal lumen within 5 minutes of intravenous challenge with worm antigen these high levels of enzyme persisted for up to 60

minutes after challenge (Chapter 5). The latter observations are perhaps the most compelling evidence that the gut is the major source of secreted RMCPII.

In summary, the present results add further support to previous studies (Miller et al., 1983; Chapter 5) suggesting that the measurement of extracellular RMCPII provides a unique and selective assay for monitoring the in vivo activity, predominantly of gastrointestinal MMC, in hypersensitivity and other immunologically-mediated responses. They do so by demonstrating that the gut mucosa is the major source of RMCPII in naive and Nippostrongylus-immune rats and it would seem likely that RMCPII released during N.brasiliensis primary infection is of intestinal mast cell origin.

CHAPTER 7 The effect of glucocorticosteroids on MMC and intestinal anaphylaxis.

Introduction

Corticosteroids are amongst the most potent and widely used drugs in the treatment of allergic and inflammatory conditions in man and domestic animals. Although they act at a number of different levels and on a variety of cell types perhaps their most significant anti-allergic effect is in preventing the generation of inflammatory mediators (rev. Fahey, Guyre & Munck, 1981; Fauci, 1978). For example, they suppress histamine release from isolated rat and murine mast cells (Marquardt & Wasserman, 1983; Daeron, Sterk, Hirata & Ishizaka, 1982) and human basophils (Schleimer, Lichtenstein & Gillespie, 1981). Corticosteroids may also act indirectly to prevent the generation of secondarily formed mediators of arachidonate metabolism by inhibition of phospholipase (Heinman & Crews, 1984a; Danon & Assouline, 1978; Blackwell & Flower, 1979).

Anaphylactic mortality in the rat is prevented by prior treatment with corticosteroid (Laddu & Sanyal, 1978), and anaphylactic bronchoconstriction is abrogated by prior treatment of sensitized rats with dexamethasone (Church, Collier & James, 1972). Steroids are also known to suppress mucosal mastocytosis in nematode infections (Jarrett et al., 1967; Olsen & Schiller, 1978) and to suppress anaphylactic pathology in N.brasiliensis-primed rats challenged intravenously with worm antigen (Urquhart et al., 1965).

In this chapter the results of measuring the concentration of RMCPII in blood and tissues are presented in order to analyse the anti-anaphylactic activity of corticosteroids and to determine their effect on MMC in rats subjected to anaphylactic shock.

Experiments and Results

Intestinal permeability and RMCPII secretion.

Rats were allocated to groups and were pretreated with saline or methylprednisolone acetate (25 mg/kg body weight) by intramuscular injection 24 and 48 hours before they were challenged intravenously with Evan's blue dissolved in either saline alone or saline containing 500 we of worm antigen. Treatment schedules are summarized in Table 1.

A highly significant ($P < 0.001$) four-fold increase in Evan's blue in the intestinal lumen of sensitized rats occurred after challenge with worm antigen (Group 1, Table 2). This response was totally suppressed in sensitized rats that had been pretreated with prednisolone (Group 2, Table 2). There was release of RMCPII into the blood and intestinal lumen of rats in Group 1, but this response was again totally suppressed ($P < 0.001$, Mann-Whitney U-test) in rats pretreated with corticosteroid (Group 2, Table 2). Saline injection of either untreated or steroid-treated sensitized rats, and antigen injection into either untreated or steroid-treated naive rats did not induce any significant alteration of mucosal permeability or release of RMCPII (Table 2).

Table 1 Treatment schedules to determine the effects of glucocorticosteroids on MMC and intestinal anaphylaxis.

Group	Status ^a	Pretreatment ^b	Challenge ^c
1	Immune	S	Ag
2	Immune	C	Ag
3	Immune	S	S
4	Immune	C	S
5	Naive	S	Ag
6	Naive	C	Ag
7	Naive	S	S
8	Naive	C	S

a Immune animals infected 1 and 3 weeks earlier with 5000 N.brasiliensis larvae

b S saline C methylprednisolone acetate

c Ag 500 we of antigen S saline

Rats were killed one hour after challenge.

Table 2 Measurement of gut permeability and RMCPII release during anaphylaxis in normal and corticosteroid treated rats.

Group ^a	Evan's blue	RMCPII	
	$\mu\text{g}/\text{perfusate}$	$\mu\text{g}/\text{ml serum}$	$\mu\text{g}/\text{perfusate}$
1	$406 \pm 41^+$	$90 \pm 19^*$	$797 \pm 250^*$
2	88 ± 8	0	0
3	104 ± 2	0	0
4	124 ± 8	0	0
5	64 ± 4	0	0
6	51 ± 6	0	0
7	72 ± 5	0	0
8	69 ± 5	0	0

+ $P < 0.001$ when compared with all other groups (Student's t-test).

* $P < 0.001$ when compared with all other groups (Mann-Whitney U-test)

a Group status and treatment as described in Table 1

Tissue distribution of RMCPII following steroid treatment.

(a) Jejunum; pretreatment of rats with methylprednisolone significantly ($P < 0.001$) depleted the jejunum of RMCPII in both immune and naive rats (Table 3). Antigen challenge of sensitized rats was without effect on the mucosal concentration of RMCPII when compared to that in immune rats challenged with saline (Table 3).

(b) Ileum; following pretreatment with corticosteroids, RMCPII was significantly ($P < 0.001$) depleted from both immune and naive rats when compared with saline injected controls (Table 4).

(c) Lung; corticosteroids reduced the concentration of RMCPII in lung homogenates from immune rats (Table 5). The concentrations in homogenates from naive rats were too low ($\leq 10\mu\text{g/g}$ tissue) to be measured by radial-immunodiffusion, but trace amounts of enzyme were detected in naive animals that had not been treated with corticosteroid (Table 5).

(d) Mesenteric lymph node; the concentrations of RMCPII in the nodes of immune rats challenged with antigen was unaltered when compared with those in immune rats given saline, but corticosteroids depleted the immunized node of RMCPII (Table 5). The values in nodes of untreated

Table 3 The effect of anaphylaxis and corticosteroid treatment on the concentration of RMCPII ($\mu\text{g/g}$ wet weight tissue) and number of MMC in the jejunum of immune and naive rats.

Group ^a	RMCPII	MMC/VCU	
		Alcian blue	Esterase
1	1780 \pm 120	29.6 \pm 1.7	30.0 \pm 2.0
2	215 \pm 25 ⁺	3.3 \pm 0.3 ⁺	3.6 \pm 0.2 ⁺
3	1840 \pm 84	32.6 \pm 1.0	32.9 \pm 1.5
4	165 \pm 33 ⁺	3.8 \pm 0.4 ⁺	4.0 \pm 0.3 ⁺
5	534 \pm 51	6.8 \pm 0.5	7.7 \pm 0.3
6	252 \pm 57 ⁺	2.1 \pm 0.2 ⁺	2.5 \pm 0.2 ⁺
7	576 \pm 51	7.5 \pm 0.5	8.3 \pm 0.5
8	210 \pm 30 ⁺	2.4 \pm 0.2 ⁺	2.4 \pm 0.2 ⁺

a group status and treatment as described in Table 1

+ $P < 0.001$ significant depletion of RMCPII and MMC following steroid treatment when compared with saline controls

Regression analysis

Alcian blue vs Esterase $y=0.45+0.99x$, $r=0.99$

Esterase vs RMCPII $y=6408+54.9x$, $r=0.97$

All data presented as mean \pm SEM

Table 4 The effect of anaphylaxis or corticosteroid treatment on the numbers of MMC and concentration of RMCPII in the ileum of immune and naive rats.

Group ^a	RMCPII ^b	MMC/VCU	
		Alcian blue	Esterase
1	471 ± 19	15.9 ± 1.5	16.0 ± 1.4
2	106 ± 10 ⁺	2.2 ± 0.4 ⁺	2.4 ± 0.4 ⁺
3	517 ± 13	17.4 ± 0.8	18.0 ± 1.0
4	98 ± 5 ⁺	2.8 ± 0.3 ⁺	2.8 ± 0.1 ⁺
5	195 ± 10	4.0 ± 0.1	4.3 ± 0.1
6	61 ± 5 ⁺	1.4 ± 0.2 ⁺	1.3 ± 0.2 ⁺
7	200 ± 6	4.2 ± 0.2	4.4 ± 0.4
8	60 ± 0 ⁺	1.4 ± 0.2 ⁺	1.4 ± 0.1 ⁺

a group status and treatments as described in Table 1

b µg RMCPII/g wet weight tissue

+ P < 0.001 significant depletion following steroid treatment (Table 3)

Regression analysis

Alcian blue vs Esterase $y=0.07+1.0x$, $r=0.99$

Esterase vs RMCPII $y=58.7+24x$, $r=0.92$

All data presented as mean ± SEM

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naive rats were, like those in lung, at the limit of the detection of the assay system (Table 5).

Mast cell changes following corticosteroid treatment.

Pretreatment of rats with corticosteroid significantly ($P < 0.001$) reduced the numbers of MMC in the jejunum (Fig.1) and ileum of naive and immune rats. The results were similar whether the cells were detected with Alcian blue or by their content of non-specific esterase (Tables 3 & 4). The numbers of MMC in the jejunal or ileal mucosa of primed rats were unaltered after challenge with worm antigen (Tables 3 & 4). Because of the anisotropic distribution of mast cells in lung and MLN, a semi-quantitative assessment of cell density was employed. Glucocorticoid treatment of sensitized rats caused an apparent reduction in density of mast cells in both tissues, whereas antigen challenge was without effect (Table 5). Relatively few mast cells were present in the lungs of naive rats and in the MLN they were distributed in the capsule with few being located in the cortex. Corticosteroids had no discernible effect on pulmonary mast cells in naive rats, but slightly reduced the density of mast cells in the cortex of MLN (Table 5).

Table 5 The concentration of RMCPII and numbers of MMC in lung and MLN of naive and immune rats after anaphylactic shock or corticosteroid treatment.

Group ^a	$\mu\text{g RMCPII/g tissue}$ (mean \pm SEM)		mast cell density	
	Lung	MLN	Lung	MLN
1	219 \pm 24	86 \pm 16	+++	+++
2	126 \pm 16 ⁺⁺	10 [*]	++ ^o	++ ^o
3	182 \pm 17	90 \pm 0	+++	+++
4	117 \pm 14 ⁺	10 [*]	++ ^o	++ ^o
5	10	10	+	+
6	0	0	+	\pm ^o
7	10	10	+	+
8	0	0	+	\pm ^o

a group status and treatment as in Table 1

+ P < 0.05 ++ P < 0.01 (Student's T-test)

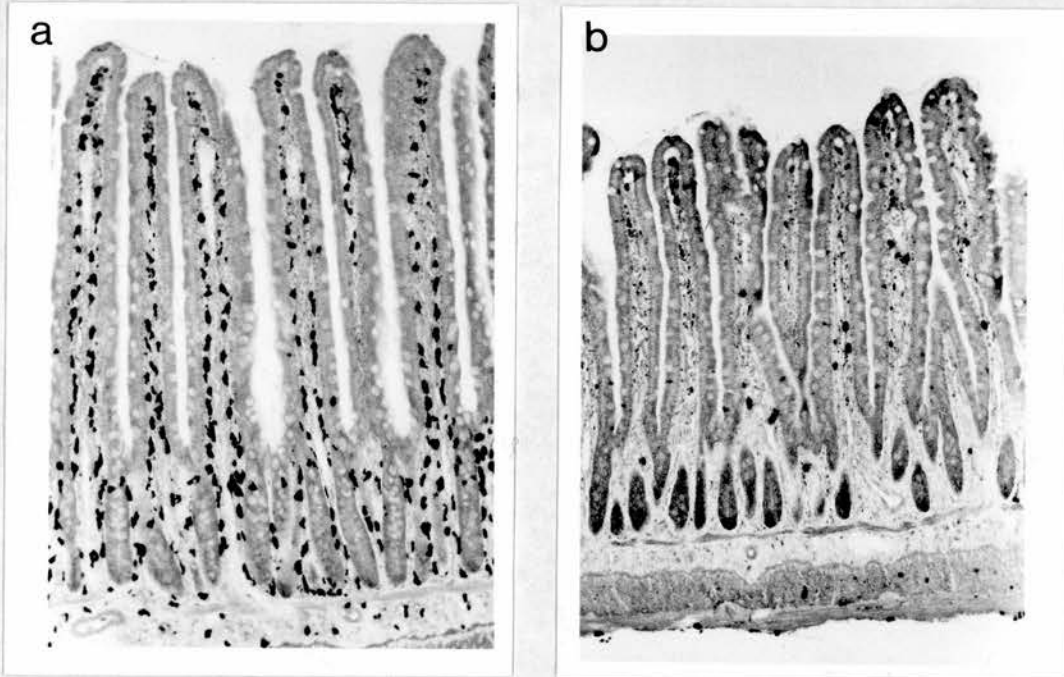
* P < 0.001 (MannWhitney U-test) significant depletion following corticosteroid treatment

subjective assessment of mast cell density:- (+++)

substantial increase when compared with naive controls (+)

o some depletion of mast cells following steroid treatment

Figure 1. Tissue sections stained with naphthol AS-D chloroacetate from (a) saline and (b) corticosteroid-treated rats. Note the substantial depletion of MMC after treatment. (Magnification x 275).



The response of CTMC to corticosteroid treatment.

Corticosteroid treatment did not alter the numbers of mast cells in the ear pinnae of naive rats (Table 6). Recipients of saline had 56 ± 4 mast cells/mm² tissue and, in corticosteroid treated rats there were 53 ± 3 mast cells/mm² tissue. Similarly, antigen challenge of naive rats was without effect on CTMC (53 ± 6 mast cells/mm²). The numbers of CTMC stained with Alcian blue when compared with the numbers containing esterase activity were, by simple regression analysis, significantly correlated ($y = 12.8 + 0.78x$, $r=0.81$, $P < 0.001$).

PCA reactivity of serum following corticosteroid treatment.

Serum taken from rats before they were treated with methylprednisolone (PCA titre 280 ± 114) and serum taken from the¹. Similarly, PCA titres measured before saline treatment of immune control rats (176 ± 39) were unaltered 48 hours later.

The effect of corticosteroid treatment on PCA.

The PCA titre (≤ 40) in naive rats injected intradermally with immune serum and then treated with methylprednisolone was significantly ($P < 0.001$) reduced when compared with similarly sensitized rats (352 ± 78) treated with saline (Fig.2).

¹ same rats immediately before antigen challenge (PCA, 280 ± 114) had identical PCA values.

Table 6 The effect of corticosteroid treatment on CTMC in the pinna of the ear of naive rats.

Treatment ^a	mast cells/mm ² tissue	
	Alcian blue	Esterase
C/Ag	55 ± 6	54 ± 6
S/Ag	53 ± 6	55 ± 6
S/S	56 ± 4	54 ± 2
C/S	53 ± 3	53 ± 1

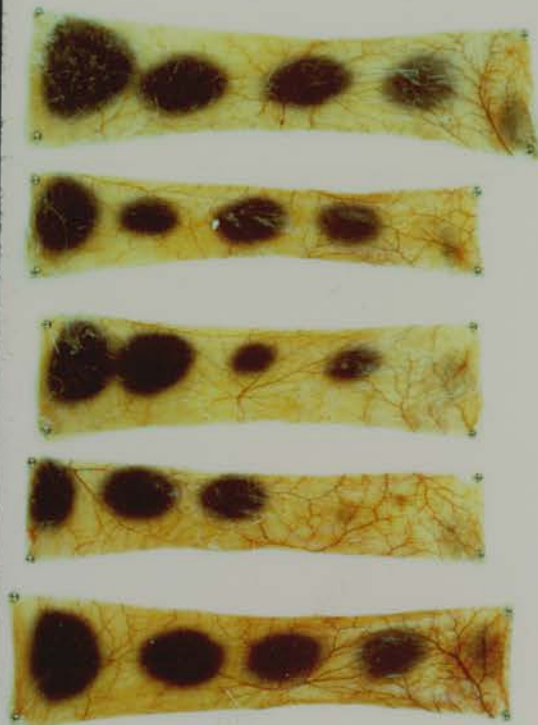
a see Table 1 for treatment schedules

Alcian blue vs Esterase $y=12.8+0.78x$, $r=0.81$, $P < 0.001$

Figure 2. The effect of corticosteroid pretreatment on rat homologous passive cutaneous anaphylaxis.

- (a) pretreated with saline.
- (b) pretreated with 25 mg/kg methylprednisolone.

(a)



x40

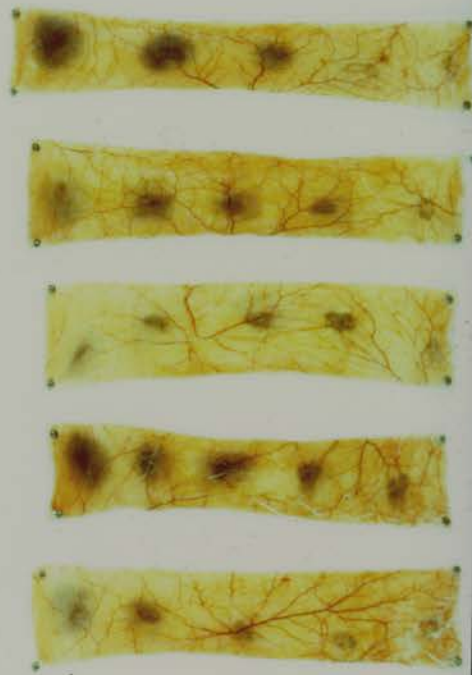
x80

x160

x320

x640

(b)



x 40

x 80

x160

x320

x640

Levels of RMCPII in blood after depletion of MMC by steroids.

In order to determine whether steroid induced depletion of MMC resulted in the release of RMCPII into blood, 15 naive rats, allocated to 3 groups, were bled via the tail vein and immediately injected intramuscularly with saline, methylprednisolone (25 mg/kg) or betamethasone (2 mg/kg). They were bled 1, 4 and 24 hours later and rats given betamethasone exsanguinated at 24 hours. The remaining 10 rats were given a second dose of saline or methylprednisolone and blood samples were taken 1 and 4 hours later; all were exsanguinated 24 hours after the second injection (Table 7).

At no time was RMCPII detected in the blood by ELISA. There was, however, depletion of MMC in rats treated with methylprednisolone (3.7 ± 0.5 MMC/VCU) or betamethasone (5.6 ± 0.2 MMC/VCU) when compared with saline treated controls (10.3 ± 0.5 MMC/VCU). The numbers of MMC detected by either esterase stain or Alcian blue were very similar and by regression analysis there was a highly significant correlation between the two staining methods ($y = -0.19 + 0.94x$, $r=0.93$, $P < 0.001$). Similarly, the concentration of RMCPII was reduced ($P < 0.001$) in the jejunum of rats treated with either methylprednisolone ($313 \mu\text{g/g} \pm 48$) or betamethasone (493 ± 33) when compared with saline controls (784 ± 24). There was, in addition, a highly significant correlation between the numbers of MMC detected

Table 7 The effect of pretreatment of naive rats with betamethasone or methylprednisolone on the concentration of RMCPII and numbers of MMC in the jejunum.

	$\mu\text{g RMCPII/g wet wt}$	MMC/VCU	
		Alcian blue	Esterase
A	493 ± 33	5.6 ± 0.2	4.4 ± 0.1
B	313 ± 48	3.7 ± 0.5	3.6 ± 0.4
C	784 ± 24	10.3 ± 0.5	9.7 ± 0.5

A 2 mg/kg betamethasone 24 hours previously

B 25 mg/kg methylprednisolone 24 and 48 hours previously

C saline 24 and 48 hours previously

Treatment with either betamethasone or methylprednisolone caused a significant ($P < 0.001$) reduction in both RMCPII and number of MMC (Student's t-test).

with esterase stain and the concentration of RMCPII in the jejunum of the three groups of rats ($y = 164 + 61x$, $r=0.87$, $P < 0.001$).

Dose-response relationship.

Rats immunized twice by infection with N.brasiliensis were injected intramuscularly with saline or with 1, 5, or 25 mg/kg methylprednisolone acetate 24 and 48 hours before they were bled out and killed. When compared with saline injected controls, methylprednisolone caused a dose-dependent depletion of RMCPII from the mucosa, (Table 8). The data fitted the following regression equation : ($y = x/-0.002 + 0.004x$, $r=0.81$) indicating that 5 mg/kg methylprednisolone was as effective as 25 mg/kg in depleting MMC from the jejunal mucosa. Furthermore, the reduction in numbers of MMC (esterase) was highly correlated with the loss of jejunal RMCPII ($y = -69 + 61x$, $r=0.98$).

Time Course of Mast Cell Changes.

Immune rats were given intramuscular injections of 25 mg/kg methylprednisolone and groups of 4 were killed 1, 4, and 24 hours after steroid treatment. An additional 4 rats were killed 24 hours after saline treatment. No significant depletion of RMCPII or MMC from the jejunum was detected until 24 hours after corticosteroid treatment (Table 9). These data would indicate that mast cell changes occur between 4 and 24 hours after steroid

Table 8 . The effect of dose of methylprednisolone on the concentration of RMCPII and number of MMC in the jejunum of immune rats.

Dose ^a	RMCPII ^b	MMC/VCU	
		Alcian blue	Esterase
Saline	1935 ± 152	33.7 ± 1.4	32.4 ± 1.0
1	487 ± 45*	12.3 ± 0.7*	10.4 ± 0.7*
5	285 ± 29*	6.3 ± 0.5*	5.9 ± 0.2*
25	292 ± 33*	4.5 ± 0.5*	4.7 ± 0.2*

* significant depletion ($P < 0.001$) when compared with saline controls (Student's t-test)

a dose of methylprednisolone (mg/kg body weight)

Regression analysis

Alcian blue vs Esterase $y = -0.45 + 0.99x$, $r = 0.99$)

b $\mu\text{g RMCPII/g tissue}$

administration.

Table 9 The effect of time after administration of methylprednisolone on the concentration of RMCPII and number of MMC in the jejunum of immune rats.

<u>Time</u> (hr)	RMCPII ^b	<u>MMC/VCU</u>	
		Alcian blue	Esterase
1	1357 ± 185	22.8 ± 1.1	22.7 ± 2.0
4	1327 ± 82	23.7 ± 1.2	22.6 ± 0.9
24	570 ± 30*	7.6 ± 0.5*	8.0 ± 0.7*
24 ^a	1395 ± 169	23.3 ± 2.2	24.9 ± 1.3

a saline treated control, all other groups were given 2 x 25 mg/kg methylprednisolone

* P < 0.001 when compared with 24 hour saline group
all data presented as mean ± SEM

Regression analysis

Alcian blue vs Esterase, $y = 0.84 + 0.96x$, $r=0.97$

b $\mu\text{g RMCPII/g tissue}$

Discussion

The two principal effects of corticosteroid treatment described in this study are (a) suppression of the anaphylactic release of RMCPII into intestinal secretions and (b) depletion of MMC and RMCPII from the intestinal mucosa. These events may, therefore, be related to the suppression of intestinal anaphylaxis.

These results confirm previous observations that corticosteroids suppress the development of anaphylactic pathology in the gut (Urquhart et al., 1965) there being no detectable permeability changes in corticosteroid treated rats challenged with worm antigen (Table 2). In addition, they extend earlier observations by showing that fully mature MMC in normal and immune rats are depleted following corticosteroid treatment. By contrast, CTMC in the pinna of the ear were apparently unaffected. The latter finding indicates a further important functional difference between CTMC and MMC in the rat.

Significant depletion of MMC and RMCPII occurred between 4 and 24 hours after injection of steroid, and may explain why in previous studies (Church & Miller, 1978) optimal inhibition of anaphylactic bronchoconstriction was not observed until 12-24 hours after administration of dexamethasone. The extreme sensitivity of MMC to steroid was indicated by the fact that as little as 1 mg/kg methylprednisolone caused a significant reduction in their numbers.

Although depletion of MMC occurred 4-24 hours after methylprednisolone treatment there was no detectable release of RMCPII into the serum at 1, 4 or 24 hours. It is possible that mast cells die without releasing granule products or are lysed following steroid treatment as has been reported for rat thymocytes (Munck & Brinck-Johnsen, 1968).

Corticosteroid treatment of rats passively sensitized intradermally with immune serum caused a significant but incomplete inhibition of passive cutaneous anaphylaxis. Since the binding of IgE is not impaired by steroids (Daeron et al., 1982; Marquardt & Wasserman, 1983) and because in this study, methylprednisolone failed to suppress the circulating levels of IgE, two mechanisms of inhibition may be considered: (i) inhibition of mediator release and (ii) modification of the response of target organs to mast cell-derived mediators. The extent to which the intestinal response was suppressed by the known capacity of steroids to decrease permeability in vascular beds (Benditt, Schiller, Wong & Dorfman, 1950) or cause vasoconstriction (Barry & Woodford, 1974) cannot be determined from the present data. It is, however, possible that both mechanisms operate in the suppression of intestinal permeability changes.

Many possible mechanisms have been put forward to explain corticosteroid-mediated inhibition of mediator release from CTMC, but it is not clear if they are

directly applicable to MMC. Changes in intracellular c-AMP, which would favour inhibition of mediator release, are unchanged in both dexamethasone treated rat and murine mast cells (Marquardt & Wasserman, 1983; Daeron et al., 1982). Effects both upon arachidonic acid metabolism (Hong & Levine, 1976) and inhibition of its release from the cell membrane by lipomodulin-like proteins (Hirata, Schiffman, Venkatsubramanian, Salomon & Axelrod, 1980; Blackwell, Carnuccio, Di Rosa, Flower, Parente & Persico, 1980; Blackwell & Flower, 1983) have been considered important in the suppression of mast cell function. Another interesting hypothesis is that steroids impair mitochondrial function (Grosman & Jensen, 1984) and it may be that MMC have a greater energy requirement than CTMC. The precise action of steroids in the inhibition of mast cell exocytosis remains obscure. However, a mechanism which takes into account many earlier observations has recently been proposed (Heinman & Crews, 1984b) suggesting that the steroid would act via a cytoplasmic receptor which when activated migrates to the nucleus and induces the synthesis of specific proteins (lipomodulin ?). These proteins would then interfere with the coupling of IgE to calcium influx. Phospholipid methylation has been implicated in the coupling of IgE to calcium influx and the release of arachidonic acid, phospholipid methylation being inhibited by steroids (Daeron et al., 1982). The result would be an inhibition of the calcium-dependent

release of mast cell mediators.

Corticosteroids are known to inhibit goblet cell differentiation (Miller & Nawa, 1979a), to prevent self-cure of N.brasiliensis infected rats (Ogilvie, 1965; Jarrett et al., 1967), to inhibit rapid expulsion of N.brasiliensis (Miller & Huntley, 1982b) and T,spiralis (Bell et al., 1982) and to abrogate both immune exclusion and mucus trapping in N.brasiliensis-primed rats (Miller & Huntley, 1982b). These mechanisms are thought to involve an immediate hypersensitivity reaction (rev. Miller, 1984) and it is therefore possible that the corticosteroid-mediated suppression of worm expulsion may be related to the capacity of corticosteroids to deplete fully mature MMC from the intestinal mucosa.

CHAPTER 8 The presence in blood of both glycosaminoglycan and RMCPII following systemic anaphylaxis in the rat.

Introduction

A theme throughout this study has been the high correlation between the numbers of MMC containing esterase and those containing glycosaminoglycan (GAG). Since the number of MMC that stain either for esterase or GAG were substantially reduced following anaphylactic shock and this was associated with the appearance, in blood, of RMCPII it follows that GAG might also be detected in the blood of shocked rats.

The results show that there is indeed a systemic release of GAG which was highly correlated with the levels of RMCPII in blood thereby suggesting that GAG released during anaphylaxis could be of MMC origin.

Experiments and Results

Fifteen female rats immunized 3 and 8 weeks earlier with 6000 N.brasiliensis larvae were randomly allocated to 3 groups of 4 and one group of 3 rats and were challenged intravenously with 1000, 500, or 250 worm equivalents (we) of adult worm antigen or saline. For control purposes 4 naive rats were challenged with 500 we intravenously. One hour after challenge the animals were exsanguinated under ether anaesthesia and the concentrations of GAG and RMCPII determined in blood plasma and serum respectively and in gut perfusates and mucosal homogenates.

RMCPII was present only in the sera of immune rats challenged with worm antigen (Table 1) and in these same rats there was a 10-fold increase in the concentration of GAG in plasma when compared with immune recipients of saline, or naive rats given antigen (Table 1, Fig.1). Although, in this particular experiment, the release of RMCPII and GAG was independent of the dose of antigen, there was a highly significant correlation between the concentrations of RMCPII and GAG ($y = 2110 + 17x$, $r=0.93$, $P < 0.001$; Fig.2) which would indicate that they may have been released from the same source.

Intestinal perfusates from primed, challenged rats contained 1144-2085 μ g RMCPII (Fig.1), whereas perfusates from control animals were devoid of enzyme (Table 2). There was, in addition, a concomitant, highly significant

Table 1 The concentrations of RMCPII and GAG in the blood of naive and immune rats challenged intravenously with worm antigen or saline.

Group	Challenge ^a	RMCPII	GAG
		($\mu\text{g/ml}$ serum)	(ng/ml plasma)
1	500 we	0	1658 \pm 358
2	Saline	0	1396 \pm 160
3	1000 we	770 \pm 65 ⁺	14655 \pm 1558 [*]
4	500 we	670 \pm 125 ⁺	15831 \pm 1630 [*]
5	250 we	635 \pm 51 ⁺	14195 \pm 898 [*]

Group 1 -naive, Groups 2-5 -immune

a rats challenged intravenously with worm antigen or saline.

+ $P < 0.001$ when compared with group 2 (Mann-Whitney U-test)

* $P < 0.001$ when compared with group 2 (Student's t-test)

all data presented as mean \pm SEM

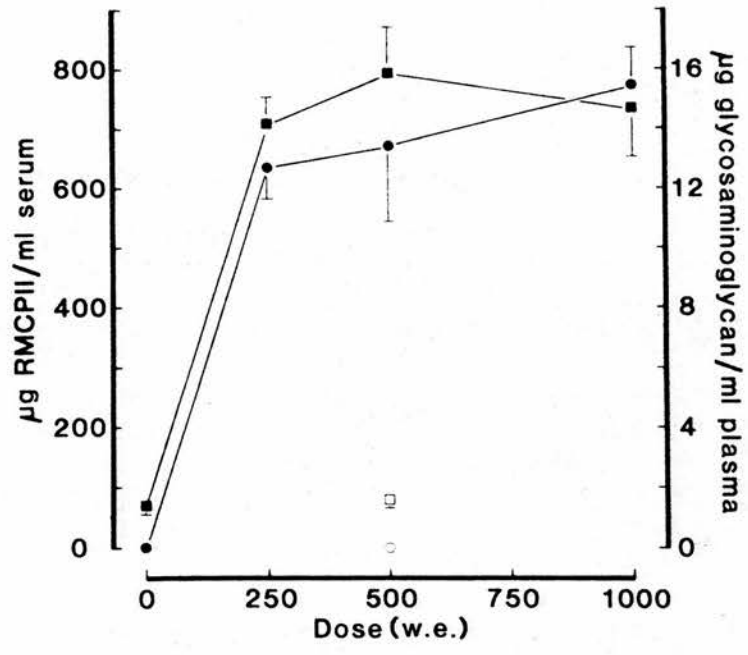
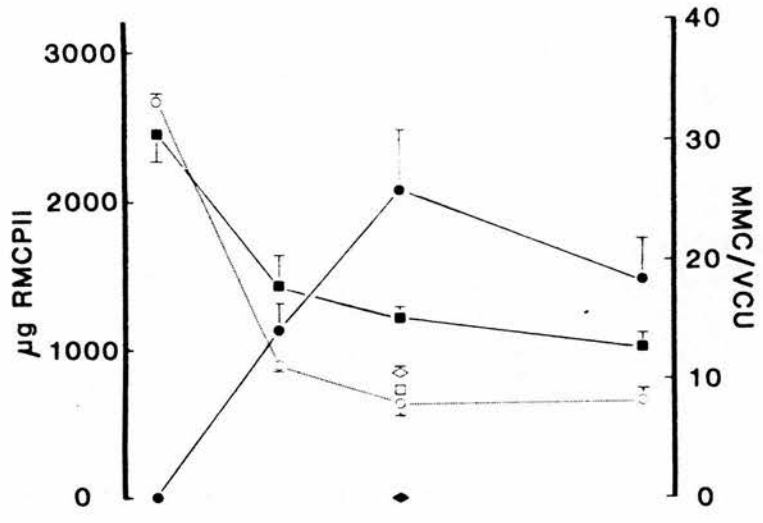


Figure 1. Top panel. Total recoveries of RMCPII (mean \pm SEM) in intestinal perfusates (\bullet - \bullet) and the concentration of RMCPII in the jejunal mucosa (μ g/g wet weight, \blacksquare - \blacksquare) of immune rats given worm antigen or saline (dose = 0). The numbers of MMC per villus crypt unit (VCU) are also shown (mean \pm SEM, \circ - \circ).

Individual symbols showing the concentration of RMCPII in perfusates (\blacklozenge) and gut homogenates (\square) as well as the number of MMC (\diamond) are also shown for naive rats given 500 we.

Lower panel. The concentrations of RMCPII in serum (mean \pm SEM \bullet) and GAG in plasma (mean \pm SEM \blacksquare) are plotted against the dose of worm antigen. Values from immune rats given saline are plotted at the zero dose and naive rats given antigen are shown as (\circ) for RMCPII and (\square) for GAG.

Table 2 The concentration of RMCPII in jejunum and gut perfusates of naive and immune rats.

Group ^a	Gut homogenate ^b	Gut perfusate ^c
1	720 ± 0 ⁺	0
2	2452 ± 179	0
3	1020 ± 90 ⁺	1471 ± 213
4	1220 ± 70 ⁺	2085 ± 383
5	1425 ± 86 ⁺	1144 ± 158

a group status and treatments as described in Table 1

b the concentration of RMCPII in jejunum (µg/g wet weight)

c the concentration of RMCPII in gut perfusates (µg)

+ P < 0.001 when compared with immune saline challenge group (2) Student's t-test.

($P < 0.001$) depletion of RMCPII and MMC from the mucosa of primed rats challenged with antigen (Fig.1, Table 2) when compared with control values. Analysis of GAG in gut perfusates and homogenates is likely to be complicated by endogenous GAG additional to that contained in mast cells, and no significant change in mucosal levels was detected, however antigen challenge caused a significant increase in GAG in perfusates from immune rats when compared with immune rats given saline (Table 4).

Mast cell depletion from the jejunal mucosa was detected both with Toluidine blue and naphthol AS-D chloroacetate (Table 3); the correlation between the two methods was highly significant ($y = -2.3 + 1.1x$, $r=0.98$, $P < 0.001$; Fig.3) suggesting that both proteoglycan and serine esterases were depleted from discharging MMC.

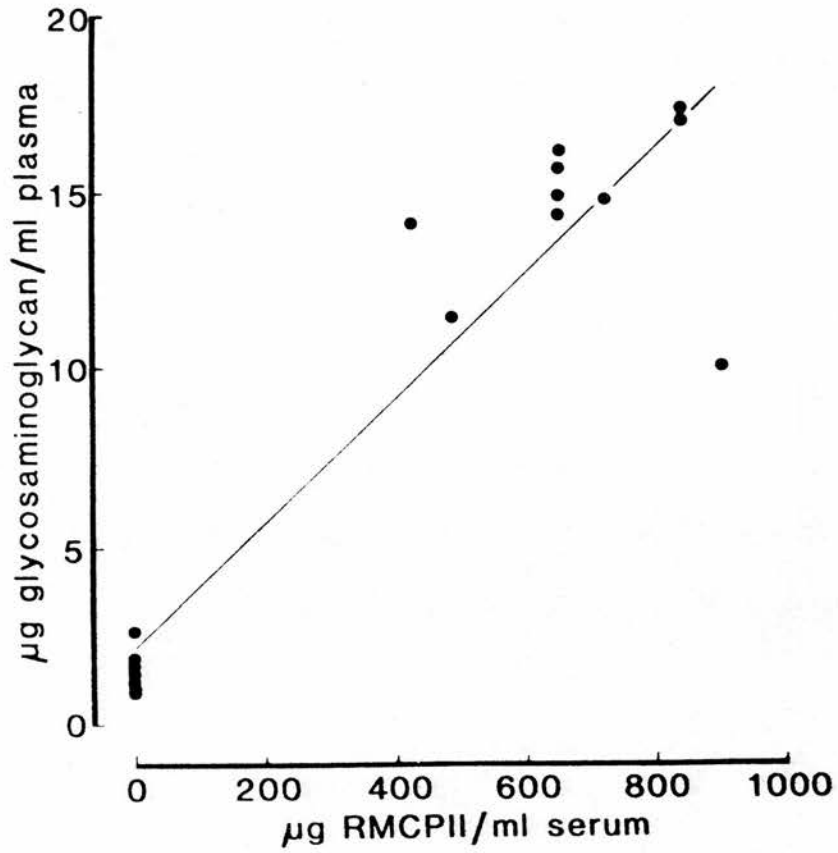


Figure 2. Regression analysis of the concentrations of RMCP II in serum against GAG in plasma ($y = 2110 + 17x$, $r=0.93$, $P < 0.001$). Plasma was not obtained from one rat given 500 we.

Table 3 Mast cell numbers as determined by their content of glycosaminoglycan or esterase in immune and naive rats.

Group ^a	MMC/VCU (mean \pm SEM)	
	Toluidine blue	Esterase
1	10.45 \pm 0.66 ⁺	10.62 \pm 0.47 ⁺
2	33.42 \pm 0.45	31.47 \pm 0.44
3	8.42 \pm 0.53 ⁺	10.60 \pm 0.53 ⁺
4	8.23 \pm 0.88 ⁺	9.66 \pm 0.63 ⁺
5	11.16 \pm 0.29 ⁺	12.06 \pm 1.01 ⁺

a treatments as described in Table 1

+ P < 0.001 when compared with group 2 (Student's t-test)

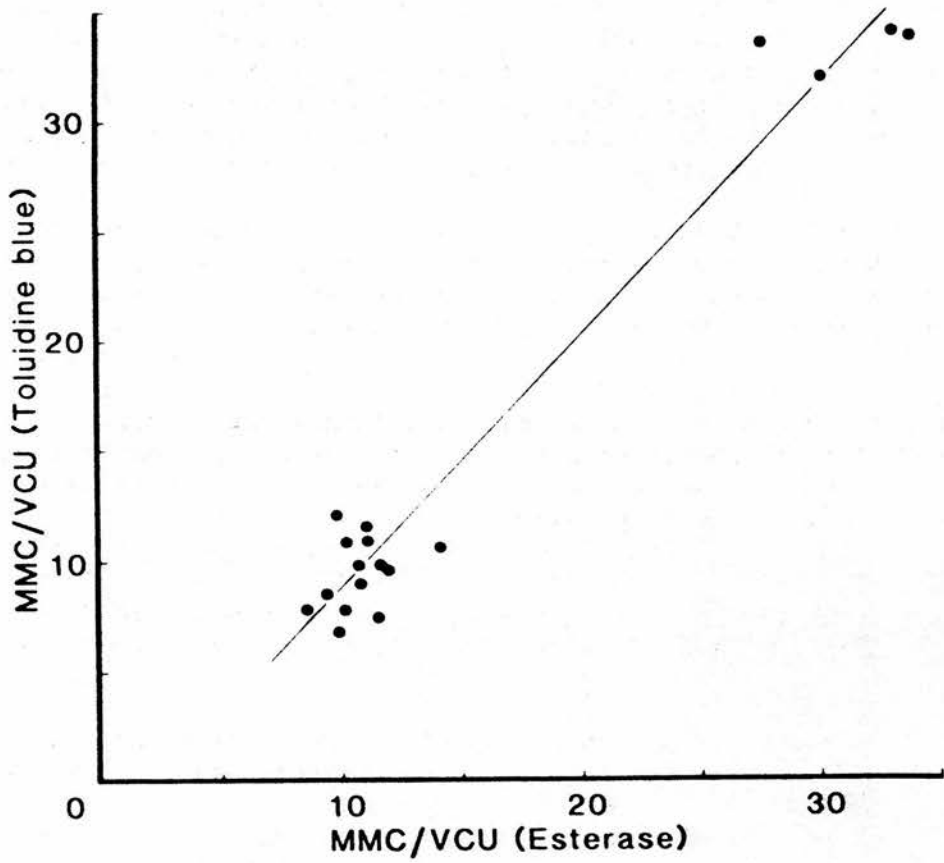


Figure 3. Regression analysis between the numbers of MMC enumerated with Toluidine blue and with NADC-esterase. ($y = -2.3 + 1.1x$, $r=0.98$, $P < 0.001$).

Table 4 The concentration of GAG in gut homogenates and perfusates.

Group ^a	GAG (ng/ml) mean \pm SEM	
	Gut Homogenate	Gut Perfusate
1	15345 \pm 1767	17419 \pm 2478 ⁺
2	15056 \pm 807	7531 \pm 4168
3	21677 \pm 3508	21061 \pm 1794 ⁺
4	17603 \pm 1586	24720 \pm 2091 ⁺
5	12268 \pm 2318	21210 \pm 681 ⁺

a treatment as described in Table 1

+ $P < 0.001$ when compared with Group 2 (Student's t-test)

Discussion

The appearance in blood of RMCPII and GAG was examined in naive and Nippostrongylus-primed rats challenged with worm antigen. The results show that the systemic release of these two products occurred only in immune recipients of antigen, there being a 10-fold increase in the concentration of GAG in the plasma of these same rats when compared with immune recipients of saline or naive rats given antigen.

There was, as described previously (Chapter 5), substantial secretion of RMCPII into the gut lumen and a concomitant fall in the mucosal concentration of this enzyme (Miller et al., 1983; Chapter 6) in primed antigen challenged rats. This was paralleled by a reduction in the number of MMC detected either with Toluidine blue or naphthol AS-D chloroacetate, there being a highly significant correlation between the two staining methods indicating that both proteoglycan and serine esterase are lost from discharging MMC.

Although these studies failed to show a change in the mucosal concentration of GAG, probably for technical reasons associated with a high background of tissue GAG, the concentration of GAG in plasma was highly correlated with the release, into serum, of RMCPII. As the major source of RMCPII is the enteric mucosa (Chapter 6), it would seem likely that GAG in plasma is also released from this site, with the MMC being the most obvious source.

More conclusive evidence that MMC are the source of secreted GAG must await further analysis. However, it is relevant to note that rat MMC proteoglycan is, like that in the murine mast cell cultured in vitro from bone marrow (Razin et al., 1982), non-heparin proteoglycan (Tas & Bernsden, 1977). Furthermore, whereas the heparin proteoglycan of rat CTMC forms an insoluble complex with neutral proteases (Schwartz, Riedel, Caulfield, Wasserman & Austen, 1981) which remains cell-associated even after the secretory granules have released soluble mediators, the chondroitin sulphate E proteoglycan of the murine cultured mast cell is not substantially retarded in its diffusion into the microenvironment (Razin et al., 1983).

Taken together the high correlation between systemic RMCPII and GAG concentrations and of MMC stained for esterase or proteoglycan would indicate that these products are released in parallel following intestinal anaphylaxis, and as the MMC is the source of RMCPII, then it would seem likely that GAG is also of MMC origin. If, as the present study suggests, proteoglycans from MMC are highly soluble, this is yet another example of the biochemical and functional differences between MMC and CTMC in the rat.

CHAPTER 9 Final discussion and conclusions.

This thesis has set out to examine the role of (a) mucus and (b) mucosal mast cells (MMC) in the expulsion of N. brasiliensis from the rat. A long standing theme in immunoparasitology is that nematode infections of laboratory animals may be expelled as a consequence of an immediate hypersensitivity reaction. As mast cells are an important component of hypersensitivity reactions it was one aim of this thesis to reevaluate the role of these cells in the rejection of N. brasiliensis, using the MMC-specific proteinase, RMCPII as a marker for the secretory activity of MMC. In view of the relationship between hypersensitivity, mast cells and mucus, especially in the lung, one chapter of this thesis was devoted to a study of the possible protective role of gastrointestinal mucus in N. brasiliensis primary infection. The possible relationship between these results and mast cell activity will be discussed.

In Chapter 3 the numbers of worms trapped in mucus during N. brasiliensis primary infection were quantitated and the results have shown that a significant number of worms are trapped in mucus on days 10 and 11 of infection. These macroscopic observations were corroborated by the use of SEM which showed worms to be embedded in a thick blanket of mucus on day 10 but not on day 6 of infection when mucus was sparse. The actual number of worms trapped was very small (less than 20) and it is important to rationalize whether mucus trapping is likely to be a

significant mechanism leading to the expulsion of N.brasiliensis from the rat.

Based solely on the proportions of worms trapped at each time point examined it would seem improbable that trapping could be responsible for the expulsion of up to 1000 worms over 2-3 days. It must be remembered that the situation is not static and the data represents those worms trapped only at the time of sampling and thus if 10 worms are trapped and expelled per hour then trapping could be of great significance. More detailed kinetic studies would be needed to clarify worm loss versus trapping, but the fact that trapping has been observed in this study suggests that mucus trapping could be a significant mechanism whereby N.brasiliensis is expelled from rats.

It is not clear whether the increased capacity of mucus on days 10 and 11 to trap worms is due to changes in quality or quantity. There is a well known association between mucus release and mediators of immediate hypersensitivity, where mast cell mediators have been shown to induce the release of mucus in the nose and lung (Kaliner et al., 1984). Mast cell secretory activity may either (a) give rise to mucus secretagogues and hence increase the quantity of mucus (b) give rise to mediators which interact with mucus and alter its properties or (c) cause an increase in the influx of plasma proteins into the gut lumen where they

may interact with mucin and affect the solubility of the mucus gel.

The second part of this thesis was an experimental investigation into the role of MMC in the rejection of N.brasiliensis. Firstly, the histochemical demonstration of MMC in parasitized tissues and their enumeration and secondly the measurement of their secretory activity in response to parasite antigens. To monitor the secretion by MMC use was made of rat mast cell protease II (RMCPII) which is uniquely found in MMC (Woodbury et al., 1978b; Gibson & Miller, 1986). This enzyme is highly soluble making its immunoassay straightforward in tissues and sera (Woodbury et al., 1981).

The results of several workers (Nawa & Miller, 1979; Woodbury & Miller, 1982; Woodbury et al., 1984) were confirmed in that during primary infection with N.brasiliensis MMC counts were low when the parasites were being expelled (Chapter 4). In agreement with the earlier studies of Woodbury et al. (1984) RMCPII secretion was maximal about 10 days after infection even though the mucosal concentration of this enzyme and numbers of MMC were depressed below control values. Neither the concentration of RMCPII in serum or numbers of MMC were affected by differing challenge doses of larvae (Chapter 4).

As MMC counts were low when maximal enzyme secretion was recorded it is possible that RMCPII was released from MMC that could not be detected using conventional

staining for glycosaminoglycan (ie Alcian blue or Toluidine blue). To examine this eventuality, sections were stained for glycosaminoglycan, for RMCPII and for naphthol AS-D chloroacetate esterase activity and the MMC counts compared. Similar cell counts were obtained irrespective of the method of demonstration, although it should be noted that staining for esterase activity makes cell counts very easy to perform because the reaction produces a dense red colour in MMC. The comparability of the cell counts obtained with these three methods reduces the likelihood of selective release of granule constituents or that secretion was from a MMC population which could not be detected by conventional staining techniques.

This experiment demonstrates that functions of MMC cannot be determined from histochemical criteria alone since release of a specific granule product was maximal even when cell numbers were at a low level. The role of MMC has, therefore to be reevaluated in the light of these observations.

What role, if any, has RMCPII and MMC in the expulsion of N.brasiliensis primary infections? It has been suggested that MMC may play a role in altering mucosal permeability (Murray, 1972), but the precise involvement of MMC is not clear. Whilst on one hand Murray et al.(1971a) showed permeability to be maximal when numbers of MMC were maximal, Nawa (1979) was

unable to agree with this relationship and demonstrated that maximal permeability occurred before MMC increased in number and that the parasites themselves were likely to be the major cause of increased mucosal permeability. However, both sets of data must be reevaluated in the light of the present findings which demonstrate the release of RMCPII in the absence of high numbers of MMC.

The possible role of RMCPII in causing permeability changes was examined in rats after anaphylactic shock, where Miller et al. (1983) have previously demonstrated an increase in mucosal permeability. The passage of Evan's blue, which was used to assess mucosal permeability, into the gut lumen was both time- and dose-dependent in primed rats challenged intravenously with worm antigen. There was a highly significant correlation between the levels of RMCPII in the serum of shocked rats and the levels of Evan's blue in the gut lumen. These results indicate that RMCPII may be responsible for anaphylactic permeability changes in the gut mucosa, and it is possible that RMCPII may cause localized changes in gut permeability during N.brasiliensis primary infection.

In Chapter 6 the results of a study of the major source of RMCPII released during anaphylaxis were presented. The results demonstrated that the jejunum contained the highest concentration of RMCPII in naive and Nippostrongylus-immune rats. Following anaphylaxis

the concentration of RMCPII was reduced only in the gut, gross depletion of protease being paralleled by a reduction in the number of MMC. No RMCPII was present in liver, kidney, spleen, bone marrow or muscle from both naive and immune rats. Both lung and MLN had increased concentrations of RMCPII following infection but there was no anaphylactic depletion from these sites and the results would therefore suggest that RMCPII present in the serum during anaphylaxis is of intestinal MMC origin.

More compelling evidence that intestinal MMC are the major source of secreted RMCPII came from a study of the kinetics of the anaphylactic release of RMCPII (Chapter 5). The release of protease into serum was time- and dose dependent in primed rats challenged intravenously with worm antigen, no protease was detected in the sera of naive rats challenged with antigen. Furthermore, RMCPII was detected in intestinal perfusates from primed-challenged rats, the release of enzyme into the gut lumen occurring as rapidly as 90 seconds after challenge with antigen. The appearance of RMCPII in the gut lumen is perhaps the most compelling evidence that intestinal MMC are the main source of secreted RMCPII during systemic anaphylaxis.

Although the concentrations of RMCPII in the sera of rats during primary infection are very much lower than those during anaphylaxis the results from Chapter 6 would

suggest that RMCPII present in the sera of rats during N.brasiliensis primary infection is of intestinal MMC origin, where it may directly affect the permeability of the intestinal mucosa. However, release from other sites especially lung cannot be excluded as a possible source of protease during primary infection.

The data from the distribution study (Chapter 6) allows for an estimation of the amount of RMCPII per MMC in stomach, jejunum, ileum and colon in naive and immune rats.

If we assume that 1 litre = 1 kg then $1\text{g} = 1\text{cm}^3$ and $1\text{mg} = 1\text{mm}^3$. Then for naive (N) and immune (I) rats we have:

		MMC/mm ³	MMC x 10 ⁶ /g	$\mu\text{g RMCPII}/10^6 \text{ MMC}$
Stomach	I	18800	18.8	10.0
	N	12600	12.6	11.1
Jejunum	N	38800	38.8	26.8
	I	116000	116	25.7
Ileum	N	35200	35.2	12.8
	I	97800	97.8	9.8
Colon	N	16200	16.2	9.8
	I	32200	32.2	6.8

This calculation shows that there is a marked difference in the amount of RMCPII per MMC at the four sites examined. The values for jejunum are very close to those reported by Haig et al. (1982) based on the

RMCP II content of cultured rat MMC ($26\mu\text{g}/10^6$ cells). Whether the differences at the four sites are 'real' or related to the amount of mucosa relative to muscle which will affect the concentration of RMCP II per gram wet weight of tissue is unclear.

The glucocorticosteroids have proved to be the most effective drugs at blocking the expulsion of primary and secondary N. brasiliensis infections (rev. Miller, 1984) and in Chapter 7 the effects of these drugs on systemic anaphylaxis and permeability changes was examined. It was found that short term treatment with corticosteroids decimated the MMC population not only in jejunum and ileum but also in lung and MLN. As little as 1mg/kg methylprednisolone caused a highly significant reduction in the numbers of mast cells and concentration of RMCP II in the jejunum. Mast cells were depleted from the mucosa between 4 and 24 hours after steroid treatment and in all instances these events were paralleled by a reduction in the levels of RMCP II. Corticosteroid treatment suppressed the development of anaphylactic pathology in the gut, there being no detectable permeability changes in rats challenged with worm antigen.

No evidence of steroid-induced mast cell degranulation was obtained but in hindsight blood samples taken between 4 and 24 hours would have been valuable. The possibility that corticosteroids cause depletion of MMC through the

action of steroids on T-cells cannot be ruled out, which would mean that MMC have an obligate dependence on T-cell-derived factors (eg IL-3) for their continuing survival. Interestingly, 1 μ M dexamethasone interrupts the proliferation of mouse bone marrow-derived mast cells after a preincubation of 24 hours (Lewis, Robin & Austen, 1985), which would support a T-cell involvement. Whatever their ultimate fate, which is well worthy of further study, MMC show an extreme sensitivity to corticosteroids not shown by their connective tissue counterparts.

That MMC are depleted and expulsion of N.brasiliensis is prevented by steroid treatment would suggest that MMC are involved in expulsion. However, the effects of corticosteroids on eosinophils, basophils and mucus secretion/synthesis must all be considered as possible explanations for the steroid-induced inhibition of worm expulsion, but the profound effects of corticosteroids on MMC would suggest that they could be crucial to worm expulsion.

If it is assumed on the basis of the secretion of RMCPII that MMC are involved in the the expulsion of a primary infection of N.brasiliensis it is important to ascertain their role and the possible interactions with other components of the mucosal immune response. The data from this thesis and other published work would suggest that day 10 is an important time in the

course of N.brasiliensis infection. Around this time RMCPII secretion is maximal, mucus trapping can be demonstrated and gut permeability is increased (Nawa, 1979). The secretion of RMCPII could, as has been proposed by others (Woodbury et al., 1984), promote changes in mucosal permeability leading to an increased translocation of antibody and complement into the gut lumen. Other mediators such as the leukotrienes and histamine could increase capillary permeability, and promote the release of mucus from goblet cells which would interact with antibody and perhaps the leukotrienes to produce a mucus gel which would trap parasites. Histamine and leukotrienes could then act on smooth muscle, increase peristalsis and purge the host of trapped parasites. This proposal is attractive and demonstrates a clear relationship between goblet cell mucus, mast cells and the mediators of hypersensitivity. However, the mode of activation of MMC in the absence of detectable IgE (Jarrett, Haig & Bazin, 1976) remains to be established.

In secondary challenge, the role of MMC and release of mast cell-derived mediators is more clear cut. There is a high level of IgE in the serum (Jarrett et al., 1976) and abundant MMC (Miller, 1970). A similar scheme of events, but of increased tempo, could operate during rapid expulsion the immediate hypersensitivity response being of greater intensity leading to the loss of

parasites over a shorter time period (Miller et al., 1981b).

MMC and goblet cell mucus would seem to provide a system to cause the expulsion of N.brasiliensis from the rat. However, it is possible that a wide range of cells including basophils and eosinophils could be involved in the mucosal immune response to N.brasiliensis infection. In as far as MMC are concerned the results of this thesis have shown that they are activated and release mediators which could promote the rejection of N.brasiliensis.

The results presented in this study have added to the list of criteria by which MMC are defined in the rat by showing an extreme sensitivity to corticosteroids and has demonstrated that the measurement of RMCPII provides a valuable marker for the assessment of the degree of MMC activation at mucosal surfaces. These results may be of particular interest to the clinician dealing with human allergy in which heterogeneity within mast cells is only now being recognised (Strobel, Miller & Ferguson, 1981; Befus, Goodacre, Dyck & Bienenstock, 1985).

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APPENDIX 1

Anaphylactic release of mucosal mast cell protease and its relationship to gut permeability in *Nippostrongylus*-primed rats

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Accepted for publication 17 October 1983

Summary. The systemic secretion of rat mucosal mast cell protease (RMCPII) was examined in *Nippostrongylus*-primed rats injected intravenously with *N. brasiliensis* whole worm antigen. The secretory response in primed rats was both time- and dose-dependent whereas no RMCPII was present in the sera of naive rats challenged with antigen. RMCPII was detected in the sol and gel phases of intestinal perfusates, the release of enzyme into the gut lumen occurring very rapidly after challenge in immune rats. Mucosal permeability, assessed by measuring the passage of Evan's blue from the blood into the gut lumen was both time- and dose-dependent and reflected the combined capillary and epithelial permeability. Although the release of RMCPII into the gut lumen occurred more rapidly than the intraluminal accumulation of Evan's blue, these two events were highly correlated. There were, in addition significant correlations between the systemic and enteric secretion of RMCPII and the enteric accumulation of Evan's blue. These results indicate that RMCPII may have a role in altering intestinal mucosal permeability during systemic anaphylaxis in the rat.

INTRODUCTION

Following infection with *Nippostrongylus brasiliensis*, rats are susceptible to anaphylactic shock induced by the injection of whole worm antigen (Ogilvie & Love, 1974). This reaction is characterized by major changes in the small intestine where increased mucosal permeability and hypersecretion of mucus are prominent features (Urquhart, Mulligan, Eadie & Jennings, 1965).

Rat mucosal mast cells contain a distinctive protease (RMCPII) (Woodbury, Gruzinski & Lagunoff, 1978a) which is present in large amounts in the intestinal tissues of rats immune to *N. brasiliensis* (Woodbury & Miller, 1982). The enzyme is secreted systemically in primed rats challenged with antigen and, in these same rats Evan's blue was observed in the intestinal lumen thus indicating substantial alterations in mucosal permeability (Miller, Woodbury, Huntley & Newlands, 1983).

The present study was undertaken to compare the systemic secretion of RMCPII by mucosal mast cells and the permeability of the intestinal mucosa using Evan's blue as an indicator. The results indicate that these two events are highly correlated and that intestinal epithelial permeability may be dissociated from intestinal mucosal vascular permeability.

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MATERIALS AND METHODS

Animals

Male outbred Wistar rats matched for age (12–20 weeks) and weight (200–400 g) were used for each experiment. They were watered and fed *ad libitum*, but fasted 24 hr before an experiment commenced.

Parasitological techniques

The methods used to culture *N. brasiliensis* and infection with third-stage larvae (L₃) are those described previously (Nawa & Miller, 1978). The preparation of whole worm antigen was as described by Nawa, Miller, Hall & Jarrett (1981).

Injection of Evan's blue antigen mixture

Rats, primed twice by infection 1 and 5 weeks earlier with 6000 *N. brasiliensis* L₃, were given i.v. by injection, Evan's blue and whole worm antigen in 1 ml of saline. The dose was calculated so that, in addition to 2.5 mg Evan's blue/100 g body weight, rats would receive 50–800 worm equivalents (w.e.) of antigen in the 1 ml dose injected.

Preparation of tissues for assay

Rats were bled out under ether anaesthesia and their small intestines removed. The intestines were divided into two equal lengths and each portion perfused with 10 ml of saline plus 10 ml of air (Nawa, 1979). Gut contents were kept for subsequent Evan's blue determination. Sera were stored at –20°.

Measurement of RMCPII

RMCPII was measured in both sera and intestinal

perfusates using minor modifications of the radial immunodiffusion assay (Woodbury & Neurath, 1978; Woodbury & Miller, 1982). A twenty-well assay gel was prepared from 15 ml of 1% agarose (BDH, Poole) in phosphate-buffered-saline (PBS) containing 0.15 ml of low titre rabbit anti-RMCPII antibody. Samples and standards were applied to the gel and precipitin rings allowed to develop for 48 hr at 4° in a humid chamber. The gel was then pressed, dried and washed with three 15 min changes of PBS and stained with 0.5% Coomassie Brilliant Blue R250 (Sigma, Poole) in ethanol:acetic acid:water (90:20:90). The gel was destained with 90:20:90 ethanol:acetic acid:water to visualize the precipitin rings. The diameters of the rings were measured and the concentrations of RMCPII in the samples determined.

Intestinal permeability

Intestinal perfusates were centrifuged for 10 min at 10,000 rev/min in a Beckman J2-21 centrifuge at 4°. The clear supernatants were removed and the pellets resuspended in 5 ml of saline, homogenized in a teflon/glass homogenizer and Evan's blue determined in both the supernatant (sol) and homogenized pellet (gel) by acetone extraction (Nawa, 1979). More than 90% of the Evan's blue from sera and perfusates was precipitable with 10% trichloroacetic acid (Table 1). The value for perfusates was corrected for background interference from normal gut contents before and after treatment with 10% trichloroacetic acid (TCA).

Statistical methods

The data were analysed by using a two-tailed Student's *t*-test and regression analysis was carried out to

Table 1. The precipitation of albumin-bound Evan's blue: the recovery of Evan's blue from sera and intestinal perfusates

	µg/ml Evan's blue					
	Serum		Sol*		Gel*	
	A	B	A	B	A	B
Immune control	405	62 (15.3)†	0.2	0 (0)	2.9	0 (0)
Immune challenge (5 min)	490	48 (9.8)	2.9	0 (0)	2.5	0.1 (4)
Immune challenge (60 min)	332	22 (6.6)	8.0	0.5 (6.25)	18	0.2 (1.1)

A = concentration of Evan's blue present in sample.

B = concentration of Evan's blue in sample following TCA precipitation.

* Values corrected for background interference at 620 nm.

† % Evan's blue retained in supernatant following TCA precipitation.

determine the equations of best fit to the data. Both analyses were carried out using a Tektronix micro-computer.

RESULTS

Time course of RMCPH release and mucosal permeability

Twenty-five immune and 10 naive rats were used in this experiment. Twenty immune and five naive rats were injected i.v. with 500 w.e. of whole worm antigen plus Evan's blue. The remaining ten rats were injected with Evan's blue in saline. Challenge control rats and immune and naive rats treated with saline were killed 30 min after injection. Immune animals injected with antigen were killed at 5, 15, 30 and 60 min after injection of antigen and Evan's blue.

No RMCPH was detected in the sera of immune rats injected with saline or of naive rats given saline or antigen. Immune rats injected i.v. with whole worm antigen had 146 µg RMCPH/ml serum after 5 min and 755 µg RMCPH/ml serum after 60 min. Regression analysis of these data (Fig. 1) shows a highly significant correlation between time after challenge and

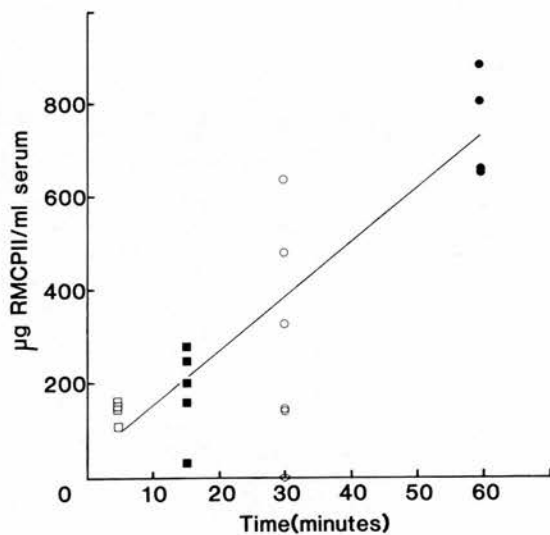


Figure 1. The levels of RMCPH in the sera of primed rats plotted against the time after challenge with 500 w.e. of whole worm antigen. On regression analysis $r=0.87$ ($P<0.001$). For challenge rats 5 (□), 15 (■), 30 (○) and 60 min (●). The mean value for serum RMCPH ± SEM for immune rats given saline shown at 30 min (◊).

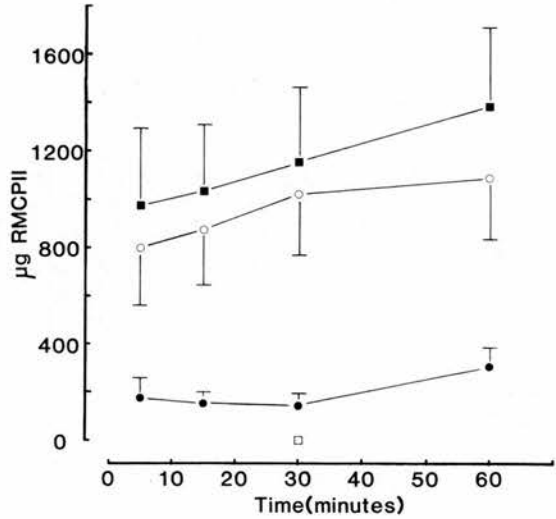


Figure 2. The partitioning of RMCPH (mean ± SEM) between the sol and gel phases of intestinal perfusates following i.v. challenge with worm antigen. Sol phase (○—○), gel phase (●—●) and total RMCPH from sol and gel phases (■—■). Immune control at 30 min (◊).

RMCPH levels in the sera of immune rats ($Y=A+BX$, $r=0.87$, $P<0.001$).

RMCPH was detected in the sol and gel phases of the intestinal perfusates from immune rats injected with antigen (Fig. 2). None was present in the sol or gel phases of perfusates from immune or naive recipients of saline or from naive rats given antigen. Although the concentration of RMCPH in the perfusates of challenged immune rats was not time-dependent it was related both to the Evan's blue accumulation in the intestine and to the serum levels of RMCPH (Table 2).

No correction was made for background values (cf. Table 1) in either challenge controls or in recipients of whole worm antigen and as a consequence some Evan's blue was detected in all intestinal perfusates. These results are in agreement with Nawa's observations on primary infections with *N. brasiliensis* (Nawa, 1979). However, immune rats given saline had significantly less Evan's blue intraluminally (34 µg/perfusate) than their challenged counterparts (187 µg/perfusate), 30 min after i.v. challenge ($P<0.001$, Fig. 3).

Five min after i.v. administration of antigen the levels of Evan's blue in the intestines of primed rats increased two-fold. Perfusates from naive rats given saline (24 µg/perfusate) contained significantly less ($P<0.001$) Evan's blue than those from naive recipients of worm antigen (55 µg/perfusate), and no

Table 2. Analysis of the relationships between time, enteric and systemic secretion of RMCPII, and enteric accumulation of Evan's blue

Data	<i>n</i>	Equation of best fit	<i>r</i>
Time vs RMCPII*	20	$Y = A + BX$	0.25
RMCPII† vs RMCPII*	20	$Y = A + B \log X$	0.73 ($P < 0.001$)
RMCPII* vs Evan's blue‡	20	$Y = A + B \log X$	0.72 ($P < 0.001$)

* RMCPII recovered from both sol and gel phases of perfusate (μg).

† RMCPII in the sera ($\mu\text{g/ml}$).

‡ Total Evan's blue from sol and gel phases of perfusate (μg).

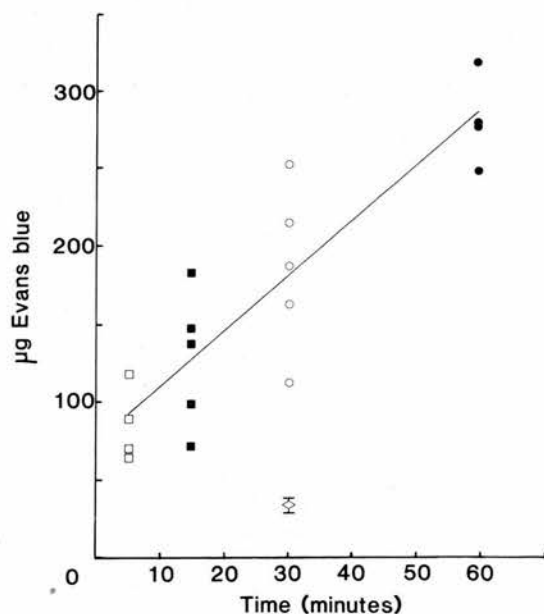


Figure 3. The levels of Evan's blue in the gut lumen against time after challenge with 500 w.e. of whole worm antigen. By regression analysis $r = 0.88$ ($P < 0.001$). For rats 5 (\square), 15 (\blacksquare), 30 (\circ) and 60 min after challenge (\bullet). Mean \pm SEM for immune controls at 30 min (\diamond).

RMCPII was detected in the sera of either group indicating that, if systemic release of RMCPII had occurred it was below the level of detection. In primed rats the accumulation of Evan's blue in the intestines was a function of the time after challenge (Fig. 3, $Y = A + BX$, $r = 0.88$, $P < 0.001$), and there was a highly significant correlation ($Y = A + BX$, $r = 0.87$, $P < 0.001$) between the concentration of RMCPII in the serum and the levels of Evan's blue in the gut lumen (Fig. 4).

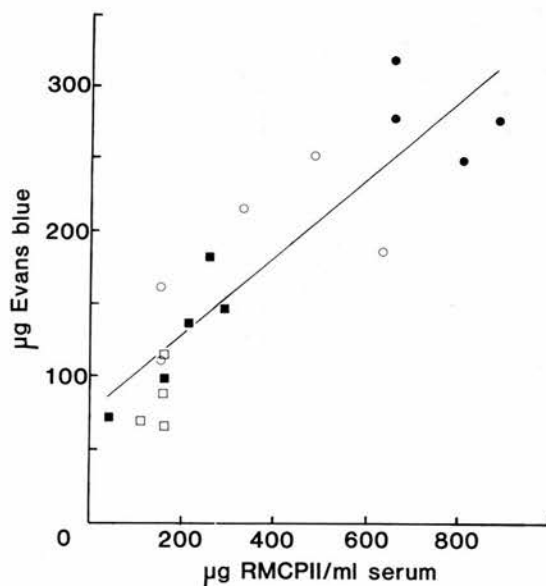


Figure 4. Regression analysis of the relationship between levels of RMCPII in the sera and Evan's blue in the gut lumen 5 (\square), 15 (\blacksquare), 30 (\circ) and 60 min (\bullet); $r = 0.87$ ($P < 0.001$).

The relative contributions of sol and gel phases to the total Evan's blue detected in the gut lumen are shown in Fig. 5. Most of the Evan's blue is present in the sol phase but the gel phase contains Evan's blue as early as 5 min after challenge. These results clearly show that Evan's blue is partitioned between the two phases of the intestinal perfusate.

Dose-response relationship

Eleven primed rats were allocated to four groups and rats in each group were injected i.v. with Evan's blue and whole worm antigen. Rats in group I were given

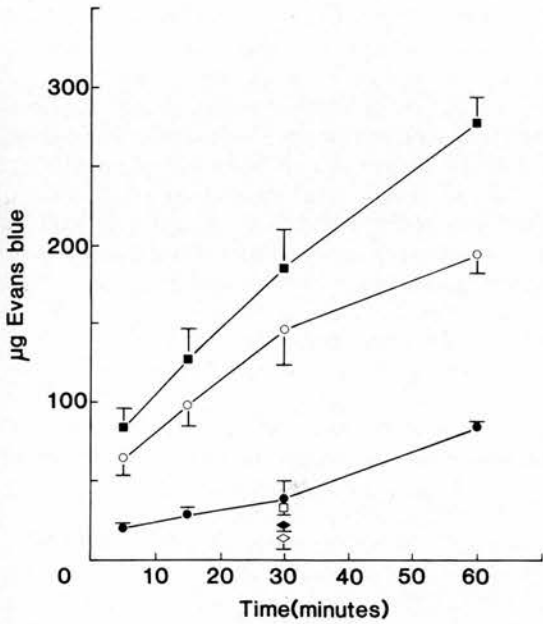


Figure 5. The relative contribution of sol and gel phases to total Evan's blue in the gut lumen plotted against time after challenge with 500 w.e. of whole worm antigen. Sol phase (○—○), gel phase (●—●) and total Evan's blue (■—■). Immune recipients of saline, sol phase (◇), gel phase (◆) and total (□) shown at 30 min (mean ± SEM).

400 w.e. of antigen, in group II 200 w.e., in group III 100 w.e. and in group IV the dose given was 50 w.e. No control group were available for this experiment. All rats were killed 1 hr after injection of antigen and Evan's blue.

The results are summarized in Table 3 and show that both the systemic secretion of RMCPH and the

Table 3. The systemic secretion of RMCPH and intraluminal accumulation of Evan's blue in the small intestine following i.v. challenge of primed rats with whole worm antigen

Group	Treatment* (w.e.)	n	Systemic RMCPH µg/ml serum (mean ± SD)	Evan's blue µg/perfusate (mean ± SD)
I	400	3	312 ± 115	113 ± 38
II	200	3	264 ± 171	76 ± 69
III	100	3	110 ± 67	24 ± 13
IV	50	2	24 ± 5	3 ± 4

* i.v. worm antigen plus Evan's blue.

intestinal accumulation of Evan's blue are dose-dependent (for RMCPH $Y = A + B \log X$, $r = 0.75$, $P < 0.01$ and for Evan's blue $Y = A + B \log X$, $r = 0.76$, $P < 0.01$). There was in addition a highly significant correlation between the concentration of Evan's blue in the perfusates and the levels of RMCPH in the sera ($Y = A + BX$, $r = 0.97$, $P < 0.001$, Fig. 6).

A second experiment was carried out using twenty-nine immune and five naive rats. These were allocated to groups and treated as described in Table 4. All

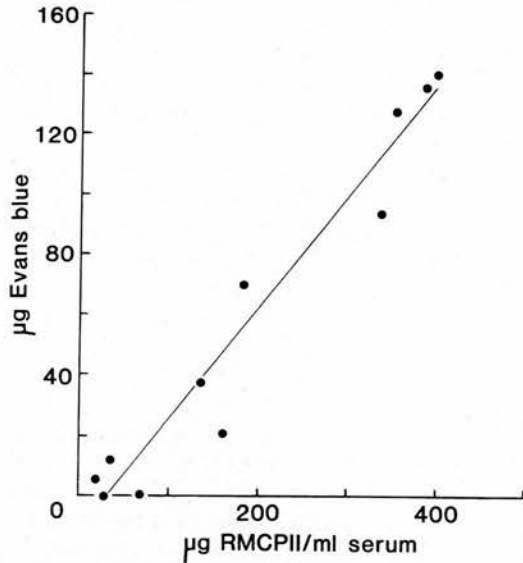


Figure 6. Regression analysis of the levels of RMCPH in the sera of primed rats and the amount of Evan's blue in the gut lumen $r = 0.97$ ($P < 0.001$).

Table 4. The systemic secretion of RMCPH and levels of Evan's blue detected in the gut lumen following i.v. challenge with whole worm antigen

Group	Treatment	n	Systemic RMCPH µg/ml serum (mean ± SEM)	Evan's blue µg/perfusate (mean ± SEM)
I	800 w.e.*	5	458 ± 148	198 ± 29
II	400 w.e.*	4	295 ± 147	138 ± 31
III	200 w.e.*	5	7 ± 2	35 ± 7
IV	100 w.e.*	5	6 ± 2	24 ± 2
V	50 w.e.*	5	11 ± 11	44 ± 11
VI	Saline*	5	0	21 ± 5
VII	400 w.e.†	5	0	24 ± 5

* Immune rats given i.v. worm antigen plus Evan's blue.

† Naive rats given i.v. worm antigen plus Evan's blue.

animals were killed 1 hr after injection and the concentrations of RMCPII in the sera and of Evan's blue in the intestinal perfusates were determined. The results are summarized in Table 4.

No RMCPII was detected in the sera of immune rats given saline (group VI) or in the sera of naive rats given antigen (group VII). There was, however, a highly significant dose-dependent ($Y=A+BX$, $r=0.71$, $P<0.001$) release of RMCPII in primed rats given whole worm antigen and Evan's blue.

The amount of Evan's blue detected in the intestinal perfusates of saline-injected immune rats and of naive recipients of antigen did not differ significantly (Table 4). Immune recipients of worm antigen responded by a dose-dependent increase in the amount of Evan's blue detected in the gut lumen. The relationship between dose of antigen and intraluminal Evan's blue levels was highly significantly correlated ($Y=A+BX$, $r=0.84$, $P<0.001$). On analysis of regression intraluminal Evan's blue levels and systemic RMCPII levels were found again to be highly significantly correlated ($Y=A+BX$, $r=0.78$, $P<0.001$).

DISCUSSION

Three major findings arise from this study: (a) RMCPII is secreted systemically in primed rats in a dose- and time-dependent manner following i.v. challenge with whole worm antigen; (b) the enzyme is present in large amounts in the intestinal lumen in these same rats; (c) systemic release of RMCPII is associated with very substantial changes in mucosal permeability as measured by the intraluminal accumulation of Evan's blue.

Our earlier studies (Miller *et al.*, 1983) indicated that systemic release of RMCPII was associated with increased intestinal mucosal permeability since Evan's blue was observed within the intestinal lumen of shocked rats. By using Nawa's technique (Nawa, 1979) of intestinal perfusion to recover the intestinal contents we have now shown that the accumulation of Evan's blue is both time- and dose-dependent. Furthermore mucosal permeability is also related to the enteric and systemic secretion of RMCPII.

The permeability of the intestinal mucosa was determined by measurement of the intraluminal accumulation of Evan's blue dye. This dye has been shown to combine preferentially with plasma albumin (Rawson, 1943) and the present results confirm those of Nawa (1979) in that >90% of the Evan's blue from

gut contents and from sera was precipitable with 10% trichloroacetic acid indicating that the dye remains bound to protein. Consequently, measurement of Evan's blue in the intestinal lumen largely represents the translocation of albumin molecules and reflects the combined vascular and epithelial leak of protein.

The method of extraction of Evan's blue (Nawa, 1979) was modified to recover the dye from both sol and gel phases of each perfusate. Data from the time course studies show that the gel phase contained Evan's blue at the earliest time point analysed although the contribution of the gel phase to the total Evan's blue in the perfusate was a minor one. However, even after homogenization and extraction of the gel phase in acetone some dye remained in the pellet, indicating that the amount of Evan's blue in the gel phase is likely to be underestimated.

RMCPII is the predominant chymotrypsin-like protease of mucosal mast cells (Woodbury & Neurath, 1978). It is highly soluble (Woodbury & Neurath, 1978) and putative protease inhibitors do not interfere with the immunoassay technique (Woodbury, Huntley & Miller, unpublished observations). The protease can be reliably measured by radial immunodiffusion down to levels of 10 $\mu\text{g/ml}$.

Relatively large amounts of RMCPII were present in the sol and gel phases of perfusates within 5 min of antigen challenge whereas none was detected in perfusates from any of the control rats. RMCPII could also be detected in perfusates within 90 sec of injection of antigen into primed rats (King, unpublished observations). Because these high levels of RMCPII were detected in the perfusates within 90 sec to 5 min of challenge they were unlikely to be grossly overestimated as a consequence of RMCPII breakdown although some catabolism of the enzyme may have occurred at later time intervals. Since the translocation of RMCPII into the gut lumen merely requires a change in epithelial permeability, and since the passage of albumin-bound Evan's blue is a consequence of both vascular and epithelial permeability it would follow that RMCPII release and epithelial permeability occurred before the development of vascular changes.

The extraction of RMCPII like that of Evan's blue from the gel phase was probably incomplete and was presumably dependent upon the nature of the interaction of RMCPII and the gel components. The gel phase in addition to containing mucin contains epithelial cells and DNA from cell breakdown (Ferencz, Orskov, Orskov & Klemm, 1980). Since RMCPII is a

highly basic protein it may interact with the acidic glycoproteins in mucin (Woodbury & Miller, 1982) and if present in an undegraded form in the gel phase it may well play some part in altering the solubility of intestinal mucin.

The intraluminal RMCP II (sol and gel phase) values correlated highly with the amount of Evan's blue in the gut lumen. The relationship was not direct and could mean that there is a rate-limiting step, possibly, for example, the development of capillary permeability.

The kinetics of systemic secretion of RMCP II were similar to those of Evan's blue accumulation in the gut lumen and these two events were highly correlated. If, as suggested above, capillary permeability is the rate-limiting step determining these kinetics, then the rapid release of RMCP II into the gut lumen is clearly independent of capillary permeability. By contrast the permeability of the mucosal epithelium, indicated by intraluminal RMCP II, is altered very rapidly and this would be compatible with histological studies demonstrating that the epithelium is shed within a few minutes of challenge (Miller & King, unpublished observations).

The high concentrations of RMCP II in the intestinal lumen are indicative of the extent of mucosal mast cell secretion from the mucosa and suggest that this could be a major source of the enzyme in blood during systemic anaphylaxis. The function of RMCP II has not been ascertained although it has proteolytic activity against basement membrane type IV collagen (Sage, Woodbury & Bornstein, 1979) and is particularly effective in releasing epithelial cells from intestinal slices *in vitro* (Woodbury, pers. comm.). It is possible, therefore, that RMCP II is responsible for epithelial shedding, although more direct experimentation would be required to confirm this.

Studies by Seppa (1979) have shown that RMCP I, biochemically a very similar enzyme (Woodbury, Katunuma, Kobayashi, Titani & Neurath, 1978) from rat connective tissue mast cells, is a potent mediator of vascular permeability in the skin and it is possible that RMCP II is likewise involved in the mediation of mucosal vascular permeability. However, monoamines and leukotrienes released from mucosal mast cells in conjunction with RMCP II are likely to be the most functionally significant mediators of mucosal vascular permeability.

In summary, systemic anaphylaxis in rats primed by injection with *N. brasiliensis* and challenged with whole worm antigen is associated with major changes

in the small intestine. By measuring the accumulation of RMCP II and Evan's blue in the gut lumen and RMCP II in the serum, it has been possible to dissociate the events of mucosal epithelial damage and permeability from mucosal vascular damage and permeability. The precise roles of mucosal mast cells and of RMCP II in the pathogenesis of these changes remain to be determined.

ACKNOWLEDGMENTS

SJK wishes to acknowledge a studentship from the Agricultural Research Council. The authors wish to thank Richard Woodbury and John Huntley for supplying reagents and for helpful advice and discussions.

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Depletion of mucosal mast cell protease by corticosteroids: Effect on intestinal anaphylaxis in the rat

(helminth infection/atypical mast cell/serine protease/glucocorticoids/immediate hypersensitivity)

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Communicated by Hans Neurath, October 11, 1984

ABSTRACT Rats primed by infection with the intestinal nematode *Nippostrongylus brasiliensis* and challenged intravenously with soluble whole-worm antigen undergo systemic anaphylactic shock. The primary lesions are in the gut and include increased permeability of the mucosa together with release, into enteric secretions, of a mucosal mast cell (MMC)-specific serine proteinase, rat mast cell protease II (RMCP-II). This enzyme is also released into the blood of shocked rats. These manifestations of anaphylaxis were abolished in rats previously treated with corticosteroids (methylprednisolone acetate, 25 mg per kg of body weight, 48 and 24 hr before i.v. challenge with antigen). Suppression of the response was associated with depletion of RMCP-II and of MMC from the intestinal mucosa. Depletion occurred 4-24 hr after treatment with as little as 1 mg of methylprednisolone per kg. By contrast, neither connective tissue mast cells nor serum levels of parasite-specific IgE were depleted in rats given 2×25 mg of methylprednisolone per kg. The capacity of unprimed treated rats to mount passive cutaneous anaphylaxis was, however, impaired.

Corticosteroids are potent, widely used anti-inflammatory drugs, and their therapeutic value in the treatment of allergic conditions in man and domestic animals is well known. Although these drugs act at a number of different levels, perhaps their most significant anti-allergic effect is in preventing the generation and release of inflammatory mediators (1). For example, they suppress histamine release from isolated rat and murine mast cells (2, 3), and they act indirectly to prevent the generation of secondarily formed mediators (4-6).

Systemic anaphylaxis in the rat is abrogated by prior treatment of sensitized animals with corticosteroids (7). The major shock organ responding to anaphylaxis in this species is the small intestine (8), and the lesions associated with intestinal anaphylaxis include hyperaemia, secretion of mucus, and epithelial shedding (9-11). The development of intestinal lesions is associated with the release, into the blood circulation and into the gut lumen, of a mucosal mast cell (MMC)-derived neutral proteinase, rat mast cell protease II (RMCP-II) (10, 11), and recent studies have implicated this enzyme in the generation of intestinal epithelial permeability (11).

Because RMCP-II is a highly soluble product of MMC (12) and is antigenically distinct from insoluble chymotrypsin-like enzyme (RMCP-I) in connective tissue mast cells (13), its release into the blood circulation or gut lumen provides a unique and highly specific marker of *in vivo* activation of MMC. We have, therefore, measured the concentration of this enzyme within the tissues, in the intestinal secretions, and in blood in order to analyze the anti-anaphylactic

activity of corticosteroids and to measure their effect on the mucosal mast cell subpopulation in rats subjected to anaphylactic shock.

MATERIALS AND METHODS

Animals. Male and female outbred Wistar rats matched for age (30-36 weeks) and weight (350-450 g) were used. They were watered and fed ad lib, but food was withdrawn 24 hr before challenge.

Parasitological Techniques. The methods used to culture the nematode parasite *Nippostrongylus brasiliensis* and to infect rats have been described (14). Adult whole-worm antigen was prepared as described (15).

Treatment of Experimental Animals. Rats were allocated to groups and were treated with saline or methylprednisolone acetate (25 mg per kg of body weight) by intramuscular injection 24 and 48 hr before they were challenged intravenously with Evan's blue (2.5 mg per 100 g of body weight). The latter was dissolved either in saline alone or in saline containing 500 worm equivalents (w.e.) (15) of worm antigen. Treatment schedules are summarized in Table 1.

Quantification of Evan's Blue and RMCP-II. One hour after intravenous challenge, rats were exsanguinated under ether anesthesia, and their intestines were excised. Each intestine was perfused with saline and the concentration of Evan's blue in the perfusate was determined spectrophotometrically (11). The following tissues were collected: jejunum (25 cm distal to the pylorus); ileum (10 cm proximal to the ileo caecal valve); mesenteric lymph node (MLN) (divided into two portions, one for histology); and the right lobe of the lung. Each specimen was weighed and homogenized in 3 vol of 0.15 M KCl (16). The concentrations of RMCP-II in the tissue homogenates, gut perfusates, and serum were measured by radial immunodiffusion (11).

Histology and Cell Counts. (i) Carnoy fixation. The following tissues were placed in Carnoy's fluid; jejunum and ileum (specimens adjacent to those used for protease determination), MLN, and the left lobe of the lung. The tissues were processed and stained with Alcian blue/Safranin as described (16).

(ii) Paraformaldehyde (4%) in phosphate-buffered saline. Portions of jejunum and ileum and ear pinnae (from naive rats) were fixed for 6 hr, transferred to 70% ethanol, and stored at 4°C overnight. The tissues were dehydrated and processed into wax, and sections were stained for non-specific esterase with the substrate naphthol AS-D chloroacetate (Fig. 1) (17). Adjacent sections were stained with Alcian blue to show mast cells (16).

Mast cells in both jejunum and ileum were counted per villus crypt unit (VCU) as described (16). Mast cells were

Table 1. Measurement of gut permeability and RMCP-II release during anaphylaxis in normal and corticosteroid-treated rats

Group	Status	<i>n</i>	Pretreatment/ challenge	Evan's blue, μg per perfusate	Systemic RMCP-II, μg per ml of serum	Enteric RMCP-II, μg per perfusate
I	Immune	6	S/Ag	406 ± 41*	90 ± 19†	797 ± 250†
II	Immune	6	C/Ag	88 ± 8	0	0
III	Immune	6	S/S	104 ± 2	0	0
IV	Immune	6	C/S	124 ± 8	0	0
V	Naive	5	S/Ag	64 ± 4	0	0
VI	Naive	5	C/Ag	51 ± 6	0	0
VII	Naive	5	S/S	72 ± 5	0	0
VIII	Naive	5	C/S	69 ± 5	0	0

Immune animals were infected 1 and 3 weeks previously with 5000 *N. brasiliensis* L₃. S, saline; C, methylprednisolone acetate; Ag, 500 w.e. of whole worm antigen. Values represent mean ± SEM.

**P* < 0.001 when compared with each group (Student's *t* test).

†*P* < 0.001 when compared with each group (Mann-Whitney U test).

not enumerated in lung and MLN, but mast cell density was scored on an arbitrary scale.

Sections of ear pinnae were examined microscopically and the total number of mast cells was enumerated. The area of tissue in which mast cells were counted, excluding cartilage, was measured by projecting the sections onto equiweight paper using a photoenlarger and then calculating the area by weight analysis of the tissue outline.

Passive Cutaneous Anaphylaxis (PCA). Rats were bled via the tail vein before saline or drug treatment and again immediately before challenge with worm antigen. Sera were harvested and double dilutions were injected intradermally into the shaved backs of recipient rats, which were challenged intravenously 48 hr later with worm antigen (1000 w.e.) and Evan's blue (18). PCA titers were expressed as the reciprocal of the highest dilution that induced blueing to a diameter of >5 mm on the underside of the skin.

The Effect of Corticosteroid Treatment of PCA. Recipient rats were injected intradermally with double dilutions of a serum sample taken from an animal immunized by two previous infections with *N. brasiliensis*. The recipients were immediately injected intramuscularly with either saline or methylprednisolone (25 mg per kg of body weight). These treatments were repeated 24 hr later, and PCA titers were determined after a further 24 hr.

RMCP-II Release as a Consequence of Corticosteroid Treatment. Fifteen naive rats, allocated to three groups, were bled via the tail vein before receiving intramuscular injections of saline, methylprednisolone acetate (25 mg per kg of body weight), or betamethasone (2 mg per kg of body weight) and were bled, 1, 4, and 24 hr later; rats given betamethasone were exsanguinated at 24 hr, and the remainder were given a second dose of saline or methylprednisolone. Blood samples were taken 1 and 4 hr later, and the remaining rats were exsanguinated 24 hr after the second injection. Samples of jejunum were collected for measurement for RMCP-II concentrations and of the numbers of MMC. Sera were analyzed for RMCP-II using an ELISA technique as described (10).

Statistical Treatment of Results. Data were analyzed using a Tektronix microcomputer using either a Student's two-tailed *t* test or, where necessary, a Mann-Whitney U test. Simple regression analysis was performed using the same computer facilities.

RESULTS

Intestinal Permeability and Secretion of RMCP-II. A highly significant (*P* < 0.001, Student's *t* test) 4-fold increase in the concentration of Evan's blue occurred in the intestinal lumen of sensitized animals (group I) challenged intravenously with worm antigen (Table 1). This response was

totally suppressed in sensitized rats that had been previously treated with methylprednisolone (group II in Table 1).

Similarly, RMCP-II was released into the blood and into the intestinal lumen of rats in group I, but this response was again totally suppressed (*P* < 0.001, Mann-Whitney U test) in rats previously treated with methylprednisolone (group II, in Table 1). Saline injection of either untreated or drug-treated sensitized rats and antigen injection into either untreated or drug-treated naive rats induced no significant alteration of mucosal permeability or release of RMCP-II (Table 1).

Tissue Distribution of RMCP-II. (i) Jejunum: pretreatment of rats with methylprednisolone acetate significantly (*P* < 0.001) depleted the jejunum of RMCP-II in both immune and naive animals (Table 2). Antigen challenge of sensitized rats was without effect when mucosal concentrations of RMCP-II were compared with those in immune rats challenged with saline (Table 2). (ii) Ileum: after treatment with corticosteroid, RMCP-II was significantly (*P* < 0.001) depleted from both immune and naive rats when compared with saline-injected controls (Table 2). (iii) Lung: corticosteroids reduced the concentration of RMCP-II in lung homogenates from immune rats (Table 2). The concentrations of RMCP-II in homogenates from naive rats were too low (<10 μg per g of tissue) to be measured by radial immunodiffusion, but trace amounts of enzyme were detected in naive animals that had not been treated with corticosteroid (Table 2). (iv) Mesenteric lymph node: the concentration of RMCP-II in the nodes of immune rats challenged with antigen was unaltered when compared with that in rats given saline, but corticosteroid depleted the immunized node of RMCP-II (Table 2). The values in nodes of untreated naive rats were similar to those in the lung at the limit of detection of the assay system (Table 2).

Mast Cell Changes After Corticosteroid Treatment. Pretreatment of rats with corticosteroid significantly (*P* < 0.001, Student's *t* test) reduced the numbers of MMC in the jejunum and ileum of naive and immune rats. The results were very similar whether the cells were detected by their content of nonspecific esterase (Fig. 1) or with Alcian blue (Table 3). The numbers of MMC in the jejunal and ileal mucosae of primed rats were unaltered after challenge with worm antigen (Table 3). Because of the anisotropic distribution of mast cells in the lung and MLN, a semi-quantitative assessment of cell density was used. Glucocorticoid treatment of sensitized rats caused an apparent reduction in the density of mast cells in both tissues, whereas antigen challenge was without effect (Table 3). Relatively few mast cells were present in the lungs of naive rats, and those present in the MLN were distributed in the capsule, with few being located in the cortex. Corticosteroids had no discernible

Table 2. Effect of anaphylaxis and corticosteroid treatment on distribution of RMCP-II in jejunum, ileum, lung, and MLN in immune and naive rats

Group	μg of RMCP-II per g (wet weight) of tissue			
	Jejunum	Ileum	Lung	MLN
I	1780 \pm 120	471 \pm 19	219 \pm 24	86 \pm 16
II	215 \pm 25*	106 \pm 10*	126 \pm 16 [†]	<10 [†]
III	1840 \pm 84	517 \pm 13	182 \pm 17	90 \pm 0
IV	165 \pm 33*	98 \pm 5*	117 \pm 14 [§]	<10 [‡]
V	534 \pm 51	195 \pm 10	<10	<10
VI	252 \pm 57*	61 \pm 5*	0	0
VII	576 \pm 51	200 \pm 6	<10	<10
VIII	210 \pm 30*	66 \pm 0*	0	0

Groups and treatments are as described in Table 1. Values represent mean \pm SEM.

* $P < 0.001$. Analysis of effect of corticosteroid treatment (Student's *t* test).

[†] $P < 0.01$.

[‡] $P < 0.001$ (Mann-Whitney U test).

[§] $P < 0.05$.

effect on lung mast cells in naive rats but slightly decreased the numbers of mast cells in the cortex of MLN (Table 3).

The Response of Connective Tissue Mast Cells to Corticosteroid Treatment. Corticosteroid treatment did not alter the numbers of mast cells in the ear pinnae of naive rats. Recipients of saline had 56 ± 4 mast cells per mm^2 ; in corticosteroid-treated rats, there were 53 ± 3 mast cells per mm^2 of tissue. Similarly, antigen challenge of naive rats was without effect on connective tissue mast cells (CTMC) (55 ± 6 mast cells per mm^2). The numbers of CTMC stained with Alcian blue when compared with the numbers containing esterase activity were, by simple regression analysis, highly significantly correlated ($y = 12.8 + 0.76x$; $r = 0.81$; $P < 0.001$).

PCA Reactivity of Serum After Corticosteroid Treatment. Serum taken from rats before they were treated with methylprednisolone (PCA titer, 280 ± 114) and serum taken from the same rats immediately before antigen challenge (PCA, 280 ± 114) had identical PCA values. Similarly, PCA titers measured before saline treatment of immune control rats (176 ± 39) were unaltered 48 hr later.

Effect of Corticosteroid Treatment on PCA. The PCA titer (40 ± 0) in naive rats injected intradermally with immune serum and then treated with methylprednisolone was signifi-

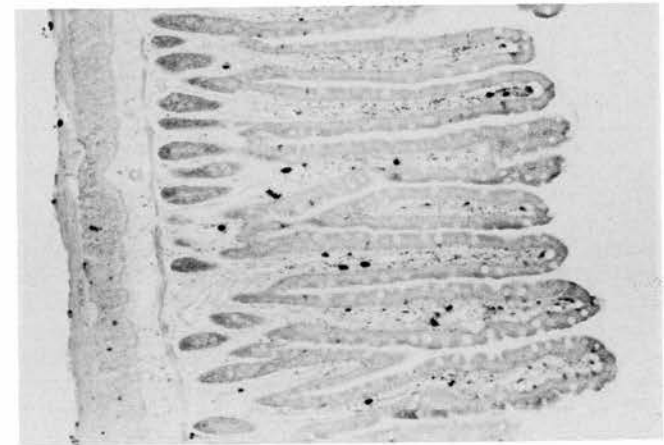
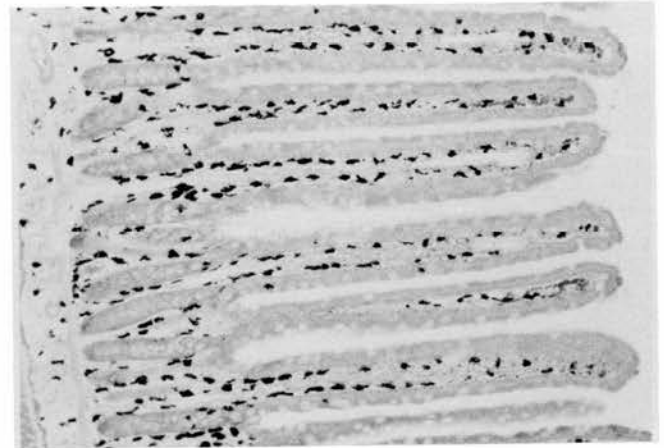


FIG. 1. Tissue sections stained with naphthol AS-D chloroacetate from (Upper) saline-treated and (Lower) corticosteroid-treated rats. Note the substantial depletion of MMC after treatment. ($\times 275$.)

cantly ($P < 0.001$) decreased when compared with similarly sensitized rats (352 ± 78) treated with saline.

Serum Levels of RMCP-II After Corticosteroid Treatment. The concentration of RMCP-II in sera obtained 1, 4, 24, and 48 hr after treatment with methylprednisolone and 1, 4, and 24 hr after treatment with betamethasone was determined by ELISA. At no time was RMCP-II detected in the blood. There was, however, depletion of MMC after treatment with either methylprednisolone (3.7 ± 0.5 MMC per VCU) or

Table 3. Quantification of MMC in jejunum, ileum, lung, and MLN in normal and corticosteroid-treated rats

Group	MMC/VCU				Mast cell density	
	Jejunum		Ileum		Lung	MLN
	Alcian blue	Esterase	Alcian blue	Esterase		
I	29.6 \pm 1.7	30.0 \pm 2.0	15.9 \pm 1.5	16.0 \pm 1.4	+++	+++
II	3.3 \pm 0.3*	3.6 \pm 0.2*	2.2 \pm 0.4*	2.4 \pm 0.4*	++ [†]	++ [†]
III	32.6 \pm 1.0	32.9 \pm 1.5	17.4 \pm 0.8	18.0 \pm 1.0	+++	+++
IV	3.8 \pm 0.4*	4.0 \pm 0.3*	2.8 \pm 0.3*	2.8 \pm 0.1*	++ [†]	++ [†]
V	6.8 \pm 0.5	7.7 \pm 0.3	4.0 \pm 0.1	4.3 \pm 0.1	+	+
VI	2.1 \pm 0.2*	2.5 \pm 0.2*	1.4 \pm 0.2*	1.3 \pm 0.2*	+	\pm [†]
VII	7.5 \pm 0.5	8.3 \pm 0.5	4.2 \pm 0.2	4.4 \pm 0.4	+	+
VIII	2.4 \pm 0.2*	2.4 \pm 0.2*	1.4 \pm 0.2*	1.4 \pm 0.1*	+	\pm [†]

Groups and treatments are as described in Table 1. Values represent mean \pm SEM. Values for MMC/VCU when stained with esterase showed a highly significant correlation with those stained with Alcian blue: for jejunum ($y = 0.45 + 0.99x$; $r = 0.99$) and for ileum ($y = 0.07 + 1.0x$; $r = 0.99$). Subjective mast cell density: (+++) substantial increase when compared with naive controls (+).

* $P < 0.001$ significant depletion of mucosal mast cells after corticosteroid treatment.

[†]Some depletion of mast cells after corticosteroid treatment.

Table 4. Effect of dose and time after administration of methylprednisolone on concentration of RMCP-II and number of MMC in jejunum of immune rats

	RMCP-II, $\mu\text{g/g}$ wet weight	MMC/VCU	
		Alcian blue	Esterase
Methylprednisolone, mg/kg*			
Saline control	1935 \pm 152	33.7 \pm 1.4	32.4 \pm 1.0
1	487 \pm 45 [†]	12.3 \pm 0.7 [†]	10.4 \pm 0.7 [†]
5	285 \pm 29 [†]	6.3 \pm 0.5 [†]	5.9 \pm 0.2 [†]
25	292 \pm 33 [†]	4.5 \pm 0.5 [†]	4.7 \pm 0.2 [†]
Time, hr [‡]			
1	1357 \pm 185	22.8 \pm 1.1	22.7 \pm 2.0
4	1327 \pm 82	23.7 \pm 1.2	22.6 \pm 0.9
24	570 \pm 30 [§]	7.6 \pm 0.5 [§]	8.0 \pm 0.7 [§]
24 (saline)	1395 \pm 169	23.3 \pm 2.2	24.9 \pm 1.3

Values represent mean \pm SEM.

*By regression analysis, Alcian blue vs. esterase: $y = -0.45 + 0.99x$; $r = 0.99$.

[†]Significant depletion when compared with saline controls ($P < 0.001$, Student's t test).

[‡]By regression analysis, Alcian blue vs. esterase: $y = 0.84 + 0.96x$; $r = 0.97$.

[§] $P < 0.001$ when compared with 24-hr saline group.

betamethasone (5.6 ± 0.2 MMC per VCU) when compared with mast cell numbers in control rats (10.3 ± 0.5 MMC per VCU) treated with saline. The numbers of MMC detected by esterase stain and by Alcian blue were similar, and by regression analysis there was a highly significant correlation between the two staining methods ($y = 0.19 + 0.94x$; $r = 0.93$; $P < 0.001$). Similarly, the concentration of RMCP-II was decreased ($P < 0.001$) in the jejunum of rats treated with either methylprednisolone ($313 \pm 48 \mu\text{g/g}$) or betamethasone ($493 \pm 33 \mu\text{g/g}$) when compared with saline controls ($784 \pm 24 \mu\text{g/g}$). There was, in addition, a highly significant correlation between the numbers of MMC detected with esterase stain and the concentration of RMCP-II in the jejunum of the three groups of rats ($y = 164 + 61x$; $r = 0.87$; $P < 0.001$).

Dose-Response Relationship. Rats immunized by two previous infections with *N. brasiliensis* were injected intramuscularly with saline or with 1, 5, or 25 mg of methylprednisolone acetate per kg of body weight 24 and 48 hr before they were killed and bled out. When compared with saline-treated controls, methylprednisolone caused a dose-dependent depletion of RMCP-II within 24 hr from the mucosa (Table 4). The data fitted the following regression equation: $y = x/ -0.002 + 0.004x$ ($r = 0.81$) and indicated that 5 mg of methylprednisolone per kg of body weight was as effective in depleting MMC from the jejunum as was 25 mg/kg. The decrease in the numbers of jejunal MMC (esterase) was highly correlated with the loss of jejunal RMCP-II ($y = -69 + 61x$; $r = 0.98$).

Time Course of Mast Cell Changes. Immune rats were allocated to groups and given intramuscular injections of 25 mg of methylprednisolone or saline per kg of body weight. Groups of four rats were killed 1, 4, and 24 hr after prednisolone treatment and four rats were killed 24 hr after saline treatment. Samples of jejunum were collected for histology and for measurement of RMCP-II concentrations. No significant depletion of either mast cells or RMCP-II was detected until 24 hr after injection of corticosteroid (Table 4). These data indicate that mast cell changes occur between 4 and 24 hr after administration of steroid.

DISCUSSION

The neutral serine proteinase RMCP-II is located uniquely within the granules of a subset of mast cells commonly

referred to as "atypical" or "mucosal mast cells" (19), and the two principal effects of corticosteroid treatment described in the present study were (i) suppression of the anaphylactic release of this enzyme into intestinal secretions and into blood and (ii) depletion of RMCP-II from the intestinal mucosa. These events may, therefore, be related to the suppression of intestinal anaphylaxis and to our failure to detect systemic or enteric release of RMCP-II in primed antigen-challenged rats.

Our results have confirmed previous reports that corticosteroids suppress anaphylactic pathology in the gut (9), there being no detectable permeability changes in the treated immune rats challenged with worm antigen (Table 1). In addition, they extend earlier observations on the ability of corticosteroids to suppress the development of intestinal mucosal mastocytosis during nematode infection (20, 21) by showing that fully mature MMC in normal and immune rats are depleted by corticosteroid treatment. By contrast, CTMC in the pinna of the ear were apparently unaffected. This latter finding indicates a further important functional difference between CTMC and MMC in the rat.

Significant depletion of MMC and RMCP-II occurred between 4 and 24 hr after exposure to steroid, and this may explain why in previous studies (7) optimal inhibition of anaphylactic bronchoconstriction was not observed until 12–24 hr after dexamethasone administration. The extreme sensitivity of MMC to steroids was indicated by the fact that treatment with as little as 1 mg of methylprednisolone per kg of body weight caused a significant decrease in their numbers (Table 4).

Depletion of MMC was in every instance paralleled by a loss of RMCP-II from the jejunal mucosa. Both MMC and CTMC were in these studies identified histologically by their content of glycosaminoglycan and of serine esterases. Neither of these granule constituents was depleted from CTMC after steroid treatment, whereas both were lost from MMC. Although depletion of MMC occurred between 4 and 24 hr after treatment, there was no detectable release of RMCP-II into the serum at 1, 4, or 24 hr. The ultimate fate of MMC was not established and must await further study.

Corticosteroid treatment of rats passively sensitized intradermally with immune serum caused a highly significant but incomplete inhibition of passive cutaneous anaphylaxis. The binding of IgE to the mast cell surface is not impaired *in vitro* by steroids (3, 22) and, in the present study, corticosteroids failed to suppress the level of circulating IgE. Consequently, two mechanisms of inhibition of intestinal anaphylaxis might operate: (i) inhibition of mediator release from mast cells by corticosteroids and (ii) modification of the response of target organs to these mast cell-derived mediators. The extent to which the intestinal response was suppressed by the known capacity of steroids to decrease permeability in capillary beds (23) or cause vasoconstriction (24) cannot be determined from the present data. It is possible that both mechanisms operate in the suppression of intestinal permeability changes.

Elevation of cAMP (3, 22) and effects on arachidonic acid metabolism (25) and its inhibition of release from the cell membrane by lipomodulin-like proteins (26–28) have been put forward to explain corticosteroid inhibition of mediator release from CTMC. However, it is not clear whether these mechanisms are applicable to MMC, as they differ biochemically and functionally (29) from CTMC. But it is interesting to note that one action of corticosteroids may be to impair mitochondrial function (2), and it is possible that MMC have a greater energy requirement than CTMC.

In summary, pretreatment of sensitized rats with corticosteroids suppresses intestinal anaphylaxis and is associated with a marked depletion of MMC. However, the possibility cannot be ruled out that the depletion of MMC is

an indirect effect resulting from the primary action by corticosteroids on other cell types, such as T lymphocytes. Also, since CTMC remain undepleted from the dermis of the ear pinna, there appears to be a very important difference between the two cell populations in terms of steroid sensitivity, which may be of potential importance in man, where MMC have only recently been identified (30).

S.J.K. wishes to acknowledge financial support from the Agricultural and Food Research Council (London).

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The Presence in Blood of Both Glycosaminoglycan and Mucosal Mast Cell Protease following Systemic Anaphylaxis in the Rat

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Abstract. The appearance in blood of rat mast cell protease II (RMCPII) and glycosaminoglycan (GAG) was examined in normal and *Nippostrongylus brasiliensis*-primed rats challenged intravenously with worm antigen. Systemic release of these two products occurred only in immune recipients of antigen; substantial levels of RMCPII were also present in the intestinal perfusates of these same rats and there was depletion of both RMCPII and mucosal mast cells (MMC) from the intestinal mucosa. Depletion of MMC was evident after staining for proteoglycan or for serine esterase and the mast cell counts with both histochemical techniques were highly correlated. Taken together, the results suggest that MMC are likely to be the principal source of secreted GAG and RMCPII.

During the immune expulsion of the gastrointestinal nematode *Nippostrongylus brasiliensis* from the rat, there is discharge of granule contents of mucosal mast cells (MMC), and depletion of proteoglycan from these cells [1]. Following intravenous challenge of primed rats with *N. brasiliensis* worm antigen, extensive mucosal damage ensues which is accompanied by the systemic and enteric release of a specific neutral proteinase: rat mast cell protease II (RMCPII) [2] derived from MMC, and depletion of MMC from the jejunal mucosa [3]. The present study was undertaken to analyse body fluids for the presence of MMC-derived proteoglycan during systemic anaphylaxis in the rat.

15 female outbred Wistar rats, immunised 3 and 8 weeks earlier by subcutaneous injection with 6,000 *N. brasiliensis* larvae [4], were allocated randomly to 3 groups of 4 rats and 1 group of 3 rats and were challenged intravenously with 1,000, 500 or 250 worm equivalents (w.e.) of adult whole worm antigen or saline. Worm antigen was prepared as described previously [5]. A further group of 4 naive rats was challenged with 500 w.e. intravenously. 1 h after intravenous challenge, animals were anaesthetised with ether and bled out. Blood was collected into the anticoagulant 'Thrombotect' (Abbott) and the plasma was separated by centrifugation and stored at -70°C. Serum,

small intestinal perfusates and samples of jejunum were collected and the concentrations of RMCPII in these samples were measured by radial immunodiffusion [2, 3]. Additionally, adjacent segments of jejunum were placed in either Carnoy's fluid or 4% paraformaldehyde prepared with phosphate-buffered saline. Tissues were embedded in wax and sections were stained with either Toluidine blue [6] or with naphthol AS-D chloroacetate to demonstrate serine esterases [7]. Plasma, gut perfusates and gut homogenates were analysed for the presence of glycosaminoglycan (GAG) by a modification of the competitive heparin binding assay previously described [8], in which polybrene-Sepharose was substituted for protamine-Sepharose as the binding reagent and the radioactive tracer and standard were heparan sulphate rather than heparin.

RMCPII was present only in the sera of immune rats challenged with worm antigen (fig. 1) and in these same rats there was a 10-fold increase in the concentration of GAG in plasma when compared with immune recipients of saline or naive rats given antigen (fig. 1). Although the release of RMCPII and GAG into the blood was, in this experiment, independent of the dose of antigen, there was a highly significant correlation between the concentrations of RMCPII and GAG ($y = 2,110 + 17x$, $r = 0.93$, $p < 0.001$; fig. 2),

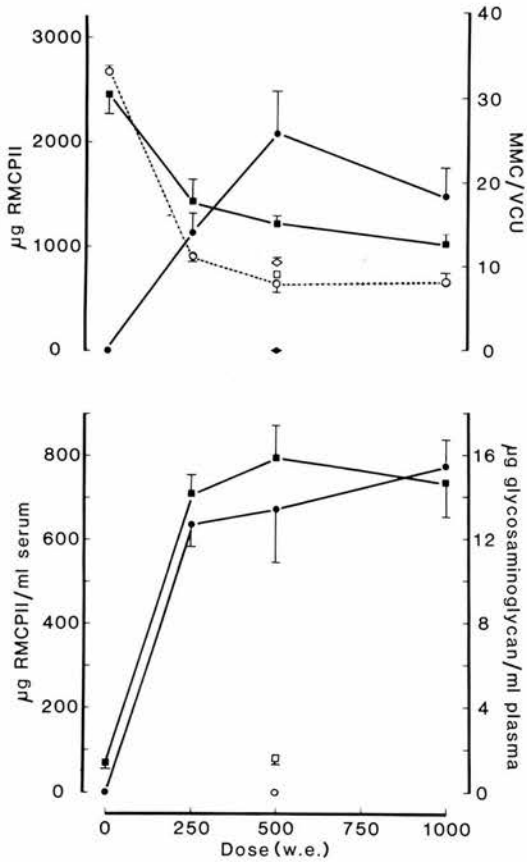


Fig. 1. a Total recoveries of RMCPII (mean ± SEM) in intestinal perfusates (●) and concentration of enzyme in jejunal mucosa (µg/g wet weight tissue, ■) of immune rats given worm antigen or saline (dose = 0). The number of mucosal mast cells (MMC) per villus crypt unit (VCU) detected with Toluidine blue (mean ± SEM, ○) are also shown. Individual symbols showing the concentrations of RMCPII in perfusates (◆) and gut homogenates (□) and the number of MMC (◇) in naive rats challenged with 500 w.e. of antigen are also plotted. **b** The concentrations of RMCPII in serum (mean ± SEM ●) and glycosaminoglycan (GAG) in plasma (mean ± SEM ■) are plotted against the dose of worm antigen. Values from immune rats injected with saline are plotted at the zero dose and in naive rats given antigen the values are plotted at 500 w.e. for RMCPII (○) and for GAG (□).

which would suggest that they may have been released from the same source.

Intestinal perfusates from primed, challenged rats contained 1,144–2,085 µg RMCPII (fig. 1), whereas perfusates from controls were devoid of this enzyme (fig. 1). There was a concomitant, highly significant ($p < 0.001$) depletion of RMCPII and MMC from the mucosa of primed, challenged rats when compared with control values (fig. 1). Analysis of gut perfusates and homogenates showed GAG to be present within

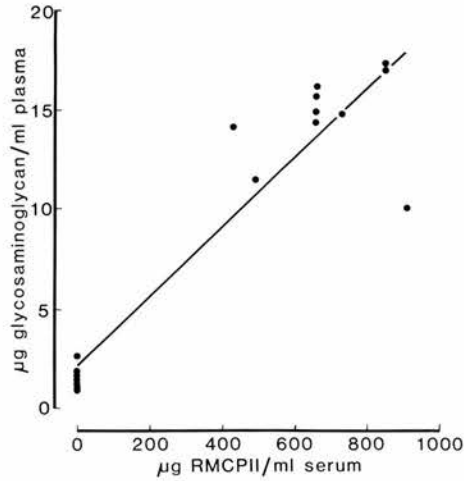


Fig. 2. Regression analysis of the concentrations of RMCPII in serum against GAG in plasma ($y = 2,110 + 17x$, $r = 0.93$, $p < 0.001$). Plasma was not obtained from one rat given 500 w.e.

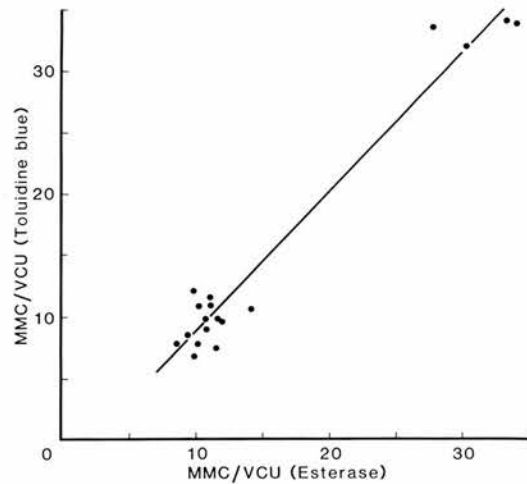


Fig. 3. Regression analysis demonstrating a highly significant correlation between the numbers of MMC enumerated after staining with Toluidine blue and with naphthol AS-D chloroacetate ($y = -2.3 + 1.1x$, $r = 0.98$, $p < 0.001$).

each sample. No decrease from jejunum or increase within the perfusate was detected. The analysis of these tissues is, however, likely to be complicated by endogenous GAG other than that contained in the mast cells.

Mast cell depletion from the mucosa was detected both with Toluidine blue (pH 0.5) and with naphthol AS-D chloroacetate; the correlation between the numbers of mast cells detected with these two methods was highly significant ($y = -2.3 + 1.1x$, $r = 0.98$, $p < 0.001$;

fig. 3), suggesting that proteoglycan and serine esterases were depleted concomitantly from discharging MMC.

The results of this study are consistent with data from previous experiments [2] except that shock was severe with the lowest dose of worm antigen and was not augmented by further increasing the challenge dose. There was, as previously described [2], substantial secretion of RMCPII into the gut lumen and a concomitant fall in the mucosal concentration of this enzyme. These changes were paralleled by a reduction in the number of MMC detected with either Toluidine blue or naphthol AS-D chloroacetate. Whilst we were unable to detect changes in mucosal levels of GAG, probably for technical reasons associated with a high background of tissue GAG, there was a tenfold increase in the concentration of GAG in plasma which was highly correlated with the release, into serum, of RMCPII. Although the distribution of RMCPII in tissues other than the gut have not yet been published, our own studies have shown that, apart from the lung in which RMCPII is increased after infection with *N. brasiliensis* and which, at most, contains 300 µg RMCPII/g wet weight, most other organs contain little or none of this enzyme [9]. Clearly, therefore, the major source of secreted RMCPII is the enteric mucosa, and it would seem likely that GAG present in plasma is also released from this site with MMC being the most obvious source of these two products.

More conclusive evidence of the mucosal mast cell as the source of secreted GAG must await further analysis. However, it is relevant to note that MMC granule proteoglycan is, like that from the murine mast cell cultured from bone marrow [10], non-heparin proteoglycan [11], and that the latter cell releases chondroitin sulphate E in response to immunological stimuli [12]. By contrast the heparin present in rat peritoneal mast cells remains within the granule matrix, apparently complexed with the insoluble chymase (RMCPI) [13]. If, as the present study suggests, granule products of MMC are highly soluble, this is yet another example of the biochemical and functional differences between MMC and connective tissue mast cells.

Acknowledgements

S.J.K. thanks the Agricultural and Food Research Council for financial support and K.R. is indebted to the Scottish Home and Health Department (grant No. K/MRS/50/660). Funding was also provided by the Medical Research Council (grant No. PG8218470).

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Received: September 7, 1984

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Gut mucosal mast cells in *Nippostrongylus*-primed rats are the major source of secreted rat mast cell protease II following systemic anaphylaxis

The distribution of the predominant chymotrypsin-like enzyme of mucosal mast cells (rat mast cell protease II: RMCP II) was examined in naive and *Nippostrongylus*-primed rats both before and after the induction of systemic anaphylaxis. Anaphylactic secretion of RMCP II following i.v. challenge of primed rats with worm antigen was accompanied by significant depletion of this enzyme from the jejunal and gastric mucosae; the concentrations were not altered in the ileum and colon. Despite significant increases in the levels of RMCP II in lung and mesenteric lymph node following infection with *N. brasiliensis* there was no anaphylactic depletion of this enzyme from these sites. No RMCP II was detected in liver, spleen, kidney or bone marrow either before or after systemic anaphylaxis. Mucosal mast cells were depleted from the jejunal, gastric and colonic mucosae following antigen challenge of primed rats. These data provide further evidence that gastrointestinal mucosal mast cells are the major source of secreted RMCP II following systemic anaphylaxis in the rat.

1 Introduction

There is now increasing evidence that mast cells constitute a heterogeneous cell system [1]. Two types of mast cell have been identified in the rat, these are "atypical" or mucosal mast cells (MMC), located in mucosal membranes such as the lamina propria of the gastrointestinal tract, and "typical" or connective tissue mast cells (CTMC) found in the serosal layers of the gut, the peritoneal cavity and in the connective tissues. These two cell types differ both functionally and biochemically [1]. For example, they contain distinctive glycosaminoglycans within the granule matrix [2] as well as different concentrations of histamine [3, 4]. They also respond differently to basic secretagogues and anti-allergic compounds [5, 6]. MMC and CTMC also differ in their content of serine proteinases; rat mast cell protease II (RMCP II) is present within the granules of MMC and RMCP I within CTMC [7, 8]. These two proteinases are distinct in their antigenic properties, in substrate specificity, and in solubility [9, 10].

The relatively high solubility of RMCP II is associated with its release into the blood circulation in rats primed with the intestinal nematode *Nippostrongylus brasiliensis* and challenged either i.v. with worm antigen, or intraduodenally with homologous parasites [11, 12]. By contrast, RMCP I remains as an insoluble complex with the proteoglycan (heparin) of the granule matrix [13]. The systemic release of RMCP II is, therefore, an excellent and specific marker for monitoring the secretory activities of MMC *in vivo*.

The major tissue source of RMCP II is not known, but published work would seem to suggest that it is the gut. Our own studies have shown that RMCP II can be detected within the gut lumen within minutes of i.v. challenge of *Nippostrongylus*-

primed rats with worm antigen [11] and that the appearance of RMCP II systemically is associated with a depletion of the jejunal concentration of RMCP II and of intestinal MMC [12]. Taken together these observations provide evidence that the gut is likely to be the major source of RMCP II.

The present study was in part derived from and is the sequel to a series of experiments published previously [11] and was designed to investigate the tissue distribution of RMCP II in both naive and immune rats and in rats undergoing anaphylactic shock. The results strongly suggest that gastrointestinal MMC are the major source of RMCP II.

2 Materials and methods

2.1 Parasitological techniques

The methods used to culture the nematode *Nippostrongylus brasiliensis*, to harvest third stage larvae and to infect rats have been described previously [14]. The preparation of whole worm antigen has also been described [15] and the soluble worm antigen (2000 worm equivalent (w.e.)/ml) was stored at -20°C .

2.2 Quantification of RMCP II

Male Wistar rats 20–30 weeks old and weighing 350–450 g were used for all experiments. They were watered and fed *ad libitum*, but food was withdrawn 24 h before challenge. Experimental animals were bled out under ether anaesthesia and the following tissues collected for RMCP II assay: jejunum, ileum, stomach, colon, trachea, thymus, mesenteric lymph node (MLN), right lobe of lung, spleen, liver, kidney, bone marrow harvested from femur, femoral muscle, skin and peritoneal exudate cells. All samples except muscle, skin, bone marrow and peritoneal cells were weighed and homogenized in 3 volumes of 0.15 M KCl [16]. The trachea was homogenized in 6 volumes of 0.15 M KCl. Muscle and skin samples were weighed, diced with scissors and homogenized in 3 volumes of 1.5 M KCl. Bone marrow was obtained by perfusing the femur with saline and then rapidly freeze-thawing the

[I 5037]

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Abbreviations: RMCP: Rat mast cell protease MLN: Mesenteric lymph node w.e.: Worm equivalent MMC: Mucosal mast cell

perfusate, cell debris was removed by centrifugation and the supernatant stored for RMCP II assay. To recover peritoneal cells, exsanguinated rats were injected i.p. with 40 ml of ice-cold saline, the peritoneal washings were aspirated and the cells recovered by centrifugation. They were washed several times with Hanks' balanced salt solution and mast cells were counted from an aliquot of cells that had been diluted 1 to 9 parts of 1% Azure A in 50% propylene glycol. The peritoneal cells were pelleted by centrifugation and resuspended in approximately 3 volumes of 0.15 M or 1.5 M KCl and freeze-thawed rapidly 5-6 times before centrifugation to remove cell debris; the concentration of RMCP II was assayed in the supernatant fluids.

The levels of RMCP II in tissues and sera of experimental animals were measured by radial immunodiffusion [11, 17]. Radial immunodiffusion plates for peritoneal mast cells, skin and muscle homogenates were set up both in standard buffer and in 1.5 M KCl, with appropriate RMCP II standard for each system.

2.3 Histology and cell counts

Samples of jejunum, tongue and left lobe of lung were placed in Carnoy's fluid. Thymus, MLN, ear pinnae and trachea were each divided into two samples, one being placed in Carnoy's fixative and the other used for the immunoassay of RMCP II. All tissues were fixed for 24 h and processed as described previously [16]. Mast cells were stained with Alcian blue/Safranin [16]. In the trachea, mast cells were enumerated per 1 mm length of mucosa and intestinal mast cells were counted per villus crypt unit [16]. Because of the anisotropic nature of the other tissues, mast cells were enumerated only in the ear pinnae and the density of mast cells in other tissues was scored on an arbitrary scale.

To demonstrate mast cells containing esterase, samples of jejunum, ileum colon and stomach were fixed for 6 h in 4% paraformaldehyde in phosphate-buffered saline and left overnight at 4°C in 70% alcohol [18]. Tissues were dehydrated in alcohol, cleared in xylene and embedded in paraffin wax. Sections were stained with naphthol As-D chloroacetate/Fast Garnet GBC [18]. To compare the density of mast cells in different regions of the gut, a calibrated grid was placed along the muscularis mucosae and the total number of MMC within the grid

area counted. The area of mucosa was calculated with reference to the calibrated grid. Mast cells numbers are expressed/mm² mucosae.

2.4 Statistical analysis

Data were analyzed using a Student's 2-tailed t-test and by simple analysis of regression using a Tektronix microcomputer.

3 Results

3.1 Kinetics of RMCP II release blood and its concentrations in enteric tissues

Ten naive rats and 30 animals primed twice by infection 5 weeks and 10 days earlier with 6000 *N. brasiliensis* third-stage larvae were used. Twenty-five immune and 5 naive rats were injected i.v. with 1 ml of saline containing 500 w.e. of antigen, the remaining 10 rats (5 naive and 5 immune) were injected with saline alone. Challenge control rats and immune and naive rats given saline were killed 30 min after injection. Immune rats were exsanguinated 5, 15, 30, 60 and 240 min after injection with antigen. The time course of appearance of RMCP II in the sera has already been published [11] but is included in Table 1 for information. Also shown in Table 1 are the concentrations of RMCP II in the jejunal mucosa and the numbers of MMC per villus crypt unit the jejunum.

Significant decreases occurred in both the numbers of MMC and the jejunal concentrations of RMCP II (Table 1), the reductions being most substantial 4 h after challenge (Table 1). Analysis of regression showed the numbers of MMC in the mucosa and the concentrations of RMCP II in the jejunum to be significantly correlated ($y = 391 + 82x$; $r = 0.84$; $p < 0.001$, Fig. 1).

3.2 Intestinal MMC as the major source of secreted RMCP II

The possibility that RMCP II had been released from sources other than the gut was considered and the distribution of RMCP II within other tissues was examined in immune and naive rats. The concentrations of RMCP II in samples of lung,

Table 1. The levels of RMCP II in the serum and jejunum and numbers of MMC in *Nippostrongylus*-primed and naive rats

Group	Challenge (i.v.)	Time ^{a)} (min)	Serum ^{b)} (μg RMCP II/ml)	Mean \pm SEM Jejunum (μg RMCP II/g wet weight)	MMC/VCU ^{c)}
Immune	Saline	30	0	3180 \pm 137	33.3 \pm 1.0
Immune	500 w.e.	5	121 \pm 27	2280 \pm 239 ^{d)}	24.3 \pm 3.6*
Immune	500 w.e.	15	190 \pm 43	2152 \pm 472	18.5 \pm 3.7**
Immune	500 w.e.	30	351 \pm 95	2280 \pm 305*	19.4 \pm 2.9***
Immune	500 w.e.	60	630 \pm 132	2184 \pm 289*	16.4 \pm 4.8**
Immune	500 w.e.	240	560 \pm 125	1275 \pm 65***	16.0 \pm 2.5***
Naive	500 w.e.	30	0	1260 \pm 60***	10.8 \pm 2.6***
Naive	Saline	30	0	796 \pm 111***	10.1 \pm 0.8***

a) Time after challenge.

b) Published data from King and Miller (1984).

c) Numbers of MMC per villus crypt unit in the jejunum.

d) * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ when compared with immune saline challenge group (Student's t test).

trachea, thymus, ear pinnae and MLN are therefore recorded in Table 2. When compared with naive controls, RMCP II levels in immune rats were significantly increased ($p < 0.001$) in both lung and MLN although it should be stressed that concentrations in the jejunum were tenfold greater than those in lung. Challenge of immune rats with antigen did not alter the concentrations of RMCP II in lung or trachea. The amount of RMCP II in thymus and MLN increased 1 h after challenge (Table 2) but the levels at 4 h were similar to those in immune rats given saline (Table 2).

Histologically, mast cell densities were increased in lung and MLN in primed rats when compared with naive controls

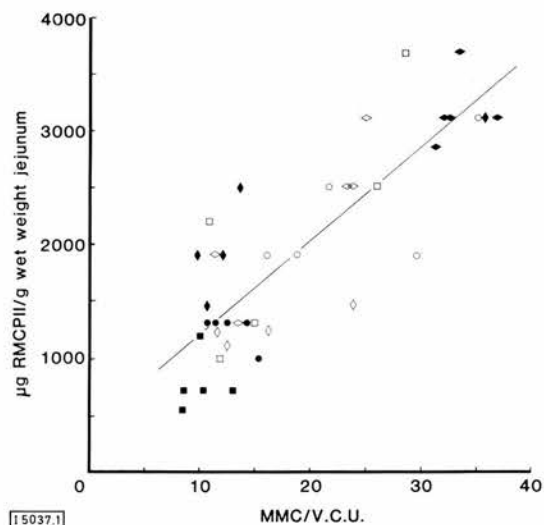


Figure 1. The relationship between mast cell density and the concentration of RMCP II in the jejunal tissues. Individual values plotted as (○) 5 min, (□) 15 min, (◇) 30 min, (◆) 60 min, (◇) 240 min, (●) immune saline, (●) naive saline, (■) naive challenge. ($y = 391 + 82 \times$; $r = 0.84$; $p < 0.001$).

(Table 3). No changes were detected in mast cells in any organ apart from the intestine (Tables 1 and 3) following challenge. Granule exocytosis was observed only in occasional mast cells in the adventitia surrounding the thymus and tongue, none was noted in ear pinnae.

Preparations of peritoneal cells containing $1 \times 10^6 - 6 \times 10^6$ mast cells from both primed and naive rats did not contain any detectable RMCP II nor did ear pinnae (Table 2). This was true for cells and tissues extracted in 0.15 M KCl and also 1.5 M KCl, and assayed by radial immunodiffusion.

A second experiment was carried out to further examine the tissue distribution of RMCP II, and to analyze release of RMCP II from sites other than the jejunum. The experimental design and challenge regimes were similar to those described in the first experiment and are summarized in Table 4. Rats were bled out under ether anesthesia 30 min after injection of antigen or saline and the concentrations of RMCP II in jejunum, stomach, ileum and colon were determined. In all four tissues there was a highly significant ($p < 0.001$) increase in the concentration of RMCP II in immune rats when compared with naive controls (Table 4). Antigen challenge of primed rats was without effect on the concentrations of RMCP II in colon and ileum. By contrast, there was significant depletion of RMCP II from both stomach and jejunum when compared with the levels in immune rats treated with saline (Table 4).

We were consistently unable to detect RMCP II in liver, kidney, spleen or bone marrow and also in homogenates of skin and muscle prepared and assayed in 1.5 M KCl, even though this concentration of salt did not alter the concentrations of RMCP II detected in the standards.

Histological examination of the four gastrointestinal mucosal tissues revealed an increase in the numbers of MMC present in immune rats when compared with naive controls (Table 4).

Table 2. The distribution of RMCP II in tissues, other than the jejunum of naive and *N. brasiliensis*-primed rats

Group status	Challenge (i.v.)	Time (min)	µg RMCP II/gm wet weight tissue (mean ± SE)				
			Ear pinna	Lung	Thymus	MLN	Trachea
Immune	Saline	30	0	300 ± 16	43 ± 2	90 ± 10	322 ± 60
Immune	500 w.e.	60	0	280 ± 25	84 ± 10***)	179 ± 5***	448 ± 48
Immune	500 w.e.	240	ND ^{b)}	237 ± 24	46 ± 1	97 ± 8	420 ± 67
Naive	Saline	30	0	45 ± 0***	40 ± 3	0 ± 0***	323 ± 18

a) ** $p < 0.01$ and *** $p < 0.001$ when compared with immune saline challenge group (Student's t-test).
b) ND = Not determined.

Table 3. Assessment of mast cell changes in tissues other than gut

Group status	Challenge (i.v.)	Time (min)	MMC per mm trachea ^{b)}	Mast cells per mm ² ear pinna ^{c)}	Mast cell density ^{a)}			
					Lung	MLN	Tongue	Thymus
Immune	Saline	30	46.2 ± 4.2	57.7 ± 2.5	+++	+++	+	+
Immune	500 w.e.	60	30.5 ± 6.7	68.7 ± 3.8	+++	+++	+	+
Immune	500 w.e.	240	31.0 ± 10.2	ND	++ ^{d)}	++ ^{d)}	+	+ ^{e)}
Naive	Saline	30	34.2 ± 1.2	74.8 ± 3.8	+	-	+	+

a) Mast cell density was a subjective assessment: (+++) substantially increased in immune rats when compared with naive controls in which mast cells were either present (+) or completely absent (-).
b) Intraepithelial mast cells counted per 1 mm length of tracheal mucosa.
c) Mast cells counted per mm² as described previously [19].
d) Mast cell depletion.
e) Exocytosis of mast cells in adventitia.

Table 4. The concentration of RMCP II (A) and number of mast cells (B) of naive and *Nippostrongylus*-primed rats at four sites along the gastrointestinal tract

		µg RMCP II/g wet weight tissue (mean ± SE)			
(A)	Group status ^{a)} /treatment	Stomach	Jejunum	Ileum	Colon
I	I/S	189 ± 5	2985 ± 235	962 ± 79	221 ± 9
II	I/Ag	155 ± 10* ^{b)}	2100 ± 132*	930 ± 18	215 ± 11
III	N/S	141 ± 11***	1042 ± 43***	453 ± 9***	159 ± 5***
IV	N/Ag	131 ± 6***	1074 ± 61***	430 ± 18***	159 ± 5***

a) I = immune; N = naive; Ag = antigen; S = saline.
 b) *p < 0.01, ***p < 0.001 when compared with group I (Student's t-test).

		Mast cells/mm ² tissue (mean ± SEM)			
(B)	Group	Stomach	Jejunum	Ileum	Colon
I	I	94 ± 3	580 ± 22	489 ± 19	161 ± 11
II	II	66 ± 7** ^{a)}	391 ± 24***	433 ± 32	113 ± 10*
III	III	63 ± 5***	194 ± 17***	176 ± 7***	81 ± 3***
IV	IV	68 ± 3***	192 ± 6***	176 ± 9***	86 ± 4***

a) *p < 0.01, **p < 0.005, ***p < 0.001 when compared with group I (Student's t-test).
 Group status and treatment as in Table 4(A).

Table 5. The relationship between mast cell density and concentration of RMCP II in stomach, jejunum, ileum and colon

Tissue	Equation of best fit	r	p
Stomach	y = 67 + 1.2x	0.72	< 0.001
Jejunum	y = 155 + 4.8x	0.97	< 0.001
Ileum	y = 188 + 1.6x	0.88	< 0.001
Colon	y = 280 + -9125/x	0.70	< 0.001

Antigen challenge was without effect on the numbers of mast cells/mm² in the ileal mucosa, whereas there were significant reductions in the numbers of mast cells in stomach, jejunum and colon following anaphylactic shock (Table 4). Regression analyses of the relationships between mast cell density and the concentration of RMCP II for each organ showed to be a highly significantly correlated. The relationships are summarized in Table 5.

4 Discussion

This study unequivocally demonstrates that the unique serine proteinase RMCP II which is a granule product of MMC [7] is most abundant in the gastrointestinal tracts of both naive and *Nippostrongylus*-primed rat. Furthermore, following i.v. challenge of immune rats with whole worm antigen the concentrations of this enzyme were reduced in the gastric and jejunal mucosae. In the absence of significant depletion from other sites, it would seem likely that the gut is the major source of secreted RMCP II following systemic anaphylaxis in the rat.

Analysis of other tissues showed RMCP II to be absent from most sites and, when present, the concentrations were much lower than those in the jejunal and ileal mucosae (Tables 2 and 4). We were consistently unable to detect RMCP II activity in muscle, skin and peritoneal mast cells, even when assayed in the presence of 1.5 M KCl, nor could RMCP II be

detected in peritoneal mast cell extracts by enzyme-linked immunosorbent assay (data not shown).

Within the gastrointestinal tract there is considerable variation in the levels of RMCP II, the highest concentration occurred in the jejunum and corresponded with maximal numbers of MMC. However, although there was a 2-3-fold difference in concentrations of RMCP II in jejunum relative to ileum, there was no such difference in density of mast cells in these organs. This is in agreement with earlier observations [4, 20] that when the numbers of MMC are expressed per unit area they are comparable in both jejunum and ileum. It may be expected that for an equal weight of tissue that there would be more mucosa relative to muscle in jejunum when compared with ileum, thus a 2-3-fold difference in RMCP II concentration may reflect a difference in the amount of mucosal tissue homogenized. Alternatively, MMC in jejunum may contain more RMCP II than their counterparts in the ileum.

The density of mast cells and the concentrations of RMCP II were, by regression analysis, highly correlated in stomach, jejunum, ileum and colon (Table 5). Within each tissue an increase in the concentration of RMCP II was accompanied by a similar increase in MMC density following infection with *N. brasiliensis*. This increase was maximal in jejunum, there being almost a 3-fold increase in both mast cell numbers and RMCP II concentrations (Table 4) following infection. Increased levels of RMCP II were also recorded in lung and MLN following infection with *N. brasiliensis*. Mast cell infiltration of MLN has been reported in *Nippostrongylus*-infected rats [21] and pulmonary mastocytosis has been observed to be associated with larval migration [22]. The results suggest that a proportion of the additional mast cells induced by parasite infection are MMC.

Depletion of RMCP II occurred only in jejunum and stomach following anaphylactic shock and was associated with a decrease in the number of mast cells in these tissues. Although there was significant depletion of MMC from the colonic mucosa this was not reflected by RMCP II concentrations

which did not alter following anaphylaxis. The reasons for this are not clear although it is possible that the released enzyme is not so rapidly cleared from colonic tissues as from stomach and jejunum. Neither the number of MMC nor the concentration of RMCP II in ileum were altered following anaphylaxis. In all four tissues (Table 4), infection with *N. brasiliensis* provoked a highly significant increase in the density of MMC when compared with uninfected controls.

One interesting finding was that MLN and thymus had increased levels of RMCP II 60 min after challenge with worm antigen. Serum RMCP II concentrations were maximal at this time and the lymphatic drainage from the gut to MLN could be a source of increased enzyme concentrations in this tissue. Since hemoconcentration is a characteristic feature of anaphylaxis in the rat [23] an alternative explanation is that the experimental animals were insufficiently exsanguinated; the increased levels in thymus and MLN might therefore reflect the accumulation of plasma-derived RMCP II.

The release of RMCP II and its depletion from the jejunum may be related to the gross mucosal damage associated with systemic anaphylaxis in the rat. The lesions occurring during anaphylactic enteropathy include epithelial shedding, hyperaemia, mucus secretion and increased mucosal permeability [11, 12, 24]. A study of the distribution of secreted RMCP II showed that epithelial permeability changes preceded penetration of enzyme into the vessels draining the villi since 1-2 mg of RMCP II were secreted into the intestinal lumen within 5 min of i.v. challenge with worm antigen whereas blood concentrations were not maximal for another 55 min [11]; these high concentrations of enzyme persisted in the gut lumen for up to 60 min after challenge [11]. The latter observations are perhaps the most compelling evidence that the gut is the major source of secreted RMCP II.

In summary, the present results add further support to previous studies [11, 12] suggesting that the measurement of extracellular RMCP II provides a unique and selective assay for monitoring the *in vivo* activity predominantly of gastrointestinal MMC in hypersensitivity or other immunologically mediated responses. They do so by demonstrating that the gut

mucosa is the major source of RMCP II in naive and *Nippostrongylus*-immune rats.

Received April 3, 1985; in revised form September 7, 1985.

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