GENERATION AND CHARACTERISATION OF MUCOSAL MAST CELLS IN NORMAL RAT BONE MARROW CULTURES

A thesis submitted for the Degree of Doctor of Philosophy in the faculty of Veterinary Medicine of the University of Glasgow

by

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This thesis is dedicated to the memory of Dr. Ellen Jarrett who initiated and supervised this project until her untimely death in January 1985. I hope the finished article is a fitting tribute to her standing as a teacher and scientist. I thank her for the example she set me as a wife, mother and scientist and hope I can live up to it.

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The work recorded in this thesis was carried out in the Department of Veterinary Parasitology at the University of Glasgow between September 1981 and September 1986. All of the results were obtained by the author unless otherwise stated in the text.

I also hereby certify that no part of this thesis has been submitted previously in any form to any university, but has been published in part as the following scientific papers.

Haig, D.M., McMenamin, C., Gunneberg, C., Woodbury, R.G. and Jarrett, E.E.E. (1983). Stimulation of mucosal mast cell growth in normal and nude rat bone marrow cultures. Proc. Natl. Acad. Sci. U.S.A. 80, 4499.

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Abbreviations

BSA	bovine serum albumin
CM	conditioned medium
Con-A	concanavalin A
CTMC	connective tissue mast cell
Dex	dexamethasone
ECF-A	eosinophil chemotactic factors of anaphylaxis
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence activated cell sorter
FCS	foetal calf serum
HBSS	Hank's balanced salt solution
HS	horse serum
IL	interleukin (e.g. IL-3 - interleukin 3)
IMLN	immune mesenteric lymph node
LTC	leukotriene
MLN	mesenteric lymph node
MMC	mucosal mast cell
Nb.	Nippostrongylus brasiliensis
NCF-A	neutrophil chemotactic factors of anaphylaxis
NMLN	normal mesenteric lymph node
PGD	prostaglandin
RMCPI & II	rat mast cell protease I & II
SRS-A	slow reacting substances of anaphylaxis
TCGF	T cell growth factor
TDL	thoracic duct lymphocytes

Summary

Mast cells with the morphological and biochemical properties of mucosal mast cells (MMC) appear and proliferate to form the predominant cell type in rat bone marrow cultures stimulated with factors from antigen or mitogen-activated lymphocytes. This thesis has been divided into two sections; in the first section, a detailed examination was undertaken of the culture conditions required, including factor dependency, to cause the selective emergence and proliferation of MMC from normal bone marrow cultures. In the second section, the author has attempted to characterise the cells growing in culture using histological, morphological and immunohistochemical techniques.

Section 1

Chapter 1: This chapter describes experiments performed which led to the adoption of the culture system that generates MMC in normal bone marrow cultures. It became apparent that immune mesenteric lymph node cells (IMLN) from Nippostrongylus brasiliensis (Nb.)-infected rats when stimulated in vitro with 2 µg/ml concanavalin A (Con-A) and the supernatant harvested after 48 hrs produced the most potent conditioned medium (CM) compared to conditioned media prepared from MLN cells of normal animals. Tritiated thymidine incorporation by the lymphocytes stimulated by Con-A could not be used as an indicator of potency for MMC growth stimulatory activity. CM is required at all times during the culture of normal bone marrow if MMC are to be stimulated to proliferate and be maintained as a viable population in vitro. A comparison was made of a number of different rat strains in their ability to produce potent CM and in their MMC bone marrow

response to CM from both syngeneic and allogeneic strains of rats. F344 rats consistently produced the most potent CM and also gave consistently higher numbers of mast cells in bone marrow cultures. This strain was therefore the one chosen for both CM and MMC production.

Chapter 2: In this chapter, the basis of the thymus-dependency of MMC was explored. Fractionation experiments of the IMLN cells indicated that the mast cell growth factor(s) was produced by immunoglobulin negative cells, maximal mast cell growth was seen in those bone marrow cultures stimulated with CM prepared from the T cell-enriched fraction of IMLN cells. MMC growth factor was not produced by IMLN cells of athymic rats, by contrast MMC precursors were present in the bone marrow of athymic rats and were normally receptive to the growth factor produced by the lymphocytes of thymus-intact rats.

Chapter 3: Having demonstrated that the thymus-dependency of MMC proliferation was based on the production of appropriate growth factor by the Con-A activated T cells present in IMLN of Nb.-infected rats, further definition of the source of MMC growth factor was provided by investigating the surface phenotype of the T cell producing the factor. Lymphocytes from the IMLN of Nb.-infected rats were separated on the fluorescence activated cell sorter into populations with and without the antigens defined by OX19, W3/25 and OX8 monoclonal antibodies: these antibodies label all T cells, T-helper cells and T-cytotoxic/suppressor cells, respectively. The supernatants were tested for the ability to induce MMC growth and differentiation in bone marrow cultures. The phenotype of the factor producing T cell was established as belonging to the helper/inducer subset i.e. $OX19^{+}$,

$W3/25^+$ and $OX8^-$.

Chapter 4: When working in in vitro systems, it must be borne in mind how the observations made may relate to phenomena observed in vivo, or how the in vitro sytem may be used to dissect the mechanisms involved in certain reactions seen in vivo. The effect of corticosteroids on the mast cell response during Nb. infection was one such reaction investigated by the in vitro culture system. Dexamethasone was found to suppress MMC growth factor production in vitro and also inhibit or arrest maturity of the mast cells growing in culture. This is discussed in the context of the phenomena observed in vivo.

Section 2

Chapter 5: The mast cells grown in culture from normal rat bone marrow exhibited the generally accepted criteria for the definition of mast cells at all stages of development, in that the granules showed metachromasia when stained with toluidine blue at pH 0.5 but shared the property of MMC in that they required special fixation techniques. The cultured mast cells were positively stained with astra blue and at no time stained with safranin. Ultrastructurally the developing cultured mast cells exhibited some of the features of developing in vivo-derived MMC. They were smaller than the classic connective tissue mast cell (CTMC) and the granules were of variable size and electron density. The time course study of the mast cell development in culture followed the pattern of maturation seen for MMC in vivo. The relationship between the cultured cells, MMC and CTMC is discussed.

Chapter 6: The heterogeneity observed in mast cell populations is difficult to define using anatomical location and detection or failure

to detect glycosaminoglycans. In this chapter, the cultured mast cells were shown to be mucosal in nature as they stained exclusively with a monospecific antibody raised against the proteinase found in MMC, rat mast cell proteinase II (RMCPII). By determining the proteinase phenotype of the mast cell granules, an alternative and reliable approach to identifying mast cell subsets has been found. The cultured mast cells were found to contain and secrete RMCPII and had the proteinase phenotype of MMC.

A system is described in this thesis whereby *in vitro* analogues of MMC can be grown *in vitro* from normal rat bone marrow under the influence of T cell products. This provides an opportunity to answer hitherto intractable questions about the factors affecting MMC growth and differentiation and the opportunity to dissect phenomena observed *in vivo* by manipulating the conditions of culture. It also provides an abundant source of these cells for further characterisation.

Table of Contents

General Introduction

The first clear description of the mast cell was made by Paul Erlich in 1877 (reviewed in Michels, 1938). He described cells whose granules showed metachromasia when stained with aniline dyes such as methyl violet and dahlia and viewed through the light microscope. The cells were variable in size and appeared in various amoeboid-like shapes in situ in the tissues. Erlich observed that the mast cell was generally found in connective tissues near blood vessels, nerves and glandular ducts as well as in inflammatory and neoplastic foci. They have subsequently been found to be widely distributed throughout the tissues although in varying numbers. In human tissues, the mast cells are relatively abundant in skin, thymus, lymphoid tissue, uterus, urinary bladder, tongue, synovia, mesentery around large and small blood vessels, and in the subserosal and submucosal layers of the digestive tract. Moderate numbers are found in the medium sized blood vessels, testes, heart and pancreas whilst reduced numbers are present in the adrenal glands, hypothalamus and placenta. Mast cells are relatively infrequent in muscle, bone and cartilage although they can be found in the surrounding connective tissue (Selye, 1965).

Mast cells are known to be involved in many reactions most notably the type I immediate hypersensitivity reaction and delayed-type hypersensitivity reaction (Metcalfe, Kaliner and Donlon, 1981; Askenase and Van Lovern, 1983). They have also been implicated in the pathogenesis of many disease states. Mast cells of the gastrointestinal tract have long been suspected of playing a physiological role in the function of this tissue and are involved in a number of inflammatory processes, including the response to parasitic infection, gastrointestinal allergy and chronic inflammatory diseases of the bowel (Bienenstock, Befus, Pierce, Denburg and

Goodacre, 1982; Lemanske, Atkins and Metcalfe, 1983; Barrett and Metcalfe,1984). The in situ histochemical studies describing the distribution and characteristics of the cells in normal and diseased tissue (Norris, Zamcheck and Gottlieb, 1963; Lloyd, Green, Fox, Mani and Turnberg, 1975; Strobel, Miller and Ferguson, 1981) along with the electron microscopic analysis of Crohn's disease indicate mast cell numbers can increase, and degranulation occur thus suggesting that mast cell mediators are involved in the pathogenesis of inflammatory bowel disease (Ranlov, Nielson and Wanstrup, 1972; Dvorak, Monahan, Osage and Dickersin, 1980). The increased prevalance of mast cells in rheumatoid synovium, pannus, and bone in rheumatoid arthritis has still to be evaluated but circumstantial evidence points to these cells being implicated in the pathogenesis of this disease also (Crisp, 1984).

A rapidly increasing body of evidence now firmly indicates that mast cells from different species, and from different tissues within the same animal exhibit marked variations in their morphological, cytochemical and functional properties (Michels, 1938; Enerback, 1966a and b; Kaliner, 1980; Pearce, 1982; Bienenstock, Befus, Denburg, Goodacre, Pearce and Shanahan, 1983; Jarrett and Haig, 1984). Most functional studies have been done with mast cells from rodents, and their tissues contain at least two distinct types of mast cell that differ in morphology, histochemical staining properties and location (Jarrett and Haig, 1984). Mast cells in the peritoneal cavity represent the connective tissue mast cell (CTMC). The other type is referred to as the mucosal mast cell (MMC) which is found in the lamina propria of the gastrointestinal tract and other mucous

membranes and is capable of rapidly increasing in number during certain immune reactions. The hyperplasia of MMC is one of the most striking cellular responses which occurs during helminth infection (Miller and Jarrett, 1971). The nature of the proliferative signals and origin of MMC has been the subject of much investigation.

Like mast cells, basophils were first recognised by Erlich in the late nineteenth century and were clearly distinguished by him from the fixed tissue mast cell (Michels, 1938), but because of similarities in the metachromatic staining properties and chemical composition of their granules, the clear distinction has sometimes become blurred. Basophils, unlike mast cells, are polymorphonuclear leukocytes which differentiate in the bone marrow, circulate in the blood and are capable of migrating into the tissues (Simpson and Ross, 1972; Dvorak and Dvorak, 1974). As well as the morphological differences, the origin, growth and differentiation of these two cell types is entirely separate. The origin of the tissue mast cell will be discussed at length later but suffice to say that mast cells are not found in the circulation and do not appear in progressive stages of development in the bone marrow although the precursor cells originally arise in the bone marrow (Kitamura, Shimada, Hatanaka and Miyano, 1977). The absence of recognisable mast cells in the blood implies that such precursors migrate to the tissues where they differentiate into mast cells. The basophils are present in the marrow in progressive stages of development and most likely arise from a stem cell in the bone marrow common to the other polymorphonuclear leucocytes, the eosinophil and neutrophil. The immature basophils arise in the bone marrow and undergo differentiation into mature cells that enter the blood but do not leave the circulation to enter the tissues under

normal circumstances. Basophils can however be recruited to the tissues as part of specific immunopathologic processes as well as increasing in numbers in the blood e.g. responses to parasites in the skin and gastrointestinal tract (Miller, 1969; Askenase, 1977; Ogilvie, Askenase and Rose, 1980). There is no evidence that basophils or mast cells are derived from each other, but because of the obvious working relationship between them their role in different allergic disease states has to be considered.

Because of the mast cell's increasingly recognised role in a variety of diseases the question of heterogeneity and control of proliferation and differentiation is crucial to our understanding of mast cell function. In this study, in vivo phenomena have been transferred to an in vitro model whereby rat bone marrow is cultured under the influence of various factors from lymph nodes of helminth infected animals. The resultant cells are then studied for their origin, differentiation, morphological and functional characterisation.

Review of the Literature

Mast cell heterogeneity

The existence of distinct subpopulations of mast cells was recognised by Maximow in 1906 and reviewed by Michels in 1938 butit wasn't till 1966 that a full systematic study of these subpopulations was performed by Enerback (1966a-d) who demonstrated the existence of "atypical" mast cells or mucosal mast cells (Mayrhofer, Bazin and Gowans, 1976) in the intestinal mucosa of the rat (Enerback, 1966a-d). These cells differ morphologically and histochemically from connective tissue mast cells which, when isolated from the peritoneal cavity, are the cells most used to study mast cell physiology and pharmacology. MMC were previously overlooked because of the special fixation procedures required for their demonstration in tissue sections (Enerback, 1966a). Mast cell heterogeneity in other species including humans (Strobel, Miller and Ferguson, 1981) has also been studied.

MMC and CTMC share the familiar staining characteristic of mast cells, namely metachromasia and dye-binding at very low pH levels, indicating the presence of sulphated glycosaminoglycans in the granules. However, the granules of MMC have a higher affinity for the copper pthalocyanine dyes such as alcian or astra blue at pH 0.3 in the astra blue/safranin sequence, while those of mature CTMC stain red: this distinction between blue and red is based on granule contents of weakly and strongly sulphated mucopolysaccharides respectively (Enerback, 1981). Staining properties cannot, however, be used as grounds for distinguishing these two subclasses of cell categorically because immature CTMC stain positively with alcian blue and become progressively more positive for safranin as they mature, until they are exclusively stained by safranin (Combs, Lagunoff and

Benditt, 1965). The indication from these findings and from the investigation by Miller and Walshaw (1972) who showed that alcianophilia of MMC granules was extinguished at a lower critical electrolyte concentration than that of the CTMC granules, was that the acid mucopolysaccharides of MMC were less strongly sulphated than those of CTMC. Confirmation of this was obtained by Tas and Berndsen (1977) who used a microspectrophotometric technique to demonstrate the absence of significant amounts of heparin in MMC and the presence of a lower sulphated glycosaminoglycan. Recently Enerback and colleagues (1985) have shown that the major polysaccharide produced by the rat MMC *in vivo* is an oversulphated galactosaminoglycan rather than heparin. They injected inorganic [^{35}S] into rats infected with *Nb.* and showed that the 5-fold increase in MMC was accompanied with a similar yield in labelled intestinal glycosaminoglycan. Analysis of the labelled material showed that 60% was oversulphated galactosaminoglycan whereas only 0.3% was heparin-related polysaccharides.

Further evidence of a difference between MMC and CTMC in the rat has been demonstrated by Woodbury and colleagues who have identified variant serine proteases in the two types of rat mast cells (Woodbury and Neurath, 1981). The first being rat mast cell protease I (RMCPI) originally isolated from skeletal muscle and found to be identical to that subsequently isolated from peritoneal mast cells. The second, a mast cell protease differing in solubility, structure and antigenicity from the first, was localised to mast cells in the small intestine and named RMCPII. Both are chymotrypsin-like in substrate specificity and molecular weight but are unusual in having relatively basic iso-electric points. Despite having a 75% sequence

identity in their first 52 amino-terminal residues (Woodbury, Everitt, Sanada, Katunuma, Lagunoff and Neurath, 1978), they apparently do not cross-react immunologically (Woodbury, Gruzenski and Lagunoff, 1978), as tested by gel diffusion. However, there is strong cross reactivity both by immunohistochemistry and by Western blotting but this can be eliminated by affinity chromatography (Gibson and Miller, 1986). The relatively high solubility of RMCPII is associated with the systemic release of this enzyme in rats primed with Nb. and challenged intraduodenally with the homologous parasite (Miller, Woodbury, Huntley and Newlands, 1983). The presence of these proteases as detected by specific antibodies and quantification by immunoassay, provides an alternative approach to identification of MMC and CTMC in the rat, as well as selectively monitoring the secretory activity of MMC in this species.

The above studies have been confined to the rat, but as MMC have been implicated in a range of diseases and allergic disorders in man as well as in helminth infections in laboratory and domestic animals, it was considered important to examine whether similar MMC enzymes were present in other species. By using enzyme histochemical methods for the demonstration of esterases within MMC of rat, mouse, sheep and man, serine esterases were detected in the granules of all four species and the mast cell identity confirmed by sequential staining with toluidine blue (Huntley, Newlands, Gibson, Ferguson and Miller, 1985). Although the function of these MMC enzymes remains unknown, the presence of similar chymotrypsin-like serine esterases within MMC of all four species studied suggests an important role for these enzymes.

Mast cells in biological reactions

Mast cells contain many biologically active substances; these are released and new mediators are generated as the end result of membrane activation. The diverse group of highly active pharmacologic materials released or generated by these cells possess all the necessary properties for a fully expressed but controlled local inflammatory response. They can be broadly grouped into vasoactive, chemotactic and enzyme moieties, depending on their physical properties and their action on surrounding tissue. It is also possible to group mast cell mediators into categories on the basis of their being preformed in the cell or subsequently generated by the cell after activation (Kaliner, 1980). The granule-associated preformed mediators released from the cell as an initial reaction are: histamine which is stored in secretory granules and is associated by ionic linkage with carboxyl groups of heparin (Uvnas, Aborg and Bergendoff, 1970); serotonin, which is stored in secretory granules of mast cells in rat and mouse (Benditt, Wong, Arase and Roeper, 1955); dopamine, being found in the mast cells of ungulates (Falck, Nystedt, Rosengren and Stenflo, 1964); the chemotactic polypeptides, eosinophil chemotactic factors of anaphylaxis (ECF-A), and in man, a neutrophil chemotactic factor of anaphylaxis (NCF-A) (Wintroub and Sotar, 1981). The stimulated mast cell then releases a number of mediators which augment the immediate effect of the above substances viz: various proteases and heparin. There is evidence that the heparin proteoglycan of rat CTMC and, by extension, the chondroitin sulphates of the rat MMC, by virtue of their charge and density, function as the storage matrix in the mast cell granule for histamine, enzymes and chemotactic factors (Uvnas, 1978). Heparin also acts as an

anticoagulant (Rosenberg, Armand and Lam, 1978) as well as inhibiting the alternative complement pathway (Weiler, Yurt, Fearon and Austen, 1978).

The RMCPI neutral protease with chymotrypsin-like activity characteristically present in rat CTMC has a tight ionic association with heparin and this results in the inhibition of protease activity against some macromolecule substrates. The proteases augment inflammation with rat mast cell protease II (RMCPII) being released very rapidly from mast cells in intestinal mucosa challenged with parasite worm antigen and being implicated in the generation of increased mucosal permeability (Miller et al, 1983; King and Miller, 1984).

Secondary mediators encompass those substances such as prostaglandins, leukotrienes and thromboxanes which are newly generated by mast cells as a direct result of their activation or are elicited from other cell types by the action of mast cell products. These mediators have agonist effects on microvasculature, smooth muscle, and inflammatory cells and can also act on mast cells to perhaps modulate the intensity of the initial activating event. Slow reacting substances of anaphylaxis (SRS-A) consists of leukotrienes C4, D4 and E4 (Murphy, Hammarstrom and Samuelsson, 1979; Lewis, Austin, Drazen, Clark, Marfat and Corey, 1980a; Lewis, Drazen, Austen, Clark and Corey, 1980b). The heterogeneity of mast cells is again emphasised by the diverse production of the arachidonate metabolites by the different cell types. The heparin containing mast cells: rat CTMC releasing PGD_2 (Lewis, Sotar, Diamond, Austen, Oates and Roberts, 1982); mouse CTMC releasing very small amounts of PGD_2 and LTC_4 (Dubuske, Stevens, Lewis and Austen, unpublished observation); human

lung mast cells releasing approximately equal amounts of PGD_2 and LTC_4 (Paterson, Wasserman, Said and Austen, 1976; Lewis et al, 1982). The non-heparin containing cells on the other hand: the rat basophilic leukaemia cell line identified as MMC-like (Seldin, Adelman, Austen, Stevens, Hein, Caulfield and Woodbury, 1985) releases substantially more LTC_4 than PGD_2 with culture-derived mouse mast cells also releasing much more LTC_4 than PGD_2 (Razin, Mencia-Huerta, Lewis, Corey and Austen, 1982). These metabolites of arachidonic acid can cause increased vascular permeability, potentiation of oedema and pain, chemotaxis of inflammatory cells and bronchoconstriction. Vasodilation is also maintained by these mediators in the later stages of the immediate allergic reactions (Lewis and Austen, 1984).

There exist many diverse agents that can provoke mast cells to degranulate and release mediators. The best known of these is the reaction of IgE antibodies with specific antigen on the surface of the cell. Antigen-dependent cross-linking of IgE which is fixed to mast cell plasma membrane receptors via its Fc region is necessary for degranulation to occur (Ishizaka, Chang, Taggart and Ishizaka, 1972). The sequence of biochemical events occurring in the target cell following antigen bridging of IgE molecules on the surface membrane involves extracellular Ca^{++} and cyclic AMP as well as esterase activity. In some instances other classes of immunoglobulin molecules can trigger mast cell mediator release but the affinity of the antibodies for receptors on the cell is much less than that of IgE. Subpopulations of mast cells do not function identically in response to secretagogues and the ability of drugs to inhibit secretion also varies with the mast cell population. In a direct comparison of

isolated rat CTMC and MMC, disodium cromoglycate and theophylline inhibited antigen-induced histamine release from CTMC but not from MMC while doxantrazole inhibited release from both populations (Pearce, Befus, Gauldie and Bienenstock, 1982). The chemical detergent compound 48/80 causes degranulation of CTMC but not MMC in the rat, and is not effective in degranulating human lung mast cells (Caulfield, Lewis, Hein and Austen, 1980; Befus, Pearce, Gauldie, Horsewood and Bienenstock, 1982). Cultured mouse mast cells release granule mediators in response to immunological activation (Razin, Mencia-Huerta, Stevens, Lewis, Liu, Corey and Austen, 1983) and ionophores (Razin, Mercia-Huerta, Lewis, Corey and Austen, 1982), but not to compound 48/80 (Sredni, Friedman, Bland and Metcalfe, 1983). Activation of either alternative or classical pathways of complement can also generate factors (C_{3a} and C_{5a}) which stimulate mediator release (Wintroub and Soter, 1981). Mast cells and their products are uniquely suited to their function in the facilitation of removal of foreign organisms or endogenous tissue breakdown products by augmentation of local venular permeability and enhancement of ingress and function of host cells.

Allergic reactions in helminth infection

Helminth parasite infection stimulates a broad based immune response involving humoral and cell mediated mechanisms. Common to virtually all helminth infections, are raised levels of antigen specific and non-specific IgE, the occurrence of immediate allergic reactions and an associated increase in the number of mast cells, basophils and eosinophils (Jarrett and Miller, 1982).

Nippostrongylus brasiliensis infection in rats

One of the most researched of all host-helminth relationships which exemplifies the IgE and associated cellular responses, is the infection of rats with N. brasiliensis (Nb.). This nematode parasite has a rapid life cycle, well documented pathological and immunological effects on the host, and is not infective for man. Rats recover rapidly and completely from a moderate infection.

Nb. is an intestinal nematode parasite of the rat. The infective larval stages (L_3) penetrate the skin of the host, migrate via the bloodstream to the lungs where they undergo a moult to the L_4 stage. They are then coughed up and swallowed and 4-5 days after initial infection form a population of adult worms inhabiting the upper portion of the small intestine (Ogilvie and Love, 1974). Ten days after infection the parasites start to be expelled and this reaction is complete by day 14 after infection (Jarrett, Jarrett and Urquhart, 1968).

The IgE response in N. brasiliensis infection

Parasite non-specific IgE is produced in infected rats before the antigen specific response can be detected (Orr and Blair, 1969; Jarrett and Bazin, 1974). The potentiated IgE response to egg albumin reaches a peak 12-14 days after infection synchronously with elevation of total IgE (Jarrett and Bazin, 1974). Nb.-specific IgE rises to a peak some 2-3 weeks later when both total IgE and the potentiated response have largely declined. Following reinfection, the parasite-specific IgE level rises steeply to around day 6 after infection followed by a slow decline (Jarrett, Haig and Bazin, 1976). The total

IgE level however, although boosted simultaneously with the specific IgE response, declines rapidly after reinfection (Jarrett, Haig and Bazin, 1976). As with all IgE responses the effect is highly T cell dependent (Jarrett and Ferguson, 1974; Jarrett, Haig and Bazin, 1976).

An extensive series of experiments was carried out in the Ishizakas' laboratory to study the soluble IgE regulatory factors produced by T cells. In fact IgE itself is involved in the regulation of IgE production. In vitro studies have identified several IgE binding factors secreted by lymphocytes of Nb.-infected rats. Around days 12-14 of an infection there is an increase in IgE bearing B cells in the MLN and spleen. These cells also exhibited IgD and IgM on their surface and produce IgE B cell generating factor which stimulates differentiation of IgM bearing cells to E, M and D triple bearing cells (Urban, Ishizaka and Bazin, 1980). It was postulated that factor production could be triggered by the cross-linking of IgE molecules by antigen on the B cell surface (Urban, Ishizaka and Ishizaka, 1978).

Yodoi and Ishizaka in 1979 discovered another subpopulation of T and B cells bearing Fc receptors for IgE ($Fc_\varepsilon R^+$ cells) in Nb.-infected rats. Associated with an increase in number of these cells was an elevation of IgE levels (Spiegelberg, 1981). In vitro studies of potentiation of IgE (Suemara and Ishizaka, 1979) have shown that $Fc_\varepsilon R^+$ T cells are the source of potentiating factor which binds to IgE B cells and stimulates IgE production (Suemara, Yodoi, Hirashima and Ishizaka, 1980; Yodoi, Hirashima and Ishizaka, 1980). IgE suppressive factor was found to abrogate the effect of the potentiating factor (Hirashima, Yodoi and Ishizaka, 1980). Both

factors have also been produced by *in vitro* culture of MLN cells from rats injected with *B. pertussis* or complete Freund's adjuvant (Hirashima, Yodoi and Ishizaka, 1980). Subsequently various T and B cell-derived factors were discovered by the use of a *Nb.*-infected rat culture system and these factors are now thought to be involved in the control of IgE synthesis in allergy (Ishizaka, Yodoi, Suemara and Hirashima, 1983).

Cellular responses in helminth infection

Since Taliaferro and Sarles (1939) first reported the involvement of what they termed connective tissue basophils and eosinophils in the mucosal response to *Nb.* several studies have documented the mast cell response in mice and rats (Askenase, 1977; 1980; Miller and Jarrett, 1982). In Hooded Lister rats infected with *Nb.* an exponential increase in mast cell numbers occurs between days 10 and 14 of a primary infection (Miller and Jarrett, 1971). However, the role of mast cells in immune expulsion is controversial as in some strains of rats these cells are only detectable following worm expulsion (Askenase, 1980). Anamnestic elevation of MMC numbers in parallel with worm expulsion occurs following reinfection (Mayrhofer, 1979). The anamnestic response of mast cells indicates the immunological control under which the cell responds to a stimulus. Detailed light and electron microscopic studies have demonstrated in rats harbouring the nematode *Nb.* that MMC arise from undifferentiated blast cells (Miller, 1971) and these findings agree well with the much earlier light microscopy study of Taliaferro and Sarles (1939).

Metachromatically stained granular cells can also be seen in the

epithelium of the small intestine of parasite-infected rats when stained with toluidine blue, these were termed globule leucocytes. These cells and their relationship to mast cells was studied extensively in the Nb. infected rat. The overwhelming conclusion reached was that globule leucocytes were mast cells that had migrated to the epithelium and degranulated (Murray, Miller and Jarrett, 1968; Miller, 1980).

The relationship between globule leukocytes and MMC in mice is less well defined. In normal mice, there are few MMC in the lamina propria, whereas cells containing basophilic granules (globule leukocytes) predominate in the epithelium in normal and parasitized mice (Askenase, 1980). A further cell type complicates the issue in mice, in that a subpopulation of intraepithelial lymphocytes has been shown to be granulated and to stain similarly to MMC, a precursor relationship of these granulated intraepithelial lymphocytes to MMC has been suggested (Guy-Grand, Griscelli and Vassalli, 1978).

A practical involvement for mast cells in helminth infection has unfolded. In Nb. infection, a mast cell-mediated local anaphylactic reaction in the gastrointestinal tract is thought to increase vascular permeability, allowing protective antibodies, cells and lymphokines access to the worms. The concept of such a "leak lesion" was put forward by Urquhart and colleagues (Urquhart, Mulligan, Eadie and Jennings, 1965; Barth, Jarrett and Urquhart, 1966). The detection and assaying of the secreted neutral proteinase RMCPII from MMC has allowed direct measurement of MMC activity. The release of this enzyme into the blood of rats infected with N. brasiliensis and T. spiralis has been described (Woodbury, Miller, Huntley, Newlands, Palliser and Wakelin, 1984). In the T. spiralis infected rats, the

majority of adult worms were expelled from the intestine between 9 and 12 days after infection. Mucosal mastocytosis was maximal at day 12 with peak concentrations of enzyme in the serum being detected on this day also. Nb. worms were expelled from infected rats 10-12 days after infection. Maximal MMC hyperplasia occurred on day 12 with maximal accumulation of RMCPII on day 13 in the jejunal mucosa. The lag of RMCPII accumulation in the jejunal mucosa behind maximal mastocytosis suggested MMC secrete rather than store the enzyme. Recently it has been demonstrated that the granule product of MMC, RMCPII, is secreted systemically in Nb.-primed rats challenged with soluble worm antigen (Miller, Woodbury, Huntley and Newlands, 1983) and the gut is the major source of the systemically secreted enzyme (King, Miller, Woodbury and Newlands, 1986). King and Miller (1984) showed that there was a significant correlation between systemic and enteric secretion of RMCPII and the enteric accumulation of Evan's blue after intravenous challenge of Nb.-primed rats with whole worm antigen. The results indicated that RMCPII could have a role in altering intestinal mucosal permeability during systemic anaphylaxis in the rat (King and Miller, 1984). These findings support the concept that mast cells have a functional role in nematode expulsion.

It was reported that in Nb.-infected rats, basophils in peripheral blood increased from 0.06% to 4.5% of total leukocytes 13 days after primary infection. Following reinfection, basophilia occurred more rapidly peaking at 5 days (Ogilvie, Hesketh and Rose, 1978). Miller (1969) observed that basophils increase in number in the small intestine from around day 8 onwards, peaking at the time of worm expulsion and declining thereafter. The basophil was thus a

prime candidate for an early anaphylactic response in the gut during expulsion whereas the mast cell role had been more controversial as mentioned previously. However, no evidence of basophil mediated anaphylaxis under these circumstances has been uncovered.

Eosinophils are much more abundant in the bone marrow, blood and the tissues of normal animals than basophils or mast cells. In mice and rats they have been shown to respond to parasite infection. Spry (1971) demonstrated that during Trichinella spiralis infection in rats, eosinophils arise from precursors in the bone marrow where they mature and travel via the bloodstream to the sites of inflammation. The response of eosinophils to stimulation is under T cell control (Walls, 1976) and an anamnestic eosinophil response to helminth infection can be demonstrated. As with mast cells, basophils and raised IgE levels, it has been difficult to ascribe any function to these cells in vivo. In vitro, however, eosinophils along with macrophages and neutrophils have been shown to attack and kill schistosomula in the presence of specific antibodies with or without complement (Askenase, 1980). Capron and colleagues have demonstrated that rat IgE antibody complexed to schistosome antigen binds to macrophages via an Fc receptor. The macrophage becomes activated and is capable of destroying schistosomula (Capron, Dessaint, Capron and Bazin, 1975; Dessaint, Capron, Joseph and Bazin, 1979). Rat eosinophils have also been demonstrated to kill schistosomula in vitro in the presence of parasite-specific IgG antibodies and complement (MacKenzie, Ramalho-Pinto, McLaren and Smithers, 1977; Ramalho-Pinto, McLaren and Smithers, 1978). The presence of mast cells has been shown to be required by eosinophils for the killing of schistosomula (Capron, Capron, Torpier, Bazin, Bout and Joseph, 1978). There is,

therefore, an intimate relationship between mast cells, basophils, eosinophils and IgE levels in parasite infection.

T cell control of mast cells

Low but near normal numbers of MMC are present in uninfected athymic animals (Mayrhofer and Bazin, 1981) so that at a basal level the progression from stem cell to this specialised cell would appear to be without the benefit of the normal complement of T cells, although some reports have been published to the contrary. Olsen and Levy (1976) noted a marked deficiency of MMC in nu/nu mice with no increase in MMC following Nb. infection, whereas previously, Jacobson and Reed (1974) showed Nb. worms persisting in nu/nu mice confirming the T dependancy of immune expulsion. It has been shown in mice infected with T. spiralis that the hyperplasia of MMC that occurs in helminth infection requires T cells (Ruitenberg and Elgersma, 1976). Wakelin and colleagues have analysed the T cell control of the effector cells of bone marrow origin in the protective response against parasitic nematodes (Wakelin, 1980; Wakelin and Donachie, 1980, 1981, 1983; Alizadeh and Wakelin, 1982), as well as the genetic control of the response to T. spiralis in mice. MLN cells transferred from immune slow responder BIOG mice to normal BIOG mice, which would normally expel T. spiralis by day 16, accelerated expulsion of worms from recipients only by day 12. However, transfer of the same cells to rapid responder NIH mice accelerated expulsion of the parasite by day 8 of infection. The reciprocal transfer of IMLN cells from NIH to BIOG mice again resulted in expulsion by day 12 (Wakelin and Donachie, 1980). Therefore the rapidity of response by the recipient to

adoptive immunization was not a function of the transferred cells but rather of the environment into which they were transferred. The slow response was found to be determined by cells derived from the bone marrow (Wakelin and Donachie, 1981). Suggestion was made of genetic control of expulsion being expressed by the cells involved in the generation of the intestinal inflammatory response, and a recipient derived cooperating lymphocyte; one of the bone marrow derived components being the mast cell. Alizadeh and Wakelin (1982) showed mastocytosis was more rapid in onset and more intense in NIH than BIO strains of mice infected with T. spiralis. The onset of mastocytosis was again determined by cells of bone marrow origin following reciprocal transfer of lymphocytes between NIH and BIOG mice and radiation chimaeras. These in vivo studies suggested that mucosal mast cells differentiated from bone marrow cells through the influence of factors originating from T cells (Alizadeh and Wakelin, 1982). In the rat, infestation with Nb. also causes an increase in gut MMC. Nawa and Miller (1979) showed that an accelerated intestinal mast cell response could be transferred with immune cells from thoracic duct lymph, or the T cell fraction (surface Ig^-), but not the B cell fraction (surface Ig^+) of these cells. Increasing numbers of T cells induced progressively greater intestinal mast cell responses. Cells obtained 10 days after infection were optimal, and antigenic stimulation provided by infection of the recipients was necessary. The MMC response was shown to be absent in rats subjected to drainage of thoracic duct lymph plus thymectomy (2-12 weeks), but could be restored by transfer of cells from thoracic duct lymph (Mayrhofer, 1979; Mayrhofer and Fisher, 1979). It was not known at this time if

stimulation of these T cells by the worm antigen was leading to direct transformation of the cells into mast cells or if factors produced by them were exerting an inductive influence on MMC precursors. An investigation of the proliferation and development of MMC will form a part of this thesis.

Mast cells in vitro

Many investigators have attempted to determine the origin of mast cells and the factors affecting their development by in vitro culture methods.

Mast cells have been cultured from many different cell populations within the haemopoietic and lymphoid tissues. As far back as 1963, Ginsburg and Sachs had grown pure suspensions of mast cells from thymocytes seeded onto mouse embryo fibroblast layers. The transformation had occurred over a period of two months and was dependant on the presence of the feeder layer. Subsequently, it was found that mesenteric lymph node and thoracic duct cells from mice hyperimmunised with horse serum developed into mast cells when grown over a mouse embryo fibroblast feeder layer in the presence of homologous antigen (Ginsburg and Lagunoff, 1967). Pursuing these earlier studies it was found that two types of mast cells arose in such cultures: one type, not dependant on T-cell factors, arose from the cells in the fibroblast monolayer; while the other type dependant on the presence of T cells or their products and resembling MMC, arose from a precursor in the lymphoid layer (Ginsburg, Ben-Shahar and Ben-David, 1982). Mast cells derived from the former are generally large and contain small homogenous granules whereas mast cells derived

from lymphoid tissue appear smaller and contain fewer and more irregular granules. In the rat, cultures of rat thymus showed that both thymus cells and feeder layers could develop into mast cells (Ishizaka, Okudaira, Mauser and Ishizaka, 1976).

In vitro, mouse mast cell lines with the biochemical and cytological properties of MMC have been grown (Sredni et al, 1983). The aforementioned authors cloned histamine-containing granulated cells from mouse bone marrow using sequential soft agar and limiting dilution techniques. They could differentiate between basophils, CTMC and MMC on functional, morphological and biochemical parameters. Ultrastructural criteria were relied on heavily for this distinction. The cloned cells bound and had an affinity for monomeric IgE similar to that of normal mast cells. They failed to ingest latex particles and contained histamine. These characteristics being consistent with the cells being basophils, CTMC or MMC. Morphologically they resembled mast cells rather than basophils, having thin, elongated microvilli or plasmalemmal ridges in contrast to short villi and blunt processes reported in mouse basophils (Dvorak, Nabel, Pyne, Cantor, Dvorak and Galli, 1982). The histamine-containing granulated cells possess the ultrastructurally distinct cytoplasmic granules found in cultured mouse mast cells, as well as the heterogeneity of size of granule which can fill the cytoplasm and which is not seen in basophils. The proteoglycans and glycosaminoglycans produced by the histamine-containing granulated cells were shown to be chondroitin sulphate, which is oversulphated, and not heparin which is present in CTMC. Their failure to respond to compound 48/80 is consistent with these cells being MMC.

Mast cells can be maintained in proliferation for prolonged

periods in the presence of the appropriate factor. The latter is associated with a lymphokine identified in the supernatant of Con-A stimulated T cells (Nabel, Galli, Dvorak, Dvorak and Cantor, 1981; Razin, Cordon-Cardo and Good, 1981; Tertian, Yung, Guy-Grand and Moore, 1981) and is also present spontaneously in the supernatant of the mouse WEHI-3 tumour (Nagao, Yokova and Aaronson, 1981; Yung, Eger, Tertian and Moore, 1981). It has been variously called IL-3 by Ihle, Rebar, Kellar, Lee and Hapel (1982), burst-promoting activity by Iscove, Roitsch, Williams and Guilbert (1982), P-cell stimulating factor by Schrader and Clark-Lewis (1982) and WEHI-3 factor by Bazill, Haynes, Garland and Dexter (1983). This factor has been extensively purified and shown to be identical to mast cell growth factor and to "histamine-producing cell stimulating factor" (Ihle, Keller, Oroszlan, Henderson, Copeland, Fitch, Prystowsky, Goldwasser, Schrader, Palyszynski, Dy and Lebel, 1983). The cDNA for the murine IL-3 gene has recently been cloned (Fung, Hapel, Ymer, Cohen, Johnston, Campbell and Young, 1984; Yokota, Lee, Rennick, Hall, Arai, Masmann, Nabel, Cantor and Arai, 1984) thus providing a source of pure IL-3 for the analysis of control of haemopoiesis and of the various lineages derived from haemopoietic tissues.

IL-3 has an amino acid sequence of 166 residues which includes a signal peptide at the N-terminal sequence, and has four potential N-glycosylation sites. The generation of the mature form of IL-3 involves a proteolytic processing step as well as removal of the signal peptide (Fung et al, 1984; Yokota et al, 1984). Kinnaird and Garland (1985) stated that the action of IL-3 appears to be the stimulation of anaerobic glycolysis. Using two IL-3 dependent cell lines, AC-2 and Ea-3, they investigated the possible existence of

different membrane epitopes on each cell line with which IL-3 interacts. Specific antibodies were raised against the IL-3 dependent cell lines and it was concluded that each line may express different receptor domains for IL-3 which are stimulated by specific antibody binding. However, the possibility that these putative antibodies may stimulate through mechanisms unrelated to an "IL-3 receptor" interaction is still under investigation.

Mast cells with the morphological features of CTMC have also been grown in culture for short periods. Precursors found in rat peritoneal washings are stimulated to proliferate by medium conditioned by an L-cell fibroblast line. These mast cells stain positively with safranin and, when stimulated with compound 48/80, release histamine, both are characteristic of CTMC (Czarnetzki, Hannich and Niedorf, 1979).

Rats infected with Nb. form the basis and source of cellular material for the experiments in cell culture carried out in this laboratory. Conditioned media capable of causing the proliferation of mast cells from normal rat bone marrow cultures were derived from cultures of antigen-stimulated MLN cells of rats infected with Nb. (Haig, McKee, Jarrett, Woodbury and Miller, 1982). The lymph nodes were removed 12-30 days after infection and incubated in culture with Nb. antigen. The resultant supernatants were then added to cultures containing normal rat bone marrow cells and the resultant total and differential cell counts analysed. The cells generated under these circumstances had the appearance and staining properties of MMC. More convincingly they both contained and secreted the specific protease RMCPII. A modified method of this culture routine is utilized to

investigate the factors involved in this model system and to provide sufficient numbers of MMC for investigation. The results obtained are documented in this thesis.

It has recently been shown that the rat basophilic leukaemia cell line, a chemically-generated tumour cell line maintained in culture, is homologous to the rat MMC in that it has similar staining properties but, more convincingly, contains RMCPII (Seldin, Adelmen, Austen, Stevens, Hein, Caulfield and Woodbury, 1985).

Attempts to culture human mast cells from various haemopoietic sources, including bone marrow (Tadakoro, Stadler and De Weck, 1983), cord blood (Ogawa, Nakahata, Leary, Sterk, Ishizaka and Ishizaka, 1983) and peripheral blood cells (Czarnetzki, Fidger, Kolde, Vroom, Aalberse and de Vries, 1984) have been reported. A plethora of conditioned media derived from concanavalin A or phytohaemagglutinin-stimulated peripheral lymphocytes (Tadakoro et al, 1983), or bladder cell carcinoma cell line supernatants (Rimmer and Horton, 1984) were used in an attempt to find a mast cell growth factor source. Despite the variety of culture techniques, no definitive liquid mast cell culture has been achieved. This has been due mainly to the inability to distinguish human basophils from mast cells in vitro. Rimmer, Turberville and Horton (1984) have produced mouse monoclonal antibodies directed against the granule components of mast cells. They are strongly reactive with human mast cells in both connective tissue and mucosal sites but not with any other infiltrating leucocyte or connective tissue cell type, which is a step in the right direction.

Origin and differentiation of mast cells in vivo and in vitro

One of the most studied aspects of mast cells concerns their origin and turnover in adult life. The possibility that the metachromatic granules, one of the visual characteristics of mast cells, need not be present in mast cell precursors makes the problem of mast cell origin very difficult, disagreement being compounded by the heterogeneity of the mast cell population. A detailed light and electron microscopic study of MMC differentiation was carried out in the rat by Miller (1971). MMC are virtually absent from rat jejunal mucosa 8 days after infection with Nb. but they reappeared on the 10th day and subsequently peaked on Day 14 (Miller and Jarrett, 1971). Initially the MMC contained small numbers of granules but by Day 14 they were fully granulated and seen to undergo mitosis throughout the course of population expansion. The sequences of differentiation and maturation beginning with the blast cell and ending with the fully granulated mast cell were readily visualised. The use of both light and electron microscopy facilitated the identification of mast cells. The granules of the mature and maturing MMC having deeply basophilic granules whereas other cell types had only faintly stained inclusions. The granules eventually filled the cytoplasm at the expense of the other cytoplasmic components and this is similar to the pattern of maturation for CTMC (Combs, 1966), eosinophils (Wetzel, Horn and Spicer, 1967) and basophils (Wetzel, Horn and Spicer, 1967; Terry, Bainton and Farquhar, 1969).

Among many theories, there is one which regards the thymus as a central source of mast cell precursors (Ginsburg, 1963; Csaba, Surjan, Fischer, Kiss and Toro, 1969). An increase in the number of mast

cells in the thymus was reported to be induced by various experimental treatments and thymic lymphocytes observed under such conditions in the blood circulation were considered to be the most plausible mast cell precursors (Csaba and Hondinka, 1970). However, Viklicky, Sima and Pritchard (1973) found it hard to reconcile the abundance of mast cells in the connective tissue of the skin and in the lymph nodes of athymic mice with the hypothesis that the precursors of these cells were of thymic origin. As mentioned previously, the work of Nawa and Miller (1979) showed that an increase in the numbers of MMC occurred in infected recipients of thoracic duct lymphocytes obtained from donor rats which had themselves been infected 10 days previously. These TDL were subsequently fractionated and immunoglobulin negative cells were the only ones capable of transferring the MMC response. It was not clear at this stage, however, if the transferred cells differentiated into the MMC or if they produced factor(s) capable of inducing proliferation and differentiation of MMC precursors in the gut itself.

A population of small lymphocytes has been isolated from the intestinal mucosa of mice, rats and rabbits (Rudzik and Bienenstock, 1974; Guy-Grand, Griscelli and Vassalli, 1978; Mayrhofer and Whately, 1983; Schrader, Scollay and Battye, 1983) These cells contain a few granules with staining properties very much like those of mast cells (Guy-Grand, Griscelli and Vassalli, 1978; Mayrhofer, 1980). It has been suggested that these granulated intraepithelial lymphocytes might represent a transitional form between lymphocytes and MMC (Guy-Grand, Griscelli and Vassalli, 1978). This has been refuted by Huntley and colleagues (1984) who demonstrated that the MMC-specific protease (RMCPII) was present only in the granules of MMC and globule

leucocytes but not the intraepithelial lymphocytes found in the gut. This study added further support to the view that MMC and GL are of common lineage and strongly suggest that the gut intraepithelial lymphocytes are unrelated to them.

Kitamura in collaboration with a number of different workers designed and executed a series of elegant experiments involving mice of the W/W^V and bg/bg genotypes. The W/W^V mice have a severe macrocytic anaemia with a decrease in granulocytes and megakaryocytes in the bone marrow and are generally regarded as being mast cell-free. The giant granules of beige (Chediak-Higashi syndrome) mice are useful as a marker for determining the origin of tissue mast cells. In 1977 Kitamura et al demonstrated that mast cells of the connective tissue type could be derived from transplanted bone marrow. Irradiated W/W^V mice were recipients of donor bone marrow cells from beige mice. Mast cells that appeared, contained giant granules of the beige type.

Mast cell precursors have been shown to be present in the foetal liver of mice on day 13 of intrauterine life (Kitamura, Shimada and Go, 1979). In irradiated recipients, the differentiation of mast cell precursors of foetal liver origin is controlled locally by the same mechanism controlling the differentiation of mast cell precursors of adult bone marrow origin. Environmental factors are implicated in the mechanisms involved, which is probably why it takes so long for the donor type mast cells to develop. It was suggested that CTMC precursors are of haemopoietic rather than lymphopoietic origin (Kitamura, Shimada, Go, Matsuda, Hatanaka and Seki, 1979). Various haemopoietic and lymphoid tissues were injected into W/W^V mice and it was found that CTMC appeared in recipient mice after bone marrow or

spleen cell injections, but lymphoid tissues were very poor sources of CTMC precursors.

Crowle and Reed (1984) wanted to determine whether MMC derived from bone marrow or from thymus. Mast cell-deficient W/W^V and athymic mice were given grafts of bone marrow, spleen cells, thymus cells, thymus glands, or anti-Thy-1 treated bone marrow or spleen cells. The grafts of thymus glands or thymus cells repaired the MMC deficiency of athymic but not W/W^V mice, suggesting that MMC precursors are not present in the thymus, although a helper or accessory cell may be found. Bone marrow, anti-Thy-1 treated bone marrow, or anti-Thy-1 treated spleen cells repaired the mast cell defects of W/W^V but not athymic mice, suggesting bone marrow contains precursors for both CTMC and MMC. Whole spleen grafts repaired the defects of both W/W^V and athymic mice, thus the spleen contains both the mast cell precursors lacking in W/W^V mice and thymus derived factors or cells needed to elicit the MMC response lacking in athymic mice.

The precursors of gut MMC were investigated by Guy-Grand, Dy, Luffau and Vassalli (1984). They found that the gut mucosal and bone marrow cells have the most MMC precursors but that these two precursors differ from each other. The bone marrow-derived MMC precursors have a striking property of homing into the gut which is not related to any T cell activity or antigenic stimulation since the high frequency of gut MMC precursor is observed in nude, germ-free and newborn mice, as well as in grafts of foetal gut. Unknown factors released <u>in situ</u> are probably responsible for the further differentiation of gut MMC precursors from bone marrow-derived MMC precursors.

With the development of semisolid culture techniques during the

past two decades, haemopoietic multipotential stem cells and most of the haemopoietic progenitor cells can produce differentiated progeny in vitro. Much of the information on haemopoiesis has been gained from primary bone marrow cultures stimulated by feeder layer or growth stimulatory factors from various cellular sources (Dexter and Testa, 1980). In particular, the soft agar technique for growing colonies of bone marrow cells has helped identify many stem cell and progenitor cells and the stimuli to which they respond (Pluznik and Sachs, 1965; Bradley and Metcalf, 1966). The cells that give rise to the colonies are termed colony forming cells. These are progenitor cells i.e. precursor cells capable of giving rise to colonies of one or two cell types (Johnson and Metcalf, 1977) with mixed colony forming cells giving rise to more than two cell types only seen very occasionally (Fauser and Messner, 1979). The pluripotent stem cell is analogous to the spleen colony forming unit. It is assayed in vivo by its ability to form colonies in the spleens of lethally irradiated animals when the pluripotent stem cell is transferred as part of a bone marrow reconstitution. The colonies formed contained all the haemopoietic cell types (Till and McCulloch, 1961). Haemopoietic stem cells are divided into three categories: the stem cell compartment, the progenitor cell compartment, and the morphologically recognisable compartment (Metcalf, 1977). There is proliferation and growth in each compartment, with specialisation occurring with progression from one compartment to the next, but cells are not able to move from mature to immature compartments (i.e. progenitors cannot revert to stem cells), or move to one of corresponding maturation in another lineage, e.g. from granulocyte/macrophage progenitor to erthyroid progenitor.

Schrader, Lewis, Clark-Lewis and Culvenor (1981) suggested a myeloid haemopoietic origin for mast cells growing in suspension cultures, by demonstrating that these cells could be generated from colonies grown in agar cultures of bone marrow cells. By direct cytologic examination of mixed haemopoietic colonies and mast cell colonies, it was clearly demonstrated that mast cells are derived from multipotential haemopoietic stem cells and share a common origin with other haemopoietic cell lineages (Nakahata, Spicer, Cantey and Ogawa, 1982). Nakahata et al (1982) concluded that the relatively differentiated cell recognisable as a mast cell did not have self-renewal capabilities. This is at variance with Schrader (1981), where homogenous populations of cells that were all granulated, showed a plating efficiency in agar of up to 10%, the limiting dilution assay confirmed this figure (Crapper and Schrader, 1983). Thus, the results of these experiments suggest that differentiated cells with many characteristics of mast cells do have significant potential for division. In vivo transfer of a single peritoneal mast cell injected into the skin of W/W^V mice resulted in a cluster of mast cells numbering ~ 2,000, this happened at 6% of injection sites. Therefore, this indicated that some peritoneal mast cells kept extensive proliferative potential even after morphological differentiation (Sonoda, Kanayama, Hara, Hayashi, Tadakoro, Yonezawa and Kitamura, 1984).

Recently, a most interesting finding was reported, where murine cultured mast cells of $WBB6F_1$, +/+ origin were transferred to mast cell deficient mice ($WBBF_1$-W/W^V) whereupon they displayed characteristics of either the connective tissue types or mucosal phenotype depending on anatomical location. Intravenous injection of

representative connective tissue type mast cells into mast cell deficient mice gave similar results to those obtained with the cultured mast cells: mast cells developing in the peritoneal cavity, skin, spleen, and glandular stomach muscularis propria of the $WBB6F_1$-W/W^V recipients stained with safranin and berberine sulphate whereas mast cells developing in the mucosa of the glandular stomach stained only with alcian blue. Thus, histochemically, it seems that both peritoneally derived mast cells and cultured mast cells are able to acquire characteristics of either CTMC or MMC, supporting the notion that the mast cell phenotype may be regulated, at least in part, by the tissue microenvironment (Nakano, Sonoda, Hayashi, Yamatodani, Kanayama, Yamamura, Asai, Yonezawa, Kitamura and Galli, 1985). It should be acknowledged, however, that histochemistry represents an indirect approach to identification of the cellular constituents of the cells and some direct biochemistry would substantiate the claims. As well as this, surface membrane characteristics of recovered cells could be compared to those of the cultured and normal peritoneal mast cells (Katz, LeBlanc and Russel, 1983; Razin, Ihle, Seldin, Mercia-Huerta, Katz, LeBlanc, Hein, Caulfield, Austen and Stevens, 1984).

In this study a number of assays have been employed to look at the origin and differentiation of rat MMC *in vivo* and *in vitro* in normal and helminth infected animals. The main objectives of this study were to generate MMC *in vitro*, using normal and infected rats as sources of haemopoietic tissue, thus analysing the effect of helminth infection on these animals. The mast cells generated under these conditions were then characterised using morphological, biochemical and histological techniques in order to try and catagorise them as has been done for their *in vivo* counterparts.

Materials and Methods

Animals and maintenance

The two principal strains of rats used in the experiments described in this thesis were (LIS x BN)F_1 and F344. Colonies of inbred Hooded Lister (LIS) and Brown Norway (BN) rats were maintained in the Wellcome Laboratories for Experimental Parasitology (WLEP). LIS females were bred with BN males, and the resulting (LIS x BN)F_1 crosses used at between 2 and 4 months of age. The F344 rats were originally purchased from OLAC Ltd. (Bicester, Oxon) and were subsequently bred from inbred colonies at WLEP. Breeding and experimental animals were housed separately, the former receiving breeding diet (Oxoid Ltd., Hampshire) and the latter Diet 41 (Hamlyn Angus Milling Ltd., Perth). Athymic rats (PVG-rnu/rnu/Ola) and rnu/+ heterozygous littermates were purchased from Olac. All animals were housed in wire bottomed cages suspended above trays containing wood shavings, except when the rats were breeding, when enclosed boxes were used. Water and food were supplied ad libitum.

Propagation of N. brasiliensis (Nb.)

Faeces from culture rats infected by the subcutaneous injection of 4000 infective Nb. larvae were collected on absorbent paper between days 7 and 9 of infection. The faecal pellets were washed under running tap water for 2-3 minutes, soaked in sufficient water to cover the pellets for 2-3 hours, and were then gently mixed to form a thick paste. Granular charcoal (10-18 mesh; BDH Chemicals, Poole, Dorset) was added until the mixture was almost dry, but still bound together (approximately one volume of faeces to two volumes of charcoal). Moist filter paper (Whatman No. 1) was put in the centre of a petri-

dish, and enough faeces/charcoal mixture was added to cover about 70% of the paper. The petri-dishes were stored covered in a moist box in a humidified incubator at 27°C for 5-7 days.

The larvae were harvested by gently layering the faeces/charcoal mixture on to a piece of filter paper (K-dex, Kleenaroll Ltd., London), placed in a fine sieve (300 mesh, Endecott) on a Baermann funnel, and larvae collected by sedimentation. Aliquots of the larval suspension were counted and the suspension adjusted in saline to give 4000 larvae per ml.

Infection of experimental rats with N. brasiliensis

Using a 20 gauge 1" needle and 1 ml syringe, 1 ml of the above suspension was administered subcutaneously in the groin region of the rat. Thus 4000 larvae were the standard infective dose.

Removal of tissues

1. Collection of blood

Animals were anaesthetised in jars containing cotton wool soaked in Trichloroethylene (BDH). Blood was obtained by cardiac puncture using a 20 gauge needle and 10 ml syringe. Serum was obtained by allowing the blood to clot at room temperature. The serum was separated from residual blood cells by centrifugation and was stored at -30°C prior to use.

2. Excision of lymphoid tissue

Anaesthetised rats were exsanguinated by cardiac puncture and killed by cervical dislocation. The rats were placed ventral side up

on tissue paper. The ventral surface was soaked with 70% ethanol in water to prevent any hair or dander from entering surgical openings or becoming airborne. The skin was cut from anus to sternum using sharp scissors, and peeled back to reveal the abdominal wall. A semi-circular flap was cut in the muscle and folded back to reveal the abdominal contents. Using fine forceps and dissecting scissors, the spleen and/or mesenteric lymph nodes were removed as desired. This operation was done as quickly and cleanly as possible. All further manipulations of the organs were performed in the microbiological safety cabinet (MAT II: Medical Air Technology, Manchester) at room temperature or on ice.

3. Collection of bone marrow

This was essentially after the method of Spry (1971). Rats were anaesthetised, exsanguinated by cardiac puncture, and killed by cervical dislocation. The left hind leg of the animal was swabbed in 70% ethanol and the skin was cut and peeled back to expose the femur. This was cut free at the hip joint releasing the entire leg. Holding the leg between the fingers and thumbs of both hands, the femur was separated from the rest of the leg at the femoro-tibial joint. The femur was transferred to the microbiological safety cabinet and all subsequent manipulations were carried out in this area. The femur was scraped clean of muscle and ligament using a scalpel blade and the hip joint end severed near its end using scissors. A 19g needle on a 10 ml syringe containing Iscove's modified Dulbecco's medium (Gibco, Paisley, Scotland) + 20% horse serum (HS) (Flow Laboratories, Irvine, Scotland) was inserted through the cartilage at the knee joint end of the femur and the plug of marrow expelled into a universal container.

A single cell suspension was obtained by aspirating gently through the 19g needle followed by filtration through 2 layers of sterile lens tissue (Whatman Ltd, Maidstone, Kent) to remove clumps of cells. The cells were sedimented at 1100 rpm for 7 minutes and resuspended in 10 ml of Iscove's MDM (Gibco) + 20% HS (Flow Laboratories). For culture purposes, strict asepsis along with sterile instruments, containers, and media were used throughout.

4. Collection of peritoneal mast cells

10 ml of warm Hanks Balanced Salt Solution (HBSS) (Gibco or Northumbria Biologicals Ltd (NBL), Cramlington, Tyneside) plus 10% HS (Flow Laboratories) were injected into the peritoneal cavity and the abdomen gently massaged for about 5 minutes. The peritoneal cavity was exposed and the medium rich in peritoneal exudate cells was collected using a 10 ml syringe. Cells were washed in medium and subsequently used as controls in a number of experiments.

Preparation of media

1. General techniques

Glass bottles were sterilized in an autoclave (Denley, Billingshurst, Sussex) at 121^{o}C for 15 minutes. Media and other labile solutions were sterilised by filtration through 0.22 µm filter units (Flow Laboratories; Millipore Ltd, Harrow, Middlesex), with the aid of a syringe.

2. Hank's Balanced Salt Solution (HBSS)

Glass bottles containing 87.5 ml distilled/deionised water were autoclaved as above. To each was added 10 ml of x 10 HBSS (Gibco or

NBL). The solution was buffered with 2 ml of 1M Hepes (Gibco or NBL) and 0.5 ml of 7.5% sodium bicarbonate solution (Flow Laboratories or NBL) to give final concentrations of 20 mM Hepes and 0.35 g/l sodium bicarbonate. This medium was normally supplemented with 10% horse serum (Flow Laboratories) or 5% foetal calf serum (NBL) prior to use.

3. Iscove's Modified Dulbecco's Medium

The above medium was obtained as stock solution in 500 ml aliquots from Gibco, it contained L-glutamine but no supplements (Cat. No. 041-1980). The medium was stored in the dark at -20°C. 10 ml of Pen/Strep (Flow Laboratories) and 50 ul 2-mercaptoethanol (No. 365-5, Sigma, Poole, Dorset) were added to each 500 ml bottle.

4. Medium Supplements

Horse serum: Batches of serum were tested for efficacy of support of growth of bone marrow cells (see liquid bone marrow culture). The batch ultimately chosen (Lot No. 29104129) was used throughout the entire study except where stated. Aliquots of heat inactivated (56°C, 45 mins) serum were stored at -20°C until needed.

Foetal Calf serum (FCS): Lot No. 10401 (NBL) was stored frozen in 100 ml aliquots. It was heat inactivated and stored as for horse serum above.

Rat serum: Pools of (LIS x BN)F_1 or F344 rat serum were obtained from normal animals of either sex. Pools were heat-inactivated and sterilised by filtration. Sera were stored at -20°C in 20 ml aliquots.

Iscoves serumless formulation additives

a. Bovine Serum Albumin (No. A-7906, Sigma)

5 g of lyophilised powder was dissolved in 100 ml distilled/deionised water, sterilised by filtration and stored at 4°C until use.

b. Transferrin (Human, No. T2252, Sigma)

100 mg of lyophilised transferrin was dissolved in 4 ml of distilled/deionised water and sterilised by filtration. The stock 25 mg/ml solution was stable for at least 6 months.

c. Soybean Lecithin (L- -phosphatidylcholine, No. P-5638, Sigma).

400 mg of soybean lecithin was dispersed in 100 ml of sterile acid HBSS (HBSS without Hepes or bicarbonate supplemented with 1% BSA and 2 ml Pen/Strep) using an ultrasonic water bath at 4°C. The suspension was then filtered through 0.45 um and 0.22 um filters. Stock solutions were stored in sterile universals at 4°C.

Components were added to stock Iscove's medium to the following concentrations, with the volume of stock solutions used per 500 ml of medium shown in parenthesis:

BSA:	1 mg/ml	(10 ml)
Transferrin:	25 µg/ml	(0.5 ml)
Soybean Lecithin:	80 µg/ml	(10 ml)

Culture Technique

1. General Techniques

Organs were removed from rats aseptically and all further manipulations were performed aseptically. Instruments were sterilised by soaking in 70% ethanol. All containers and pipettes were either sterile plastic disposables (Sterilin, Feltham, Middlesex) or sterilised glassware. All manipulations except organ removal from rats were performed in the microbiological safety cabinet.

Nucleated Cell Count

0.1 ml of the cell suspension (around 10^6 cells/ml) was added to 0.1 ml of white cell diluting fluid (Turk's solution; 0.01% Gentian Violet (BDH Ltd) in 3% acetic acid (BDH Ltd) in distilled water) in a small tube and mixed. An aliquot of this was transferred to a haemocytometer (Improved Neubauer) and the cells were counted in the central large divided area. The number obtained was multiplied by the dilution factor (2) and 10^4. For a more accurate count a duplicate count was made in the opposite chamber of the Neubauer, or all 5 large squares in any single chamber were counted and the mean value taken. Turk's solution lyses red blood corpuscles, but not nucleated cells, the nuclei of which are stained with Gentian Violet.

Viable Cell Count

Exclusion of the stain nigrosin (BDH Ltd) has been the method of choice as this dye is non-toxic to cells. The cell suspension was adjusted to 2-20 x 10^6 cells/ml. A stock solution of nigrosin (1% w/v in distilled water, filtered through Whatman No. 1 filter paper) was diluted 1:10 in HBSS + 5% FCS just prior to use and 0.1 ml cell suspension added to 0.1 ml nigrosin dilution in a small tube. After

5-10 minutes, cells were placed in a Neubauer chamber and the number of unstained (viable) and black-stained (dead) cells were counted.

2. Culture of Lymphoid Cells

Spleen or lymph nodes were excised as described in Removal of Tissues (No.2). A maximum of 3 mesenteric lymph nodes (MLN's) or spleens were placed in a sterile universal containing HBSS + 5% FCS or HS. MLN's or spleens were then decanted with 10 ml of HBSS into sterile plastic stomacher bags and the contents were "stomached" in a Colworth Stomacher (Seward Laboratory, Bury St. Edmonds, Suffolk) for 20 seconds.

The cell suspension, thus obtained, was filtered through 2 thicknesses of sterile lens tissue into universal containers, was made up to 20 ml with HBSS and was sedimented at 150 x g for 7 minutes at 4°C. The cell pellet was resuspended with HBSS using a Pasteur pipette and the washing procedure was repeated until the medium above the cell pellet was clear. Following the final wash, cells were resuspended in the desired amount of tissue culture medium and counted.

3. Preparation of Feeder Cells

Spleens were taken from normal syngeneic rats and irradiated with 3000 rad from a ^{60}Co source (Dept. Physiology, University of Glasgow Veterinary School). The spleens, like the MLNs, were stomached, and the cells washed, and plated at 2×10^2 cells/well in 96-well tissue culture plates (Nunc, Paisley, Scotland).

4. Preparation of MLN Cell Conditioned Medium (CM)

Single cell suspensions of MLN from Nb.-infected rats were prepared in HBSS. The cells were counted and resuspended in Iscove's serumless medium at 4 x 10^6 viable nucleated cells per ml. The cells were stimulated with 2 µg/ml Concanavalin A (Sigma Ltd or Miles-Yeda, Slough). The cell suspension was placed in 120 cm^2 tissue culture flasks (Nunc) and capped. The flasks were placed in a 37°C incubator for 48 hr.

The CM was harvested after sedimenting the cells by centrifugation, sterilised by filtration and stored at -20°C.

In one experiment (Section 1, Chapter 3) IMLN cells separated by the fluorescence-activated cell sorter (FACS IV: Becton Dickinson, Sunnyvale, CA), were plated at 4 x 10^5 cells/well and Con-A added at 2 µg/ml in 96-well tissue culture plates (Nunc) to which 2 x 10^2 irradiated syngeneic spleen feeder cells/well had been added. The cells were incubated for 48 hr in a humidified incubator at 37°C flushed with 5% CO_2. The CM were harvested and treated as above.

5. Liquid Bone Marrow Culture

This was modified after the method of Sumner (Sumner, Bradley, Hodgson, Cline, Fry and Sutherland, 1972) as described for the culture of murine cells. Bone marrow cells were suspended in Iscove's medium supplemented with 20% HS at 2.5 x 10^5 viable nucleated cells/ml. 1 ml aliquots of the cell suspension were added to sterile 24 well tissue culture plates (Linbro Spacesaver, Flow Laboratories) together with 0.25 ml conditioned medium, or 0.25 ml medium as a control. For experimental purposes, a pool of three bone marrows was used and set up in triplicate. Plates were incubated at 37°C in a humidified

incubator flushed with 5% CO_2 in air. Routinely, cells were harvested after 4 and 7 days of incubation. Plates were gently shaken to suspend settled non-adherent cells. The cells were aspirated 3 times using a pasteur pipette and then transferred to small plastic tubes (LP3, Luckham Ltd, Burgess Hill, Sussex) and after gentle mixing, samples were counted in Neubauer chambers for a total cell count.

To obtain a differential count, cell suspensions in the LP3 tubes were centrifuged at 150 x g for 7 minutes. The cell concentration was adjusted to within the range $5 \times 10^5 - 2 \times 10^6$/ml by removal of the appropriate quantity of supernate, the cells were resuspended and 3-5 drops were added to the cuvettes of the cytocentrifuge (Cytospin 2, Shandon Southern Ltd, Runcorn, Cheshire). Following centrifugation at 600 rpm for 5 minutes, slides were removed and smears allowed to air dry before staining with Leishman stain or with one of the mast cell specific stains (see Histology).

Cell smears obtained using the cytocentrifuge tended to show an increasing cell density from the centre to the periphery of the smear. This, coupled with the fact that mast cells tend to aggregate, meant that care had to be taken to select representative areas of the smear for a differential count to avoid a bias for or against any particular cell type. At least 500 cells were counted moving from the periphery of the smear to the centre.

To assay the supernatants from the FACS sorted IMLN cells, a further modification of this technique was carried out. A pool of two rat bone marrows was used for each assay. The bone marrow cells were prepared in Iscove's medium + 20% HS, adjusted to a concentration of 5×10^5 cells/ml. 100 µl volumes of cell suspension were distributed in 96-well flat-bottomed tissue culture plates (Nunc) to which were

added 50 µl of CM from FACS-sorted cells and 50 µl of Iscove's medium + 20% HS. Each supernatant was tested in triplicate. The cells were incubated at 37°C in a humidified incubator flushed with 5% CO_2, and harvested after 7 days, total cell counts were then performed. Cytocentrifuge preparations were made and stained as previously described.

6. Bulk Cultures of Bone Marrow Cells

5×10^5 viable nucleated cells/ml in Iscove's medium + 20% HS + 25% conditioned medium were dispersed into 25 cm^2 or 120 cm^2 capped tissue culture flasks (Nunc). These were incubated at 37°C for 7 days. At this time, the cultures were restimulated with conditioned medium and re-fed with fresh Iscove's medium + 20% HS by centrifuging the cell suspension and resuspending the cells in the medium. Such cultures could be maintained for up to 6 weeks after plating and routinely contained >99% mast cells after 3 weeks in culture.

Histology

1. Bone Marrow Cells

Differential Cell Count

2-5 drops of a bone marrow cell suspension of between 5×10^5 and 5×10^6 cells/ml in buffer or medium which contained a percentage of serum were added to cuvettes of a cytospin centrifuge. The cells were then spun on to a clean glass slide at 600 rpm for 5 minutes. Smears were allowed to air dry for 10 minutes before staining with Leishman stain (Gurr, BDH Ltd). Slides were flooded with Leishman stain and left for 5 minutes. An equal volume of buffered water (0.05M

phosphate buffer, pH 6.8) was added to the slide and staining was allowed to proceed for a further 20 minutes. The slides were rinsed under tap water, blotted dry, mounted and covered using "DPX" (BDH Ltd) mountant. Standard procedure for counting bone marrow has been outlined under the paragraph of liquid bone marrow cultures above. A x 40 objective was used and a minimum of 500 cells were counted.

Specific Mast Cell Staining

Fixation: Bone marrow cytospin smears were air-dried for 10 minutes and then fixed in Carnoy's (6 parts absolute alchohol, 3 parts chloroform, 1 part glacial acetic acid) for 10 minutes, 4% paraformaldehyde for 30 minutes, or methanol for 10 minutes. The slides were then rinsed in water.

Toluidine Blue: Slides were covered with 0.1% solution of toluidine blue (Gurr, BDH) in 0.5N HCl (pH 0.5) for 15 minutes then rinsed in 0.125N HCl. Smears were mounted as above. Mast cells were characterized by the presence of purple, metachromatically-stained granules in the cytoplasm.

Astra Blue/Safranin: This technique was after Enerback (1966b). Smears were stained with 0.1% astra blue (Gurr) in 0.7N HCl (pH 0.3) for 30 minutes followed by 10 minutes in 0.7N HCl to aid differentiation. 0.1% safranine-O (Gurr) in 0.125N HCl was added for 15 minutes as counterstain. Mast cells contained blue-staining or orange-red-staining granules depending on proteoglycans present (see text).

2. Immunoperoxidase Staining of RMCPI and RMCPII

Cytospin preparations of peritoneal wash cells and cultured cells were fixed in 4% paraformaldehyde, Carnoy's fluid or methanol. The sections were either used fresh or were frozen and stored at -20°C until use. Cell smears were rehydrated. Endogenous peroxidase activity was inhibited with periodic acid (1% w/v, 10 min) and the sections treated with 10% BSA in PBS for 30 minutes before being incubated overnight with monospecific rabbit $F(ab')_2$ anti-RMCPI or rabbit $F(ab')_2$ anti-RMCPII (8 µg/ml diluted in 2% BSA in PBS) in a humid chamber. Development was accomplished using sheep F(ab) anti-rabbit $F(ab')_2$-peroxidase conjugate and peroxidase activity was revealed with the substrate 3-amino-9-ethyl-carbazole or 3,3 diaminobenzidine (Newlands, Huntley and Miller, 1984). Washing between steps was with TRIS-HCl pH 7.4. Positively stained cells were coloured red or brown depending on staining technique used (Gibson and Miller, 1986).

3. Electron Microscopy

Pelleted cells were fixed overnight at 4°C by resuspending in 3% gluteraldehyde (BDH) in cacocylate buffer (pH 7.4). They were then rinsed in buffer and post-fixed by resuspending in 1% osmium tetroxide for 1 hour. The cells were then rewashed in buffer. The samples were dehydrated through an ascending series of alcohols, treated with propylene oxide, and embedded in Araldite in standard fashion. Semithin sections (1 µm) and ultrathin sections (60-90 nm) were cut with a Reichart 301 ultratome. The 1 µm sections were stained with toluidine blue and the ultrathin sections mounted on copper grids and double stained with uranyl acetate and lead citrate. They were

examined with a Philips 301 transmission electron microscope. The processing of the cells for electron microscopy and operation of the microscope was carried out by Dr. Paul McMenamin (Dept. of Anatomy, University of Glasgow) to whom I am most grateful.

4. Fluorescent Antibody Labelling of Cells

Monoclonal and Fluorescent Antibodies

The mouse anti-rat monoclonal antibodies OX19, W3/25, W3/13, OX6, OX7 and OX8 were a generous gift of D.W. Mason (MRC Unit for Cellular Immunology, Sir William Dunn School of Pathology, University of Oxford). Subsequent batches of the above monoclonal antibodies were purchased from Seralab (Crawley-Down, Sussex).

Mouse IgG was kindly provided by A. Williamson, Department of Biochemistry, University of Glasgow. Fluorescent goat anti-mouse IgG was prepared according to the method of Barclay and Hyden (1978). Goat anti-mouse IgG was passed through a rat globulin immunosorbent column to remove reactivity for rat IgG. The antiserum was then purified by immunosorption to mouse IgG, and after elution was subsequently digested with pepsin according to Nisonoff, Wissler, Lipman and Woernley (1960). The purified $F(ab')_2$ fragments were isolated by gel filtration on AcA 44 and conjugated with fluorescein isothyocyanate by the method of Barclay and Hyden (1978) to obtain fluorescein $F(ab')_2$ molar ratios of between 2 and 4. Batches of this antibody were stored at $-20^{o}C$ and each batch was titrated to obtain optimum fluorescence before use. Fluorescein conjugated sheep $F(ab')_2$ anti-mouse IgG (New England Nuclear, Stevenage, Herts) was also used in certain experiments (see text).

Labelling of IMLN Cells with Monoclonal and FITC labelled antibodies

A single cell suspension of 10^8 cells/ml was incubated with 2.5-5.0 µl/ml of the monoclonal antibody (OX19, W3/25 or OX8) i.e. 50-100 µl/10^7 of monoclonal cells for 30 minutes at 4°C. After three washes in HBSS + 10% normal rat serum (NRS) + 2 mM NaN_3, the cells were further incubated for 30 minutes at 4°C with FITC-labelled goat anti-mouse IgG (1:10 or 1:20 dilution, depending on the monoclonal being used). The cells were washed again three times in HBSS + 10% NRS + 2 mM NaN_3 and maintained on ice prior to sorting on the FACS.

Cell Separation Techniques

1. Removal of Adherent Cells

Plastic adherent cells were removed from mesenteric node preparations by incubation in plastic tissue culture flasks. 2 x 10^7 cells/ml in Iscove's medium + 10-20% heat inactivated FCS were incubated at 37°C in 25 cm^2 plastic tissue culture flasks (Nunc) for 2 hours. Nonadherent cells were removed by gently rocking the flasks and decanting the suspension. Adherent cells were cultured *in situ* if desired, by first washing 3 times with warm HBSS + 5% serum and then counting 4 x 1 cm^2 areas of the dish under an inverted microscope (Olympus) for an estimate of adherent cell numbers. These cells were then incubated in the appropriate volume of Iscove's medium. Occasionally, an estimate of phagocytic cells was performed on adherent cells using their ability to phagocytose latex particles. 0.3 ml of a suspension of latex spheres (0.93 µm diameter, 4 x 10^8 spheres/ml, Polyscience Ltd, Warrington, Pennsylvania, U.S.A.) in medium + 25% FCS were added to the cells. Cells and particles were

gently agitated for 1 hour at 37°C. The cells were sedimented and washed 3 times in medium or HBSS. Phagocytic cells were identified by their content of latex particles either by phase contrast microscopy (Leitz Orthomat) or by ordinary light microscopy on fixed and Leishman stained preparations.

2. Removal of Immunoglobulin Bearing Cells

Immununoglobulin bearing cells (Ig^+) were depleted by immunoadsorption to sheep $F(ab')_2$ anti-rat Ig coupled to CNBr-activated Sepharose 6MB (Pharmacia Ltd, Uppsala, Sweden).

Sterile buffers and aseptic technique were used throughout. 1 g of gel was swollen in a beaker and washed for 15 minutes on a glass filter with 200 ml of a 10^{-3} M HCl solution. 4 mg of antibodies dissolved in 5 ml of 0.1 M sodium bicarbonate buffer containing 0.5 M sodium chloride were added to the gel in a test tube and the mixture was rotated end over end for 2 hours at room temperature. After 3 washes in coupling buffer, the remaining active groups were reacted with 0.2 M ethanolamine pH 8.0 for 1 hour. This was followed by 3 washing cycles to remove non-covalently absorbed protein, each cycle consisting of a wash at pH 4.0 (0.1 M acetate buffer containing 0.5 M NaCl) followed by a wash at pH 8.0 (0.2 M carbonate/bicarbonate buffer containing 0.5 M NaCl). The gel was placed in a sterile 10 ml, 1 cm diameter column to a bed volume of 5 ml.

10^8 adherent cell depleted MLN cells in 1 ml of HBSS + 0.01% sodium azide and 0.3% BSA was added to the column and allowed to penetrate the gel. After 15 minutes incubation, unbound cells were eluted and collected and the column was washed with 10 volumes of HBSS. Bound cells were eluted by the addition of 5 ml normal rat

gamma globulin. After 15 minutes incubation, the cells were collected and the column washed as previously. Bound gamma globulin was removed by passing 0.1 M glycine HCl buffer pH 3.0 through the column until no more protein could be detected spectrophotometrically in the eluted buffer. The column was then given 2 cycles of washes using 0.1 M TRIS buffer (Sigma Ltd) pH 8.0 and 0.1 M acetate buffer pH 4.0 and finally stored at $4^{o}C$ in the presence of 0.1 M TRIS buffer pH 8.0 + 0.2% sodium azide.

3. Fluorescent Identification of Immunoglobulin-Bearing Cells

50 µl of a 2 x 10^6/ml cell suspension in HBSS was incubated with 50 µl of a FITC labelled $F(ab')_2$ preparation of sheep anti-rat Ig antibodies (courtesy of Dr. E. Jarrett). After 1 hour at $4^{o}C$, the cells were washed twice in HBSS and 10 µl placed on to a slide, covered, and examined for fluorescence at x 500 on a Leitz Orthomat microscope.

4. IMLN Cell Separation on the FACS

The IMLN cells were prepared and fluorescently labelled as previously described. Separation was carried out on a fluorescence activated cell sorter (FACS IV: Dept. Zoology, University of Edinburgh). Labelled lymphocytes were analysed and sorted fractions collected on ice in Iscove's serumless medium. Cells were sorted at an average rate of 2000-2500 per second. Appropriate forward angle light scatter gating excluded dead cells. Under these conditions, the average recovery (of viable cells) was 58% and the purity of labelled cells 94-97% of total collected. I am most grateful to Andrew Sanderson for operating the FACS IV.

Quantitation of Rat Mast Cell Protease II (RMCPII)

1. Preparation of Extracts

Isolation of RMCPII from the small intestine and the preparation of a specific antiserum were as previously described (Woodbury and Neurath, 1978). RMCPII was extracted from cultured cells by resuspending pellets in 0.15M KCl. The cells were disrupted by ultrasonication, freezing and thawing. The debris was removed by centrifugation at 1500 x g for 15 minutes.

Serum samples were prepared as described previously. RMCPII content of extracted cells or culture supernatants was assayed by an enzyme-linked immunosorbent assay (ELISA) of which two methods were used (see 2 and 3 below). Both methods were set up by Dr. J. Huntley (Moredun Institute, Edinburgh).

2. Competitive Inhibition ELISA

Plastic 96-well microtiter plates (Linbro) were incubated overnight at 4°C with 200 ul of RMCPII at 1 µg/ml per well in carbonate/bicarbonate buffer (pH 9.6). The plates were washed four times with buffer (phosphate-buffered saline/1% BSA/0.5% Tween 20). Dilutions of a standard (range 5-200 ng/ml RMCPII) or of culture extracts or supernatants (3-5 dilutions per sample) were incubated separately with a 1:3000 dilution of rabbit anti-RMCPII in buffer for 1 hour at 37°C. The reaction mixtures were transferred to the antigen sensitized plates (200 µl/well) and were incubated for 2 hours at 37°C. The wells were washed three times with buffer before incubation for 2 hours with 200 µl of horseradish peroxidase-conjugated sheep $F(ab')_2$ anti-rabbit Fab, diluted 1:1100 with buffer. After three further washes, wells were incubated for 20-30 minutes with O-

phenylenediamine dihydrochloride (Sigma Ltd) in phosphate/citrate buffer (pH 5.0). The reaction was stopped by adding 50 µl of 12.5% sulphuric acid per well. The $O.D._{492}$ read by a Dynatech Microelisa plate reader, was plotted against the standard and sample dilutions and the concentration of RMCPII in culture extracts was calculated from the formula:

(Dilution of standard giving 50% inhibition of positive control/Dilution of unknown giving 50% inhibition of positive control) x 1 µg/ml.

The results were expressed as µg of RMCPII per ml of supernatant.

3. Capture ELISA

Plastic 96-well plates (Costar, NBL) were incubated overnight with affinity purified sheep anti-RMCPII at 1 µg/ml in carbonate/bicarbonate buffer at 4°C in a humidified box. A stock solution of RMCPII was prepared and dilutions of 10-80 ng/ml prepared from it in PBS/0.1% Tween 20/2% FCS. Appropriate dilutions of the test samples were also prepared. The plates were washed three times in buffer (PBS/0.1% Tween 20/2% FCS). 150 µl of standard and test sample were added to the plate and incubated for 1.5-2 hours at 37°C in a humidified box. The plates were again washed three times. 150 µl of sheep anti-RMCPII conjugated to horseradish peroxidase were added at a 1:2000 dilution to the plates and they were further incubated for 1.5-2 hours at 37°C in a humidified container. The plates were washed five times with the washing buffer as before. The substrate was as in (2), the reaction time being 10 minutes. A

standard curve was drawn and the test sample values calculated from this curve. Four dilutions were prepared per sample and the mean of the four values calculated from the standard curve was expressed in µg/ml.

All RMCPII standard preparations and antibody preparations used in (2) and (3) were generous gifts from Drs. Steven Gibson and John Huntley (Moredun Institute, Edinburgh) to whom I am most grateful.

Tritiated Thymidine Assay

For the assay of mitogen-stimulated DNA synthetic responses, 0.2 ml cultures of 4×10^6 cells were set up in 96 well microculture plates (Nunc) and 0.2 µCi of [^{3}H]dThd (2 Ci/mmol; 1 Ci = 3.7×10^{10} Bq: Amersham International) was added to each well 24 hr before harvesting. The cells were harvested with a Titertek cell harvester on to glass fibre filter pads and [^{3}H]dThd incorporation was determined after liquid scintillation spectrometry of the pads in a Packard B spectrometer.

SECTION 1

Chapter 1

Generation of mucosal mast cells in vitro

Introduction

In this chapter, the experiments performed which led to the culture system adopted to generate mucosal mast cells will be described. The development of liquid and soft agar bone marrow cultures using CM from Nb.-infected rat MLN cells has been described previously (Haig, 1982; Haig et al, 1982). In that system, CM was made by antigen stimulation of MLN taken from Nb.-infected rats. The potency of the CM to induce growth and differentiation of MMC from rat bone marrow depended on the ability of the antigen to stimulate the MLN cells to produce the necessary factor(s). Since the production of batches of potent antigen was highly variable in that some stimulated the MLN cells to produce factors capable of stimulating mast cell growth in normal rat bone marrow and some did not, it was decided to try and stimulate MLN from normal and infected animals with the T cell mitogen concanavalin-A. This is of some importance as mitogen stimulated lymphocytes are known to produce a variety of biologically active substances similar to those produced by antigen activated lymphocytes. In particular, T cells secrete lymphokines with a broad spectrum of activity including the ability to stimulate the differentiation of other T and B cells and the regulation of haemopoiesis (Schrader, Arnold and Clark-Lewis, 1980). Thus it was of interest to compare the activity of the various CM on bone marrow cells in liquid culture.

The variability of the bone marrow response to those CM and culture requirements which might most effectively maximise mast cell growth and differentiation was also investigated.

Experiment 1

Some difficulty was encountered in preliminary cultures in provoking normal MLN (NMLN) and immune MLN (IMLN) to produce a potent CM with Con-A. Various doses of the mitogen and various incubation periods were tried. Ultimately the best results were obtained with NMLN stimulated by 2-10 µg/ml Con-A for three days in culture and IMLN stimulated by 2-10 µg/ml Con-A in a two day culture. Three MLN were pooled per group from each normal (LIS x BN)F_1 and infected (LIS X BN)F_1 rat and stimulated with the various doses of Con-A, these were then harvested after the appropriate time. Standard 1 ml bone marrow cultures were stimulated with the various CM added at 25%, including a known potent CM from allergen-stimulated IMLN cells as a positive control and medium alone (negative control). Cells were harvested on days 4 and 7 of culture and the results represent the mean of triplicate samples, from a representative experiment. (See appendices 1 and 2 for data from repeat experiments).

Results

The CM prepared using NMLN activated by 5 µg/ml Con-A, stimulated mastocytopoiesis and total cell growth to almost the same extent as the antigen stimulated IMLN cells (positive control) (Figure 1). The other CM were not as effective. Figure 2 shows that IMLN cells stimulated with 2 µg/ml Con-A gave a total cell and mast cell growth which far surpassed the ability of the other CM including the positive control to stimulate normal rat bone marrow to grow and differentiate. It was therefore apparent that both NMLN and IMLN could be stimulated with an appropriate dosage of Con-A to produce mast cell growth factor

of similar or superior potency to that produced by allergen-activated immune rat MLN.

Experiment 2

A comparison was made between the stimulatory effect of Con-A on normal and immune rat MLN by measuring the level of incorporation of tritiated thymidine [^{3}H]dThd. This was done to see if a measure of the potency of the CM to stimulate growth of MMC from normal rat bone marrow could be predicted. For this assay, MLN were removed from (LIS X BN)F_1 normal and immune rats (3 per group). The cells were cultured in the presence of 2 and 5 µg/ml Con-A. The details for assay of mitogen-stimulated DNA synthetic responses can be found in the Materials and Methods. The results are expressed as the mean counts per minute (cpm) on day 3 in triplicate cultures. Bone marrow cultures were harvested on day 7 and cell numbers expressed as the mean of triplicate cultures, from a representative experiment. (See appendix 3 for data from repeat experiment).

Results

In the experiment shown in Table 1, the effect of Con-A on NMLN and IMLN was assessed by measuring incorporation of [^{3}H]dThd into the MLN cells. The DNA synthetic responses to Con-A stimulation were considerably greater in immune than in normal MLN. However, it was evident that the level of incorporation of [^{3}H]dThd could not be taken as an indicator of the potency of the CM to induce total and mast cell growth in normal bone marrow.

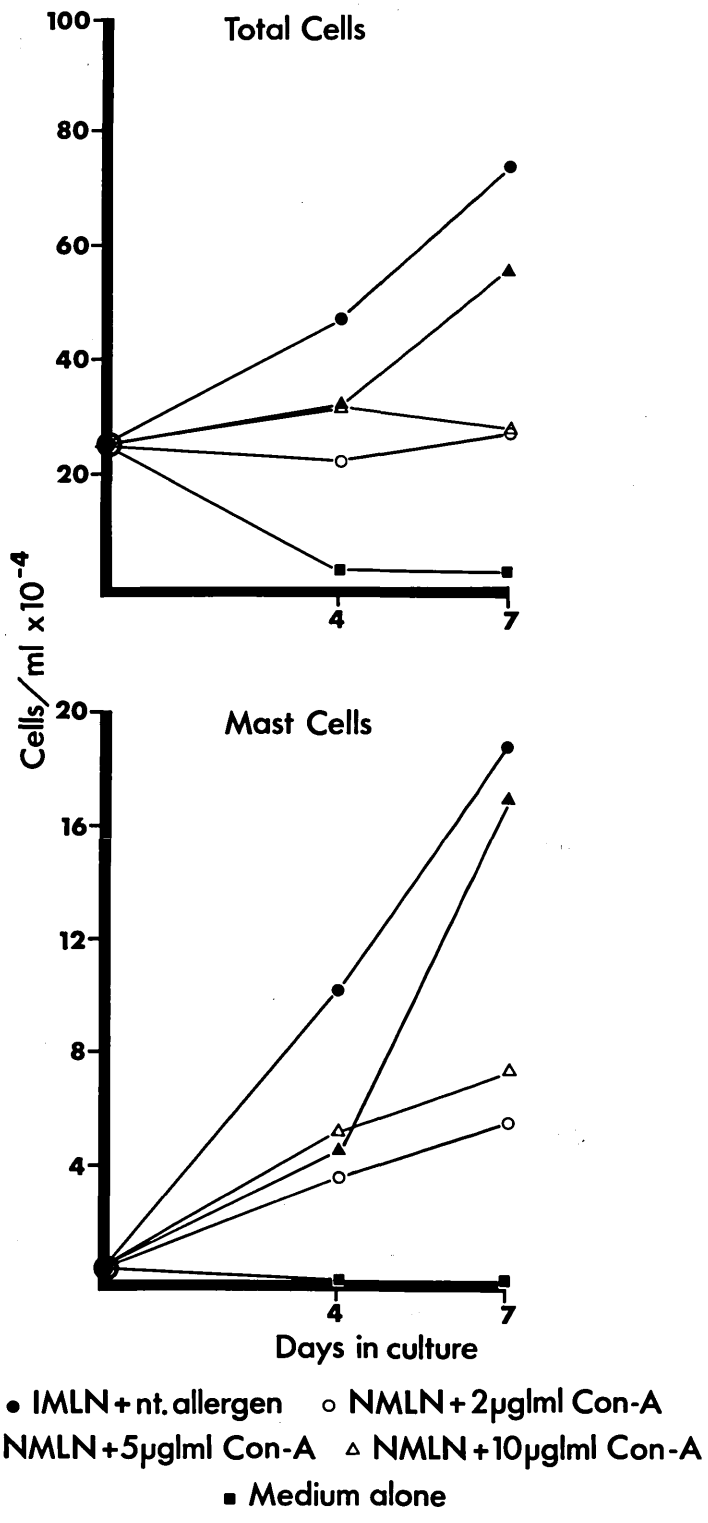


Figure 1: Total cell and mast cell growth in normal bone marrow cultures stimulated with conditioned media made from NMLN stimulated with various concentrations of Con-A

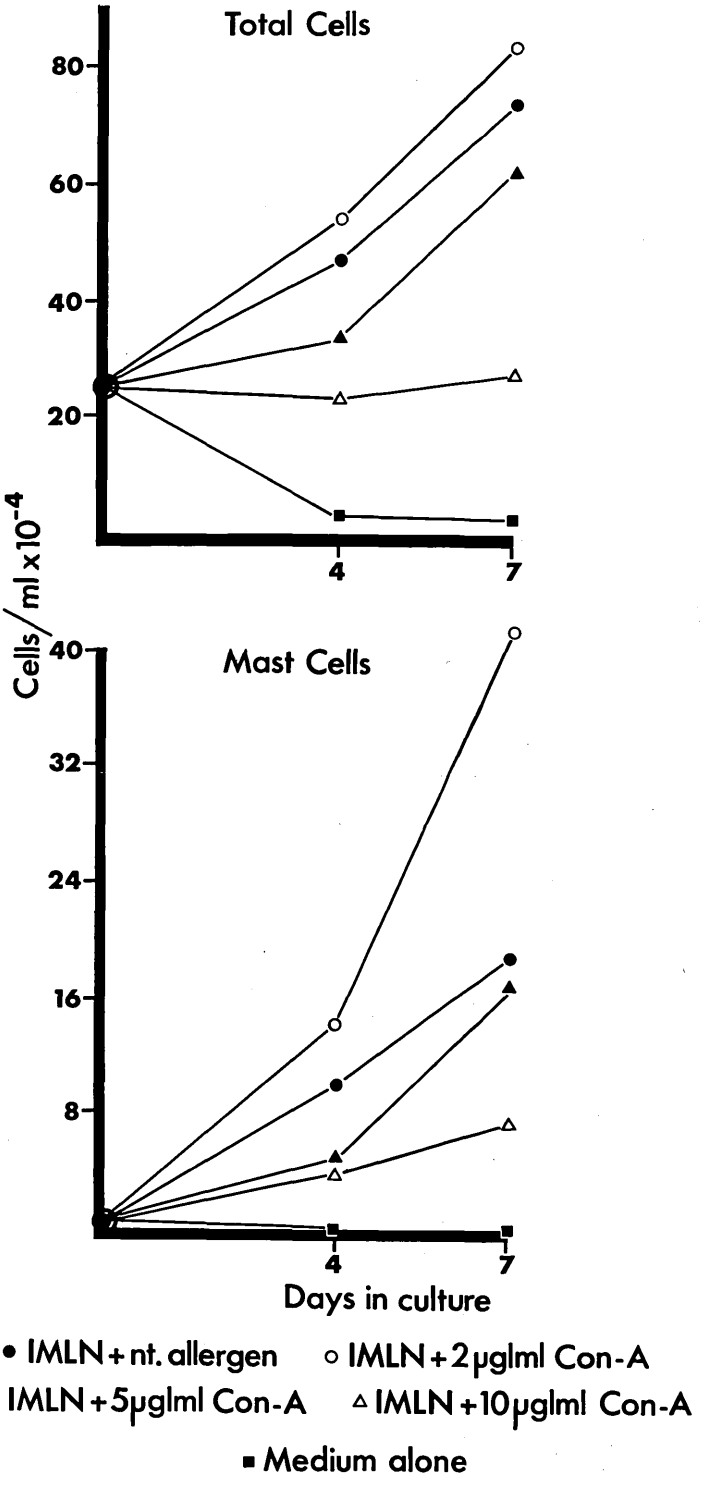


Figure 2: Total cell and mast cell growth in normal bone marrow stimulated with conditioned media made from IMLN stimulated with various concentrations of Con-A.

Table 1: Total cell and mast cell growth in bone marrow cultures stimulated with CM from Con-A activated normal or immune MLN cells

Source of CM	[^{3}H]dThd incorporation in MLN cultures cpm x 10^{-3}	Bone Marrow Cultures	
		Total Cells no x 10^{-4}	Mast Cells no x 10^{-4}
Normal MLN			
+ 2 µg Con-A/ml	11.0	28.0	5.8
+ 5 µg " "	1.0	56.0	16.8
Unstimulated	2.2	3.0	0
Immune MLN			
+ 2 µg Con-A/ml	5.5	79.0	40.1
+ 5 µg " "	72.3	63.0	14.1
Unstimulated	0.4	5.0	0

Experiment 3

In investigating the growth and differentiation of the mast cells which appeared in the cultures, it was of interest to look at the CM requirements of these cells; would initial pulsing of the cells with CM give the necessary stimulation for growth and differentiation or does CM have to be present throughout the period of culture? One ml standard bone marrow cultures were set up with a known potent CM added to each well excluding the negative control. After 24 and 48 hrs in culture, cells were removed from the wells, washed in medium and re-plated without CM. Control groups included; cells which were washed and then re-plated with CM and the normal positive control which did not undergo this treatment. The bone marrow cultures were harvested on days 4 and 7 after plating and cell numbers expressed as the mean of triplicate cultures from a representative experiment. (See appendix 4 for data from repeat experiments).

Results

Clearly CM is required at all times during culture of normal rat bone marrow if it is to grow and differentiate to give any appreciable total cell and mast cell growth above initial plating values (Figure 3). Compared to the cells which were left in culture with CM and those replated with CM (the washing procedure having no detrimental effect on the cells) the total cell and mast cell growth in the two pulsed groups was negligible. The results show the mean of triplicate cultures harvested on days 4 and 7 after plating.

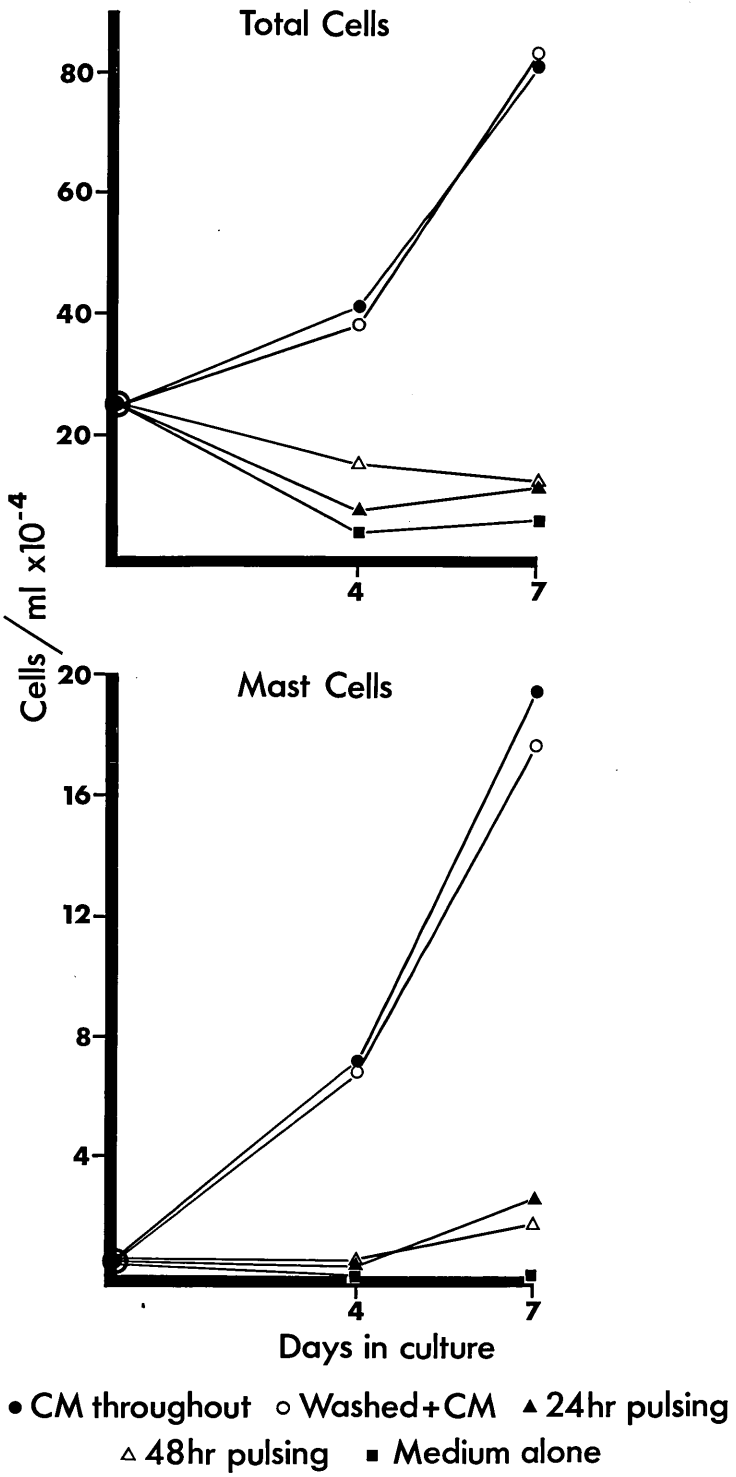


Figure 3: Total cell and mast cell growth in normal bone marrow cultures stimulated with CM throughout culture, after washing and pulsed for 24 hr and 48 hr.

Experiment 4

Having established the culture system using (LIS X BN)F_1 rats it was of interest to see if other strains of rats were able to produce potent CM. CM was made from immune MLN taken from 4 strains of rats, F344, CBH, AUG and AO, D14 after infection with Nb. The IMLN cells were stimulated with 2 µg/ml Con-A and harvested after 24, 48 and 72 hours. The CM were tested on bone marrow taken from the same strain from which the CM had been made and (LIS X BN)F_1 marrow as a control.

The strain variability of the bone marrow response to a known potent CM was also investigated. (LIS x BN)F_1 CM was used to stimulate the BM taken from the above mentioned strains. The results show the mean of triplicate cultures harvested on day 7 after plating. (See appendices 5-7 for data from repeated experiments).

Results

Table 2 shows the total and mast cell growth obtained from bone marrow stimulated with CM made from IMLN taken from syngeneic strains of rats, including the standard (LIS X BN)F_1 strain used routinely in the laboratory. F344 CM with F344 bone marrow gave the highest total cell growth and mast cell growth. Compared to the other strains, (LIS X BN)F_1 gave a mediocre total cell count and few mast cells, therefore the culture system had not been being used to its full potential as far as production of growth factors and mast cells was concerned. The results shown are from CM harvested after 48 hrs as these had optimal growth stimulatory capacity compared to those harvested after 24 or 72 hr (see appendix 5 for this data). The bone

Table 2: Total cell growth and mast cell growth in normal bone maroow cultures stimulated with CM made from a syngeneic strain of rat

Source of CM	Source of BM	TotalCells no. x 10^{-4}	TotalMast Cells no. x 10^{-4}
F344	F344	135	62.3
CBH	CBH	84	35.9
AUG	AUG	93	27.4
AO	AO	62	16.7
(LIS x BN)F_1	(LIS x BN)F_1	79	22.1

marrow cultures were plated in triplicate and harvested after 7 days. The bone marrow results are the mean of triplicate cultures.

Comparison between CM made from the different strains of rats tested on the (LIS X BN)F_1 bone marrow is documented in Table 3. The highest mast cell growth was obtained in the culture stimulated with syngeneic CM i.e. (LIS X BN)F_1 CM but F344 CM stimulated the mast cell growth to almost the same extent. AUG and CBH gave slightly lower numbers of mast cells with AO CM generating less than half the numbers of mast cells produced by the (LIS x BN)F_1 CM. Obviously it was desirable to maximise the total mast cell numbers in the culture, thus F344 CM used with F344 bone marrow was adopted to give the best yield.

To look at the variability of the bone marrow response between strains, (LIS X BN)F_1 CM was used to stimulate bone marrow taken from the 4 other strains used. Again F344 bone marrow gave the highest total cell count and mast cell growth with (LIS X BN)F_1 bone marrow also giving a reasonable response (Table 4). The other 3 strains gave a much poorer response.

Table 3: Total cell and mast cell growth in normal (LIS x BN)F_1 bone marrow cultures stimulated with CM made from allogeneic strains of rats

Source of CM	Source of BM	Total Cells no. x 10^{-4}	Total Mast Cells no. x 10^{-4}
F344	(LIS x BN)F_1	63	20.8
CBH	"	51	14.7
AUG	"	92	15.9
AO	"	46	9.7
LIS x BN	"	79	22.1

Table 4: Total cell and mast cell growth in normal bone marrow cultures stimulated with CM made from an allogeneic strain of rat

Source of CM	Source of BM	Total Cells no. x 10^{-4}	Total Mast Cells no. x 10^{-4}
(LIS x BN)F_1	F344	87	29.1
	CBH	54	17.3
	AUG	60	13.8
	AO	40	7.6
	(LIS x BN)F_1	79	22.1

Discussion

A variety of experimental protocols have been used to establish successful liquid rat bone marrow cultures. Bone marrow cells can be stimulated to grow and differentiate in the presence of CM prepared with MLN cells from *Nb.*-infected rats stimulated with various concentrations of Con-A. Figures 1 and 2 illustrate that medium from such cultures enriches for mast cells. Similarly, in the mouse, mast cells developed in haemopoietic tissue cultures stimulated with mitogen-activated splenocyte CM (Hasthorpe, 1980; Nabel *et al*, 1981; Razin, Cordon-Cardo and Good, 1981; Schrader *et al*, 1981; Tertian *et al*, 1981). The best total cell and mast cell growth was achieved in the rat system by culturing 4 x 10^6/ml IMLN cells taken D14 after infection with 2 µg/ml Con-A for 48 hrs. This optimal period of 48 hrs is similar to the *in vitro* kinetics reported for murine and human IL-2 production (Gillis, Ferry, Orr and Smith, 1978) but different from the kinetics of factor production in the antigen-induced response (Haig *et al*, 1982). Maximal levels of mast cell growth stimulatory activity was obtained only after 4-6 days in culture in the presence of 1-10 µg/ml of a crude *Nb.* worm secretory-excretory product (Haig *et al*, 1982). It seems the use of *in vivo* activated MLN cells from infected rats enlarges the population of cells potentially able to produce growth factors capable of causing selective proliferation and differentiation of rat bone marrow *in vitro*. This may be because those MLN cells are expressing more receptors for Con-A induced growth factors (Larsson and Coutinho, 1979) and hence undergo a more rapid *in vitro* expansion than the resting MLN cells of normal animals. In this particular case the MLN greatly increase in size and cellularity

during infection. The MLN has proven to be the most potent source of mast cell growth stimulatory activity compared to the other tissues evaluated (spleen and peripheral blood), indicating that this is a major source of activated lymphocytes during Nb. infection (Haig, Jarrett and Tas, 1984). In time course experiments it was found that mast cell growth stimulatory activity could be detected in CM from day 10 of infection with Nb. onwards, reaching maximal levels at around day 14 and persisting for at least 30 days (Haig, Jarrett and Tas, 1984). This coincides with the kinetics of appearance in vivo of MMC in the lamina propria of infected rats (Jarrett, Jarrett, Miller and Urquhart, 1967; Nawa and Miller, 1979; Denburg, Befus and Bienenstock, 1980; Haig, 1982). Investigations to identify the cells responsible for the production of the factor(s) causing growth and differentiation of mast cells from normal bone marrow will be described in subsequent chapters.

There are various parameters which can affect the potency of CM in stimulating the growth and differentiation of mast cells from normal rat bone marrow. It is evident from Experiment 4 that IMLN cells taken from different strains have a varying capacity to be stimulated by Con-A to produce the necessary factor(s) for mast cell growth. (LIS X BN)F_1 rats had been chosen initially because they were readily available, were good breeders and were able to tolerate helminth infection well. However, MLN cells from F344 rats secreted a very potent CM and have subsequently proved to be consistently better than MLN cells from other strains. Therefore, F344 rats are the strain of choice for CM production. There is obviously still some variation between batches of CM but this can be minimised by excising the nodes on the same day after infection, stimulating with the same

brand of Con-A and by preparing the components of the serumless medium according to the strict protocol detailed in the Materials and Methods. The preparation of the soybean lecithin (phosphatidylcholine) in particular seems to be of crucial importance and any deviation in quality or preparation of this component affects the potency of the CM. It should also be noted that for any particular experiment, one batch of CM should be used throughout and this has been done in the experiments included in this thesis.

The varying response of the different rat strains to Nb. infection as far as worm burden and mast cell response in the intestine was investigated by Nawa and Miller (1979). Both the worm burden kinetics and mast cell responses were different in each strain of rat used. In addition they found that the intestinal mast cell response could be transferred using thoracic duct lymphocytes (TDL) which were slg^- and that the increase in intestinal mast cell numbers was closely related to the final stage of the rapid phase of worm expulsion. It was also noted that the timing of transfer of the TDL after infection was also important, day 10 TDL being the most effective in conferring the response. These in vivo phenomena appear to be being mirrored in the in vitro culture system where the ability to stimulate mast cells in normal bone marrow cultures is dependent upon the strain of rat used to make the CM and also the time after infection when the nodes are removed.

Reactivity to a given CM by different bone marrow pools was slightly variable from one pool to another. This had previously been observed by Bradley and Siemienowicz (1968). Therefore it was not considered valid for experimental results to be expressed as the mean

of values from repeated experiments using different bone marrow pools. Representative experiments are presented, supported by results from repeated experiments, using both different bone marrow pools and different batches of CM. This cross checking procedure was followed for all the experiments contained in this thesis. The results of repeated experiments are tabulated in the appendix.

In studying the variability of bone marrow to respond to a given CM, F344 rats gave consistently higher mast cell numbers than the other strains. It should be noted that on this occasion the bone marrow responded better to CM made from syngeneic than from allogeneic IMLN cells. Results in Table 2 and Table 4 confirm this although it must be said that in general, this is not always the case because so many factors may affect the potency of CM that many more detailed experiments would be necessary to determine the involvement of histocompatibility barriers in the action of CM on mast cell growth.

Again it is interesting to compare these findings to the results obtained *in vivo*. It was shown that although reciprocal adoptive transfers of IMLN cells between rapid (NIH) and slow (BIO.G) responder strains of mice to the nematode *Trichinella spiralis*, could enhance the mast cell response in normal recipients and partially restore the response in sub-lethally irradiated recipients, the magnitude of the response in each case was determined primarily by the phenotype of the recipient (Wakelin and Donachie, 1980). As well as this, studies using chimaeric mice show clearly that bone marrow cell phenotype plays a major role in determining the level of response to infection whether or not IMLN cells are transferred (Alizadeh and Wakelin, 1982). The *in vitro* system established in this laboratory provides an opportunity to further investigate the mechanisms at play in the rat

and compare it to the findings in the mouse.

The main objective of the experiments in this chapter was to find the optimum conditions for culture of maximum numbers of mast cells in vitro. There was a number of interesting and puzzling trends which became apparent and, given time, could be followed up.

Eosinophils, monocytes/macrophages and neutrophils (but not lymphoid or erythroid cells) are also stimulated by CM in bone marrow cultures but these cells disappear during the first 10 days of culture (Haig, 1982). There are no progressive stages of mast cell development in unstimulated rat bone marrow but in stimulated cultures, cells with sparse granulation can be seen by day 2 and these progress by the end of the first week to form cells which satisfy the usually accepted criteria of mast cells i.e. metachromatic granular dye-binding when stained with toluidine blue, ultrastructural appearance and uptake of IgE (Galli and Dvorak, 1979; Haig et al, 1982). However cultured mast cells differed morphologically from any cell seen in normal uncultured marrow. When stained with toluidine blue or Leishman, the sparsely granulated, red-purple staining mast cells bore little resemblance to the occasional densely granulated, blue-black staining cells seen in uncultured marrow. This suggests that the precursors of the cultured mast cells are very different from their progeny. No reliable histological technique at present can identify mast cell precursors which are, however, thought to resemble lymphoblasts (Miller, 1971). Thus for the purpose of this thesis, the number of mature CTMC found in bone marrow represent the day 0 value for the cultured cells, even though these cells may not be related to the cells ultimately growing in culture, and they die off within 7

days of culture.

The experimental results represented in Table 3 shows that the mast cells require the presence of CM continually in the culture in order to grow and differentiate. Restimulation and refeeding leads to cultures containing almost 100% mast cells by 2-3 weeks after plating. Bulk cultures of these mast cells may henceforth be maintained for at least 8 weeks in the presence of CM. The characteristics and properties of these cultured mast cells will be described in Section 2.

Chapter 2

Stimulation of MMC growth in normal and nude rat bone marrow cultures

Introduction

In both rats and mice, the MMC response to helminth infection is immunologically mediated. It is dependent on the integrity of the T-lymphocyte system of the host (Ruitenberg and Elgersma, 1976; Mayrhofer, 1979) and can be accelerated in infected animals by the adoptive transfer of immune lymphocytes (Befus and Bienenstock, 1979) or immune T cells (Nawa and Miller, 1979). It was speculated that the T cells in this response could be acting in two ways: they might develop into mast cells as hypothesised by Burnet (1977), and seemingly supported by growth of mast cells from thymus cell cultures, or they may produce a lymphokine which induces proliferation and development of MMC precursors.

The experiments described in this chapter were performed to explore the basis of the thymus dependency of MMC proliferation.

Experiment 5

Cell fractionation experiments were undertaken to examine which population within the MLN was producing the factor(s) responsible for the selective growth and differentiation of MMC in normal bone marrow cultures. Phagocytic cells were removed from immune MLN cells, taken day 14 after infection, by adherence to plastic, and the remaining cells were fractionated on a sheep $F(ab')_2$ anti-rat Ig-Sepharose 6MB column. CM were prepared from unfractionated cells and from each of the fractions by stimulating the cells with 2 µg/ml Con-A. The CM were then used to stimulate normal bone marrow cultures. The cell counts represent the mean numbers in triplicate cultures on day 7.

Results

Maximal cell growth, including MMC growth, was seen in bone marrow cultures stimulated with CM prepared from the T cell-enriched fraction of immune MLN (Table 5). Adherent cell-depleted MLN and the Ig^- fraction contained <1% phagocytic cells, as identified by their capacity to ingest 1 μm latex spheres (see Materials and Methods). The CM produced by the IMLN depleted of adherent cells had a greater mast cell stimulatory capacity than unfractionated IMLN. CM prepared from the adherent cell or Ig^+ cell fractions did not stimulate bone marrow growth (Table 5). These results are from a representative experiment. (See appendix 8 for results from repeated experiments).

Experiment 6

To explore the nature of the T cell dependance of mast cell growth stimulatory activity, as indicated in the last experiment, we tested the stimulatory capacity of the CM prepared with athymic rat lymphocytes and the ability of the athymic rat bone marrow to generate MMC. Thus, CM were prepared from rnu/rnu and rnu/+ MLN 14 days after infection with 2,000 Nb. larvae, using 2 μg/ml Con-A to stimulate the cells in culture. These CM were tested on rnu/rnu and rnu/+ bone marrow in standard culture. A CM from Nb.-infected (LIS x BN)F_1 rat MLN was used as a positive control. Different batches of CM were tested against different bone marrow samples in separate experiments. The bone marrow cells were harvested on days 4, 6, 8 and 10 of culture and the results represent the mean of triplicate cultures from a representative experiment. (See appendix 9 for additional data).

Results

(LIS x BN)F_1 CM contained the most potent activity for total bone marrow and mast cell growth in both rnu/rnu and rnu/+ BM cultures (Figure 4). CM from rnu/+ rats stimulated total cell and mast cell growth in both rnu/rnu and rnu/+ bone marrow cultures whereas CM from rnu/rnu animals contained no growth stimulatory activity. It is also observed that both (LIS x BN)F_1 CM and rnu/+ CM can stimulate mast cell growth in rnu/rnu bone marrow cultures to the same extent as rnu/+ bone marrow.

Experiment 7

In order to verify that the mast cells growing in cultures from rnu/rnu bone marrow were of a similar type to those grown from (LIS x BN)F_1 marrow i.e. mucosal type, athymic and intact rat bone marrow cells were cultured in the presence of CM from immune MLN stimulated with 2 µg/ml Con-A. Cultures were harvested on the days shown for total and differential cell counts. The measurement of RMCPII (the MMC marker) in the culture supernatant was performed by an enzyme-linked immunosorbent assay (as detailed in Materials and Methods). Comparison between the two bone marrow types was assessed by these criteria. (See appendix 10 for additional data).

TABLE 5: Total cell and mast cell growth in bone marrow cultures stimulated with fractionated immune MLN cells.

Source of CM	Total Cells no. x 10^{-4}	Mast Cells no. x 10^{-4}
Unfractionated immune MLN cells	59.0	25.9
Immune MLN without adherent cells	94.9	45.7
Adherent cells alone	8.0	0.6
Ig^+ cells	6.5	0.2
Ig^- cells	106.5	66.1
Medium alone	7.5	0.1

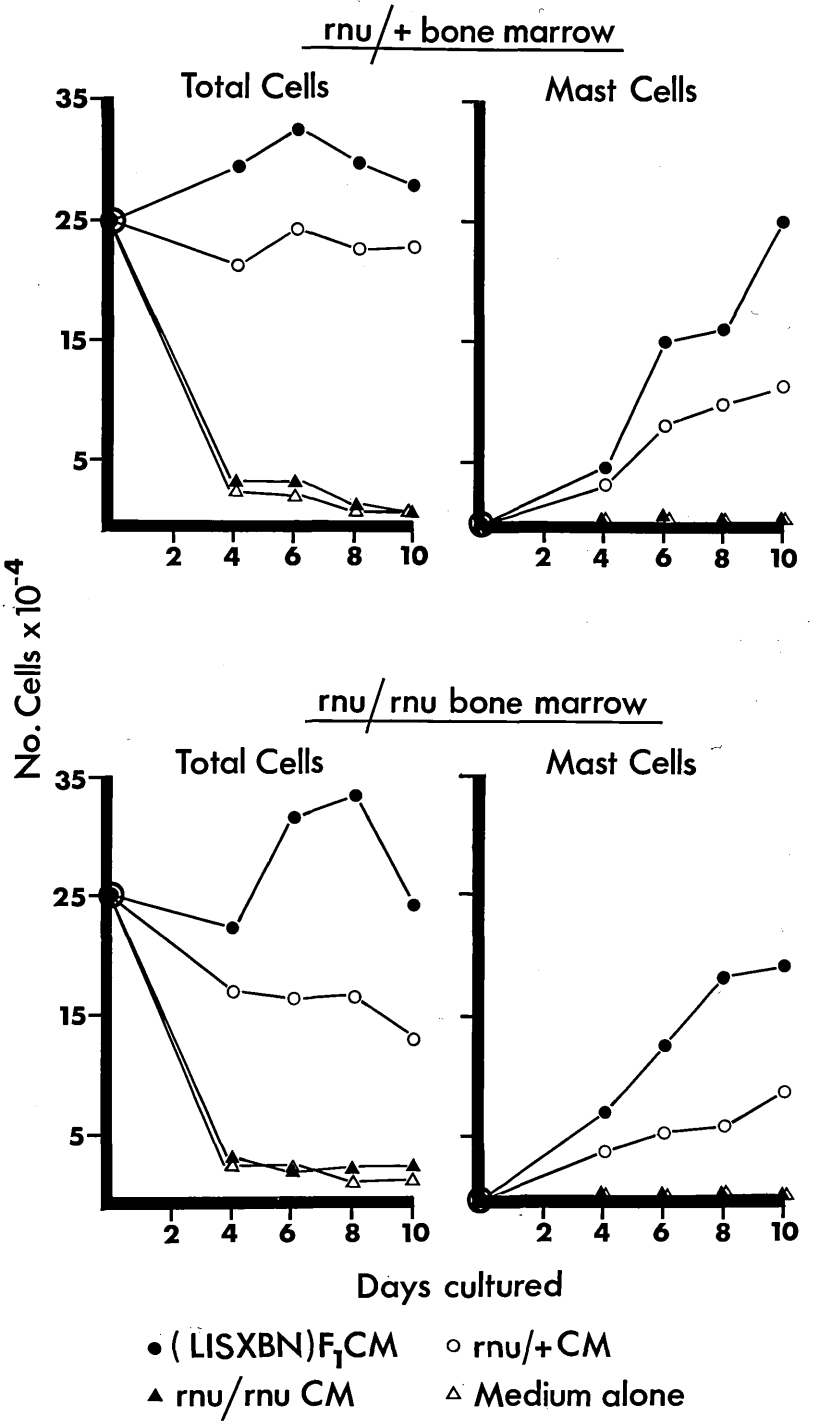


Figure 4: Total cell and mast cell growth in rnu/rnu and rnu/+ rat bone marrow cultures stimulated by various CM

Results

Concentrations of RMCPII in supernatants from both (LIS x BN)F_1 and rnu/rnu mast cell cultures were comparable (Table 6). Additionally, the mast cells of rnu/rnu origin had identical staining characteristics to those of (LIS x BN)F_1 origin; both populations stained with astra blue in preference to safranin at pH 0.3 after Carnoy's fixation.

TABLE 6: Total cell growth, mast cell growth and RMCPII production in normal and nude rat bone marrow cultures stimulated with (LIS x BN)F_1 CM.

Days of culture	Total cells, no. x 10^{-4}		Mast cells, no. x 10^{-4}		RMCPII in culture fluid, µg/ml	
	Nu/nu	(LIS x BN)F_1	Nu/nu	(LIS x BN)F_1	Nu/nu	(LIS x BN)F_1
2	7.0	11.0	0.2	0.7	0.06	0.04
4	13.7	14.3	4.1	5.5	0.36	0.52
6	18.3	30.0	8.5	15.4	0.99	1.30
8	59.3	77.0	34.2	47.2	2.99	0.73

Discussion

It has previously been shown that mast cells with the characteristics of MMC appear and proliferate to form the predominant cell type in rat bone marrow cultures stimulated with factors from antigen reactive IMLN (Haig et al, 1982) and, in the present study, with Con-A stimulated normal or immune MLN (Haig et al, 1983).

The factor was produced uniquely by immunoglobulin-negative non-adherent lymphocyte-enriched preparations and not by adherent or immunoglobulin-positive cell-enriched fractions (Table 5). It should be noted that the adherent cells seem to have a suppressive effect on factor production for when they were removed, CM from non-adherent MLN preparations generated higher total cell and mast cell growth. Since the Ig^- adherent cell-depleted fraction was contaminated by only small numbers of adherent cells (~1%) this yielded the best total cell and mast cell growth. T cells as the source of mast cell growth factor was confirmed in the experiments with rnu/rnu and rnu/+ rats where supernatants from MLN cell cultures from rnu/rnu rats failed to generate mast cells in bone marrow cultures, whereas CM from rnu/+ rats did contain mast cell growth stimulatory activity. Differences in the capacity to produce mast cells between the control (LIS x BN)F_1 rat IMLN CM and rnu/+ CM may be a consequence either of strain differences or of the reduced antigenic priming of the rnu/rnu and rnu/+ rats. Thus (LIS x BN)F_1 rats used for CM production were infected with 4,000 Nb. larvae whereas rnu/rnu or rnu/+ rats were given 2,000 larvae in order not to compromise their health.

The extensive in vivo studies of the striking hyperplasia of mast cells which occurs during helminth infection have shown that this

hyperplasia does not occur in T cell-deficient animals i.e. nude mice (Ruitenberg and Elgersma, 1976) or T cell-deficient rats (Mayrhofer, 1979). More importantly, this present study agrees with the in vivo results of Nawa and Miller (1979) who transferred fractionated TDL from Nb. infected rats and found that the intestinal mast cell response could only be conferred by cells lacking surface immunoglobulin.

One of the questions at issue is whether MMC are derived from a T cell precursor (Burnet, 1977) or whether T cells induce growth and differentiation from a thymus-independent precursor. The results in this chapter have now shown that the numbers of MMC generated in rnu/rnu rat bone marrow cultures by factors derived from the lymphocytes of intact rats is of the same order as the numbers arising in rnu/+ or (LIS x BN)F_1 bone marrow cultures. In the experiment reported here, the presence of MMC precursors in nude rat bone marrow indicates that the normal cycling and production of the precursor is not under thymic control, whereas proliferation and differentiation in culture is. In vivo, nude mice and normal mice contain similar numbers of intestinal MMC when examined under optimal fixation and staining conditions indicating that the presence of MMC under physiological conditions is not thymus-dependent but that proliferation in response to parasite infection is under thymic control (Ruitenberg and Elgersma, 1976).

The results expressed in this chapter agree with studies of cultured mouse mast cells which have shown that mast cell growth factor is produced by activated T cells (Hasthorpe, 1980; Nabel et al, 1981; Nagao, Yokova and Aaronson, 1981; Razin et al, 1981; Schrader et al, 1981; Tertian et al, 1981; Yung et al, 1981). Murine mast cells

can be maintained in proliferation for prolonged periods in the presence of the appropriate factor(s). The factor(s) are associated with a lymphokine IL-3 which has been shown to be an absolute requirement for the growth of a number of haemopoietic cell lines as well as a promoter of the cycling of multipotential stem cells (Ihle et al, 1982; Iscove et al, 1982; Schrader and Clark-Lewis, 1982; Bazill et al, 1983). It may also be associated with the novel murine mast cell and T cell growth factor (TCGF) identified and partially purified from the supernatants of the activated helper T cell line Cl.Ly1^{+}2^{-}/9 (Smith and Rennick, 1986). This factor has been shown to enhance the IL-3 dependant growth of mast cells and demonstrates that an interaction between factors may be required for optimal mast cell growth. Cl.Ly1^{+}2^{-}/9 cells also produce high levels of B cell stimulating activity. Further studies using cDNA clones that encode a lymphokine with all these activities have been carried out and it has been proposed the factor be designated IL-4 (Lee, Yokota, Otsuka, Meyerson, Villaret, Coffman, Mosmann, Rennick, Roehm, Smith, Zlotnik and Arai, 1986).

The presence of RMCPII in supernatants from the rnu/rnu bone marrow cultures demonstrates that these cells are of the mucosal type. The cells are morphologically identical to and had the same staining characteristics as the MMC growing in rnu/+ and (LIS x BN)F_1 cultures. The rat and mouse mast cells described above have several features in common. In addition to showing a T-cell dependent hyperplasia the mouse bone marrow-derived mast cell is astra blue positive and can be grown from nude or T cell depleted bone marrow (Schrader et al, 1981; Tertian et al, 1981). The rat MMC contains non-heparin

glycosaminoglycan and RMCPII (Tas and Berndsen, 1977; Woodbury et al, 1978) in distinction to CTMC and, similarly, the cultured mouse mast cells synthesize a non-heparin proteoglycan, identified as chondroitin sulphate E. In the absence of a specific marker for mouse MMC, these criteria suggest the cultured mouse mast cells are of the mucosal subtype.

Thus the regulation of a MMC response can be studied in detail and in later chapters the phenotype of the T cell producing the mast cell growth factor as well as the nature of the mast cells themselves will be further characterised.

Chapter 3

Surface phenotype of T cells producing growth of mucosal mast cells in normal bone marrow culture

Introduction

Mast cells emerge and selectively proliferate in normal bone marrow cultures under the influence of factors from antigen or Con-A stimulated MLN cells from Nb.-infected rats (Haig et al, 1982, Chapter 1). Cell fractionation studies showed that factor(s) were derived from cells lacking surface immunoglobulin and, the use of tissues from athymic rats demonstrated that the thymus-dependency of MMC proliferation is based on the production of an appropriate growth factor by the Con-A activated T cells (Haig et al, 1983; Chapter 2).

In order to further define the source of MMC growth factor, the surface phenotype of the cell(s) which produced it was investigated. Studies in the mouse showed that T cells which selectively induce morphologically normal mouse mast cell clones to proliferate have the phenotype $Ly1^{+}2^{-}3^{-}$ (Nabel et al, 1981). As mentioned previously mouse mast cells generated in culture have some of the features of rat MMC and may be of a similar type (Chapter 2).

In the rat, monoclonal antibodies which recognize T cell associated antigens include OX19 which labels all T cells (Dallman, Mason and Webb, 1982), W3/25 (Williams, Galfre and Milstein, 1977) and OX8 (Brideau, Carter, McMaster, Mason and Williams, 1980) which label non-overlapping T cell subpopulations. Rat helper and cytotoxic/suppressor T cell subsets are distinguished by the monoclonals W3/25 and OX8 respectively.

Experiment 8

IMLN cells removed day 14 after infection with Nb. were separated in the fluorescence-activated cell sorter into populations with or without the antigens defined by OX19, W3/25 and OX8 monoclonal antibodies. The selected subpopulations were then cultured in vitro with Con-A and irradiated spleen feeder cells (as detailed in Materials and Methods). The supernatants were tested for their ability to induce MMC growth in a micro liquid bone marrow assay. All supernatants were tested in triplicate and the cultures were harvested after 7 days. The results are expressed as the mean of triplicate cultures. (See appendix 11 for repeated experiment).

Results

OX19 labelling

Table 7a shows that cells which label with the monoclonal antibody OX19 are capable of producing MMC growth factor, whereas those which are negative for this marker are not. Maximal cell growth, including MMC growth, was seen in the bone marrow cultures stimulated with the conditioned medium prepared from the OX19 positive cells (i.e. the T cell enriched fraction). The CM prepared from the OX19 negative cells did not stimulate the bone marrow above the level of the medium-alone control.

W3/25 labelling

Cells which stained positively for this monoclonal antibody also produced a factor capable of stimulating MMC growth in rat bone marrow cultures. Table 7b shows that supernatants from W3/25 positive cells

TABLE 7a: Total cell and mast cell growth in bone marrow cultures stimulated with conditioned media from OX19-labelled IMLN

Source of CM	Total cells ($\times 10^{-4}$)	Mast cells ($\times 10^{-4}$)
Unfractionated IMLN cells	48	26.8
OX19-positive IMLN cells	70	42.8
OX19-negative IMLN cells	8	0.8
Medium alone	4	0.4

TABLE 7b: Total cells and mast cell growth in bone marrow cultures stimulated with conditioned media from W3/25-labelled IMLN

Source of CM	Total cells ($\times 10^{-4}$)	Mast cells ($\times 10^{-4}$)
Unfractionated IMLN cells	56	23.5
W3/25-positive IMLN cells	52	21.8
W3/25-negative IMLN cells	8	0.7
Medium alone	3	0.1

and unfractionated MLN cells generated comparable numbers of MMC.

OX8 labelling

Table 7c demonstrates that the factor is not produced by cells which stain positively for the monoclonal antibody OX8, the marker for the T cytotoxic/suppressor cell. Cells which were negative for this marker produced the factor that induced MMC in normal bone marrow cultures.

Supernatants from the appropriate control cultures which included various combinations of labelled and unlabelled cells ± feeder cells, ± Con-A and medium alone did not cause any significant MMC growth in normal rat bone marrow. Only those cultures which contained FACS sorted MLN cells (either labelled or unlabelled depending on the monoclonal used) with feeder cells and stimulated by Con-A, produced MMC growth stimulatory activity.

Discussion

The experiments described here analysed the phenotype of the T cells which produce a factor able to stimulate MMC growth in normal rat bone marrow cultures. The results demonstrate that the cells express antigens defined by OX19 and W3/25 but not by the OX8 monoclonal antibodies.

The positive control comprising CM from unfractionated MLN cells generated the expected total cell and mast cell growth in the cultures but the CM from the OX19-positive fraction, which was T cell enriched, promoted an even greater total cell count and a larger proportion of mast cells. The removal of the macrophages and other non-T cells had an enhancing effect on MMC growth factor production and this is in

TABLE 7c: Total cell and mast cell growth in bone marrow cultures stimulated with conditioned media from OX8-labelled IMLN

Source of CM	Total cells ($\times 10^{-4}$)	Mast cells ($\times 10^{-4}$)
Unfractionated IMLN cells	60	25.2
OX8-positive cells	4	0.1
OX8-negative cells	64	31.4
Medium alone	4	0.4

agreement with earlier results (Haig et al, 1983; Chapter 2). Unfractionated and W3/25 positive MLN cell supernatants gave approximately the same total cell growth and numbers of mast cells, although both parameters were less than those obtained from the cultures stimulated by the OX19 positive fraction supernatant. As W3/25 also labels macrophages (Barclay, 1981) this again supports the earlier observation (Chapter 2) that their inclusion in culture has a detrimental effect on MMC growth factor production. IL-2 is a product of W3/25-positive cells which reinforces the idea that this subset plays the helper/inducer role in the rat (Cantrell, Robins and Baldwin, 1982). T-cytotoxic/suppressor cells, which are labelled by OX8 monoclonal antibody, did not produce the factor in amounts large enough to generate mast cells in normal bone marrow culture. Supernatants from the non-labelled population was, on the other hand, equivalent in potency to the supernatants from unfractionated MLN cells. The small increase observed in total cell growth may have been caused by the removal of suppressor cells.

As mentioned previously, the factor(s) produced in this culture system would seem to be comparable with murine IL-3. The murine WEHI-3b cell line which produces IL-3 constitutively has provided the means to purify and analyse IL-3 (Ihle, 1984), a lymphokine with numerous effects on haemopoietic cell lines. IL-3 is normally a product of mitogen- or antigen-stimulated $Thy1^+$, $Lyt1^+,2^-$ T cells (Nabel et al, 1981) which also produce IL-2 (Miller and Stutman, 1983).

On the basis of molecular weight, tissue distribution and functions of cells reactive with the monoclonal antibodies, it appears that in the rat, man and mouse, the antigens OX19, Leu1, T1 and Ly1 are homologous as are, respectively OX8, Leu2a, T5 and Ly2 antigens

are similarly so (Mason, Arthur, Dallman, Green, Spickett and Thomas, 1983). The isolation of the rat T cell subset responsible for the production of MMC growth factor was the first step in providing a source of the factor itself. However, it is now recognised that the sequential expression of genes by a subset of T cells, or even by a single clone, may alter the expression of immune function, and phenotype of the cell as it differentiates. In this way, the cells may gain or lose a function in culture with time or in response to different environmental signals (Freeman, Clayberger, Dekruyff, Rosenblum and Cantor, 1983; Pawelec, Schneider and Wernet, 1983). This feature clearly limits the usefulness of cloning T cells and makes the cloning of IL-3 genes necessary instead.

The cDNA for murine IL-3 has now been cloned (Fung, Hapel, Ymer, Cohen, Johnson, Campbell and Young, 1984; Yokota, Lee, Rennick, Hall, Arai, Masmann, Nabel, Cantor and Arai, 1984) and more recently the IL-3 gene itself (Campbell, Ymer, Fung and Young, 1985). The expression of IL-3 from IL-3 cDNA clones in eukaryotic cells has provided an opportunity to examine the range of biological activities possessed by IL-3 without some of the problems associated with the use of purified factors from "normal" biological sources (Hapel, Fung, Young, Johnson and Metcalf, 1985; Rennick, Lee, Yokota, Arai, Cantor and Nabel, 1985). The activities exhibited by this material includes all those previously attributed to IL-3. As was expected, the rat IL-3 gene has now been cloned and sequenced and expressed in non-human primate cells (Cohen, Hapel and Young, 1986). This will provide excellent pure material to compare activities with the CM generated by the T cell subclass $OX19^{+}$, $W3/25^{+}$, $OX8^{-}$ in this system.

Chapter 4

Effect of dexamethasone on the production of mucosal mast cell growth factor and the growth and differentiation of MMC in vitro

Introduction

Physiologically, glucocorticoids are both immunosuppressive and anti-inflammatory, although the cellular and molecular basis for these effects are poorly understood. These drugs suppress histamine release (Daeron, Sterk, Hirata and Ishizaka, 1982; Grosman and Jenson, 1984) and prevent the generation of secondarily formed mediators from mast cells (Danon and Assouline, 1978; Flower and Blackwell, 1979; Heinman and Crews, 1984).

Cortisone treatment of rats prior to Nb. infection completely suppresses previously acquired resistance to Nb. and the initiation of immunity is stopped completely or at a very early stage (Ogilvie, 1965). In studying the mechanism of expulsion of the parasite, and in particular the kinetics of the mast cell response during Nb. infections (Jarrett, Jarrett and Urquhart, 1968), it was observed that daily administration of cortisone commencing 5 days before infection produced a marked reduction in mast cell numbers (Jarrett et al, 1967). After withdrawal of the steroid, mast cells reappeared and the globule leucocyte reaction occurred, whereupon self-cure took place. When cortisone was introduced at day 10 after infection, there was no hyperplasia of mast cells, in fact mast cell numbers fell until the cortisone was withdrawn. The self-cure reaction then followed 6 days later. Cortisone can also interrupt the expulsion process when administered during the self-cure reaction. The mast cell hyperplasia again occurred after cortisone withdrawal and the self-cure followed some days later (Jarrett et al, 1967). Rats primed by infection with Nb. and challenged intravenously with soluble whole worm antigen undergo systemic anaphylactic shock (Jarrett and Miller, 1982). The

major shock organ responding to anaphylaxis in this species is the small intestine, and associated with the intestinal lesions is the release into the blood and lumen of the gut of the MMC proteinase, RMCPII (Miller et al, 1983; King and Miller, 1984). The release of RMCPII provides a unique and specific marker of in vivo activation of MMC. The concentration of RMCPII in the tissues, intestinal secretions and blood was measured to analyse the anti-anaphylactic activity of corticosteroids, on the mucosal mast cell subpopulation (King, Miller, Newlands and Woodbury, 1985). The manifestations of anaphylaxis were abolished in rats treated with corticosteroids 24 and 48 hr before intravenous challenge with antigen. Suppression of the response was associated with depletion of RMCPII and of MMC from the intestinal mucosa.

Both the direct effect of corticosteroids on MMC and their effect on T cells were of interest because MMC hyperplasia during helminth infection is an apparently T cell-dependent event (Mayrhofer, 1979; Nawa and Miller, 1979). Furthermore, dexamethasone exerts an inhibitory effect on lymphokine production by T lymphocytes in vitro (Gillis, Crabtree and Smith, 1979a; 1979b; Kelso and Munck, 1984).

Experiments were designed therefore to investigate the activity of dexamethasone in the culture system, using a dual approach: firstly to investigate the influence of dexamethasone on the production of MMC growth factor (Experiment 9) and secondly, to determine its effect on the growth and differentiation of the cultured mast cells (Experiment 10).

Experiments 9

IMLN cells taken day 14 after infection with Nb. were plated at 4 x 10^6/ml and stimulated with 2 µg/ml Con-A as for normal production of CM. In addition to this, dexamethasone was added to the cultures at 10^{-6}M, 10^{-7}M and 10^{-8}M with and without Con-A. The cultures were harvested after 48 hr and the supernatants dialysed against three changes of PBS over 48 hrs to remove the dexamethasone. The supernatants were then checked for MMC growth stimulatory activity using the bone marrow assay. On harvesting, the bone marrow cultures were extracted with 0.15M KCl and the levels of RMCPII were measured using the ELISA in order to verify that growth of MMC was being stimulated. The results shown are those obtained from a representative experiment harvested on day 7 and are the mean of triplicate cultures. (See appendix 12 for additional results).

Results

Dexamethasone inhibited the production of both total cell and MMC growth stimulatory activity in a dose-dependent fashion (Table 8). Total cell growth and mast cell growth in the positive control (IMLN 14 + 2 µg/ml Con-A) were, respectively 5 and 16 times that of the 10^{-8}M dexamethasone + Con-A treated culture. With increasing concentration of dexamethasone both total cell and mast cell numbers decreased. The RMCPII content of the bone marrow cultures also reflected the numbers of mast cells present, with the positive control again containing the highest concentration of RMCPII and the level decreasing with increasing concentration of dexamethasone.

TABLE 8: Effect of dexamethasone (Dex) on the production of MMC growth factor by IMLN cells, stimulated by Con-A, and assayed by growth of MMC in normal bone marrow cultures.

Conditioned Medium	Total Cells $x10^{-4}$/ml	Mast Cells $x10^{-4}$/ml	RMCPII content µg/ml
IMLN 14 + 10^{-8}M Dex + Con-A	12	1.68	0.68
" 10^{-7}M " "	10	0	0.16
" 10^{-6}M " "	1	0	0.01
IMLN 14 + 10^{-8}M Dex alone	3	0	0.01
" 10^{-7}M " "	2	0	0.02
" 10^{-6}M " "	1	0	0.02
IMLN 14 + 2 µg/ml Con-A	60	27.0	3.46
Medium alone	<1	0	0.01

Experiment 10

Normal bone marrow cultures were set up according to materials and methods. Dexamethasone was added to the cultures at 10^{-6}M, 10^{-7}M and 10^{-8}M with and without CM. The cultures were harvested after 7 days. Total cell counts were performed and differential cell counts carried out on Leishman stained cytospin preparations. On harvesting, the cells were pelleted by centrifugation and extracted with 0.15M KCl for RMCPII analysis. The cell culture supernatants were also analysed for RMCPII content. The results presented are the mean of triplicate cultures and are from a representative experiment (see appendix 13 for repeated experiments).

After 7 days, both dexamethasone treated and untreated cultures were washed and replated with fresh medium + CM and cultured for a further 7 days, whereupon, they were harvested and cytospin preparations made. The slides were stained with Leishman and the morphology of the mast cells compared in those cultures previously treated with dexamethasone and in untreated cultures.

Three week old normal rat bone marrow cultures which contained >90% mast cells were treated with dexamethasone at 10^{-6}, 10^{-7} and 10^{-8}M for 24 and 48 hrs. After this time the cells were harvested, cytocentrifuge preparations made, and RMCPII analysis carried out on cell extracts and supernatants. Triplicate cultures were treated for each concentration of dexamethasone and the mean of these individual results is presented (repeat experiments are detailed in appendix 14).

Results

Dexamethasone inhibited the total cell growth in the normal bone marrow cultures compared to the positive control (NBM + CM), although the proportion of mast cells per culture did not vary significantly between the different doses of dexamethasone, or the positive control (Table 9). The concentration of RMCPII per cell decreased with increasing concentration of dexamethasone. The content of the culture supernatants followed a similar pattern.

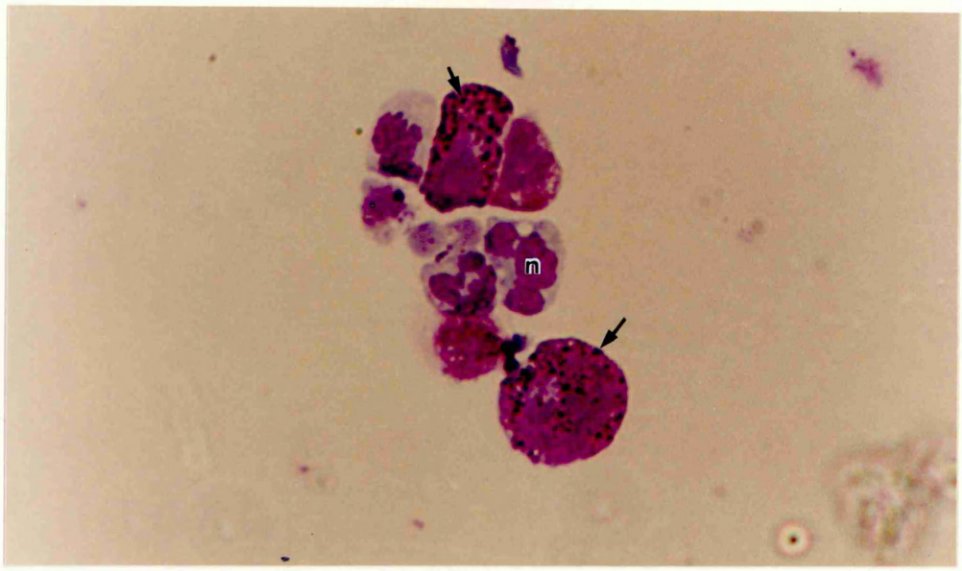
The mast cells in the dexamethasone-treated cultures differed from the positive control culture in that the granules in the drug-treated cells were much smaller than those of untreated cells (Figures 5,6). The mast cells from the treated cultures had the appearance of immature cultured mast cells taken from cultures less than 4 days old (see Section 2, Chapter 1). Differential cell counts revealed that as the concentration of dexamethasone increased, the proportions of neutrophils and macrophages became inversely related. Thus the percentages of neutrophils and macrophages were approximately equal at 23% in the presence of 10^{-8}M dexamethasone and were, respectively, 36% and 9% in 10^{-7}M dexamethasone and 44% and 4% in 10^{-6} dexamethasone. In the positive control the proportions of neutrophils and macrophages were 8% and 40%. This trend was seen in the repeated experiments.

The dexamethasone-treated cultures which were refed and restimulated yielded mast cells which had larger granules, looked more mature, and were similar in morphology to the mast cells from the untreated cultures (Figures 7,8). The addition of dexamethasone to mature mast cell cultures did not cause any change in cell numbers,

TABLE 9: Effect of dexamethasone (Dex) on the growth, differentiation and RMCPII content of normal bone marrow cultures.

CM and conc. Dex.	Total Cells $x10^{-4}$/ml	Total Mast Cells $x10^{-4}$/ml (% of total culture)	RMCPII content of extracted cells (µg/ml)	RMCPII content of supernatant (µg/ml)
NBM+CM+10^{-8}M Dex	30	16 (53)	3.7 (23 pg/cell)	0.6
" " 10^{-7}M "	28	15 (53)	3.3 (22 pg/cell)	0.6
" " 10^{-6}M "	25	13 (52)	2.0 (15 pg/cell)	0.5
NBM+10^{-8}M Dex	4	0	0.01	+
" 10^{-7}M "	6	0	0.02	+
" 10^{-6}M "	7	0	0.02	+
NBM+CM	59	30 (51)	8.4 (28 pg/cell)	1.5
NBM+MA	1	0	<0.01	+

+ <10 ng/ml detected.



Figures 5: 10^{-7}M dexamethasone treated normal bone marrow culture, harvested on day 7. Note the small granules of the mast cells (arrows). Also present are neutrophils (n). Leishman stain (x733).

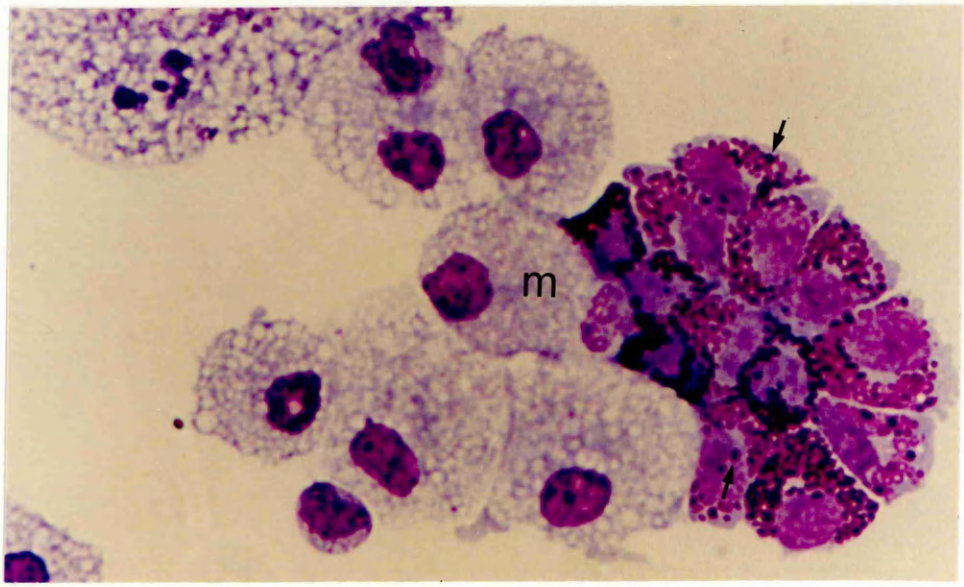
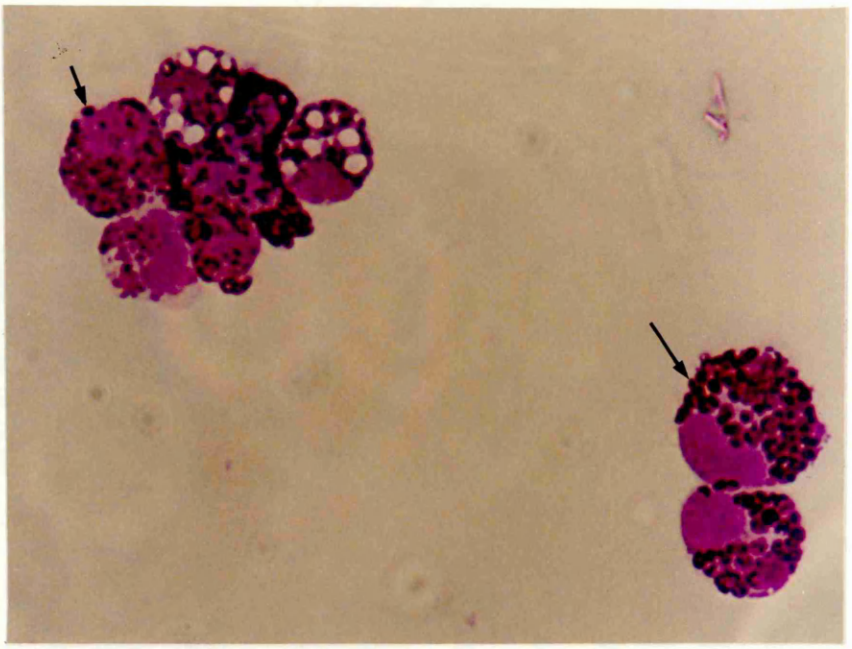


Figure 6: Normal bone marrow after 7 days in culture in presence of CM. The granules of the mast cells are much larger than those in Fig.5 (arrows). Note also the presence of macrophages (m). Leishman stain (x733).



Figures 7: Seven day old 10^{-7}M dexamethasone treated culture, washed, refed, restimulated and cultured for a further 7 days. The cells are much more heavily granulated, also the granules themselves are now much bigger (arrows) than in Fig.5. These cells form >90% of culture. Leishman stain (x733)

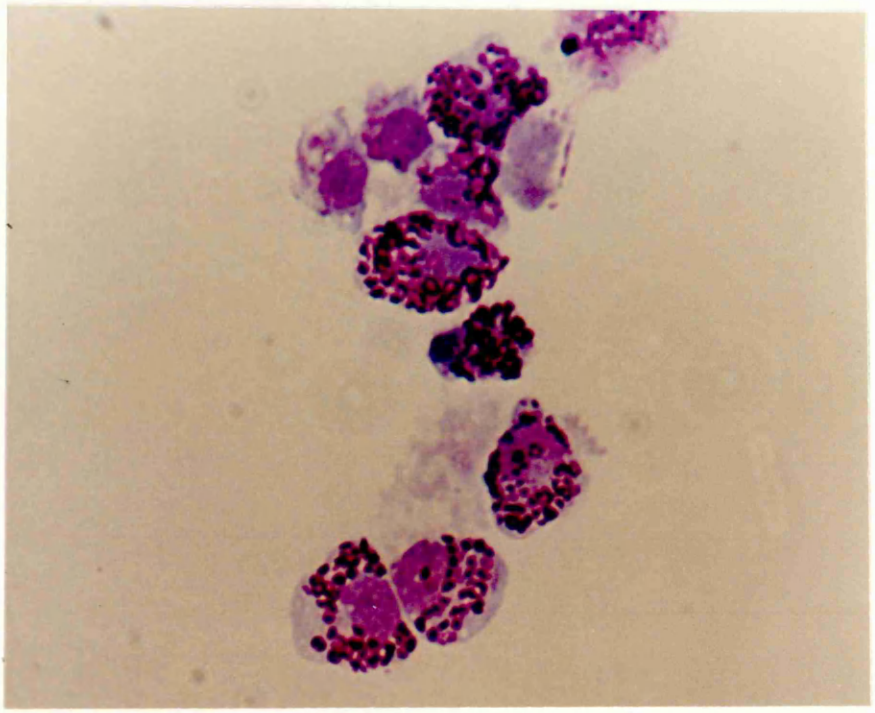


Figure 8: Seven day old normal bone marrow culture, refed, restimulated and cultured for a further 7 days. The mast cells form >90% of the culture. Leishman stain (x733).

TABLE 10: Effect of dexamethasone (Dex) on 3 week old normal rat bone marrow cultures.

Conc. of Dex.	Total cell no. $x10^{-4}$	Mast cell no. $x10^{-4}$	RMCPII content cell extract (µg/ml)	RMCPII content cell supernatant (µg/ml)
Before treatment	50	49.5	14.85 (30pg/cell)	2.41
24 hr treatment				
10^{-8}M Dex	52	51.2	14.33 (28pg/cell)	2.23
10^{-7}M Dex	49	48.4	14.51 (30pg/cell)	2.53
10^{-6}M Dex	50	49.4	15.82 (32pg/cell)	2.56
48 hr treatment				
10^{-8}M Dex	50	49.5	14.35 (30pg/cell)	2.32
10^{-7}M Dex	47	46.5	13.96 (30pg/cell)	2.26
10^{-6}M Dex	49	48.8	14.14 (29pg/cell)	2.29

in the RMCPII content of the cells or supernatants (Table 10), or in mast cell morphology as judged by Leishman staining of cytocentrifuge preparations.

Discussion

In addition to exerting anti-inflammatory and immunosuppressive effects *in vivo*, glucocorticoids inhibit and enhance a wide range of cell types and functions *in vitro* by specific receptor-mediated mechanisms (Fahey, Guyre and Munck, 1981). Since generation of MMC in culture from normal rat bone marrow is a T cell-dependent phenomenon (Haig *et al*, 1983; Chapter 2) and, more specifically, is dependent on a factor supplied by helper T cells (McMenamin, Jarrett and Sanderson, 1985; Chapter 3), it was of interest to determine the effects of dexamethasone on the production of the MMC growth factor. It had been shown previously that proliferative and cytolytic responses of T lymphocytes to antigens and mitogens were profoundly inhibited by glucocorticoids (Nowell, 1961; Tormey, Fudenberg and Kamin, 1967; Baxter and Harris, 1975). Similarly, Culpepper and Lee (1985) found that dexamethasone inhibits the appearance of IL-3 activity in supernatants of murine helper T cell clones.

The present results show that MMC growth stimulatory activity is greatly reduced in those CM derived from IMLN cultures to which dexamethasone has been added. Since it seems that an IL-3 like factor (Chapters 2 and 3) is normally generated in these cultures, the present results add further support to the data cited above.

The actual mechanism of inhibition of mitogen-induced proliferation has been investigated and a number of conflicting

results obtained. Initially it was shown that glucocorticoids had an inhibitory effect on T lymphocytes by a dual mechanism: that is, a direct inhibitory effect on T-cell proliferation as well as an inhibition of IL-2 production which resulted in the control of clonal expansion of activated T-cells (Gillis, Crabtree and Smith, 1979a; 1979b). Direct inhibition of IL-1 production by macrophages may also be important since IL-1 is probably obligatory for inducing and promoting the maturation of IL-2-producing cells; inhibition of IL-1 synthesis could, therefore, be the essential step in glucocorticoid-mediated inhibition of T lymphocyte proliferation (Smith, Crabtree, Gillis and Munck, 1980; Stosic-Grujicic and Simic, 1982). However further experiments revealed two important dissociations. Glucocorticoid inhibition of factor secretion was dissociated from clonal IL-2 production, because inhibition could be observed even in the presence of excess IL-2, and inhibition could be dissociated from effects on cell proliferation, because clones were found in which factor production, but not growth, was inhibited (Kelso and Munck, 1984). It has also been observed that the generation of factors, chemotactic for eosinophils and macrophages from Con-A-stimulated lymphocytes, is inhibited by the release of a suppressive factor by dexamethasone-treated T lymphocytes and that the latter acts directly on the cells producing the chemotactic lymphokines, but not on the eosinophil or macrophage chemotactic factors themselves (Hirashima, Sakata, Tashiro, Yashimura and Hayashi, 1985).

Investigations at a more molecular level have revealed that dexamethasone directly inhibits transcription of the IL-3 gene (Culpepper and Lee, 1985). Several pieces of evidence support this: the kinetics of dexamethasone action on IL-3 mRNA levels are very

rapid and consistent with inhibition of transcription, and since no detectable precursor or mature IL-3 mRNA accumulated in the nuclei of dexamethasone-treated cells, corticosteroid inhibits neither transport nor processing of mRNA. These findings demonstrate that dexamethasone can inhibit the production of several lymphokines that are produced in response to mitogen activation thus modulating the immune response in a variety of ways.

Treatment with glucocorticoids *in vitro* inhibits IgE-dependent granule secretion from rat CTMC (Heiman and Crews, 1984), mouse CTMC (Daeron *et al*, 1982) and human basophils (Schleimer, MacGlashan, Gillespie and Lichtenstein, 1982) and suppresses the IgE-dependent release of preincorporated radiolabelled arachidonic acid and its metabolites (Heiman and Crews, 1984). Furthermore, *in vivo* administration of dexamethasone to rats inhibits IgE-dependent release of histamine and arachidonic acid when the rat CTMC are subsequently labelled and activated *in vitro* (Marquardt and Wasserman, 1983). Preincubation of mouse bone marrow-derived cells cultured *in vitro* with dexamethasone inhibited subsequent IgE antigen-dependent exocytosis of secretory granules and the *de novo* biosynthesis of arachidonic acid metabolites (Robin, Seldin, Austen and Lewis, 1985).

The effects of glucocorticoids, both *in vitro* on the above mentioned cells and *in vivo* on the rat MMC (Introduction), prompted the investigation of the effect of dexamethasone on the development of the *in vitro* cultured, bone marrow derived rat mast cell. The neutral serine proteinase RMCPII is located uniquely within the granules of rat MMC and is present within the cultured mast cells also. Dexamethasone treated cells contained less RMCPII than control cells

as well as secreting less RMCPII into the culture supernatant. The granule formation of the treated cells was also affected in that, compared to untreated control cells, their granules were much smaller and the treated cells looked generally immature. Removal of dexamethasone from the bone marrow cultures permitted maturation of the mast cells which were then indistinguishable from those in control cultures. These observations extend earlier findings on the ability of corticosteroids to suppress the development of intestinal mucosal mastocytosis during nematode infection (Jarrett et al, 1967; Olsen and Schiller, 1978) by showing that the putative in vitro counterpart of rat MMC is also suppressed in its development by corticosteroids.

A novel observation made on the differential cell counts of the dexamethasone-treated normal bone marrow cultures revealed that the numbers of neutrophils in the cultures increased with increasing dexamethasone concentration and the number of macrophages decreased. The CM added to the normal bone marrow cultures contains a heterogenous mixture of growth factors capable of stimulating various cell types and, in some instances, macrophages and not mast cells have been the major cell type growing in culture (unpublished observation). Culture conditions during CM production can drive the T cells to produce factors favouring macrophage growth and differentiation. It may be that dexamethasone inhibits the responsiveness of macrophages to growth factors present in the CM so that neutrophil growth and differentiation is favoured. It has been observed that systemic glucocorticoid treatment induced monocytopenia (Parrillo and Fauci, 1978) and neutrophilia (Parrillo and Fauci, 1979) in humans. The mechanisms of these actions have not been elucidated but increased

release of neutrophils by the bone marrow has been observed (Parrillo and Fauci, 1979). There are obviously many complicated regulatory circuits involved in haemopoiesis, and the effects of glucocorticoids on the different cell types have still to be unravelled. Perhaps the culture system described in this thesis could be utilized to investigate these various effects.

The present study also throws light on the findings of King et al (1985), who observed that methylprednisolone treatment causedthe depletion of mature MMC and of RMCPII from the rat jejunum. Addition of dexamethasone to 3 week old rat bone marrow cultures which comprise >90% mast cells did not, however, in the continuing presence of CM, cause depletion of these cells nor depletion of RMCPII and the cells maintained their well developed granular morphology. Collating these in vivo and in vitro data, it would seem that the effects of glucocorticoids on MMC are two-fold: firstly, they inhibit the production of MMC growth factor(s) on which the in vitro generated cells are absolutely dependent for their growth and differentiation and secondly, they act directly on the mast cells themselves inhibiting the normal pattern of granule formation. If the hyperplasia of MMC and their persistence in the parasitised gut is due to the production of MMC growth factors by T-cells, then treatment of these animals with glucocorticoids would cause factor production to cease or be at too low a level to sustain or stimulate and maintain the population of MMC or their precursors. In addition, the drug also acts on the MMC precursor directly inhibiting development. These observations go some way to providing an explanation for the disappearance of MMC from the gut of corticosteroid treated rats.

It is important to note the usefulness of the in vitro culture

system in investigating the phenomena found *in vivo*. It enables dissection of the responses to reveal the component parts of an integrated response, especially in this particular case where dexamethasone is acting on different cell types which vary the immune response.

SECTION 2

Characteristics of mast cells grown in vitro from normal rat bone marrow

Introduction

In the previous section it was shown that cells with the staining characteristics of mast cells grow and differentiate in normal rat bone marrow cultures stimulated with CM obtained from the MLN cells of Nb. infected rats activated *in vitro* by Con-A. The staining characteristics of the mature cultured mast cells have been described by Haig (1982; Haig *et al*, 1982). A detailed morphological and histochemical analysis of the developing cultured mast cells was undertaken, using light and electron microscopy.

In the rat, two populations of mast cells have been demonstrated. Cytochemical distinction between CTMC found in skin, muscle and peritoneal cavity, and MMC which predominate in the mucosa of the gastrointestinal tract, is based on the absence of granule heparin in MMC, and on the failure to detect MMC glycosaminoglycans in formalin fixed tissues (Enerback, 1981; Newlands *et al*, 1984). An alternative approach to identifying mast cell subsets is to determine the proteinase phenotype of mast cell granules. Biochemical studies have shown that RMCPI predominates in CTMC and RMCPII in MMC (Woodbury *et al*, 1978b) and more recently immunohistochemical analysis has confirmed that RMCPII is located exclusively in cells of the MMC phenotype and RMCPI is present in CTMC (Gibson and Miller, 1986). Using the above mentioned criteria, the *in vitro* cultured mast cells were compared to the mast cells identified *in vivo*.

Chapter 5

Time course of mast cell development in vitro by light and electron microscopy

Experiment 11

In order to obtain as many cells as possible for analysis, bulk cultures were set up as described in the Materials and Methods, using 25% of a known potent CM produced from MLN cells from Nb. infected rats + 2µg/ml Con-A. After 7 days, the BM cells were pelleted and resuspended with fresh nutrient medium containing CM, this was repeated at least every 7 days or as required as assessed by the pH of the medium. Aliquots were removed from the flasks every 2 days and cytocentrifuge preparations made from the cell culture samples. Some of the slides were stained with Leishman and some were fixed in either 4% buffered neutral formalin, methanol or, Carnoy's fluid, and subsequently stained with either toluidine blue or astra blue/safranin according to the Materials and Methods. Aliquots of cells were also taken for examination by electron microscopy and were processed according to the Materials and Methods.

Results

Leishman staining

Figure 9a shows normal bone marrow cells stained with Leishman, in the centre are a number of mast cells with granules stained an intense deep purple-black colour, these have the appearance of the classic CTMC obtained from peritoneal washings (Figure 13a). Also present are neutrophils, eosinophils and various other haemopoietic precursors. After culturing bone marrow for 2 days, cells with sparse granulation appeared and became progressively more granulated with time (Figures 9b-g). The CTMC were not stimulated to grow and could not be maintained in culture much past day 7, when one or two could

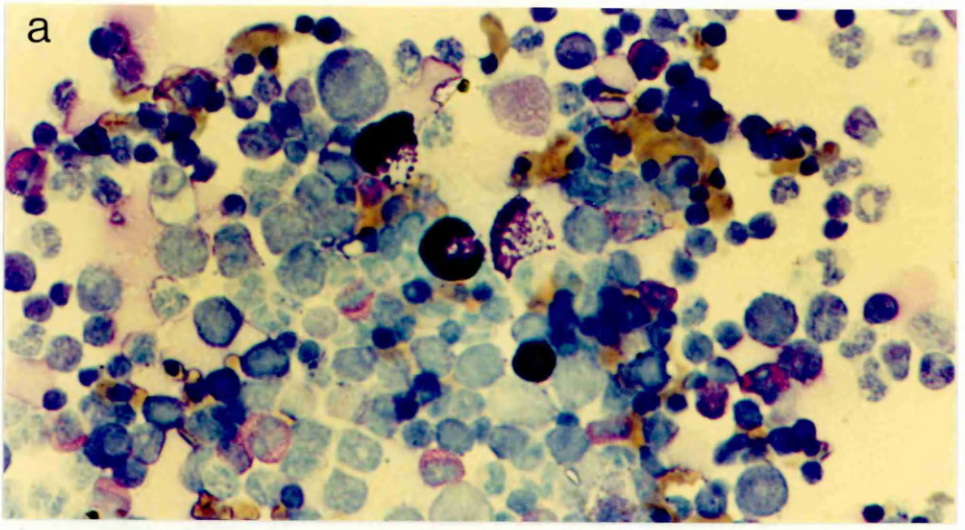


Figure 9a: Cytospin preparation of normal bone marrow before culture and stained with Leishman (x500). At the centre are densely stained mast cells.

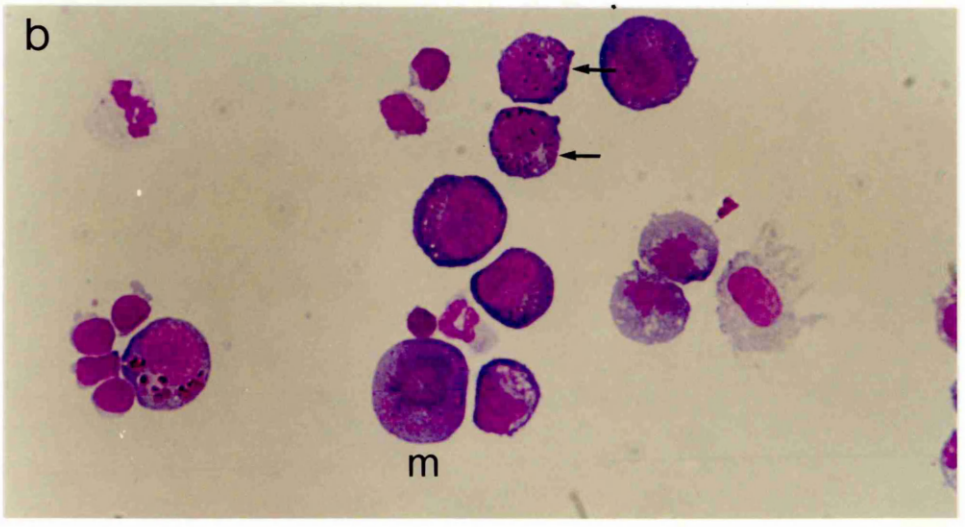


Figure 9b: Cytospin preparation of normal bone marrow cells after 2 days in culture. Note the sparse granulation of the cultured mast cells (arrows), and the mitotic figure (m). Leishman stain (x500).

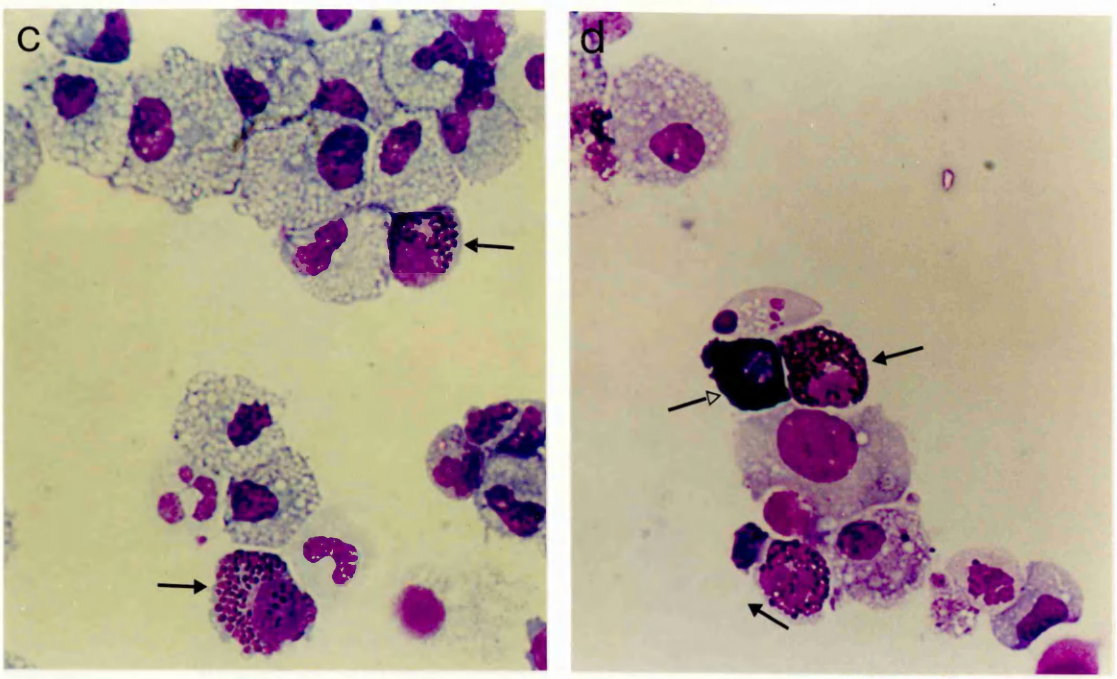


Figure 9 c & d: Cytocentrifuge preparations of cultured bone marrow harvested on day 4 (c) and day 6 (d) of culture. The cells become more granulated with time (arrows). Note the residual CTMC (open arrow) in the day 6 culture. Leishman stain (x500).

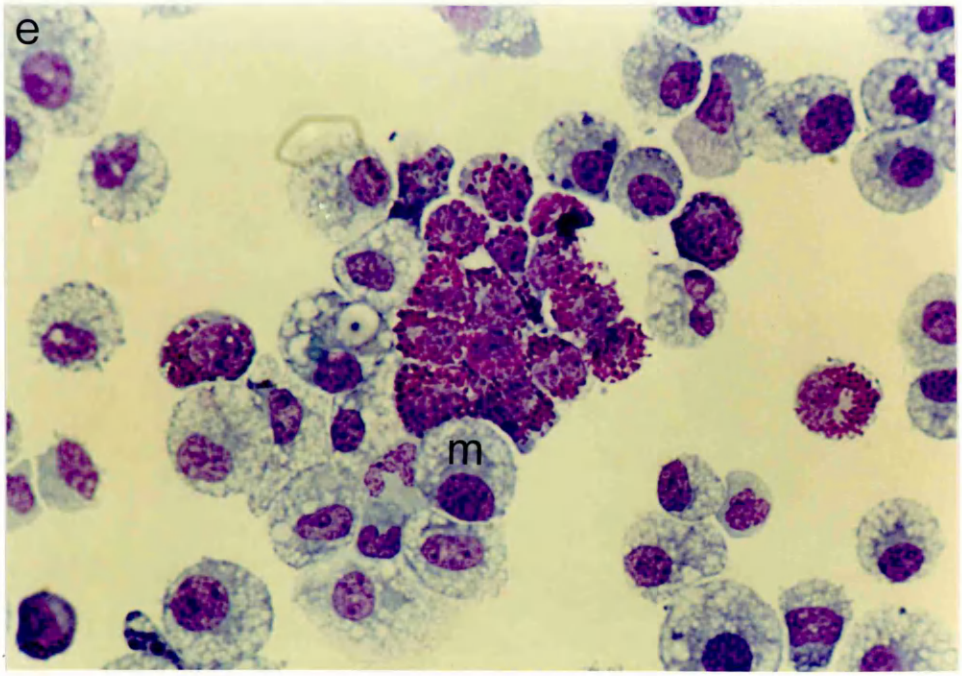


Figure 9e: Leishman stained cytocentrifuge preparation of a normal bone marrow culture harvested on day 8. The clump of mast cells is typical of the cell formation seen at this stage of culture, macrophages (m) being the other major cell type (x500).

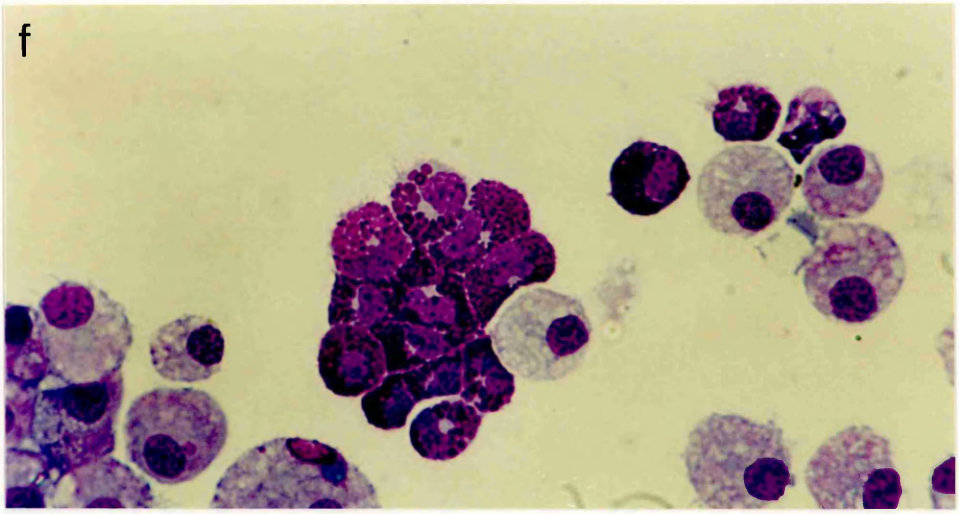


Figure 9f: Cytocentrifuge preparation of a normal bone marrow culture, day 10 after plating. The mast cells are heavily granulated. Note the heterogeneity of granule size within the population of cells. Leishman stain (x500).

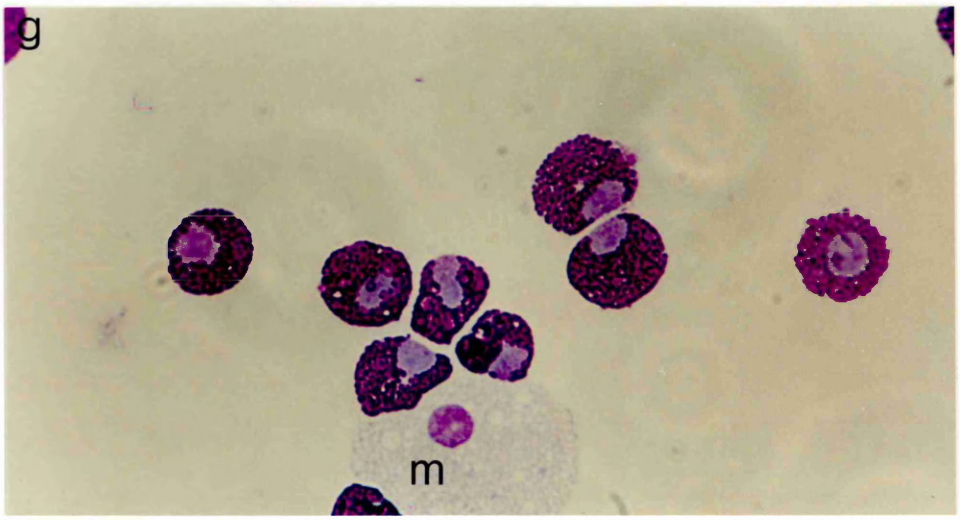


Figure 9g: Normal bone marrow culture day 16 after plating. Mast cells show the characteristic purple granules and eccentrically placed nucleus. A large macrophage is also present (m). Leishman stain (x500).

still be seen at this time (Figure 9e). Eosinophils, monocytes/macrophages and neutrophils (but not lymphoid or erythroid cells) were also stimulated by CM in these cultures for a short period but disappeared approximately by day 10 of culture . Cultures refed and restimulated were, by 2-3 weeks, composed of relatively pure populations (>99%) of heavily granulated mast cells (Figure 9g) which were maintained for at least 8 weeks in the presence of CM. The only remaining contaminating cells were macrophages (Figure 9g). Cultured mast cells were larger than the other granulocytes and had large round or oval eccentric nuclei. Basophilic cytoplasmic granules of variable size and number were visible and stained deep blue-purple in colour with Leishman's stain. By comparison, identically stained peritoneal exudate mast cells appeared densely granulated, with granules often obscuring the nucleus (Figure 13a). The granules appeared smaller and more uniform and stained an intense deep purple-black colour.

Toluidine blue staining

Peritoneal exudate cells fixed by any of the three fixatives, buffered neutral formalin, methanol or Carnoy's, displayed a mass of purple metachromatically stained granules in the cytoplasm which obscured the nucleus (Figure 10a). Cultured cells harvested on day 14 of culture were only clearly stained when fixed in either methanol or Carnoy's. They had readily visible pale blue nuclei and purple metachromatically stained granules which were much sparser than those of the peritoneal exudate cells (Figure 10b).

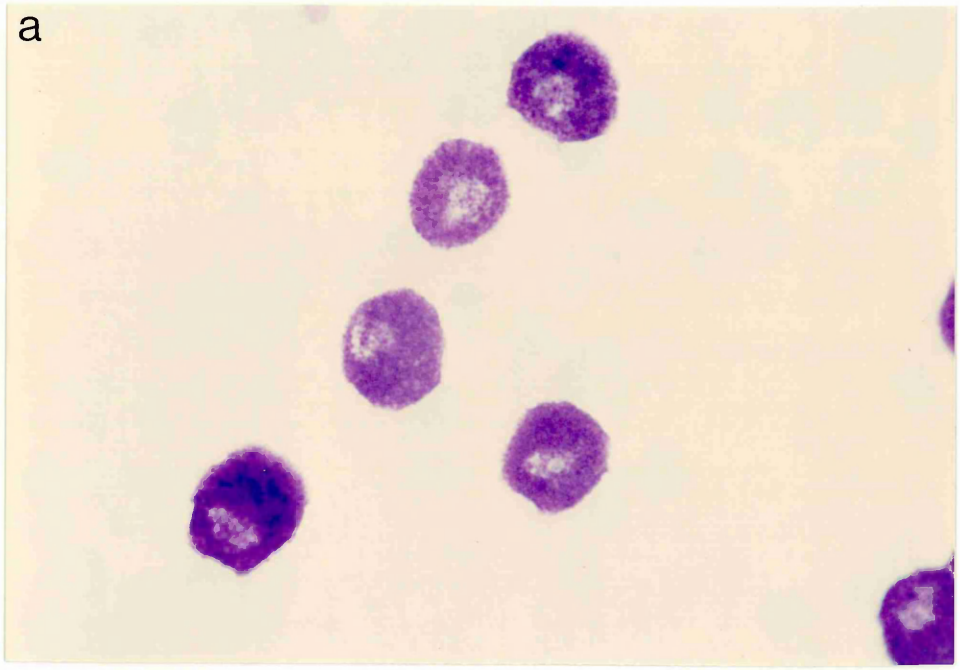


Figure 10a: Toluidine blue stained mast cells isolated from the peritoneal cavity, showing characteristic purple-black metachromasia of the granules. (x625).

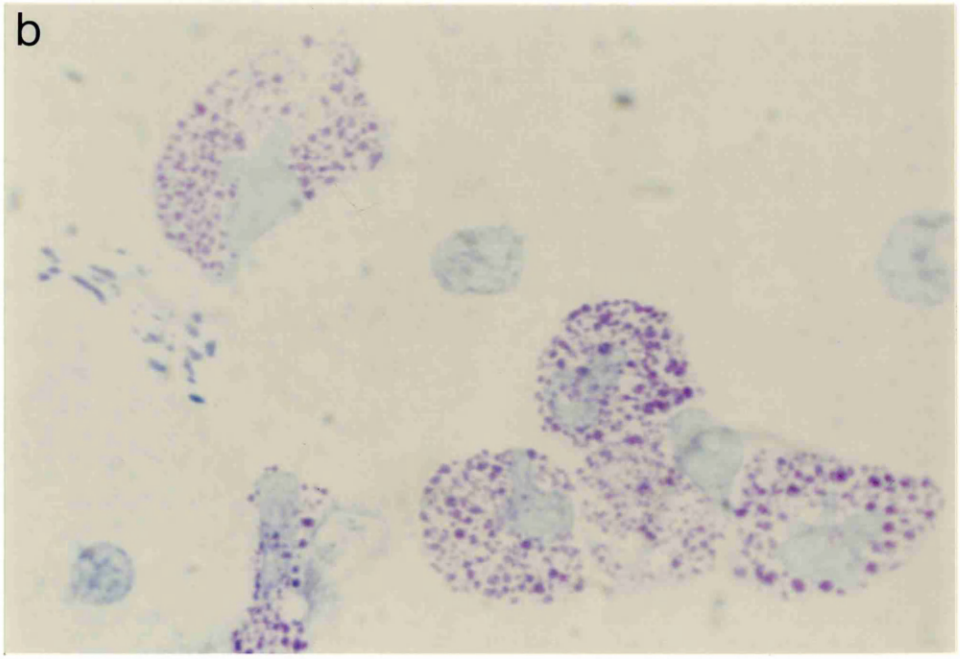


Figure 10b: Methanol fixed cultured bone marrow cells, day 14 after plating, stained with toluidine blue (pH 0.5). These cells are sparsely granulated compared to mast cells from peritoneal cavity but also display metachromasia (x1250).

Astra blue/safranin

The granules of mast cells in normal bone marrow displayed a spectrum of staining with astra blue/safranin from exclusively blue through a mixture of both blue and orange to exclusively orange granules (Figures 11a-c). All three fixatives gave similar results. The staining characteristics of marrow mast cells were identical to those of peritoneal mast cells (Figure 13b).

The sparsely granulated cells which appeared in the culture from day 2 onwards were stained with astra blue/safranin dyes only when fixed in either methanol or Carnoy's. They could not be stained after fixation in neutral buffered formalin. On Day 2 of culture the cells had only very few astra blue positive granules and the nuclei stained pale pink with safranin (Figure 12a). By day 6 of culture, the mast cells were much more granulated (Figure 12b). The cells were heterogenous with regard to size and shape and displayed various degrees of granularity (Figure 12b). Mature cultured mast cells, day 14 after plating showed heavy granulation (Figure 12c). The granules displayed the typical heterogeneity of size associated with MMC. The granules stained positively with astra blue throughout the culture period and at no time took up safranin in preference to astra blue in contrast to the CTMC.

Electron microscopy

By day 2 of culture, blast cells could be seen which contained occasional small electron-dense inclusions dispersed in their abundant cytoplasm particularly in the vicinity of the Golgi apparatus (Figure 14a). Larger vesicles of varying shapes which contained more electron-lucent granular material were also occasionally present

(Figure 14a). The nuclei which were large, oval or bean-shaped demonstrated margination of the heterochromatin and usually contained a prominent nucleolus. The cell membrane was characterised by some small stout pseudopodia. Macrophages were very abundant at this point in culture and could be differentiated from the early mast cells by the numerous electron-lucent cytoplasmic inclusions of varying sizes, and occasional lipid droplets. Their cytoplasm was also paler because they contained fewer free ribosomes and polyribosomes than maturing mast cells. However these macrophages contained relatively few lysosomal and residual bodies (Figure 14b).

By day 4 of culture, cells with a number of large electron-dense granules in their cytoplasm were present (Figure 14c). The increase in granule content was accompanied by a decrease in the volume of the other cytoplasmic organelles. The perinuclear Golgi apparatus was usually still a characteristic feature of these cells. The heterogeneity of the granule matrix was of interest at this stage in culture (Figure 14d). They ranged in size and electron-density; some very irregular vacuoles contained very electron-lucent particulate or condensed granular material whereas other generally smaller granules were electron-opaque. The delineating membrane could be seen quite clearly in some of the granules (Figure 14d). There appeared to be a series of vacuoles in varying stages of maturation. The smallest membrane bound vesicular structures possessed a granular matrix of low electron density. As the granules enlarged, their matrix became more electron-dense and often contained circular inclusions. They were usually spherical in profile (Figure 14d).

In more mature cultures of 3-4 weeks of age, the mast cell's

cytoplasm was full of electron-dense granules at the expense of most other organelles (Figures 14e-h) such as Golgi complexes and rough endoplasmic reticulum. The granules were more pleomorphic than in previous stages. Most of the granules were more or less uniformly electron-dense. However, some granules near the plasma membrane showed evidence of dissolution of their matrix. Some of the granules at these later stages of culture also exhibited a fibrillar or crystalline substructure to their matrix. Electron-lucent halos or demilunes were also present around some granules. However it is unclear whether these are artefactual or not since impregnation of large cell pellets with resin was often a problem.

The peritoneal mast cell (CTMC) contained granules of much more uniform size and shape than those of the cultured mast cells (Figure 15). The granules of this cell were also very electron-opaque.

Discussion

Basophilic granular cells were described growing in liquid bone marrow cultures in the presence of CM made from IMLN cells from Nb. infected rats stimulated in vitro by Nb. antigen or Con-A (Haig, 1982; Haig et al, 1982; Section 1). It appeared that they exhibited some of the characteristics of MMC (Haig, 1982; Haig et al, 1982) and so a more detailed analysis of their histological and ultrastructural development in culture was undertaken.

The mature cultured cells exhibited the generally accepted criteria for the definition of mast cells. When stained with Leishman, the cells showed a pale round eccentrically placed nucleus with well defined basophilic staining granules. In addition, when

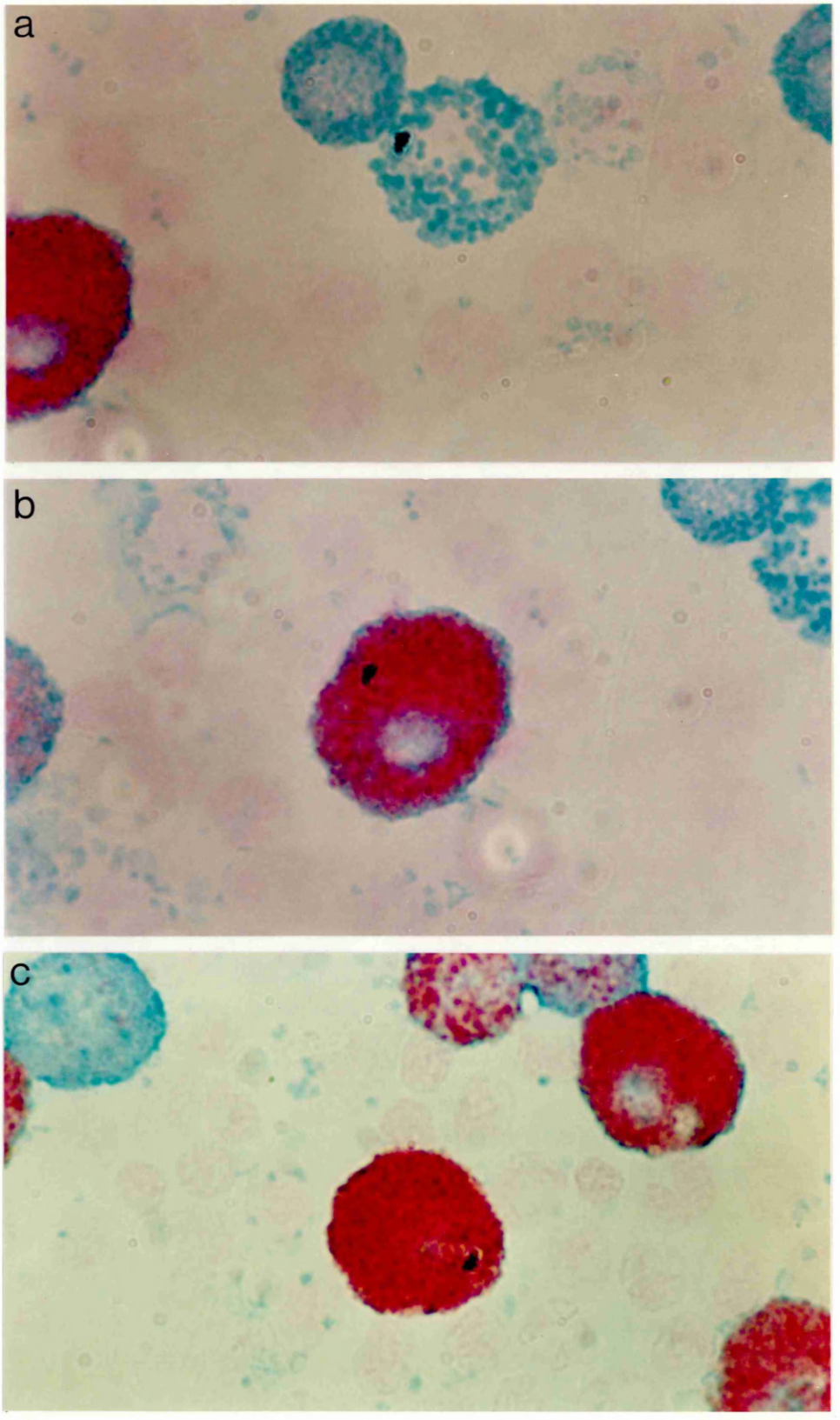


Figure 11: Astra blue/safranin stained normal bone marrow displaying CTMC in various stages of maturity, from the immature cell stained exclusively blue (a), to the intermediate cell (b) stained both blue and orange, to (c) the most mature cell stained exclusively orange (a-c) (x1250).

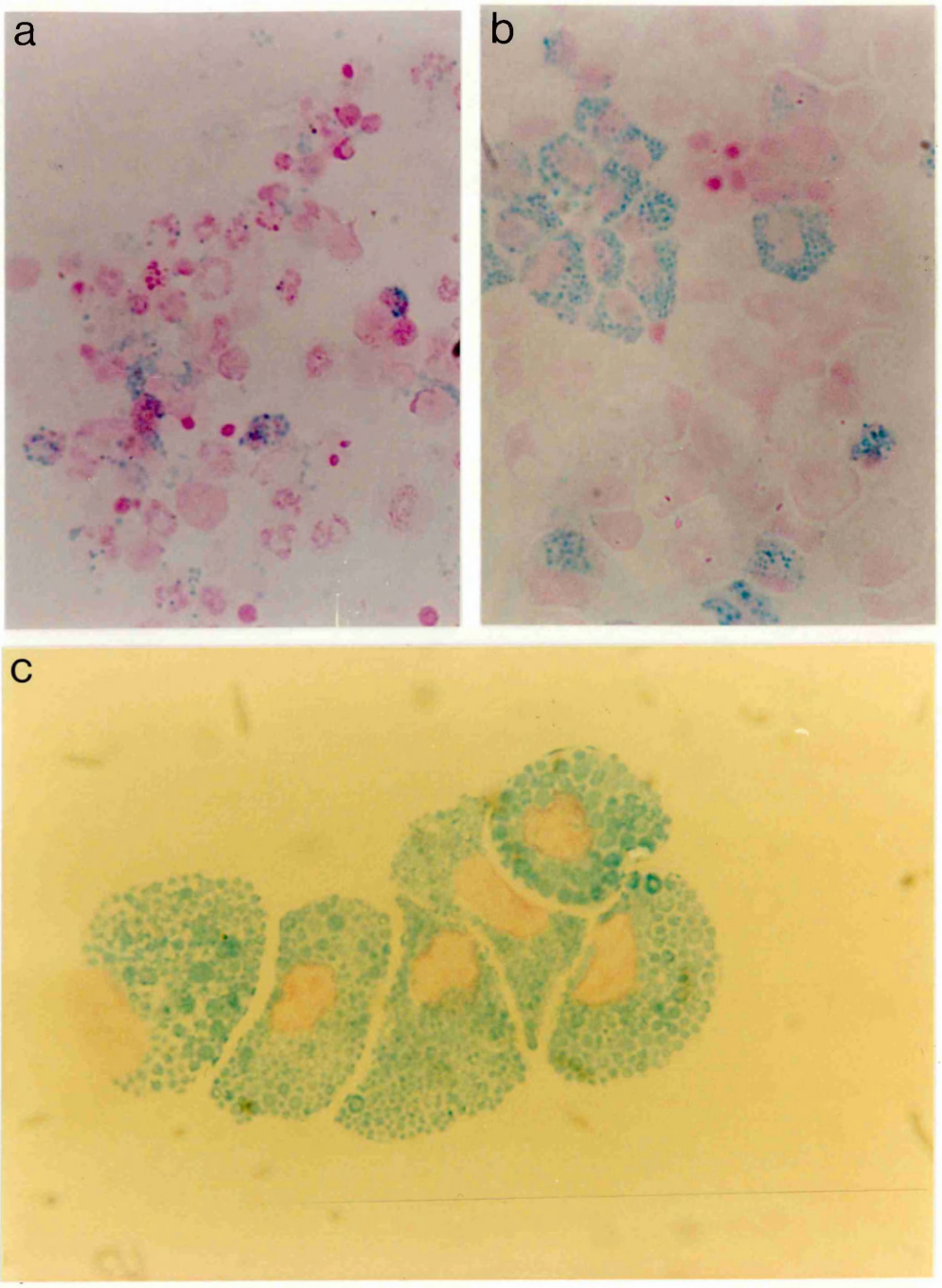


Figure 12: Methanol fixed cultured bone marrow cells stained with astra blue/safranin on day 2 (a) x320 (blue filter), day 6 (b) x500 (blue filter) and day 14 (c) x1250. Note the progressive granulation of the cells as the cultures mature and that none of the mast cell granules stain with safranin.

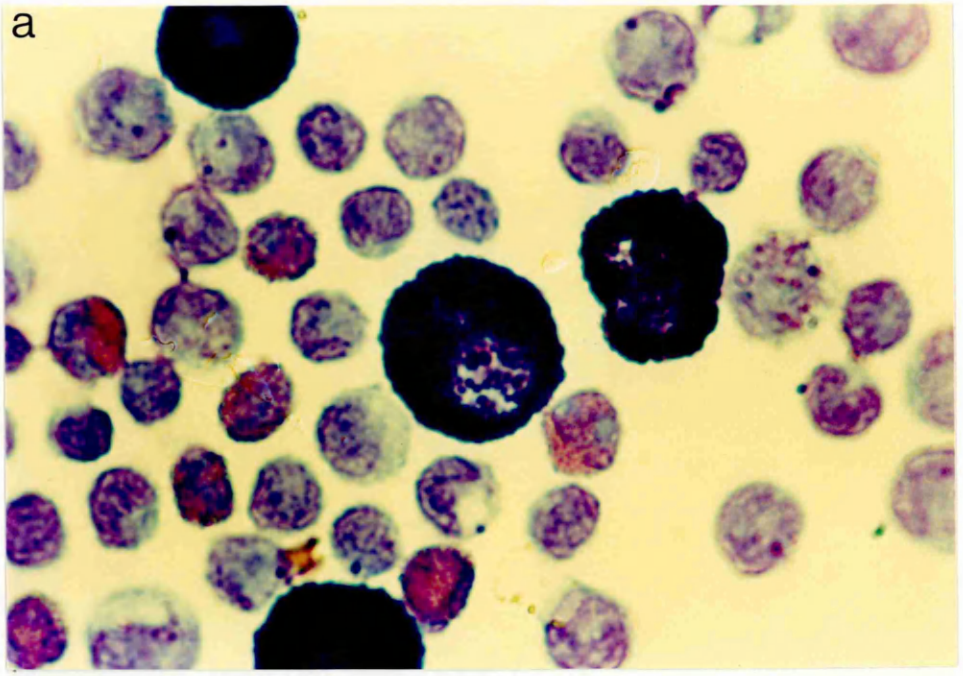


Figure 13a: Leishman stained mast cells isolated from the peritoneal cavity. The cells are so heavily granulated the nucleus is obscured in many of the cells. (x1250).

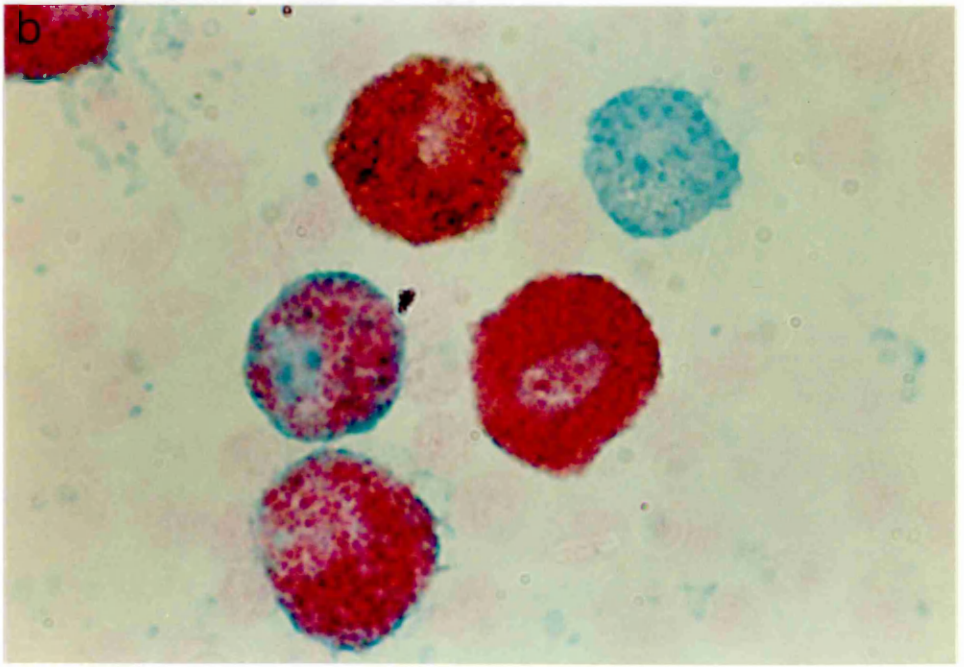


Figure 13b: Mast cells isolated from the peritoneal cavity and stained in the astra blue/safranin sequence. Note the spectrum of staining of the granules from astra blue positive, to a mixture of both astra blue and safranin, to staining exclusively with safranin (x1250).

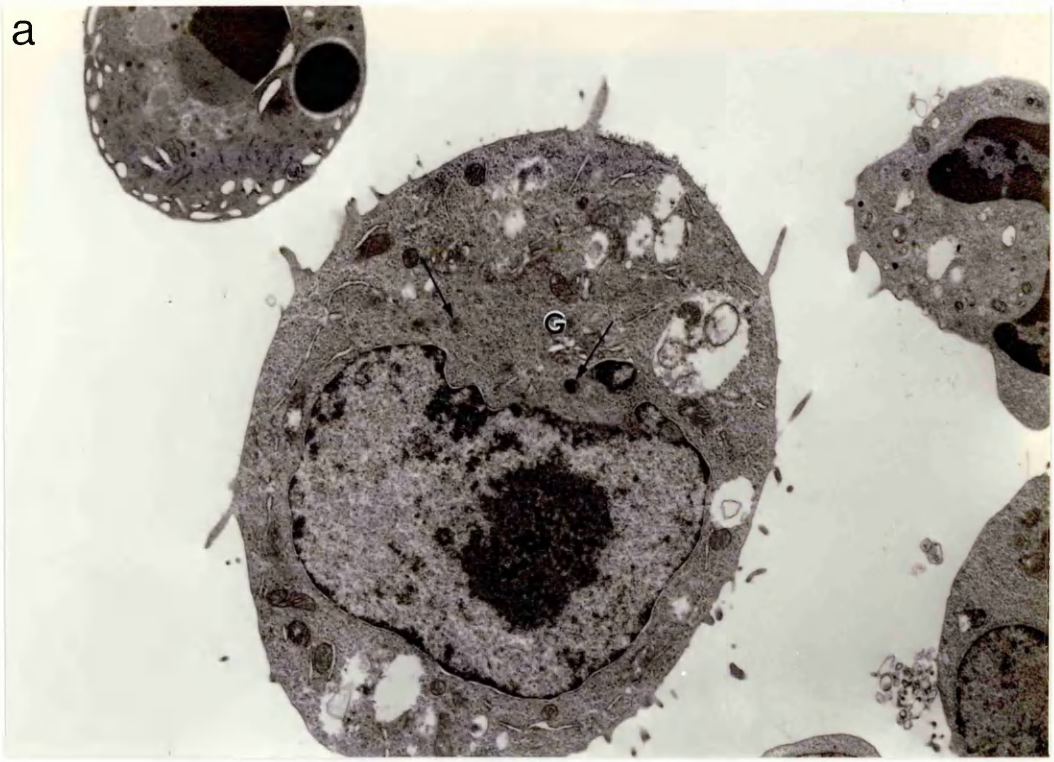


Figure 14a: Electron micrograph of normal bone marrow cells 2 days after plating, showing a possible immature mast cell with a large nucleus and prominent nucleolus. A few small electron-dense granules are indicated (arrows) adjacent to the Golgi apparatus (G). The cytoplasm contains several profiles of rough endoplasmic reticulum (x5,700).

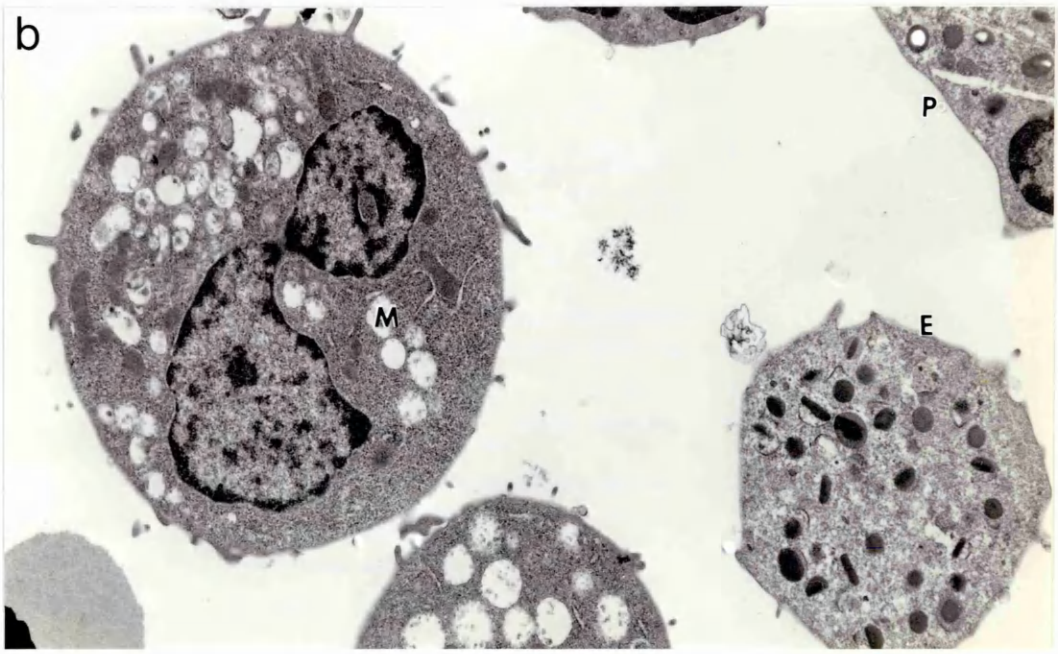


Figure 14b: Electron micrograph of day 2 culture showing a variety of cell types including an eosinophil (E), a polymorphonuclear leukocyte (P) and macrophage (M). The latter are characterised by large numbers of electron-lucent phagolysosomes, and complex nuclear profiles (x7,600).

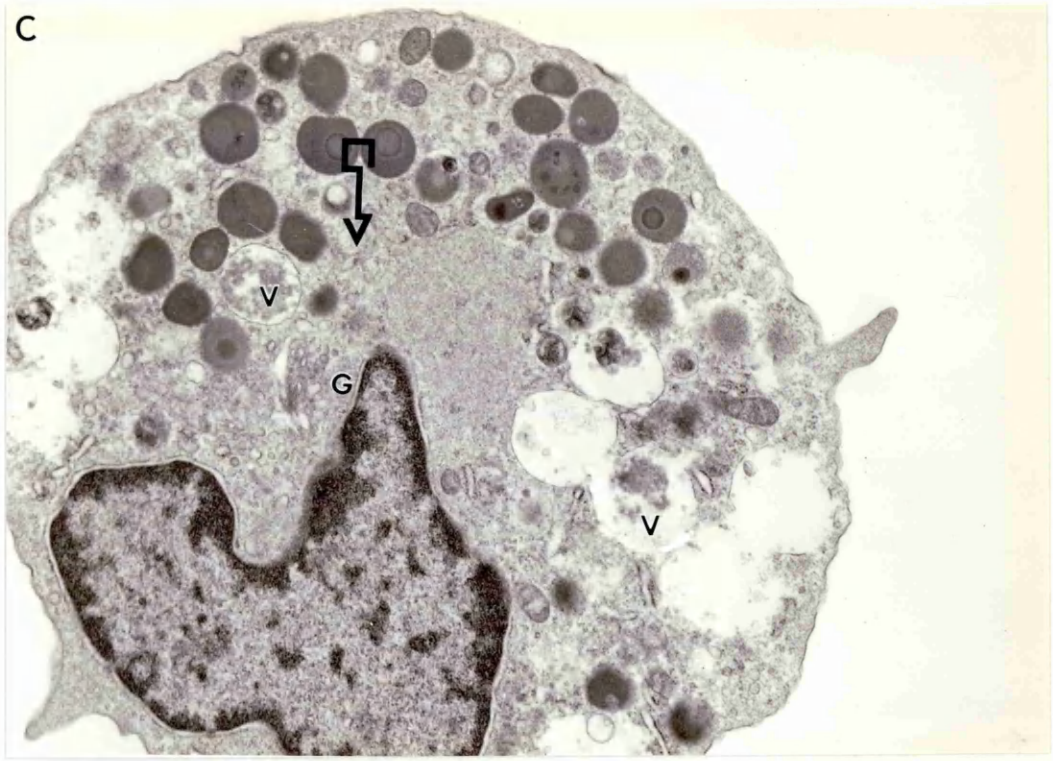


Figure 14c: Electron micrograph of cultured mast cell day 4 after plating. Note the prominant perinuclear Golgi apparatus (G) and numerous granules of variable morphology including several large electron-lucent membrane bound vesicles (V), some containing flocculent material (x15,500).

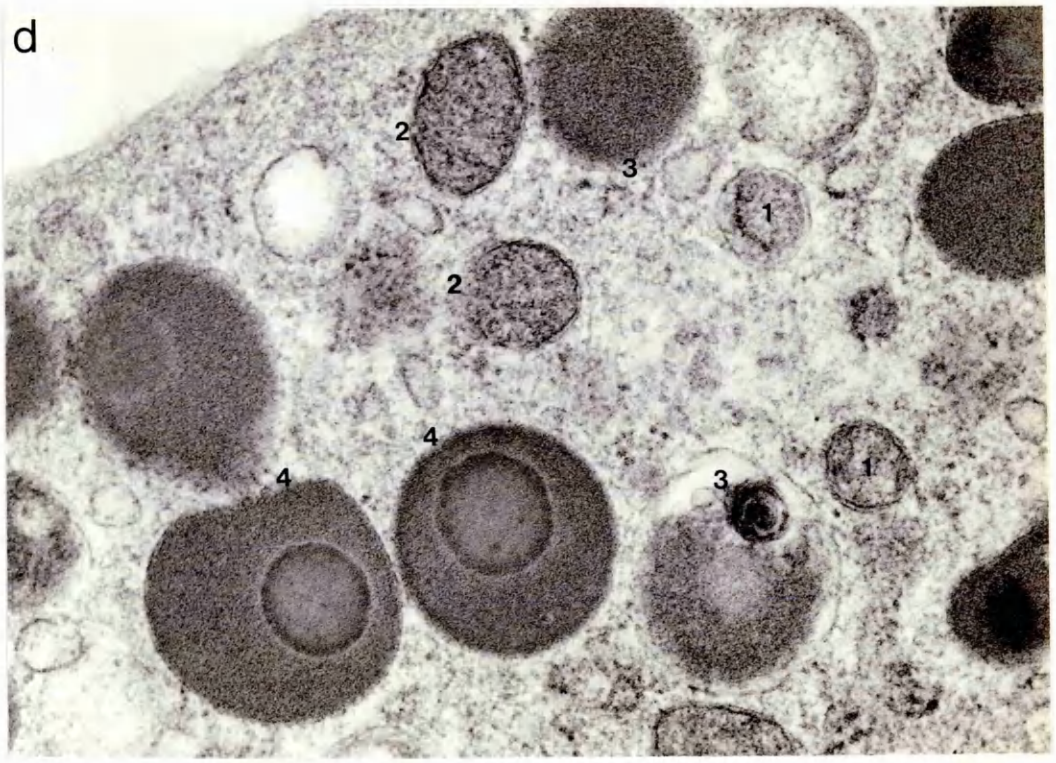


Figure 14d: Higher power of boxed area from Fig.14c showing more detail of the membrane bound electron-dense granules. Possible stages of granule maturation (1-4). (x57,000).

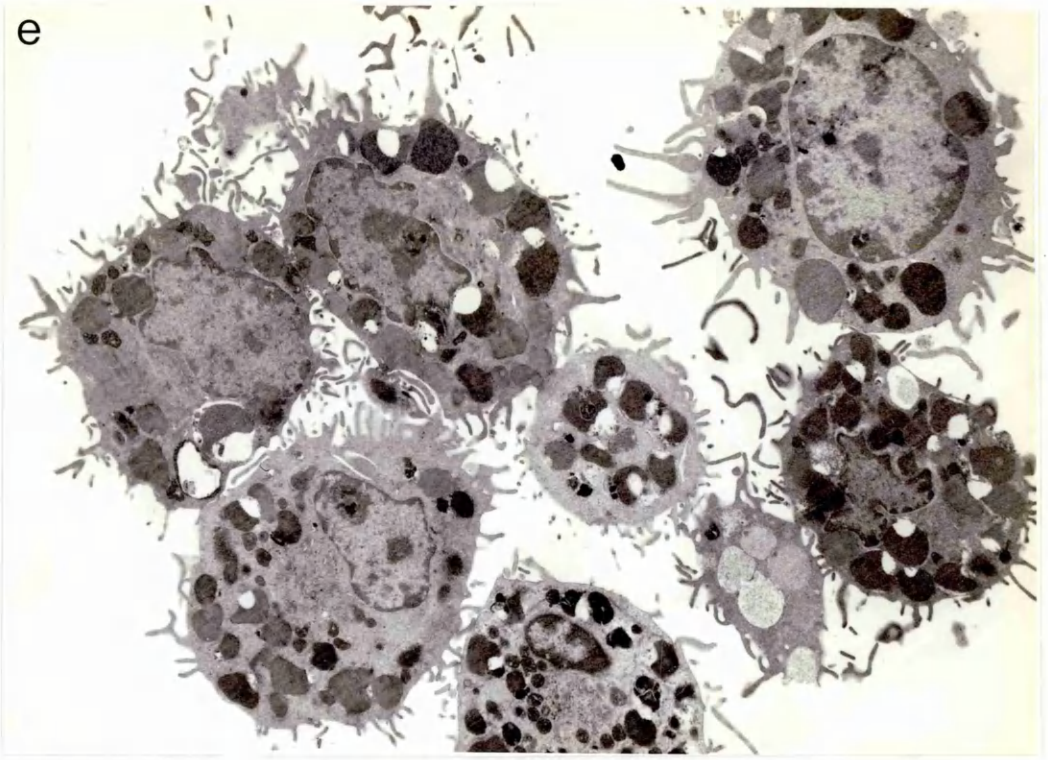


Figure 14e: Electron micrograph of normal bone marrow culture day 24 after plating illustrating several mature mast cells. The granules are numerous and of varying sizes, shapes and electron densities. Note the numerous filopodia on the cell membranes (x4,200).

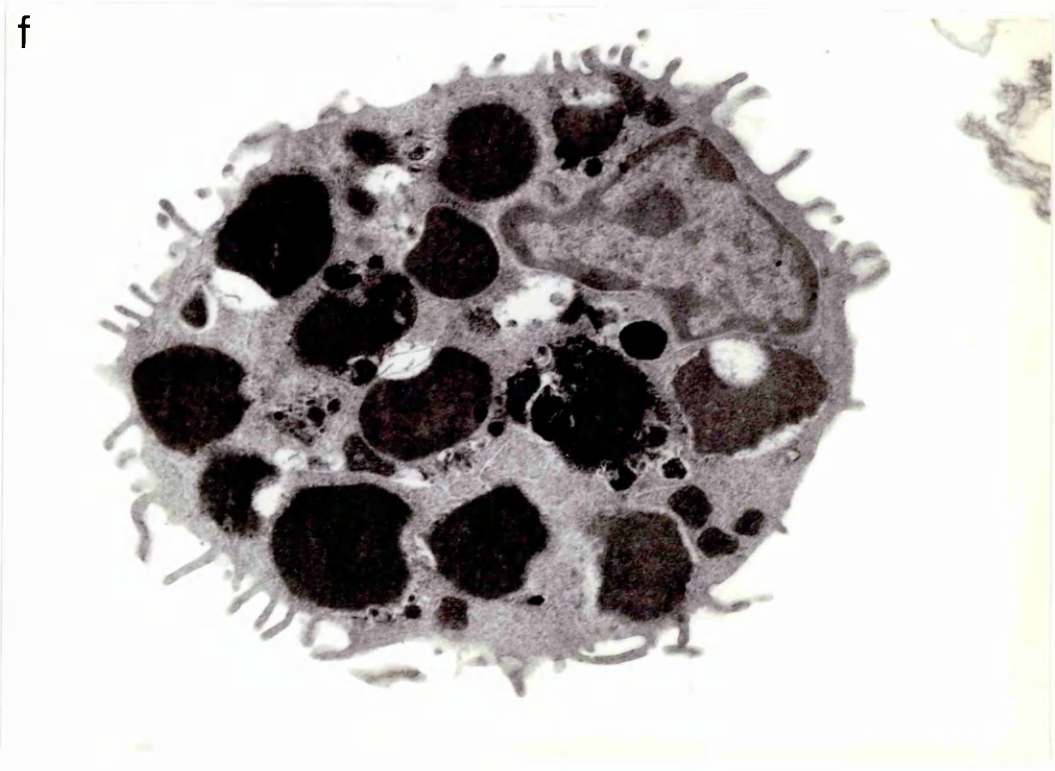


Figure 14f: Higher power of mast cell from day 24 culture, to show the pleomorphic nature of the granules (x12,000).

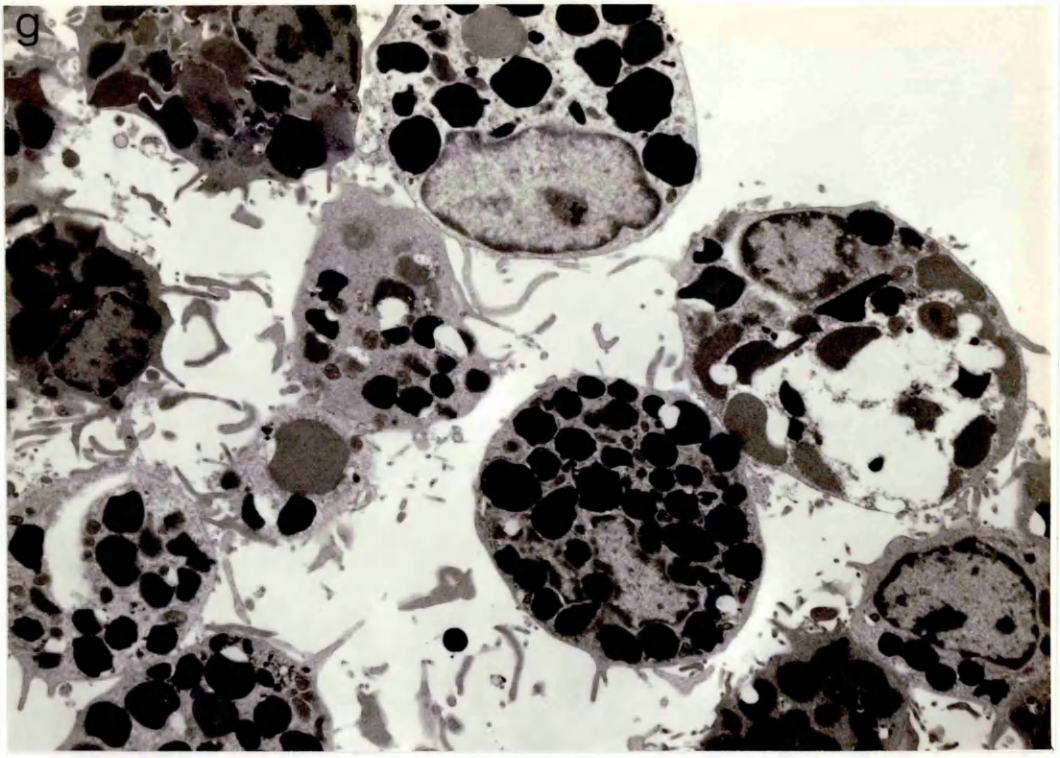


Figure 14g: Electron micrograph of day 28 cultured mast cells which are replete with large electron-dense granules (x4,200).

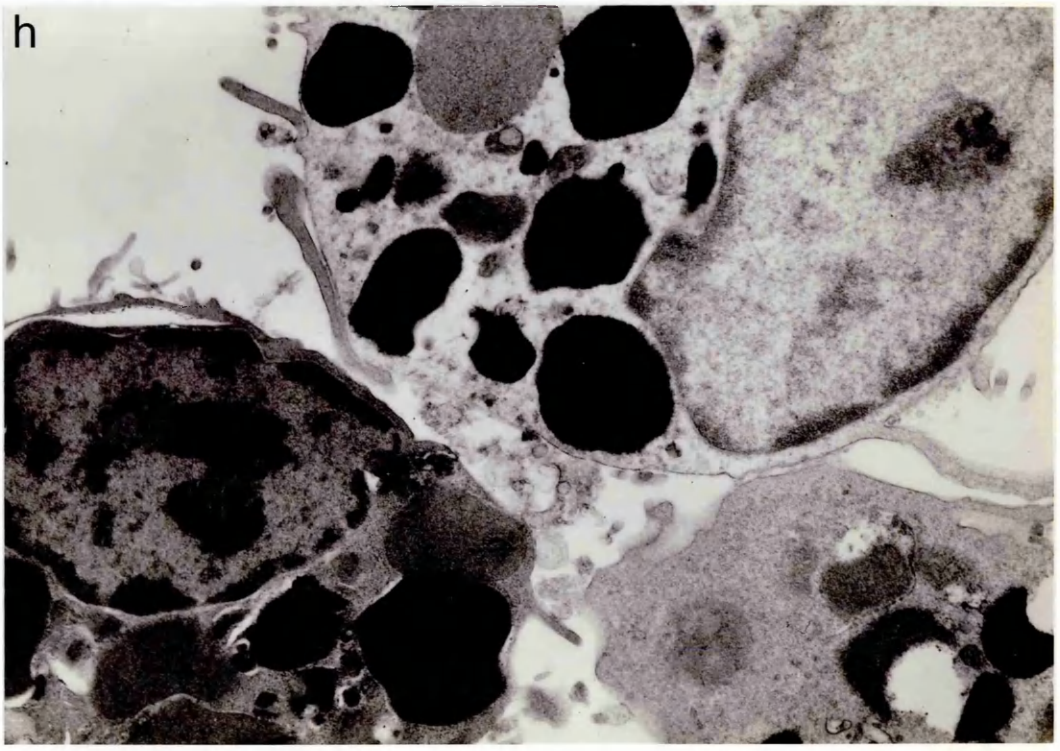


Figure 14h: Higher power of Fig.14g illustrating the range in dimensions and morphological appearance of the granules. Electron-lucent holes around some of the granules could possibly be artefactual. Other identifiable cytoplasmic organelles are infrequent (x12,000).

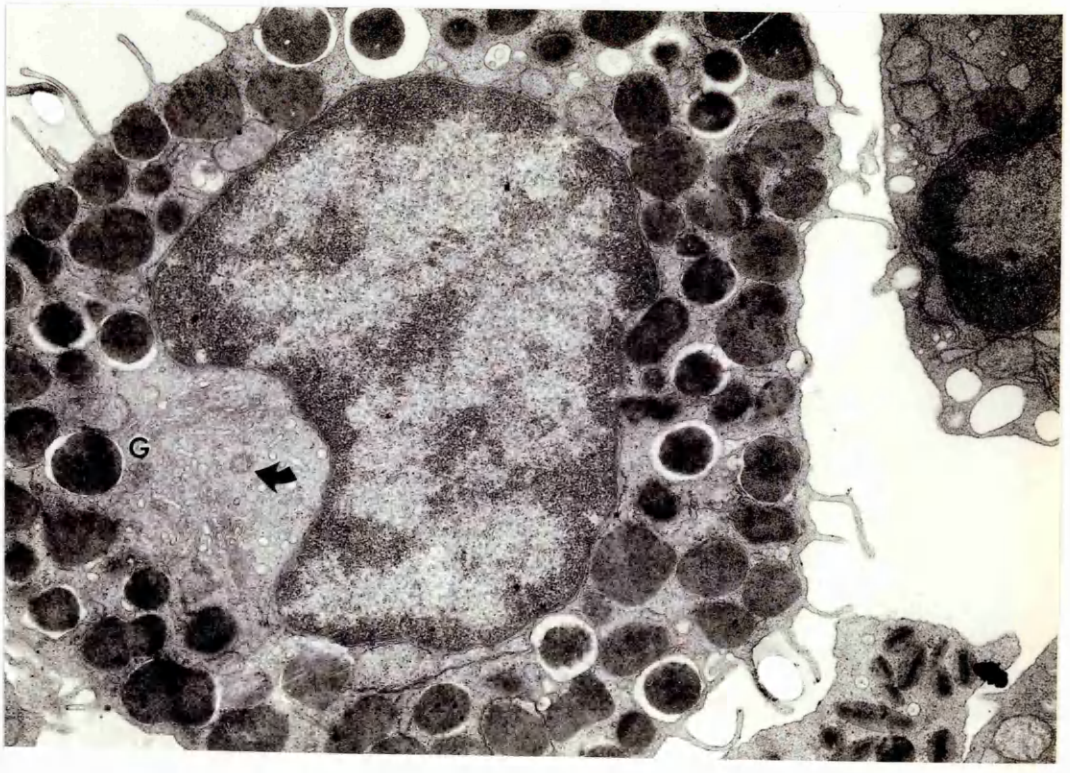


Figure 15: Electron micrograph of an isolated CTMC from the peritoneal cavity. Note the more uniform size and osmophilia of the granules which also have a more circular profile compared to the cultured mast cell. Note the prominant Golgi apparatus (G) and centriole (arrow). (x9,000).

stained with toluidine blue at pH 0.5, the granules showed metachromasia, this has been, historically, the distinguishing feature of the mast cell. Thus the cultured cells shared with the MMC and CTMC the basic staining characteristic of mast cells and indicated that they contained sulphated glycosaminoglycans (GAG) in the granules (Haig, 1982; Haig et al, 1982).

The special fixation techniques required to demonstrate MMC (Enerback, 1966a) were also found to be necessary for the demonstration of cultured mast cells throughout their period of development in vitro, with both toluidine blue staining and astra blue/safranin staining. It had been originally suggested that the failure of normal aldehyde fixatives to preserve the stainability of MMC granules was due to the extraction of a soluble GAG by the fixative rather than to a blocking of dye binding groups of the GAG by the aldehyde (Enerback, 1966a). Wingren and Enerback (1983) have found that dye is taken up by MMC in conventionally fixed tissues after staining for 5-7 days or after trypsinization of the tissue sections, thus confirming Miller and Walshaw's (1972) observation that aldehyde fixation of MMC granule protein presents a barrier to the diffusion of stain.

The cultured mast cell granules at all stages of development , when appropriately fixed, stained positively with astra blue at pH 0.3, the nuclei staining pink with safranin counterstain as did the mature in vitro-derived mast cells (Haig, 1982; Haig et al, 1982). CTMC granules, on the other hand, took up the dye safranin preferentially. This is in agreement with the studies of Enerback (1966b) who showed that MMC took up the blue stain exclusively whereas identically fixed and stained dermal mast cells showed a preferential

uptake of safranin: this distinction is based on granule contents of weakly and strongly sulphated mucopolysaccharides respectively. The indication from these findings that the proteoglycan of MMC has a different structure to that of CTMC was confirmed by Tas and Berndsen (1977) who used a microspectrophotometric technique to demonstrate an absence of significant amounts of heparin in MMC and the presence of a lower sulphated GAG. This GAG has been identified as an oversulphated galactosaminoglycan (Enerback et al, 1985). Preliminary studies have identified an oversulphated chondroitin sulphate as the major GAG present in the mature cultured mast cells (R. Stevens, personal communication) thus agreeing with the previous data of Enerback et al. Analysis of the cultured murine mast cells has shown that they also contain an oversulphated chondroitin sulphate as opposed to heparin (Razin, Stevens, Akiyama, Schmid and Austen, 1982). Rat peritoneal mast cells may show a mixed blue/red granulation with astra blue/safranin, reflecting mixed granule contents of weakly and strongly sulphated glycosaminoglycans, respectively. Maturation of rat peritoneal mast cells involves an ultrastructural reorganisation of the granule matrices. The process of granule formation reflecting the synthesis and accumulation of a heparin precursor in the astra blue positive granules followed by N-sulfation of these precursors associated with a shift to safranin staining (Combs, Lagunoff and Benditt, 1965). No equivalent granule reorganisation occurs during the progression of rat intestinal mast cells in vivo (Miller, 1980) or in vitro (Haig, 1982; Haig et al, 1982). The newly developing mast cells in culture continue to demonstrate astra blue staining granules throughout the period of culture. Using the criteria of proteoglycan

fixation and staining to distinguish the mast cell subtypes present in the rat has proven to be difficult as the CTMC displays a range of proteoglycan phenotypes (Combs, Lagunoff and Benditt, 1965). According to the aforementioned criteria, in vivo and in vitro-derived MMC show the staining characteristics of immature CTMC.

After 4 days of culture, the granulated cells which appeared in normal rat bone marrow cultures exhibited some of the ultrastructural characteristics previously attributed to MMC by in vivo studies (Murray, Miller and Jarrett, 1968; Miller, 1971a,b; Enerback and Lundin, 1974) as well as resembling MMC isolated from the intestine of the rat (Befus et al, 1982). The in vivo-derived MMC are smaller than the CTMC isolated from the peritoneal cavity and contain granules which are much more variable in size than those of CTMC, and although many granules are homogenously electron-dense, others show various degrees of electron-lucency. The nucleus of MMC in situ or isolated from the rat intestine is variable in shape, sometimes being oval, or it can be irregular with indentations. From as early as day 4 of culture, cells with electron-dense and electron-lucent granules were apparent. The granules displayed the marked variation seen in the in vivo-derived MMC with regard to size and shape. The nuclei of cultured MMC were generally ovoid or reniform and nucleoli were commonly present, a feature often seen in MMC of rat small intestine (Miller, 1971a). It has already been shown that MMC derived from 3 week old normal bone marrow cultures have the ultrastructural features of in vivo-derived MMC (Haig et al, 1982) and this is confirmed in the present study.

Mastoblasts were present in the rat intestine from day 10 after infection with Nb.. These cells had abundant cytoplasm in which

there were numerous ribosomal aggregates as well as a few profiles of rough endoplasmic reticulum. Small electron-dense granules were present in the area of the Golgi apparatus. It was suggested that these cells gave rise to MMC found in rat intestine (Miller, 1971a). It was observed that a few electron-dense granules were present in cells with a blast-like appearance by day 2 of normal bone marrow culture. These cells had abundant cytoplasm containing many free ribosomes and polyribosomes and a well developed Golgi apparatus. The nuclei of these cells was large and contained a prominent nucleolus. The cultured mast cells at this time therefore bore a resemblance to the "mastoblast" referred to by Miller (1971a). The pattern of maturation of the cultured mast cells whereby the granules eventually filled the cytoplasm at the expense of the other cytoplasmic organelles seemed to follow that described for both CTMC (Combs, 1966) and intestinal MMC (Miller, 1971a). The prominant Golgi apparatus and numerous fragments of rough endoplasmic reticulum which were seen in the early developing mast cells of all types being reduced by the formation of large and numerous granules.

It is not clear if the heterogeneity of the granule matrix observed during development of the cultured mast cells was due to the process of maturation of the granules or if discharge of the matrix was occurring. In CTMC progranules fuse to form dense aggregates which become precursors of the mast cell granule (Combs, 1966). However, no aggregates of this type were seen in the intestinal MMC (Miller, 1971a). Instead the majority of the granules in the maturing cell were large and had electron-dense matrices, although progranules were seen to fuse with large granules. Dense progranule-

like structures were evident within the borders of developing cultured mast cells (Figure 14d). Ultrastructural alterations of the granule matrices has been described during the discharge process of MMC (Murray, Miller and Jarrett, 1968). The intracellular discharge observed by Miller (1971b) where the perigranular membrane breakdown was followed by a gradual loss of matrix which became less dense and more granular, eventually disappearing, and leaving paracrystalline structures in empty vacuoles, was observed in cultured mast cells harvested throughout the period of culture. It is probable that both formation and discharge of granules is being observed in the cultured mast cells. The variable morphology of the granule matrix could be associated with the secretion of RMCPII into the culture supernatant (Chapter 6) as MMC can secrete granule bound substances without apparently degranulating. The possibility that the observations are artefactual due to the processing involved for electron microscopy cannout be ruled out.

There was difficulty identifying the cultured mast cells at early stages of development when few granules were present, and as well as this, the precursor cells from which these putative MMC developed were not readily identifiable. In order to identify these immature mast cells it is proposed to study them at both light and electron microscopic level using an immunogold technique. Monospecific $F(ab')_2$ antibodies raised against the specific MMC marker RMCPII (see Chapter 6) will be used to label the cells and this will be visualised using a gold conjugated anti-antibody. The localisation of RMCPII within the granules of the developing cultured mast cell would identify these cells categorically as MMC and provide a marker to investigate their development both *in vivo* and *in vitro*.

The newly developing mast cells in culture had the fixation and staining characteristics of the mature MMC cultured *in vitro* by Haig *et al* (1982) and bore the features of *in vivo*-derived MMC rather than CTMC. The culture system provides an opportunity to produce MMC in sufficient numbers to characterise them more fully.

Chapter 6

Analysis of mast cell granule proteinases in normal rat bone marrow cultures

Introduction

Cytochemical studies of mast cell glycosaminoglycans have detected two major subpopulations of mast cells in the rat: CTMC found in skin, muscle and peritoneal cavity, and MMC which predominate in the mucosa of the gastrointestinal tract (Enerback, 1981). MMC glycosaminoglycans cannot be detected in formalin-fixed tissues and this forms the basis for the cytochemical distinction between CTMC and MMC (Enerback, 1981; Newlands, Huntley and Miller, 1984). Clearly, neither of these techniques adequately defines mast cell heterogeneity. By determining the proteinase phenotype of the mast cell granules, an alternative approach to identifying mast cell subsets has been found. RMCPI has been shown to be predominant in CTMC by biochemical analysis, whereas RMCPII predominates in MMC (Woodbury et al, 1978b). Immunohistochemical staining of rat tissues has shown that RMCPII is present only in MMC and RMCPI in CTMC (Gibson and Miller, 1986).

The cultured mast cells described in Section 1 have been shown to exhibit the morphological and biochemical properties of MMC (Haig et al, 1982, Chapter 5). Further analysis of the mast cells grown in culture from normal rat bone marrow was undertaken to investigate their proteinase content and phenotype.

Experiment 12

Bulk cultures of normal rat bone marrow were prepared according to the Materials and Methods. A time course study on the appearance of RMCPII in cell extracts and culture supernatants was performed. Cells and supernatants were harvested on days 2, 4, 6, 8, 10, 14 and

30 of culture. Extracts of cells were prepared for RMCPII analysis by the 'capture' ELISA method according to the Materials and Methods.

Results

RMCPII was detected in the cultured cell extracts in increasing amounts from day 2 of culture onwards (Table 11). This increase was also reflected in the amount of RMCPII being secreted into the culture supernatant. Thus the RMCPII levels in cell extracts and supernatants paralleled the increase and decline of total mast cell numbers in the culture. The cell kinetics followed the same pattern as those shown previously (Section 1, Chapter 1) with the total cell and mast cell numbers increasing within 7 days and levelling off between days 10 and 14. After 3 weeks in culture the cells declined in number from the peak values of day 10, and stabilised at around 5×10^5 per ml. The RMCPII content of the cells increased as they became more granulated and more mature (Table 11).

Experiment 13

The phenotype of the cultured cells was characterised using monospecific $F(ab')_2$ antibodies which were employed to demonstrate, immunohistochemically, the presence of RMCPI and RMCPII. For this purpose cells were harvested from bulk bone marrow cultures on days 7, 16 and 30 of culture. Total cell counts were performed and cytospin preparations were made. The slides were then fixed in methanol, Carnoy's or 4% paraformaldehyde, and stored at -20°C. Peritoneal exudate cells were obtained by peritoneal lavage of F344 rats (a pool of 3 animals was used per experiment). A total cell count was

Table 11: Time course of RMCPII content and secretion by cultured mast cells

Day of culture	Total cells $x10^{-4}$/ml	Mast cells $x10^{-4}$/ml	(% total culture)	RMCPII content cell extract (pg/cell)	RMCPII content supernatant (µg/ml)
0	25	0.2	(0.9)*	<1	<0.01
2	32	7.0	(22.0)	7	0.30
4	79	36.3	(46.0)	15	1.50
6	92	43.7	(47.5)	22	2.20
8	135	68.5	(50.8)	28	2.51
10	137	77.1	(56.3)	32	3.10
14	121	80.0	(66.1)	35	3.15
30	51	50.1	(98.2)	42	2.56

* Cells with staining characteristics of CTMC

performed and cytospin preparations were made and fixed as for the cultured cells.

RMCPI and RMCPII were demonstrated in the cell preparations using affinity purified rabbit $F(ab')_2$ anti-RMCPI and anti-RMCPII which had been rendered monospecific for each enzyme by affinity chromatography and cross absorption (Gibson and Miller, 1986). Development was accomplished using sheep Fab anti-rabbit $F(ab')_2$-peroxidase conjugate and 3-amino-9-ethyl carbazole as substrate (Newlands, Huntley and Miller, 1984) (for details, see Materials and Methods). The aforementioned procedure was carried out by Steve Gibson and George Newlands (Moredun Institute, Edinburgh) to whom I am most grateful. Toluidine blue (pH 0.5) and Leishman staining were also performed on the cytospin preparations. The numbers of mast cells stained with toluidine blue/Leishman were counted. Comparable slides were then assessed for numbers of mast cells stained with anti-RMCPI and anti-RMCPII after the immunoperoxidase reaction. Five fields of view were counted per slide with at least 100 cells counted per field. The results are expressed as a percentage of total cells counted per slide.

Results

On day 0 when the bone marrow was plated, cells containing RMCPII were not present and those few mast cells (1.8% of total) which stained with toluidine blue had the histological appearance of CTMC and contained RMCPI exclusively (Table 12). By day 7, cells containing RMCPII made up almost half of the total culture with a small percentage of anti-RMCPI stained cells still remaining

Table 12: Comparison of mast cell subsets stained with toluidine blue/Leishman, anti-RMCPI or anti-RMCPII in peritoneal exudate cells (PEC) and cultured normal bone marrow (NBM)

Day of culture	Cell Type	% cells stained Tol. blue/Leishman	% cells stained anti-RMCPI	% cells stained anti-RMCPII
NA	PEC	11.6	10.8	0
0	NBM	1.8	1.6	0
NA	PEC	9.6	7.7	0
7	NBM	48.7	1.6	44.4
NA	PEC	10.2	9.3	0
16	NBM	88.7	0	88.0
NA	PEC	13.9	15.6	0
30	NBM	94.1	0	93.0

NA Not applicable

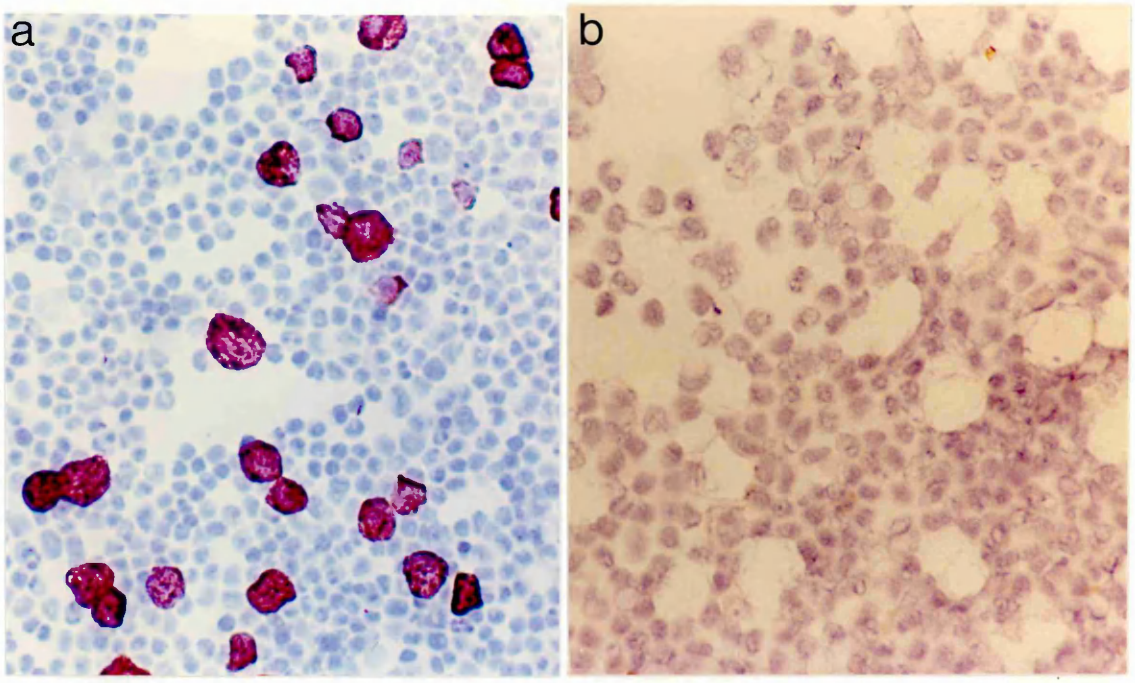


Figure 16a & b: Peritoneal exudate cells stained by an immunoperoxidase method utilising monospecific rabbit $F(ab')_2$ anti-RMCPI or II. a). anti-RMCPI (blue filter, x320); b). anti-RMCPII (x500). The CTMC stained exclusively with the anti-RMCPI antibodies.

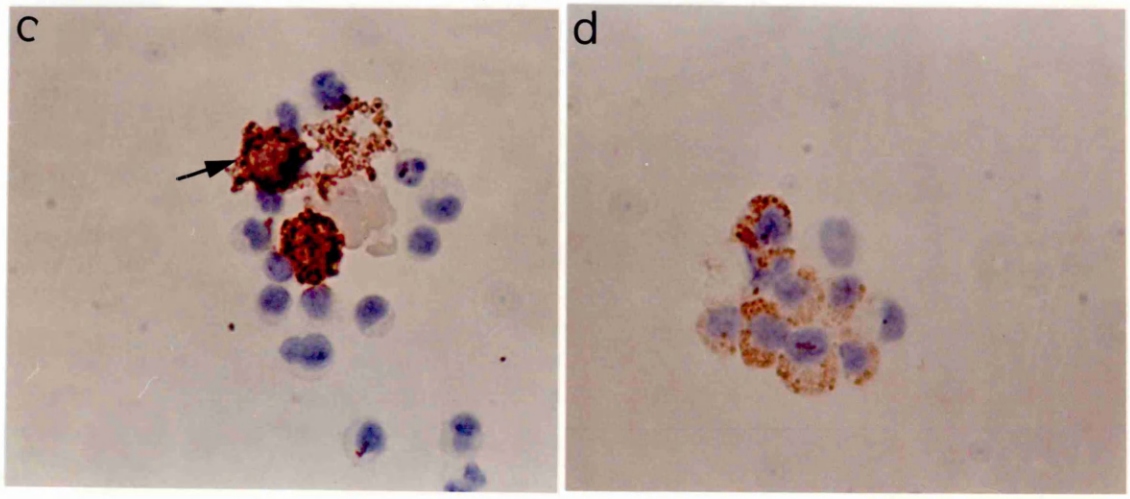


Figure 16 c & d: Normal bone marrow cells, day 7 after plating, stained as above. c). anti-RMCPI (x500), these are residual CTMC (arrows). d). anti-RMCPII (x500), cultured mast cells stained exclusively by these antibodies.

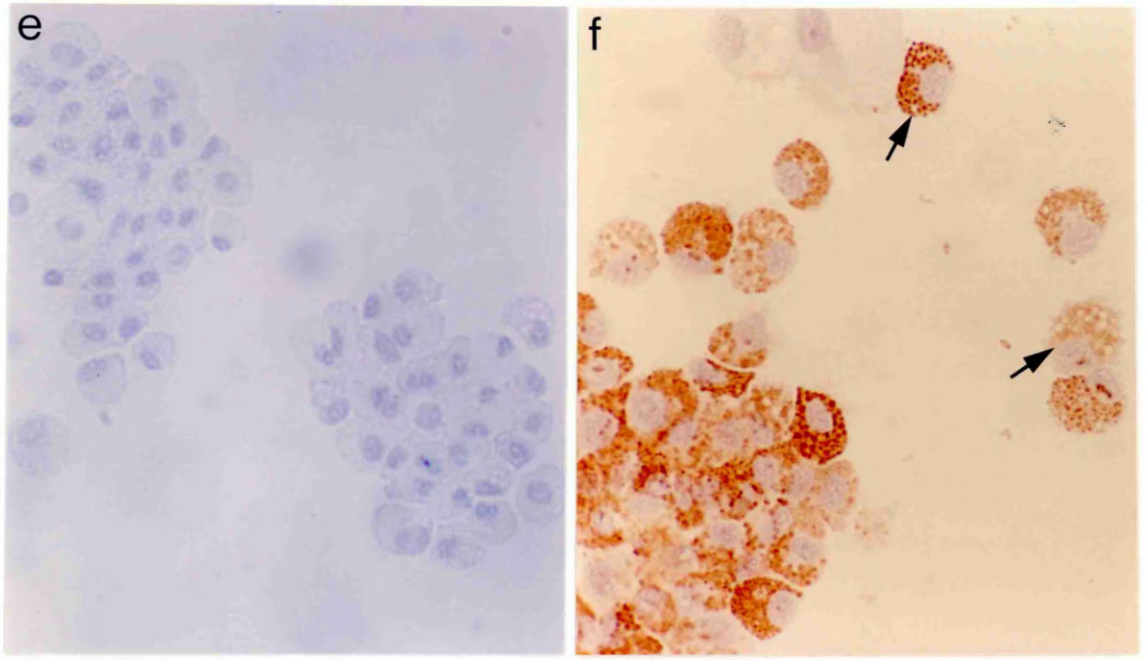


Figure 16e & f: Normal bone marrow cells, day 16 after plating, stained as in Fig.16a-d. e). anti-RMCPI (blue filter, x320), no cells containing RMCPI remained in culture by this time. f). anti-RMCPII (x500), >88% of cultured cells contained RMCPII. Note the varying degrees of staining within the population (arrows).

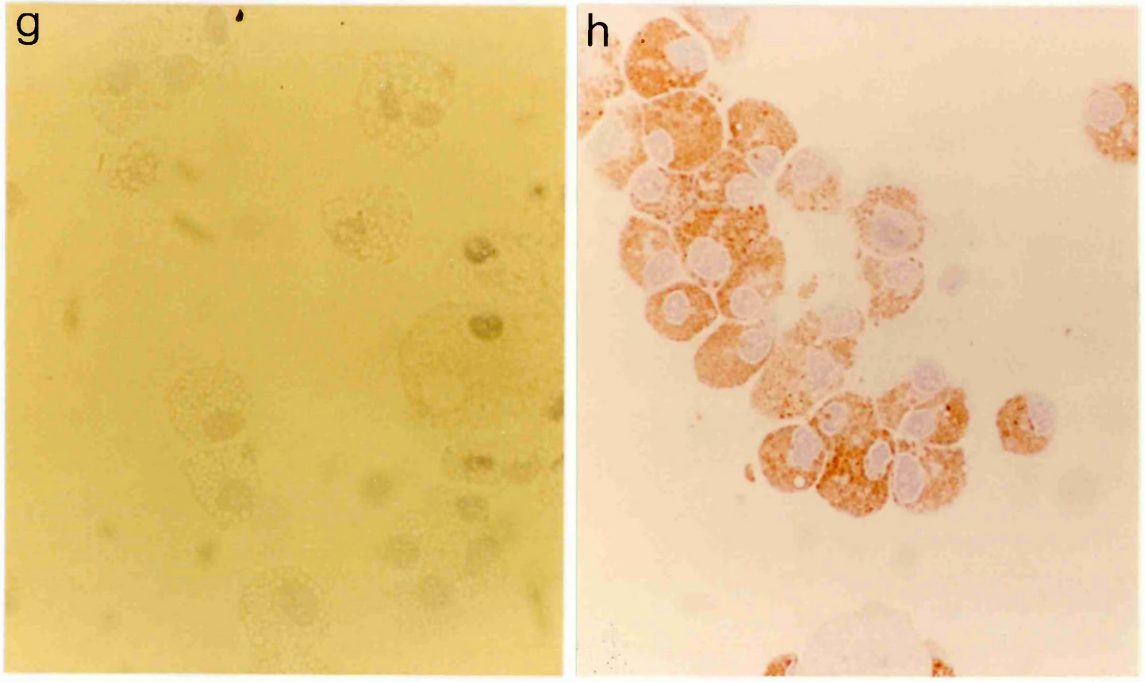


Figure 16g & h: Normal bone marrow cells, day 30 after plating, stained as above. g). anti-RMCPI (x500), there were no remaining RMCPI containing cells at this stage of culture. h). anti-RMCPII (x500), >90% of cells in the culture stained with this antibody but again to varying degrees.

(Table 12, Figures 16c & d). The latter were residual CTMC present in the normal bone marrow but these had virtually disappeared within 7-10 days in culture. Both the CTMC and cultured mast cells could be stained with the antibodies after fixation in all three fixatives tried, but methanol preserved the structure of the cells better than the Carnoy's fixative or paraformaldehyde. By day 16 of culture, RMCPII-containing cells made up >88% of the culture and no RMCPI positive staining cells remained (Figures 16e & f). The only remaining contaminating cells were macrophages. Cells stained by anti-RMCPII antibodies made up >90% of the culture by day 30 and, as with the cells taken from day 16 cultures, displayed varying degrees of staining (Figures 16g & h).

The connective tissue mast cells in peritoneal exudates stained with anti-RMCPI and this was used as the control (Figures 16a & b). The CTMC present in the day 7 culture were, like peritoneal mast cells, typically large cells densely packed with uniform granules. By comparison, the granules of the RMCPII-containing cells at day 7, 16 and 30 of culture were sparser and varied in size (Figure 16).

Discussion

A mast cell protease which showed chymotrypsin-like activity was first characterized by Benditt and Arase (1959). Subsequently a mast cell enzyme which behaved similarly to trypsin was described (Glenner and Cohen, 1960). However, most interesting was the discovery that an intracellular serine protease isolated from rat small intestine differed from a similar enzyme isolated from skeletal muscle or liver (Katanuma, Kominami, Kobayashi, Banno, Suzuki, Chichibu, Hamaguchi and

Katsunuma, 1975). Woodbury and colleagues have demonstrated these variant serine proteases in the two types of rat mast cells (Woodbury, Gruzenski and Lagunoff, 1978b). RMCPI, originally isolated from skeletal muscle as mentioned above being identical to that subsequently isolated from peritoneal mast cells, and RMCPII, differing in solubility, structure and antigenicity from RMCPI, was localised to mast cells in the small intestine. The protease RMCPII appears to be the real hallmark of the MMC subtype in the rat and is contained in substantial amounts in the cultured mast cells. It is also secreted by them, without apparent degranulation, into the culture fluid at detectable levels by day 2 of culture and in μg quantities/ml within 1 week of culture. This finding supports the proposal (Woodbury et al, 1984) that MMC have a secretory as well as a degranulatory function. Exactly what RMCPII is doing being produced and secreted in such quantities is not yet known but it has been shown to catabolize type IV collagen of basement membrane (Sage, Woodbury and Bornstein, 1979). Miller and colleagues have confirmed this (Miller, King, Gibson, Huntley, Newlands and Woodbury, 1986) and have presented evidence to suggest that RMCPII may be involved in the generation of mucosal epithelial permeability (King and Miller, 1984). As far as their involvement in the elimination of gut dwelling parasites is concerned, MMC may play a role by causing the enteric mucosal epithelium to be sloughed off and so exclude the parasites which live on or penetrate the epithelium.

Despite the 75% sequence identity in their 52 amino terminal residues, cross-reactivity between the enzymes RMCPI and II and their respective antibodies could not be detected on gel diffusion (Woodbury et al, 1978b) nor by ELISA (Miller et al, personal communication).

However, a strong cross-reactivity was demonstrated when the enzymes were separated on SDS-PAGE under reducing conditions and transferred to nitrocellulose, and then probed with the same antibodies (Gibson and Miller, 1986). Affinity absorption procedures eliminated all cross-reactivity detectable by Western blot analysis. A definitive pattern of immunohistochemical localisation of each enzyme was revealed with RMCPII present only in the mast cells of the intestinal mucosa, and tracheal and bronchial epithelium. Mast cells in skin, ear pinna, tongue, tracheal submucosa and lung parenchyma contained uniquely RMCPI.

Using previously available criteria to distinguish MMC from CTMC (Enerback, 1981; Jarrett and Haig, 1984) it appeared that the mast cells growing in culture were of the mucosal type. However, none of these criteria on its own was sufficient to identify the cells as MMC with any degree of certainty. Having shown biochemically that the cultured mast cells contain and secrete RMCPII, it has been demonstrated immunohistochemically using monospecific $F(ab')_2$ antibodies that the cells which grew and differentiated in the normal bone marrow cultures were exclusively stained by anti-RMCPII, and RMCPI was present only in cells which by previous histochemical criteria had been defined as CTMC i.e. the peritoneal exudate cells and the mast cells present in normal bone marrow before culture.

Thus from the results detailed in this chapter it can be stated that the mast cells grown in culture from normal rat bone marrow stimulated by factors present in conditioned medium made from mesenteric lymph node cells from Nb. infected rats are *in vitro* analogues of the cells identified *in vivo* by comparable technique (Gibson and Miller, 1986) as mucosal mast cells.

General Discussion

In this thesis a rat bone marrow culture system has been described, whereby normal bone marrow cells were cultured *in vitro* in the presence of conditioned medium to yield an almost pure population of mucosal mast cells, and this system provided an opportunity to look at these cells in detail and investigate the factors influencing their growth and differentiation.

Numerous reports have described the growth of mast cells in mouse haemopoietic tissue cultures stimulated with mitogen-activated splenocyte CM (Hasthorpe, 1980; Nabel *et al*, 1981; Razin, Cordon-Cardo and Good, 1981; Schrader *et al*, 1981; Tertian *et al*, 1981). Similarly it was shown that the cultured MMC in the rat are dependent on a factor produced by antigen or concanavalin-A stimulated T cells present in MLN taken from *Nb.*-infected rats (Haig *et al*, 1982;1983: Chapters 1 and 2).

It is clear from experiments *in vivo* that MMC precursors are strongly T cell-dependent for their hyperplasia in immune reactions and especially in helminth infection (Mayrhofer, 1979; Nawa and Miller, 1979). *In vitro* experiments involving nude rats show why this is the case (Haig *et al*, 1983; Chapter 2). CM made from rnu/rnu MLN does not contain growth stimulatory activity for MMC in normal or athymic bone marrow cultures. On the other hand, CM from rnu/+ or control (LIS x BN)F_1 IMLN stimulates total and mast cell growth in both rnu/+ and rnu/rnu bone marrow cultures. In conjunction with the information that the rnu/rnu genotype is associated with congenital aplasia of the thymus and a gross deficiency of mature T cells (Festing *et al*, 1978; Brooks *et al*, 1980), this experiment says two things: that MLN from rnu/rnu rats, having insufficient T cells, simply does not produce MMC growth factor but that a population of

bone marrow cells from these rats is capable of responding to the factor(s) if it is presented to them. Subsequently, the cell surface phenotype of the T cell responsible for producing the MMC growth factor(s) was established and the cells found to belong to the helper/inducer subset (McMenamin, Jarrett and Sanderson, 1985; Chapter 3). This agreed with Nabel et al (1981) who found that murine mast cell growth factor activity was a product of mitogen or antigen-stimulated $Thy1^+$, $Lyt1^+,2^-$ T cells, these also being of the helper/inducer subset.

Bone marrow-derived murine mast cells proliferate in the presence of the haemopoietic growth factor IL-3 (Ihle, Pepersack and Rebar, 1981) which has been purified to homogeneity (Ihle et al, 1982; Bazill et al, 1983; Clark-Lewis, Kent and Schrader, 1984). The cDNA for IL-3 has been cloned (Fung et al, 1984; Yokota et al, 1984) and the pure and expressed product shown to support the proliferation of not only T-dependent mast cells but also multipotential haemopoietic progenitor cells (Ihle et al, 1983; Hapel et al, 1985). More recently, murine mast cell growth stimulatory activity distinct from IL-3 has been identified and partially purified from the supernatant of the activated T-cell line $Cl.Ly1^+2^-/9$ (Lee et al, 1986). This mast cell growth factor supports only low levels of proliferation of several IL-3 dependent mast cell lines and synergistically enhances the growth of mast cells in the presence of IL-3. In addition, this clone produces B and T cell growth factor activity. The lymphokine with these activities has been collectively called IL-4 (Lee et al, 1986).

Fractionation of whole CM derived from mitogen-stimulated IMLN has revealed IL-3-like activity and this is based on several

observations: firstly, rat CM stimulates the incorporation of ^{3}H-Thymidine into the IL-3-dependent murine cell line, AC-2; secondly, both the AC-2 cell line growth supporting activity and MMC growth activity in CM are heat stable and elute from a DEAE sepharose column in the 5-10 mM buffer pH 8.3 breakthrough fraction (D.M. Haig, personal communication). Murine IL-3 behaves in a similar fashion after such treatment, as does the pure rat IL-3 obtained by the cloning of the rat IL-3 gene and expressed in non-human primate cells (Cohen, Hapel and Young, 1986). The "IL-3 enriched" fraction of rat CM, as well as retaining MMC growth factor activity, also supported the growth of multipotential colonies which is a typical property of IL-3.

In addition, a further fraction of rat CM was obtained which was capable of stimulating mature cultured MMC and maintaining the IL-3 dependent cell line AC-2 and was separate and different from the IL-3 activity (D.M. Haig, personal communication). This is interesting in the light of the findings in the murine system, where additional mast cell/T cell growth factor activity has been identified (Lee et al, 1986; Smith and Rennick, 1986). Having shown that T cells can regulate the growth of mast cells through the production of IL-3 (Clark-Lewis and Schrader, 1981; Nabel et al, 1981; Ihle, Keller, Henderson, Frederick and Palasyznski, 1982), it seems that mast cells and T cells are linked in a complex immunoregulatory circuit. The activities of the IL-4 lymphokine, as designated by Lee et al (1986), adds another dimension to the circuit since the latter may coordinately regulate aspects of mast cell and T cell growth and differentiation. It seems, therefore, that more than one factor is involved in the growth and differentiation of mast cells. Future

experiments utilizing the culture system described in this thesis are planned to test the pure rat IL-3 obtained by expression of the cloned gene product to see if it has the ability to initiate and maintain the growth of MMC from normal rat bone marrow in a similar fashion to the CM produced by the mitogenic stimulation of MLN cells from Nb.-infected rats. The fractions obtained from the partial purification of whole CM will also be tested.

Whilst working with in vitro systems it must always be borne in mind how the results compare with those obtained in vivo. Evidence that IL-3 is released from activated T cells during immunological reactions in vivo is indirect, and has been based upon the observation of localised increases in numbers of mast cells and IL-3 from cells of the ipsilateral draining lymph node of mice injected in one footpad with KLH, but not from cells of the untreated contralateral node. Increases in IL-3 secreting T cells and MMC precursors were detected in draining but not distal lymph nodes following antigen stimulation of mice (Crapper, Clark-Lewis and Schrader, 1984) or parasitic infection of rats (Haig, Jarrett and Tas, 1984) although it is not certain this is the only factor active in the latter situation. However mice injected subcutaneously with the WEHI-3b IL-3 secreting tumour developed detectable circulating levels of IL-3 and this was associated with an increase in MMC precursors, megakaryocytes, and neutrophils in distal organs (Crapper, Clark-Lewis and Schrader, 1984; Crapper, Thomas and Schrader, 1984; Guy-Grand, Dy, Luffau and Vassalli, 1984). The experiments involving localised immune reactions of moderate intensity provided evidence that the effects of IL-3 were confined to the immediate vicinity of the activated T cells (Crapper,

Clark-Lewis and Schrader, 1984; Haig, Jarrett and Tas, 1984). However, during Nb. infection of mice, IL-3 activity was transiently observed in the serum (Filho, Dy, Lebel, Luffau and Hamburger, 1983). This particular parasitic infection may have provided a stronger stimulus for IL-3 production, similarly induction of Graft-versus-host disease in mice induced vigorous and widespread activation of donor T cells reflected by the presence of IL-3 in the serum (Crapper and Schrader, 1986).

A general scheme has been proposed for MMC development in vivo (Haig, 1982; Crapper and Schrader, 1983; Guy-Grand et al, 1984; Haig, Jarrett and Tas, 1984). In rats and mice, MMC precursors in a morphologically unrecognisable form migrate via the blood to the tissues, principally the small intestine. These then differentiate into granulated MMC under as yet undefined local tissue influences. Since comparable numbers of MMC develop in T cell-depleted or athymic animals, and in normal animals (Ruitenberg and Elgersma, 1976; Mayrhofer, 1979), T cell derived IL-3 may not be responsible for MMC recruitment under normal conditions. However, in athymic animals the possibility of activity from other cell types or from a few residual T cells cannot be ruled out. Following helminth parasite infection, there is a marked increase in both the number of T cells producing IL-3 and MMC appearing in the small intestine and local lymph nodes (Guy-Grand et al, 1984; Haig, Jarrett and Tas, 1984) implicating IL-3 as the mediator of MMC hyperplasia. The IL-3 activity detected in the sera of Nb.-infected mice supports the biological importance of this molecule in helminth infection (Filho et al, 1983).

IL-3 acts on stem cells to expand populations of committed progenitors for eosinophil, megakaryocyte, neutrophil, macrophage and

erythroid lineages in culture (Prystowsky, Otten, Naujokas, Vardiman, Ihle, Goldwasser and Fitch, 1984; Hapel et al, 1985; Rennick et al, 1985). Following helminth infection, an in vivo increase in MMC is almost invariably associated with recruitment of eosinophils, neutrophils, monocytes and lymphocytes to the parasitized tissue (Miller, 1984). All of these cell types have potential helminthicidal activities (Ogilvie, Love, Jarra and Brown, 1977; Capron, Dessaint, Haque and Capron, 1982) that might be regulated by MMC either by the elaboration of chemotactic factors (Wasserman, 1983) and/or by allowing the cells access to parasites via an IgE-mediated local immediate hypersensitivity reaction (Urquhart, Mulligan, Eadie and Jennings, 1965). Studies on T cell products including IL-3 generated during helminth infection has led to the conclusion that one of their principle actions is to stimulate production of MMC. Further investigations are required to reveal at which level of haemopoiesis these factors act to expand the pool of precursor cells involved in the local reaction.

It was observed that production of MMC growth factors was suppressed when IMLN cells were cultured in vitro in the presence of dexamethasone during CM production (Chapter 4). It had already been shown in vivo that mast cell hyperplasia was suppressed by the pretreatment of these animals with cortisone (Jarrett et al, 1967). A possible explanation for this could be that the treatment of these animals with glucocorticoids caused the factor production to be suppressed to such a low level that the population of MMC or their precursors could not be sustained or stimulated and maintained. Since the MMC growth stimulatory activity in the rat has been shown to

have certain properties in common with IL-3 (D.M. Haig, personal communication), these results lend further support to the evidence that IL-3 is one of the mediators of mast cell hyperplasia. Glucocorticoids also act on the MMC themselves inhibiting the normal pattern of granule formation (Chapter 4). These observations go some way to providing an explanation for the effects of glucocorticoids on MMC *in vivo*.

A particularly important point to consider in a study of mast cell differentiation is the relationship between CTMC and MMC. Although both CTMC and MMC are derived from precursors that reside in the bone marrow (Hatanaka, Kitamura and Nishimune, 1979; Kitamura, Matsuda and Hatanaka, 1979; Kitamura, Yokoyama, Matsuda, Ohno and Mori, 1981; Sonoda, Ohno and Kitamura, 1982; Crowle and Reed, 1984) it is not clear whether CTMC and MMC are the products of separate mast cell lineages or represent different stages in a single pathway of mast cell differentiation. To address this question, Nakano *et al* (1985) injected cultured murine mast cells, which expressed some phenotypic similarities to MMC, into different sites of mast cell deficient mice $WBB6F_1$-W/W^V and observed that connective tissues were subsequently populated by CTMC-like safranin positive cells of donor origin whereas the gastrointestinal mucosa contained alcian blue positive cells. It seemed, therefore, that cultured cells with the apparent phenotype of MMC were capable of giving rise to either CTMC or MMC depending on the *in vivo* microenvironment. However, since these cells were not cloned, there was the possibility that they contained two distinct sets of mast cell precursors and since the cultures were derived from bone marrow, one would expect them to contain haemopoietic stem cells capable of generating CTMC and MMC

populations. Taking these issues into consideration, further studies were done where mast cell cultures were isolated in methyl cellulose, clones of cells were divided and injected into both the skin and stomach of individual mast cell-deficient $WBB6F_1$-W/W^V mice. Mast cells arose in these tissues which seemed to have the features of CTMC and MMC depending on the anatomical location (Kobayashi, Nakano, Nakahata, Asai, Yagi, Tsuji, Komiyama, Akabane, Kojima and Kitamura, 1986). This work highlights some of the problems associated with the study of mast cell heterogeneity. Firstly, no definitive markers to demonstrate mast cell heterogeneity exist in the mouse, and the use of just one parameter, that of proteoglycan content, based on histological staining techniques, is not adequate. Secondly, and related to the above, is the fact that although cultured murine mast cells are compellingly similar to the alcian blue staining gastrointestinal population of mast cells which, in turn, may be analogues of rat MMC, this has not been proven.

From the experiments described above, the importance of the influence of the microenvironment on mast cell phenotype has been stressed. Further experiments to investigate this have been done *in vitro* (Levi-Schaffer, Austen, Caulfield, Hein, Bloes and Stevens, 1985; Levi-Schaffer, Austen and Stevens, 1986). It was discovered that isolated rat CTMC would survive *ex vivo* for at least 30 days if they were co-cultured on monolayers of living mouse fibroblasts, but would die within 2 days if they were cultured alone or co-cultured with macrophages (Levi-Schaffer *et al*, 1985). It was concluded that living fibroblasts were required for survival of the *in vivo* differentiated CTMC as neither fibroblast CM nor the fibroblast matrix

devoid of living fibroblasts supported the viability of this mast cell subtype. Effects of fibroblast co-culture on mouse bone marrow-derived mast cells has been investigated (Levi-Schaffer, Austen and Stevens, 1986). Cultured murine mast cells were cultured for up to 14 days on a fibroblast layer. Although all of the granules of the starting cultured mast cells were, like MMC *in vivo*, alcian blue$^+$/safranin$^-$, almost all of the mast cells were observed to possess some granules which were safranin$^+$ after 6 days in co-culture. After 10-14 days in co-culture, all the mast cells had granules both alcian blue$^+$/safranin$^+$. The fibroblasts apparently induced a change in phenotype of the mast cell from MMC to CTMC.

The histological techniques based on the detection of glycosaminoglycans cannot be relied upon to give a true picture of mast cell heterogeneity. Since several genes are likely to regulate the synthesis of the highly complex proteoglycans in mast cell granules, and because local environmental factors seem to influence post-translational events in the synthesis and assembly of proteoglycans, histochemical analysis of mast cell granule glycosaminoglycans, however sophisticated, is likely to produce dubious phenotypic identification of mast cells. In the rat, an alternative approach is to determine the distribution of other major granule products, the chymotrypsin-like enzymes RMCPI and RMCPII. When monospecific anti-RMCPI and anti-RMCPII were applied in an immunohistochemical study of mast cell proteinases in the duodenum and respiratory tract, RMCPI and II were apparently located in different mast cell subsets (Gibson and Miller, 1986). The present study has shown the immature and mature mast cells grown in culture from normal rat bone marrow under the influence of CM contain RMCPII exclusively

in their granules (McMenamin, Haig, Gibson, Newlands and Miller, 1986; Chapter 6). In order to determine if the apparent in vitro transformation of cultured murine mast cells to the CTMC phenotype also occurs in the rat, the bone marrow-derived rat MMC could be cultured on rat skin fibroblast layers and stained both with the monospecific antibodies raised against the proteinases, and histochemically to characterise granule glycosaminoglycans.

The questions posed with regard to the developmental and functional relationship of the different mast cell types have not been answered. The production of monoclonal antibodies against specific determinants on both MMC and CTMC, in conjunction with the markers provided by the specific protease and proteoglycan, histochemistry would be invaluable in studying the development of the mast cell lineage. Identification of MMC precursors by a membrane determinant would allow separation of these cells which could then be labelled for in vivo studies of their migratory properties. It is hoped to exploit the culture system described in this thesis to further investigate the many unanswered questions relating to rat mast cell heterogeneity.

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Appendices

Appendix 1 (Figure 1)

Tabulated is the raw data used to generate Fig.1, hereafter only the mean of triplicate values are given as the individual results obtained in this experiment were typical of the spread of values one routinely observed.

Figure 1(a): Total cell and mast cell growth in normal bone marrow cultures stimulated with CM from NMLN stimulated with various concentrations of Con-A

CM	Day Counted	Total Cells $x10^{-4}$ (Mean)	Actual Mast Cell/ Total Cell Counts	Mast Cells %	Mast Cells $x10^{-4}$ (Mean)
IMLN + nt. allergen	D4	46	132/602	22	10.12
	"	47 (47)	154/740	21	9.72 (10.15)
	"	48	162/744	22	10.45
	D7	65	132/664	20	13.00
	"	80 (74)	232/832	28	22.40 (18.83)
	"	77	220/801	27	21.10
NMLN + 2µg/ml Con-A	D4	19	104/640	16	3.09
	"	25 (22)	120/634	19	3.16 (3.57)
	"	22	116/636	18	4.01
	D7	25	124/640	19	4.84
	"	29 (27)	142/622	23	5.75 (5.53)
	"	27	138/620	22	6.01
NMLN + 5µg/ml Con-A	D4	28	136/800	17	4.76
	"	36 (32)	68/612	11	3.96 (4.57)
	"	32	116/744	16	4.99
	D7	58	200/672	30	17.40
	"	54 (55)	180/616	30	16.20 (16.87)
	"	55	202/654	31	17.00
NMLN + 10µg/ml Con-A	D4	39	112/690	16	6.32
	"	26 (32)	118/692	17	4.43 (5.19)
	"	30	114/710	16	4.82
	D7	26	160/544	29	7.54
	"	29 (28)	144/596	24	7.01 (7.33)
	"	28	150/564	27	7.45
Medium alone	D4	2			
	"	2			
	"	3	Too few cells to evaluate		
	D7	3			
	"	3			
	"	2			

Appendix 2 (Figure 2)

Total cell and mast cell growth in normal bone marrow stimulated with conditioned media from IMLN stimulated with various concentrations of Con-A.

CM	Day Counted	Total Cells $x10^{-4}$ Experiment a	b	c	Mast Cells % Experiment a	b	c	Mast Cells $x10^{-4}$ Experiment a	b	c
IMLN + nt. allergen	D4	47	46	46	22	23	23	10.15	10.37	10.75
	D7	74	74	71	25	26	27	18.83	19.45	19.23
IMLN + 2 µg/ml Con-A	D4	54	54	58	27	27	28	14.48	15.76	15.89
	D7	84	81	85	48	49	50	40.13	39.83	42.77
IMLN + 5 µg/ml Con-A	D4	34	35	37	15	16	15	5.13	5.52	5.67
	D7	62	62	65	27	27	28	16.79	16.63	18.47
IMLN + 10 µg/ml Con-A	D4	23	24	24	17	17	17	3.83	4.03	4.17
	D7	27	28	28	27	26	26	7.32	7.34	7.40
Medium alone	D4	3	3							
	D7	3	3					Too few cells to evaluate		

Appendix 3 (Table 1)

Repeat experiment of Table 1.

Total cell and mast cell growth in bone marrow cultures stimulated with CM from Con-A activated normal or immune MLN cells.

Source of CM	[^{3}H]dTdh incorporation in MLN cultures cpm x 10^{-3}	Bone marrow cultures Total cells no. x 10^{-4}	Mast cells no. x 10^{-4}
Normal MLN			
+ 2 µg/ml Con-A	21.3	31	6.1
+ 5 µg/ml Con-A	10.9	39	15.2
Unstimulated	0.6	4	0
Immune MLN			
+ 2 µg/ml Con-A	15.8	59	33.7
+ 5 µg/ml Con-A	51.3	47	16.3
Unstimulated	3.7	4	0

Appendix 4 (Figure 3)

Total cell and mast cell growth in normal bone marrow cultures stimulated with CM throughout culture, after washing, and pulsed for 24hr and 48hr.

Duration of CM treatment	Day Harvested	Total Cells $x10^{-4}$ Experiment			Mast Cells % Experiment			Mast Cells $x10^{-4}$ Experiment		
		a	b	c	a	b	c	a	b	c
Total time of culture	D4	41	40	42	17.6	18.3	17.2	7.2	7.3	7.3
	D7	81	82	80	24.2	25.1	26.0	19.5	20.6	20.7
Before and after washing	D4	38	37	37	17.9	17.5	17.7	6.8	6.5	6.5
	D7	83	82	82	21.2	21.7	22.3	17.6	17.8	18.3
24hr pulse	D4	7	8	8	3.6	3.5	4.5	0.3	0.3	0.4
	D7	11	11	10	21.8	22.1	20.5	2.4	2.5	2.1
48hr pulse	D4	15	15	18	4.0	4.8	5.1	0.6	0.7	0.9
	D7	12	10	10	14.2	14.9	15.3	1.7	1.5	1.5
None	D4	4	4	4	<1	<1	<1	<0.1	<0.1	<0.1
	D7	6	6	7	<1	<1	<1	<0.1	<0.1	<0.1

Appendix 5 (Table 2)

Total cell growth and mast cell growth in normal bone marrow cultures stimulated with CM from a syngeneic strain of rat. Additional data from different CM harvest times and repeat experiment.

Source of CM	Source of BM	Time of harvest of CM (hrs)	Total Cells $x10^{-4}$ Experiments a	Total Cells $x10^{-4}$ Experiments b	Mast Cells $x10^{-4}$ Experiments a	Mast Cells $x10^{-4}$ Experiments b
F344	F344	24	30	41	5.4	7.9
"	"	48	135	132	62.3	59.9
"	"	72	54	49	19.5	17.8
CBH	CBH	24	58	51	17.1	15.4
"	"	48	84	89	35.9	37.2
"	"	72	47	46	13.3	11.9
AUG	AUG	24	30	22	9.9	10.1
"	"	48	93	89	27.4	29.8
"	"	72	75	63	18.2	16.4
AO	AO	24	8	6	2.7	3.2
"	"	48	62	54	16.7	19.1
"	"	72	32	35	12.4	14.2
(LISxBN)F_1	(LISxBN)F_1	24	47	38	11.5	13.1
"	"	48	79	77	22.1	25.2
"	"	72	52	43	11.7	9.7

Appendix 6 (Table 3)

Total cell and mast cell growth in normal (LIS x BN)F_1 bone marrow cultures stimulated with CM made from IMLN of allogeneic strains of rats.

Source of CM	Source of BM	Total Cells $x10^{-4}$	Total Mast Cells $x10^{-4}$
F344	(LISxBN)F_1	59	23.2
CBH	"	42	13.5
AUG	"	102	17.8
AO	"	39	8.9
(LISxBN)F_1	"	77	23.4

Appendix 7 (Table 4)

Total cell and mast cell growth in normal bone marrow stimulated with CM made from IMLN of an allogeneic rat strain (LIS x BN)F_1

Source of CM	Source of BM	Total Cells $x10^{-4}$	Total Mast Cells $x10^{-4}$
(LISxBN)F_1	F344	92	31.1
"	CBH	64	17.9
"	AUG	46	13.7
"	AO	36	8.3
"	(LISxBN)F_1	77	25.7

Appendix 8 (Table 5)

Total cell and mast cell growth in normal bone marrow cultures stimulated with CM from fractionated immune MLN cells.

Source of CM	Total Cells $x10^{-4}$	Mast Cells $x10^{-4}$
Unfractionated immune MLN cells	62.0	27.1
Immune MLN without adherent cells	87.2	43.7
Adherent cells alone	5.0	0.4
Ig^+ cells	4.7	0.1
Ig^- cells	97.5	55.2
Medium alone	3.0	<0.1

Appendix 9 (Figure 4)

Total cell and mast cell growth in rnu/rnu and rnu/+ rat bone marrow cultures stimulated by various CM. (Results depicted in Figure 4(a), and repeat experiments (b & c)

rnu/+ bone marrow

Source of CM	Day Harvested	Total Cells $x10^{-4}$ Experiment			Mast Cells $x10^{-4}$ Experiment		
		a	b	c	a	b	c
$(LISxBN)F_1$	D4	29.6	24.9	28.0	4.5	3.1	5.5
	D6	32.6	26.0	30.0	14.9	17.7	15.4
	D8	30.0	28.1	38.5	16.0	19.1	19.8
	D10	27.3	28.5	36.2	24.9	19.4	18.6
rnu/+	D4	21.3	20.0	25.0	3.1	6.7	4.6
	D6	24.3	25.4	26.4	7.0	8.5	4.9
	D8	22.6	28.6	27.0	9.9	9.6	5.0
	D10	22.6	29.5	27.0	11.3	9.9	5.0
rnu/rnu	D4	3.0	1.5	2.1	0.1	<0.1	0.1
	D6	3.0	1.7	2.0	0.2	<0.1	0.1
	D8	1.3	2.0	1.6	<0.1	<0.1	<0.1
	D10	<0.1	1.3	1.3	<0.1	<0.1	<0.1
medium alone	D4	2.3	2.3	2.0	<0.1	0.1	0.1
	D6	2.0	1.6	1.5	<0.1	<0.1	0.1
	D8	<1.0	<1.0	<1.0	<0.1	<0.1	<0.1
	D10	<1.0	<1.0	<1.0	<0.1	<0.1	<0.1

Appendix 9 (cont)

rnu/rnu bone marrow

Source of CM	Day Harvested	Total Cells $x10^{-4}$ Experiment			Mast Cells $x10^{-4}$ Experiment		
		a	b	c	a	b	c
$(LISxBN)F_1$	D4	22.3	21.2	14.0	6.9	5.4	4.1
	D6	31.6	22.3	18.0	12.6	8.4	8.9
	D8	33.0	26.4	29.5	18.3	12.2	17.1
	D10	24.3	25.7	29.0	19.0	14.4	17.0
rnu/+	D4	17.0	12.0	13.0	3.6	1.4	2.2
	D6	16.3	13.6	14.0	5.1	5.0	3.4
	D8	16.6	14.9	14.0	5.8	5.5	4.0
	D10	13.0	14.1	14.0	8.6	5.2	4.0
rnu/rnu	D4	3.2	2.0	1.7	<0.1	0.1	0.1
	D6	2.0	1.5	2.0	<0.1	<0.1	0.1
	D8	2.3	1.5	1.5	<0.1	<0.1	<0.1
	D10	2.5	1.2	1.5	<0.1	<0.1	<0.1
medium alone	D4	2.6	1.5	2.0	<0.1	<0.1	<0.1
	D6	2.3	1.0	1.5	<0.1	<0.1	<0.1
	D8	<1.0	<1.0	<1.0	<0.1	<0.1	<0.1
	D10	1.3	<1.0	<1.0	<0.1	<0.1	<0.1

Appendix 10 (Table 6)

Total cell growth, mast cell growth and RMCPII production in normal and nude rat bone marrow cultures stimulated with (LIS x BN)F_1 CM (repeated experiments).

Day of Culture	Total Cells $x10^{-4}$				Mast Cells $x10^{-4}$				RMCPII content of culture supernatant (µg/ml)			
	Nu/Nu		(LISxBN)F_1		Nu/Nu		(LISxBN)F_1		Nu/Nu		(LISxBN)F_1	
	a	b	a	b	a	b	a	b	a	b	a	b
2	13.0	10.2	14.1	11.5	3.9	3.0	0.7	0.3	0.34	0.29	0.06	0.02
4	14.7	11.5	32.0	15.7	4.1	3.2	9.3	3.9	0.41	0.44	1.20	0.29
6	18.5	21.7	79.1	67.4	8.9	10.4	41.9	35.0	0.98	1.04	2.20	2.01
8	29.5	43.5	89.0	72.6	17.0	25.1	52.5	41.4	1.27	1.53	2.50	1.98

Appendix 11 (Table 7a-c)

Repeat experiment of CM made from FACS-sorted IMLN cells labelled with Oxford monoclonal antibodies.

Source of CM	Total Cells ($x10^{-4}$)	Mast Cells ($x10^{-4}$)
Unfractionated IMLN cells	42	23.5
OX19-positive IMLN cells	61	37.3
OX19-negative IMLN cells	5	0.6
Medium alone	5	0.4
Unfractionated IMLN cells	72	33.1
W3/25-positive IMLN cells	65	25.4
W3/25-negative IMLN cells	6	0.8
Medium alone	4	0.4
Unfractionated IMLN cells	57	28.3
OX8-positive cells	5	0.2
OX8-negative cells	63	31.4
Medium alone	5	0.4

Appendix 12 (Table 8)

Repeat experiment of effect of dexamethasone (Dex) on the production of MMC growth factor by IMLN cells stimulated by Con-A, assayed by growth of MMC in normal bone marrow cultures.

Conditioned Medium and Dex. conc.	Total Cells ($\times 10^{-4}$)		Mast Cells ($\times 10^{-4}$)		RMCPII content µg/ml	
	Experiment		Experiment		Experiment	
	a	b	a	b	a	b
IMLN14 + 10^{-8}M Dex + Con-A	10	8	0.9	0.7	0.28	0.20
" 10^{-7}M " "	8	8	0	0	0.13	0.10
" 10^{-6}M " "	2	1	0	0	0.03	0.02
IMLN14 + 10^{-8}M Dex alone	4	3	0	0	0.02	0.01
" 10^{-7}M " "	2	1	0	0	0.02	0.01
" 10^{-6}M " "	1	1	0	0	0.03	0.01
IMLN14 + 2 µg/ml Con-A	64	56	28.8	29.1	6.33	7.57
Medium alone	1	1	0	0	0.02	0.01

Appendix 13 (Table 9)

Repeat experiments to show the effect of Dex on the growth, differentiation and RMCPII content of normal bone marrow cultures.

CM and conc. Dex.	Total Cells ($x10^{-4}$)/ml		Mast Cells ($x10^{-4}$)/ml		RMCPII cont. of extracted cells (µg/ml)		RMCPII cont. of supernatant (µg/ml)	
	Experiment		Experiment		Experiment		Experiment	
	a	b	a	b	a	b	a	b
NBM + CM + 10^{-8}M Dex	30	36	15	15	3.6	3.7	0.9	0.5
" " 10^{-7}M "	31	29	16	15	4.2	3.7	0.9	0.6
" " 10^{-6}M "	25	25	13	12	2.6	2.3	0.5	0.6
NBM + 10^{-8}M Dex	4	9	0	0	0.01	0.04	+	+
" 10^{-7}M "	10	7	0	0	0.02	0.02	+	+
" 10^{-6}M "	7	8	0	0	0.02	0.02	+	+
NBM + CM	64	56	29	29	6.3	7.6	2.2	1.7
NBM + Medium alone	2	1	0	0	0.02	0.01	+	+

+ <10 ng/ml detected

Appendix 14 (Table 10)

Repeat experiment to show effect of Dex on 3 week old normal rat bone marrow cultures

Conc. of Dex.	Total Cells ($\times 10^{-4}$)	Mast Cells ($\times 10^{-4}$)	RMCPII content cell extract (µg/ml)	RMCPII content cell supernatant (µg/ml)
Before treatment	54	49.7	12.92 (26 pg/cell)	2.72
24 hr treatment				
10^{-8}M Dex	49	45.1	13.53 (30 pg/cell)	2.32
10^{-7}M "	51	48.5	12.59 (26 pg/cell)	2.27
10^{-6}M "	54	51.8	16.58 (32 pg/cell)	2.15
48 hr treatment				
10^{-8}M Dex	55	52.8	14.26 (27 pg/cell)	2.51
10^{-7}M "	47	45.6	14.14 (31 pg/cell)	2.27
10^{-6}M "	52	49.4	14.82 (30 pg/cell)	2.92