The Role of Mast Cells during Experimental Schistosomiasis mansoni in Mice

Parviz Kermanizadeh

A thesis submitted for the degree of Doctor of Philosophy

Division of Infection and Immunity,
Institute of Biomedical and Life Sciences
Faculty of Medicine and Faculty of Science, University of Glasgow

June 1997

Summary

Mast cells are important immune cells which reside in crucial sites in all organs of the body. The large number of potentially active substances which are released from the mast cell upon cell degranulation play a central role in the pathology of parasitic infections, allergic and other chronic inflammatory disorders. The influence of mast cell-derived mediators may well be important factors in atherosclerosis, anaesthesia, arthritis, angiogenesis, lipid metabolism, fibroblast interactions and other events.

The association between mast cells, eosinophils and IgE antibody (three hallmarks of helminth infections and some other diseases) is of great interest. An increase in our knowledge of the processes in this association would be beneficial for the treatment and prevention of those diseases.

Development of reliable methods for better investigation of mast cells and eosinophils both separately and together in tissues and body fluids was the first target of the study and a prolonged investigation resulted in the development of improved methods for fixation and staining of mast cells and eosinophils simultaneously in internal organ and skin tissues. The investigation also resulted in improving the fixation and staining of those cells separately in internal tissue.

The common method of counting the cells in intestinal tissues suffers from various limitations. A new simple counting method, which could be applied to counting of the cells in the intestine and any other diffuse tissue, was developed during this investigation.

The kinetics of mast cell populations in the anterior, middle and posterior portions of the small intestine and the kinetics of the mast cells and eosinophils in the liver granuloma and the association and the effect of mast cells and eosinophils on egg destruction and granuloma production were studied during a 16 week experimental *Schistosoma mansoni*-infection. The distribution of mast cells was always greater in the anterior than posterior portion of intestine and the number of the mast cells was greater in the intestine than in the liver (with relatively equal numbers of granulomas). On the basis of these observations and information obtained from a review literature, the possibility of mast cell attraction to nerve-derived material was proposed. The notion is that continuous

stimulation of the nerve receptors or damage and degeneration of the nerve fibres by rough foreign particles (stimulants) are terminats in the release of chemoattractant material (s) from irritated nerve cells. Consequently mast cells migrate toward the injured tissue as a result of nerve-induced chemotaxis.

This hypothesis not only explains the unequal distribution of mast cells in inflamed tissues but also gives a reasonable framework for consideration of possible influences on T helper cell activation. In this way mast cells which are attracted to inflamed tissues are triggered through cross-linking of high affinity IgE receptors on their surface plasma membranes. Degranulation and release of a collection of Th-1 down-regulating and Th-2 up-regulating cytokines from mast cells would lead to Th-2 type helper cell activation.

For a test of this hypothesis, *in vitro* and *in vivo* experiments were designed. Pure mast cells were prepared by bone marrow culture and *in vitro* chemotaxis assays (culture, micro-pore and polarisation) were carried out. Promising results were achieved from polarisation assays, but because of time limitations the investigation had to be curtailed. Continuation of the *in vitro* and the *in vivo* experiments are believed to be worth considering.

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Declaration

During the course of this research, some aspects of the work have been reported elsewhere, either as published scientific papers, or in conference presentations. The presentations and the thesis chapters to which they relate are listed below.

Published papers

Kermanizadeh, P., Hagan, P. & Crompton, D.W.T. (1995). A differential staining method for mast cells and eosinophils in murine intestine, liver, and spleen. *Parasitology Today* **11**, 194-196. (Chapter 3)

Kermanizadeh, P., Hagan, P. & Crompton, D.W.T. (1995). Fixing and staining mast cells and eosinophils in murine tissues. *Parasitology Today* **11**, 464. (Chapter 3)

Kermanizadeh, P., Hagan, P. & Crompton, D.W.T. (1997). A simple method for counting cells in diffused tissue. *Parasitology Today* (in press) (Chapter 4)

Conference presentations

Communications

Kermanizadeh, P., Hagan, P. & Crompton, D.W.T. The role of mast cells in *schistosoma mansoni*-infected mice. presented at the British society for Parasitology Spring Meeting, held at the University of Edinburgh., 10h-12h April, 1995 (Chapter 5and 6)

Poster

Kermanizadeh, P., Hagan, P. & Crompton, D.W.T. Differential staining of mast cells and eosinophils in murine, intestine and spleen. Presented at the Royal Society of Tropical medicine and Hygiene, Scottish Branch, Laboratory Meeting, Edinburgh, 12 May 1994 (Chapter 3)

I declare that the research described in this thesis has been carried out by myself unless otherwise cited or acknowledged. It has not, in whole or part, been submitted for any other degree. All animal procedures were carried out under licence from the Home Office: Personal Licence PIL 60/4735.

Parviz Kermanizadeh

June 1997

Acknowledgements

I express my deepest gratitude and most sincere thanks to my supervisors, Professor D.W.T. Crompton and Dr Paul Hagan for their continued encouragement, advice, support, profound enthusiasm, and particularly for their patience in reading and commenting on this thesis. Their enthusiasm and interest made this study very enjoyable and also educational. I greatly appreciate the help of Professor Coombs for use of the laboratory facilities.

I most sincerely thank Professor J. R. Kusel and Dr J. Modha for their support and kind nature during all my work. I also wish to thank Professor P. C. Wilkinson for all his help. I am also very thankful to Ms Kate. Orr for her all collaboration and help during my work and John Laurie and Willie Orr for their technical help. My thanks also go to Raymond Stoddart in particular for helping me with photography, Liz Denton and all staff members for their help during the work.

I most sincerely thanks my family for their patience and support which helped me throughout all my studies.

Finally, I am indebted to the University of Medical Sciences of Tabriz and Ministry of Health and Medical Education of Islamic Republic of Iran for awarding me a scholarship to pursue my Ph.D. in Britain.

Chapter 1

General Introduction

1.1. Introduction to *Schistosoma mansoni*

Schistosoma mansoni is a species of digenetic trematode. As adult worms they settle in the mesenteric and portal veins around the large intestine where they may survive for several years (Pearce *et al* 1994). The female worms are held in the gynaecophoric canal of the male worms and each may produce 100 - 300 laterally spined eggs, approximately 61x140 μm containing an embryo. The eggs move through the tissues with the help of their spines and lytic enzymes which pass through micropores in the egg-shells. These lytic enzymes are highly immunogenic and are responsible for the induction of the characteristic host cellular infiltration or granuloma which encapsulates the eggs (Weinstock 1992). The granuloma may prevent toxic components contained in egg secretions from reaching and damaging other tissues and organs but may also serve as a dynamic cellular mass which helps to transport the eggs through the tissues. Eggs which reach the lumen of the intestine are carried in the faeces to the environment. If they reach fresh water the eggs hatch releasing the highly motile ciliated miracidium. If the miracidium encounters a suitable intermediate snail host such as *Biomphalaria* spp, it will penetrate the soft tissue and continue its development through two sporocyst generations before cercarial stages develop and are shed from the snail. The cercaria is capable of penetrating the skin of the definitive human host and as it does so it sheds its tail, transforming to the schistosomulum stage. This enters the circulation and after passing through the heart and lungs eventually settles in the mesenteric and portal blood vessels where it matures (Schmidt *et al.*1989).

1.2. Pathology of schistosomiasis mansoni

Schistosomiasis mansoni has three important features (i) a dermatitis when cercariae enter the skin (ii) an acute phase of infection which may appear some weeks later when the adult female worms have produced large numbers of eggs which are deposited in various sites around the body and (iii) a chronic phase involving the gastro-intestinal tract and liver. The main clinical manifestation at this stage consists of fever, weakness, weight loss, bloody diarrhoea and hepatosplenomegaly and most of these symptoms are considered to arise from immunologic hyperactivity (Gazzinelli *et al.* 1985; Newport *et al.* 1993). The large number of granulomas present in the tissues is the result of a delayed type hypersensitivity to the antigens released from the eggs which have lodged in the tissues.

Most frequently fibrosis and polypoid inflamed lesions are seen in the intestine containing granulomatous tissues. *S. mansoni* eggs can be found in these lesions, polyps develops with intestinal bleeding (Olds & Wiest 1995) and diameter of the intestinal lumen may be reduced (Curts & Sleisenger 1973). The infected liver may be enlarged during infection with *Schistosoma mansoni* and its surface may become nodular in severe infection. Granulomas frequently replace the intraportal small sinusoids and causes the obstruction of the lumen, endophlebitis of intraportal sinusoids may also induce vascular obstruction (Olds & Wiest 1995). The thrombi that arise from the destruction of the inflamed vessel walls may leads to the anastomosis between newly formed blood vessels and those local vessels passing across the area occupied by thrombus. This phenomenon "recanalization", may extend to dilation and vascular lesions " liver telangiectasia" (Marcial *et al.*1990). The thrombi can also be broken off and cause further blockage in the vessels elsewhere. Peripylephlebitis in the liver causes extensive fibrosis around the veins, calcified and thickened fibrosis along the affected veins is known as "pipestem". The existence of frequent pylephlebitis and peripylephlebitis in the

liver eventually causes portal hypertension (Newport *et al.* 1993), by decreasing the out flow and increasing the in flow of the liver vein circulation. Portal hypertension then increases the back-pressure to the circulation and splenomegaly is the consequence of this event. Accumulation of the blood in the mesenteric venules leads to ascites by allowing leakage of fluid into the peritoneal cavity. The high resistance to blood flow in the liver causes the portal circulation to shunt around and bypass the liver, connecting to the systemic veins (vena cava) through the connection between cervical oesophageal veins and left gastric vein. These collateral bridges can cause oesophageal varices, under overloaded circulation pressure. The rupture of the enlarged veins in this part of the oesophagus may lead to the fatal bleeding (Wilson & Lester 1992).

1.3 Resistance to *Schistosoma mansoni*.

There is clear evidence of acquired resistance to *S. mansoni* in animal models. In rhesus monkeys and mice there is substantial immunity against later infection but in all cases hosts endure the first infection for a lengthy period (Wakelin 1984). In rats, in which the worms do not usually mature, there is no egg production and strong resistance is achieved after worm destruction (Butterworth 1993). In humans there is resistance against *S. haematobium* (Hagan *et al.* 1985) and *S. mansoni* infection during the second decade in an endemic area (Butterworth *et al.* 1984). Th2- cells are known preferentially to generate prominent responses to helminth infections whereas Th1-cells are responsible for dealing with intracellular pathogenic invaders such as bacteria, protozoa and viruses. Th1 cells release IFN-γ, interleukin 2 (IL2), tumour necrosis factor (TNF-β) and produce both humoral and cellular immunity. Th2 cells, on the other hand, generate interleukins (IL4, IL5, IL6, IL9, IL10, IL13) which stimulate IgE synthesis, mast cell and eosinophil production (three hallmark elements of helminth infection) and mucosal immunity (Romagnani 1994; Reiner & Locksley 1993). Larvae, adult worm and egg stages

of the worms life cycle all influence the immunological response during schistosomiasis. The general view is that eggs are not strictly main inducers of protection in schistosomiasis, but at least in humans the adult worm can probably produce protective resistance (Pearce 1994).

It is thought that resistance to reinfection is achieved partly through increased numbers of circulating eosinophils (Hagan *et al.* 1985 & 1987) and the association of eosinophils with IgE (Hagan *et al.* 1991 & 1993a). IgE levels in resistant people were 6-8 times higher than in susceptible people (Dessein *et al.* 1992). It is also stated that a high level of IgE gave 10 times more protection (Hagan *et al.* 1991; Rihet *et al.* 1991; Dunne *et al.* 1992). Th2-phenotype helper T cells mediate IgE switching and synthesis through IL4 and IL13 secretion (Pears 1994; Vries 1994). IgM, IgG_4, IgG_2, which are produced partly in response to egg antigens, may play a role as blocking antibody and preventing the development of protection (Dunne *et al.*1995; Hagan *et al.* 1991; Capron & Dessaint 1992).

Neutrophils, macrophages and monocytes may all contribute to killing schistosomula *in vivo*, but eosinophils are most likely to be the most important killer cells in this regard (Capron *et al.* 1982). Macrophages may kill the schistosomula by non-specific mechanisms or more specifically with immune complexes (IgE or IgG and parasite antigen). Neutrophils may affect the schistosomula by producing a variety of enzymes following adherence to schistosomula's tegument (Dean *et al* 1974). Platelets also may cause some damage to schistosomula through association with parasite specific IgE (Joseph *et al.* 1983).

Eosinophils have low affinity receptors for IgE antibody (Capron *et al.* 1981a & 1984) and in the presence of IgE can damage schistosomula (Capron *et al.* 1981b; Butterworth *et al.* 1977). Eosinophils flatten and make intimate contact with the worm's surface and then degenerate releasing their secretions onto the tegument. Eosinophils produce major basic protein, cationic protein, phospholipase and peroxide which destroy the tegument. Eosinophils penetrate under the tegument,

separate the tegument from the muscle layer and kill the larva (see Wakelin 1984). Mast cells are also involved directly and indirectly with schistosomula effector mechanisms. (see the role of the mast cells in schistosomiasis (section 1.4.5.6)).

Egg production by *S. mansoni* reduces from a peak at 8 to 12 weeks to lower levels later. Adult *S. mansoni* may evade the host's immune response and protect its tegument from destruction. They can mask the surface covering it with host's antigen that hides the worm's antigens from the exposure to the host immune response (Smithers *et al.* 1969b; Goldring *et al.* 1977) or they can break down the host's immune effectors by producing proteases (Auriault *et al.* 1981) or other substances which modify mast cells, granulocytes or other immune functions. While resident in the blood stream schistosomes lose their surface antigens as the surface membrane turns over (Newport *et al.* 1993). The host responds to many of these antigens but appears unable to eliminate the adult worms which produce them (Pearce *et al.* 1986) and adult worms can live for up 30 years (average, 5-10) (Pearce & Simpson 1994) in human. However, newly invading schistosomula may suffer severe damage by efficient immediate or delayed-hypersensitivity and thus further reinfection may be prevented.

When mice are exposed to live cercariae of *S. mansoni*, attenuated by radiation, they develop immunity against the challenge infection, despite never being exposed to adult worms or eggs of the parasite as irradiated larvae fail to become mature. This immunity is T-cell dependent and, in particular, depends on IL2 and IFN-γ production (Smythies *et al.* 1992). Normal cercariae may also induce a Th1-type responses in mice (Sher *et al.* 1992a ; Pearce 1994; Wilson 1993), but when egg deposition begins this Th1-type response switches to a Th2-type response (Sher *et al.* 1992a & 1992b). The precise reasons why helminths induce T-cell responses of particular types have yet to be determined, though it has been hypothesised that products derived from helminths trigger cells other than T-cells to produce cytokines

that may influence the differentiation of helper T-cells in either a Th1 or Th2 direction (Urban *et al.* 1992; Finkelman & Urban 1992).

All studies support the view that the Th1 responses may be stimulated during the early phase of schistosomiasis and change to Th2 responses during the chronic phase of the infection, as a result of adult worm or egg activities (Pearce 1994). Although the worms induce Th2 responses, these may not be effective against the adult worms which continuously survive in the bloodstream, evading the host immune responses by different mechanisms. Th2 responses appear to be preferentially effective against the newly penetrated larvae through mast cell and IgE associated immediate hypersensitivity and through eosinophil and monocyte associated cell-mediated immunity (Dessein *et al.*1992).

1.4. Immunology

Immunology is the study of the those mechanisms that contribute to maintaining the homeostasis of the body by operating against internal abnormality and external invasion. The discrimination of self from non-self is the underlying principle of the immune response. Although a functional immune response is necessary for a healthy life, the result of both too much activity, autoimmune diseases or allergy or too little activity, overwhelming infection would be life threatening.

The protection against infection and disease which is afforded by the immune system can be enhanced through vaccination. However, in other situations immune responses may favour harmful allergic reactivities and, of course, tissue transplantation may be hampered by the recognition of 'non-self' and attempts at immune rejection. The range of immune reactivities which are possible can be both potentially harmful and potentially beneficial. In many cases of infection, the responses which are generated turn out to be a mixture of both, with some degree of

pathology being associated with the development of a protective immune response. Various factors (including products of the nervous system) may influence the nature, types and magnitude of immune responses which are generated (Blalock 1994).

1.4.1. Antigen presentation.

Recognising self from non-self is the basic function of the immune system and this is achieved in part by the process of antigen presentation, involving the properties of the complex of molecules known as the Major Histocompatibility Complex (MHC). This is a complex of linked genetic loci involved in non-self tissue rejection in mice, called antigen 2 by Peter Gorer (Elgert 1996a), and subsequently changed to histocompatibility 2 (H2). The genetic loci analogous to mice H2 was found in human leukocytes and called the human leukocyte antigen (HLA) complex (Elgert 1996b). Regions of chromosomes 17 and 6, of mice and humans respectively, are responsible for the production of these molecules (Trowsdale *et al.* 1993). The products of these genes appear as "class I and class II antigens "on the surface of cells. Association of these antigens with the T-cells' receptors plays the essential role of self and non-self discrimination. Class I molecules are expressed on all nucleated cells, but class II molecules are found only on the antigen presenting cells. The T cell receptor cannot recognize antigen unless the antigen associated with class I or class II Major Histocompatibility Complex molecules, CD8 molecules on cytotoxic T cells interact only with class I antigens and CD4 molecules on T-helper cells interact only with class II antigens. T cells with CD4 or CD8 molecules and other receptors, complex with antigen and class I or class II molecules on antigen presenting cells (Elgert 1996b).

Mononuclear phagocytes, dendritic cells, B cells and more recently mast cells (Frandji *et al.* 1993) have been shown to present antigen (antigen presenting cell-APC) by exposing "semi-digested" antigens on their surface membrane. All three cell types carry the class I and class II molecules on their surface. In ideal

conditions, phagocytes break down the complicated protein materials and expose the partially catabolized segments consisting of 9-24 amino acids, (antigen) on the cell surface.

The complex formed between class II with the exogenous antigen (processed e.g. by B-cell) can activate helper T-cells. Cytotoxic T-cells on the other hand are activated in the similar way when endogenous antigen is associated with class I molecules (Unanue & Allen 1987). Antigen presentation is increased by the influence of INF-γ or INF-γ like molecules (Steeg *et al.*1982) and is decreased by prostaglandin interference (Snyder *et al.*1982).

1.4.2. Inflammation

Inflammation is the first response of the body to an infection, tissue injury or disturbance. Inflamed tissues are characterised by redness, heat, swelling, pain, cell infiltration and with pus collection mostly during bacterial infection. Vasodilation and extravasation are the main features of inflammation. The plasma fluid delivered to the inflamed tissues dilute the toxic materials and promote cellular infiltration. An inflammatory process can be described in four separate phases. (1) The vascular phase, which happens within 5 to 10 minutes and is completed by 15 to 30 minutes, unless the stimulus continues. Vasodilation and increased blood flow in this phase causes redness, and increased vascular permeability resulting in post capillary fluid leakage, oedema and pain. (2) The acute cellular phase which takes place over the next few hours and involves cell infiltration, clot formation and eventually pus production. (3) The chronic phase in which, dead cells and debris are cleaned and remodelling begins. (4) If the destruction of infectious material is not possible or needs a prolonged time, the injured part of the tissue will be walled off from the surrounding tissue and a granuloma will be produced (Austyn & Wood 1993).

How does inflammation takes place? Inflammation is mediated by mast cells. Mast cells are involved in the process of inflammation by producing cytokines, histamine, leukotrienes, prostaglandin and other mediators. Agents from injured tissues activate mast cells to release kallikrein-like enzyme (Sigal & Ron 1994) which generates bradykinin, a very potent vasodilator. Bradykinin is a spasmogenic peptide, which increases vascular permeability and produces pain at the inflamed site. Histamine and serotonin are the most important mediators in inflammation released from mast cells. Histamine causes arteriole vasodilatation and venule contraction through H1 receptors, and consequently increases the permeability of post-capillary venules by increasing the gaps between the endothelial cells (Weaver 1995). Also, histamine modulates neutrophil and eosinophil chemotaxis through H2 receptors (Cavanah & Casale 1993).

Mast cells contribute to both immediate and delayed type hypersensitivity (Askenase & Loveren 1983). By producing IL-6, TNF, histamine, serotonin and arachidonic acid derived mediators mast cells play an important role in both early and late phase of inflammation (Austyn & Wood 1993).

Tissue repair is initiated at the time of injury and is inseparable from inflammation. An effect of mast cell on tissue repair depends on heparin's ability to protect growth factors from degeneration, consequently enhancing angiogenesis and fibroblast production (Roberts *et al.* 1988). Moreover mast cells may contribute directly to the building up of the basement membrane by the production of laminin, collagen type IV and heparan sulphate (Thompson *et al.* 1991). Some of the key factors in inflammatory responses especially during helminth infection are described below.

1.4.3. IgE antibody

IgE or reaginic antibody is a homocytotropic immunoglobulin and 57-2000 (ng/ml) of this antidody is found in normal serum (Solomon 1992). IgE synthesis is markedly increase during parasitic infections and contributes to allergic reactions (Befus 1995; Wardlaw 1993). There are two kind of Fc receptors for IgE molecules. FcE RI the high-affinity receptor which are found only on mast cells and basophils. FcεRII the low affinity receptor is found on eosinophils (Elgert, 1996). IgE is known to protect against helminth parasites (Hagan *et al* 1991). IgE molecules on mast cell surfaces are responsible for initiation of type 1 hypersensitivity reactions (from minor atopic allergy to anaphylaxis) (Elgert, 1996).

1.4.4. Eosinophils

Eosinophils were first studied in detail by Ehrlich (Spry, 1993). Eosinophils are derived from bone marrow, are easily detectable in the peripheral circulation and normally reside in the various tissues and organs. IL-5 not only stimulates the production of the eosinophils but also increases their survival (Tai 1991; Finkelman *et al.* 1991).

Eosinophil-derived cationic protein has a strong cytotoxic effect on the schistosomula of *S . mansoni* through surface disruption (Ackerman *et al.*1985). The eosinophil peroxidase (EPO) forms hydrogen peroxide and then in the presence of a halogen is directly toxic to schistosomula (Jong *et al.* 1981). EPO also increases the capacity of neutrophils to kill schistosomula (Jong *et al.* 1984).

Eosinophil derived major basic protein (MBP) is highly toxic to schistosomula of *S . mansoni* (Butterworth *et al.* 1979). Eosinophils have been found to kill IgE-coated schistosomula (Capron *et al.* 1984). In addition, eosinophils, by production of a large number of other mediators including IL-8

(Braun *et al.* 1993), tumor necrosis factor-α (TNF-α), macrophage inflammatory protein-1 α (Costa *et al.* 1993), transforming growth factors (TGF) α and β1 (Elovic *et al.* 1994) and IL-5 (Desreumaux *et al.* 1992), may interact with other cells such as mast cells (O'Donnell *et al.*1983) and these interactions may lead to activation of the remaining effector functions of inflammation.

1.4.5. Mast cells

1.4.5.1. Introduction

Mast cells were first recognised in unstained tissue in 1863 by Friedrich von Recklinghausen (Pepys *et al.* 1979). Later in 1878 a young medical student, Paul Ehrlich, described these cells and distinguished them from other cells by observing granules that stain red or blue with basic aniline dyes. Ehrlich called these cells "Mastzellen" because he believed that the large cytoplasmic metachromatic granules actually resulted from over eating (in German "Masstung" means to chew and zelle means cell). The shape of the mast cells varies greatly among the species and even between different tissues of the same individual. They can be seen to be small or large, round, spindle or star-shaped depending on the surrounding tissues. The typical mast cell has a mean diameter of about 15 μm (Policard & Collet 1959; Selye 1965). Mast cells contain a single nucleus. The granules of the mast cells are about 0.1-0.4 μm in diameter (Wasserman 1983) and consist up to 53% of the total cytoplasmic volume (Helander & Bloom 1974). Metachromasia, the important characteristic of the mast cell granules, results from a changing light absorption during the processes of staining with a basic dyes such as toluidine blue. If the dye is bound to the monomeric state of the polyanionic polymer of 'polyglycan' in the mast cells (dilute situation) it stains an orthochromatic or blue colour, but existence of the polyglycan in the dimeric or polymeric condition causes more dye to aggregate, lower wavelength absorption and consequently a colour shift to 'red

colour' (Padykula 1993). Depending on the nature of the polyglycan, mast cells can be stained red to blue or both colours at the same time (Huntley 1992).

Mast cells are positioned preferentially around the blood vessels in the connective tissue of glandular ducts, skin and the gastrointestinal mucosa (Metcalfe 1983), mast cells gather near any portals where noxious materials may enter. They are observed near to the nerves in various normal and diseased organs and tissues (Newson *et al.* 1983; Stead *et al.* 1989; Nennesmo & Reinholt 1986; Tanaka *et al.* 1977; Olssen 1968; Dasgupta & Maiti 1979).

1.4.5.2. Origin of the mast cell

Mast cells and basophils are produced from the multipotent haemopoietic progenitor cells (Kitamura *et al.* 1981; Zuker- Franklin 1980) but their specific - precursor cells are still unknown. Unidentified immature mast cell precursors may migrate to various tissue sites, including bone marrow and under the influence of different growth and micro-environmental factors they can then develop to be specific mast cells (Metcalfe 1983 ; Kirshenbaum 1993). Basophils mature in bone marrow and the mature cells enter the blood circulation. Basophils may have a common precursor with other granulocytes. There are more similarities between basophils and eosinophils than between basophils and neutrophils (Galli 1990). Mast cells, by having the ability to synthesise some specific lymphokines and monokines, have more affinities with mononuclear than polymorphonuclear cells (Sigal & Ron 1994).

1.4.5.3. Mast cell heterogeneity

There are at least two major populations of mast cells in mammalian tissue; these are connective tissue mast cells (CTMC) and mucosal mast cells (MMC). They have been recognised on the basis of their fixation, staining characteristics,

and mediator differences. Distinctive neutral proteases are a prominent factor in this regard (Kitamura 1989).

RMCPI (chymase) is found in rat connective tissue mast cells (Woodbury *et al.* 1978a; Seppa & Jarvinen 1978) and RMCPII (chymase) is found in rat mucosal mast cells (Haig *et al.* 1982; Woodbury *et al.* 1978). There are great similarities between rat and mouse proteases (Miller *et al.* 1988 & 1989). In humans, chymase is found only in connective tissue mast cells (Schwartz 1993). Tryptase in contrast is found in both human mucosal and connective tissue mast cells (Sigal & Ron 1994).

The proteoglycan of mouse and rat connective tissue mast cells is heparin. Chondroitin sulphate on the other hand is known to be the dominant proteoglycan of the mouse and rat mucosal mast cells. Chondroitin sulphate and heparin are found in both human MMC and CTMC (Schwartz 1993).

Kitamura *et al.* (1979) stated that a single haemopoietic stem cell is responsible for both MMC and CTMC development, but whether their differentiation needs another divergent cell line in the same cell lineage is still a subject to be studied. Injection of interleukin 3 into nude mice increased the number of the MMC (Abe *et al.* 1988) and IL-3-dependent mice bone marrow culture changed to connective tissue type mast cells when co-cultured with 3T3 fibroblasts (Levi-Schaffer *et al.* 1986).

Mouse bone-marrow derived mast cells have similarities to mucous mast cell (Levi-Schaffer *et al.* 1986). It is possible that the maturation of mast cells depends on the stage of development or particular factor (s) in the local tissue environment that controls the heterogeneity of the tissue specific mast cells.

1.4.5.4. Mast cell mediators

Mast cell products can be considered in two important categories, performed and newly formed mediators (Schwartz *et al.* 1981a & 1981b).

Histamine

Histamine, an important mediator in mast cells, is formed from the amino acid histidine. The histamine levels are reported to be about 1-3 pg in human mucous and connective tissue mast cells, and similar levels are known in rat and mouse mast cells (Schwartz 1993). Histamine is stored in the mast cells bound to heparin or chondroitin proteoglycan. Three percent of total histamine production is expelled by urination and the other 97% is metabolised and converted to methylhistamine and imidazole acetic acid (Sigal & Ron 1994). Histamine exerts its biological influence through three kinds of histamine-sensitive surface receptors (H_1, H_2, H_3). Stimulation of H_1 receptor causes constriction of the bronchial and gastrointestinal smooth muscles, increased vasodilation and vasopermeability, increased mucus secretion and increased cyclic GMP. Histamine increases the C3b receptor expression of eosinophils (Anwar & Kay 1980) through the H_1 receptor and increases eosinophil activation, chemotaxis through the H_1 and H_2 receptors (Clark *et al.* 1975). Histamine in contrast inhibits neutrophil (Seligman *et al.* 1983) and basophil (Lett-Brown *et al.* 1877) chemotaxis and stimulates T cells (Lee *et al.* 1986) through the H_2 receptor.

Stimulation of the H_2 receptor increases gastric acid secretion and induces the endothelial cells to release platelet aggregation inhibitory factor. Histamine can effect the release of neurotransmitters from the nervous system through the H_3 receptor (Arrang *et al* 1988).

Chemotactic factors

At least four chemotactic factors are recognised in mast cell granules: (1) eosinophil chemotactic factor of anaphylaxis (ECF-A) of 360-390 molecular weight; (2) ECF of 1300-2500 molecular weight; (3) neutrophil chemotactic factor (NCF) with molecular weight of 750000; (4) histamine which is a chemotactic factor for eosinophils and a chemotactic inhibitor for neutrophils (Wasserman 1979).

Serotonin

Serotonin (5-hydroxytryptamine) is found in rodent mast cells, but not in human mast cells. Serotonin acts like histamine and causes vasodilation and increases vasopermeability.

Proteoglycans

Proteoglycans consist of a large core protein molecule with repeating serine and glycine residues. Highly sulphated glycosaminoglycan is attached to this core as a branch. The nature of these negatively-charged side chains distinguishes the variants of proteoglycans such as heparin and chondroitin (Holgate *et al.* 1993). Histamine and enzymes are attached to the side chains by ionic bonds, low charged cationic mediators such as histamine are easily dissociated by ionic exchange (Ca^{2+} or Na^{+}), but highly charged mediators such as neutralised proteases are dissociated slowly. Heparin has effects on lipid metabolism (Kovanen 1995) and angiogenesis (Meininger 1995). Heparin releases a growth factor from endothelium and by binding to these molecules protects them from protease degradation involved in coagulation cascade (Redington 1995). Heparin can inhibit smooth muscle proliferation, protect against oncogenic agents and protect cells against mitogenic stimulation and increase plasmogen and elastase activation (Sigal & Ron 1994). Heparin can also induce osteoporosis (Arnason & Malone 1995).

Enzymes

Mast cells contain a large quantities of enzymes some of which are described below.

Acid hydrolyses

Hydrolyses in mast cells are associated with granules and, despite their putative phagocytic capability (Padawer 1969; Daeron *et al.* 1994; Daeron *et al.* 1993; Otani *et al.* 1982), they may have extra-cellular functions (Sher 1976). Arylsulphatase A and B hydrolyse the sulphated esters of aromatic substrates. These enzymes have been reported in rat mast cells (Schwartz *et al.* 1984). Hexosaminidase and glucuronidase are found in human and rat mast cells granules (Schwartz *et al.* 1979, 1981b, 1984). Elastase is a serine protease which degrades collagen and elastin (glycoprotein in connective tissue) and has been found in human mast cells (Meier *et al.* 1989).

Kininogenase

Kininogenase cleaves the inactive kininogen to form biologically active kinins which increase vasodilation and vasopermeability. This enzyme has been reported in human mast cells (Proud *et al.* 1985).

Proteases

Chymase I, which is found in rat connective tissue mast cells, is distinctive from chymase II from rat mucosal mast cells although there is a considerable homology between the two chymases (Woodbury *et al.* 1978a). The chymases of the human mast cells, on the other hand, are antigenically unrelated to rat mast cell chymases (Wintroub *et al.* 1984).

Tryptase, the most prominent protease of the human mast cells, cleaves the human C3 into its complement peptides C3a, C3b and inactivates the kininogen

(Maier *et al.* 1983). This latter action along with heparin anticoagulation activity may enhance the role of the mast cells on clot inhibition (Schulman 1993).

Carboxypeptidase is a metalloexopeptidase protease which is found in rat, mouse and human mast cells and cleaves COO-terminal amino acid residues (Schulman 1993).

Oxidative enzymes of mast cells

Superoxide dismutase converts superoxide radicals (O_2^-) to hydrogen peroxide (H_2O_2). This enzyme can be obtained from mast cell granules (Henderson *et al.* 1979). The mast cell peroxidase is functionally similar to eosinophil peroxidase (Henderson & Kaliner 1979), and catalyses the formation of water from hydrogen peroxide.

Arachidonic acid metabolites (Eicosanoids)

Membrane disturbances of mast cells produce arachidonic acid from phospholipids. Catabolism of arachidonic acid occurs by two pathways; (1) the cyclooxygenase pathway which extends to prostaglandin and thromboxane production and (2) the lipoxygenase pathway resulting in leucotriene production.

Leucotrienes

The lipoxygenase pathway, following mast cell activation, leads to the production of four leucotrienes LTB4, LTC_4, LTD_4 and LTE_4. The leucotrienes LTC_4, LTD_4 and LTE_4, previously called slow-reacting substance of anaphylaxis (SRS-A), cause venule and capillary dilation.

Prostaglandin and thromboxanes

Along the cyclooxygenase pathway, PGD_2, PGE_2 and small quantities of thromboxanes are generated (Stewart *et al.*1996; Wasserman *et al.* 1979). PGD_2 causes smooth muscle contraction, platelet aggregation, is chemotactic for

neutrophils and causes neutrophil infiltration. PGD_2 also causes vasodilation and increased vasopermeability. Thromboxanes, on the other hand, cause broncho-vaso constriction (Stewart *et al.* 1996).

Acetylated phospholipid metabolites

Platelet activating factor (PAF) is produced by mast cells and some other cells from 2-acetylated phospholipids. PAF is a potent spasmogen (Findlay *et al.* 1981), causes platelet aggregation (O'Flaherty & Wykle 1983) is also a chemotactic factor for eosinophils (Wardlaw *et al.* 1986).

Interleukins

Cytokines are proteins or glycoproteins which exert their effect by means of cytokine receptors on target cell surfaces. Cytokines play a communication role in the immune system and keep immune elements in touch. Cytokine participation is important for the regulation of the immune response and for modulation of inflammation. Cytokines act in concert with other substances. Mast cells are a rich source of cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF), tumour necrosis factor (TNF-α), interferon (IFN-γ), interleukins (IL-1, IL-2, IL3, IL4, IL5, IL6,) and macrophage inflammatory proteins (MIP-family) (Metcalfe *et al.* 1992, Gordon *et al.* 1990a), IL8 (Moller *et al.* 1993). Upon immunological stimulation mast cells generate an array of multifunctional cytokines, and it is not surprising that mast cells can contribute to the immunology and pathology of a large variety of diseases such as parasite infection, allergic disorder, fibrosis, neoplasm, physiology of tissues remodelling, wound repair, angiogenesis and clotting.

1.4.5.5. Mast cell activation

Cross-linking a pair of IgE-receptors on mast cells with appropriate antigen and IgE antibody, triggers the membrane associated enzymes such as

methyltransferase and adenylate cyclase. Methylation of the membrane phospholipids facilitates the Ca^{+} intake and histamine release through the mast cell plasma membrane. Increases in cyclic AMP and activation of adenylate cyclase and cyclic AMP-dependent kinase may causes the histamine release from mast cells (Holgate *et al.* 1980). Additionally phospholipids in the plasma membrane can be changed (through methylation) to phosphatidylcholine, the cleavage of phosphatidylcholine by phospholipase terminates in arachidonic acid production (Ischizaka & Ishizaka 1984).

1.4.5.6. The role of mast cells in schistosomiasis

Mast cells are involved in schistosome immunopathology as multifunctional cells. They may also be involved in immune resistance (Dean *et al.* 1976). The elevation of IgE level, eosinophils and mast cell numbers is a hallmark of schistosomiasis mansoni. Large arrays of preformed materials such as histamine, serotonin, heparin, enzymes and newly formed lipid derived mediators and a large number of cytokines released from mast cells through high affinity receptor FcεRI activation by IgE antibody association (Capron & Capron 1994), can orchestrate the host's immune response in schistosomiasis (Lee *et al.* 1986). Destruction of the schistosomula during skin penetration may be an important immune reaction against the schistosomiasis. Mast cells, by association with the protective IgE antibody (Hagan 1992) may produce local anaphylactic reaction and significantly hinder a large proportion of viable schistosomula during skin migration (Gerken *et al.* 1984 & 1990a & 1990b). IL-2 and TNF-α clearly play an important role in granuloma formation (Amiri *et al.* 1992; Chikunguwo *et al.* 1991), mast cells may be contribute directly in granuloma formation through TNF-α production (Gordon & Galli 1990b). In the schistosome induced granuloma (Epstein *et al.* 1979; Weinstock 1992) mast cells increase the size of granuloma in the presence of cimetidine (Weinstock *et al.* 1982) These experiments offer further evidence for involvement of

mast cells and histamine in granuloma formation. Histamine affects lymphocytes, macrophages and eosinophils through H1 receptor and suppressor T cell responses through H_2 receptors in granulomas (Weinstock 1992). Mast cells, by producing the IL-5 (Gordon 1990a) are involved in eosinophil proliferation and migration. Mast cells can express ligands for CD40 (CD40L) on its surface and in the presence of IL-4 can stimulate the B cells directly to IgE production. Basophils can do this without exogenous IL-4 (Gauchat *et al.* 1993). The important role of mast cells in ADCC has been observed already (Butterworth *et al.* 1977, 1975). Mast cells contribute to schistosomiasis by, macrophage mediated cytotoxicity through its granulocyte / macrophage colony-stimulating factor (GM-CSF) production (Marshal & Bienenstock 1990a). neutrophils recruitment (Anwar *et al.* 1979; Dean *et al.* 1974) through IL-8 production (Moller *et al.* 1993). Mast cells assist the platelets in killing schistosomula (Joseph *et al.* 1983) by PAF production (Lee *et al.* 1985). The involvement of mast cells in fibrosis (Claman 1985; Gordon *et al.* 1990a) emphasises the important role of these cells in the processing of the granulomas. Mast cells by having an array of putative capabilities such as phagocytic activities (Lobell *et al.* 1994; Daeron *et al.* 1994 & 1993; Otani *et al.* 1982), antigen presenting (Frandji *et al.* 1993), nerve growth factor production (Leon *et al.* 1994), regulation of immune responses (Garovoy *et al.* 1983) may play a crucial role in immunology and immunopathology of the schistosomiasis, allergic disorders and infectious diseases.

1.5. Neuronal influence

1.5.1. Nerve system

Collectively, the neurones which are found in the brain and spinal cord are called the central nervous system, whilst those lying in other parts of the body are considered the peripheral nervous system. The sympathetic and parasympathetic

nervous system which work involuntarily are called the autonomic nervous system. The sympathetic system originates from thoracic and upper lumbar spinal cord Thoracolumbar system and the parasympathetic system originates from brain and sacral cord (Craniosacral) system. The sympathetic and parasympathetic nervous system make up the peripheral nervous system.

On the other hand the voluntary or somatic peripheral nerves, consist of 12 pairs of cranial and 31 pairs of spinal afferent and efferent nerves. Cranial nerves are confined to the neck and head. The vagus nerve in this group is the only exception, it is a part of the parasympathetic system and acts on the heart and gut by giving off branches to those organs through the thorax and abdomen. Complex constructions of synapses called ganglia are established along the pathways of each sympathetic and parasympathetic nerve fibre passing toward the organs in the body.

The position of these ganglia relates to the structural differences between the sympathetic and parasympathetic nerve systems, in the sympathetic nerve system that ganglia lie much nearer to the spinal cord so that the length of the pre-ganglionic fibres (distance between central nervous system and ganglion) is less than postganglionic fibres (distance between ganglion and effector organ), whilst in parasympathetic nerves ganglia are situated in the wall of the effector organs so that the pre-ganglionic fibres are much longer than the postganglionic fibres. Acetylcholine is the major transmitter substance of the parasympathetic and sympathetic fibres while noradrenaline is the neurotransmitter of the postganglionic sympathetic fibres.

Sympathetic postganglionic and parasympathetic preganglionic fibres are extrinsic nerves for the enteric nervous system. The sensory nerves project from the visceral organs to form the afferent nerves of autonomic nervous system. These afferent nerves are transmitted by the vagus and the pelvic nerves and are called parasympathetic afferents (Schmidt 1976a; Brown 1994). The enteric nervous system consists of those nerves that have their cell bodies situated within the

gastrointestinal tract (McKay *et al.* 1994a). The ends of both pre-and postganglionic fibres of the extrinsic nerves are joined to the enteric nervous system. The enteric nervous system comprises of sensory neurones, interneurons and motor neurones. The motor neurones of the enteric nervous system are the postganglionic parasympathetic neurones which receive nerve pulses from the enteric sensory neurones, enteric interneurons and extrinsic sympathetic and parasympathetic fibres (Noback 1996). Myenteric plexuses (which exist between the longitudinal and circumferential muscle layer) and submucosal plexus (situated in the submucosa of the intestine) are the two major plexuses in the enteric nervous system (Neutra 1993). The cell bodies of the sensory neurones are located in the myenteric and Meissner's plexuses and processes of the cells from the plexuses are distributed in the submucosa and mucosa layers. Intrinsic neurones maintain the alimentary activities either by integration with the central nerve system or independently. The digestive system enteroendocrine cells which are laid between the epithelium and exposed to the lumen of the digestive tube, are like sensory cells when stimulated with antigens as they release neuropeptides that co-ordinate with the intrinsic nerve fibres (reviewed by Schmidt 1976b; Gershon 1981; McKay 1994b; Wilson 1992). Close morphological associations between mast cells and neuropeptide-containing myelinated and non-myelinated nerves (Stead *et al.* 1989; Newson *et al.* 1983; Stead *et al.* 1987; Skofitsch *et al.* 1985; Weisner-Manzel *et al.* 1981; Tanaka *et al.* 1977; Hukkanen *et al.* 1991; Dimlich 1984), the effect of neuropeptides on mast cell degranulation (Assem *et al.* 1989; Dimitriadou *et al.* 1991; Shanahan *et al.* 1985). The influence of mast cell induced nerve growth factor NGF on the function of the nervous system (Leon *et al.* 1994), the differentiation of certain neurones (Marshall & Bienenstock 1994) by mast cell produced leukemia inhibitory factor (LIF) (Marshall *et al.* 1993), and the release of as many as 20 neuropeptides and neurotransmitters such as acetylcholine (ChAT), norepinephrine, epinephrine, calcitonin gene-related peptide (CGRP), cholecystokinin (CCK), neuropeptide-Y (NPY), histamine, somatostatin (SOM),

substance-p (SP), serotonin, neurokinin A, neurotension, vasoactive intestinal peptide (VIP), enkephalins, gamma amino butyric acid (GABA) from the end of the autonomic neuronal fibres (Cuello *et al.* 1978; Dalsgaard *et al.* 1983; Hartschuh *et al.* 1983; Landis *et al.* 1986; Hubel 1989 & McKay *et al.* 1994), strongly supports the interaction of mast cells with the nervous system during inflammation and suggests a much wider role for mast cells in both health and disease. Although mast cells are known to be prominent cells in the inflammatory reaction such during parasitic and allergic disease, the role of factor or factors in the process of mast cell accumulation from progenitor populations in either the peripheral blood or tissues remain a matter worthy of additional research.

1.6. Research objectives

From the above review it is clear that mast cells respond in a variety of ways in inflammation and in the function of the immune system. The manner in which mast cells have their effects remains unclear and not always well understood, a conclusion that applies during infection with endoparasitic helminths including schistosomes. By means of experimental infections of *Schistosoma mansoni* in laboratory mice, the following research objectives were set, in an attempt to elucidate the role of mast cells during schistosomiasis:

1- Develop a suitable histological method for studying mast cells and eosinophils in tissues;

2- Develop and evaluate a histological method for studying mast cells and eosinophils in the tissues simultaneously;

3- Develop quantitative methods for counting mast cells in various tissues;

4- Apply these techniques to study the distribution and dynamics of mast cells during schistosomiasis mansoni;

5- Investigate the migration of mast cells toward *Schistosoma*-induced tissue injury under the influence of the nervous system.

Chapter 2

Material and Methods

2.1 Histological methods

2.1.1. Stains and their preparation

Alcian blue

1g Alcian blue (BDH, Gurr).

100 ml 0.7N HCl.

Stir, filter through Whatmans No.1 filter paper adjust to pH 0.3.

Alcian green

1g Alcian green (BDH, Gurr).

100 ml 0.7N HCl.

Stir, filter through Whatmans No.1 filter paper adjust to pH 0.3.

Aniline blue

0.5 g Aniline blue.

100 ml distilled water.

Stir, filter through Whatmans No.1 filter paper.

Astra blue

1g Astra blue (BDH, Gurr).

100 ml 0.7N HCl.

Stir, for several hours.

Filter through Whatmans No.1 filter paper adjust to pH 0.3.

Brilliant blue

0.5 g Brilliant blue.

100 ml distilled water.

Stir, filter through Whatmans No.1 filter paper.

Chromotrope R

Phenol	1g
Chromotrope 2R (BDH Gurr 3402)	0.5g
Absolute ethanol	10ml
Distilled water	100ml

Melt the phenol by warming in a flask in a water bath. Add the chromotrope and mix to form a slurry. Add the alcohol and continue mixing, finally add the distilled water and filter through Whatmans No. 1 filter paper.

Eosin Y

Eosin Y	1g
Potassium dichromate	0.5g
Absolute ethanol	10ml
distilled water	80ml
Saturated aqueous picric acid	10ml

Dilute 1 : 3 in distilled water before use.

Fast green

0.5 g Fast green.

100 ml distilled water.

Stir, filter through Whatmans No.1 filter paper.

Haematoxylin

Haematoxylin	2.5g
Absolute ethanol	25ml
Potassium alum	50g
Distilled water	500ml
Mercuric oxide	1.25g
Glacial Acetic acid	20ml

Light green

0.5 g Light green.

100 ml distilled water.

Stir, filter through Whatmans No.1 filter paper.

Lugol's solution

Iodine	1g
Potassium iodine	2g
distilled water	100ml

Safranin

Safranin O (BDH, Gurr 34067)	0.5g
0.125 N HCl	100ml

Toluidine blue (pH 0.5)

Toluidine blue (BDH, Gurr)	0.5-1g	(depending on required concentration)
0.5 N HCl	100ml	

Stir, filter through Whatmans No.1 filter paper.

2.1.2 Staining methods

AAC (Astra blue, HCl, Chromotrope)

1. Deparaffinise in xylene 8 min.
2. Rehydrate in graded alcohols to distilled water.
3. Stain for 30 min in 1% astra blue pH 0.3.
4. Rinse in tap water.
5. Wash in 0.7 N HCl for 5 -10 min.
6. Rinse in tap water.
7. Flood the surface of the slide with 0.5 % chromotrope for 30 min.
8. Briefly wash in tap water.
9. Dehydrate the sections quickly not more than 30 s in each alcohol.
10. Blot on soft tissue.
11. Place in xylene, 3 min.
12. Mount in DPX.

AC (Astra blue, Chromotrope)

1. Deparaffinise in xylene 8 min.
2. Rehydrate in graded alcohol: absolute / 90%/ 70%/ 50%/ 30%; 2.5 min, in each

then into distilled water for not more than 1.5 min.

3. Transfer the sections into 1 % Astra Blue for 30 min.

4. Rinse the sections in tap water until slide is clear and extra stain has been removed.

5. Wash for 1 min in alkaline solution, such as Scott's tap water.

6. Rinse in tap water.

7. Flood the surface of the slide with chromotrope and leave for 30 min.

8. Briefly wash in tap water to remove excess stain.

9. Dehydrate the sections quickly, not more than 30 s in each of 30 %/ 50 %/ 70 %

/90% and absolute ethanol.

10. Blot in soft tissue.

11. Place in xylene 3 min.

12. Mount in DPX.

ACH (Astra blue, Chromotrope, Haematoxylin)

1. Deparaffinise in xylene 8 min.

2. Rehydrated in graded alcohol to distilled water.

3. Stain for 30 min in 1% astra blue pH 0.3.

4. Rinse in tap water.

5. Stain with chromotrope 30 min.

6. Wash in tap water, briefly.

7. Stain with haematoxylin, 1 min.

8. Wash in tap water.

9. Dehydrate the sections quickly not more than 30s in each of 30 %/ 50 %/ 70 %/ 90% and absolute ethanol.

ACL (Astra blue, Chromotrope, Lugol's)

1. Deparaffinise in xylene 8 min.

2. Rehydrate in graded alcohol to distilled water.

3. Stain for 30 min in 1% astra blue pH 0.3.

4. Rinse in tap water.

5. Stain with chromotrope 30 min.

6. Wash in tap water.

7. Place in Lugol's solution, 1 min.

8. Wash in tap water.

9. Dehydrate in graded alcohol to distilled water.

10. Place in xylene, 3 min and mount.

ACS (Astra blue, Chromotrope I)

1. Deparaffinise in xylene 8 min and rehydrate in graded alcohol: absolute/ 90%/ 70%/ 50%/ 30%; 2.5 minutes in each, then into distilled water for not more than 1.5 minutes.

2. Transfer into 1 % Astra Blue pH 0.3 for 30 min.

3. Rinse the sections in tap water until the slide is clear and extra stain has been removed.

4. Flood the surface of the slide with chromotrope and leave for 30 min.

5. Briefly wash in tap water to remove excess stain.

6. Place the sections into 1 % sodium thiosulphate for 30 s.

7. Dehydrate the sections quickly not more than 30 s in each of 30 %/ 50 %/ 70 %/ 90% and absolute ethanol.

8. Blot on soft tissue.

9. Place in xylene, 3 min.

10. Mount in DPX.

AHAC (Astra blue, Haematoxylin, Chromotrope II)

1. Deparaffinise in xylene 8 min and rehydrate in graded alcohol to distilled water.

2. Stain with 1% Astra blue pH 0.3 for 30 min.

3. Rinse in tap water.

4. Wash in 0.7 N HCl, for 5 -10 min.

6. Rinse in tap water.

7. Stain with haematoxylin 2.5 min.

8. Wash in Scott's water 1 min.

9. Stain with chromotrope 30 min.

10. Dehydrate in graded alcohol, quickly, not more than 1 min in each alcohol.

11. Blot on saft tissue.

12. Place in xylene 3 min.

13. Mount in DPX.

AHC (Astra blue, Haematoxylin, Chromotrope I)

1. Deparaffinise in xylene 8 min and rehydrate in graded alcohol to distilled water.

3. Staine with 1% astra blue pH 0.3 for 30 min.

4. Rinse in tap water.

5. Stain with haematoxylin,1 min.

6. Wash in tap water.

7. Counter stain with 0.5% chromotrope for 30 min.

8. Briefly dehydrate in graded alcohol not more than 1 min in each alcohol.

AHS (Astra blue, Haematoxylin)

1. Deparaffinise and rehydrate in graded alcohol to distilled water.

2. Stain with 1 % Astra Blue, for 30 min.

3. Rinse the sections in tap water.

4. Stain with haematoxylin for 1 min.

5. Wash in Scott's tap water 1 min.

6. Dehydrate.

7. Blot on soft tissue.

8. Place in xylene, 3 min.

9. Mount in DPX.

ALC (Astra blue, Lugol's, chromotrope)

1. Deparaffinise in xylene 8 min and rehydrate in graded alcohols to distilled water.

2. Stain for 30 min in 1% astra blue.

3. Rinse in water.

4. Stain with Lugol's 1 min.

5. Wash in tap water.

6. Counter stain in 0.5% chromotrope for 30 s.

7. Briefly wash in tap water.

8. Dehydrate in graded alcohol 1 min each.

AlGC (Alcian green, Chromotrope)

1. Deparaffinise in xylene 8 min and rehydrate in graded alcohols: absolute/ 90%/ 70%/ 50%/ 30%; 2.5 minutes in each, then into distilled water for not more than 1.5min.

2. Transfer into 1 % Alcian green for 30 min.

3. Rinse in tap water to remove excess stain.

4. Wash in Scott's tap water, 1 min, if this is necessary.

5. Rinse in tap water.

6. Flood the surface of the slide with chromotrope and leave for 30 min.

7. Briefly wash in tap water to remove excess stain.

8. Dehydrate the sections quickly, not more than 30 s in each of, 30 %/ 50 %/ 70 %/ 90% and absolute ethanol.

9. Blot on soft tissue.

10. Place in xylene 3 min.

11. Mount in DPX.

AIGS (Alcian green,Safranin)

1. Deparaffinise in xylene 8 min and rehydrate in graded alcohols to distilled water.

2. Stain with 1% alcian green, for 30 min.

3. Rinse in tap water.

4. Wash in 0.7 N HCl for 5 -10 min.

5. Rinse in tap water.

6. Counter stain with 0.5% safranin, for 30 s.

7. Dehydrate in graded alcohol.

8. Blot on soft tissue.

9. Place in xylene 3min.

10. Mount in DPX.

ANC (Aniline blue, Chromotrope)

1. Deparaffinise in xylene 8 min and rehydrate in graded alcohols to water.

2. Transfer into 0.5 % Aqueous aniline Blue for 30 min.

3. Rinse in tap water.

4. Flood the surface of the slide with 0.5 % chromotrope for 30 min.

5. Briefly wash in tap water and dehydrate the sections.

6. Blot on soft tissue.

7. Place in xylene 3 min.

8. Mount in DPX.

AS (Astra blue, HCl, Safranin)

1. Deparaffinise in xylene 8 min and rehydrate in graded alcohols to distilled water.

2. Stain with 1% astra blue for 30 min.

3. Rinse in tap water.

4. Wash in 0.7 N HCl for 5 -10 min.

5. Rinse in tap water.

6. Counter stain with 0.5% safranin for 30 s.

7. Dehydrated in graded alcohol 1 min in each.

8. Blot on soft tissue.

9. Place in xylene 3 min.

10. Mount in DPX.

BRC (Brilliant blue, Chromotrope)

1. Deparaffinise in xylene 8 min and rehydrate in graded alcohols to distilled water.

2. Stain with 0.5% Aqueous Coomassie brilliant blue R (Sigma B-0630) for 10 min.

3. Rinse in tap water.

4. Wash in 0.7 N HCl for 5 -10 min.

5. Rinse in tap water.

6. Counter stain with 0.5% Chromotrope for 30 min.

7. Dehydrate in graded alcohols.

8. Blot on soft tissue.

9. Place in xylene 3 min.

10. Mount in DPX.

Ch (Chromotrope)

1. Deparaffinise in xylene 8 min and rehydrate in graded alcohols to distilled water.

2. Flood the surface of the slide with 0.5 % chromotrope for 30 min.

3. Briefly wash in tap water

4. Dehydrate quickly, not more than 30 s in each of 30 %/ 50 %/ 70 %/ 90 % and absolute ethanol.

5. Place in xylene 3 min.

6. Mount in DPX.

FC (Fast green)

1. Deparaffinise in xylene 8 min and rehydrate in graded alcohols to distilled water.

2. Stain with 0.5% Aqueous Fast green (Michrome-135) for 10 min.

3. Rinse in tap water.

4. Wash in 0.7 N HCl for 5 -10 min.

5. Rinse in tap water.

6. Counter stain with 0.5% Chromotrope for 30 min.

7. Dehydrate in graded alcohols.

8. Blot on soft tissue.

9. Place in xylene 3 min.

10. Mount in DPX.

HAC (Haematoxylin, Chromotrope)

1. Deparaffinise in xylene 8 min and rehydrate in graded alcohols to distilled water.

2. Stain with haematoxylin, 3-4 min.

3. Wash in tap water.

4. Differentiate in acid alcohol.

5. Wash in tap water.

6. Place in Scott's tap water until slide changes to blue.

7. Wash in tap water.

8. Examine using microscope (differentiate stain further, if necessary).

9. Counter stain with 0.5 % chromotrope, 30 min.

10. Differentiate in tap water.

11. Dehydrate through graded alcohols.

12. Place in xylene 3 min.

13. Mount in DPX.

HAE (Haematoxylin, Eosin)

1. Deparaffinise in xylene 8 min and rehydrate in graded alcohols to distilled water.

2. Stain with haematoxylin, 3-4 min.

3. Wash in tap water.

4. Differentiate in acid alcohol.

5. Wash in tap water.

6. Place in Scott's tap water until slide changes to blue.

7. Wash in tap water.

8. Examine using microscope (differentiate stain further, if necessary)

9. Counter stain with eosin, 3 min.

10. Differentiate in tap wate.

11. Dehydrate through graded alcohols.

12. Place in xylene 3 min.

13. Mount in DPX.

LGC (Light green, Chromotrope)

1. Deparaffinise in xylene 8 min and rehydrate in graded alcohols to distilled water.

2. Stain with 0.5% Aqueous light green (Gurr 22501) for 10 min.

3. Rinse in tap water.

4. Wash in 0.7 N HCl for 5 -10 min.

5. Rinse in tap water.

6. Counter stain with 0.5% Chromotrope for 30 min.

7. Dehydrate in graded alcohols.

8. Blot on soft tissue.

9. Place in xylene 3 min.

10. Mount in DPX.

PAC (Astra blue, Chromotrope II)

1. Deparaffinise in xylene 8 min and rehydrate in graded alcohols to distilled water.
2. Stain with 1 % Astra Blue, pH 0.3 for 30 min.
3. Rinse in tap water.
3. Flood the surface of the slide with 0.5 % chromotrope for 30 min.
4. Briefly wash in tap water.
5. Dehydrate through graded alcohols.
6. Blot on soft tissue.
7. Place in xylene 3 min.
8. Mount in DPX.

TO (Toluidine blue 0.5 %, pH 0.5)

1. Deparaffinise in xylene 8 min and rehydrate in graded alcohols to distilled water.
2. Stain with 0.5% Toluidine blue for 30 min.
3. Wash in tap water.
4. Differentiate in 95 % alcohol.
5. Dehydrate in graded alcohol.
6. Blot on soft tissue.

7. Placed in xylene 3 min.

8. Mount in DPX.

TO1 (Toluidine blue 1 %, pH 0.5), modification of Yam *et al.* 1971

1. Fixed in MOT, 1 min.

2. Stain with 1 % aqueous toluidine blue, for 5 min.

3. Wash in tap water.

4. Dry in air.

5. Mount in DPX.

2.1.3. **Tissue processing** (Gordon 1990)

1. Tissues are placed in a tissue processing baskets and washed under tap water.

2. The baskets were transferred to an automatic tissue processing machine (Shandon) consisting of 10 chambers. The chambers move vertically and rotate the tissues into different solutions. Processing takes 24 h and the time tissues spend in each solution is pre-programmed (Routine processing was as follows).

3. 70% Alcohol, 3 hours.

4. 70% Alcohol, 3 hours.

5. 90% Alcohol, 3 hours.

6. 8% phenol and 99 % Alcohol, 2 hours.

7. Absolute alcohol, 2 hours.

8. Absolute alcohol, 2 hours.

9. Histoclear I, 2 hours.

10. Histoclear II, 1 hour.

11. Wax I, 3 hours.

12. Wax II, 3 hours.

13. Wax blocks were made by placing the tissues in liquid paraffin.

14. Wax blocks containing tissue were fixed to wooden blocks.

15. Sections were cut from the blocks with a microtome.

16. Sections were laid on the surface of warm water in a 50° C water bath.

17. Flattened sections were lifted to a glass slide and kept at 60° C one hour

2.2.3.1. Rehydration

1. Xylene, 10 min.

2. Absolute alcohol, 1.5 - 2.5 min.

3. 90 % alcohol, 1.5 - 2.5 min.

4. 70 % alcohol, 1.5 - 2.5 min.

5. 50 % alcohol, 1.5 - 2.5 min.

6. 30 % alcohol, 1.5 - 2.5 min.

7. Distilled water, 3 min.

2.1.3.2. Dehydration

1. Distilled water, 1 min.
2. 30 % alcohol, 1 min.
3. 50 % alcohol, 1 min.
4. 70 % alcohol, 1 min.
5. 90 % alcohol, 1 min.
6. Absolute alcohol, 1 min.
7. Xylene, 3 min.

2.1.3.3. Swiss roll method

The small intestine was dissected from the mouse abdomen and placed on a piece of tissue paper soaked with saline. The proximal and distal ends of the intestine are marked on the paper. Care was taken to cut the small intestine longitudinally along the mesenteric border. The inside of the intestine was immediately cleaned and the whole intestine was immersed in Carnoy's fixative two seconds. This helped to harden the tissue making it easier to form a roll. Some infected intestines are very thick and bosselated (granulated). As rolling up of the whole intestine and processing in one piece was impractical due to the quantity of tissue which would make the latter stages of blocking and sectioning difficult, the intestine was divided in two and the Swiss roll technique was carried out for each piece of infected intestine (control intestines

generally did not need to be divided). Great care was taken to ensure that the orientation of the intestine was not lost. The outer surface (serosal) of the duodenal end of the small intestine was attached to the end of an orange stick " wooden applicator " (the end of the stick must be cleaned and dried with a piece of paper prior to use) two tiny points of the intestine must be pressed using forceps on to the surface of the stick until they adhered firmly. The intestine was rolled up around the stick by carefully and slowly rotating the stick around its axis. Meanwhile gradually the stick was moved toward the distal part of the intestine. Care was taken to ensure that the intestine turned around the stick in a tidy and concentric fashion. To prevent damage of the villi the concentric layers should not be coiled too tightly and the intestine should be layered evenly around the axis of the stick. When the action of rolling was completed two points on each side of the width of the intestine were pressed onto the lower layer. The rolled intestine was then placed on its side on the wet paper. By carefully turning the stick in the opposite direction, the stick was released and pulled out from the rolled intestine. Each piece of the intestine was marked carefully and placed in a circular metal mesh basket 2cm in diameter and 2.5 cm in depth. They were then ready to be put into the chosen fixatives.

2.1.4. Cell suspension

2.1.4.1. Nerve cell suspension

The brain and large part of the spinal cord of a female *Schistosoma mansoni*-infected BALB/ C mouse was removed under sterile conditions after killing the animal. The tissue was homogenised in a mortar for a half an hour and the supernatant was collected by centrifugation. The solution was stored in a refrigerator and used within 24 h. Serial dilution's were made from the extract of brain and spinal cord as 1/3, 1/9, 1/27, 1/81, 1/243, 1/729, 1/2187, 1/6561, 1/19683.

2.1.4.2. Leukocyte preparation

2.1.4.2.1. Blood collection

The *Schistosoma mansoni*-infected female mice were killed with CO_2 inhalation and the carcasses were placed on their back on the table. A 20 G needle and a heparin loaded syringe were used. The syringe was rinsed with heparin and the plunger checked for free movement before the needle was inserted at 45° from the tip of the sternum and directed forward into the heart until the blood entered into the syringe. Withdrawal of blood continued slowly.

2.1.4.2.2. Leukocyte isolation

0.5 ml heparinized blood was collected and diluted with saline to 6 ml. This was layered down the wall of a 15 ml centrifuged tubes on to 3ml of NycoPrep^{TM} 1.077 Animal (Nycomed). The tube was capped and centrifuged at 2400 rpm (600 xg) for 15 min, the upper layer was decanted and the white cells were collected from the interface and bottom layer. Cells were then washed twice with saline by centrifugation at 400 x g.

2.1.4.3. Other organ suspensions

Organs such as brain, heart, lung, liver, spleen and mesentery were removed from the *Schistosoma mansoni*-infected animals and teased apart as described for the spleen cell preparation. Smears were made from the cell suspensions using a cytospin centrifuge.

2.1.4.4. Tissue culture cell suspension

Culture medium was collected from the flask into 15 ml plastic capped sterile tubes, centrifuged and washed 200 x g 10 min. Pelleted cells were resuspended in approximately 100 µl of medium.

2.2. Animal techniques

2.2.1. Animals

Mice were obtained as required from Harland UK Ltd., all were kept in the J.A.F licenced by the UK Home Office. Further details are set out in table.1.

Table 1. Animal (mice).

Strain of mice	Sex	Age when used	Use
BALB/C	Male and Female	8-16 week	Histology Bone marrow culture
C57B1/6J	Male	8-12 week	Spleen media
C3H	Male	8-12 week	Spleen media
CFLP	Female	10 week	Granuloma, mast cell and eosinophils kinetics

2.2.2. Infection with *Schistosoma mansoni*

The mice were anesthetized by injecting with 0.01 ml / g of body weight of Sagatal (see buffers and solutions). The skin of the stomach was shaved completely with an electric shaver and the animals were laid between grooved wooden supports and the skin was soaked with water. A metal ring was put on the shaved part of the skin of each animal and about 100 live cercariae (kindly supplied by Professor John Kusel, Division of Biochemistry and Molecular Biology) were placed into each ring.

Cercariae were left in contact with the skin for approximately 30 min after which animals were returned to their cages to recover from anaesthesia.

2.2.3. Perfusions

Mice were killed by CO_2 inhalation. The abdominal and thoracic cavities of the mice were opened. Animals were fixed on a Perspex sheet, supported in the vertical position by a metal clamp. The hepatic portal vein was cut and the blood circulation was flushed with by 50 ml of saline by inserting with a 20 - g needle into the heart (left ventricle) of animal. The worms were collected on the two plastic and metal meshes which were positioned horizontaly below the apparatus.

2.3. Parasitological methods

2.3.1. Worm collection

Worms recovered by perfusion were maintained in RPMI medium for morphological study and preparation of crude or fractionated worm antigens.

2.3.2. Egg isolation (Coker & Lichtenberg 1956)

Schistosoma mansoni-infected BALB/C mice were killed for perfusion, the carcasses were kept 4 days in refrigerator. The livers were removed and crushed completely in a electrical blender with 50 ml of 1.7 % NaCl aqueous solution. The resulting sticky white homogenate was poured through 40 and 80 mesh sieves into a glass flask. The mesh was flushed with more NaCl solution until the flask was filled. Thirty minutes later the contents of the flask were removed by pipetting until only about 3 cm remain. The remainder was washed 5 times with 1.7 % NaCl. After washing the mixture 5 ml was overlaid upon a filtered solution of freshly made 0.5 M

sucrose in a 50 ml standard burette. The end tip of the burette had been cut off for easy flow of the solution through the burette. Five ml of the burette solution was discarded after 15 min of setting up and after 60 min the first 15 ml and the second 15 ml were collected and centrifuged at 500 x g for 1.5 min and washed in 1.7 % NaCl. This clean egg preparation was concentrated in a small volume of 1.7 % NaCl.

2.4. Cell culture

2.4.1. Buffers and solutions

Acid alcohol (Concentrated HCl, 95% Ethanol, 1 : 99).

Bleaching agent (Sodium thiosulphate BDH 30235, distilled water, 1 : 100).

Bicarbonate buffer 0.5 M (Na H CO3 21g / 500 ml distilled water, Na_2 CO_3 26.5 g / 500 ml distilled water, 1 : 1).

Coating solution (Na H CO_3, 0.1 M pH 8.2).

Concanavalin A Sigma (5 mg / 1ml RPMI).

HCl 5 N solution (50 ml of 10N HCl BDH, diluted with 50 ml double distilled water).

HBSS / MOPS solution (HBSS without Ca and Mg Sigma H4641-14H2312 500 ml x 10, Sterile distilled water, HCO3 7.5% Gibco 043-05080, MOPS Sigma 33H57141, 100 : 895.3 : 4.7 : 2) pH 7.2 adjusted with 5N- Na OH.

IL-3 was kindly supplied by Dr Richard Grencis from the School of Biological Science, University of Manchester and Dr Mary Freshney from Department of Medical Oncology, CRC Beatson Laboratories, University of Glasgow.

L- Glutamine (Gibco 043 - 05030 D, 200 m M).

2 **Mercaptoethanol** Sigma M-6250, 14.3 M (diluted to 0.05 M, One microlitre in 1 ml will yield a 50 μ M solution, this solution was filtered through a 0.22 μm pore filter prior to use).

Na OH 5 N solution (50 ml of 10N Na OH BDH, diluted in 50 ml sterile double distilled water).

1.7% Na Cl solution (Na Cl, double distilled water, 17 : 1000) filtered through Whatmans No 1 filter paper.

Non-essential amino acid Gibco-11140-035, 100 ml 9.99 mM , (having the final concentration of 0.1 mM 0.01 ml should be used in 1 ml).

PBS / Tween solution Tween-20 Sigma P-1379, (0.5 ml / 1L of PBS pH 7.0).

Penicillin and streptomycin 100 ml, Gibco 043-05140 D (10000 unit penicillin / 10000 μg streptomycin).

Phosphate buffer saline 0.15 M, pH 7.0, (NaCl 8g, Na_2HPO_4 1.16g, KH_2PO_4 0.2g, KCl 0.2g, Distilled water 1L pH 7.0).

Phosphate buffer saline 0.15 M, pH 7.4, (NaCl 8g, Na_2HPO_4 1.16g, KH_2PO_4 0.2g, KCl 0.2g, Distilled water 1L pH 7.4), solution is autoclaveable.

Primary anti-mouse IgE capture m Ab (kindly supplied by Professor John Kusel, Division of Biochemistry and Molecular Biology).

RPMI 1640 (with-L-glutamine, Gibco 041-01875 H).

Sagatal (Rhone Merieux 910014) solution, (Sagatal 5ml , Ethanol 5ml, Sterile distilled water 50ml).

Scott's tap water (Sodium bicarbonate 7g, Magnesium sulphate 40g, Distilled Water 1L).

Secondary biotinylated Anti-mouse IgE m Ab (kindly supplied by Professor John Kusel, Division of Biochemistry and Molecular Biology).

Substrate solution 0.1 M Citrate buffer, (Citric acid 1.05g, Disodium hydrogen phosphate 0.89g, H_2O 100ml, Hydrogen peroxide 50µl, and 34mg Ortho-phenylenediamine are added prior to use).

0.5 M Sucrose solution, (17.11g Sucrose Analar 10274, Double distilled water 100ml).

Sulphuric acid (12 % solution).

2.4.2. Culture media

Mast cell culture medium 200ml, (RPMI medium 86ml, FCS 10ml, non-essential amino acid 1ml, L-glutamine 1ml, 2-mercaptoethanol 100µl, spleen-derived medium 100ml, penicillin & streptomycin 2ml,100U/ml) mixed and pH adjusted to 7.2.

2.4.3. Spleen cell preparation (Mishell & Shiigi 1980)

C3H and C57B1/6J male mice were killed in a CO_2 chamber then immersed in a beaker containing 70% ethanol. The mice were then placed on a paper towel soaked in 70% ethanol in a sterile laminar air flow cabinet and a transverse incision was made in the abdominal skin, the skin was then deflected by pulling towards the head and tail of the animal. The muscle surface was flooded with 70% ethanol. The stomach muscles were cut using forceps and scissors dipped in 95% ethanol and flamed. The spleen was taken out aseptically and was placed in a sterile petri dish containing about 10ml BSS solution (Gibco 14060-040.500 x10), the spleens were crushed with forceps and were pressed between two frozen slides and a single cell suspension was

obtained by sucking the spleen cell solution in and out through a sterile Pasteur pipette. The suspension was transferred to a graduated 15ml sterile centrifuge tube left, for 5-6 min to allow spleen cell clumps to settle to the bottom of the tube and the remaining fluid was transferred to another 15ml centrifuge tube and the cells were centrifuged 10 min at 200 xg. The pelleted cells were resuspended with medium at the required volume. All procedures were done under sterile conditions.

2.4.4. Bone marrow preparation

Animals were killed in a CO_2 chamber, and then dipped in 70% ethanol. In a sterile laminar air flow cabinet the skin was cut across the abdomen and deflected back to expose the hind legs. These were then flooded with 70% ethanol. The legs were disconnected from the body and the feet were cut off. The muscles were then dissected away completely from the femur and tibia in a petri dish containing BSS solution (Gibco 14060-040). The femur was separated from the tibia. The epiphyses were cut and the ends of the bones were punctured by a 20 gauge needle. A 25 gauge needle was then inserted and BSS flushed through the bone using a syringe to free the bone marrow, this being indicated by the white appearance of the bone following the perfusion. The bone marrow was then separated using the needle and syringe, until a single cell suspension was formed. Cells were washed in BSS by centrifugation at 200 xg for 10 mins.

2.4.5. Cell counting

Cells were counted in an improved Neubauer cell counting chamber with Trypan blue solution as diluent to assess cell viability (see below).

2.4.5.1. **Counting of nucleated cells** (with Acetic Acid)

The number of the nucleated cells was counted using acetic acid (3% acetic acid in distilled water). Although acetic acid destroys red blood cells, cell viability can and must be confirmed with trypan blue stain exclusion or microscopic examination.

2.4.5.2. Cell viability

1. Dye

Trypan blue 0.2g

Distilled water 100 ml

Filtered through Whatmans No1 filter paper.

2. Saline 5 N

Mix 4 parts of dye with 1 part of saline, and mix one part cell suspension with one part of diluted dye (solution was made freshly). Cell viability was confirmed by microscopic examination.

2.4.6. Spleen cell culture for spleen cell derived supplemented medium

Mix 879 ml RPMI-medium, 100ml (10%) FCS, 1ml stock 2-Mercaptoethanol (50μM), 10ml stock L-glutamine solution (2mM), 10ml non-essential amino acids (0.1mM), and 0.4ml stock concanavalin A (2μg/ml), pH was adjusted to 7.2. Divided into the 20, 75-cm^2 tissue culture flasks, each 50ml. Add $5x10^7$ nucleated spleen cells from C57B1/6J - C3H male mice to each flask.

Incubate at 37°C in humidified 5% CO_2 / 95% air for 45 hours. Centrifuge the suspension 1000 g, 20 mins and remove the supernatant. Filter through a 0.45 μm filter and divide this spleen-derived medium into aliquots, store frozen until use.

2.4.7. Cell culture for mast cell production.

2.4.7.1. Using spleen-derived medium (Razin *et al.*1981)

Forty ml of mast cell culture medium was put into 75-cm^2 tissue culture flasks (Greiner-labortechnik, 250 ml) and 1 x 10^5 / ml nucleated bone marrow cells from either male or female BALB/C mice (8-16 weeks old) were added into each flask. Incubate at 37°C in humidified 5 % CO_2 and 95% air for 7 days. After 1 week the medium was centrifuged x 200 g 10 min and the pelleted cells resuspended in 1-2 ml medium with a small sample being taken for cell counting and viability determination. 1 x 10^5 / ml of cells were transferred into other flasks containing fresh mast cell culture medium and incubated for another week. At the end of the second week the cells were collected by centrifugation 200 g, 10 min and used after examination for viability.

2.4.7.2. Using IL-3 supplemented medium (Razin *et al.* 1984; Thompson *et al.* 1989).

IL-3 supplemented medium was prepared by mixing 87 ml RPMI, 10ml FCS, 1ml non-essential amino acid, 1ml L-glutamine, 100μl stock 2-mercaptoethanol, 1ml stock penicillin and streptomycin together with 10, 20, 30 or 40 U/ml (IL-3) pH being adjusted to 7.2.

Tissue culture flasks (75-cm^2) were loaded with 40 ml of IL-3 supplemented culture medium together with 1 x 10^5 cells ml^{-1}nucleated bone marrow cells derived from 8-

16 week old BALB/C mice (male and female). The flasks were cultured at 37°C in humidified 5 % CO_2 / 95% air for 7 days. At this time the flasks contents were centrifuged 200 g, 10min and the cells pelleted in 1-2 ml of medium for cell counting and viability assessment.

Cells were re-distributed at 1 x 10^5 cells ml^{-1} in fresh IL-3 supplemented medium and the process repeated at the end of the second week of culture. When IL-3 supplemented medium was used cells were cultured for 3 weeks for mast cell production.

2.5. Immunological methods

2.5.1. Serum preparation

Mouse blood was collected into a small glass cylinder and allowed to clot at room temperature for 1h. Once the clot formed it was loosened from the glass wall and left at 4°C overnight before serum was exracted after centrifuging (200 g,10 min) and then frozen in small aliquots until required.

2.5.2 Total serum IgE measurement. (ELISA, Double Ab sandwich method)

1. Dilute purified anti-mouse IgE capture m Ab (primary Ab, PharMingen Cat No.02111D, clone R35-72) to 2µg / ml in coating solution.

2. Add 50 µl of above antibody solution to wells of an enhanced protein-binding ELISA plate (Nunc immuno plate F96 Maxisorb).

3. Cover the plates and incubate overnight at 4 °C.

4. Wash 2 x with PBS / Tween, wells are filled with PBS / Tween solution and allowed to stand for 1 minute prior to emptying. Pound inverted plate on paper towels.

5. Block plates with 5% dried milk in PBS / Tween solution using 200 µl per well.

6. Cover plate and incubate at room temperature for 2 h.

7. Wash 3 x with PBS / Tween as in step 4.

8. Leave column 1 for blank wells (100 µl PBS /Tween).

9. Add serum samples at various dilutions at 100 µl per well (diluted in 0.5 % dried milk + PBS / Tween), 1/50, 1/100, 1/200 dilutions were used.

10. Cover and incubate for at least 3 h at room temperature or overnight at 4°C.

11. Wash 4 x with PBS / Tween as step 4.

12. Dilute biotinylated anti-mouse IgE m Ab (secondary Ab, PharMingen cat. No. 02122D, clone R35-92 or cat.no. 02132D, R35-118. 1mg/ml) to 2 µg/ml in PBS/Tween (50 µl/25 ml), add 100 µl per well.

13. Cover and incubate at room temperature for 1 hr.

14. Wash 4 x with PBS / Tween as step 4.

15. Dilute avidin-peroxidase in PBS / Tween (Sigma A-3151, 1: 1000 of 1mg / ml solution). Add 100 µl per well.

16. Cover and incubate at room temperature for 30 min.

17. Wash at least 6 x with PBS / Tween as step 4.

18. Substrate (0.1 M citrate buffer), immediately prior to use add 100 µl per well and allow to develop at room temperature (20-30 min). Colour reaction can be stopped by adding 50 µl of Sulphuric acid (12%) which denatures the enzyme.

19. Read plate at OD 492 nm.

2.5.3. Chemotaxis

2.5.3.1. Beads culture

2.5.3.2. Bead preparation

Using the method described by Goes *et al.* (1991), 0.02 g of Biogel P-4 polyacrylamide beads (Bio Gel, Bio RAD 150-4124) were autoclaved in a little acidic distilled water pH 6.0 then swollen in sterile double distilled water for 48 hours at room temperature. The beads were washed twice with bicarbonate buffer 200g, 10min and were then suspended in 45ml bicarbonate buffer shaking gently in a 63°C water bath for 4 h. They were washed twice with sterile double distilled water, bringing the final volume to 1ml bead suspension. Next they are divided in two 0.5ml Eppendorf tubes (each containing 10mg bead) one of these tubes was coated with Substance-P and the other was used as the control. Four and half ml slightly acidic double distilled water pH 6.5-6.8 and 5mg EDAC (Sigma E.6383) was added to the control tube.

One millilitre (1mg) of substance-P and 3.5ml acidic double distilled water pH 6.5 - 6.8 and 5mg EDAC was added to other tube for coating. Both tubes were fastened firmly and were rotated for 18 hours at 4°C. Eventually the beads were washed with 0.15 M sterile PBS pH 7.4 and stored at 4°C, without preservative, until required.

2.5.3.3. Estimation of the bead number

0.02 g beads were suspended in 1ml double distilled water for 48 h then 10 µl of this bead suspension was added to 490 µl water (1/50). 10µl of diluted suspension was used for bead counting. The average of 7 counts was taken and bead numbers were estimated at 5.55×10^5 ml $^{-1}$.

2.5.3.4. Substance-P coated beads and mast cell culture

Mast cell bead cultures were prepared using the method of Bentley *et al.* 1985 and Goes *et al.* 1991. About 50-60 substance-P coated beads in 10µl PBS (pH 7.5) were added into each of the 24 well tissue culture plates. Then, 150 µl RPMI medium containing 10% FCS, 1.6% L-Glutamine and 3% antibiotic was added into each well. The same procedure was followed for control experiments, except that Substance-P free beads were added into each of the wells in 10 µl PBS. 2.5×10^5 viable mast cells in 40µl were collected from the culture and were added into each well. The plates were covered and maintained under humidified conditions at 37°C in 5% CO_2 in 95% air for 5 days. The number of mast cells binding to 50 individual beads in each well was counted. Fifteen wells from the experimental and 15 wells from control group were studied.

2.5.3.5. Polarisation assay

The polarisation assay was carried out using the method of Haston & Shields (1985).

1. Hanks's balanced salt solution (without Ca^{++} and Mg^{++}) with 10 mM morpholinopropanesulphonic acid (MOPS) was made freshly as described in the

chemicals section. The substances to be tested were prepared in appropriate concentrations in 1ml of HBSS / MOPS and were added into each of the 50 conical capped plastic 15ml sterile tubes.

2. Mast cells collected from the culture were washed twice with HBSS / MOPS solution 200 x g . Pelleted cells were resuspended in suitable volume. An aliquot of 50µl was taken for cell counting and cell viability examination.

3. 5×10^5 cells were added to each tube and incubated for 30 min at 37° C.

4. 1ml of 2.5 % glutaraldehyde in HBSS / MOPS was added to the tubes. After 15min the fixed cells were washed twice, 200xg with HBSS / MOPS and resuspended in the remaining HBSS / MOPS and stored at 4° C. About 300-400 cells were examined by phase contrast microscopy under a x 40 objective. Any cell which was longer in one axis than the other, was considered as polarised.

2.5.3.6. Micropore chemotaxis filter assay

2.5.3.6.1. Apparatus

Chemotaxis chambers and filters were provided kindly by Dr Roger Parton from the Microbiology laboratory, Division of Infection and Immunity. The chemotaxis chamber consist of a perspex multiwell (48 wells) tray split horizontally and the filter can be placed between the two pieces. The two pieces can be screwed to each other giving a suitable seal (Neuroprobe).

2.5.3.6.2. Method

1. Cut off the upper left hand side of a filter using scissors. The dull side of the filter is laid uppermost.

2. Affix the rubber seal above the bottom section of the chemotaxis chamber.

3. Introduce 30 µl of putative chemo-attractant into each well and fill showing a slight meniscus. Bubble formation must be avoided because bubbles interfere with cell migration.

4. Transfer the filter using fine forceps onto the bottom plate, (dull side uppermost). Line up the filter in the centre of the plate.

5. Secure the top half of the chemotaxis apparatus to the bottom half by screwing the metal heads on the opposite ends, and leave at 37°C for 10 min in humidified 5% CO_2 incubator

6. Fill the top wells with 51 µl mast cell solution (3×10^5/ml).

7. Leave the plate at 37° C for 45 min in humidified 5 % CO_2 incubator.

8. Remove the screw heads, rise the top plate away and carefully attach the extreme outer edges of the filter with the wide edge clips.

9. Wash the back side (dull side) of the filter with PBS and rub with a rubber policeman or a stick with cotton. Scrape off excess water.

10. Hang the filter up to dry.

11. Fix the filter in methanol for 2 min.

12. Stain the filter in 100% Leishman's stain for 6 min and counter stain in 50 % Leishman's stain for 6 min, wash the filter with tap water.

13. Air dry the filter.

14. Mount on suitable slide with DPX.

15. Count the cells in 5 fields per well.

Chapter 3

Histological Methods for Studying Mast Cells and Eosinophils

3.1. Introduction

Despite the demonstration that mast cells and eosinophils have the capacity to act as effector cells in the immune response to helminth infection (Lee *et al.* 1986; Ferguson & Miller 1979 ; Butterworth *et al.* 1975; Capron & Dessaint 1992), a clear-cut functional role for mast cells during intestinal nematode infections remains questionable. Since in the absence of mast cells, effector mechanisms against intestinal helminths still functions but their action is prolonged (Reed 1989).

On the other hand IgE-mediated activation and degranulation of mast cells with recent evidence of the release of a large number of preformed potent mediators including histamine, heparin and enzymes, and newly formed mediators such as prostaglandin and leukotrienes and a variety of cytokines IL-3, IL-4, IL-5, IL-6, IL-8, TNF-α GM-CSF (Wodnar-Filipowicz *et al.* 1989; Galli *et al.* 1989; Plaut *et al.* 1989; Moller *et al.* 1993; Gordon & Galli 1991), chemokines MIP-1, MCP-1, RANTES (Selvan *et al.* 1994) has renewed interest in the protective and pathologic capacity of mast cells during helminth infection and the involvement of these cells in allergic reactions.

Similarly, the role of eosinophils in protection against infection and their involvement in helminth-induced immunopathology remains a matter for debate (Sher *et al.* 1990). There is evidence that high peripheral blood eosinophil counts

are associated with resistance to reinfection with *S. mansoni* and *S. haematobium* in older children (Sturrock *et al.* 1983; Hagan *et al.* 1985 & 1987). Products released from eosinophils are important in the immunopathology of allergy, particularly in the severe lesions found in the lung. Also eosinophils have the capacity to kill schistosomula *in vitro*, but their function *in vivo* remains unclear.

The fixation and staining of mast cells is based on the nature of the granules they contain. The matrix of these granules consist of strongly polyelectrolyte heparin or chondroitin sulphate which are negatively charged (anionic). The immobilisation of the matrix by an appropriate fixative and its presentation by a suitable cationic dye has been the aim of many scientists since Ehrlich's pioneering studies. As a result structural differences between MMC and CTMC have been determined and the variations in mast cells between species documented. Nowadays most techniques employed to fix and stain mast cells are based on the methods of Enerback (1966a & 1966b, and Bloom & Kelly 1960). Modifications of their methods by Miller & Jarrett 1971; Jarrett *et al.* 1969; Miller & Walshaw 1972; Strobel *et al.* 1981 have improved on the results. Eosinophils have two kind of granules. Azurophilic granules which are few in number and basophilic in character, and eosinophilic granules which contain a dense cationic matrix constitute the majority. Savage & Colley (1980) suggested Maximov's or Bouin's fluids for fixation and Dominici stain for staining of the eosinophils, whilst Bentley *et al.* (1981) suggested 5% glutaraldehyde for fixation and 1% methylene blue for staining. Later Incani & McLaren (1984) used 10% neutral buffered formalin as fixative and either haematoxylin and eosin or haematoxylin and chromotrope for staining (Lendrum 1944)

The demonstration of mast cells and eosinophils in tissues has until recently, required separate fixation and staining methods to differentiate these cell types. Simple staining methods have been described but these have the drawback that the differentiation of the cells requires high magnification (Fisher 1989; Marsh & Hinde

1985). More complicated staining protocols have also had some success (Ball & Hay 1990) including the combined staining method of (Duffy *et al.* 1993) using Astra Blue and Vital Red with formaldehyde fixed human oesophagus.

Given the inherent drawbacks of these methods and the need to be able to differentiate mast cells and eosinophils in tissue sections for studies of their role in the immune response in the intestine, attempts were made to improve on established staining methods for these cell types.

A variety of fixatives and staining protocols have been thoroughly investigated using a number of tissues including liver, spleen, intestine and skin. A modification of the staining methods of Bloom & Kelly (1960) for mast cells, and of Lendrum (1944) for eosinophils, using new fixatives, permits the simultaneous identification and differentiation of eosinophils and mast cells in a single tissue section. While the method will be of value to those interested in cellular responses to helminth infections, it may also find wider application in the study of the immunopathology arising from other aetiologies.

3.1.1. Aims of study

The main objectives of this study were:

1. To determine methods for mast cell staining;

2. To determine methods for eosinophil staining;

3. To determine methods for simultaneous staining of mast cells and eosinophils in tissue sections;

4. To determine methods for simultaneous staining of mast cells and eosinophils in cell suspensions;

5. To determine methods for simultaneous staining of basophils and eosinophils in cell suspensions and blood smears;

6) To determine suitable methods for simultaneous staining of mast cells in cell culture.

3.2. Results

The fixation and staining protocols which have been tested in these studies are set out below. Since many studies were carried out, the results are presented in a series of Tables (3.1-3.68). In order to simplify the lay out of the tables, most reagents names have been abbreviated. Explanations for the abbreviations are given in the Appendix.

3.2.1. Staining to identify mast cells

The steps taken to improve and develop satisfactory histological techniques are described chronologically as a series of Experiments (Exp3.1-3.2)

Exp-3.1 The intestines and livers from *Trichinella* infected mice were fixed in IFAA, Carnoy's and Bouin's solutions for 24 h at room temperature and stained with (TO) and (AS) staining as described at table 3.1.

Table 3.1 Exp 3.1

Tissue	Fixative	Stain	Cell type	Results
Intestine	IFAA 24h	TO	MMC CTMC	+- -
Liver	IFAA 24h	TO	CTMC	-
Intestine	IFAA 24h	AS	MMC CTMC	+- +-
Liver	IFAA 24h	AS	CTMC	-
Intestine	CAR 24 h	TO	MMC CTMC	+- +-
Liver	CAR 24 h	TO	CTMC	-
Intestine	CAR 24 h	AS	MMC CTMC	+- +-
Liver	CAR 24 h	AS	CTMC	-
Intestine	BOU 24 h	TO	MMC CTMC	- +-
Liver	BOU 24 h	TO	CTMC	-
Intestine	BOU 24 h	AS	MMC CTMC	- +-
Liver	BOU 24 h	AS	CTMC	-

(++) Cells well stained, numbers high, background is clear. (+) Cells well stained, number lower, background good. (+-) Weak staining limited number of cells stained, background obtrusive. (-) Inadequate staining, no cells, background poor.

Table 3.1. Only a few MMC and CTMC were seen with all three fixatives when stained with (TO) and (AS) methods. Bouin's was the least efficient, and Carnoy's gave the best result with both staining methods.

Exp-3.2 Small and large intestine *Schistosoma mansoni* infected mice were fixed in Carnoy's and Bouin's solutions for 24 h at room temperature and stained with (AS) as described in table 3.2.

Table 3.2, Exp 3.2

Tisues	Fixative	Stain	Cell type	Results
Intestine	CAR 24 h	AS	MMC CTMC	++ +
Intestine	BUO 24 h	AS	MMC CTMC	+- +-

(++) Cells well stained, numbers high, background is clear. (+) Cells well stained, number lower, background good. (+-) Weak staining limited number of cells stained, background obtrusive. (-) Inadequate staining, no cells, background poor

Table 3.2. Both MMC and CTMC could be seen in Carnoy's fixed intestine but Bouin's was found to be a less effective fixative in comparison. Steps were taken to stain the eosinophils as well.

3.2.2. Staining to identify mast cells and eosinophils

A series of experiments using different fixatives and staining protocols have been tested in an attempt to develop a method which would stain both mast cells and eosinophils in a single tissue section these are described below (Exp 3.3-3.19).

Exp-3.3 Intestines from *Schistosoma*-infected mice were fixed in Bouin's solution for 24 h at room temperature stained as described in table 3.3.

Table 3.3 Exp 3.3

Stain	Cell type	Results
AHAC	MMC	-
	CTMC	-
	EOS	-
AHS	MMC	-
	CTMC	-
AHC	MMC	-
	CTMC	-
	EOS	-
ACL	MMC	-
	CTMC	-
	EOS	-
ALC	MMC	-
	CTMC	-
	EOS	-
ACH	MMC	-
	CTMC	-
	EOS	-
PAC	MMC	-
	CTMC	-
	EOS	-

(+++) Cells well stained, numbers high, background is clear. (+) Cells well stained, number lower, background good. (+-) Weak staining limited number of cells stained, background obtrusive. (-) Inadequate staining, no cells, background poor

Table 3.3 No Satisfactory results were obtained from these experiments. Using an old batch of chromotrope may be the reason for the lack of a positive response with PAC staining.

Exp-3.4 With the intention of staining mast cells and eosinophils a new fixative (PAChA) was made and intestine, liver and ear from *Schistosoma*-infected mouse were placed in Bouin's, Carnoy's, Glutaraldehyde, IFAA and the new fixative for 24 h at room temperature and stained as described in table 3.4

Table 3.4 Exp 3.4.

Tissue	Fixative	Stain	Cell type	Cell No / Tissue Quality
Intestine	BOU 24 h	TO	MMC	- +
			CTMC	+
Intestine	BOU 24 h	AAC	MMC	- +
			CTMC	+-
			EOS	+-
Intestine	CAR 24 h	AS	MMC	+ ++
			CTMC	+
			EOS	-
Intestine	CAR 24 h	AAC	MMC	+ ++
			CTMC	+
			EOS	-
Intestine	IFAA 24 h	AAC	MMC	+ ++
			CTMC	+
			EOS	-
Intestine	GLU 24 h	AAC	MMC	- +
			CTMC	-
			EOS	-
Intestine	PAChA 24 h	AAC	MMC	+ +
			CTMC	-
			EOS	-
Intestine	CAR 24 h	TO	MMC	+ ++
			CTMC	++
			EOS	-
Liver	BOU 24 h	AS	CTMC	- +-
			EOS	-
Ear	CAR 24 h	TO	CTMC	++ ++
			EOS	-
Ear	CAR 24 h	AS	CTMC	+ +
			EOS	-
Ear	IFAA 24 h	TO	CTMC	+ +
			EOS	-
Ear	IFAA 24 h	AS	CTMC	+- +
			EOS	-
Intestine	CAR 24 h	Ch	MMC	- +
			CTMC	-
			EOS	-

(++) Cells well stained, numbers high, background is clear. (+) Cells well stained, number lower, background good. (+-) Weak staining limited number of cells stained, background obtrusive. (-) Inadequate staining, no cells, background poor.

Table 3.4. with Carnoy's fixed intestine both types of mast cells were visible with AS, AAC and TO staining, Carnoy's also showed the skin mast cells with both TO and AS staining. IFAA with TO and AS staining showed mast cells but to a lesser extent, in both skin and intestine. PAChA and glutaraldehyde gave unsatisfactory results with the staining protocols, Bouin's allowed a weak staining of CTMC and

eosinophils, depending on the tissue when TO and AAC staining was used. Bouin's fixed intestine revealed both mast cells and eosinophils together when AAC stained was used. The most important outcome of these experiments was that astra blue and chromotrope are suitable for staining of both mast cell and eosinophil granules. Only a suitable fixative for fixing the naturally different granules of both cell types would need to be found. In additional experiments using Carnoy's fixed intestine and staining with chromotrope only, red blood cells in blood vessels were clearly stained. In appropriate circumstances chromotrope can therefore be employed as a selective stain for red blood cells and eosinophils. This needs more study.

Exp-3.5 Intestine, Liver and spleen from *Schistosoma mansoni*-infected mouse fixed in new fixatives MPA, MPS, MB, IB, IBR, CB and Carnoy's, Bouin's, and IFAA fixatives for 24 h at room temperature and stained as described in table 3.5

Table 3.5, Exp 3.5.

Tissue	Fixative	Stain	Cell type	Cell No / Tissue Quality
Intestine	MPA 24 h	AAC	MMC CTMC EOS	- - -	+-
Intestine	MPS 24 h	AAC	MMC CTMC EOS	- - -	+-
Intestine	MB 24 h	AAC	MMC CTMC EOS	- - -	+-
Intestine	IB 24 h	AAC	MMC CTMC EOS	- +- -	+-
Intestine	IBR 24 h	AAC	MMC CTMC EOS	- - -	+-
Intestine	CB 24 h	AAC	MMC CTMC EOS	- +- -	+-
Intestine	CAR 24 h	AAC	MMC CTMC EOS	++ + -	+
Intestine	CAR 24 h	AS	MMC CTMC EOS	+ + -	++
Intestine	BOU 24 h	AAC	MMC CTMC EOS	+- +- +	+
Intestine	IFAA 24 h	AAC	MMC CTMC EOS	+- + -	+
Intestine	IFAA 24 h	AS	MMC CTMC EOS	+- + -	+
Spleen	CAR 24 h	AS	CTMC EOS	++ -	+-
Spleen	BOU 24 h	AS	CTMC EOS	- -	+-
Liver	CAR 24 h	AS	CTMC EOS	++ -	+-
Liver	BOU 24 h	AS	CTMC EOS	- -	+-

(++) Cells well stained, numbers high, background is clear. (+) Cells well stained, number lower, background good. (+-) Weak staining limited number of cells stained, background obtrusive. (-) Inadequate staining, no cells, background poor.

Table 3.5 MPA, MPS, MB and IBR gave unsatisfactory results, mast cells but not eosinophils were detected with IB and CB fixatives. Carnoy's and IFAA fixed intestines gave results consistent with those observed earlier. Carnoy's fixed liver and spleen demonstrated mast cell with (AS) but Bouin's was unsatisfactory with these tissues and stains. Bouin's was successful in showing mast cells and eosinophils when staining intestines with the (AAC) method.

Exp-3.6 Intestine from *Schistosoma*-infected mice fixed in Bouin's solution stained with AAC at room temperature and stained as described at table 3.6.

Table 3.6, Exp 3.6

Tissue	Fixative	Stain	Cell type	Cell No/ Tissue quality
Intestine	BOU 24 h	AC	MMC CTMC EOS	+ - + + +

(++) Cells well stained, numbers high, background is clear. (+) Cells well stained, number lower, background good. (+-) Weak staining limited number of cells stained, background obtrusive. (-) Inadequate staining, no cells, background poor.

Table 3.6. A variety of investigations were made and small changes were made in the AAC processing, the final modification yielded better results and is here called AC.

Exp-3.7 *Schistosoma* infected intestines were placed in a series of new fixatives. PA, PA1, PAF, PF, PA3, TA, TAP, TAPF, TP, PAE, Ph, PhA, PAF2, PAF3, PhP, PhAPFEChl, PhT, MPF, MA, MAPh, MP, MT, DP, MuA, MuAT, MPd and Bouin's and Spleen, skin and lung were fixed in Bouin's for 24 h at room temperature and stained with AC, table 3.7

Table 3.7, Exp 3.7

Tissue	Fixative	Cell type	Cell No / Tissue quality
Intestine	PA	MMC	+- +
		CTMC	+
		EOS	-
Intestine	PA1	MMC	+- +
		CTMC	+
		EOS	-
Intestine	PAF	MMC	+- +
		CTMC	+-
		EOS	-
Intestine	PF	MMC	+- -
		CTMC	+-
		EOS	+-
Intestine	PA3	MMC	+ +
		CTMC	-
		EOS	-
Intestine	TA	MMC	+ +
		CTMC	+
		EOS	-
Intestine	TAP	MMC	+ +
		CTMC	+
		EOS	-
Intestine	TAPF	MMC	- +-
		CTMC	-
		EOS	-
Intestine	TP	MMC	+- +
		CTMC	+-
		EOS	+-
Intestine	PAE	MMC	++ +
		CTMC	++
		EOS	-
Intestine	Ph	MMC	- +
		CTMC	-
		EOS	-
Intestine	PhA	MMC	+- +
		CTMC	+
		EOS	-
Intestine	PAF2	MMC	+ +
		CTMC	-
		EOS	-
Intestine	PAF3	MMC	- +
		CTMC	-
		EOS	+
Intestine	PhP	MMC	- +-
		CTMC	-
		EOS	-
Intestine	BOU	MMC	+- +-
		CTMC	+
		EOS	+-

Table 3.7, Exp 3.7 Continued

Tissue	Fixative	Cell type	Cell No / Tissue quality
Intestine	PhAPFEChl	MMC	- -
		CTMC	-
		EOS	-
Intestine	PhT	MMC	+ +
		CTMC	+-
		EOS	+-
Intestine	MPF	MMC	- +-
		CTMC	+-
		PAN	++
		EOS	+
Intestine	MA	MMC	+ +
		CTMC	+
		EOS	-
Intestine	MAPh	MMC	+ +
		CTMC	+
		EOS	-
Intestine	MP	MMC	- -
		CTMC	-
		EOS	-
Intestine	MT	MMC	++ +
		CTMC	++
		EOS	++
Intestine	DP	MMC	- -
		CTMC	-
		EOS	-
Intestine	MuA	MMC	+ +
		CTMC	+
		EOS	-
Intestine	MuAT	MMC	+ -
		CTMC	+
		EOS	-
Intestine	MPd	MMC	+ +-
		CTMC	+
		EOS	+
		PAN	++
Spleen	BOU	CTMC	+ +-
		EOS	+
Lung	BOU	CTMC	- +-
		EOS	-
Skin	BOU	CTMC	- -
		EOS	-

(++) Cells well stained, numbers high, background is clear. (+) Cells well stained, number lower, background good. (+-) Weak staining limited number of cells stained, background obtrusive. (-) Inadequate staining, no cells, background poor.

Table 3.7. PA, PA1, TA, TAP, PhA, MA, MAPh, PAE, MuA, MuAT and PAF all showed mast cells to some degree. PA3 and PAF2 preferentially fixed MMC. PAE was found to be almost as good as Carnoy's fixative for the demonstration of

MMC. Ph, PhP, PhAPFEChl, TAPF, DP, MP gave unsatisfactory results. MPF and MPd showed both eosinophil and Paneth cells. PAF3 stained only eosinophils. MPd, PF, TP, PhT, BOU and MT showed mast cells and eosinophils to different degrees but MT gave excellent results with both cell types. Bouin's showed mast cells and eosinophils granules to a lesser extent in the spleen but not in the lung or skin tissue.

Exp-3.8 Intestine from *Schistosoma mansoni* -infected mice were fixed with the M, T, MTA, MT1, MT2, MPF, PAF3, MT for 24 at room temperature and stained with AC. table 3.8.

Table 3.8, Exp 3.8

Fixative	Cell type	Cell No / Tissue quality	
M	MMC	+-	+-
	CTMC	+-	
	EOS	+	
T	MMC	+-	-
	CTMC	+-	
	EOS	+-	
MTA	MMC	-	-
	CTMC	-	
	EOS	-	
PAF3	MMC	-	+
	CTMC	-	
	EOS	+	
MT1	MMC	-	-
	CTMC	-	
	EOS	-	
MT	MMC	++	+
	CTMC	++	
	EOS	++	
MT2	MMC	+-	+-
	CTMC	+-	
	EOS	+-	
MPF	MMC	-	+
	CTMC	-	
	EOS	+-	
	PAN	+	

(++) Cells well stained, numbers high, background is clear. (+) Cells well stained, number lower, background good. (+-) Weak staining limited number of cells stained, background obtrusive. (-) Inadequate staining, no cells, background poor.

Table 3.8 MTA and MT1 were unsatisfactory as fixatives. MT2 and M gave poor results in comparison with MT. PAF3 gave good fixation for the demonstration of eosinophils. Eosinophils and paneth cells were adequately fixed by MPF. Acetic

acid was not a good fixative for eosinophils while tannic acid gave good results with mast cells and eosinophils.

Exp-3.9 Intestine from *Schistosoma mansoni* infected mice fixed in MF, MT3, MT, MT4, MT5, M, M1, MPd1 and BUO for 24 h at room temperature and stained with AC, table 3.9

Table 3.9, Exp 3.9

Fixative	Cell type	Cell No/Tissue quality	
MF	MMC	-	-
	CTMC	-	
	EOS	-	
MT3	MMC	+	+
	CTMC	+	
	EOS	+	
MT	MMC	+	+-
	CTMC	+	
	EOS	++	
MT4	MMC	+	+
	CTMC	+	
	EOS	+	
MT5	MMC	+	+
	CTMC	+	
	EOS	+	
M	MMC	+-	+
	CTMC	+-	
	EOS	+-	
M1	MMC	+-	+
	CTMC	+-	
	EOS	+-	
MPd1	MMC	+-	+
	CTMC	+	
	EOS	+	
BOU	MMC	-	+
	CTMC	-	
	EOS	+-	

(++) Cells well stained, numbers high, background is clear. (+) Cells well stained, number lower, background good. (+-) Weak staining limited number of cells stained, background obtrusive. (-) Inadequate staining, no cells, background poor.

Table 3.9 MT3, MT4, MT5, MPd1, gave good results to some extent, the results with MT were the best . In this experiment background with MT was not very clear, but it did not interfere with viewing of cells.

Exp-3.10 Intestine from *Schistosoma mansoni* infected mice fixed in MT, MT3, MTMu, M1, M, MPd1, MPd2, MPd3, MT6, MT7, MT4, MT5 and CAR 24 h at room temperature and stained with AC. table 3.10

Table 3.10, Exp 3.10

Fixative	Cell type	Cell No / Tissue quality	
MT	MMC	++	+
	CTMC	++	
	EOS	++	
MT3	MMC	++	+-
	CTMC	+	
	EOS	+	
MTMu	MMC	+-	+-
	CTMC	+-	
	EOS	+-	
M1	MMC	-	-
	CTMC	+-	
	EOS	+-	
M	MMC	-	-
	CTMC	-	
	EOS	-	
MPd1	MMC	-	-
	CTMC	+-	
	EOS	+-	
MT6	MMC	+-	-
	CTMC	+-	
	EOS	+-	
MT7	MMC	+-	-
	CTMC	+-	
	EOS	+-	
MT4	MMC	+	-
	CTMC	+	
	EOS	+	
MT5	MMC	+	-
	CTMC	+	
	EOS	+	
MPd2	MMC	+	+
	CTMC	+	
	EOS	+	
MPd3	MMC	+	+
	CTMC	+	
	EOS	+	
CAR	MMC	++	+
	CTMC	++	

(++) Cells well stained, numbers high, background is clear. (+) Cells well stained, number lower, background good. (+-) Weak staining limited number of cells stained, background obtrusive. (-) Inadequate staining, no cells, background poor.

Table 3.10. MT3, MT4, MT5, MPd2 and MPd3 all showed both cell types, MT was much better and background staining was very good.

Exp-3.11. Intestine from *Schistosoma mansoni* infected mice fixed in MT, MPd3, MT6, MT3 for 24 h at room temperature and stained with AC. table 3.11

Table 3.11, Exp 3.11

Fixative	Cell type	Cell No / Tissue quality	
MT	MMC	++	+
	CTMC	++	
	EOS	++	
MPd3	MMC	+	+
	CTMC	+	
	EOS	+	
MT6	MMC	+-	-
	CTMC	+-	
	EOS	+-	
MT3	MMC	+-	+
	CTMC	+-	
	EOS	+	

(++) Cells well stained, numbers high, background is clear. (+) Cells well stained, number lower, background good. (+-) Weak staining limited number of cells stained, background obtrusive. (-) Inadequate staining, no cells, background poor.

Table 3.11 MT6 fixed tissues had excessive background staining. Background staining was less of a problem with MPd3. MT3 fixed intestine revealed fewer mast cells in this experiment. Both mast cell and eosinophils were clearly stained with MT and there was little precipitation of background staining.

Exp-3.12. Intestine from *Schistosoma mansoni* infected mice fixed in MT, MT5, MT3, MPd3, MPd4, MT8, MT9, MT2, MT10, 24 and 48 h at room temperature and stained with AC.table 3.12

Table 3.12, Exp 3.12

Fixative	Cell type	Cell No / Tissue quality	
MT 24 h	MMC	+-	+
	CTMC	+	
	EOS	++	
MT5 24 h	MMC	+	+
	CTMC	+	
	EOS	++	
MT3 24 h	MMC	+	+
	CTMC	+	
	EOS	+	
MPd3 24 h	MMC	+	+
	CTMC	+	
	EOS	+	
MPd4 24 h	MMC	+-	+
	CTMC	+-	
	EOS	+	
MT8 24 h	MMC	+-	+
	CTMC	+-	
	EOS	+-	
MT9 24 h	MMC	+	+
	CTMC	+	
	EOS	+-	
MT2 24 h	MMC	+-	+-
	CTMC	+-	
	EOS	+-	
MT10 24 h	MMC	+	+
	CTMC	+	
	EOS	+	
MT 48 h	MMC	+	+
	CTMC	+	
	EOS	+	
MT5 48 h	MMC	+	+
	CTMC	+	
	EOS	+	
MT3 48 h	MMC	+	+
	CTMC	+	
	EOS	+	
MPd3 48 h	MMC	+-	+
	CTMC	+-	
	EOS	+-	
MPd4 48 h	MMC	-	-
	CTMC	-	
	EOS	-	
MT8 48 h	MMC	-	-
	CTMC	-	
	EOS	-	
MT9 48 h	MMC	-	-
	CTMC	+-	
	EOS	+-	

Table 3.12, Exp 3.12. Continued

Fixative	Cell type	Cell No / Tissue quality
MT2 48 h	MMC	- -
	CTMC	-
	EOS	-
MT10 48 h	MMC	+ +
	CTMC	+
	EOS	+-

(++) Cells well stained, numbers high, background is clear. (+) Cells well stained, number lower, background good. (+-) Weak staining limited number of cells stained, background obtrusive. (-) Inadequate staining, no cell, background poor

Table 3.12. MT, MT3, MT5, MPd3 and MT10 gave excellent results for mast cells and eosinophils after 24 hours fixation. MT and MT5 showed eosinophils more clearly. MPd4 gave good results for the staining of eosinophils. Similar results were obtained for mast cells when tissue has stained with MT9 for 24 hours. The results with MT2 and MT8 were not satisfactory. Good results were observed for mast cells and eosinophils after 48 hours fixation in MT, MT5 and MT3 fixatives. MT10 fixed tissue showed mast cells but the results with MPd4, MPd3,MT8, MT9 and MT2 were not satisfactory.

Exp-3.13. Intestine from *Schistosoma mansoni* infected mice fixed in MT, MT5, MT3, MPd3, MT9, MT10 for 24, 48 and 72 h and meanwhile samples from heart, and diaphragm were fixed in MPd3 and intestine in CAR at room temperature and were stained as described in table 3.13.

Table 3.13, Exp 3.13

Tissue	Fixative	Stain	Cell type	Cell No / Tissue quality	
Intestine	MT3 24 h	AC	MMC	+	+/-
			CTMC	+	
			EOS	+/-	
Intestine	MT3 48 h	AC	MMC	+	-
			CTMC	+	
			EOS	+/-	
Intestine	MT3 72 h	AC	MMC	+	-
			CTMC	+	
			EOS	+/-	
Intestine	MT5 24 h	AC	MMC	+	+
			CTMC	+/-	
			EOS	+	
Intestine	MT5 48 h	AC	MMC	+	-
			CTMC	+/-	
			EOS	+	
Intestine	MT5 72 h	AC	MMC	+	-
			CTMC	+/-	
			EOS	+	
Intestine	MT 24 h	AC	MMC	+	+
			CTMC	+/-	
			EOS	+	
Intestine	MT 48 h	AC	MMC	+	+/-
			CTMC	+	
			EOS	+	
Intestine	MT 72 h	AC	MMC	+/-	+/-
			CTMC	+/-	
			EOS	+	
Intestine	MPd3 24 h	AC	MMC	+/-	+/-
			CTMC	+/-	
			EOS	+	
Intestine	MPd3 48 h	AC	MMC	+/-	+/-
			CTMC	+	
			EOS	+	
Intestine	MPd3 72 h	AC	MMC	+/-	+/-
			CTMC	+	
			EOS	+	
Intestine	MT10 24 h	AC	MMC	+	+
			CTMC	+	
			EOS	+/-	
Intestine	MT10 48 h	AC	MMC	+	-
			CTMC	+	
			EOS	+/-	
Intestine	MT10 72 h	AC	MMC	+	-
			CTMC	+	
			EOS	+/-	
Intestine	MT9 24 h	AC	MMC	+	+
			CTMC	+	
			EOS	+	

Table 3.13, Exp 3.13. Continued

Tissue	Fixative	Stain	Cell type	Cell No / Tissue quality	
Intestine	MT9 48 h	AC	MMC CTMC EOS	+- +- +-	+-
Intestine	MT9 72 h	AC	MMC CTMC EOS	+- +- +-	+-
Intestine	MT	ALC	MMC CTMC EOS	- - -	+-
Intestine	MT	ANC	MMC CTMC EOS	- - -	-
Intestine	CAR	AlS	MMC CTMC	++ ++	+
Intestine	CAR	AlGS	MMC CTMC	++ ++	+
Intestine	MT	AlGC	MMC CTMC EOS	++ ++ ++	+
Intestine	MT	BRC	MMC CTMC EOS	- - -	-
Intestine	MT	FC	MMC CTMC EOS	- - -	-
Intestine	MT	LGC	MMC CTMC EOS	- - -	-
Heart	MPd3	AC	CTMC EOS	+- +-	+
Diaphragm	MPd3	AC	CTMC EOS	+- +-	+
Heart	MPd3	AlGC	CTMC EOS	+- +-	+
Diaphragm	MPd3	AlGC	CTMC EOS	+- +-	+

(++) Cells well stained, numbers high, background is clear. (+) Cells well stained, number lower, background good. (+-) Weak staining limited number of cells stained, background obtrusive. (-) Inadequate staining, no cells, background poor.

Table 3.13 A reduced number of eosinophils was visible with MT10, MT3 fixatives staining with (AC) at 24 h. With the same fixatives at 48h and 72h the colour of the tissue was unsuitable for clear detection of the cells. MPd3 and MT5 gave poor results at 24, 48, and 72 h. MPd3 fixed heart and diaphragm also

showed few mast cells and eosinophils when stained with AC and AlGC. At 24h MT and MT9 gave better results than MT3 and MT10 but the background colour changed later. MT fixed intestine gave poor results with ALC, ANC, BRC, FC, LGC staining. Mast cells and eosinophils were well stained with AlGC though the staining of goblet cells in the intestine limits the use of this stain, excluding its use from intestinal tissue when trying to detect mast cells and eosinophils. Carnoy's fixed intestine showed MMC and CTMC when stained with AlS and AlGS.

Exp-3.14. Intestine, spleen, liver of *Schistosoma mansoni* infected mice were placed in MT and MT3 for 24 and 48 h and trachea, lung, skin, urinary bladder and tongue for 24 h at room temperature and stained as described in table 3.14

Table 3.14, Exp 3.14

Tissue	Fixative	Stain	Cell type	Cell No / Tissue quality	
Intestine	MT 24 h	AC	MMC CTMC EOS	++ + +	+
Spleen	MT 24 h	AC	CTMC EOS	+ +	+
Liver	MT 24 h	AC	CTMC EOS	+ +	+
Intestine	MT3 24 h	AC	MMC CTMC EOS	++ +- +-	+
Spleen	MT3 24 h	AC	CTMC EOS	- -	+
Liver	MT3 24 h	AC	CTMC EOS	- -	+
Intestine	MT 24 h	AlGC	MMC CTMC EOS	+ + +	+
Spleen	MT 24 h	AlGC	CTMC EOS	+ +	+
Liver	MT 24 h	AlGC	CTMC EOS	+ +	+
Intestine	MT3 24 h	AlGC	MMC CTMC EOS	+ +- +-	+
Spleen	MT3 24 h	AlGC	CTMC EOS	- -	+
Liver	MT3 24 h	AlGC	CTMC EOS	- -	+

Table 3.14, Exp 3.14. Continued

Tissue	Fixative	Stain	Cell type	Cell No / Tissue quality	
Intestine	BOU	AlGC	MMC CTMC EOS	+ + +	+-
Intestine	MT 48 h	AC	MMC CTMC EOS	+ + +	+-
Spleen	MT 48 h	AC	CTMC EOS	+ +	+-
Liver	MT 48 h	AC	CTMC EOS	+ +	+-
Intestine	MT3 48 h	AC	MMC CTMC EOS	+ +- +-	+-
Spleen	MT3 48 h	AC	CTMC EOS	- -	+
Liver	MT3 48 h	AC	CTMC EOS	- -	+-
Skin	MT 24h	AC	CTMC EOS	- -	+-
Trachea	MT 24h	AC	CTMC EOS	- -	+-
Tongue	MT 24h	AC	CTMC EOS	- -	+-
Urinary blad	MT 24h	AC	CTMC EOS	- -	+-
Lung	MT 24h	AC	CTMC EOS	- -	+-
Skin	MT3 24 h	AC	CTMC EOS	- -	+-
Trachea	MT3 24 h	AC	CTMC EOS	- -	+-
Tongue	MT3 24 h	AC	CTMC EOS	- -	+-
Urinary blad	MT3 24 h	AC	CTMC EOS	- -	+-
Lung	MT3 24 h	AC	CTMC EOS	- -	+-

(++) Cells well stained, numbers high, background is clear. (+) Cells well stained, number lower, background good. (+-) Weak staining limited number of cells stained, background obtrusive. (-) Inadequate staining, no cells, background poor.

Table 3.14. MT fixed intestine, liver and spleen and MT3 fixed intestine showed both mast cells and cosinophils at 24 h but results were poor with longer fixation when stained with the AC method. Substitution of Alcian green in the AC staining

method yielded similar results to Astra blue in MT fixed tissue but had the drawback that goblet cells were also stained limiting its use to tissues other than intestinal tissue. Both cell types were observed, though to a lesser extent in Bouin's fixed intestine stained with Alcian green. Mast cells could not be detected in Trachea, Skin, tongue urinary bladder and lung when these tissues were fixed in MT and MT3, at room temperature, and stained with AC. At 24 and 48 h MT3 fixed liver and spleen did not give good results when staining with AC or with AlGC, BOU fixed intestine gave poorer results.

Exp-3.15. Intestine, trachea, lung from *Schistosoma mansoni* infected mice were placed in MT and MT3 for 24h at room temperature and stained with AC as described in table 3.15

Table 3.15, Exp 3.15

Tissue	Fixative	Stain	Cell type	Cell No / Tissue quality	
Intestine	MT 24 h	AC	MMC CTMC EOS	++ ++ ++	+
Lung	MT 24 h	AC	CTMC EOS	- -	+
Trachea	MT 24 h	AC	CTMC EOS	- -	+
Intestine	MT3 24 h	AC	MMC CTMC EOS	+ +- +	+
Lung	MT3 24 h	AC	CTMC EOS	- -	+
Trachea	MT3 24 h	AC	CTMC EOS	- -	+

(++) Cells well stained, numbers high, background is clear. (+) Cells well stained, number lower, background good. (+-) Weak staining limited number of cells stained, background obtrusive. (-) Inadequate staining, no cells, background poor.

Table 3.15. Mast cell and eosinophils were observed in MT fixed intestine but not in lung and trachea.

Exp-3.16. Intestine, liver, spleen from *Schistosoma mansoni* infected mice were placed in MT, CAR and intestine only in BOU 24h at room temperature stained as in table 3.16

Table 3.16, Exp 3.16

Tissue	Fixative	Stain	Cell type	Cell No / Tissue quality	
Intestine	MT 24 h	AC	MMC CTMC EOS	+- +- ++	-
Liver	MT 24 h	AC	CTMC EOS	+- +	+
Spleen	MT 24 h	AC	CTMC EOS	+ +	+
Intestine	BOU 24 h	AC	MMC CTMC EOS	+- +- +-	+
Intestine	CAR 24 h	AS	MMC CTMC	++ +	+
Liver	CAR 24 h	AS	CTMC	+	+-
Spleen	CAR 24 h	AS	CTMC	-	+-
Intestine	CAR 24 h	TO	MMC CTMC	+ +	+-
Liver	CAR 24 h	TO	CTMC	+	+-
Spleen	CAR 24 h	TO	CTMC	+	+-

(++) Cells well stained, numbers high, background is clear. (+) Cells well stained, number lower, background good. (+-) Weak staining limited number of cells stained, background obtrusive. (-) Inadequate staining, no cells, background poor.

Table 3.16. MT fixed intestine was unsuitable for mast cell detection due to the background (Swiss role treated intestine). Mast cells were seen in Carnoy's fixed intestine, liver and spleen when stained with TO despite some precipitation. AS did not identify mast cells in this experiment with Carnoy's fixed spleen , but mast cells were observed in intestine and liver stained with AS method.

Exp-3.17. Intestine, liver and spleen from *Schistosoma mansoni* infected mouse were fixed in MT for 9 h at room temperature and stained as described in table 3.17.

Table 3.17, Exp 3.17

Tissue	Fixative	Stain	Cell type	Cell No / Tissue quality	
Intestine	MT 9 h	AC	MMC CTMC EOS	- - +	-
Liver	MT 9 h	AC	CTMC EOS	- +	-
Spleen	MT 9 h	AC	CTMC EOS	- +-	-

(++) Cells well stained, numbers high, background is clear. (+) Cells well stained, number lower, background good. (+-) Weak staining limited number of cells stained, background obtrusive. (-) Inadequate staining, no cells, background poor.

Table 3.17. Tissue fixed in MT for 9 h had an intense blue back ground staining with AC. This made identification of mast cells particularly difficult but eosinophils were clearly observed.

Exp-3.18 (I) Intact (unopened) intestine, liver and spleen from *Schistosoma mansoni* infected mouse, (II) Opened and washed intestine, liver and spleen from *Schistosoma mansoni* infected mouse, (III) Non-perfused intestine, liver and spleen from *Trichinella spiralis* infected mouse, (IV) Non-perfused intestine, liver and spleen from *S. mansoni* infected mouse, (V) perfused (RPMI medium) intestine, liver and spleen from *S, mansoni* infected mouse, (VI) citrated saline perfused intestine, liver and spleen from *S. mansoni* infected mouse. All were fixed in MT for 24h at room temperature and stained with AC and some intestine was fixed in M or T for 24h at room temperature and also stained with AC as described in table 3.18

Table 3.18, Exp 3.18

Tissue	Fixatives	Stain	Cell type	Cell No / Tissue qaulity	
Intestine I	MT 24 h	AC	MMC CTMC EOS	+ + +	+
Liver I	MT 24 h	AC	CTMC EOS	+ +	+-
Spleen I	MT 24 h	AC	CTMC EOS	+ +	+
Intestine II	MT 24 h	AC	MMC CTMC EOS	+ + +	-
Liver II	MT 24 h	AC	CTMC EOS	+ +	+-
Spleen II	MT 24 h	AC	CTMC EOS	+ +	+-
Intestine III	MT 24 h	AC	MMC CTMC EOS	+ + +	+
Liver III	MT 24 h	AC	CTMC EOS	- -	+
Spleen III	MT 24 h	AC	CTMC EOS	+- +-	+

Table 3.18 Exp 3.18 Continued

Tissue	Fixatives	Stain	Cell type	Cell No /Tissue quality
Intestine IV	MT 24 h	AC	MMC CTMC EOS	+ + +
Liver IV	MT 24 h	AC	CTMC EOS	+ +
Spleen IV	MT 24 h	AC	CTMC EOS	+ +
Intestine VI	MT 24 h	AC	MMC CTMC EOS	+ + +
Liver VI	MT 24 h	AC	CTMC EOS	- -
Spleen VI	MT 24 h	AC	CTMC EOS	+ +
Intestine V	MT 24 h	AC	MMC CTMC EOS	+- +- +
Liver V	MT 24 h	AC	CTMC EOS	+- +-
Spleen V	MT 24 h	AC	CTMC EOS	+ +
Intestine	M 24 h	AC	MMC CTMC EOS	+- + -
Intestine	T 24 h	AC	MMC CTMC EOS	- - -

(++) Cells well stained, numbers high, background is clear. (+) Cells well stained, number lower, background good. (+-) Weak staining limited number of cells stained, background obtrusive. (-) Inadequate staining, no cells, background poor.

Table 3.18. The non-perfused tissues gave satisfactory results, but perfusion with citrated buffered saline had no effect on the quality of fixation and staining of the intestine and spleen. Liver quality was affected. Intact, and unwashed intestine gave the best tissue quality in this series. M and T individually were not suitable for fixing mast cells and eosinophils.

Exp-3.19. Intestine and liver from *S. mansoni* infected mice fixed in Carnoy's, liver in MT and lung in BOU solutions for 24h at room temperature and stained as described in table 3.19

Table 3.19, Exp 3.19.

Tissue	Fixatives	Stain	Cell type	Cell No/Tissue quality	
Intestine	CAR 24 h	HAE	MMC CTMC	- -	+
Intestine	CAR 24 h	HAC	MMC CTMC	- -	+
Intestine	CAR 24 h	TO	MMC CTMC	+- ++	+
Liver	CAR 24 h	AS	CTMC	+	+
Liver	MT 24 h	AS	CTMC EOS	+ -	+
Lung	BOU 24 h	TO	CTMC	-	-

(++) Cells well stained, numbers high, background is clear. (+) Cells well stained, number lower, background good. (+-) Weak staining limited number of cells stained, backgroumd obtrusive.(-) Inadequate staining, no cells, background poor.

Table 3.19. Carnoy's fixed intestine yielded CTMC in Submucosal and muscle layer which were very prominent with TO staining. MT and CAR fixed liver showed mast cells in liver granulomas to the some extent with AS stain. Carnoy's fixed intestine when stained with HAC only the cells nuclei of the tissue context were evident, Chromotrope failed to stain anything in this tissue. Perhaps the results are in agreement with the specificity of the chromotrope for eosinophil granules. The results of the Carnoy's fixed intestine and BOU fixed lung was not satisfactory with HAE and TO stain respectively.

3.2.3. Improving the methods

Other steps were taken to improve and develop satisfactory staining methods for mast cells and eosinophils (Exp 3.20-3.22)

Exp-3.20. The intestines from *Schistosoma mansoni* infected mice were fixed in MOT fixative and in newly designed fixatives for 24 hours at room temperature and stained with AC. table 3.20.

Table 3.20, Exp 3.20

Fixative	Stain	Cell type	Cell No / Tissue quality	
MOT	AC	MMC	+	+-
		CTMC	+	
		EOS	+	
CITM	AC	MMC	+	+
		CTMC	+	
		EOS	+-	
CITM1	AC	MMC	+	+
		CTMC	+	
		EOS	+-	
LME	AC	MMC	+	+
		CTMC	+	
		EOS	+	
MOM	AC	MMC	+	+
		CTMC	+	
		EOS	+	
LEMT	AC	MMC	-	+-
		CTMC	-	
		EOS	+	
MOMT	AC	MMC	+-	-
		CTMC	+	
		EOS	+	
LP	AC	MMC	+	+
		CTMC	+	
		EOS	-	
MTF	AC	MMC	+-	+-
		CTMC	+-	
		EOS	+	
		PAN	+	
MT	AC	MMC	+	+
		CTMC	+	
		EOS	+	
LM	AC	MMC	++	++
		CTMC	++	
		EOS	++	
MLC1	AC	MMC	+	+-
		CTMC	+-	
		EOS	+	
TC1	AC	MMC	-	-
		CTMC	+-	
		EOS	-	
LM1	AC	MMC	-	-
		CTMC	+-	
		EOS	+-	

Table 3. 20, Exp 3.20 continued.

Fixative	Stain	Cell type	Ce.. No / Tissue qu ity
MCl	AC	MMC	+-
		CTMC	+-
		EOS	-
CaCl	AC	MMC	-
		CTMC	-
		EOS	-

(++) Cells well stained, numbers high, background is clear. (+) Cells well stained, number lower, background good. (+-) Weak staining limited number of cells stained, background obtrusive. (-) Inadequate staining, no cells, background poor.

Table 3.20. With MOT fixative MMC, CTMC and eosinophils were visible. Goblet cells were distinct as gaps in the intestinal epithelium. However the pink background staining made this combination unsuitable. With ClTM and ClTM1 the granules of the eosinophils were distributed across a wider range than normal but the results were better than with MOT fixative. With LME and MOM the tissue background was clear and mast cell and eosinophils were quite distinct. LEMT fixation resulted in a blue background staining, suitable for eosinophils but not for mast cells. LMT1, LMT3, LMT2 and LT all gave a jelly-like precipitation when mixed, these and TCl, LM1, MCl and CaCl all proved to be impractical. MOMT had an unsuitable background staining but mast cells and eosinophils were still visible. LP was unsuitable for the demonstration of eosinophils. MTF was not suitable for mast cells but proved to be good for the presentation of eosinophils and paneth cells. MT was as effective as ClTM, ClTM1 and LME in this experiment .

With LM fixative eosinophil granules remained concentrated and background staining was clear. MOM, LME, LM, ClTM1, ClTM and MT were suitable fixatives but of these LM and LME were superior.

Exp-3.21 Liver and spleen from *Schistosoma mansoni* infected mice was placed in LM, ClTM, LME, MLCl fixatives for 24 h at room temperature also two different mercuric chloride solutions were compared in this experiment staining with AC. table 3.21, 3.21.1

Table 3.21, Exp 3.21

Tissue	Fixative	Cell type	Cell No / Tissue qua y
Liver	LM 24 h	CTMC	++ +
		EOS	++
Spleen	LM 24 h	CTMC	++ +
		EOS	++
Liver	CITM 24 h	CTMC	+ +
		EOS	++
Spleen	CITM 24 h	CTMC	++ +
		EOS	+
Liver	LME 24 h	CTMC	++ +
		EOS	++
Spleen	LME 24 h	CTMC	++ +
		EOS	++
Liver	MLCl 24 h	CTMC	+ +
		EOS	+
Spleen	MLCl 24 h	CTMC	+ +
		EOS	+

(++) Cells well stained, numbers high, background is clear. (+) Cells well stained, number lower, background good. (+-) Weak staining limited number of cells stained, background obtrusive. (-) Inadequate staining, no cells, background poor.

Table 3.21.1 Precipitation when old (Ol M) and new (N M) mercuric chloride are used. (Stain AC).

Table 3.21.1.

Fixative	Cell type	Precipitation
Liver	LM (N M)	+-
Spleen	LM (N M)	-
Liver	CITM (N M)	+
Spleen	CITM (N M	-
Liver	LME (N M)	+
Spleen	LME (N M)	-
Liver	MLCl (N M)	+
Spleen	MLCl (N M)	+-
Liver	LM (Ol M)	-
Spleen	LM (Ol M)	-
Liver	CITM (OlM)	+-
Spleen	CITM (OlM)	-
Liver	LME (Ol M)	-
Spleen	LME (Ol M)	-
Liver	MLCl (OlM)	+-
Spleen	MLCl (OlM)	-

(++) Cells well stained, numbers high, background is clear. (+) Cells well stained, number lower, background good. (+-) Weak staining limited number of cells stained, background obtrusive. (-) Inadequate staining, no cells, background poor.

Table 3.21. Both mast cells and e inophils were seen in liver and spleen with all four fixatives. Black precipitatio was found in the liver when the new mercuric chloride was used. Using the old (och Light) mercuric chloride there was little or no precipitation.

Table 3.21.1. To determine the effect of the old and new mercuric chloride, liver and spleen were fixed in LM, CITM, LME, MLCl fixatives (using old and new mercuric chloride) and stained with AC stain. More precipitation was found when new mercuric chloride was used in the fixatives.

Exp-3.22. Intestines from *Schistosoma mansoni* infected mice were placed in LM, CITM, LME, MLCl fixatives for 1,2,3,4, weeks and liver and spleen for 1 week at room temperature and stained With AC table 3.22

Table 3. 22, Exp 3.22

Tissue	Fixative	Cell type	Cell No / Tissue quality
Intestinal	LM 1 w	MMC CTMC EOS	++ + ++ ++
Liver	LM 1 w	CTMC EOS	++ + ++
Spleen	LM 1 w	CTMC EOS	++ + ++
Intestinal	LM 2 w	MMC CTMC EOS	+ + + +
Intestinal	LM 3 w	MMC CTMC EOS	+ + + +
Intestinal	LM 4 w	MMC CTMC EOS PAN	+ + + + +
Intestinal	CITM 1 w	MMC CTMC EOS	+ + + +
Liver	CITM 1 w	CTMC EOS	+ +- +
Spleen	CITM 1 w	CTMC EOS	+ + +

Table 3. 22, Exp 3.22. Continued

Tissue	Fixative	Cell type	Cell No / Tissue quality
Intestinal	CITM 2 w	MMC	+
		CTMC	+
		EOS	+
		PAN	+
Intestinal	CITM 3 w	MMC	+
		CTMC	+
		EOS	+
		PAN	+
Intestinal	CITM 4 w	MMC	+
		CTMC	+
		EOS	+
		PAN	+
Intestine	LME 1 w	MMC	+
		CTMC	+
		EOS	+
Liver	LME 1 w	CTMC	+
		EOS	+
Spleen	LME 1 w	CTMC	+
		EOS	+
Intestinal	LME 2 w	MMC	+
		CTMC	+
		EOS	+
Intestinal	LME 3 w	MMC	+
		CTMC	+
		EOS	+
Intestinal	LME 4 w	MMC	+
		CTMC	+
		EOS	+
Intestinal	MLCI 1 w	MMC	+
		CTMC	+
		EOS	+
Liver	MLCI 1 w	CTMC	+
		EOS	+
Spleen	MLCI 1 w	CTMC	+
		EOS	+
Intestinal	MLCI 2 w	MMC	+
		CTMC	+
		EOS	+
Intestinal	MLCI 3 w	MMC	+
		CTMC	+
		EOS	+
Intestinal	MLCI 4 w	MMC	+
		CTMC	+
		EOS	+

(++) Cells well stained, numbers high, background is clear. (+) Cells well stained, number lower, background good. (+-) Weak staining limited number of cells stained, background obtrusive. (-) Inadequate staining, no cells, background poor.

Table 3.22. With ClTM extension of the fixation time resulted in pink background staining which did not interfere with mast cell or eosinophil identification. As fixation time lengthened paneth cells detection became more marked. After 4 weeks LME showed concentrated eosinophil granules. Paneth cells were visible after 4 weeks fixation in LM. LM gave better results than ClTM, LME. MLCl gave clear background and good results for both mast cells and eosinophils. Taken together LM, MLCl and ClTM gave good results, but in these experiments LM proved superior.

3.2.4. Staining of skin to identify mast cells and eosinophils (Exp 3.23-3.30)

Exp 3.23. Skin from *Schistosoma*-infected mice were fixed in ClTM, LME, LM for 24 h at room temperature and stained with AC. table 3.23

Table 3.23, Exp 3.23

Fixative	Cell type	Cell No / Tissue quality	
ClTM 24 h	CTMC	-	+-
	EOS	-	
LME 24 h	CTMC	+-	+-
	EOS	+	
LM 24 h	CTMC	++	+-
	EOS	++	

(++) Cells well stained, numbers high, background is clear. (+) Cells well stained, number lower, background good. (+-) Weak staining limited number of cells stained, background obtrusive. (-) Inadequate staining, no cells, background poor.

Table 3.23. Poor results were obtained when skin was fixed in ClTM. LME and LM gave good quality fixation and staining of mast cells and eosinophils but the respective blue and red background staining limited their value for detection of these cells.

Skin from *S. mansoni* infected mice was fixed in LM for 24h at room temperature and stained as described in table 3.24.

Table 3. 24

Exp. No	Tissue	Fixative	Stain	Continue	Tissue quality
3.24	Skin	LM	Rehydration, in 3% Aquatic thiosulphate 1 min, Rinse in water, 1% Astra blue 30 min, Rinse in water, Scott's tap water 1 min, Rinse in water, Chromotrope 30 min, Briefly wash in water,	Quick dehydration, Blot, Xylene 3 min, Mount in DPX.	-
3.25	Skin	LM	Rehydration, Rinse in water, 1% Astra blue 30 min, Rinse in water, 3% thiosulphate 1 min (Aquatic), Rinse in water, Chromotrope 30 min, Briefly wash in water,	Quick dehydration, Blot, Xylene 3 min, Mount in DPX.	-
3.26	Skin	LM	Rehydration, Rinse in water, 1% Astra blue 30 min, Rinse in water, Chromotrope 30 min, Briefly wash in water, 3% aquatic thiosulphate 1 min,	Quick dehydration, Blot, Xylene 3 min, Mount in DPX.	-
3.27	Skin	LM	Rehydration, Rinse in water, 1% Astra blue 30 min, Rinse in water, Chromotrope 30 min, Briefly wash in water, 2% aquatic thiosulphate 1 min,	Quick dehydration, Blot, Xylene 3 min, Mount in DPX.	-
3.28	Skin	LM	Rehydration, Rinse in water, 1% Astra blue 30 min, Rinse in water,Chromotrope 30 min, Briefly wash in water, 2% aquatic thiosulphate 0.5 min,	Quick dehydration, Blot, Xylene 3 min, Mount in DPX.	+
3.29	Skin	LM	Rehydration, Rinse in water, 1% Astra blue 30 min, Rinse in water, Chromotrope 30 min, Briefly wash in water, 1% aquatic thiosulphate 1 min,	Quick dehydration, Blot, Xylene 3 min, Mount in DPX.	+
3.30	Skin	LM	Rehydration, Rinse in water, 1% Astra blue 30 min, Rinse in water, Chromotrope 30 min, Briefly wash in water, 1% aquatic thiosulphate 0.5 min, (ACS)	Quick dehydration, Blot, Xylene 3 min, Mount in DPX.	++

(++) Cells well stained, numbers high, background is clear. (+) Cells well stained, number lower, background good. (+-) Weak staining limited number of cells stained, background obtrusive. (-) Inadequate staining, no cells, background poor.

Table 3.24. The results of Exp 3.28, 3.29 were satisfactory and Exp 3.30 proved best of all. Manipulations of the AC staining method led to staining of mast cells and eosinophils in the skin of the *Schistosoma*-infected mouse in an absolutely clear background using LM fixative, the method called ACS.

3.2.5. Staining mast cells and eosinophils in cell suspensions

3.2.5.1. Producing of cell suspension from different organs of the body

The brain, heart, lung, thymus, liver, spleen, mesentery, kidney and heparinized blood from 9 weeks *S.mansoni*-infected mice were collected and teased for cell suspension preparation as has been described for spleen cell preparation (see Chapter 2).

3.2.5.2. Producing of cell suspension from tissue culture

A pellet of cells was prepared by centrifugation of the suspension cultures. This was used for diluting with stain for studying mast cells in suspension or was mixed with small amount of FCS for smear preparation.

3.2.5.3. Staining to identify mast cells in cell culture

Examination of smears is the most reliable way to identify cell types from a suspension culture. However, the quality of the smear is important and can be affected by a number of factors. Generally fixatives used for the preparation of mast cells are relatively strong chemicals which readily remove the cells from the surfaces of the slides. Experiments were set up to test a variety of fixatives and stains which would permit the preparation of high quality stained smears of cultured mast cells.

Smears were prepared manually. Within one minute of addition of IFAA the smear was removed from the slide. The cells which remained attached to the slide were inadequately fixed. Carnoy's removed cells from the slide surface within 30 secs.

A system for fixing slides with hot Carnoy's vapour was found to be highly effective (Gas chamber, CARG) and staining was of high quality. Synchronised with this progress, lead subacetate as a strong fixative for mast cell granules was found and the Mota formulation of lead acetate used. It was able to fix the mast cells within 1 minute with out removing the smear from the surface of the slide. By employing a cytospin centrifuge the quality of the slides improved. Additional steps were also taken to improve on the staining of mast cells in smears and suspension cultures. The experiments are described in table 3.25.

Table 3.25

Exp No	material	Fixative	Staining Method
3.31	Suspension	Unfixed	1 part of 1% Toluidine blue (pH. 5 adjusted with hydrochloric acid) was mixed with 19 parts of cell suspension 5min and cell study was done in a hemocytometer
3.32	Suspension	Unfixed	1 part of 0.2 % Toluidine blue (pH 3 adjusted with hydrochloric acid) was mixed with 1 parts of cell suspension 5min and cell study was done in a hemocytometer
3.33	Suspension	Unfixed	1 part of 0.2 % Toluidine blue (pH. 3 adjusted with hydrochloric acid) was mixed with 2 parts of cell suspension 5 min and cell study was done in a hemocytometer
3.34	Suspension	Unfixed	1 part of 0.2 % Toluidine blue (pH 3 adjusted with hydrochloric acid) was mixed with 5 parts of cell suspension 5 min and cell study was done in a hemocytometer
3.35	Suspension	Unfixed	11 ml of 0.05 % Toluidine blue (20% ethanol and 80 % saline), 0.5 ml of saturated saponin in 50 % ethanol and 5ml of 0.1 M acetic acid were mixed (1part of above stain with 10 parts of cell suspension were mixed for 5 min and suspension was studied for mast cell), Result was not satisfactory
3.36	Suspension	Unfixed	11 ml of 0.05 % Toluidine blue (20% ethanol and 80 % saline), 0.5 ml of saturated saponin in 50 % ethanol and 5ml of 0.1 M acetic acid were mixed (1part of above stain with 20 parts of cell suspension were mixed for 5 min and suspension was studied for mast cell), Result was not satisfactory
3.37	Smears	CARG	Smears fixed stained with 0.25 % toluidine blue 5 min, (pH adjusted to 3 with hydrochloric acid)
3.38	Smears	Unfixed	air dried smears stained with 0.25 % toluidine blue 5 min, pH adjusted to 3 with hydrochloric acid. (Smear came off)
3.39	Smears	CARG	Smears fixed stained with 1 % toluidine blue in 50 % ethanol 5 min, pH. adjusted to 3 with hydrochloric acid), Results were good.
3.40	Smears	Unfixed	air dried smears stained with 1 % toluidine blue in 50 % ethanol 5 min, pH. adjusted to 3 with hydrochloric acid. (smear came off).

Table 3.25, Continued

Exp No	Material	Fixative	Staining methods
3.41	Suspension	Unfixed	0.3 ml cultured mast cells mixed with 100 µl FCS, 10 µl suspension added to 90 µl of 0.025% toluidine blue, pH adjusted to 3.5 with acetic acid (Benveniste 1977), shaken gently after 5min mast cells studied in haemocytometer the results were not satisfactory .
3.42	Suspension	IFAA 24h	0.3 ml of cultured mast cells was fixed in 0.3 ml fixative, pelleted cells stained with toluidine blue (0.1%/50% ethanol, pH.3.0) 5min (1:1).
3.43	Suspension	CAR 24h	0.3 ml of cultured mast cells was fixed in 0.3 ml fixative, pelleted cells stained with toluidine blue (0.1%/50% ethanol, pH.3.0) 5min, (1:1). CAR fixed suspension did not showed mast cell.
3.44	Suspension	GLU 1h	0.3 ml of cultured mast cells was fixed in 0.3 ml fixative,10µl of pelled fixed cells stained with 10µl (0.1% toluidine blue/50 % ethanol, pH.3.0) 3 min, using 10µl FCS or without using FCS.
3.45	Suspension	MT 3h	0.3 ml of cultured mast cells was fixed in 0.3 ml fixative,10µl of pelled fixed cells stained with 10µl (0.1% toluidine blue/ 50 % ethanol, pH. 3.0) 3 min, using 10µl FCS or without using FCS. Results were not satisfactory with MT fixative.
3.46	Suspension	CAR 1h	0.3 ml of cultured mast cells was fixed in 0.3 ml fixative,10µl of pelled fixed cells stained with 10µl (0.1% toluidine blue/ 50 % ethanol, pH. 3.0) 3 min, using 10µl FCS or without using FCS. Results were not satisfactory with CAR fixative.
3.47	Suspension	BOU 1h	0.3 ml of cultured mast cells was fixed in 0.3 ml fixative,10µl of pelled fixed cells stained with 10µl (0.1% toluidine blue/ 50 % ethanol, pH. 3.0) 3 min, using 10µl FCS or without using FCS. Results were not satisfactory with BOU fixative.
3.48	Suspension	Unfixed	1part toluidine blue (0.1% pH. 3) with 19 parts of reference cell are mixed and shakened gently after 5 min the mast cells are studied in haemacytometer. (Bray *et al.* 1961).
3.49	Suspension	Unfixed	Cell suspension resuspended in small volume of fetal calf serum and stained with 0.1% toluidine blue in 50% ethanol pH. 3.0, (Razin *et al.* 1981) 1:1
3.50	Smear	CARG	Pelleted cells resuspend in small volume of FCS, fixed and stained with 0.1 %, 0.2 %, 0.5% toluidine blue in 50 % ethanol 5 min, pH. adjusted to pH 3 with hydrochloric acid.
3.51	Smear	Unifxed	Pelleted cells resuspend in small volume of FCS, stained 0.1 %, 0.2 %, 0.5% toluidine blue in 50 % ethanol 5 min, pH. adjusted to pH 3 with hydrochloric acid (smears came off).

Table 3.25. All of the methods were able to stain mast cells in the smears with a dark blue staining colour. The internal components of the cells were not visible.

3.2.5.4. Staining to identify mast cells and basophils

Steps were taken to identify mast cells in cell suspensions in Experiments (Exp 3.52-3.60). Smears were made from brain, heart, lung, thymus, liver, spleen, mesentery, kidney and heparinized blood cell suspensions and stained as described in table 3.26.

Table 3.26

Exp No	Tissue	Fixative	Stain	Cell No. Mast cell
3.52	Heart	MOT 1 min	Washed in water, 1% toluidine blue 5 min, washed in water (TO1)	-
3.53	Brain	MOT 1 min	Washed in water, 1% toluidine blue 5 min, washed in water (TO1)	-
3.54	Lung	MOT 1 min	Washed in water, 1% toluidine blue 5 min, washed in water (TO1)	+-
3.55	Thymus	MOT 1 min	Washed in water, 1% toluidine blue 5 min, washed in water (TO1)	++
3.56	Liver	MOT 1 min	Washed in water, 1% toluidine blue 5 min, washed in water (TO1)	++
3.57	Spleen	MOT 1 min	Washed in water, 1% toluidine blue 5 min, washed in water (TO1)	++
3.58	Mesentery	MOT 1 min	Washed in water, 1% toluidine blue 5 min, washed in water (TO1)	+-
3.59	Kidney	MOT 1 min	Washed in water, 1% toluidine blue 5 min, washed in water (TO1)	-
3.60	Blood	MOT 1 min	Washed in water, 1% toluidine blue 5 min, washed in water (TO1)	+- (Basophil)

(++) Cells well stained, numbers high, background is clear. (+) Cells well stained, number lower, background good. (+-) Weak staining limited number of cells stained, background obtrusive. (-) Inadequate staining, no cells, background poor.

Table 3.26. Few small mast cells were detected in mesentery and lung suspension. Mast cells of varying sizes were apparent in the liver and abundant in the spleen and thymus. No mast cells were found in any of the cell suspensions from other organs. A few basophils were found in blood smears.

3.2.5.5. Staining to identify mast cell and eosinophils (Exp 3.61-3.68)

Smears were made by hand or by using the cytospin centrifuge, from *S. mansoni* infected mouse thymus, liver, spleen and heparinized blood cell suspensions. These were fixed in LM, LME, L, CITM fixatives and stained as described in table 3.27.

Table 3. 27

Exp No	Tissue	Fixative	Stain	Mast cell	Eosinophil
3.61	Liver, Spleen, and Thymus	LM 1min	Washed in water, 1% astra blue 6 min, washed in water, Chromotrope 6 min, Rinsed in water.	-	-
3.62	Liver, Spleen, and Thymus	LM 2min	Washed in water, 1% astra blue 6 min, washed in water, Chromotrope 6 min, Rinsed in water.	-	-
3.63	Liver, Spleen, and Thymus	LME 2min	Washed in water, 1% astra blue 6 min, washed in water, Chromotrope 6 min, Rinsed in water.	-	-
3.64	Liver, Spleen, and Thymus	LME 2min	Washed in water, 1% astra blue 10 min, washed in water, Chromotrope 5 min, Rinsed in water.	-	-
3.65	Liver, Spleen, and Thymus	L 2min	Washed in water, 1% toluidine blue 5 min, washed in water (TO1), Chromotrope 5 min, Rinsed in water.	-	-
3.66	Liver, Spleen, and Thymus	L 2min	Washed in water, 1% Astra blue 20 min, washed in water, Chromotrope 5 min, Rinsed in water.	-	-
3.67	Liver, Spleen, and Thymus	CITM 4min	Washed in water, 1% Astra blue 10 min, washed in water, Chromotrope 5 min, Rinsed in water.	-	-
3.68	Liver, Spleen, and Thymus	LME 3min	Washed in water, 1% Astra blue10 min, washed in water, Chromotrope 1.5 min, Rinsed in water.	-	-

(++) Cells well stained, numbers high, background is clear. (+) Cells well stained, number lower, background good. (+-) Weak staining limited number of cells stained, background obtrusive. (-) Inadequate staining, no cells, background poor.

Table 3.27. The methods proved not to be useful and neither mast cells nor eosinophils were observed in these experiments.

3.2.6. Staining to identify basophils and eosinophils in blood samples

Smears were made from *S. mansoni* infected mouse blood using the cytospin centrifuge. Smears were fixed in CITM, LME, MT and stained as described in table 3.28.

Table 3.28

Exp No	Smear	Fixative	Stain	Basophil	Eosinophil
3.69	Blood smears	CITM 3min	Washed in water, 1% Astra blue 10 min, washed in water, Chromotrope 1.5 min, Rinsed in water.	+-	+-
3.70	Blood smears	LME 3min	Washed in water, 1% Astra blue 10 min, washed in water, Chromotrope 1.5 min, Rinsed in water.	-	-
3.71	Blood smears	MT 3min	Washed in water, 1% Astra blue 10 min, washed in water, Chromotrope 1.5 min, Rinsed in water.	-	-
3.72	Blood smears	CITM 3.5min	Washed in water, 1% Astra blue 10 min, washed in water, Chromotrope 1.5 min, Rinsed in water.	-	-
3.73	Blood smears	CITM 4min	Washed in water, 1% Astra blue 10 min, washed in water, Chromotrope 2 min, Rinsed in water.	-	-

(++) Cells well stained, numbers high, background is clear. (+) Cells well stained, number lower, background good. (+-) Weak staining limited number of cells stained, background obtrusive. (-) Inadequate staining, no cells, background poor.

Table 3.28. When cytospin or hand made blood smears were fixed in CITM for 3 min, stained with 1% astra blue for 10 min and counter stained with chromotrope for 1.5min a few eosinophil and mast cell granules were found separately. However these results were not reproducible. Neither mast cells nor eosinophils were found in Exp 3.70, 3.71, 3.72, 3.73. Cell suspensions prepared from mouse organs did not show mast cell or eosinophils with these methods, more investigation will be required.

3.2.7. Staining to identify basophils and eosinophils in leukocyte suspensions

Smears made from leukocytes (see chapter 2) were fixed in LM, ClTM, LME, MLCl and stained as described in table 3.29.

Table 3.29

Exp No	Smear	Fixative	Stain	Basophil	Eosinophil
3.74	Leukocytes	LM	Washed in water, 1% astra blue 5-10 min, washed in water, Chromotrope 2-5 min, Rinsed in water.	-	-
3.75	Leukocytes	ClTM	Washed in water, 1% astra blue 5-10 min, washed in water, Chromotrope 2-5 min, Rinsed in water.	-	-
3.76	Leukocytes	LME	Washed in water, 1% astra blue 5-10 min, washed in water, Chromotrope 2-5 min, Rinsed in water.	-	-
3.77	Leukocytes	MLCl	Washed in water, 1% astra blue 5-10 min, washed in water, Chromotrope 2-5 min, Rinsed in water.	-	-

(++) Cells well stained, numbers high, background is clear. (+) Cells well stained, number lower, background good. (+-) Weak staining limited number of cells stained, background obtrusive. (-) Inadequate staining, no cells, background poor.

Table 3.29. The presence of excessive numbers of erythrocytes in smears which stain red with chromotrope interfered with the detection of eosinophils which also stained red.

3.3. Discussion

For the detection of mast cells, tissues are normally fixed in Carnoy's fixative or an isotonic formaldehyde-acetic acid mixture and stained with toluidine blue, Alcian blue or Astra blue (Bloom & Kelly 1960; Enerback 1966b). The detection of eosinophils generally requires that tissues be fixed in formalin-buffered

saline (Incani & McLaren 1984), Bouin's fluid or Maximov (Savage & Colley 1980) followed by staining in Dominici, haematoxylin and eosin, or chromotrope (Lendrum 1944).

The combined method of staining mast cells and eosinophils reported by Duffy *et al.* (1993) has the advantage that it works on formalin-fixed human tissue. The alternative method described in this chapter of demonstrating the presence of mast cells and eosinophils is designed primarily for use on murine tissues. It offers several advantages over existing methods and allows the examination of the relationships between these cell types in a single tissue specimen. The current protocol has permitted the staining of mast cells in intestine, liver and spleen but it may be possible to adapt the technique to allow the demonstration of these cells in other sites. Also, the quality of the staining of eosinophils represents an improvement on existing methods and should ease their identification (Kermanizadeh *et al.* 1995a) (refer to Fig 3.1.).

The principle limitation of the above methods was the short period during which tissue could be left in the fixative. Further modifications of MT fixative with 2% aqueous calcium chloride solution resulted in the preparation of a new fixative (referred to here as CITM). With CITM, Paneth cell granules in the crypts of the intestine were also clearly visible and easily distinguished from eosinophil granules in liver and spleen, the erythrocytes are also easily discriminated from eosinophil granules as they have a uniform red colour.

Another new fixative was developed, LM. With LM, excellent results have been obtained when staining mast cells and eosinophils in murine intestine, liver and spleen. An important advantage of LM is that it has permitted the staining of mast cells and eosinophils in murine skin (refer to Exp. 3.30). LM fixative is currently favoured over the others which have been investigated (Kermanizadeh *et al.* 1995b) refer to Fig 3.2 & 3.3

Using these new fixatives, the blue colour of the granules of the mast cells is probably attributable to the binding of the positively charged Astra Blue dye to the polyanionic groups in their granules. The crimson red of the granules of eosinophils probably results from the binding of anionic chromotrope to their granules.

As with all histology, quality observation is improved with a clear tissue background. With the above methods, the use of freshly prepared materials is advisable. Some mercuric chloride precipitation has been observed but not interfering with the cells identification in mouse tissue. Winter *et al.* (1995) have extended the methods used above to ovine tissues, reporting excellent results. They also introduced a modification to remove mercuric chloride precipitation with 0.5 % alcoholic iodine solution. In addition, development of PAF3 and MPd4 fixatives for demonstration of the eosinophils, and MPF, MPd and MTF for observation of the eosinophils and paneth cells, were also improvements which arose from this recent investigation.

The staining of mast cells and eosinophils in cell suspensions or basophils and eosinophils in blood smears and leukocyte suspensions remains problematic. The excess of erythrocytes in blood smears and leukocytes suspensions, leads to background staining which makes the identification of eosinophils extremely difficult. However mast cells were detectable in both leukocyte and organ cell suspensions and additional experiments are required.

3.4. Summary

Methods have been established which permit the staining and identification of mast cells and eosinophils together in single tissue sections. In addition a variety of new fixatives have been evaluated. Using these methods, eosinophils and mast

cells can be readily detected in liver, spleen, intestine and skin. The quality of fixation and staining is much improved with these new methods. The work on methods to distinguish eosinophils and basophils together in cell suspensions and blood smears needs further development. A series of histological procedures have been identified for fixation and staining of the mast cells and eosinophils either separately or together in tissue sections.

Fig 3.1 Section of the intestine of 8 week *S.mansoni*-infected mice. Mast cells and eosinophils are evident in blue and red colour respectively. Fixing in MT and staining with astra blue, magnification x 500

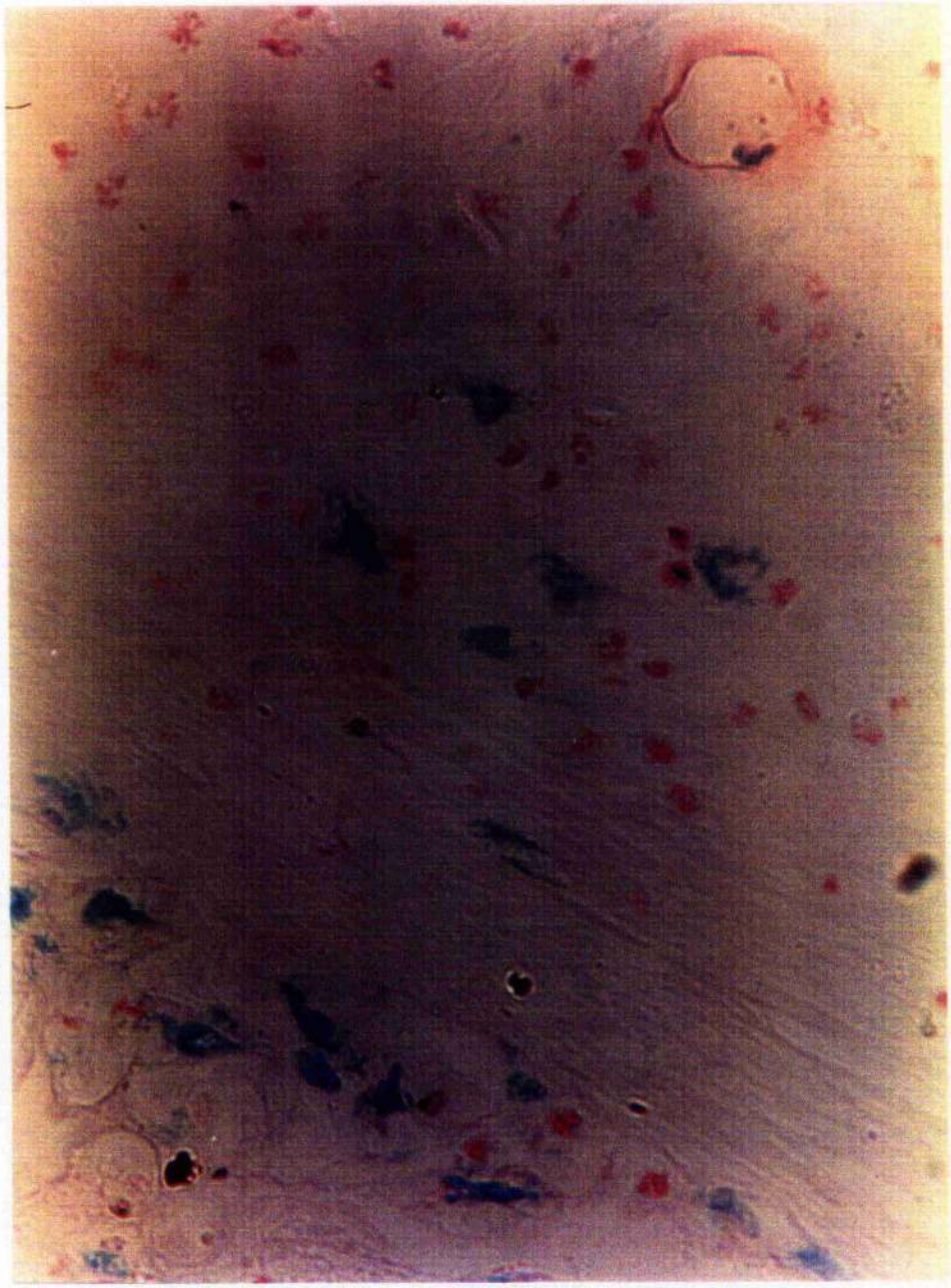
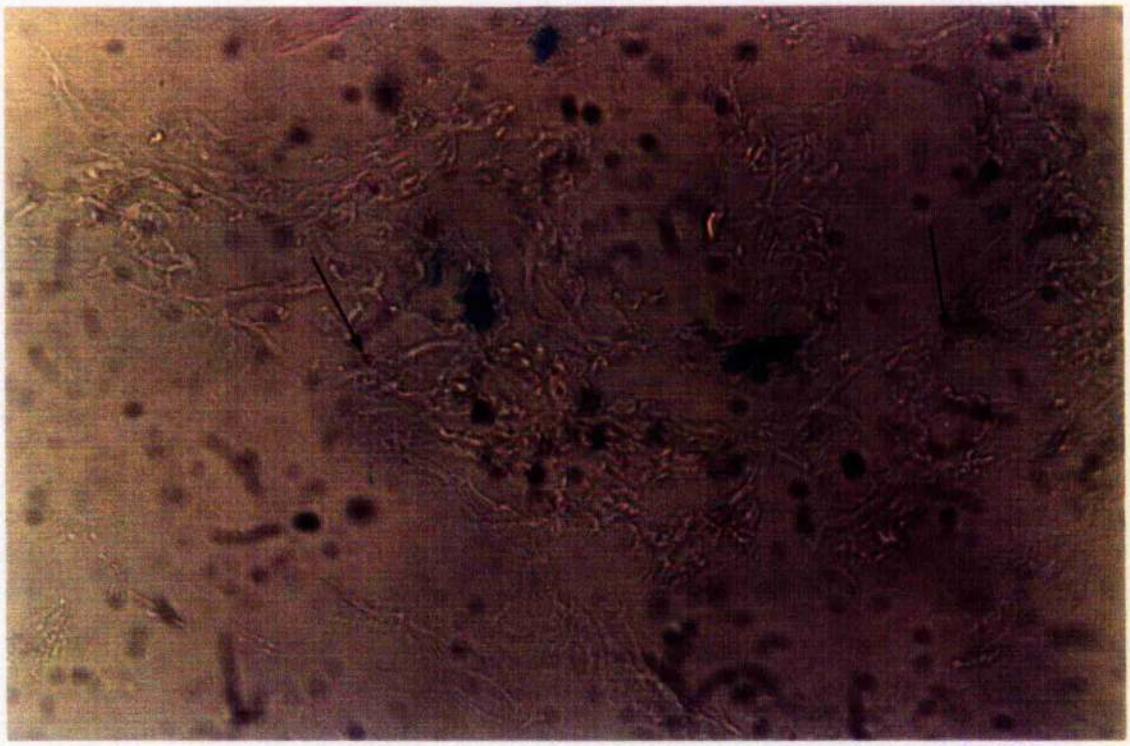
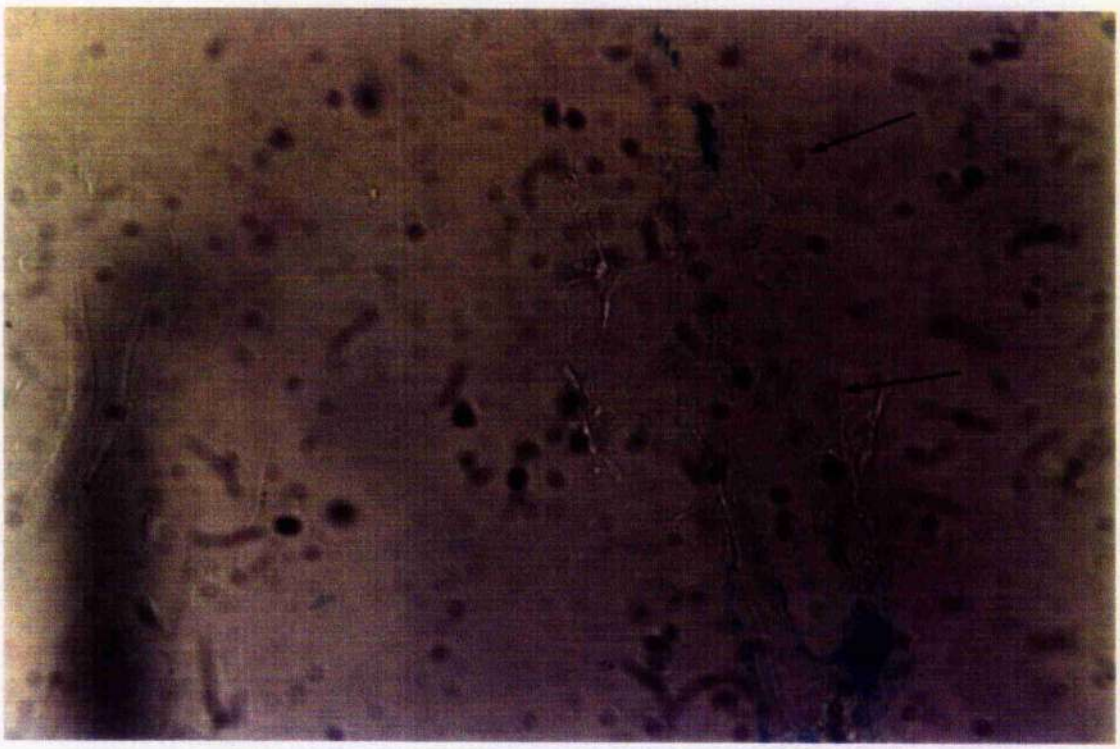


Fig 3.3 Section of the skin from a mouse infected with *S.mansoni* for 8 weeks. Three mast cells and a few eosinophils are evident in blue and red colour respectively. Arrows point to eosinophils. Fixing in LM and staining with ACS, magnification x 250

Fig 3.2 Section of the skin from a mouse infected with *S.mansoni* for 8 weeks. Few mast cells and two eosinophils are evident in blue and red colour respectively. Arrows point to eosinophils. Fixing in LM and staining with ACS, magnification x 250





Chapter 4

A Simple Method for Counting Cells in Tissue Sections

4.1. Introduction

The accurate recognition of particular cell types in a tissue section can be a straight forward procedure requiring nothing more than a calibrated microscope and a well trained observer. Generally such methods readily lend themselves to the study of cellular infiltrates into uniform tissue sections such as are found in the liver or spleen. Obtaining accurate quantitative data on cell counts from less compact tissues such as the lung, or the mucosa of the small intestine, is much more difficult and there is no method which has gained universal acceptance.

Computer assisted image analysis remains costly in terms of hardware and software and is restricted to a limited number of laboratories. In places where schistosomiasis is endemic, such equipment is not readily available. The development of a simple, inexpensive, reliable and quantitative method for counting cell numbers in tissue sections would be a useful technique in this regard.

The aim of the work described in this chapter was to develop a simple method for estimating the numbers of cell types per unit area of stained tissue section. As to preliminary considerations, it was decided to base the method on the use of a graticule designed by Weibel (1979).

4.2 Materials and methods

4.2.1 Details of microscope

The microscope used was a Leitz Wetzlar SM-LUX type 020-441, 003.

4.2.2 Weibel graticule

The weibel graticule was kindly provided by Dr V. Moss, physiologist, the University of Glasgow. Sadly Dr Moss died before this chapter was written.

4.2.3 Calibrated linear slide

Graticules LTD of, Tonbridge, Kent England. (100 x 0.01= 1mm).

4.2.4 Description of the method

A Weibel graticule is placed into one of the microscope eyepieces, with the image being superimposed on a calibrated microscope slide. The 1mm calibrated microscope slide with (0.01 mm) divisions is most suitable. Measuring the length of the two sides of the graticule the area covered by Weibel graticule is determined in any desired magnification.

With the above microscope system, the area covered by graticule at x100 and x400 magnifications is 0.6889 mm^2 and 0.0484 mm^2 respectively. Next a slide containing the tissue section is put in place, the number of the cells of interest covered by the graticule can then be counted.

The Weibel graticule is marked with 21 lines with interruptions as long as the lines themselves (Fig 4.1.), the ends of these lines can be considered as 42 distinct

points. When the graticule is superimposed over the tissue the number of the points which lie over the tissue section should be determined (at the same time some points may lie not on the tissue). From the number of the points which lie over the tissue and total number of points in the graticule the ratio of tissue to space within the graticule can be calculated.

For example, if 18 of the 42 points lie on the tissue the ratio of tissue to space within the graticule is therefore 18 / 42. At x 400 magnification, the area covered by the tissue in the total graticule covered area is then 18 / 42 x 0.0484 mm^2 = 0.0207 mm^2 and if 5 cells are observed in this area, this is the equivalent of 5 / 0.0207 = 241 cells / mm^2.

If the counting of the cells in a unit volume desired, the thickness of the section should be multiplied by the area covered by graticule and number of the cells per / mm^3 can then be estimated.

4.3. Results

This method has been used to count the mast cells in both infected and uninfected mouse intestine in an experimental *Schistosoma mansoni* infection. The number of the MMC and CTMC mast cells are counted by this method and the results of the study are demonstrated in Chapter 5 of this thesis.

The method has been successfully applied on muscularis mucosa surrounding the lumen of the eosophagus of *S. mansoni*-infected mice and the nuclei per mm^2 present in this area has been counted (L.Rennie-personal communication).

4.3 Discussion

Current methods of counting the numbers of cells in a tissue section from small intestine (Miller *et al* 1971; Murray 1979; MacDonald *et al* 1980) suffer from a number of drawbacks. The numbers of cells present in gut tissue, for example, are often expressed in relation to villus crypt units, but since the shape and dimension of individual villi and crypts may vary, particularly during inflammatory processes, this may not give results directly comparable between different experiments or different investigators. Furthermore given that the villus crypt unit is being used, there might be a tendency to select suitable tissue sections on which to make the counts, introducing an additional bias.

Use of this method increases the accuracy of counting and, since it can be applied on small sections of tissue, removes any bias which might arise from the selection of areas of small intestinal tissue which have complete villi and crypts. Since this process of selection requires that many tissue sections be screened, the new method will reduce the time needed to make an accurate determination of cells in any given tissue sections. In addition direct comparisons between the results of different investigators and their experiments can be made.

4.5 Summary

An improved method of counting cells in tissue sections is described. The method uses a Weibel graticule and permits the rapid quantification of cells in tissues with complex morphology.

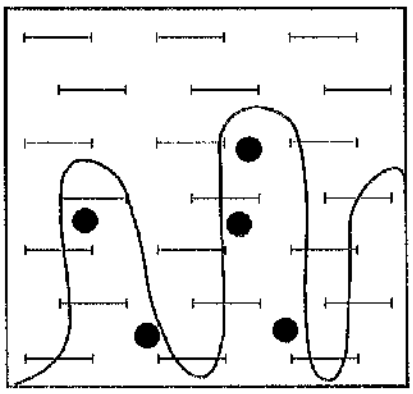


Fig. 4.1 **A suppositional view of a graticule which was superimposed on imaginary tissue. 18 of 42 points are lying on the tissue.**

Chapter 5

Mast Cell Kinetics and IgE During Experimental Schistosomiasis mansoni in Mice

5.1. Introduction.

An important hallmark of host responses to parasitic helminth infections in the intestine is an increase in the numbers of mast cells in the mucosa (Miller 1980 &1987). Detailed descriptions of this feature have come from studies of the nematode *Nippostrongylus brasiliensis* in rats in which the increase in the numbers of mast cells is associated with worm expulsion from the gut; interestingly the increase in mast cell numbers is found through the length of the gut and not just at the sites occupied by the worms (McDonald *et al.* 1980). Studies with other parasites have suggested that localised increases in mast cell numbers can occur. During infection with *Fibricola seoulensis* in rats for example, the proximal region of the small intestine was found always to contain higher numbers of mast cells than the middle and distal regions (Kho *et al.* 1990). This trematode is generally found in the proximal small intestine. Accumulation of mast cells locally in parasitized tissues can be used as a reason in favour of these cells in producing immunity. Also secondary challenge experiments have confirmed that basophils (Rothwell & Dineen 1972) and mast cells (Rothwell 1989) may be contribute to the prevention of reinfection in gastrointestinal parasitism and the early stage of schistosomiasis (Li Hsu *et al.* 1979). The importance of mast cells on parasitic infection has been investigated extensively (Charbon *et al.* 1991; Askenase 1980; Lee *et al.* 1986).

Depletion of mast cells has been shown to cancel the cytotoxic activity of eosinophils on schistosomula (Capron *et al.* 1978b). The protective response of the mast cells particularly has been focused on gastrointestinal parasites, since mast cell-induced immediate-type hypersensitivities at secondary challenge would produce oedema, increase mucous secretion and intestinal peristalsis, and change the local condition consequently leading to larval entrapment (Miller *et al.* 1981) worm migration inhibition (Douch *et al.* 1983) and worm damage (Miller & Huntley 1982).

Mast cells are involved not only in the pathology of parasitic infections but also in other gastrointestinal inflammatory diseases such as food induced hypersensitivity, ulcerative colitis, coeliac disease, *Helicobacter pylori*-induced gastritis or ulcer, and also ethanol-induced gastritis or ulcer (Wershil 1995). The role mast cells play during schistosome infection is less well defined and remains a subject for debate. Recent evidence shows that mast cells produce a variety of multifunctional cytokines, including IL3, IL4, IL5, IL6 and TNF-α and has renewed interest in the activity of these cells in the inflammatory response and the way in which they may contribute to the pathology and immune response to parasitic diseases. The major role of IgE in *Schistosoma*-infection has been demonstrated, by association of high levels of specific anti-schistosome IgE with resistance to reinfection with *S. haematobium* (Hagan *et al.* 1991) and *Schistosoma mansoni* (Rihet *et al.* 1991, Dunne *et al.* 1992), and the involvement of IgE in mast cell stimulation may be important in *Schistosoma* infection. According to the literature, most attention has been focused on studying the role of the mast cells on gastrointestinal dwelling parasites, very little attention has been applied to the blood resident, *Schistosoma* in this regard. Therefore studying the kinetics of mast cells during the acute and chronic phase of infection was of great interest, particularly as a new cell counting method was available for use.

5.1.1. Aims of study

The work described in this chapter was carried out to understand the following points:

1) The kinetics and distribution of mast cells in *S. mansoni*-infected small intestine;

2) The relationship between IgE antibodies and mast cells during *schistosomiasis mansoni* in mice.

5.2. Materials and methods

5.2.1. Animals

A total of 68 outbred female CFLP mice, weighing between 30g and 37g, were infected percutaneously with 100 cercariae of a laboratory maintained Puerto Rican strain of *Schistosoma mansoni*. After inducing anaesthesia with 0.01 ml / g body weight of a solution of Sagatal (*i.p.*), the mice were exposed to the cercariae (see chapter 2).

5.2.2. Post-mortem examination

The infected and control mice were killed according to the schedule described in Table 5.1 and the intestine was prepared for histological examination, as described below. One mouse was perfused to give an indication of the adult worm burden. Body weights were recorded for all animals at *post-mortem*. The weights of the liver and spleen and the length of small intestine were recorded.

Table. 5.1 Post mortem schedule

Weeks post infection	No of infected mice	No of control mice	No of perfused mice	No of the worms
0	0	3	0	0
1	4	0	0	0
2	4	0	0	0
3	4	4	0	0
4	4	4	1	0
5	5	4	1	0
6	5	0	1	1 NI
7	5	4	1	8 F,M
8	5	0	1	4 F,M
9	5	4	1	3 F,M
10	5	0	1	12 F,M
11	4	4	1	3 F,M
12	5	0	1	12 F,M
14	5	0	1	10 F,M
16	5	4	1	6 F,M

F (Female), M (Male), NI (Not identifiable)

5.2.3. Tissue processing

At post-mortem tissues were fixed in different fixatives Table 5.2. The small intestine was removed and all mesenteric tissue was trimmed away and treated using the modification of the Swiss role technique (Chapter 2), originally described by Domingo & Warren (1969). Briefly the intestine was placed in Carnoy's fixative for 1 min and then laid on clean filter paper soaked in 0.85% NaCl solution. The intestine was opened along the mesenteric border and the inside gently washed with saline to remove any undigested food and other debris. Careful manipulation allowed the intestine to be flattened against the filter paper without damaging the villus structure. The small intestine was then rolled on a stick from the pyloric end. At times, with the thickened intestine of infected animals, the intestine was prepared in two halves, two rolls being made from each intestine. The intestinal tissues were returned to Carnoy's fixative for 24 h, embedded in paraffin wax (50°C) and sectioned at 5 μm thickness. Sections were stained with (AS) methods.

5.2.4. Identification and counting of cells

The numbers of mast cells were counted at three parts of the intestine using the new method already described (Chapter 4). Ten to 20 fields were counted with the graticules for each third of the intestine. Two types of mast cells have been identified in the intestinal tissue. Those found in the crypts and in the villi are considered as mucosal mast cells (MMC) whereas those in the submucosa and muscle layers are considered to be connective tissue mast cells (CTMC). For simplicity the results are described for each cell type in each section of the intestine that was examined.

Table.5.2. Processing of the *S. mansoni*-infected mice

Tissue	tissue processing	Fixative	Stain	Results
Intestine	Swiss role	CAR 24 h	AS	(Chapter 5)
Liver	Section	MT 24 h	AC	(Chapter 6)
Liver	Section	NBF still are being fixed	-	tissues have not been used during this examination
spleen	Section	NBF still are being fixed	-	tissues have not been used during this examination

5.2.5. IgE antibody measurement

The amount of IgE antibody in *S. mansoni* infected and control mouse serum was measured by ELISA assay at weeks 3, 4, 5, 7, 9, 11 and 16 post infection using the anti IgE, (Chapter 2). Sera was diluted 50X, 100X and 200X respectively. The strongest response was obtained with the 50 fold dilution.

5.2.6. Statistical analysis

Since the animal sample size and optical density (OD) readings of the ELISA assay were small, the estimation of the distribution of the samples was not possible therefore a distribution-free or non-parametric (Mann-Whitney) statistical test was used for data analyses.

5.3. Results

5.3.1. MMC in the anterior portion of the small intestine

In control mice MMC numbers were estimated as 130 MMC per mm^2 on week 3. The numbers increased in control animals and by week 16 had reached 644 per mm^2. Such an increase may be a function of the age of the mice. In infected mice at the start of the experiment, most of the MMC to be detected were found in the *lamina propria* but by week 7 post infection MMC were present throughout the whole width of the intestine from the *lamina propria* to the tips of the villi. In relation to the control mice, higher numbers of MMC were found in infected mice at week 7 post infection (21144 per mm^2). Thereafter the numbers of MMC decreased steadily. There were 5227 MMC per mm^2 at week 16 the end of this experiment (Fig 5.1, 5.2, 5.13).

5.3.2. MMC in the middle and posterior portions of the small intestine

In the control mice MMC numbers did increase slightly, perhaps associated with the ageing of the mice, from 284 to 883 and from 105 to 380 in the middle and posterior gut sections respectively. In *S. mansoni* -infected mice, MMC numbers in the middle and posterior sections followed a pattern similar to that of the anterior section except that these reached a peak of 18427 in per mm^2 in the middle section and 8998 per mm^2 in the posterior section by week 7 post infection Thereafter the numbers of MMC decreased to 5000 and 4000 per mm^2 by week 16 post infection (Fig 5.3, 5.4, 5.5, 5.6).

Fig 5.1 Mean numbers of MMC at the anterior portion of the small intestine for infected (n = 4-5) and control (n = 3-4) mice. (*) Mann-Whitney test shows that difference between medians of the infected and controls are statistically significant ($P < 0.05$)

Fig 5.3 Mean numbers of MMC at the middle portion of the small intestine of mice (*) Mann-Whitney test shows that difference between medians of the infected (n = 4-5) and controls (n = 3-4) are statistically significant ($P < 0.05$)

Fig 5.4 Mean numbers of MMC at the posterior portion of the small intestine of mice (*) Mann-Whitney test shows that difference between medians of the infected (n = 4-5) and controls (n = 3-4) are statistically significant ($P < 0.05$)

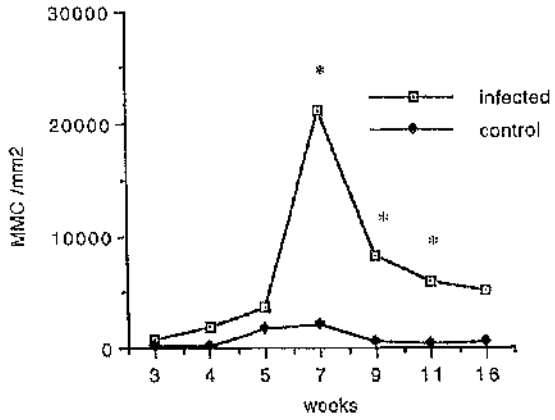


Fig. 5.1

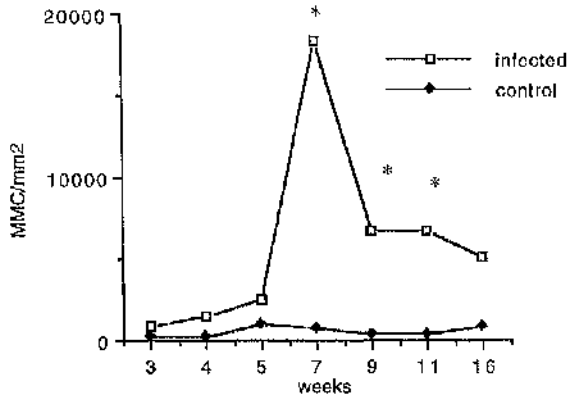


Fig. 5.3

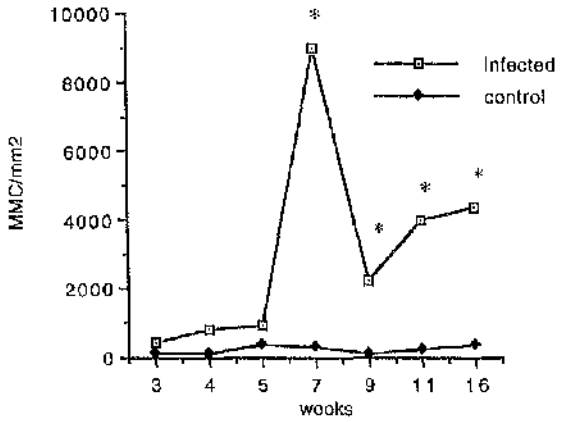


Fig. 5.4

5.3.3. CTMC in the anterior portion of the small intestine

In both infected and control mice, CTMC were located in the submucosa and muscle layers. In control mice, their numbers increased from 158 per mm^2 at week 3 to 761 per mm^2 at week 16.

In *S. mansoni* -infected mice, larger numbers of CTMC were observed at 6 weeks post infection, increasing from 336 per mm^2 to 2232 per mm^2 at weeks 7 and 3134 per mm^2 at week 11 post infection. CTMC numbers continued to increase until the end of the experiment (Fig 5.7, 5.8).

5.3.4. CTMC in the middle portion of the small intestine

In control mice CTMC numbers increased from 58 per mm^2 at week 3 to 105 per mm^2 at week 16 with most of the rise being observed to occur after week 11. In *S . mansoni* -infected mice, the pattern was similar to that found in the anterior small intestine with 2850, 2586 and 4744 CTMC per mm^2 being counted at weeks 7, 11 and 16 post infection respectively (Fig 5.9, 5.10).

5.3.5. CTMC in the posterior portion of the small intestine

In control mice, CTMC were not seen during first three weeks of the experiment. By week 16 there were 281 CTMC per mm^2. In *S. mansoni*-infected mice the numbers of CTMC in the posterior part of the small intestine increased, reaching a maximum of 3746 per mm^2 at the end of the experiment (Fig 5.11, 5.12).

Fig 5.7. Mean numbers of CTMC at the anterior portion of the small intestine of mice (*) The difference between medians of the infected (n =4-5) and controls (n =3-4) are statistically significant ($P < 0.05$).

Fig 5.9. Mean numbers of CTMC at the middle portion of the small intestine of mice (*) The difference between medians of the infected (n =4-5) and controls (n =3-4) are statistically significant ($P < 0.05$)

Fig 5.11. Mean numbers of CTMC at the posterior portion of the small intestine (*) The difference between medians of the *S. mansoni*-infected (n =4-5) and controls (n = 3-4) are statistically significant ($P < 0.05$)

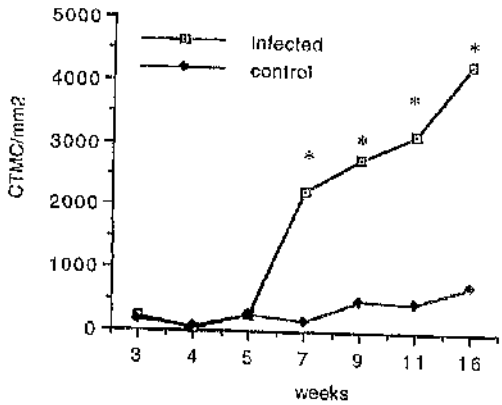


Fig. 5.7

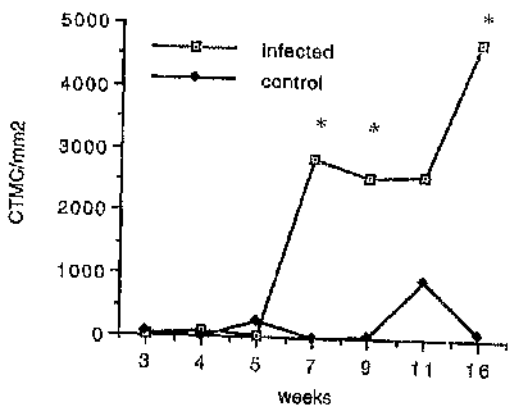


Fig. 5.9

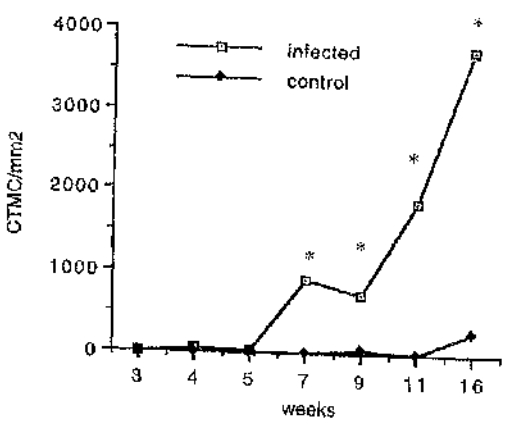


Fig. 5.11

5.3.6. IgE response

The total IgE concentration in the serum was measured by ELISA assay. An increase in the IgE level was detected after week 5 post infection and reached a maximum at week 9 post infection and thereafter decreased, it remained considerably higher compared to the control mice until the end of the study (Fig 5.14).

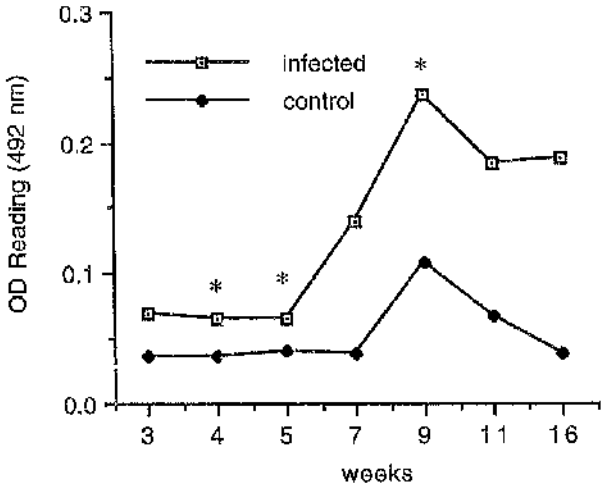


Fig 5.14. Mean IgE concentration in *S. mansoni* infected and control mice.(*) The difference between medians of the infected (n =2-5) and controls (n =3-4) are statistically significant ($P < 0.05$).

5.3.7. Weight of the mice during the experiment

The weight of both infected and control mice declined during the first 4 weeks of the experiments. The loss of weight in the infected mice could be attributed to the infection. The loss of weight in control animals is difficult to be explain. Thereafter the weight of both infected and control animals increased. The weight of infected mice was always higher than controls but the differences were not found to be statistically different (Fig 5.15).

5.3.8. Length of the intestine during the experiment

In control mice the length of the small intestine remained steady with only minor fluctuations throughout the duration of the experiment. In infected mice the length of the small intestine decreased from 43 cm to 37 cm though the decline was marked by large fluctuations. The decrease in length of the intestine may be a consequence of granuloma formation and inflammation affecting the muscles of the intestine (Fig 5.16).

5.3.9. Weight of the liver during the experiment

The weights of the livers of control mice changed little during the course of the experiment. On the other hand, livers from infected mice showed large changes in weight increasing from 2.3 g at week 3 to 4.2 g at week 11 and then decreased towards the end of the study (Fig 5.17).

5.3.10. Weight of the spleen during the experiment

The weights of the spleens of control mice remained steady throughout the study. Spleens from infected mice increased in weight reaching a maximum at week 11 post infection. The weights then fluctuated downwards until the end of the experiment (Fig 5.18).

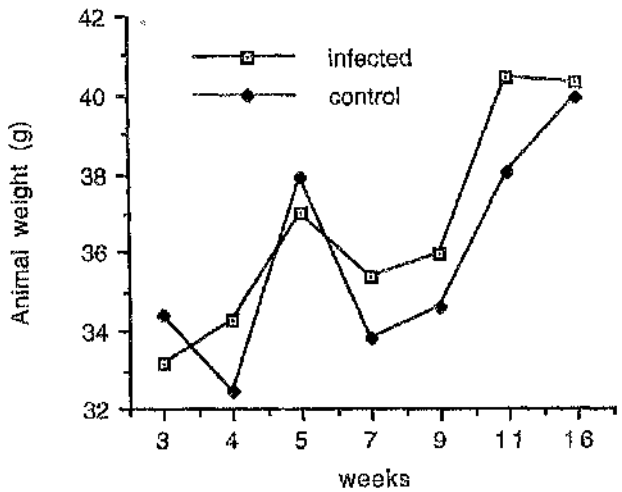


Fig 5.15. Mean weights of *S. mansoni*-infected mice and control. Differences between infected (n = 4-5) and control (n = 3-4) mice were not statistically significant.

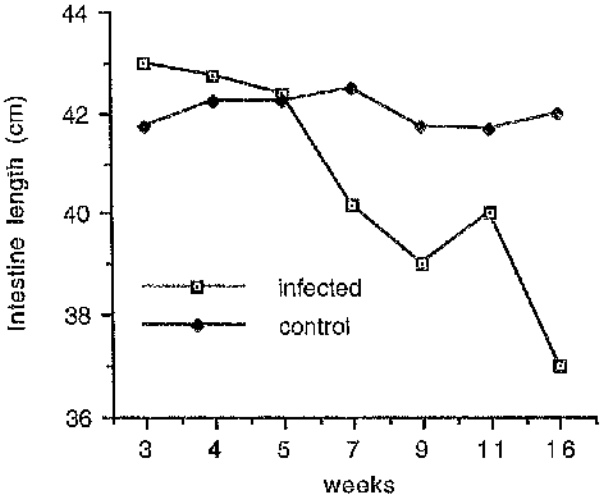


Fig 5.16. Mean length of small intestines of *S. mansoni* -infected (n = 4-5) and control mice (n = 3-4). Despite obvious differences between the lengths of infected and control intestines, there was never any statistically significant difference between the two groups.

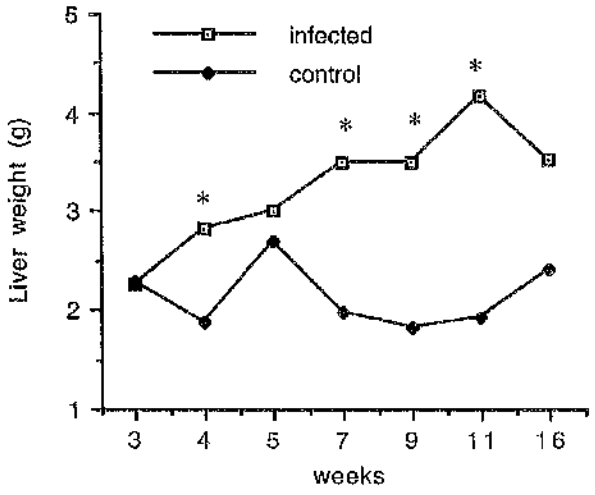


Fig 5.17. Mean weights of the livers of *S. mansoni* -infected and control mice (*) The difference between medians of the infected (n = 4-5) and controls (n = 3-4) are statistically significant ($P < 0.05$).

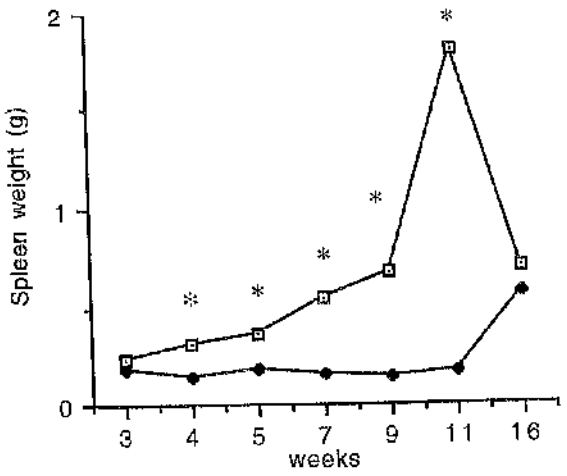


Fig 5.18. Mean spleen weight of *S. mansoni* -infected and control mice. (*) The difference between medians of the infected (n = 4-5) and controls (n = 3-4) are statistically significant ($P < 0.05$)

5.4. Discussion

In addition to their participation in inflammatory responses mast cells, by producing tumour necrosis factor (Benyon *et al.* 1991; Steffen *et al.* 1989) and other cytokines (Gordon & Galli 1991, 1990a), are potentially important in the regulation of inflammatory processes and other immune reactivities.

Crucial to the participation of mast cells in these activities are the degranulation events which occur through cross-linking of specific IgE on their surfaces. Degranulation can lead to immediate hypersensitivity and acute inflammation.

The close association of mast cells and IgE and their activities during helminth infection has been the subject of much study and fierce debate. Most of these studies have involved investigations of responses to intestinal parasites and in particular to intestinal nematodes. Additional studies have

been conducted on schistosomes and especially on *Schistosoma mansoni* but despite the central role of IgE in resistance to *Schistosoma* (Hagan *et al.* 1991; Dunne *et al.* 1992) and its participation in antibody-dependent cellular cytotoxicity mechanisms associated with mast cells, basophils, eosinophils and platelets (Joseph *et al.* 1983; Capron *et al.* 1986 & 1980; Miller 1987) less attention has been focused on the effect of schistosomes on intestinal tissue. Although *S. mansoni* is a blood-dwelling parasite its intimate association with the mesentery and gut tissue, across which eggs have to pass to reach the environment suggests that a fuller understanding of the biology of this parasite and of the pathological consequences of infection, could be obtained, from the more detailed examination of the dynamics of mast cells and IgE levels during infection and in particular during the intestinal phase of infection. In this study the induction of mucosal and connective tissue mast cells by *S. mansoni* infection has been examined in CFLP mice. Total IgE levels have been monitored during the course of infection.

For the detection of MMC and CTMC, Carnoy's fixative generally is used. In particular it has been recommended for MMC fixation by some investigators (Strobel *et al.* 1981; Enerback 1966a). In recent experiments it has been found useful for both MMC and CTMC fixation. The most frequently used stain for mast cells is toluidine blue, a metachromatic stain which stains the mast cells granules purple-red or violet. Astra blue has also high affinity for mucopolysaccharides present in the mast cell's granules (Bloom & Kelly 1960).

Since the nature of the proteoglycans in mast cells granules are different, by using a non-metachromatic astra blue and safranin dye MMC and CTMC can be differentiated (The MMC stain blue and CTMC stain red). Astra blue on its own has been shown in recent study to be a general stain for mast cell granules, since, in addition to gut dwelling mast cells, skin resident

mast cells also were observable using this dye. Using the astra blue and safranin, most of the CTMC in the muscle layer of the intestine stained red while the rest of the mast cells stained a mixture of red and blue colours (Miller & Walshaw 1972; Enerback 1966b) (Fig 5.8, 5.10). This change of colours being attributed to the amount of the heparin which is found in connective tissue mast cell granules. CTMC were also stained with toluidine blue to confirm the accuracy of astra blue staining for mast cell detection.

Since the method described above stains the granules of the cells, cytoplasm is rarely visible and each cluster of mast cell granules is considered to have arisen from an individual mast cell. In keeping with the classification of other authors (Marshall 1990) all the mast cells found in the submucosa and muscle layer, (which are stained similar to the peritoneal mast cells) are considered to be connective tissue mast cells (CTMC) and those in the epithelium, lamina propria, and mucosal glands to be mucosal mast cells (MMC).

MMC were increased slowly during week 1-2 post infection and first observed at the bottom of villi in the *lamina propria* and only later in the epithelium and at the tops of the villi. MMC increased about week 3 post-infection and reached their maximum numbers at week 7 post infection and then decreased and remained at a steady number between week 11 to 16 post infection. The number of MMC was higher in the anterior than the posterior of the small intestine.

CTMC also started to increase at week 6 post infection and remained around the same level until the end of the experiment. The earlier increase of the MMC at week 3 post infection in this experiment looked to be schistosomula dependent, rather than the adult worm dependent but the increase of the CTMC coincided with development of adult worms and egg production. As the infection matured the numbers of the MMC reduced.

While the CTMC remained at a high level during the chronic phase of *S. mansoni* infection. The results described are consistent with those of (Lenzi *et al.* 1987) who reported an increase in peritoneal mast cells between days forty and fifty five post infection. Unfortunately they did not continue their experiments beyond this time.

What is the functional role of the mast cells during infection ? Although it is impossible to know exactly what the relationships between the mast cells and parasites really are, the temporal relationships of parasite maturation and egg deposition do allow room for some speculation. According to some investigators MMC proliferation in the intestine of parasitised animals is a localised response dependent upon the presence and level of the offending helminth (Kho *et al.* 1990). *S. mansoni* is present in the portal veins of the intestine and access of parasite eggs to the large intestine and the posterior small intestine is greater than to the middle and anterior small intestine. This was not reflected in the numbers of mast cells which were higher in the anterior small intestine than in posterior small intestine. The increased blood supply to the anterior small intestine may be one reason why numbers there are higher, since blood flow to this site is higher.

The results from these experiments illustrate some points. (1) MMC increased earlier, at about week 3 and CTMC at week 6 post infection. (2) The distribution of MMC and CTMC were not equal over the intestine, always greater numbers of mast cells were found at the anterior part of the intestine than the posterior. (3) The numbers of MMC reached a peak and then reduced through the duration of the infection, but the numbers of CTMC on the other hand remained stable as the infection matured. (4) All the infected mice suffered from splenomegaly and hepatomegaly, and the length of the small intestine decreased as the infection matured. However the difference between

infected and control animals was never statistically significant. (5) IgE levels increased during the infection.

On the basis of the above observations it is possible to speculate that I) mast cells may be involved in the immune resposes to *S. mansoni* through local inflammatory responses probably through leucocyte recruitment, mast cells may reduce the chance of worms and eggs survival by altering the tissue environment, diverting the young worms pathway to unsuitable sites within the host body, during tissue migration and delaying or preventing their development. Non-hospitable tissues have been described to play a crucial role in parasite reinfection (Gerkin *et al.* 1990b & 1984).

II) The existence of mast cell hyperplasia and high levels of IgE during early and at the chronic phase of the infection in this study, is in line with the statement of Marshall (1993a) " continuous doses of *S. mansoni* antigen cause a hyperplasia of mast cells" and all are consistent with concomitant immunity described by Smithers (Smithers & Terry 1969a), since the inflammatory processes needed to be active may require continous stimulation.

III) Mast cells may proliferate in response to *S. mansoni* stimulation but their distribution and accumulation within the body and injured areas may be determined by other factors. Since these cells take up positions at the periphery of the host tissues where attack and ingress of external substances is probable.

5.5. Summary

The kinetics of MMC and CTMC responses were studied in the intestines of 68 female CFLP mice infected with 100 *S. mansoni* cercaria. Four or five infected mice were examined on weeks 1,2,3,4,5,6,7,8,9,11,12,14,16 post infection, control mice were examined only on weeks 3,4,5,7,9,11,16 post infection. Whole intestines were histologically examined after (AS) staining. MMC and CTMC numbers increased from week 3 and week 6 respectively. While the number of CTMC were still increasing during the chronic phase of the infection the MMC number continuously decreased as the infection matured. The numbers of both cells were always greater in the proximal part of the small intestine.

Fig 5.2. Section of intestinal mucosa of mice, 7 week after infection with *S. mansoni.* Mast cells are evident at all levels of proximal portion of intestine. Astra blue / safranin x 100

Fig 5.5 section of intestinal jejunal mucosa of mice, 7 week after infection with *S. mansoni.* Mast cells are evident at all levels of this part of the intestine. Astra blue / safranin x 100

Fig 5.6. Section of ileal mucosa of mice, 7 week after infection with *S. mansoni.* Mast cells are evident at all levels of this part of the intestine. Astra blue / safranin x 100

Fig 5.8 Section of proximal portion of mice intestine, 7 week after infection with *S. mansoni.* Mast cells are evident in the submucosa and the muscularis. Astra blue / safranin x 100

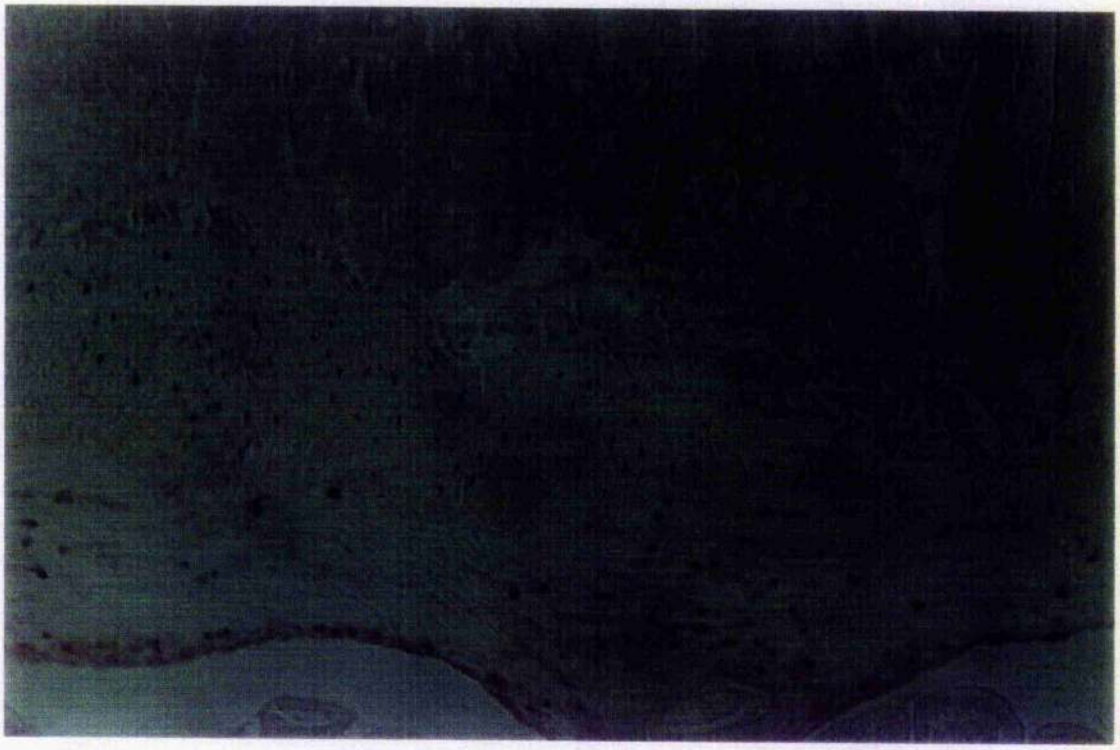
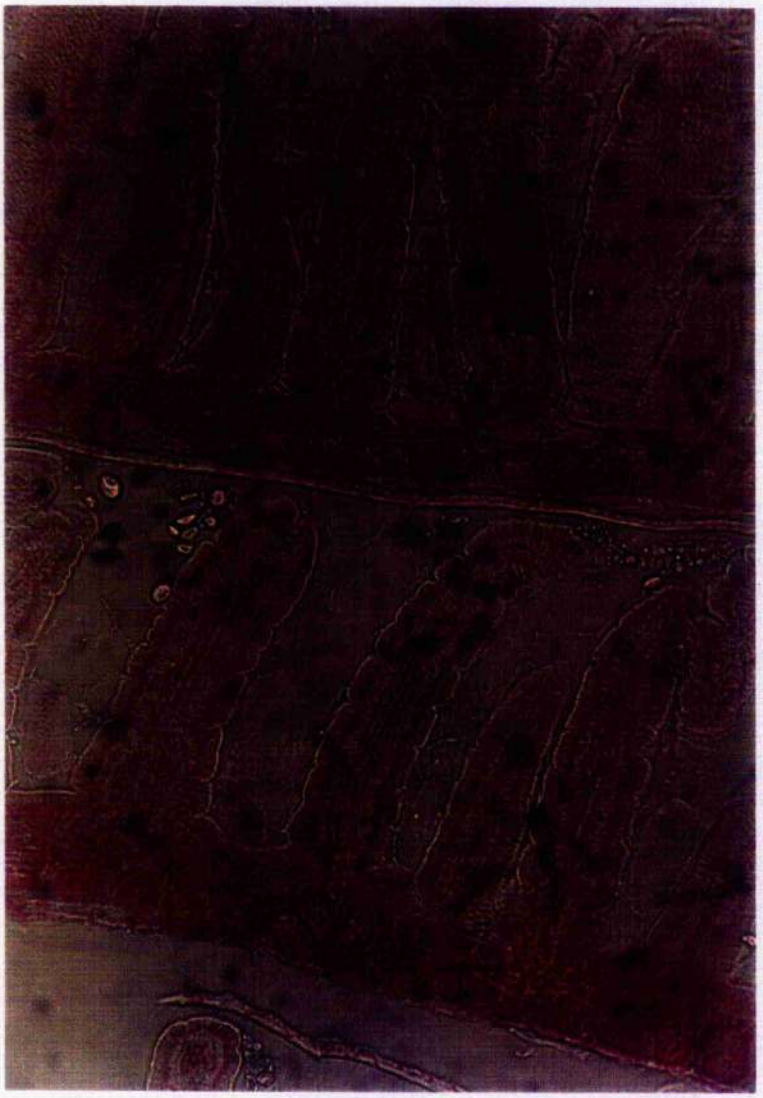


Fig 5.12. Section of ileal portion of mice intestine, 7 week after infection with *S. mansoni*. Mast cells are evident in the submucosa and the muscularis. Astra blue / safranin x 100

Fig 5.10. Section of jejunal portion of mice intestine, 7 week after infection with *S. mansoni*. Mast cells are evident in the submucosa and the muscularis. Astra blue / safranin x 100

Fig 5.13 Section of jejunal portion of uninfected mice intestine, 7 week after starting the experiments (compare with tissue from infected intestine). Only small numbers of mast cells are found at all levels of uninfected intestine.

Astra blue / safranin x 100



Chapter 6

The Kinetics of Mast Cells and Eosinophils in the Livers of Mice Experimentally Infected with *Schistosoma mansoni*

6.1. Introduction

Adult *Schistosoma mansoni* which live in the inferior mesenteric veins, deposit eggs containing embryo, and these move into the intestine. Some eggs, however, are transported to the liver, predominantly lodging in portal areas while others are carried to other organs where they become trapped. The eggs of schistosomes are responsible for the major symptoms of schistosomiasis. The embedded ova in the tissues produce a variety of toxic (Byram & Von Lichtenberg 1977) and antigenic substances which are released through micropores in the egg shells into the surrounding tissues. The toxic materials destroy the cells of the host tissue and antigenic materials stimulate the host to develop large inflammatory reactions (granuloma) around the egg material during the acute phase of infection (Warren *et al.* 1977). Because the eggs are not phagocytosed easily in the tissue, the inflammation extends to the chronic stage of infection. Granuloma formation decreases during the chronic phase of infection and this is known as granuloma modulation (Boros 1986).

A variety of cell types appear to contribute to granuloma modulation, and humoral modulation is also possible by idiotypic / anti-idotypic and other as yet undefined mechanisms (Perrin & Phillips 1989; Weinstock 1992). Granuloma are considered to be principally a result of cell mediated immunity.

However the neutralization of IL-4 results in the formation of considerably smaller granuloma while the induction of IL-2 and IL-4 results in the development of larger granuloma (Pearce 1995). Granuloma formed in this way are unlikely to be exclusively influenced by classic cell mediated immune responses (Henderson *et al.* 1991). However, Th1 cells may be involved in the early phase of granuloma formation by producing IFN-γ and IL-2, but Th2-type T cells are replaced with Th1 cells. Th2 subset of T cells by producing large amounts of IL-4 and IL-5 affect later stages of the granuloma process (Grzych *et al.* 1991).

The granuloma is considered to serve as a protective barrier produced by the immunological response to sequester toxic and antigenic substances secreted continuously from the schistosomes eggs so preventing further diffusion of these harmful materials. Eggs, and embryos therein, if not expelled, are eventually destroyed, and residual debris is absorbed (Weinstock 1992). Through its dynamic process of development the granuloma may also serve to facilitate egg excretion (Doenhoff *et al.* 1979; Damian 1987).

On the other hand granulomas may replace the damaged parenchyma of the organs in which they are found by producing and setting down collagen between the normal cells and converting the functional tissue to the dead fibrotic tissue. *Schistosoma mansoni*-induced granulomas consequently initiate periphlebitis and fibrosis in the liver (DeCock 1986; Newport *et al.* 1993). Portal hypertension, splenomegaly, ascites and oesophageal and gastric varices develop due to the resulting impaired blood circulation.

Despite the fact that granulomas are modulated in part by T cell regulation, the existence of the IL-4, IL-5, IL-10 and other cytokines in mast cells suggests that, locally resident mast cells may contribute strongly to granuloma formation, pathology and regulation. Eosinophils are also known to be a source of IL-3, IL-1, IL-5, and MIP-1 cytokine production (Weller *et al.*

1993; Kita *et al.* 1991; Deseumeaux *et al.* 1992; Costa *et al.* 1993). Eosinophil-associated pathogenesis is attributed to the release and properties of four cationic proteins. MBP (major basic protein) and ECP (eosinophil cationic protein) have cytotoxic effects on parasites and mammalian cells (Ackerman *et al.* 1985; Motojima *et al.* 1989). EDN (eosinophil-derived neurotoxin) induces neurotoxic reactions (Durack *et al.* 1981) and EPO (eosinophil peroxidase) is cytotoxic for mammalian and tumour cells (Jong *et al.* 1980; Motojima *et al.* 1989). Furthermore EPO has been found to increase the mast cell-induced tumour cytotoxicity and basophil defence against parasites (Brown *et al.* 1982). Eosinophils also limit mast cell and basophil-induced histamine and the slow-reacting substances of anaphylaxis effects in tissues (Zeiger & Colten 1977; Wasserman *et al.* 1975) by releasing histaminase and arylsulfatase from their granules.

To study the kinetics of mast cells and eosinophils during the process of granuloma formation the newly established methods for staining of both cell types simultaneously in the same tissue section proved to be invaluable (see Chapter 3).

6.2. Aims of study

The distribution and accumulation of mast cells along the small intestine during experimental *Schistosoma mansoni* infection are described in Chapter 5. Since the granuloma is the basic lesion for all life-threatening cases of schistosomiasis and has a major impact on liver architecture and haemodynamics, the study of the kinetics of mast cells and eosinophils in granuloma formation is of interest for both pathologists and parasitologists. The study of mast cells and eosinophils simultaneously in the acute and chronic phase of liver granuloma was the main purpose of this investigation.

6.3. Materials and methods

A total of 68 female outbred CFLP mice each weighing between 30g and 37g were infected percutaneously with 100 cercariae of a laboratory-maintained Puerto Rican strain of the *Schistosoma mansoni*. The animals were processed for study as described in Table 6.1. For histology, 0.2g of liver tissue was fixed in MT fixative for 24 h at room temperature, tissues were processed and embedded in paraffin. 5µm thick sections were cut and stained with AC (see Chapter 2 and 3). For each granuloma, the major and minor diameters were measured using a calibrated eyepiece graticule and the area was calculated for each granuloma section. A large majority of the granulomas were found to be ovoid, a few were spherical in which case the area was calculated as for a circle. At least ten granuloma were measured and were subjected to cell counting for each sample of liver tissue. In conditions where fewer than ten granuloma were present, their total number was considered. The number of mast cells and eosinophils were counted in each granuloma at x400 magnification and the mean number of all counted cells (per mm^2) were plotted arithmetically. The number of granuloma which contained eggs, those without eggs and the total number of granulomas in a minimum of 10 graticules of each infected liver section were selected randomly and the mean number each type of granuloma was computed in 100 mm^2.

The length of the long and short sections of 10-20 eggs in each liver sample were measured, the size of eggs as an ellipse were calculated and means were computed.

Table. 6.1 post-mortem schedule

Weeks post infection	No of infected mice
0	0
1	4
2	4
3	4
4	4
5	5
6	5
7	5
8	5
9	5
10	5
11	4
12	5
14	5
16	5

6.4. Results

From week 3 post infection eosinophils were observed in the liver samples, but by week 5 post infection clusters of eosinophils could be seen, predominantly in peri-vascular areas of the liver tissue (Fig 6.1). No mast cells were observed at these times. At week 6, granulomas started to form and rapidly became prominent in tissue sections. The number of granuloma increased from 39 per 100 mm^2 at week 6 to 309 per 100 mm^2 at week 12, decreasing to 165 per 100 mm^2 by week 16 post-infection the end of the experiment (Fig 6.2). The size of the granuloma also increased gradually from 0.018 mm^2 at week 6 post-infection to 0.037 mm^2 at week 9 post-infection and decreased later to 0.025 mm^2 at the end of the experiment (Fig 6.3). As the number of granuloma increased, the number of the granuloma without eggs also increased. The number of granuloma with eggs reached 133 and without eggs 176 per 100 mm^2 at week 12 post-infection (Fig 6.4).

In contrast to the intestine tissue generally, a very small number of mast cells was observed in liver granuloma (Fig 6.5). Most of the granuloma were found to be located in sites around the perimeter of the liver. Compared with

the number of eosinophils, mast cells comprised only a small per cent of the total granuloma cell number in this experiment. Mast cells were observed in liver tissue by week 6 post-infection. The number of the mast cells increased from 23 per mm^2 per area of the granuloma at week 6 post-infection reaching a maximum of 105 per mm^2 area of the granuloma at week 9 post-infection The number of mast cells decreased to about 90 per mm^2 at the end of the experiment (Fig 6.6).

In contrast, the number of eosinophils was seen to be high ranging from 3299 per mm^2 of the granuloma at week 6 post-infection to 4545 per mm^2 by week 8. Eventually decreasing to 2160 per mm^2 at week 16, the end of our experiment (Fig 6.7, 6.8).

The size of the eggs in the liver tissue showed some fluctuation but seemed to reach a stable state during the chronic phase of the infection (Fig 6.9).

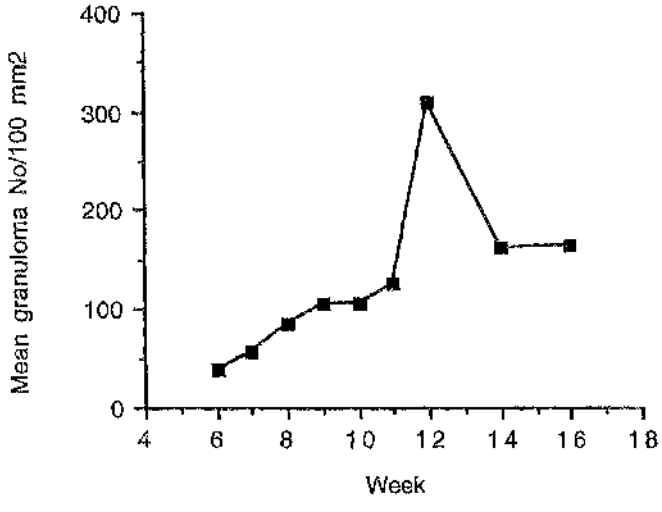


Fig 6.2 Chronological changes in the number of granulomas in the liver of mice infected with *Schistosoma mansoni*.

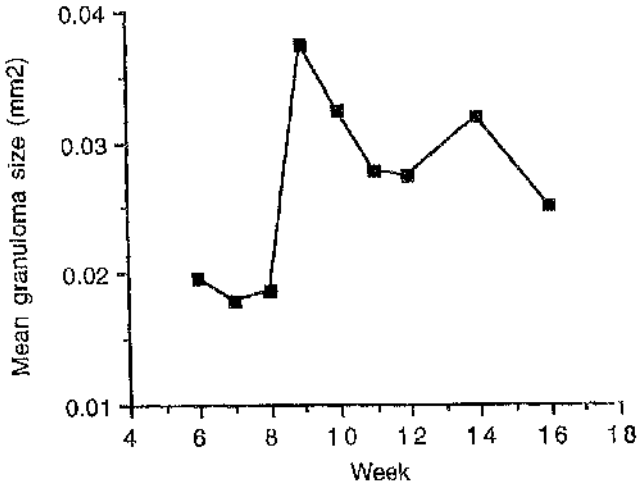


Fig 6.3 Chronological changes in the granuloma size in the liver of mice infected with *Schistosoma mansoni.*

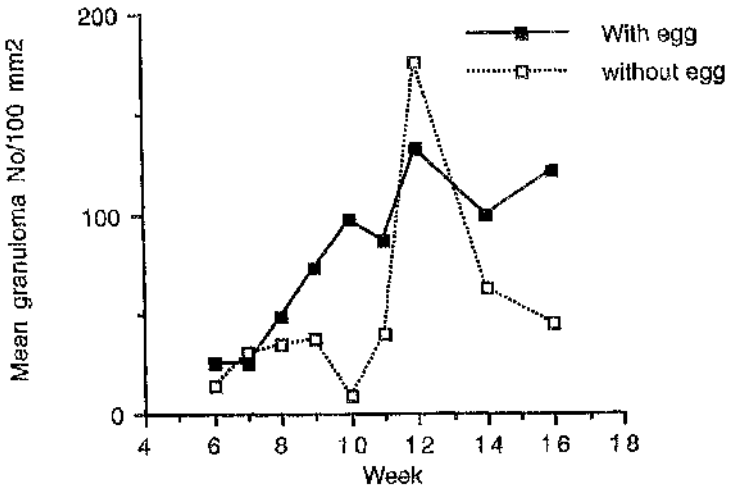


Fig 6.4 Chronological changes in the number of granuloma with and without eggs in the liver of mice infected with *Schistosoma mansoni.*

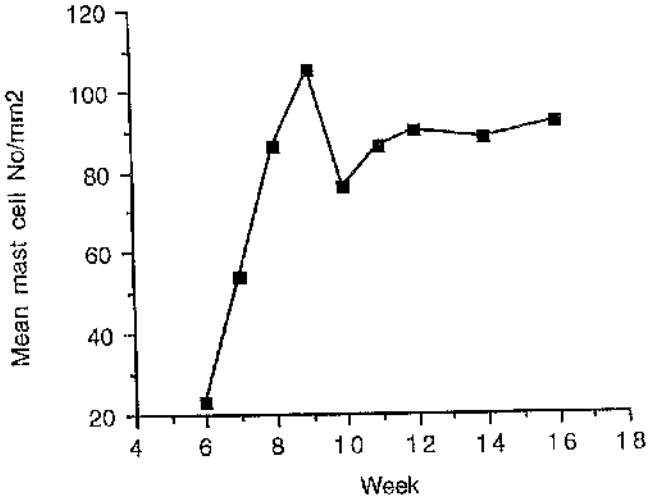


Fig 6.6. Chronological changes in the number of the mast cells in the liver granuloma of the mice infected with *Schistosoma mansoni.*

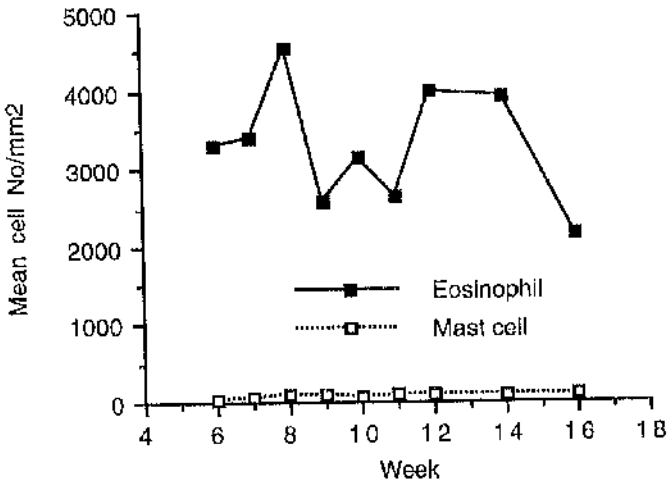


Fig 6.7. Chronological changes in the number of mast cells and eosinophils in thc liver granuloma of mice infected with *Schistosoma mansoni.*

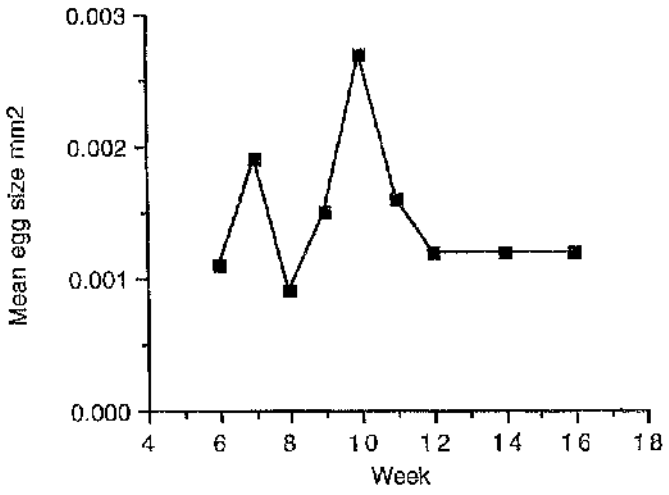


Fig 6.9. Chronological changes in the egg size in the liver of mice infected with *Schistosoma mansoni.*

6.5. Discussion

Hepatic schistosomiasis and the cellular organisation of schistosome granulomas have been studied by many investigators (Andrade 1965; Cheever 1961; Weinstock & Boros 1983c). In general, T cells have been considered to be the most important cells in the regulation of granuloma formation (Phillips & Lammie 1986) especially during infection with *Schistosoma mansoni* (Pearce 1995). Macrophages comprise up to 30 % of the cells within a granuloma and different morphological and presumably functional types of macrophage are known to play distinct roles in the formation and regulation of schistosome granulomas (Epstein *et al.* 1979). In addition, eosinophils constitute as much as 50% of the cells present in the granuloma (Weinstock 1992) and significant numbers of mast cells and their effects on granuloma formation (Epstein *et al.* 1979; Owhashi *et al.* 1990) have also been reported. Eosinophils have been shown to participate in egg destruction (James *et al.* 1978; De Brito *et al.* 1984) and mediators derived from mast cells have an

effect on granuloma regulation (Weinstock & Boros 1983b; Kunkel *et al.* 1984; Beer & Rocklin 1984). In earlier work (Chapter 5), it was shown that large numbers of both MMC and CTMC can be found in the intestinal tissues of *Schistosoma mansoni* -infected mice.

According to the results obtained in the current study, granuloma numbers increased from 6 weeks post-infection and reached a peak by 12 weeks post-infection. This peak at 12 weeks post-infection may be the time of peak egg production by adult *S. mansoni* female worms, granuloma numbers remained constant thereafter. The size of the granuloma varied with time reaching their peak diameter at 9 weeks post-infection becoming smaller by 16 weeks post-infection. A finding in keeping with ideas on the down-regulation of the granulomatous response (Domingo & Warren 1968). By week 16 there was also evidence of repair and healing of damaged tissue. The mast cell dynamics fit well with the increasing size of the granuloma. The number of granulomas which were observed not to contain eggs peaked at week 12 post-infection. This may have been due to the activity of eosinophils which have been shown to participate in egg destruction (Fig 6.10). Eosinophil numbers were observed to increase after week 8 and their number remained high throughout the experiment. Eosinophils could be found surrounding eggshells or closely adhered to them also, eggs full of eosinophils were often observed. It was uncertain how the eosinophils gained entry to the eggshells but evidence of damage to eggs was frequently linked to adjacent clusters of eosinophils (Fig 6.11).

Unlike eosinophils, mast cell numbers were seen to increase from week 6 post-infection, reaching a maxium 105 per mm^2 per area of granuloma by week 9 post-infection. At week 10 mast cell numbers had declined to 90 per mm^2 per area and remained at that level until week 16, the end of the experiment, by which time 3% of the cells present were mast cells and 97 % were eosinophils. Finally it seemed that the schistosome eggs, or antigenic

products derived from the eggs attracted eosinophils (Owhashi *et al.* 1983 & 1985; Tsudo *et al.* 1979) rather than mast cells.

The results confirm that eosinophils have an important role in egg destruction (James *et al.* 1978; De Brito *et al.* 1984). It is reasonable to speculate, from the complicated cell dynamics, that mast cells probably did not contribute to egg destruction, but conversely they may have played a central supporting role in granuloma formation. Mast cells were frequently observed near the middle or even towards the periphery of the cellular response making up the granuloma, but they were never observed inside or near to the eggshell. One interpretation is that whilst the eosinophil may be an effector cell actively participating in egg destruction the mast cell is more important in maintaining control of tissue repair (Fig 6.12) and regeneration and may therefore have a subsidiary role in fibroblast activation, regulating their activity in collagen production (Russell *et al.* 1977). Fibrosis is probably of some value to the host since it serves to isolate the egg and it's products from the remainder of the tissues. Fibrotic insulation of the granuloma occurred whether or not the eosinophils succeed in completely destroying the egg. However when granulomatous fibrosis was evident the number of eosinophils found in these granulomas was reduced to a few or even to zero. Other changes were evident. The staining properties of the granuloma altered, the granuloma becoming blue (Fig 6.12). Eosinophils could be observed outside the layers of fibrosis around the granuloma and the granuloma itself was largely devoid of cells with only a few mast cells in the middle or external layer of the granuloma. Only rarely were eosinophils found in contact with the granuloma at this stage. During the phase of acute inflammation, eosinophil and mast cell numbers increase to a maximum, at weeks 8 to 9 post-infection respectively. This increase was proportional to the increase in the size of the granuloma but was not proportional to the increase in the number of the granuloma and may represent a limitation on the capacity of the animals to manufacture these cell

types during phases of severe and acute inflammation resulting from infection with *S. mansoni*. The results of this study are in the line with those of Lenzi *et al.* (1987) in that they report increasing numbers of mast cells and eosinophils in liver from mice infected with *S. mansoni* between weeks 5 and 8 post-infection.

Several points can be gleaned from these studies. First, eosinophils are important in the destruction of eggs of *S. mansoni*. Second, the observed increase in mast cell numbers always followed the increase in eosinophil numbers. Third, in the liver of mice infected with *S. mansoni* mast cell numbers remained constant and the results are consistent with the view that the mast cell serves as an accessory cell during infection with *S. mansoni*. Mast cells may play a dominant role in recruiting the inflammatory cells and they may take part in granuloma regulation by releasing histamine (Weinstock *et al.* 1983a) or producing cytokines (Gordon & Galli 1991 & 1990a). Fourth, in heavy infection, as judged by the number of granulomas in the tissue, the numbers of mast cells are relatively lower than in light infection. Finally, the distribution of mast cells in *S. mansoni* infected mice seems to be independent of the parasite antigen and the inflammation itself. In the current experiments, despite finding high numbers of parasite eggs in the livers of the infected mice, the number of the mast cells in the livers was always much lower than found in the small intestine and at heavy infection as judged by egg number during these experiments, relatively fewer mast cell were found in tissue. On the basis of these observations, other factors appear to be important in distribution of mast cells within the tissues. Parasite antigen may be a stimulus in mast cell production but it is of secondary importance in determining the final tissue sites occupied by the mast cells.

6.6. Summary

Livers from *S. mansoni*-infected mice were studied during 16 weeks of infection with regard to granulomas, and the kinetics of mast cells and eosinophils in the granulomas. The tissues were stained with a newly developed method designed to detect mast cells and eosinophils simultanously.

Granulomas appeared from week 6 post-infection and both the number and size of the granulomas increased gradually in the acute phase of the infection, and then reduced as the infection matured. As the infection passed through its chronic phase, fewer of granulomas were seen to contain any egg material.

Eosinophils comprised 97% of the cell types in the granuloma and probably play an important role in *Schistosoma* egg destruction. At all times during the infection mast cells comprised approximately 3% of the cells in the granuloma. The appearance of mast cells in the granuloma may be dependent on factors other than those associated directly with the granuloma.

Fig 6.1. Section from the liver of 5 week *S. mansoni*-infected mice. A cluster of eosinophils (arrows) are evident between the two vessels are pointed by arrows and many red blood cells are also evident into the vessels x 100

Fig 6.5. Section from the liver of 9 week *S. mansoni*-infected mice. A large number of eosinophils and a few mast cells are evident around a big granuloma containing some particles of the egg which has been already destroyed. x 400

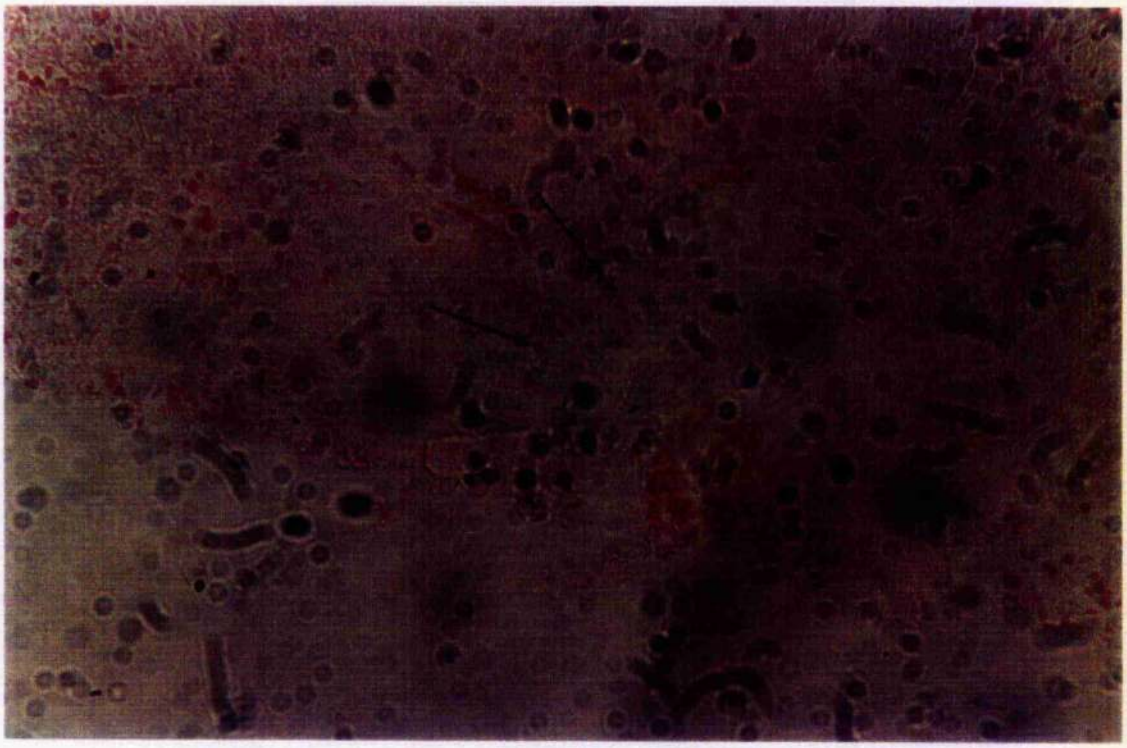


Fig 6.8. Section from the liver of 11 week *S. mansoni*-infected mice. A considerable number of eosinophils are seen around the two granuloma, more eosinophils are collected near to the intact egg on the right. x 250

Fig 6.10 Section from the liver of 11 week *S. mansoni*-infected mice. Eosinophils are seen invading an egg, it appears that one side of the egg has been destroyed completely. x 250

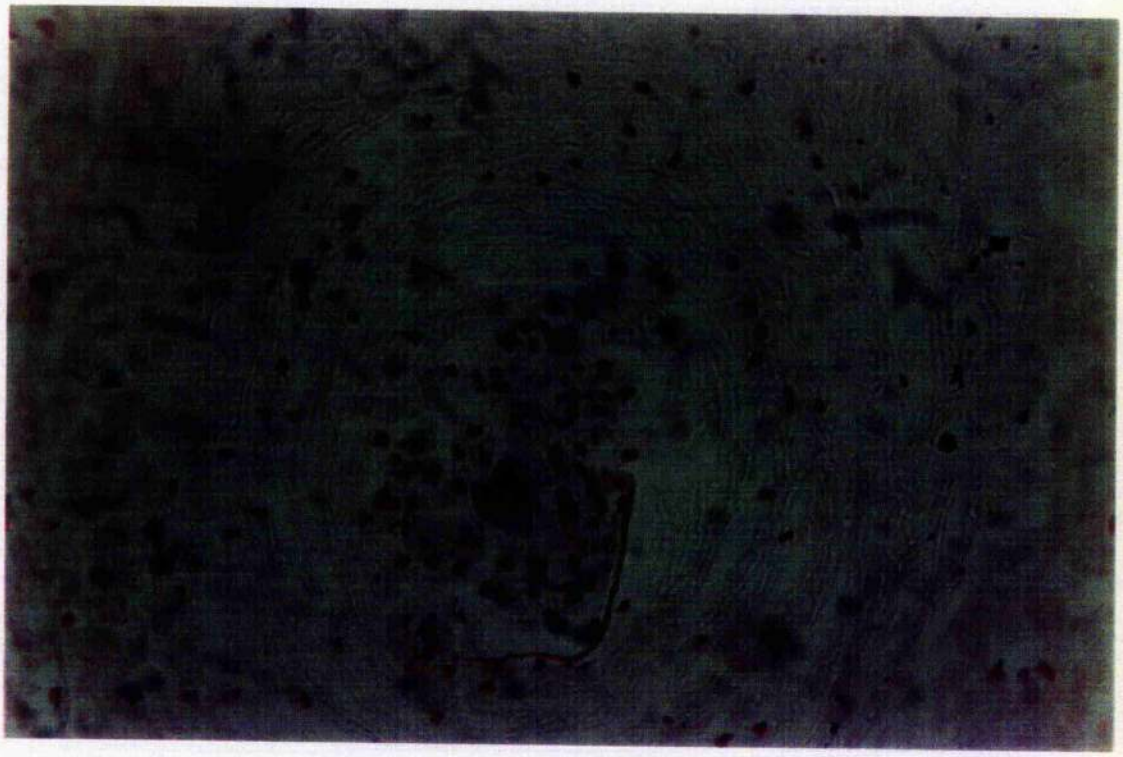
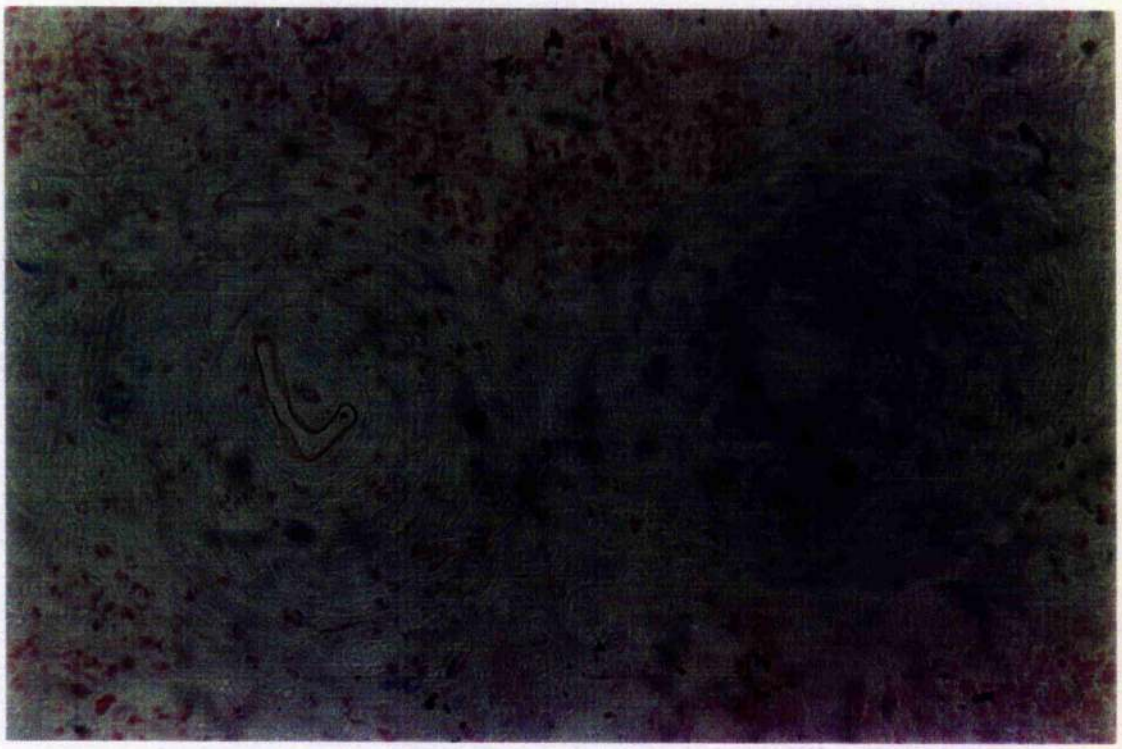


Fig 6.11 Section from the liver of 9 week *S.mansoni*-infected mice. A large number of eosinophils are evident in the infected liver. Some eosinophils are seen invading an egg. Eosinophils appear to have passed into one egg across a damaged egg. x 250

Fig 6.12. Section from the liver of 10 week *S. mansoni*-infected mice. Two granuloma are seen in different phase of processing, at left granuloma the mast cells are busy repairing the damaged area and in the other granuloma appear to be in the final stages of destroying the egg. In addition large numbers of eosinophils surrounding the granulomatous area. x 250



Chapter 7

Influence of Nerve tissue and Mediators on Mast Cell Migration

7.1. Introduction

The enteric nervous system can be considered as a third component of autonomic nervous system (ANS). The autonomic nervous system (sympathetic and parasympathetic) receives sensory input from the viscera and sends motor nerves to visceral smooth muscles and the smooth muscles of blood vessels in all parts of the body. The autonomic nerves, including those of the enteric nervous system, terminate in large branching fibres and neuromuscular junctions are made through nerve projections. The target cells are controlled by help of a variety of co-transmitters, in particular sympathetic nerves contain noradrenaline, ATP and neuropeptide Y, parasympathetic nerves containing acetylcholine and vasoactive intestinal polypeptide VIP and sensory-motor nerves containing substance P and calcium gene-related peptide CGRP (Burnstock 1993). Motor nerves of the autonomic nervous system function involuntarily, pairs of sympathetic and parasympathetic fibres are sent to each target organ. Sympathetic nerves deal with emergency excitatory situations and are adrenergic (using noradrenaline for stimulation of the effector activity). Parasympathetic nerves generally keep the body in a steady state condition and are cholinergic (using acetylcholine for stimulation of the effector activity).

The enteric nervous system refers to that category of neurones whose cell bodies are located in the gastrointestinal system (Cook 1986). The enteric nervous system is independent of and differs from the sympathetic and parasympathetic systems. It consists of two major myenteric and submucosal

plexuses. The myenteric plexus is located between the longitudinal and circular muscle layers and the submucosal plexus is located in submucosa of the gastrointestinal trunk. Both plexuses are interconnected. The submucosal nerves project into the adjacent muscularis mucosa, surrounding the crypts and the villous cells. Most of the myenteric neurones ramify within the muscular layer. Fibres derived from the myenteric and extrinsic ganglia also end in the submucosal and mucosal layers (Furness & Costa 1987; Cook 1986; Noback *et al.* 1996). On the basis of the shape, the enteric neurones have been classified into 3 classes (Furness & Costa 1987)

Sympathetic and parasympathetic nerves enter the enteric plexus and form synapses with enteric nerves. Both type I and type II neurones are found in the myenteric plexus of the small intestine, but type II are in the majority. Submucous neurones are stated to be of type III in the small intestine. All three types of adrenergic, cholinergic and peptidergic nerves are found in the myentric plexus of the intestine (Gabella 1979).

The concept of psychological effects, mediated through the activities of the nervous system, on immune functioning has to a large extent been described by several groups of researchers (Backen 1992; Brosschot 1992; Herbert & Cohen 1993; Knapp 1992). The intimate collaboration between the nervous system and the immune system has changed the view that immune responses are isolated from neural influences. Lymphoid tissue has been shown to be directly innervated by peptide-containing nerve fibres (Weihe *et al.* 1991; Blalock 1994; Shanahan & Anton 1988; Solomon 1987; Jankovic 1989) and common physiological characteristics between lymphocyte-derived cytokines and neurotransmitters have been observed (Blalock & Stanton 1980). Noradrenergic nerve fibres have been demonstrated in primary and secondary lymphoid organs (Bellinger 1990). Peptidergic innervation has been described in the lymph nodes of a variety of animal organs (Fink & Weihe 1988; Popper 1988; Kurkowski 1990) including

the gut (Cook 1986) and direct anatomic association between nerves and lymphocytes has been demonstrated in the spleen (Felten 1987).

It is well established that mast cells are critical in IgE-dependent hypersensitivity reactions involving the release of multifunctional cytokines including IL1, IL2, IL3, IL4, IL5, IL6, GM-CSF, TNF-α, TGF-β, MIP-1α, MIP-1β, JE, TCA3, IFN-γ (Galli 1992). Also, it is now well established that mast cells are associated with nerves in many tissues (Williams *et al.* 1981, Stead *et al.* 1987) in a variety of species, with evidence for mast cells cooperating with the autonomic nervous system and with sympathetic ganglia in guinea pigs (Weinreich & Unden 1987), cat (Hollinshead & Sheldon 1967), rat (Shanthaveerappa & Bourne 1964) and cow (Trop 1961).

Neuroanatomical associations between mast cells and noradrenergic and peptidergic nerves in the thymus and lymphoid tissues have been studied (Weihe *et al* 1989; Mullar & Weihe 1991; Felten *et al.* 1988). The neuroanatomical association of mast cells and nerves in the gastrointestinal system (Stead *et al.* 1989 & 1987; Newson *et al.* 1983; Yonei 1987; Dvorak *et al.* 1980) and the close apposition of the mast cells and nerves has been observed in liver (Dimlich 1984), normal skin (Alving *et al.* 1991) diseased skin (Wiesner-Menzel *et al.* 1981) diseased tissue (Dvorak & Monahan 1983) the respiratory system, (Nilsson *et al.* 1990; Kakuta *et al.* 1989) and in several culture models (Blennerhassett *et al* 1987).

Additional studies of the associations between noradrenegic nerve and mast cells have been made on knee joint synovial membranes (Tanaka *et al.* 1977) and iris tissue (Nyiri *et al.* 1977). Flood & Kruger (1970) described the close association between mast cells and myelinated axons and those with non-myelinated nerve fibres in human and sheep ureters have also been reported (Ugaily-Thulesius *et al.* 1988).

Mast cells are present in perineural and endoneural spaces of the peripheral nerves fibres (Ryan *et al.* 1994; Kimura *et al.* 1988) and the densities of mast cells around normal peripheral nerve fibres in a variety of animals, including humans, rats (Rosenheim 1986; Bienenstock *et al.* 1991; Enerback *et al.* 1965), rat, cow and guinea pigs (Trop 1961; Olssen 1966a) are well documented. The hyperplastic gathering of mast cells around injured and damaged nerves has been investigated by numerous investigators (table 7.1).

Table 7.1 Published observation on accumulation of mast cells near injured nerves

Animal	Perturbation	Location	Observation	Reference
Rat	*Nipostrongylus brasiliensis*-infection	Small intestine	Mast cell increased	Arizono, *et al.* 1990
Rat	Hyperglycaemia	Distal sciatic nerve	Two-fold increase of mast cells	Dasgupta & Maiti 1979
Rat	Electrically stimulation	Trigeminal ganglion	Mast cells increased	Dimitriadou, *et al.* 1991
Human	Crohn's Disease	Gut	Mast cells increased	Dvorak & Monahan 1983
Rat	Nerve sectioning	Distal part of sciatic nerve	Five-fold Mast cells increase	Enerback, *et al.* 1965
Rat	Vagotomy	Gastric Mucosa	Mucosal mast cell increase	Ganguly, *et al.* 1978
Cat	Cervical sympathetic truck sectioning	Superior cervical ganglion	Mast cells increased	Hollinshead & Sheldon 1967
Rat	Poly arthritis	Ankle joints	Both mast cell and nerve depletion	Hukkanen, *et al.* 1991
Human	Neurofibroma	Nerve sheath	Mast cells increased	Isaacson, 1976
Human	Neurofibroma	Cranial nerve & peripheral nerve	Mast cells increased	Johnson, *et al.* 1989
Mice	Sciatic nerve sectioning	Distal part of the nerve	Mast cells increased	Nennesmo & Reinholt 1986
Rat	Nerve sectioning	Distal degeneration part of the nerve	Mast cells increased	Olssen, 1965
Rat	Trauma	Distal part of the sciatic nerve	Mast cells increased	Olssen, 1966b
Rat	Neuropathy induced by Isonicotinic acid hydrazide (INH) in treatment of tuberculosis	Peripheral nerve system	Mast cells increased	Olssen, 1967
Human	Diabetes	Femoral nerve	Mast cells increased	Olssen, *et al* 1968
Human	Trauma	Peripheral nerve	Mast cells increased	Olssen, 1971
Human	Tumors	Peripheral nerve	Mast cells increased	Pineda & Calif 1965

Membrane-membrane contact between mast cells and nerves (Blennerhassett *et al.* 1991) with a direct proportional relationship between nerve and mast cell populations has been observed. Increasing sympathetic activity, but

not parasympathetic increased mast cell populations in the rat synovial joint (Levine *et al* 1990).

Stead (1992) has reported a close correlation between mast cell and nerve densities in normal mucosal samples and co-ordinated changes of both occur during disease process, the negative relationship between mast cells and old nerves and positive relationship of mast cells with newly regenerating nerves in these experiments can be considered as additional evidence of migration of the mast cells toward inflamed tissue and association of the them with inflamed tissue, as result of the activity of material being produce by nerves during nerve irritation or regeneration. The coexistence of the nerves and mast cells in normal rat synovium and the parallel disappearance of nerves and mast cells in arthritic rat synovium has been reported also by Hukkanen *et al.* (1991).

Based on these observations and the growing list of chemicals, neuropeptides and neurotransmitters and cytokines including IL1, IL6, TNF-α, CSF which are found in enteric neurones (Benveniste 1992; Malipiero *et al.* 1990; Costa & Furness 1989; Brown 1994) and enteric plexus resident (Gabella 1972; Yamamoto 1977) glial cells, a direct influence of the nerve system and neuroendocrine peptides on the immune system and immunocompetent cells (Blalock 1994; Payan *et al.* 1984; Felton *et al.* 1987) and functional interaction between mast cells and the nervous system has been proposed (Stead *et al.* 1990; Mathison *et al.* 1992; Ansel *et al.* 1993).

Additional evidence for an influence of nerve tissue on mast cells has come from experiments in which the trigeminal nerve was electrically stimulated. Application of 0.02 - 0.1mA to the trigeminal nerve resulted in increased levels of blue staining mast cells in the area of the tongue served by the nerves (Dimitriadou *et al.* 1991). However, such an increase in young mast cells may have resulted from either proliferation of resident mast cells or migration to the site of stimulation (which can be judged by Astra blue and Safranin staining method).

Stem cell factor SCF which can be produced by Schwann cells in both normal and damaged nerves fibres (Ryan *et al.* 1994) is known to affect stem cells (Keshet *et al.* 1991) and mast cell migration (Kanemoto *et al.* 1992; Meininger *et al.* 1992; Nilsson *et al.* 1994) through c-kit receptors (Wershil *et al.* 1992). Additionally, the production of Transforming Growth Factor β (TGF-β) from astrocytes in the (CNS) of AIDS patients (Wahl *et al.* 1991) and the secretion of nerve growth factor (NGF) by schwann cells (Stead 1992) may also be significant. TGF-β has been shown to be a chemo-attractant for mast cells (Gruber *et al.* 1994) and NGF induces mast cell hyperplasia (Aloe & Levi-Montalcini 1977; Marshall *et al.* 1990b). In the light of this information and the histological observations of nerve tissue associated with tissue pathology and mast cell infiltration in tissue sections (Chapters 4 and 5) the possibility that other substances derived from nerve might serve to attract mast cells was investigated.

The effect of neurotransmitters on mast cell attraction were of particular interest as the effect of neuropeptides and neurotransmitters on lymphocyte and monocyte traffic has already been described (Ottaway 1984; Moore 1984; Ruff *et al.* 1985) and recently the role of neuropeptides from autonomic fibres on lymphocyte migration has been observed (Ottaway & Husband 1994).

7.1.1. Aims of the investigation

The present study was designed to investigate the potential role of nerve-derived substances (neurotransmitters) in attracting mast cells to tissue sites which may have been exposed to helminth antigen or damaged by parasites.

Before the experiments could be conducted several techniques had to be established in the laboratory and these are described in chapter 2.

7.2. Materials and methods

7.2.1. Substance-p conjugated bead and mast cell culture

A full description of the techniques for preparation of spleen-derived conditional medium, bone marrow culture for preparation of the mast cells and bead preparation and conjugation are given in Chapter 2. About 50-60 substance-p coated beads in 10 µl PBS (pH 7.5) were added to each well of a 24 well tissue-culture plates. 150 µl RPMI medium containing (10% FCS, 1.6% L-glutamine, 3% stock penicillin and streptomycin) was added to each of the wells. For the control groups about 50-60 substance-p free beads in 10 µl PBS (pH7.5) were added in each well. 2.5 x 10^5 cells (95-98 % mast cells) were added to each well. After incubation for 5 days (37°C, 5% CO_2 / Air) the number of cells bound to 50 individual beads was counted. Fifteen experimental and 15 control wells were examined in each experiment and mean mast cell binding values calculated. A similar protocol to that used for mast cells was followed for substance-p conjugated beads and spleen cell culture. In these experiments spleen cells from a *S. mansoni*-infected mouse were used instead of mast cells.

7.2.2. Cell polarisation assay

For the cell polarisation assay the following potential active substances were diluted in HBSS/MOPS to the following concentration, table 7.2.

Table 7.2. Concentration of substances

Adrenaline (M)	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
Substance-p (M)	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	10^{-12}
Histamine (M)	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{7}	10^{-8}	10^{-9}
serotonin (M)	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
DOPA (M)	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{9}	10^{-10}
Acetylcholine (M)	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
Norepinephrine(M)	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{10}	10^{-11}	10^{-12}
Aspartic acid (M)	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
Nerve suspension	1 /	1 /	1 /	1 /	1 /	1 /	1 /	1 /	1 /
(concentration)	3	9	27	81	243	729	2187	6561	19683

A full description of the technique is given in Chapter 2. Briefly, 5 x10^5 mast cells were added to the final concentrations / dilutions of test substances shown above. In all substances control tubes contained HBSS / MOPS were set up without test substances. After incubation for 30 min at 37 °C, cells were fixed for 20 min with 2.5 % glutaraldehyde / HBSS / MOPS. The cells were then washed twice in HBSS / MOPS. The morphology of 300-400 cells was assessed under phase-contrast microscopy and expressed as the percentage of cells examined. polarised cells were those which were elongated rather than rounded. Preliminary polarisation tests were carried out with aspartic acid, adrenaline and nerve cell suspension.

7.2.3. Micropore filter chemotaxis methods

7.2.3.1. Substance-p induced chemotaxis

Full details of the chemotaxis chambers and methods are described in Chapter 2. Five μm polycarbonate filters (Nucleopore) were dried, fixed and stained prior to counting. Assays were run in triplicate.

7.2.4 Statistical analysis

Student's t-test was used for comparisons of experimental and control groups. $P < 0.05$ was considered to be significant.

7.3. Results

7.3.1. Culture

7.3.1.1. Spleen cell culture for spleen-derived medium preparation

After culture all spleen cells were removed by centrifugation at 1000 x g for 20 min after which the medium was filtered (0.45 μm) and stored at - 20°C. The medium remained effective in inducing mast cell development from bone marrow cell culture for at least one year after preparation.

7.3.1.2. Bone marrow cell culture for mast cell production

7.3.1.2.1. Bone marrow culture supplemented with spleen-derived medium

Bone marrow cell culture supplemented with spleen-derived medium. Generally cultures produced good results. After 1 week 30% of the cells stained metachromatically. After 14 days 2 x 10^5 mast cells / ml could be obtained of which 96-98 % contained metachromatic granules (Fig7.11,7.12)

7.3.1.2.2. Bone marrow cell culture supplemented with IL-3

IL-3 proved to be less effective than spleen-derived medium in inducing mast cell production. Even after 21 days of culture, fewer mast cells were produced. This has to be balanced against the ease of use of IL-3. Though stockpiling quantities of spleen-derived medium could be an alternative approach. IL-3 also has the advantage of being a defined additive unlike the complex contents of spleen-derived medium.

Table.7.3. 3000 U / ml of purified IL-3

IL-3 U / ml	Mast cell number at 7 days %	Mast cell number at 14 days %	Mast cell number at 21 days %
10	20-21	24-25	80-85
20	20-22	25-27	95-97
30	20-30	70-75	96-97
40	20-22	23-24	90-93

Using the 30 U / ml IL-3, the number of the metachromatically stained cells reached about 20-30 % at day 7 of culture, increasing to 70 % until day 14 and to 96-97 %, at 21 days (table 7.3.).

7.3.1.3. Substance-p conjugated bead and mast cell culture

Significant differences were observed between experimental and control groups using the substance-p conjugated beads and mast cell culture methods to determine the effect of substance-p on mast cell attraction (Fig 7.1)

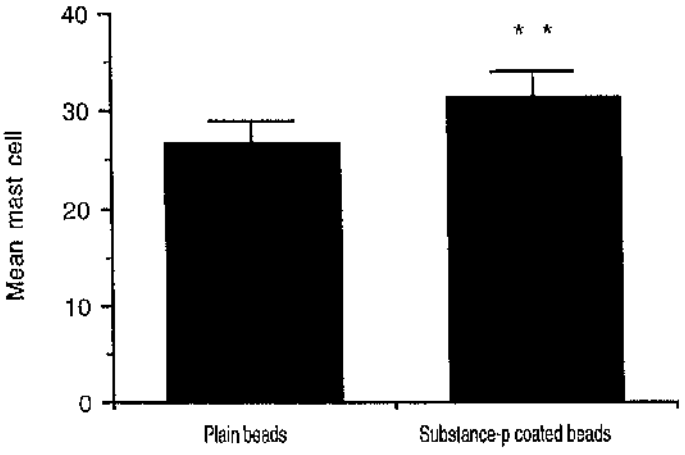


Fig.7.1 Mean number of mast cells around beads coated with substance-P and plain beads $\bar{x} \pm$ SE. The difference between experimental and control groups was found to be significant (** $0.001 < P < 0.01$, using an unpaired t-test n = 750)

7.3.1.4. Substance-p conjugated bead and spleen ells culture

No significant difference was found between the groups when the same assay was carried out with spleen cells (Fig.7.2.).

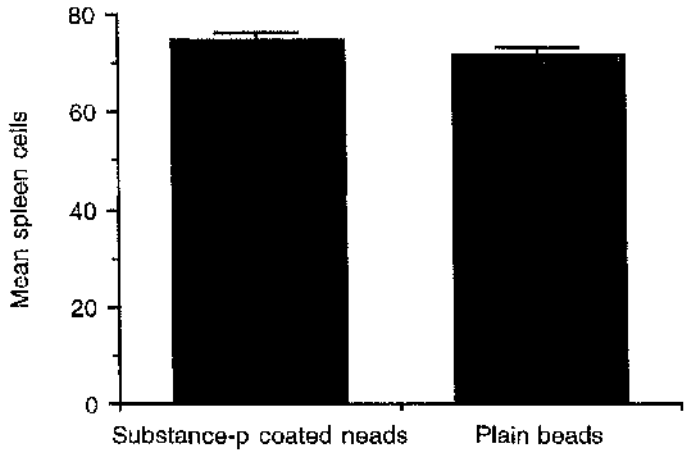


Fig.7.2 Mean number of the spleen cells bounded to the beads coated with substance-p and plain beads $\bar{x} \pm$ SE. The difference between experimental and control groups was not significant (using an unpaired t-test n = 57)

7.3.2. Cell polarisation assay

7.3.2.1 Substance-p induced mast cell polarisation

In spite of the positive results of the substance-p on mast cell attraction in culture significant differences were not observed between the experimental and control groups (when using the cell polarisation assay) (Fig.7.3.).

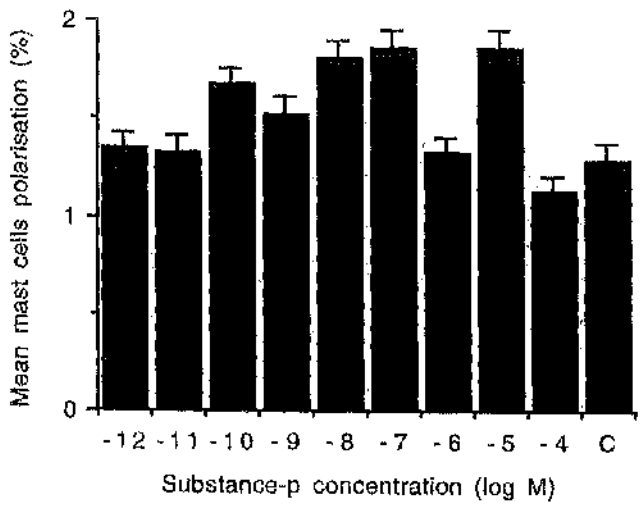


Fig.7.3. Dose response studies of the efficiency of polarisation of mast cells in different concentrations of substance-p. No significant polarisation was observed. each column represents mean $\bar{x} \pm$ SE. The difference between experimental and control groups was not significant using an unpaired t-test (n=5).

7.3.2.2. Histamine-induced mast cell polarisation

Using the cell polarisation assay for investigation of a possible chemoattractant effect of histamine on mast cells, a significant percentage of cell polarisation was observed with 10^{-3}, 10^{-4} and 10^{-5} molarity of histamine. Greater differences were obtained with 10^{-3} molar histamine (Fig.7.4).

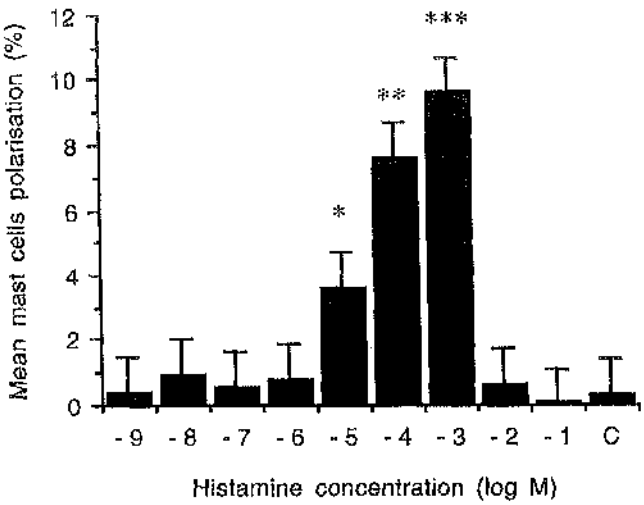


Fig. 7.4 Dose response studies of the efficiency of polarisation of mast cells in different concentrations of histamine suggest that at 10^{-3} concentration there is maximum cell polarisation, as the concentration is reduced to 10^{-5} a minority of cells polarise. Concentration of 10^{-5} M histamine and lower appears to be too low to produce cell polarisation (first hit). At concentrations of 10^{-2} M histamine and above multiple pseudopod formation results in poor polarisation. The difference between experimental and control groups was found to be significantly different (* $P < 0.05$, ** $0.001 < P < 0.01$, *** $P < 0.001$, using an unpaired t-test, n=5), each column represents mean $\bar{x} \pm$ SE.

7.3.2.3. Serotonin-induced mast cell polarisation

The cell polarisation assay investigating a possible chemoattractant effect of serotonin on mast cells demonstrated that most polarisation was obtained with 10^{-9} and 10^{-10} M serotonin.(Fig.7.5)

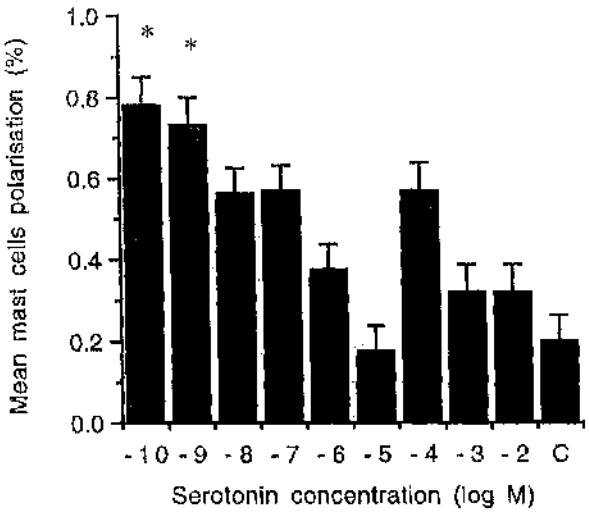


Fig. 7.5. Dose response studies of the efficiency of polarisation of mast cells in different concentrations of serotonin suggest that at 10^{-10} concentration there is maximum cell polarisation, each column represents mean $\bar{x} \pm$ SE. The difference between experimental and control groups was found to be statistically significant (* $P < 0.05$, using unpaired t-test n=5). However the rate of polarisation is not high.

7.3.2.4 Dopa-induced mast cell polarisation

The cell polarisation assay investigating a possible chemoattractant effect of Dopa on mast cells, most polarisation was obtained with 10^{-2} M of Dopa (Fig.7.6).

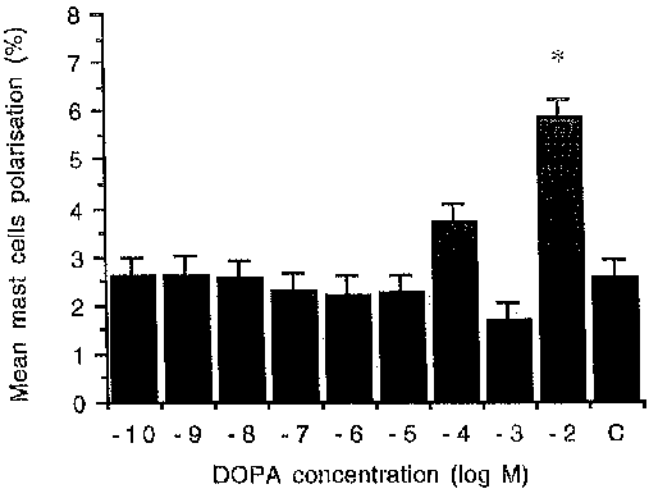


Fig. 7.6 Dose response studies of the efficiency of polarisation of mast cells in different concentrations of DOPA suggest that at 10^{-2} M concentration there is maximum cell polarisation, as the concentration is reduced to 10^{-3} M a minority of cells polarise. DOPA concentration of less than 10^{-2} M appear to be too low to produce cell polarisation while concentrations higher than 10^{-2} M killed mast cells, each column represents mean $\bar{x} \pm$ SE. The difference between experimental and control groups was found to be significantly different (* $P < 0.05$, using an unpaired t-test, n=5)

7.3.2.5 Noradrenaline and acetylcholine-induced mast cell polarisation

No significant differences between experimental and control groups regardless of concentration with noradrenaline and acetylcholine (Fig 7.7 / 7.8).

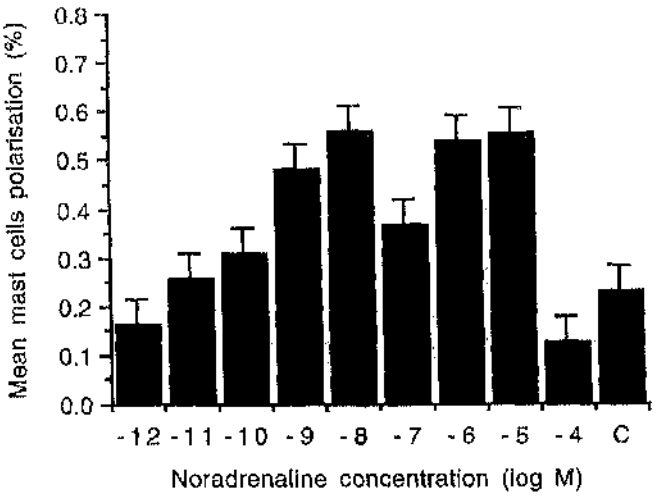


Fig.7.7. Dose response studies of the efficiency of polarisation of mast cells in different concentrations of noradrenaline. Each column represents mean $\bar{x} \pm$ SE. The difference between experimental and control groups was found to be not significant (unpaired t-test, n=5)

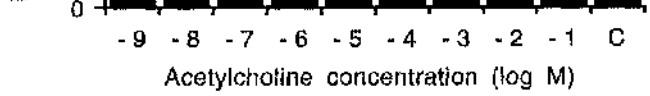


Fig.7.8. Dose response studies of the efficiency of polarisation of mast cells in different concentrations of acetylcholine. Each column represents mean $\bar{x} \pm$ SE. The difference between experimental and control groups was found to be not significant (unpaired t-test n=5)

7.3.2.6. Aspartic acid-, epinephrine- and nerve suspension-induced mast cell polarisation

The polarisation assay was used for the investigation of a possible chemoattractant effect of aspartic acid, epinephrine and nerve suspension. Differences were observed between experimental and control groups with epinephrine and nerve suspension (Fig 7.9 / 7.10).

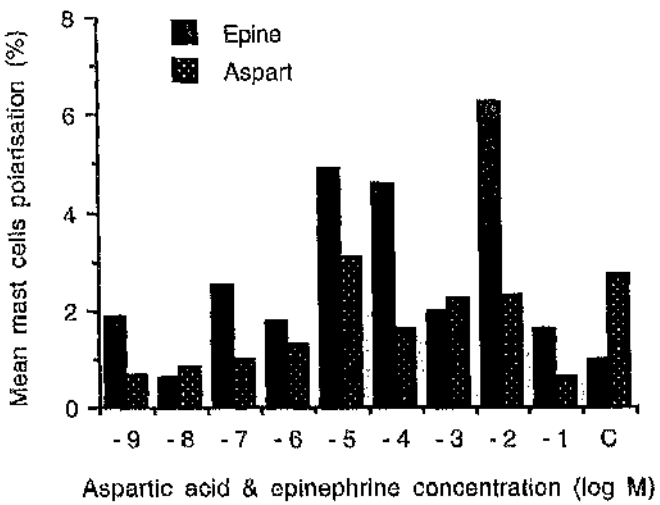


Fig.7.9. Preliminary dose response studies of the efficiency of polarisation of mast cells in different concentrations of epinephrine suggest that at 10^{-2} M concentration there is maximum cell polarisation, as the concentration is reduced to lower concentrations the minority of cells polarise. The concentration of more than 10^{-2} M of epinephrine may produce multiple pseudopods and poor polarisation, preliminary tests showed no differences between the aspartic acid treated and control groups.

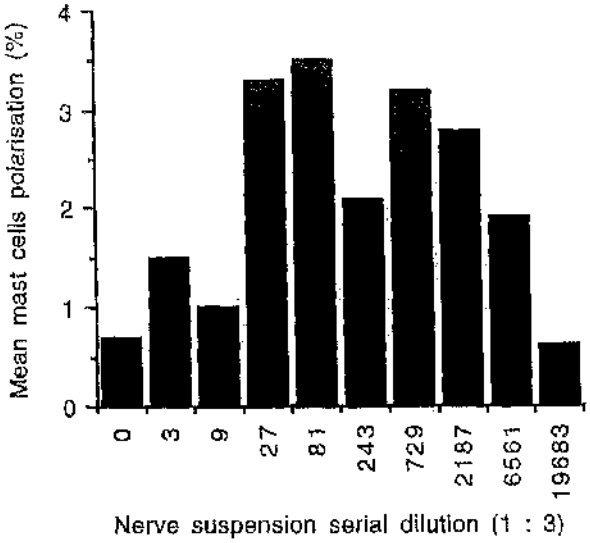


Fig.7.10 Preliminary dose response studies of the efficiency of polarisation of mast cells in different dilutions of nerve suspension suggest that at 81 time dilution there is maximum cell polarisation, as the concentrations are reduced or increased only a minority of cells polarise to nerve suspension.

7.3.3. Micropore filter chemotaxis method.

The micropore filter chemotaxis assay gave uniformly negative results. At no time were sufficient cells detected, after migrating through the filters, to allow a reliable assessment of a chemotactic effect of substance-p on mast cells.

7.4. Discussion

Cultured mast cells were used in all the experiments. Bone marrow from male and female BALB/ C mice 2-4 month old mice were used for mast cell production. More than $2x10^5$ mast cells were yielded per ml of culture. This was twice as much as had been reported previously (Razin *et al.* 1981). Extra L-

glutamine in the medium may be the reason for increasing the efficiency of the culture.

Perhaps using mast cells collected from the peritoneum or other sources would have been more appropriate but mast cells from such sources would have been pre-sensitised with neurotransmitters in the body under physiological conditions. This may have resulted in the production of more surface membrane receptors and increased their responses to additional stimulation as has been suggested to occur with leukocytes (McKay *et al.* 1994a)

The clustering of mast cells around peripheral neurones (Bienenstock *et al.* 1991; Rosenheim 1986; Enerback *et al.* 1965; Trop 1961) and the increase of mast cells around injured nerves (Olsson 1971; Isaacson 1976) and abnormal tissues (Baroni 1964) has been well demonstrated.

The enteric nerve system (ENS) consists of an estimated 10^8 nerve cells a number equivalent to that in the spinal cord (McKay *et al.* 1994a). In addition, the neuronal distribution in the muscular layer of small intestine in some animals, including guinea pig (Irwin 1931; Matsuo 1934; Ohkubo 1936) rabbits (Maslennikova 1962) and cats (Christensen & Riick 1985) confirms that there is a greater neuronal density in the duodenum than in the ileum.

In recent studies, greater numbers of mast cell numbers have been observed in the anterior part of the small intestine than the posterior (Chapter 5) and more mast cells have been observed in small intestine than in the liver (Chapter 6). Together, all these observations offer support for the hypothesis that the nervous system may be involved directly in mast cell attraction. This proposition is further supported by the observations that Schwann cell produced stem cell factor SCF (Ryan *et al.* 1994) can cause *in vitro* mast cell migration (Kanemoto *et al.* 1992; Nilsson *et al.* 1994) and Transforming growth factor-β (TGF-β), which is

chemotactic for mast cells (Gruber *et al.* 1994), can be produced by astrocytes in disease conditions (Wahl *et al.* 1991).

In spite of temporary axonal sprouting during nerve regeneration and the innervation of the damaged tissues (Stead *et al.* 1991), the numbers of nerves in peripheral tissues are often supposed to be stable (Stead 1992) and because mast cells are migratory cells, therefore the innervation of mast cells by nerve fibres does not seem to be highly probable. Particularly in the light of the observations, that mast cells have been found to project their cytoplasmic projections intimately into nerve fibres (Wiesner-Menzel *et al.* 1981). All this evidence is consistent with the nerve-induced mast cell migration.

A modification of the method for coating beads with *S. mansoni* egg antigen to induce *in vitro* granuloma formation (Bentley *et al.* 1985; Goes *et al.* 1991) was designed with the intention of studying the possible migration of mast cells toward the beads which were coated with substance-p. Although the conjugation of substance-p, a well known nerve fibre-end secretion, on beads had not been confirmed quantitatively, significantly greater numbers of cells were gathered around the substance-p coated beads. This modified method proved to be highly successful.

The polarisation (which represents the initial morphological response of the cell to chemotactic stimuli) assay was selected as a reliable means of studying the response and locomotion of the cells to chemotactic substances (Haston & Shields 1985; Shields & Haston 1985; Wilkinson 1986). The assay was reasonably simple to perform and could be used with cells in suspension.

A chemotactic effect of substance-p on mast cells using the polarisation assay and chemotaxis micropore filter method was not detected. Concentrations of 10^{-4} M to 10^{-12} M of substance-p was used in this experiment and concentrations greater than 10^{-4} were found to be toxic to mast cells. Using the polarisation

assay, mast cells showed a significant morphological response to 10^{-3}, 10^{-4} and 10^{-5} M histamine, 10^{-9} M serotonin and 10^{-2} M L-3-4- dihydroxyphenylalanine (DOPA). Histamine was used at 10^{-1} - 10^{-9} M concentration and serotonin was used at 10^{-3} - 10^{-10} M concentration. DOPA was used at 10^{-2} - 10^{-10} M concentration. DOPA at greater than 10^{-2} M concentration killed the mast cells. Mast cells did not showed significant change of shape over 10^{-1} - 10^{-9} M acetylcholine concentration or over 10^{-4} - 10^{-12} M noradrenaline concentration. Noradrenalin at greater than 10^{-4} M concentration killed the mast cells.

The approximate *in vivo* levels of some neurotransmitters are given below (see table 7.4).

Table 7.4 *In vivo* level of neurotransmitters

Neurotransmitters	Concentration (*In vivo*)	Reference
Histamine	10^{-7}	(Muller, *et al.* 1977)
Serotonin	10^{-8}	(Muller, *et al.* 1977)
DOPA	10^{-10}	(Goshima, *et al.* 1988)
Adrenaline	10^{-9}	(Skok, 1973)
Noradrenaline	10^{-8}	(Skok, 1973)
Acetylcholine	10^{-7}	(Feldberg & Vogt 1948)
Aspartic acid	10^{-6}	(Davidson, 1976)
Substance-p	10^{-10}	(Lajtha, 1982)

Preliminary polarisation assays using epinephrine, nerve extracts and aspartic acid induced some shape changes in mast cells *in vitro* with 10^{-2} M epinephrine concentration, and 27, 81 and 729 times stock dilution of nerve solution. Aspartic acid did not induced shape changes in mast cells. Only a few, of more than 20, recognised neurotransmitters (Costa & Furness 1989) were examined during this study. The remainder are still to be tested for an effect on mast cell attraction.

In spite of these preliminary promising results more experiments need to be carried out. Even if the effect of neurotransmitters on mast cell motility is

confirmed directional cellular mobility must be verified by either the micropore filter or agarose, collagen gel assay (Wilkinson 1986).

Using a chemotaxis chamber (nuclepore Inc) and a 5μm polycarbonate micropore filter, the chemotactic effect of substance-p on mast cells was investigated. The negative results of the test were in keeping with the polarisation assay. However because of the lack of availability of the appropriate pore size of filter, the micropore filter assay was not used for other neurotransmitter examinations.

By adhering closely to specified culture conditions the process of mast cell production has been highly successful and reproducible. This was particularly important as large numbers of pure mast cells were required for the chemotaxis assays. Nevertheless, a suitable reliable and accurate method of identifying mast cells was necessary. Some methods for identifying mast cells in culture have already been described (Bray & Vanarsdel 1961; Ishizaka *et al.* 1973 & 1976; Jarboe & Huff 1989a; Jarboe *et al.* 1989b; Haisa *et al.* 1992). While these methods may be of use in laboratories with excellent microscopy facilities and with skilled observers they are not satisfactory for relative beginners. In addition experience of trying to observe mast cell granules in culture samples under light microscopy led to the considered view that this was more of an art than a science. Electron microscopy studies of the type described by Rein & Karasek (1992) are valuable but time consuming and can not be used as readily as light microscopy. It was decided to study the mast cells in smears prepared from the cultured material but two major difficulties were identified. When using the majority of recognised mast cell fixatives, such as Carnoy's, manually prepared smears detached from the slide. An exclusive stain was also needed. Satisfactory results were eventually described for both (Chapter3).

Nerve and mast cell co-culture (Blennerhassett *et al.* 1991) are experiments which would help define the hypothesis regarding the migration of mast cells

towards neurones and help explain the observed correlation of mast cells in the vicinity of nerve tissue.

Stabilising the mast cells functionally with a stabiliser such as disodium cromoglycate or fenoterol a "selective beta 2 adrenoreceptor stimulant" and using stabilised mast cell and nerve co-culture might be a useful technique to confirm the idea of mast cell migration. Because stabilised mast cells would not be able to induce any tropism for nerve tissue attraction.

7.4.1. The importance of nerve-induced mast cell migration

7.4.1.1. Choice of mast cells on immune system functions

According to the literature, cellular granuloma are considered to be classical cell-mediated immune responses which are regulated by the Th1 subset of T cells. The participation of mast cells in granulomatous responses is obviously of interest because of the intense capacity these cells have for Th2 cytokine production. Furthermore the deposition of eggs in murine schistosomiasis is associated with a switching of the response from a predominant Th1 type to a predominant Th2 type. If mast cells increase the size of schistosome egg induced granulomas then this would be further evidence against the view that Th1 responses were the dominant type in inducing granuloma formation. Indeed the balance of Th1 to Th2 cytokines is likely to determine the outcome of granuloma formation and both subsets of cells and their cytokines are likely to be involved in the response.

It is well established that cytokine profiles are important in mediating the resistance to various infections (Sher 1992b). T cells are a rich source of cytokines. T cell precursors (CD+4) may terminally differentiate to either the Th1 subset, producing IL-2, INF-γ, and lymphotoxin involved in cell mediated inflammatory functions, or differentiate to the Th2 subset producing IL-4, IL-5,

and IL-10 which are involved in antibody, particularly IgE production and eosinophil production and proliferation.

IL-4, IL-10 and TGF-β are important down-regulators of Th1 type responses (Sher 1995; O'Garra & Murphy 1996) and INF-γ, IL-12 are powerful cytokines which can down-regulate Th2 type responses (Sher 1995). Despite our current understanding of the basic effector role of Th1 and Th2 T cells on immune responses, the full extent of the pattern of Th1 or Th2 selection during immune responses is not yet clear and full of contradictory statements (O' Garra & Murphy 1996; Mosmann & Sad 1996). Since mast cells are a source of IL-4, IL-10 and TGF-β together (Gordon *et al.* 1990a; Sher *et al.*1992a), the primary role of mast cells in early Th2 switching may be important.

One hypothesis to explain why the immune response moves towards Th1 or Th2 type is described below. If the inducing antigen is small or soluble and is taken up by macrophages or B cells, IL-12 production by macrophages results in Th1 helper cell activation and further production of IFN-γ and lymphotoxin by Th1 cells producing macrophage activation and cell-mediated immunity. In contrast when the foreign particle is coarse or resistant to destruction (pollens, allergens, worms or even a foetus in the uterus Th1 response are suppressed systematically during pregnancy Mosmann & 1996) they are more likely to impinge on the nervous system. As a consequence the release of mediators which influence and attract mast cells will result in the involvment of cytokines which favour Th2-type responses.

Any physical, chemical or indeed mechanical stimulation of nerve receptors may cause release of mast cell attracting substances from the nerve cells and consequently local accumulation of mast cells. Later, activation and then degranulation of mast cells by nerve derived substances or by antigen stimulation through IgE on the surfaces of mast cells may result in the release of a large

number of mediators including IL-4, IL-10 and TGF-β which cause Th1 down regulation and up regulation of Th2-type responses.

Th2-dependent responses could be down-regulated by Th1-derived cytokines or through the activity of IgG4 antibodies (Hagan 1992) or β2 adrenergic agents receptors on mast cells (Wardlaw 1993). Th1-type and Th2-type cytokines modulate one other (Windhagen *et al.* 1996).

7.4.1.2. On parasitology (schistosomiasis)

Many stages of the *Schistosoma* life-cycle intensively irritate the host tissues. Young larval schistosomes penetrate the skin and actively migrate through the tissues to the venous system. The eggs of the schistosome also move or are moved through the tissues. To a lesser extent adult worms moving through the blood vessels may also irritate the endothelium. Irritated or damaged nerves may release SFC, histamine or other substances which may attract mast cells to the site of injury. Once mast cells degranulate, Th2-type immune responses are favoured. Biologic amines such as histamine and serotonin are released affecting vascular permeability, increasing local blood circulation, recruiting leukocytes (Lee *et al.* 1986) and may make the host tissue environment inhospitable to the parasite (Gerken *et al.* 1984 & 1990a).

IL-4 and IL-5 are also released by mast cells and induce IgE synthesis and eosinophil proliferation respectively (De Vries 1994). Schistosomula are killed by eosinophils (Butterworth *et al.* 1993 & 1979), IgE also protects against reinfection with schistosomes (Hagan *et al.* 1991; Hagan 1993a; Rihet *et al.* 1991; Dunne *et al.* 1992). Th2 derived cytokines intensify granuloma formation (Pearce 1995) which isolates the pathogen and reduces the risk of toxic material reaching the remote tissues (Weinstock 1992). Eggs are destroyed by eosinophils, James *et al.*

1978 and (chapter 5). Mast cells through IL-4 cytokine production can also be involved in granuloma regulation.

By favouring Th2 responses the nerve system appears to play a key role in controlling parasitic helminth infection.

7.4.1.3 Psychosomatic aspects

At first glance, it is difficult to comprehend how infectious diseases are influenced by psychosomatic factors. But it is well established that psychological factors influence many diseases, including infectious diseases (Plaut & Friedman 1981), neoplasia (Riley *et al.* 1981), and autoimmune diseases (Solomon 1981). But how psychological factors contribute to the process of diseases is not yet clear; recent hypotheses, which point to a functional link between the nervous and immune system might be an acceptable answer to this question. The connection between nervous system and immune system may be facilitated in part by mast cells migration.

The effect of the nervous system upon Th2 cells may be a consequence of mast cell degranulation. In the case of parasites, a direct effect of the pathogen may be the initiating stimulus. In the case of psychological effects the same mechanism may be involved though the initiating stimulus may be different.

7.5. Summary

In vitro culture systems using bone marrow cells from BALB/ C mice were set up in medium supplemented with either IL-3 or spleen-derived medium. Bone marrow cells grown in spleen-derived medium gave rise to cultures containing > 97% mast cells. These cells were used in bead culture, polarisation and micropore

filter chemotaxis assays with the intention of determining the effect of substance-p on mast cell migration. Polarisation assays were used also to examine the effect on mast cells of other neurotransmitters. Histamine and to a lesser extent DOPA caused mast cell polarisation.

Fig.7.11. Pure bone marrow cultured mast cells stained with toluidine blue. x 500

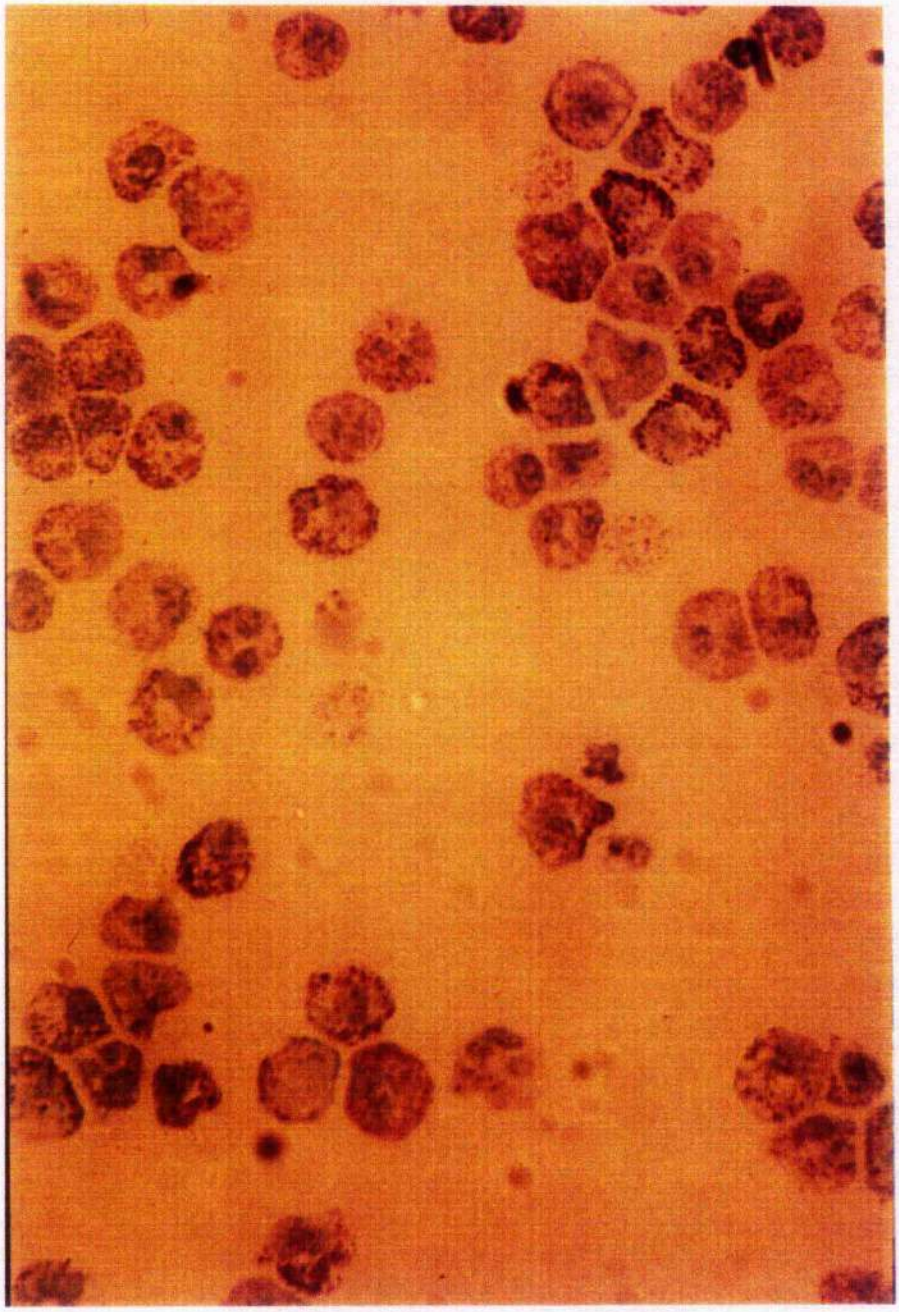
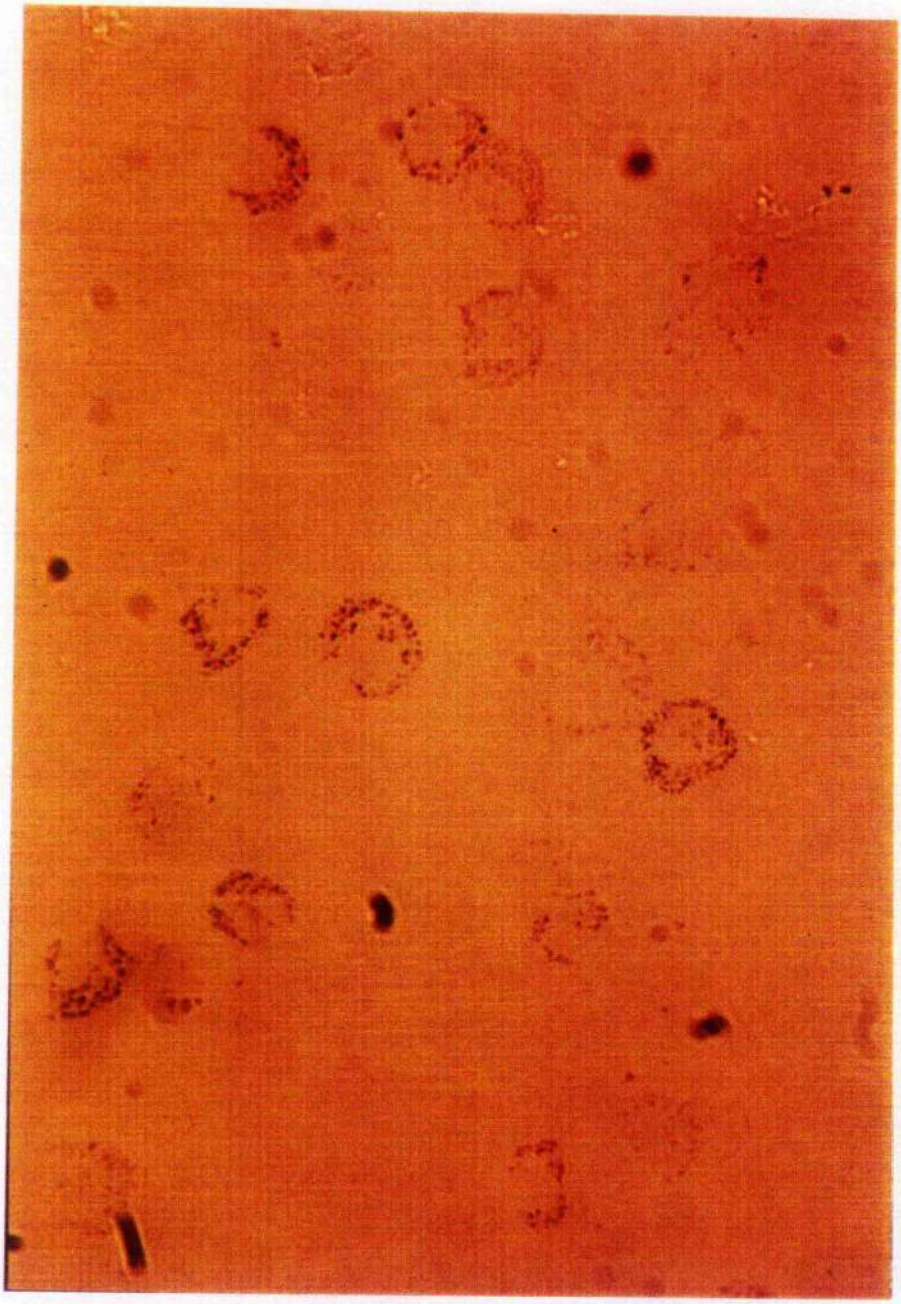


Fig.7.12. Pure bone marrow cultured mast cells diluted for better identification, stained with toluidine blue. x 500



Chapter 8

General Discussion and Conclusion

8.1. General discussion

Mast cells are haemopoietic cells which store potent mediators, including histamine, proteases and proteoglycans, and contain newly synthesised mediators including leukotrienes, prostaglandins, platelet-activating factor, and a number of multifunctional cytokines within their granules.

Mast cells are involved in inflammatory and allergic reactions. In spite of their well established potential range of functions there is still no conclusive data on the relative contribution of these cells to inflammatory pathology. An increase in knowledge of mast cell development and function may lead to methods which will allow modulation of mast cell functions to the benefit of the treatment of various diseases.

Mast cells are characterised by their granules and the proteoglycan which causes the histologically diagnosed difference between MMC and CTMC (Chapter 3). Carnoy's, IFAA (Isotonic Formaldehyde Acetic Acid) fix the granules of the both types of mast cell but these fixatives are exclusive more by MMC than CTMC (Enerback, 1986). The degree to which the protoglycan is negatively sulphated in the mast cell granules is responsible for the metachromatic properties of these cells when stained with toluidine blue and is also responsible for orthochromasia or metachromasia, of MMC and CTMC respectively, when stained with cationic dyes such as the Astra blue sequence and safranin (Enerback, 1966b). Accumulation of the mast cells and eosinophils within infected tissue and elevated levels of IgE in

serum are a common feature of metazoan infections including the blood dwelling trematode *Schistosoma mansoni.*

Recent studies have concentrated on the study of mast cells and to a lesser degree eosinophils and IgE which all are the hallmarks of the *Schistosoma mansoni* infection. Activated eosinophil antibody-dependent cell-mediated cytotoxicity against schistosomula, involvement of the eosinophils in egg destruction (James & Colley 1978; De Brito *et al.* 1984) and IgE-induced resistance to schistosomes (Hagan *et al.* 1991; Dunne *et al.* 1992) have been described.

During this study, the first experiments were done to develop suitable methods for fixation and staining of the mast cells and eosinophils separately, then a series of experiments was carried out to develop the techniques to allow the observation of both cell types together in *S. mansoni* infected tissue.

Until now, fixing the tissue in Bouin's, Helly's (Tronchin *et al.* 1979) or Maximov (Savage & Colley 1980) and staining with chromotrope or haematoxylin and eosin are the general methods for the histological investigation of eosinophils. A method developed to demonstrate mast cells and eosinophils together offers advantages over existing methods (Duffy *et al.* 1993). The method developed herein shows both cell types in intestine, liver and spleen of the *S. mansoni*-infected mice (Kermanizadeh *et al.* 1995a).

Further investigation overcame the short period during which tissue could be left in fixative. Development of an another fixative (LM) and modification of the previous staining procedure permitted the staining of mast cells and eosinophils in intestine, liver, spleen and skin of the *S. mansoni*-infected mice (Kermanizadeh *et al.* 1995b).

This latter method is currently favoured over others which have been investigated. Using mercuric chloride, black precipitation was observed in tissue sections but this did not interfere with cell identification. Winter *et al.* (1995) have

extended the methods used above to ovine tissues, reporting excellent results, they also introduced a modification to remove mercuric chloride precipitation.

The method developed here demonstrates mast cells with blue granules and eosinophils with red granules against clear tissue background. The quality of the staining of the eosinophils represents an improvement on existing methods and eases their identification. In addition if the efficiency of the method is confirmed using tissue from other animals, the concept of the histological differences of mast cell heterogeneity may be questioned. However this method may ease the identification of the mast cells in various tissues with a unique fixation and staining method.

The kinetics of the MMC and CTMC were studied in experimentally *S. mansoni*-infected mice during 16 weeks of infection. CTMC appeared 3 weeks later than MMC in the tissue sections. Although the CTMC numbers were still increasing during the chronic phase of the infection, MMC numbers reduced as the infection matured. The greater migratory capacity of the MMC (Enerback & Lundin 1974) may be a reason for early increase of these cell types in the mucosal tissue or, alternatively, the presence of more sensory nerve receptors in the mucosal layer may be another reason.

The reduction in MMC numbers as compared to CTMC numbers during the chronic phase of infection, may be due to the decline in egg production and the lack of eggs in the mucosa resulting in less nerve stimulation or damage to the mucosa layer. Alternatively the reduction may result from the adaptation of the nerve cells to continuous stimulation in this area.

Despite a more or less equal distribution of the *Schistosoma* eggs in the posterior portion of the small intestine, the anterior portion of the small intestine always contained greater numbers of mast cells. This also can be explained by the presence of more sensory nerves in this area.

An increase in the numbers of granuloma until week 12, the size of the granuloma until week 9 and the eosinophils until week 11 post infection and the gradual decrease of all elements later, are consistent with the slow development of immunity during schistosome infection. Adherence and penetration of eosinophils into the egg shell, which has been often observed during these experiments, is also consistent with other studies demonstrating destruction of the schistosome eggs by eosinophils.

Unlike eosinophils, mast cells comprise 3 % of the total cells in the granuloma and their dynamics fit well with the development of the granuloma. Mast cells may play a supporting role in granuloma formation, tissue repair and fibrosis.

Despite the presence of high numbers of parasite eggs in the liver of infected mice the number of the mast cells in the liver were always much lower than those found in the small intestine.

Since the villus crypt unit, used in current counting methods, suffers from some severe limitations, for example the size of the villi and crypts are not always equal, or there is a tendency to select "suitable" tissue sections which introduces an additional bias, a new method for counting the cells in tissues sections was developed. This is simple and useful method was used for counting mast cells in the intestine. But, it can be applied on any less compact tissue such as lung and glandular tissues even on a small piece of tissue such as a biopsy.

The enteric nerve system is a tissue rich in neurones (McKay *et al.* 1994a) and shows a greater neuronal density in duodenum than ileum in some animals which have been studied (Christensen & Rick 1985; Irwin 1931; Maslennikova 1962).

Mast cells are reported to be present in the endoneural and in the perineural space of the peripheral neurones (Olsson 1971) and an increase of these cells has

been demonstrated in nerve injury, nerve repair and neurofibroma (Isaacson 1976). A good correlation between mast cell and nerve densities in normal mucosal tissue samples and disease processes (Stead 1992; Dvorak *et al.* 1992) and an increase of mast cell numbers in the injured area in a variety of diseases such as hyperglycaemia (Dasgupta & Maiti 1979), nerve sectioning (Enerback *et al.* 1965), vagotomy (Ganguly *et al.* 1978), and trauma (Olssen 1966b) has been observed.

Taken together this information and the results of mast cell kinetics during recent experiments offers support for a hypothesis that the nervous system and its secretions may be involved directly in mast cell attraction.

Bead culture, micropore filter chemotaxis, and polarisation assays were employed to investigate the possible attractive effects of the neurotransmitters on mast cell migration, using mast cells prepared by bone-marrow culture, supported with spleen-derived medium. IL-3 supplemented culture medium proved to be less effective than spleen-derived medium. Significant differences were observed between experimental and control groups using the substance-p coated beads and mast cell cultures. Using the cell polarisation and micropore chemotaxis assay substance-p was shown not to have attractive effects on mast cell migration. Having the optimal concentration of the substance-p on coated beads but not in the chemotaxis assay may be the reason for the lack of positive results in the latter assays. Using the polarisation assay histamine and Dopa showed polarising effects on mast cells.

8.2. Conclusion and future work

In conclusion this work has:

1. investigated a series of experiments about mast cell preparation (*in vivo* and *in vitro*) and related histology methods;

2. developed a new method for fixing and staining of the mast cells (MMC and CTMC) and eosinophils simultaneously in the intestine, liver and spleen tissue;

3. developed a new method for fixing and staining of the mast cells and eosinophils simultaneously in the skin tissue;

4. developed a new method for counting of the mast cells, eosinophils and other cells in diffuse tissues;

5. developed improved methods for fixing and staining of eosinophils and paneth cells in internal organs;

6. improved culture methods for pure mast cell production;

7. investigated a series of chemotaxis methods (polarisation, micropore-filter and culture) for mast cells;

8. developed the bead coated antigen method for purposes of chemotaxis assays;

9. propounded a new hypothesis for involvement of the nervous system in mast cell attraction and consequently the positive effect of the mast cells on activation of the Th2-type helper cells.

In spite of the preliminary promising results in favour of the hypothesis a definitive conclusion requires additional experimentation, possible avenues of research are described below.

1. use mast cells from the primary source (peritoneal cavity or lung);

2. compare of the nerve density in different parts of the intestine, liver, and skin of the normal, W/W^V and SI / SI mice (both are mutants and genetically mast cell-deficient the W/W^V can develop mast cells if receives bone marrow but SI / SI cannot develop mature mast cells after injection of the mast cell precursors);

3. determination of substance-p and bead conjugation quantitatively;

4. investigation of the mast cell attractive effects of the other neurotransmitters;

5. use of micropore and collagen chemotaxis assay;

6. injection of crude or fractionated nerve suspension to mice skin tissue and peritoneum;

7. stabilised mast cell and nerve cell culture;

8. organ and mast cell culture;

9. investigations of methods for the staining of mast cells and eosinophils in cell suspension.

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Abbreviations

Cell types.

CTMC (Connective tissue mast cell)

EOS (Eosinophils)

MMC (Mucosal mast cell)

PAN (Paneth cell)

Stains

AAC (Astra blue, HCl, Chromotrope)

AC (Astra blue, Chromotrope)

ACH (Astra blue, Chromotrope, Haematoxylin)

ACL (Astra blue, Chromotrope, Lugol's)

ACS (Astra blue, Chromotrope I)

AHAC (Astra blue Haematoxlin, Chromotrope II)

AHC (Astra blue, Haemasoxlin, Chromotrope I)

AHS (Astra blue, Haematoxylin)

ALC (Astra blue, Lugol's, Chromotrope)

AIGC (Alcian green, Chromotrope)

AIGS Alcian green, safranin)

AIS (Alcian blue, Safranin)

ANC (Aniline blue, Chromotrope)

AS (Astra blue, HCl, Safranin)

BRC (Brilliant blue, Chromotrope)

Ch (Chromotrope)

FC (Fast green)

HAC (Haematoxylin, Chromotrope)

HAE (Haematoxylin and eosin)

LGC (Light green, Chromotrope)

PAC (Astra blue, Chromotrope II)

TO (Toluidine blue 0.5%)

TO1 (Toluidine blue 1%)

Fixatives

BOU, Bouin's solution (Saturated aqueous Picric acid 75 ml, 40% formaldehyde 25 ml, Glacial acetic acid 5 ml)

CITM (Calcium Chloride 2%, Tannic acid 20%, Mercuric chloride 5 %, 1 : 1 : 1)

CITM1 solution, (Aqueous Calcium Chloride 2% , Aqueous Tannic acid 20% , Aqueous Mercuric chloride 5%, 1 : 2 : 2)

CaCl solution, (Aqueous Calcium Chloride 2%)

CAR Carnoy's solution, (Absolute alcohol 60 ml, Chloroform 30 ml, Glacial acetic acid 10 ml)

CARG (Gas chamber fixation method)

CB solution, (Carnoy's 25 %, Bouin's 50 %)

DP (Potassium dichromate 5%, Picric acid, 1 : 1)

GLU (Glutaraldehyde, 0.1M Sodium Cacodylate buffer, 31.5 : 100)

IB solution, (IFAA, Bouin's, 1 : 1)

IBR solution, (IFAA, Bouin's, 1 : 2)

IFAA Isotonic Formaldehyde-Acetic Acid (Formaldehyde 0.6%, Acetic acid 0.5 %, Distilled water 98.9)

L solution, (Aqueous Basic lead acetic solution 1%)

LEMT solution, (Aqueous Basic lead acetic 5 %, Absolute ethanol, Aqueous Mercuric chloride 5%, Aqueous Tannic acid 20%, 1 : 1 : 1 : 2)

LM solution, (Aqueous Basic lead acetic 5%, Aqueous Mercuric chloride 5%, 1 : 1)

LM1 solution, (Aqueous Basic lead acetic 1%, Aqueous Mercuric chloride 1%, 1 : 1)

LME solution, (Aqueous Basic lead acetic 5%, Aqueous Mercuric chloride 5%, Absolute Ethanol, 1 : 1 : 1)

LMT1 solution, (Aqueous Basic lead acetic 5%, Aqueous Mercuric chloride 5%, Aqueous Tannic acid 20%, 1 : 1 : 1)

LMT2 solution, (Aqueous Basic lead acetic 5%, Aqueous Mercuric chloride 5%, Aqueous Tannic acid 20%, 1 : 1 : 2)

LMT3 solution, (Aqueous Basic lead acetic 5%, Aqueous Mercuric chloride 5%, Aqueous Tannic acid 5%, 1 : 1 : 1)

LP solution, (Aqueous Basic lead acetic 5%, Saturated Picric acid / 95% ethanol, 1 : 2)

LP1 solution, (Aqueous Basic lead acetic 5%, Absolute Ethanol, Saturated Picric acid / 95% ethanol, 1 : 1 : 2)

LT solution, (Aqueous Basic lead acetic 5%, Aqueous Tannic acid 20%, 1 : 1)

M solution, (Aqueous Mercuric chloride 5%)

M1 solution, (Aqueous Mercuric chloride 0.5%)

MA solution, (Aqueous Mercuric chloride 5%, Acetic acid, 2 : 1)

MAPh solution, (Aqueous Mercuric chloride 5%, Acetic acid, Phenol 6%, 2 : 1 : 1)

MB solution, (Aqueous Mercuric chloride 2 g, Bouin's solution 50 ml)

MCl solution, (Aqueous Calcium Chloride 2%, Aqueous Mercuric chloride 5%, 1 : 1)

MF solution, (Aqueous Mercuric chloride 5% , Formaldehyde, 4 : 1)

MLCl solution, (Aqueous Mercuric chloride 5%, Aqueous Basic lead acetic 5%, Aqueous Calcium Chloride 2%, 1 : 1 :1)

MOM solution, (I : Basic lead acetic 1g, Absolute ethanol 50 ml, Distilled water 50 ml, Acetic acid 0.5 ml, II : Aqueous Mercuric chloride 5%, 1 : 1)

MOMT solution, (I : Basic lead acetate 1g, Ethanol 50 ml, Distilled water 50 ml, Acetic acid 0.5 ml, II : Mercuric chloride 5%, III : Aqueous Tannic acid 20%, 1 : 1 : 1)

MOT solution (Mota's Fixative: Basic lead acetate 1g, Ethanol 50 ml, Distilled water 50 ml, Acetic acid 0.5 ml)

MP solution, (Aqueous Mercuric chloride 5%, Saturated Picric acid, 1 : 1)

MPA solution, (Mercuric chloride 2.5g, Potassium dichromate 1.25g, Acetic acid 1 ml, Double distilled water 50 ml)

MPd solution, (Aqueous Mercuric chloride 5%, Aqueous Potassium dicromate 5%, 1 : 1)

MPd1 solution, (Aqueous Mercuric chloride 0.5%, Aqueous Potassium dichromate 0.5%, 1 : 1)

MPd2 solution, (Aqueous Mercuric chloride 0.5%, Aqueous Potassium dichromate 0.25%, 1 : 1)

MPd3 solution, (Aqueous Mercuric chloride 1%, Aqueous Potassium dichromate 0.25%, 1 : 1)

MPd4 solution, (Aqueous Mercuric chloride 5%, Aqueous Potassium dichromate 0.25%, 1 : 1)

MPF solution, (Mercuric chloride 0.3 g, Potassium dichromate 0.3 g, Formaldehyde 2 ml, Distilled water 25 ml)

MPS solution, (Mercuric chloride 2.5g, Potassium dichromate 1.25g, Sodium sulfate 0.5g, Double distilled water 50 ml)

MT solution, (solution I : Aqueous 5 % Mercuric chloride, solution II : 20 % Tannic acid filtered through Waumans No.1 filter, 1 : 1)

MT_1 solution: (Aqueous Mercuric chloride 5%, Aqueous Tannic acid 10%, 1 : 1)

MT_2 solution, (Aqueous Mercuric chloride 5%, Aqueous Tannic acid 5%, 1 : 1)

MT3 solution, (Aqueous Mercuric chloride 0.5%, Aqueous Tannic acid 20%, 1 : 1)

MT4 solution, (Aqueous Mercuric chloride 0.5%, Aqueous Tannic acid 10%, 1 : 1)

MT5 solution, (Aqueous Mercuric chloride 5%, Aqueous Tannic acid 10%, 1 : 1)

MT6 solution, (Aqueous Mercuric chloride 1%, Aqueous Tannic acid 20%, 1 : 1)

MT7 solution, (Aqueous Mercuric chloride 1% , Aqueous Tannic acid 10%, 1 : 1)

MT8 solution, (Saturated Aqueous Mercuric chloride 10%, Aqueous Tannic acid 20%, 1 : 1)

MT9 solution, (Saturated Aqueous Mercuric chloride 10%, Aqueous Tannic acid 10%, 1 : 1)

MT10 solution, (Aqueous Mercuric chloride 0.5%, Aqueous Tannic acid 5%, 1 : 1)

MTA solution, (Aqueous Mercuric chloride 5%, Aqueous Tannic acid 20%, Acetic acid 0.5 %, 2 : 2 : 1)

MTF solution, (Aqueous Basic lead acetate 5%, Aqueous Tannic acid 20%, 10% Neutral formaldehyde, 1 : :1 : 1)

MTMu solution, (Aqueous Mercuric chloride 5%, Aqueous Tannic acid 20%, Mustard powder 1%, 2 : 2 : 1)

MuA solution, (Aqueous Mustard Solution 20 %, Acetic acid, 1 : 1)

MuAT solution, (Mustard Solution 20 %, Acetic acid, Tannic acid, 2 : 2 : 3)

NBF, formaldehyde 40% 100 ml, Acid sodium phosphate monohydrate 4 g, Anhydrous disodium phosphate 6.5 g, Distilled water 900 ml)

PA solution: (Saturated Picric acid, Acetic acid, 3 : 1)

PA_1 solution, (Saturated Picric acid, Acetic acid, 3 : 4)

PA_3 solution, (Saturated Picric acid, Acetic acid, 1 : 1)

PAChA solution, (Saturated picric acid, Absolute alcohol, Chloroform, Acetic acid, 5 : 3 : 2 : 1)

PAE solution, (Saturated Picric acid, Acetic acid, Absolute ethanol, 4 : 1 : 6)

PAF_2 solution, (Saturated Picric acid, Acetic acid, Formaldehyde, 98.9 : 0.5 : 0.6)

PAF_3 solution, (Saturated Picric acid, Acetic acid, Formaldehyde,19 : 1 : 1)

PAF solution, (Saturated Picric acid, Acetic acid, Formaldehyde, 20 : 5 : 1)

PF solution, (Saturated Picric acid, Formaldehyde, 4 : 1)

Ph solution, (Phenol 6%)

PhA solution, (Phenol 6%, Acetic acid, 2 : 1)

PhAPFEChl solution, (Phenol 6%, Acetic acid, Saturated Picric acid, Formaldehyde, Ethanol, Chloroform, 5 : 5 : 20 : 1 : 2 : 2)

PhP solution, (Phenol 6%, Saturated Picric acid, 1 : 1)

PhT solution, (Phenol 6%, Tannic acid 20%, 3 : 1)

PP solution, (Aqueous Potassium dichromate 5%, Saturated Aqueous Picric acid, 1 : 1)

T solution, (Aqueous Tannic acid 20%)

TA solution, (Tannic acid 20%, Acitic acid, 2 : 1)

TAP solution, (Tannic acid 20% , Acetic acid, Acid picric, 2 : 1 : 2)

TAPF solution, (Tannic acid 20%, Acetic acid, Saturated picric acid, Formaldehyde, 2 : 1 : 3 : 1)

TCl solution, (Aqueous Calcium Chloride 2% , Aqueous Tannic acid 20%, 1 : 1)

TP solution, (Tannic acid 20%, Saturated picric acid, 1 : 1)