THE UNIVERSITY OF MANITOBA

ESTABLISHMENT AND CHARACTERIZATION OF HYBRID RAT MAST CELLS

BY

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A Thesis

Submitted to the Faculty of Graduate Studies in Partial Fulfilment of the Requirement for the Degree of Doctor of Philosophy

Department of Immunology
Winnipeg, Manitoba
1994

ISBN 0-315-99141-0

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A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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ACKNOWLEDGMENTS

I am very indebted to my supervisor, Dr. Arnold Froese for his excellent guidance, support and encouragement in the course of this study. His unmoving trust in my effort to learn in scientific research is deeply appreciated and his friendship and understanding will be a constant source of strength in my career.

I wish to thank Dr. Bosco Chan for his introduction to the mast cell research field and hands-on training for the techniques used in this project.

I wish to thank Ms. K. McNeill for her excellent technical assistance in the preparation of various reagents used in this project.

I am grateful to my Ph.D advisory committee members, Drs. A. Becker, I. Berczi, F. Kisil for their constant guidance in the research project and reviewing of my thesis; to Dr. E. Rector for cell sizing and DNA analysis, to Dr. C.R. Braekevelt for allowing me to use the microscope.

I am thankful to Ms. K. Risk, Y. Hein for their help during the course of study.

I also wish to acknowledge the support provided by a Medical Research Council of Canada fellowship.

ABSTRACT

Using polyethylene glycol, rat peritoneal mast cells (RPMC) were fused with 6-thioguanine resistant, HAT (hypoxanthine, aminopterin, thymidine) sensitive RBL-CA10.7 or RBL-CK2 cells, yielding the hybrid rat mast cells (HRMC). While the parent RPMC and RBL cells, are representatives of the connective tissue type (CTMC) and mucosal type (MMC) mast cells, respectively; most hybrid cells from the first two fusions are found to be of the MMC phenotype, as determined by differential histochemical staining. Only one cell line showed a few cells with CTMC staining characteristics which, even after cloning, changed to an MMC phenotype. Another fusion was then performed and it confirmed that cells of the CTMC phenotype can indeed be obtained. Four out of 14 primary cell lines contained significant amounts of CTMC-like granules as demonstrated by histochemical staining with either safranine O or berberine sulfate. The presence of the CTMC phenotype was also confirmed by the presence of rat mast cell protease I (RMCPI) and by mediator release triggered by compound 48/80. The CTMC phenotype vanished after culturing established cell lines for two weeks (eight weeks after fusion). The disappearance of this phenotype was accompanied by a loss of counterstaining with safranine O, RMCPI and

release of serotonin due to compound 48/80. The change in phenotype did not significantly alter the mediator release due to calcium ionophore A23187. Repeated cloning did not yield a cloned line of cells expressing the CTMC phenotype only, although it prolonged the persistence of this phenotype. At about the same time that the loss of the CTMC phenotype is taking place, loss of DNA from HRMC lines and clones was observed.

When the HRMC were analyzed for Fc receptors for IgE, the high affinity receptor, FcϵRI(α), and low affinity receptor, FcϵR_L were detected. All fusions gave rise to some HRMC showing significant variations in the relative Mr. of either FcϵRI(α) or FcϵR_L. One fusion gave rise to some HRMC with two molecular forms of FcϵR_L with approximate Mr. of 55 kDa and 45 kDa. One HRMC cell line examined carried both these molecules while others appeared to be associated primarily with one or the other molecule. These results suggest that these two molecules may be the rat equivalents of mouse FcγRIII and FcγRII-1, respectively.

LIST OF ABBREVIATION

BMMC: bone marrow-derived mast cells

BSA: bovine serum albumin

cAMP: cyclic adenosine 3'5'-monophosphate

CMEM: complete Eagle's minimum essential medium

CTMC: connetive tissue mast cells

DNP: 2,4-dinitrophenyl

DSCG: disodium cromoglycate

EDTA: ethylenediaminetetraacetic acid

FcϵRI: high affinity receptor for IgE on basophils and mast cells

FcϵRI(α): alpha chain of FcϵRI, also named R in previous studies

FcϵR_L: low affinity receptor for IgE on RBL and mast cells, also named as H in previous studies

FcγRII: Fc receptor for IgG II

FcγRIII: Fc receptor for IgG III

FCS: fetal calf serum

GM-CSF: granulocyte/macrophage colony stimulating factor

HARE: monospecific horse anti-rat IgE

HEPES: N-2-hydroxy-piperazine-n-2-ethanesulfonic acid

HRMC: hybrid rat mast cells

5-HT:	5-hydroxytryptamine binoxalate
IL-3:	interleukin 3
IL-4:	interleukin 4
IL-5:	interleukin 5
IL-9:	interleukin 9
IL-10:	interleukin 10
IMMC:	intestinal mucosal mast cells
kDa:	kilodaltons
LT:	leukotriene
MMC:	mucosal mast cells
Mr:	relative molecular mass
OPT:	O-phthaldialdehyde
PBS:	phosphate buffered saline
PG:	prostaglandin
PI:	phosphatidylinositol
PKC:	protein kinase C
PMA:	phorbol 12-myristate 13-acetate
RCMC:	rat tissue cultured mast cells
RBL:	rat basophilic leukemia
RMCP:	rat mast cell protease
RPMC:	rat peritoneal mast cells
SDS-PAGE:	polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate
TfR:	transferrin receptor
w/v:	weight/volume

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CHAPTER I

LITERATURE REVIEW

DEVELOPMENT AND DIFFERENTIATION OF MAST CELLS

Ever since mast cells were first discovered by Paul Ehrlich over a hundred years ago, they have been a source of fascination to innumerable investigators. At that time, Paul Ehrlich proved unequivocally that these cells represent a unique cell category in body tissue. Another contribution made by Paul Ehrlich was that he coined the term "mast cells" which meant "fat cell" (Ehrlich, 1878). At that time, the presumed functions of mast cells and related basophils was confined solely to the "fixed store house" or "recruitable store house" of some chemical materials. It was not until the mid-1960's that the heterogeneity of mast cells was noticed and their functions were realized to be more complex than being merely a "store house". Subsequent studies had shown that mast cells and basophils are critical participants in a variety of clinically important acute and chronic disorders with components of immediate hypersensitivity, such as certain forms of anaphylaxis and asthma. The progress of mast cell research achieved in recent years such as : the characterization of the structure and function of mast cells and basophil constituents at the molecular level, the development of techniques for growing mast cells and basophils in <u>in vitro</u> tissue culture and the realization of potentially

critical interactions between these cells and other tissue elements, have brought our understanding of the biology of mast cells to a higher level. At the same time, these efforts have also extended our understanding of the functions of mast cells and basophils as part of a greater diversity of biological responses which are independent of the immune system. It is a new start in a sense that mast cells are realized to participate importantly in non-immunological processes such as wound healing, fibrosis, angiogenesis, and reactions to environmental toxins. For example, the possible roles of mast cells in the symptoms of food allergy, inflammatory bowel disease and irritable bowel syndrome were suggested in one of the most recent studies (Marshall, 1993a).

Subpopulations of mast cells:

To some extent, today's knowledge of mast cell biology is critically influenced by a finding made almost 100 years ago: the observation that mast cells do not represent terminally differentiated cells of uniform characteristics but are heterogeneous in many aspects of their phenotype (Maximow, 1905). It is now clear that "heterogeneity" of mast cell phenotype encompasses potentially significant variations in their morphology, biochemical constituents and products, susceptibility to proliferation and regulation of maturation

by cytokines and sensitivity to the effects of both secretogogues and drugs affecting mast cell release of mediators. Generally speaking, two populations of mast cells have been recognized and named mucosal mast cell (MMC) and connective tissue mast cells (CTMC). Such mast cell subpopulations have been observed in rat, mouse and human systems although the classification in humans is not been yet as clear as that in rodents. Maximow observed that certain mast cells in the intestinal mucosa were "atypical " in their histochemical staining characteristics and they differed from those of mast cells observed in other anatomical sites such as skin and peritoneal cavity (Maximow, 1905; Galli, 1990). Enerbäck then greatly extended the concept of mast cell heterogeneity by defining in great detail the conditions of fixation and histochemical staining which discriminated between the mucosal mast cell type (MMC) observed primarily in the intestinal lamina propria and the connective-tissue mast cells (CTMC) of skin, peritoneal cavity and other tissues in the rat (Enerbäck, 1966a; 1966b). Subsequent studies showed that rat and mouse MMC and CTMC appear to differ in many aspects including morphologic and histochemical characteristics, natural history, biochemistry, and roles in inflammation and immunity (Bienenstock et al., 1986; Enerbäck, 1986). Among the most striking differences is that MMC can expand remarkably during T cell-dependent immune responses to certain intestinal parasites while CTMC exhibit little or no

T Cell dependent response and they can occur in athymic mice or rats in numbers similar to those present in normal counterparts (Ruitenberg et al., 1976).

The continuous use of potentially-confusing terms like "mucosal" and "connective tissue-type" mast cells (i.e., MMC and CTMC) as the designations for mast cell "subtypes" are still frequently encountered in the literature. One of the reason that such unclear terms being used is that the study of mast cell and basophil biology have not progressed enough to provide sufficient information for the construction of a new, unifying nomenclature (Bienenstock et al., 1989).

Histochemical properties of mast cells:

As mentioned above, early studies by Maximow show that certain mast cells in the rat intestinal mucosa are atypical in their staining characteristics and differ from those mast cells in other anatomical sites (Maximow, 1905; Galli, 1990). The classic studies of Enerbäck clearly defined the conditions of fixation and histochemical staining that discriminated between such atypical or mucosal mast cells (MMC) and the connective tissue-type mast cells (CTMC) of the skin, peritoneal cavity and muscularis propria of the digestive canal (Enerbäck, 1966a; 1966b; 1966c; 1981). Enerback's study resolved a long-running dispute over the existence of

mast cell in intestinal mucosa because the granules of MMC are not fixed with the most commonly used fixative, 10% formalin. It was shown that after adequate fixation and staining, the gastrointestinal mucosa in rats is one of the tissues richest in mast cells. Carnoys solution (containing methanol, chloroform and acetic acid) or a combination of 0.6% formaldehyde and 0.5% acetic acid is necessary for the fixation of MMC. When appropriately fixed sections are stained, rat MMC stain blue with alcian blue and can not be counterstained by safranine, while the granules of rat CTMC stain red with safranine (Combs et al., 1965; Enerbäck, 1966c; Seldin et al., 1985). Only CTMC can be stained by the fluorescent dye berberine sulphate (Berlin and Enerbäck, 1983).

The differential staining properties between MMC and CTMC are due to the different proteoglycans in mast cell granules. Proteoglycans consist of a peptide core covalently linked to glycosaminoglycan side-chains composed of repeating disaccharide units. The nature of the alternating sugar groups and their sulphation determines the class of proteoglycan. Heparin accounts for the majority of the proteoglycan synthesized by rat serosal CTMC while little is produced by rat intestinal MMC and mouse bone marrow-derived mast cells (Lagunoff et al., 1976; Yurt et al., 1977; Metcalfe et al., 1980). These latter two types of mast cells contain the oversulphated chondroitin sulphate di-B and E

proteoglycans (Enerback, 1985; Stevens et al., 1986a). The higher degree of sulphation of heparin (2.5 sulphate and 1 carboxyl per disaccharide) imparts a higher negative charge density to this compound, which results in the different metachromatic staining properties of CTMC vs. MMC. However it is interesting to note that rat CTMC normally also contain small amounts of oversulphated chondroitin sulphate proteoglycans (Katz et al., 1986). It has been shown that a single gene encodes the core peptide of both types of proteoglycans and the post-translational modification by glycosyltransferase decides their final chemical nature (Tantravahi et al., 1986; Stevens et al., 1986b).

Serine proteases of mast cells:

Different types of proteases have been found in both rat and mouse mast cell populations. Two serine proteases with chymotrypsin-like activity are found in rat mast cells, named rat mast cell protease I and II (RMCPI and RMCPII) with Mr. of 29 kDa and 25 kDa, respectively (Woodbury et al., 1981; Gibson and Miller, 1986). RMCPI has been localized to CTMC in the submucosa of the rat small intestine, skin, tongue, intestinal serosa and lung parenchyma. RMCPII is an antigenically distinct serine protease which is found in MMC of the rat intestine and bronchial epithelium (Woodbury et al., 1978a;

1978b; 1981). The availability of monospecific polyclonal antibodies to RMCPI and RMCPII allows for the phenotypic differentiation of CTMC and MMC according to the presence of the respective serine protease (McMenamin et al., 1987). RMCPII has been cloned and the result suggests that RMCPI is likely to be encoded by a separate gene highly homologous to that of RMCPII (Benfey et al., 1987). Recently, Rouleau et al. (Rouleau et al., 1994) cloned the RMCPI gene by reverse transcriptase-polymerase chain reaction (RT-PCR) using oligonucleotide primers corresponding to the RMCPII gene. The RMCPI amino acid identity with RMCPII is 74%, and 76%, 65% and 90% with the mouse proteases MMCP1, MMCP2 and MMCP4, respectively. In addition to RMCPI, CTMC also contain carboxypeptidase A which is an enzyme that cleaves C-terminal aromatic amino acids (Reynolds et al., 1989).

So far, seven mouse serine proteases have been described and termed "mouse mast cell protease" (MMCP). Their relative Mr. ranges from 26 to 32 kDa and the substrate specificity indicates that MMCP-1 through MMCP-5 are chymases while MMCP-6 and MMCP-7 are tryptases (Huang et al., 1991; McNeil et al., 1991; Reynolds et al., 1991; Serafin et al., 1991; Castells et al., 1992; Stevens et al., 1994). The amino acid sequence of MMCP-1 shows a high degree of homology with RMCPII and both proteases are significantly expressed in MMC (Huang., et al., 1991). MMCP-4 shows a high degree of homology with RMCPI which may indicate an evolutionary relation between these

enzymes (Serafin et al., 1991). It is interesting to note that the genes encoding MMCP-1, 2, 4, and 5 have been cloned and their genomic organization are very similar to T lymphocyte granzymes and neutrophil elastase and cathepsin G (Huang.,. et al., 1991; McNeil et al., 1991; Reynolds et al., 1991; Serafin et al., 1991). Carboxypeptidase A has also been isolated from mouse CTMC and a cDNA encoding this exopeptidase has been isolated from a mouse KiSV-MC derived cDNA library (Reynolds et al., 1989). In summary, mouse CTMC contain carboxypeptidase A, MMCP-4, MMCP-5 and MMCP-6 and mouse MMC express MMCP-1 and MMCP-2. Mouse bone marrow derived mast cells (BMMC) are found to express carboxypeptidase A, MMCP-5, MMCP-6 and MMCP-7 but not MMCP-1, 2 or 4 which indicates indirectly that BMMC may represent a unique stage of mast cell differentiation (Katz et al., 1991; Castells et al., 1992).

Biogenic amines and arachidonic acid metabolites in mast cells:

Histamines and 5-Hydroxytryptamine are present in mast cells and play important role in mediating type I hypersensitivities and other mast cell functions (Schwartz and Austen, 1984). Histamine is ubiquitous among mast cells of different species, although the concentrations may vary depending on specific type of cells. It is generally

recognized that mast cells of CTMC phenotype have higher histamine content than those of the MMC phenotype (Enerbäck and Wingren, 1980; Befus et al., 1982). Rat peritoneal mast cells, typical of CTMC, contain about 15-20 pg of histamine per cell whereas rat MMC contain only about 1-2 pg per cell. Mouse peritoneal mast cells contain about 10 pg of histamine per cell and mouse BMMC about 0.1 pg per cell. Some significant differences in histamine concentration have been noticed among peritoneal mast cells harvested from different inbred strains of mice and also from mice of different ages but of the same strain (Enerbäck and Wingren, 1980; Ahlstedt and Haard, 1987).

Unlike histamine, the concentration of serotonin in mast cells shows a remarkable species difference and both rat and mouse mast cell contain serotonin while the serotonin content is very low or non-existent in mast cells isolated from human lung (Wingren et al., 1983; Enerbäck, 1986).

When they are stimulated, the activated mast cells will not only release so-called "preformed" mediators such as histamine , serotonin or serine protease they also produce pharmacologically active metabolites from arachidonic acid and release them (Heavey et al., 1988). Rat peritoneal mast cells will mainly metabolize arachidonic acid through the cyclo-oxygenase pathway and the chief products are prostaglandins D_2 and E_2. On the other hand, rat mucosal mast cells mainly produce leukotriene C_4, leukotriene B_4 and some

prostaglandin D_2. Arachidonic acid metabolism in mouse mast cells is found to be similar to that of rat mast cells (Razin et al., 1983; Hamasaki and Tai, 1984).

Mediator release properties of mast cells:

The intestinal mast cell hyperplasia induced by *Nippostrongylus brasiliensis* and other enteric parasites in rats provides a useful model for the study of the rat mucosal mast cells. Using an enzymatic digestion technique, it has been possible to isolate relatively pure MMC for functional analysis (Lee et al., 1985b). The isolation of rat peritoneal mast cells by peritoneal lavage technique and subsequent density gradient centrifugation with Ficoll or Percoll made pure rat CTMC available for detailed studies (Bach et al., 1971; Conrad et al., 1975; Enerbäck and Svensson, 1980; Nemeth and Rohlich, 1980). It has been shown that in addition to the morphological and histochemical differences, MMC differ from CTMC in their profile of responsiveness to a variety of secretagogues and anti-allergic compounds. Although calcium ionophore A23187, multivalent antigen and anti-IgE can induce histamine release from CTMC or MMC, MMC do not respond to polyamine secretagogues: compound 48/80, polymyxin or bee venom peptide 401 (Enerbäck, 1966c; Befus et al., 1982; Shanahan et al., 1984,1986). Similarly, phosphatidyl serine

which enhances antigen-induced histamine secretion from peritoneal mast cells has no effect on MMC, whereas secretion from both cell types is potentiated by adenosine (Befus et al., 1982). Certain anti-allergic compounds like disodium cromoglycate and theophylline which inhibit antigen-induced CTMC secretion are ineffective in MMC (Pearce et al., 1982).

It has been shown that neuropeptides can induce secretions of mediators by mast cells (Theoharides and Douglas, 1978; Kruger et al., 1982). When MMC and peritoneal CTMC from the same animals were compared, somatostatin, substance P, vasoactive intestinal polypeptide and neurotensin had a potent secretagogue effect on CTMC, but only substance P induced significant histamine release from MMC (Shanahan et al., 1984, 1985). With regard to the influence on mediator release properties of other naturally occurring substances, endorphin including dynorphin and beta-endorphin are far more potent than exogenous opiate in inducing CTMC secretion, but have very little effects on MMC (Shanahan et al., 1985).

Expression of Fcε receptors on mast cells

The expression of high affinity Fc receptors for IgE (FcεRI) is one of the prominent features of mast cells and basophils. In addition, a low affinity receptor for IgE

($Fc\epsilon R_L$) has been described on rat mast cells (Froese, 1980; Chan et al., 1990) and rat basophilic leukemia (RBL) cells (Conrad and Froese, 1978). The latter should not be confused with $Fc\epsilon RII$ or CD23 which is found primarily on B cells (Kikutani et al., 1986). CD23 will be discussed in a separate section. The receptors were initially characterized in the rat system due to the availability of both RBL cells and rat peritoneal mast cells (RPMC). The two classes of receptors for IgE, the α chain of high affinity receptor, designated now as $Fc\epsilon RI(\alpha)$, and the low affinity receptor $Fc\epsilon R_L$ were previously named R and H, respectively, and were isolated from RBL cells and RPMC and characterized (Froese, 1980). Recently both $Fc\epsilon RI(\alpha)$ and $Fc\epsilon R_L$ were also isolated from rat intestinal mucosal mast cells (Swieter et al., 1989).

Quantitative studies on the number and density of $Fc\epsilon R$ on mast cells were carried out in several studies. RPMC were found to have from 1×10^5 to 3×10^5 receptors per cell with a Ka of 8×10^9 M^{-1} (Conrad et al., 1975) while rat IMMC carry 3×10^4 receptors per cell but with similar affinity constant (Sterk and Ishizaka, 1982; Lee et al., 1985a; 1985b).

Origin and development of mast cells:

The recognition of mast cell subtypes and the emergence

of the concept of mast cell heterogeneity has opened up many new questions concerning the lineage and maturation of mast cells in different tissues. The ontogeny of mast cells and their subpopulations have been the subject of extensive research. Many different hypotheses have been postulated and one of them suggested mast cells were derived from basophils with a relationship similar to that between tissue macrophages and circulating blood monocytes. However, recent advances in mast cell research, especially the studies with mast cell deficient mutant mice have indicated mast cells are derived from precursors which originate from haematopoietic cells in the bone marrow.

The generation of mast cells from haematopoietic cells was first shown by using giant granules of beige (C57BL/6-bg^j/bg^j) mice as a marker. The bone marrow cells from C57BL/6-bg^j/bg^j were transferred to irradiated normal congenic mice (C57BL/6-+/+) mice and resulted in development of beige-type mast cells with giant granules (Kitamura et al., 1978). Two other mutant mice are also used in the investigation of mast cell differentiation. Both $WBB6F_1$-W/W^v and $WCB6F_1$-Sl/Sl^d are deficient in mast cells and their absence is attributed to a defect of precursors cells in W/W^v mice and to a defect of the tissue environment necessary for differentiation of mast cells in Sl/Sl^d mice. One of the critical observations was that adult $WBB6F_1$-W/W^v mice can develop tissue mast cells populations if they receive bone marrow cells derived either

from the normal littermates ($WBB6F_1$-+/+ mice), or from semisyngeneic C57BL/6-bg/bg mice. Because mast cells derived from beige mice contain giant cytoplasmic granules, the mast cells which develop in $WBB6F_1$-W/W^v mice transplanted with C57BL/6-bg/bg bone marrow cell can be identified unambiguously as being of donor origin. This approach was used to establish that mouse mast cells develop from circulating, bone marrow derived precursors, and that a common precursor cell can give rise to both mast cells and granulocytes. Most of the progeny of multipotential stem cells such as erythrocytes, platelet, neutrophils, eosinophils, and basophils leave the haematopoietic tissue after they differentiate except mast cells . Mast cells do not complete their differentiation in haematopoietic tissue until they reach different tissues sites. This notion was further supported by the fact that no mast cells or their precursors can be detected in blood. It seems that colony forming mast cell precursors will invade the connective or mucosal tissues, proliferate,and differentiate into morphologically identifiable mast cells, i.e, either MMC or CTMC. Further studies have indicated that the life span of most MMC appears to be limited to one or two weeks. In contrast, CTMC in the skin and peritoneal cavity of mice can live as long as one year. Although the proliferation potential of MMC has been unequivocally established the proliferation of CTMC is not very clear. Some studies indicated that some morphologically identifiable CTMC have an

appreciable proliferative potential. When single murine CTMC cells were injected into the skin of WBB6F_1-W/W^v mice and mast cell colonies containing about 2000 mast cells developed in some injection sites (Kitamura et al., 1978; Kitamura, 1989). Subsequent in vitro experiments supported the in vivo finding and indicated that both IL-3 and IL-4 were necessary for development of colonies from CTMC (Tsuji et al., 1990).

Cytokines and mast cell development:

Complicating our efforts to understand the biology of mast cells is their heterogeneous nature. Accumulated evidence suggests that mast cell heterogeneity may largely be due to the effects of site-specific blending of cytokines produced within different local environment, hence cytokines could be critical factors influencing mast cells development and differentiation. On the other hand, it has been shown that mast cells produce and secrete several different cytokines and these cytokines can function as mast cell mediators and play important role in mast cell biology (Galli et al., 1991). Several cytokines have been recognized to regulate mast cell development:

Interleukin 3. IL-3 is a glycoprotein with a Mr. of 28-kDa which is mainly produced by T cells after antigen and

mitogen stimulation. It has multipotential effects and can influence the survival, proliferation and differentiation of haematopoietic progenitor cells of several lineages, including erythrocytes, lymphocytes, monocytes, granulocytes, megakaryocytes, and mast cells (Schrader, 1986; Ihle and Weinstein, 1986). Accumulated evidence suggests that IL-3 is one of the most important cytokines for mast cell development in both in vivo and in vitro conditions. In the 1980's, several groups reported that mast cells of the MMC phenotype could be derived by culturing bone marrow cells in WEHI-3-conditioned medium (WEHI-3-CM) or in concanavalin A (ConA) splenocyte-conditioned medium (Razin et al., 1981; Nabel et al., 1981). Subsequent studies confirmed the above findings and further clarified that it was the IL-3 in the conditioned medium which induced the MMC development (Razin et al., 1984). In vivo, IL-3 effects on mast cell development were exemplified by studying the mast cell response of nude mice to parasite infection. Ruitenberg et al. and Olson et al. reported that, unlike normal murine rodents infected with nematodes such as N. brasiliensis, athymic or nude mice do not undergo a T cell-dependent hyperplasia of mast cells in the intestinal mucosa. However, nude mice do respond with mucosal mastocytosis similar in degree to that of parasite-infected normal animal when being given repeated intra-peritoneal injections of purified IL-3 (Ruitenberg and Elgersma, 1976; Olson and Levy, 1976; Abe et al.,1988). These

studies suggested that in vivo IL-3 was critical for the proliferation of mast cells in small intestinal mucosa of mice and rats during parasitic infestations.

Interleukin 4. IL-4, previously known as B-cell stimulatory factor 1 (BSF-1), T-cell growth factor 2 (TCGF2) or mast cell growth factor 2 (MCGF2), has multiple effects on the proliferation and functions of B cells, T cells and mast cells (Paul,W., 1983; Smith and Rennick, 1986). Several in vitro experiments have shown that IL-4 has synergistic or costimulatory effects with IL-3 in the proliferation of both bone marrow derived and peritoneal mast cells (Mosmann et al., 1986; Hamaguchi et al., 1987; Tsuji et al., 1990). Tsuji et al. studied the effects of IL-3 and IL-4 on murine peritoneal mast cells , representative of mast cells of the CTMC phenotype. Although both IL-3 and IL-4 failed to induce extensive proliferation of CTMC, they stimulated CTMC proliferation synergistically in a dose-dependent manner (Tsuji et al., 1990). In the above mentioned study of the mast cell response in nematode infected mice, it was shown that anti-IL-4 antibodies administered to mice infected with N. Brasilinesis cause an approximately 50% reduction in the ensuing intestinal mastocytosis (Madden et al., 1991). When both anti-IL-3 and IL-4 antibodies were used , 85-90% of the intestinal mastocytosis was inhibited. Thus, IL-4 is a significant modulator of mast cell proliferation in vivo as

well as in vitro.

Interleukin 9. IL-9, also known to have a novel mast cell enhancing activity and to be a T cell growth factor, is a 35-kDa glycoprotein produced by T cells and mitogen-activated spleen cells that enhances the proliferation of IL-3-dependent mouse mast cell lines (Hultner et al., 1989; 1990). Extensive work has been done by Hultner and co-workers. A series of permanent IL-3-dependent mast cells lines were established from normal BALB/c or C3H mouse bone marrow using PWM-stimulated spleen cell-conditioned medium as a source of IL-3. They found that in vitro growth of these mast cells lines was not only dependent on IL-3 and synergistically enhanced by IL-4, but in addition was regulated by a mast cell growth enhancing activity (MEA) present in spleen cell-conditioned medium and acting in concert with IL-3. MEA could be partially purified and completely separated from IL-4 and IL-3 (Moeller et al., 1989). Subsequent studies indicated that MEA was structurally identical to mouse IL-9 (Hultner et al., 1990). It should be mentioned that , when used alone, IL-9 has no growth-promoting activity on primary mast cell cultures, although it does prolong their survival. Since IL-3, IL-4 and IL-9 are all produced by activated cloned CD4+ T lymphocytes and by spleen cell stimulated with mitogen, it seems likely that they act together to induce mast cell proliferation during certain inflammatory responses.

Interleukin 10. IL-10, previously known as murine cytokine synthesis inhibitory factor, is produced by activated T cells and normal B cells (Moore et al., 1990; O'Garra et al., 1990). Studies from Thompson-Snipes and colleagues showed that IL-10 alone failed to support the growth of either IL-3-dependent mouse mast cell lines or mast cell progenitors from mesenteric lymph nodes of N. brasilinesis-infected mice. However, IL-10 dramatically enhances mast cell proliferation when used in combination with IL-3 or IL-4. It is speculated that mast cell growth can be engendered by IL-4 and IL-10 in the complete absence of IL-3 suggesting another pathway for mast cell production in circumstances when IL -3 would be unavailable. It is further shown the combination of these three cytokines together is a very potent growth promoting stimulus for either primary cultured mast cell or IL-3-dependent mouse mast cells. Since these three cytokines are coordinately synthesized by the same T cell population and appear to be produced during N. brasiliensis parasite infection, they may constitute a network of overlapping mast cell growth-promoting activities needed to ensure appropriate levels of mast cells as a host response to infection (Thompson-Snipes et al., 1991). Recently, Rennick et al.(1994) from the same group reconfirmed the mast cell growth promoting effect of IL-10.

Interferon-γ, TGF-β_1 and GM-CSF. Preliminary studies indicated that these three cytokines may have negative effects on mast cell proliferation. IFN-γ is a 25 kDa glycoprotein produced mainly by T cells and some natural killer (NK) cells and has extensive immunoregulatory and anti-proliferative effects in addition to being an anti-viral agent. Mouse bone marrow-derived mast cells were shown to express a small number of high affinity binding sites for γ-interferon (Nafziger et al., 1990). Takagi et al. (1990) reported a negative effect of IFN-γ on the proliferation of both BMMC and peritoneal mast cells. Adding murine INF-γ to mouse bone marrow cells cultured in cytokine-laden media induced a marked reduction in the number of mast cells that develop. Similarly, murine IFN-γ, when added to IL-3 and IL-4-containing cultures of mouse peritoneal mast cell inhibited proliferation of these cells.

Broide et al. (1989) reported that TGF-β_1 could selectively inhibit IL-3-dependent mouse bone marrow derived mast cell proliferation without affecting their function or differentiation. TGF-β_1 can significantly decrease [^{3}H]thymidine uptake by IL-3-dependent BMMC in a dose-dependent manner and the inhibitory effect is reversible and not cytotoxic. They concluded that TGF-β_1 is an important negative regulator of IL-3-dependent mast cell proliferation <u>in vitro</u>.

Bressler et al. (1989) studied whether GM-CSF would

affect IL-3-dependent growth of mast cells from mouse bone marrow cells (BMMC). BMMC grown in the presence of 50 U/ml of GM-CSF gave rise to cultures containing primarily macrophages and granulocytes with less than 1% mast cells. Addition of increasing amounts of GM-CSF to BMC cultures resulted in a decrease in the number of mast cells present in culture at two to three week, and the inhibitory effect was not abrogated by the addition of Indomethacin to cultures. They concluded that the suppressive effect of GM-CSF on IL-3-dependent mast cell growth may indicate an important role for GM-CSF in the down-regulation of mast cell proliferation in tissue.

So far, the above cytokines have been clearly shown to have important regulatory effects on mast cell proliferation and development. On the other hand, it has also been reported that mast cells themselves can produce and secrete a broad panel of multifunctional cytokines and it is assumed that such mast cell cytokines may be important mediators in biological responses associated with the activation of mast cells (Plaut et al., 1989; Galli et al., 1991).

Early evidence on the cytokine production by mast cells came from the study using the Abelson murine leukaemia virus (A-MuLV)-transformed tumorigenic mouse mast cell lines. It was shown that some of these lines can constitutively produce granulocyte-macrophage colony stimulating factor (GM-CSF) mRNA and release GM-CSF (Chung et al., 1986). Then, it was reported that some of these A-MuLV-transformed mouse mast cell

lines also constitutively expressed mRNA for interleukin 4 and some constitutively release IL-4 bioactivity (Brown et al., 1987). Subsequently, several cytokine bioactivities were reported among the non-transformed mast cells. Several groups demonstrated that in vitro derived IL-3-dependent mouse mast cells, rat basophilic leukaemia (RBL) cells, or freshly isolated mouse or rat peritoneal mast cells expressed cytolytic activity against certain cellular targets, and some examples of this mast cell-dependent cytotoxicity were partially inhibited by antibodies to TNF-α (Gordon et al., 1990a; Galli et al., 1991). One study showed that mouse peritoneal mast cells and IL-3-dependent and IL-3-independent mouse mast cells generated in vitro, contained a cytoplasmic granule-associated cytokine with immunologic and cytotoxic activities very similar to those of TNF-α (Young et al.,1987). Gordon et al. (1990b) confirmed that this TNF-α-like cytotoxic mediator was a product of the TNF-α gene and that unstimulated mouse peritoneal mast cells constitutively contained approximately twice as much TNF-α bioactivity as did lipopolysaccharide-stimulated mouse peritoneal macrophages.

It has been shown that stimulation of mast cells through the FcεRI can also induce increased levels of cytokine mRNA and give rise to protein products. Plaut et al. (1989) demonstrated that IgE-dependent stimulation of long-term IL-3-dependent mouse mast cell lines resulted in increased levels of mRNA for IL-3, IL-4, IL-5, and IL-6 and the release of

bioactivity for IL-3, IL-4 and IL-6. In addition to changes in the mRNA for these cytokines, others had found increased mRNA levels for GM-CSF and for IL-1, macrophage inflammatory protein-1α (MIP1α), MIP1β (Wodnar-Filipowicz et al., 1989; Burd et al., 1989;). These findings, once again, suggest that mast cells are important in terms of effector functions.

Several studies have suggested that mouse splenic non-B, non-T cells can produce IL-4 and IL-3 in response to cross-linking of high-affinity FcϵRI (Ben-Sasson et al., 1990; Gros et al., 1990; Conrad et al., 1990). Ben-Sasson et al. (1990) reported that spleen cell populations depleted of both B and T lymphocytes produce IL-4 in response to stimulation with immunoglobulin bound to the surface of culture dishes. In the presence of IL-3, plate-bound IgE and IgG1 are excellent stimulants suggesting that Fc receptor cross-linking is required for IL-4 production. It is interesting to note that later it was found that the splenic or bone marrow non-B,non-T cell populations, which express high affinity FcϵRI(α) and have IL-4-producing capability, were highly enriched in basophils (Sedar et al., 1991). So it is possible that mouse basophils may be able to produce IL-4 and perhaps IL-3 upon IgE-dependent and other forms of activation, but the fact that the basophil-enriched populations also contained rare immature cells of the mast cell lineage, make the precise identity of the IL-4 producing cells unclear.

Other biological factors: Leukemia inhibitory factor (LIF). In one of their recent studies, Marshall et al. (1993) examined the production and secretion of Leukemia inhibitory factor (LIF) from different mast cells. LIF is a pluripotent cytokine of importance in the regulation of immune and inflammatory responses, in neuronal developmnet and bone metabolism. It was shown that two mucosal mast cell lines RBL-2H3 and RCMC9 endogenously produce low levels of LIF bioactivity and the production could be enhanced by calcium ionophore A23187. Freshly isolated and purified peritoneal cells also expressed the mRNA for LIF. They suggested that the findings may have important implications for the role of mast cells in neuronal development, hematopoiesis, bone metabolism and the acute phase response (Marshall et al., 1993b).

<u>C-kit protein, stem cell factor and mast cell development:</u>

It was in the study of mast cell-deficient mice, W/W^v and Sl/Sl^d mutants, that the so-called c-kit protein and its ligand stem cell factor, emerged as another important contributor to mast cell growth. Mice with two mutant alleles at either the dominant white spotting locus on Chromosome 5 (W) or at the Steel locus on Chromosome 10 (Sl) display several characteristics such as specific abnormal pigmentation

of fur, sterility secondary to deficiency in germ cells and macrocytic anaemia (Russel et al., 1979; Silver et al., 1979; Gregory et al., 1978). In 1978 and 1979, Kitamura and colleagues reported the critical observation that both strains of mutant mice, W/W^v and Sl/Sl^d mice, were mast cell deficient with less than 1% of the number of mast cells present in the tissue of the congenic normal (+/+) mice (Kitamura et al., 1978; 1979). Further studies linked the mast cell deficiency in W mutants to a defect in haematopoietic progenitors, because bone marrow from normal congenic mice transplanted into W/W^v mice cures the mast cell defect (Kitamura et al., 1978). Detailed studies indicated that the nature of the problem with bone marrow does not appear to involve a lack of mast cell precursors or their trafficking, but mast cell deficiency is likely due to a defect inherent in the mast cell precursor itself, which affect both its presence and differentiation in the tissue (Suda et al., 1985). Thus, mast cell deficiency in Sl mutant mice has been linked to a defect in the tissue microenvironment because mast cells could be generated from Sl/Sl^d bone marrow, and transplantation of Sl/Sl^d bone marrow into W/W^v mice cured the mast cell deficiency (Kitamura et al., 1979; Fujita et al., 1989). Genetic studies revealed that the W locus was allelic with the proto-oncogene c-kit, which encodes a 145- to 160- kDa cell surface receptor with tyrosine kinase activity (Chabot et al., 1988; Geissler et al., 1988; Majumder et al., 1988). It is

a member of the receptor tyrosine kinase family that includes the platelet-derived growth factor receptor and the receptor for colony stimulating factor-1 on macrophages (Qiu et al., 1988). Shortly after these studies on the c-kit protein, several groups reported simultaneously that the mouse Sl locus encodes the ligand for c-kit protein which is now being referred to by many names such as kit ligand, steel factor or stem cell factor (Williams et al., 1990; Zsebo et al., 1990; Huang et al., 1990). The relationship between the c-kit protein and stem cell factor may thus explain the similarities in the defects caused by mutations at the W and Sl loci.

Both c-kit protein and its ligand, stem cell factor, have been cloned and the protein structures are also known. The c-kit protein has three domains: an extracellular ligand-binding domain with five immunoglobulin-like loops, a small transmembrane segment, and a C-terminal intracellular tyrosine kinase region (Majumder et al., 1988; Qiu et al., 1988). Different forms of stem cell factor, soluble and membrane attached, secondary to alternative mRNA splicing have been reported (Flanagan et al., 1991; Anderson et al., 1990). SCF can be produced by fibroblasts, bone marrow stromal cells and possibly also by Schwann cell or rat osteosarcoma cells (Huang et al., 1990; Flanagan and Leder, 1990; Anderson et al., 1990; Ryan et al., 1991).

As predicated from the phenotypic abnormalities associated with mutant mice, SCF has multiple effects

including promotion of haematopoiesis, survival and proliferation of primordial germ cells, and the development of melanocytes (Huang et al., 1990; Anderson et al., 1990; Godin et al., 1991; Funasaka et al., 1992). Analysis of the effects of soluble SCF on mast cell in vitro and in vivo clearly indicated it has extensive and critical effects for mast cell proliferation and development. Tsai et al. (1991a; 1991b) reported that recombinant SCF can induce proliferation of both IL-3-dependent BMMC and mature peritoneal mast cells. It can also promote the maturation of bone-marrow derived cultured mast cells in vitro: SCF-treated cells had higher histamine content, incorporated [^{35}S]sulfate into heparin, contained granules with reactivity for safranin and/or berberine sulphate. In vivo effects of SCF were demonstrated in an experiment where rrSCF was administered intravenously to rats. It was shown that mast cells with the CTMC phenotype and the MMC phenotype markedly increased in anatomical locations which ordinarily contain CTMC and MMC, respectively. In addition, it was recently shown that soluble recombinant SCF may directly induce mediator release from some mouse or human mast cell populations (Columbo et al., 1992; Bischoff et al., 1992; Coleman et al., 1993). Coleman et al. examined the effects of rrSCF, IL-3 and IL-4 on mouse peritoneal mast cell mediator release. Challenge of purified mast cells with rrSCF induced a modest release of serotonin (5-HT), whereas IL-3 or IL-4 did not directly stimulate serotonin release. SCF, but

not IL-3 or IL-4, had an additive effect on the 5-HT release induced by cross-linking membrane-bound IgE. Koike et al. (1993) studied the SCF-induced signal transduction as a mechanism involved in the peritoneal mast cell mediator release. They found that SCF could induce serotonin release from RPMC and the SCF treatment failed to produce inositol 1,4,5-triphophate which indicted the absence of involvement in the phosphoinositide-specific phospholipase C pathway. They further showed that the phosphatidylcholine-specific phosphalipase D (PLD) pathway is the main pathway for the production of 1,2-diacylglycerol (1,2-DG) in SCF-treated rat peritoneal mast cells. They concluded that SCF induces the activation of PLD through the protein kinase pathway without activation of phosphoinositide-specific phospholipase C (Koike et al., 1993).

Since it has been shown that both IL-3 and SCF are the two principle cytokines effecting mast cell proliferation and maturation, the interaction between these two factors was studied and reported (Mekori et al., 1993). It was found that IL-3 dependent mast cells would undergo programmed cell death (apoptosis) on removal of IL-3 from either primary bone-marrow -derived cultured mast cells (BMMC) or from the growth factor dependent mast cell line MCP5. Apoptosis could be prevented by SCF and they suggested that SCF exerted its effect via interacting with c-kit protein, not through the induction of other cytokines. They concluded that SCF and IL-3 may act in

concert to regulate mast cell numbers under physiological conditions (Mekori et al., 1993). Recently, Iemura et al. (1994) also reported that SCF can promote the survival of SCF-dependent mouse mast cells by suppressing apoptosis.

It should be mentioned that the complete functions of c-kit and stem cell factor still remain to be fully delineated and some questions are not answered yet, such as whether SCF can directly activate mast cell differentiation from a genuine precursor, whether specific effects observed with a particular mast cell population can be generalized to mast cells in different stages require further investigation.

In vitro cell culture of mast cells:

Accumulated experimental data indicates that CTMC and MMC can be distinguished by a variety of characteristics which includes their different anatomical locations. Thus, CTMC exist in the skin, peritoneal cavity and the muscularis propria of the stomach while MMC are abundant in the intestinal lamina propria (Enerbäck and Rundquist, 1981; Bienenstock et al., 1982). Due to the relative scarcity and limited purity of mast cells from the lamina propria of the gut and the fact that the CTMC from peritoneal cavity can not survive in cell culture without losing their specific phenotypic characteristics, research on mast cells have

largely been depended on several in vitro derived cultured mast cell lines. The in vitro approach makes it possible to carry out various precise biochemical analysis of mast cells and to address some fundamental questions about the proliferation and interactions of mast cells with other cell types in the in vivo environment.

Ginsburg and colleagues obtained murine mast cells by growing mouse thymocytes on monolayers of mouse embryo cells consisting of a mixture of fibroblasts and haematopoietic progenitor cells (Ginsburg, 1963). A similar approach was taken in which rat thymus cells could give rise to mast cells when being cultured on rat fibroblast monolayers (Ishizaka et al., 1976). Subsequently, murine mast cells resembling MMC were generated from bone marrow cells in vitro in the presence of conditioned medium from mitogen-stimulated splenocytes (Nabel et al., 1981; Razin et al, 1981; Tertian et al, 1981). Later it was shown that these bone marrow-derived mast cells (BMMC) were dependent on IL-3 in the conditioned medium (Ihle et al., 1983; Razin et al., 1984). BMMC have been used to identify and characterize mouse mast cell proteoglycans and considered to be the representative of mast cells of MMC phenotype (Razin et al., 1984). However, as will be discussed in the section of mast cell enzymes, the expression of CPA, MMCP-5, MMCP-6 and MMCP-7 in BMMC, but not MMCP4 of CTMC or MMCP-1 and MMCP-2 of MMC indicates that BMMC represent a unique stage of mast cells differentiation relative to MMC and

CTMC (Castells et al.,1992). Similarly, rat BMMC were derived by culturing rat haematopoietic tissue cells in the presence of T lymphocyte cytokines (Haig et al., 1982).

In this laboratory, rat tissue cultured mast cell (RCMC) lines were established by prolonged culturing of rat peritoneal mast cells in the absence of exogenous growth factors and fibroblast feeder cells (Chan et al., 1988; 1990). Phenotypic characterization of RCMC lines revealed that they were of the MMC phenotype based on the histochemical staining properties, the presence of serine protease RMCPII, and the low level of histamine content (Chan et al,, 1990). Although the nature of cells that gave rise to RCMC is not clear yet (Chan et al., 1990), it should be pointed out here that there are significant differences between the RCMC and HRMC of this project and therefore, they are different cells. The major differences between RCMC and HRMC will be discussed in Chapter V.

In 1973, rat basophilic leukaemia (RBL) cells were generated by treating rats with the chemical carcinogen, β-chlorethylamine, and maintained in tissue culture without any growth factors (Eccleston et al., 1973). Phenotypic analysis clearly showed that these cells are phenotypically related to rat intestinal mucosal mast cells (Seldin et al., 1985).

Relationship between mast cells and basophils:

In history, both mast cells and basophils were identified almost a century ago (Ehrlich, 1879). By definition, basophils are granulocytes and constitute less that 1% of the circulating leucocytes. It is obvious that mast cells and basophils share several notable features in addition to the characteristic metachromatic staining property.

Both mast cells and basophils are derived from bone marrow haematopoietic precursor cell, express plasma membrane IgE Fc receptors (FcϵR) (Kitamura, 1989; Froese, 1984) and are major source of histamine and other potent chemical mediators (Befus et al., 1982; Enerback, 1981). After sensitization with IgE, exposure to specific multivalent antigen triggers both cell types to release both preformed mediators stored in the cytoplasmic granules (such as histamine, serotonin, protease, heparin or other sulphated proteoglycans) and newly-synthesized mediators such as prostaglandins and/or leukotrienes. Other immunologically nonspecific molecules such as complement C3a or C5a , calcium ionophore can also trigger similar mediator release from them (Ishizaka, 1988; Schwartz and Austen, 1984). Therefore, they both play a role in immediate hypersensitivity reactions and other inflammatory events.

Despite such remarkable similarities, mast cells and basophils are not identical. Basophils normally differentiate in the bone marrow and enter the circulation as mature,

functionally active cells. In contrast, mast cells mature in the tissue where they reside. Basophils are not primarily found in connective tissues although some of them can be recruited into tissue during inflammatory responses. The life span of basophils is in the range of days in contrast to weeks or months for mast cells. Cytogenetic analysis revealed that basophils share common precursors with other granulocytes and bear more similarities with eosinophils than with mast cells. The morphological difference between mature basophils and mast cells have been characterized in all mammalian species and especially in human mast cells. Basophils are generally smaller, have irregular short processes on their surface and a segmented nucleus .The cytoplasmic granules in basophils are fewer and larger than in mast cells and there are some aggregates of cytoplasmic glycogen. Studies in several mammalian species suggest an inverse quantitative relationship between basophils and mast cells; e.g. species that have abundant mast cells have few basophils except in human situation where significant numbers of both are present. So far there has been no evidence to suggest that basophils can transform into mast cells (Galli et al., 1984; Galli and Lichtenstein, 1988; Dvorak et al., 1982; 1983a; 1983b).

Heterogeneity of human mast cells:

The heterogeneity of mast cells in rodent system has been relatively well established. The two subtypes of mast cells, MMC and CTMC, differ in the anatomical distribution, histochemical staining, histamine content, serine protease, and mediator release properties. Analogous to the rodent mast cell systems, it has been assumed that human mast cells do not represent a homogeneous population and some degree of heterogeneity exists, but the direct application of rodent mast cell heterogeneity to human mast cell system is not satisfactory. Human mast cell heterogeneity was indirectly suggested based on stainability with basic dyes after fixation of comparable sections of lung or intestine in neutral formalin versus other fixatives such as Carnoy's solution (Strobel et al., 1981; Shanahan et al., 1987). When attempts were made to visualize mast cells in the intestinal mucosa, it was shown that the method of tissue fixation is of great importance. When fixation was done with formalin, human mast cells in the mucosa failed to display the metachromatic staining characteristics and they were, thus, termed "formalin sensitive", while mast cells in the submucosa were "formalin resistant"(Strobel et al, 1987).

So far, two subpopulation of human mast cells have been identified based on the neutral protease composition, ultrastructure and secretory response to immune and nonimmune stimuli. It is known that the presence of serine proteases in rat and mouse mast cells is an important criterion for

designation of mast cells to a particular type. Extensive research on the enzymes has been done on human mast cells and it turned out that they also contain certain serine proteases, i.e. tryptase and chymase. The terms of "mast cell chymase" and " mast cells tryptase" were first introduced by Lagunoff and Benditt for the proteolytic chymotrypsin-like and trypsin-like enzymes in mast cells (Lagunoff and Benditt, 1963). Schwartz and colleagues purified and characterized human mast cell tryptase and they showed that high amounts of tryptase are present in all human mast cells and only negligible amounts exist in other inflammatory cells (Schwartz et al., 1981; 1987). Tryptase is a tetrameric serine protease with a molecular weight of 134 kDa that contains subunits of 31 kDa-35 kDa, each with an active site. It is stored in secretory granules of mature mast cells in an active form probably bound to sulphated proteoglycans and accounts for a substantial portion of the total mast cell protein and for at least 90% of the trypsin-like activity in mast cells derived from lung and skin. The mean level of tryptase in a mast cell from adult lung was calculated to 12 pg and from adult foreskin was 35 pg. Chymase, a serine protease, is a monomer with a molecular weight of 30 kDa and stored in an active form in mast cell granules. The mean level of chymase, as determined by immunoassay, in mast cells derived from adult foreskin is calculated to be 4.5 pg per mast cell and is approximately 10-fold greater than amounts observed in dispersed human lung

mast cells. Thus, two subpopulation of human mast cells were demonstrated by immunohistochemical studies. Human mast cells are designated as MC^{TC} if both tryptase and chymase are present and as MC^{T} if only tryptase are detected. MC^{T} mainly exist at human lung alveolar wall, bronchial epithelium, small intestine mucosa and nasal mucosa; MC^{TC} mainly in skin and small intestinal submucosa. It has not been convincingly concluded that MC^{T} and MC^{TC} are the counterparts of MMC and CTMC of rodent mast cells. It is important to notice that all of these different anatomical sites have representatives of either subtypes and human mast cells cannot be described as MC^{TC} or MC^{T} based on the tissue location itself.

Ultrastructure analysis of human mast cells by electron microscopy techniques has revealed different patterns of the cytoplasmic granules such as scrolls, particulate and crystal patterns (Dvorak et al., 1983a; 1983b). The granules in MC^{T} cells are smaller than those of MC^{TC} and contain characteristic discrete scrolls that are either compact or loosely coiled and contain either electron-dense or electron lucent cores. On the other hand, the granules in MC^{TC} appear round or oval, compact and uniformly electron dense and characteristic grating and lattice patterns were evident (Craig et al., 1988).

Rodent mast cell of MMC phenotype show a T cell dependent proliferation tendency whereas CTMC do not (Mayrhofer et al., 1981; Mayrhofer, 1973; Ruitenberg and Elgersma, 1976).

Analogous to the situation in rodents, human mast cells in the gastrointestinal mucosa of patients with congenital combined immunodeficiency disease and those with the AIDS, where CD4+ T cells are preferentially destroyed, were examined and compared to normals (Irani et al., 1987). In each case, the MC^T cells were selectively and markedly reduced in number in the mucosa and submucosa of otherwise normal appearing bowel. MC^{TC} cells numbers in the mucosa and submucosa were not significantly different from normal.

Both human mast cell subpopulations undergo coupled activation-secretion mediated by IgE and by calcium ionophore, but the response of each cell type to other secretagogues appears to differ (Church et al., 1982; Lawrence et al., 1987). Only MC^{TC} cells respond to C5a, f-met peptides, substance P, Compound 48/80 and morphine. The mediator release of MC^T cells, but not MC^{TC}, can be inhibited by disodium cromoglcate (DSCG). It should be noted that mast cells used in most of these studies were not 100% pure and thus variations or different results might be anticipated when pure subpopulation of mast cells are used.

Very limited data have been accumulated regarding the quality and quantity of different mediators in human mast cells. The histamine content in either human mast cell subtype was shown to be similar in one study (Schwartz et al., 1987). One study with human lung mast cells indicated that both the cyclooxygenase pathway and the 5-lipoxygenase pathway

products of arachdonic acid metabolism are produced which include : PGD_2, LTC_4 and LTD_4 (Peter et al., 1984). Although the nature of proteoglycans in human mast cells is not as clear as that of rodent mast cells, a recent study indicated that human lung mast cells synthesize both heparin and chondroitin sulphate E proteoglycans in roughly 2:1 ratio (Stevens et al., 1988)

IgE Fc RECEPTORS Of MAST CELLS AND BASOPHILS

For the purpose of this thesis, the so-called IgE-binding molecules include : (1) the high affinity IgE receptors (FcϵRI) found on mast cells and basophils; (2) the low afffinity receptor for IgE (FcϵR_L) on rat mast cells; (3) the CD23 molecule or the low affinity receptor for IgE (FcϵRII) on cell types other than mast cells and basophils and (4) the soluble IgE binding factors from T cell and B cells. The latter factors, mainly involved in the IgE response regulation, are not related to this research project and thus will not be discussed here. Although considerable knowledge on the structure and function of CD23 has been accumulated, both are not yet fully understood. The significance of IgE binding to CD23 is not clear and some even suggest that the binding of IgE to CD23 may be "fortuitous" (Spiegelberg, 1991). The structure and functions of CD23 will be discussed very briefly in a following separate section. The high and low affinity receptor for IgE on rat mast cells will be the main topic of discussion.

For years it has been known that the high affinity receptor for IgE are present on mast cells and basophils, but recently evidence has emerged to indicate that other cell types can also express FcϵRI (Bieber et al., 1992; Bieber and

Ring, 1992; Wang et al., 1992; Grabbe, et al., 1993; Osterhoff et al., 1994; Gounni et al., 1994). In the first four closely related studies, human epidermal Langerhans cells (LC) were found to express the high affinity receptor for IgE which can bind monomeric IgE molecules. They reported that IgE binding to Langerhans cells could not be prevented by preincubation with monoclonal antibodies against either FcεRII/CD23 or FcγRII/CD32. However, the binding could be entirely abrogated by preincubation with anti-FcεRI(α) mAb15-1, which interferes with IgE binding to FcεRI(αγ) transfectants (Wang et al., 1992; Bieber et al., 1992). Therefore, it was concluded that the IgE binding to epidermal Langerhans cells was mediated by FcεRI rather than by CD23, CD32, or D-galactose-specific IgE - binding protein. This conclusion was further supported by additional findings which indicated that (1) most of the epidermal Langerhan cells showed some degree of surface immunolabelling with the anti-FcεRI(α) mAbs, but not with several different anti-CD23 mAbs; and (2) the transcripts for α,β, and γ chains of FcεRI could be amplified by the polymerase chain reaction from RNA preparations of LC-enriched, but not of LC-depleted epidermal cell suspensions. Similar strategies lead to the demonstration that FcεRI are also present on monocytes and eosinophils (Maurer et al., 1994; Gounni et al., 1994).

The affinities of IgE Fc receptors on mast cells and basophils:

The FcεR on mast cells and basophils have attracted extensive studies due to the extremely important role of IgE-FcεR interaction in hypersensitivity reactions. Such studies, particularly in the rat system, have been further enhanced by the availability of rat basophilic leukaemia (RBL) cells (Eccleston et al., 1973) and of sufficient quantities of relatively pure monoclonal IgE molecules (Bazin et al., 1974). The recognition of the existence of IgE Fc receptors and studies on the interaction between IgE and these receptor were mainly carried out in the 1970s' and early 1980s'. Early investigations on IgE binding to mast cells and basophils by Ishizaka and colleagues suggested the existence of high affinity IgE Fc receptors on mast cells and basophils and their results suggested the value of the equilibrium constant for the human basophils-IgE interaction might be as high as 10^8 to 10^9 M^{-1} (Ishizaka and Ishizaka, 1971; Ishizaka et al., 1973). In one of a series of studies, Conrad et al. (1975) compared molecular parameters for rat IgE binding by both normal rat peritoneal mast cells (RPMC) and RBL cells and found by Scatchard analysis that the equilibrium constant for both cell types was of the order of 10^9 M^{-1}. They also found that RBL cells had a higher number of receptors sites per cell and a higher receptor density. Kulczycki and colleagues also

studied the quantitative aspects of IgE binding to RBL cell surface receptors (Kulczycki et al., 1974). They found that the reaction mechanism appeared to consist of a simple reversible binding reaction with $k_{12} = 9.6 \times 10^4\ M^{-1}\ sec^{-1}$ and $k_{21} \leq 1.6 \times 10^{-5}\ sec^{-1}$ at 37^0C. The calculated K was therefor $\geq 6 \times 10^9\ M^{-1}$. The average number of receptors per RBL cell varied between $3x10^5$ to $1x10^6$ and the binding was insensitive to pH's between 6-8; but at pH 3.0 complete dissociation of bound IgE occurred in about one minute at 0^0C and left the receptors for IgE intact. The rate constants for the interaction of mouse IgE and peritoneal mouse mast cells were found to be of a very similar magnitude to those for the rat system (Sterk et al., 1982).

Isolation of FcεR from mast cells and basophils:

Since the number of FcεR on the cell surface of mast cells are relatively limited and in order to study them more efficiently, FcεR need to be radioactively labelled before subsequent solubilization and isolation. Generally speaking, two different methods of receptor labelling have been applied. The first method involves tagging of surface molecules using either lactoperoxidase-catalysed radioiodination of surface proteins (Conrad et al., 1976; Kulczycki et al.,1976) or labelling of surface carbohydrate residues using a galactose

oxidase-catalysed reaction with ^{3}H-$NaBH_4$ (Pecoud et al., 1981). The second method involves culturing cells in the presence of ^{3}H-amino acids or ^{3}H- or ^{14}C-sugars as protein or carbohydrate precursors and this approach will label all cell proteins or carbohydrates and not only cell membrane molecules (Kulczycki et al., 1976; Helm and Froese, 1981;). Alternatively the isolated FcεR can be labelled by means of N-succinimidyl 3-(4-hydroxy, 5-[^{125}I] iodophenyl) propionate (Bolton-Hunter reagent) which labels proteins (Kanellopoulos et al., 1979).

Following surface labelling of the FcεR, cells are disrupted and receptors are solubilized, using detergents which do not interfere with the IgE-binding capacity of the receptors. Thus, receptor solubilization was achieved with the aid of the non-ionic detergent Nonidet P-40 (NP-40) (Conrad et al., 1976) or the chemically related detergent Triton X-100 (Rossi et al., 1977). Another detergent , Rennex 30 could also be used and it has the advantage that the receptors can be iodinated in its presence without the detergent itself being labelled (Pecoud et al., 1981).

A number of different approaches have been used to isolate and analyze the labelled receptors and these have been summarized in great detail (Froese, 1984). Briefly the following approaches have been used: (1) IgE-receptor complexes and anti-IgE immunoprecipitation. IgE molecules will interact with detergent solubilized receptors and the

IgE-receptors complexes are then isolated using rabbit anti-rat IgE and goat anti-rabbit Ig (Conrad and Froese, 1976). The anti-IgE and IgE-receptors complexes can also be insolubilized with the aid of protein A-Sepharose (Conrad and Froese, 1978a). (2) Affinity chromatography with IgE-Sepharose. The bound receptors can be eluted with 3M potassium thiocyanate (KSCN), 0.5M acetic acid or 6M guanidine-hydrochloride (Conrad et al.,1976; Kulczycki and Parker, 1979;). (3) Haptenated IgE and anti-hapten antibody immunoprecipitation. Dinitrophenylated IgE will interact with FcεR and the IgE-receptor complexes will be isolated with anti-dinitrophenol (DNP) antibodies coupled to Sepharose CL-4B. DNP-IgE-receptor complexes are eluted with 2,4-dinitrophenol (Conrad and Froese, 1978b). (4) Receptor isolation with anti-receptor antibodies. The solubilized receptors will interact with anti-receptor antibodies and this immunocomplex will be rendered insoluble with protein A-Sepharose and later the receptors can be dissociated with SDS (Conrad et al., 1978; Conrad et al., 1979).

The isolated receptors are subsequently characterized by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS-PAGE) (Conrad and Froese, 1976; Kulczycki and Parker, 1979; Holowka et al., 1980).

Characterization of IgE Fc receptors:

Previous studies from this laboratory indicated that there are two IgE Fc receptors on RBL cells and mast cells with different affinities in IgE binding. The high affinity receptor, previously named "R", was found to have an Mr. of 45 kDa and is now known to be the α-component of the high affinity IgE receptor complex and is thus named FcϵRI(α). The low affinity receptor, previously named "H" or "FcϵRII", has an Mr. of 55 kDa and is termed as FcϵR_L (Conrad and Froese, 1978a; Froese, 1980; LaCroix and Froese, 1993). Although both FcϵRI(α) and FcϵR_L can be isolated by IgE-Sepharose affinity chromatography, they show some intrinsic differences in binding to IgE because only FcϵRI(α) can be immunoprecipitated by anti-IgE from IgE containing cell extracts. Similarly, FcϵRI(α), but not FcϵR_L could be isolated by means of DNP-IgE and anti-DNP-Sepharose.

As mentioned earlier, original identification of these two receptors using IgE-Sepharose was carried out on RBLwpg cells. Studies using RBL cell sublines maintained in different laboratories gave rise to different receptor patterns and pointed to only one type of receptor on the cell surface represented by an SDS-PAGE peak corresponding to Mr. values from 45 kDa to 60 kDa (Kulczycki et al., 1976; Iserky et al.,1978; Kanallopoulos et al., 1979). At same time the dependence of the apparent molecular weight on the acrylamide concentration was demonstrated (Kulczycki et al.,1976; Kanellopoulos et al., 1980). To establish that such

differences between different sublines were real and not due to subtle difference in methodology, receptors from surface-iodinated RBL cells originating in the various laboratories were isolated by identical techniques and analyzed by using the same SDS-PAGE system. The low affinity receptor for IgE could be detected in all cell lines after absorption of the high affinity receptor FcϵRI(α) with IgE and anti-IgE - Sepharose (Froese, 1984).

Receptor analysis carried out on mouse peritoneal mast cells indicated there were two kinds of IgE-binding receptors (Sterk and Ishizaka, 1982). One of these receptors binds both rat and mouse IgE with equal affinity and the other will bind mouse IgE only. Recently, both high and low affinity IgE receptors on rat intestinal mucosal mast cells (IMMC) were characterized and compared with those on rat peritoneal mast cells and RBL cells (Swieter et al., 1989). It was shown that both FcϵRI(α) and FcϵR_L were present on IMMC and the FcϵRI(α) had an apparent Mr. of 59 kDa, somewhat higher than that of RPMC (51kDa), RBL-2H3 cells (51kDa) or RBL-CA10.7 cells (45 kDa). IMMC also differed from peritoneal mast cells (PMC) in the number of forms of FcϵR_L isolated; two forms of FcϵR_L with 50 kDa and 58 kDa were obtained, whereas RPMC yielded most often a single 55 kDa band. This FcϵR_L pattern on IMMC was similar to that on RBL-2H3 cells where two forms of receptor with Mr. of 46 kDa and 55 kDa were identified.

In addition to FcϵRI(α) and FcϵR_L, a third but minor

receptor molecule on RBL cells was discovered in this laboratory. It was found to have an apparent molecular weight of 71,000 daltons, and it was simply named 71K (Helm and Froese, 1981). The 71K molecule resembles FcϵRI(α) in IgE binding because it can be isolated either by means of IgE-Sepharose, IgE and anti-IgE, or haptenated IgE and the appropriate anti-hapten antibodies. Subsequent studies indicated that this molecule consists of FcϵRI(α) crosslinked by disulphide bonds to another, as yet unidentified polypeptide chain and that it was induced by Mycoplasma hyorhinis infection when this microorganism contaminated RBL cell cultures (Roth et al., 1986; Chan et al., 1986; 1988).

IgE Fc receptors are glycoproteins:

It was established relatively early that the receptors for IgE are glycoproteins (Froese, 1984). This notion has been supported by the following findings: (1) Receptors could be labelled with radioactive iodine and were found to be sensitive to trypsin degradation in the solubilized form (Conrad and Froese, 1976); (2) Receptors could also be labelled by radioactive sugars or so-called biosynthetic labelling with [^{14}C]-glucosamine, [^{3}H]-fucose and [^{3}H]-galactose (Kulczycki et al., 1976; Helm and Froese, 1981a); (3) Lectin binding studies also supported the glycoprotein nature (Helm

and Froese, 1981b). It was shown that both FcϵRI(α) and FcϵR_L are associated with fucose, mannose, galactose and N-acetylglucosamine and the carbohydrate content of FcϵRI(α) was estimated to be 32% (Kanellopoulos et al.,1980). The extent of glycosylation of FcϵR_L has not been established yet.

The nature and microheterogeneity of the carbohydrate moiety of both FcϵRI(α) and FcϵR_L on RBL cells were further described in a recent report (LaCroix and Froese, 1993). Cells were treated with the glycosylation processing inhibitors, castanospermine (CN), 1-deoxymannojirimycin (DMJ), swainsonine (SW) and the receptors were isolated and analyzed by SDS-PAGE. The treated cells exhibited a decrease of relative molecular mass of both FcϵRI(α) and FcϵR_L and it was shown that both of receptors were composed primarily of complex oligosaccharides with a single high mannose, N-glycosylated site. Deglycosylation studies with N-glycanase revealed that in the case of RBL-CA10 cells, a cloned line of RBLwpg, the N-linked carbohydrate comprised 38-42% and 32-37% of FcϵRI(α) and FcϵR_L, respectively. In the case of the FcϵRI(α), this amounted to most of the carbohydrate, suggesting that the amount of O-linked sugars is , at best, small (Lacroix and Froese, 1993).

Although the precise function of carbohydrates moiety on FcϵRs is not clear yet, it was suggested that it is involved in receptor transport and membrane incorporation since RBL cells treated with tunicamycin, an antibiotic which can

inhibit the glycosylation of proteins, reduces receptor incorporation into the plasma membrane (Pecoud et al., 1981; Hempstead et al., 1981).

The cross-reactivity of IgE Fc receptors:

Several studies suggested that mast cells and basophils carried on their surface receptors capable of interacting with IgG (Ishizaka et al., 1977). In addition to IgE, mouse IgG_1 and rat IgG_{2a} were also found to be involved in mediator release (Daeron et al., 1975;). A few early experiments suggested that IgG and IgE may indeed interact with the same receptors. In one study, it was shown that the addition of IgG_{2a} antibody to rat sera containing IgE decreased the amount of IgE removed by cell-free particles of RPMC origin (Bach and Brashler, 1977). Another study demonstrated that mediator release from RPMC by rat IgG_{2a} alloantibodies could be inhibited by IgE and the alloantibodies in turn blocked IgE mediated histamine release (Mossmann et al., 1976). Segal and colleagues compared the interactions of RBL-2H3 cells with rat IgE and different forms of IgG and found that RBL cells carried two different kinds of receptors (Segal et al., 1981). One of these binds IgE very tightly and this binding cannot be inhibited by monomeric IgG or aggregated IgG; the other binds oligomers of rat IgG with relatively high affinities and the

binding of dimers could be inhibited by both monomeric rat IgE and IgG.

Studies in this laboratory made a similar finding to that of Segal et al.(Kepron et al., 1982). It was shown that two molecules having an apparent molecular weight identical to FcϵRI(α) and FcϵR_L could be isolated from NP-40 extracts of ^{125}I-labelled RBL cells by using IgE-free rat IgG coupled to Sepharose. Absorption of NP-40 extracts with IgE-Sepharose completely removed both molecules capable of binding to IgG-Sepharose. Thus, it became clear that both FcϵRI(α) and FcϵR_L were capable of binding to IgG-Sepharose. Inhibition studies revealed that binding of FcϵRI(α) to IgG-Sepharose can be inhibited by both IgE and IgG with IgE being the better inhibitor. Binding of FcϵRI(α) to IgE-Sepharoes was inhibited by IgE and not IgG. Binding of FcϵR_L to either IgE-Sepharose or IgG-Sepaharose could be inhibited by either IgE or IgG, IgE being the better inhibitor. In another study, the inhibition of binding of FcϵRI(α) and FcϵR_L to rat IgG-Sepharose by various homologous and heterologous immunoglobulins was used to assess their relative affinities for the two receptor molecules (Kepron et al., 1988). Ranking of rat Ig in order of their affinities for the FcϵRI(α) yielded: IgE >> IgG_{2a} > IgG_1 > IgG_{2b}; and for FcϵR_L : IgE > IgG_{2b} > IgG_{2a}. The affinities of mouse IgG subclass interacting with FcϵRI(α) were ranked as: IgG_1 > IgG_{2a} > IgG_{2b}; and for the FcϵR_L: IgG_1 > IgG_{2b} > IgG_{2a}.

Structure of the high affinity receptor for IgE

Substantial progress in the characterization of IgE Fc receptor has been made in recent years with the introduction of molecular biology techniques. All three subunits of FcεRI have been cloned, this in return has led to new insights in the structure and functions of FcεRs.

Although the initial studies by affinity chromatography identify a single polypeptide with an apparent Mr. of 45kDa as the high affinity receptor for IgE (Conrad and Froese, 1976; Kulczycki et al., 1976), subsequent studies suggested that there might be more than one subunit for FcεRI. Holowka and his colleagues labelled RBL-2H3 cells with a mixture of ^{3}H-amino acids, isolated the receptors with haptenated IgE and detected two molecules by SDS-PAGE (Holowka et al., 1980). One of them corresponded to the FcεRI(α) previously isolated using surface-labelled cells, while the other had a lower molecular weight of 30-35 kDa. Further studies indicated that this 30 kDa peptide was associated with the 45 kDa FcεRI(α) in a 1:1 ratio and this peptide was named the β chain of FcεRI (Holowka et al., 1980). The fact that β-chain can be detected by biosynthetic incorporation of [^{3}H]-amino acids but not by surface iodination and can be labelled with hydrophobic probe 5-iodonaphthyl-1-azide (INA) implied that this peptide is not exposed on cell surface and is mainly membrane embedded (Holowka et al., 1981). Biosynthetic experiments with

labelled carbohydrate precursors showed no evidence for incorporation into the β-chain, suggesting an apparent absence of carbohydrate in this peptide (Holowka and Metzger, 1982). A similar molecule was observed in ^{3}H-tyrosine-labelled receptor preparations isolated from RBLwpg by the same approach and the apparent Mr. was 26 kDa (Helm and Froese, 1981b). This peptide was also detected in receptor preparation isolated from ^{35}S-methionine-labelled RPMC by repetitive affinity chromatography (Hempstead et al., 1981). Rivnay and colleagues reported that the β-chain tended to dissociate from the α chain during purification even at neutral pHs and physiological ionic strengths. However, the interaction of α and β chains can be stabilized by maintaining an appropriate phospholipid to detergent ratio (Rivnay et al., 1982).

Perez-Montfort and colleagues reported that under conditions that stabilized the interaction between the α and β subunits of the FcϵRI, a new component was recovered having apparent molecular weights of 20 kDa and this molecule was demonstrated to consist of a disulphide-linked dimer of two 10-kDa polypeptides which had all the characteristics expected for subunits of the receptor. It shared many of the labelling properties of the β chain and is likely to be embedded in the plasma membrane and exposed on the internal but not the external surface of the membrane bilayer (Perez-Montfort et al., 1983). Thus, the new 10-kDa-peptide was named γ chain of

the high affinity receptor for IgE which consists of one α, one β and two γ chains.

Complementary DNAs for the α subunit have been isolated from three species, rat (Kinet et al., 1987), mouse (Ra et al.,1989) and human (Kochan et al., 1988; Shimizu et al., 1988). In rat, the cDNA library was constructed from the mRNA of RBL cells and the sequence studies indicated the α chain contained a 180-residue extracelluar portion with two immunoglobulin-related domains of 40 and 42 residues, a 20-residue transmembrane segment and a 27-residue cytoplasmic portion. The sequence showed no homology with the low-affinity receptor for IgE on lymphocytes but had over 30% homology with FcγRIII (Kinet et al., 1987).

The cDNA for the β component of FcϵRI has also been characterized. The rat cDNA was also constructed from mRNA of RBL cells and it was found that the gene encoded a protein of 243 residues with no leader sequence. The hydropathicity plot suggested that the polypeptide crossed the plasma membrane four times and epitope studies with mAb suggested both ends of the β subunit were cytoplasmic (Kinet et al.,1988; Ra et al., 1989).

The cDNA sequence of rat, mouse, and human γ chain have been reported (Ra et al., 1989; Blank et al., 1989; Kuster et al., 1990). The sequence studies of rat γ chain showed there is a short segment of five residues extracellularly, a single transmembrane domain and followed by a long intracellular

portion. There are two cysteine residues in the sequence and dimerization of γ chain occurs via the N-terminal cysteine only. Unlike the case for α and β chains the transcripts for γ chain have been identified in many haematopoietic cell lines, such as T cells.

The surface expression of FcϵRI on transfected COS-7 cells by gene transfer supported the four-chain model of FcϵRI (Blank et al., 1989). In this study, COS-7 cells transfected with different receptor cDNAs were tested for IgE-binding using an IgE-rosetting assay. It was found that the rosette forming cells were only detected after co-transfection of the full set of the cDNAs. It should be pointed out the expression of human FcϵRI may be somewhat different from that of the rodent system because it was shown that co-transfection of human α and γ is sufficient for functional expression of the receptor (Miller et al., 1989).

Although FcϵR_L has not been cloned and the protein stucture is not clear, accumulated data suggests that it may be related to the FcγRII or FcγRIII. This hypothesis is supported by several observations. It was shown that mast cells and basophils carried on their surface receptors capable of interacting with IgG and it was also shown that mouse IgG_1 and IgG_{2a} were also involved in the mediator release in addition to IgE (Daeron et al., 1975; Ishizaka et al., 1977). Segal and collegues suggested that RBL cells may carry two

different kinds of receptors for IgE and IgG, respectively (Segal et al., 1981). Studies from this laboratory indicated that both FcϵRI(α) and FcϵR_L are capable of binding to IgG-Sepharose (Kepron et al., 1982). The ranking of rat Igs in order of their affinities for the FcϵR were established as following: (1) for FcϵRI(α), IgE >> IgG_{2a} > IgG_1 >IgG_{2b}; (2) for FcϵR_L, IgE >> IgG_{2b} > IgG_{2a}. Interaction of mouse IgG subclasses with FcϵRI(α) are ranked as: IgG_1 > IgG_{2a} > IgG_{2b}; and for the FcϵR_L : IgG_1 > IgG_{2b} > IgG_{2a} (Kepron et al., 1988).

Studies from Takizawa and colleagues show that FcγRII and FcγRIII on mouse mast cells and macrophages can bind IgE-immune complexes and the binding can be blocked by monoclonal antibody against the extracellular homologous region of both FcγRII and FcγRIII. The IgE-immune complexes bind specifically to FcγRII or FcγRIII transfected into COS-7 cells with an estimated association constants of $3.1 \times 10^5 M^{-1}$ for FcγRII and $4.8 \times 10^5 M^{-1}$ for FcγRIII. Engagement of FcγRII and FcγRIII with IgE-immune complexes after blocking access to FcϵRI or with IgG-immune complexes can trigger these mast cells to release serotonin and it was suggested that the low affinity receptor for IgE on mouse mast cells and macrophages is represented by FcγRII and FcγRIII (Takizawa et al., 1992).

Bocek and Pecht (1993) cloned and sequenced the rat FcγRII from a cDNA library of the rat MMC line RBL-2H3 cell. The predicated amino acid sequence is highly homologous with mouse FcγRII as well as rat FcγRIII.

The above evidence strongly suggests the $Fc\epsilon R_L$ on rat mast cells identified in this laboratory may be related or identical to the rat $Fc\gamma RII$ or $Fc\gamma RIII$.

Activation of mast cells and basophils via $Fc\epsilon R$ cross-linking:

It is well-known that one of the major functions of $Fc\epsilon R$ on mast cell and basophils involves the triggering of these cells to release a variety of pharmacological mediators which can subsequently lead to allergic reactions. The classic allergic reaction occurs when antigen-specific IgE bound to the high affinity receptor on the surface of mast cells is aggregated by binding to a multivalent antigen. Activated mast cells will release at least the following mediators: histamine and other preformed mediators of immediate hypersensitivity; secretion of newly synthesized eicosanoid mediators such as leukotrienes and prostaglandins; synthesis and secretion of different cytokines. Later it was shown that IgE is not necessarily required for the triggering of mediator release from target cells, provided the $Fc\epsilon R$ can be cross-linked by means other than the interaction of antigen with receptor-bound IgE (Ishizaka et al., 1978). It has been demonstrated that histamine release can be triggered by any mechanisms that results in aggregation of IgE receptors such as: anti-receptor antibodies; addition of preformed oligomers

of IgE; addition of lectin to cells (Ishizaka et al., 1978).

CD23: a low affinity receptor for IgE on other cell types:

Historically, the CD23 molecule was discovered twice. At first it was identified by Spiegelberg and associates as a low affinity IgE receptor on lymphocytes and macrophages (FcεRII) (Gonzalez-Molina and Spiegelberg, 1983). Subsequently and independently it was described as B cell activation marker (Kintner and Sugden, 1981). In 1987 it became clear that this B cell FcεRII and the B cell activation marker are the same molecule (Bonnefoy et al., 1987; Yukawa, et al., 1987). The only common property between this FcεRII and the high affinity IgE receptor (FcεRI) is that both of them can bind to the Fc portion of IgE molecule although with different affinities. FcεRI binds IgE with an association constant of 10^{-9} to 10^{-10} M and binding is Ca^{++} independent; CD23 binds IgE with an association constant of 10^{-6} M and the binding needs Ca^{++} (Conrad, 1990). Compared with the function of FcεRI on mast cell and basophils, the function of CD23 on B cells and other cell types remain to be fully determined.

CD23 is present on B cells, T cells, monocyte, macrophages, eosinophils, platelet and EBV (Epstein-Barr virus)-transformed B cells (Suemura et al., 1986; Richards and Katz, 1991). Several biological molecules can affect the

expression of CD23 and these include: IL-4, IgE, IFNγ, IFNα, TGFβ and glucocorticoids. Of them IL-4 is the most potent inducer of CD23 expression and the effect is due to increased CD23 protein synthesis (Yokota et al., 1988).

Both the human and mouse CD23 genes have been cloned and functionally expressed (Ikuta et al., 1987; Wendel-Hansen et al., 1990). The human CD23 gene is present on chromosome 19 and contains eleven exons separated by 10 introns and the gene is approximately 13 kilobases long. The CD23 gene exons correlate very well with the putative functional domains of the protein. The big division between exons 9 to 11 from the first eight exons reflects the dimorphic structure of this protein products: the membrane bound CD23 and soluble CD23. The CD23 gene structure studies also confirms the existence of two forms of CD23, CD23a and CD23b (Yokota et al., 1988).

It has been shown that human CD23 has an Mr. of 45 kDa. The presence of carbohydrate moieties such as sialic acid residues, both O-linked and N-linked carbohydrates on its polypeptides chain, classify CD23 molecule as a glycoprotein (Spiegelberg, 1984; Nakajima and Delespesse, 1986). Its amino acid sequence was deduced from nucleotide sequence of the cDNA and found to contain 321 residues coding for a protein of 36 kDa (Bonnefoy et al., 1988). The lack of signal peptide sequence at the amino terminus indicates that CD23 has an unusual membrane orientation, with the amino terminus inside and the carboxyl terminus outside the cytoplasmic membrane.

The CD23 is, therefore, composed of a short cytoplasmic domain of 23 mainly hydrophillic amino acids, followed by a transmembrane domain and by a large extracelluar domain of 277 amino acids.

In contrast to other Fc receptors, the FcεRII/CD23 has not evolved as a member of the immunoglobulin supergene family but has substantial homology with several animal lectins (Suter et al., 1987; Drickamer, 1988). The homologous domain is found entirely within the soluble 25-kDa IgE-binding factor. It is possible that, besides binding to IgE, CD23 has additional functions where carbohydrate binding could be important.

Both human and murine CD23 are labile proteins in a sense that the soluble forms (sCD23) are generated and released into the extracelluar environment continuously. The formation of soluble CD23 is associated with a process so-called "autoproteolysis" in which the membrane 45 kDa CD23 is cleaved into different fragments (Nakajima et al.,1987; Letellier et al., 1990). The human soluble CD23 (sCD23) can still bind IgE and is thus called B cell-derived IgE-binding factor (IgE-BF). Whether murine B cell-derived sCD23 can bind IgE remains to be determined although some results suggest it may have lost the activity (Conrad, 1990).

The role of CD23 may well relate to the cell type on which it is expressed. It was shown that CD23 on macrophages, platelets and eosinophils mediates IgE-dependent cytotoxicity

and promotes phagocytosis of IgE-coated particles. The function of CD23 on B cells is more complex and may be involved in B cell growth regulation, IgE production regulation and B cell antigen presentation (Snapper et al., 1988; Kehry and Yamashita, 1989; Luo et al., 1991).

CHAPTER II

CHARACTERIZATION OF HYBRID RAT MAST CELLS

SUMMARY

Using polyethylene glycol, RPMC were fused with 6-thioguanine resistant, HAT (hypoxanthine, aminopterin, thymidine) sensitive RBL-CA10.7 or RBL-CK2 cells, yielding eleven hybrid rat mast cell lines (HRMC). The hybrid cells exhibited different size and cytoplasmic granularity and had more variable receptor patterns than the parent lines. Differential histochemical staining with alcian blue and safranine O dyes indicated the hybrids to be predominantly of the MMC type; however, a few cells of one of these uncloned hybridomas were found to be of the CTMC type. Attempts to isolate the CTMC hybridomas by cloning yielded one culture which was predominantly of the CTMC phenotype. This also contained cells expressing simultaneously both the CTMC and the MMC phenotype. After three more weeks in cell culture, all hybridomas expressed only the MMC histochemical phenotype, had a low histamine content and contained RMCPII only. These results indicate that somatic cell hybrids expressing the MMC and CTMC phenotype could be produced by the fusion of RBL and RPMC.

INTRODUCTION

In the rat there exist two phenotypically distinct subpopulation of mast cells, the connective tissue type (CTMC) represented by rat peritoneal mast cells (RPMC) and the mucosal type (MMC) represented by intestinal mucosal mast cells (IMMC), rat basophilic leukaemia (RBL) cells, and tissue-cultured mast cells (RCMC) (Enerbäck, 1966; Wingren and Enerbäck, 1983; Enerbäck, 1981; Befus et al.,1982; Seldin et al., 1985; Chan et al., 1988).

These two types of mast cells differ in anatomical distribution (Enerbäck, 1966; Kitamura et al.,1979; Ginsburg et al., 1982), histochemical staining properties (Enerbäck, 1966), total histamine content (Chan et al., 1988; Schrader et al., 1981; Levi-Schaffer et al., 1986), and serine proteases (Woodbury et al., 1981; Gibson and Miller, 1986). The histamine content of the cultured variety of MMC is significantly lower than that of RPMC. The difference in the histochemical staining properties of CTMC and MMC probably reflects the difference in their proteoglycan content with heparin accounting for the majority of the proteoglycans synthesized by CTMC (Lagunoff and Pritzl, 1976) while the over-sulphated chondroitin sulphate proteoglycans di-B and E predominate in MMC (Enerbäck et al., 1985; Stevens et al., 1986). Both subpopulation of rat mast cells have been shown

to possess high (FcϵRI) and low affinity (FcϵR_L) receptors for IgE. These receptors have previously been found on MMC-like RBL cells (Conrad and Froese, 1978; Froese et al., 1982), RCMC (Chan et al., 1988; Chan et al., 1990), and IMMC (Swieter et al., 1989), and on CTMC-like rat peritoneal cells (RPMC) (Froese, 1980).

When the present study was initiated, RBL cells and RCMC have served as in vitro models for rat MMC (Seldin et al., 1985; Chan et al., 1988). Both can be propagated in tissue culture in the absence of added factors or feeder layers. Unfortunately, at the time, no similar model was available for rat CTMC, although such a cell line has since been produced in the mouse by co-culture of splenocytes with fibroblasts producing a Ki-ras-containing murine sarcoma virus (Reynolds et al., 1988). Other CTMC-like mouse mast cell generated in vitro do require the continuous presence of fibroblasts (Levi-Schaffer et al., 1986) or fibroblast factors (Jarboe et al., 1989). The phenotypic changes in mouse mast cell subpopulations may be attributable (at least in part) to the effect of multiple cytokines in each specific microenvironment including stem cell factor (SCF) which is also named c-kit ligand (Tsai et al., 1991; Wershil et al., 1991).

An attempt was therefore made to produce a cell line which would have characteristics of CTMC by fusing RPMC and RBL cells. Among the desired characteristics were the high histamine content of RPMC and the presence of FcϵRI(α) and

$Fc\epsilon R_L$ free of bound IgE. It was also anticipated that the establishment of such hybrid mast cells and their availability might aid in the elucidation of the nature of mast cell differentiation.

MATERIALS AND METHODS

Buffers and solutions:

The following buffers are used in this part of study:

(1) Phosphate buffered saline (PBS): 0.14M NaCl, 0.01M PO_4, pH 7.4;

(2) PBS/BSA: PBS with 0.05% bovine serum albumin (BSA);

(3) SDS sample buffer: 0.0625M Tris-(hydroxymethyl) aminomethane (Tris), 4% sodium dodecyl sulphate (SDS), 10% glycerol, 0.005% bromophenol blue, pH 6.8;

(4) Trans-blot buffer (TBB): 0.025M Tris, 0.19M glycine, 20% methanol;

(5) Tris-NaCl buffered saline (TBS): 0.02M Tris, 0.5M NaCl, 0.024M Na_3N, pH 7.5;

Immunoglobulins and antibodies

Rat IgE was purified from the ascitic fluid of rats bearing the IR-162 immunocytoma as described earlier (Conrad et al., 1975; Chan et al., 1986). Rat IgE in the ascites

fluid of Lou/M/Wsl rats bearing the IR-162 immunocytoma (Bazin et al., 1974) was precipitated at 50% saturation with $(NH_4)_2SO_4$ and collected by centrifugation and washed once with 50% $(NH4)_2SO_4$. The precipitate was dissolved and dialysed against 0.2M Tris-HCl, 0.15M NaCl, 0.5% NaN_3, pH 8.0, overnight at 4^0C. IgE was purified by gel filtration using Ultragel AcA 34 (LKB, Broma, Sweden), pre-equilibrated in the same Tris-HCl buffer. The second peak, as measured by absorption at 280nm and identified as IgE, was pooled, concentrated and dialysed against PBS at 4^0C overnight. The IgE concentration was determined using an extinction coeffient of $E^{1\%}/280=13.6$ (Carson and Metzger, 1974) and stored at -70^0C until further use. This batch of IgE was used for the preparation of IgE-Sepharose conjugates. For experiments where IgE was required for selective isolation of FcϵRI(α), it was further purified by preparative isoelectric focusing at 600V for 48 hours using 1% carrier ampholytes of pH 4.0 to 6.5 (LKB, Bromma, Sweden) in a 0 to 40% sucrose gradient. The IgE is known to have an isoelectric point of pH 5.9 and the protein at this pH was then collected and dialysed against PBS before use or stored at -70^0C. Horse anti-rat IgE was a gift from Dr. K.A.Kelly and prepared as described previously (Kelly et al., 1979).

The $F(ab)_2$ fragments of rabbit anti-RMCPI and sheep anti-RMCPII were kindly provided by Dr. H.R.P. Miller. The former were monospecific for RMCPI, while the latter exhibited minor cross-reactivity with RMCPI.

A rabbit antiserum to the low affinity receptor for IgE ($Fc\epsilon R_L$) was previously prepared by Dr. P.A.Roth of this laboratory as described previously (Chan et al., 1990). Briefly, serum was collected from rabbits, six to eight months after immunization and boosting with purified $Fc\epsilon R_L$ in complete Freund's adjuvant. The immunoglobulin fraction was precipitated with ammonium sulphate at 50% saturation. The washed precipitate was dissolved and dialysed against PBS. It was subsequently absorbed two times with IgE-Sepharose and stored at -70^0C until use.

Cells and cell culture

RBL-CA10.7 and RBL-CK2 were clones derived from RBL_{wpg} (Chan et al., 1986). Both were identified to be free of mycoplasma contamination and were cultured in cMEM according to methods established in this laboratory (Conrad et al., 1976). The medium consisted of Eagle's minimum essential medium with Earle's salts (Difco Laboratories, Detroit, MI), 15% fetal calf serum (FCS), nonessential amino acids, vitamins, dextrose, antibiotics, and L-glutamine (GIBCO, Grand Island, NY). Aliquots of these lines were rendered 6-thioguanine resistant and highly sensitive to HAT medium by sequentially culturing the cells in cMEM containing increasing concentrations of 6-thioguanine (6TG, Sigma, St. Louis, MO)

(Uchida et al., 1985). Initially, RBL cells were cultured for two weeks in the presence of 6TG at concentrations of 0.5, 1, 2, 4, 7.5, 15, or 30μg/ml. Subsequently, it was established that 15μg/ml of 6TG were sufficient to yield complete sensitivity to HAT medium (cMEM containing 136μg/ml hypoxanthine, 0.19μg/ml aminopterin, and 3.88μg/ml thymidine). The adapted cell lines were designated RBL-CA10.7-TG and RBL-CK2-TG. Whenever 6TG resistant cells were recovered from frozen stock, they were grown for at least two weeks in 15μg/ml of 6TG.

Peritoneal mast cells from Wistar-ICI rats, which were maintained in the Animal Care Facility, University of Manitoba, were purified as described previously (Conrad et al., 1975) but under sterile conditions. Ether anaesthetized rats were bled by cardiac puncture and 15ml of ice-cold THM solution (140mM NaCl, 2.7mM KCl, 1.3mM $CaCl_2$, 1mM $MgCl_2$. 0.4mM KH_2PO_4, 5mM HEPES, 5mM Mes, 5.5mM Glucose and 1.2mM $NaHCO_3$, pH 7.3) containing 0.05% BSA and heparin (100 units/ml) was injected i.p. After one minute of gentle massage of the abdomen, peritoneal fluid was withdrawn and cells were collected and washed three times with cold THM/BSA. Mast cells were purified by Percoll gradients, rendered isotonic with a 10-fold concentration THM solution. The purity was found to be above 98%.

Hybridization of RPMC and RBL cells

The initial fusions of the 6-thioguanine resistant RBL cells with RPMC was performed by Dr. Bosco Chan of this laboratory according to established procedures by using polyethylene glycol (PEG, MW 4,000, Merck, Darmstadt, FRG) (Zola and Brooks, 1982). Briefly, equal numbers ($5x10^6$) of RBL cells and RPMC were mixed in cMEM without FCS. The cell pellet thus obtained by centrifugation was loosened by gentle shaking and a 0.5ml volume of PEG (50%) in GKN-solution (8g NaCl, 0.4g KCl, 1.77g $Na_2HPO_4.2H_2O$, 0.69g $NaH_2PO_4.H_2O$, 2g glucose, and 0.01g phenol red/liter) was gradually added in 30 seconds at 37^0C. After an additional 90 seconds of gentle shaking, one ml of cMEM without FCS was added over a period of one minute followed by the addition of another nine ml in five minutes at 37^0C. Cells were then pelleted by centrifugation and gently resuspended in eight ml of cMEM. One millilitre aliquots of the cell suspension thus obtained were transferred into 24-well tissue culture plates (Linbro, Mclean, VA) and were then incubated at 37^0C with 5% CO_2 overnight before changing to the HAT medium for selection of hybrids. For controls, RBL-CA10.7-TG, RBL-CK2-TG, and RPMC were treated under identical culture conditions.

After two weeks of culture in HAT medium, aminopterin was withdrawn and only cMEM was used after the third week. Once cell growth was established, the primary cultures were

immediately expanded and stored in liquid nitrogen for future use. Subsequently, cell lines were partially cloned at three cells per well and recloned at 0.5 cells per well.

Histochemical staining

The established method using alcian blue 8GX and safranine O (Sigma Chemical Co., St. Louis, MO) for differential staining of CTMC and MMC was followed (Seldin et al., 1985). Cells were centrifuged in a Shandon Cytospin Centrifuge (Shandon Southern Instrument, Sewickley, PA) and then fixed for one minute in Mota's solution prepared by dissolving one gram of lead subacetate (Aldrich, Milwaukee, WI) in a 50% ethanol solution containing 0.5% acetic acid. Slides were then stained with alcian blue at 5mg/ml in 0.3% acetic acid for five minutes and counter-stained with safranine O at one mg/ml in 1% acetic acid for five minutes. Then slides are rinsed in distilled water and air-dried. Permanent slides are prepared by using cover glass and Permount solution (Fisher, Canada).

The staining with berberine sulfate (Sigma Chemical Co., St.Louis, MO) was carried out according to an established procedure (Enerbäck, 1974). Slides are prepared in same way as above. Cells are stained in 0.02% berberine sulfate solution for 20 minutes at pH of 4.0 titrated with 1% citric acid. Then slides are rinsed in distilled water with a pH of

4.0 and air-dried. Permanent slides are then prepared as above.

Western blot analysis of rat mast cell proteases (RMCP)

Rat mast cell proteases, enzyme markers for rat mast cells, were analyzed by means of immunoblotting. Standard RMCPI was prepared from purified peritoneal mast cells (RPMC) according to the published procedures (Schick et al., 1984). Briefly, RPMC were disrupted in 0.01M MES containing 0.5M NaCl, pH 6.0, by thawing and freezing. The supernatant was then pooled and loaded onto a Dowex 1-X2 column (Bio-Rad Laboratories, Richmond, CA) pre-equilibrated with MES buffer. The column was washed with MES buffer containing 0.6M NaCl. RMCPI was eluted stepwise with the washing buffer containing 1.0M NaCl. The eluates were pooled, concentrated and dialysed against 0.0625M Tris-HCl, pH 6.5, and stored at -70^0C for further use.

Iodination of anti-RMCPI and II antibodies were performed on the day before the experiment with ^{125}I by using insolubilized chloramine-T conjugated as described by Markwell (Markwell, 1982). To 50μg of antibody in 0.5ml PBS, 0.25MBq of ^{125}I was added. Two Chloramine-T polystyrene beads (Iodobeads; Pierce, Rockford, IL) were added to initiate the iodination reaction which was then terminated after 15 minutes by immediate removal of the beads and subsequent Sephadex G-25

column chromatography to remove the free ^{125}I. Labelled protein was then stored at -20^{0}C until use.

Extracts of solubilized cells ($2x10^5$) were electrophoresed by SDS-PAGE under reducing conditions using 12.5% gels (Laemmli, 1970). Proteins were then transferred onto a nitrocellulose membranes with a Bio-Rad Transblot cell according to the manufacturer's instructions (Bio-Rad Laboratories, Richmond, CA). Blocking of unreacted sites was performed by incubation of the membrane for six hours with Tris-buffered saline (TBS) containing 10% FCS and 0.05% Tween-20. The blocked membrane was then incubated overnight with ^{125}I-labelled $F(ab)_2$ fragments of sheep anti-RMCPII or rabbit anti-RMCPI antibody, in TBS containing 5% FCS and 0.05% Tween 20. After rinsing with TBS, the membrane was dried and autoradiography was performed using a hypersensitized Kodak X-OMAT AR film (Eastman Kodak, Rochester, NY).

Determination of total histamine content of HRMC:

The total histamine content of the various HRMC lines and clones was assayed by a modification of spectrofluorometric method (May et al., 1970). A volume of 100μl of perchloric acid (14%) was added to $1x10^6$ cells in 1.10ml PBS/BSA followed by heating in a boiling water bath for two minutes. After 30 min at 4^{0}C, aggregates were pelleted by centrifugation and one ml of the supernatant was extracted by alkaline n-butanol,

2.5ml n-butanol, 0.2ml NaOH (2.5N) and 0.5g NaCl for eight minutes. Two milliliters of the butanol extract was then immediately subjected to an acid extraction of 0.6ml HCl (0.1N) in 2.5ml heptane for eight minutes. The o-phthaldialdehyde (OPT) (Aldrich, Milwaukee, WI) reaction solution was prepared by dissovling OPT in methanol (1mg/ml) and mixing this freshly prepared OPT solution with 0.33N NaOH at 1:2 volume ratio. Of this OPT solution, 0.3ml was immediately added to 0.5ml of the acid extract. Reaction was continued in the dark for 40 minutes at 4^0C, then terminated by the addition of 0.2ml of 1M H_3PO_4, and the fluorescence was measured at 450nm with excitation at 360nm (Aminco-Bowman Spectrophotofluorometer, American Instrument, Silver Spring, MD). Total histamine content of each HRMC line was measured in quadruplicates and compared with standards of known histamine concentration which had been subjected to an identical extraction procedure.

Analysis of size and granularity of HRMC

This analysis was carried out by Dr.E.S. Rector of this department. Cells were suspended in cMEM, adjusted to $1x10^6$ cells/ml and analyzed using an Epics 742 fluorescence-activated cell sorter (Coulter Electronic Inc., Hialeah, FL) equipped with an argon laser emitting 500mW at 488nm. Forward angle light scatter and peak 90^0 light scatter measurements

were used to estimate the relative size and cytoplasmic granularity, respectively. The histograms to be shown utilize linear scales of 256 channel resolution and represent the analysis of 10,000 cells.

Karyotype analysis

This was kindly performed by Dr. David Cox, (Alberta Children's Hospital, Calgary, Alberta, Canada) using standard procedures (Worton and Duff, 1979).

RESULTS

Establishment of hybrid rat mast cells (HRMC):

Evidence of cell growth was observed after two to three weeks in all eight primary cultures (HRMC1-8) from the fusion of RBL-CK2-TG with RPMC, while only three (HRMC9-11) of the eight culture wells derived from RBL-CA10.7-TG exhibited cell growth. None of the control cultures, RBL-CK2-TG, RBL-CA10.7-TG, or RPMC at $5x10^5$/ml exhibited any cell growth throughout the entire period of hybrid selection in the HAT medium. The 11 cell lines were surface-iodinated and Fcϵ receptor profiles were established following receptor isolation by IgE-Sepharose and compared to those of RBL cell lines. The majority of these lines exhibited a normal receptor pattern showing the presence of both low (FcϵR_L) and high affinity (FcϵRIα) receptors for IgE in positions corresponding to those of parent RBL-CK2-TG and RBL-CA10.7-TG cells (Fig.1). Only HRMC3, 9, 10, and 11 yielded somewhat different receptor patterns. A more detailed analysis of the Fcϵ receptors of the primary cell lines as well as some of their clones and subclones is presented in Chapter IV.

Figure 1. Isolation of receptors for IgE by IgE-Sepharose from HRMC lines and analysis by SDS-PAGE under nonreducing conditions.

TfR–
FcεR$_L$–
FcεRI(α)–
RBL–CK2–TG
HRMC 1
HRMC 2
HRMC 3
HRMC 4
HRMC 5
HRMC 6
HRMC 7
HRMC 8
RBL–CA10.7–TG
HRMC 9
HRMC 10
HRMC 11

Three HRMC lines were selected for further study. Lines HRMC5 and HRMC8 were chosen since they exhibited a normal receptor pattern characteristic of RBL cells and, upon inspection by light microscopy, appeared to be significantly larger than all the other lines. On the other hand, line HRMC9 was chosen because of its rather odd receptor pattern and the fact that its cells looked smaller than cells of other lines.

Comparison of size and granularity of HRMC lines:

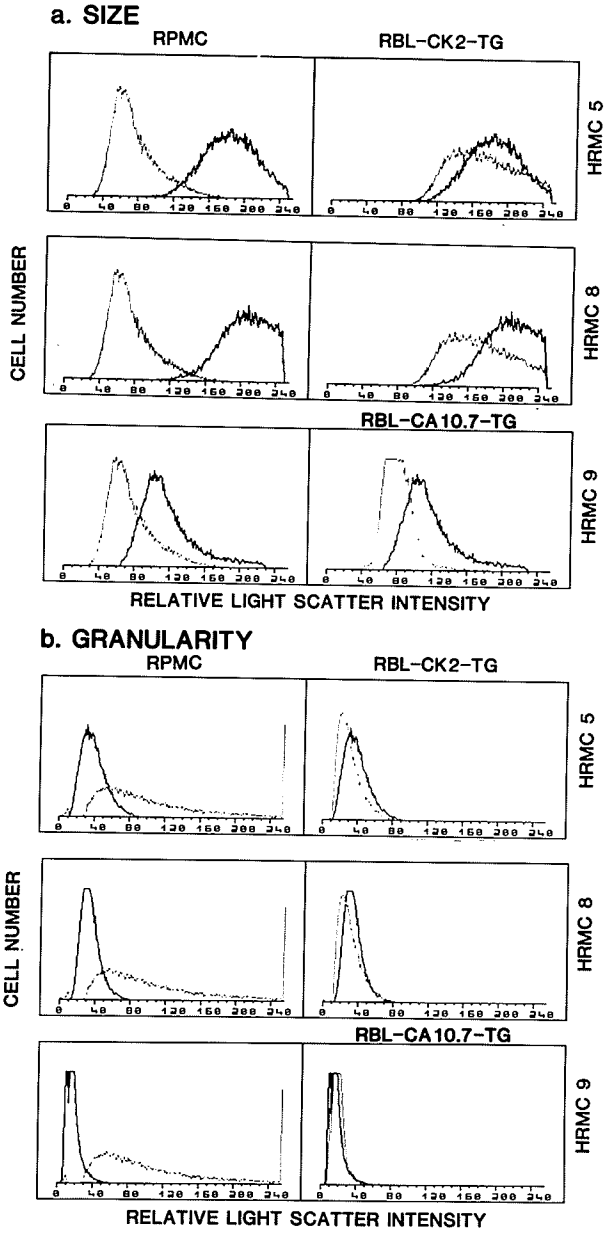
Cells of all three selected HRMC lines were found to be larger than their respective fusion partners as judged by their forward angle light scatter (Fig. 2a).

Both HRMC5 and 8 were found to have very similar cytoplasmic granularity which is slightly higher than that of RBL-CK2-TG but is considerably lower than that of RPMC (Fig. 2b). In contrast, HRMC9 is larger than either RPMC or RBL-CA10.7-TG, but in granularity it ranks below either fusion partner (Fig. 2b).

Histochemical properties of HRMC

It is known that CTMC and MMC differ in histochemical

Figure 2. Analysis of relative size (a) and cytoplasmic granularity (b) of selected HRMC lines using a fluorescence-activated cell sorter. The HRMC (dark lines) are compared to either RPMC or RBL-CK2-TG (light lines) in the case of HRMC5 and HRMC8, RBL-CA10.7 in the case of HRMC9.

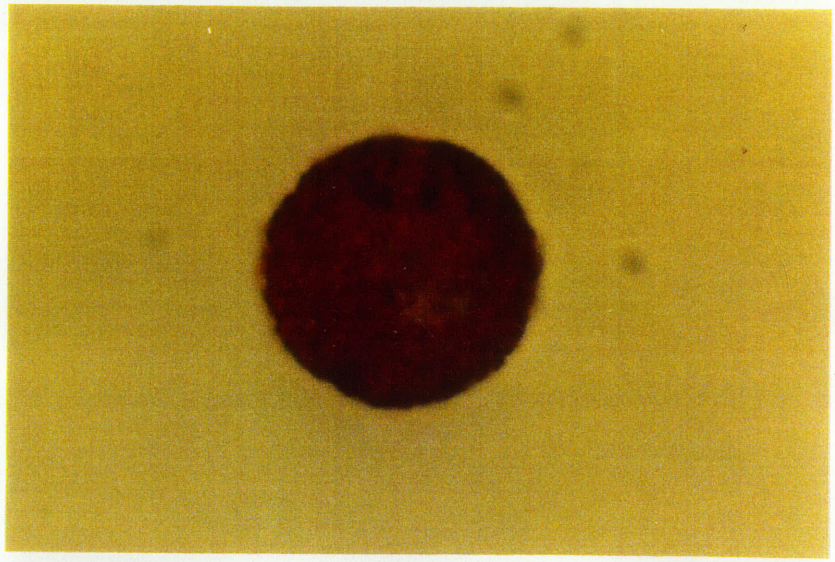

a. SIZE
RPMC
RBL-CK2-TG
HRMC 5
HRMC 8
CELL NUMBER
RBL-CA10.7-TG
HRMC 9
RELATIVE LIGHT SCATTER INTENSITY
b. GRANULARITY
RPMC
RBL-CK2-TG
HRMC 5
HRMC 8
CELL NUMBER
RBL-CA10.7-TG
HRMC 9
RELATIVE LIGHT SCATTER INTENSITY

staining. Thus, CTMC can be stained with alcian blue and counterstained with safranine O as well as stained with berberine sulphate; while MMC are usually alcian blue positive but are not stained by safranine O or berberine sulphate. This difference in histochemical staining is a reflection of the different proteoglycan content of two cell types (Enerbäck, 1966c; Seldin et al., 1985a).

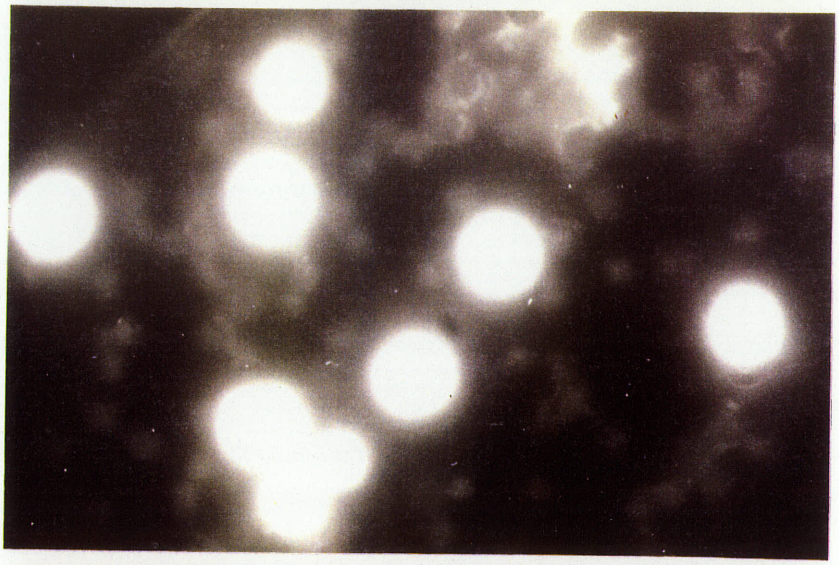
Typical histochemical staining of peritoneal mast cells and RBL cells is shown in Figure 3. When the three selected HRMC lines were stained with alcian blue and counter-stained with safranine O, HRMC8 and 9 could not be counter-stained, indicating that they are of the MMC phenotype (Fig. 4b, 4C). In the case of HRMC5 the vast majority of cells also had staining characteristics of MMC, however, there appeared to be a few isolated cells which were counter-stained with safranine O (Fig. 4a). Therefore, an attempt was made to isolate the CTMC-like cells by cloning. However, in order to increase the chances of actually finding these cells, only partial "cloning" of three cells per well was undertaken. Only one partial "clone", ie., HRMC5.1, consisted predominantly of cells counter-stained with safranine O (Fig. 5a), five contained some such cells (Fig. 6a, 6b) while no cells in the remaining 18 partial clones could be counter-stained (Fig. 5c). In Figure 5b and 6c, a single CTMC-like cell of HRMC5.1 is shown in larger magnification. Interestingly, there were also some cells which contained both blue and red granules

Figure 3. Differential staining with alcian blue and safranin O of (a) RPMC, 500x, (c) RBL-CK2-TG, 400x, (b) positive staining of RPMC with berberine sulfate, 200x.

a



b



c

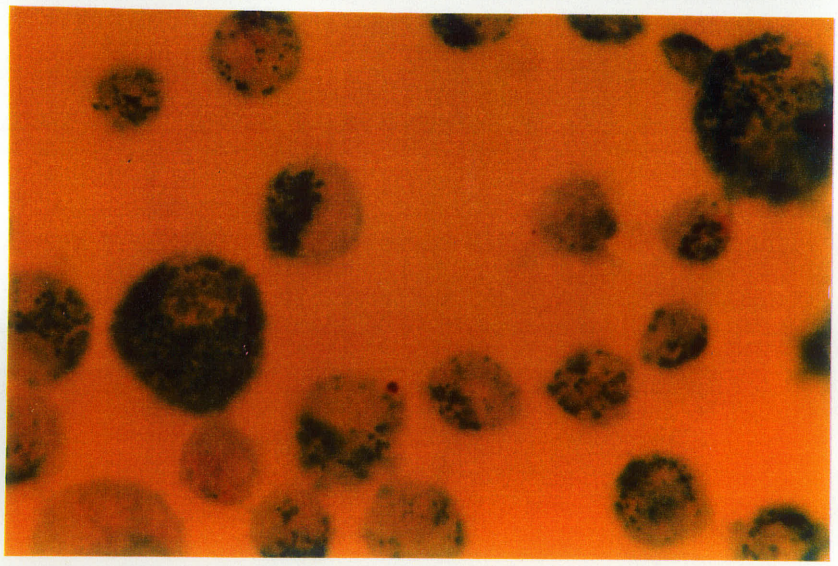
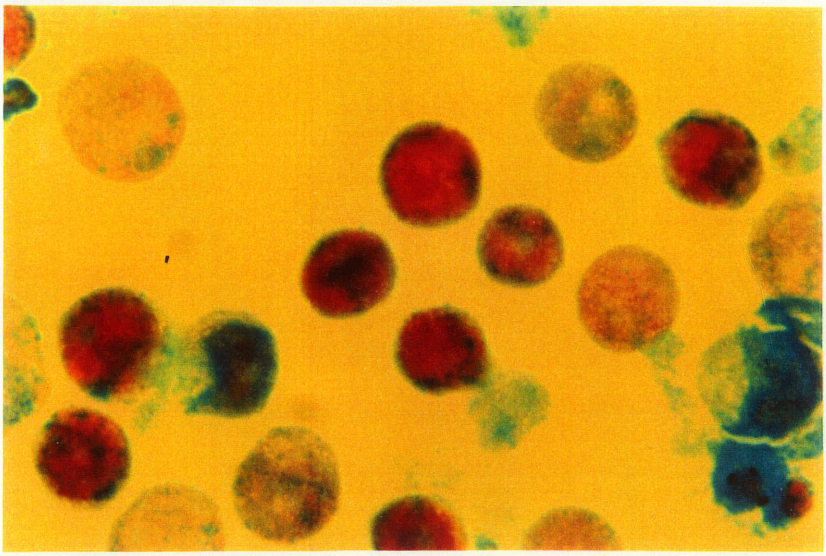


Figure 4. Differential staining with alcian blue and safranin O of (a) HRMC5, 400x, (b) HRMC8, 400x, (c) HRMC9, 400x.

a



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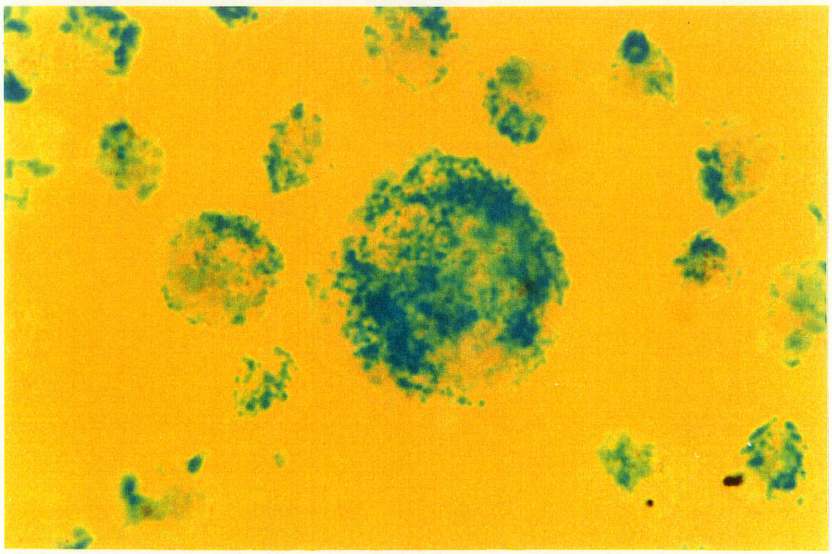


c

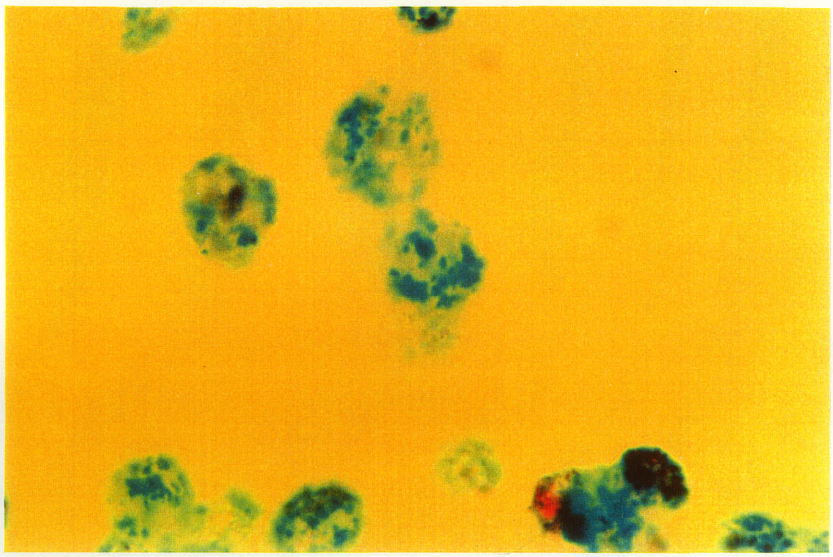
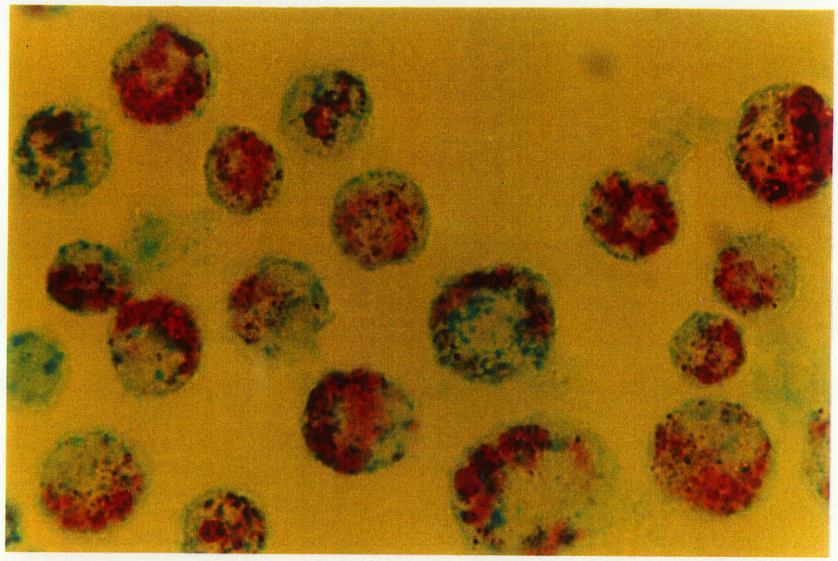


Figure 5. Differential staining with alcian blue and safranin O of (a) HRMC5.1, 400x, (b) HRMC5.1, 1000x, (c) HRMC5.2, 1000x.

a



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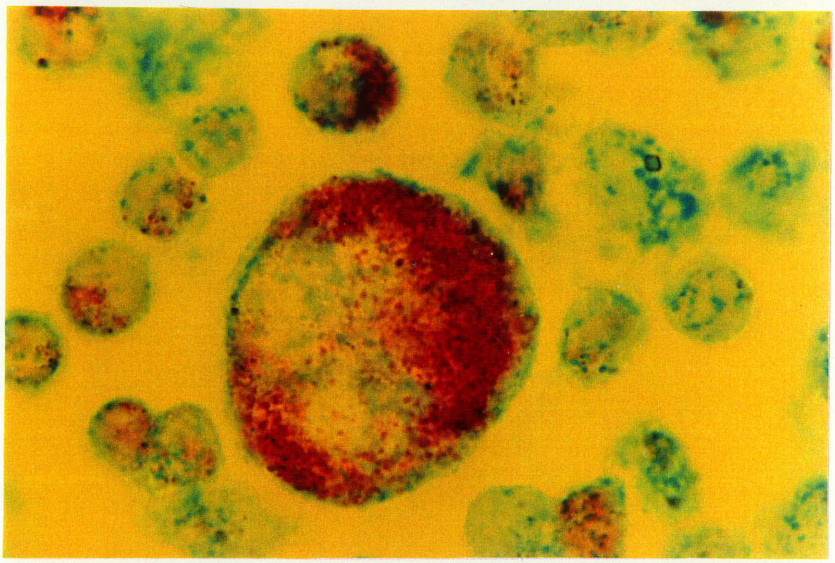

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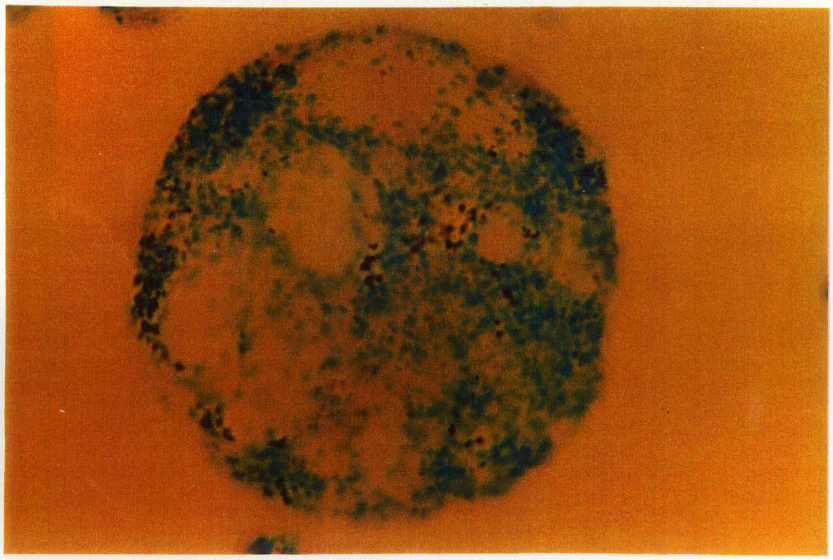
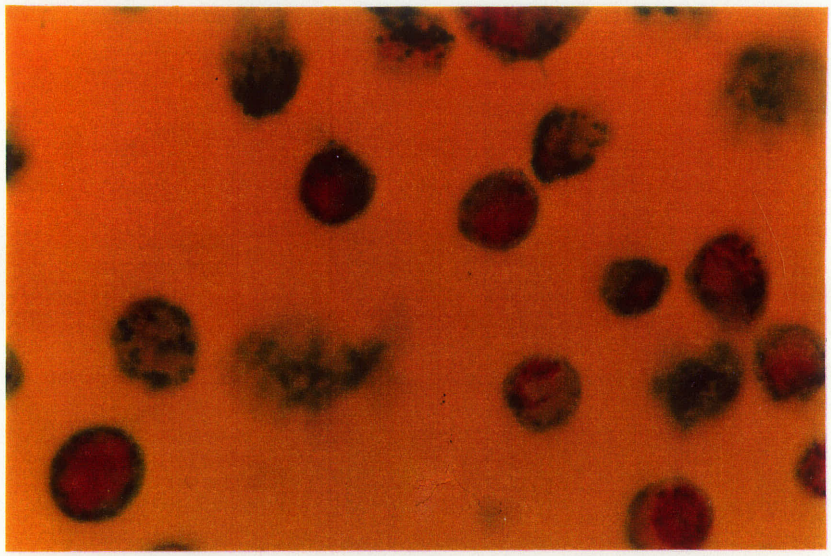
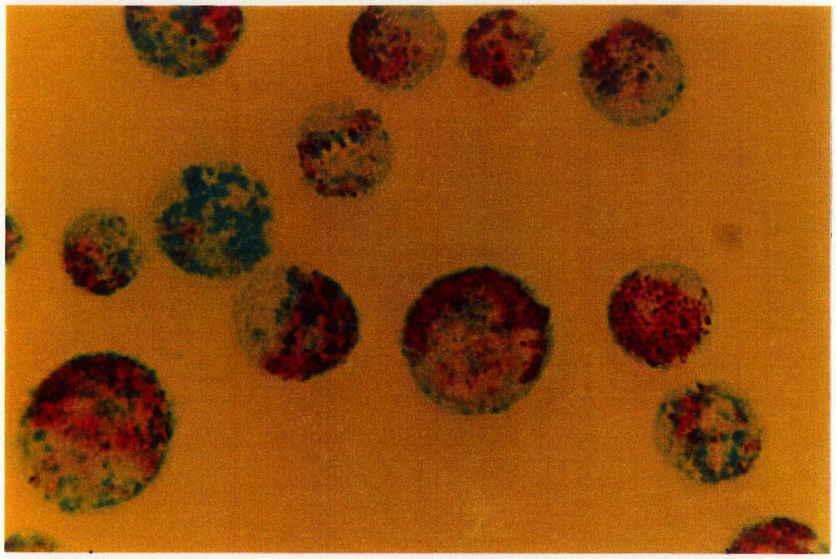


Figure 6. Differential staining with alcian blue and safranin O of (a) HRMC5.1, 100x, (b) HRMC5.1, 400x, (c) HRMC5.1, 1000x.

a



b



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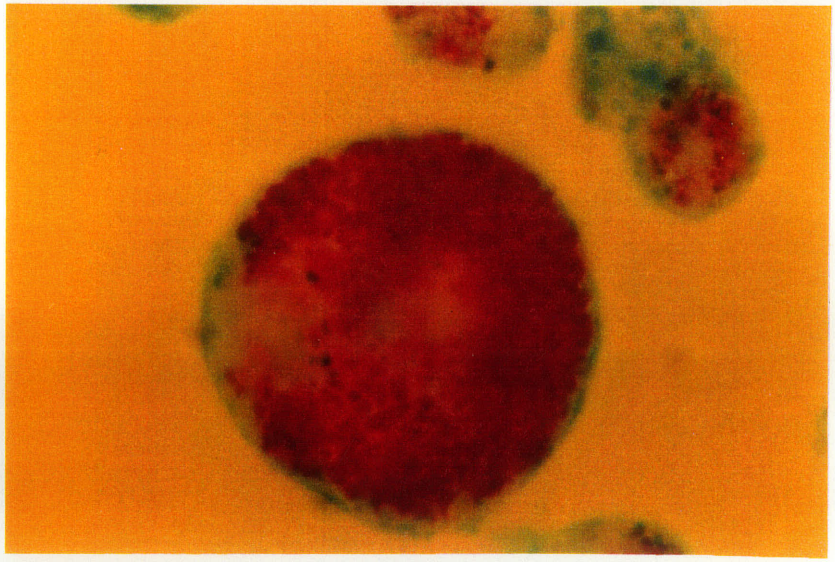


(Fig. 7, 8a, 8b). However, after about three weeks in culture, no cells present in HRMC5.1 could any longer be counter-stained with safranine O (Fig. 8c, 9).

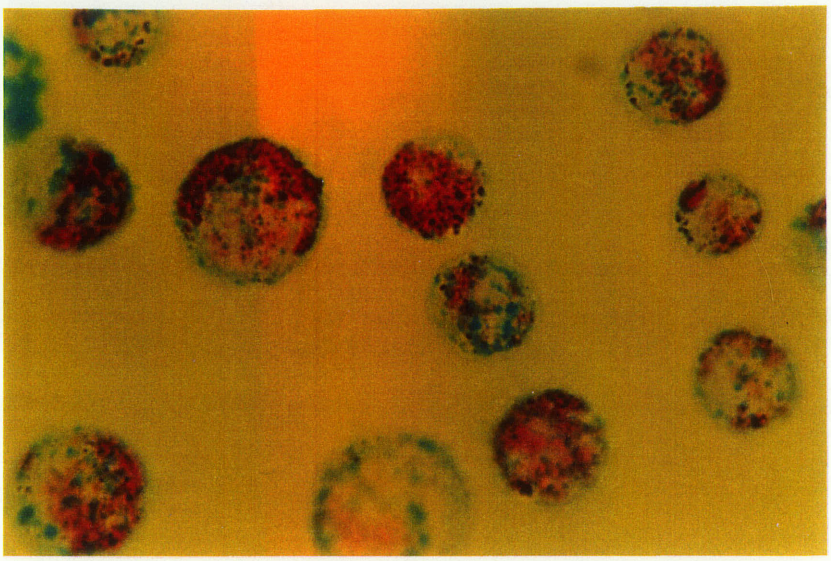
Line HRMC 5.1 was subsequently cloned again at a density of 0.5 cells per well. However, when the clones were analyzed, all cells of all 13 clones had the appearance of the cell shown in Figure 9, pointing solely to the MMC phenotype. Some cell nuclei were also nonspecifically stained pinkish in Figure 9a.

Characterization of rat mast cell proteases (RMCP):

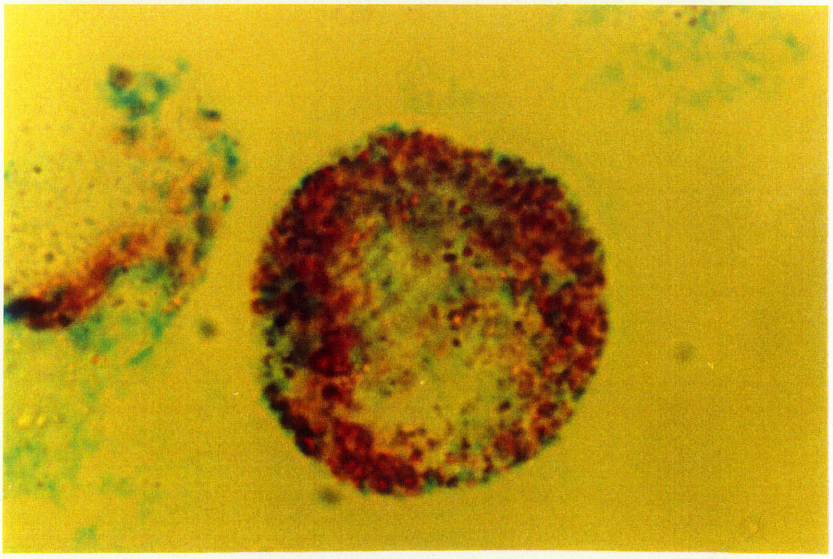
Characterization of mast cell proteases of HRMC5, 8, 9 and "clone" HRMC 5.1 and subclones, by means of Western blotting, were carried out after another three-four weeks period of culture, a time at which HRMC5.1 was no longer safranine O positive. All cell lines and clones as well as RBL-CA10.7 were found to contain RMCPII but no RMCPI (Fig. 10, 11, 12) . Attempts to demonstrate the presence of RMCPI using an antiserum monospecific for this protease were completely negative although a sample of RMCPI isolated from RPMC could easily be detected (Fig. 10, lane b). This confirmed histochemical staining data on HRMC5.1 using alcian blue and safranine O. No evidence for the CTMC phenotype could be found at this point in the development of the hybrid mast

Figure 7. Differential staining with alcian blue and safranin O of (a) HRMC5.1, 400x, (b) HRMC5.1, 1000x, (c) HRMC5.1, 1000x. Cells with both "blue" and "red" granules.

a



b



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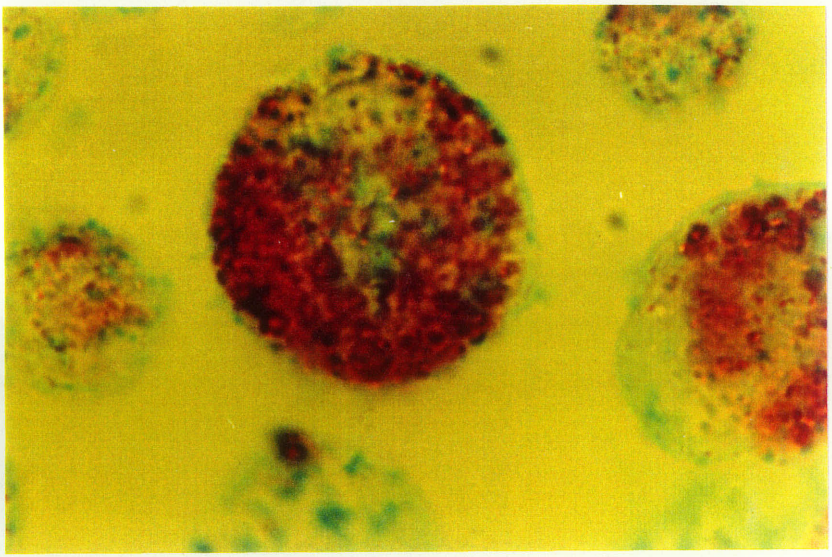
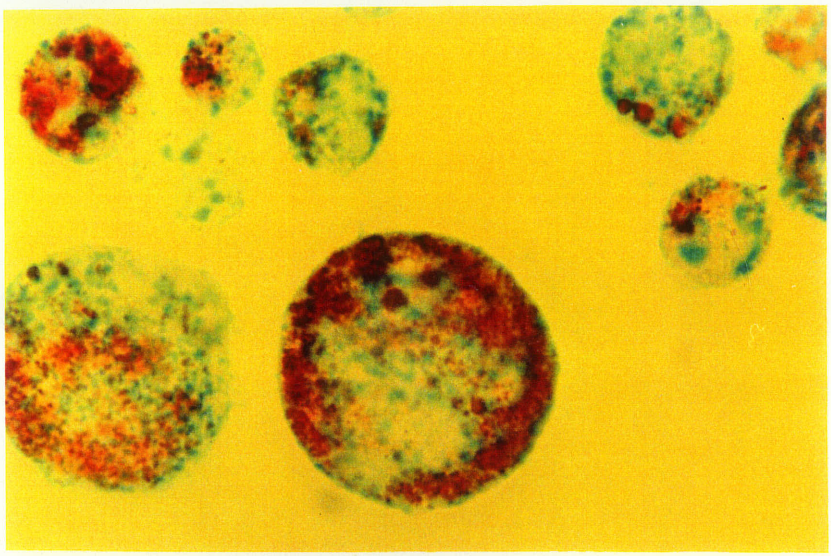
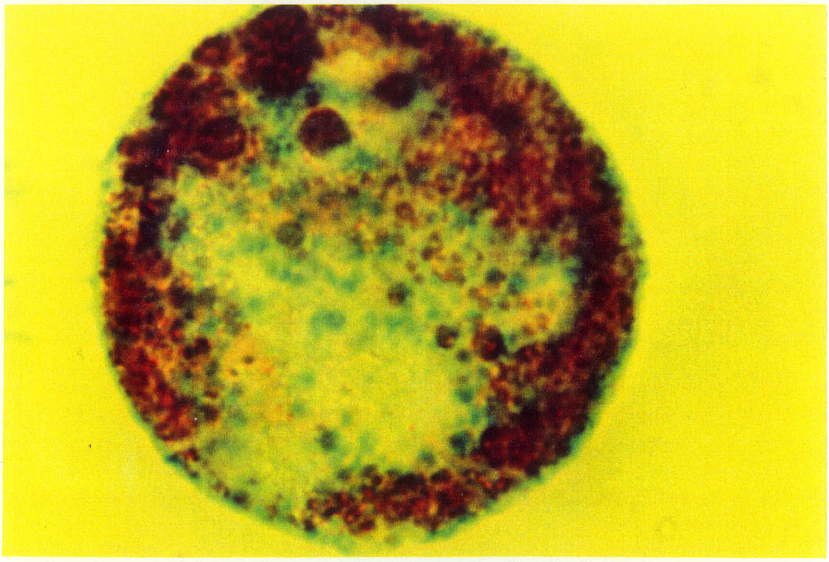


Figure 8. Differential staining with alcian blue and safranin O of (a) HRMC5.1, 500x, (b) HRMC5.1, 1000x : Cells with both "blue" and "red" granules. (3) HRMC5.1, 1000x, after another three weeks in cell culture.

a



b



c

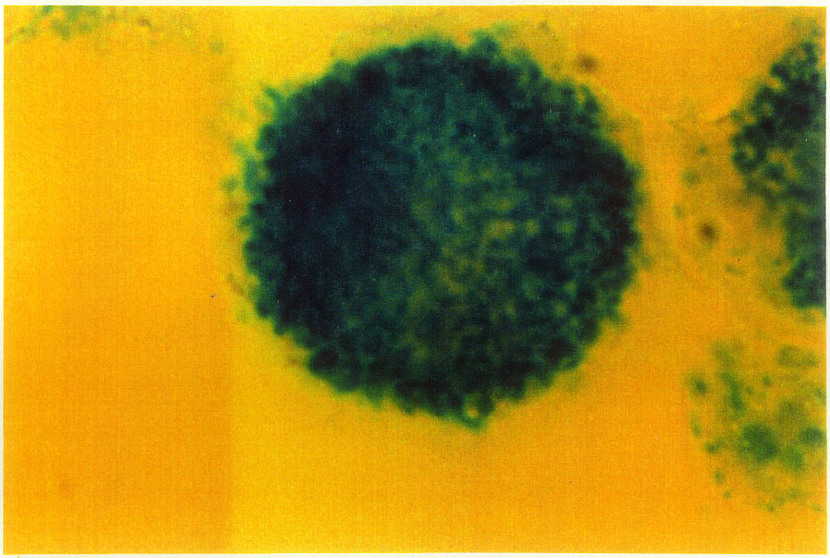
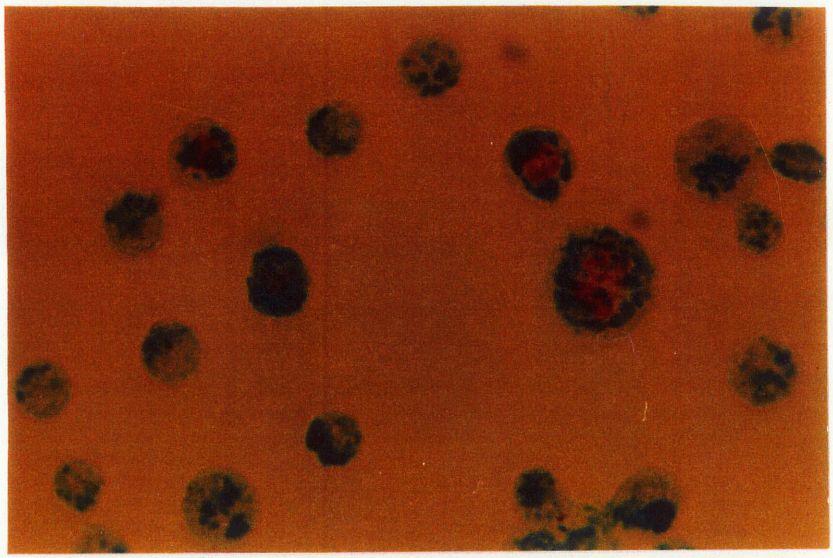


Figure 9. Differential staining with alcian blue and safranin O of (a) HRMC5.1, 100x, (b) HRMC5.1, 500x : after another three weeks in cell culture.

a



b

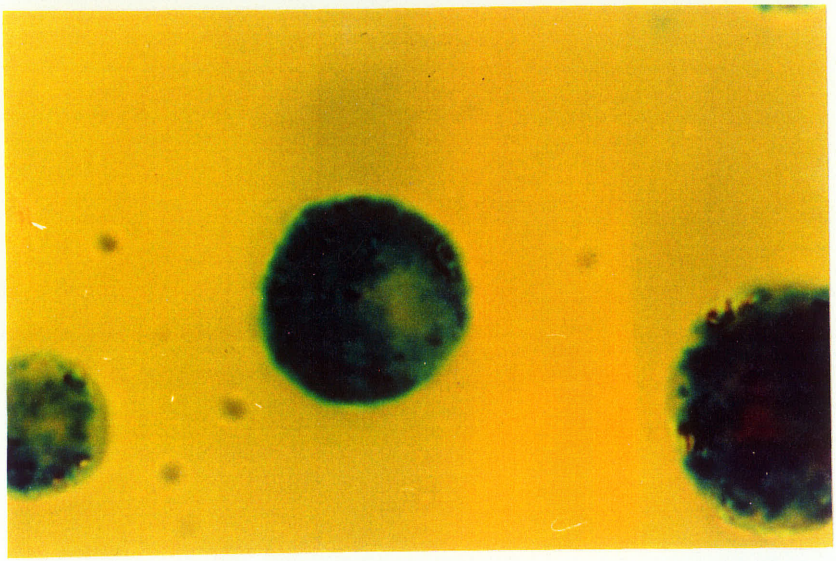


Figure 10. Western blot analysis of RMCPI of (a) RBL-CK2-TG, (b) purified RMCPI, (c) HRMC5, (d) HRMC8, (e) HRMC9, (f) HRMC5.1, (g) HRMC5.2, (h) HRMC5.3, (i) HRMC5.4, (j) HRMC5.5, (k) HRMC5.6, (l) HRMC5.7, (m) RCMC2.

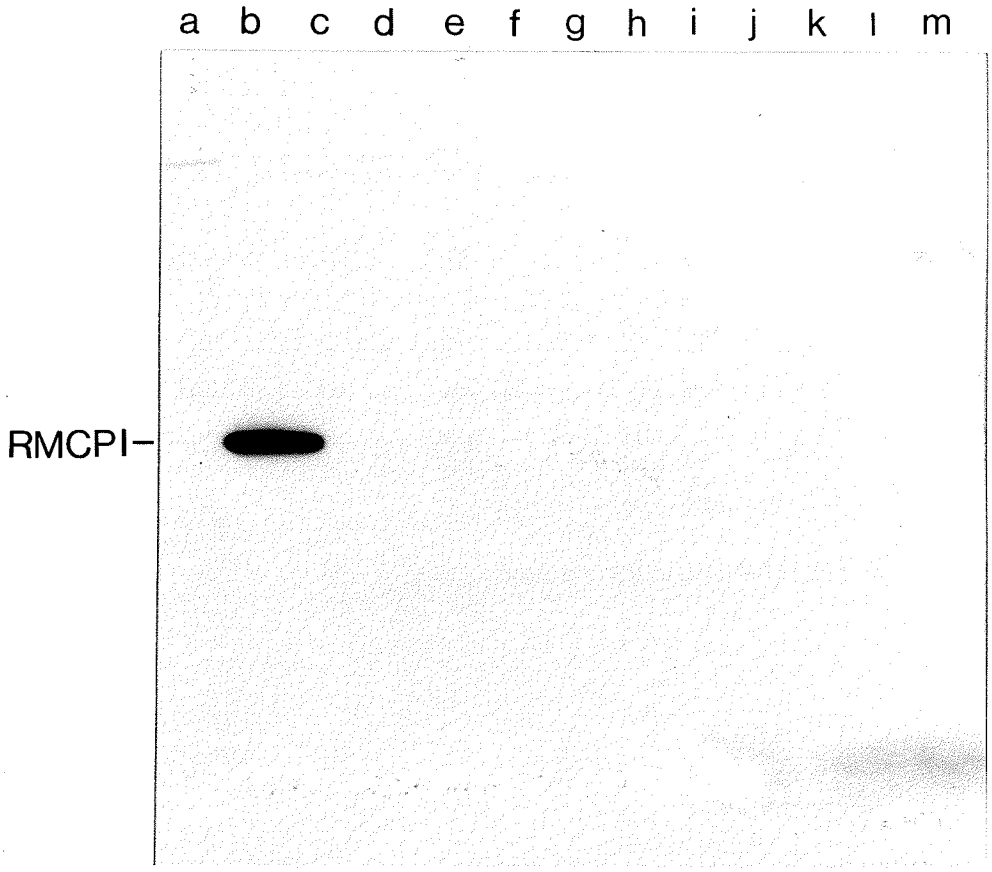

a b c d e f g h i j k l m
RMCPI

Figure 11. Western blot analysis of RMCPII of (a) RBL-CK2-TG, (b) purified RMCPI, (c) HRMC5, (d) HRMC8, (e) HRMC9, (f) HRMC5.1, (g) HRMC5.2, (h) HRMC5.3, (i) HRMC5.4, (j) HRMC5.5, (k) HRMC5.6, (l) HRMC5.7, (m) RCMC2.

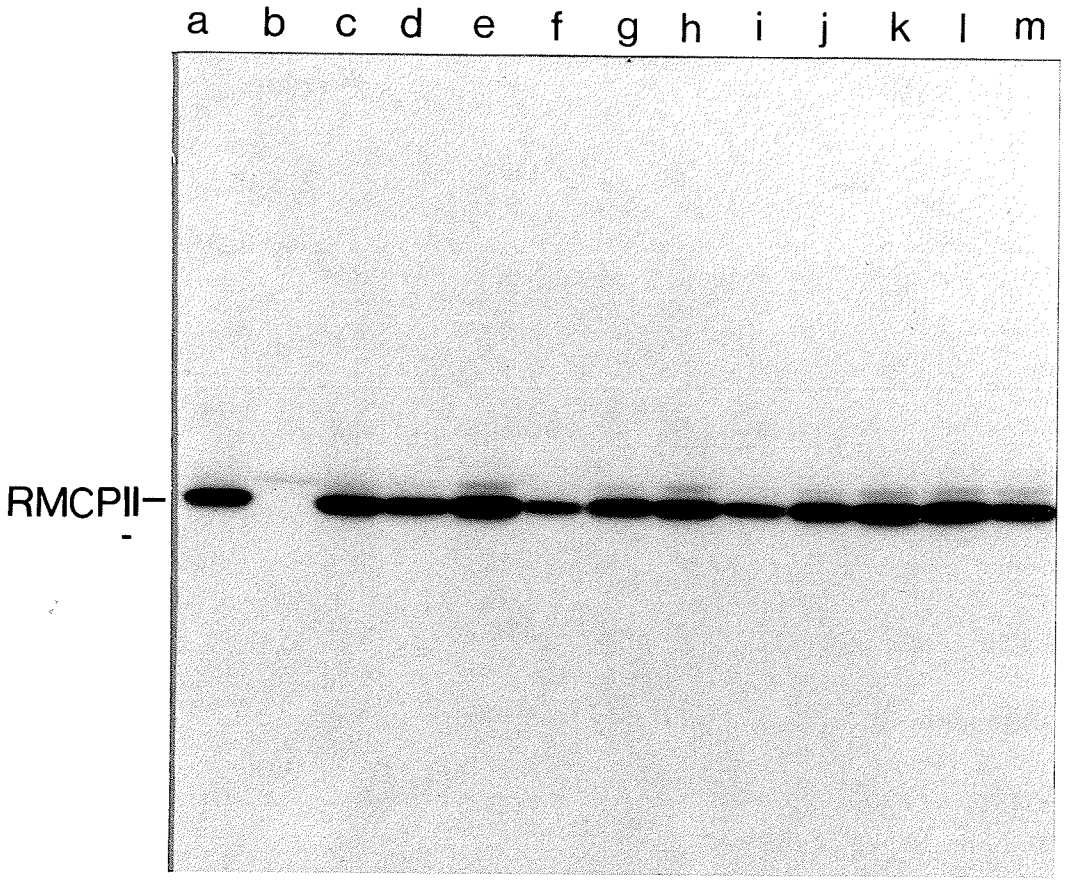
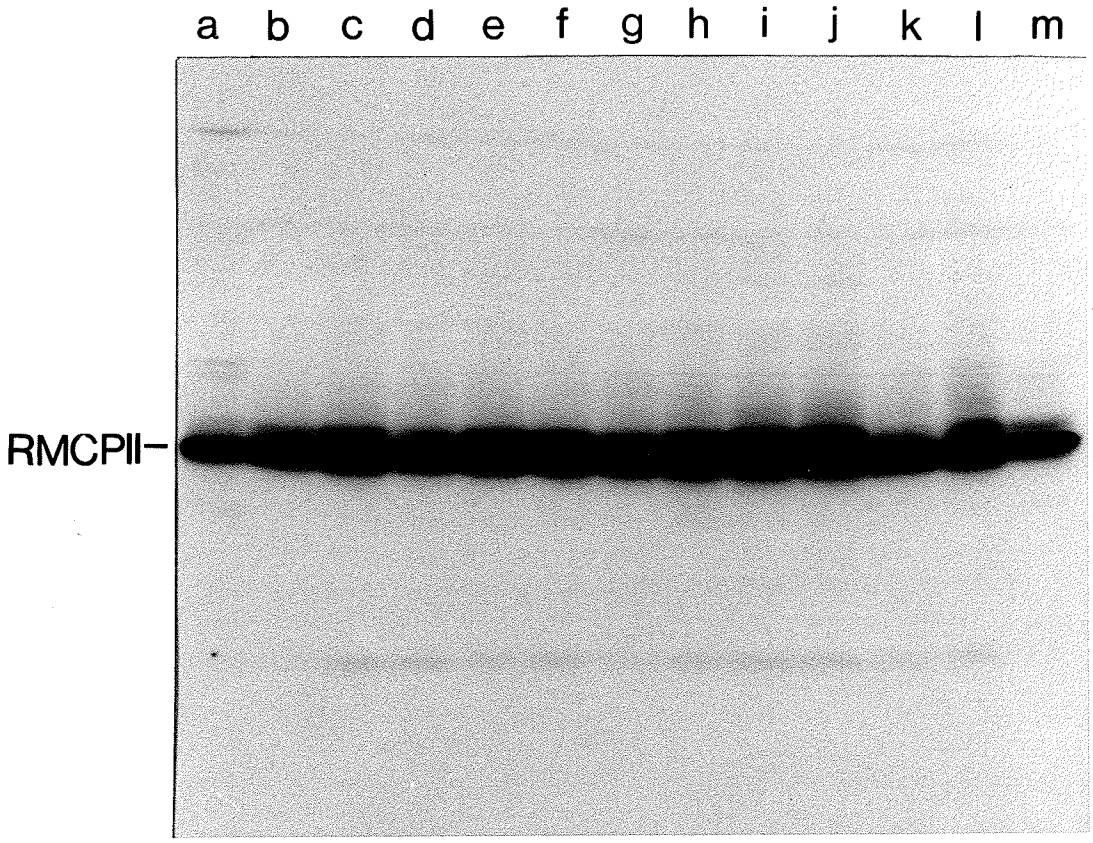

a b c d e f g h i j k l m
RMCPII

Figure 12. Western blot analysis of RMCPII of (a) RBL-CK2-TG, (b) HRMC5, (c) HRMC5.1, (d) HRMC5.1.1, (e) HRMC5.1.2, (f) HRMC5.1.4, (g) HRMC5.1.5, (h) HRMC5.1.6, (i) HRMC5.1.7, (j) HRMC5.1.8, (k) HRMC5.1.9, (l) HRMC5.1.10, (m) HRMC5.1.12.


a b c d e f g h i j k l m
RMCPII

cells.

Histamine content of HRMC:

In this analysis the three selected HRMC lines and partially cloned HRMC5 cells were compared to RBL-CK2-TG and RCMC2, the latter being cultured rat mast cell line developed in this laboratory (Chan et al., 1988; Chan et al.,1990). As can be seen in Figure 13, the histamine content of HRMC5 was found to be intermediate between the values for RBL-CK2-TG and RCMC2, while that of HRMC8 was practically identical to RBL-CK2-TG. Interestingly, "clones" of HRMC5 exhibited a considerable range of histamine content, many of them containing more histamine than RBL-CK2-TG, the parent of HRMC5.

Karyotype analysis of selected HRMC lines:

In a previous study it was demonstrated that RBL-CA10.7 cells carry a metacentric marker chromosome (M8) which is not present in rat tissue cultured mast cells (RCMC) (Chan et al., 1990). Interestingly, the same marker chromosome was also detected in RBL-CK2-TG and HRMC5.1, HRMC8, and HRMC9 (Fig. 14, 15; HRMC9 data not shown). The modal chromosome number for

Figure 13. Total histamine content of RBL-CK2-TG, selected HRMC lines and clones. The results show the mean of quadruplicate determination and the standard deviation.

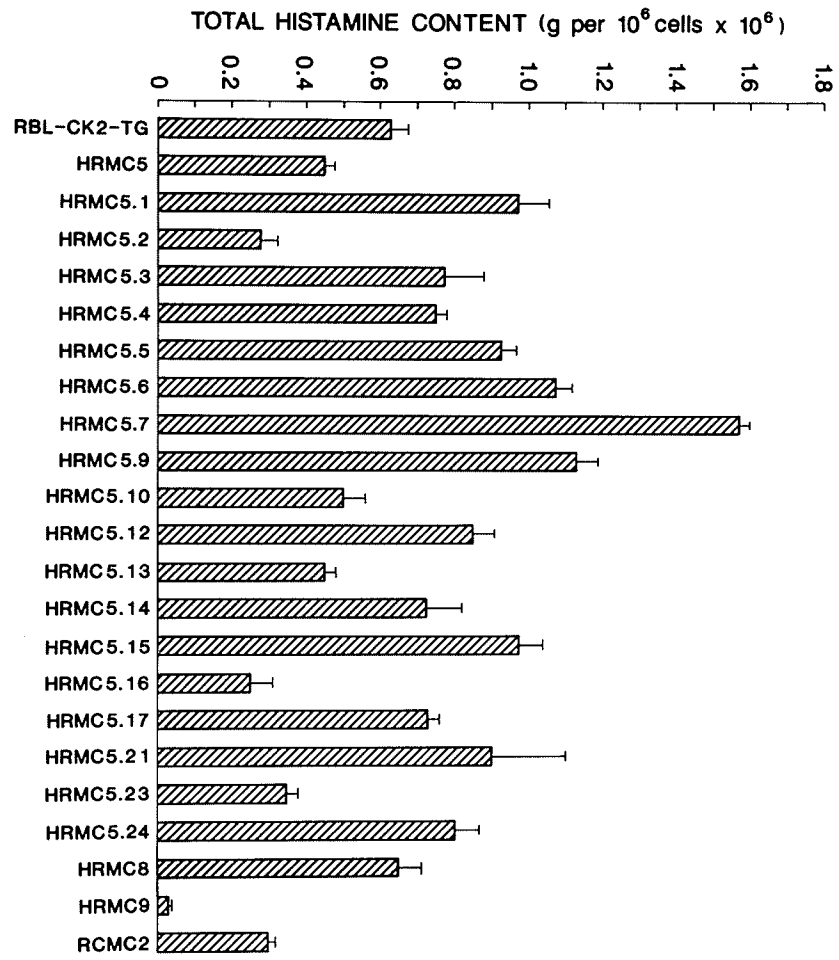
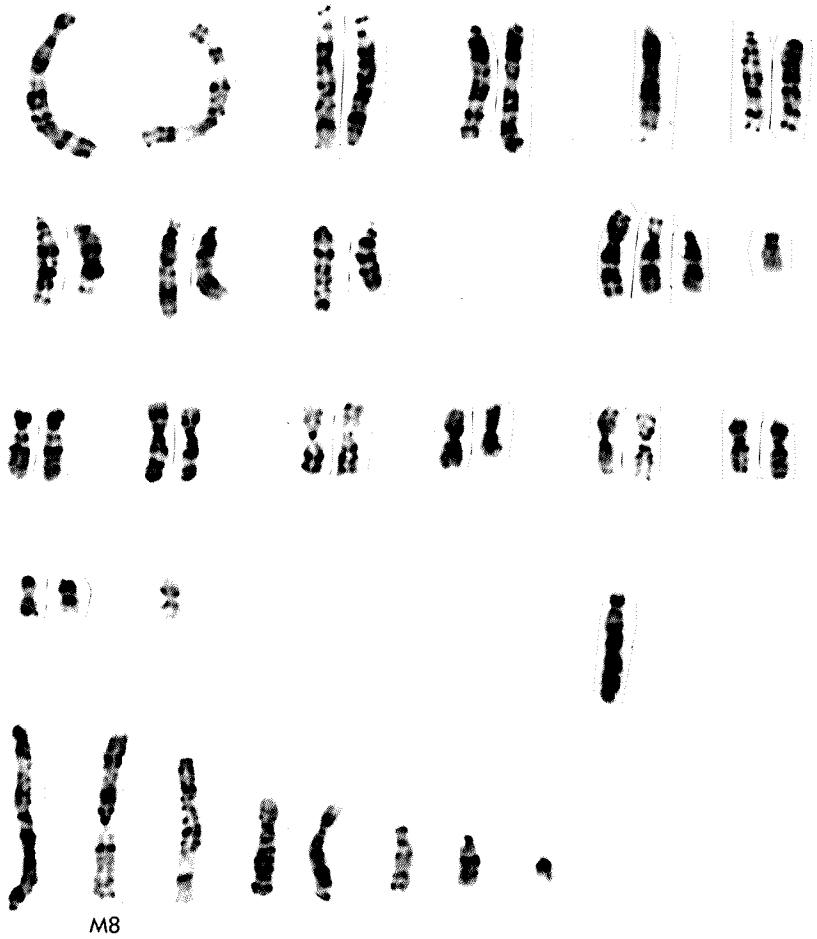

TOTAL HISTAMINE CONTENT (g per 10^6 cells x 10^6)
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
RBL-CK2-TG
HRMC5
HRMC5.1
HRMC5.2
HRMC5.3
HRMC5.4
HRMC5.5
HRMC5.6
HRMC5.7
HRMC5.9
HRMC5.10
HRMC5.12
HRMC5.13
HRMC5.14
HRMC5.15
HRMC5.16
HRMC5.17
HRMC5.21
HRMC5.23
HRMC5.24
HRMC8
HRMC9
RCMC2

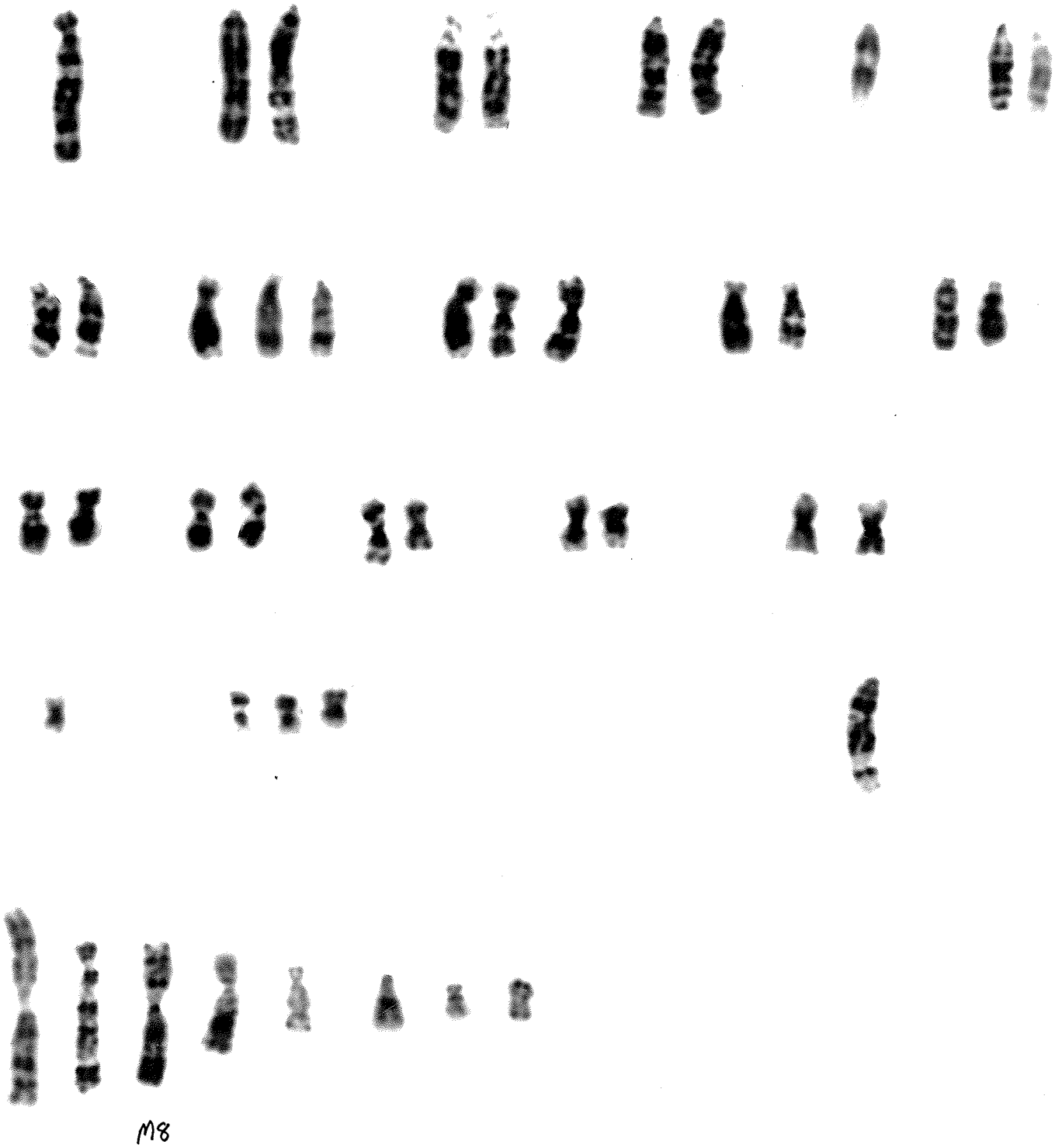
Figure 14. Karyotype analysis of RBL-CK2-TG and HRMC5.1.

a. HRMC5.1



b. RBL-CK2-TG

HRMC 8



these cells was found to be 45, 44, 45, and 48, respectively.

DISCUSSION

Mycoplasma-free cell lines RBL-CK2 and RBL-CA10.7 (Chan et al., 1986) were chosen for the fusions described in this study. Initially, 8-azaguanine-resistant RBL-CK2 cells were produced, but they were found not to be sensitive to HAT medium. However, when the cells were rendered resistant to 6-thioguanine, they no longer survived in HAT medium. Two to three weeks after fusion, when cell growth became apparent in HAT medium, all control wells containing either RBL-CK2-TG, RBL-CA10.7-TG, or RPMC did not show any sign of growth. However, previous work from this laboratory has shown that RPMC can give rise to RCMC but only after a period of eight to ten weeks in culture (Chan et al., 1988; Chan et al., 1990). To exclude the possibility that the cells described here were of the RCMC type, HRMC5.1, 8, and 9 were analyzed and found to express the metacentric M8 marker chromosome as was RBL-CK2-TG. Previously, this marker was found in RBL-CA10.7 cells but not in RCMC (Chan et al., 1990). The modal chromosome number of HRMC 5.1, 8, and 9 was found to be 44, 45, and 48, respectively, compared to 45 for RBL-CK2-TG and 42 (2N) for RPMC. Recently, hybrid mouse mast cells were found to have a tetraploid chromosome number (Diamantis et al., 1989);

however, it should be pointed out that when the karyotyping of the HRMC lines was performed, the cells had been in continuous culture for about one year and thus may have lost most of their extra chromosomes.

Histochemical staining of HRMC5.1 in early culture revealed cells of both the CTMC and MMC phenotype and even some which had a mixed phenotype, clearly pointing to a fusion product, particularly since the cells also carried the M8 marker chromosome. Moreover, HRMC5 and HRMC8 are significantly larger than either parent and their granularity falls between RPMC and RBL-CK2-TG. HRMC9 is larger in size and less granular than either of its parents. It deviates considerably from them as far as the FcεR pattern is concerned, and has a very low histamine content.

It is rather significant that about five to seven weeks after fusion, relatively little evidence for the presence of the CTMC phenotype could be found among HRMC. In HRMC5 only a few cells could be counter-stained with safranine O and even though partial cloning of this cell line gave rise to a "clone" of CTMC-like cells, cells of the CTMC phenotype were no longer present three weeks later. A complete absence of the CTMC phenotype among all subclones as well as HRMC5.1, HRMC5, HRMC8, and HRMC9, harvested at about the same time, was confirmed by Western blotting with anti-RMCPI which showed no evidence for the presence of this protease. These findings, therefore, suggest several possible but not mutually exclusive

alternatives: (i) The CTMC phenotype was lost because it can only be maintained in the proper microenvironment. This possibility is indirectly supported by the facts that prolonged culture of RPMC gives only rise to RCMC of the MMC phenotype (Chan et al., 1988; Chan et al., 1990) and that bone marrow derived MMC can be converted to the CTMC phenotype by co-culture with fibroblasts (Levi-Schaffer et al., 1986); (ii) cells of the CTMC phenotype grow much more slowly than those of the MMC phenotype and since HRMC5.1, most likely, was not monoclonal, cells of the latter phenotype outgrew those of the former; (iii) the loss of chromosomes involves primarily those derived from the RPMC parent and that, as a consequence, the phenotype of these cells is lost; and (iv) finally, the overwhelming majority of HRMC of the MMC phenotype may be the result of successful fusions in which the RPMC partner consisted primarily of immature mast cells. Such immature cells make up 1-4% of the peritoneal mast cell population (Mendonca et al., 1986) and, at present, their phenotypic expression is still obscure. It could conceivably resemble that of MMC. The rare occurrence of the CTMC phenotype among HRMC and its disappearance when found suggest that phenotypic characterization and karyotype analysis of hybrid cells should, perhaps, have been performed sooner after fusion, and some of the fused cells should have been cultured in the presence of fibroblasts. It should also be pointed out that the presence of some cells of the CTMC phenotype in early HRMC

cultures, because of the subsequent rapid disappearance of this phenotype, was established only on the basis of histochemical staining with alcian blue and safranine O. However, as will be shown in Chapter III, based on another fusion, the presence of the CTMC phenotype among early HRMC could be confirmed on the basis of other parameters as well.

The histamine content of the hybrid cell lines is much lower than that of RPMC (shown previously to be about 25×10^{-6} g/10^6 cells) (Chan et al., 1990) and in the case of HRMC5 and HRMC8 is close to or identical to that of RBL cells. Line HRMC9 contains extremely little histamine. Thus, there is some correlation between histamine content and granularity as measured by 90^0 light scatter using a fluorescence-activated cell sorter. It should, however, be pointed out that the size and shape of the nucleus of a given cell can also influence the 90^0 light scatter and that a precise correlation between granularity as determined by FACS and total histamine content should not be necessarily expected. Many of the partially cloned lines of HRMC5 contain significantly more histamine than either RBL-CK2-TG or RCMC2. It should be noted that the histamine analysis was performed at a time when HRMC5.1 no longer exhibited the CTMC phenotype. While these results have shown that cells of somewhat higher histamine content than that of RBL cells can be produced by fusion of RBL cells and RPMC, for HRMC to approach the histamine content of the latter, the CTMC phenotype may have to be preserved.

Conceivably, HRMC containing large amounts of histamine may have rather slow growth characteristics and may have been crowded out by faster growing cells. Thus, it has been shown that sodium butyrate which increases the histamine content of mast cells also decreases their growth rate (Galli et al., 1982).

CHAPTER III

PHENOTYPIC CHANGES AMONG HYBRID RAT MAST CELLS

SUMMARY

In Chapter II, it was shown that hybrid rat mast cells (HRMC) can be produced by the fusion of rat peritoneal mast cells (RPMC) and rat basophilic leukaemia (RBL) cells. While the parent RPMC and RBL cells, are representatives of the connective tissue type (CTMC) and mucosal type (MMC) mast cells, respectively; most hybrid cells were found to be of the MMC phenotype, as determined by differential histochemical staining. Only one cell line showed a few cells with CTMC staining characteristics which, even after cloning, changed to an MMC phenotype. Another fusion was performed and it was confirmed that cells of the CTMC phenotype can indeed be obtained. Four out of 14 primary cell lines contained significant amounts of CTMC-like granules as demonstrated by histochemical staining with either safranine O or berberine sulfate. Two cell lines, one of the CTMC (predominantly) and the other of the MMC phenotype were selected for further study. The presence of the CTMC phenotype was also confirmed by detecting rat mast cell protease I (RMCPI) and by mediator release triggered by compound 48/80. The CTMC phenotype vanished after culturing established cell lines for two weeks (eight weeks after fusion). The disappearance of this phenotype was accompanied by a loss of counterstaining with

safranine O, RMCPI and release of serotonin due to compound 48/80. The change in phenotype did not significantly alter the mediator release due to calcium ionophore A23187. Repeated cloning did not yield a cloned line of cells expressing the CTMC phenotype only, although it prolonged the persistence of this phenotype. At about the same time that the loss of the CTMC phenotype was taking place, loss of DNA occurred, suggesting that it may, at least partially, have been responsible for the phenotypic changes.

INTRODUCTION

In Chapter II, the establishment of hybrid rat mast cells (HRMC) was described. They were generated by the fusion of rat peritoneal mast cells (RPMC) and HAT (hypoxanthine, aminopterin, thymidine) sensitive RBL cells in the presence of polyethylene glycol. Only relatively few cells of CTMC phenotype could be detected among the hybrid cells by differential staining using alcian blue and safranine O. Attempts to confirm the presence of cells of this phenotype by other methods failed. However, by the time these characterizations were performed, histochemical staining also no longer revealed CTMC-like cells. As discussed in Chapter II, there were several possible explanations : (i) cells with CTMC-like phenotype usually proliferate much more slowly than cells with MMC phenotype. Since HRMC5.1, the "clone" with some CTMC-like granules, may not be truely monoclonal and the cells with later phenotype can outgrow those of the former; (ii) the chromosomes lost in the culture period involves primarily those from the RPMC parent and thus, as a consequence, the CTMC phenotype is lost; (iii) the overwhelming majority of HRMC with MMC phenotype may be the

result of successful fusion in which the RPMC partner consisted primarily of immature mast cells which may make up 1-4% of the peritoneal mast cell population (Mendonca et al., 1986); and (iv) more likely, the CTMC phenotype was lost because it can only be maintained in the proper microenvironment including the effects of multiple cytokines (Galli, 1990; Swieter et al., 1992) and in vitro culture in RPMI 1640 medium alone can not provide the critical factors.

Now the fusion experiment using RPMC and RBL-CK2-TG was repeated and the presence of CTMC phenotype among several resultant HRMC lines and clones was screened for at entry stages in culture by monitoring different phenotypic parameters. The changes in different phenotypic characteristics with continuous growth in tissue culture are described.

METHODS AND MATERIALS

Preparation of DNP-BSA conjugates:

Bovine albumin coupled with DNP (DNP_{17}-BSA) was prepared by reacting BSA with 2,4-dinitrophenylsulfonate as described by Carsten and Eisen (Carsten and Eisen, 1953). Briefly, 500mg of BSA and 500mg of dinitrobenzensulfonate (DNBS) were mixed in 0.4M Na_2CO_3 and stirred gently in a 18^0C water bath for six hours. During the incubation, small amount of reactants were removed at intervals, passed through a Sephadex G25 column pre-equilibrated with PBS and the optical densities were measured at 280nm and 360nm in spectrophotometer. The number of groups of DNP coupled per molecule of BSA was then determined.

Preparation of mouse DNP-specific IgE:

Mouse monoclonal IgE specific for DNP was prepared from the ascitic fluid of mice injected with hybridoma cells using published procedures (Liu et al., 1980). Briefly, BALB/c mice

(eight weeks old) were primed with 0.5ml Pristane (Aldrich Chemical Co., Milwaukee, WI) by i.p. injection. After two weeks, $1x10^7$ anti-DNP IgE H_1-DNPϵ.26.82 hybridoma cells (Liu et al., 1980), were injected i.p. into the peritoneal cavity of each primed mouse. After another two weeks, ascitic fluid was collected and centrifuged to remove any cells. DNP_{40}-BSA-Sepharose was washed extensively with 0.1M acetic acid, followed by PBS, mixed with the ascitic fluid and the mixture was kept on a rotator overnight at 4^0C. Then, the mixture was applied to a column and was washed extensively with PBS to remove any unbound material. The bound DNP-specific IgE was eluted with 0.1M 2,4-dinitrophenol (DNPOH). The eluates were then passed through a Dowex 1x8 column to separate the IgE from DNPOH. Proteins in each fraction were measured and tubes containing the protein peak were pooled and dialysed against PBS for several changes at 4^0C to remove traces of DNPOH. The concentration of purified IgE was determined by using extinction coefficient of 16.2.

Proteins and immunoglobulins:

The $F(ab')_2$ fragments of rabbit anti-RMCPI and sheep anti-RMCPII were kindly provided by Dr. H.R.P. Miller. The former were monospecific for RMCPI, while the latter exhibited minor cross-reactivity with RMCPI.

Cells and cell culture:

RBL-CK2 is a cloned cell line of RBL_{Wpg} (Chan et al., 1986). It was identified to be free of mycoplasma contamination and cultured under conditions established in this laboratory (Conrad et al., 1976) as described in Chapter II.

Hybrid rat mast cells (HRMC) were maintained in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS), 1mM L-glutamine, 1mM sodium pyruvate, 0.05mM 2-mercaptoethanol and 100μg/ml kanamycine monosulfate as suggested by the manufacturer.

RBL-2H3 cells were kindly provided by Dr. R.P. Siraganian. They were cultured under conditions identical to those for HRMC. Rat peritoneal mast cells (RPMC) were harvested from Wistar-ICI rats, maintained in the Animal Care Facility of the University of Manitoba, and purified according to procedures described in Chapter II, yielding a purity above 98%.

Mouse 3T3 fibroblasts were obtained from ATCC (Rockville, MD) and the rat fibroblast cell line RC1 was kindly provided by Dr. J. Gauldie, McMaster University. Both cell lines were cultured in complete RPMI 1640 medium containing 10% FCS.

For co-culture experiments of HRMC with either 3T3 or RC1 fibroblasts the procedures outlined by Levi-Schaffer et al. were used (Levi-Schaffer et al., 1986). In a standard

experiment, fibroblasts (10^3 cells) were added to each well of a 24-well plate and cultured to confluence. HRMC ($5x10^4$ cells per well) were added and cultured for two or four weeks. At the end of culture period, HRMC were harvested and their phenotype analyzed by differential staining.

Hybridization of RPMC and RBL cells:

Fusion of 6-thioguanine resistant RBL-CK2-TG cells and RPMC was essentially carried out as described previously in Chapter II, except that polyethylene glycol (PEG) of MW 1500 (Merck, Darmstadt, FRG) was used and the fusion was carried out in RPMI 1640 medium without added FCS instead of MEM medium. Once cell growth was established, the primary cultures were immediately expanded and aliquots were frozen for storage in liquid nitrogen. Subsequently, selected cell lines were cloned at 0.1 cells per well.

Histochemical staining:

To obtain differential staining of CTMC or MMC, alcian blue and safranine O as well as berberine sulfate (Sigma Chemical Co., St. Louis, MO) were used and experiments were carried out as described in Chapter II.

Propidium iodide DNA staining :

The staining was carried out according to the published procedures (Grissman and Steinkamp, 1973). Briefly, cells obtained from the tissue culture were washed once with phosphate buffered saline (PBS). An aliquot of $3x10^6$ cells was transferred into another test tube and centrifuged. The supernatant was removed and 1ml of cold PBS was added and followed by mixing, the addition of 2.33ml cold 100% ethanol and incubation at 4°C for one hour. Cells were then washed with PBS one more time before they were treated with RNase (1mg/ml, Sigma Chemical Co., St. Louis, MO) at 37°C for 30 minutes. After the RNase treatment, cells were washed with PBS and then incubated with 2.5ml propidium iodide (50μg/ml, Sigma Chemical Co., St. Louis, MO) at room temperature for one hour. The DNA analysis was carried out by Dr.E.S.Rector of this department. Samples were analyzed with an Epics 753, fluorescence activated cell sorter (Coulter Electronics Inc., Hialeah, FL), equipped with an argon ion laser emitting 300mW at 488nm. Fluorecence was observed through a 610nm long pass absorbance filter. Fluorecence histograms were double-gated on forward angle vs. 90^0 light scatter, and linear vs. peak fluorecence histograms was generated in order to eliminate debris and cell aggregates and include only single cell events. The fusion parent cell line RBL-CK2-TG was used as

standard to allow the comparison of DNA histograms obtained at different times during the course of this study. Accordingly minor adjustments to the high voltage of the fluorecence detector were made as necessary, to position the G_0G_1 peak of this cell line in channel number 78. All fluorescence histograms in Figure 29 were based on 10,000 gated events.

Western blot analysis of rat mast cell proteases (RMCP):

Experiments were carried out using same procedures described in Chapter II.

Determination of total histamine content:

Experiment was carried out using same procedures as described in Chapter II.

[^{3}H]-5-hydroxytryptamine (^{3}H-5HT) release from HRMC:

This was achieved essentially according to published procedures (Fewtrell and Metzger 1980). Cells were harvested by trypsinization and were plated at $1x10^5$ cells/ml/well in complete RPMI 1640 medium in 24-well plate (Gibco). Eighteen

hours later, the medium was removed and cells were sensitized for two hours at 37°C with 3μCi ^{3}H-5HT (NEN, Dupont Canada Inc., Markham, ON) either in the presence of DNP-specific IgE (for IgE-antigen mediated release) or in the absence of IgE (for Calcium ionophore A23187 or compound 48/80 induced release). The medium was removed, wells were washed (4x) with HEPES buffer, pH 7.2, containing 10mM HEPES, 135mM NaCl, 5mM KCl, 1.0mM $MgCl_2$, 1.8mM $CaCl_2$, 5.6mM glucose, BSA 0.05%. Cells were stimulated with either DNP_{17}-BSA or calcium ionophore A23187 or compound 48/80 for 30 minutes at 37°C and the reaction was terminated by centrifugation. Supernatants were collected, counted in a Canberra Packard Model 2200CA liquid scintillation counter and the percentage of ^{3}H-5HT release was determined.

RESULTS

Histochemical properties of HRMC:

First signs of viable cells were observed about two weeks after fusion, an observation which was similar to that made previously when the first two fusions were carried out as described in Chapter II. Among the primary cultures, established growth was eventually found in 14 wells. The resultant cell lines were named HRMC12 to HRMC25. At six weeks post fusion, all lines could be stained with alcian blue but only four, namely HRMC12, 14, 15 and 18 could be counterstained with safranine O (Table I, Fig. 16, 17). The same four cell lines also could be stained with berberine sulfate while the remainder could not (Table I). Two of the lines, HRMC12 and HRMC13, were selected for further detailed studies.

When line HRMC12 was cloned at 0.1 cells per well, of the 19 clones, only five could be counterstained with safranine O (Table II, Fig. 19, 21). In Table II, the staining characteristics of HRMC12 and some of its selected clones are depicted. It is clear that the parent line, HRMC12, lost its

Table I
Histochemical Staining Characteristics of Hybrid Rat Mast Cells

Mast Cells	Alcian blue (AB)/Safranin O (SO) Staining		Berberine sulfate (B) Staining	
	AB^+/SO^+	**AB^+/SO^-**	**B^+**	**B^-**
RPMC	+[a]	-[b]	+	-
RBL-CK2TG	-	+	-	+
HRMC12[c]	+	+	+	+
HRMC14	+	+	+	+
HRMC15	+	+	+	+
HRMC18	+	+	+	+
HRMC13[c]	-	+	-	+
HRMC16	-	+	-	+
HRMC17	-	+	-	+
HRMC19	-	+	-	+
HRMC20	-	+	-	+
HRMC21	-	+	-	+
HRMC22	-	+	-	+
HRMC23	-	+	-	+
HRMC24	-	+	-	+
HRMC25	-	+	-	+

[a] Indicates phenotype is present
[b] Indicates phenotype is absent
[c] Selected for further study

Table II
Changes in Histochemical Characteristics of HRMC12 and Selected Clones

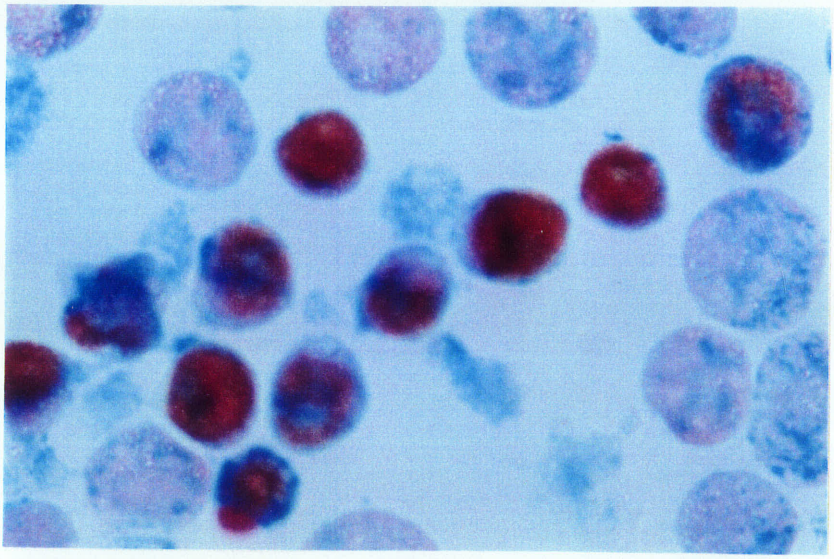
Mast Cells	Alcian blue (AB)/Safranin O (SO) Staining[a]	
	AB^+/SO^+	**AB^+/SO^-**
RPMC	+++	-
RBL-CK2TG	-	+++
HRMC12 (6w[b])	++	+
HRMC12 (8w)	-	+++
HRMC12.1 (8w)	-	+++
HRMC12.1 (10w)	-	+++
HRMC12.4 (8w)	++	+
HRMC12.4 (10w)	-	+++
HRMC12.5 (8w)	+	++
HRMC12.13 (8w)	-	+++
HRMC12.14 (8w)	-	+++
HRMC12.18 (8w)	+	++

[a] Scoring system:
- \- Negative
- \+ < 50% Cells
- ++ > 50% Cells
- +++ 100% Cells

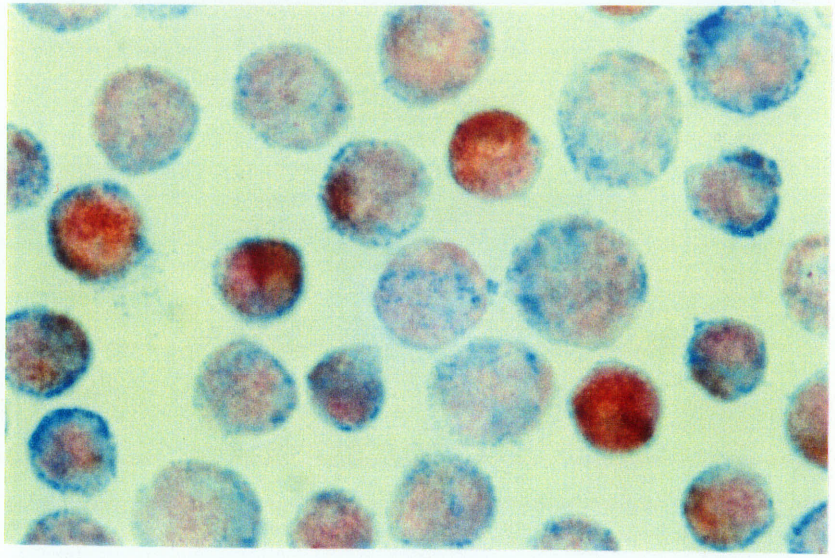
[b] Indicates number of weeks in culture

Figure 16. Differential staining with alcian blue and safranin O of (a) HRMC12 (6 weeks), (b) HRMC14 (6 weeks), (c) HRMC15 (6 weeks). Magnification: 400x.

a



b



c

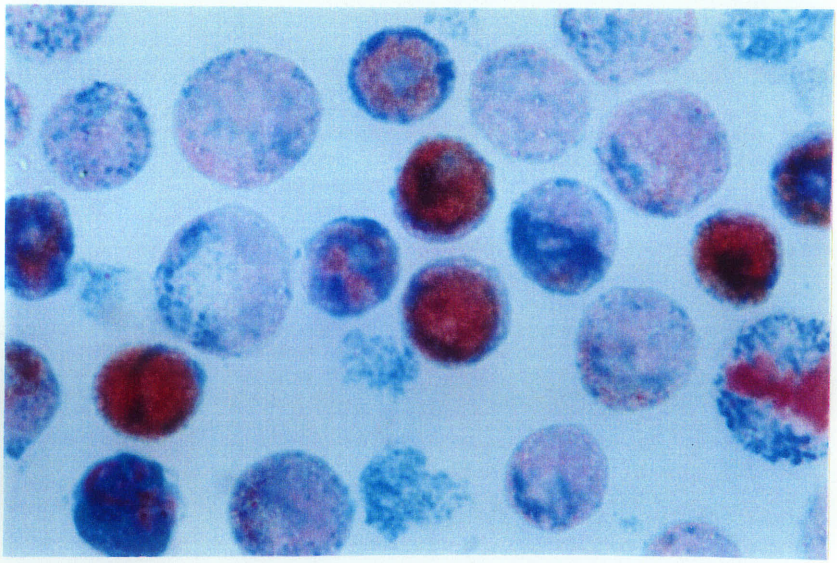
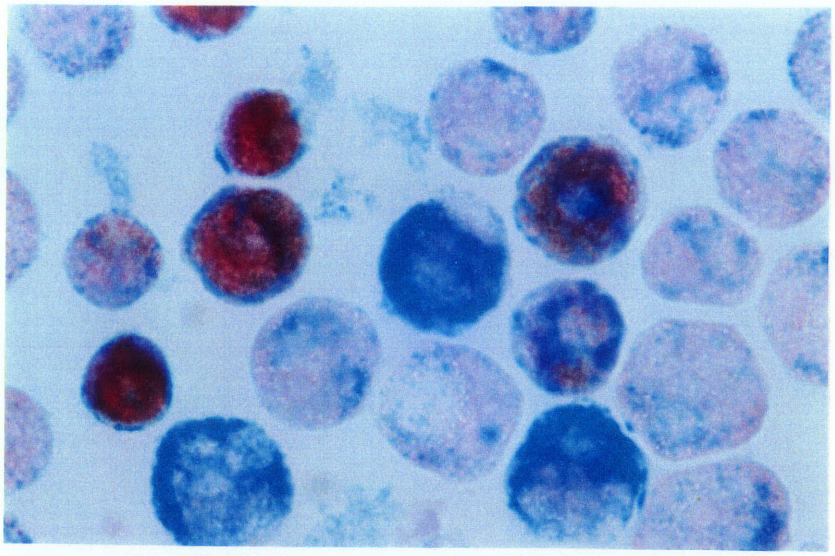
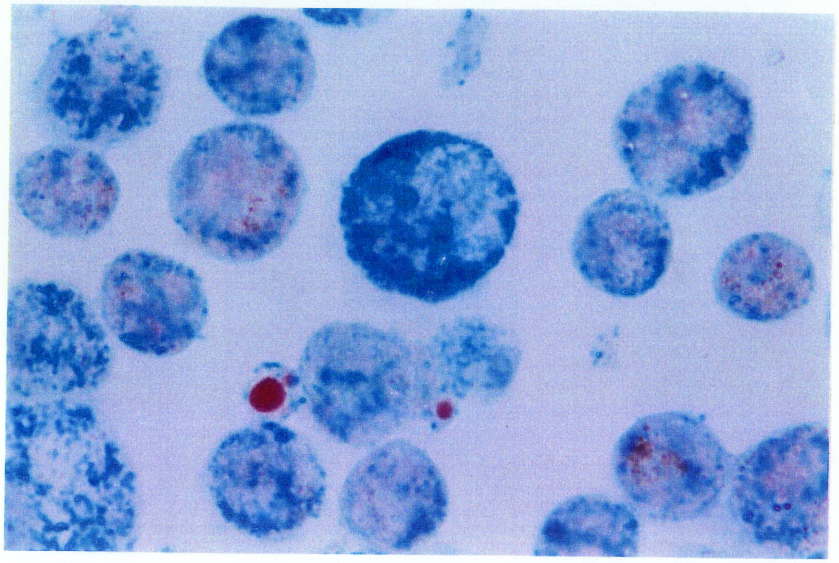


Figure 17. Differential staining with alcian blue and safranin O of (a) HRMC18 (6 weeks), (b) HRMC13 (6 weeks) Magnification: 400x.

a



b

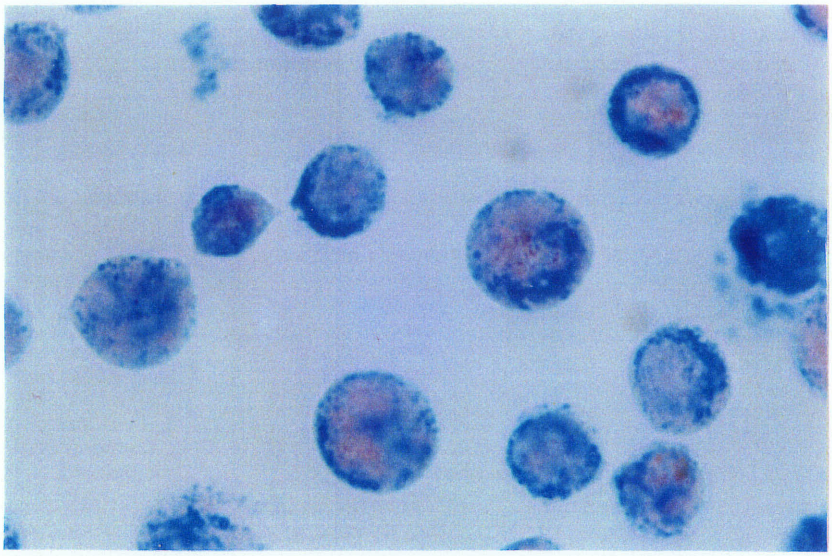


capacity to be counterstained by safranine O by eight weeks after fusion (two weeks after they were initially tested) (Fig. 18). Cloning could prolong the presence of safranine O positive (SO^+) cells, but two weeks later the cloned cells had also lost their SO^+ characteristic (Fig. 19, 20, 21). As indicated by the scoring system in Table II, not all cells in HRMC12 and HRMC12.4, HRMC12.5 and HRMC 12.18 were SO^+. Cloning could not significantly increase the relative number of SO^+ cells, it simply seemed to extend their persistence with time in continuous culture. Subcloning of HRMC12.4 led to a similar pattern, in as much as SO^+ cells could be preserved in some subclones without achieving an enrichment of such cells (data not shown). It should be noted that, as observed among cells of the first fusions (Chapter II), primary cell lines and cloned lines which were SO^+ contained some cells which exhibited a mixed phenotype, i.e., some granules were SO^+ and other SO^-.

It is also interesting to point out that an effort was made to maintain SO^+ or CTMC phenotype of HRMC by co-culture of SO^+ cells with rat or mouse fibroblasts. These attempts failed.

Figure 18. Differential staining with alcian blue and safranin O of (a) HRMC12 (8 weeks), (b) HRMC14 (8 weeks) Magnification: 400x.

a



b

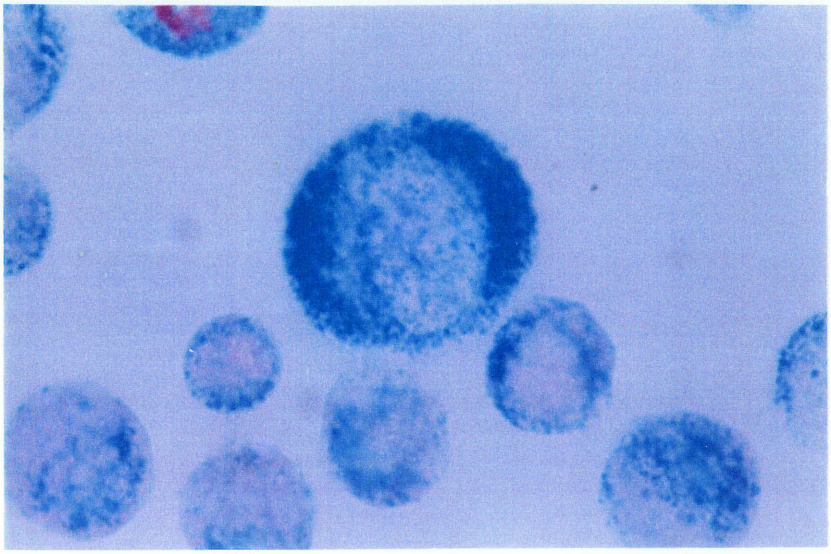
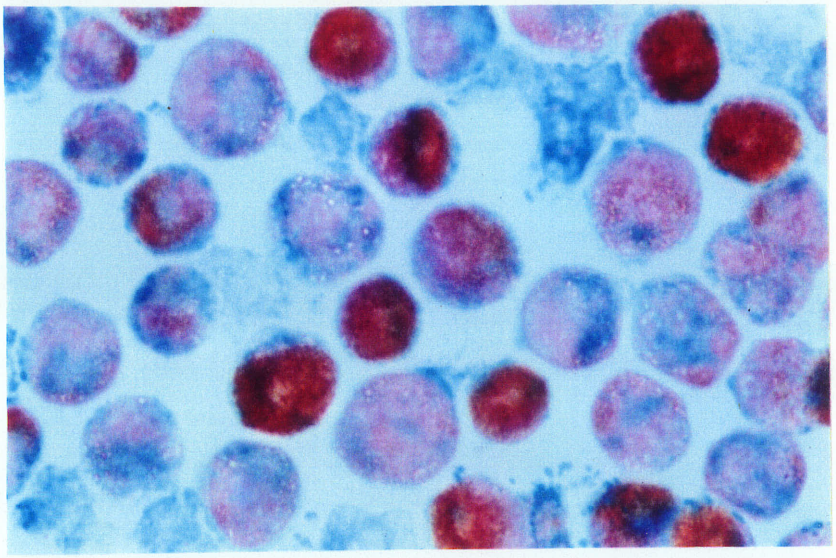
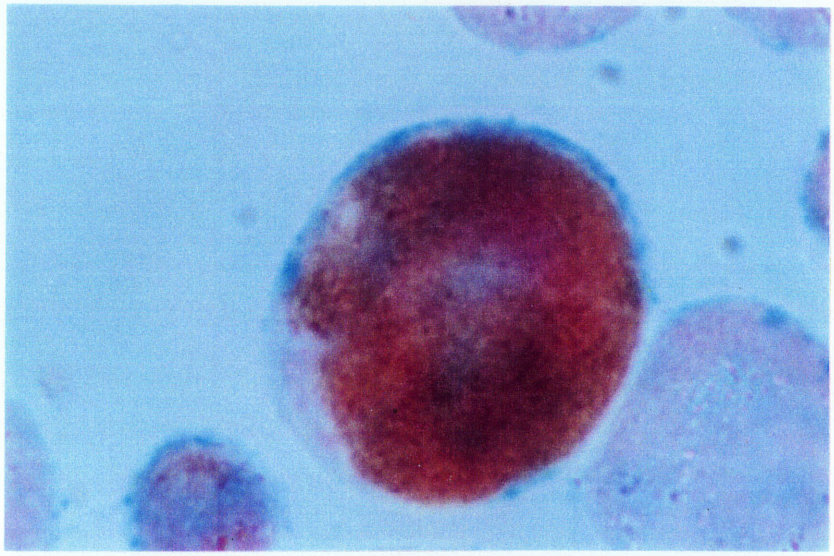


Figure 19. Differential staining with alcian blue and safranin O of (a) HRMC12.4 (8 weeks), 400x, (b) HRMC12.4 (8 weeks), 1000x, (c) HRMC12.4 (10 weeks), 1000x.

a



b



c

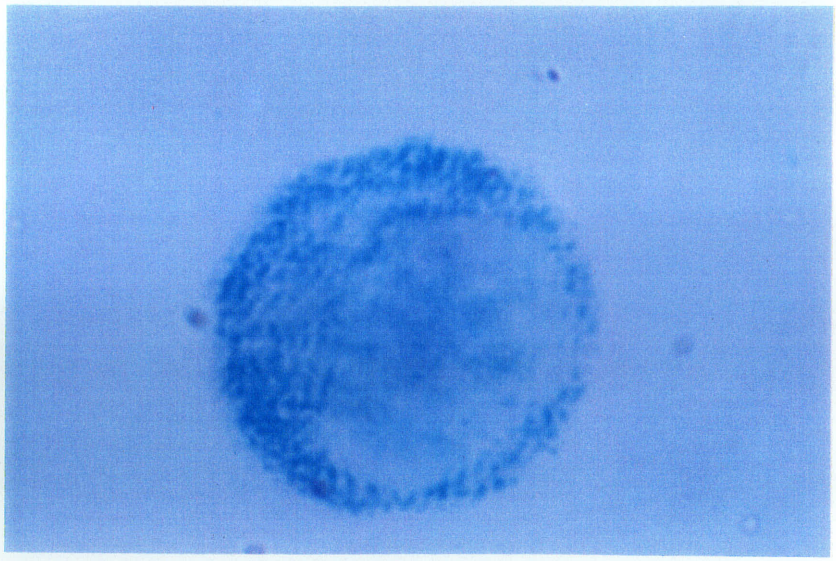
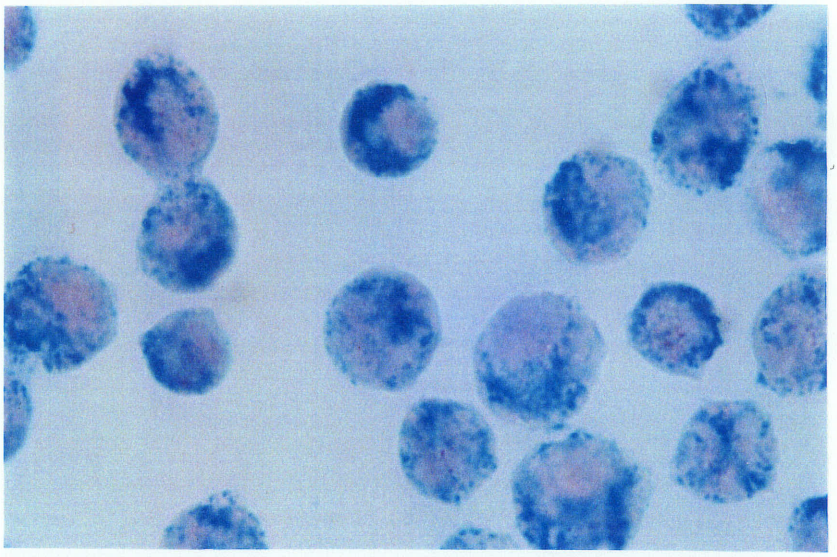


Figure 20. Differential staining with alcian blue and safranin O of (a) HRMC12.4 (10 weeks), 400x, (b) HRMC12.4 (10 weeks), 1000x.

a



b

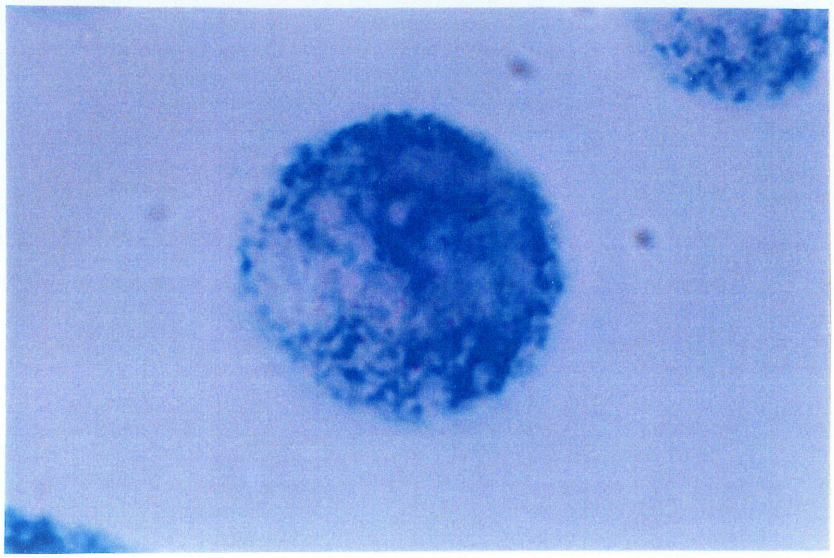
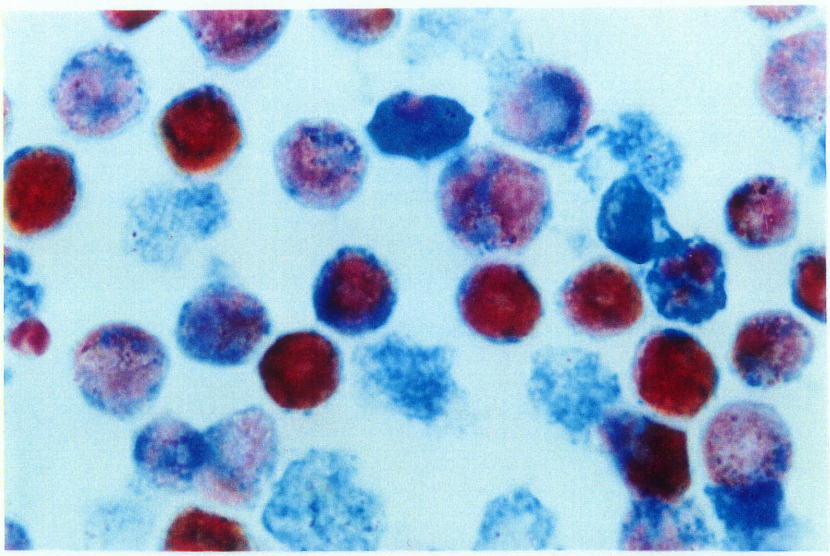
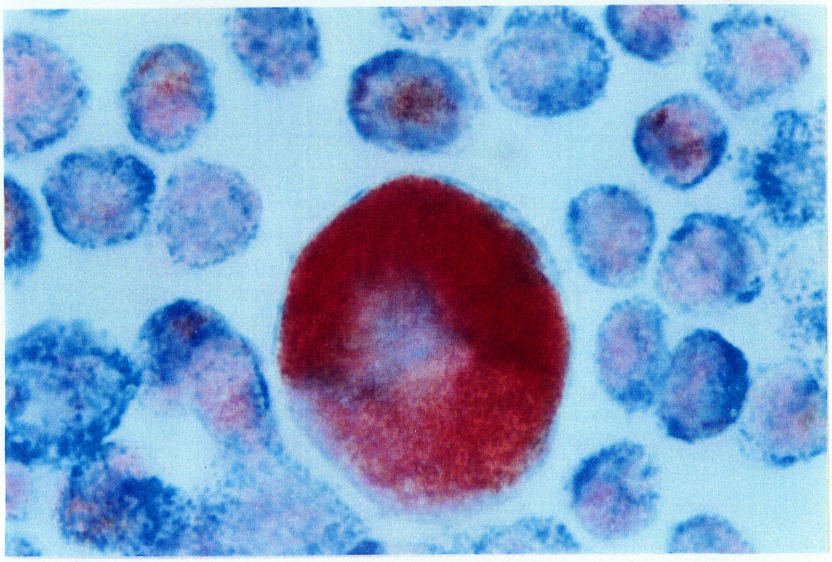


Figure 21. Differential staining with alcian blue and safranin O of (a) HRMC12.5 (8 weeks), 400x, (b) HRMC12.5 (8 weeks), 1000x, (c) HRMC12.5 (10 weeks), 1000x.

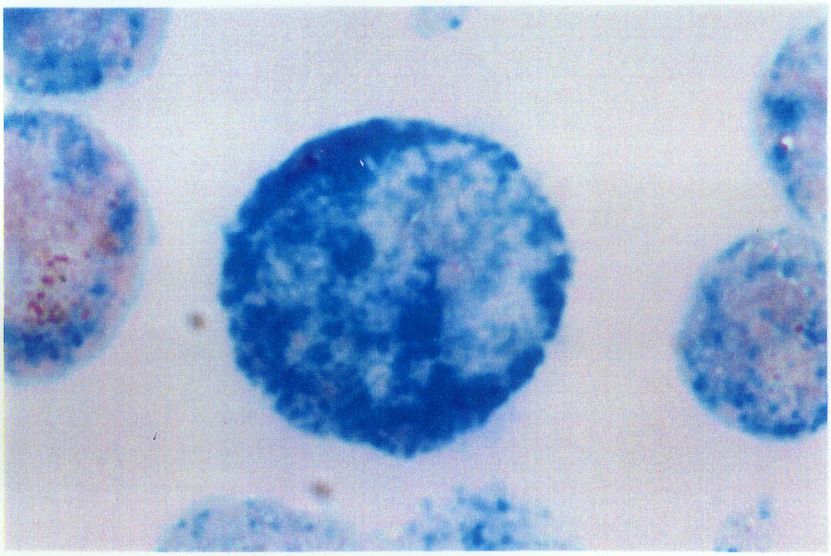
a



b



c



Characterization of mast cell proteases:

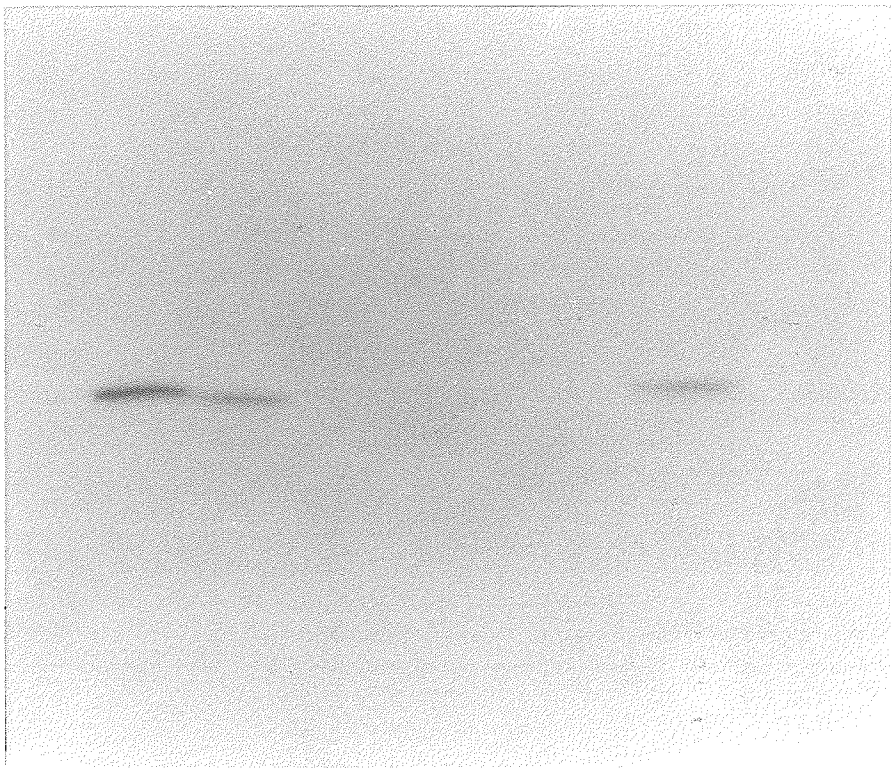
To confirm the phenotype of cells in a given cell line, these were assayed for the presence of RMCPI and RMCPII by Western blotting. It is clear from Figures 22 and 23, that the cell lines which were shown to be SO^+ were found to contain both RMCPI and RCMPII, indicating further the presence of both the CTMC and the MMC phenotypes. At the 12th week of *in vitro* cell culture, every HRMC line tested contained RMCPII (Fig. 24) and no RMCPI could be detected (data not shown).

Histamine content determinations:

The histamine content of a few selected cell lines was examined. Similar to observations described in Chapter II, the primary hybrid mast cell lines generally contained more histamine than the parent RBL-CK2-TG (Fig. 25) and lines which showed the presence of the CTMC phenotype (HRMC12 and HRMC12.4) had a higher histamine content then those expressing the MMC phenotype only (HRMC12, HRMC12.1, HRMC12.2 and HRMC12.3). None of the cell lines approached the histamine content of RPMC which was previously found to be about 25×10^{-6} g/10^6 cells (Chan et al., 1990). In general, all the values shown in Figure 25 are somewhat lower than those of HRMC produced by previous fusions (Chapter II).

Figure 22. Western blot analysis of RMCPI (upper panel) and RMCPII (lower panel) of RBL-CK2-TG(a), purified RMCPI(b), HRMC12 (6 weeks)(c), HRMC12 (8 weeks)(d), HRMC12.1(e), HRMC12.2(f), HRMC12.3(g), HRMC12.4 (8 weeks)(h), HRMC12.4 (10 weeks)(i) and HRMC13(j).

a b c d e f g h i j



a b c d e f g h i j

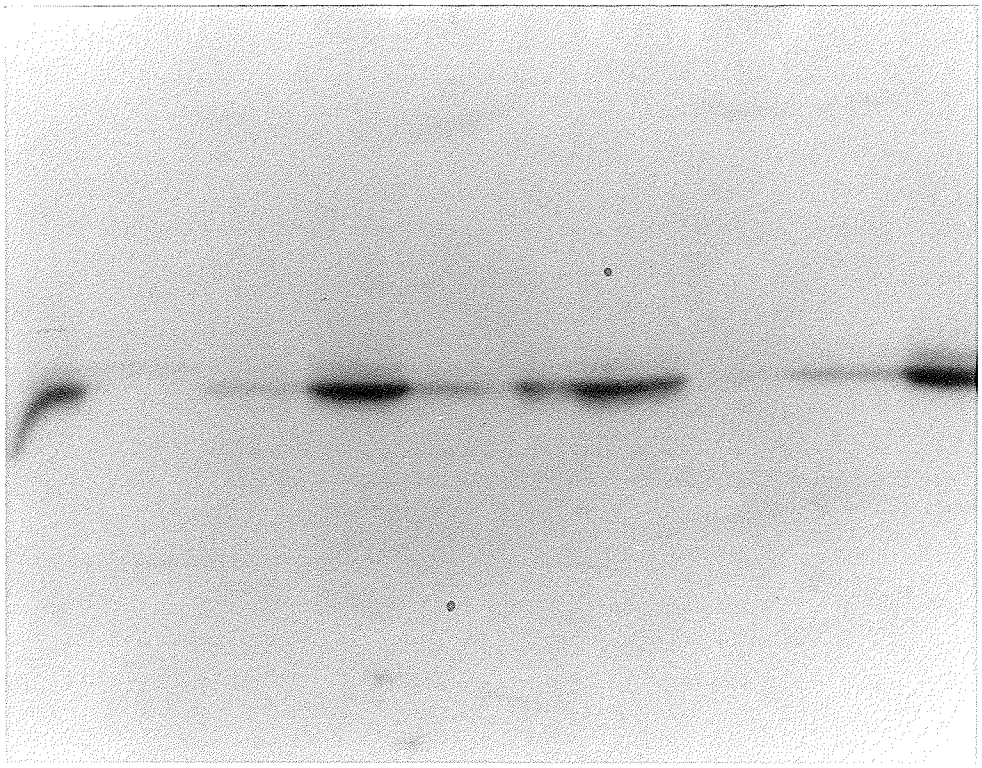


Figure 23. Western blot analysis of RMCPI of (a) RBL-CK2-TG(a), (b) purified RMCPI, (c) HRMC14 (6 weeks) (d), HRMC14 (8 weeks), (e) HRMC16 (6 weeks), (f) HRMC16 (8 weeks), (g) HRMC17 (6 weeks), (h) HRMC17, (8 weeks), (i) HRMC19 (6 weeks), (j) HRMC19, (8 weeks), (k) HRMC21 (6 weeks), (l) HRMC21, (8 weeks), (m) HRMC24 (6 weeks).

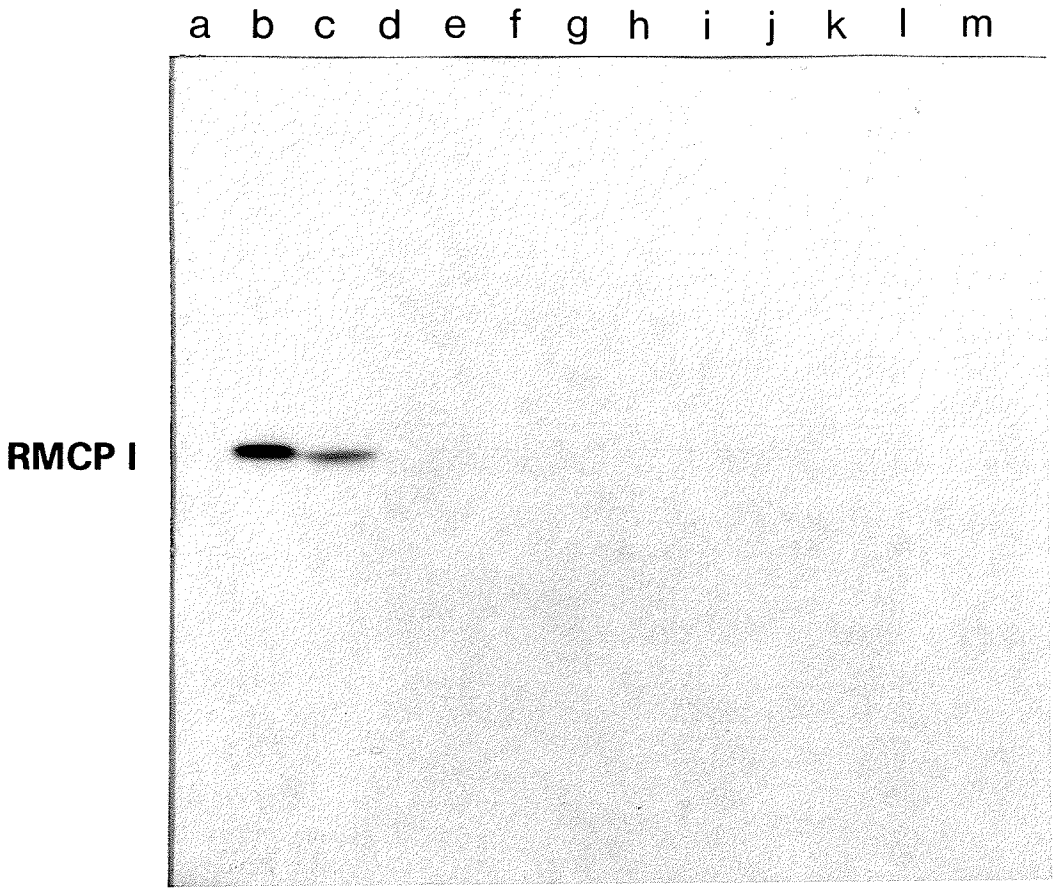

a b c d e f g h i j k l m
RMCP I

Figure 24. Western blot analysis of RMCPII (at 14th weeks) of (a) RBL-CK2-TG, (b) HRMC12, (c) HRMC13, (d) HRMC14 (e) HRMC16, (f) HRMC17, (g) HRMC18, (h) HRMC19, (i) HRMC20, (j) HRMC21, (k) HRMC22, (l) HRMC23, (m) HRMC24.

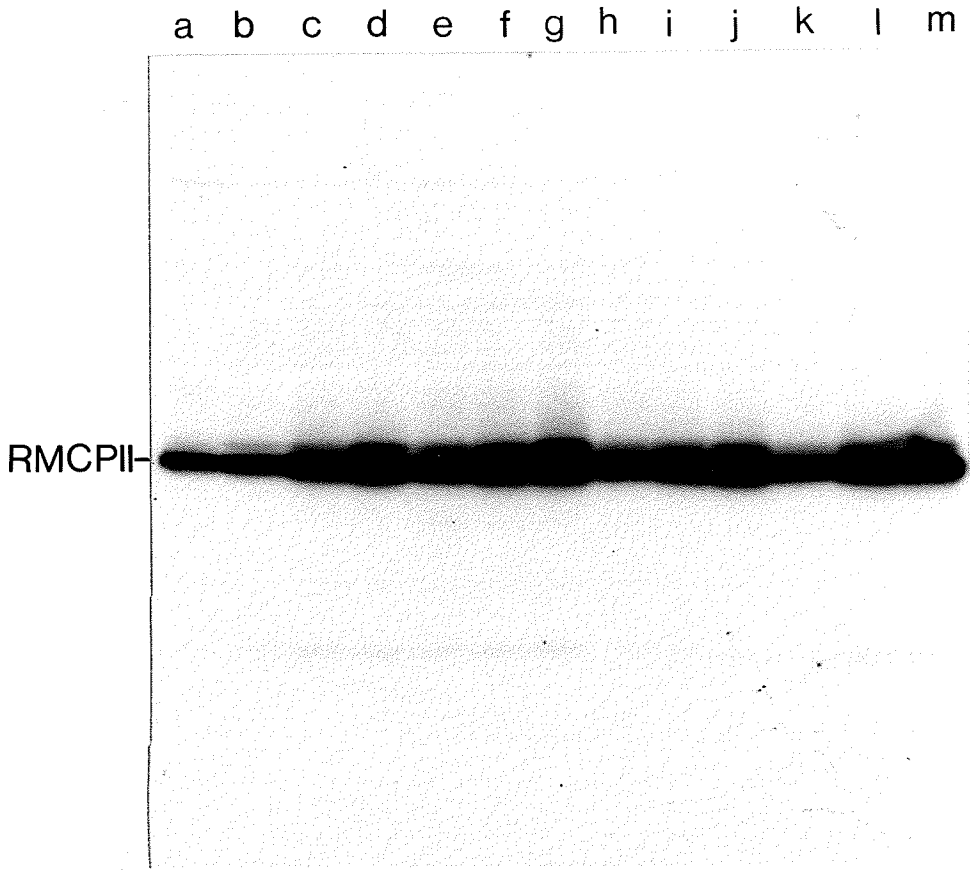
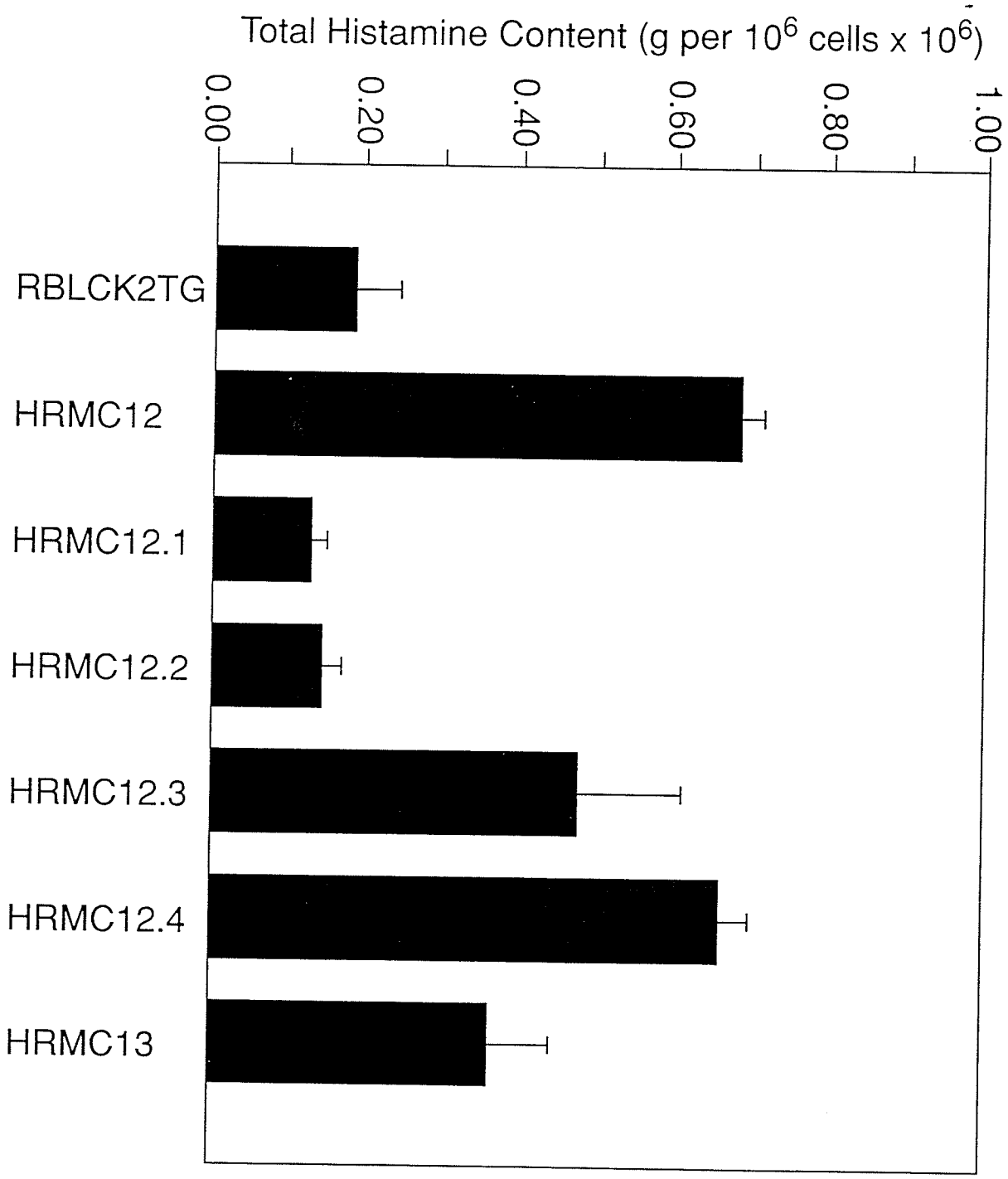

a b c d e f g h i j k l m
RMCPII

Figure 25. Total histamine content of RBL-CK2-TG and selected HRMC lines. The results show the mean of quadruplicate determination and the standard deviation.


Total Histamine Content (g per 10^6 cells x 10^6)
1.00
0.80
0.60
0.40
0.20
0.00
RBLCK2TG
HRMC12
HRMC12.1
HRMC12.2
HRMC12.3
HRMC12.4
HRMC13

Mediator release properties of HRMC:

In view of the fact that HRMC lines exhibited a relatively low histamine content, histamine release experiments were not attempted. Instead, the release of [^{3}H]-5-hydroxytryptamine binoxalate (^{3}H-5HT), previously taken up by the cells, was studied. Selected cell lines and clones were first treated with calcium ionophore A23187. The best results were obtained with HRMC12, HRMC12.4, HRMC13 and HRMC17. In Figure 26, the results obtained with HRMC12 are depicted and compared to those obtained with RPMC and RBL-2H3. As can be seen, release by the various cell types was comparable, showing a similar dose response. The release by RBL-CK2-TG was lower than that of the releasing HRMC lines, with the exception of HRMC17. It should also be noted that the change in the phenotype of HRMC12, which was shown to take place between weeks six and eight of continuous culture, did not seem to have a significant effect on the release due to the calcium ionophore. The change in phenotype also did not seem to influence the IgE mediated release of ^{3}H-5HT (Fig. 27). In contrast to HRMC, RBL-2H3 cells release ^{3}H-5HT over a much wider range of antigen concentrations.

An entirely different picture emerged when the release of ^{3}H-5HT due to compound 48/80 was measured (Fig. 28). Thus

Figure 26. Release of ^{3}H-5HT as a consequence of treating selected HRMC lines with calcium ionophore A23187. The release is compared to that of RBL-2H3, RBL-CK2-TG and RPMC. The results shown in (a) were obtained on one occasion and those in (b) and (c) on another occasion.

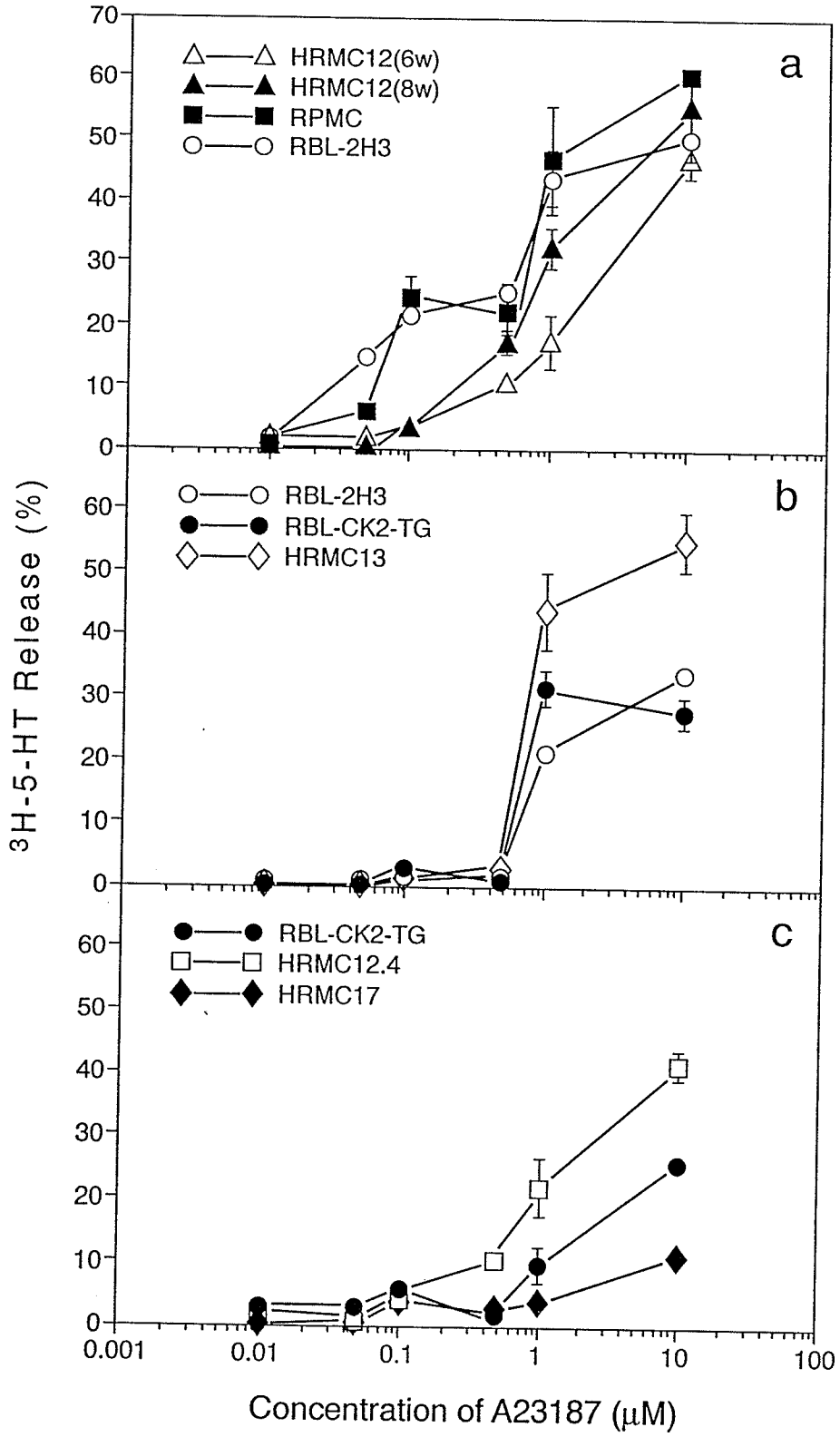
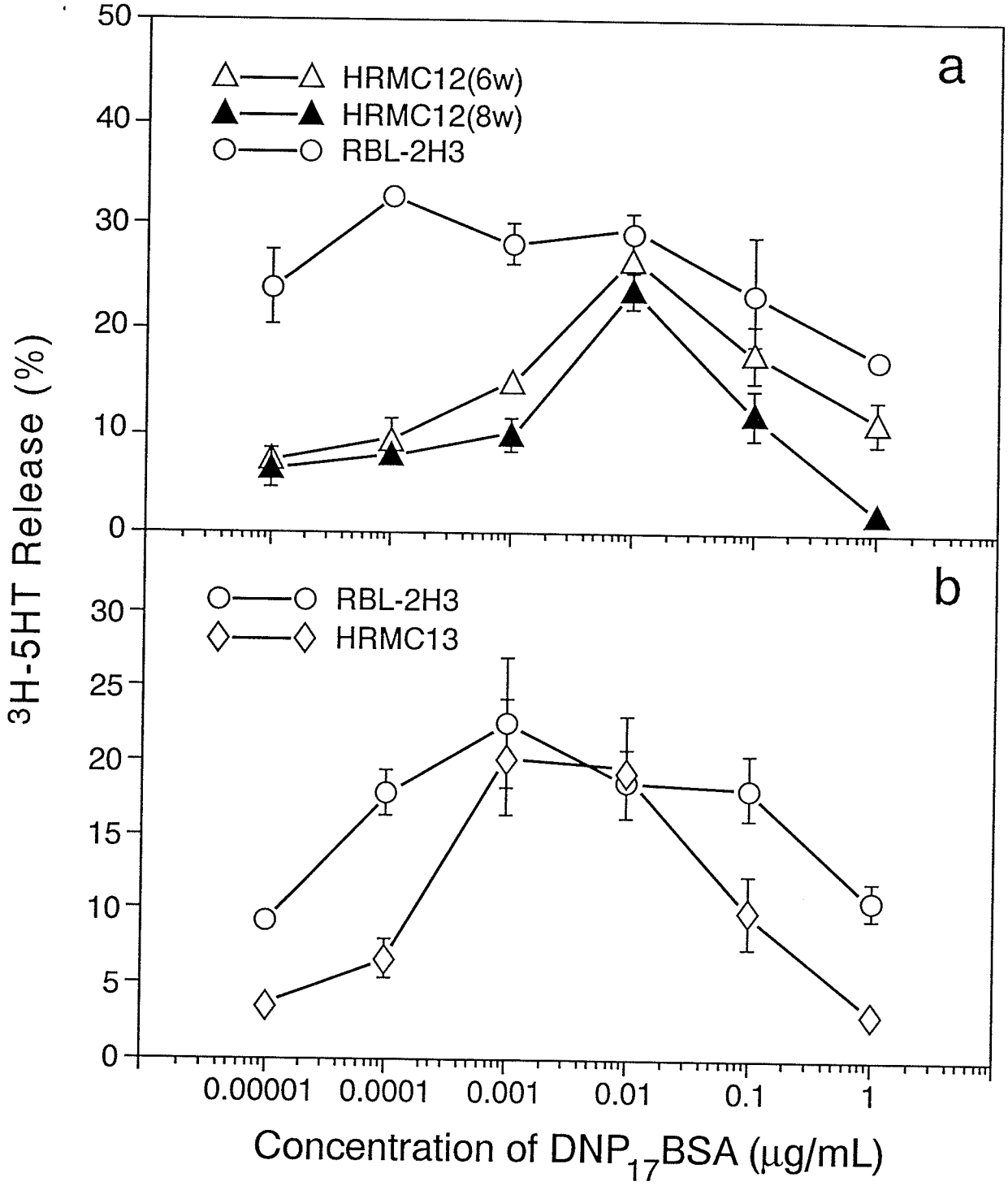

a
HRMC12(6w)
HRMC12(8w)
RPMC
RBL-2H3
b
RBL-2H3
RBL-CK2-TG
HRMC13
c
RBL-CK2-TG
HRMC12.4
HRMC17
^{3}H-5-HT Release (%)
Concentration of A23187 (μM)
0.001
0.01
0.1
1
10
100

Figure 27. Release of ^{3}H-5HT from HRMC12 after 6 and 8 weeks in continuous culture as a consequence of treating these cells first with mouse anti-DNP IgE, followed by various concentrations of DNP_{17}-BSA. The results shown in (a) and (b) were obtained on different occasions and RBL-2H3 cells served as positive control.

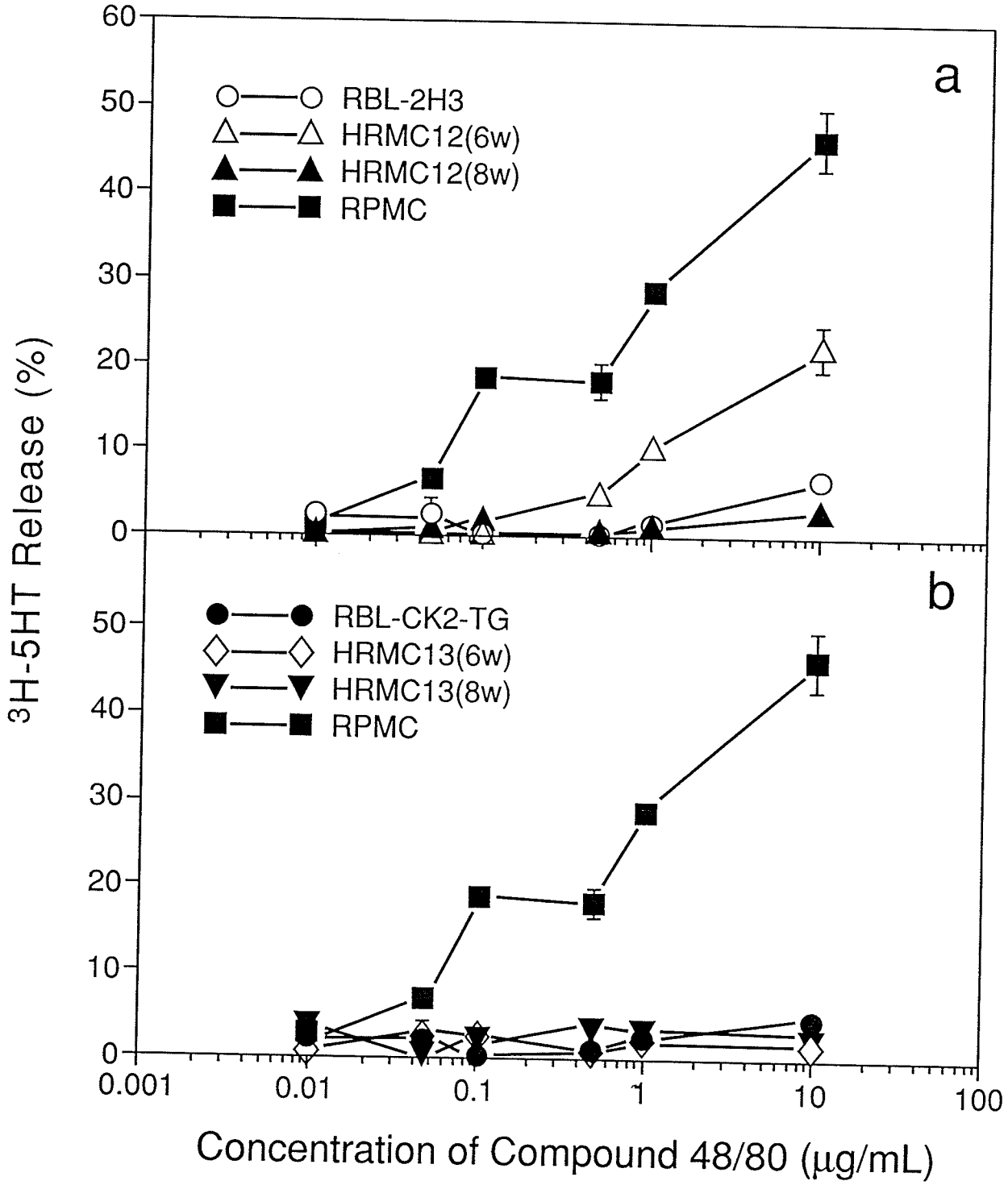

a
HRMC12(6w)
HRMC12(8w)
RBL-2H3
b
RBL-2H3
HRMC13
^{3}H-5HT Release (%)
Concentration of DNP$_{17}$BSA (μg/mL)
0.00001
0.0001
0.001
0.01
0.1
1

RPMC released much more of the ^{3}H-5HT which they had taken up. After 6 weeks in culture, HRMC12 released the mediator. However, at 8 weeks, when these cells no longer were SO^+, this release did not take place. As expected, RBL-2H3 and RBL-CK2-TG which are both of the MMC phenotype were not subject to ^{3}H-5HT release by compound 48/80 (Fig. 28). Similarly, HRMC13 at 6 and 8 weeks in culture could not be triggered by compound 48/80.

DNA contents of HRMC:

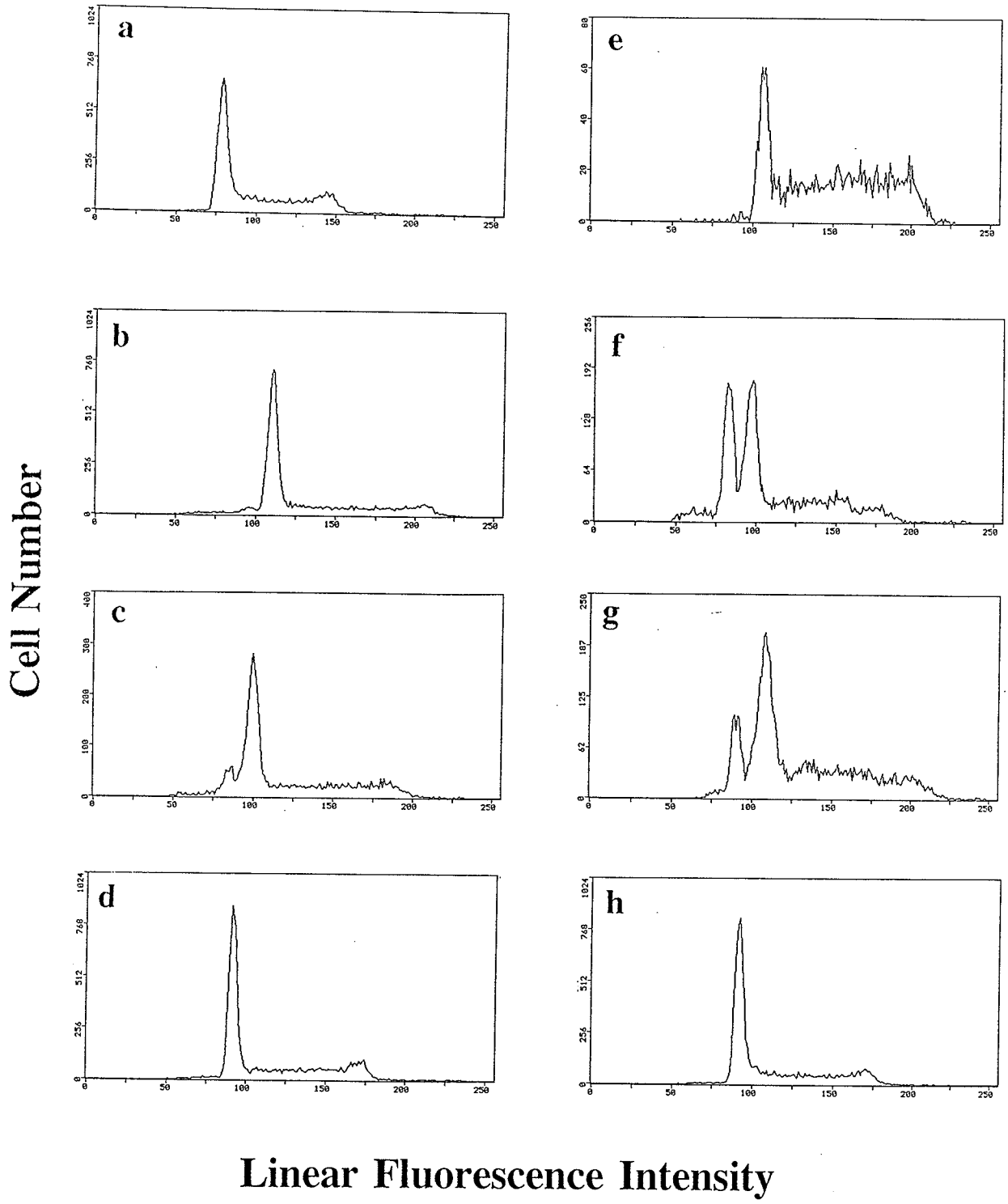
In order to establish if phenotypic changes, observed among HRMC, could possibly occur as a consequence of chromosome or DNA loss, cells were stained with propidium iodide and analyzed by flow cytometry. As can be seen in Figure 29, HRMC12 exhibited a significant change in staining pattern between week six and week eight in culture (Fig. 29. b,c). The major peaks representing the G_0G_1 phase of the cell cycle seen at week six (Fig. 29b) had split into two peaks by week eight (Fig. 29c). The peak of lower linear fluorescence intensity appeared at a position closer to that of the parent RBL-CK2-TG cells (Fig. 29a). These RBL cells had previously been shown to have a modal chromosome number of 45 (Chapter II), suggesting that the hybrid cells were undergoing chromosome loss. It should be noted that HRMC12.4 which were

Figure 28. Release of ^{3}H-5HT from HRMC12 and HRMC13 after 6 and 8 weeks in continuous culture as a consequence of treating these cells with increasing concentrations of compound 48/80. The results are compared to those obtained with RBL-2H3-CK2-TG and RPMC.


a
RBL-2H3
HRMC12(6w)
HRMC12(8w)
RPMC
b
RBL-CK2-TG
HRMC13(6w)
HRMC13(8w)
RPMC
3H-5HT Release (%)
Concentration of Compound 48/80 (µg/mL)
0
10
20
30
40
50
60
0.001
0.01
0.1
1
10
100

cloned from HRMC12 started with a single G_oG_1 peak of fluorescence intensity close to that of HRMC12 (Fig. 29e), and two weeks later exhibited a second peak of lower fluorescence intensity (Fig. 29f). Interestingly HRMC13, which at six weeks in culture, only exhibited the MMC phenotype, yielded already two G_oG_1 peaks (Fig.29g). It should also be noted that other lines such as HRMC14 (Fig. 25d) and HRMC15 (Fig. 25h) showed a single G_oG_1 peak of intensity between the two peaks seen in Figure 29c for HRMC12 at eight weeks in culture. As noted previously, these lines contained some cells of the CTMC phenotype. However, other HRMC lines and clones which exhibited the alcian blue$^+$/ safranine O$^+$ phenotype only, had a single G_oG_1 peak at about the same fluorescence intensity.

Figure 29. Histograms of linear fluorescence intensity of propidium iodide stained cells as determined by measurements using an Epics 742V fluorescence activated cell sorter (a) RBL-CK2-TG, (b) HRMC12 (6w), (c) HRMC12 (8w), (d) HRMC14, (e) HRMC12.4 (8w), (f) HRMC12.4 (10w), (g) HRMC13, (h) HRMC15.


a
b
c
d
e
f
g
h
Cell Number
Linear Fluorescence Intensity

DISCUSSION

The findings described in this chapter confirmed those made earlier that a fusion between RBL cells and RPMC is possible and that upon continuous culture of the hybrid mast cells, the CTMC phenotype, which is characteristic of RPMC, disappears. There are, however, a number of findings which extend and complement the earlier ones. Thus, several primary cultures (HRMC12, 14, 15 and 18) which contained a significant number of cells of the CTMC phenotype were now found. Previously, only one primary culture (HRMC5) contained such cells and, at that, only very few (Chapter II). A possible reason for this difference may be the fact that in the earlier study all primary cultures were frozen as soon as they had been expanded. They were then re-established from frozen stock for analysis. In the most recent fusion, the cells were maintained in continuous culture, at least for most of the phenotypic analysis. Only aliquots were frozen for future use.

Primary cultures described in this chapter were analyzed for phenotypic expression, not only histochemically by staining with alcian blue and counterstaining with safranine O, but also by staining with berberine sulfate and analysis of

rat mast cell proteases and by mediator release using secretagogues such as calcium ionophore A23187 and compound 48/80. The former triggers mediator release both from CTMC and MMC-like cells, while the latter does so only from CTMC (Shanahan et al., 1984). Thus, primary cultures and clones which could be counterstained with safranine O were also found to contain RMCPI, a protease which is characteristic of CTMC, but as expected they also contained RMCPII confirming the presence of MMC in these cultures (Woodbury et al., 1978). Further evidence for the presence of CTMC in early primary cultures came from ^{3}H-5HT release due to compound 48/80 (Fig. 28). All phenotypic characteristics of CTMC disappeared by the time HRMC had been in continuous culture for eight weeks. Interestingly, the persistence of the CTMC phenotype could be prolonged by cloning. However, no clones containing 100% of CTMC could be obtained, even after a second cloning, suggesting that the cells were undergoing continuous change.

Propidium iodide staining which measures DNA content (Grissman and Steinkamp, 1973; Kraemaer et al., 1971) confirmed that between six and eight weeks in continuous culture, a significant loss in DNA content had occurred. This does not prove that the change in phenotype had occurred as a consequence of chromosome loss, but it certainly strengthens this possibility. It is particularly interesting that in the case of HRMC13, a primary line which expressed only the MMC phenotype even at only six weeks in culture, the propidium

iodide staining pattern already was associated with the double peak representing the G_0/G_1 phase of the cell cycle. Although some HRMC lines, which exhibited only the MMC phenotype in terms of histochemical staining, yielded only one peak upon propidium iodide staining, the linear fluorescence intensity of that peak was lower than that for HRMC12 at six weeks in culture. It is conceivable that the DNA loss which occurs as HRMC are being cultured involves primarily chromosomes of the RPMC parent.

Another possible explanation for the failure of HRMC to express or maintain the CTMC phenotype may, of course, be the lack of an appropriate microenvironment. Thus it was shown in the mouse that co-culturing of mouse BMMC with mouse fibroblasts can yield mast cells with some CTMC phenotypic characteristics, i.e., staining with alcian blue and safranine O and the presence of heparin (Levi-Schaffer et al., 1986), a change which has been attributed to SCF (Tsai et al., 1991; Wershil et al., 1991). However, in the present study, an attempt to prevent a change in histochemical staining from the CTMC to the MMC phenotype using either rat or mouse fibroblasts in co-culturing experiments, proved unsuccessful. Also, preliminary experiments (data not shown) using recombinant mouse SCF could not convert MMC-like HRMC to CTMC-like cells. However, since the amino acid sequences of mouse and rat SCF are not completely identical (Anderson et al., 1990; Martin et al., 1990), species differences may account

for the negative results. Moreover, it now appears that, at least in the mouse, a c-Kit mediated signal may not be required to yield the CTMC-phenotype, at least as far as proteases are concerned (Eklund et al., 1994).

As reported in Chapter II, the HRMC generated were also found to have a much lower histamine content than RPMC, but some of the primary lines and their clones contained more of this mediator than the parent RBL-CK2-TG cells (Fig. 25). In general, the histamine content of HRMC observed at this time as well as that of RBL-CK2-TG appeared to somewhat lower than that observed in the earlier experiments (Chapter II).

It is interesting to note that all the new HRMC lines examined were capable of releasing ^{3}H-5HT either due to the secretagogue calcium ionophore A12387 or by way of an IgE-mediated mechanism. To obtain cells capable of releasing mediators was one of the original goals when fusions were initiated. The mediator release capacity (percent released) of some of the HRMC was similar to that of RBL-2H3 cells and, when using A23187, the percentage of triggered release even approached that of RPMC. The loss of the CTMC phenotype of HRMC12 did not seem to affect the capacity of this cell line to release ^{3}H-5HT upon treatment with the calcium ionophore. In the case of HRMC12, the IgE mediated release exhibited a much narrower concentration range than RBL-2H3. This may be related to the number of high affinity receptors for IgE

(FcϵRI) expressed on these cells. Once again, these newly generated HRMC were found to express both FcϵRI as well as FcϵR_L, the low affinity receptor for IgE described previously on HRMC as well as RBL cells and rat cultured mast cells (RCMC) (Chan et al, 1990). The expression of Fc receptors for IgE on these cells will be described in Chapter IV.

CHAPTER IV

IgE Fc RECEPTORS OF HYBRID RAT MAST CELLS

SUMMARY

Selected hybrid rat mast cells (HRMC) produced by somatic fusion of rat peritoneal mast cells (RPMC) and RBL-CK2-TG cells were analyzed in terms of their Fc receptor patterns by SDS-PAGE. The initial fusions generated eleven HRMC lines and receptor analysis indicated the presence of both the high affinity receptor, FcϵRI(α), and low affinity recepter, FcϵR_L. It also indicated significant variations of the relative Mr. among the HRMC3,HRMC9 HRMC10 and especially the different clones and subclones of HRMC5. Repeated fusion was carried out and receptor patterns of HRMC were characterized. In addition to the presence of both FcϵRI(α) and FcϵR_L on every cell line examined, two molecular forms with approximately Mr. of 55 kDa and 45 kDa were isolated by means of anti-FcϵR_L antibodies. One HRMC cell line examined carried both these molecules while others appeared to be associated primarily with one or the other molecule.

INTRODUCTION

In the rat, rat basophilic leukemia (RBL) cells and rat peritoneal mast cells (RPMC) are representatives of mucosal (MMC) (Seldin et al., 1985) and connective tissue type (CTMC) (Enerbäck, 1981) mast cells, respectively. Both subpopulations of rat mast cells have been shown to possess high (FcϵRI) and low affinity (FcϵR_L) receptors for IgE. Thus, these two kinds of receptors have previously been found on MMC-like RBL cells (Conrad and Froese, 1978; Froese et al., 1982), RCMC (Chan et al., 1988; Chan et al., 1990), IMMC (Swieter et al., 1989), and on CTMC-like rat peritoneal cells (RPMC) (Froese, 1980). In fusing RPMC and RBL cells to obtain HRMC (Chapter II and III), it was hoped to produce cells which can be grown in tissue culture, have the high histamine content of RPMC and carry FcϵRI and FcϵR_L free of bound IgE. In Chapter II and Chapter III, it was shown that these two types of cells can be fused using polyethylene glycol to yield hybrid rat mast cells (HRMC) and most of the HRMC were shown to have the phenotypic characteristics of MMC, and even when phenotypic expression of CTMC was observed at early times after fusion, it always was found to disappear in favor of MMC.

In Chapter II, it was pointed out that HRMC carry both

FcϵRI and FcϵR_L. In this Chapter, FcϵR on various HRMC are described in detail. The first part will deal with FcϵR found on HRMC obtained from the first two fusion experiments (Chapter II) while the second part will deal with HRMC described in Chapter III. The cell lines or clones chosen for more detailed FcϵR analysis were those which exhibited the more unique phenotypic charateristics. This normally turned out to be the cell lines which exhibited some characteristics of CTMC, since it was expected that such cells might also have the higher histamine content.

MATERIALS AND METHODS

Buffers and solutions:

The following buffers are used in this part of study:

(1) Phosphate buffered saline (PBS): 0.14 M NaCl, 0.01 M PO_4, pH7.4

(2) PBS/BSA: PBS containing 0.05% bovine serume albumin (BSA)

(3) SDS sample buffer: 0.0625M Tris-(hydroxymethyl) aminomethane (Tris), 4% sodium dodecyl sulfate (SDS), 10% glycerol, 0.005% bromophenol blue, pH 6.8;

Immunoglobulins:

Antibodies to the low affinity receptor for IgE($Fc\epsilon R_L$) were prepared as described previously (Chan et al., 1990) and in Chapter II. $F(ab')_2$ fragments of these antibody or of normal rabbit IgG (Sigma Chemical Co, St. Louis, MO) were prepared by digestion with pepsin. Briefly, the immunoglobulin was dissolved or dialyzed against acetate buffer pH 5.5 to yield a solution of 100mg/ml. Pepsin was added at 3% by weight of the immunoglobulin and the digestion was allowed to proceed at 37°C for six hours. The reaction was terminated

by adjusting the pH to 8.0 with 1N NaOH and the digested protein was applied onto an Ultragel AcA44 column (LKB, France) equilibrated with Tris-HCl buffer consisting of 0.15M NaCl, 0.02M Tris and titrated to pH 8.0 with HCl. The first major peak was collected, concentrated and dialyzed against PBS it was then passed through a column of Protein-A-Sepharose (Pharmacia, Uppsala, Sweden) to remove traces of undigested immunoglobulin.

Rat IgE was purified from the ascitic fluid of rats bearing the IR-162 immunocytoma as described in Chapter II; the horse anti-rat IgE was a gift from Dr. K.A. Kelly of this Department.

Cell culture and hybridization of RPMC and RBL cells

The fusion between 6-thioguanine resistant RBL cells and RPMC of Wistar-ICI rats was performed and all cell lines were maintained as described in Chapter II and III.

Preparation of affinity gel conjugates:

Conjugates of IgE-Sepharose, HARE-Sepharose and transferrin-Sepharose were prepared by the cyanogen bromide activation method (Cuatrecasas and Anfinsen, 1971). Sepharose

CL-4B (Pharmacia, Uppsala, Sweden) was washed and resuspended in equal volume of distilled water. The activation reaction was initiated by the addition of CNBr dissolved in acetonitrile (0.8g/ml) at a final concentration of 40mg CNBr per ml Sepharose. The pH of the reaction mixture was maintained between 9 and 11 by the addition of 0.5N NaOH for 15 min. The activated Sepharose was then washed consecutively with 0.05M carbonate buffer, pH10, and PBS. Sufficient amounts of proteins were added to yield conjugates of 2.5mg/ml Sepharose for HARE and, 5mg/ml Sepharose for both IgE and transferrin (Sigma, St. Louis, MO). The final volume of the reaction mixture was adjusted with PBS to yield a 50% slurry. Protein coupling was continued overnight at 4^0C with gentle mixing. Conjugates were then quenched with 1M ethanolamine in 0.2M carbonate buffer containing 0.1M NaCl, pH 8.0, followed by washing with PBS. A coupling efficiency of 90% to 97% was generally achieved and conjugates thus prepared were stored in PBS containing 0.1% NaN_3 at 4^0C until use.

Cell iodination and solubilization:

The labelling of cell surface proteins with ^{125}I (Amersham, Oakville, Ont.) was performed according to established procedures using the lactoperoxidase catalysed reaction (Conrad and Froese, 1976). Aliquots of $1x10^7$ cells

in 0.5ml volumes were labelled after washing three times with PBS. Cell surface iodination was achieved by the addition three times of three 10ul aliquots of both lactoperoxidase (Calbiochem, San Diego, CA) at 166IU/ml and 0.03% H_2O_2 at 1 min intervals. The iodination reaction was terminated by transferring the labelled cell suspension into PBS/BSA at 4^0C followed immediately by centrifugation. The resulting cell pellet was resuspended in 1ml of PBS/BSA and incubated at 37^0C for 1h with gentle mixing. Iodinated cells were then washed by centrifugation through a 2ml volume of FCS. After a final wash with PBS/BSA, $1x10^7$ labelled cells were solubilized in 200 μl of PBS containing 0.5% Nonidet-P40 (NP40) (Particle Data Laboratories, Elmhurst, IL) at 4^0C for 20 min. Particulate material was removed by centrifugation at 12,000g at 4^0C for two minutes. The supernatant thus obtained was used for the subsequent isolation of FcϵR.

Affinity isolation of receptors:

The simultaneous isolation of FcϵRI(α) and FcϵR_L was achieved by means of IgE-Sepharose conjugates (Conrad and Froese, 1978a). A solubilized cell extract from $1x10^7$ test cells was added to 200μl of IgE-Sepharose, pre-washed three times with 0.1% NP-40/PBS. After 3h with mixing at 4^0C, the affinity gel was washed three times with 0.1% NP-40/PBS

followed by a final wash with 0.0625M Tris-HCl, pH 6.8. Bound receptor material was then eluted by heating the washed affinity gels in SDS-PAGE sample buffer in a boiling water bath for 90 seconds. For the analysis of samples under reducing condition, an elution buffer containing 5% 2-mercaptoethanol was used.

Previous studies have demonstrated that only FcϵRI(α) are selectively isolated by means of IgE and HARE-Sepharose (Conrad and Froese, 1978). Thus, rat IgE purified by preparative isoelectric focusing was added to the NP-40 cell extract from $1x10^7$ cells. After one hour at 4^0C, the receptor-IgE complexes were isolated using 400 μl of HARE-Sepharose and allowing the reactants to mix for another three hours at 4^0C. Receptors were subsequently isolated as described above.

The low-affinity receptor for IgE (FcϵR_L) was isolated by rabbit anti-FcϵR_L and protein-A-Sepharose (Pharmacia, Uppsala, Sweden) as described previously (Conrad et al., 1979; Chan et al., 1990). Briefly, 50μg of rabbit anti-FcϵR_L was added to $5x10^6$ cell extracts. After one hour incubation at 4^0C, the receptor-antibody complex was isolated using 200μl of protein-A-Sepharose and the reactants were mixed for another three hours at 4^0C. Subsequent isolation procedures are as described above.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Eluates containing labelled receptors were analyzed by SDS-PAGE (10% gels) according to established procedures (Laemmli, 1970) using a "Protean" slab gel apparatus (Bio-Rad Lab., Richmond, CA). The electrophotogram was developed by autoradiography using a hypersensitized Kodak X-OMAT AR film (Eastman Kodak, Rochester, NY).

RESULTS

IgE Fc receptors of hybrid rat mast cells (HRMC):

The 11 HRMC lines, generated in the first two fusion experiments using RBL-CK2-TG and RBL-CA10.7-TG as described in Chapter II, were surface-iodinated and Fcε receptor profiles were established following receptor isolation by IgE-Sepharose and compared to those of RBL cell lines. Of the HRMC lines obtained by fusion of RPMC and RBL-CK2-TG, most exhibited a receptor pattern identical to the RBL cell parent, yielding both FcεRI(α) and $Fc\epsilon R_L$ (Fig. 1, Chapter II). HRMC3 appeared to be associated with FcεRI(α) only and this molecule was found to have a somewhat lower Mr. than other cell lines. Of the hybrid lines produced by fusing RPMC with RBL-CA10.7-TG, none appeared to have a receptor profile identical to the parent RBL cell line. HRMC9 and 10 both yielded a pattern in which both receptor molecules had a Mr. significantly different from those of the RBL parent, while HRMC11 was found to exhibit what appeared to be a single molecule, most likely FcεRI(α), having a somewhat higher Mr. than that of all the other lines.

Analysis of IgE Fc receptors of HRMC5 and its clones:

As pointed out in Chapter II, this hybrid cell line was chosen for more detailed characterization because it was the only one containing some CTMC-like cells. Initially, IgE-Sepharose was used for receptor isolation an approach which isolates both FcϵRI(α) and FcϵR_L (Conrad and Froese, 1978). As can be seen in Figure 30, the receptor patterns of most of the selected partial clones of HRMC5 are nearly identical, corresponding in mobility to those of RBL-CA10.7 and RBL-CK2-TG. Only HRMC5.1 shows a band of higher mobility which is absent from all other patterns.

To more precisely identify this extra broad band, receptors were isolated by means of IgE and anti-IgE-Sepharose, a procedure which isolates FcϵRI(α) only (Conrad and Froese, 1978). It is apparent from Figure 31, that for HRMC5.1 there are two bands representing FcϵRI(α). Also seen in this figure is the FcϵRI(α) band for HRMC9. As already seen in Figure 1, this cell line exhibits a rather unusual receptor pattern, suggesting that the Mr. of its FcϵRI(α) may be lower than that of RBL cells and most other HRMC lines.

Since HRMC5.1 may not be monoclonal in nature, the two forms of FcϵRI(α) may have had their origin on the same or on different cells. Therefore, receptors were isolated from various subclones of HRMC5.1 and analyzed by SDS-PAGE. It is

Figure 30. Isolation of Fc receptors for IgE (FcϵR) by IgE-Sepharose from HRMC5 clones and analysis by SDS- PAGE on 10% gel under nonreducing conditions.

TfR
Fc$_\epsilon$R$_L$
Fc$_\epsilon$RI(α)
RBL-CA10.7
HRMC 5.11
HRMC 5.10
HRMC 5.9
HRMC 5.8
RBL-CK2-TG
HRMC 5.1
HRMC 5.7
HRMC 5.6
HRMC 5.5
HRMC 5.4
HRMC 5.3
HRMC 5.2

Figure 31. Isolation of Fc receptors for IgE (FcϵR) by IgE and horse anti-IgE-Sepharose from HRMC8, HRMC9 and HRMC clones and analysis by SDS-PAGE on 10% gel under nonreducing conditions.

TfR–
FcεRI(α)–
HRMC 8
RBL–CA10.7
HRMC 5.11
HRMC 5.10
HRMC 5.9
HRMC 5.8
RBL–CK2–TG
HRMC 5.1
HRMC 5.7
HRMC 5.6
HRMC 5.5
HRMC 5.4
HRMC 5.3
HRMC 5.2
HRMC 9

apparent from Figure 32 and Figure 33 that the two forms of FcϵRI(α) are, in fact, associated with different cells. In another experiment, only the low affinity receptors for IgE (FcϵR_L) were isolated by means of anti-FcϵR_L-Sepharose. The receptor patterns shown in Figure 34 revealed that clones of HRMC5.1 express FcϵR_L of slightly different Mr. Also shown in this figure are FcϵR_L bands for HRMC8 and 9. While the mobility of FcϵR_L of HRMC8 corresponds in Mr. to that of HRMC5 and some of its clones that of HRMC9 is slower yet. Interestingly, the transferrin receptor band (TfR) isolated from HRMC9 also has a slower mobility. The same phenomenon is also seen in Figure 31.

Analysis of IgE Fc Receptor of HRMC12:

As pointed out in Chapter III, HRMC, when in continuous culture for prolonged periods of time, exhibit exclusively the mucosal mast cell (MMC) phenotype, even though the CTMC phenotype was found to be present among cells of some lines at early times in culture, (i.e., six weeks after fusion of parent cells). The cells chosen for the present study had been in culture for three months or more and had characteristics of MMC only. Only a few HRMC cell lines from the fusion described in Chapter III were chosen for receptor analysis, in particular HRMC12, some clones of HRMC12 and

Figure 32. Isolation of Fc receptors for IgE (FcϵR) by IgE-Sepharose from HRMC5 clones and subclones and analysis by SDS-PAGE on 10% gel under nonreducing conditions.

TfR–
FcεR$_L$–
FcεRI(α)–
RBL–CA 10.7
RBL–CK2–TG
HRMC 5
HRMC 5.1
HRMC 5.1.1
HRMC 5.1.2
HRMC 5.1.3
HRMC 5.1.4
HRMC 5.1.5
HRMC 5.1.6
HRMC 5.1.7
HRMC 5.1.8
HRMC 5.1.9

Figure 33. Isolation of Fcε receptors by IgE and horse anti-IgE-Sepharose and analysis by SDS-PAGE on 10% gel under nonreducing conditions from RBL-CK2-TG(a), HRMC5(b), HRMC5.1(c), HRMC5.1.1(d), HRMC5.1.2(e), HRMC5.1.3(f), HRMC5.1.4(g), HRMC5.1.5(h), HRMC5.1.6(i), HRMC5.1.7(j), HRMC5.1.8(k), HRMC5.1.9(l), RBLCA10.7(m).

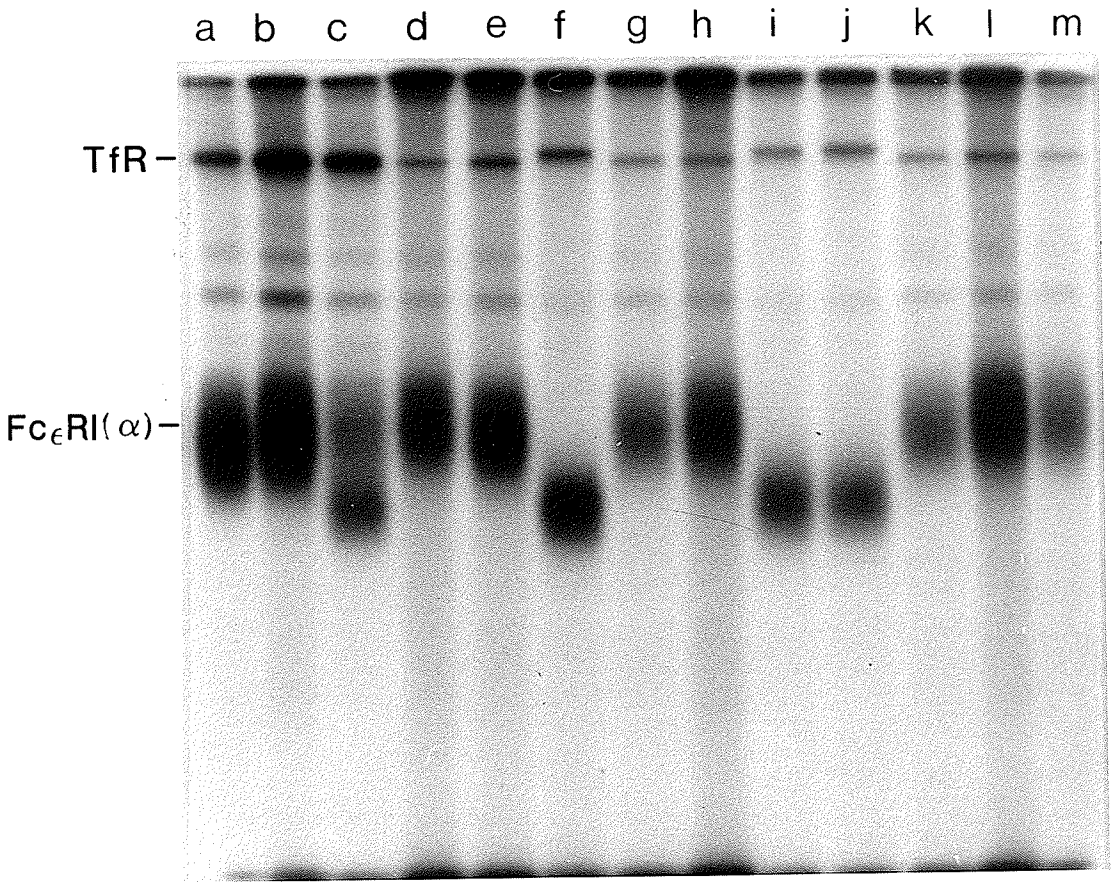

a b c d e f g h i j k l m
TfR
FcεRI(α)

Figure 34. Characterization of FcϵR from HRMC8, HRMC9, HRMC5 and clones by SDS-PAGE on 10% gel under nonreducing conditions, isolated by means of anti-FcϵR_L and protein-A-Sepharose.

TfR
FcεRL
RBL-CA 10.7
RBL-CK2-TG
HRMC 5
HRMC 5.1
HRMC 5.1.2
HRMC 5.1.6
HRMC 5.1.13
HRMC 5.1.8
HRMC 5.1.11
HRMC 5.1.12
HRMC 9
HRMC 8

HRMC13. These cells have been characterized in terms of their phenotypic as well as mediator release properties (Chapter III).

When FcεR were isolated using IgE-Sepharose, the patterns seen in Figure 35a-e were obtained for RBL-CK2-TG, HRMC12, HRMC12.6, HRMC12.4 and HRMC12.9, respectively. Only RBL-CK2-TG yielded a pattern suggesting the presence of both FcεRI(α) and $FcεR_L$. HRMC12, HRMC12.6 and HRMC12.4 appeared to be associated primarily with bands corresponding to FcεRI(α), while HRMC12.9 appeared to exhibit two bands but of somewhat slower mobility.

Isolation of receptors by means of IgE and anti-IgE-Sepharose confirmed that all the HRMC examined were associated with FcεRI(α) (Fig 35f-j) with that of HRMC12.9 having a somewhat slower mobility (Fig. 35j). When FcεRI(α) was isolated from some additional clones of HRMC12 and from HRMC13 and analyzed by SDS-PAGE, most bands were once again broad and had a mobility similar to that of RBL-CK2-TG. Only the mobility of FcεRI(α) of HRMC13 appeared to be somewhat faster (Fig. 36).

When receptors were isolated by means of anti-$FcεR_L$ and protein A-Sepharose and analyzed by SDS-PAGE, some rather surprising results were obtained (Fig. 37). Thus, two major bands of different mobility were observed. One of the bands seen in the case of RBL-CK2-TG, HRMC12 (trace), HRMC12.6, HRMC12.4 and HRMC12.9, corresponded in mobility to $FcεR_L$ seen

Figure 35. Characterization of FcεR isolated by means of IgE-Sepharose by SDS-PAGE on 10% gels (a,b,c,d,e) and IgE and horse anti-IgE-Sepharose (f,g,h,i,j) from RBL-CK2-TG (a,f), HRMC12 (b,g), HRMC12.6 (c,h), HRMC12.4(d,i) and HRMC12.9 (e,j).

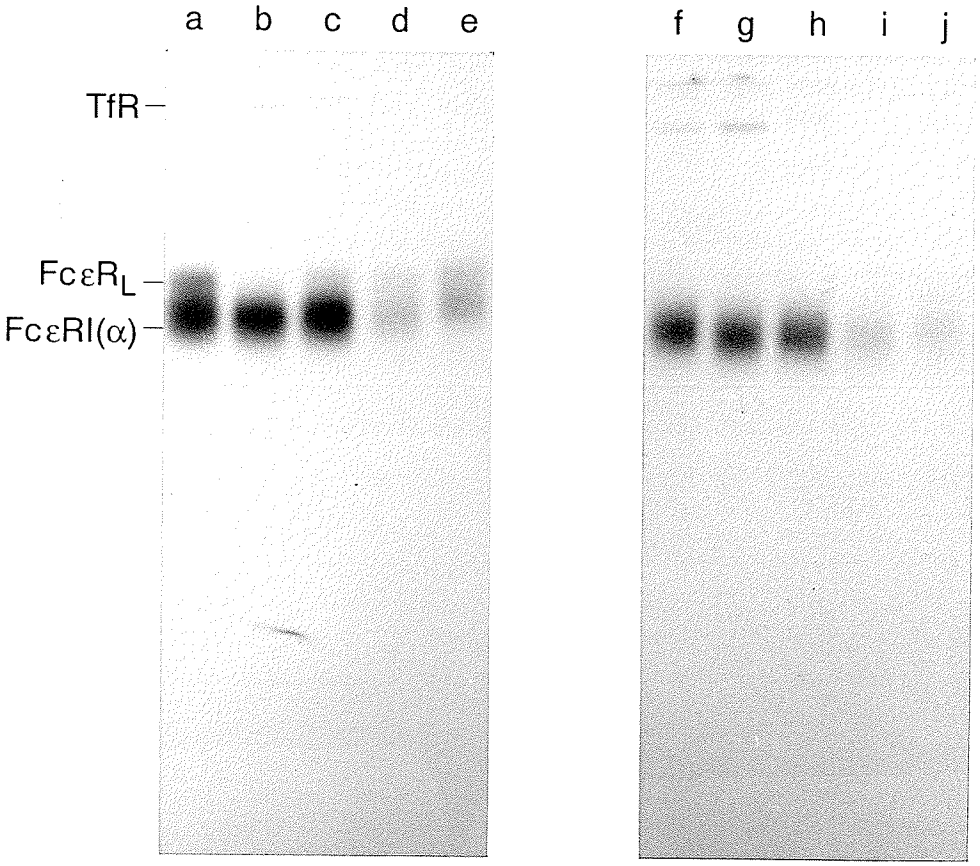

a b c d e
f g h i j
TfR
FcεR$_L$
FcεRI(α)

Figure 36. Characterization of FcϵR(α) from RBL-CK2-TG (a), HRMC12.1 (b), HRMC12.2 (c), HRMC12.3 (d), HRMC12.7 (e), HRMC12.8 (f), and HRMC13 by SDS-PAGE on 10% gel under nonreducing conditions, isolated by means of IgE and horse anti-IgE-Sepharose.

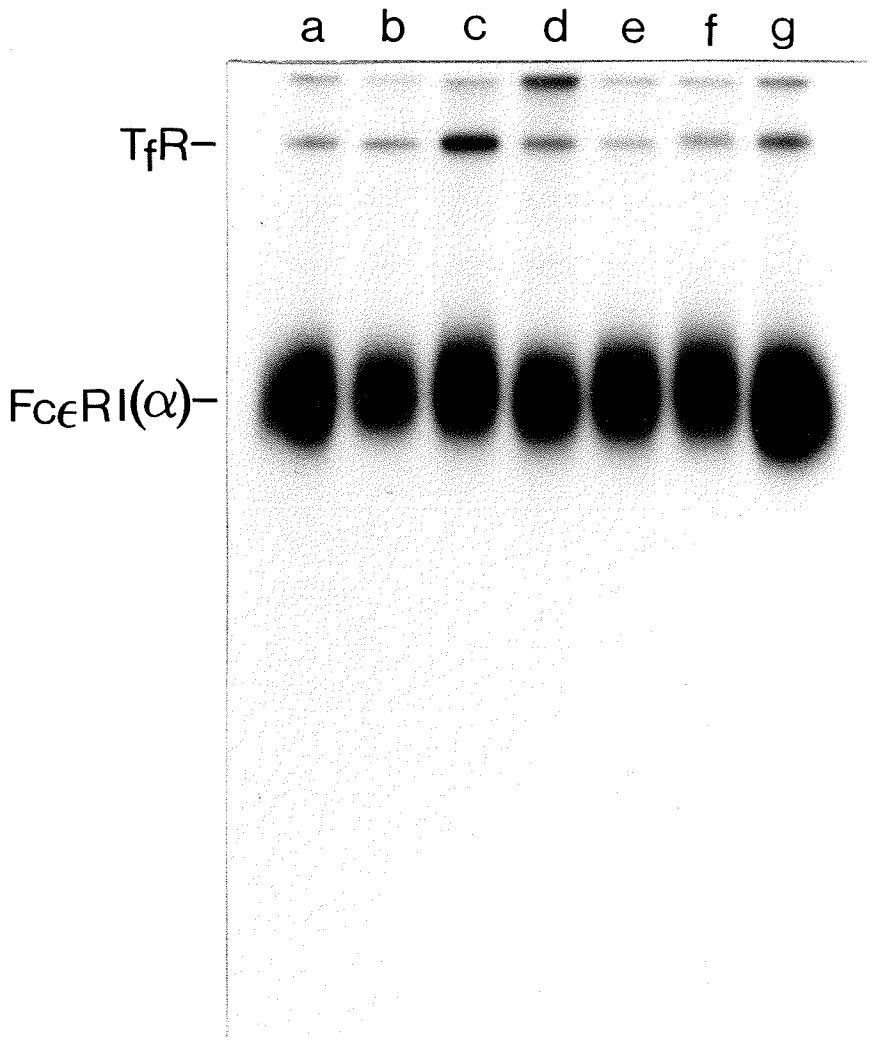
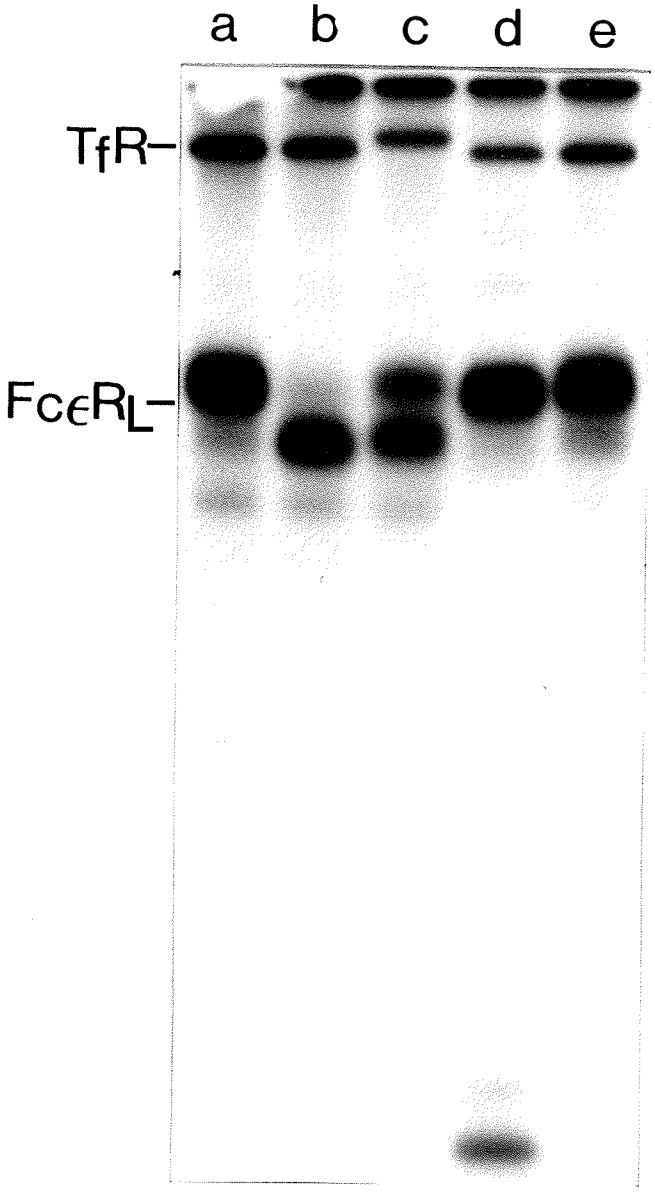

a b c d e f g
TfR–
FcεRI(α)–

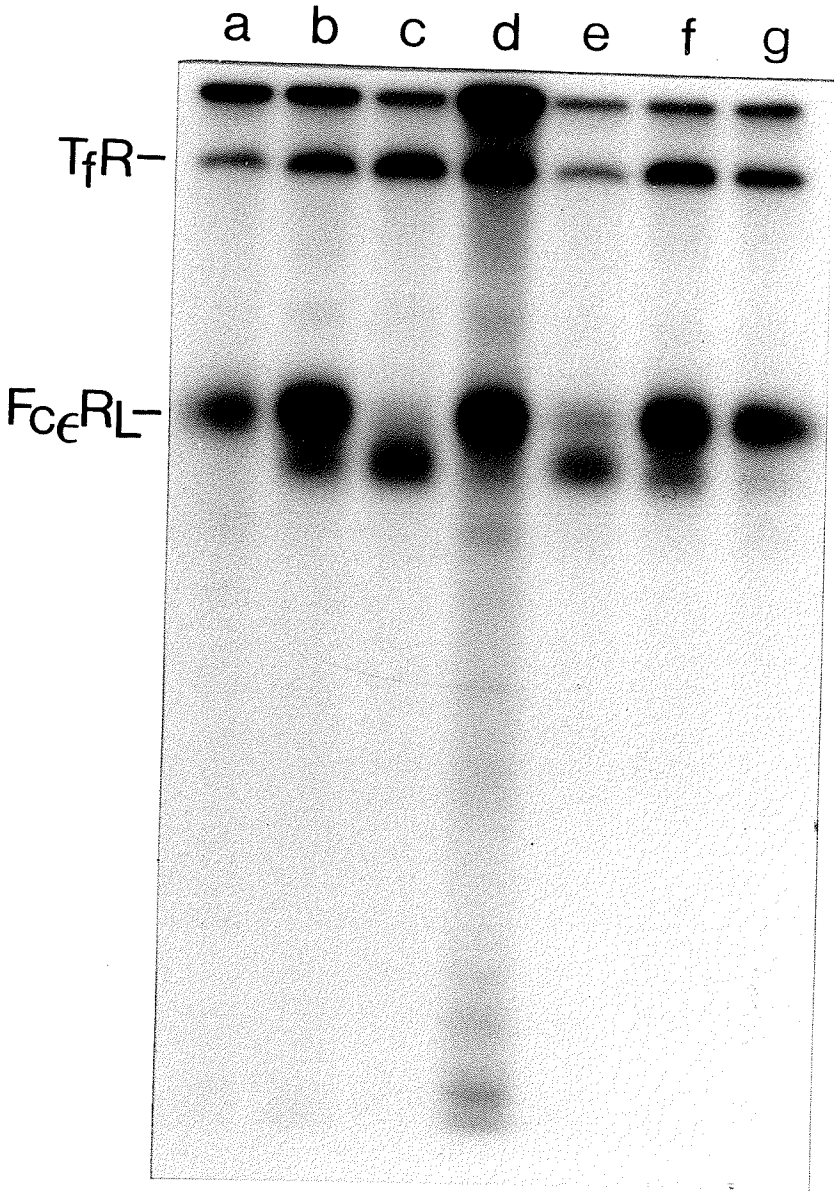
Figure 37. Characterization of FcϵR from RBL-CK2-TG (a), HRMC12(b), HRMC12.6 (c), HRMC12.4 (d) and HRMC12.9 (e) by SDS-PAGE on 10% gel under nonreducing conditions, isolated by means of anti-FcϵR_L and protein-A-Sepharose.


a
b
c
d
e
TfR–
FcεRL–

previously with RBL-CA10.7, RBL-CK2-TG and most HRMC lines derived from the first fusion with RBL-CK2-TG (Fig.1 and Fig.34). The mobility of the band for HRMC12.9 was perhaps slightly slower than the other bands. A band of significantly faster mobility was seen in the case of HRMC12 and HRMC12.6. The mobility of this band corresponded very nearly to that usually seen for FcϵRI(α) and its M_r would thus be approximately 45 kDa (Conrad and Froese, 1978). It is quite obvious that HRMC12.6 has two different cell surface molecules (Fig. 37c), both of which are recognized by anti-FcϵR_L. The intensity of these two bands appears to be nearly equal. It can be seen that the other HRMC lines may also be associated with a second band isolated by anti-FcϵR_L but these bands were significantly lower in intensity. RBL-CK2-TG, HRMC12 and HRMC12.6 also exhibited traces of yet a third band of even faster mobility, the identity of which is unknown.

An analysis of receptors isolated by measns of anti-FcϵR_L from additional clones of HRMC12 and from HRMC13 was also indicative to the presence of two kinds of receptors reactive with this antibody (Fig. 38). Thus all HRMC the FcϵR of which were analyzed, are associated with both types of FcϵR_L. The type of higher Mr. which was associated with RBL-CK2-TG, predominated in the case of HRMC12.1, HRMC12.3, HRMC12.8 and HRMC13 while that of lower Mr. was more prevalent in the case of HRMC12.2 and HRMC12.7 (Fig. 38).

Figure 38. Characterization of FcϵR from RBL-CK2-TG (a), HRMC12.1 (b), HRMC12.2 (c), HRMC12.3 (d), HRMC12.7 (e), HRMC12.8 (f), and HRMC13 by SDS-PAGE on 10% gel under nonreducing conditions, isolated by means of anti-FcϵR_L and protein-A-Sepharose.


a b c d e f g
TfR–
FcεRL–

DISCUSSION

In contrast to the other phenotypic data on HRMC and their clones, the FcϵR patterns of these cells are found to exhibit quite a degree of variability over long-term culture conditions even though the parent cells (i.e., the RBL cells and RPMC) are known to possess FcϵRI(α) and FcϵR_L which, in terms of Mr., are nearly identical (Froese, 1980). This variability is also greater than that observed in the case of various RCMC lines and their clones (Chan et al., 1988; Chan et al., 1990).

Among the HRMC lines generated in the first two fusions, HRMC9 and 10 exhibit some unique receptor patterns. While FcϵRI(α) of these two cell lines migrates faster than that of its parent RBL-CA10.7-TG; FcϵR_L has a slower mobility (Fig. 1). Different receptor patterns were also found for HRMC3 and HRMC11. Only FcϵRI(α), with a Mr. somewhat lower than that of the parent RBL-CK2-TG and most of the other HRMC lines, is seen in the case HRMC3. The pattern of HRMC11 resembles that of RBL-2H3 cells (Froese et al., 1982; Chan et al., 1990; Swieter et al., 1989) which were previously shown to have FcϵRI(α) and FcϵR_L which overlap in terms of Mr. (Froese et al., 1982).

Interesting receptor patterns were also seen in the case of HRMC5.1 and its clones. HRMC5.1 clearly yielded two

FcϵRI(α) bands differing significantly in Mr. (Figs. 30 and 31). Thus, HRMC5.1.1, 5.1.2, 5.1.4, 5.1.5, 5.1.8, 5.1.9, and 5.1.10 express a FcϵRI(α) receptor band of "normal" Mr., corresponding to that of RBL-CA10.7 and RBL-CK2-TG, while HRMC5.1.3, 5.1.6, 5.1.7 possess one of much lower Mr. These findings demonstrate further that HRMC5.1 is not monoclonal but consists of at least two clones of cells. Differences in the Mr. of FcϵRI(α) have previously been observed among RBL-WPG and RBL-CA10.7 on the one hand and RBL-NIH on the other. They were attributed to differences in the glycosylation of these molecules and not to differences in their polypeptide chains (Chan et al., 1990; Froese, 1984).

Variations in the Mr. were also observed among the FcϵR_L of the clones of HRMC5.1; some have the Mr. of the parent RBL cells, while others appear to have a lower one. Both forms of this receptor differ from that on HRMC9 (Fig. 32).

The receptor analysis of HRMC described in Chapter III once again revealed some interesting differences for different cell lines or clones. While the FcϵRI(α) band appeared to be nearly identical for the different cells examined, the receptors isolated by anti-FcϵR_L fell into two categories which are easily distinguishable in terms of mobility on SDS-PAGE gels and thus in terms of Mr.. These differences appear to be larger than those seen in HRMC9 and HRMC5.1 clones. It is interesting to note that some cells, i.e., HRMC12.4 and

HRMC12.9, carry primarily the $Fc\epsilon R_L$ molecule of higher Mr., while HRMC12 is associated primarily with the molecule of lower Mr.. HRMC12.6 appears to be associated with both molecules. Unfortunately, in spite of the fact that HRMC12.6 is a cloned line of HRMC, it cannot be concluded that individual HRMC12.6 cells carry both molecules on their surface. These HRMC were cloned from HRMC12 when the cells were still undergoing changes in DNA content (Fig. 29). Therefore, it is conceivable that cells of this "clone" differentiated in different ways some to express one of the anti-$Fc\epsilon R_L$ binding molecules and some to express the other.

It should be pointed out that different forms of $Fc\epsilon R_L$ have been described previously (Froese et al., 1982; Swieter et al., 1989), although in those cases the two forms were derived from the same population of either RBL-NIH (Froese et al., 1982) or RBL-2H3 (Swieter et al., 1989) and intestinal mucosal mast cells (IMMC) (Swieter et al., 1989). In the latter two instances, the possibility was raised that one of the two forms of the receptors may have represented a degradation product of the other. The present finding that different cells can carry different forms of this receptor lends more evidence to the possibility that the two $Fc\epsilon R_L$ bands seen previously may actually have been due to distinct low affinity receptors on different subtypes of cells present in the same population. Once again, it is conceivable that differences among $Fc\epsilon R_L$ may reside in their carbohydrate

moiety. In view of the recent finding that FcγRII and FcγRIII receptors are present on mouse mast cells (Benhamou et al., 1990; Katz et al., 1990), the data presented here and described previously (Swieter et al., 1989) may have to be interpreted differently. Thus, it would be reasonable to suggest that the more slower migrating form of FcϵR_L which had been shown earlier to have a Mr. of 55 kDa is, in fact, FcγRII or more precisely the rat equivalent of mouse FcγRII-1. The band of faster mobility would then be FcγRIII which, on mouse serosal mast cells, was shown to have an Mr. of 43 kDa (Katz et al., 1990). In fact, a study from this labarotory has also shown (LaCroix and Froese, 1993) that when NP-40 extracts of RBL-CA10 cells are treated with N-glycanase to remove N-linked oligosaccharides, one major band of Mr. of 38 kDa is isolated by means of anti-FcϵR_L and traces of a band of Mr. 29-30 kDa are seen. In the mouse system, these Mr. values would correspond to the deglycosylated forms of FcγRII-1 and FcγRIII, respectively (Katz et al., 1990).

The conclusion that the two bands, isolated from HRMC by means of anti-FcϵR_L are rat equivalents of mouse FcγR is further supported by the preliminary data of [^{3}H]-5HT release experiments (data not shown). These experiments which, at this point, must be considered preliminary, suggest that the faster migrating band is involved in mediator release as demonstrated by results obtained with HRMC12 and HRMC12.6, while the receptor represented by the slower migrating band,

corresponding to Mr. of about 55 kDa does not participate in triggering [^{3}H]-5HT release, as seen through experiments with HRMC12.4 and HRMC13.

It should be noted that in the present study, [^{3}H]-5HT release experiments were carried out using $F(ab')_2$ fragments of anti $Fc\epsilon R_L$. This was done to avoid any binding of the Fc fragment to $Fc\epsilon R_L$. That such an interaction is possible in the case of rabbit IgG was previously shown (Kepron et al., 1988). Since whole IgG and not specifically purified antibodies were used, it is conceivable than an Fc-FcR interaction might have inhibited the cross-linking of $Fc\epsilon R_L$ through anti-$Fc\epsilon R_L$.

In conclusion, it thus appears that the rat receptor which we have so far designated $Fc\epsilon R_L$ or H is, in fact, the rat equivalent of mouse FcγRII-1 and that on some rat mast cells a second Fc receptor which is antigenically closely related to $Fc\epsilon R_L$ can be found. This Fc receptor is most likely analogous to mouse FcγRIII. There can, however, be no doubt that signal transduction on mast cells can take place not only via FcεRI but also via a second receptor, most likely FcεRIII and that the latter can transmit signals either via an IgE or IgG mediated mechanism.

CHAPTER V

GENERAL DISCUSSION

Although mast cells were discovered long ago, they are certainly no less interesting today and our understanding of mast cell biology is far from complete. The development of mast cells from haematopoietic precursor cells is a complex process resulting in two phenotypically distinct populations, CTMC and MMC. Such definition by no means represents two fixed, uniform, unrelated cellular entities, it would rather indicate they are at two different stages in the mast cell development spectrum (Bloom, 1984; Galli, 1990). Both in vitro and in vivo interconversions between these two subtypes have been reported in the literature. Thus, it was shown in the mouse that co-culturing of mouse BMMC, MMC-like cells, with mouse fibroblasts 3T3 can yield mast cells with a CTMC phenotype: i.e., cells become alcian blue$^+$/safranin O$^+$ (AB$^+$/SO$^+$), synthesize heparin proteoglycans and have high histamine content (Levi-Schaffer et al., 1986; 1987; Dayton et al., 1988). When WBB6F$_1$-W/W^v mice are reconstituted with factor-dependent MMC cultured from the bone marrow of congenic +/+ mice, histochemical studies show mast cells have different staining properties depending on the anatomical locations; mast cells stained AB$^+$/SO$^+$ in the skin, peritoneal cavity and AB$^+$/SO$^-$ in the intestinal mucosa (Nakano et al., 1985; Kitamura, 1989). Injection of peritoneal mast cells into WBB6F$_1$-W/W^v mice results in a similar mast cell distribution (Kobayashi et al., 1986; Sonoda et al., 1986; Kitamura, 1989). While these findings may suggest that the phenotype of a

given mast cell is entirely dependent on the microenvironment in which it finds itself, it is even more likely that each population of mast cells used in these studies contained precursor cells capable of differentiating into either phenotype. Actually, BMMC may not represent fully differentiated cells, and RPMC may contain a small percentage of immature mast cells (Mendonca et al., 1986).

As described in Chapter II and III, hybrid mast cells do express some CTMC-like phenotype at certain stages of development, i.e., positive staining with alcian blue and safranine O, presence of RMCPI, release of [^{3}H]-5HT in response to compound 48/80, although eventually the MMC phenotype predominates. There are several possibilities to explain such phenotypic changes. It has been suggested that the specific microenvironments in which mast cell reside will have decisive influence on the mast cell phenotypes (Kitamura, 1989; Galli, 1990). Thus, it has been suggested that most, if not all, aspects of mast cell development, including growth, proliferation and the differentiation/maturation are regulated by two important induction mechanisms: the T cell derived lymphokines such as IL-3, IL-4, IL-9, and IL-10; the fibroblast-dependent mast cell development mediated mainly by SCF through c-kit receptor tyrosine kinase (Galli, 1990; Swieter et al., 1992; Tei et al., 1994; Galli et al., 1994). Rat recombinant SCF (rrSCF) can stimulate the proliferation of both CTMC and BMMC either <u>in vivo</u> or <u>in vitro</u> and can also

induce some maturation of MMC-like cells. IL-3 stimulates BMMC growth to a lesser extent than does SCF, and SCF and IL-3 together have synergistic effects on the growth of both BMMC and CTMC (Tsai et al., 1991a; 1991b; Wershil et al., 1991; Haig et al., 1994). The fusion of RPMC (CTMC-like) and RBL-CK2-TG (MMC-like) in this project may not provide all the necessary microenvironmental factors needed in maintaining the CTMC phenotype. However, in the present study, attempts to prevent a change from the CTMC to the MMC phenotype and to reverse the changed MMC to CTMC phenotype using either rat or mouse fibroblasts in co-culturing experiments proved unsuccessful. Also, preliminary experiments (data not shown) using recombinant mouse SCF could not convert MMC-like HRMC to CTMC-like cells. However, since the amino acid sequences of mouse and rat SCF are not completely identical (Anderson et al., 1990; Martin et al., 1990), species differences may account for the negative results. An absence of the c-kit receptor on HRMC could also explain why fibroblasts or SCF failed to maintain the CTMC phenotype or to re-establish it. Indeed, c-kit negative mast cells have been reported. Recently, a rat mutant with a 12-base deletion in the tyrosine kinase domain of the c-kit gene was identified and the homozygous Ws/Ws rats are deficient in both CTMC and MMC (Tsujimura et al., 1991; Tei, et al., 1994). In addition, Dvorak and colleagues assessed the cell surface expression of FcεR and c-kit in mouse basophils and mast cells present in

the short-term culture of mouse bone marrow cells in IL-3 with or without SCF (Dvorak et al., 1994). Although most mast cells are found to be $Fc\epsilon R^+$ and c-kit$^+$ in this study, a second population of $Fc\epsilon R^+$, c-kit$^-$ mast cells was present after culture in IL-3 and SCF and this population was less mature and contains significantly fewer granules. In this laboratory, exploratory reverse transcriptase-polymerase chain reaction (RT-PCR) experiments revealed the presence of mRNA for the c-kit receptors in HRMC12, HRMC13 but not in HRMC12.4; thus yielding no conclusive correlation between the early phenotype of these cells and the potential presence or absence of the c-kit receptor. In view of these preliminary experiments, unequivocal evidence for the involvement (or absence thereof) of SCF and its receptor in the phenotypic expression of HRMC will have to await more detailed studies.

It has been demonstrated in Chapters II and III that there is a significant loss in DNA content between six and eight weeks of continuous cell culture and karyotype analysis showed that the chromosome number of selected HRMC is close to 2N after prolonged culture. These results cannot prove conclusively that the phenotypic changes are a consequence of a chromosome loss, they do , however, point in that direction. They also suggest that the chromosome loss involves primarily those of the RPMC parent. Moreover, if a chromosome loss is responsible for the disappearance of the CTMC phenotype, it, most likely, involves the loss of a regulatory gene. It is

much less likely that it involves several individual genes which control the outcome of histochemical staining, mediator release due to compound 48/80 or RMCPI expression.

It should be pointed out that the hybrid rat mast cells (HRMC) generated in this project are different from the rat tissue-cultured mast cells (RCMC) previously established in this laboratory (Chan et al., 1988; 1990). RCMC were produced by extended in vitro cell culture, 10-12 weeks on average, of purified rat peritoneal mast cells (RPMC) in the absence of any conditioned medium, exogenous cytokines or feeder cells. They are continuously proliferative cell lines and have been maintained in cell culture for more than five years. Both high affinity receptor for IgE (FcϵRI) and low affinity receptor (FcϵR_L) are present on RCMC lines or clones. Although RCMC lines are derived from CTMC, they exhibited phenotypic characteristics of MMC only throughout the five-year study period, i.e., they contain RMCPII instead of RMCPI, have relatively low levels of histamine, stained alcian blue$^+$/Safranine O$^-$ and fail to release [^{3}H]-5HT upon exposure to compound 48/80. Karyotype analysis of RCMC indicated the absence of a large abnormal metacentric marker chromosome, M8, first discovered among RBL-CA10.7. The nature of cells which gave rise to RCMC is not clear (Chan et al., 1990). On the other hand, HRMC are produced by fusing RPMC and 6-thioguanine resistant, HAT sensitive RBL-CK2-TG or RBL-CA10.7-TG cells.

Viable hybrid cells were found after five or six weeks in cell culture. Most importantly, several cell lines or their clones exhibit some CTMC-like phenotypes at certain development stages, i.e., stained alcian blue$^+$/safranine O$^+$, contain RMCPI, and respond to compound 48/80 stimuli by releasing [^{3}H]-5HT. Moreover, the karyotyping revealed that the HRMC lines and RBL-CK2-TG examined were associated with the M8 chromosome marker (Chapter II).

Based on the phenotypic characterization, it is possible that it was the same small population of RPMC, which was reported to be morphologically immature (Mendonca et al., 1986), that gave rise to RCMC. It is also conceivable that these immature cells , as fusion partner of RBL cells, gave rise to the majority of HRMC, particularly since the most of the hybrid cells screened, exhibited only the MMC phenotype even at early times in culture (Chapter III).

Four species of mouse FcγR have been identified so far: FcγRI, which binds IgG_{2a} with high affinity, and FcγRII1, FcγRII2 and FcγRIII, all of which bind IgG_1, IgG_{2a}, and IgG_{2b} with low affinity (Amigorena et al., 1992). FcγRII1 and FcγRII2 are formed by alternative splicing of the FcγRII gene transcript and they are identical except for a 47 amino acid insertion in the cytoplasmic domain of FcγRII1. FcγRIII is 95% homologous in the extracellular domain with that of FcγRII (Ravetch et al., 1986; Ra et al., 1989). Only recently was

it shown that these receptors also interact with IgE (Takizawa et al., 1992), albeit to lesser extent than with IgG. Previous studies from this laboratory have shown, using soluble receptors, that $Fc\epsilon R_L$ also reacts with rat IgG subclasses but less effectively than with IgE (Kepron et al., 1988). Whether these rat and mouse receptors differ in their specificity for IgE and IgGs will have to await a comparative study using an identical experimental approach. As discussed in Chapter IV, there are two forms of $Fc\epsilon R_L$ with different Mr. on SDS-PAGE analysis and these two may represent the rat FcγRII-1 and FcγRIII, respectively. It has been shown by using RBL cells transfected with either FcγRII or FcγRIII, of the mouse that only the latter receptor is involved in serotonin release (Daeron et al., 1992). It is now known that in the mouse FcγRIII is associated with the γ-chain (Ra et al., 1989) which is also part of FcεRI (Perez-Montford et al., 1983) and that it is the γ chain which mediates signal transduction (Wirthmueller et al.,1992). It should be noted that it is unlikely that the release of [^{3}H]-5HT from HRMC12 and HRMC12.6 was mediated by FcεRI since the anti-$Fc\epsilon R_L$ antibody did not react with FcεRI(α) (Kepron et al., 1988). Moreover, this receptor was also found on the HRMC lines which did not release [^{3}H]-5HT. These latter lines have been shown to release [^{3}H]-5HT when they are exposed to either calcium ionophore A23187 or IgE and antigen (Chapter III).

From the results obtained here it can be inferred that

the putative FcγRII and FcγRIII of the rat, like the corresponding receptors of mouse, have extracytoplasmic domains which exhibit considerable homology (Ravetch et al., 1986), since they both react with the same antibody (Katz et al., 1990). The anti-FcϵR_L used in this study is a polyclonal antibody and therefore its reactivity with two different receptors is less surprising than the reactivity of monoclonal antibody 2.4G2 with the two FcγR of the mouse (Katz et al., 1990).

In summary, the present investigation has achieved at least some of the objectives of the project. Thus fusion of RPMC with RBL-CK2-TG and RBL-CA10.7-TG has successfully yielded hybrid rat mast cells which could be maintained in the tissue culture without added factors or feeder layer of fibroblasts. Although none of the HRMC came near to parent RPMC in histamine content , some of the hybrids contained more of this mediator than the parent RBL cells. Moreover, some of the lines were found to have an intact mediator release mechanism. These latter cells do have an advantage over the releasing RBL-2H3 cells in that they carry FcϵR_L and FcϵRI(α) which are clearly distinguishable on the basis of Mr. while those of RBL-2H3 are not (Froese et al., 1982).

The different HRMC showing notable differences in the Mr. of FcϵRI(α) and FcϵR_L should prove useful in studies on difference in the carbohydrate moiety of these receptors and thus contribute to the understanding of microheterogeneity of

these receptors (LaCroix and Froese, 1993). Moreover, the discovery of HRMC with two different forms of $Fc\epsilon R_L$ which may turn out to be the rat equivalent of mouse $Fc\gamma RII$ and $Fc\gamma RIII$ should help to gain an understanding of the biological functions of these two receptors on rat mast cells.

Finally, although the phenotypic changes found to occur among HRMC are as yet not clearly understood, once solved they may provide some insight into the differentiation of mast cells.

BIBLIOGRAPHY

Abe, T., Ochiachi, H., Minamishima, Y and Nawa, Y. (1988) Int. Arch. Allergy Arch. Immunol. 86:356

Ahlstedt, s., and Haard, J. (1987) Int. Archs. Allergy Appl. Immnunol. 84:359

Amigorena, S., Bonnerot, C., Drake, J.R., Choquet, D., Hunziker, W., Guillet, J.G., Webster,P., Sautes, C., Mellman, I., and Fridman, W.H. (1992) Science 256:1808

Anderson, D. M., Lyman, S. D., Baird, A., Wignall, J. M., Eisenman, J., Rauch, C., March, C. J., Boswell, H. S., Gimpel, S. D., Cosman, D., Williams, D. E. (1990) Cell 63:235

Bach, M. K., Bloch, K. J., and Austen, K.F. (1971) J. Exp. Med. 133:752

Bach, M. K. and Brashler, J. R. (1977) Int. Archs. Allergy Appl. Immunol. 55:255

Bazin, H., Querijean, P., Beckers, A., Heremans, J. F., and Dessy, F. (1974) Immunology 26:713

Befus, A.D., Pearce, F.L., Gauldie, J., Horsewood, P., Bienenstock, J. (1982) J. Immunol. 128:2475

Benfey, P.N., Yin, F.H., and Leder, P. (1987) J. Biol. Chem. 262:5377

Benhamou, M., Bonnerot, C., Fridman, W.H. and Daëron, M. (1990) J. Immunol. 144:3071

Berlin, G. and Enerbäck, L. (1983) Int. Archs Allergy appl. Immunol. 71:332

Bieber, T., and Ring, J. (1992) Int. Arch Allergy Immunol. 99:204

Bieber, T., Salle, H. D. L., Wollenberg, J. H., Chizzonite, R., Ring, J., Hanau,D., Salle, C. D. L. (1992) J. Exp. Med. 175:1285

Bienenstock, J., Befus, A.D., and Denburg, J.A. (1986) in "Mast Cell Differentiation and Heterogeneity", (Ed. by Befus, A.D., Bienenstock, J., and Denburg, J.A.), Raven Press, New

York, p.391

Bienenstock, J., Austen, K.F., and Galli, S.J.(1989) in "Mast cells and Basophils Differentiation and Function in Health and Disease" (Ed. by Galli, S.J. and Austen, K.F.), Raven Press, New York, p.329

Bischoff, S.C., and Dahinden, C.A. (1992) J. Exp. Med. 175:237

Blank, U., Ra, C., Miller, L., White, K., Metzger, H., Kinet, J.-P. (1989) Nature 337:187

Bocek, P., and Petcht, I. (1993) FEBS Letters 331:86

Bonnefoy, J. Y., Aubry, J. P., Peronne, C., Wijdense, J., and Banchereau, J. (1987) J. Immunol. 138:2970

Bonnefoy, J. Y., Guillot, O., Spits, H., Blanchard, D., Ishizaka, K., and Banchereau, J. (1988) J. Exp. Med. 167:57

Broide, D.H., Wassermann, S.I., Alvaro-Gracia, J., Zvaifler, N.J., and Firestein, G.S. (1989) J. Immunol. 143:1591

Burd, P. R., Rogers, H. W., Gordon, J. R., Martin, G. A., Jayaraman, S., Wilson, S. D., Dvorak, A. M., Galli, S. J.., and Dorf, M. E. (1989) J. Exp. Med. 170:245

Carson, D.A., and Metzger, H. (1974) J. Immunol. 113:1271

Carsten, D.H. and Eisen, H.N. (1953) J. Ameri. Chem. Soc. 75: 4451

Castells, M., Katz, H.R., and Austen, K.F. (1992) Int. Arch Allergy Immunol. 99:189

Chabot, B., Stephenson, D. A., Chapman, V. M., Besmer, P., Bernstein, A. (1988) Nature 335:8889

Chan, B. M. C., McNeill, K., Berczi, I., and Froese, A. (1986) Eur. J. Immunol. 16:1319

Chan, B. M. C., McNeill, K., and Froese, A. (1988) Immunol. Lett. 18:37

Chan, B. M. C., Ra, C., Hu, W., McNeill, K., Miller, H.R. P., Befus, D., Kinet, J. P., and Froese, A. (1990) Eur. J. Immunol. 20:185

Coleman, J.W., Holliday, M.R., Kimber, I., Zsebo, K.M., and Galli, S.J. (1993) J. Immunol. 150:556

Columbo, M., Horowitz, E.M., Botana, L.M., MacGlashan, D.W.Jr,

Bochner, B.S., Gillis, S., Zsebo, K.M., Galli, S.J., and Lichtenstein, L.M. (1992) J. Immunol. 149:599

Combs, J. W., Lagunoff, D. and Benditt, E. P. (1965) J. Cell Biol. 25:577

Conrad, D. H., Bazin, H., Sehon, A. H., and Froese, A. (1975) J. Immunol. 114:1688

Conrad, D. H., Berczi, I., and Froese, A. (1976) Immunochemistry 13:329

Conrad, D. H., and Froese, A. (1976) J. Immunol. 116:316

Conrad, D. H., and Froese, A. (1978a) J. Immunol. 120:429

Conrad, D. H. and Froese, A. (1978b) Immunochemistry 15:283

Conrad, D. H., Froese, A., Ishizaka, T., and Ishizaka, K. (1978) J. Immunol. 120:507

Conrad, D. H., Helm, R. M., Yiu, S. H., Froese, A., Ishizaka, T., and Ishizaka, K. (1979) J. Immunol. 122:2238

Conrad, D. H. (1990) Ann. Rev. Immunol. 8:623

Conrad, D. H., Ben-Sasson, S. Z., Gros, G. L., Finkelman, F. D., and Paul, W.E. (1990) J. Exp. Med. 171:1497

Copeland, N. G, Gilbert, D. J., Cho, B. C., Donovan, P. J., Jenkins, N. A., Cosman, D., Anderson, D., Lyman, S. D., Williams, D. E. (1990) Cell 63:175

Daeron, M., Duc, H. T., Kanellopoulos, J., Lebouteiller, P., Kinsky, R. and Voisin, G. A. (1975) Cell. Immunol. 20:133

Daëron, M., Latour, S., Hückel, C., Bonnerot, C., and Fridman, W.H. (1992) Immunobiol. 185:159

Dayton, E.T., Pharr, P., Ogawa,M., Serafin, W.E., Austen, K.F., Levi-Schaffer, F., and Stevens, R.L. (1988) Proc. Natl. Acad. Sci. USA. 85:569

Diamantis, I. D., Nair, A. P. K., Hirsch, H. H., and Moroni, C. (1989) Proc. Natl. Acad. Sci. USA 86:9299

Drickamer, K. J. (1988) J. Biol Chem. 263:9557

Dvorak, A.M., Nabel, G., Pyne, K., Cantor, H., Dvorak, H.F. and Galli, S.J.(1982) Blood 59:1279

Dvorak, A.M., Dvorak, H.F., and Galli, S.J. (1983a) Am. Rev.

Respir. Dis. 128:s49

Dvorak, A.M., Galli, S.J., Schulman, E.S., Lichtenstein, L.M., and Dvorak, H.F. (1983b) Fed. Proc. 42:2510

Dvorak, A.M., Seder, R.A., Paul, W.E., Morgan, E.S., and Galli, S.J. (1994) Am. J. Pathol. 144:160

Eccleston, E., Leonard, B. J., Lowe, J. S.and Welford, H. J. (1973) Nature (New Biol.) 244:73

Ehrlich,P. (1878) Beiträge zur Theorie und Praxis der histologischen Färbung. Leipzig; 1878 Thesis

Eklund, K.K., Ghildyal, N., Austen, K.F., Friend, D. S., Schiller, V., Stevens, R.L. (1994) J. Exp. Med. 180:67

Enerbäck, L. (1966a) Acta Pathol. Microbiol. Scand. 66:289

Enerbäck, L. (1966b) Acta Pathol. Microbiol. Scand. 66:303

Enerbäck, L. (1966c) Acta Pathol. Microbiol. Scand. 66:313

Enerbäck, L. (1974) Histochemistry 42:301

Enerbäck, L., and Svensson, I. (1980) J. Immunol.Method 39:135

Enerbäck, L. and Wingren, U. (1980) Histochemistry 66:113

Enerbäck, L. (1981) Monogr. Allergy 17:222

Enerbäck, L. and Rundquist, I. (1981) Histochemistry 71:521

Enerbäck, L., Kolset, S. O., Kusche, M., Hjerpe, A., and Lindahl, U. (1985) Biochem J. 227:661

Enerbäck, L. (1986) in "Mast Cell Differentiation and Heterogeneity", (ed. by Befus, A.D., J. Bienenstock and J.A. Denburg) Raven Press, New York, p. 1.

Fewtrell, C. and Metzger, H. (1980) J. Immunol. 125:701

Flanagan, J. G., Chan, D. C., Leder, P. (1991) Cell 64:1025

Flanagan, J. G. and Leder, P. (1990) Cell 63:185

Froese, A. (1980) J. Immunol. 125:981

Froese, A., Helm, R. M., Conrad, D. H., Isersky, C., Ishizaka, T., and Kulczycki, A., Jr. (1982) Immunology 46:107

Froese, A. (1984) Progr. Allergy. 34:142

Fujita, J., Onoue, H., Ebi, Y., Nakayama, H., Kanakura, Y. (1989) Proc. Natl. Acad. Sci. USA 86:2888

Funasaka, Y., Boulton, T., Cobb, M., Yarden, Y., Fan, B., Lyman, S.D., Williams, D.E., Anderson, D.M., Zakut, R., Mishima, Y., and Halaban, R. (1992) Mol. Biol. Cell 3:197

Galli, S.J., Dvorak, A. M., Marcum, J. A., Ishizaka, T., Nabel, G., DerSimonian, H., Pyne, K., Goldin, J. M., Rosenberg, D. R., Cantor, H., and Dvorak, H. F. (1982) J. Cell. Biol. 95:435

Galli, S.J., Dvorak, A.M., and Dvorak, H.F. (1984) Prog. Allery 34:1

Galli, S.J. and Lichtenstein, L.M. (1988) in "Allergy. Principles and Practice", (Ed. by Middleton Jr, E., Reed, C.E.,Ellis, E.F., Adkinson Jr, N.F., and Yunginger, J.W.), The CV Mosby Company, St.Louis, p.106

Galli, S.J. (1990) Laboratory Investigation 62:5

Galli, S. J., Gordon, J. R., and Wershil, B. K. (1991) Current Opin. Immunol. 3:865

Galli, S.J., Tsai, M.,and Wershil, B.K. (1992) Int. Arch Allergy Immunol. 99:234

Galli, S.J., Tsai, M. and Wershil, B.K. (1993) Amer. J. Path. 142:965

Galli, S.J., Zsebo, K.M., and Geissler, E.N. (1994) Advan. Immunol. 55:1

Geissler, E.N., Ryan, M.A., Housman, D.E. (1988) Cell 55:185

Gibson, S., and Miller, H. R. P. (1986) Immunology 58:101

Ginsburg, H., Ben-Shahar, D., and Ben-David, E. (1982) Immunology 45:371

Godin, I., Deed, R., Cooke, J., Zsebo, K.M., Dexter, M., and Wylie, C.C. (1991) Nature 352:807

Gonzalez-Molina, A., and Spiegelberg, H.L. (1976) J. Immunol. 117:1983

Gordon, J. R.and Galli, J. S. (1990) Nature 346:274

Gounni, A. S., Lamkhioued, B., Ochiai, E., Tanaka, Y., Delaporte, E., Capron, A., Kinet, J-P., Capron, M. (1994) Nature 367:183

Grabbe, J., Haas, N., Hamann, K., Kolde, G., Hakimi, J., Czarnetzki, B. M. (1993) Brit. J. Dermatol. 129:120

Gregory, C. J., Eaves, A. C. (1978) Blood 51:527

Grissman, H.A. and Steinkamp, J.A. (1973) J. Cell.Biol. 59:766

Gros, G.L., Ben-Sasson, S.Z., Conrad, D.H., Clark-Lewis, I., Finkleman, F.D., Plaut, M., and Paul, W.E. (1990) J. Immunol. 145:2500

Haig, D.M., McKee, T.A., Jarrett, E.E.E.(1982) Nature 300:188

Haig, D.M., Huntley, J.F., MacKeller, A., Newlands, G.F.J., Inglis, L., Sangha, R., Cohen, D., Hapel, A., Galli, S.J., and Miller, H.R.P. (1994) Blood 83:72

Hamaguchi, Y., Kanakura, Y., Fujita, J., Takeda, S. I., Nakano, T., Tarui, S., Honjo, T. and Kitamura, Y. (1987) J. Exp. Med. 165:268

Hamasaki, Y. and Tai, H-S. (1984) Biochim. Biophys. Acta 793:393

Heavey, D. J., Ernst, P. B., Stevens, R. L., Befus, A. D., Bienenstock, J. and Austen, K. F. (1988) J. Immunol. 140:1953

Helm, R. M. and Froese, A. (1981a) Immunology 42:629

Helm, R. M. and Froese, A. (1981b) Int. Archs. Allergy appl. Immunol. 65:81

Hempstead, B. L., Parker, C. W. and Kulczycki, A Jr. (1981) J. Biol. Chem. 256:10717

Holgate, S.T., Robinson, C., and Church, M.K. (1988) in "Allergy. Princinples and Practice, Ed.3" (Ed. by Middleton Jr. Yunginger, J.W.), The CV Mosby Company, St. Louis, p.135

Holowka, D., Hartmann, H., Kanellopoulous, J. and Metzger, H. (1980) J. Receptor Res. 1:41

Holowka, D., Gitler, C., Bercovici, T., Metzger, H. (1981) Nature 289:806

Holowka, D. and Metzger, H. (1982) Mol. Immunol. 19:219

Huang, E., Nocka, K., Beier, D. R., Chu, T. Y., Buck, J., Lahm, H. W., Wellner, D., Leder, P., Besmer, P. (1990) Cell 63:225

Huang, R., Blom, T., Hellman, L. (1991) Eur. J. Immunol.

21:1611

Hultner, L., Druez, C., Moeller, J., Uyttenhove, C., Schmitt, E., Rude, E., Dormer, P. and Van Snick, J. (1990) Eur. J. Immunol. 20:1413

Hultner, L., Moeller, J., Schmitt, E., Jager, G., Reisbach, G., Ring, J and Dormer, P. (1989) J. Immunol. 142:3440

Iemura, A., Tsai, M., Ando, A., Wershil, B.K., and Galli, S.J. (1994) Am. J. Pathol. 144:321

Ihle, J.N. and Weinstein, Y. (1986) Adv. Immunol. 39:1

Ikuta, K., Takami, M., Kim, C. W., Honjo, T., Miyoshi, T., Tagaya, Y., Kawabe, T., and Yodoi, J. (1987) Proc. Natl. Acad. Sci. U.S.A 86:7566

Isersky, C., Taurog, J. D., Poy, G.and Metzger, H. (1978) J. Immunol. 121:549

Ishizaka, K. and Ishizaka, T. (1971) in"Biochemistry of the Acute Allergic Reactions", (Ed. by Austen, K.F. and Becker, E), Blackwell Scientific Publ., Oxford, p. 13.

Ishizaka, T., Soto, C. S. and Ishizaka, K. (1973) J. Immunol. 111:500

Ishizaka, T., Okudaira, H., Mauser, L.E., and Ishizaka, K. (1976) J. Immunol. 116:747

Ishizaka, T., Chang, T. H., Taggart, M and Ishizaka, K. (1977) J. Immunol. 119:1589

Ishizaka, T., Ishizaka, K., Conrad, D. H. and Froese, A. (1978) J. Allergy Clin. Immunol. 61:320

Ishizaka, T. and Ishizaka, K. (1984) Progr. Allergy 34:188

Ishizaka, T. (1988) in "Allergy. Principle and Practice, Ed.3" (Ed. by Middleton Jr, E., Reed, C.E., Ellis, E.F., Adkinson Jr.,N.F. and Yunginger, J.W.), The CV. Mosby Company, St.Louis, p.71

Jarboe, D. L., Marshall, J. S., Randolph, T. R., Kukolja, A., and Huff, T. F. (1989) J. Immunol. 142:2405

Kane, P., Holowka, D., Baird, B. (1990) Immunol. Invest. 19:1

Kanellopoulos, J. M., Liu, T-Y., Poy, G.and Metzger, H. (1980) J. Biol. Chem. 255:9060

Kanellopoulos, J., Rossi, G. and Metzeger, H. (1979) J. Biol. Chem. 254:7691

Katz, H.R., Austen, K. F., Caterson, B. and Stevens, R. L. (1986) J. Biol. Chem. 261:13393

Katz, H.R., Arm, J.P., Benson, A.C. and Austen, K.F. (1990) J. Immunol. 145:3412

Katz, H.R., Kaye, R.E., and Austen, K.F. (1991) Transplant. Proc. 23:2900

Kehry, M. R., and Yamashita, L. C. (1989) Proc. Natl. Acad. Sci. USA. 86:7556

Kepron, M. R., Conrad, D. H. and Froese, A. (1982) Mol. Immunol. 19:1631

Kepron, M. R., Bazin, H., and Froese, A. (1988) Mol. Immunol. 25:599

Kikutani, H., Inui, S., Sato, Barsumian, E.L., Owaki, H., Yamasaki, K., Kaisho, T., Uchibayashi, N., Tsunnsawa, S., Sakiyama, F., Suemura, M., Kishimoto, T. (1986) Cell 47:657

Kinet, J-P., Metzger, H., Hakimi, J. and Kochan, J. (1987) Biochemistry 26:4605

Kinet, J.-P., Blank, U., Ra, C., White, K., Metzger, H., and Kochan, J. (1988) Proc. Natl. Acad. Sci. USA 85:6483

Kintner, C., and Sugden, B. (1981) Nature 294:458

Kitamura, Y., Go, S., and Hatanaka, S. (1978) Blood 52:447

Kitamura, Y., Shimada, M., Go, S., Matsuda, H., Hatanaka, K., and Seki, M. (1979) J. Exp. Med. 150:482

Kitamura, Y.and Go, S. (1979) Blood 53:492

Kitamura, Y. (1989) Ann. Rev. Immunol. 7:59

Kobayashi, T., Nakano, T., Nakahata, T., Asai, H., Yagi, Y., Tsuji, K., Komiyama, A., Akabane, T., Kojima, S., Kitamura, Y. (1986) J. Immunol. 136:1378

Kochan, J., Pettine, L.F., Hakimi, J., Kishi, K, and Kinet, J.-P. (1988) Nucleic Acids Res. 16:3584

Koike, T., Hirai, K., Morita, Y., and Nozawa, Y. (1993) J. Immunol. 151:359

Kraemer, P.M., Petersen, D.F., and VanDilla, M.A. (1971) Science 174:714

Kraft, A. S., and Anderson, W. B. (1983) Nature 301:621

Kruger, P. E., Aas, P., Onarheim, J. and Helle, K. B. (1982) Acta Physiol. Scand. 114:467

Kulczycki, A. Jr. and Metzger, H. (1974) J. Exp. Med. 140:1676

Kulczycki, A. Jr., McNearney, T. A.and Parker, C. W. (1976) J. Immunol. 117:661

Kulczycki, A. Jr. and Parker, C. W. (1979) J. Biol. Chem. 254:3187

Kuster, H., Thompson, H., Kinet, J.-P. (1990) J. Biol. Chem. 265:6448

LaCroix, E.L. and Froese, A. (1993) Mol. Immunol. 30:321

Laemmli, U. K. (1970) Nature 227:680

Lagunoff, D., Pritzl, P. (1976) Arch. Biochem. Biophys. 173:554

Lee, T. D. G., Sterk, A., Ishizaka, T., Bienenstock, J., and Befus, A. D. (1985a) Immunology 55:363

Lee, T. D. G., Shanahan, F., Miller, H. R. P., Bienenstock, J. and Befus, A. D. (1985b) Immunology 55:721

Letellier, M., Nakajima, T., Pulido-Cejudo, G., Hofstetter, H., and Delespesse, G. (1990) J. Exp. Med. 172:693

Levi-Schaffer, F., Austen, K. F., Gravellese, P. M., and Stevens, R. L. (1986) Proc. Natl. Acad. Sci. USA 83:6485

Levi-Schaffer, F., Dayton, E.T., Austen, K.F., Hein, A., Caulfield, J.P., Gravallese, P.M., Liu, F.T., and Stevens,R.L. (1987) J. Immunl. 139:3431

Liu, F.T., Bohn, J.W., Ferry, E.L., Yamamoto, H., Molinaro, C.A., Sherman, L.A., Klinman, N.R. and Katz, D.H. (1980) J. Immunol. 124:2728

Lobell, R.B., Austen, K.F., and Katz, H.R. (1994) J. Immunol. 152:811

Luo, H., Hofstetter, H., Banchereau, J., and Delespesse, G. (1991) J. Immunol. 146:2122

Madden, K. B., Urban, J. F. Jr., Ziltener, H. J., Schrader, J. S., Finkelman, F. D., and Katona, I. M. (1991) FASEB J. 5:A1012

Majumder, S., Brown, K., Qiu, F. H., Besmer, P. (1988) Mol. Cell. Biol. 8:4896

Markwell, M.A.K. (1982) Anal. Biochem. 125:427

Marshall, J.S. (1993a) Gastroenterology 105:391

Marshall, J.S., Gauldie, J., Nielsen, L., and Bienenstock, J. (1993b) Eur. J. Immunol. 23:2116

Martin, F.H., Suggs, S.V., Langley, K.E., Lu, H.S., Ting, J., Okino, K.H., Morris, C.F., McNiece, I.K., Jacobsen, F.W., Mendiaz, E.A., Birkett, N.C., Smith, K.A., Johnson, M.J., Parker, V.P., Flores, J.C., Patel, A.C., Fisher, E.F., Erjavec, H.O., Herrera, C.J., Wypych, J., Sachdev, R.J., Pope, J.A., Leslie, I., Wen, D., Lin, C.-H., Cupples, R.L. and Zsebo, K.M. (1990) Cell 63:203

Maurer, D., Fiebiger, E., Reininger, B., Wolff-Winiski, B., Jouvin, M-H., Kilgus, O., Kinet, J-P., Sting, G. (1994) J. Exp. Med. 179:745

May, C. D., Lyman, M., Alberto, R., and Cheng, J. (1970) J. Allergy 46:12

Maximow, A. (1905) Uber die Zellformen des lockeren Bindegewebes. Arch mikrosk Anat Entw Mech 67:680

McMenamin, C., Haig, D. M., Gibson, S., Newlands, G. F. J., Miller, H. R. P. (1987) Immunology 60:147

McNeil, H. P., Austen, K. F., Somerville, L. L., Gurish M. F., Stevens, R. L. (1991) J. Biol. Chem. 266:20316

Mekori, Y., Oh, C.K., and Metcalfe, D.D. (1993) J. Immunol. 151:3775

Mendonca, V. O., Vugman, I., and Jamur, M. C. (1986) Cell. Tissue Res. 243:635

Metcalfe, D. D., Smith, J. A., Austen, K. F.and Silbert, J. E. (1980) J. Biol. Chem. 255:11753

Miller, L., Blank U., Metzger, H., and Kinet, J.-P. (1989) Nature 244:334

Moeller, J., Hultner, L., Schmitt, E., Dormer, P. (1989) J. Immunol. 142:3447

Moore, K. W., Vieira, P., Fiorentin, D., Trounstine, M., Khan, T., Mosmann, T. (1990) Science 248:1230

Mosmann, T. R., Bond, M. W., Coffman, R. L., Ohara, J. and Paul, W. E. (1986) Proc. Natl. Acad. Sci. USA 83:5654

Mossmann, H., Lauer, W., Vortisch, Z., Gunther, E., Bazin, H. and Hammer, D. K. (1976) Eur. J. Immunol. 6:591

Nabel, G., Galli, S. J., Dvorak, A. M., Dvorak, H. F. and Cantor, H. (1981) Nature (Lond.) 291:332

Nafziger, J., Arock, M., Guillosson, J. J., Wietzerbin, J. (1990) Eur J. Immunol. 20:113

Nakajima, T., Sarfati, M., and Delespesse, G. (1987) J. Immunol. 139:848

Nakajima, T., and Delespesse, G. (1986) Eur. J. Immunol. 16:809

Nakano, T., Sonoda, T., Hayashi, C., Yamatodani, A., Kanayama, Y., Yamamura, T., Asai, H., Yonezawa, T., Kitimura, Y. and Galli, S.J. (1985) J. Exp. Med. 162:1025

Nemeth, A., and Rohlich, P. (1980) Eur. J. Cell Biol. 20:272

O'Garra, A., Stapleton, G., Dhar, V., Pearce, M., Schumacher, J., Rugo, H., Barbis, D., Stall, A., Cupp, J., Moore, K., Vieira, P. (1990) Int. Immunol. 2:821

Olson, C. E. and Levy, D.A. (1976) Fed. Proc 35:491

Osterhoff, B., Rappersberger, K., Wang, B., Koszik, F., Ochiai, K., Kinet, J-P., Sting, G. (1994) J. Invest. Dermatol. 102:315

Pearce, F. L., Befus, A. D. Gauldi, J. and Bienenstock, J. (1982) J. Immunol. 128:2481

Pecoud, A. R., Ruddy, S. and Conrad, D. H. (1981) J. Immunol. 126:1624

Pecoud, A.R. and Conrad, D. H. (1981) J. Immunol. 127:2208

Perez-Montfort, R., Kinet, J-P.and Metzger, H. (1983) Biochemistry 22:5722

Peters, S.P., MacGlashan, D.W., Schulman, E.S. (1984) J. Immunol. 132:1972

Plaut, M., Pierce, J. H., Watson, C. J., Hanley-Hyde, J.,

Nordan, R. P., Paul, W. E. (1989) Nature 339:64

Qiu, F., Ray, P., Brown, K., Barker, P. E., Jhanwar, S., Ruddle, F. H., Besmer, P. (1988) EMBO J. 7:1003

Ra, C., Jouvin, M.-H.E., Kinet, J.-P.(1989) J. Biol. Chem. 264:15323

Ra, C., Jouvin, M.-H.E., Blank, U. and Kinet, J.P. (1989) Nature 341:752

Ravetch, J.V., Luster, A.D., Weinshank, R., Kochan, J., Pavlovec, A., Portnoy, D.A., Hulmes, J., Pan, Y-C.E. and Unkeless, J.C. (1986) Science 234:718

Razin, E., Cordon-Cardo, C. and Good, R. A. (1981) Proc. Natl. Acad. Sci. USA 28:2559

Razin, E., Mencia-Huerta, J-M., Stevens, R. L., Lewis, R. A., Liu, F-T., Corey, E. J.and Austen, K. F. (1983) J. Exp. Med. 157:189

Razin, E., Ihle, J. N., Seldin, D., Mencia-Huerta, J-M., Katz, H. R., Leblanc, P. A., Hein A., Caulfield, J. P., Austen, K. F. and Stevens, R. L. (1984) J. Immunol. 132:1479

Rennick, D., Hunte, B., Dang, W., Thompson-Snipes, L., and Hudak, S. (1994) Exp. Hematol. 22:136

Reynolds, D. S., Serafin, W. E., Faller, D. V., Wall, D. A., Abbas, A. K., Dvorak, A. M., Austen, K. F., and Stevens, R. L. (1988) J. Biol. Chem. 263:12783

Reynolds, D. S., Stevens, R. L., Gurley, D. S., Lane, W. S., Austen, K. F., Serafin, W. E. (1989) J. Biol. Chem. 264:20094

Reynolds, D. S., Gurley, D. S., Austen, K. F., Serafin, W. E. (1991) J. Biol. Chem. 266:3847

Richards, M. L., and Katz, D. H. (1991) Critical Rew. Immunol. 11:65

Rivnay, B., Wank, S. A., Poy, G. and Metzger, H. (1982) Biochemistry 21:6922

Rossi, G., Newmann, S. A., and Metzger, H. (1977) J. Biol. Chem 252:704

Roth, P. A., Rao, M. and Froese, A. (1986) Immunology 58:671

Rouleau, A., Garbarg, M., Schwartz, J.C., and Ruat, M. (1994) Biochem. Biophysic. Res. Communication. 199:593

Ruitenberg, E. J. and Elgersma, A. (1976) Nature 264:258

Russell, E. (1979) Adv. Genet. 20:357

Ryan, J. J. and Huff, T. F. (1991) FASEB J. 5:A1006

Schick, B., Austen, K.F., and Schwartz, L.B. (1984) J. Immunol. 132:2571

Schrader, J. W. (1986) Annu. Rev. Immunol. 4:205

Schrader, J.W., Lewis, S. J., Clark-Lewis, I., and Culvenor, J. G. (1981) Proc. Natl. Acad. Sci. USA 78:323

Schwartz, L. B. and Austen, K. F. (1984) Progr. Allergy 34:271

Schwartz, L.B. (1989) in "Mast Cell and Basophil Differentiation and Function in Health and Disease", (Ed. by Galli, S.J., Austen, K.F.), Raven Press, New York, p.93

Sedar, R. A., Paul, W. E., Dvorak, A. M., Sharkis, S. J., Kagey-Sobotka, A., Niv, Y., Finkelman, F. D., Barbieri, S. A., Galli, S. J., and Plaut, M. (1991) Proc. Natl. Sci. USA 88:2853

Segal, D. M., Sharrow, S. O., Jones, J. F.and Siraganian, R. P. (1981) J. Immunol. 126:138

Seldin, D.C., Adelman, S., Austen, K.F., Stevens, R. L., Hein, A., Caulfield, J. P., and Woodbury, R. G. (1985) Proc. Natl. Acad. Sci. USA 82:3871

Serafin, W. E., Sullivan, T. P., Conder, G. A., Ebrahim, A., Marcham, P., Johnson, S. S., Austen, K. F., Reynolds, D. S. (1991) J. Biol. Chem. 266:1934

Shanahan, F., Denburg, J. A., Bienenstock, J. and Befus, A. D. (1984) Can. J. Physiol. Pharmacol. 62:734

Shanahan, F., Denburg, J. A., Fox, J., Bienenstock, J. and Befus, D. (1985) J. Immunol. 135:1331

Shanahan, F., Lee, T. D. G., Denburg, J. A., Bienenstock, J. and Befus, A. D. (1986) Immunology 57:455

Shimizu, A., Tepler, I., Benfey, P.N., Berenstein, E.H., Siraganinan, R.P., and Leder, P. (1988) Proc. Natl. Acad. Sci. USA. 85:1907

Smith, C. A. and Rennick, D. M. (1986) Proc. Natl. Acad. Sci. USA 83:1857

Snapper, C. M., Finkelman, F. D., and Paul, W. E. (1988) J. Exp. Med. 167:183

Sonoda, S., Sonoda, T., Nakano, T., Yonezawa, T., Kitamura, Y. (1986) J. Immunol. 137:1319

Spiegelberg, H. L. (1984) Adv. Immunol. 35:61

Spiegelberg, H. L. (1991) Monogr. Allergy 29:1

Sterk, A. R. and Ishizaka, T. (1982) J. Immunol. 128:838

Stevens, R. L., Lee, T. D. G., Seldin, D. C., Austen, K. F., Befus, A. D., Bienenstock, J. (1986a) J. Immunol. 137:291

Stevens, R. L., Katz, H. R., Seldin, D. C. and Austen, K. F. (1986b) in "Mast Cell Differentiation and Heterogeneity", (Ed. by Befus, A. D., Bienenstock, J. and Denburg, J. A.) Raven Press, New York, p. 183

Stevens, R.L., Fox, C.C., Lichtenstein, L.M., and Austen, K.F. (1988) Prot. Natl. Acad. Sci. USA 85:2284

Stevens, R.L., Friend, D.S., McNeil, H.P., Schiller, V., Ghildyal, N., and Austen, K.F. (1994) Proc. Natl. Acad. Sci. U.S.A. 91:128

Suda, T., Suda, J., Spicer, S. S., Ogawa, M. (1985) J. Cell Physiol. 122:187

Suemura, M., Kikutani, H., Barsumian, E. L., Hattori, Y., Kishimoto, S., Sato, R., Maeda, A., Nakamura, H., Owaki, H., Hardy, R. R., and Kishimoto, T. (1986) J. Immunol. 137:1314

Suter, U., Bastos, R., Hofstetter, H. (1987) Nucleic Acids Res. 15:7295

Swieter, M., Chan, B. M. C., Rimmer, C., McNeill, K., Froese, A., and Befus, D. (1989) Eur. J. Immunol. 19:1879

Swieter, M., Mergenhagen, S. E., and Siraganian, R. P. (1992) Proc. Soci. Exp. Biol. Med. 199:22

Takagi, M., Koike, K.,Nakahata, T. (1990) J. Immunol. 145:1880

Takizawa, F., Adamczewski, M. and Kinet, J.-P. (1992) J. Exp. Med. 176:469

Tantravahi, R. V., Stevens, R. L., Austen, K. F. and Weis, J. H. (1986) Proc. Natl. Acad. Sci. USA 83:9207

Tei, H., Kasugai, T., Tsujimura, T., Adachi, S., Furitsu, T.,

Tohya, K., Zsebo, K.M., Newlands, G.F.J., Miller, H.R.P., Kanakura, Y., and Kitamura, Y. (1994) Blood 83:916

Theoharides, T. C. and Douglas, W. W. (1978) Endocrinology 102:1637

Thompson-Snipes, L., Dhar, V., Bond, M. W., Mosmann, T. R., Moore, K. W., Rennick, D. M. (1991) J. Exp. Med. 173:507

Tsai, M., Takershi, T., Thompson, H., Langley, K. E., Zsebo, K. M., Metcalfe, D. D., Geissler, E. N., Galli, S. J. (1991a) Proc. Natl. Acad. Sci. USA 88:6382

Tsai, M., Shih, L., Newlands, G.F.J., Takeishi, T., Langley, K.E., Zsebo, K.M., Miller, H.R.P., Geissler, E.N., and Galli, S.J. (1991b) J. Exp. Med. 174:125

Tsuji, K., Nakahata, T., Takagi, M., Kobayashi, T.,Ishiguro, A., Kikuchi, T., Naganuma, K., Koike, K., Miyajima, A., Arai, K.I. and Akabane, T. (1990) Blood 75:421

Tsujimura, T., Hirota, S. Nomura, S., Niwa, Y. Yamazaki, M., Tono, T., Morii, E., Kim, H.-M., Kondo, K., Nishimume, Y. and Kitamura, Y. (1991) Blood 78:1942

Uchida, T., Ju, S.-T., Liu, Y. N., and Dorf, M. E. (1985) J. Immunol. 134:772

Wang, B., Rieger, A., Kilgus, O., Ochiai, K., Maurer, D., Foderer, D., Kinet, J-P., Sting, G. (1992) J. Exp. Med. 175:1353

Wendel-Hansen, V., Riviere, M., Uno, M., and Rosen, A. (19900 Somatic Cell Mol. Genet. 16:283

Wershil, B.K., Tsai, M., Geissler, E.N., Zsebo, K.M. and Galli, S.J. (1991) J. Exp. Med. 175:245

White, J. R., Ishizaka, T., Ishizaka, K., Sha'afi, R. I. (1984) Proc. Natl. Acad. Sci. USA 81:3978

White, J. R., Pluznik, D. H., Ishizaka, T., Ishizaka, K. (1985) Proc. Natl. Acad. Sci. USA 82:8193

Williams, D. E., Eisenman, J., Baird, A., Rauch, C., Van Ness, K., March, C. J., Park, L. S., Martin, U., Mochizuki, D. Y., Boswell, H. S., Burgess, G. S., Cosman, D., Lyman, S. D. (1990) Cell 63:167

Wingren, U., and Enerback, L. (1983) Histochem. J. 15:571

Wingren, U., Wasteson, A. and Enerback, L. (1983) Int. Archs.

Allergy appl. Immunol. 70:193

Wirthmueller, U., Kurosaki, T., Murakami, M.S. and Ravetch, J.V. (1992) J. Exp. Med. 175:1381

Wodnar-Filipowicz, A., Heusser, C. H., Moroni, C. (1989) Nature 339:150

Woodbury, R. G., Everitt, M. T., and Neurath, H. (1981) In Methods in Enzymology (Lorand, L., Ed.), Vol. 80, pp.588, Academic Press, New York.

Woodbury, R. G., Gruzenski, G. M. and Lagunoff, D. (1978a) Proc. Natl. Acad. Sci. USA 75:2785

Woodbury, R. G., Everitt, M., Sanada, Y., Katunuma, N., Lagunoff, D. and Neurath, H. (1978b) Proc. Natl. Acad. Sci. USA 75:5311

Worton, R. G., and Duff, C. (1979) in Methods in Enzymology (Jakoby, W. B. and Pastan, I. H., Eds.), Vol. 58, pp. 322, Academic Press, New York.

Yokota, A., Kikutani, H., Tanaka, T., Sato, R., Barsumian, E. L., Suemura, M., and Kishimoto, T. (1988) Cell 55:611

Yukawa, K., Kikutani, H., Owaki, H., Yamasaki, K., Yokota, A., Nakamura, H., Barsumian, E. L., Hardy, R. R., Suemura, M., and Kishimoto, T. (1987) J. Immunol. 138:2576

Yurt, R. W., Leid Jr, R. W., Austen, K. F. and Silbert, J. E. (1977) J. Biol. Chem. 252:518

Zola, H., and Brooks, D. (1982) in Monoclonal Hybridoma Antibodies: Techniques and Applications (Hurrell, J. G. R., Ed.), pp.20, CRC Press Boca Raton, FL.