

The University of Manitoba

**DEMONSTRATION OF CLONAL VARIATIONS IN MAST CELL
DIFFERENTIATION AMONG RAT TISSUE CULTURED
MAST CELL CLONES**

by

Weining Hu

A Thesis

Submitted to the Faculty of Graduate Studies in

Partial Fulfillment of the Requirements for the

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ABSTRACT

Previous results had shown that rat tissue cultured mast cell (RCMC) lines can be generated by culturing rat peritoneal mast cell in the absence of added growth factors or feeder layers. Clones were generated from one of these mast cells lines by limiting dilution. Selected clones were cultured continuously for a period of 24 weeks during which the surface expression of the high ($\text{Fc}_\epsilon\text{RI}$) and low ($\text{Fc}_\epsilon\text{R}_\text{L}$) affinity receptors for IgE was monitored at regular intervals. Initially, the chosen clones exhibited varying receptor expression. Some showing no receptors at all while others carried either $\text{Fc}_\epsilon\text{R}_\text{L}$ or both types of receptors. During culture, $\text{Fc}_\epsilon\text{R}_\text{L}$ expression increased on most clones, remaining nearly constant on the one clone which had the highest starting expression of this receptor. $\text{Fc}_\epsilon\text{RI}$ expression showed more clonal variation: decreasing, increasing and remaining absent on some clones. This suggested that some clones may have represented mast cells at different stages of differentiation.

In order to test further the latter possibility, clones showing the most significant differences in receptor expression were subjected to long-term modulation with the differentiation agents: phorbol 12-myristate 13-acetate (PMA), dexamethasone (DM) and retinoic acid (RA). The effects of the modulators on $\text{Fc}_\epsilon\text{R}$ expression and total histamine content were monitored. The most varied clonal changes, both in terms of $\text{Fc}_\epsilon\text{R}$ expression and histamine content, were brought about by PMA and DM. When effective, these agents up-regulated the expression of $\text{Fc}_\epsilon\text{R}_\text{L}$ and histamine. Their effect on $\text{Fc}_\epsilon\text{RI}$ was marginal. RA consistently down-regulated the expression of both receptors and the histamine content. However, subtle differences among some clones were observed. These results suggested further that most of the clones chosen for

the modulation study were, in fact, at different stages of differentiation. PMA and DM, most likely, induced a maturation process. Nevertheless, this process was not accompanied by a phenotypic change from mucosal to connective tissue type mast cells.

In most cases, the expression of $\text{Fc}\epsilon\text{RI}$ could not be up-regulated, suggesting that other factors may be required. One such factor might be IgE itself. When added to the tissue culture medium of some clones, it up-regulated the expression of both receptors for IgE on the majority of clones. Similar experiments with the F(ab')_2 or Fab' fragments of anti- $\text{Fc}\epsilon\text{R}_\text{L}$ antibodies demonstrated that the former was virtually ineffective while the latter generally increased the expression of either $\text{Fc}\epsilon\text{R}$. Thus, it does appear that while the long-term culture experiment and the use of the differentiation agents PMA and DM point to independent regulatory mechanisms for the expression of the two receptors, those with Fab' indicate that there may be at least some common aspects to the otherwise different mechanisms involved in the regulation of the expression of these two receptors for IgE. Even though, IgE and the F(ab')_2 and Fab' fragments of anti- $\text{Fc}\epsilon\text{R}_\text{L}$ had very similar effects on the different cloned RCMC lines, some minor clonal variations were observed.

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LIST OF ABBREVIATION

| | |
|------------|--|
| BM: | bone marrow |
| BMMC: | bone marrow-derived mast cells |
| BSA: | bovine serum albumin |
| cAMP: | cyclic adenosine 3':5'-monophosphate |
| CMEM: | complete Eagle's minimum essential medium |
| Con A-SCM: | Con A-stimulated rat spleen cell conditioned medium |
| CTMC: | connective tissue-type mast cells |
| DMSO: | dimethylsulfoxide |
| DNBS: | dinitrobenzensulfonate |
| DNP: | 2,4-dinitrophenyl |
| DSCG: | disodium cromoglycate |
| EDTA: | ethylenediaminetetraacetic acid |
| FCS: | fetal calf serum |
| GM-CSF: | granulocyte/macrophage colony stimulating factor |
| HEPES: | <i>N</i> -2-hydroxy-piperazine- <i>N'</i> -2-ethanesulfonic acid |
| 5-HT: | 5-hydroxytryptamine binoxalate |
| IgE-BF: | IgE binding factor |
| IL-3: | interleukin 3 |
| IMMC: | intestine mucosal mast cells |
| LT: | leukotriene |
| MMC: | mucosal mast cells |
| Mr: | a relative molecular mass |
| OPT: | O-phthaldialdehyde |
| PG: | prostaglandin |
| PI: | phosphatidylinositol |
| PKC: | protein kinase C |
| PMA: | phorbol 12-myristate 13-acetate |
| PMSF: | phenylmethylsulfonyl fluoride |
| PMC: | peritoneal mast cell |
| RCMC: | rat tissue cultured mast cells |

RBL: rat basophilic leukemia
rDNA: recombinant DNA
RMCP: rat mast cell protease
rIL-3: recombinant interleukin 3
SDS-PAGE: polyacrylamide gel electrophoresis in the presence of SDS
v/v: volume/volume
w/v: weight/volume

CHAPTER I

REVIEW OF THE LITERATURE

ONTOGENY AND HETEROGENEITY OF MAST CELLS

Evidence has been provided to suggest that rodent mast cells are derived from precursors located in the bone marrow (Kitamura et al., 1979a; 1981). Characterization of mast cells in various tissues has identified two types of mast cells which show marked heterogeneity in their histochemical, morphological and functional properties. The nomenclature used for distinguishing these two types of mast cells varies depending on the species, the tissue source or the biochemical characteristics. In rodents, they are known as the connective tissue mast cells (CTMC) or typical mast cells and the mucosal mast cells (MMC) or atypical mast cells, respectively. In addition to the above systems, human mast cells have also been defined according to their tissue of origin and neutral protease content. An internationally agreed nomenclature is so far unavailable to reflect the current knowledge on mast cells.

Phenotypic differences between mast cells:

The predominant morphological feature of the mast cell is the presence of large numbers of metachromatic cytoplasmic granules. Evidence for the presence of two histochemically distinct phenotypes of mast cells in the rat was first provided in 1966 by Enerback (1966a). Mast cells in the mucosa of the small intestine were found to require fixation in Carnoy's solution, a mixture of isotonic formaldehyde and acetic acid, or Mota's basic lead acetate in order to be stained by cationic dyes; while fixation with typical aldehyde fixatives blocked the binding of these cells to these

dyes. In contrast, mast cells found in the submucosa of the small intestine could be stained regardless of the type of fixatives used. The former type of mast cells were subsequently termed the atypical or mucosal mast cells (MMC), and the latter conventional mast cells were also identified in the serosal cavities, submucosa of the small intestine and the skin and were therefore named typical or connective tissue type mast cells (CTMC) (Enerback, 1981; Bienenstock et al., 1982; Guy-Grand et al., 1984). A nearly homogeneous CTMC population can be conveniently obtained from the peritoneal cavity of mice or rats and such cells form the basis of much of our knowledge on the connective tissue type of mast cells. More recently, the distribution of MMC and CTMC was studied in greater detail throughout the rat gastrointestinal system (Saavedra-Delgado et al, 1984). Although the two phenotypes of mast cells are intermixed in most tissues, CTMC were found to be the exclusive phenotype seen in the cheek, tongue, oesophagus and non-glandular stomach, whereas MMC were detected in up to 20-fold larger numbers than CTMC in the glandular stomach, small and large intestine and caecum (Saavedra-Delgado et al., 1984).

Under Carnoy's fixation, berberine sulphate, a basic fluorescent dye, forms a strong yellow fluorescent complex with heparin, the highly sulphated glycosaminoglycan of CTMC but not MMC. Similarly, both MMC and CTMC can be stained blue with alcian blue while only CTMC can be counterstained red with safranin O (Enerback, 1966b). Since the differences in binding to cationic dyes are attributed to the negative charge density and isoelectric point of granule proteoglycan, it is important to mention here that the immature CTMC with lower level of sulphation also stain blue in the Alcian blue/Safranin sequence (Combs et al., 1965).

Mast cells in tissues are usually ovoid or irregularly elongate and contain a round or ovoid nucleus. The mucosal mast cells are usually smaller in size and more variable in shape than the connective tissue mast cells. The morphology of the cytoplasmic granules may vary from species to species and in different tissues within the same species. In the rat, the mature CTMC typically contains 1,000 separate granules, each surrounded by an individual membrane. Under the electron microscopy the granules appear homogeneous in structure and uniformly electron-dense with an average diameter of 0.2-0.4 μm , the matrix of the granules tends to contain less soluble proteoglycans (Behrendt et al., 1978; Enerback, 1981). The other type of mast cells MMC are found to contain smaller and amorphous granules filled with less electron-dense materials and a relatively soluble matrix with poorly sulphated glycosaminoglycans which requires strong acid conditions to penetrate for rapid fixation (Enerback, 1981).

Heterogeneity in granule content:

Anaphylactic mediators are generally divided into two major classes: the pre-formed mediators, which are stored in the secretory granules and released upon mast cell activation; and the newly generated mediators, which are synthesized *de novo* and released following cell activation. The preformed mediators include vasoactive amines, hydrolytic enzymes and chemotactic factors, among which proteoglycans, neutral proteases, and biogenic amine are demonstrated to vary in the two phenotypes of mast cells and are reviewed in detail. The newly synthesized mediators are produced by the oxidative metabolism of arachidonic acid which is liberated from the cell membrane upon stimulation.

Proteoglycans consist of a peptide core linked covalently to glycosaminoglycan side-chains of repeating disaccharide units. The heterogeneity is attributable to the nature of the sugar groups and the degree of sulphation. Rat CTMC are found to synthesize predominantly the protease-resistant heparin proteoglycans with a relative molecular mass (M_r) of 650 KDa (Lagunoff and Pritzl, 1976; Holgate and Church, 1982), while rat MMC produce little, if any of it (Tas and Berndsen, 1977). The heparin carries a high density of negative charge from sulphation of sugar residues and constitutes approximately one-third of the granule by dry weight. On the other hand, two types of less sulphated chondroitin sulphate proteoglycans of M_r between 150-350 KDa have been identified in the mucosal mast cells (Otsu, et al., 1987). Rat intestinal mucosal mast cells were characterized to contain chondroitin sulphate di-B (Stevens et al., 1986). A distinct proteoglycan, chondroitin sulfate E, was identified in mouse bone marrow derived mast cells (BMMC) (Razin et al., 1982). The heparin proteoglycan can be degraded by heparinase and nitrous acid, while the chondroitin sulphates are sensitive to chondroitinase ABC. Normally, rat CTMC contain small amounts of over-sulphated chondroitin sulphate proteoglycans (Katz et al., 1986) and the mucosal-like rat basophilic leukemia cells contain both heparin and chondroitin sulphate E glycosaminoglycans (Seldin et al., 1985a). In the presence of β -D-xyloside which acts as an exogenous acceptor for glycosaminoglycans and competes with the peptide core protein, rat serosal mast cells demonstrated the capacity to polymerize chondroitin sulphate E onto the exogenous acceptor while they continue the synthesis of heparin onto the endogenous peptide core (Stevens et al., 1983). The results indicated the presence of the enzymatic pathways for both classes of glycosaminoglycans in rodent

mast cells and raised the possibility that the existence of distinct core peptides direct the type of glycosaminoglycan produced. Thereafter, it was found that a single gene encodes the core peptides for both heparin and chondroitin sulfate proteoglycans (Tantravahi et al., 1986). The synthesis of proteoglycans is thus known to be regulated by the activation of the two enzymatic pathways involving the polymerization of the side-chains of glycosaminoglycan.

Mast cell granules are associated with a few neutral proteases. Among them, two serine proteases with chymotrypsin-like activity in substrate specificity and molecular weight, are found in mast cells (Woodbury et al., 1978a) and are generally termed as rat mast cell protease (RMCP) I and II. Immunofluorescence analysis has identified the presence of the insoluble RMCP I in the connective tissue mast cell population (Woodbury et al., 1978b) and the soluble RMCP II in the mucosal mast cells (Woodbury and Miller, 1982). Both types of RMCP are unusual among the neutral proteases in having relatively basic isoelectric points (Woodbury et al., 1981). This property enables them to form complex with the negatively-charged proteoglycans in the secretory granules (Schwartz et al., 1981). Both RMCP I and RMCP II are single polypeptides in nature, with Mr of 29 KDa and 25 KDa, respectively. The two types of enzymes share 75% sequence homology in their first 52 amino-terminal residues and polyclonal rabbit antisera raised against RMCP I or II showed extensive cross-reactivity on western blot although not by gel filtration. (Woodbury et al., 1981; Gibson and Miller, 1986). The gene coding for RMCP II has been demonstrated to be expressed exclusively in MMC, and a separate gene codes likely for RMCP I (Benfey et al., 1987). The availability of specific polyclonal antibodies to the two types of RMCP respectively, allows for

the phenotypic differentiation of CTMC and MMC by the presence of the respective serine protease. In addition, a carboxypeptidase A-like enzyme appears to be selectively concentrated in the rat peritoneal CTMC (Everitt and Neurath, 1980; Schwartz et al., 1982). In the mouse system, the neutral proteases have been characterized more recently. The mouse intestinal mast cell protease (MIMCP) was characterized to have a Mr of 28 KDa and to share 74-75% amino acid sequence homology not only with the rat counterpart RMCP II but also with the rat RMCP I (Le Trong et al., 1987). Similarly, an insoluble mouse serosal mast cell protease (MSMCP) of 25 KDa was extracted from serosal mast cells and was found to cross-react with MIMCP (Miller et al., 1988).

Histamine and 5-hydroxytryptamine (5-HT, also known as serotonin) are the major mediators causing symptoms of anaphylaxis. Histamine is present in all mast cells of all species also and virtually all of their granules, while mast cells of certain species contain 5-HT (Benditt et al., 1955; Gustafsson and Enerback 1978; Ritzen, 1966;). The turnover of these amines was studied *in vivo* with their labeled precursors, ³H-histidine and ³H-5-hydroxytryptophan. The synthesis, reflected by uptake of the precursors, was rapid, i.e. a few hours; but the elimination was slow, with a half-life for radiolabeled histamine of 23 days and one for 5-hydroxytryptamine of 25 days (Wingren et al., 1983). Normally rat CTMC contain 50-100 times as much histamine as 5-HT (Enerback and Wingren, 1980). However, mast cells have been observed to actively take up the 5-HT from the extracellular fluid (Frisk-Holmberg and Uvnas, 1972; Furano and Green, 1964; Jansson, 1970; Ritzen, 1967). The uptake of extracellular 5-HT was balanced by the displacement of the histamine in the granules

in a 1:1 molar ratio. The results indicated that histamine and 5-HT are complexed to identical storage sites in mast cell granules. Furthermore, the incorporated 5-HT was found to be subsequently released by either non-specific stimuli or specific ones involving the cross-linking of IgE by antigen (Morrison et al., 1974; Stechschulte et al., 1974). Since the percent of the released exogenous 5-HT was almost identical to that of the endogenous histamine, ³H-labeled 5-HT release has been used as a simple convenient alternative for the traditional histamine release assay in both rat and murine systems (Mazingue et al., 1978; Otsuki et al., 1976). The major difference in biogenic amine between CTMC and MMC of rats is their quantity in the granules. MMC, dispersed from the small intestinal lamina propria, were found to contain 1.3 pg histamine per cell while dispersed peritoneal CTMC contain 15 pg per cell (Befus et al., 1982; Enerback and Wingren, 1980). The state of mast cell maturity and activation or the local environment might also account for the variations in the amount of the amines.

The newly synthesized mediators of anaphylaxis are produced by the oxidative metabolism of arachidonic acid liberated from the cell membrane upon stimulation (Lewis et al., 1981; 1982). The generation of each mediator can reflect the presence of the corresponding specific enzyme: the lipoxygenase pathway leading to the production of leukotrienes and the cyclooxygenase pathway leading to the generation of prostaglandins and thromboxanes. Rat peritoneal mast cells (PMC) process arachidonic acid almost exclusively through cyclooxygenase to form prostaglandin D₂ with little or no generation of leukotrienes (Heavy et al., 1988; Lewis et al., 1982b; Roberts et al., 1979). Rat intestinal MMC generate the leukotrienes LTC₄ and LTB₄ as well as

PGD₂ (Heavy et al., 1988), while mouse BMMC with the characteristics of mucosal mast cells preferentially synthesize LTC₄ and LTB₄ (Mencia-Herta et al., 1983; Razin et al., 1982b; Robin et al., 1985).

Functional differences between mast cells:

The functional heterogeneity of mast cells is illustrated by their responses to different secretagogues and their susceptibilities to anti-allergic drugs.

Mast cells of either phenotype can be triggered to release mediators following an IgE-dependent stimulus (Barrett and Pearce, 1982; Befus et al., 1982). Similarly, mast cells from different species and tissues all respond to challenge with calcium ionophore, although the levels of responsiveness varies. This observation indicates that the rise in the intracellular concentration of calcium ions serves as the general signal for mast cell activation (Pearce, 1982a). Other secretagogues exhibit a higher degree of selectivity. An early observation made by Enerback (1966c) revealed that gastrointestinal MMC failed to respond to compound 48/80 which has been a potent activator of rat CTMC (Barrett et al., 1983; 1985). *In vitro* studies using enzymically dispersed single cell suspensions provided the same results (Befus et al., 1982; Barrett et al., 1985). The less widely studied peptide 401, the mast cell degranulating peptide derived from bee venom, closely resembles the specific action of compound 48/80 (Pearce, 1982b). In addition, dextran induces the histamine release exclusively from rat CTMC, in the presence of phosphatidylserine which was demonstrated to enhance histamine release from rat CTMC but not MMC (Mongar and Svec, 1972; Saavedra-Delgado et al., 1984). Finally, rodent CTMC can be selectively activated by all neuropeptides except

substance P, the latter triggers mediator release in both phenotypes of mast cells (Shanahan et al., 1986).

Functional heterogeneity also extends to the mast cell susceptibility to inhibition of their mediator release by antiallergic drugs. The best known agents, disodium cromoglycate (DSCG) and theophylline, inhibit the antigen-induced histamine release from rat CTMC but not MMC (Pearce et al., 1982c), with DSCG being highly species- and tissue-specific to rat serosal mast cells (Barrett and Pearce, 1983; Church, 1978). Other pharmacological compounds such as the flavonoid quercetin, tetrazole and doxantrazole, are equally effective in inhibiting histamine release from both types of mast cells (Pearce et al., 1982c; 1984).

Origin of Mast Cells:

Multipotential hematopoietic stem cells were first demonstrated as the precursors of mast cells in the murine system by Kitamura and his associates (1977; 1979a; 1981). Intravenous injection of bone marrow cells, from normal WBB6F₁(+/+) or from beige mice in which mast cells contain characteristic giant cytoplasmic granules, into mast cell-deficient WBB6F₁-W/W^v mice resulted in the development of mast cells of donor origin in the skin, stomach, caecum and mesentery. Subsequently, the growth of mast cells was observed in the bone marrow cultures from normal and athymic *nude* rats (Haig et al., 1983), indicating that the generation of precursors is thymus-independent. Recently, in irradiated WBB6F₁-W/W^v mice spleen colonies were produced from individual haematopoietic stem cells of WBB6F₁-+/+ origin (Kitamura et al., 1987) and single colony subsequently transferred intravenously to WBB6F₁-W/W^v mice resulted in the development of mast cells in various tissues,

indicating not only that the stem cells are the precursors of mast cells but also that these precursors further populate the tissue mast cell sites.

Unlike most of the progeny of multipotential stem cells, mast cells do not complete their differentiation in haematopoietic tissues. No mast cells have been detected in the blood, however, large colonies consisting of mast cells (CFU-mast) appear when blood mononuclear cells are plated in methylcellulose with appropriate growth factors (Kitamura et al., 1979c; Zucker-Franklin et al., 1981). The mast cell precursors that produce such colonies resemble lymphoid cells as seen by light microscopy (Kanakura et al., 1988b). A series of studies in mouse by Ginsburg and his associates (1962; 1963; 1967) found that the mononuclear lymphoid-like precursor cells were also located in the thymus, spleen, and lymph nodes. These precursors in haematopoietic tissues and in the blood stream subsequently enter various tissues where they differentiate into morphologically identifiable mast cells, i.e., the atypical or mucosal mast cells (MMC) and the connective tissue mast cells (CTMC), as observed in the classical *in situ* studies of Maximow (1906) and Enerback (1966a). Although most progenies of multipotential stem cells lose the proliferative ability when they fully differentiate, some morphologically identifiable mast cells have been found to have significant proliferative potential (Sonada et al., 1984).

Although the precise differentiation pathways of the mast cells are uncertain, a general idea on the development of mast cell could be obtained from the above studies. Mast cells are derived from the precursors in the bone marrow which are capable of proliferating in the absence of thymic influence. The undifferentiated precursors leave the haemopoietic tissues and circulate in the peripheral blood. They then home to the

target tissues and acquire distinct phenotypic and functional characteristics according to the effects of the potential growth and differentiation factors in the local tissue microenvironment. Comparative studies have revealed that mast cells from different sites show marked heterogeneity in their ultrastructural, histochemical, and granule biochemical properties (Galli, 1990).

Mast cell maturation and differentiation:

Once the multihemopoietic stem cells become committed to mast cell precursors, the further maturation will then lead to heterogeneous populations of mature mast cells. The general maturation process has been identified using histochemical and biochemical analyses. In 1965, Combs et al. found that mast cell differentiation in the course of maturation is associated with the progressive sulphation of heparin; the accumulation of histamine; the synthesis of an alkaline-active protease; and the loss of the capacity for cell division in the later stages of maturation. By distinguishing weakly sulphated mucopolysaccharides from strongly sulphated mucopolysaccharides, four sequential stages in maturation were defined using the Alcian blue-safranin staining technique at pH 1.0 (Combs et al, 1965).

The size of the immature mast cells was found to be smaller than that of the mature mast cells (Pretlow and Cassady, 1970). Rat peritoneal mast cells (PMC), which normally exhibit the characteristic CTMC phenotype, were separated into fractions differing in cell sizes and each of them was further characterized by Beaven et al. (1983). As defined by histochemical staining of nonsulphated heparin precursors and of heparin with Alcian blue and safranin, respectively, fractions containing small mast cells (<12 μm diameter) were predominantly immature mast cells with few

granules, whereas fractions of large cells (14-17 μm) contained mostly mature cells with numerous heparin-containing granules. Fractions in between contained mast cells correlated with intermediate stages in the four stages defined by Combs et al (1965). With increase in cell size, mast cell maturation was found to also associate with a decrease of histamine synthesis and an increase in histamine content. In addition, a functional maturation was observed by the increased responsiveness of histamine release to Compound 48/80 (Beaven et al., 1983).

Immature cloned mast cells, resembling the MMC phenotype, were modulated to mature *in vitro* by exposing them to sodium butyrate (Galli et al., 1982; Dvorak et al., 1987), an agent which had been shown to promote granulogenesis and augment the mediator content of mast cell tumor lines (Mori et al., 1979; 1981). Cloned mast cells cultured without butyrate were a rapidly dividing population with little or no electron-dense granule content. By contrast, butyrate-treated mast cells stopped proliferating and acquired large amounts of electron-dense granules, similar in ultrastructure to that of mature mast cells. In addition, these cells contained up to 50-fold more histamine than rapidly dividing mast cells in the absence of butyrate, and degranulated more completely in response to IgE and antigen. However, the cloned mast cells, although having matured in the presence of sodium butyrate, incorporated $^{35}\text{SO}_4$ into chondroitin sulfates rather than heparin in the case of connective tissue type mast cells.

An important aspect of mast cell differentiation process is the development of the Fc receptors for IgE. In the earlier studies, mast cells were generated by long-term culture of thymus cells on embryonic fibroblast monolayers (Ginsburg and Sachs,

1962; Ishizaka et al., 1976). Several days after seeding the thymus cells, receptors for IgE were detected on the surface of mastoblasts which contained a few metachromatic granules. This finding was significant in that the demonstration of the receptors would facilitate the identification of precursors of mast cells. Evidence was also provided that the number and/or affinity of the receptors for IgE increased as the amount of the metachromatic granules increased with time in culture (Ishizaka et al., 1976). Judging from the method used in the isolation of the receptors it is now known that only the development of the high affinity receptors for IgE was recorded. Recently, mast cells (BMMC) are generated from culturing bone marrow cells in the presence of T cell growth factor interleukin-3 (Prystowsky et al., 1984; Ihle and Weinstein, 1986). When the expression of the high affinity receptors for IgE ($\text{Fc}_\epsilon\text{RI}$) was investigated during the *in vitro* differentiation of BMMC it was found that the RNA transcripts for $\text{Fc}_\epsilon\text{RI}$ subunits and membrane-associated receptors were apparent by 1 week (Thompson et al., 1990). In parallel with the cell population being granulated progressively to finally resemble mast cells, the expression of transcripts and the number of receptor-positive cells continued to increase over 3 wk of culture.

The precise maturation pathway of mast cell has not been elucidated due to the difficulties in purifying the immature mast cells at different stages to homogeneity. Furthermore, there has not been a specific method to identify the immature mast cells at different stages of maturation, for instance, whether specific surface markers are associated with mast cell maturation and differentiation has not been investigated.

Transdifferentiation between mast cell phenotypes:

The characteristics of mouse bone marrow-derived mast cells (BMMC) have been

suggested to resemble MMC or to reflect the immaturity of the mast cells in many aspects (Galli et al., 1982; 1984). Experiments were initiated by Galli's group to investigate the interrelationship between the *in vivo* mature mast cells and the *in vitro* BMMC (Nakano et al., 1985). BMMC were injected into genetically mast cell deficient WBB6F₁-W/W^v mice intracutaneously, intraperitoneally, and intravenously. Mast cells populated the peritoneal cavity, skin, spleen, and glandular stomach muscularis propria exhibited properties of CTMC. By contrast, the transferred mast cells identified in the mucosa of the glandular stomach resembled MMC. These results suggested that BMMC can give rise to mast cells of either connective tissue type or mucosal phenotype, depending on anatomical locations. The changing process from BMMC to CTMC type was further analyzed following the injection of BMMC into the peritoneal cavity of the deficient mice (Nakano et al., 1987). An increase in the proportion of dense mast cells was found to parallel with the increase in histamine and in the percentage of heparin-containing mast cells. Moreover, the mast cells recovered 10 wk after transfer almost lost the proliferation activity in the culture medium used to establish the cultured BMMC. Both the increase in heparin-containing cells and the arrest of proliferation activity indicate a differentiation process towards CTMC.

The phenotypic change from CTMC to MMC was further investigated. A single PMC identified under the phase-contrast microscope, was picked up and injected into the stomach wall of the W/W^v mice (Sonoda et al., 1986). The progenies of this mast cell found in the mucosa revealed the MMC properties by microscopical and histochemical analyses, while those developed in the muscularis propria showed CTMC features. However, these results can be interpreted either as a change of

CTMC to MMC or as the development of a bipotent precursor into both CTMC and MMC under different microenvironments. The unambiguous change of CTMC to MMC and then MMC to CTMC were demonstrated in another study (Kanakura et al., 1988a). Mouse PMC, containing heparin proteoglycans as indicated by staining berberine sulphate-positive, were purified and further cultured in the presence of IL-3 and IL-4. The percentage of the berberine sulphate-negative population among the cultured mast cells gradually increased to 100% and the cells switched to synthesize chondroitin sulphate proteoglycans predominantly. After adoptive transfer to the peritoneal cavity of the mast cell-deficient mice, the mast cell population became 100% berberine sulphate-positive. Thus, a bidirectional alteration between CTMC and MMC was demonstrated by mast cells derived from a CTMC population.

Taken together, these studies provide evidence that the apparently different CTMC and MMC can interchange under the influence of the anatomical environment in which their final differentiation occurs. The results imply that the two distinct mast cell phenotypes represent different expressions of the same cell lineage. It would be very important to determine the factors in the microenvironment which promote mast cell differentiation to either direction.

Heterogeneity in the expression of IgE receptors

The heterogeneity of the mast cells is also reflected in the expression of the receptors for IgE ($Fc\epsilon R$). The receptor expression is best characterized and compared in the rat system since methods are only available in rat to obtain relatively pure populations of not only PMC but also of MMC from rat intestinal mucosa (Befus et al., 1982). A single rat PMC was found to express approximately 3×10^5 receptors with

a K_a of $8 \times 10^9 \text{ M}^{-1}$ (Stern and Ishizaka, 1982). In contrast, rat intestinal mast cells carry only $3.6 \times 10^4 \text{ Fc}_\epsilon\text{RI}$ per cell (Lee et al., 1985), although their affinity is as high as that on PMC. *In vitro* cultured mast cells derived from the murine intestine were reported to carry $4.8 \times 10^4 \text{ IgE}$ receptors, a number which closely resembles that of rat intestinal mast cells (Ernst et al., 1985). However, the numbers of receptors estimated for BMDC varies among different laboratories: $6.0 \times 10^4/\text{cell}$ (Sredni et al., 1983); $0.7\text{--}1 \times 10^5/\text{cell}$ (Galli et al., 1982); $6.0 \times 10^5/\text{cell}$ (Razin et al., 1982). Considering that the size of MMC is significantly smaller than that of PMC, the receptor expression is further compared by the receptor density. The calculated receptor density of MMC is 1.2×10^2 receptors per μm^2 , while that of PMC is considerably higher, being in the range of $2.5 \times 10^2\text{--}2.8 \times 10^2$ per μm^2 (Conrad et al., 1975; Stern and Ishizaka, 1982; Lee et al., 1985).

Two types of surface receptors for IgE with different affinities and molecular weight, designated now as $\text{Fc}_\epsilon\text{RI}(\alpha)$ and $\text{Fc}_\epsilon\text{R}_\text{L}$ for the previous H and R, respectively, have been isolated from rat PMC and RBL cells (Conrad and Froese, 1978, Froese, 1980). Recently, both high and low affinity receptors were isolated from rat intestinal mucosal mast cells and further compared with those expressed on PMC (Swietler et al., 1989), and the heterogeneity in receptor expression on these mucosal mast cells is reflected in both molecular form and molecular weight. In the analysis using polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE), rat MMC yielded a 59 KDa $\text{Fc}_\epsilon\text{RI}(\alpha)$ band, which is higher than the 51 KDa band identified on PMC, and two bands of the low affinity $\text{Fc}_\epsilon\text{R}_\text{L}$ at 50 KDa and 58 KDa, whereas a single $\text{Fc}_\epsilon\text{R}_\text{L}$ band of 56 KDa was obtained from PMC. The

low affinity receptor dimorphism was considered to originate from the differences in degradative enzyme capability and not from the genes encoding the receptors. The differences in the receptor density and receptor expression extend the evidence for possible functional heterogeneity between the two types of mast cells.

Other surface antigens on mast cells:

Surface markers other than the receptors for IgE but characteristic of mouse mast cell phenotypes have been defined by a panel of rat monoclonal antibodies (Katz et al., 1982; 1985). The Forssman glycolipid globopentaosylceramide, which is recognized by the B1.1 antibody, is expressed on mouse CTMC but not MMC. On the other hand, an epitope present on MMC but absent on CTMC is identified by antibody B23.1. A similar epitope is detected by B54.2 on both CTMC and MMC. The epitopes recognized by all three monoclonal antibodies, however, are also expressed on the surface of activated mononuclear phagocytes. In the rat system, PMC were found to express leukocyte common antigen (LCA), Thy1.1 and MHC class I surface markers (Mowat et al., 1989). Unfortunately, no specific surface markers for rat mast cells, have been identified to recognize mast cells of either phenotype or immature mast cells at various maturation stages.

The *in vitro* growth of mast cells:

A persistent problem that investigators face in studying mast cell functions in various tissues has been the extreme difficulty in obtaining large numbers of purified cells free of contamination with other cell types. One approach to this problem has been the attempt to develop tissue culture systems for growing mast cells. Ginsburg (1963) enriched the first mast cell population from cultures of murine thymus cells

plated on embryonic fibroblast monolayers. A similar method has been used to grow mast cells from mouse lymph node cells (Ginsburg and Lagunoff, 1967) and rat thymus cells (Ishizaka et al., 1976). The phenomena demonstrated a link between the generation of mast cells and the presence of T cells. In fact, *in vivo* studies also observed a T cell-dependent mucosal mast cell hyperplasia following nematode infection, notably by *Nippostrongylus brasiliensis* (Mayrhofer, 1979a). The lack of intestinal mucosal mast cells during infections was also demonstrated in *nude* mice (Ruitenbergh and Elgersma, 1976) or T-cell-depleted rats (Mayrhofer and Fisher, 1979b). Although these results originally had suggested the derivation of the intestinal mucosal mast cells from a T-related precursor cell (Burnet, 1977), the later finding that mastopoietic activity could be transferred with immune serum, makes a requirement for a T-cell-derived soluble factor more likely (Befus and Bienenstock, 1979). Indeed, lymphocytes of helminth-infected rats, upon *in vitro* stimulation with specific antigen, release factors causing pronounced mucosal mastocytosis in normal rat bone marrow (Haig et al., 1982). In addition, mast cells are also stimulated to grow *in vitro* from bone marrow cultures with conditioned medium from either pokeweed mitogen- or concanavalin A-stimulated splenocytes (Hasthorpe, 1980; Razin et al., 1981; Nabel et al., 1981), indicating the involvement of non-antigen-specific mast cell growth factors. These bone marrow derived mast cells (BMMC) appear immature by ultrastructure but resemble MMC in their low level of histamine and the synthesis of chondroitin sulfate E.

The nature of the growth factors in the conditioned medium has been extensively characterized by a number of groups. Two distinct T cell factors have been identified

to play major roles in supporting the proliferation of cultured mast cells. The first one was purified from the supernatant of WEHI-3 cells which were originally designated as a myelomonocyte cell line (Ihle et al., 1982; 1983), and was identified to be interleukin-3 (IL-3) after the cDNA for murine IL-3 was cloned (Fung, et al., 1984; Yokota et al., 1984). Similarly, the mast cell factors produced by concanavalin A-stimulated T cell lines was also found to be identical to IL-3. Both recombinant IL-3 (rIL-3) and purified IL-3 have been shown to promote proliferation of not only T-dependent murine MMC but also multi-potential haemopoietic progenitor cells (Ihle et al., 1983; Hapel et al., 1985). Subsequently, rat IL-3 gene was cloned, but the product revealed unexpectedly low homology in amino acid sequence (54%) with its murine counterpart and demonstrated little cross-reactivity (Cohen et al., 1986). Rat rIL-3 has also shown to stimulate and maintain rat MMC growth from BM precursors (Haig et al., 1988). The other mast cell growth factor with different protein properties from IL-3 was identified and partially purified from a helper T cell line (Smith and Rennick, 1986). Its cDNA was cloned (Lee et al., 1986) and shown to be identical to the cDNA encoding the IgG1-inducing factor (Noma et al., 1986). Moreover, the N-terminal amino acid sequence of the peptide deduced from the cDNA was in agreement with the amino acid sequence of B-cell stimulating factor-1. This factor was thereafter uniformly designated as IL-4 because of its multiple biological activities (Lee et al., 1986).

Only recently, another cytokine in addition to IL-3 and IL-4 which also supports the growth of mouse BMMC was detected in the conditioned medium from pokeweed mitogen-stimulated mouse spleen cells (Hultner et al., 1988; Moeller et al., 1989). It

was termed MEA: mast cell growth enhancing activity. Initially MEA was characterized as a factor synergizing with IL-3 to enhance the proliferation of BMMC lines in the presence of saturating levels of IL-3 and was found later that it also acts independent of IL-3 and IL-4. The cytokine was subsequently purified and characterized as a glycoprotein with a Mr range from 37 KDa to 43 KDa (Moeller et al., 1990). Partial amino acid sequence determination revealed complete homology of this cytokine with a recently purified and cloned novel T cell growth factor, P40 or TCGF III. The latter is known now as the murine homologue of human IL-9 as defined by a striking sequence homology at both the protein and the nucleotide levels (Yang et al., 1989). The subsequent functional characterization of the relationship between MEA and P40 found that both factors have identical activities on either P40- or MEA-dependent cell lines and they interact with the same surface receptors (Hultner et al., 1990). MEA and P40 are therefore designated as murine IL-9.

Lately, studies in mice have provided evidence that another hematopoietic growth factor was encoded by the steel (*Sl*) locus (Huang et al., 1990; Zsebo et al., 1990). This factor, designated stem cell factor, was shown to be the ligand of the *c-kit* tyrosine kinase receptor which is coded by the murine white spotting (*W*) locus (Chabot et al., 1988; Geissler et al., 1988). Mice harboring mutations at the *W* and *Sl* loci exhibit several phenotypic abnormalities, including a virtual absence of mast cells. The *in vivo* administration of recombinant rat SCF led to local development of mast cells of the CTMC phenotype in the skin of mice and a systemic development of mast cells of both MMC and CTMC phenotypes in the rat (Tsai et al., 1991). Earlier findings have pointed to the presence of a growth factor in fibroblast conditioned medium (Jarboe et

al., 1989; Jarbo and Huff, 1989). This factor is capable of supporting the proliferation and differentiation of mast cell-committed progenitors in the absence of IL-3 and IL-4. It was found that this factor was absent in the fibroblast conditioned medium from monolayers prepared from *Sl/Sl^d* mice (Jarboe and Huff, 1989), indicating that this factor is probably the stem cell factor.

As suggested by Levi-Schaffer and her colleagues, the differentiation of murine bone marrow precursors to BMMC is dependent on the presence of IL-3, and the further differentiation of BMMC toward CTMC is induced by coculture with fibroblasts (Levi-Schaffer et al., 1986; Dayton et al., 1988). Others found that the synergistic effect of IL-4 and IL-3 is essential for *in vitro* clonal proliferation of the CTMC from the murine peritoneal mast cell population, with minimal response observed by the presence of either factor alone (Nakahata et al., 1986; Hamaguchi, et al., 1987). In addition, many have demonstrated that a connective tissue environment provided by coculturing with skin-derived living 3T3-fibroblasts is also necessary for maintaining the phenotype and the functional ability of the connective tissue mast cells (Levi-Schaffer et al., 1985; 1989; Reynolds et al., 1988). More recently, it was found that cross-linking of surface-bound IgE molecules by multivalent Ag also induces the proliferation of CTMC in the presence of IL-3 (Takagi et al., 1989). One explanation for this phenomenon is the finding by Plaut et al. (1989) on the release of endogenous IL-3 and IL-4 subsequent to triggering. The further investigation by the same group of researchers suggested that activation of protein kinase C after cross-linking resulted in the proliferation of CTMC in the presence of IL-3, since proliferation was observed again by using phorbol esters to activate protein kinase C (Tsuji et al., 1990a).

In addition to BMMC, mast cell lines have been established for *in vitro* chemical and functional analyses of pure mast cells. Murine mast cell lines (KiSV-MC) were derived by coculturing splenocytes with fibroblasts producing a Ki-*ras*-containing murine sarcoma virus (Reynolds et al., 1988). Some of the transformed KiSV-MC lines showed characteristics of CTMC in that they synthesized heparin proteoglycans and contained high contents of histamine. However, rat tissue-cultured mast cell (RCMC) lines were established recently in the absence of T cell growth factors or fibroblast feeder layers, from rat peritoneal mast cell population yielded cells of the MMC phenotype (Chan et al., 1988). As stated in the section on mast cell maturation and differentiation, although the peritoneal cavity normally contains the characteristic CTMC phenotype, subpopulations of immature mast cells were also isolated and defined by the negative staining with safranin O, the low histamine content, and the few cytoplasmic granules (Beaven et al., 1983). Characterization of RCMC cell lines and of clones derived from one line, demonstrated their immaturity in terms of histochemical staining, serine protease analysis, and histamine content (Chan et al., 1990). Thus, these cells could be stained with alcian blue but not be counter-stained with safranin O, pointing to the presence of chondroitin sulfate instead of heparin proteoglycans. The granules of these cells contained the serine protease type II (RMCP II) and had very low level of histamine. The fact that they were established as continuous cell lines in the absence of exogenous growth factors suggested the presence of self-supporting growth system. In association with the recent finding that mast cells produce a group of growth and differentiation factors including IL-3, IL-4, IL-5 and IL-6, in response to cross-linkage of the surface IgE receptors or to calcium ionophores (Plaut et al.,

1989; Wodnar-Filipowicz et al., 1989), makes it highly plausible that the proliferation of the *in vitro* cultured mast cells might be supported by the self-produced growth factors, pointing to a possible autocrine system.

Characteristics of human mast cells:

Although human mast cells resemble in many aspects the mast cells in rodents, the differences between the two systems are large enough that a separate discussion is needed. Like rodent mast cells, human mast cells are demonstrated to be heterogeneous in morphology, granule content, and functions. Two types of human mast cells analogous to the rodent MMC and CTMC have been identified, and in many studies they are additionally designated as tryptase positive (T) mast cells and tryptase-and-chymase positive (TC) mast cells, respectively, according to their differences in neutral protease content. Human mast cells are detected in tissues but unlike rodent mast cells their immature forms can not be recognized in blood. In addition, the *in vitro* derivation of mast cells from precursors in the haemopoetic tissues has yet been unsuccessful although the growth of mast cell-like cells was obtained from human fetal liver cell cultures in the presence of murine T cell factors (Seldin et al., 1986). The differences in the growth requirement of the two types of mast cells was observed by the immunohistological analysis on the mast cell distributions in immune deficient patients. A selective diminishment of the T mast cells in association with normal or slightly increased numbers of TC mast cells in gastrointestinal tissues was found in subjects whose T cell functions are hampered due to congenital or acquired immune deficiency syndromes (Irani et al., 1987), indicating that the growth of the T mast cells is dependent on the functional integrity of the T cells.

The phenotypic heterogeneity in human mast cells was first detected by Strobel et al. (1981) in that the visualization of certain mast cells in gastrointestinal mucosa was shown to be dependent on the methods of fixation. Under fixation with neutral buffered formalin, human mucosal-type mast cells failed to display metachromatic staining and are therefore referred to as formalin sensitive or atypical, while the human connective tissue-type mast cells were formalin resistant or typical (Befus et al., 1985). The two types of mast cells can also be distinguished by the ultrastructural features of their granules. Human MMC were the only type to have discrete scrolls in the granules, while grating or lattice substructures were observed exclusively in CTMC (Craig et al., 1988). Similar to the situations in rodents, the granules of human CTMC are found to be larger and more uniform in electron dense materials than the granules of human MMC. However, the cell sizes of the two types of mast cells are variable with extensive overlaps, therefore, the type of human mast cells can not be distinguished on the basis of cell size alone (Castells et al., 1987). The distribution of human MMC and CTMC is intermixed in most tissues examined, although MMC are predominant in the mucosa and lamina propria of the intestine, and the epithelium of the respiratory tract, while CTMC predominate in the submucosa of the intestine and bronchi (Befus et al., 1985; Shanahan et al., 1987). The anatomical nomenclature of CTMC and MMC does not reflect the differences of the two types of mast cells but tends to confuse the concept on the distributions of these mast cells. Suggestions have been made to have the present terms replaced with more precise names.

The granule mediators of the human mast cells are not as well characterized as those in rodent mast cells at present. The histamine contents of the MMC and CTMC

exhibit similar levels and therefore do not reflect the mast cell heterogeneity (Fox et al., 1985; Schwartz LB et al., 1987). The major compositional differences are derived from the content of neutral proteases, i.e., tryptase and chymase (Irani et al., 1986; Schwartz et al., 1987). The presence of tryptase in MMC and of both tryptase and chymase in CTMC reflect the situation in rats where antigenically distinct neutral proteases, RMCP I and II, are defined in CTMC and MMC, respectively. A major development was the method to distinguish the two types of mast cells in the same tissue section based on their content of neutral proteases. Carnoy's fixed tissue sections are first stained immunoenzymically with antibodies to chymase and tryptase by an immunoalkaline phosphatase technique, resulting in a blue color for T mast cells and a brown color for TC mast cells (Castells et al., 1987; Schwartz et al., 1987). In addition, a human carboxypeptidase has been found to be mainly synthesized in the TC mast cells and may be used as another marker for TC type of mast cells (Goldstein et al., 1987). As for the proteoglycan compositions, whether a heterogeneity also exists in human system has not yet been clarified. The major difficulty in characterizing human mast cells has its origin in the fact that preparations of mast cells obtained from various tissues often contain a mixture of T and TC mast cells. Recently, both heparin and chondroitin sulphate E were identified in the highly purified human lung mast cells (Metcalf et al., 1979; Stevens et al., 1988; Thompson et al., 1988); however, chondroitin sulphate E was found to be present not only in mast cells but also present in cultured human peritoneal macrophages and human leukaemic basophils (Koisset, 1986; Rothenberg et al., 1987). Similarly, the relative levels of the newly generated mediators are largely dependent on the ratios of mast cell subsets between different preparations. Human

skin mast cells, which are predominantly TC mast cells, generated PGD_2 but little or no LTC_4 upon stimulation (Lawrence et al., 1987).

The functional heterogeneity of the human mast cells is based on studies which used dispersed preparations of mast cells from different anatomical sites. Again, the impurity of the preparations results in difficulties in the interpretation of the results. Perhaps the most reliable studies are the ones using human foreskin since the tissue contains almost solely TC mast cells. In the response to secretagogues, mast cells isolated from foreskin can be triggered by both immunological stimuli through IgE receptors and a variety of non-immunological agents such as calcium ionophore, compound 48/80, substance P, the anaphylatoxin C_{5a} , and certain polypeptides (Benyon et al., 1987; Lawrence et al., 1987). In contrast, mast cells isolated from human lung and colonic mucosa can be activated only by IgE-dependent stimuli and calcium ionophore among the agents listed above (Church et al., 1982; Fox et al., 1985). However, the percentage of each type of mast cells in these preparations is dependent on the pathological condition of the donor organs (Irani et al., 1987). The sensitivity of the human mast cells to the inhibition of mediator release by anti-allergic drugs has been tested. Mast cells isolated from the tissues predominant in T type are generally inhibited by disodium cromoglycate (DSCG) although the percentage of inhibition is relatively low and also variable with the tissue origin (Flint et al., 1985; Schmutzler et al., 1985; Church and Hiroi, 1987). In contrast, DSCG has no effect on the histamine release from mast cells obtained from human skin, demonstrating opposite effector cells than the rodent's in which CTMC but not MMC are affected (Pearce et al., 1974; Clegg et al., 1985).

THE RECEPTORS FOR IgE ON MAST CELLS AND BASOPHILS

The early availability of the method to isolate rat peritoneal mast cells in high purity (Humphrey et al., 1963) and of the *in vitro* cultured rat basophilic leukemia (RBL) cell lines (Eccleston et al., 1973), as well as the availability of rat immunocytoma cell lines which produce a sufficient quantity of monoclonal IgE (Bazin et al., 1974), have lead to detailed characterization of the receptors for IgE in the rat system. RBL cells were originally induced in one Wistar rat after feeding with the carcinogen β -chloroethylamine (Eccleston et al., 1973). Later they were identified to be homologous to rat mucosal mast cells (Seldin et al., 1985b).

Isolation of the solubilized receptors for IgE:

In order to isolate the relatively small amounts of the surface receptor molecules, they are usually labeled with radioactive tracers before cell solubilization. Labeling of cell surface proteins is achieved by a lactoperoxidase catalysed reaction with ^{125}I (Conrad and Froese, 1976; Kulczycki et al., 1976), while that of surface carbohydrate residues makes use of galactose oxidase and ^3H - NaBH_4 (Pecoud et al., 1981). Biosynthetic labelling makes use of either ^3H or ^{14}C labelled amino acids or sugar residues (Kulczycki et al., 1976; Helm and Froese, 1981a). The solubilization of receptors utilizes the non-ionic detergents which retain the IgE-binding ability of the receptors. The commonly used detergents are Nonidet P-40 (NP-40) (Conrad et al., 1976); Triton X-100 (Rossi et al., 1977); and Rennex 30 (Pecoud and Conrad, 1981).

The solubilized receptors are further isolated with a number of procedures which make use of immunoprecipitation and affinity chromatography, as reviewed in great

detail by Froese (1984). Briefly, the following approaches have been used: [1] isolation of IgE-receptor complexes with anti-IgE antibodies coupled to Sepharose (Conrad and Froese, 1976); [2] IgE-Sepharose (Conrad and Froese, 1978a; Kulczycki and Parker, 1979); [3] isolation of haptenated IgE-receptor complex with anti-hapten-Sepharose (Conrad and Froese, 1978b; Kanellopoulos et al., 1979); [4] anti-receptor antibodies either directly coupled to Sepharose or used in association with protein A-Sepharose (Conrad et al., 1978; Conrad et al., 1979). Once isolated, the receptors are further characterized by polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) (Conrad and Froese, 1976; Kulczycki et al., 1976).

Identification of two types of receptors for IgE:

In this laboratory two types of receptors for IgE have been isolated with IgE-Sepharose from RBL cells and rat PMC, which were originally designated as H and R (Conrad and Froese, 1978a, Froese A, 1980; Froese et al., 1982). The R receptor molecule is now known to represent the IgE-binding subunit α of the high affinity $\text{Fc}_\epsilon\text{RI}$ complex, $\alpha\beta\gamma_2$, and therefore is referred as $\text{Fc}_\epsilon\text{RI}(\alpha)$, while H is currently named as $\text{Fc}_\epsilon\text{R}_\text{L}$ (Chan et al., 1990) in order to distinguish from the low affinity $\text{Fc}_\epsilon\text{RII}$ expressed on other cell types. $\text{Fc}_\epsilon\text{R}_\text{L}$ exhibits properties of a low affinity receptor for IgE (Kepron et al., 1982) and is not isolated with IgE and anti-IgE-Sepharose conjugate or with haptenated IgE and antibody coupled to Sepharose (Conrad and Froese, 1978a; 1978b).

SDS-PAGE analysis in this laboratory has identified the relative molecular mass (M_r) of $\text{Fc}_\epsilon\text{RI}(\alpha)$ and $\text{Fc}_\epsilon\text{R}_\text{L}$ to be 45 KDa and 55 KDa, respectively (Conrad and Froese, 1978a). $\text{Fc}_\epsilon\text{RI}(\alpha)$ isolated from RBL cell lines maintained in other laboratories

yielded a much higher Mr, ranging from 53 KDa to 58 KDa (Kanellopoulos et al., 1979; Pecoud et al., 1981). However, it is necessary to point out here that the receptor molecular weight as determined by SDS-PAGE is higher because of the abnormal mobility of glycoproteins on polyacrylamide gels (Kulczycki et al., 1976). Kanellopoulos et al. (1980) demonstrated that the molecular weight of Fc ϵ RI(α) depends on the acrylamide concentration of the gels and that an extrapolation of a plot of apparent molecular weight versus the reciprocal of the gel concentration to zero gel concentration yielded to a decrease in molecular weight by about 10 KDa. It was later demonstrated that the tryptic maps of the Fc ϵ RI(α) from the various RBL cell lines and rat PMC yielded identical spots, indicating that the differences in Mr are attributable to the various degrees of protein glycosylation (Pecoud and Conrad, 1981). Recently, the expression of receptors for IgE on intestinal mucosal mast cells (IMMC) was characterized and both types of Fc ϵ R were isolated (Swieter et al., 1989). Fc ϵ RI(α) had a Mr of 59 KDa, while dimorphism was detected for Fc ϵ R_L with Mr being 50 KDa and 58 KDa.

In addition, a minor surface molecule, having a Mr of 71 KDa (71K) (Helm and Froese, 1981), could be reversibly induced on RBL cells by their infection with *Mycoplasma hyorhinis* (Chan et al., 1986). The properties of this molecule appeared to resemble those of Fc ϵ RI(α). Indeed, it was demonstrated that 71K is derived from mycoplasma modulation of partial surface Fc ϵ RI(α) (Chan et al., 1986). However, no biological functions have yet been assigned to either Fc ϵ R_L or 71K.

The surface receptors for the Fc portion of the IgE (Fc ϵ R) on mast cells and basophils receive signals from IgE together with its specific antigen and form a link

between the extra-cellular environment and the intracellular mechanisms. Generally, a divalent antigen which can simply bridge the $Fc\epsilon R$ through the receptor bound IgE will be sufficient to trigger mediator release (Siraganian et al., 1975). Therefore, IgE is not always required since cross-linking of the receptors is the critical step in the release function. It has been shown that antiserum specific for $Fc\epsilon RI(\alpha)$ has the same effect as the antigen-IgE complex (Ishizaka et al., 1978).

Distribution of the IgE receptors:

The high affinity receptors for IgE expressed on both rat peritoneal mast cells (PMC) and rat basophilic leukemia (RBL) cells were shown to distribute independently of each other and randomly over the entire surface, as a smooth ring stain by immunofluorescence and immunoenzymatic analyses (Sullivan et al., 1971; Lawson et al., 1975; Mendoza and Metzger, 1976a; Schlessinger et al., 1976). These receptors were demonstrated in 1970's to be laterally mobile across the membrane (Schlessinger et al., 1976) and to redistribute into caps at room temperature by the treatment with divalent anti-IgE antibodies (Sullivan et al., 1971). However, capping but not patching was inhibited when anti-IgE was introduced either at low temperatures or in the presence of cytochalasin B which inhibits the microfilament formation, indicating that capping is an energy dependent process (Carson and Metzger, 1974).

Extensive studies have been carried out in the late 1970's to quantitate the number of receptors on one intact cell. It was demonstrated by a number of techniques that generally the tumour cell line RBL carried approximately two-fold more receptors than rat PMC, with the mean values around $6 \times 10^5/\text{cell}$ and $3 \times 10^5/\text{cell}$, respectively (Conrad et al., 1975; Mendoza and Metzger, 1976b; Sterk and Ishizaka, 1982); while

the number of $\text{Fc}_\epsilon\text{RI}$ on rat MMC was found to be an order of magnitude lower than that on PMC, with the mean value as $3 \times 10^4/\text{cell}$ (Lee et al., 1985). The surface receptor density was calculated from an assumed spherical shape of the cell and the average cell size. Since rat PMC have about double of the cell size than RBL and rat MMC ($19.6 \mu\text{m}$, $9.5 \mu\text{m}$ and $9.7 \mu\text{m}$, respectively), the receptor density of RBL cells are much higher than that of either PMC or MMC.

In addition, the number of receptors on RBL cells was shown to vary with the stages of maturation and growth cycle. More interestingly, it was found that the number of receptors on human basophils correlated with the level of the serum IgE in normal and atopic individuals (Malveaux et al., 1978;), suggesting an inducing effect of the serum IgE for the expression of $\text{Fc}_\epsilon\text{R}$. Indeed, RBL cells cultured in the presence of IgE showed increased binding to IgE and the effect was demonstrated to be the result of an 80% decrease in the elimination of the receptor from surface with continuous insertion of previously synthesized receptor (Furuichi et al., 1985). Similarly, mast cells, from helminth infected rats in which serum IgE level increases, were found to possess more surface IgE binding sites than those from normal rats (Ishizaka et al., 1975). However, the intrinsic mechanisms in the regulation of the receptor expression has not been clarified.

Interaction of IgE with its receptors:

The binding of monomeric IgE to the receptors of mast cells and basophils does not cause any known perturbation by itself. This binding ability of the $\text{Fc}_\epsilon\text{RI}$ to IgE is not lost when the cell membrane is fragmented by lysis with distilled water or by sonication (Bach and Brashler, 1973; Konig and Ishizaka, 1974). In humans, the

receptors on basophils were found to be partially occupied by IgE (Ishizaka et al., 1973) and the amount of IgE bound correlated with the individual's serum IgE level (Conroy et al., 1977; Skov et al., 1977). In addition, as stated before, a positive correlation between the total number of $Fc\epsilon R$ on each human basophil and the serum levels of IgE was observed (Malveaux et al., 1978).

The kinetics of the interaction of IgE and its receptor on RBL were demonstrated to be bimolecular by Kulczycki and Metzger (1974). The association rate constant was found to be $0.96 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, while the dissociation rate constant was of the order of $1.6 \times 10^{-5} \text{ s}^{-1}$, which theoretically proved the fact that once IgE binds to a receptor, it remains attached for a prolonged period of time. Although it is not clearly understood, it is noteworthy to mention that the rate of dissociation of the solubilized receptors from RBL was found to be $0.026 \times 10^{-5} \text{ s}^{-1}$, which is much lower than that for the membrane-bound receptors. This slow dissociation rate is of great advantage for the *in vitro* isolation of the solubilized $Fc\epsilon$ receptors. The equilibrium or the binding constant used to express the affinity of the receptors was derived either from a calculation using the two rate constants, or from the Scatchard plots of IgE-binding measurements. High affinity constants were obtained from both approaches, varying from 1.1×10^8 to $2.8 \times 10^{10} \text{ M}^{-1}$ (Froese, 1980).

The univalency of the $Fc\epsilon RI$ was clearly demonstrated by Mendoza and Metzger in a classical study (1976) which used normal and tumour mast cells of rat and mouse. Principally, the cells were reacted with a mixture of fluorescein-labeled IgE (F-IgE) and rhodamine-conjugated IgE (R-IgE) followed by anti-fluorescein serum. The green fluorescence (F-IgE) appeared in patches while the red fluorescence (R-

IgE) remained diffuse, indicating that the receptors binding R-IgE did not comigrate with the receptors occupied by F-IgE. However, a comigration of F-IgE and R-IgE into patches was observed on all the cells examined when anti-IgE was applied. Thus, it was concluded that one unit of receptor binds one molecule of IgE and each receptor is not connected with one another in any way.

The structure of an IgE molecule is known to consist of two heavy (ϵ) and two light chains which are held together by disulphide bonds as well as by noncovalent interactions, similarly to other classes of immunoglobulins. Amino acid sequencing of human ϵ (Bennich and von Bahr-Lindstrom, 1974) and DNA sequencing of the rodent ϵ gene (Ishida et al., 1982; Liu et al., 1982) have shown IgE to have an extra domain, $C_{\epsilon}2$, in the place of the hinge region of IgG and therefore to exhibit less segmental flexibility (Oi et al., 1984). What is of great significance in understanding the mechanism of action of the receptor and in inhibiting of mediator release in therapeutic implications is to identify the part of the IgE molecule responsible for binding with $Fc_{\epsilon}R$'s and the structure of the bound IgE on the cell surface.

In early studies, involving the denaturation of different parts of the human IgE molecule, Dorrington and Bennich (1978) suggested that the binding site of IgE is contained within the $C_{\epsilon}3$ and/or $C_{\epsilon}4$ domains. The rodent IgE binding site was studied by Perez-Montfort and Metzger (1982) using proteolytic digestions of either IgE or the IgE-receptor complexes purified from RBL cells. Two sites of cleavage were identified in an IgE molecule in two species, the interdomain regions between $C_{\epsilon}3:C_{\epsilon}4$ and $C_{\epsilon}2:C_{\epsilon}3$ in mouse and within $C_{\epsilon}3$ and between $C_{\epsilon}2:C_{\epsilon}3$ in rat. Notably, when bound to the receptor, the rate of cleavage was inhibited approximately three-

fold for the first site and forty-fold for the second site. The results clearly implicate a region close to the C ϵ 2:C ϵ 3 junction as the principal site of interaction between rodent IgE and its receptor. Additional information has come from the use of two monoclonal antibodies generated against rat IgE, one specific for a site in the Fab (V $_H$ -C ϵ 1) domains and one for a site in the Fc (C ϵ 3-C ϵ 4) domains (Conrad et al., 1983). Both these antibodies appear to bind to the same extent whether the IgE is soluble or bound to receptors on RBL cells, indicating that these sites are not obscured by the receptor. The recent application of recombinant DNA (rDNA) technology has generated the cDNA encoding all or part of the Fc region of IgE (Kenten et al., 1984; Liu et al., 1984). The unglycosylated active fragment produced from bacteria by Liu et al. is missing the one third of the C ϵ 2 from the NH $_2$ -terminal, indicating that the deleted region and the carbohydrate are not required for the binding of the receptors. The recombinant rodent IgE with the C ϵ 4 domain replaced by the homologous C γ 3 appeared to be not to differ in binding and in triggering mediator release from the original IgE (Baird et al., 1989), indicating that the C-terminal end of IgE does not contribute in the interaction between IgE and its receptor. Consistent results were obtained in the case of human IgE, in which a segment of 76-amino acids containing portions of the C ϵ 2 and C ϵ 3 domains could be used as a very potent inhibitor of native IgE binding in an *in vivo* assay (Helm et al., 1988). This segment was found to fit into the proposed three-dimensional structure for human IgE Fc (Padlan and Davies, 1986) at the interface between the C ϵ 2 and C ϵ 3 domains.

The three-dimensional structure of the membrane-bound IgE-receptor complex was characterized by Holowka and Baird using the fluorescence resonance energy

transfer technique. They placed donor probes at several different sites on the IgE molecule and measured the distance between each of these sites and acceptors at the plasma membrane surface when IgE was bound to receptor. The comparison of the distances at each dimension with the soluble IgE molecule provided information on the orientation and conformation of the receptor-bound IgE (Holowka and Baird, 1983). With the information obtained from the energy transfer studies and from the previous reports, a model was constructed (Baird et al., 1988). Briefly, IgE binds to its high-affinity receptor in a bent conformation, with the Fab arms sticking up and the C-terminal end of the Fc segment sticking out. The idea was also supported by the finding that a change in IgE conformation in association with loss of some segmental flexibility occurs when IgE binds to receptor (Holowka et al, 1985).

Structure of the high affinity receptor for IgE:

The most thoroughly characterized receptor for IgE is the high affinity receptor $Fc\epsilon RI$ of the RBL cell lines. The rodent $Fc\epsilon RI$ has been identified to be a tetrameric complex of non-covalently attached subunits. The α -subunit is a glycoprotein with a M_r of approximately 45 KDa, which can be labeled by external iodination of the intact cells and isolated by affinity chromatography after solubilization with mild detergents (Conrad and Froese, 1976; Kulczycki et al., 1976). Using cross-linking reagents the 33-KDa β -subunit was found to be associated with the α -subunit in 1:1 molar ratio (Holowka et al., 1980; Holowka and Metzger, 1982). Subsequently, it was found that the β -component could be co-purified with the α -subunit by adding phospholipids to the nonionic detergent containing solvent (Rivnay et al., 1982). Under these conditions, the third component, a 20-KDa dimer of disulphide-linked γ subunits, was

also observed (Perez-Montfort et al., 1983). Although the β and the γ dimer are easily dissociated from α , the three subunits were found to be synthesized and degraded coordinately as a minimal unit of the $\text{Fc}\epsilon\text{RI}$ (Quarto et al., 1985). More data on the relationship between each unit have been provided recently. It was found that β and the γ dimer dissociate from α as a unit before dissociating from each other in detergent solutions (Rivera et al., 1988). Conversely, the α and β subunits interact with each other before association with the two γ chains during biosynthesis, as suggested by Rivera and Metzger from their preliminary observation (Blank et al., 1989).

Earlier studies on the topology of $\text{Fc}\epsilon\text{RI}$ revealed that unlike the α -subunit, the β and γ could only be labeled extrinsically on the lysed plasma membrane vesicles or at the cytoplasmic side of the membrane (Holowka et al., 1981; Holowka and Baird, 1984). These subunits were presumed to be intramembrane proteins with exposure on the internal surface of the bilayer but with no or minimal exposure on the cell surface. The recent rDNA approaches further elucidated the possible arrangement of the subunits. The complementary DNA encoding the three subunits have been isolated from the cDNA library constructed from the mRNA of RBL cells (Kinet et al., 1987; 1988; Shimizu et al., 1988; Blank et al., 1989). Analyses of the nucleotide sequences predicted the amino acid residues of the putative extracellular, transmembrane and cytoplasmic portions of each receptor chain. The cDNA for the three subunits were further cotransfected and the rat IgE-binding activity was revealed in the transfectants by an IgE-rosetting assay (Blank et al., 1989). When various combinations of the cDNA were transfected, rosette-forming cells were only detected after transfection of all three cDNAs, indicating that only the full receptor complexes may incorporate into

the plasma membrane and function as a unit. However, the human α subunit could be expressed on transfected cells in the presence of rat β subunit alone, indicating that the β subunit has the function of assisting the expression of the high affinity IgE receptors (Miller et al., 1989). In the proposed model, the large extracellular portion of the α -subunit at the top, containing two homologous domains possibly linked by disulphide bonds, covers the short extracellular extensions of the β and γ chains. The β subunit is located in between the remaining portion of the α at the right and the two γ chains at the left, with both of the C- and N-terminal portions in the cytoplasm; while the low Mr γ chains form long tails in the intracytoplasmic domains at the C-terminal.

Cross-linking of the receptors as initial signal:

The bridging of the surface receptors for IgE is the initial signal in triggering degranulation of mast cells, basophils and RBL cells. The cross-linking can be achieved by anti-receptor antibodies, oligomers of IgE, anti-IgE antibodies or multivalent antigens, however, accumulating evidence shows that there may be some specific requirements for the cross-linked state in order to generate a transmembrane signal. Early studies had suggested that aggregates of IgE as small as a dimer might be sufficient to induce degranulation (Siraganian et al., 1975). It was later found that with RBL cells, dimers were virtually inactive in the absence of D₂O, and even in its presence were considerably less effective than trimers, and the latter less than higher oligomers (Fewtrell and Metzger, 1980). However, the data provided by studies with small oligomers of IgE suggest that the binding of a dimer may initiate interactions with other components that constrain receptor mobility, and eventually induce the coalescence

and aggregation of the receptors into large clusters and the subsequent internalization (Menon et al., 1984).

Rigid bivalent ligands of different lengths, made with polymers of avidin cross-linked by bis-biotin and capped with DNP at the two biotins at each end of the linear polymer, were used to study the structural constraints for cross-linking the receptors. These ligands bind to monoclonal anti-DNP IgE in solution and efficiently trigger degranulation by cross-linking the receptor-bound IgE on the cell surface (Kane et al., 1990). It was found that dose-response curves for dimers, trimers, tetramers, pentamers, and hexamers are nearly superimposable. Since the hexamers are nearly 240 Å long, it is unlikely that at this distance the directly cross-linked receptors could come into stable physical contact. In association with the finding that limited cross-linking of IgE receptors induces small clusters to coalesce into large aggregates (Menon et al., 1984), it was presumed that two noncross-linked receptors may be brought into proximity and hence initiate the stimulus for degranulation although the directly cross-linked receptors may be far apart. In addition, the monomers of the bivalent ligands were found to stimulate a lower level of degranulation than the dimers and higher polymers. It was thought that the cross-linker was too short so that the bound receptors did not have sufficient flexibility to transduce signals (Kane et al., 1990). As suggested by the authors, this phenomenon could be interpreted to reflect either a structural constraint or kinetic constraint with respect to the lifetime of the cross-linked IgE-receptor complex or the rate of cross-linking. It appeared from studies that a long lifetime is not sufficient to generate a signal and the rate of cross-linking is more important than the extent of cross-linking (Baird, et al., 1989).

The precise role of the function of receptor aggregation in transduction and regulation of biological signals of mast cells leading to degranulation still remains to be determined, although possible mechanisms have been considered (Metzger, 1983). Upon aggregation, the receptor itself may perform certain functions, such as forming a channel for ions or carrying out an enzymatic activity. A distal membrane component may also be activated by interaction with the aggregated receptor. Finally, one general aspect of the role of receptor aggregation is whether a particular perturbation can persist in the absence of the original signal after its initiation. Whatever the roles of the perturbation may be, it appears that only the internalization of the ligand-receptor complex at the late stage shows time-dependency in virtually all receptor-mediated responses.

Activation of mast cells and basophils:

Mast cells and basophils activated through the cross-linkage of the $\text{Fc}_\epsilon\text{RI}$ release a variety of preformed and newly synthesized biologically active substances, such as histamine and PGD_2 , causing allergic inflammatory reactions. Recently, the secretion of a series of lymphokines classically produced by T helper cells was detected simultaneously by three independent groups (Burd et al., 1989; Plaut et al., 1989; Wodnar-Filipowicz et al., 1989), including mast cell growth factor IL-3, IgE switch factor IL-4, eosinophil differentiation factor IL-5, immunoglobulin secretion factor IL-6 and a granulocyte/macrophage colony-stimulating factor GM-CSF. The latter response was speculated to play roles either in the late phase of the allergic reactions (Burd et al., 1989; Plaut et al., 1989) or in local tissue defense (Wodnar-Filipowicz et al., 1989). In addition, a cell population lacking B- and T-cell markers, in the

spleen of normal mice, produces IL-4 when stimulated by cross-linkage of IgE Fc receptors (Ben-Sasson et al., 1990). This population of cells probably resembles the mast cell-committed progenitors which are detected in haemopoietic tissues and in the mesenteric lymph nodes (Ashman et al., 1991). These progenitors are capable of developing into the connective tissue phenotype when cultured on a fibroblast monolayer and the mucosal phenotype when cloned in the presence of conditioned medium from PWM-stimulated spleen cells (Jarboe et al., 1989). The biochemical events involved in the mediator release have been extensively characterized and will be reviewed here.

Bridging of IgE receptors like other surface stimulators sends signals to the cells and activates a series of intracellular events. It is generally accepted that calcium plays an important role in mast cell activation. The development of sensitive new method to monitor changes in cytoplasmic calcium concentration using the fluorescent probe Quin-2 made an important finding that mobilization of intracellular Ca^{2+} occurs within 5 seconds after antigen challenge and was believed to be the earliest intracellular change (White et al., 1984). In the event of mediator release, calcium of both intracellular and extracellular sources are utilized (Stump et al., 1987). Calcium ionophores which transport Ca^{2+} from the incubation medium to the cell interior can bypass the surface receptor and induce mediator release directly (Foreman et al., 1973). Following receptor activation a variety of membrane-associated enzymes, such as serine protease, phospholipase C, and adenylate cyclase, are also activated. The activated enzymes stimulate phosphatidylinositol (PI) turnover (Cockcroft and Gomperts, 1979; Kennerly et al., 1979) and metabolize the inositol phospholipids to release the products, 1,2-diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3),

into the cytoplasm (Streb et al., 1983, Kurosawa and Parker, 1989). The former product serves as a second messenger for mobilization of intracellular Ca^{2+} from the endoplasmic reticulum (Burgess et al., 1984), while the accumulation of the latter correlates with histamine release. Rapid changes in phospholipid methylation were observed by Ishizka's group (1987) but not by others (Benyon et al., 1986).

The increased intracellular calcium and diacylglycerol activate the phospholipid-dependent protein kinase C which plays an important role in protein phosphorylation (Nishizuka, 1986; White et al., 1985). In support of this was the evidence that phorbol esters which directly activates the protein kinase C can promote mediator release from mast cells (Heiman and Crews, 1985). In addition, staurosporine isolated from cultures of *Streptomyces* has been shown to inhibit protein kinase C (Tamaoka et al., 1986) and also inhibit mediator release from mast cells (Kurosawa et al., 1989).

Chemical nature of the receptors for IgE:

The glycoprotein nature of the surface receptors for IgE was demonstrated by their sensitivity to trypsin digestion (Conrad and Froese, 1976), the incorporation of radioactive precursors into both protein and carbohydrate (Kulczycki et al., 1976; Helm and Froese, 1981a), and lectin-binding ability (Helm et al., 1979). $\text{Fc}_\epsilon\text{RI}(\alpha)$ has been estimated to consist of about 30% carbohydrate (Kanellopoulos et al., 1980) which is composed of fucose, mannose, galactose, N-acetylglucosamine and sialic acid (Kanellopoulos et al., 1980; Goetze et al., 1981, Helm and Froese, 1981a; 1981b), while the other subunits of $\text{Fc}_\epsilon\text{RI}$ were demonstrated to be unglycosylated (Holowka et al., 1981; Holowka and Metzger, 1982; Perez-Montfort et al., 1983). Although the total amount of carbohydrate present on $\text{Fc}_\epsilon\text{R}_\text{L}$ has not been determined, most

sugar residues identified in $\text{Fc}_\epsilon\text{RI}(\alpha)$ also exist in $\text{Fc}_\epsilon\text{R}_\text{L}$ except sialic acid (Helm and Froese, 1981a; 1981b). The presence or absence of sialic acid in $\text{Fc}_\epsilon\text{R}_\text{L}$ has not been established. Recent studies in this laboratory using glycosylation processing inhibitors and endoglycosidases demonstrated that both receptors for IgE are composed primarily of complex oligosaccharides with a single N-linked high mannose residue, and a small amount of O-linked oligosaccharides in the case of $\text{Fc}_\epsilon\text{RI}(\alpha)$ (LaCroix, personal communication). Functional characterization of the carbohydrates pointed to a role in the incorporation of the receptors into the plasma membrane (Hempstead et al., 1981; Pecoud et al., 1981).

As described earlier, the cDNAs coding for the $\text{Fc}_\epsilon\text{RI}$ tetramer ($\alpha\beta\gamma_2$) have been isolated (Kinet et al., 1987; Shimizu et al., 1988; Kinet et al., 1988; Blank et al., 1989) and used for transfection in order to obtain complete expression of $\text{Fc}_\epsilon\text{RI}$ (Blank et al., 1989). The nucleotide sequences predict a 237-, a 243-, and a 68-residue polypeptides for the subunits α , β , and monomeric γ , respectively. Based on the recent data and previous biochemical information a model for the complete receptor was proposed (Blank et al., 1989). Seven transmembrane segments of about 20 residues each presumably in an α -helical conformation were identified for the complex, i.e., one, four and one for the α , β , and monomeric γ , respectively. Two homologous domains comprising disulphide loops have been identified in $\text{Fc}_\epsilon\text{RI}(\alpha)$ at the extracellular portion of $\text{Fc}_\epsilon\text{RI}(\alpha)$ (Kinet et al., 1987).

Less information exists for the polypeptide composition and sequence of $\text{Fc}_\epsilon\text{R}_\text{L}$. Nevertheless, peptide mapping analysis revealed entirely different patterns for $\text{Fc}_\epsilon\text{R}_\text{L}$ and $\text{Fc}_\epsilon\text{RI}(\alpha)$ (Pecoud and Conrad, 1981), indicating the structural distinction of these

two receptors for IgE. Evidence derived from controlled digestion of the solubilized receptors suggested only a single extracytoplasmic domain for $\text{Fc}\epsilon\text{RL}$ (Roth and Froese, 1982).

Cross-reactivity of the receptors for IgE with other immunoglobulins:

Anaphylactic reactions have been recognized to be induced not only by IgE molecules but also by certain subclasses of IgG in several species, including rat (Morse et al., 1968); mouse (Ovary et al., 1970); and human (Parish, 1970). In the rat system, IgG_{2a} was shown to compete with IgE for receptor sites on rat PMC, however, with rather low affinity (Mossmann et al., 1976). The direct binding of IgG_{2a} to RBL cells was only detected in the form of immune complex (Halper and Metzger, 1976). It was demonstrated by Segal et al. (1981) that RBL cells carry two different kinds of receptors both of which could bind to rat IgG-Sepharose. However, one was found to bind IgE with high affinity, whereas the other bound both IgE and IgG weakly, with relatively higher affinity for IgE.

Kepron et al. (1982) have suggested that the two types of receptors identified by Segal et al. (1981) are identical to those isolated from RBL and rat PMC cells with IgE-Sepharose in this laboratory, i.e. the high affinity $\text{Fc}\epsilon\text{RI}(\alpha)$ and the low affinity $\text{Fc}\epsilon\text{RL}$ (Conrad and Froese, 1978a; Froese, 1981). Inhibition studies revealed that both IgE and IgG inhibited the binding of $\text{Fc}\epsilon\text{RI}(\alpha)$ to IgG-Sepharose but only IgE inhibited the binding of this receptor to IgE-Sepharose, while both IgE and IgG inhibited the binding of $\text{Fc}\epsilon\text{RL}$ to either IgG-Sepharose or IgE-Sepharose, with the former being always the more effective inhibitor (Kepron et al., 1982). Thus, $\text{Fc}\epsilon\text{RL}$ is more of a receptor for IgE than for IgG. More recently, the cross-reactivities of the

two receptors for IgE with various subclasses of IgG were further compared (Kepron et al., 1988). For $\text{Fc}_\epsilon\text{RI}(\alpha)$ they were ranked as $\text{IgG}_{2a} > \text{IgG}_1 > \text{IgG}_{2b}$ and for $\text{Fc}_\epsilon\text{R}_L$ as $\text{IgG}_{2b} > \text{IgG}_1 > \text{IgG}_{2a}$. It has been known for some time that mouse IgE and rat IgE bind to the same IgE receptors on rat PMC with comparable affinity (Sterk and Ishizaka, 1982). In the study carried by Kepron et al. (1988) the binding affinity of mouse IgG subclasses to $\text{Fc}_\epsilon\text{RI}(\alpha)$ was examined to rank as $\text{IgG}_1 > \text{IgG}_{2a} > \text{IgG}_{2b}$ and to $\text{Fc}_\epsilon\text{R}_L$ as $\text{IgG}_1 > \text{IgG}_{2b} > \text{IgG}_{2a}$.

Similarly, two types of Fc receptors capable of binding IgE were observed to exist on the mast cells in mouse (Ishizaka et al., 1979; Daeron et al., 1980) and the basophils in human (Sterk and Ishizaka, 1982). In addition to the high affinity receptor for IgE, the other receptor was found to interact with IgG and may therefore be comparable to the low affinity $\text{Fc}_\epsilon\text{R}_L$ in rat system.

The low affinity receptors for IgE on other cell types:

The high affinity receptor for IgE ($\text{Fc}_\epsilon\text{RI}$) is exclusively expressed on mast cells and basophils, a low affinity receptor for IgE is found, on the other hand, not only on mast cells and basophils but also on a variety of haematopoietic cells, including lymphocytes (Fritsche and Spiegelberg, 1978), monocytes (Melewicz et al., 1982), macrophages (Capron et al., 1975), eosinophils (Capron et al., 1981), and platelets (Joseph et al., 1983). The latter group of low affinity IgE Fc receptors is generally referred as $\text{Fc}_\epsilon\text{RII}$. In normal individuals, approximately 8-10% of peripheral blood mononuclear cells are $\text{Fc}_\epsilon\text{RII}^+$ and more than 90% of these cells are B lymphocytes (Delespesse et al., 1986), while the B cells in allergic patients carry a higher density

of Fc ϵ RII although the percentage is similar (Spiegelberg et al., 1979; Suemura and Kishimoto, 1987).

Fc ϵ RII on murine hybridoma B cells has been determined to be a single chain glycoprotein with a Mr of 49 KDa (Conrad and Peterson, 1984; Conrad et al., 1987; Keegan and Conrad, 1987). The human Fc ϵ RII is very similar to its rodent counterpart, it is also a single chain glycoprotein with Mr of 45 KDa. Monomeric IgE binds to Fc ϵ RII on various cell types with an equilibrium association constant in the order of 10^7M^{-1} (Spiegelberg, 1984). Subsequently, Fc ϵ RII was demonstrated to be identical to a B cell-restricted activation antigen CD23 (Bonney et al., 1987; Yukawa et al., 1987).

Recently, the structure of Fc ϵ RII expressed on various cell types has been analyzed and two species of Fc ϵ RII (Fc ϵ RIIa and Fc ϵ RIIb) have been identified (Yokota et al., 1988). Sequence analysis revealed that they differ only at the N-terminal cytoplasmic region, but share the same C-terminal extracellular portion. Fc ϵ RIIa is constitutively, but cell type-specifically, expressed in normal B cells and B cell lines, whereas Fc ϵ RIIb is detectable in various cell types and in peripheral blood lymphocytes in atopic individuals. Normally, Fc ϵ RIIb are undetectable in B cells but its expression could be induced by IL-4.

Fc ϵ RII is expressed transiently on B cells at a certain stage of differentiation, i.e., virtually all B cells bearing sIgM and sIgD express Fc ϵ RII but not the cells which have switched to IgG $^+$, IgA $^+$, or IgE $^+$ expression (Kikutani et al., 1986b). This surface antigen is therefore known as a unique differentiation antigen of B lymphocytes. In addition, certain monoclonal antibodies to CD23 were shown to trigger DNA synthesis

and to induce proliferation in activated B lymphocytes (Gordon et al., 1986). In fact, CD23 was proposed to be the receptor for a T cell-derived low molecular weight (12 KDa) B cell growth factor (BCGF) (Gordon et al., 1986; Guy and Gordon, 1987). These observations point to the importance of this molecule in cell growth and differentiation.

Unlike $\text{Fc}_\epsilon\text{R}_\text{L}$, $\text{Fc}_\epsilon\text{R}_\text{II}$ can be released as fragments, which have been identified as IgE-binding factors (Ishizaka, 1985; Keegan and Conrad, 1987). Recently, the IgE-binding factor (IgE-BF) generated from $\text{Fc}_\epsilon\text{R}_\text{II}$ has been identified as an autocrine growth factor for EBV-transformed B lymphoblastoid cells (Swendeman and Thorley-Lawson, 1987). Moreover, the IgE-BF was speculated to be identical to the 25-30 KDa B cell-derived BCGF described in several other studies (Gordon et al., 1984; Jurgensen et al., 1986; Muraguchi et al., 1986). In the human system, a higher serum level of IgE-BF is detected in patients with atopic diseases and with parasitic infections (Delespesse et al., 1988), which is in agreement with the previous observations on the augmented expression of $\text{Fc}_\epsilon\text{R}_\text{II}$ on B cells in such situations (Spiegelberg et al., 1979). Semi-purified preparations of the B cell-derived IgE-BF have been demonstrated to regulate the production of IgE by *in vivo* preactivated B cells (Delespesse et al., 1988; Pene et al., 1988).

The gene coding for $\text{Fc}_\epsilon\text{R}_\text{II}$ in human B lymphoblastoid cells has been cloned by three independent groups (Kikutani et al., 1986; Ikuta et al., 1987; Ludin et al., 1987). Subsequently, the cDNA encoding the murine $\text{Fc}_\epsilon\text{R}_\text{II}$ has also been isolated from activated B cells (Bettler et al., 1989). The predicted murine $\text{Fc}_\epsilon\text{R}_\text{II}$ shares a 57% identity with the human $\text{Fc}_\epsilon\text{R}_\text{II}$. Both human and murine $\text{Fc}_\epsilon\text{R}_\text{II}$ demonstrate

marked sequence homology with animal lectin family, including rat asialoglycoprotein receptors, chicken hepatic lectin, and rat mannose binding proteins. These lectins have specific sugar binding activities. The homology among the lectins and Fc ϵ RII is confined to the carboxyl-terminal portion, which may contain the ligand-binding domain.

The predicted sequences of Fc ϵ RII from the isolated cDNA clones (Kikutani et al., 1986a; Ikuta et al., 1987; Ludin et al., 1987) showed no homology to that of the Fc ϵ RI(α) subunit (Kinet, et al., 1987). Recent studies on gene mapping of the three subunits of Fc ϵ RI, revealed that the genes for α and γ subunits are closely linked to the distal end of mouse chromosome 1 where the gene coding for Fc ϵ RII is located (Hupp et al., 1989), indicating a close association between these three genes. In addition, the structural interactions of IgE with Fc ϵ RII appear to be different from those with the high affinity receptor (Baird et al., 1989). The relationships between Fc ϵ RII and Fc ϵ R_L is still under investigation. The gene coding Fc ϵ R_L has not yet been cloned and therefore it is impossible to compare sequence homology between these two receptors. It would be of significance to determine whether the gene expression of the two receptors are associated, especially since the function of Fc ϵ R_L is still unknown.

The expression of Fc ϵ RII on B cells is known to be regulated by T cell-derived lymphokines (Galizzi et al., 1988). These Fc ϵ R bearing lymphocytes play important roles in IgE-mediated immune responses (Spiegelberg et al., 1979; Yodoi and Ishizaka, 1979). The proportion of Fc ϵ R-bearing cells in normal lymphocyte suspensions also increased by culture of the cells with IgE (Yodoi et al., 1979). Recently, the Fc ϵ R expression on human monoblast cell line U-937 and EBV-transformed B cell line

RPMI 8866 was shown to be regulated by a monoclonal anti-Fc ϵ RII antibody (H107), PMA, IFN- γ and dexamethasone (Mayumi et al., 1988). It was found that PMA, IFN- γ and H107 enhanced Fc ϵ R expression on both types of cell lines. Interestingly, dexamethasone suppressed 50% surface expression of Fc ϵ RII on U-937 cells but had little effect on the expression of the receptors on RPMI 8866. In association with the recent findings on the two species of human Fc ϵ RII, the observation in this study may reflect the functional differences between these two subtypes of receptors.

A role for the Fc ϵ RII has been reported by Capron et al. (1975; 1981), who showed that the receptors for IgE on eosinophils and macrophages are directly involved in the effector function of IgE-mediated immunity to parasitic infections. Subsequently, it was reported that pulmonary eosinophils could kill parenchymal cells in lung diseases (Davis et al., 1984), which suggested an additional effective function of these eosinophils in pathological situations.

THE EFFECTS OF DIFFERENTIATION AGENTS

A variety of biologically active compounds is used in many situations to promote cell differentiation as well as to investigate regulatory mechanisms involved in this process. The most common types of these agents are phorbol esters, retinoids and glucocorticoids.

Phorbol esters:

Tumor-promoting phorbol esters can elicit a variety of biological effects in tissues and cultured cells, including alterations in the synthesis and turnover of phosphatidylcholine, prostaglandins, polyamines, proteins, RNA and DNA. Their action starts with binding to a class of specific receptors with high affinity, one of them was identified as the phospholipid- Ca^{2+} -dependent protein kinase, the protein kinase C (PKC) (Ashendel et al., 1983; Kikkawa et al., 1983; Leach et al., 1983). The phorbol esters are analogues of diacylglycerol, the natural activator of protein kinase C (Nishizuka, 1984). This effector function is reflected by the induction of histamine release from rat peritoneal mast cells (Chakravarty et al., 1990; Heiman and Crews, 1985) or human basophilic leukocytes (Schleimer et al., 1981) and by the secretion of various cellular constituents from diverse cells. The histamine release mechanism is known to involve the activation of PKC, which was also demonstrated recently by the inhibition of IgE-dependent mediator release with the PKC inhibitors, H-7 and staurosporine (Gilfillan et al., 1990). Activation of protein kinase C serves as the first intracellular messenger in the transduction of many surface signals, but it is unknown how PKC subsequently leads to activation of many cellular responses important for both cell growth and cell functions (Nishizuka, 1984).

Protein kinase C, a multifunctional serine and threonine protein kinase, is widely distributed in tissues and organs. Most of the protein kinase activity is in the cytosol for cells at normal state, with total the PKC activity varying considerably among different cell lines (Chida et al., 1988). With the aid of molecular biology, genes closely related, yet distinct were identified to encode various bovine and human PKC, which are classified into three major types, α , β and γ (Coussens et al., 1986). Most cells so far examined were found to co-express several PKC subspecies, however, in different ratios (Nishizuka, 1988). This, in fact, may partially explain the diversity in the signal routes leading to the activation of PKC and in the regulatory roles of PKC within different cell types.

The action of phorbol esters starts with the initial binding of phorbol esters to cytosolic PKC. The affinity of PKC for calcium is then dramatically increased, resulting in the full activation of the enzyme at physiological calcium concentration. The activation of PKC by phorbol esters is reflected by the rapid translocation of cytosolic PKC to the cytoplasmic membrane (Kraft and Anderson, 1983). The translocation of PKC from cytosol to membrane was found to be concentration-dependent on the phorbol esters (Ballester and Rosen, 1985). It was found that less PKC was associated with the membrane when the cells were treated with 20 nM phorbol 12-myristate 13-acetate (PMA) than when they were exposed to 400 nM PMA. However, the maximum translocation was observed after 15 min of incubation with either concentration of PMA. The process was followed by a subsequent degradation of the membrane-associated enzyme at a rate independent of the PMA concentration. Therefore, a relatively short exposure to a high dose of phorbol esters may result in the

temporary activation PKC whereas a prolonged exposure would cause the continuous association of PKC with the membrane and eventually lead to the depletion of PKC activity due to the subsequent degradation of membrane-bound enzyme. Indeed, 18 hour treatment of rat basophilic leukemia cells (RBL) with 200 ng/ml PMA could completely deplete the PKC activity (White and Metzger, 1988). Nevertheless, evidence has been provided that there is also an increase in the rate of synthesis of PKC after treatment of cells with PMA (Krug et al., 1987). In this report, 14 hour incubation with 160 nM PMA caused an increase in the rate of PKC synthesis which was masked by a greater increase in the rate of degradation of the membrane-associated enzyme.

Activation of PKC by phorbol esters is the earliest intracellular event in the signal transduction pathway. The biological effects could be further mediated either directly by PKC or by its interaction with other intracellular messengers. Most tissues have at least two signal transduction systems: one involves the generation of cAMP as a second messenger and the other of PKC. It is important to note that these two systems have been realized to sometimes control each other in either a bidirectional or monodirectional mode (Bell et al., 1985; Dixon et al., 1988), possibly resulting in contrary effects.

One of many biological effects of PKC activation by phorbol esters has been shown to be the regulation of cell surface expression of various receptors: the Fc receptors for IgE of both low affinity $Fc_{\epsilon}RII$ or CD23 and the high affinity $Fc_{\epsilon}RI(\alpha)$ (Mayumi et al., 1988; and Ra et al., 1989), transferrin receptors (May et al., 1986), asialoglycoprotein receptors (Fallon and Schwartz, 1987), and muscarinic receptors (Liles et al., 1986).

A dose of PMA of 20 ng/ml caused 50-60% internalization of the transferrin receptor of K562 cells in about 20-60 min (Klausner et al., 1984). Similar results were obtained with IgE receptors, presumably the high affinity $\text{Fc}_\epsilon\text{RI}(\alpha)$, on RBL 2H3 cells (Ra et al., 1989). The intracellular mechanism of the PMA effects in these two cases was demonstrated to be mediated through the activation of PKC. However, PMA of 1 ng/ml enhanced the expression of $\text{Fc}_\epsilon\text{RII}$ (CD23) on a $\text{Fc}_\epsilon\text{RII}$ positive human monoblast cell line U937 (Mayumi et al., 1988). As indicated in the paper, the level of $\text{Fc}_\epsilon\text{RII}$ mRNA was found to have increased in these cells, suggesting that PMA actually induced an enhancement of the biosynthesis of the receptor. Moreover, it was revealed in other studies that the increased receptor expression was accompanied by the induced differentiation of U937 cells towards macrophage-like cells (Hsu and Hsu, 1985). Therefore, it was indicated that $\text{Fc}_\epsilon\text{RII}$ expression may be associated with the differentiation of B lymphocytes (Suemura et al., 1986). In addition to the receptors for IgE, the regulation of PMA on the expression of Fc receptors for IgG on U937 cells was investigated by another group (Nambu et al., 1989). PMA was found to be synergistic with dibutyl cAMP in inducing the low affinity $\text{Fc}\gamma\text{RII}$ expression, but to have no effect on the high affinity $\text{Fc}\gamma\text{RI}$ which was down-regulated by dibutyl cAMP, indicating that $\text{Fc}\gamma\text{RI}$ and $\text{Fc}\gamma\text{RII}$ expression on U937 was regulated by different mechanisms possibly involving cAMP rather than PKC.

The effect of phorbol esters on cell activation and proliferation is mediated by the activated PKC through the phosphorylation of proteins whose activity may be critical in the induction in gene expressions. Those genes particularly related to the regulation of cell growth would lead to cell proliferation (Nishizuka, 1986), on the

other hand, the activation of oncogenes would promote abnormal cell proliferation leading to carcinogenesis (Housey et al., 1988). Phorbol esters have been shown in *in vitro* experiments to induce differentiation of a variety of human cell lines. Treatment of human myelomonocytic leukemia cell lines, such as U937 and HL-60, with phorbol esters demonstrated a differentiation process involving morphological and functional changes towards mature monocytes (Hsu and Hsu, 1985; Marques-Silva, et al., 1990; Paulin-Levasseur et al., 1989). The importance of PKC in the induction of differentiation was recently demonstrated in HL-60 variants (HL-60R) which were found to be resistant to differentiation induction by phorbol esters (Nishikawa et al., 1990). A decrease in the expression of a PKC isoform, which appears to be a major PKC isozyme in HL-60 cells, was demonstrated to be associated with the resistance in HL-60R cells.

Glucocorticoids:

The glucocorticoids are commonly used as anti-inflammatory substances. They are particularly effective in the treatment of immediate hypersensitivity reactions. Treatment with glucocorticoids *in vitro* inhibits, in a dose- and time-dependent fashion, the release from granules of the preformed mediators from rat mast cells (Walajtys-Rode et al., 1988) and from human basophils (Bergstrand et al., 1984); as well as the synthesis of the newly formed lipid mediators by preventing the release of their precursor, i.e., arachidonate (Hong and Levine, 1976).

The effects of glucocorticoids on the response of diverse cell types are mostly attributed to the stimulated biosynthesis of regulatory proteins, particularly a family of inhibitory proteins of the phospholipases involved in the intracellular signal

transduction (Flower and Blackwell, 1979; Hirata et al., 1980). Like other steroid hormones, the action of glucocorticoids begins with binding to a cytoplasmic receptor and the drug-receptor complex is then translocated to the nucleus to initiate the biosynthesis of regulatory protein (Thompson and Lippman, 1974). Nevertheless, no satisfactory explanation has been provided so far on the actions of glucocorticoids at molecular levels other than certain isolated observations in the biochemical changes after glucocorticoid treatment. Some cell lines have been found to have increased expression of β -adrenergic receptors as well as of the adenylate cyclase regulatory GTP-binding proteins (G-proteins) (Chang and Bourne, 1987; Collado-Escobar et al., 1990b; Rodan and Rodan, 1986), resulting in an enhanced adenylate cyclase response to β -adrenergic agonists in these cells. Another consistent observation has been the inhibition of phospholipase A_2 in various types of cells treated with dexamethasone (Davidson et al., 1987). A recent study on the mechanism of action of dexamethasone on RBL-2H3 cells found that the IgE receptor-mediated signals were down-regulated whereas the adenosine receptor-mediated signals were up-regulated (Collado-Escobar et al., 1990a).

One study on the binding of dexamethasone to purified rat peritoneal mast cells showed that ^3H -dexamethasone bound directly to the specific receptors in the fashion of sigmoidal dependence on concentration (Walajtys-Rode et al., 1988). This may reflect either negative cooperation or the presence of a different classes of binding sites. Two saturation plateaus were observed at $20\text{-}30 \times 10^{-9}$ M and $70\text{-}90 \times 10^{-9}$ M. The equilibrium dissociation constant for the higher affinity binding sites K_{d1} was 1.9×10^{-8} M and represented 25,290 sites/cell, whereas the apparent K_{d2} for lower affinity

sites amounted to 5.5×10^{-8} M and represented about 120,000 sites/cell.

Lately, dexamethasone (DM) has been used in a number of studies to modulate properties of bone marrow-derived mast cells (BMMC). Generally, dexamethasone treatment on mouse bone marrow-derived mast cells (BMMC) caused a reduction in the surface expression of $Fc\epsilon R$ (Benhamou et al., 1986; Robin et al., 1985), which may account partially for its inhibition of mediator release. More interestingly, 1 μ M DM could also induce a time-dependent increase in cellular histamine content in mouse BMMC, possibly due to an increase in the synthesis of histamine (Pitton et al., 1988). In this study, the untreated BMMC were rapidly dividing populations which appeared to be ultrastructurally similar to immature mast cells, containing only a few cytoplasmic vacuoles devoid of electron-dense granule matrix. By contrast, BMMC treated with DM stopped dividing and developed more mature cytoplasmic granules, some partially filled with electron-dense material and some filled with uniform electron-dense matrix. Morphologically, the DM-treated cells transformed into an Alcian blue⁺/Safranin⁺ phenotype. The results indicated that DM promoted a cellular maturation process from immature BMMC towards cells with characteristics of CTMC. However, what regulatory protein is involved in the induction of differentiation has not been determined. In addition, a contrary effect of dexamethasone on mast cell growth and differentiation was reported by another group (McMenamin et al., 1987). In this study, the addition of dexamethasone to cultures of lymphocytes from *Nippostrongylus brasiliensis* (Nb)-infected rats in conditioned medium resulted in a suppression in the generation of MMC. There has been findings in the previous study that cells of monocyte-macrophage lineage early in differentiation were insensitive

to dexamethasone treatment (Koehler et al., 1989). The difference in sensitivity to dexamethasone may also apply to BMMC and the lymphocyte population containing mast cell precursors.

Retinoic acid:

Retinoids, vitamin A and its natural and synthetic derivatives, are potent agents for control of both cellular differentiation and proliferation. Retinoic acid (RA) plays a fundamental role in regulating the normal development of many endodermally, ectodermally, and mesodermally derived cells (Sporn and Roberts, 1983). The RA effect is well characterized in human hematopoietic cell proliferation and differentiation. Two continually proliferating human myeloid leukemia cell lines have provided good model systems. The HL-60 cell line consists predominantly of promyelocytes with a slight tendency to spontaneous differentiation and U937 is a human histiocytic lymphoma cell line comprised of monoblast cells.

Retinoic acid is known to induce terminal differentiation of HL-60 cells into morphologically and functionally mature granulocytes (Breiman et al., 1980). Among the changes induced by RA was the increase in the percentage of cells expressing IgG Fc receptors (Fc γ R). Like HL-60, U937 and THP-1, although being more mature myelomonocytic cell lines, could also be induced to form monocyte-like cells with functional characteristics of monocytes (Olsson and Breitman, 1982). However, the differentiation process was associated with a decreased surface expression of Fc γ R (Breitman et al., 1983). A recent study on the regulation of Fc γ R expression by RA demonstrated that the effects of RA on Fc γ R expression, either increase or decrease, depend on the stage of myeloid differentiation (Nakamura and Hemmi, 1988). RA

was found to increase the Fc γ R expression on the promyelomonocytic cell line HL-60, but to decrease it on the myelomonocytic cell lines, such as U937 and THP-1. On cells which are derived from very immature stage in myeloid cell development, such as the myeloblast cell line KG-1, RA had no effect on the Fc γ R expression.

It was found by Olsson et al. (1982) that the differentiation effect of RA could be potentiated by agents known to increase intracellular cyclic adenosine 3':5'-monophosphate (cAMP) levels, indicating that the RA effect might be mediated through cAMP-dependent protein kinase. Recently, RA, dibutyryl cAMP (cAMP analog) and some other inducers were found to increase cytosolic PKC activity along the differentiation pathway (Tanaka et al., 1990). However, the differentiation pathway induced by RA is unique in that RA only markedly enhanced the PKC-mediated phosphorylation of certain cytosolic proteins. A more recent study demonstrated differential changes in PKC subspecies, increase or decrease, during the differentiation process of HL-60 cells induced by RA, suggesting that PKC subspecies in HL-60 cells have distinct functions in cell differentiation (Hashimoto et al., 1990). Recently, it was reported that HL-60 cell differentiation induced by RA was facilitated by using the PKC inhibitor, sphinganine (Stevens et al., 1990). A number of types of PKC inhibitors become available lately but their PKC subspecies-specificity has not been defined. Therefore, an apparently contradictory result may be explained when the effects of PKC inhibitors are further investigated to give more detailed information in terms of PKC subspecies.

The HL-60 cell line was found to express the specific retinoic acid receptors α and β (RAR- α and RAR- β), which possess discrete DNA-binding and ligand-

binding domains (Hashimoto et al., 1989). The RARs (α , β and γ) belong to the superfamily of steroid-thyroid hormone receptors, which regulate cellular functions by directly activating gene expression (Brand et al, 1988; Giquereet al., 1987, 1990; Petkovich et al., 1987). It has been proposed that the ligand-receptor complex of RA with its receptors could induce a cascade of regulatory events by activating specific gene networks (Giquereet al., 1987). Indeed, RAR- α was demonstrated to directly mediate RA-induced granulocytic differentiation of HL-60 cells (Collins et al., 1990). However, the exact molecular mechanism of the differentiation process, especially the relationship between the intracellular signal transduction pathway and RAR, has not yet been identified. A recent report indicated that certain GTP-binding proteins are involved in the signal transduction pathway in RA-induced differentiation of HL-60 cells (Tohkin et al., 1989).

In contrast to cells of the myelomonocytic lineage, the differentiation of mouse mast cells from haematopoietic precursor cells in the presence of IL-3 was inhibited when the cells were exposed continually to RA at 1 μ M-10 μ M concentration. A mastoblast-like population, instead of mast cells with granules, was generated in the presence of RA (Arock et al., 1988), indicating that RA has an opposite effect on mast cells in terms of cell differentiation. The intracellular changes initiated by RA in mast cells were investigated by its effect on histamine release (Cantwell and Foreman, 1989). It was concluded in this study that the actions of retinoic acid on rat peritoneal mast cells were mediated through the inhibition of protein kinase C. In fact, several studies in other systems have also demonstrated that the retinoids exert their action through the inhibition of PKC (Cope et al., 1986; Taffet et al., 1983).

In summary, the mechanism of action of retinoids is not completely understood. Collectively, the intracellular events involved in the action of retinoids include the receptors for RA (RARs) (Collins et al., 1990; Giguere et al., 1990; Hashimoto et al., 1989), the GTP-binding protein (Tohkin et al., 1989), the increase in cAMP-dependent protein kinase activity (Ludwig et al, 1980; Plet and Evain, 1982), either stimulation (Tanaka et al., 1990) or inhibition (Cantwell and Foreman, 1989; Taffet et al., 1983) of the protein kinase C, and finally the activation of certain gene expression involved in cell growth and differentiation (Sporn and Roberts, 1983). This possibly indicates that the regulatory mechanism of retinoids is rather complicated.

SCOPE OF THE PRESENT INVESTIGATION

The present investigation was aimed at characterizing the differences of the rat tissue-cultured mast cell (RCMC) clones in terms of their stages of mast cell differentiation as measured by the expression of the high ($\text{Fc}_\epsilon\text{RI}$) and low ($\text{Fc}_\epsilon\text{R}_\text{L}$) affinity receptors for IgE and their histamine content

The thesis work started with characterization of the receptor expressions on clones and subclones of RCMC1. A systematic study was carried out to monitor the *in vitro* receptor development on selected subclones of RCMC for a period of 24-26 weeks. Variable development in the expression of $\text{Fc}_\epsilon\text{RI}(\alpha)$ and $\text{Fc}_\epsilon\text{R}_\text{L}$ was exhibited in the subclones studied during the time-dependent studies. The results suggested that these RCMC cell lines may represent mast cells at different stages of mast cell maturation. This part of the work will be presented in Chapter II.

The RCMC lines which exhibited variable receptor development during the time-dependent studies were further subjected to the differentiation agents: phorbol ester, dexamethasone, and retinoic acid, which have been used in previous studies to promote cell growth and differentiation of a variety of cell lines at different stages of maturity. The effects of these agents on the expression of $\text{Fc}_\epsilon\text{RI}(\alpha)$ and $\text{Fc}_\epsilon\text{R}_\text{L}$ and on the total cellular histamine content were measured. This approach demonstrated differences in the response to differentiation agents among the RCMC cell lines, indicating that they indeed were derived from various stages of mast cell maturation. This section will be described in Chapter III.

In Chapter IV, the clones were further modulated with biological factors which are possibly involved in influencing mast cell maturation and differentiation in the *in vivo*

environment. Immunoglobulin E, the ligand of both $Fc\epsilon RI$ and $Fc\epsilon RL$ as well as an *in vivo* environmental factor both in normal situation and in parasite infection, was added to the culture medium. In addition, polyclonal antibodies to $Fc\epsilon RL$ were added to the culture medium in the form of either Fab' monomers or dimers to modulate the low affinity $Fc\epsilon RL$. Thus, it was hoped that they would possibly mimic the effect of cross-reactive IgG ligands and their immune complexes. In addition to the immunoglobulins, the T cell growth factors, the rat recombinant IL-3 and Con A-stimulated condition medium (Con A-SCM) from rat spleen cell culture, were also used.

Chapter V contains the overall discussion of the present investigation.

CHAPTER II

DIFFERENTIAL IgE RECEPTOR DEVELOPMENT ON RAT TISSUE CULTURED MAST CELLS

INTRODUCTION

Mast cells are generally divided into two major subpopulations, the connective tissue type mast cells and the mucosal mast cell type according to their differences in morphology, granule content, histochemical staining and anatomical location (Enerbach, 1966b; Gibson and Miller, 1986; Nakano et al., 1985; Stevens et al., 1986). In the rat, the former are represented by peritoneal mast cells (PMC) (Gibson et al., 1986), while examples of the latter are intestinal mucosal mast cells (IMMC) (Stevens et al., 1986), rat basophilic leukemia cells (RBL) (Seldin et al., 1985b) and rat tissue cultured mast cells (RCMC) recently established in this laboratory (Chan et al., 1988; 1990). It is now recognized that both mast cell types have their origin in the bone marrow (Nakano et al., 1985), but the exact lineage relationship between them remains to be established.

In the rat, mast cells of both phenotypes have been shown to bear on their surface both high ($Fc_\epsilon RI$) and low affinity ($Fc_\epsilon R_L$) receptors for IgE (Conrad and Froese, 1978a; Froese, 1980; Conrad et al., 1975). Recently, receptor analysis of RCMC line 1 (RCMC1) derived from rat peritoneal mast cells but expressing the mucosal phenotype revealed changes in surface receptor pattern upon continuous culture (Chan et al., 1988). Previous experiments have demonstrated that the *in vitro* maturation of mast cells is associated with an increased IgE binding to the cells (Ishizaka et al., 1976; Thompson et al., 1990). However, the relationship of mast cell maturation

and the expression of receptors for IgE, especially the low affinity $Fc\epsilon R_L$ which has been ignored in most of the studies, has not been elucidated to date due to the lack of homogeneous mast cell populations established from various stages of cell differentiation.

The present study was undertaken in order to establish whether the changes in the cell line were inherent or involving changes in the cell population, and whether the changes vary among the clones and the subclones derived from RCMC1 since the descendants of RCMC1 expressed variable patterns of the two types of receptors for IgE (Chan et al., 1990). The differences in the receptor expression and the *in vitro* receptor changes could potentially be an indication that such clones represent different stages of differentiation.

MATERIALS AND METHODS

Buffers and solutions:

The following buffers were used where indicated:

1. Phosphate buffered saline (PBS): 0.14M NaCl, 0.01M PO₄, pH 7.4.
2. PBS/BSA: PBS with 0.05% bovine serum albumin.
3. Elution/sample buffer: 0.0625M Tris-HCl, 4% sodium dodecyl sulfate (SDS), 10% glycerol, 0.005% bromophenol blue, titrated to pH 6.8 with NaOH.
4. Bis-acrylamide: 30% (w/v) acrylamide, 0.8% (w/v) methyl bis-acrylamide.
5. Stacking gel buffer: 0.5M Tris-HCl, titrated pH 6.8.
6. Separation gel buffer: 1.5M Tris-HCl, titrated to pH 8.8.
7. Electrode buffer: 0.025M Tris, 0.192M Glycine, 0.1% SDS, pH 8.3.
8. Fixing solution: 12.5% (w/v) trichloroacetic acid, 4% (w/v) 5-sulfosalicylic acid.
9. Tanning solution: 4% (v/v) glycerin, 7% (v/v) acetic acid, 12% (v/v) 2-propanol.
10. HEPES buffer: NaCl 135mM, KCl 5mM, MgCl 1mM, CaCl 1.8mM, glucose 5.6mM, HEPES 10mM, gelatin 0.5mg/ml, BSA 0.05%, titrated pH 7.2.

Antibodies:

A rabbit antiserum specific for the low affinity Fc_εR_L was prepared by Dr. P. Roth from this laboratory and was tested by Dr. B.M.C. Chan as outlined before (Chan et al., 1990). Briefly, Fc_εR_L was prepared by absorbing NP-40 extracts of RBL-Wpg cells with rat IgE and anti-IgE-Sepharose to remove Fc_εRI followed by isolation of Fc_εR_L by means of rat IgE-Sepharose. Rabbits were initially immunized with Fc_εR_L in complete Freund's adjuvant, followed by boosts with the antigen in incomplete adjuvants. The immunoglobulin fraction was precipitated with ammonium

sulfate at 50% saturation. The washed precipitate was dissolved and dialyzed against PBS. It was subsequently absorbed two times with IgE-Sepharose and the resulting antiserum was found to interact with $\text{Fc}_\epsilon\text{R}_\text{L}$ only. In experiments to selectively isolate $\text{Fc}_\epsilon\text{R}_\text{L}$, the intact antibodies were either coupled to Sepharose or used in association with Protein A-Sepharose.

Monospecific horse anti-rat IgE (HARE) was a gift from Dr. K.A. Kelly and was prepared as described previously (Kelly et al., 1979). The antibodies were coupled to Sepharose in order to isolate the receptor-IgE complex.

Preparation of affinity gel conjugates:

The procedures of Cuatrecasas and Anfinsen (1971) were used to prepare IgE-Sepharose, anti- $\text{Fc}_\epsilon\text{R}_\text{L}$ -Sepharose, HARE-Sepharose and DNP_{40} -BSA-Sepharose. Briefly, Sepharose CL-4B (Pharmacia, Uppsala, Sweden) was activated by the addition of cyanogen bromide to a final concentration of 40mg per ml Sepharose in acetonitrile (0.8g/ml) and the reaction was allowed to proceed for 15 min. During this time the pH of the reaction mixture was maintained between 9 and 11 by adjusting it with 0.5N NaOH. The Sepharose was then washed with 0.05M carbonate buffer (pH 10), and with PBS. For coupling appropriate amounts of protein in PBS were added to packed and activated Sepharose and the reaction was allowed to proceed overnight at 4°C with gentle mixing. Unreacted groups were quenched with buffered 0.5M ethanolamine, pH 8.0. IgE-Sepharose and anti- $\text{Fc}_\epsilon\text{R}_\text{L}$ -Sepharose were coupled at 5mg of protein per ml of packed Sepharose, HARE-Sepharose at 2.5mg/ml. The coupling efficiency was found to be 90% to 98% with different preparations. The affinity gel conjugates were stored for further use in PBS/0.1% sodium azide at 4°C.

Cell lines:

The establishment of rat tissue cultured mast cell line 1 (RCMC1) in the absence of growth factors and feeder layers has been described previously (Chan et al., 1988; 1990). The cells were cloned under the same culture conditions at 0.25 cells per well and subcloned at 0.5 cells per well in 96-well plates (Linbro, McLean, Va., USA) by Dr. B.M.C. Chan. RCMC lines Briefly, peritoneal mast cells from Wistar-ICI rats were purified and cultured in multiwell dishes containing 1×10^6 cells/ml in the same culture medium used in the present study, as described below. Cell growth became apparent in all primary cultures after an initial latent period of about 10 weeks, at which time most cells had died. Thereafter, the cells were cultured under the conditions which have also been used in the present study which are described below. The established clones were maintained in a 3% CO₂ conditioned and humidified incubator at 37°C in 50-ml culture flasks (Falcon, Becton Dickinson, Oxnard, Calif.) in Eagle's minimum essential medium (MEM) (Difco Laboratories, Detroit, Mich., USA) as previously described (Conrad et al., 1976). The culture medium (CMEM) was completed by supplementing MEM with 15% fetal calf serum (FCS), 1% nonessential amino acids (Gibco, Grand Island, NY), 1% vitamins (Gibco, Grand Island, NY), dextrose, and 5% L-glutamine (Gibco, Grand Island, NY). The cell cultures were passaged regularly every four days by gentle mechanical stripping and seeding flasks with about 5×10^5 cells. For time-dependent studies of receptor development, aliquotes were removed every two weeks, frozen in 9% (v/v) dimethylsulfoxide (DMSO) in CMEM, and maintained in liquid N₂ until needed. For receptor analysis, frozen cell aliquotes were thawed, washed once with culture medium, cultured to confluence and passaged once,

cultured again to confluence, and used for analysis. The mycoplasma-free cell line RBL-CA10.7 established from the parent RBL-Wpg (Chan et al., 1986) was cultured under identical condition.

Cell surface iodination and solubilization:

Cells obtained from the tissue culture were washed three times with PBS and suspended at a concentration of 1.0×10^7 cells/ml. Aliquotes of 5×10^6 cells were surface iodinated according to the established procedures using a lactoperoxidase catalyzed reaction (Conrad and Froese, 1976). The iodination was initiated by the addition of 0.3 mCi of the carrier-free of sodium salt of ^{125}I (Amersham, Oakville, Ont.). This was followed by the addition of $10 \mu\text{l}$ of 166IU/ml lactoperoxidase (Calbiochem, San Diego, CA) and $10 \mu\text{l}$ of 0.03% hydrogen peroxide. The addition of these reagents was repeated twice at one minute intervals. At the end of three minutes, the reaction was stopped by transferring the cells to 5ml of cold PBS/BSA. After centrifugation, the cell pellet was resuspended in 1ml PBS/BSA and subsequently incubated for 60 min at 37°C with gentle mixing in order to reduce the presence of labeled low molecular weight lipids. The iodinated cells were then centrifuged through 2ml FCS, leaving any ^{125}I not bound to the cells on top of the FCS. After one more wash with PBS/BSA, cells were solubilized by mixing the pellet with 0.1ml of 0.5% NP-40 in PBS for 20 min at 4°C . Particulate material was removed by centrifugation in an Eppendorf microfuge at 12,000 g for 2 min and the supernatant material was used for receptor isolation. When comparing RCMC subclones harvested at different time points in continuous culture, all cells were re-established from frozen aliquotes at the same time and labeled on the same day using the same batch of ^{125}I .

Affinity chromatography for receptor isolation:

The receptors for IgE were isolated from the solubilized, surface iodinated cells by means of affinity gel conjugates. To isolate both $\text{Fc}_\epsilon\text{RI}(\alpha)$ and $\text{Fc}_\epsilon\text{R}_\text{L}$, IgE-Sepharose was used as previously reported (Conrad and Froese, 1978a). To an aliquot of 0.1ml packed IgE-Sepharose stored in PBS/sodium azide, washed three times with PBS/0.1% NP-40, was added the solubilized cell extract from 5×10^6 cells. After mixing for a minimum of 2 hours at 4°C , the Sepharose was washed three times with PBS/0.1% NP-40 and once with 0.0625M Tris-HCl (pH 6.8). The isolated material was then eluted by boiling the affinity gel in 0.1 ml sample buffer for 90 seconds.

For the selective isolation of $\text{Fc}_\epsilon\text{R}_\text{L}$, either anti- $\text{Fc}_\epsilon\text{R}_\text{L}$ -Sepharose was used directly or the anti- $\text{Fc}_\epsilon\text{R}_\text{L}$ antiserum was used in association with Protein A-Sepharose (Sigma, St. Louis, MO). The former isolation involved identical procedures as those with IgE-Sepharose. The latter was divided into two steps. The soluble extract of the solubilized cells was first mixed with 0.1 mg of intact anti- $\text{Fc}_\epsilon\text{R}_\text{L}$ for 1h at 4°C . The receptor-antibody complex was then transferred to 0.1 ml of packed Protein A-Sepharose and incubated at 4°C for a minimum of two hours. Washing and elutions were identical to those used with IgE-Sepharose.

To selectively isolate $\text{Fc}_\epsilon\text{RI}(\alpha)$, early studies had demonstrated that this could be achieved by isolating receptor-IgE complexes with HARE-Sepharose (Conrad and Froese, 1978a). Briefly, 12.5 μg of isoelectrically focused IgE was added to the soluble cell extract from 5×10^6 iodinated cells and further mixed for 1h at 4°C . Packed HARE-Sepharose of 0.2 ml volume was used to isolate the $\text{Fc}_\epsilon\text{RI}(\alpha)$ -IgE complex. After a minimum of 2h mixing, the receptors were eluted as described above using 0.1ml

sample buffer. Eluates from affinity gels were kept frozen at -20°C till use.

Polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE):

Receptor characterization using SDS-PAGE was performed according to the procedures established by Laemmli (1970) using a Protean II slab gel apparatus (Biorad, Mississauga, Ont.). One day before the experiment, 10% separation gel was prepared by appropriately diluting the stored bisacrylamide solution with Tris buffer and by adding 10% SDS and the polymerized gel was stored at 4°C. The stacking gel of 3% was poured on top of the separation gel on the day of the electrophoresis and was allowed to polymerize for approximately 3h at room temperature. Eluates of 50 μ l from the affinity gels were applied to the wells on the stacking gel and electrophoresis was carried out at 20mA/gel in electrode buffer. When the tracking dye reached the bottom of the gels the electrophoresis was stopped and the gels were immediately fixed in the fixing solution for 2h, and then in the tanning solution for 2h. Gels were dried on a gel dryer (Biorad, Mississauga, Ont.). Autoradiographs were obtained by exposing dried gels to hypersensitized X-OMAT AR film (Eastman Kodak, Rochester, NY) at -70°C. Relative concentrations of receptors were obtained by densitometric scanning of autoradiographs using a Helena Quick Scan R&D densitometer (Helena Laboratories, Beaumont, TX) as previously described (Kepron and Froese, 1987).

Proliferation assay:

Proliferation assays have been performed using the standard procedures. Cells of variable clones of RCMC1 were diluted with CMEM into 5×10^4 cells/ml and 0.2ml aliquots were added to 96-well plates (Falcon, Oxnard, CA). They were incubated for 24h at 37°C in a 3% CO₂-conditioned humidified chamber and were pulsed for 12h

with 1 μ Ci per well of ^3H -thymidine (Amersham, Oakville, Ont.). Cells were harvested onto glass fiber mats using a PHD cell harvester (Cambridge Technology Inc., MA). The glass fiber discs added with scintillation fluid were counted in scintillation counter (Beckman).

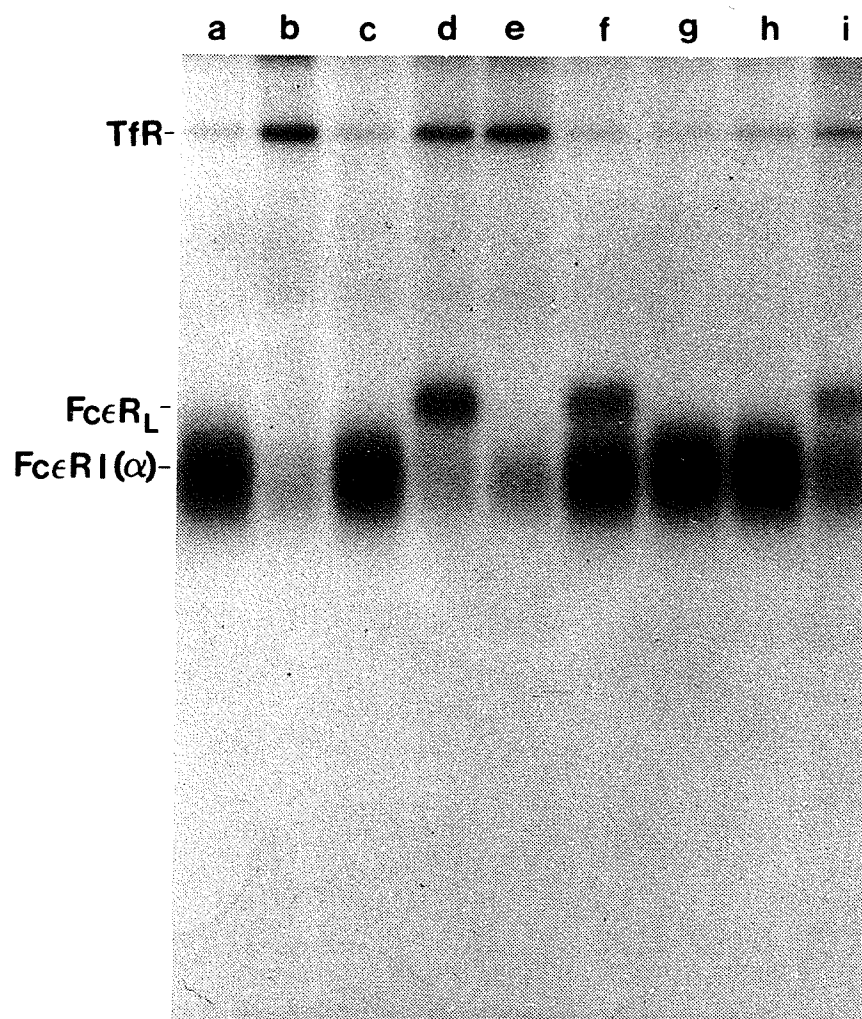
RESULTS

Expression of surface receptors for IgE on RCMC1 subclones:

The cell line RCMC1 established in the absence of growth factors and feeder layers (Chan et al., 1988) was found originally to be associated with only $\text{Fc}\epsilon\text{RI}(\alpha)$ on the cell surface. However, after 6 months in culture, the low affinity receptor $\text{Fc}\epsilon\text{R}_\text{L}$ began to show up. The RCMC1 cells in early culture were cloned by Bosco Chan of this laboratory and yielded 22 proliferative clones which showed variable expressions of the two types of receptors for IgE. Two clones expressed only low levels of $\text{Fc}\epsilon\text{R}$, primarily the α chain of the high affinity receptor for IgE [$\text{Fc}\epsilon\text{RI}(\alpha)$], fourteen clones, like the parent RCMC1 expressed only $\text{Fc}\epsilon\text{RI}(\alpha)$, two, expressed mainly $\text{Fc}\epsilon\text{R}_\text{L}$ while the remaining four were associated with both receptors. Receptor patterns of representative clones are shown in Figure 1 (kindly provided by Dr. Bosco Chan). Four of these clones, RCMC1.2, 1.3, 1.9, and 1.11, were found to have the most distinctive receptor patterns and therefore chosen for further study. Each was recloned into several subclones. Receptor analyses of each group of subclones were performed immediately after enough cells were expanded in tissue culture. In some cases subclones from the same parent showed very different surface expression of the two types of receptors for IgE among them and from the parent. The results of some representative subclones are shown in Figure 2. It should be noted that this is a composite figure derived from several different SDS-PAGE runs. Moreover, relative intensities of receptor bands can only be compared for subclones derived from the same parent. Only one subclone from each of RCMC1.2 and RCMC1.11 is shown, since the receptor patterns of other subclones were very similar. Six clones were

Fig.1. SDS-PAGE analysis on 10% gels of receptors for IgE isolated by IgE-Sepharose from RCMC1 in early culture (lane a) and clones derived from it: RCMC1.2 (lane b), RCMC1.3 (lane c), RCMC1.9 (lane d), RCMC1.10 (lane e), RCMC1.11 (lane f), RCMC1.15 (lane g), RCMC1.20 (lane h), and RCMC1.21 (lane i).

Fig. 1



selected for studies on receptor development with time in culture. Clones RCMC1.2.5 and RCMC1.3.1 appeared to be devoid of receptors for IgE (Fig. 2, lanes a and g). RCMC1.3.2 and RCMC1.3.3 exhibited only $\text{Fc}_\epsilon\text{RI}(\alpha)$ (Fig. 2, lanes h and i), and the remaining two clones, RCMC1.9.3 and RCMC1.11.2, carried both $\text{Fc}_\epsilon\text{RI}(\alpha)$ and $\text{Fc}_\epsilon\text{R}_\text{L}$ (Fig.2, lanes d and j), but at varying densities.

Time-dependent receptor development:

The preliminary observations described above led to a more systematic study on receptor development with time in culture. Six subclones were selected: RCMC1.2.5 and 1.3.1 apparently with no receptors (Fig. 2, lanes a and g), RCMC1.3.2 and 1.3.3 with only $\text{Fc}_\epsilon\text{RI}(\alpha)$ (Fig. 2 lanes h and i), and the remaining two clones, RCMC1.9.3 and 1.11.2 (Fig. 2, lanes d and j) carrying both $\text{Fc}_\epsilon\text{RI}(\alpha)$ and $\text{Fc}_\epsilon\text{R}_\text{L}$ but at varying densities.

Time-dependent receptor development studied over a period of 24-26 weeks yielded rather interesting results. The receptor profile of each subclone was established at two-week intervals by SDS-PAGE in combination with autoradiography. The receptor pattern for RBL-CA10.7 was also determined by IgE-Sepharose in every experiment to indicate the positions of the two types of IgE receptors. To show the time-dependent changes in receptor expression, the peak height of either $\text{Fc}_\epsilon\text{RI}(\alpha)$ or $\text{Fc}_\epsilon\text{R}_\text{L}$ was determined and plotted versus time in culture. An autoradiograph of results obtained with RCMC1.2.5 is shown in Figure 3, demonstrating the gradual increase with time in the expression of $\text{Fc}_\epsilon\text{R}_\text{L}$. However, at the latter time points new bands which appeared as trails of $\text{Fc}_\epsilon\text{R}_\text{L}$ started to show at a position where it did not quite correspond to $\text{Fc}_\epsilon\text{RI}(\alpha)$. Attempts were made to isolate $\text{Fc}_\epsilon\text{RI}(\alpha)$ by means of

Fig. 2

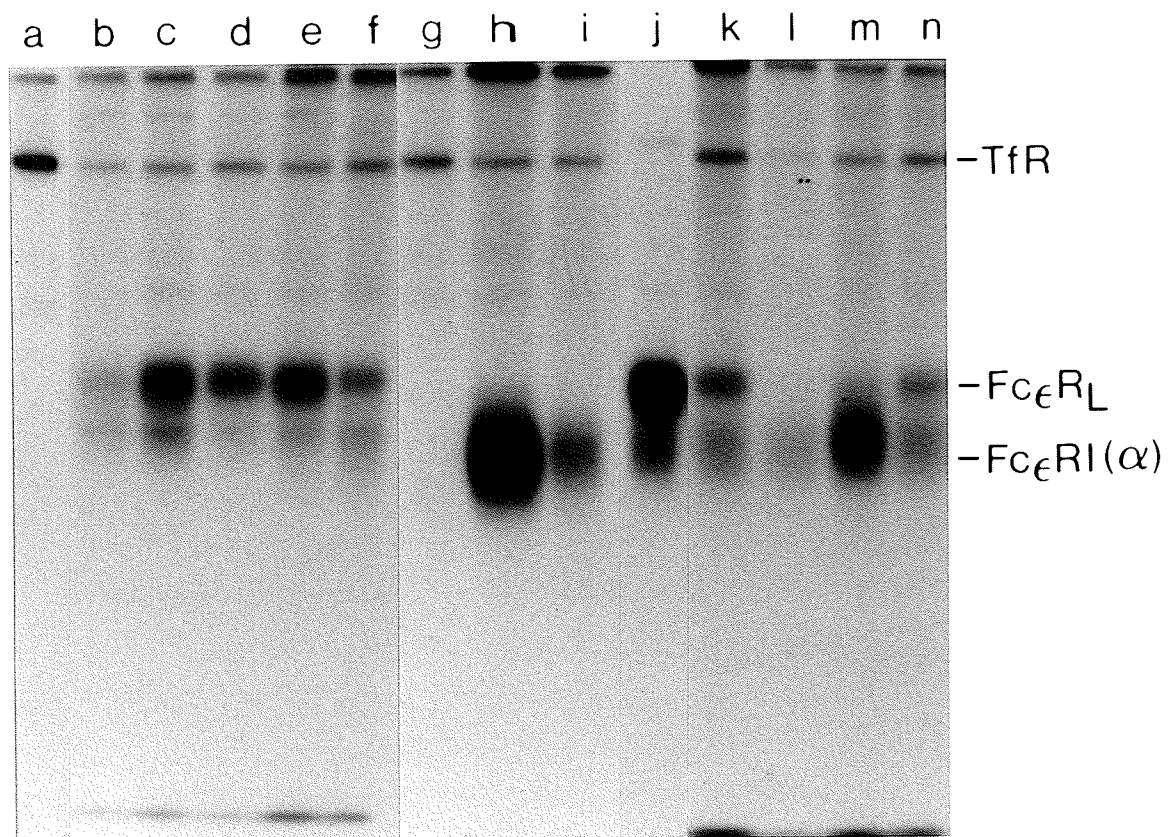


Fig. 3. Changes in the receptors for IgE on clone RCMC1.2.5 as a function of time in culture. Cells were maintained in tissue culture and cell aliquots of every two-week intervals were ^{125}I labelled, as described under Materials and Methods. The receptors were isolated by means of IgE-Sepharose and analyzed by SDS-PAGE on 10% gels.

Fig. 3

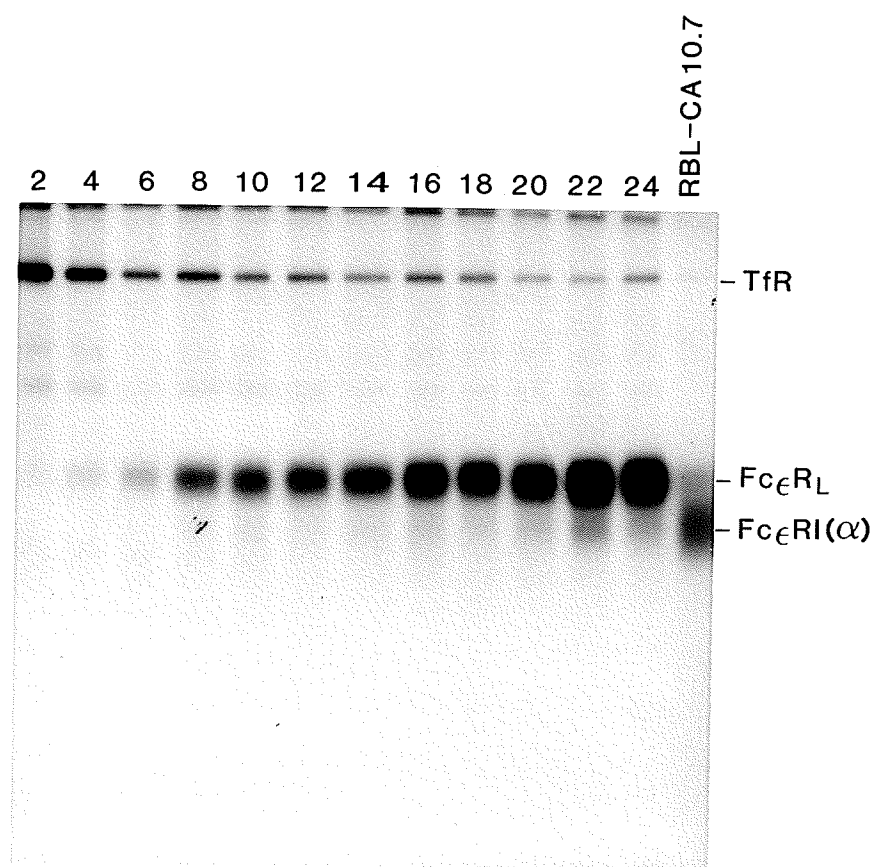


Fig.4. Changes in the density of the receptors for IgE on clone RCMC1.2.5 as a function of time in culture. The autoradiograph shown in Fig. 3 were scanned by densitometry and the peak heights determined.

Fig.5. Changes in the density of the receptors for IgE on clone RCMC1.3.1 as a function of time in culture.

Fig. 4

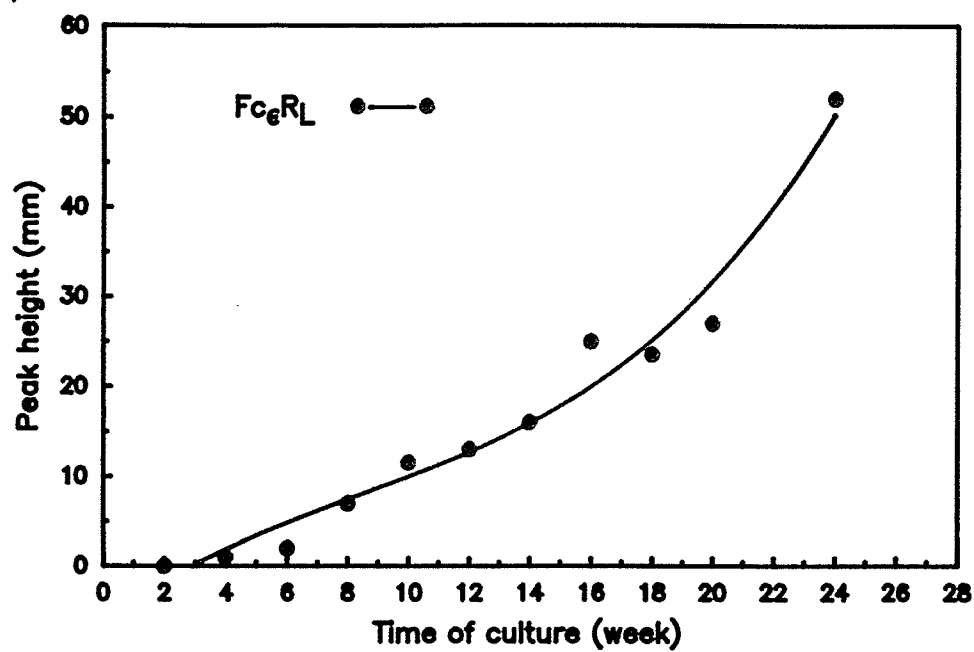


Fig. 5

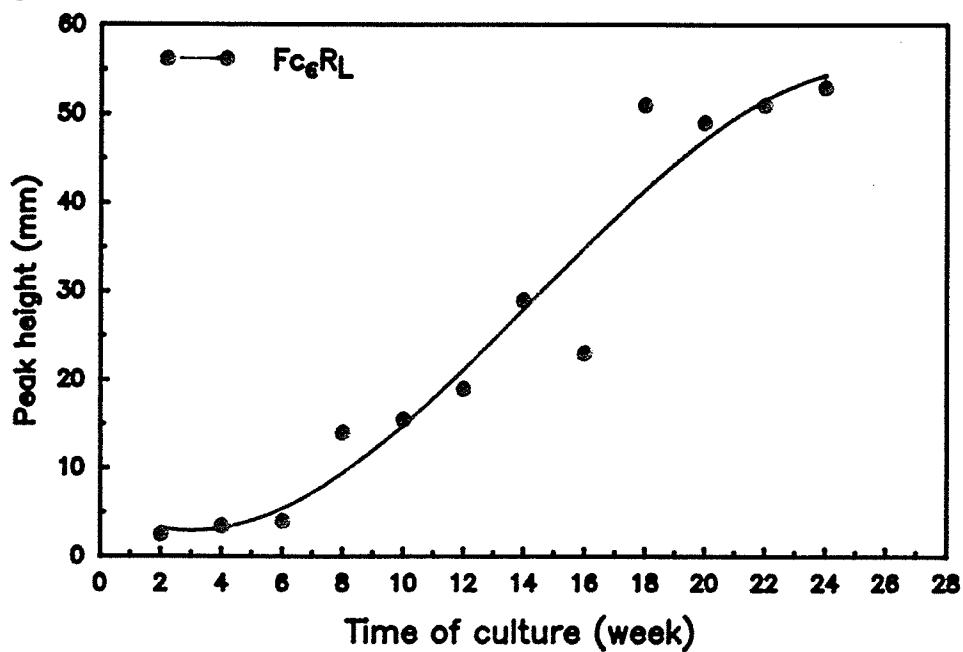


Fig.6. Changes in the receptors for IgE on clone RCMC1.3.2 as a function of time in culture. Cells were maintained in tissue culture and cell aliquots of every two-week intervals were ^{125}I labelled, as described under Materials and Methods. The receptors were isolated by means of IgE-Sepharose and analyzed by SDS-PAGE on 10% gels.

Fig. 6

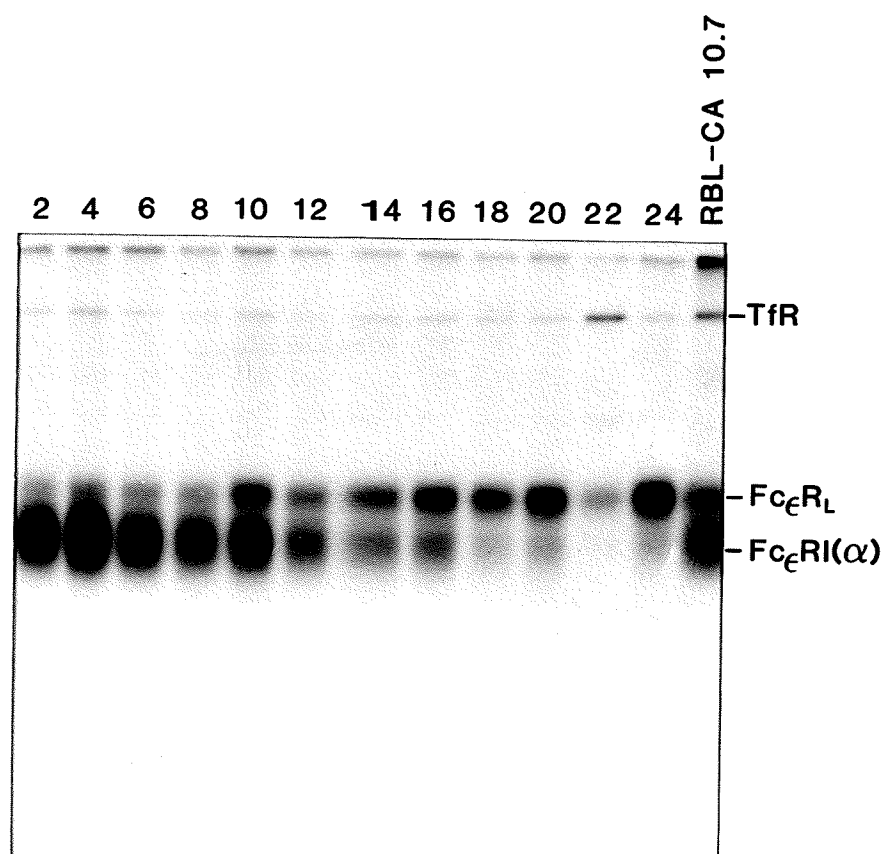


Fig.7. Changes in the density of the receptors for IgE on clone RCMC1.3.2 as a function of time in culture. The autoradiograph shown in Fig. 6 were scanned by densitometry and the peak heights determined.

Fig. 7

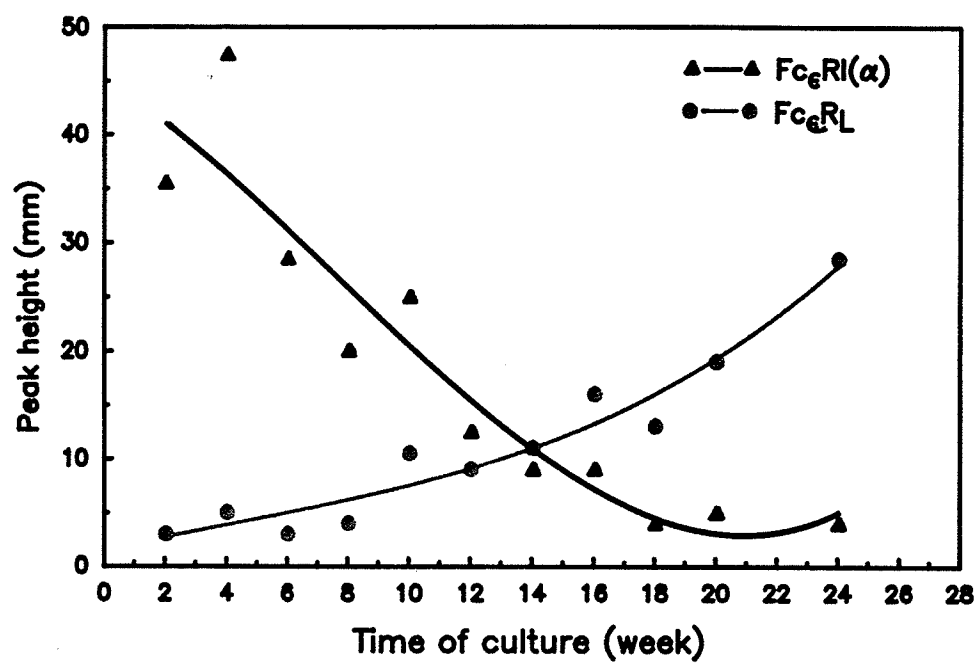


Fig.8. Changes in $\text{Fc}_\epsilon\text{RI}(\alpha)$ on clone RCMC1.3.2 as a function of time in culture. The receptors were isolated by means of IgE and anti-IgE-Sepharose.

Fig. 8

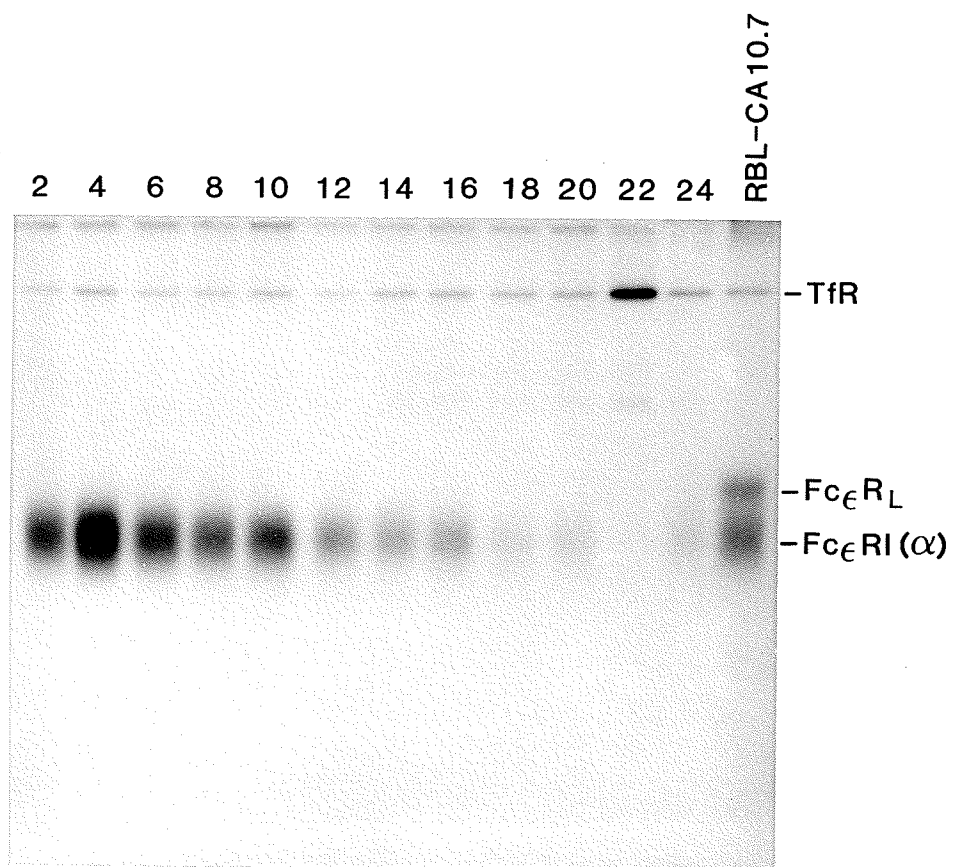


Fig.9. Changes in $\text{Fc}\epsilon\text{R}_\text{L}$ on clone RCMC1.3.2 as a function of time in culture. The receptors were isolated by means of anti- $\text{Fc}\epsilon\text{R}_\text{L}$ and Protein A-Sepharose.

Fig. 9

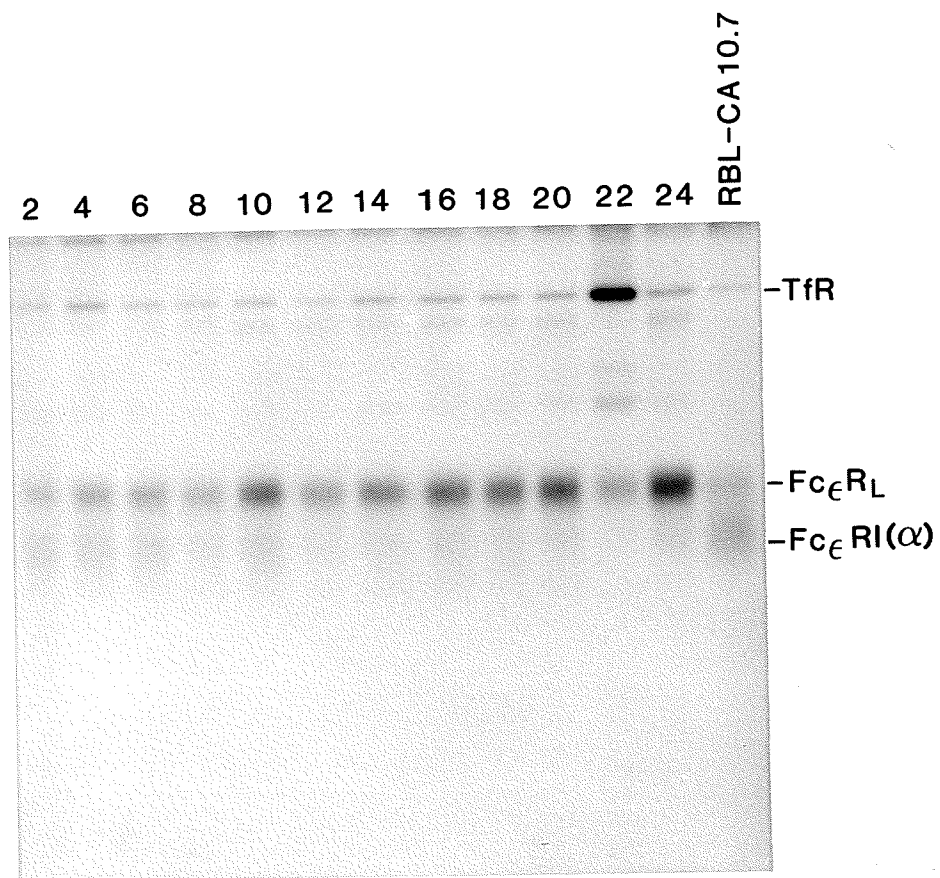


Fig.10. Changes in the density of $Fc_{\epsilon}RI(\alpha)$ and $Fc_{\epsilon}R_L$ on clone RCMC1.3.2 as a function of time in culture. The autoradiographs shown in Figs. 8 and 9 were scanned by densitometry and the peak heights determined.

Fig.11. Changes in the density of the receptors for IgE on clone RCMC1.11.2 as a function of time in culture.

Fig. 10

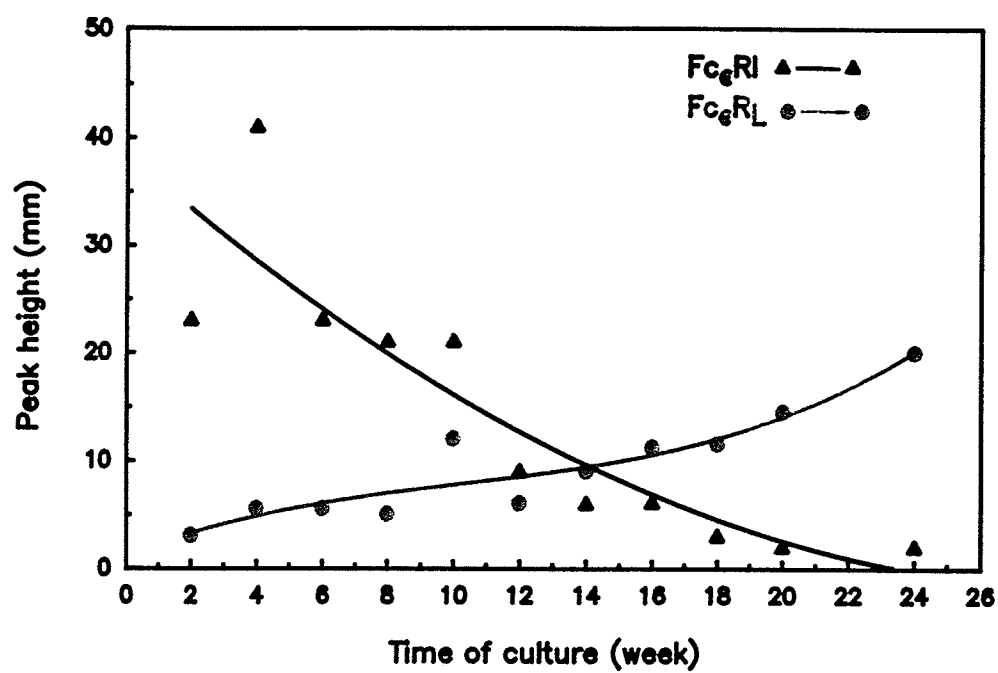
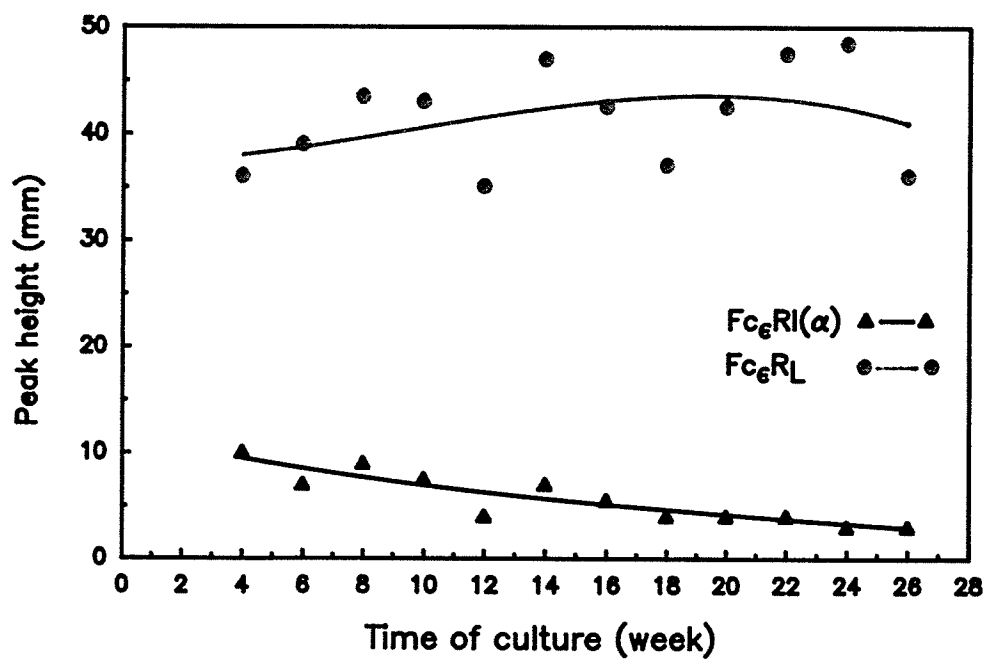


Fig. 11



of $\text{Fc}_\epsilon\text{R}_\text{L}$ and the expression increased throughout the experiment. The expression of $\text{Fc}_\epsilon\text{RI}(\alpha)$ decreased dramatically during the same time-period. To verify these results the two receptors were also isolated separately. In Figure 8, the results obtained by isolating $\text{Fc}_\epsilon\text{RI}(\alpha)$ only by means of IgE and anti-IgE-Sepharose, are shown in the form of an autoradiograph of receptor analysis by SDS-PAGE. A comparable autoradiograph of a receptor profile obtained by isolating $\text{Fc}_\epsilon\text{R}_\text{L}$ only by means of anti- $\text{Fc}_\epsilon\text{R}_\text{L}$ and protein A-Sepharose is shown in Figure 9. A plot of receptor peaks from densitometry scans of both autoradiographs of Figures 8 and 9 are shown in Figure 10. A comparison of this figure with Figure 7 showed a similar change of receptor expression with time.

The situation for RCMC1.11.2 resembled that of RCMC1.3.2 except that the surface $\text{Fc}_\epsilon\text{R}_\text{L}$ numbers were high to begin with and remained fairly constant, while those of $\text{Fc}_\epsilon\text{RI}(\alpha)$ were low at the start of the experiment and decreased slightly (Fig. 11). Finally, RCMC1.3.3 started with the absence of $\text{Fc}_\epsilon\text{R}_\text{L}$ and little $\text{Fc}_\epsilon\text{RI}(\alpha)$. In this case, the expression of both receptors was found to increase with time (Fig. 12). The data demonstrating the expression of both types of receptors shown in Figures 11 and 12 were obtained using IgE-Sepharose for receptor isolation. The changes in receptor expression were also seen when $\text{Fc}_\epsilon\text{RI}(\alpha)$ and $\text{Fc}_\epsilon\text{R}_\text{L}$ were isolated separately using IgE in association with HARE-Sepharose and anti- $\text{Fc}_\epsilon\text{R}_\text{L}$ -Sepharose, respectively (data not shown).

Growth rates of the RCMC clones:

Cell growth rate could potentially affect protein synthesis and therefore the synthesis and surface expression of the Fc receptors as well. In order to determine if

Fig.12. Changes in the density of the receptors for IgE on clone RCMC1.3.3 as a function of time in culture.

Fig.13. The growth rates of RCMC clones at various time points of time-dependent study. Cells of each clone at 4 wk, 12 wk and 20 wk were plated into 96-well plates at 1×10^4 cells/well and pulsed with 1 μ Ci/well of ^3H -thymidine after 24 hr of culture. The ^3H -thymidine incorporation was determined after 12 hrs of culture.

Fig. 12

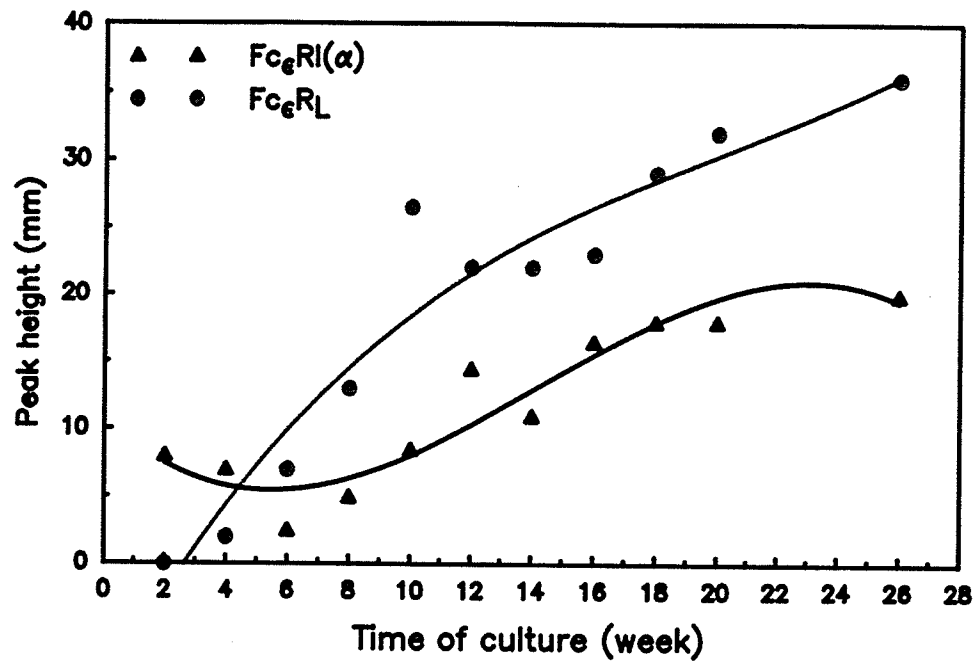
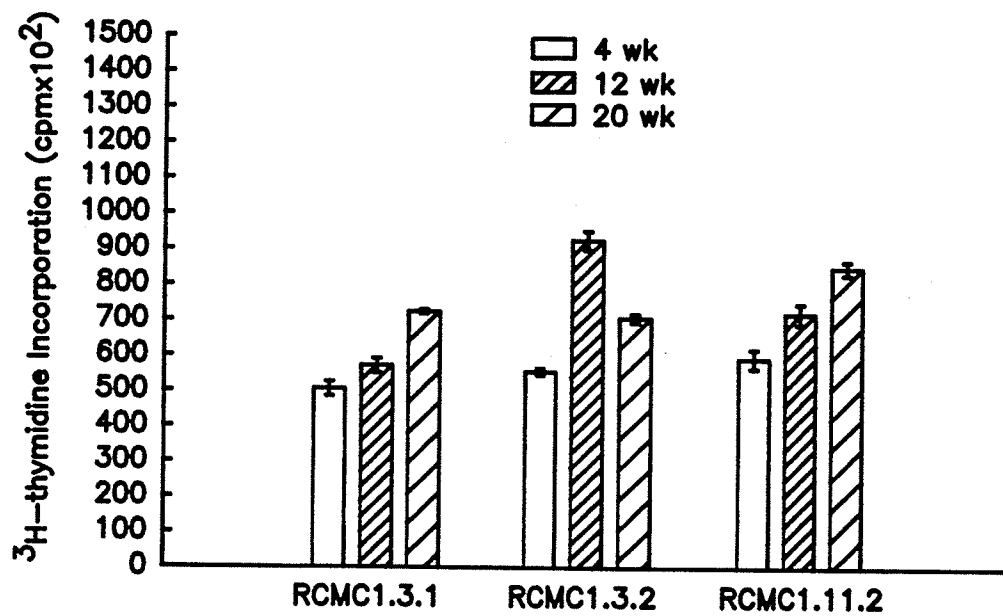


Fig. 13



the variations in receptor patterns of the RCMC clones could have resulted from the differences in the rate of cell growth ^3H -thymidine incorporation was compared among clones with different receptor patterns and for each of the cloned line at several time points along its continuous growth in culture. As can be seen in Figure 13, RCMC1.3.1 and 1.11.2 showed a small, but steady increase in their rate of proliferation as they continued to grow in culture, while RCMC1.3.2 appeared to pass through a maximum at week 12.

DISCUSSION

This study had its origin in the observation that some of the RCMC lines generated in this laboratory and particularly RCMC1 were subject to changes in $Fc_{\epsilon}R$ patterns, as they continued to grow in culture (Chan et al., 1988; 1990). In particular, it was observed that RCMC1, which in early culture exhibited only $Fc_{\epsilon}RI(\alpha)$ on its surface, began to show $Fc_{\epsilon}R_L$ as time in culture progressed. Therefore, it seemed important to differentiate between two mutually not exclusive possibilities: (1) the observed changes were a consequence of cell population changes, i.e., in the case of RCMC1, a minor population carrying many $Fc_{\epsilon}R_L$ may have become more prominent relative to cells expressing mainly $Fc_{\epsilon}RI(\alpha)$, and (2) the expression of $Fc_{\epsilon}R_L$ on cells of RCMC1 was upregulated. It demonstrated that changes in receptor expression do take place in all clones of RCMC1 which were investigated and that these changes apply particularly to $Fc_{\epsilon}R_L$ which invariably increased in numbers, as time in culture progressed. This, therefore, most likely explains the previously seen changes in RCMC1 (Chan et al., 1988). Nevertheless, the current investigation has also revealed, as seen in Figures 1 and 2, that RCMC1 does not consist of cells with a uniform receptor pattern. Cloning has yielded cells with receptor patterns which vary considerably, particularly as far as the relative amounts of $Fc_{\epsilon}RI(\alpha)$ and $Fc_{\epsilon}R_L$ are concerned. The majority of clones (Fig. 1), as expected, expressed primarily $Fc_{\epsilon}RI(\alpha)$. However, it should be pointed out that although $Fc_{\epsilon}RI$ is the predominant Fc_{ϵ} receptor of RCMC1 and its clones (Fig. 1), this does by no means hold if one considers all the RCMC lines generated from rat peritoneal mast cells (Chan et al., 1988).

It should be pointed out that the data on $Fc_{\epsilon}R$ presented in this and subsequent

chapters do not deal with absolute receptor numbers. The results are expressed as cpm peaks measured for a given receptor. Since the specific activities for each receptor are not known equal peak heights for the two receptors do not necessarily mean equal numbers of receptor. To make it possible to compare relative numbers of either $\text{Fc}\epsilon\text{R}_\text{L}$ or $\text{Fc}\epsilon\text{RI}(\alpha)$ cells of a given clone frozen after different times in culture were all thawed at the same time, reestablished in culture and ^{125}I -labeled on the same day using the same batches of reagents. Receptor preparations of one clone were always analyzed on the same gel slab.

To ascertain that changes in receptor patterns were not actually due to changes in the growth rate of cells, rates were established for cloned lines which had shown the most diverse changes in receptor pattern upon prolonged tissue culture. Generally, the rates of cell growth increased slightly with increased continuous time in culture. Therefore, it is unlikely that the changes seen in receptor expression are due to growth rate changes, particularly since the synthesis and expression of surface molecules could be expected to decrease as the rate of cell growth increases.

It is rather interesting to note that while the expression of $\text{Fc}\epsilon\text{R}_\text{L}$ increased for all subclones of $\text{Fc}\epsilon\text{R}$ tested, that of $\text{Fc}\epsilon\text{RI}(\alpha)$ varied. It decreased in two instances and increased in another. Moreover, several of the subclones did not show any detectable $\text{Fc}\epsilon\text{RI}(\alpha)$ at all. This, of course, suggests that the regulation of the two receptors for IgE is not linked. It is to be expected that the regulatory mechanism of the expression of $\text{Fc}\epsilon\text{RI}$ is quite complex, as this receptor consists of α -, β -, and γ -subunits, and the complex is incorporated into the plasma membrane as a unit only and the subunits are synthesized and degraded in a coordinated fashion (Quarto et al., 1985). This

should make the expression of $\text{Fc}_\epsilon\text{RI}$ subject to more complex regulatory influences. Considering the changes in $\text{Fc}_\epsilon\text{RI}$ and $\text{Fc}_\epsilon\text{R}_\text{L}$ expression together, one can conclude that the two receptors are subject to different regulatory mechanisms. In this context, it is interesting to note, once more, that if one looks at the different RCMC cell lines described previously (Chan et al., 1988), one can conclude that on the average $\text{Fc}_\epsilon\text{R}_\text{L}$ is the more prevalent receptor. However, if one compares these patterns with those of rat peritoneal mast cells and intestinal mucosal mast cells (Froese 1980; Swieter et al., 1989), it becomes immediately apparent that, among the latter cells, $\text{Fc}_\epsilon\text{RI}$ is the more abundant molecule. It has been observed in the past that when mast cells are derived from cultures of thymocytes, the number of $\text{Fc}_\epsilon\text{R}$ per cell increases with time in culture (Ishizaka et al., 1977). Increased binding of IgE was also observed in the case of peritoneal cells which had been depleted of rat peritoneal mast cells and which had subsequently been cultured in medium containing culture supernatants from L cell fibroblasts (Czarnetzki et al., 1982). In neither case was it established that the authors were actually dealing with $\text{Fc}_\epsilon\text{RI}$. However, based on the method used for binding studies, one can assume that it was this receptor. Taken together, these results and those obtained with rat peritoneal mast cells and intestinal mucosal mast cells would suggest that $\text{Fc}_\epsilon\text{RI}$ is a marker of mature mast cells. However, the relative low expression of this $\text{Fc}_\epsilon\text{R}$ on most RCMC lines (Chan et al., 1988) and the fact that its expression decreases in two of three clones of RCMC1 and is absent in others indicate that certain microenvironmental factors may be required to induce the expression of this receptor and to maintain it. The presence of IgE itself may be one of these regulatory factors. This aspect will be dealt with in Chapter IV

when studies in the role of IgE on Fc ϵ R are described. It remains to be established to what extent an enhanced Fc ϵ RI expression can be maintained once environmental factors, such as IgE, are removed. The results presented here can not answer this question. In addition, previous studies (Chan et al., 1990) on the RCMC lines have shown the presence of the mRNA for the α , β and γ subunits of the Fc ϵ RI in the cell line which did not express the receptor at the time of the studies. A similar situation could apply to the RCMC1.2.5 and 1.3.1. Thus, Fc ϵ RI expression decreased in the case of RCMC1.3.2 and RCMC1.11.2 and increased in the case of RCMC1.3.3. On the other clones, no Fc ϵ RI was detected. Perhaps it depends on the precise state of differentiation whether or not high expression of this receptor can be preserved in the absence of IgE and/or other factors. Indeed, cloned cell lines exhibiting different changes in Fc ϵ RI expression may have originated from mast cells at different stages of differentiation. Since the expression of Fc ϵ R_L of all RCMC1 clones increased with time in culture and since it is present on most RCMC lines generated, it may actually turn out that it is widely distributed among mast cells.

CHAPTER III

MODULATION OF RAT TISSUE CULTURED MAST CELL CLONES WITH DIFFERENTIATION AGENTS

INTRODUCTION

Studies described in Chapter II have shown that RCMC clones vary not only in their surface IgE receptor patterns but also in their *in vitro* receptor development. The results clearly demonstrated that the expression of $Fc\epsilon RI$ and $Fc\epsilon R_L$ is regulated by different mechanisms. In this context, it would be important to learn more about factors involved in the regulation of the expression of the two types of Fc receptors for IgE. Moreover, experimental evidence obtained in Chapter II suggested that RCMC clones of various receptor expressions may have originated from mast cells at different stages of differentiation.

Phorbol esters, glucocorticoids and retinoids are well known differentiation agents which have been used to induce cell differentiation of a variety of cell types. Tumor-promoting phorbol 12-myristate 13-acetate (PMA), a protein kinase C activator, was reported to regulate the expression of the low affinity IgE Fc receptors ($Fc\epsilon RII$) on a human monoblast cell line U937 (Mayumi et al., 1988) and to induce a differentiation process of these cells to macrophage-like cells (Hsu and Hsu, 1986; Koehler et al., 1989). Dexamethasone (DM), a glucocorticoid, is known to down-regulate antigen dependent exocytosis of secretory granules and the *de novo* synthesis of arachidonic acid metabolites of mouse bone marrow derived mast cells (BMMC) by decreasing $Fc\epsilon RI$ expression (Robin et al., 1985). Moreover, DM treatment elevates the granule histamine content and promotes a maturation process of these cells (Pitton et al.,

1988). Finally, retinoic acid (RA) has been extensively used in myelomonocytic cell lines and found to promote their differentiation to cells having characteristics of mature monocytes (Breitman et al., 1980; Olsson and Breitman, 1982). The effect of RA on Fc γ R expression on these cell lines depends on the stage of myeloid differentiation. Thus, RA increases Fc γ R expression on promyelocytes of HL-60, decreases it on myelomonocytic cells, such as U937 and THP-1-T cells which are blocked at relatively late stages and has no effect on the very immature myeloblast cell line KG-1 (Nakamura and Hemmi, 1988).

Based on the assumption that cells of various stages of maturity would show different properties in the induction of differentiation, the primary objective of the present study was to obtain further evidence that some cloned lines of RCMC1 represent mast cells at different stages of differentiation, by exposing them to PMA, DM and RA and monitoring changes in surface Fc ϵ R expression and histamine content. In addition, it was hoped that these studies may also provide some information on the regulatory mechanisms of Fc ϵ R expression.

MATERIALS AND METHODS

Reagents:

Phorbol 12-myristate 13-acetate (PMA), dexamethasone (DM) and retinoic acid (RA) were all purchased from Sigma Chemical Co. (St. Louis, MO). PMA was dissolved in dimethyl sulphoxide (DMSO) at a concentration of 100 μ g/ml. DM and RA were dissolved in ethanol at concentrations of 1mM and 3.3mM, respectively. The stock solutions were diluted to the desired concentrations with complete culture medium when used for cell culture. Alcian blue, safranin O, 1-(5-Isoquinolinylsulfonyl)-2-methyl-piperazine (H-7), staurosporin, benzamidine, leupeptin and phenylmethylsulfonyl fluoride (PMSF) were also obtained from Sigma. PMSF was dissolved in isopropanol at 200mM and stored at -20°C and diluted into the buffer immediately before experiment. The protein kinase C assay kits and [γ -³²P]ATP (1.0 mCi/ml, 3000Ci/mmol) were purchased from Amersham (Oakville, Ont.) and lead subacetate was from Aldrich (Milwaukee, WI).

The purification of rat monoclonal IgE and the preparation of IgE-Sepharose were described in Chapter II.

Cell culture in the presence of differentiation agents:

Cloned lines of RCMC1 exhibiting differential expressions of the receptors for IgE were described in Chapter II. These cells at both early (0-4 weeks) and late (20-24 weeks) time points of the time-dependent receptor development study described in Chapter II were subjected to modulation with PMA, DM and RA. Cells were cultured for a prolonged period ranging from 3-4 weeks in the presence of differentiation agents in the same culture medium as described in the previous chapter. Every week aliquots

of modulated cells and control cells were frozen and kept in liquid N₂. At the end of a set of experiments with a given differentiation agent, the cell aliquots frozen after different times in culture were revived and expanded in the presence of differentiation agents until enough cells were obtained for receptor isolation and the histamine assay. Normally, this process involved 4-5 days of tissue culture. Receptor isolation was done in quadruplicate at one time point in each experiment. Cells at this time point were revived and expanded to yield enough cell to make modulation on quadruplicate samples possible. In the case of PMA modulation, 10 μ M of H-7 was occasionally added to the culture to inhibit the PKC activity.

Dose-dependent responses of receptor expression due to the presence of differentiation agents were carried out for each modulator using clone RCMC1.3.2 at an early time point. Cells were incubated for 72 hr in the presence of various concentrations of differentiation agents and analyzed for receptor expression. The effect of PMA was tested at concentrations of 0.1ng/ml, 1ng/ml and 10ng/ml, dexamethasone was tested at 0.1 μ M, 1 μ M and 10 μ M while RA was used at 10nM, 100nM and 1000nM concentrations.

Assay of surface receptor expression:

The cells of selected RCMC clones modulated with the differentiation agents were iodinated and solubilized according to the established procedures described in Chapter II. The receptors in solubilized cell preparations were isolated by using IgE-Sepharose and further characterized on SDS-PAGE followed by autoradiographic analysis and densitometry as described in Chapter II. Standard errors of the peak heights of each

experiment carried out were calculated at the time points where quadruplicates were performed.

Assay of cellular histamine content:

The cellular histamine content was determined with the aid of a modified spectrofluorometric method (May et al., (1970). Quadruplicates of 1×10^6 cells, washed three times with 0.05% BSA/PBS, were lysed with 0.1 ml of 14% perchloric acid and then boiled for 2 min. The cooled samples were centrifuged at 2000 rpm for 15 min at 4°C to sediment the precipitate. The supernatant was transferred to glass tubes containing 2.5ml butanol, 0.2ml of 2.5 N NaOH and approximately 0.5 gm of NaCl, and mixed at a 45° angle for 8 min on a horizontal shaker (New Brunswick) at 300 rpm. The extracted material in the butanol layer was immediately transferred to tubes containing 2.5ml heptane and 0.6ml of 0.1N HCl and mixed again for 8 min as above. The tubes were left to sit in the dark at 4°C for a minimum of 30 min. A volume of 0.5ml of the lower HCl layer containing the extracted histamine was allowed to react at 4°C in the dark with a mixture of 0.1ml fresh solution of 1mg/ml O-phthalaldehyde (OPT) in methanol and 0.2ml of 0.33N NaOH for 40 min. The reaction was terminated by the addition of 0.2ml 1M H_3PO_4 . The fluorescence was measured at 450nm with excitation at 360nm (Aminco-Bowman Spectrophotofluorometer, American Instrument, Silver Spring, MD). The total histamine content was determined from a set of histamine standards which were carried out with each experiment.

Statistical analysis:

The modulation data on receptor expression and histamine content were analyzed to establish the statistical significance between controls and experimental values. A

common t-statistic test procedure was used according to the description by Pankratz (1983). First, the standard errors of both the control and the modulation were calculated from the results of quadruplicates and the mean of the two standard errors indicated the sigma range. Second, the values of the difference between control data and modulated data were obtained. Third, if the absolute value of a difference between a control and a modulation was greater than two times of the sigma range they were considered significantly different from each other with a probability higher than 95%. Otherwise they were not significantly different.

Assay of protein kinase C activity:

Cells were harvested in the logarithmic growth phase and washed twice in serum-free medium. The membranous and cytosolic fractions used for PKC assays were prepared using a modified method of White and Metzger (1988). A total of 1×10^7 cells were reconstituted in 1ml of sonication medium which was composed of 50mM Tris/HCl, pH7.5, 5mM EDTA, 10mM EGTA, 0.3% (w/v) β -mercaptoethanol, 10mM benzamidine, 10ug/ml lupeptin and 50ug/ml PMSF, and disrupted using a W225R sonicator at output 4 for 30 sec at 4°C. The sonicated cells were fractionated into membrane and supernatant portions by centrifugation on a superspeed centrifuge (Sorval RC2-B) at 17,000 rpm (35,000g) for 60 min at 4°C. The supernatant was stored on ice for the cytosolic PKC assay. The membrane portion was resuspended in sonication medium containing 0.5% NP-40 by sonication to yield 1×10^7 cell equivalent/ml, and used for the membrane PKC assay.

The PKC activity was measured and calculated according to the procedures outlined in the assay kit by the manufacturer (Amersham). The reaction was characterized

by the transfer of γ - ^{32}P -phosphate of adenosine-5'-triphosphate (ATP) to the threonine group on a PKC-specific peptide. Briefly, a volume of 25 μl samples was added to 50 μl of a component mixture, containing 225 μM peptide, 5mCi/ml [^{32}P]ATP, 3mM calcium, 2M phosphatidyl-L-serine, and 7.5mM dithiothreitol in 50mM Tris/HCl at pH7.5, and allowed to react for 15 min at 25°C. The reaction was terminated by adding 100 μl dilute acidic quenching reagent. The phosphorylated peptide was precipitated onto the peptide binding paper. After washing two times with 5% (v/v) acetic acid the papers were counted in a scintillation cocktail (Amersham) using a liquid scintillation counter (Beckman).

Alcian blue/Safranin staining:

Mast cells were histochemically stained according to established methods (Seldin et al., 1985b) in order to distinguish between the MMC and CTMC phenotypes. Cells were centrifuged (Shandon Southern Instrument, Sewickley, PA) unto glass slides at 10,000 rpm for 1 min. The slides were fixed for 1 min in Mota's lead acetate solution which was composed of 1% (w/v) lead subacetate, 50% (v/v) ethanol, 0.5% (v/v) acetic acid. After rinsing in 0.3% acetic acid solution, the slides were stained with 5mg/ml alcian blue in 0.3% acetic acid for 5 min and counter-stained with 1mg/ml safranin O in 1% acetic acid for 5 min. The cells were serially dehydrated with 75%, 95% and 100% alcohol for 45 sec each and finally in Xylene for 45 sec, then air-dried, and examined under a microscope.

Preparation of DNP-BSA conjugates:

The conjugates of DNP and bovine serum albumin (BSA) were prepared according to procedures described by Carsten and Eisen (1953). Thus 500mg of BSA and 500mg

of dinitrobenzensulfonate (DNBS) were mixed in 0.4M Na₂CO₃ and stirred gently in a 18°C water bath for 6 hours. During the incubation small aliquotes were removed at intervals, passed through a Sephadex G25 column equilibrated with PBS and the optical densities measured at 280nm and 360nm in Zeiss PMQ II spectrophotometer. The number of groups of DNP coupled per molecule of BSA was determined by the relationship of moles: E₂₈₀ of BSA of 6, the molecular weight of BSA of 69,000, the constant 2.89 derived from the ratio between the optical density (OD) of DNP at 360nm and the OD of DNP at 280nm, and the molar extinction coefficient of DNP at 360nm of 1.753x10⁴:

$$\text{Moles of BSA} = \left[\left(OD_{280} - \frac{OD_{360}}{2.89} \right) / 6 \right] / 6.9 \times 10^4$$

$$\text{Moles of DNP} = \frac{OD_{360}}{1.753 \times 10^4} \quad .$$

Preparation of mouse DNP-specific IgE:

Mouse monoclonal IgE with anti-2,4-dinitrophenyl (anti-DNP) specificity was produced from the murine hybridoma H₁-DNP- ϵ -26.82 originally prepared by Liu et al. (1980) and kindly provided to Dr. A. Sehon. BALB/c mice (7-9 weeks old) were primed with 0.5 ml Pristane (Aldrich Chemical Co., Milwaukee, WI) by i.p. injection. Two weeks later, 1x10⁷ of tumour cells in 1ml RPMI medium 1640 were injected into the peritoneal cavity of each primed mouse. The tumour cells were allowed to grow in the ascites fluid for approximately 2 weeks, after which the ascites fluid was collected and centrifuged. The supernatant recovered from such ascites were kept frozen for further purification.

To isolate IgE from the ascites fluid, DNP₄₀-BSA-Sepharose was washed extensively with 0.1M acetic acid until no protein was detected in the effluent as measured with a Zeiss PMQII spectrophotometer. The conjugate was then neutralized by washing extensively with PBS. Twice the volume of ascites was added and the mixture was agitated on a rotator overnight at 4°C. The next morning the affinity gel was applied to a column and was washed extensively with PBS to remove unbound material. The bound DNP-specific IgE was eluted with 0.1M 2,4-dinitrophenol (DNPOH). The latter was prepared by titrating 2% (w/v) of DNPOH with 1N NaOH until the pH reached 8. To separate IgE from DNPOH the eluates were then passed through a Dowex 1x8 column and the eluate collected in a fraction collector. Each tube from the fraction collector was then measured for its protein content using a spectrophotometer. The tubes containing the protein peak were pooled and dialyzed against PBS for several changes at 4°C to remove traces of DNPOH. The IgE concentration was determined using an extinction coefficient of 16.2 (E₂₈₀) and a molecular weight of 184,000 (Liu et al., 1980).

Assay of incorporated ³H-serotonin (³H-5-HT) release:

The ³H-serotonin release assay was performed using a modified method of Taurog et al. (1977). The assay system was established with using the releasing cell line RBL-2H3. This cell line was found to release 50-70% of ³H-serotonin which it had taken up. Cells were mechanically stripped from the tissue culture flask and plated in 24-well tissue culture plate (Linbro, McLean, VA) at 1x10⁵ cells/ml/well in complete culture medium. After overnight incubation at 37°C in an atmosphere of 3% CO₂, the culture supernatant was removed and 0.3μg of purified mouse DNP-specific IgE was added

to each well simultaneously with 3mCi of ^3H -hydroxytryptamine binoxalate (^3H -serotonin) (New England Nuclear-Du Pont Co., Markham, Ont.) in 1ml of complete culture medium supplemented with 2% FCS. The plate was incubated at 37°C for 2.5h. Cells were carefully washed four times with HEPES buffer and then triggered with a serial dilution of $\text{DNP}_{17}\text{-BSA}$ for 35 min at 37°C . The spontaneous release was assayed by adding buffer as a control. The released ^3H -serotonin was determined by removing 0.2ml supernatant from each well and counted in 5ml Aquasol scintillation fluid (New England Nuclear-Du Pont Co. Markham, Ont.) in Beckman scintillation counter. The values were derived from the averages of triplicate samples.

Proliferation assay:

RCMC clones modulated with differentiation agents were tested for their growth rates as described in Chapter II.

RESULTS

Modulation of $Fc\epsilon R$ expression on RCMC clones:

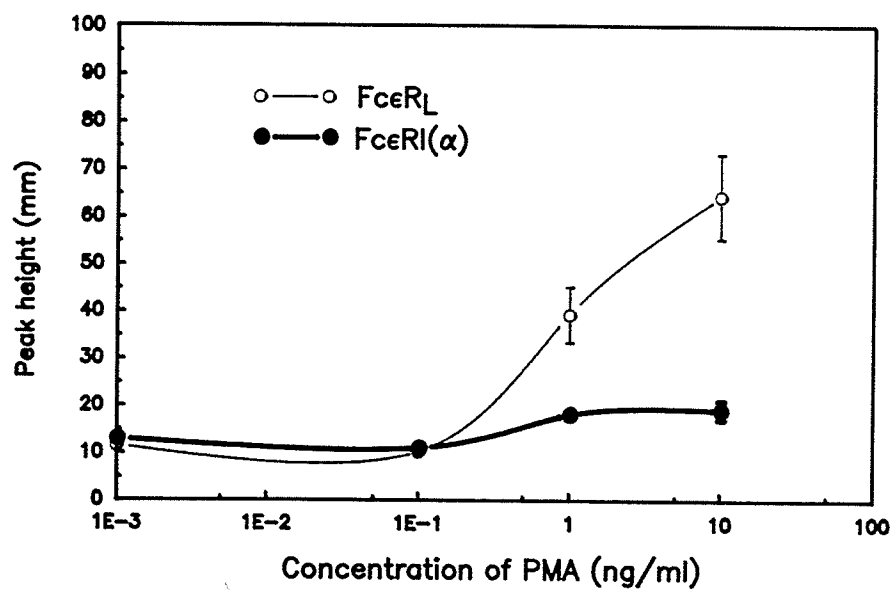
The dose response curve of PMA modulation indicated that after 72 hr in culture PMA at both 1 ng/ml and 10 ng/ml concentrations increased the expression of $Fc\epsilon R_L$ but had no effect on $Fc\epsilon RI(\alpha)$ (Fig. 14a). However, PMA at 10 ng/ml resulted in obvious cell death. The PMA concentration of 1 ng/ml was chosen for prolonged modulation of RCMC cloned lines. Long-term culture (3-5 weeks) with 1 ng/ml PMA had a cell viability of over 90%. Dexamethasone at 0.1 μM , 1 μM and 10 μM concentrations after 72 hr culture significantly increased the expression of $Fc\epsilon R_L$ on RCMC1.3.2 (Fig. 14b), and this effect was dose-dependent. The concentration of 1 μM DM was chosen for long-term modulation since at 10 μM concentration the DM appeared to be toxic to the cells which became enlarged, filled with large vacuoles, and nearly stopped proliferation. The effectiveness of RA on the cloned lines of RCMC was tested with 10 nM, 100 nM and 1000 nM concentrations of RA on RCMC1.3.2. All three concentrations of RA were found to induce a decrease in the expression of both $Fc\epsilon RI(\alpha)$ and $Fc\epsilon R_L$ (Fig. 14c). Retinoic acid at 300 nM was selected for the modulation of various RCMC clones since it was in the dose range (100-1000nM) within which the cells appeared to be more sensitive to RA concentrations.

The phorbol ester, PMA in this case, generally elevated the surface expression of $Fc\epsilon R_L$ while the effect on the surface expression of $Fc\epsilon RI$, as monitored by its α subunit, was minimal. Most importantly, variations in response to PMA were observed among the RCMC clones and between the early and late stages of certain clone(s). The effects of PMA on RCMC1.3.1 which carried $Fc\epsilon R_L$ only, were different for cells

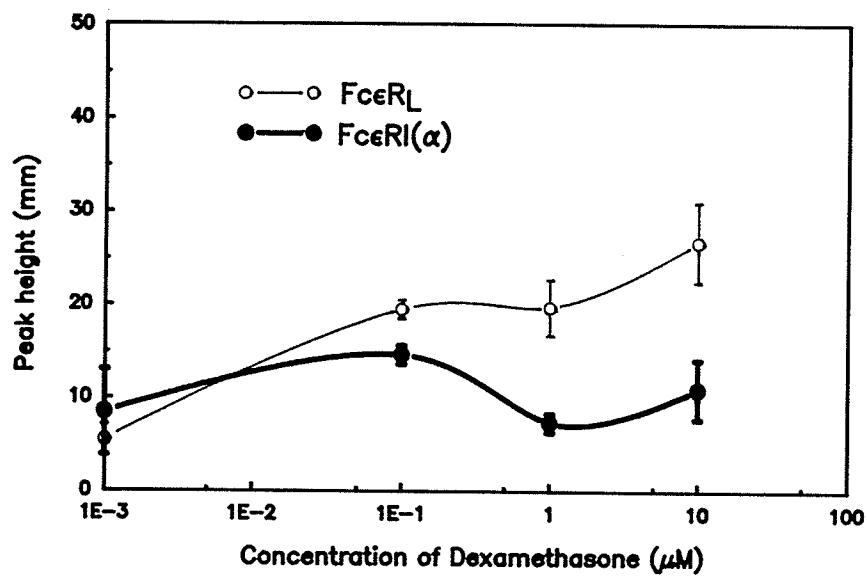
Fig. 14. Dose-dependent response of RCMC1.3.2 at early stage to (a) PMA, (b) dexamethasone and (c) retinoic acid treatment in terms of the $Fc_\epsilon R$ expression. The X-axis Log scale indicates the doses of the differentiation agents. The effect on receptor expression is shown as peak height which was derived by densitometric analysis of receptor density on autoradiographs.

Fig. 14

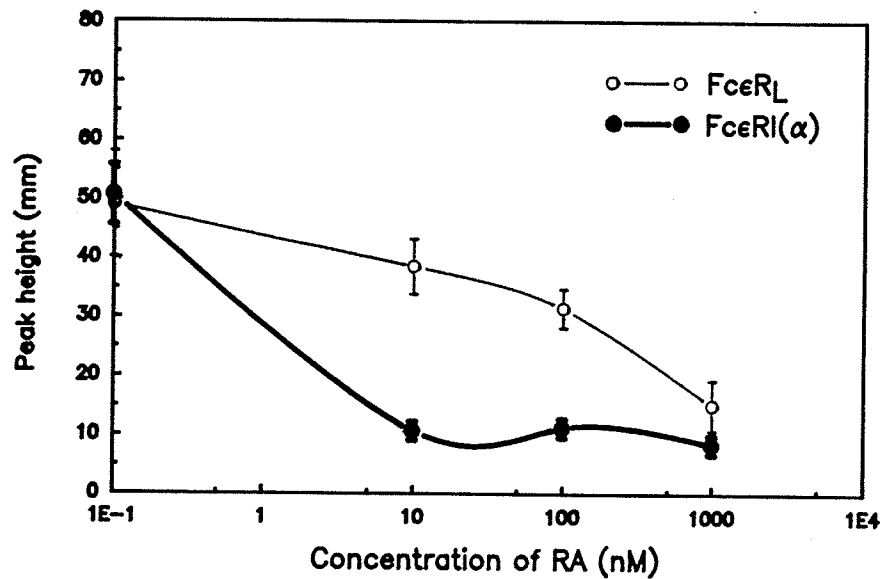
a)



b)



c)



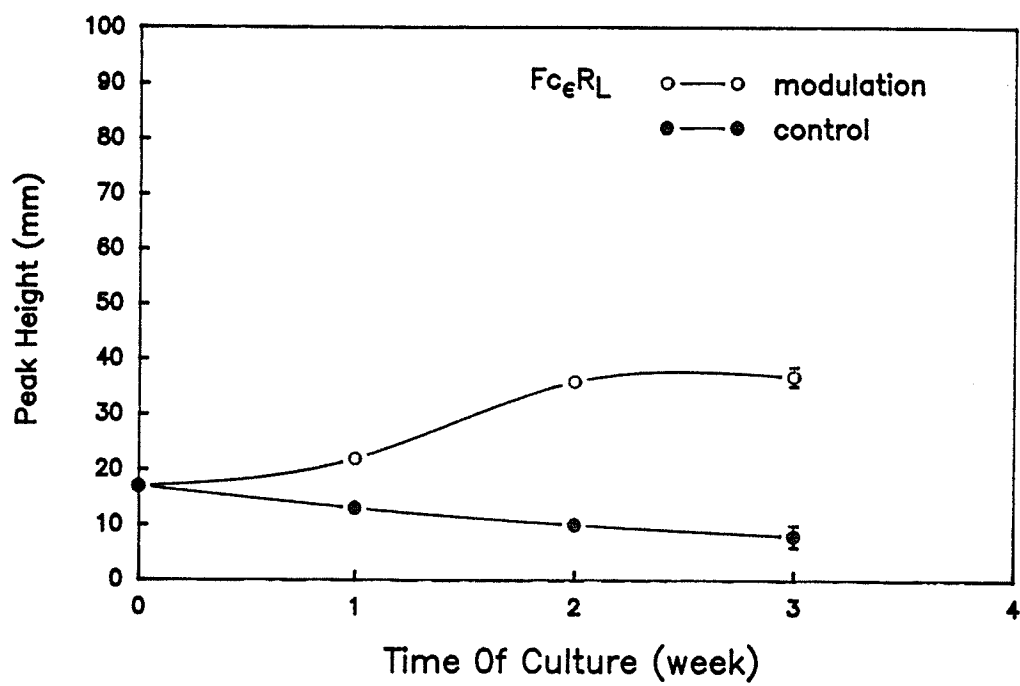
obtained from the early and late stages of the time studies (Chapter II). PMA increased the expression of $\text{Fc}_\epsilon\text{R}_\text{L}$ (Fig. 15a) of the cells at the early stage when minimal surface receptors were expressed, but it had no effect on the receptor expression (Fig. 15b) in the case of the cells at the late stage, when they had almost reached maximum in the expression of $\text{Fc}_\epsilon\text{R}_\text{L}$ (compare Fig. 5). In the presence of PMA, clone RCMC1.11.2 which had relatively stable expression of $\text{Fc}_\epsilon\text{R}_\text{L}$ in the previous study, at either stage of receptor development, showed minor variations in their receptor expression (Fig. 16a, b) which, however, were not found to be statistically significant. PMA modulation on RCMC1.3.3, at both early (Fig. 17a) or late stage (Fig. 17b), up-regulated their expression of $\text{Fc}_\epsilon\text{R}_\text{L}$. Yet the modulated receptor expression for cells at late stage reached a plateau within the first week of modulation, whereas the receptor modulation on cells at the early stage reached a plateau of a greater magnitude at a later modulation point (week 3). The increased $\text{Fc}_\epsilon\text{R}_\text{L}$ expression by PMA was found to be reversible. When PMA was removed from the culture medium of RCMC1.3.3 cells after a 4 week modulation period the elevated surface receptor expression (Fig. 17a) returned to near control levels within two weeks. Similar results to those RCMC1.3.3 were observed in the case of clone RCMC1.3.2. At its early stage the expression of $\text{Fc}_\epsilon\text{R}_\text{L}$ was greatly increased by PMA (Fig. 18a) whereas at late stage the increase of $\text{Fc}_\epsilon\text{R}_\text{L}$ was relatively constant after week 1 (Fig. 18b). Of all the three clones which expressed $\text{Fc}_\epsilon\text{RI}$, RCMC1.11.2, 1.3.3 and 1.3.2, PMA showed little effect on the expression of this receptor.

In previous reports, DM has been shown to modulate mouse bone marrow-derived mast cells (BMMC) into connective tissue type of mast cells and decrease the

Fig. 15. PMA modulation of $\text{Fc}\epsilon\text{R}$ expression on RCMC1.3.1 (a) at early stage and (b) at late stage. A dose of 1 ng/ml PMA in culture medium was used for modulation. Cells cultured in the culture medium only were used as control. Cells at either the early or the late stage of culture were cultured either in the culture medium described under Materials and Methods or in the same medium supplemented with the differentiation agent. Cell aliquots were removed every week from tissue cultures, frozen and stored in liquid N_2 . At the end of the study period the frozen aliquots from one clone were revived and expanded in the same culture medium till enough cells were obtained for receptor analysis.

Fig. 15

a)



b)

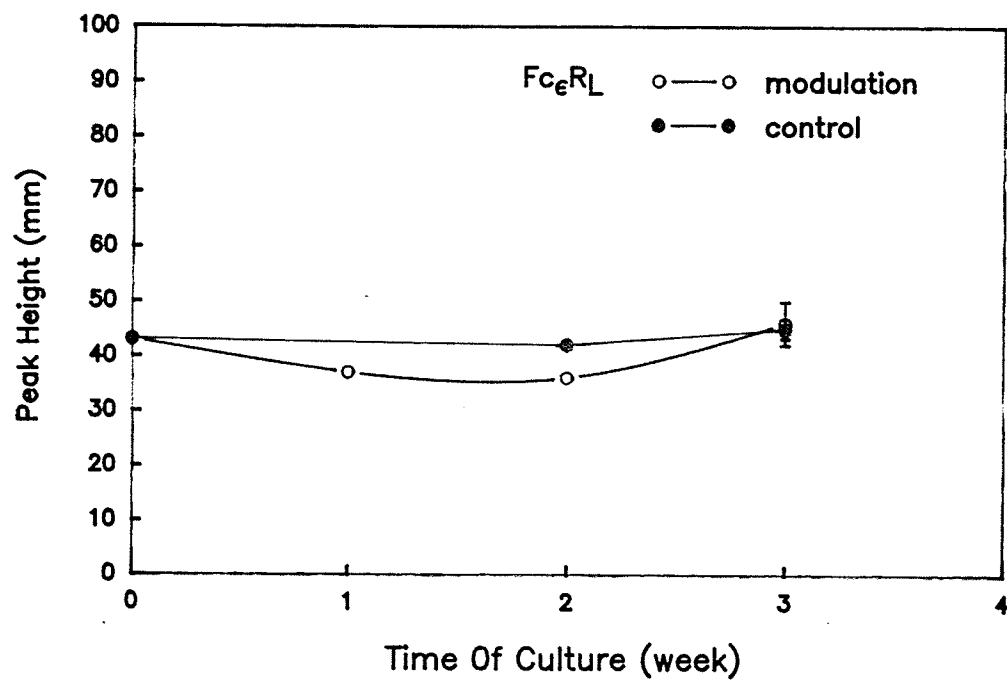
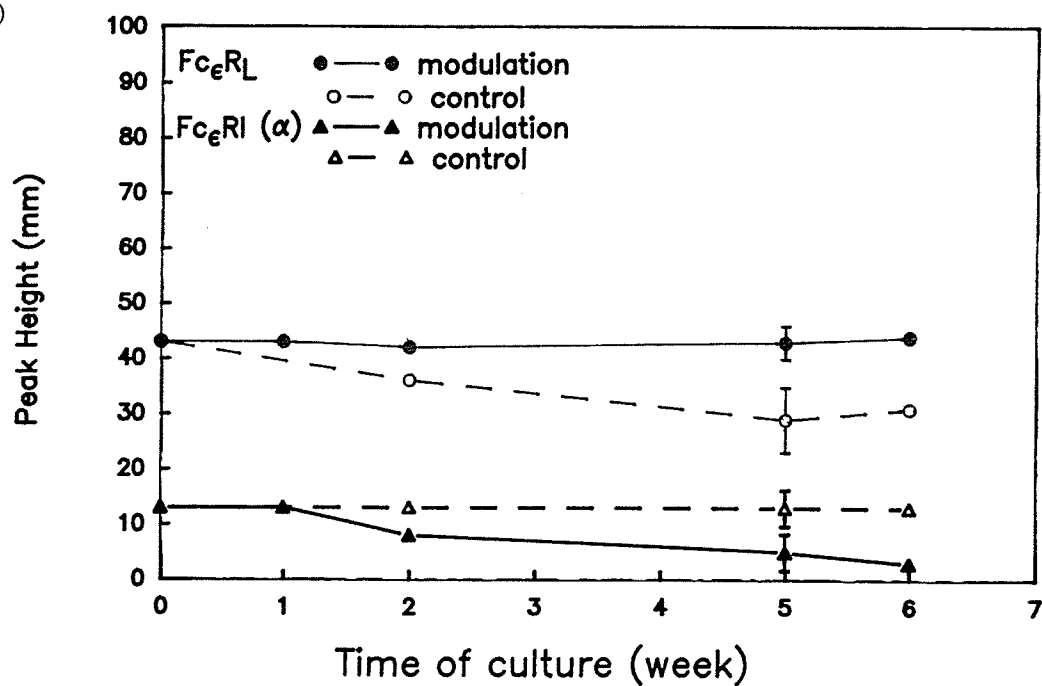


Fig. 16. PMA modulation of $\text{Fc}\epsilon\text{R}$ expression on RCMC1.11.2 (a) at early stage and (b) at late stage.

Fig. 16

a)



b)

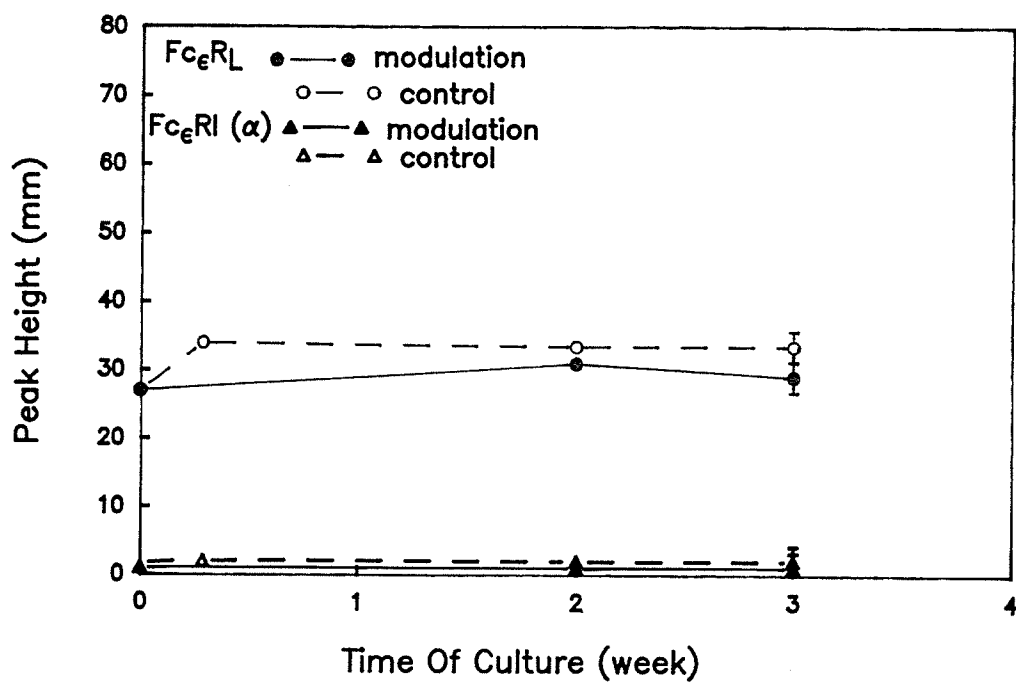
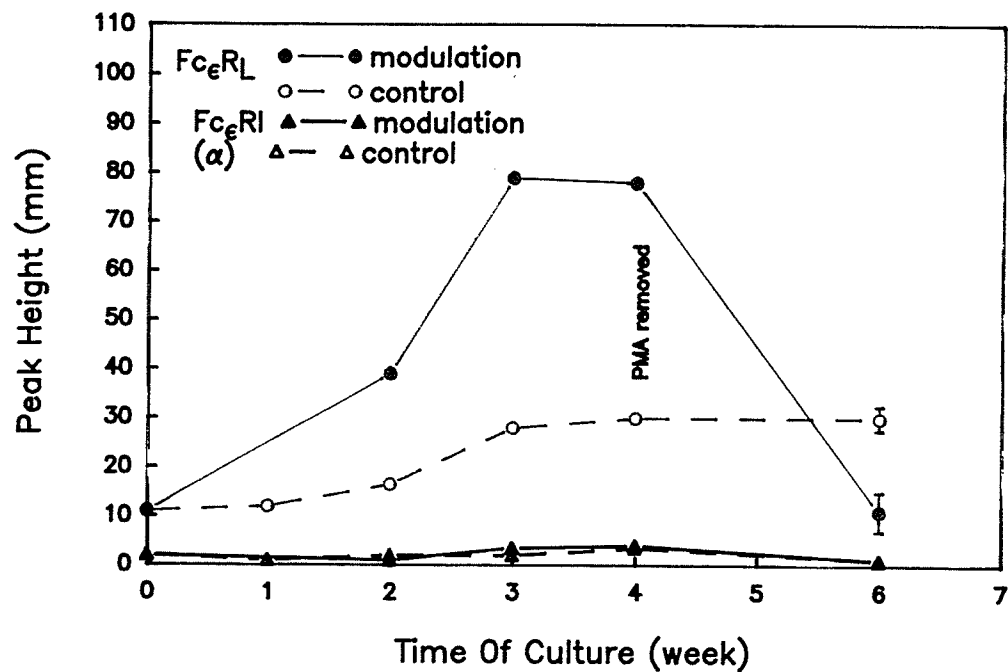


Fig. 17. PMA modulation of $\text{Fc}\epsilon\text{R}$ expression on RCMC1.3.3 (a) at early stage and (b) at late stage. In Fig. 17a PMA was removed from culture medium at 4 week modulation and the cells were further cultured in culture medium only for two more weeks.

Fig. 17

a)



b)

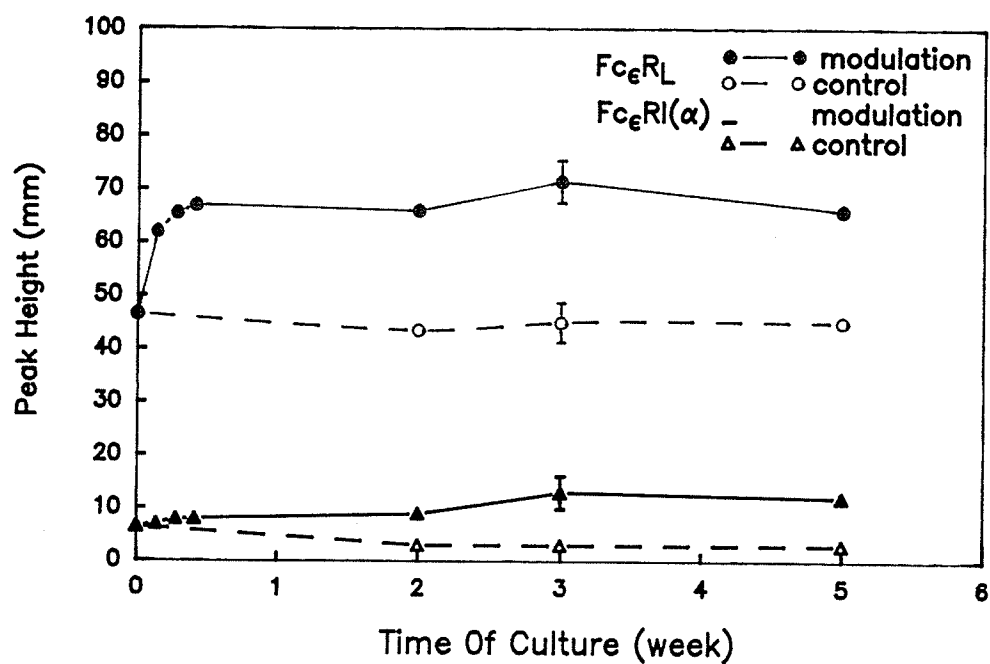
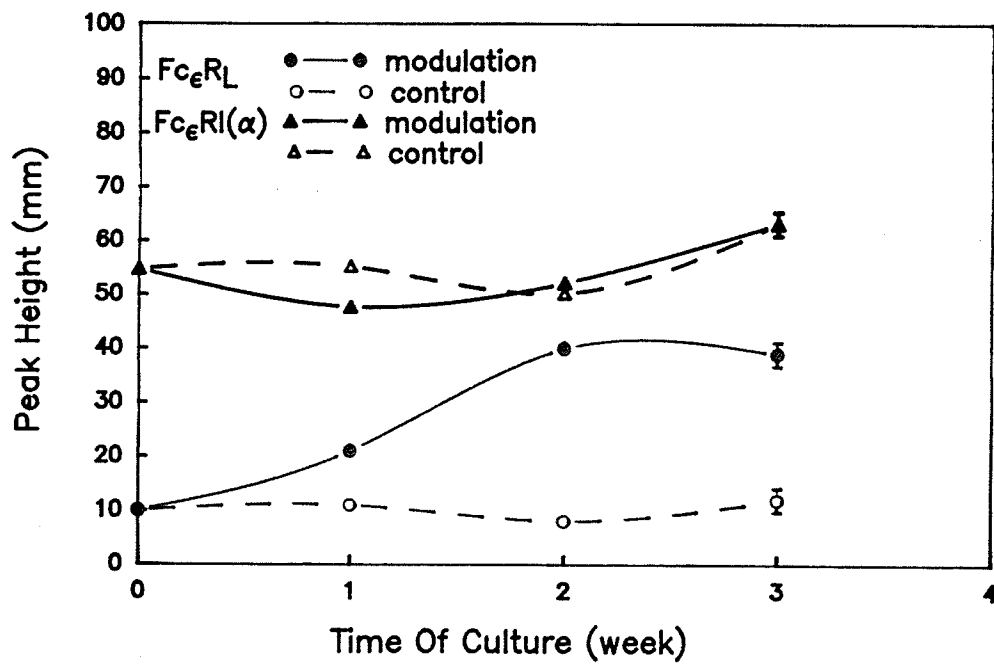


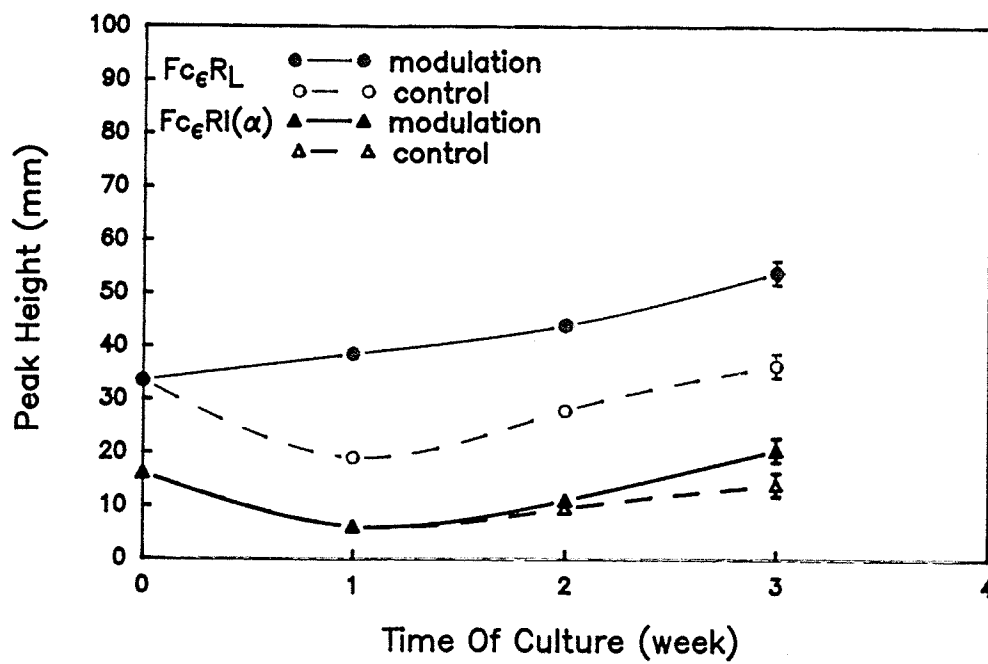
Fig. 18. PMA modulation of $\text{Fc}_\epsilon\text{R}$ expression on RCMC1.3.2 (a) at early stage and (b) at late stage.

Fig. 18

a)



b)

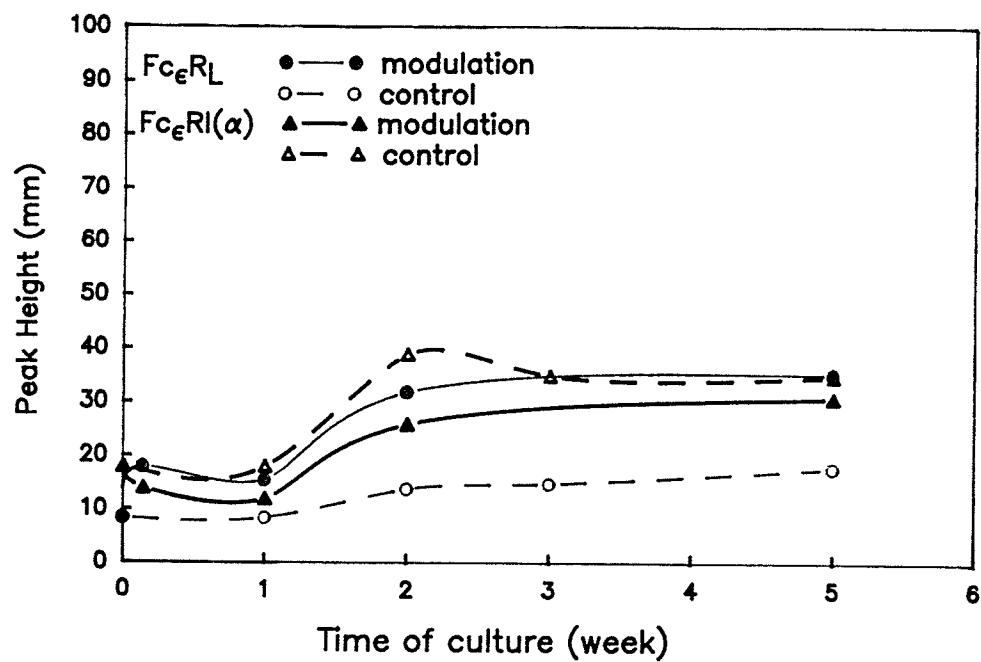


expression of the high affinity $\text{Fc}_\epsilon\text{RI}$ on these cells (Pitton et al., 1988; Robin et al., 1985). In this study DM was generally found to increase the expression of the low affinity $\text{Fc}_\epsilon\text{R}_\text{L}$ on the clones which also expressed $\text{Fc}_\epsilon\text{RI}$. Statistical analysis on the expression of $\text{Fc}_\epsilon\text{RI}(\alpha)$ revealed that DM slightly but significantly decreased the expression of $\text{Fc}_\epsilon\text{RI}(\alpha)$ on certain clones, and at some modulation time points of others when the cells expressed relatively higher levels of this receptor. The DM modulation on the early stage of RCMC1.3.2, as shown in Fig. 19a, resulted in an increase in the expression of $\text{Fc}_\epsilon\text{R}_\text{L}$ and a slight decrease in $\text{Fc}_\epsilon\text{RI}$ expression at all modulation points except one at which the effect of DM was more pronounced. The latter agreed with the existing data on receptor modulation. At the late stage of this clone, when the cells expressed more $\text{Fc}_\epsilon\text{R}_\text{L}$ and less $\text{Fc}_\epsilon\text{RI}$ (see Figs. 6 and 7), DM only increased the expression of $\text{Fc}_\epsilon\text{R}_\text{L}$ and the effect of DM on $\text{Fc}_\epsilon\text{RI}(\alpha)$ became undetectable (Fig. 19b). Similar results were obtained from modulation studies on RCMC1.3.3. In this case, cells at the early stage were up-regulated in their $\text{Fc}_\epsilon\text{R}_\text{L}$ expression by DM and slightly down-regulated in the $\text{Fc}_\epsilon\text{RI}$ expression, but only at one modulation point (Fig. 20a) and cells at late stage showed similar response to DM as did RCMC1.3.2 at the late stage (Fig. 20b), although at this stage the cells expressed relatively more $\text{Fc}_\epsilon\text{R}_\text{L}$ and $\text{Fc}_\epsilon\text{RI}$ (see Fig. 12). For clone RCMC1.11.2, at both stages, DM significantly increased the expression of $\text{Fc}_\epsilon\text{R}_\text{L}$ and had no effect on $\text{Fc}_\epsilon\text{RI}$ expression (Fig. 21a, b). Interestingly, DM was found to have no effect on the expression of $\text{Fc}_\epsilon\text{R}_\text{L}$ on RCMC1.3.1 at both early and late stages. This clone was different from the others in that $\text{Fc}_\epsilon\text{R}_\text{L}$ were the only receptors expressed on cells of this clone (Fig. 22a, b). Although at one modulation point of cells at the late stage

Fig. 19. Dexamethasone modulation of Fc ϵ R expression on RCMC1.3.2 (a) at early stage and (b) at late stage. A dose of 1 μ M DM was used throughout the modulation experiments.

Fig. 19

a)



b)

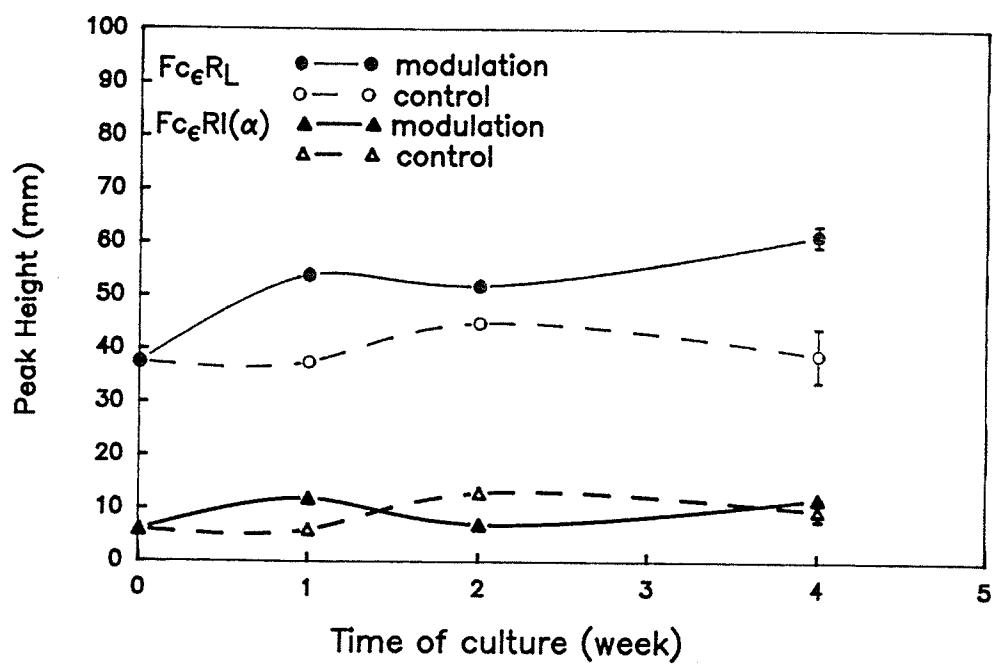
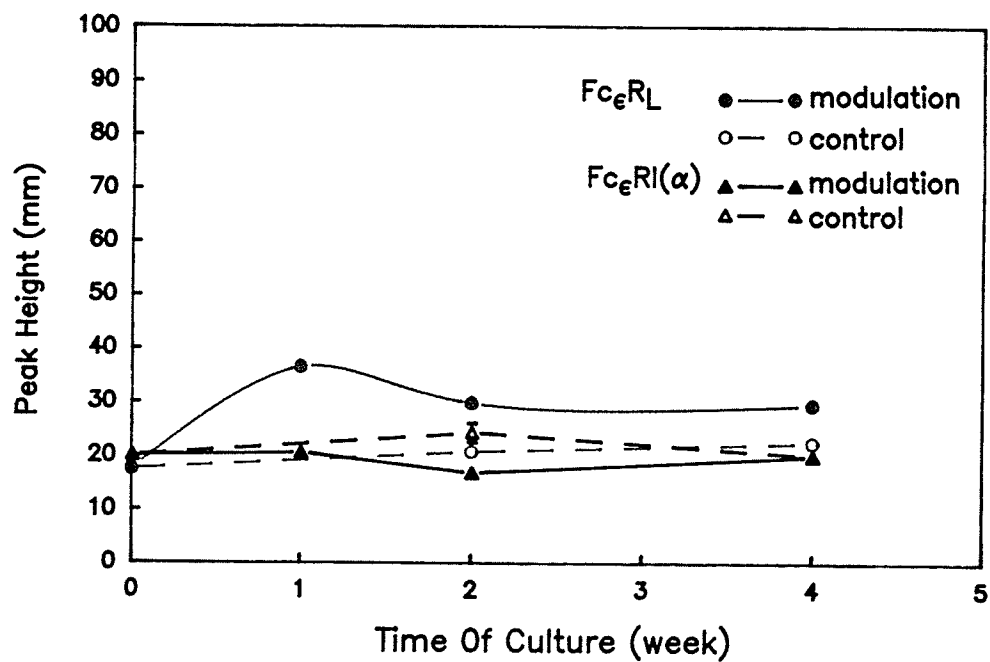


Fig. 20. Dexamethasone modulation of $\text{Fc}\epsilon\text{R}$ expression on RCMC1.3.3 (a) at early stage and (b) at late stage.

Fig. 20

a)



b)

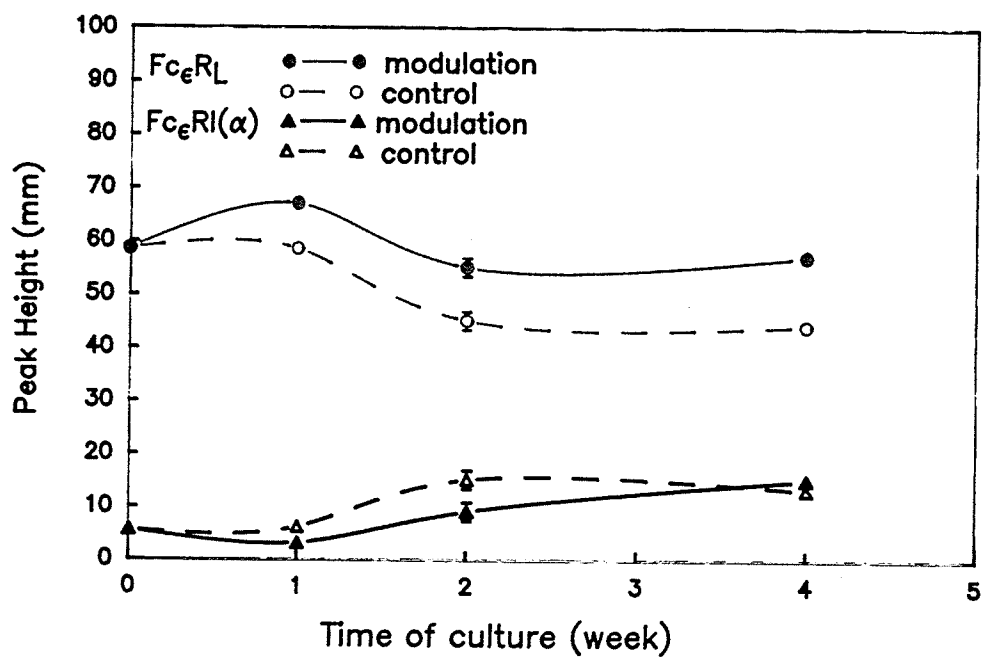
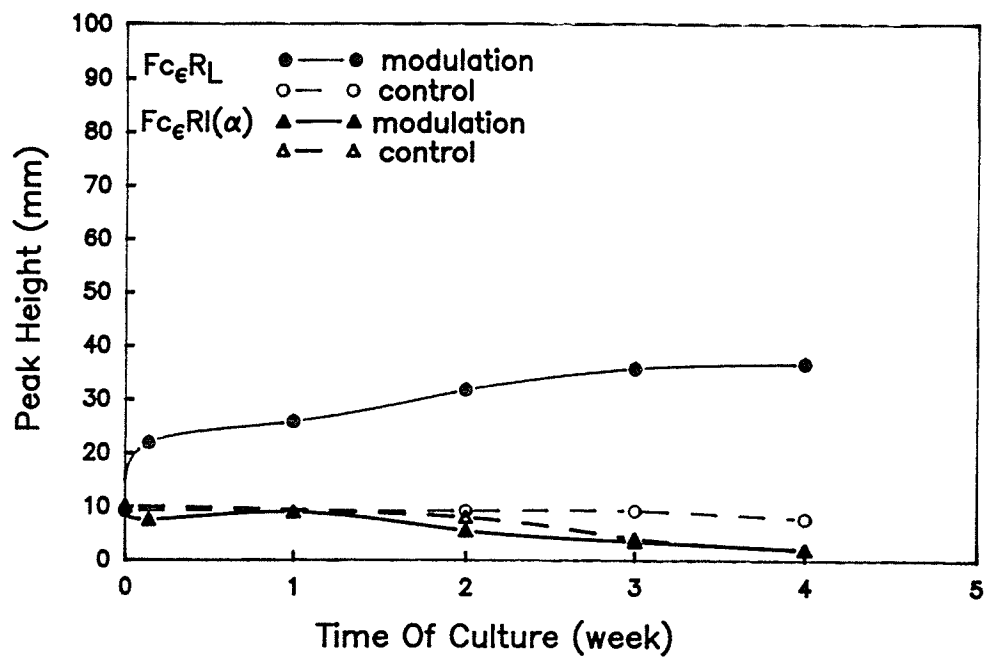


Fig. 21. Dexamethasone modulation of $\text{Fc}_\epsilon\text{R}$ expression on RCMC1.11.2 (a) at early stage and (b) at late stage.

Fig. 21

a)



b)

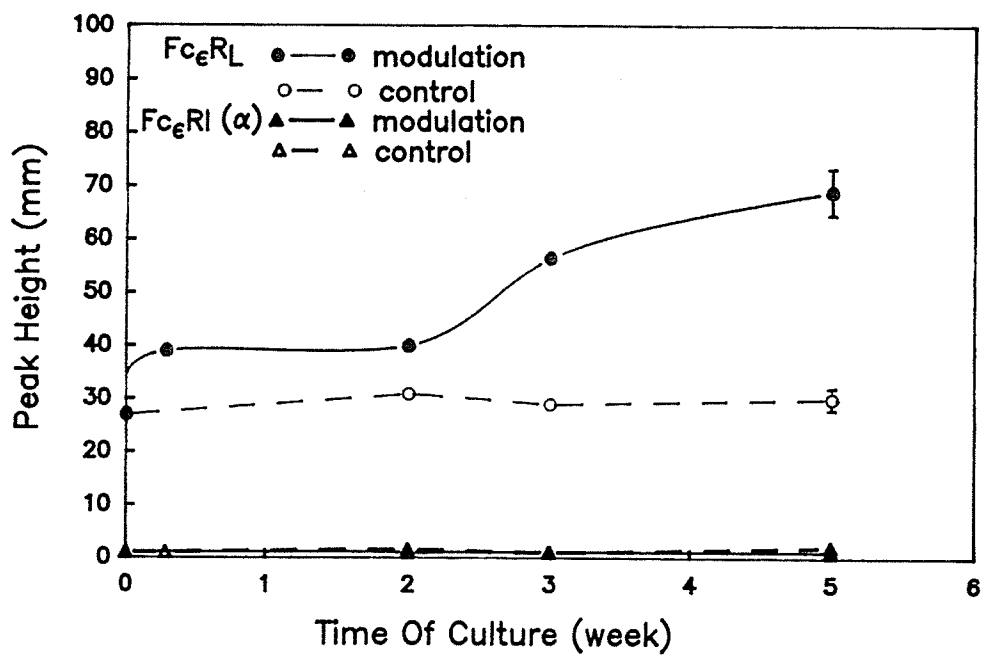
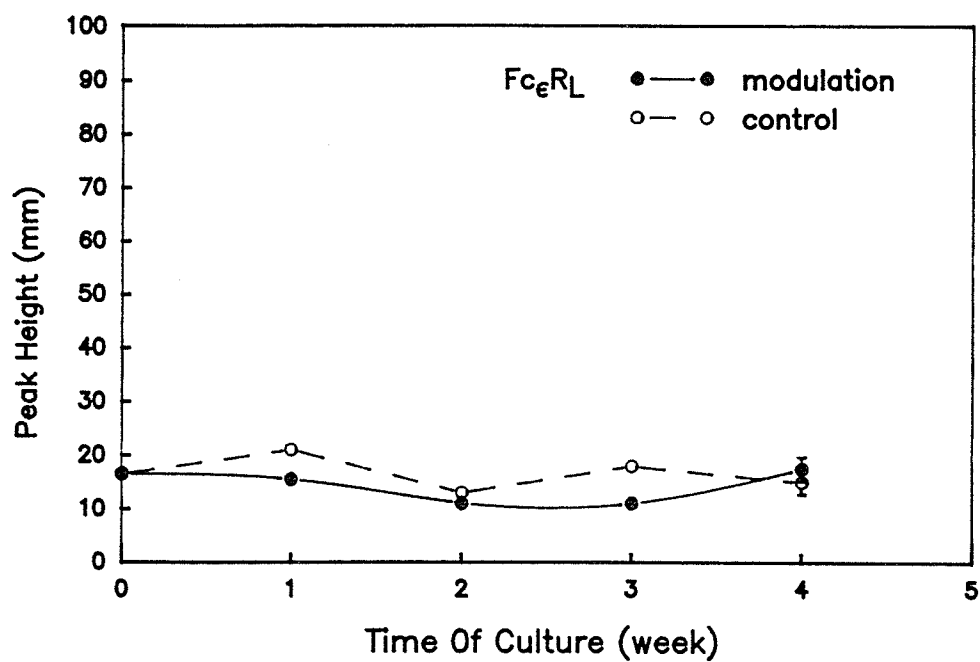


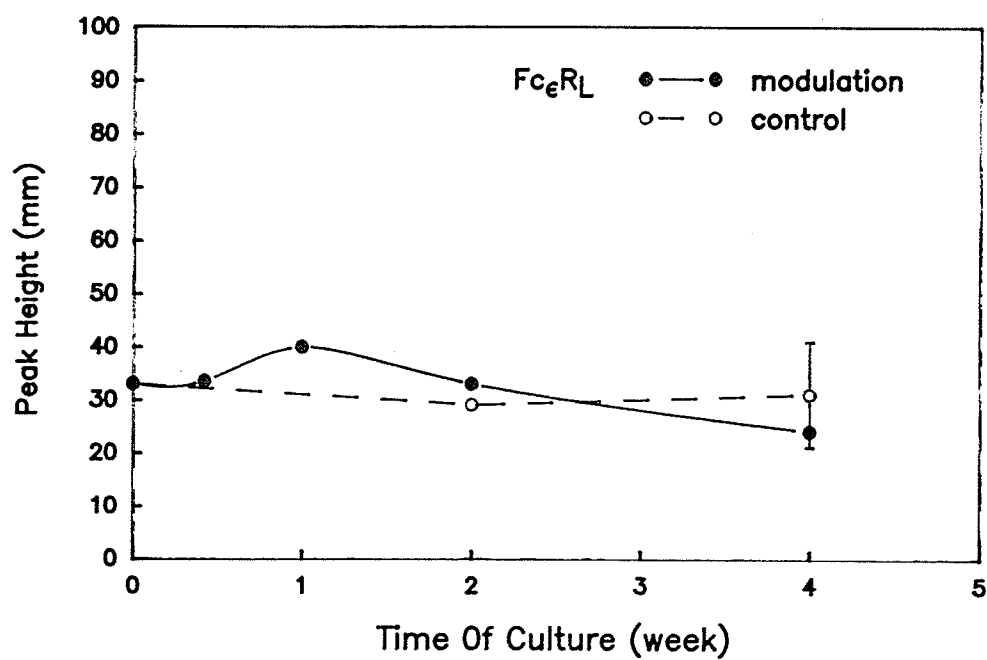
Fig. 22. Dexamethasone modulation of $\text{Fc}_\epsilon\text{R}$ expression on RCMC1.3.1 (a) at early stage and (b) at late stage.

Fig. 22

a)



b)



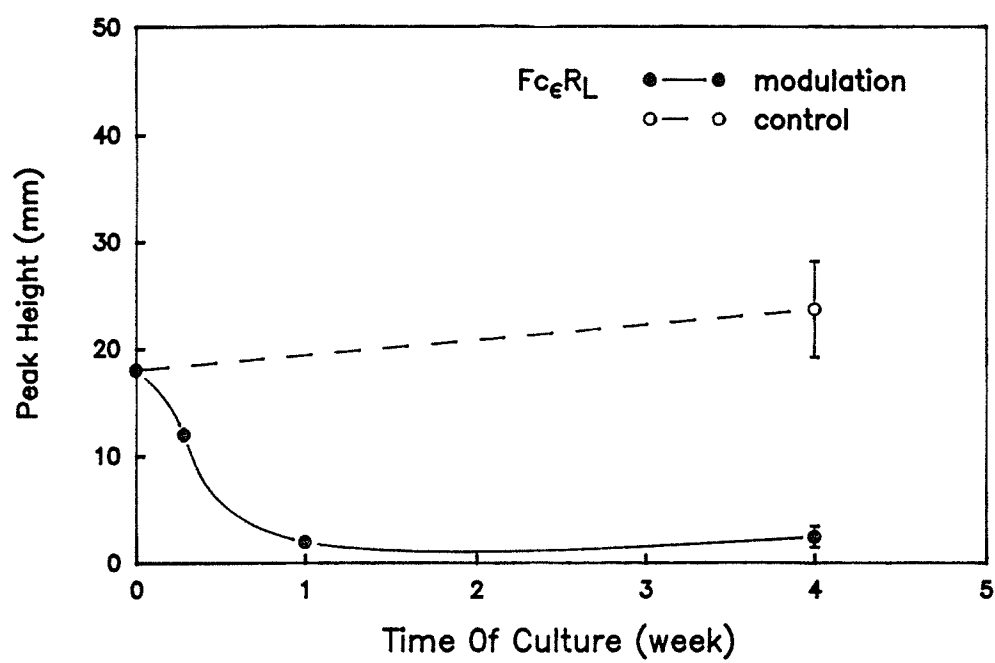
an increase in receptor expression was noticed, statistical analysis found that the variation was within the two-sigma range of standard deviation.

The reason why RA was used in this study to modulate RCMC clones was because RA has been shown to differentially regulate myelomonocytic cell lines at various stages along the maturation pathway. However, despite the differences in receptor expression among the RCMC clones, RA indiscriminately down-regulated the expression of both $\text{Fc}\epsilon\text{RI}(\alpha)$ and $\text{Fc}\epsilon\text{R}_\text{L}$ on all of the clones studied and for each clone at both early and late stages as defined in Chapter II after continuous growth in tissue culture. Even for the early stage of RCMC1.3.1 which did not express a high density of $\text{Fc}\epsilon\text{R}_\text{L}$, RA modulation still decreased the receptor expression two-fold (Fig. 23a), whereas for cells expressing a higher number of this receptor at the late stage, RA lowered the surface receptor number about three-times (Fig. 23b). The high affinity receptor, $\text{Fc}\epsilon\text{RI}$, which was expressed at a higher density on the early stage RCMC1.3.2 (Fig. 24a) and the higher expression of the low affinity $\text{Fc}\epsilon\text{R}_\text{L}$ at the late stage of this clone (Fig. 24b) were substantially decreased by RA. In the case of $\text{Fc}\epsilon\text{R}_\text{L}$ of the early stage RCMC1.3.2, it gradually merged with the slightly decreasing control $\text{Fc}\epsilon\text{R}_\text{L}$. On the other hand, $\text{Fc}\epsilon\text{RI}$ at late stage of this clone expressed at a lower density was only slightly down-regulated by RA. However, it is apparent from these graphs (Figs. 23, 24) that at 1 wk of modulation, both types of IgE receptors were markedly down-regulated. Somewhat different modulation results were obtained with clone RCMC1.3.3, on which both receptors at the early stage were drastically decreased by RA (Fig. 25a). $\text{Fc}\epsilon\text{R}_\text{L}$ at the late stage, expressed at a relatively higher density, was also down-regulated by RA but at a slower rate and $\text{Fc}\epsilon\text{RI}$, modulated

Fig. 23. Retinoic acid modulation of $Fc\epsilon R$ expression on RCMC1.3.1 (a) at early stage and (b) at late stage.

Fig. 23

a)



b)

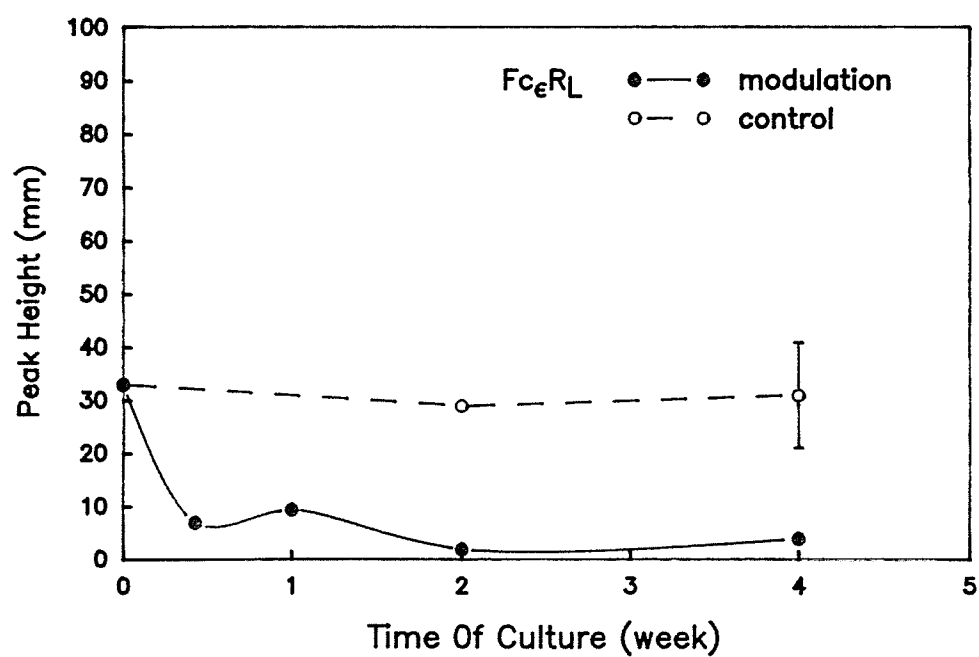
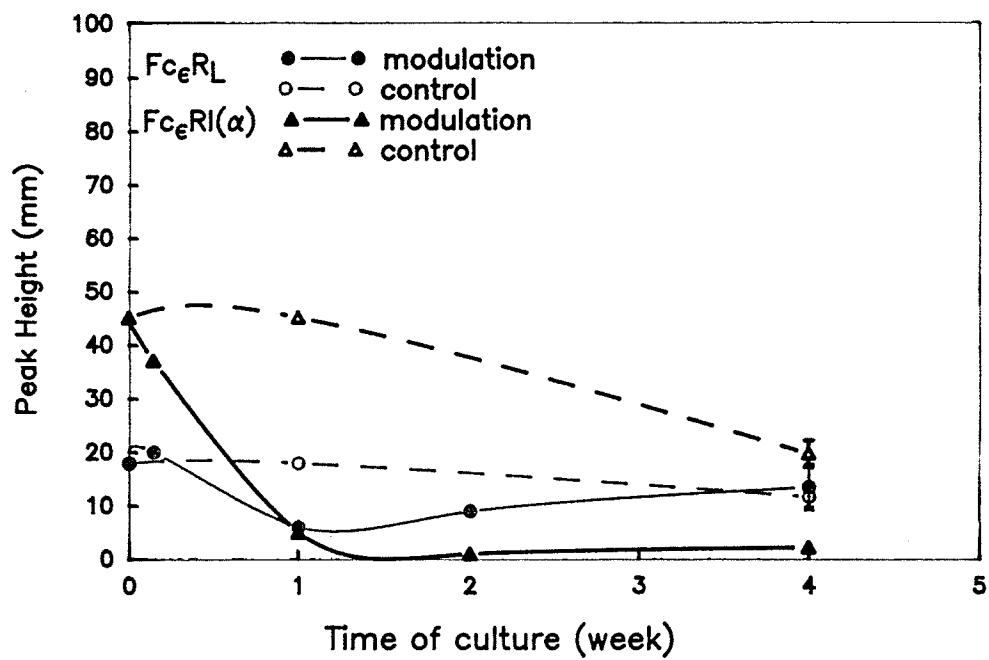


Fig. 24. Retinoic acid modulation of $\text{Fc}\epsilon\text{R}$ expression on RCMC1.3.2 (a) at early stage and (b) at late stage.

Fig. 24

a)



b)

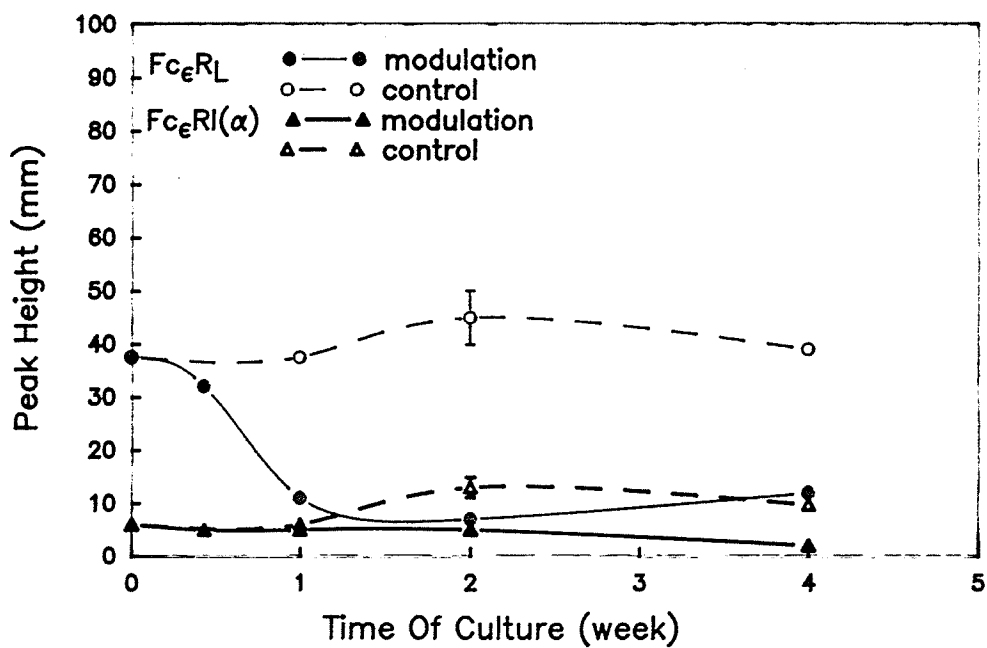
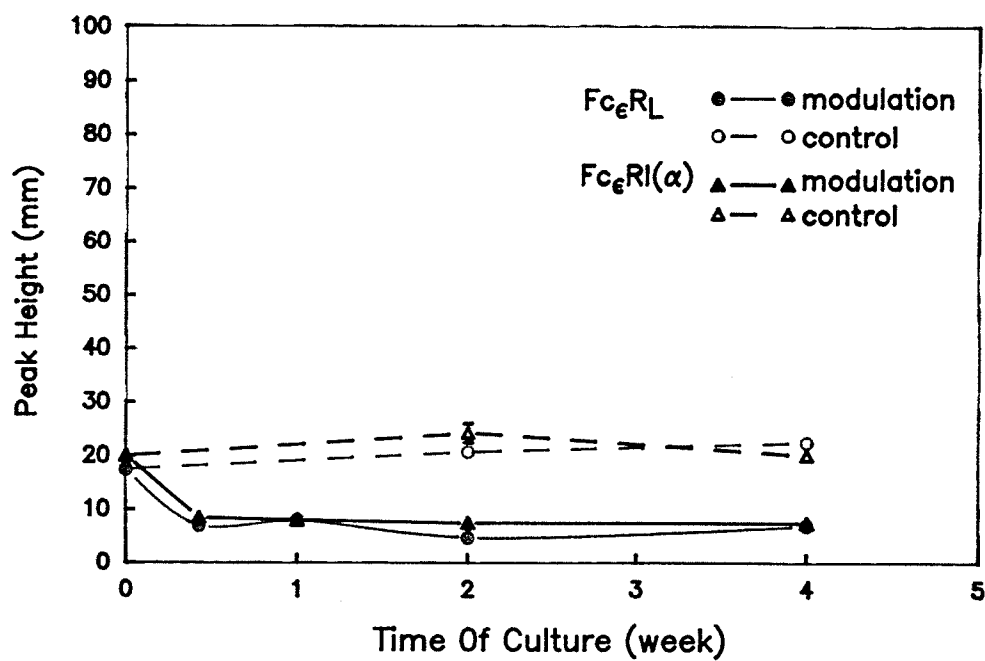


Fig. 25. Retinoic acid modulation of Fc ϵ R expression on RCMC1.3.3 (a) at early stage and (b) at late stage.

Fig. 25

a)



b)

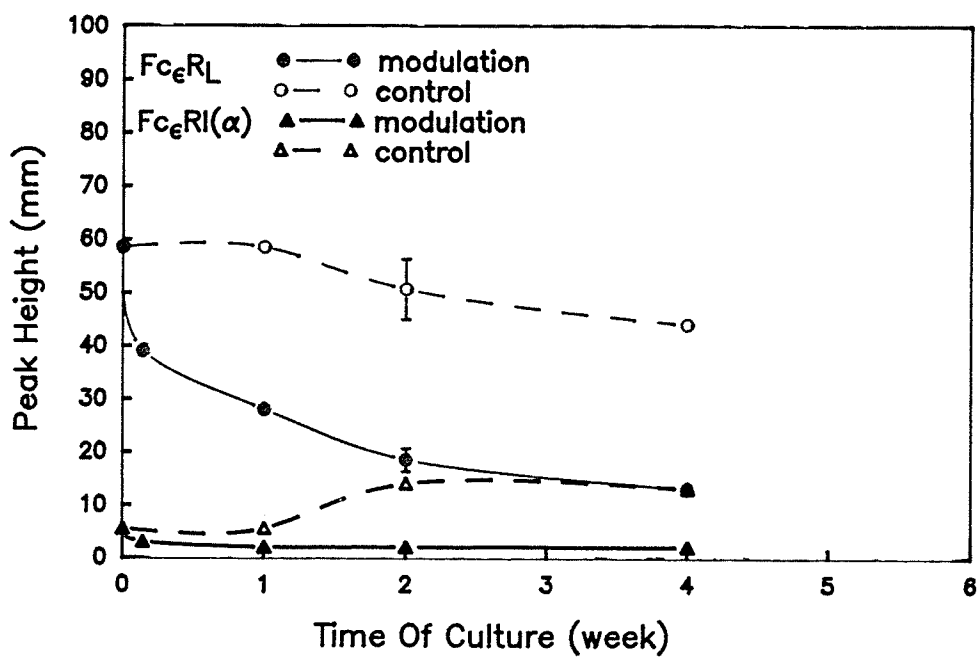
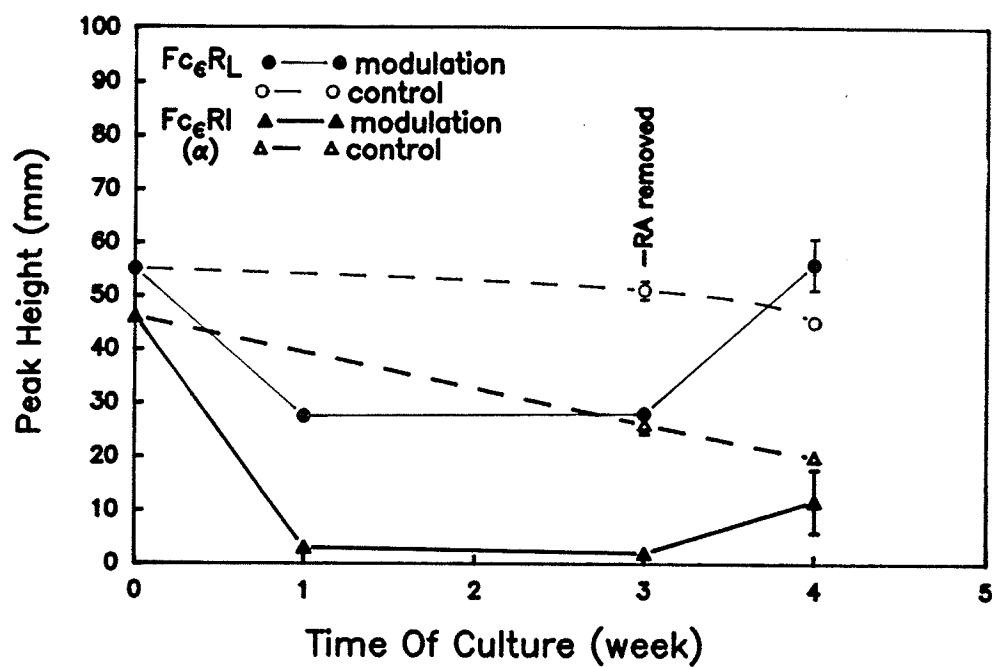


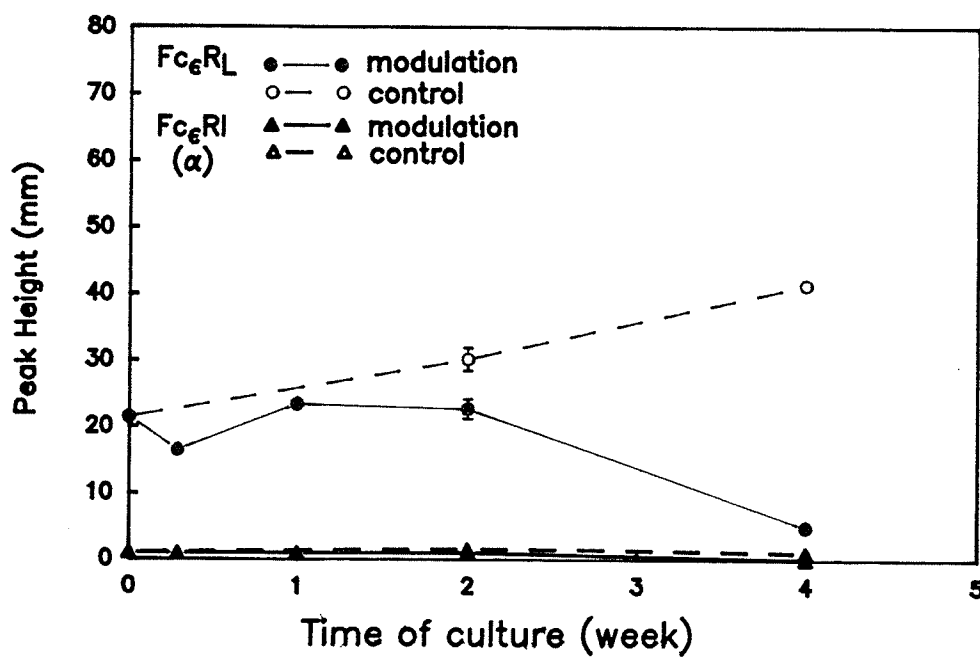
Fig. 26. Retinoic acid modulation of $\text{Fc}\epsilon\text{R}$ expression on RCMC1.11.2 (a) at early stage and (b) at late stage. In Fig. 26b RA was removed from culture medium at 3 wk modulation and the cells were cultured in the absence of RA for another week.

Fig. 26

a)



b)



at this stage, stayed at a very low density while the control $\text{Fc}_\epsilon\text{RI}$ appeared to increase slightly (Fig. 25b). Clone RCMC1.11.2 at the early stage, showed a drastic down-regulation of both $\text{Fc}_\epsilon\text{RI}$ and $\text{Fc}_\epsilon\text{R}_\text{L}$ after 1 wk of RA modulation (Fig. 26a), but at the late stage its $\text{Fc}_\epsilon\text{R}_\text{L}$ surface expression persisted at near control levels for two weeks of RA modulation and then decreased quickly to a low level (Fig. 26b). After removing the RA from the tissue culture, the effect of RA was reversed, as both $\text{Fc}_\epsilon\text{RI}$ and $\text{Fc}_\epsilon\text{R}_\text{L}$ of RCMC1.11.2 near returned to about control levels (Fig. 26a).

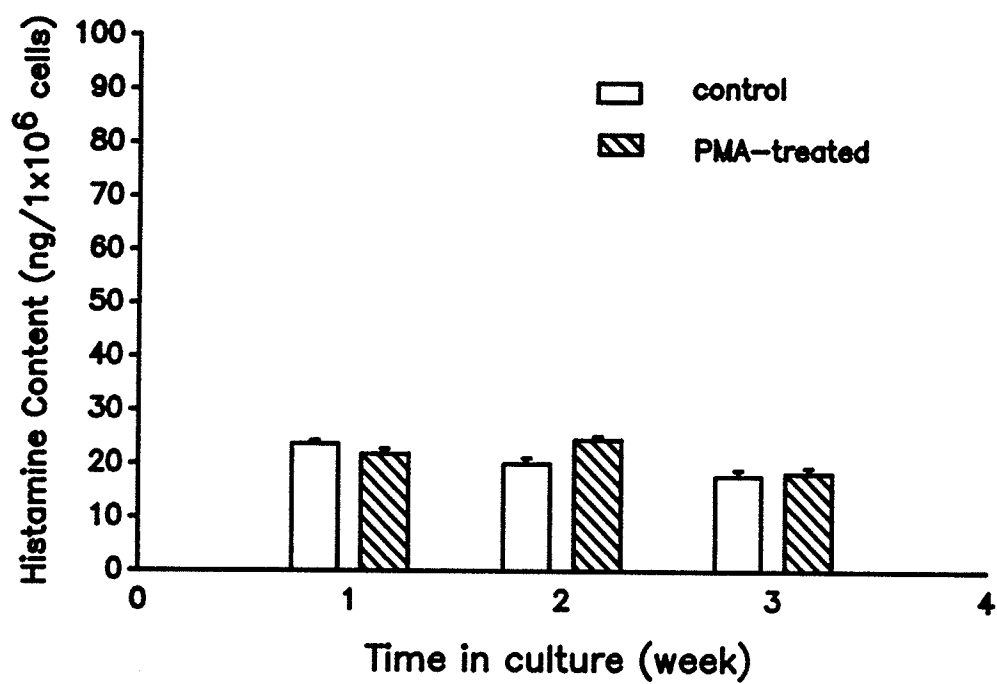
Effects of differentiation agents on histamine content:

The histamine content of the modulated RCMC clones were determined in the hope that they might reflect a change, if any, in mast cell differentiation status under the modulation of the differentiation agents. PMA, which has been found to induce differentiation in a human monoblast cell line U937 (Hsu and Hsu, 1985), did not seem to affect the histamine content of some of the RCMC clones but increased the histamine content in others. Two clones, RCMC1.3.1 expressing $\text{Fc}_\epsilon\text{R}_\text{L}$ only and RCMC1.11.2 expressing a relatively stable $\text{Fc}_\epsilon\text{R}_\text{L}$ and a decreasing $\text{Fc}_\epsilon\text{RI}(\alpha)$, did not show any changes in histamine content during a period of 2-3 weeks of PMA modulation (RCMC1.3.1 shown in Fig. 27a, b; RCMC1.11.2 shown in Fig. 28a, b). The other two clones, RCMC1.3.2 and 1.3.3, responded to PMA with an increase in histamine content. By 1 week, PMA modulation significantly increased the histamine content of early stage RCMC1.3.2 while the control histamine levels stayed relatively constant (Fig. 29a). In the case of cells at the late stage, an increase in histamine was observed only in the third week of PMA modulation (Fig. 29b). Both early (Fig. 30a)

Fig. 27. The effect of PMA on histamine content of clone RCMC1.3.1 (a) at early stage and (b) at late stage. PMA of 1 ng/ml was used for modulation studies.

Fig. 27

a)



b)

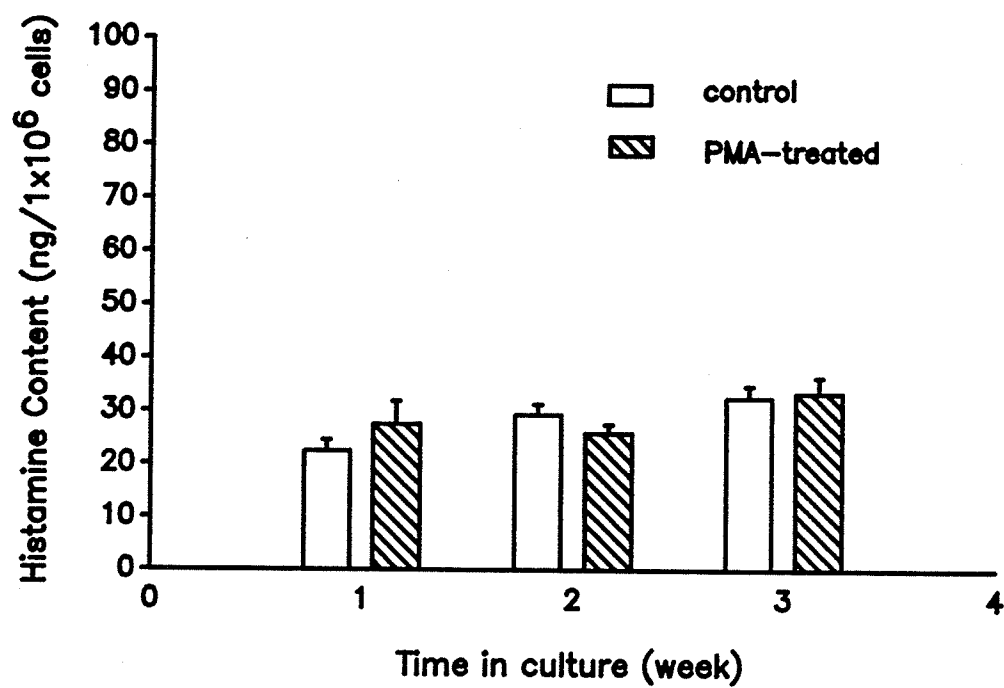
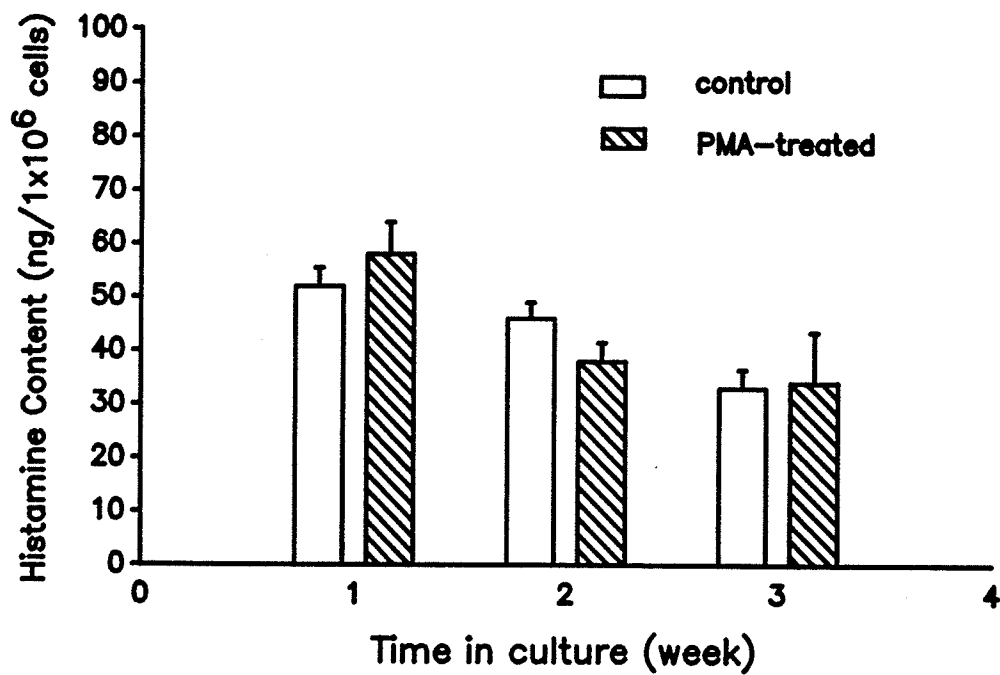


Fig. 28. The effect of PMA on histamine content of clone RCMC1.11.2 (a) at early stage and (b) at late stage.

Fig. 28

a)



b)

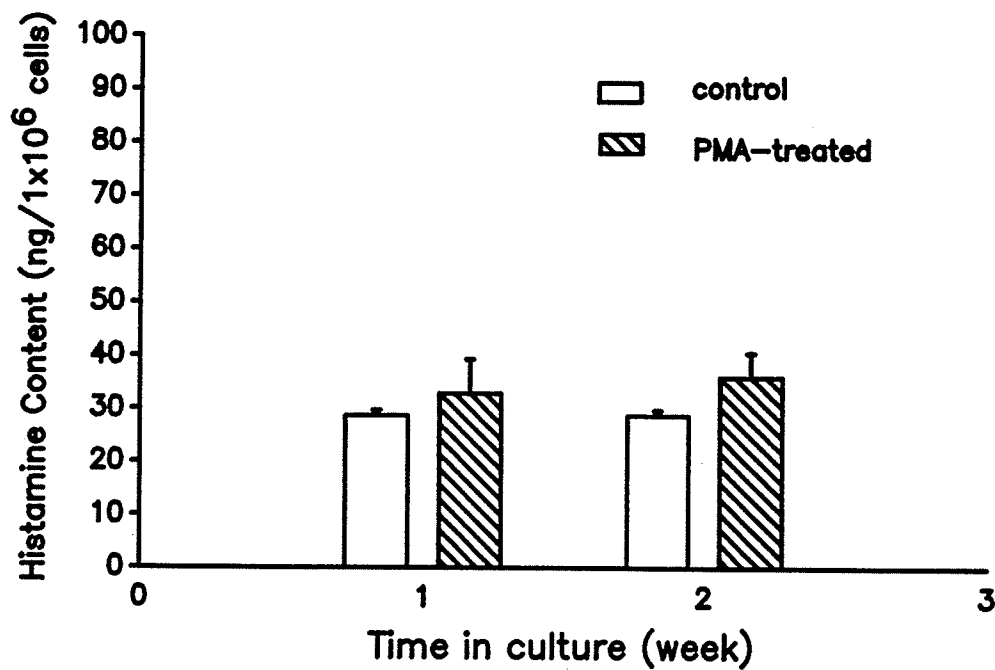
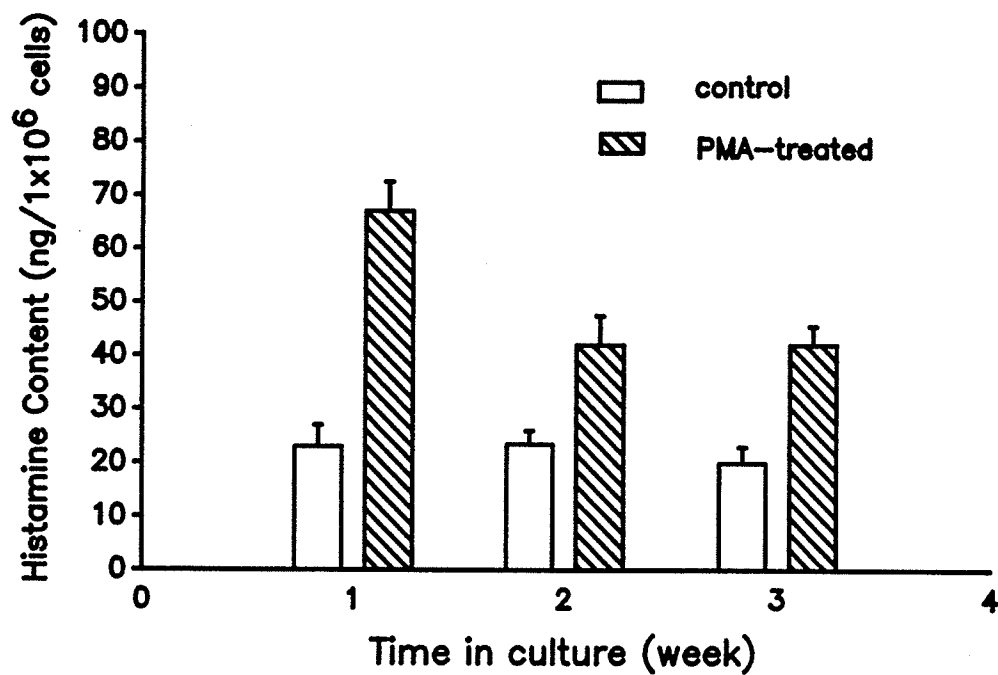


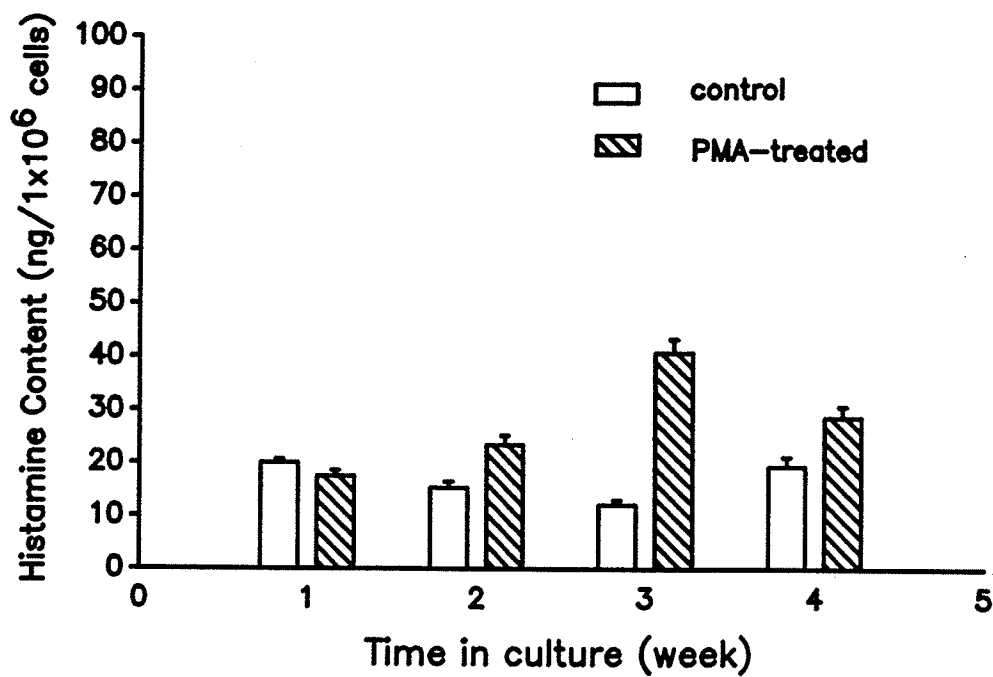
Fig. 29. The effect of PMA on histamine content of clone RCMC1.3.2 (a) at early stage and (b) at late stage.

Fig. 29

a)



b)



and late stage RCMC1.3.3 (Fig. 30b) showed significant increases in histamine content during the period of 3-4 weeks of PMA modulation. Furthermore, the increased histamine content was found to return to control level after 2 weeks of continuous culture in the absence of PMA (Fig. 30a).

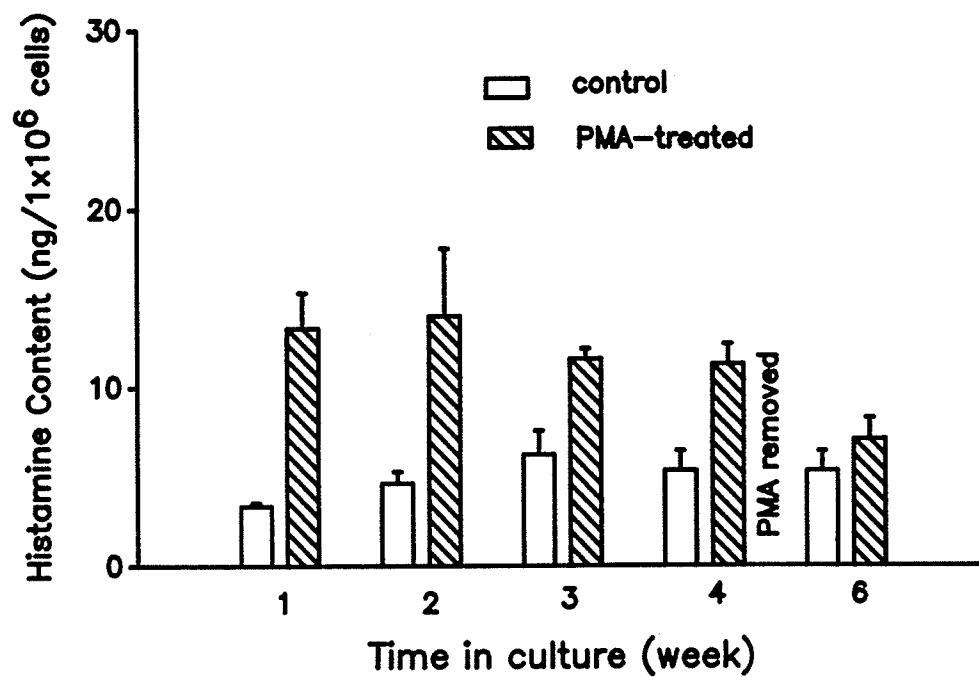
Dexamethasone has been shown previously to up-regulate histamine content in mouse BMMC and induce a phenotypic change from MMC-like cells to CTMC (Pitton et al., 1988). Modulation of RCMC1.3.1 with dexamethasone increased the histamine content of the early stage cells (Fig. 31a) but did not significantly affect the late stage cells (Fig. 31b), although after the third week of modulation, the histamine content appeared to have increased but it was within the two-sigma range of statistical analysis. A gradual increase in histamine content was observed on dexamethasone modulation of clone RCMC1.3.2 at early (Fig. 32a) and late stages (Fig. 32b). Clone RCMC1.3.3 showed a similar response to DM as RCMC1.3.2 in that the histamine content of the cells at early and late stages was gradually up-regulated (Fig. 33a, b). DM modulation of early RCMC1.11.2 generally increased the histamine content as shown in Fig. 34a, although at week 2 the effect was not significant. Late RCMC1.11.2 showed an initial high increase in histamine content followed by a lower level of increase at weeks 3 and 4 (Fig. 34b).

In addition to the extensive application of RA on monocytic lineage cells, RA has also been used to regulate mouse mast cell growth and differentiation (Arock et al., 1988). RA treatment of RCMC clones generally induced a down-regulation of histamine content in all the clones studied. Early and late stage RCMC1.3.1 (Fig. 35a, b), 1.3.2 (Fig. 36a, b), and 1.3.3 (Fig. 37a, b) showed an immediate decrease

Fig. 30. The effect of PMA on histamine content of clone RCMC1.3.3 (a) at early stage and (b) at late stage. PMA was removed from culture medium at 4 wk modulation and the modulated cells were cultured for two weeks in the absence of PMA.

Fig. 30

a)



b)

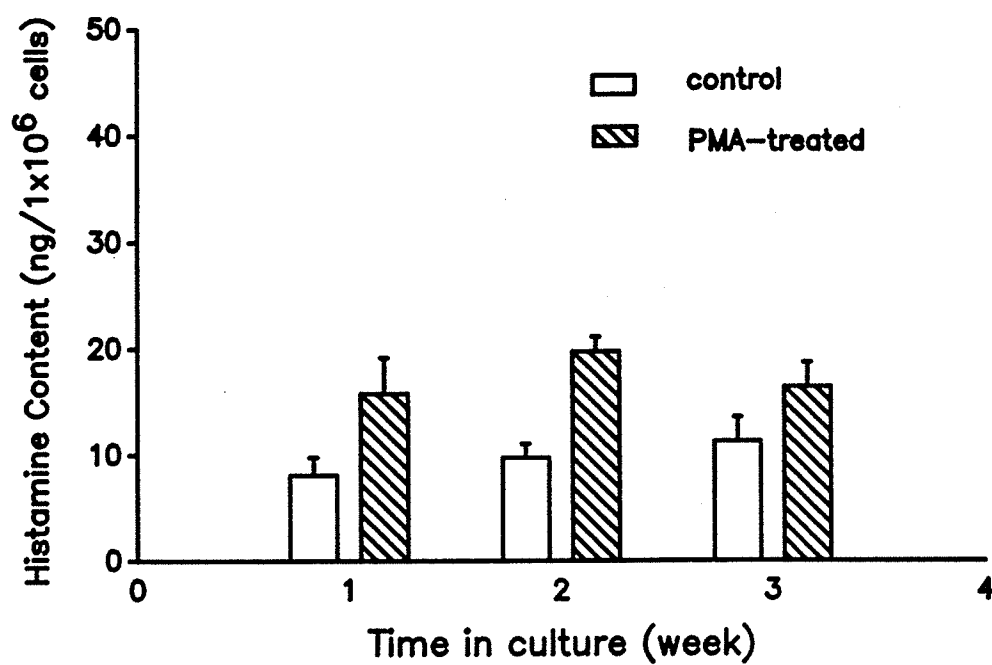
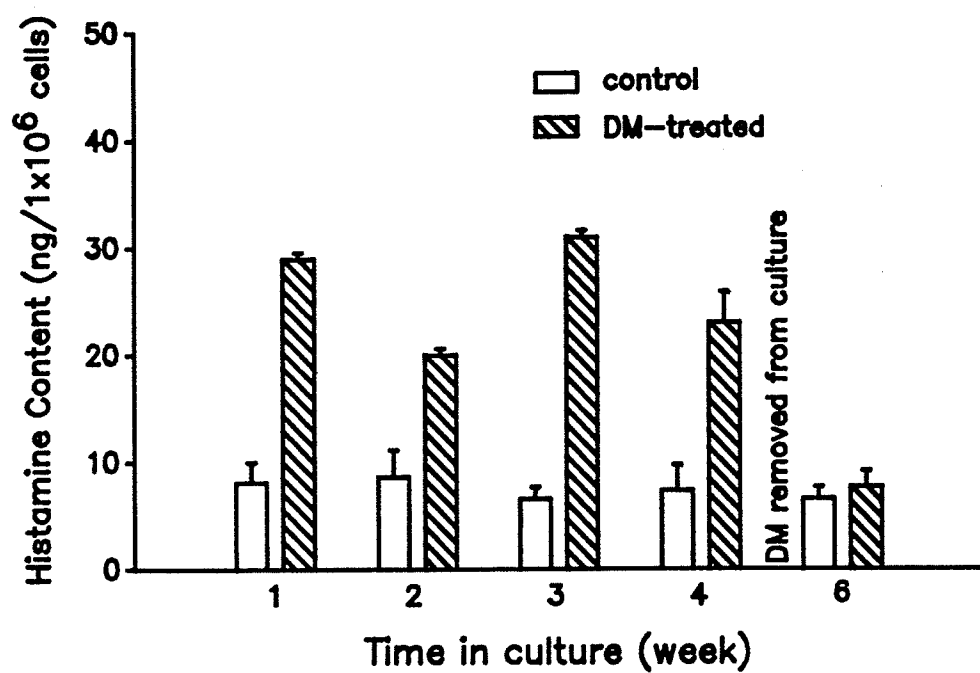


Fig. 31. The effect of dexamethasone on histamine content of clone RCMC1.3.1 (a) at early stage and (b) at late stage. In Fig. 31a DM was removed from culture medium at week 4 modulation and the modulated cells were cultured in the absence of DM for two weeks.

Fig. 31

a)



b)

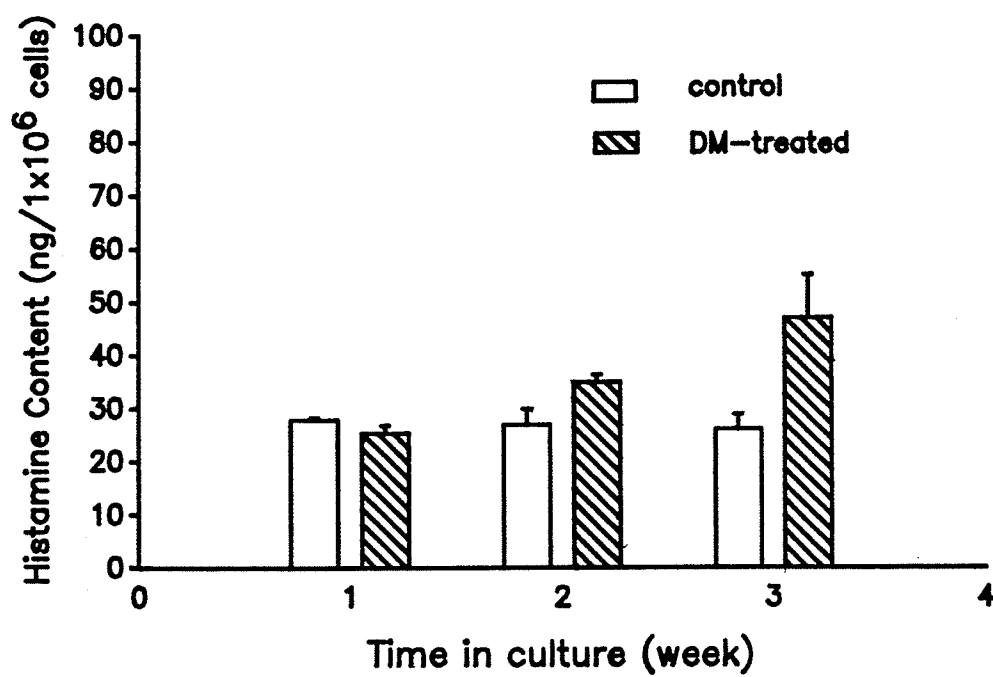
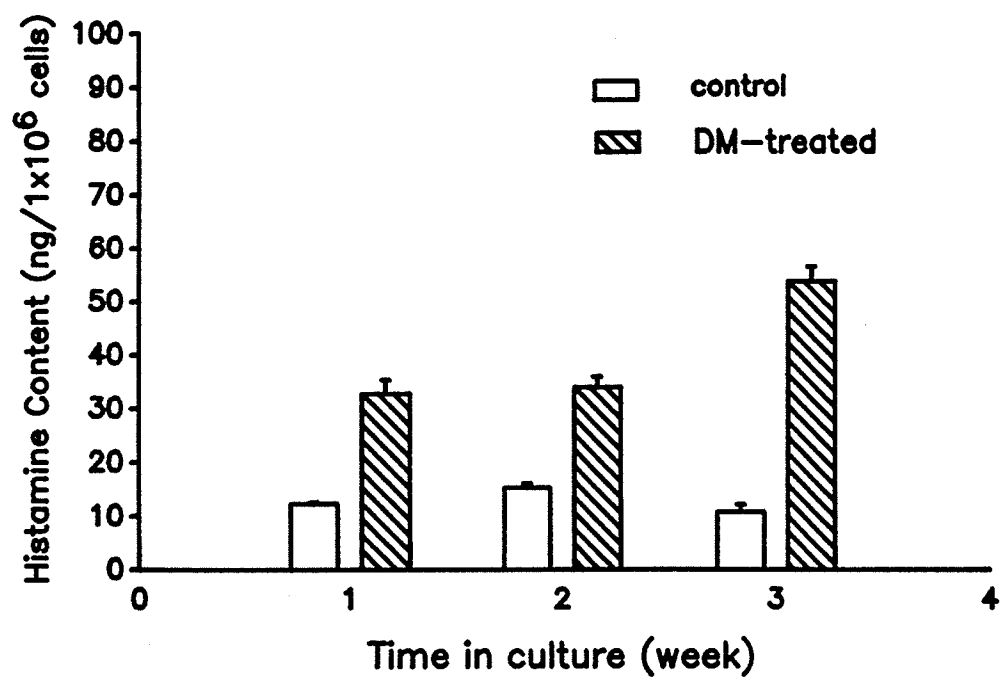


Fig. 32. The effect of dexamethasone on histamine content of clone RCMC1.3.2 (a) at early stage and (b) at late stage.

Fig. 32

a)



b)

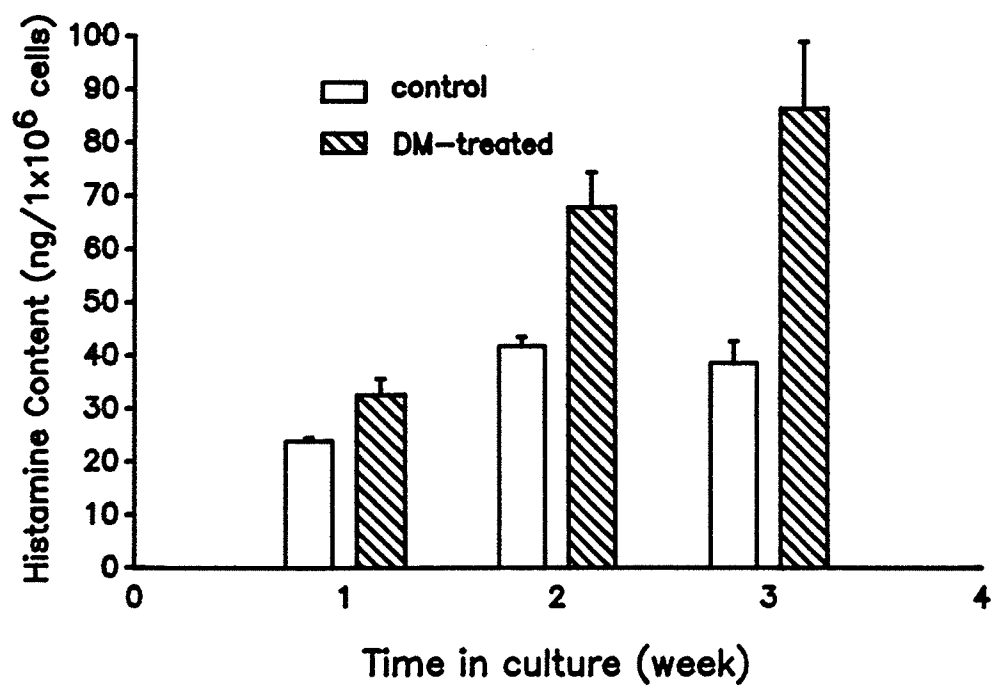
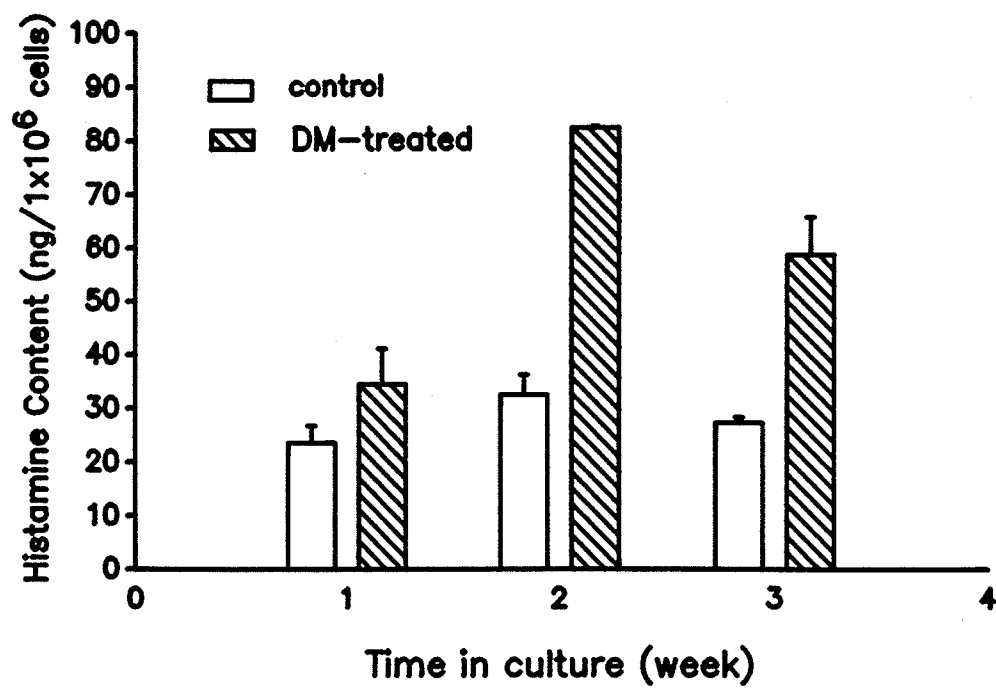


Fig. 33. The effect of dexamethasone on histamine content of clone RCMC1.3.3 (a) at early stage and (b) at late stage.

Fig. 33

a)



b)

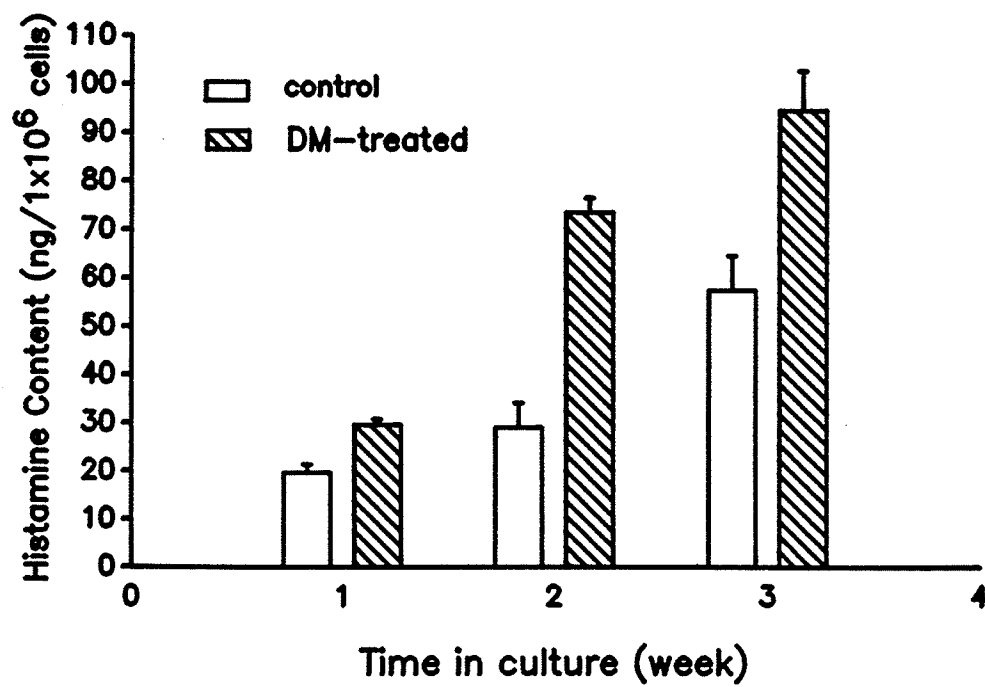
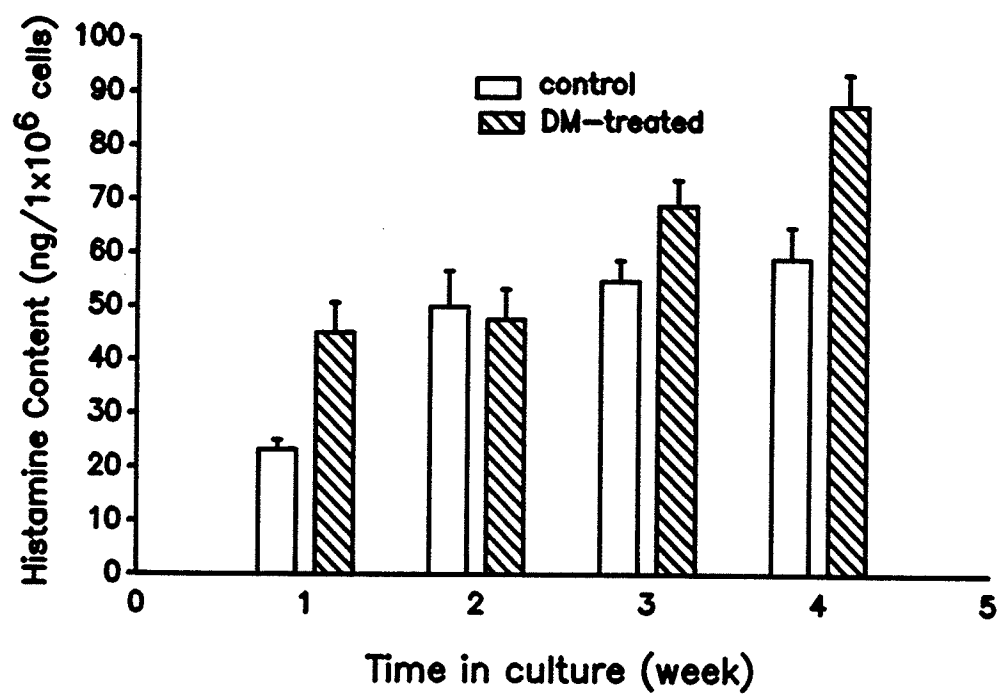


Fig. 34. The effect of dexamethasone on histamine content of clone RCMC1.11.2 (a) at early stage and (b) at late stage.

Fig. 34

a)



b)

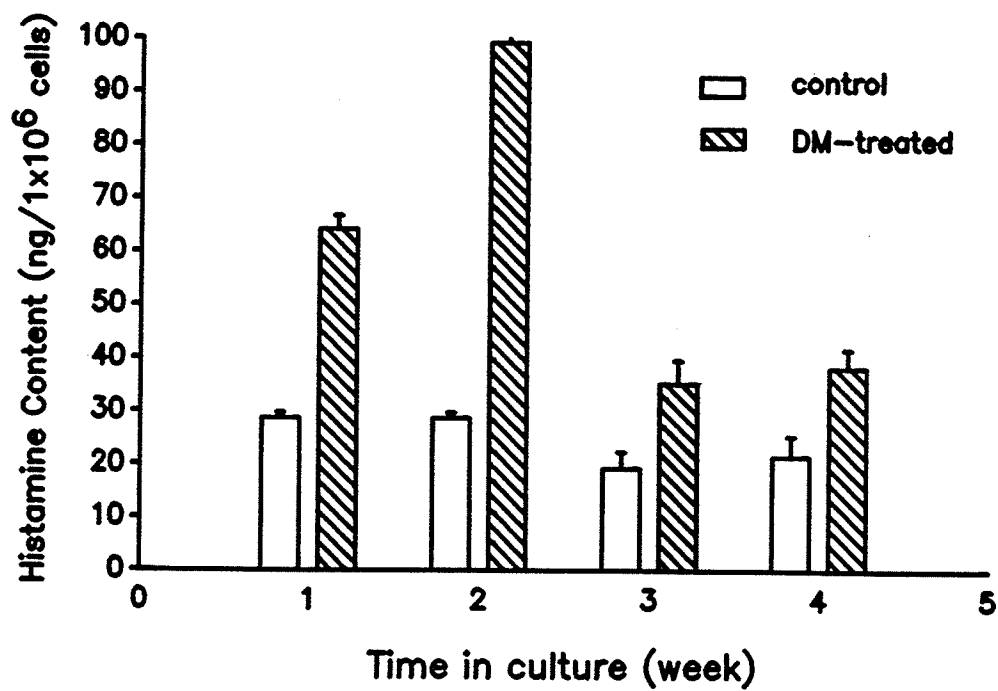
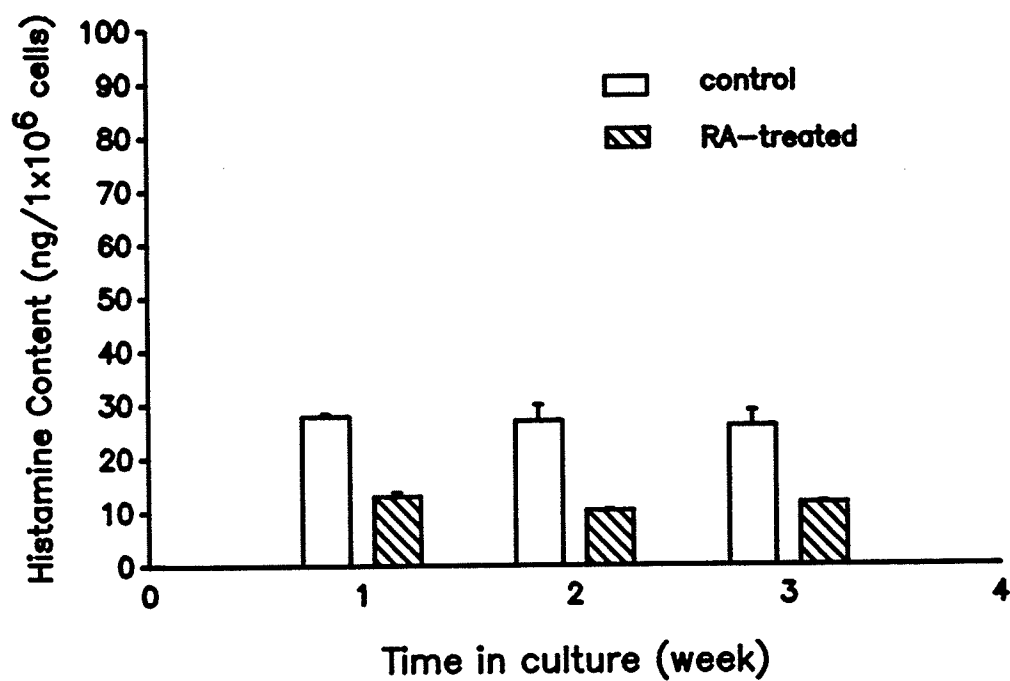


Fig. 35. The effect of retinoic acid on histamine content of clone RCMC1.3.1 (a) at early stage and (b) at late stage.

Fig. 35

a)



b)

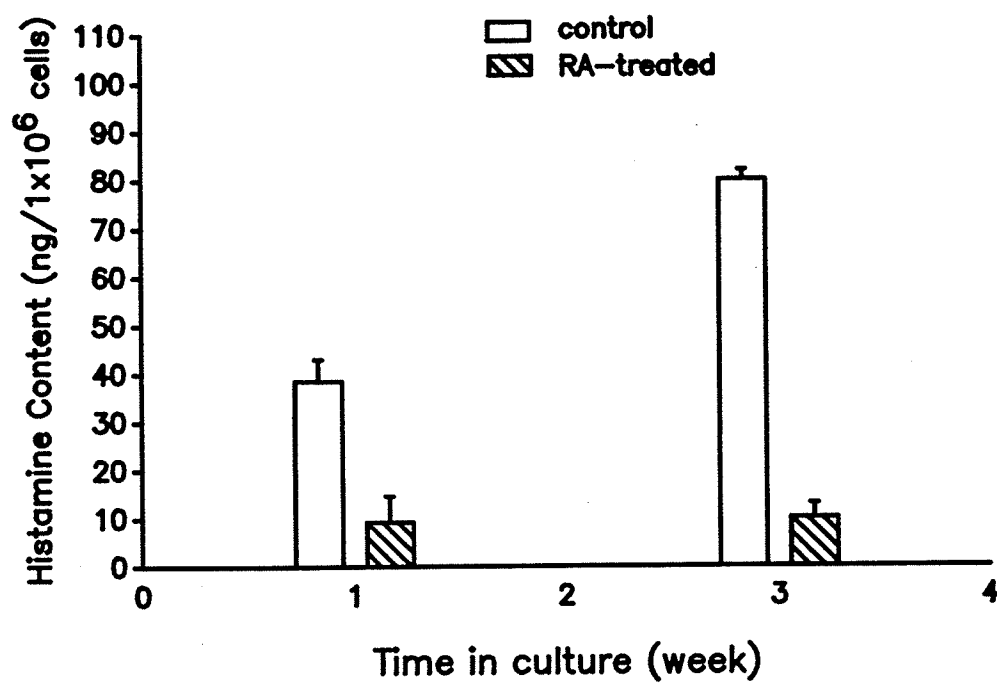
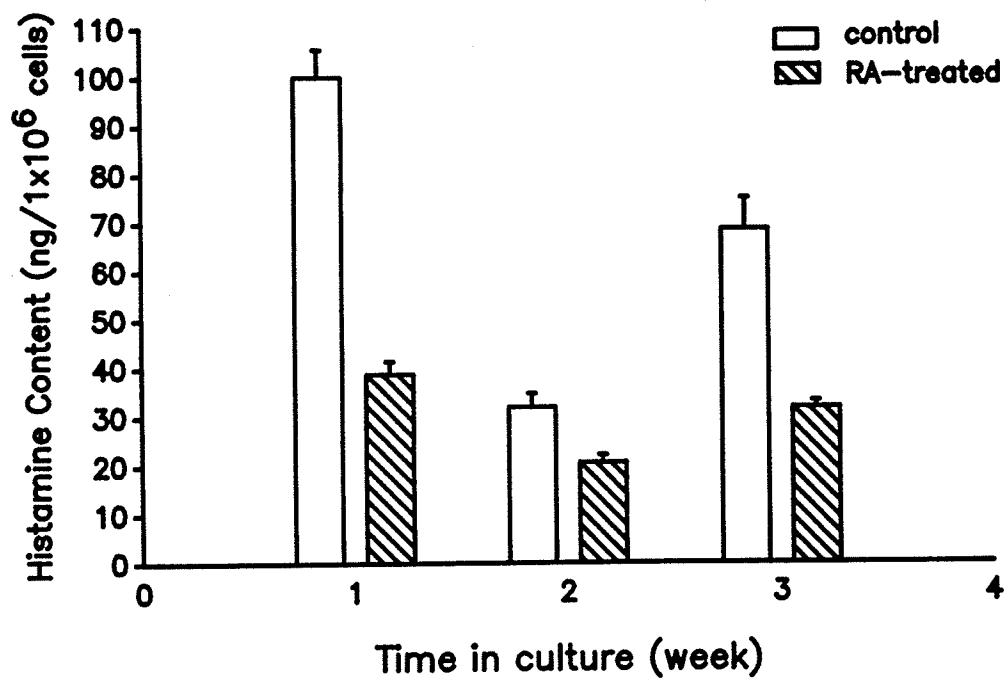


Fig. 36. The effect of retinoic acid on histamine content of clone RCMC1.3.2 (a) at early stage and (b) at late stage.

Fig. 36

a)



b)

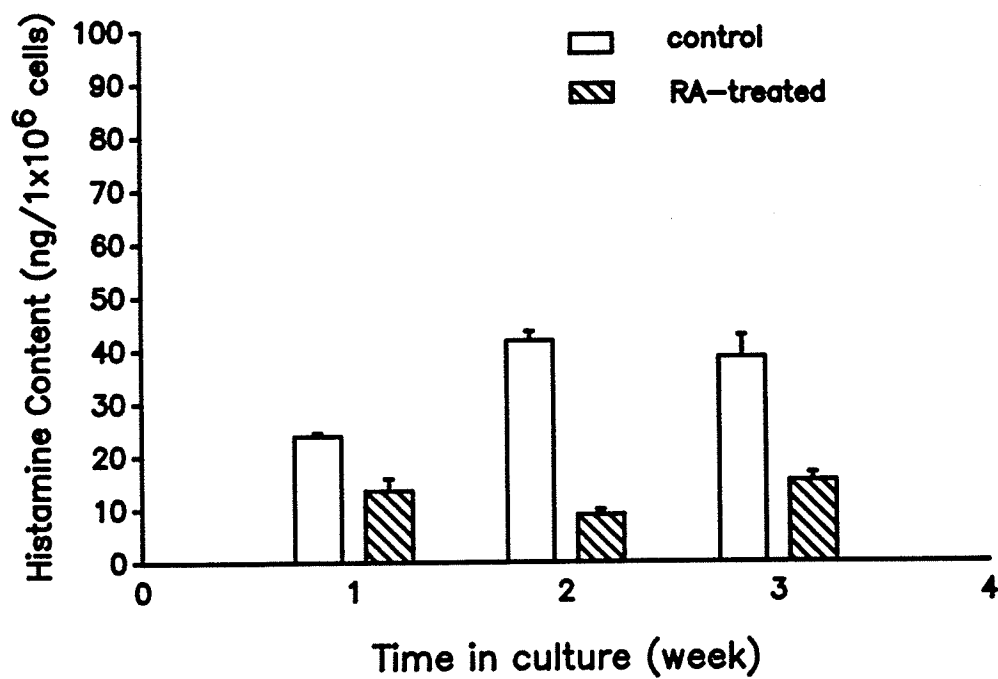
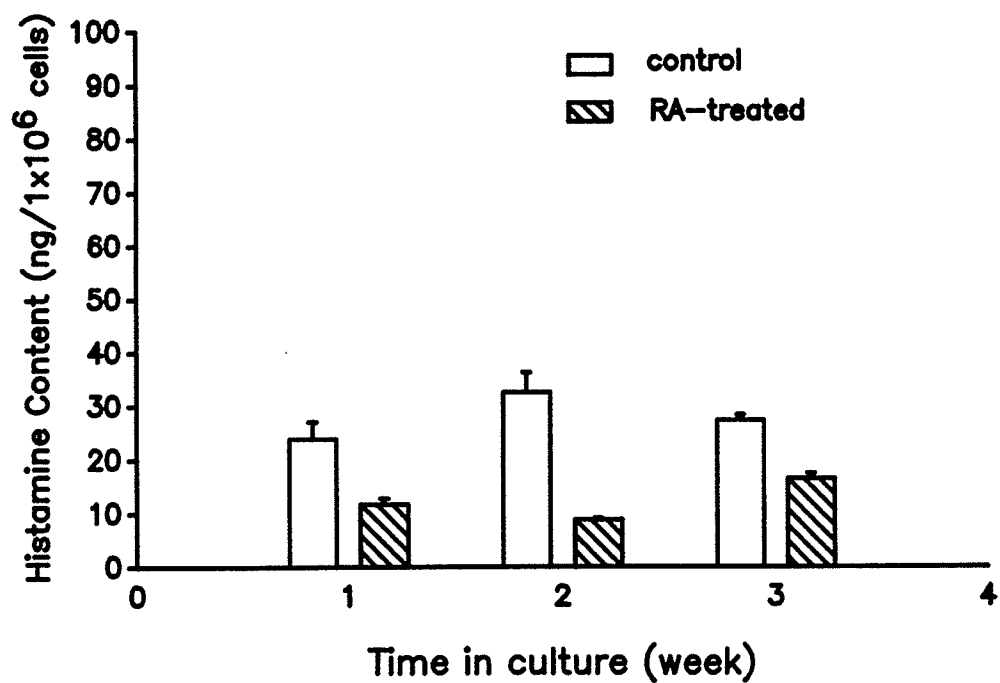


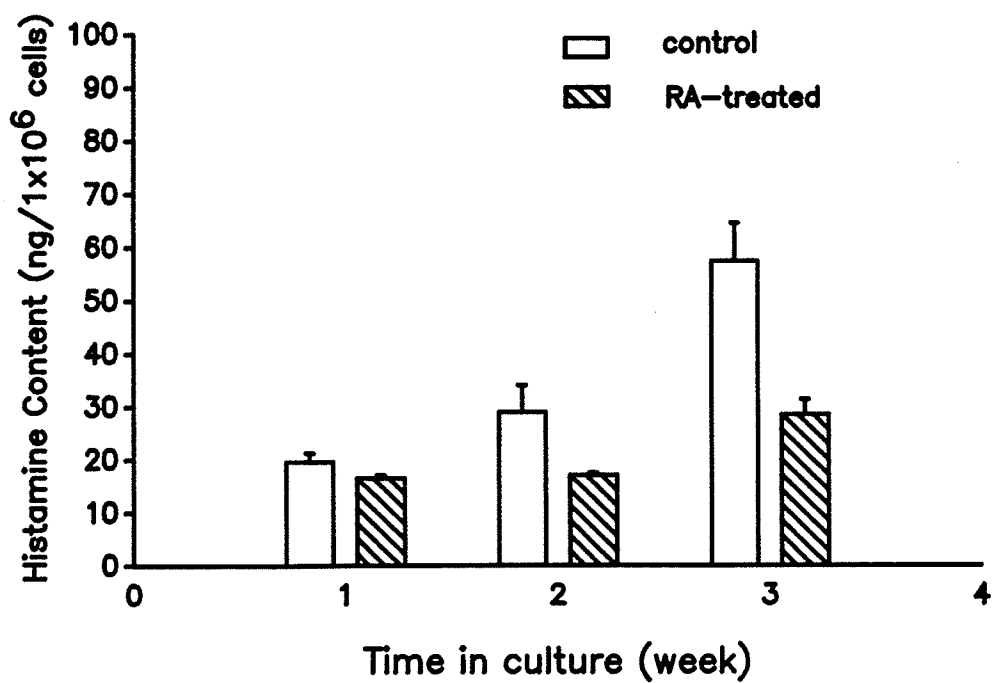
Fig. 37. The effect of retinoic acid on histamine content of clone RCMC1.3.3 (a) at early stage and (b) at late stage.

Fig. 37

a)



b)



in histamine content upon RA modulation. Only early RCMC1.11.2 exhibited a more gradual decrease in histamine content (Fig. 38a) upon RA modulation, and the late stage cells of this clone yielded a quick decrease in histamine content like the other clones (Fig. 38b). Despite the differences in the control levels of histamine, the modulated histamine level stayed at approximately 10ng/10⁶ cells for all the clones at both stages except the early stage RCMC1.3.2 and 1.11.2. This may reflect background fluorescence since this histamine level was very close to the lowest limit of this assay. The reversibility of RA effect on histamine content was examined on early RCMC1.11.2. As shown in Figure 38a, the decreased histamine content returned to the control level after one week continuous culture in the absence of RA.

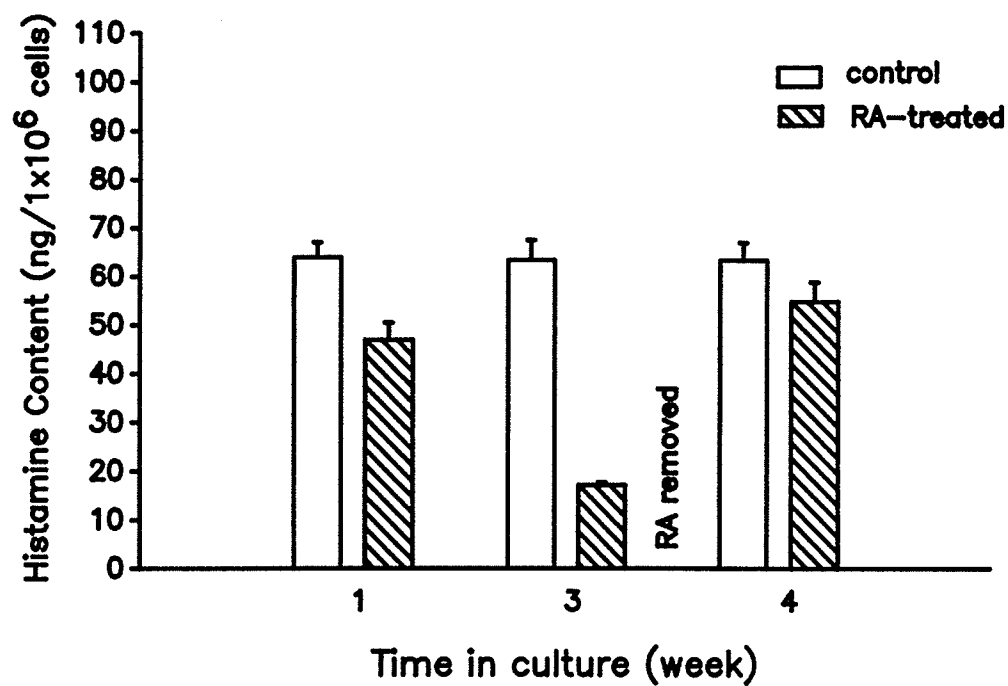
Changes in cell growth rate of the RCMC clones:

Most of the differentiation agents are known to regulate cell growth in addition to their differentiation-inducing effect. The growth rate of the RCMC clones in the presence of modulators was examined in order to study whether the changes in growth rate were related to the modulatory effects on receptor expression and histamine content. The proliferation assays on clones RCMC1.3.1, 1.3.2 and 1.11.2, which revealed the most differential response in the modulation studies, demonstrated that both DM and RA generally reduced the growth rates while PMA did not seem to change the cell proliferation significantly (Fig. 39). Although the percentage of growth inhibition varied slightly among the three clones, each differentiation reagent had similar effect on all the three clones studied.

Fig. 38. The effect of retinoic acid on histamine content of clone RCMC1.11.2 (a) at early stage and (b) at late stage. In Fig. 38a RA was removed from culture medium at week 3 modulation and the modulated cells were cultured for one more week in the absence of RA.

Fig. 38

a)



b)

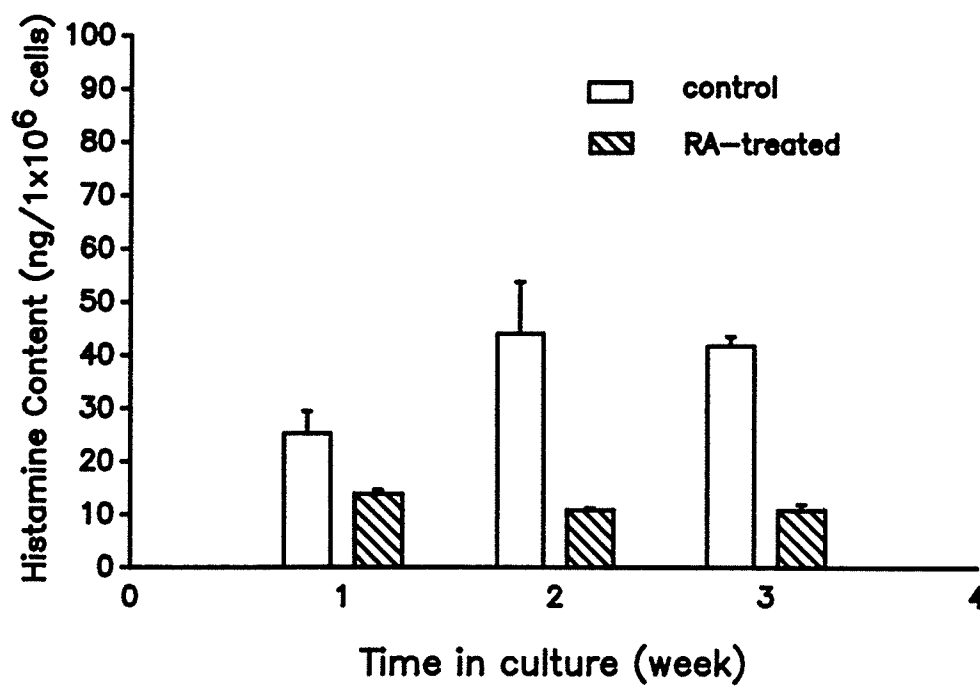
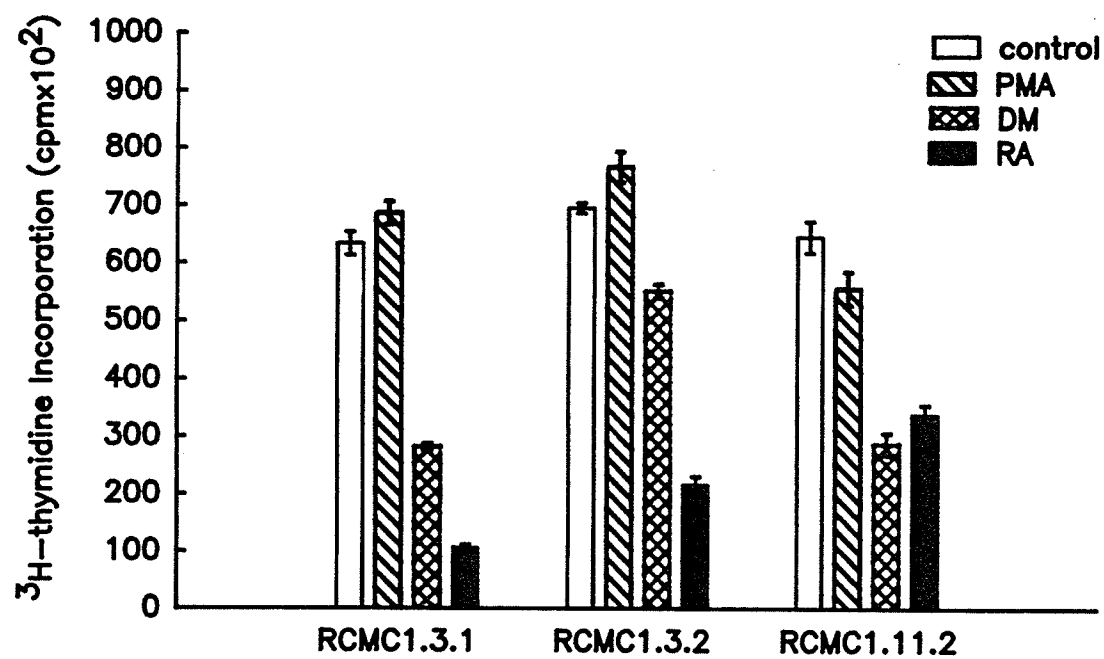


Fig. 39. The growth rates of RCMC clones in the presence of the differentiation agents, PMA, DM and RA. [^3H]-thymidine incorporation was measured after the cells had been cultured in the differentiation agents for one week.

Fig. 39



Protein kinase C activity:

To determine whether the effect of PMA is mediated through the activation of intracellular protein kinase C, the modulated cells were analyzed for PKC activity. It was found that prolonged culture of RCMC1.3.2 in the presence of 1 ng/ml PMA had induced a gradual accumulation of cytosolic PKC activity in these cells (Fig. 40), which reached a plateau after 3 weeks of modulation. To further determine if the increased PKC activity actually mediated PMA effects on receptor expression and histamine content it was subsequently examined by adding 10 μ M 1-(5-Isoquinolinsulfonyl)-2-Methyl-piperazine (H-7), a PKC inhibitor, to PMA-containing cell cultures. The results (Fig. 41) show that this inhibitor did not affect the induction of PKC activity by PMA. However, the PMA-mediated increases in $Fc\epsilon R_L$ expression (Fig. 42) and histamine content (Fig. 43) were abrogated. Both the receptor expression and the histamine content of RCMC1.3.2 in the presence of PMA and H-7 were comparable to the control levels. In Fig. 43 at wk2 and wk3 PMA did not significantly increase the histamine, therefore, no inhibitory effect by H-7 can be expected. The inhibitory effect on the PMA-enhanced histamine content was also demonstrated with using 100 nM staurosporin, a more potent PKC inhibitor (data not shown). When the basal PKC activities of the RCMC clones studied were compared with the histamine-releasing basophilic leukemia cell line RBL-2H3, it became apparent that the cytosolic PKC activities of the RCMC clones were markedly lower than those of RBL-2H3 (Fig. 44).

3 H-serotonin release:

Functionally active mast cells can incorporate 3 H-serotonin into the granules from the extracellular media and release it upon immunological stimulations. The cell line

Fig. 40. The protein kinase C activities in the cytosol and membrane of RCMC1.3.2 at early stage when cultured in culture medium (control) and in the presence of PMA in the culture medium (modulation).

Fig. 41. The protein kinase C activities in the cytosol and membrane of RCMC1.3.2 at early stage when cultured in culture medium (control), PMA and PMA with a PKC inhibitor, H-7.

Fig. 40

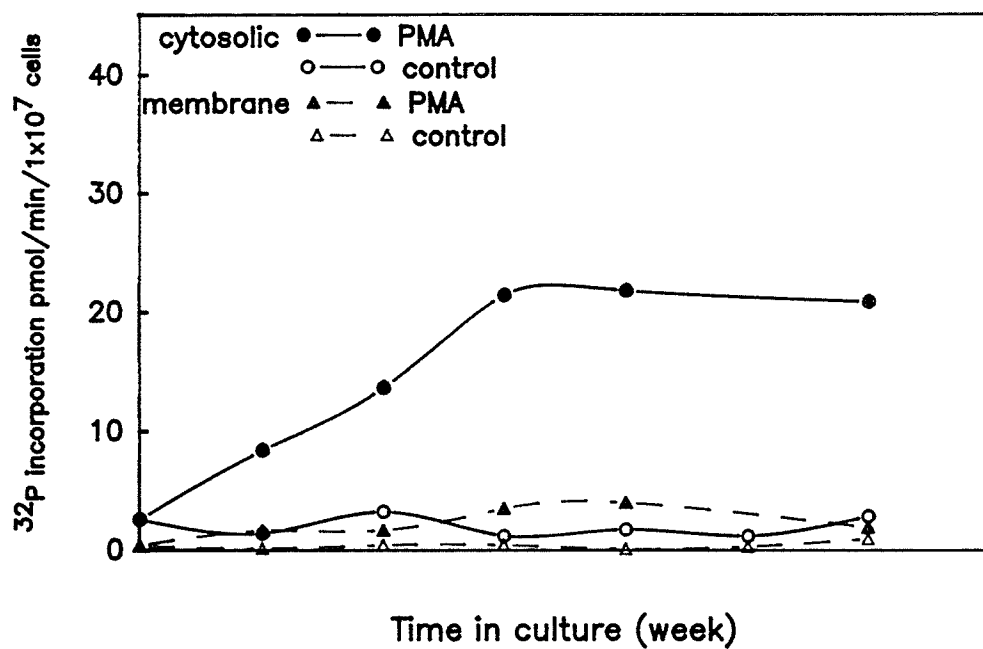


Fig. 41

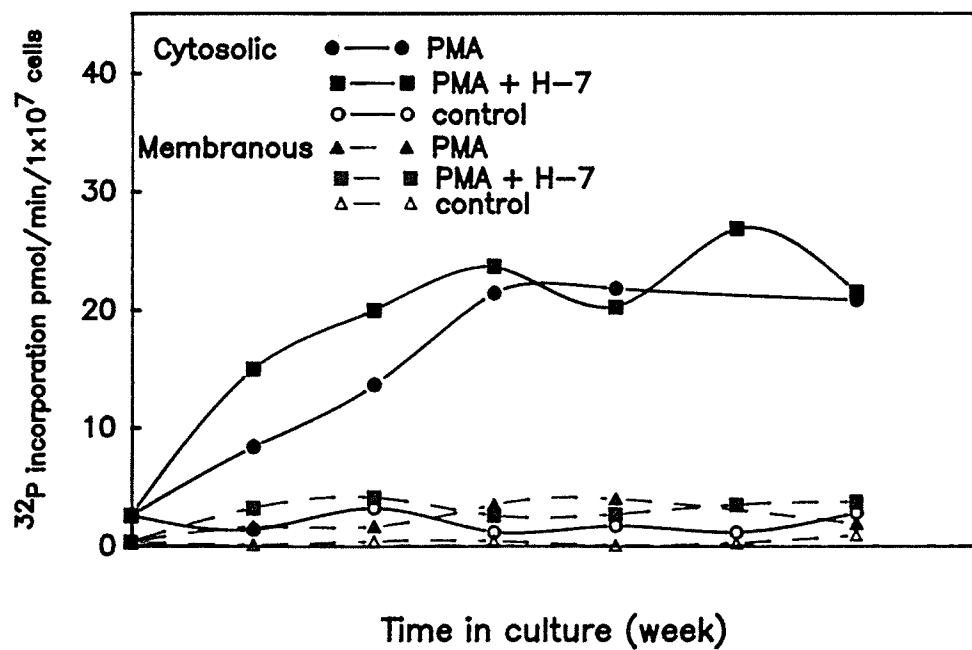


Fig. 42. The effect of PMA and PMA with H-7 on the expression of $Fc\epsilon Rs$ of RCMC1.3.2 at early stage.

Fig. 43. The effect of PMA and PMA with H-7 on histamine content of RCMC1.3.2 at early stage.

Fig. 42

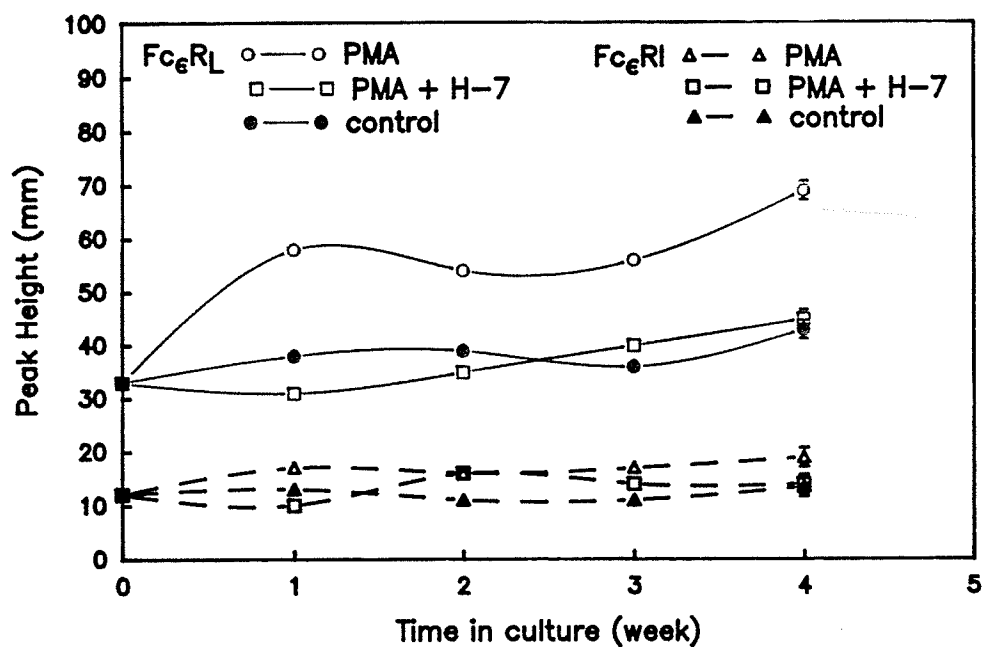


Fig. 43

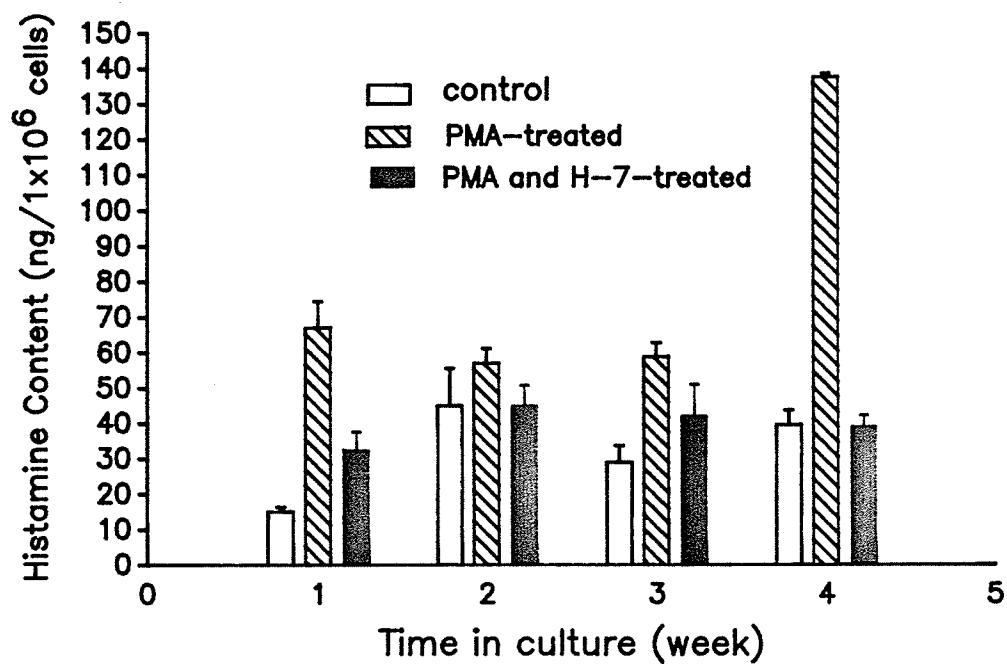


Fig. 44. Protein kinase C activities in the cytosol and membrane of the RCMC clones and the cell line RBL-2H3.

Fig. 44

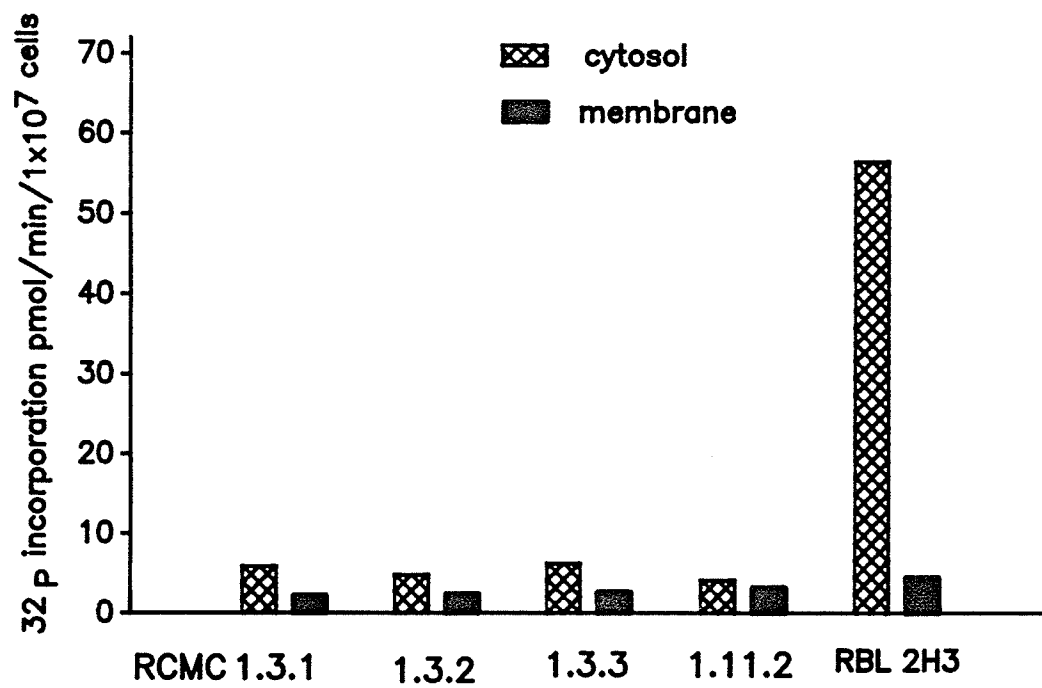


Table 1 ^3H -Serotonin Release of RCMC Triggered by DNP-IgE and DNP₁₇-BSA

| DNP ₁₇ -BSA μg/ml | RCMC1 | RCMC1.2.5 | RCMC1.3.2 | RCMC1.9.3 | RCMC1.11.2 |
|---------------------------------|-------|-----------|-----------|-----------|------------|
| | cpm | cpm | cpm | cpm | cpm |
| 10 | 9058 | 10423 | 1698 | 9697 | 7192 |
| 1 | 7349 | 9807 | 2268 | 7390 | 8509 |
| 0.1 | 7337 | 8982 | 2096 | 5807 | 7834 |
| 0.01 | 6173 | 7178 | 1980 | 4303 | 9800 |
| 0.001 | 8460 | 6297 | 1575 | 7205 | 6240 |
| 0.0001 | 9197 | 12785 | 1305 | 7111 | 8897 |
| 0 | 8452 | 11821 | 2204 | 7488 | 8713 |
| uptake | 44342 | 54179 | 12170 | 80365 | 60607 |

RCMC1 and some of its clones were assayed for serotonin release. The cells were found to be capable of incorporating serotonin which is shown as the uptake, however, neither RCMC1 nor its clones were able to actively release it upon stimulation with DNP-specific IgE and DNP₁₇-BSA (Table 1). The table merely indicates the spontaneous releases of the tested cells, ranging from 5% to 24%. The degranulation of mast cells by means of antigen-antibody reaction is the result of the influx of calcium into mast cells following a change in the calcium permeability of the cell membrane induced by the surface signal. Calcium ionophores can bypass the surface signal and selectively increase the permeability of the cell membranes to calcium, leading to the secretory process. However, RCMC1 and some of the clones again failed to release serotonin when triggered by calcium ionophore A23187 (data not shown).

Histochemical staining:

Characterization of RCMC lines and clones has shown that RCMC, although derived from rat PMC, are MMC-like, stain Alcian blue⁺/Safranin O⁻ and have a histamine content comparable to bone marrow-derived MMC (Chan et al., 1988; 1990). Classically, the histamine content has been used as an indicator of mast cell maturity and differentiation and in the case of mouse mast cells, DM has been shown to induce a phenotype change from MMC to CTMC. Modulation of RCMC cloned lines with PMA and DM induced an increase in the total histamine content as shown above. However, histochemical staining of the modulated cloned cells demonstrated that they could be stained with alcian blue but they were not counter-stained with safranin O (data not shown), suggesting that the modulation process did not involve a phenotypic change.

DISCUSSION

As demonstrated in Chapter II some RCMC clones vary in receptor expressions in terms of both ratios of $Fc_{\epsilon}R$ and their time-dependent development. It was suggested in that study that the differences observed may actually have reflected differences in the status of maturity and thus various stages of differentiation of these mast cells. In order to confirm the findings described in Chapter II, clones, RCMC1.3.1, 1.3.2, 1.3.3 and 1.11.2, which exhibited the most varied changes in $Fc_{\epsilon}R$ expression upon prolonged culture, were subjected to differentiation agents. Since clones at early and late stages of the time-dependent study may have represented different stages of differentiation, representative samples of each stage were used for the experiments. To monitor the effects of the differentiation agents, changes in $Fc_{\epsilon}R$ expression and histamine content were analyzed since these parameters have been used in previous studies as measures of mast cell development.

PMA modulation distinguished between the different clones and in the case of some clones between the early and the late stages in culture as summarized in Table 2. In the case of clone RCMC1.3.1 which expressed $Fc_{\epsilon}R_L$ only, PMA increased the receptor expression at the early time points when the cells had very few surface receptors but not at the late time points when the receptor expression had reached the plateau as shown in the time-dependent study. The differences between RCMC1.3.1 and RCMC1.11.2 observed in Chapter II were that the former's $Fc_{\epsilon}R_L$ expression increased with time, while that of the latter was relatively stable. $Fc_{\epsilon}RI$ expression of RCMC1.11.2 decreased slightly. Interestingly, the early stage of RCMC1.11.2 responded to PMA with only small changes in the expression of both $Fc_{\epsilon}R_L$ and

Table 2 Modulation of FcεR Expression by Differentiation Agents

| | | RCMC1.3.1 | | RCMC1.3.2 | | RCMC1.3.3 | | RCMC1.11.2 | |
|-----|-------|-----------|------|-----------|------|-----------|------|------------|------|
| | | early | late | early | late | early | late | early | late |
| PMA | FcεRL | ++ | 0 | ++ | + | ++ | ++ | 0 | 0 |
| | FcεRI | NA | NA | 0 | 0 | 0 | 0 | 0 | 0 |
| DM | FcεRL | 0 | 0 | + | + | + | + | ++ | ++ |
| | FcεRI | NA | NA | (-) | 0 | 0 | 0 | 0 | 0 |
| RA | FcεRL | -- | -- | (-) | -- | - | -- | -- | - |
| | FcεRI | NA | NA | -- | (-) | - | - | -- | 0 |

++, + relative increase in FcεR expression
 --, - relative decrease in FcεR expression
 0 no change
 (-) denotes marginal change
 NA not applicable

Fc ϵ RI and these were statistically not significant, while the late stages did not respond at all. Thus it would appear that both early and late RCMC1.11.2 were at a stage of differentiation where they would no longer be influenced significantly by PMA. The other two clones, RCMC1.3.2 and 1.3.3, the Fc ϵ R_L expression of which increased with time in culture, while that of Fc ϵ RI changed in opposite directions, responded to PMA treatment by increasing Fc ϵ R_L, suggesting that the two clones were at a similar stage of differentiation.

The effect of PMA on the histamine content of RCMC clones, as summarized in Table 3, made a distinction among them. PMA was found not to affect the histamine content of clones RCMC1.3.1 and 1.11.2 at both stages of the time-dependent studies, whereas the presence of PMA in the medium increased the histamine content of RCMC1.3.2 and 1.3.3. These results correspond to the effect of PMA on the Fc ϵ R expression (Table 2) of which RCMC1.3.1 and 1.11.2 were affected to a lesser extent than RCMC1.3.2 and 1.3.3. Among the cell lines studied, it appears from the PMA modulation that RCMC1.3.1 and 1.11.2 on one hand, and RCMC1.3.2 and 1.3.3 on the other, are more closely related. Mast cells residing in the peritoneal cavity have been extensively studied because of the easy accessibility to these cells. It has been observed that as immature mast cells mature the histamine content of these cells increases simultaneously with granule maturation (Beaven et al., 1983). No comparable studies with intestinal mucosal type mast cells have been described. Recently, bone marrow-derived mast cells have been established in tissue culture and characterized to resemble MMC phenotype (Sredni et al., 1983). In the presence of sodium butyrate these MMC were demonstrated to be induced to proceed in cellular maturation, also showing

Table 3 Modulation of Histamine Content by Differentiation Agents

| | RCMC1.3.1 | | RCMC1.3.2 | | RCMC1.3.3 | | RCMC1.11.2 | |
|-----|-----------|------|-----------|------|-----------|------|------------|------|
| | early | late | early | late | early | late | early | late |
| PMA | 0 | 0 | + | + | + | (+) | 0 | 0 |
| DM | + | (+) | + | + | + | + | (+) | + |
| RA | - | -- | - | - | - | - | - | - |

++, + relative increase in histamine content
 --, - relative decrease in histamine content
 0 no change
 () denotes marginal change

increased cytoplasmic granules concomitant with increased histamine content (Galli et al., 1982). The results obtained with RCMC1.3.2 and 1.3.3 may therefore represent a maturation process of the mucosal type of mast cells. However, no changes in phenotype, as determined by histochemical stainings were associated with the increase in histamine content. Perhaps, this is not surprising, since the histamine content was still well below that of peritoneal mast cells.

As a differentiation agent for mast cells, dexamethasone yielded somewhat different results from PMA. DM modulation made, in terms of its effect on $\text{Fc}_\epsilon\text{R}_\text{L}$, a distinction between RCMC1.3.1 on one hand and RCMC1.3.2, 1.3.3 and 1.11.2, on the other (Table 2). Thus, $\text{Fc}_\epsilon\text{R}_\text{L}$ on clone RCMC1.3.1, were not affected, while those on clones RCMC1.3.2, 1.3.3 and 1.11.2, were up-regulated at both early and late time points. The effect of DM on $\text{Fc}_\epsilon\text{R}_\text{I}$ was minimal, showing an effect only on the early stage RCMC1.3.2, a clone which had one of the highest levels of $\text{Fc}_\epsilon\text{R}_\text{I}$. Therefore, the majority of clones differed from mouse bone marrow derived mast cells (BMMC) which upon treatment with $1\mu\text{M}$ DM showed a 55% decrease of surface $\text{Fc}_\epsilon\text{R}_\text{I}$ (Benhamou et al., 1986; Robin et al., 1985). It has also been shown previously that $1\mu\text{M}$ dexamethasone treatment of mouse BMMC induces an increase in cellular histamine content and a change in histochemical staining, indicating a maturation process and phenotypic change from immature BMMC to more mature CTMC (Pitton et al., 1988). In the present study DM modulation was found again to increase the histamine content of all clones tested at both early and late stages, however, no changes in histochemical staining were detected. This may further indicate that the RCMC clones can only be induced into a mucosal mast cell maturation-like process and that other factors are

required to convert RCMC into cells of the CTMC phenotype. It should be pointed out that so far there is no report in the literature that an *in vitro* phenotypic change from MMC to CTMC can take place among rat mast cells. The actions of glucocorticoids have not been satisfactorily explained at the biochemical level, however, as other steroid hormones, DM, may exert its effects by initiating the biosynthesis of regulatory proteins (Blachwell et al., 1982) .

Retinoic acid was reported to have distinct effects on myelomonoblast cells at various stages in terms of regulation on $\text{Fc}\gamma\text{R}$ expressions. It increases the $\text{Fc}\gamma\text{R}$ expression on the promyelocytes, such as HL-60 cells, decreases it on the more mature myelomonocytic cell lines, such as U937 and THP-1, but has no effect on the very immature myeloblast cell line KG-1 (Nakamura and Hemmi, 1988). However, when it was applied to the RCMC clones, RA resulted in a decrease in the expression of both $\text{Fc}\epsilon\text{R}_\text{L}$ and $\text{Fc}\epsilon\text{R}_\text{I}$ of all clones (Table 2). This may be due to different effects of RA on a different kind of cells or because the RCMC clones are not different enough for RA to distinguish. Nevertheless, subtle differences were also observed among these cells. Thus, for clones RCMC1.3.3 and 1.11.2, as shown in Figures 25 and 26, cells at late stage appeared to have a slower response to RA in terms of $\text{Fc}\epsilon\text{R}_\text{L}$ expression than the cells at early stage.

The effect of RA on histamine content, as shown in Table 3, was more uniform, causing the down-regulation of all the RCMC clones studied. The best known function of RA is its support for cells of many types to proliferate and differentiate (Sporn and Roberts, 1983). Yet, RA was found in a previous study to induce a dedifferentiation of mouse mast cells (Arock et al., 1988). In association with the recent findings that

the different subtypes of retinoic acid receptors (RAR) may perform unique biological functions and the combinations of RAR subtypes may conduct different functions (deTh et al., 1989) mast cells may therefore express different combinations of RAR from the myelomonoblast cells and exhibit a different response to RA. In this study, in contrast to PMA and DM, retinoic acid down-regulated the expression of $Fc\epsilon R_L$ and the histamine content. The drastic decrease in surface receptor expression and histamine content of all the RCMC clones suggests that RA again induced a dedifferentiation process in these mast cells. Furthermore, the intracellular effect of RA on mast cells has been shown to be its inhibition of protein kinase C activity (Cantwell and Foreman, 1989), which is opposite to the mechanisms of PMA on the RCMC clones as demonstrated in the study. Unlike phorbol esters the mode of RA action may not be direct on PKC but through the binding to the nuclear fractions of the cells and the induction of the synthesis of inhibitory proteins, as suggested in the recent findings of Takishita et al. (1990).

In this study, the effects of the three modulators were found to be reversible, confirming that the changes were due to the actions of the differentiation compounds. Results obtained on the proliferation rates of these RCMC clones suggest that the clonal variations in response to either PMA, DM or RA modulations were not derived from differences in cell growth rates. Thus, PMA did not significantly modulate the growth rates whereas DM inhibited the cell growth of the three clones which responded differently to PMA and DM, respectively (Fig. 39). By the same token, the modulatory effects of the differentiation agents on receptor expression and histamine contents did not seem to be related to changes in growth rate since the cell growth

rates of the RCMC clones were inhibited by both DM and RA, but DM generally increased the expression of $\text{Fc}_\epsilon\text{R}_\text{L}$ and histamine content whereas RA down-regulated both parameters of all the clones. It is, however, possible that at least the increase in histamine content in the various clones due to DM may have had its origin in a decreased rate of cell growth. In the case of mast cells which had been treated with sodium butyrate, the cells responded with a decreased growth rate and an increased histamine content (Galli et al., 1982).

Two of these three modulators, PMA and DM, have previously been used to regulate the surface expression of $\text{Fc}_\epsilon\text{R}_\text{II}$ (CD23) on U937 cells (Mayumi et al., 1988). The present study found that PMA had similar effect on the expression of $\text{Fc}_\epsilon\text{R}_\text{L}$ as on $\text{Fc}_\epsilon\text{R}_\text{II}$, but DM had an opposite effect on $\text{Fc}_\epsilon\text{R}_\text{L}$ as on $\text{Fc}_\epsilon\text{R}_\text{II}$. This may imply that DM could have induced the synthesis of regulatory proteins which differ between monoblast cells and mast cells. Since the $\text{Fc}_\epsilon\text{R}_\text{II}$ is known to be a growth and differentiation factor for B cells, its regulatory mechanism may differ from that of $\text{Fc}_\epsilon\text{R}_\text{L}$. Preliminary evidence (D. H. Conrad and A. Froese, personal communication) indicates that there is no hybridization between the total mRNA of RCMC cells expressing $\text{Fc}_\epsilon\text{R}_\text{L}$ and the mouse cDNA probes for $\text{Fc}_\epsilon\text{R}_\text{II}$. Furthermore no cross-reactivity was observed between either polyclonal or monoclonal antibodies to mouse $\text{Fc}_\epsilon\text{R}_\text{II}$ and $\text{Fc}_\epsilon\text{R}_\text{L}$ nor did a polyclonal antibody to $\text{Fc}_\epsilon\text{R}_\text{L}$ react with mouse $\text{Fc}_\epsilon\text{R}_\text{II}$. Therefore, it is unlikely that $\text{Fc}_\epsilon\text{R}_\text{L}$ is the rat equivalent of mouse $\text{Fc}_\epsilon\text{R}_\text{II}$.

The PKC assays have revealed that long-term culture with low doses of PMA actually induced a gradual increase in cytosolic PKC activity. In reviewing the literature it becomes apparent that PMA stimulates a rapid association of the cytosolic

PKC with the plasma membrane and a subsequent degradation of the membrane-bound PKC, eventually leading to a depletion of the cytosolic PKC activity after a continuous exposure to PMA. It should be pointed out that in those cases the effect was observed with at least 20-fold higher concentrations of phorbol ester after at most 24 hr treatment (Ballester and Rosen, 1985; Koopmann and Jackson, 1990). Since the translocation of cytosolic PKC to the membrane is concentration-dependent, less PKC was translocated to the membrane when lower concentrations of PMA were used (Ballester and Rosen, 1985). The low dose of PMA in prolonged culture used in this study could, therefore, have constantly activated and induced a relatively small percentage of cytosolic PKC to become associated with the membrane. In turn, the membrane-bound, active form of PKC could have been degraded. This continuous cycle could, in principle, have eventually depleted the cytosolic PKC if the synthesis of the enzyme were at the normal rate. However, an earlier study has demonstrated that PMA treatment can also increase the synthesis of PKC (Kruget al., 1987). Taken together, this evidence, along with the observations made in the present study, allow the speculation that the persistent activation of a small amount of cytosolic PKC with the low dose PMA might have triggered a positive feedback in the synthesis of the enzyme, resulting in an accumulated level of PKC activity in the cytosol. Since the degradation rate for the membrane-bound enzyme is independent of PMA concentration, the reason for not having detected the changes in enzyme activity in the plasma membrane might be due to the constant degradation of the small amount of the bound enzyme, which might have reached an equilibrium with the translocation of the cytosolic enzyme to the membrane.

By comparing the cytosolic activity between the histamine-releasing cell line RBL-2H3 and the four RCMC clones, it was found that before PMA modulation, the RCMC clones had a significantly lower PKC activity (Fig. 44). Yet after 3 week PMA modulation RCMC1.3.2 contained 3-4 fold higher PKC activity in the cytosol than the unmodulated cells but still a considerably lower level than RBL-2H3 (Fig. 40). PKC is known to play an important role in mast cell mediator release. Examination of ^3H -serotonin release on these RCMC cell lines revealed the inability of these cells in conducting mediator release function. The results may be explained by the fact that these RCMC lines have a much lower PKC activity than the functional cell line RBL-2H3. This also provides evidence that the RCMC clones are functionally immature mast cells and the induction of PKC activity by PMA may actually lead a differentiation process towards functionally more mature mast cells.

The fact that the PKC inhibitor eliminated the PMA effects demonstrated that the increase in receptor expression and histamine content is at least partially mediated by protein kinase C. The inhibitor H-7 is known to interact with the ATP-binding site of protein kinase C and not to interfere with the binding of PMA to PKC (Hidaka et al., 1984). The results agree with the mechanism of inhibition in that H-7 did not inhibit the induced increase in PKC activity by PMA (Fig. 41).

The activation of PKC by PMA is the first step in many cellular responses. The enzyme appears to interact with many signalling pathways either positively or negatively, as reviewed in detail by Nishizuka (1986; 1988). The negative-feedback control or the down-regulation of various receptors by PKC is exerted presumably through coupling factors in the membrane (Sagi-Eisenberg, 1989). The immediate

effect of a high dose of PMA (20ng/ml) leading to the internalization of $\text{Fc}_\epsilon\text{RI}$ on RBL-2H3 cells (Ra et al., 1989) is likely attributable to this function of PKC. This effect of PMA was also shown in this study on RBL-2H3 and RCMC 1.3.3 (data not shown). The major roles of the PKC transduction pathway may be extended to the regulation of cell growth and differentiation. It is becoming clear that sustained activation of PKC induces the expression of genes, especially those related to the actions of growth factors, leading eventually to cell proliferation (Berry et al., 1990; Nishizuka, 1986). Unlike the physiological PKC activators which activate PKC transiently, phorbol esters are metabolized more slowly, resulting in a persistent activation of PKC. The effects of PMA on receptor expressions could be induced, either directly on receptor recycling or by the action on the biosynthesis of the receptors through gene activation. It would be interesting to check if the mRNA for $\text{Fc}_\epsilon\text{R}_\text{L}$ in the RCMC clones is also up-regulated once that the cDNA probes for this receptor become available. In fact, in a previous study PMA modulation on U937 did result in increased mRNA level for $\text{Fc}_\epsilon\text{RII}$ (Mayumi et al., 1988). Finally, the mode of action of PMA has also been found to be the result of interactions of PKC with other signal transduction systems which normally co-exist with the PKC regulatory pathway, in fashions of either counteracting or potentiating the others (Bell et al., 1985; Dixon et al., 1988; Marquardt and Walker, 1989). The effects observed in this study could also be mediated by PKC through subsequent regulation on other signalling pathways involving cell activation and proliferation.

CHAPTER IV

EFFECTS OF BIOLOGICAL FACTORS ON RAT TISSUE CULTURED MAST CELL CLONES

INTRODUCTION

A study outlined in Chapter II demonstrated that RCMC cloned lines exhibit a variety of receptor patterns in terms of the high affinity $\text{Fc}_\epsilon\text{RI}$ and the low affinity $\text{Fc}_\epsilon\text{R}_\text{L}$ and a differential development of the two types of receptors. Generally, the expression of $\text{Fc}_\epsilon\text{R}_\text{L}$ was found to increase in all the clones while that of $\text{Fc}_\epsilon\text{RI}$ was found to vary, being absent in two clones and decreasing in two of three clones. Modulation of the cloned RCMC lines with differentiation agents, PMA, DM and RA, as described in Chapter III, demonstrated variations in the effects of the agents on the clones, suggesting that some lines were at different stages of differentiation. PMA and DM generally elevated the surface expression of $\text{Fc}_\epsilon\text{R}_\text{L}$ and the histamine content, while neither had a major effect on the expression of $\text{Fc}_\epsilon\text{RI}$. Although RA modulated both types of receptors and the histamine content it induced drastic decreases in the expression of the receptors and the histamine content. The high affinity $\text{Fc}_\epsilon\text{RI}$ are known to be the major receptors expressed on peritoneal mast cells (Froese, 1980) and on intestinal mucosal mast cells (Swieter et al., 1989). The expression of $\text{Fc}_\epsilon\text{RI}$, as judged by binding studies, has been observed to increase on mast cells derived from thymocytes when cultured in the presence of fibroblast feeder layers (Ishizaka et al., 1976) and on peritoneal cells depleted of PMC when cultured in medium containing supernatant from L cell fibroblasts (Czarnetzki et al., 1982). The results obtained in the present investigation and in previous studies suggest that certain environmental

factors which are present in mast cell maturation sites may be necessary for the surface expression of $\text{Fc}_\epsilon\text{RI}$ on mast cells.

IgE is present at very low levels under normal physiological conditions and is elevated in allergic diseases. The presence of IgE has been shown to influence the expression of Fc_ϵ receptors. A good positive correlation was observed between the total number of $\text{Fc}_\epsilon\text{R}$ on human basophils and the serum levels of IgE (Malveaux et al., 1978) and in cultures numbers of $\text{Fc}_\epsilon\text{R}$ on RBL were shown to increase in the presence of IgE, primarily due to protection of these receptors from degradation by the presence of bound IgE (Furuichi et al., 1985). Thus, while IgE is known to play some role in the expression of $\text{Fc}_\epsilon\text{RI}$, no information exists on its involvement in the expression of $\text{Fc}_\epsilon\text{RL}$. Nor is it known what other environmental factors play a role in the regulation of expression of the latter receptor. Potentially, IL-3 and IL-4 could be involved in the regulation of $\text{Fc}_\epsilon\text{R}$ expression on mast cells since these cytokines are known to promote development of MMC-like cells from bone marrow cells (Levi-Schaffer et al., 1986; McMenamin et al., 1987). Both are also implicated in the support of survival and proliferation of mast cell growth (Tsuji et al., 1990b). Interestingly mast cells are known to produce both of these interleukins themselves (Plaut et al., 1989).

This part of the investigation deals with the growth and differentiation of the cloned RCMC lines and the expression of their Fc_ϵ receptors as a consequence of their exposure to environmental factors, including rat IgE, Fab' and F(ab')_2 of anti- $\text{Fc}_\epsilon\text{RL}$, rat rIL-3 and concanavalin A-stimulated rat spleen cell conditioned medium (Con A-SCM).

MATERIALS AND METHODS

Immunoglobulin:

Rat monoclonal IgE was purified from the ascitic fluid of Lou/M/Wsl rats (maintained in the Animal Care Facility of the University of Manitoba) bearing the IR-162 immunocytoma (Bazin et al., 1974) according to established procedures (Conrad et al., 1975). The immunoglobulins of the ascites were precipitated twice at 50% saturation with $(\text{NH}_4)_2\text{SO}_4$. The precipitate was dissolved and dialyzed against 0.2M Tris-HCl, 0.15M NaCl, and 0.5% NaN_3 (pH 8.0), overnight at 4°C. IgE was isolated by gel filtration using Ultrogel AcA 34 (LKB, Bromma, Sweden) which was pre-equilibrated in the same Tris-HCl buffer above. The purified IgE was identified by absorbance at 280 nm and pooled, concentrated and dialyzed against PBS overnight at 4°C. The IgE concentration was determined using an extinction coefficient of $E^{1\%}_{280}=13.6$ (Carson and Metzger, 1974) and stored at -70°C for further use. This preparation of IgE was used for conjugating IgE-Sepharose. For experiments where IgE was required for selective isolation of $\text{Fc}_\epsilon\text{RI}(\alpha)$ and for modulation of RCMC clones in tissue culture, further purification was done by preparative isoelectric focusing at 600 volts for 48h using 1% carrier ampholytes of pH 4.0 to 6.5 (LKB, Bromma, Sweden) in a 0 to 40% sucrose gradient. The IgE focused at pH5.9 was collected and dialyzed against PBS before use.

Preparation of F(ab')_2 and Fab' fragments of anti- $\text{Fc}_\epsilon\text{R}_\text{L}$ antibodies:

The rabbit antiserum specific for $\text{Fc}_\epsilon\text{R}_\text{L}$ was prepared and tested as outlined in Chapter II. The F(ab')_2 preparations of the antiserum and of normal rabbit IgG were prepared by pepsin digestion. Briefly, anti- $\text{Fc}_\epsilon\text{R}_\text{L}$ antiserum or normal rabbit IgG

(Sigma) were either dissolved in or dialyzed against acetate buffer which was made of 0.07M acetic acid and 0.05M NaCl. Pepsin of 3% by weight of the amount of immunoglobulin, dissolved at 100mg/ml in acetate buffer, was added to the protein solution. The enzymatic reaction was allowed to proceed for 6 hr at 37°C and was then terminated by adjusting the pH to 8 with 1N NaOH. The digested protein was immediately applied onto an Ultrogel AcA 44 column (LKB, France) equilibrated with Tris-HCl buffer, consisting of 0.15M NaCl, 0.02M Tris and 0.05% NaN₃ titrated to pH 8 with HCl. The first major protein peak of a complete digestion, consisting the F(ab')₂, was collected, concentrated and dialyzed against PBS, and the material was run through a Protein A-Sepharose column to remove traces of intact antibody.

An aliquot of the F(ab')₂ preparations of both anti-Fc_εR_L antibodies and rabbit IgG was reduced to Fab' fragments by the addition of 2-mercaptoethanol at a final concentration of 0.01M. The reaction was carried out for 100 min at 37°C, followed by alkylation for 1 hr at 0°C with iodoacetamide at a 20% molar excess over 2-mercaptoethanol, with the pH of the reaction mixture being adjusted to 8.5. The reaction mixture was separated using an Ultrogel AcA 44 column equilibrated with Tris-HCl buffer. The second peak containing the Fab' fragments was collected, dialyzed and concentrated, and stored at -80°C till use.

Culture media:

Eagle's minimum essential medium supplemented to make the complete culture medium (CMEM) as described in Chapter II, was used throughout the study unless other medium was specified. Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Burlington, Ont.) supplemented with 10% FCS was used in IL-3 modulation.

Medium RPMI 1640 (Gibco), supplemented with 10% FCS, 1mM L-glutamine, 1mM sodium pyruvate, 0.05mM 2-mercaptoethanol and 100 μ g/ml kanamycine monosulfate, was used to prepare Con A-SCM with the addition of 5 μ g/ml concanavalin A (Con A) and as the control medium for Con A-SCM.

Preparation of Con A-SCM:

Con A-SCM was kindly prepared by Ms. C. Lee according to previously established procedures (Rosenberg et al., 1978). Briefly, spleen cells of Spague-Dawley rats were cultured at 5×10^6 cells/ml in 200 ml portions of RPMI 1640 medium, supplemented with 10% FCS, 5 μ g/ml concanavalin A (Con A), 1mM L-glutamine, 1mM sodium pyruvate, 0.05mM 2-mercaptoethanol and 100 μ g/ml kanamycine monosulfate, at 37°C in a humidified 5% CO₂ environment. The culture supernatants were collected after 40 hrs and sterilized by passage through 0.22 μ m filters. The Con A activity in the conditioned medium was then inactivated by adding 20mM methyl- α -D-mannopyranoside. The preparation was stored at -80°C till use.

Bone marrow cell proliferation in the presence of rIL-3:

Rat rIL-3 which had been prepared according to Cohen et al (1986) was kindly provided by Dr. A. J. Hapel. Bone marrow cells were obtained by flushing the cavity of the tibias of male 4-week-old Wistar rats (Animal Care Facility, University of Manitoba) with RPMI 1640 medium. After washing once with the same medium, 5×10^4 bone marrow cells were plated into each well of 96 well microtiter tissue culture plates (Falcon, Becton Dickinson, Oxnard, CA). Serial dilutions of rIL-3 were added to constitute a final volume of 200 μ l per well. On day 3, the bone marrow cultures were pulsed overnight with ³H-thymidine (Amersham, Oakville, Ont.). As described

previously (Cohen et al., 1986), rIL-3 activity was defined as the reciprocal of the titration endpoint after which the support for bone marrow cell proliferation was no longer significant. In addition, the proliferation of bone marrow cells was measured in the presence of the culture supernatant of the cloned RCMC lines.

Cell culture:

The IEF-focused rat monoclonal IgE and the rabbit anti-Fc ϵ R_L antibodies of both Fab' fragments and F(ab')₂ were deaggregated by ultracentrifugation at 35,000 rpm for 90 min before addition to the culture medium. Cloned lines of RCMC1.3.1, 1.3.2, 1.3.3 and 1.11.2 were cultured in the presence of 1 μ g/ml of either rat IgE, anti-Fc ϵ R_L antibodies of either Fab' fragments or F(ab')₂, respectively, for 4-7 wk in complete culture medium as described in Chapter II. A set of controls was included the normal rabbit IgG F(ab')₂ and Fab'. Briefly, the cell cultures were passaged every 5-7 days and about 5x10⁵ of cells were seeded into new flasks at the time of passage. Cell aliquots from confluent cultures were frozen every week in 9% DMSO culture medium and kept in liquid N₂. In order to isolate the receptors for IgE, the surface-bound Igs were diluted out by culturing the cells in the absence of the Igs for two more passages after they were revived and before the cells were subjected to receptor analysis. Clone RCMC1.3.2 was also cultured in the presence of 10 units/ml rIL-3 or 25% Con A-SCM for 10 days, respectively. Since rIL-3 was produced in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS (Cohen et al., 1986), the IL-3 preparation was either dialyzed against CMEM using a dialysis tubing with a 10KDa cut-offing or a control using DMEM with 10% FCS was used. A 25% supplemented

RPMI 1640 as described above was included as the control medium for 25% Con A-SCM modulation medium.

FITC staining of surface $\text{Fc}_\epsilon\text{R}_\text{L}$:

The interaction of rabbit anti- $\text{Fc}_\epsilon\text{R}_\text{L}$ Fab' and F(ab')_2 with the receptors on intact RCMC cells was revealed by using a fluorescein isothiocyanate (FITC)-conjugated goat antibody with specificity to rabbit IgG F(ab')_2 (Cappel, West Chester, PA). The staining procedures were carried throughout at 4°C or on ice. A total of 5×10^5 RCMC1.3.2 was added into each V-bottom well of 96-well plates (Falcon, Becton Dickinson, Oxnard, CA). The deaggregated F(ab')_2 or Fab' of anti- $\text{Fc}_\epsilon\text{R}_\text{L}$ were added in excess amount at 50 $\mu\text{g}/\text{well}$ and incubated for 60 min on ice. After washing with RPMI 1640 supplemented with 1% HEPES solution (Gibco) and 15% FCS, the deaggregated FITC-conjugated goat anti-rabbit IgG was added at 20 $\mu\text{g}/\text{well}$ and incubated for 60 min. After washing, the cells were fixed with a 1% paraformaldehyde saline solution for examination under a fluorescence microscope. Photographs were taken using Kodak TriX-400 films (Rochester, NY).

Specificity of anti- $\text{Fc}_\epsilon\text{R}_\text{L}$ antibodies:

To test the specificity of the rabbit anti- $\text{Fc}_\epsilon\text{R}_\text{L}$ antibodies 5×10^5 of RCMC1.3.2 were labelled with ^{125}I as described in Chapter II. After washing the cells with PBS/BSA and FCS as previously described procedures (Chapter II) the cells were incubated with 100 μg of the antibody for 2h at 4°C with gentle mixing. The cells were washed three times with PBS to remove the unbound antibodies and then solubilized with 0.5% NP-40. The antibody-receptor complexes were isolated with Protein A-Sepharose.

Receptor analysis:

RCMC clones modulated with immunoglobulins and growth factors as described above were analyzed for the changes in the expression of receptors for IgE. The procedures were outlined in Chapter II.

Assay of total cellular histamine content:

The total cellular histamine content of the modulated RCMC clones was measured to reveal the intracellular changes according to the procedures described in Chapter II.

Proliferation assay of growth factor-modulated RCMC:

The growth rates of RCMC1.3.2 in the presence of rIL-3 and Con A-SCM were measured according to the previously described procedures in Chapter II.

RESULTS

Modulation of RCMC clones with IgE:

RCMC clones demonstrating variable expressions of the two types of receptors for IgE were subjected to long-term culture in the presence of 1 $\mu\text{g/ml}$ rat monoclonal IgE. After culturing the cells for two passages in the absence of IgE in order to decrease the concentration of surface-bound IgE, the cells were analyzed for their expression of $\text{Fc}_\epsilon\text{R}_\text{L}$ and $\text{Fc}_\epsilon\text{RI}$ in terms of its α subunit. Despite the differences in their surface receptor patterns the RCMC clones studied all showed a gradual increase in the expression of $\text{Fc}_\epsilon\text{RI}(\alpha)$ and $\text{Fc}_\epsilon\text{R}_\text{L}$ in the presence of IgE in culture. As shown in Figure 45, clone RCMC1.3.1 which expressed only $\text{Fc}_\epsilon\text{R}_\text{L}$ was enhanced in the expression of the receptor, yet, no surface $\text{Fc}_\epsilon\text{RI}$ was induced. Clones RCMC1.3.2, 1.3.3, 1.11.2, which expressed both types of receptors for IgE, were modulated to express increased $\text{Fc}_\epsilon\text{RI}$ and $\text{Fc}_\epsilon\text{R}_\text{L}$, although to varying degrees (Fig. 46, 47, 48). It is rather interesting to note that for each clone, changes in $\text{Fc}_\epsilon\text{RI}$ and $\text{Fc}_\epsilon\text{R}_\text{L}$ were similar in magnitude and for most clones also, in terms of kinetics. It is also important to point out that RCMC1.3.2 differed from others in having a slower response to IgE, showing an obvious increase in both receptors at six weeks of modulation. The histamine contents of the IgE-modulated RCMC lines were also assayed. No significant changes were found in all the RCMC lines studied (data not shown).

Modulation of RCMC clones with anti- $\text{Fc}_\epsilon\text{R}_\text{L}$ antibodies:

Three RCMC cloned lines, expressing both types of receptors for IgE, were modulated with the Fab' and F(ab')_2 fragments of antibodies to $\text{Fc}_\epsilon\text{R}_\text{L}$. The effects of these reagents on $\text{Fc}_\epsilon\text{R}$ expression were rather interesting. The Fab' fragment of

Fig. 45. Modulation of $\text{Fc}_\epsilon\text{R}$ expression with IgE on clone RCMC1.3.1. Cells cultured in the presence of 1 $\mu\text{g/ml}$ of IgE for the period of time shown, followed by two passages in the absence of IgE. They were then labelled with ^{125}I and the receptors were isolated by means of IgE-Sepharose and analyzed by SDS-PAGE as described under Materials and Methods (filled circles). In control experiments cells were treated identically, except that no IgE was used.

Fig. 46. Modulation of $\text{Fc}_\epsilon\text{R}$ expression with IgE on clone RCMC1.3.2.

Fig. 45

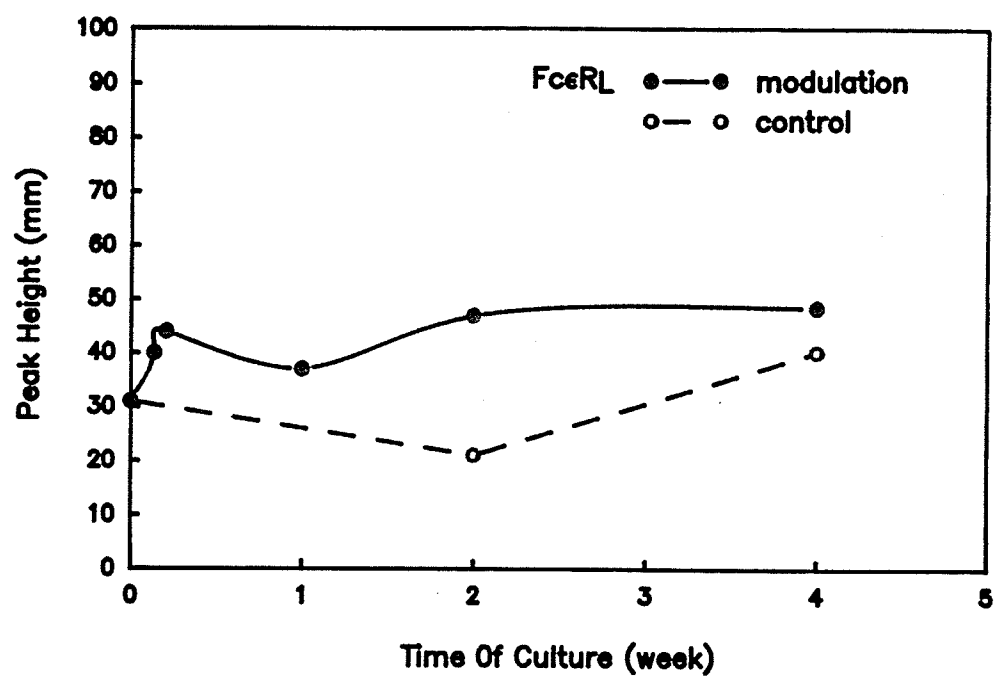


Fig. 46

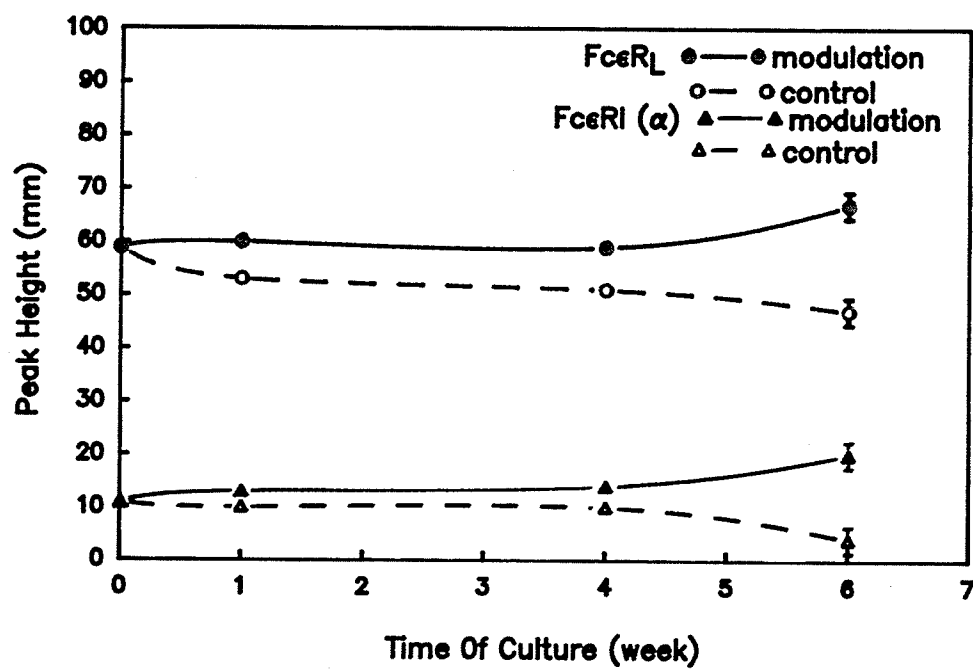


Fig. 47. Modulation of $\text{Fc}_\epsilon\text{R}$ expression with IgE on clone RCMC1.3.3.

Fig. 48. Modulation of $\text{Fc}_\epsilon\text{R}$ expression with IgE on clone RCMC1.11.2.

Fig. 47

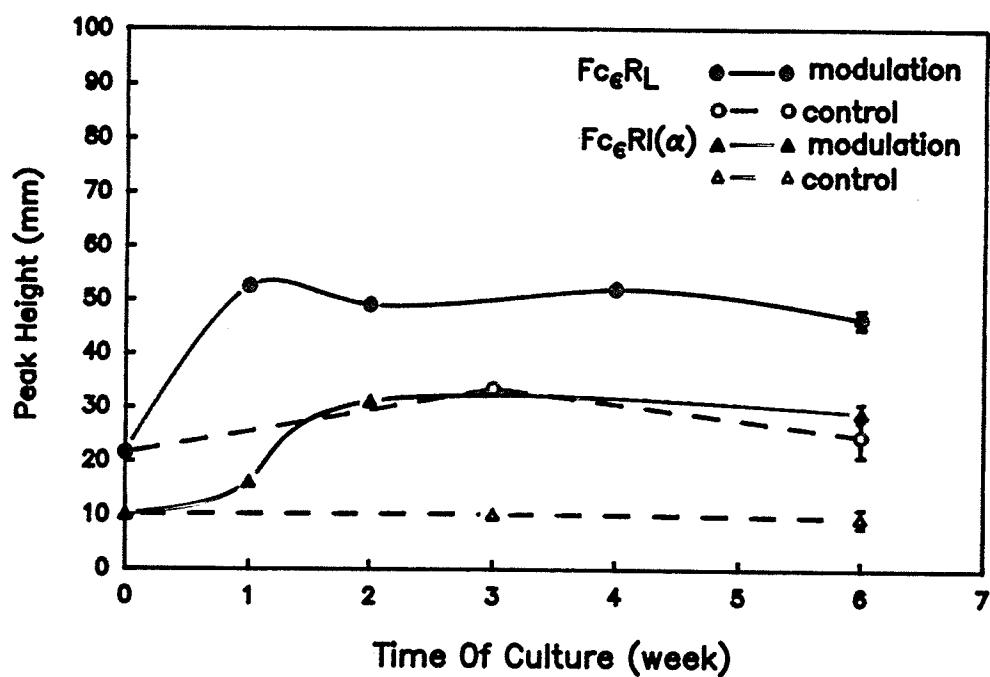
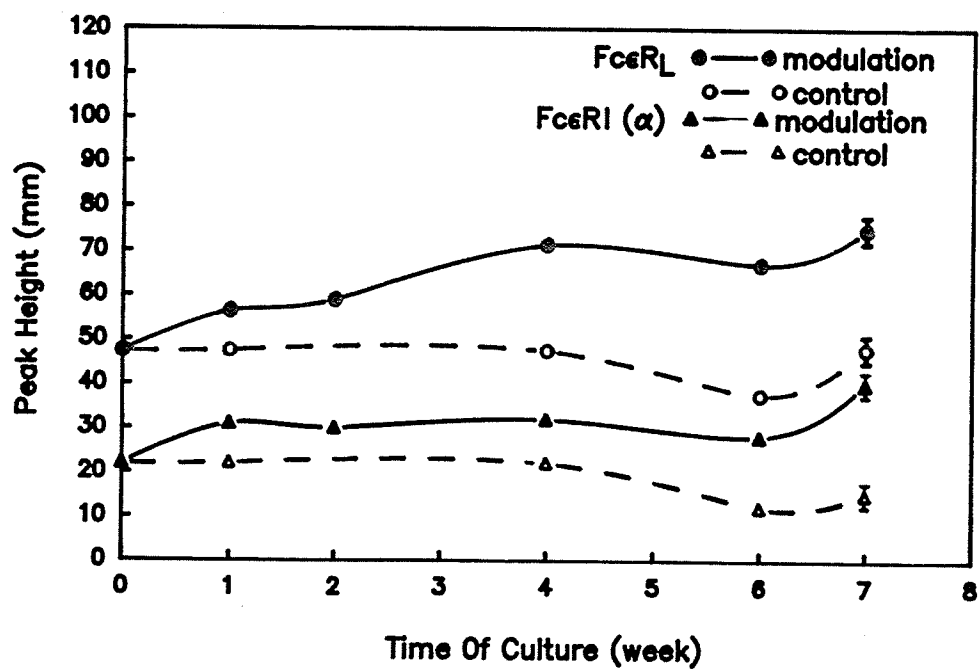


Fig. 48



anti-Fc ϵ R_L generally increased the expression of Fc ϵ R_L on all three clones, but the kinetics and the magnitude of the response varied among them (Fig. 49a, 50a, 51a). A more variable effect was observed in the case of Fc ϵ RI expression. Fc ϵ RI was not affected on RCMC1.3.2 (Fig. 49a), increased slightly on RCMC1.11.2 (Fig. 51a), and was significantly enhanced on RCMC1.3.3 (Fig. 50a). However, anti-Fc ϵ R_L in the form of F(ab')₂ was found to show a significant effect only in the case of Fc ϵ R_L expression which was decreased, but only on RCMC1.3.2 (Fig. 49b). No effect was seen on Fc ϵ RI(α) of this clone. The expression of either Fc ϵ RI or Fc ϵ R_L on the other clones was not changed (Fig. 50b, 51b). The histamine contents of the cloned lines modulated with either Fab' or F(ab')₂ of anti-Fc ϵ R_L was not significantly affected (data not shown).

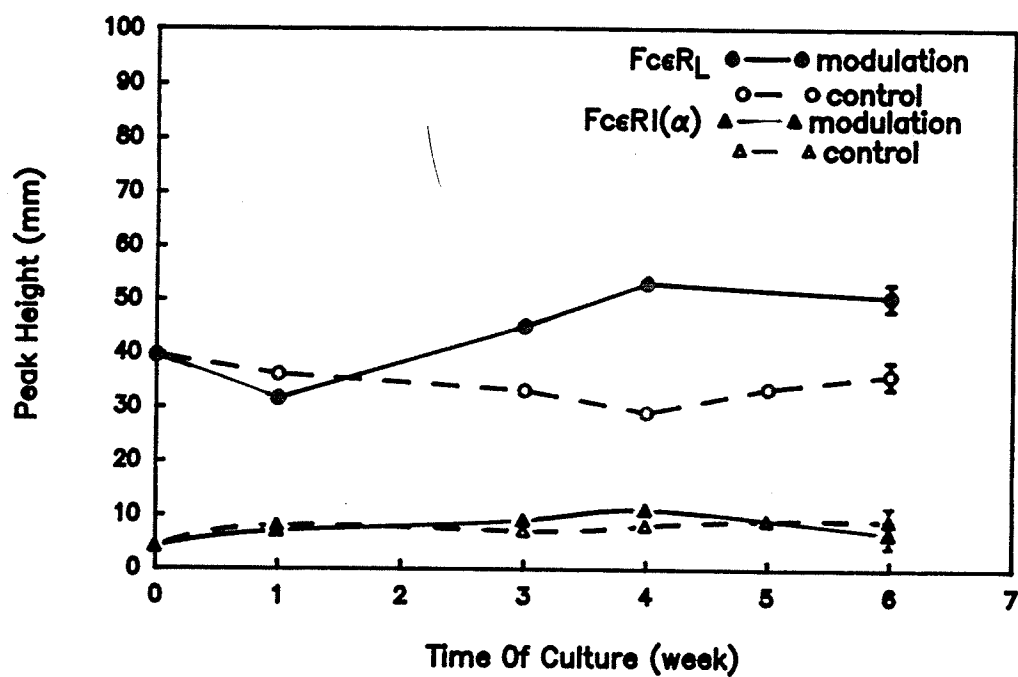
The interaction of the anti-Fc ϵ R_L with RCMC cells was revealed by fluorescence staining. As shown in Figure 52, both the Fab' (Fig. 52a) and F(ab')₂ fragments (Fig. 52c) of the antibody bound to the surface Fc ϵ R_L uniformly showed a ring-shaped homogeneous staining pattern. The corresponding controls using rabbit IgG Fab' (Fig. 52b) and F(ab')₂ (Fig. 52d) showed no fluorescein on the cell surface except for background staining. Since excess amounts of Fab' and F(ab')₂ of the antibody were used in order to show sufficient fluorescein intensity, the bivalent antibodies, therefore, would bind to the receptors monovalently in high concentration and function as Fab' fragments. No apparent differences were observed between the staining results using Fab' or F(ab')₂ of the antibody.

The specificity of the rabbit anti-Fc ϵ R_L antibody and its capacity to bind to Fc ϵ R_L on the cell surface were examined by allowing the antibody to interact with the receptor

Fig. 49. Modulation of $\text{Fc}_\epsilon\text{R}$ expression on clone RCMC1.3.2 with 1 $\mu\text{g/ml}$ of (a) Fab' and (b) $\text{F(ab}')_2$ fragments of anti- $\text{Fc}_\epsilon\text{R}_\text{L}$ antibody. Fab' and $\text{F(ab}')_2$ fragments of normal rabbit IgG were used at 1 $\mu\text{g/ml}$ as controls.

Fig. 49

a)



b)

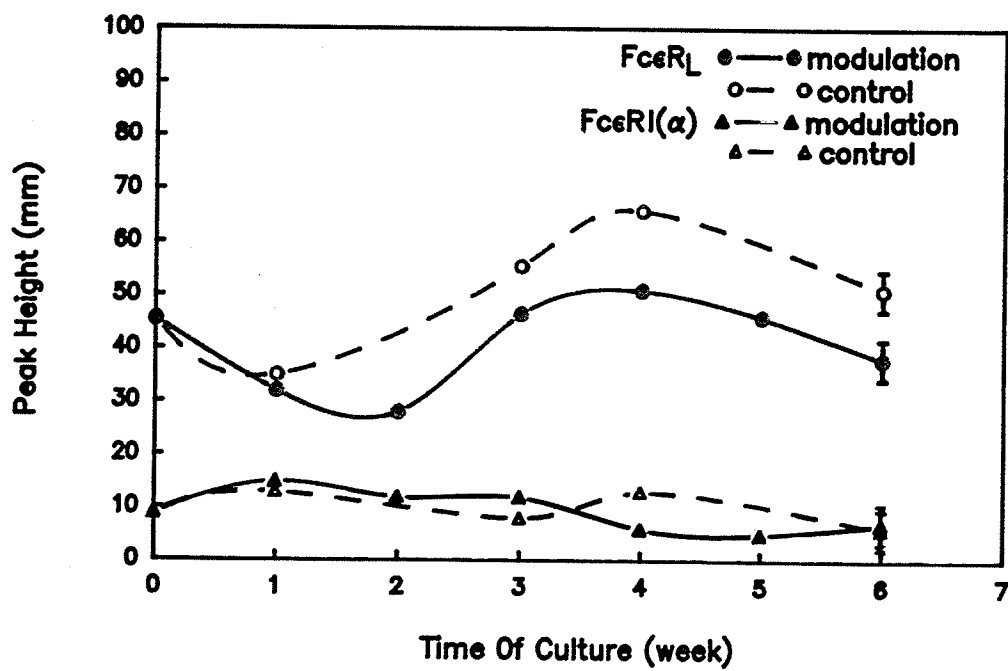
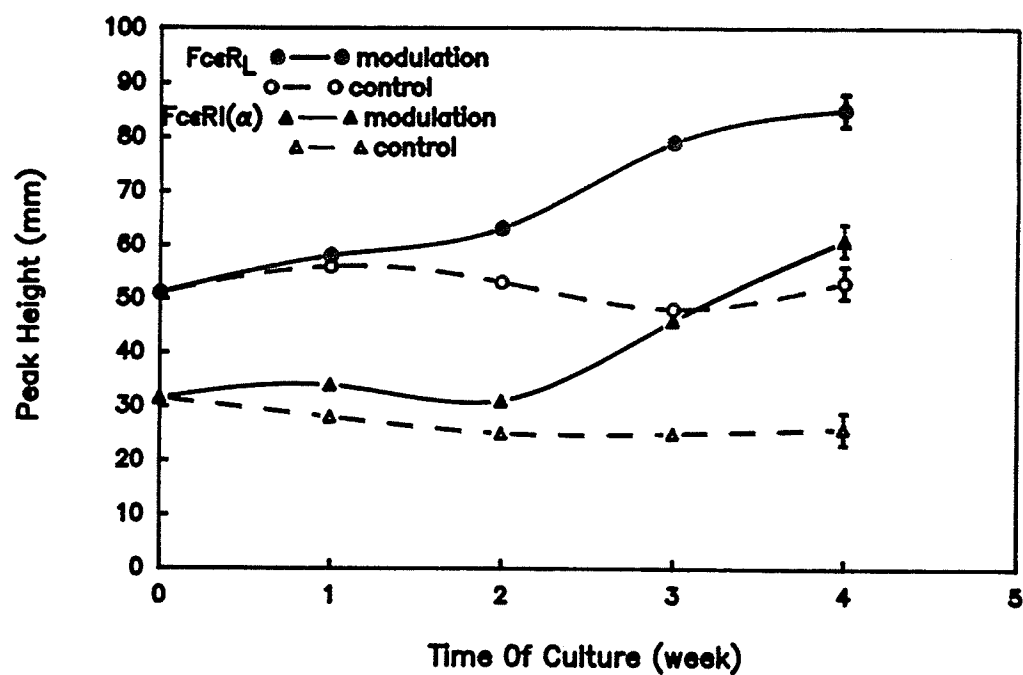


Fig. 50. Modulation of $\text{Fc}_\epsilon\text{R}$ expression on clone RCMC1.3.3 with (a) Fab' and (b) $\text{F(ab}')_2$ fragments of anti- $\text{Fc}_\epsilon\text{R}_\text{L}$ antibody. Concentrations and controls as in Fig. 49.

Fig. 50

a)



b)

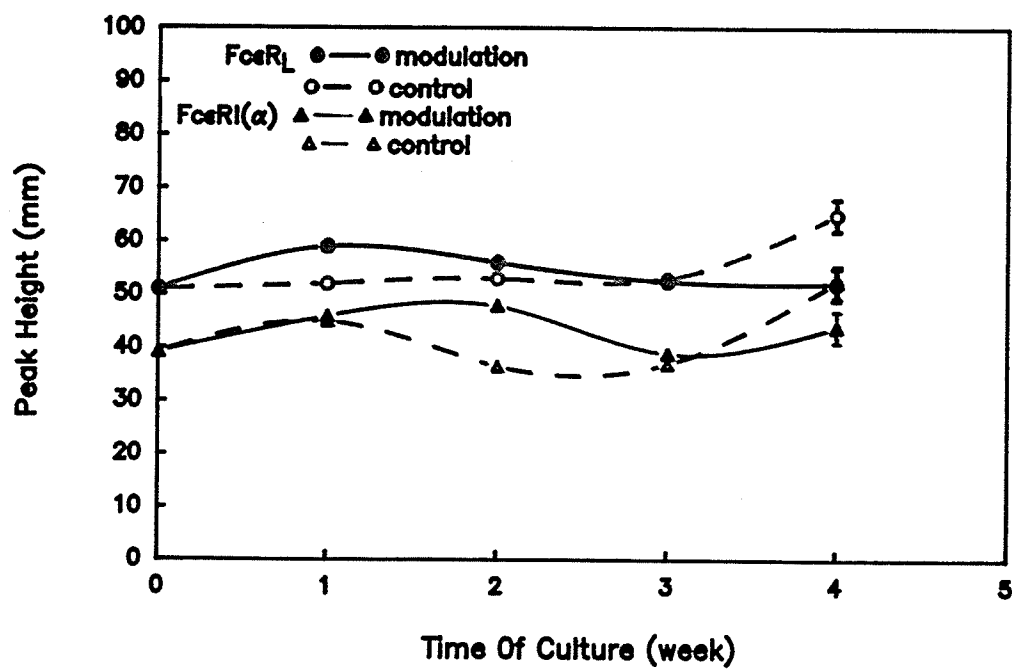
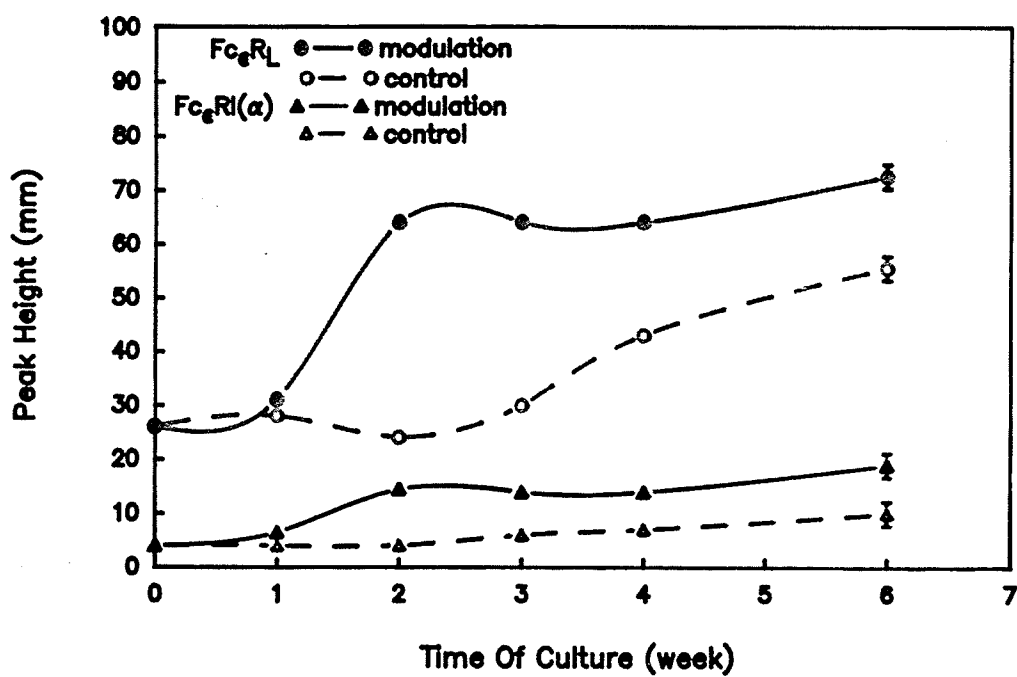


Fig. 51. Modulation of $\text{Fc}_\epsilon\text{R}$ expression on clone RCMC1.11.2 with (a) Fab' and (b) F(ab')_2 fragments of anti- $\text{Fc}_\epsilon\text{R}_\text{L}$ antibody. Concentrations and controls as in Fig. 49.

Fig. 51

a)



b)

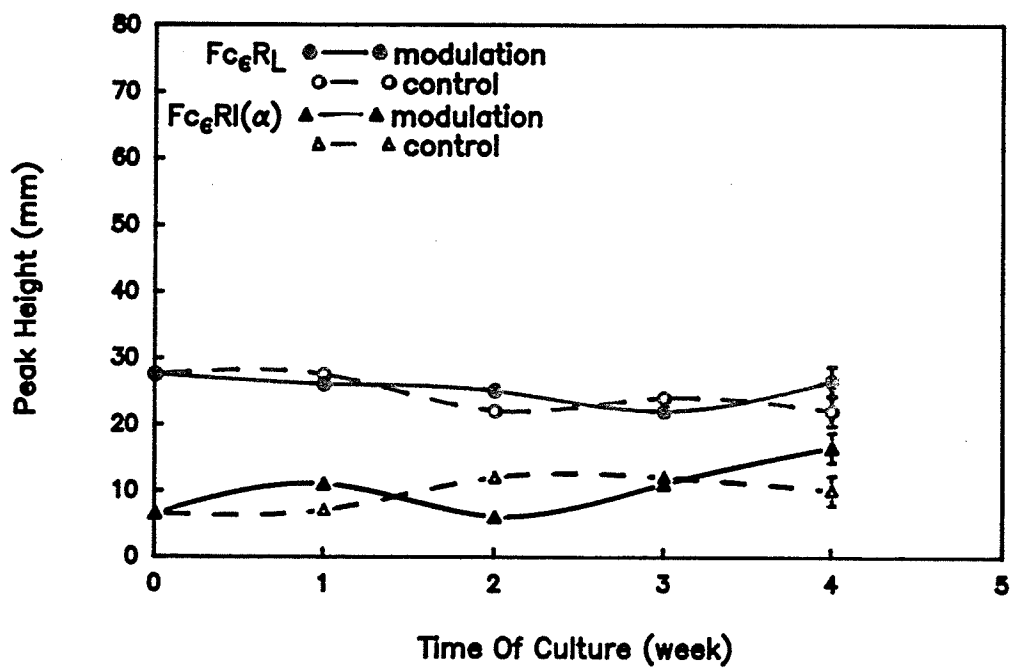
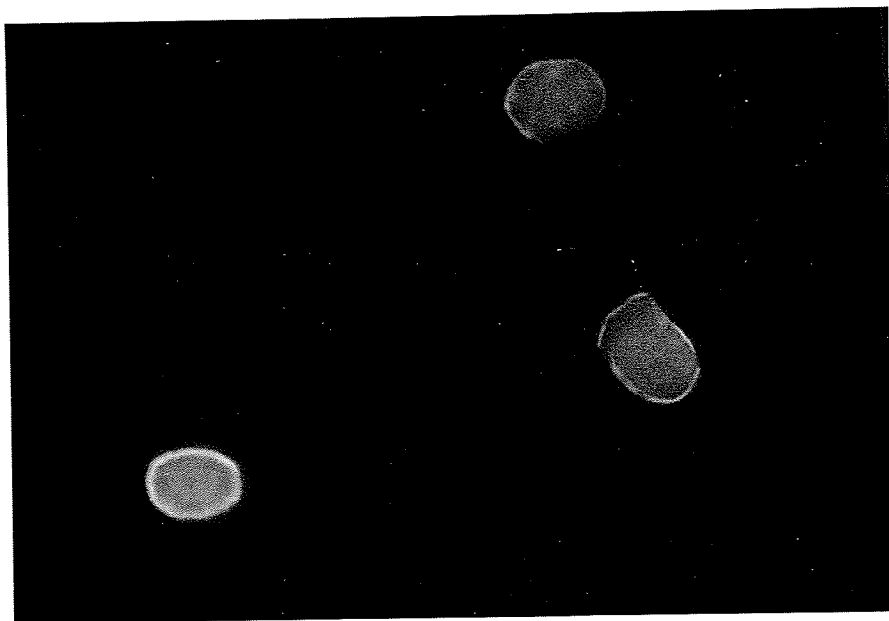


Fig. 52. The interaction of anti-Fc ϵ R_L (a) Fab' and (c) F(ab')₂ with the surface Fc ϵ R_L on RCMC1.3.2. The fluorescence staining was carried out using a second FITC-conjugated goat anti-rabbit IgG F(ab')₂. Normal rabbit IgG, (b) Fab' and (d) F(ab')₂ fragments were used under identical conditions for control purposes.

Fig. 52

a)



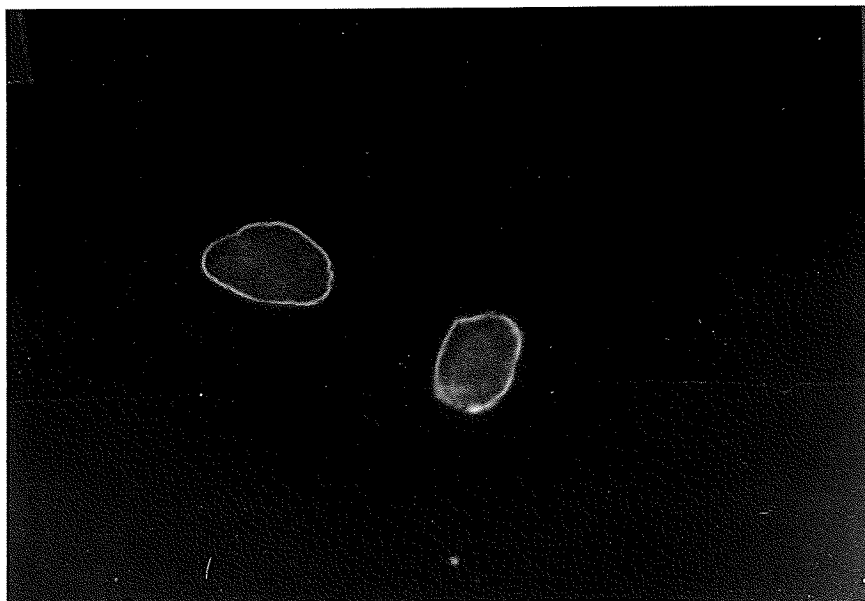
b)



165a

Fig. 52

c)



d)

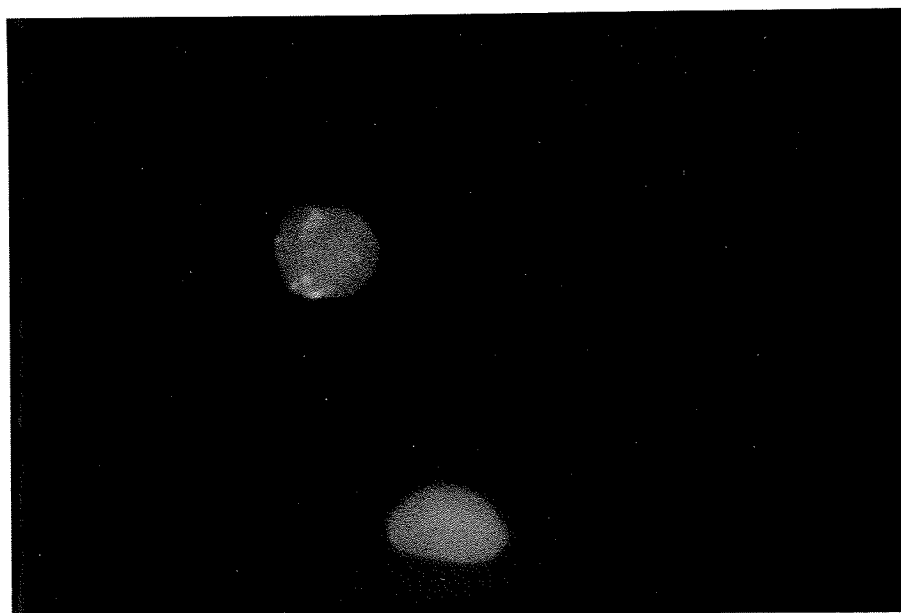


Fig. 53

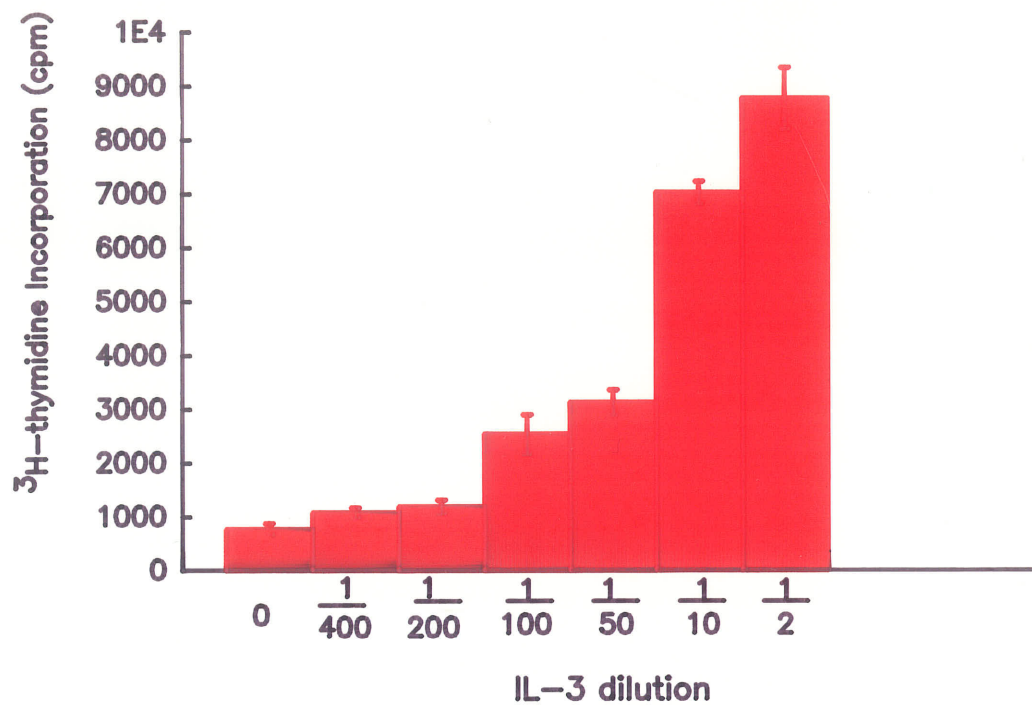
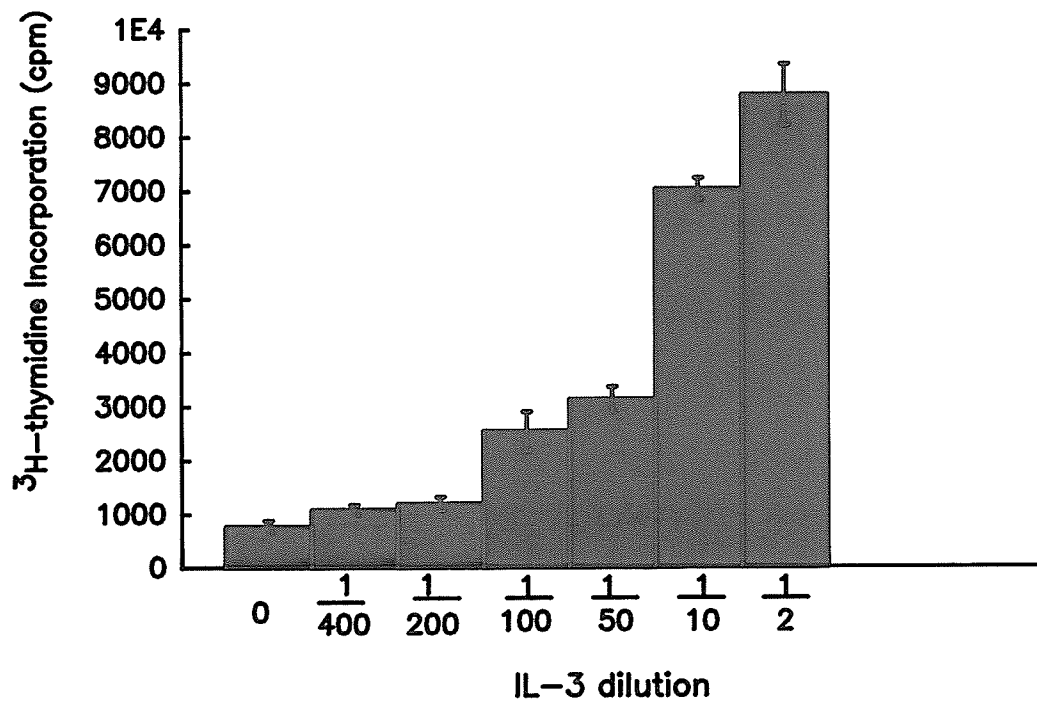


Fig. 53. The proliferation of bone marrow cells in the presence of a serial dilutions of rat rIL-3.

Fig. 53



Clone RCMC1.3.2 was cultured in rIL-3 either in CMEM or in DMEM and the corresponding media in the absence of rIL-3 were used as controls. Similar experiments were carried out in CMEM containing either 25% Con A-SCM or 25% RPMI. After 10 days of culture the cells were analyzed for their Fc ϵ receptor expression and histamine content. The growth factors did not affect the expression of either Fc ϵ RI or Fc ϵ RL (Fig. 54a). However, it appears that DMEM, even though used at only 10% (the remainder was CMEM), significantly increased the expression of either Fc ϵ RI or Fc ϵ RL. Both factors significantly increased the histamine content (Fig. 54b) and this increase was largest when cells were cultured in CMEM. Nevertheless, histochemical staining revealed no phenotypic change due to exposure to these factors, showing alcian blue⁺/Safranin O⁻ cells in their presence or absence (data not shown). The growth factors have been shown to support mast cell proliferation, particularly the generation of mast cells from bone marrow cultures (Haig et al., 1988). Therefore, the growth rates of RCMC1.3.2 in the presence of these modulators were measured. Interestingly, both rIL-3 and Con A-SCM were found to decrease the proliferation rates of RCMC1.3.2 (Fig. 54c). Since the cloned lines of RCMC were generated in the absence of growth factors and fibroblast feeder layer it seemed possible that the cells could secrete the lymphokines needed for their growth, especially when it was found that mast cells produce IL-3, IL-4, IL-5 and IL-6 upon cross-linkage of Fc ϵ RI (Plaut et al., 1989). Therefore, the culture supernatant of RCMC1.3.2 was added to the bone marrow cultures to test for the possible presence of growth factors as measured by ³H-thymidine incorporation. The results showed no apparent growth-supporting activity on bone marrow cells (data not shown).

Fig. 54.

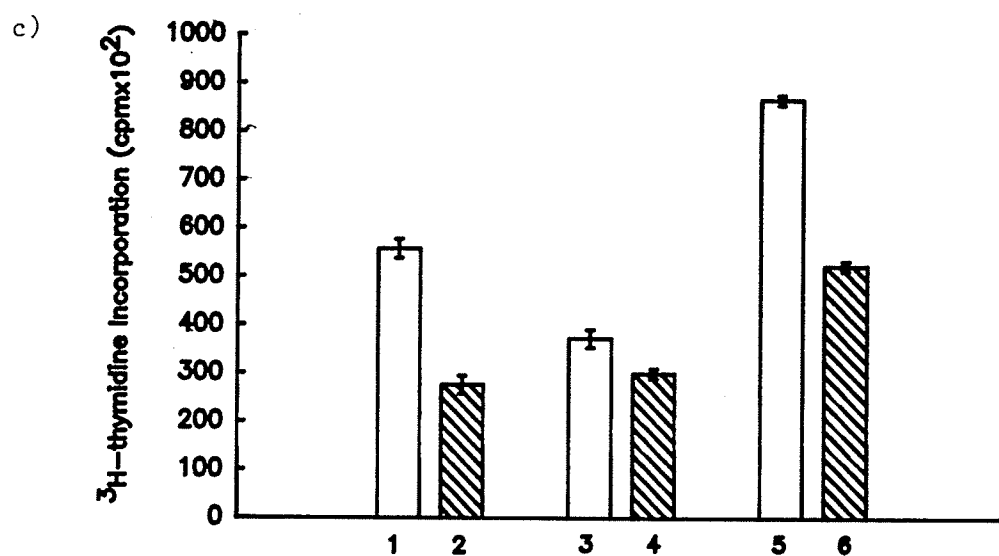
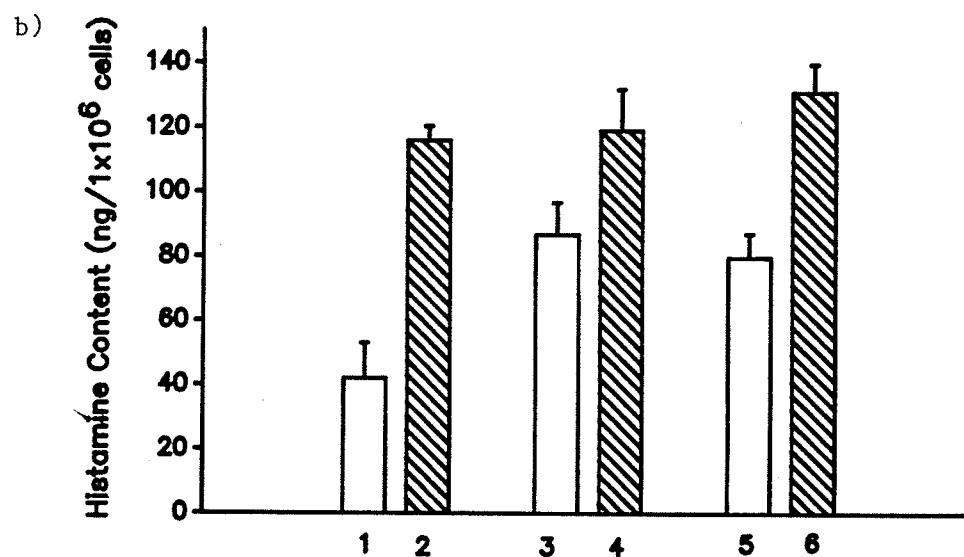
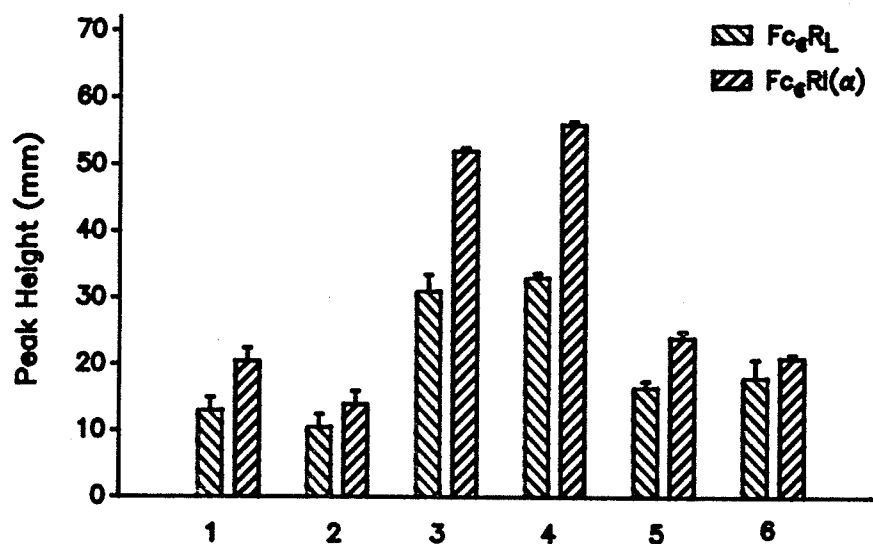
(a) The effect of growth factors on Fc_εR expression on RCMC1.3.2.

(b) The effect of growth factors on total histamine content of RCMC1.3.2.

(c) The growth rates of RCMC1.3.2 in the presence of growth factors.

Groups 1, 3, and 5 are controls for groups 2, 4, and 6, respectively. Cells were cultured under the conditions of (1) CMEM only, (2) dialyzed rat rIL-3 in CMEM, (3) DMEM only, (4) rat rIL-3 in DMEM, (5) 25% RPMI in CMEM, (6) 25% Con A-SCM in CMEM.

Fig. 54
a)



DISCUSSION

Results presented in the time-dependence study in Chapter II have shown that $\text{Fc}_\epsilon\text{R}_\text{L}$ appears to be expressed in greater numbers on all RCMC lines than $\text{Fc}_\epsilon\text{RI}$, and that the expression of the latter receptor is lacking on some of the lines and decreased in most cases. This part of the study, has shown that the addition of IgE to cultures of RCMC cloned lines leads to an increased expression of both $\text{Fc}_\epsilon\text{RI}$ and $\text{Fc}_\epsilon\text{R}_\text{L}$ (Fig. 45-48). The ligand IgE molecules bind to both high ($\text{Fc}_\epsilon\text{RI}$) and low ($\text{Fc}_\epsilon\text{R}_\text{L}$) affinity receptors with their Fc portions. The binding itself is not known to cause any readily perceivable perturbation to the cells before the cross-linkage of the ligands by antigens. But, the presence of IgE on the cell surface of RBL cells was reported to increase the number of $\text{Fc}_\epsilon\text{RI}$ on the cell surface by preventing the elimination of the Fc_ϵ receptors from the cell surface, with the newly synthesized receptors continuing to be incorporated into the membrane (Furuichi et al., 1985). In addition, IgE was used in tissue cultures to induce the expression of the low affinity receptors for IgE on lymphocytes (Yodoi et al., 1979), indicating that the presence of the ligands in the environment regulates the expression of their receptors. The results presented here, further support these observations and those of Malveaux et al. (1978) who have shown a positive correlation between the serum level of IgE and the number of $\text{Fc}_\epsilon\text{R}$ per human basophil. It is interesting to note that the kinetics in the induction of $\text{Fc}_\epsilon\text{RI}$ and $\text{Fc}_\epsilon\text{R}_\text{L}$ were correlated within each clone although the clones expressed variable numbers of the two receptors. This may indicate that IgE has similar effect on the expression of $\text{Fc}_\epsilon\text{RI}$ and $\text{Fc}_\epsilon\text{R}_\text{L}$. The differences observed in the extent and kinetics of receptor changes among different clones may be derived from the clonal variations as

discussed in Chapter III. In this context, it should be pointed out that RCMC1.3.1 did not respond to IgE in the culture medium with an appearance of $\text{Fc}_\epsilon\text{RI}$. This would suggest that this clone was indeed devoid of these receptors and that their number was not simply too low to be detected. The fact that $\text{Fc}_\epsilon\text{RI}$ did not appear, may indicate that cells are at a stage of differentiation at which they cannot respond to IgE in the microenvironment, or alternatively that they may be defective in that they have, perhaps, been subject to a mutation which makes it impossible for them to produce or express $\text{Fc}_\epsilon\text{RI}$. The defect may actually reside in the translation mechanism since it was shown in the case of one RCMC line that it contained mRNA for all three subunits of $\text{Fc}_\epsilon\text{RI}$, yet, did not express this receptor (Chan et al., 1990).

The increase in $\text{Fc}_\epsilon\text{R}$ expression observed in this study was most likely not due to a protection of receptors from degradation. In the case of $\text{Fc}_\epsilon\text{R}_\text{L}$ this is an unlikely mechanism in any event because the statistical chance of having a major fraction of these receptors occupied can be expected to be low, because of their low affinity for IgE. Because modulation experiments with IgE involved two passages of the target cells in the absence of IgE, it can be expected that even occupied $\text{Fc}_\epsilon\text{RI}$ would have been diluted by a factor of 400 and thus bound IgE could have played only a minor role in the protection of $\text{Fc}_\epsilon\text{RI}(\alpha)$ from degradation. Moreover, the receptor numbers measured in these experiments were only those which were not occupied at the time of the experiment, since occupied receptors would not have been isolated by IgE-Sepharose, nor would they have been effectively labelled by ^{125}I (Conrad and Froese, 1976). Actually, the nature of the modulation of $\text{Fc}_\epsilon\text{R}$ expression, may vary with the state of differentiation or maturity of the mast cells. Thus, in the case of relatively

immature cells, interaction of IgE with the $\text{Fc}_\epsilon\text{RI}$ may lead to increased synthesis of this receptor while in the case of more mature cells an increase in the rate of receptor synthesis may no longer be possible and increases in receptor numbers can only be achieved by protecting receptors occupied by IgE.

The use of IgE to modulate the expression of $\text{Fc}_\epsilon\text{RI}$ and $\text{Fc}_\epsilon\text{R}_\text{L}$ had shown that its presence in tissue culture has an effect on both. This is not really surprising since IgE is a ligand for both. Other environmental factors normally present *in vivo* but largely absent *in vitro* are immunoglobulins in general and IgG in particular. A previous study from our laboratory had shown that both types of $\text{Fc}_\epsilon\text{R}$ can cross-react with at least three subclasses of IgG through their Fc moiety (Kepron et al., 1988). Therefore, the addition of any of these IgGs to cultures of RCMCs could have potentially modulated the expression of both $\text{Fc}_\epsilon\text{Rs}$. Therefore, it was decided not to use rat IgG, but to study instead the effect of rabbit anti- $\text{Fc}_\epsilon\text{R}_\text{L}$ which was shown to react specifically with $\text{Fc}_\epsilon\text{R}_\text{L}$ only on intact RCMC1.3.2 cells. However, since it was also shown that rabbit IgG can interact with $\text{Fc}_\epsilon\text{R}_\text{L}$ through the Fc portion of the antibody molecules, the Fc portion of anti- $\text{Fc}_\epsilon\text{R}_\text{L}$ was removed. Binding studies to intact cells using Fab' and F(ab')_2 fragments of anti- $\text{Fc}_\epsilon\text{R}_\text{L}$ along with FITC labelled anti-mouse Fab' have clearly shown specific binding to RCMC1.3.2 cells (Fig. 52a,c), while corresponding fragments of normal rabbit IgG did not react (Fig. 52b,d).

The most striking observation made when studying the effects of the Fab' and F(ab')_2 fragments of anti- $\text{Fc}_\epsilon\text{R}_\text{L}$ on $\text{Fc}_\epsilon\text{R}$ expression was that, in general, the former elicited an increased expression of both $\text{Fc}_\epsilon\text{RI}$ and $\text{Fc}_\epsilon\text{R}_\text{L}$ while the latter appeared to have no such effect. Only early RCMC1.3.2 deviated somewhat from this general

response pattern. Thus, these cells responded to $F(ab')_2$ with a decrease in $Fc\epsilon R_L$ expression and no change in $Fc\epsilon RI$ expression. The reasons for this response are not clear. However, it should be noted that expression of $Fc\epsilon R_L$ showed considerable oscillation during the six weeks in culture, even with control $F(ab')_2$ fragments. These cells did not show such behavior in $Fc\epsilon R_L$ expression when used for modulation studies in Chapter III, nor did they do so when the effects of Fab' fragments were studied. However, since these cells also deviated from the other clones in their $Fc\epsilon RI$ expression upon modulation with Fab', clonal variation may be implicated. Thus, RCMC1.3.2 and 1.11.2 showed a much slower response to IgE than RCMC1.3.3. This may be due to the fact that the former two decreasing $Fc\epsilon RI$ expression whereas the latter had an increasing expression of both the receptors in the time studies on the receptor development.

Anti-receptor antibodies would not be expected to be in the normal environment of mast cells. However, the Fab' fragment may be considered to mimic monomeric Igs (IgE or subclasses of IgG) which would be expected to interact with $Fc\epsilon R_L$ without cross-linking them. $F(ab')_2$ fragment, on the other hand, could be expected to do so, thereby mimicking immune complexes. Therefore, it is clear that the signal provided by the types of fragments and their naturally occurring analogs can be expected to differ. In *in vivo* situations, monomeric Igs could be expected to give chronic stimulation while immune complexes would normally appear only transiently and give rise to mast cell degranulation and perhaps internalization of the complex along with the receptors.

As indicated above, Fab' fragments of anti- $Fc\epsilon R_L$ may therefore interact with

Fc ϵ R_L to yield a signal similar or identical to that given by IgE and thereby up-regulate the expression of Fc ϵ R_L. The observation that it also has an effect on Fc ϵ RI could, perhaps, be explained by two mutually not exclusive possibilities. (i) The anti-Fc ϵ R_L antibodies were not absolutely monospecific for Fc ϵ R_L, i.e., antibodies to Fc ϵ RI(α) were present but their numbers were too low to be detected and (ii), the initial triggering signal may be at least particularly identical for the two receptors even though, as proposed in Chapter II, the regulatory mechanism, for the two receptors differ. It must, however, be pointed out that the presence of some anti-Fc ϵ RI antibodies is rendered less likely if one takes into consideration that anti-Fc ϵ R_L antibodies are incapable of triggering mediator release from RBL-2H3 cells (unpublished observations) even though these cells did release mediators by an IgE-mediated mechanism. It is also unlikely that Fc ϵ R_L and Fc ϵ RI(α) are intimately associated on the plasma membrane of mast cells. Studies with bifunctional cross-linking agents have not shown any association between these two molecules (Holowka et al., 1980; Lao and Froese, unpublished observations).

In contrast to most of the effects of the differentiation agents described in Chapter III, changes in Fc ϵ R expression were never accompanied by changes in histamine content. This is an indication that the stimulation provided by anti-Fc ϵ R_L is of a much more restricted nature. Therefore, the maturation and differentiation of mast cells *in vivo* may require a number of factors which all contribute to the generation of mature mast cells of a given type.

Rat rIL-3 and Con A-SCM had no apparent effect on IgE receptor expression of clone RCMC1.3.2 (Fig. 54a). However, the cellular histamine content was up-

regulated somewhat by both rIL-3 and Con A-SCM (Fig. 54b). The increase in histamine content of RCMC1.3.2 due to rIL-3 varied depending on the medium in which the cells were cultured even though an increase due to rIL-3 was observed in either medium. Cells were cultured in one or the other medium since the addition of 10% DMEM to RCMCs grown in CMEM resulted in significant changes in receptor expression and histamine content of the cells, obscuring the effect of rIL-3. It is interesting to note that both rIL-3 and Con A-SCM had similar effects on the histamine contents and that in both instances this effect was accompanied by a decrease in cell proliferation (Fig. 54c). This phenomenon is thus similar to the effect seen when mast cells are grown in sodium butyrate (Galli et al., 1982), an agent which also reduced mast cell growth. However, in the case of sodium butyrate the increase in histamine content was much more substantial and the process involved a mast cell phenotypic change from BMMC to CTMC. The relatively small increase obtained in this study, could, perhaps be indicative of a minor change towards maturation of the cells. It certainly was too small to suggest that a phenotypic change from MMC to CTMC had taken place. Indeed, histochemical staining with alcian blue and safranin O indicated that the rIL-3 or Con A-SCM treated cells could not be counter-stained with the latter dye (data not shown). It would have been of interest to treat the cells with both IL-3 and IL-4, since Nakata et al (1988) have shown that both cytokines are essential for the growth of CTMC. Unfortunately, rat IL-4 is not yet available. Con A-SCM is known to contain a variety of lymphokines, possibly including IL-3 and IL-4, however, without a better knowledge on the lymphokine composition it is difficult to interpret the results more precisely.

A somewhat surprising fact was the observation that both rIL-3 and Con A-SCM decreased the ^3H -thymidine incorporation of RCMC1.3.2. Haig et al. (1988) had made a similar observation but at higher concentrations of lymphokines. Thus, they found that 50 units of rIL-3 compared to 10 units used in this study, were required to start reducing the growth rate of bone marrow derived MMC. These authors could not provide a definitive explanation for their observation, but suggested that the action of IL-3 may be similar to that of IL-2 which, at high concentration, down-regulates its own receptors (Smith and Cantrell, 1985).

It has been suggested that RCMC may be able to grow in the absence of added cytokines because they secrete their own and thus regulate the growth in an autocrine fashion (Chan et al., 1990). Thus any added rIL-3 together with the endogenous IL-3 may have resulted in a down-regulation of cell proliferation. To check for this possibility, culture supernatants of RCMC1.3.2 cells were used in an attempt to support the growth of rat bone marrow-derived cells. This could not be achieved, suggesting that the RCMC1.3.2 did not secrete any IL-3. Mouse mast cells were shown to have the capacity to secrete IL-3 among other cytokines when appropriately stimulated (Plaut et al., 1989).

It is also conceivable that, as suggested by Huff and Justus (1988), that RCMC cells may no longer require IL-3 for growth since they had already passed through the IL-3 dependent stage *in vivo* before having been established in tissue culture, thereafter the cells may, perhaps, respond to IL-3 differently compared to BM-derived cells which require IL-3 to proliferate *in vitro*.

CHAPTER V

GENERAL DISCUSSION

Mast cells have been demonstrated to originate from multipotential bone marrow stem cells (Kitamura et al., 1977; 1979a; 1981). Mast cell precursor cells mature and differentiate into the two phenotypes of mast cells, MMC and CTMC. It was found that as CTMC mature they show increased histamine content but decreased histamine synthesis, progressive sulphation of heparin, and the arrest of cell division in the later stages (Combs et al., 1965). The two phenotypes of mast cells have been found to interchange under the influence of different anatomical environments, however, the exact conditions that promote mast cell transdifferentiation have not been clearly identified. Another aspect which has not yet been clearly established is the factors responsible for the development of the IgE receptors, especially of the low affinity $Fc_{\epsilon}RL$. The high affinity $Fc_{\epsilon}RI$, as judged by the experimental methods used, has been found to be expressed on mastoblasts and the number or the affinity of the receptor was found to increase with the maturation of mast cells (Ginsburg and Sachs, 1962; Ishizaka et al., 1976). More recently it was shown that bone marrow derived MMC contain mRNA for $Fc_{\epsilon}RI$ component chains after about one week in culture (Thompson et al., 1990). However, since there have been difficulties in identifying and isolating pure populations of immature mast cells at different stages of mast cell maturation and more importantly in maintaining them *in vitro*, little is known about the natural pathways of mast cell differentiation *in vivo*.

Another aspect of mast cell research has dealt with the structure and function of IgE receptors. Those of $Fc_{\epsilon}RI$ have been extensively studied and much of the basis

of our knowledge on IgE receptors has been provided. Relatively little attention has been paid to $\text{Fc}_\epsilon\text{RL}$ which is now being actively investigated in this laboratory. It has been demonstrated that $\text{Fc}_\epsilon\text{RL}$, like $\text{Fc}_\epsilon\text{RI}$, is a glycoprotein but in contrast to the latter appears to be comprised of single polypeptide chain (Roth and Froese, 1982). It differs from $\text{Fc}_\epsilon\text{RI}$ in having a much lower affinity for IgE but like $\text{Fc}_\epsilon\text{RI}$ it cross-reacts with various subclasses of rat and mouse IgG (Kepron et al., 1988). However, its importance if any, in mast cell mediator release, and its expression during mast cell maturation and differentiation are not known.

Ginsburg (1963) enriched murine mast cell populations by seeding mouse thymus cells on mouse embryonic fibroblasts and Ishizaka et al. (1976) obtained rat mast cells by long-term culture of rat thymus cells on rat fibroblast monolayers. Subsequently, murine mast cells resembling MMC have been generated from bone marrow cells *in vitro* in the presence of conditioned medium from mitogen-stimulated splenocytes (Hasthorpe, 1980; Razin et al., 1981). Recently, murine mast cell lines (Kisv-MC) of CTMC phenotype were obtained by co-culturing splenocytes with fibroblasts producing a Ki-ras-containing murine sarcoma virus (Reynolds et al., 1988). Thus *in vitro* cultured mast cells have been proven useful for studying the conditions that support the growth of mast cells, and IL-3, IL-4 and IL-9 have been identified as mast cell growth factors (Fung et al., 1984; Lee et al., 1986; Hultner et al., 1988).

However, in this laboratory, rat tissue-cultured mast cell (RCMC) lines were established from peritoneal mast cell populations in the absence of exogenous growth factors and fibroblast feeder cells (Chan et al., 1988) and since then they have been maintained under the same culture conditions. Characterization of the RCMC lines

revealed that they were of the MMC phenotype as they stained Alcian blue⁺/ Safranin O⁻, contained the serine protease type II (RMCP^{II}) and had low levels of histamine content (Chan et al., 1990). These results suggested that these cells had originated from a small subpopulation of immature mast cells (Chan et al., 1990) known to be present in the peritoneal CTMC population (Beaven et al., 1983). The RCMC lines became of great interest to the laboratory because they not only showed varying receptor patterns but also demonstrated changes with time in culture in the ratio of the surface expression of the two types of IgE receptors. The cell line RCMC1 was cloned twice and both the clones and subclones showed various patterns in terms of relative numbers of the two receptors as well as in receptor densities.

The present investigation was aimed at making use of the RCMC cells to study the IgE Fc receptor development at the clonal level during *in vitro* tissue culture and to establish whether some of the subclones of RCMC might represent mast cells at different stages of maturation and differentiation.

Changes in receptor expression observed in RCMC were investigated systematically in time-dependent study of the IgE receptor development on RCMC subclones which appeared to differ most in terms of Fc_εR expression. The results, presented in Chapter II, demonstrated that the Fc_ε receptor expressions on all the clones studied invariably changed during the prolonged tissue culture period. However, clones with different receptor patterns at the beginning of the study exhibited diverse changes in the surface receptor numbers during the *in vitro* culture. Clones with little or no receptors, like RCMC1.2.5 and 1.3.1, started to express the low affinity Fc_εR_L and continued to do so as time in culture progressed. Clones expressing both Fc_εR_I and Fc_εR_L also began to

show an increasing expression of $\text{Fc}_\epsilon\text{RL}$, however, the $\text{Fc}_\epsilon\text{RI}(\alpha)$ expression was found to be variable, decreasing on RCMC1.3.2 and 1.11.2 but increasing on RCMC1.3.3. The results demonstrated that despite the differences in the original receptor pattern all the clones tested invariably had increased expression of $\text{Fc}_\epsilon\text{RL}$ but showed variation in the expression of $\text{Fc}_\epsilon\text{RI}(\alpha)$. The high affinity $\text{Fc}_\epsilon\text{RI}$ are the predominant receptors expressed on mature mast cells from the peritoneal cavity of the rat (Froese, 1980) and the intestinal mucosa (Swieter et al., 1989). It has also been shown in the previous studies that mast cells derived from thymocyte cultures demonstrated an increased IgE binding ability as they matured (Ishizaka et al., 1977). Since the IgE bound to the low affinity $\text{Fc}_\epsilon\text{RL}$ has been demonstrated to be removed by washing the increased IgE binding of these cells actually pointed to an increase in the expression of the high affinity $\text{Fc}_\epsilon\text{RI}$. Thus, $\text{Fc}_\epsilon\text{RI}$ expression appeared to be an indicator of mast cell maturity. Thus differences in the expression of this receptor on RCMC may be a reflection of difference in the state of differentiation of these mast cells.

The presence of IgE has been shown both *in vivo* and *in vitro* to influence the expression of $\text{Fc}_\epsilon\text{RI}$ (Malveaux et al., 1978; Furuichi et al., 1985). Indeed, addition of IgE to the culture medium, as described in Chapter IV, induced an elevated expression of both types of IgE receptors on RCMC clones which originally expressed them. Clone RCMC1.3.1, which expressed very little $\text{Fc}_\epsilon\text{RL}$ to begin with but showed increased levels as time in culture progressed, never developed $\text{Fc}_\epsilon\text{RI}(\alpha)$ expression either as time in culture progressed (Fig. 5) or upon the addition to the culture medium of IgE (Fig. 45) or the differentiation agents, PMA (Fig. 15), DM (Fig 22) or RA (Fig. 23). The inability of RCMC1.3.1 in expressing surface $\text{Fc}_\epsilon\text{RI}$ may therefore indicate that

this cell line is at an earlier stage of differentiation than the other cell lines studied. However, it cannot be excluded that RCMC1.3.1 may be defective in expressing $\text{Fc}_\epsilon\text{RI}$ due to mutation. It is possible that RCMC1.2.5 which like RCMC1.3.1 expressed no $\text{Fc}_\epsilon\text{RI}$ in culture would have reacted similarly to IgE and the differentiation agents. It is also tempting to speculate that RCMC1.3.1 may require another differentiation signal before it will respond to IgE with the appearance of $\text{Fc}_\epsilon\text{RI}$. Thus it may prove useful in future experiment to treat RCMC1.3.1 first with differentiation agents or growth factors followed by exposure to IgE. Alternatively, the cells could be treated with these agents and IgE simultaneously. The fact that bone marrow derived mouse MMC after one week of culture in the presence of IL-3 contained mRNA for all three chains of $\text{Fc}_\epsilon\text{RI}$ and expressed this receptor on their surface (Thompson et al., 1990) does suggest that RCMC1.3.1 may indeed be very immature. This interpretation is supported by the fact that RCMC1.3.1, like RCMC1.2.5 also expressed little or no $\text{Fc}_\epsilon\text{R}_\text{L}$ when first established in tissue culture.

Studies on the $\text{Fc}_\epsilon\text{R}$ development during long-term culture of RCMC clones described in Chapter II had already shown clonal variations and had suggested that the different clones may represent mast cells at different stages of differentiation. These cells, most likely, had their origin among the less mature cells which at the time of transfer to tissue culture may have reached different stages of maturity before becoming the mature CTMC and before losing their capacity to divide. Therefore, it was hoped that the various clones might respond differently to various physiological and pharmacological stimuli. Indeed, two of the differentiation agents used, PMA and DM, could already elicit different clonal responses as measured in terms of $\text{Fc}_\epsilon\text{R}$ expression and

histamine content of these cells.

The most interesting clone was RCMC1.3.1 which, at early stages in culture, was not modulated by PMA in terms of histamine content and by DM in terms of receptor expression; at late stage, was not modulated for both receptor expression and histamine content. Since $\text{Fc}_\epsilon\text{R}_\text{L}$ was found to be the first receptor to be expressed on this originally surface receptor negative clone and was also expressed on all the other clones, it may indicate again that RCMC1.3.1 represented cells at an early stage of differentiation. The intracellular regulatory mechanisms of $\text{Fc}_\epsilon\text{R}$ expression and histamine synthesis in these cells may not be fully developed and therefore these cells are not responsive to some of the modulators. On the other hand, the clone RCMC1.11.2 which, as shown in Chapter II, expressed relatively constant levels of $\text{Fc}_\epsilon\text{R}_\text{L}$ and a slightly decreasing $\text{Fc}_\epsilon\text{R}_\text{I}$, was not significantly modulated by PMA in terms of both receptor expression and histamine content. In this case the unresponsiveness of RCMC1.11.2 to PMA may have been due to the fact that receptor expression had already reached levels which no longer be up-regulated by the differentiation agents. Therefore this clone possibly represents mast cells at a more mature stage. The two other clones RCMC1.3.2 and 1.3.3, which with time in culture expressed increasing $\text{Fc}_\epsilon\text{R}_\text{L}$ numbers, exhibited similar responses to PMA and DM in increasing the $\text{Fc}_\epsilon\text{R}_\text{L}$ expression and histamine content and to RA in decreasing $\text{Fc}_\epsilon\text{R}_\text{L}$ expression and histamine content. These two clones may therefore represent clones intermediate to RCMC1.3.1 and 1.11.2. However, the two clones are obviously not quite identical since, as shown in Chapter II, upon long-term culture $\text{Fc}_\epsilon\text{R}_\text{I}$ numbers on RCMC1.3.2 decreased while those on RCMC1.3.3 increased. Therefore, the use of biological factors in the culture medium of the different RCMC

clones, as described in Chapter IV, had further pointed to subtle differences among these clones. The presence of IgE in culture medium induced an increased expression of both $\text{Fc}_\epsilon\text{R}_\text{L}$ and $\text{Fc}_\epsilon\text{RI}$ on RCMC1.3.2 and 1.3.3, however, the former showed a much slower response. In addition, other than the enhancing effect of anti- $\text{Fc}_\epsilon\text{R}_\text{L}$ Fab' fragment on the expression of $\text{Fc}_\epsilon\text{R}_\text{L}$ on all the clones studied, it showed no effect on $\text{Fc}_\epsilon\text{RI}$ expression on RCMC1.3.2 but increased the expression of this receptor on RCMC1.3.3. The evidence further supports the possibility that they represent mast cells at different stages of differentiation.

The increase in $\text{Fc}_\epsilon\text{R}_\text{L}$ and either increase or decrease in $\text{Fc}_\epsilon\text{RI}$ observed on the RCMC cell lines during the time-dependent studies (Chapter II) strongly suggested that the two receptors are regulated by independent mechanisms. However, the simultaneous increase in $\text{Fc}_\epsilon\text{R}_\text{L}$ and $\text{Fc}_\epsilon\text{RI}$ expression due to the presence of the Fab fragments of anti- $\text{Fc}_\epsilon\text{R}_\text{L}$ antibodies as well as IgE indicates that modulation of $\text{Fc}_\epsilon\text{R}_\text{L}$ also triggers a signal to increase the expression of $\text{Fc}_\epsilon\text{RI}$. This, therefore, suggests that the two types of receptors may have regulatory systems with at least one common pathway. However, the fact that $\text{Fc}_\epsilon\text{RI}$ is the major receptor expressed on PMC (Froese, 1980) and IMMC (Swieter et al., 1989) but $\text{Fc}_\epsilon\text{R}_\text{L}$ was found to be expressed at a higher density on most of the RCMC and its expression increased with time in culture, suggests that $\text{Fc}_\epsilon\text{R}_\text{L}$ expression may be actively suppressed by certain factors specific for the regulation of $\text{Fc}_\epsilon\text{R}_\text{L}$ during mast cell maturation *in vivo*. These suppressive factors may be absent in tissue culture system, thereby allowing for a relatively high expression of $\text{Fc}_\epsilon\text{R}_\text{L}$.

Certain lymphokines, such as IL-3, IL-4 and Con A-SCM which contain a variety of T cell factors, have been shown to support mast cell growth and differentiation.

Treatment of RCMC1.3.2 with rat rIL-3 and Con A-SCM on RCMC1.3.2 did not seem to affect the IgE receptor expression but increased the histamine content of these cells. As stated before, the histamine content has been used as one of the criteria to indicate mast cell maturity; the increase in histamine content in conjunction with the arrest of proliferation could indicate a maturation process in these RCMC cells. However, neither of the modulators, including PMA, DM, IL-3 and Con A-SCM which all increased the histamine content of the RCMC lines in the present investigation, induced a phenotypic change of the RCMCs from the MMC-like mast cells to CTMC. These results suggest that other factors are probably needed to trigger a phenotype switching process of the RCMC clones. A phenotype switch has been demonstrated by the *in vivo* transfer of mast cells of either phenotype (Nakano et al., 1985; Sonada et al., 1986) In *in vitro* studies, Levi-Schaffer and colleagues demonstrated that IL-3 is required for the differentiation of murine bone marrow precursors to BMMC and fibroblasts coculture induces further differentiation of BMMC to CTMC (Levi-Schaffer et al., 1986; Dayton et al., 1988). Therefore, it appears that a connective tissue environment provided by fibroblasts is important for the switch from MMC to CTMC. Fibroblast factors appear to be required for maintaining the CTMC phenotype (Jarboe et al, 1989). In maintaining mast cells in the *in vitro* system, others found that IL-3 has been necessary to just support the survival of mast cells whereas both IL-3 and IL-4 are essential for the *in vitro* growth of connective tissue-type mast cells (Hamaguchi et al., 1987; Tsuji et al., 1990). It would be very interesting to use both IL-3 and IL-4 to study the maturation and differentiation of RCMC lines when the rat IL-4 becomes available. In addition to these two cytokines a novel factor has recently been discovered to possess mast cell growth enhancing activity in both murine and human systems (Moeller et al., 1990;

Hultner et al., 1990). This factor, designated IL-9, has been shown to act independently of IL-3 and IL-4 and to support the growth of murine BMMC lines. It still needs to be established if an equivalent factor exists in the rat and if so, it could be another potential candidate for modulating the RCMC lines in future studies.

The RCMC cloned lines could be used as a nice model system to study the biochemical and transcriptional changes associated with mast cell maturation processes, especially in the expression of the low affinity receptor $Fc_{\epsilon}RL$. The two types of mast cells, MMC and CTMC, are known to interchange under different microenvironments (Nakano et al., 1985; 1987), however, the exact conditions required to induce these changes are yet to be defined. Since the RCMC clones are adapted to continuous tissue cultures in the absence of T cell growth factors or fibroblast feeder layers, exogenous factors could be added to the cultures in an attempt to induce these immature mast cells to differentiate into functionally mature mast cells and to define the conditions required to bring about the switch from MMC to CTMC.

To date there are only few known mast cell markers. One is $Fc_{\epsilon}RI$ which does not distinguish between MMC and CTMC but it may perhaps be absent from very immature mast cells. The only true differentiation markers are the mast cell proteases which in the rat are known as RMCPI and RMCPII and which distinguish CTMC and MMC, respectively (Woodbury and Miller, 1982; Woodbury et al., 1978b). However, on the basis of these two enzymes it seems only possible to distinguish between mature CTMC and all other mast cells. In other words, among the cells which contain RMCPII there may be some which are at different stages of differentiation. These cells may be characterized by different surface markers. Therefore if some of the RCMC clones

represent mast cells at different stages of differentiation, it may be possible to generate monoclonal antibodies to different surface markers on these clones. The mABs could then be used to identify the *in vivo* differentiating mast cells.

In summary, the present investigation has achieved the major objective in demonstrating that the clonal variations in IgE receptor expression and in their time-dependent development observed among the RCMC cloned lines are derived from their differences in cell maturity and stages of mast cell differentiation, through the modulation of these lines with differentiation agents and biological factors. In addition, this study provides more information on the expression of $Fc_{\epsilon}R$, especially the low affinity $Fc_{\epsilon}R_L$. It is found that $Fc_{\epsilon}R_L$ is expressed more widely among the RCMC clones than mast cells isolated from different sites *in vivo*, and possibly $Fc_{\epsilon}R_L$ expression is actively suppressed by certain factors present in the *in vivo* environment. The expression of the two types of IgE receptors was shown to be subject to independent regulatory mechanisms with perhaps one common initial pathway. The increase in the PKC activity and in the expression of $Fc_{\epsilon}R_L$ by PMA modulation indicates the involvement of protein kinase C in the regulation of $Fc_{\epsilon}R_L$ expression. The use of differentiation agents, i.e., PMA, DM, and growth factors, i.e., Con A-SCM and IL-3, in the culture medium have induced an increase in histamine content of most of the RCMC clones and possibly a maturation-like process. However, none of them, also including the natural ligand IgE and anti- $Fc_{\epsilon}R_L$ antibodies which may be equivalent to the functions of IgG *in vivo*, appears to trigger a phenotypic switch of the RCMC clones from MMC to CTMC, indicating that either additional factors or a combination of the factors are required to bring about the phenotype switch.

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