REGULATION OF INTERLEUKIN-6 AND TUMOR NECROSIS FACTOR-α PRODUCTION IN RAT PERITONEAL MAST CELLS BY LIPOPOLYSACCHARIDE, PROSTAGLANDIN E_1, PROSTAGLANDIN E_2, AND CHOLERA TOXIN

by

IRENE LEAL-BERUMEN, QBP., M. Sc.

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AUTHOR: Irene Leal-Berumen, QBP (University of Chihuahua, Mexico)

M. Sc. (CINVESTAV-IPN, Mexico)

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ABSTRACT

The mast cell has been implicated as an initiating cell in the immediate responses to allergen challenge where preformed mediators such as histamine play an important role. However, the role of mast cells is less well understood in severe allergic disorders such as asthma where chronic inflammatory changes are present. Results presented in this thesis demonstrate that freshly isolated and highly purified rat peritoneal mast cells can release IL-6 without any necessity for histamine release. These observations were determined with the use of bacterial products such as LPS and CT which significantly enhanced IL-6 production in our system.

Prostanoids of the E family (PGE_1 and PGE_2) were also used as stimulating agents which may also participate in inflammation. We observed that these prostaglandins selectively enhanced IL-6 production while TNF-α synthesis was significantly inhibited CT was observed to have very similar effects to PGE_1 on mast cells . These findings illustrate the potential role that mast cells may have during chronic inflammation and infectious disease.

PREFACE

Chapters 2, 3 and 4 of this thesis are in manuscript form and represent published papers (chapters 2 and 3) or manuscript accepted for publication (chapter 4). The practical work and the majority of the intellectual contribution presented in each of these chapters was provided by Irene Leal-Berumen. These research papers were also all written in thier entirety by the author of this thesis. The supervision of research progress, provision of reagents or specific areas of pharmacological expertise resulted in the inclusion of other authors in each of the manuscripts.

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Finally, I would like to thank my husband Edmundo for his support, understanding, and because he always believed that I could finish my Ph.D., but most of all for his love and for our wonderful son who gave me enough pressure to finish the writing of this thesis.

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Abbreviations:

BAL	Bronchoalveolar lavage
CAM	Cell adhesion molecules
cAMP	3':5'-cyclic adenosine monophosphate
CT	Cholera toxin
CTMC	Connective tissue mast cells
DG	Degranulated mast cell
DTH	Delayed-type hypersensitivity
G	Granulated
GPI	Glycosylphosphatidyl inositol
hIL-6	Human IL-6
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cells
IgE	Immunoglobulin E
IL-	Interleukin-
IP_3	Inositol phosphate
LPS	Lipopolysaccharide
LBP	Lipid binding protein
LT	Leukotriene
MMC	Mucosal mast cell
PAF	Platelet activating factor
PBMC	Peripheral blood mononuclear cells

PG	Prostaglandin
PGEs	Prostaglandin of the E series
PMCs	Peritoneal mast cells
RA	Rheumatoid arthritis
RMCP	Rat mast cell protease
rmTNF-α	Recombinant mouse TNF-α
TNF	Tumor necrosis factor

Aims of the thesis:

I intend with this thesis to share the experience I obtained working and studying on mast cells *in vitro* . In this project we decided to study the cytokine production of freshly isolated and purified peritoneal mast cells obtained from Brown Norway rats with no previous treatment. Once the cells were isolated we performed *ex-vivo* studies where highly pure mast cells were cultured (usually for 18 hr) with different agents such as LPS, anti-IgE, PGE_2, CT, or media as control. We mainly examined mast cell cytokine production in the context of inflammation. You will find that our studies mainly showed the effect of a number of agents which could mimic a cell response during inflammation on mast cell IL-6 and TNF-α production by these cells. Histamine release by mast cells was used as a cell degranulation control.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Mast Cells.

1.1.1 Description, distribution and origin.

The term mast cell come from German "**mastzellen**" which means "stuffed cell" (Ehrlich, 1878). Ehrlich (1879) was the first to identify mast cells in human connective tissue, and basophils, a type of circulating leukocyte, by using the metachromatic staining properties of their cytoplasmic granules. Although these two cells share staining properties and several notable features there are many important differences which lead to the differentiation of mast cells and basophils as totally separate cell types (Galli and Lichtenstein, 1988);(Table 1).

Table 1. Differences between Mast cells and Basophils.

Characteristic	Basophils	Mast cells
Origin of precursor cell	Bone marrow	Bone marrow
Site of maturation	Bone marrow	Connective tissues
Mature cells in circulation	Yes	No
Mature cells in connective tissue	No	Yes
Life span	Days	Weeks to months
c-Kit expression	low/negative	positive
Mast cell protease expression	low/negative	positive

Note: most data for basophils are derived from studies in humans and guinea pigs, whereas for mast cells the data comes from rodents. (Modification from Galli, S.J.) (1990).

The morphological features of human mast cells have been well documented by Ann M. Dvorak and others (1986; 1988). Mature human mast cells have a round or oval shape, and are approximately 6-12 μM in diameter, their nucleus is round or oval but not normally segmented. Mast cells also contain small but numerous metachromatic granules in their cytoplasm which may fuse during degranulation (fig 1).

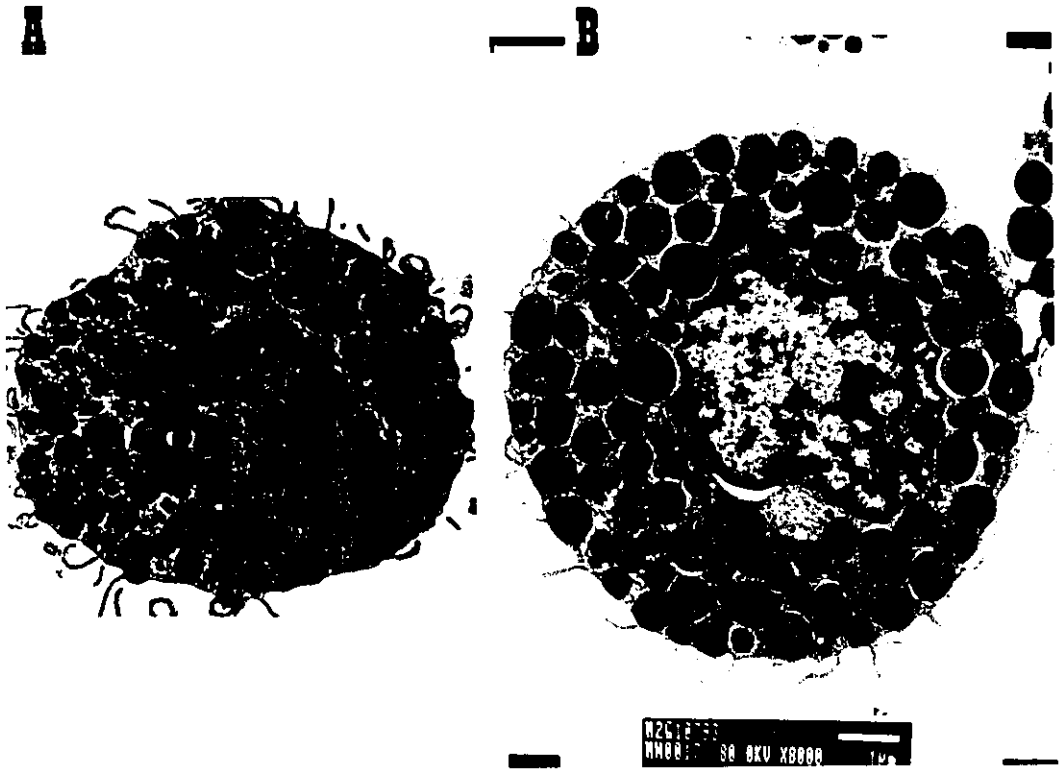

Fig.1 A) Electron microscopy of a human lung mast cell (Dvorak, 1986), B) Electron microscopy of a rat peritoneal mast cell (our studies).

Mast cells are distributed throughout normal connective tissue, often localized adjacent to blood and lymphatic vessels, near nerves, and beneath epithelial surfaces (Selye, 1965; Metcalfe *et al.* 1981; Galli *et al.* 1984; Bienenstock *et al.* 1991). Mast cells are most frequent at body sites which interact directly with the external environmental antigens such as skin, respiratory and gastrointestinal tracts (Enerback *et al.* 1989; Harper *et al.* 1989). Unlike basophils, mast cells do not normally circulate in the blood.

Kitamura et al. (1978; 1979) have shown that mast cells are derived from bone marrow precursors by using genetically mast cell-deficient mutant mice (W/W^v) and their congenic normal (+/+) littermates. In vitro studies also have confirmed the hematopoietic origin of mast cells demonstrating that their development is regulated by a number of factors including IL-3 (Ihle *et al.*), IL-4 (Hamaguchi *et al.* 1987), IL-9 (Hultner *et al.* 1990), IL-10 (Thompson-Snipes *et al.* 1991), and c-kit ligand or stem cell factor (SCF) (Zsebo *et al.*).

1.1.2 Mast cell heterogeneity

The concept of mast cell heterogeneity is nearly one hundred years old. Maximow described in 1905 that mast cells from the rat intestinal mucosa stained differently than mast cells from other anatomical sites. It is now generally believed that such distinct mast cell populations may be the result of different environmental conditions which will influence the cell to express different functional and morphological characteristics. There is much recent evidence which demonstrates that mast cells from different sites can have substantial differences in mediator content, sensitivity to secretagogues and

responses to pharmacologic agents (Galli and Lichtenstein, 1988; Kitamura, 1989; Schwartz, 1989).

In rodents there are two well defined mast cell populations: the "mucosal mast cells" (MMC) observed in the lung and intestinal lamina propria, and the "connective tissue mast cells" (CTMC) which are found in the skin, peritoneal cavity, and other sites (Enerback, 1981; Enerback, 1986). These two populations differ in a number of histochemical and functional features. MMC can only be visualized following Carnoys or basic lead acetate fixation, while CTMC can easily be stained following fixation with formalin (Enerback, 1987). Both cells stain with Alcian blue but only CTMC stain with Safranin O. These cell types have a different content of proteoglycans and proteases, MMC contain mainly chondroitin sulphate di-B and RMCP II (rat mast cell protease), whereas CTMC contain heparin and RMCP I (Lee *et al.* 1985). MMC also have substantially less histamine and 5-hydroxy tryptamine than CTMC (Lee *et al.* 1985). However, the differences between CTMC and MMC are not limited to their mediator content, functional differences have also been recognized. For example, MMC isolated from the intestinal mucosa of a previously *Nippostrongylus brasiliensis* infected rat do not respond to compound 48/80 and the bee venom peptide 401, while mature CTMC isolated from the peritoneal cavity will rapidly degranulate in response to these agents (Lee *et al.* 1985; Befus, 1986) (Table 2).

Table 2. Mast cell heterogeneity in rats.

	CTMC	MMC
Proteoglycans		
Heparin	+++	0
Chondroitin sulphate di-B	+	+++
Other mediators		
Histamine	+++	+
5-Hydroxy tryptamine	++	+
Serotonin	+++	+
Serine Proteases		
RMCP I	+++	0
RMCP II	+	+++
Response to		
48/80	rapid degranulation	no degranulation
Bee venom peptide 401	rapid degranulation	no degranulation
Staining with Alcian blue	+	+
Staining with Safranin O	+	-

Adapted from Lee et al. (1985), Miller et al. (1989), and Marshall et al. (1990).

Another important difference between these two mast cell populations is that MMC hyperplasia is T cell dependent during an immune response to certain nematode parasites such as *Nippostrongylus brasiliensis or Trichinella spiralis*. In contrast, CTMC hyperplasia shows little or no T cell dependence (Ruitenberg and Elgersma, 1976; Mayrhofer and Fisher, 1979). Although there are clear differences to classify rodent mast cells in two groups it is important to emphasize that there is evidence of mast cells with intermediate features between those of MMC from the intestinal mucosa and CTMC such as those obtained from the peritoneal cavity under certain circumstances (Ruitenberg and Elgersma, 1976; Mayrhofer and Fisher, 1979). These findings may further reflect the important influence of the surrounding environment in the expression of functional and mediator content characteristics. In the studies described in this thesis we have examined the mature peritoneal mast cell exclusively which is an example of the rodent CTMC type.

Human mast cells also show significant variability and have been classified into two main groups accordingly to their enzymatic content: mast cells which contain human mast cell tryptase but not mast cell chymase (MC_T) and those with both tryptase and chymase enzymes (MC_{TC}) (Schwartz *et al.* 1987). MC_T could be considered in some ways as analogous to the rodent MMC type because they can be found in the mucosa of the small intestine, whereas the MC_{TC} (which can be observed in the skin) in some ways resembles CTMC of the rodent (Irani *et al.* 1986). However the correlation between these enzyme markers and human mast cell functional properties of these cells is not as strong as is observed in rodents. The degree of heterogeneity that is seen in human mast

cells may reflect a greater complexity in cell development and a more diverse array of environmental stimuli than that observed in laboratory rodents.

1.1.3 IgE mediated mast cell activation

Mast cells can be activated by a wide variety of stimuli. The classic allergic reaction (fig. 2), where mast cells are known to play an important role, is initiated when an individual is in contact with a specific antigen that binds to IgE, which is attached to the high affinity IgE receptor ($Fc_{\varepsilon}RI$) on the mast cell surface (Hohman and Hultsch, 1993). A crosslinking of cell-bound IgE will aggregate the $Fc_{\varepsilon}RI$ causing a biochemical signalling cascade which leads to mast cell activation. There are $1x10^4$ to $1x10^6$ FcεRI receptors per mast cell (Coleman and Godfrey, 1981) and degranulation can occur with aggregation of 1-15% of these receptors (Oelz *et al.* 1977). This event in turn leads to the release of many mast cell mediators throughout three possible routes: granule release, de novo lipid metabolism, or transcriptional and translational activation (Siraganian, 1988).

Beaven and Metzger (1992) have reviewed the signal transduction by Fc receptors where they summarized the main biochemical events associated with the FcεRI which include (adapted from the AAAI 1995 meeting): enhanced arachidonic acid metabolism, phospholipase C activation, phospholipase D activation, Ca^{+2} mobilization, activation of tyrosine kinase, protein kinase C activation, granule fusion and release, transcriptional activation, cytokine production and release.

Metzger et al. (1986) have shown that the FcεRI from rat basophilic leukemia (RBL) cells is a tetramer consisting of a single IgE-binding α subunit, a single β

subunit, and two disulfide linked γ subunits. A similar structure is observed in the human (Kinet, 1989a). Moreover, Oliver and others (1985; 1988) have reported that during the IgE mediated cross-linking of FcεRI, at least two receptor-associated protein tyrosine kinases are activated (Syk and Lyn), assembly of F-actin is elicited, and membrane ruffling, increased spreading and adhesion is observed in RBL-2H3 cells. FcεRI plays a central role in the initiation of allergic reactions. Understanding the biochemical signals which mediate the activation of the high affinity receptor and the processes that are initiated in the cell are being widely studied with a view to developing new therapeutic approaches to allergy.

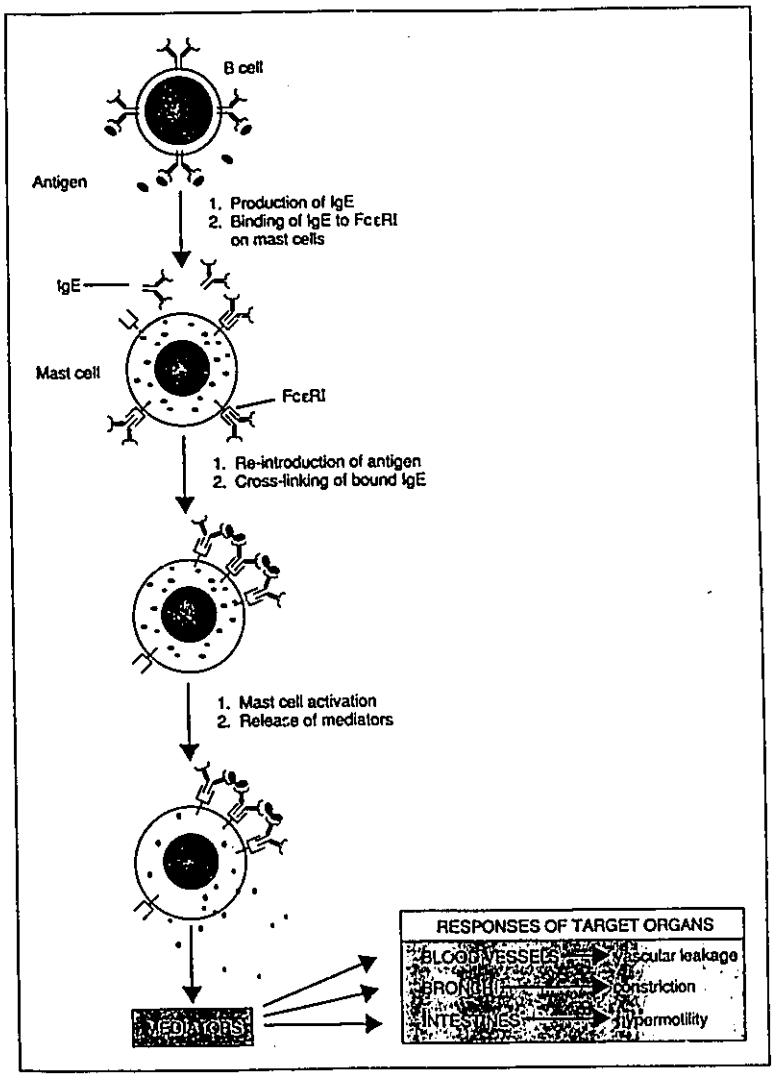

Fig. 2. Events that take place during an immediate mast cell response. Adapted from Abbas et al. (1991)

1.1.4 Mast cell mediators (fig 3)

The first events during mast cell activation occur within minutes after stimulation, whereas new cytokine production involves new protein synthesis and this is maximal several hours later (Hohman and Hultsch, 1993). However, it has been shown that mast cells contain preformed TNF-α which could be released immediately after stimulation (Galli *et al.* 1991). It remains to be determined if other cytokines may be stored by mast cells in a similar way. From our *ex-vivo* studies presented in this thesis, we know that IL-6 is not a preformed cytokine in rat peritoneal mast cells (PMCs), but rather it is a newly formed and can be detected as early as three hours after mast cell stimulation.

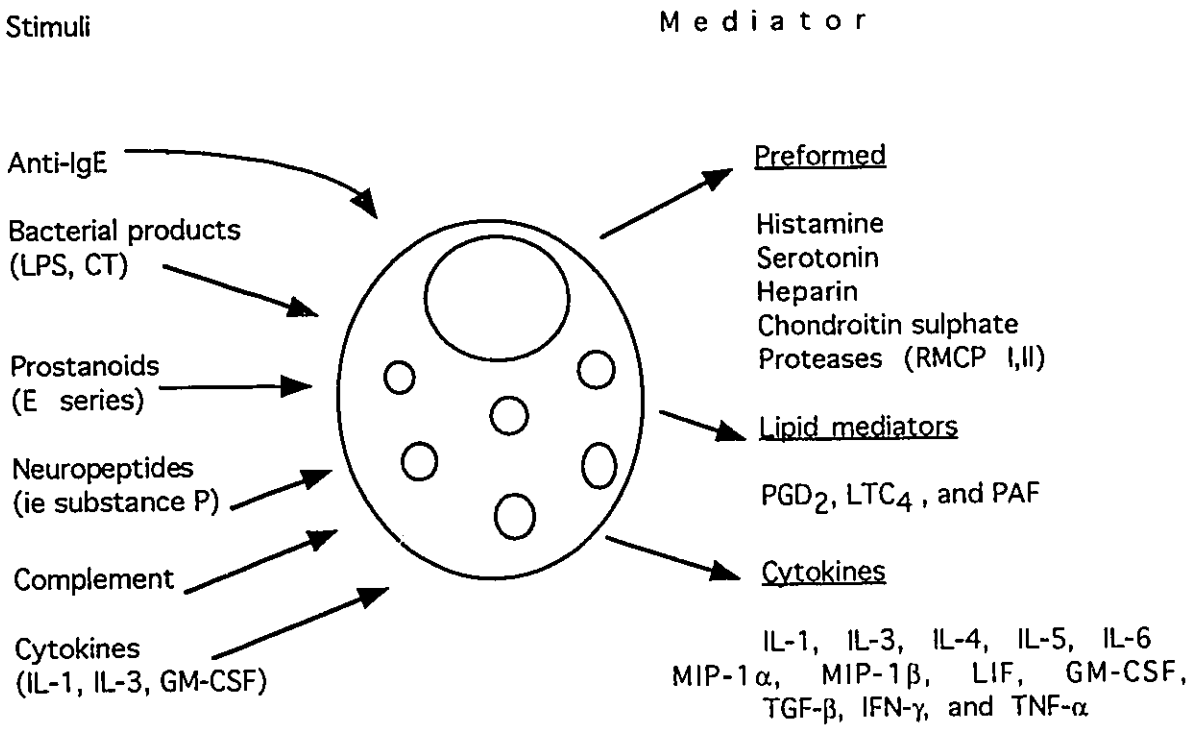

Fig. 3. Mediators that can be released from rodent mast cells in response to different stimuli.

1.1.4.1 Preformed mediators

Mast cells contain a wide variety of granule associated mediators including histamine, protease enzymes, and proteoglycans, which are released upon cell degranulation. However, we will discuss histamine in greater detail because we used histamine release as a degranulation marker in our studies.

Histamine (β-imidazolyl-ethylamine)(fig. 4) was first described by Windaus and Vogt in 1907. It is formed during decarboxylation of histidine by a pyridoxal phosphate-dependent enzyme, histidine decarboxylase (Falus *et al.* 1992). Most histamine in mammals is stored in mast cells (human mast cells contain 4-10 pg/cell) and basophils (human basophils contain 1pg/cell) (Casale, 1988). An unstimulated cell has an acidic pH which keeps histamine associated through ionic linkage with carboxyl groups of proteoglycans and/or proteins (Johnson *et al.* 1980; Lagunoff and Rickard, 1983). Following degranulation, histamine is dissociated from the proteoglycan-protein complex. Its rapid metabolism suggests that histamine may have its major actions near the site of release (Nilsson, 1989).

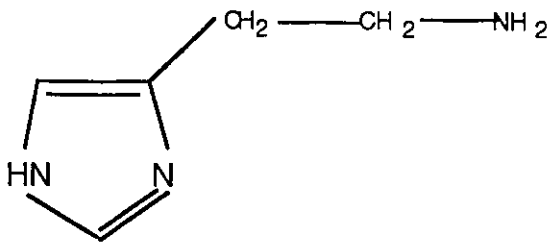

Fig. 4. The structure of histamine. Adapted from Cavanah and Casale (1993).

The role of histamine has been well characterized in mediating allergic reactions. Histamine is known to participate in numerous physiological and pathophysiological processes including vasodilation and vasoconstriction, gastric acid secretion, ureagenesis, and neuro-transmission (Falus *et al.* 1992). Histamine also plays an important role in immune and inflammatory reactions through its action on different histamine receptors. So far, three types of histamine receptor have been described H_1, H_2, and H_3 which are differentially expressed on specific tissues and cell types (Ash and Schild, 1966; Black *et al.* 1972; Arrang *et al.* 1983). Histamine can regulate cytokines, for example, acting through the H_2 receptor it downregulates endotoxin-induced IL-1 and TNF-α production by human peripheral blood mononuclear cells (Dohlsten *et al.* 1988). Histamine it also has a direct inhibitory effect on IL-2 production by the same cells (Carlsson *et al.* 1985). Cytokines can modulate histamine production by mast cells and basophils as well. IL-1 has been shown to potentiate the effect of histamine on the release of prostaglandin by endothelial and hematopoietic precursor cells (Revtyak *et al.* 1988). During an allergic response histamine is an important mediator in the early response through its physiological actions. In combination with other mediators it may also play an important role initiating a late phase response and in modulating ongoing immune and inflammatory events.

1.1.4.2 Lipid mediators

Lipid mediator production is initiated immediately after classical mast cell activation. Production of three main classes of lipid mediators, prostaglandins (PG), leukotrienes (LT), and platelet activating factor (PAF) have been described by mast

cells. Hydrolysis of membrane bound phospholipids yield free arachidonate through enzymatic pathways involving phospholipases A_2 and C (Davidson and Dennis, 1989; Dennis, 1990). Arachidonic acid metabolism then follows either the cyclooxygenase or lipooxygenase pathways which finally lead to lipid mediators (fig 5).

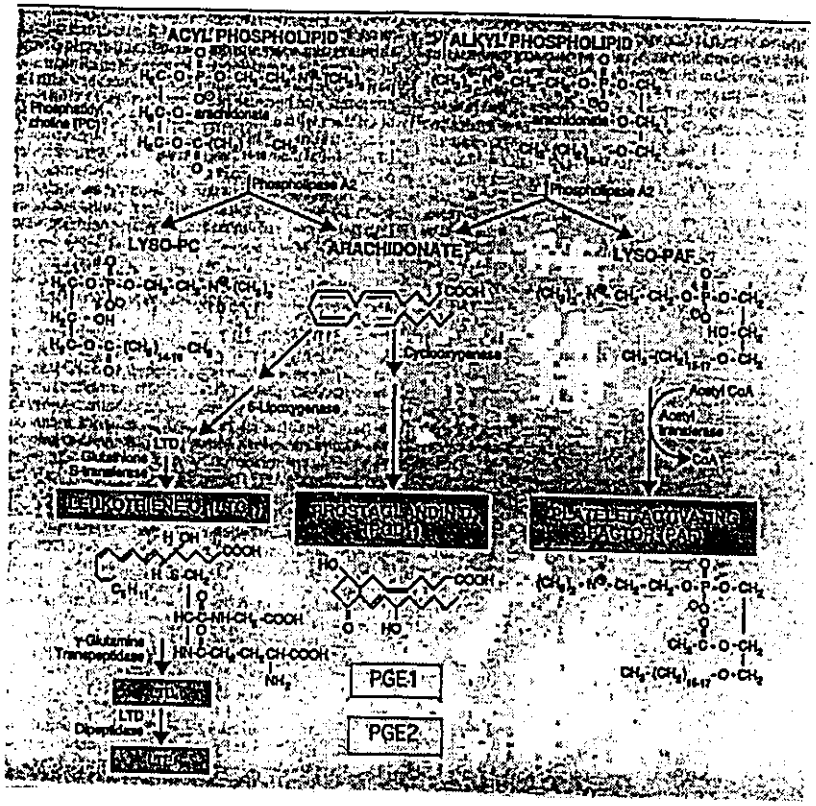

Fig. 5. Biosynthesis of lipid mediators that occurs in a number of cell types. Adapted from Abbas et al. (1991).

In rodents, CTMC such as the PMC we used in our studies, produce mainly prostaglandin D_2 (PGD_2) with little or no synthesis of PG of the E series (PGEs) detected following IgE mediated activation, whereas LTC_4 is the predominant lipid mediator in MMC (Schwartz, 1994). Both mast cell types produce PAF.

PGD_2 can be produced by a number of cell types other than mast cells, which include activated human alveolar macrophages (MacDermot *et al.* 1984), platelets (Oelz *et al.* 1977), and CNS neurons (Hayaishi, 1991). PGD_2 is chemoattractant for neutrophils (Goetzl and Pickett, 1981), it is a potent inhibitor of platelet aggregation (Mills and Macfarlane, 1974). Exogenous PGD_2 does not alter histamine release from human pulmonary and CTMC (Lewis, 1981), however, it enhances histamine release from human basophils in response to antigen, phorbol esters, and calcium ionophore (Lichtenstein *et al.* 1984). PGD_2 has been shown to be released from lung mast cells during asthmatic bronchoconstriction (Beasley *et al.* 1987).

The effects that PGD_2 may have on mast cell cytokine production have not been well studied. However, results from this thesis (*see chapter 3)* shows that although PGEs have an effect on IL-6 and TNF-α production by rat PMCs, PGD_2 does not. The use of flurbiprofen, which blocks the arachidonic acid pathway, supports these results because it showed a significant decrease in PMCs PGD_2 production in response to anti-IgE. However, flurbiprofen had no effect upon anti-IgE mediated IL-6 or TNF-α production. We also observed that lipopolysaccharide (LPS) does not induce PGD_2 production in PMCs (data not shown).

LTC_4 has bronchoconstriction activities in normal subjects (Holroyde *et al.* 1981) and elevated levels of LTC_4 have been recovered from the bronchoalveolar lavage fluid of subjects with stable asthma (Pliss *et al.* 1990) suggesting that this lipid mediator may play an important role in the airway narrowing which is seen in asthma.

PAF has been suggested to participate in allergic disorders and inflammation because of its ability to increase vascular permeability and the accumulation of inflammatory cells, most of which have specific binding sites for PAF (Pretolani, 1995).

1.1.4.3 Mast cell cytokines

Cytokines are proteins that exert their biological effects by interaction with specific surface receptors on target cells (Costa, 1993). Cytokines possess a broad spectrum of bioactivities that play a role in cell growth, repair, inflammation, and the immune response (Schleimer *et al.* 1989; Galli, 1990). Cytokines are mainly pleiotropic molecules and there is evidence of some redundancy in their function.

Mast cells as a potential source of cytokines was first described in mast cell lines in response to IgE-dependent activation (Wodnar-Filipowicz *et al.* 1989). However, it is now well recognized that mast cell lines and freshly isolated mast cells do not always produce the same cytokine repertoire (Marshall *et al.* 1993). From our studies we can give a clear example: RBL cells (a mucosal mast cell line) do not produce IL-6 (data not shown). A wide range of cytokines has been reported in human mast cells including TNF-α, IL-3, IL-4, IL-5, IL-6 and IL-8 (Benyon *et al.* 1991; Bradding *et al.* 19; Bradding *et al.* 1993; Moller *et al.* 1993). In cultured rodent mast cells the

production of IL-1, IL-3, IL-4, IL-5, IL-6, MIP-1α, MIP-1β, LIF, GM-CSF, TGF-β, IFN-γ, IL-10, IL-12 and TNF-α have been confirmed either at the mRNA or protein level (Marshall *et al.* 1987; Burd *et al.* 1989; Kulmburg *et al.* 1992; Plaut *et al.* 1989; de Waal Malefyt *et al.* 1992; Wodnar-Filipowicz *et al.* 1989; Marshall *et al.* 1993; Tan *et al.* 1993; Smith *et al.* 1994). Although there is not definitive evidence to confirm that different mast cell populations (CTMC vs MMC, or MC_T vs MC_{TC}) produce different cytokine profiles in response to the same stimuli, we should consider the potential influence of other local surrounding factors such as cell contact and cytokine influence on mast cell cytokine expression.

Studies of mast cells has been mainly performed in rodents, and although interesting advances in understanding the role of mast cells in health and disease have emerged from these studies, it is not always possible to extrapolate these results to human. Therefore, intensive studies on human mast cell cytokine production have been carried out recently. Several groups have reported the localization and release of IL-4 by human mast cells and basophils (Bradding *et al.* 19; MacGlashan *et al.* 1994). IL-4 appears to play an important role in the switching of B cells to synthesize IgE (Coffman *et al.* 1986) and it has been suggested by Kay et al. (1991) that IL-4 is upregulated in human allergic disease. Th_2 cells are a good source of IL-4, however they require a pulse of IL-4 to initiate this production (Le Gros *et al.* 1990; Swain *et al.* 1990). Bradding et al. (19) proposed that mast cell activation during an allergic response could provide the local pulse of IL-4 necessary to trigger Th_2-IL-4 production and to initiate the cell recruitment and activation which is seen during inflammation. Moreover, IL-4 induces VCAM-1 expression on endothelial cells, an adhesion molecule which is

important in the selective recruitment of eosinophils observed in allergic inflammation (Masinovsky *et al.* 1990; Thornhill *et al.* 1991). Moller et al. (1993) have demonstrated IL-8 production in human mast cell lines (HMC-I cell line) and in human skin mast cells following IgE mediated activation. IL-8 is another cytokine which could be important during immediate as well as in late phase allergic reactions, because it is a potent neutrophil and lymphocyte chemoattractant (Moller *et al.* 1993).

Some of the increased cytokine expression observed in tissues of individuals with allergic disease may originate from the mast cell but the contribution of mast cells compared to other cells has not been properly evaluated. Bradding et al. (1993) recently immunolocalized IL-4, IL-5 and IL-6 in human mast cells of normal and perennial rhinitic subjects, indicating the importance of mast cell cytokines during the chronic mucosal inflammation that is seen during allergic rhinitis. TNF-α has been observed in human lung and skin mast cells (Walsh *et al.* 1991), such mast cell derived TNF-α can enhance ELAM-, ICAM-1 and VCAM-1 expression on vascular endothelial cells which could contribute to the leukocyte infiltration observed during a late phase response (Klein *et al.* 1989b).

There are many limitations to much of the work examining human mast cell cytokine expression. Many studies are limited to immunohistochemical analysis which can not distinguish between production, uptake or receptor binding of the cytokine in question. *In vitro* studies of cytokine production are limited by the lack of good reproducible techniques to obtain large numbers of purified human mast cells.

The concept of mast cells as an important source of cytokines has changed our view regarding the potential role that these cells may have in different diseases.

Moreover, the potential for unique mechanisms of cytokine regulation and induction in mast cells increases the interest in these cells as a therapeutic target.

1.1.5 Role of mast cells in inflammation and in different diseases.

Inflammation is a complex process where several cell types play an important role and a cascade of different factors may orchestrate the response of these cells. However, in order to understand the potential role of each cell type it is also important to study them in isolation.

Mast cells can no longer be regarded simply as cells that initiate acute allergic reactions through the release of preformed mediators such as histamine. If we consider the diversity of mediators and multifunctional cytokines that can be produced by mast cells in response to different stimuli and the broad spectrum of biological responses which can be influenced by these mediators or cytokines, it is not difficult to suggest a major role of mast cells during inflammation. Mast cells have been implicated in a number of other chronic inflammatory conditions such as inflammatory bowel disease (Beck *et al.* 1989; Tillie-Leblond *et al.* 1994) and rheumatoid arthritis. Despite these clinical observations there is little information regarding the consequences of repeated mast cell activation on mast cell mediator production *in vivo.* Moreover, the actual methodology to detect mast cells histologically depends on the dye binding properties of mast cell granules, however, after mast cell degranulation we can no longer easily detect the "phantom" mast cells which could remain in the tissue in an active state. This phenomenon has been well described in Scleroderma patients (Hawkins *et al.* 1985).

1.1.5.1 Mast cells and inflammatory bowel disease

Inflammatory bowel diseases are chronic inflammatory disorders of the intestine of unknown origin which at a histological level are characterized by an intense active inflammatory infiltrate of macrophages and lymphocytes, including large numbers of plasma cells (Schreiber *et al.* 1992).

Controversial results have been reported regarding the relationship between mast cell numbers in IBD, some groups have observed an increase number of mast cells in ulcerative colitis, proctitis and Crohn's disease (Sommers, 1966; Dvorak *et al.* 1978; Dvorak *et al.* 1980) while others disagree (Lloyd *et al.* 1975). These results may not be surprising due to the potential for inaccurate quantitation of activated mast cells. Degranulated mast cells, mentioned previously, may not be seen by regular staining, or stains like Toluidine Blue (Enerback, 1966) may not be specific since other cells including eosinophils and basophils may be stained with this dye. A further problem in many studies has been the use of inappropriate fixation conditions, for example, when aldehyde fixatives are used instead of Carnoy's solution the number of human mast cells visualized with toluidine blue staining decreases (Schwartz, 1987) .

Goldsmith et al. (1990) found a significant decrease in tryptase-containing mast cells in patients with IBD which were treated with steroids compared with non treated patients and controls. To avoid the problems we mentioned above regarding mast cell quantification the authors used a monoclonal antibody against human mast cell tryptase to estimate mast cell numbers. Moreover, their results also suggest that glucocorticoids may mediate improvement in IBD through mast cell depletion.

Inflammatory mediators and cytokines have been viewed as particularly interesting research areas for both increasing our understanding of chronic inflammation in IBD and as targets for therapeutic intervention (Schreiber *et al.* 1992). Mast cell mediators such as histamine may have an important role in IBD by inducing the synthesis of leukotrienes, prostaglandins, and thromboxanes (Platshon and Kaliner, 1978; Fox *et al.* 1990). Raithel et al. (1994) demonstrated that both active Crohn's disease and ulcerative colitis patients' intestinal mucosa released greater amounts of histamine than did tissue from normal controls and patients with quiescent IBD. For example, several groups have shown that administration of recombinant IL-1 or TNF-α to rodents induced changes in the intestine similar to those observed in IBD (Patton *et al.* 1987; Remick *et al.* 1987; Butler *et al.* 1989). Others have reported that IFN-γ increases mucosal permeability by injuring epithelial tight junctions and inducing adhesion molecule expression which enhances cellular transmigration across the endothelial monolayers (Kelly *et al.* 1992; Colgan *et al.* 1993). Circulating monocytes from patients with active IBD, particularly those with Crohn's disease, spontaneously secrete IL-1, IL-6, and IL-8 (Hyams *et al.* 1993). Tissue levels of IL-1, IL-6 and IL-8 correlate well with endoscopic and histological evidence of inflammation in ulcerative colitis, but a lower degree of correlation was observed in Crohn's disease (Isaacs *et al.* 1992). However, the role of mast cells relative to other sources for these mediators still needs to be further studied.

1.1.5.2 Mast cells in Rheumatoid arthritis (RA)

The joint destruction that is seen in chronic arthritis involves the action of many cell types. Inflammed synovium is a mast cell-rich tissue, however the mechanisms of synovial mast cell participation in arthritis is not well understood (Malone, 1993). Rheumatoid arthritis is considered to have many similarities to a delayed-type hypersensitivity (DTH) reaction in synovium, which may result from antigen deposition through the relatively "leaky" synovial fenestrated capillary bed. Histologic studies have shown a correlation between synovial mast cell number and the clinical inflammatory index of tissue seen in rheumatoid arthritis patients (Crisp *et al.* 1984; Malone *et al.* 1987). As discussed above, mast cells could contribute to the disease process by their release of a broad variety of mediators and cytokines. Several groups have shown in animal models that the acute and chronic phases of rat adjuvant arthritis can be abrogated by blocking the histamine receptor (Al-Haboubi and Zeitlin, 1982; Vickers and Sykes, 1982). The potential role of cytokines in RA has also been considered by different groups. TGF-β has been localized in rheumatoid synovium (Lotz *et al.* 1990; Wahl *et al.* 1990) and Gruber et al. (1994) have shown that TGF-β can induce mast cell chemotaxis. Wahl et al. (1991) also observed that the local administration of cytokines, such as TGF-β or IFN-γ, promote synovial hyperplasia and erosive destruction of bone and cartilage. IL-6 can stimulate chondrocyte proliferation (Guerne and Lotz, 1991) and LIF has been shown to be overexpressed in human arthritis (Lotz *et al.* 1992). Both cytokines can be produced by synoviocytes, however we should also consider mast cells as a good source of these cytokines or as a target cell

where other cytokines may exert an important effect in potentiating the chronic inflammatory response in RA.

1.1.5.3 Mast cells and allergic inflammation of the airways

Activation of mast cells and basophils through receptors for IgE on their cell surfaces leads to the release of a variety of chemical mediators and this property has lead many groups to believe these cells are important participants in the immediate hypersensitivity response seen in airway disorders (allergy, asthma, rhinitis). However, activation of mast cells was not considered relevant to the late phase inflammatory response until recently. Attention by scientists examining the late phase response has focused on the ability of T lymphocytes to synthesize and release cytokines particularly the Th_2 cytokines which may favor the induction and maintenance of allergic inflammation (Ricci, 1992). Redington and Howarth (1994) recently reviewed the current evidence which supports the observation that airway mast cells synthesize, store and release cytokines relevant to allergic inflammation and consider their potential in the late phase response as well as in fibrotic structural changes that occur in chronic disease.

Asthma has been described as "a condition characterized by widespread airway narrowing varying in calibre over short periods of time either spontaneously or in response to treatment" (CIBA Foundation Guest Symposium., 1959). There is to much information for a precise definition to be agreed upon. However, it is clear that asthma can not be considered as an inflammatory airway disorder dependent upon activation of one single cell population. Recent studies have made it possible to propose that the

immediate mediators released by mast cells could initiate the acute phase response, while later on, cytokine production by mast cells and T cells may contribute to the late phase response. These effects could be mediated in part by inducing changes in the microvasculature, up-regulating adhesion molecules on endothelial cells, which may contribute to the migration and activation of inflammatory cells (Ricci, 1992). Recent studies have shown that there is a sequence of events that involves the expression of adhesion molecules in order to recruit cells to the area of inflammation. The rolling of leukocytes along the endothelium is modulated by P-, L- and E- selectins that can be rapidly expressed after exposure to short acting mediators such as histamine, PAF, and leukotrienes (Holgate, 1995) which can be produced by mast cells. Upregulation of ICAM-1 and VCAM-1 is known to arrest leukocytes thereby facilitating transendothelial migration (Vonderheide and Springer, 1992) and these molecules can be induced by cytokines, such as IL-1, TNF-α and IFN-γ (Gundel *et al.* 1991; Vonderheide and Springer, 1992). Bentley et al. (1992) have shown 24 hr after allergen challenge, an increased influx of T cells and eosinophils bearing the integrin VLA-4 which binds to VCAM-1. Thus, mast cells have the ability to initiate and prolong allergen induced inflammatory responses. Interestingly mast cells themselves also express VLA-4 (Fox *et al.* 1990) and may therefore assist in further mast cell recruitment. Mast cells after allergen activation may release preformed TNF-α that could upregulate E-selectin, ICAM-1, and VCAM-1. In addition the participation of other cytokines such as IL-4 which promotes the upregulation of VCAM-1, and IL-5 that promotes eosinophil chemotaxis will enhance the response. Although beyond the scope of this thesis, it is important to consider that in asthma the epithelium also plays a very important role in

protecting the airways from environmental insults to which we can be continuously exposed.

Numerous findings have implicated mast cells in rhinitis (Otsuka *et al.* 1985; Simons and Simons, 1988). The total number of mast cells in the nasal mucosa has been found to be increased in exacerbations of perennial as well as of seasonal allergic rhinitis. Elevated levels of mast-cell mediators have been reported in the nasal lavage fluid after experimental allergen challenge (Castells and Schwartz, 1988). Moreover, application of histamine (Svensson *et al.* 1989) and PGD_2 (Howarth *et al.* 1991) to the nasal mucosa of healthy people can reproduce various symptoms of rhinitis. And again, we must consider the broad spectrum of cytokines produced by mast cells which may participate in this disease.

The presence of different mast cell populations within upper airway tissues and that their differential response may be modulated by the continued antigen challenge present in this environment has been proposed by Finotto et al. (1994a). Thus, it is possible that mast cells, under conditions of chronic antigen challenge and inflammation, may persistently produce a number of immunomodulatory and proinflammatory cytokines which may act in turn on other surrounding cells (paracrine effects) or back to themselves (autocrine effects).

1.2 TNF-α

1.2.1 General information

The name tumor necrosis factor (TNF) was originated from studies of Carswell et al. (1975) who showed that a factor produced in mice pretreated with *Bacillus*

Clamette-Guerin (BCG) and subsequently challenged with lipopolysaccharide was capable of causing hemorrhagic necrosis of the meth A sarcoma grown in the skin of a recipient animal. Cachectin, a factor known for its ability to promote wasting (Theologides, 1974; Lawson *et al.* 1982) in humans affected by cancer or various infectious diseases was found to have the same amino acid sequence as TNF-α in 1985 by Beutler et al. (1985; 1987). TNF-α is a polypeptide with a subunit size of approximately 17 kilodaltons. Aggarwal et al. (1985) were able to purify TNF-α by using an L-929 cell cytotoxicity assay and the same group also succeeded in purifying lymphotoxin (now known as TNF-β) (Aggarwal *et al.* 1984).

TNF-α and TNF-β are synthesized as precursor proteins with amino-terminal extensions that are cleaved from the mature sequence prior to secretion (Beutler *et al.* 1986). TNF-α is initially expressed as a 233-amino acid membrane-anchored precursor that is proteolytically processed to its mature form: a 157-amino acid cytokine (Kriegler *et al.* 1988). The enzyme(s) involved in TNF-α processing are unknown. However, recent data suggest that metalloproteinase-like enzymes may play an important role in controlling TNF-α processing. For example, Gearing et al. (1994) recently observed that several matrix metalloproteinase enzymes have the ability to cleave TNF-α precursor *in vitro* into its mature form. The same authors and other groups (Mohler *et al.*) examined the effect of metalloproteinase inhibitors which prevented the release of TNF-α into the circulation of endotoxin challenge rats and protected mice from a lethal dose of endotoxin.

TNF-α has been shown to exist as a trimer by different techniques (Smith and Baglioni, 1987; Eck *et al.* 1988) and although it is relatively resistant to detergents,

acid conditions, and organic solvents, it is sensitive to proteases. It is important to consider these properties because the cytotoxic assay which is commonly used to detect TNF-α, only detects the bioactive form (trimer), and *in vitro* studies where supernatants are obtained from long term cultures could easily have less detectable TNF-α due to the action of proteases.

The mechanisms that regulate TNF-α biosynthesis in mast cells have not been extensively studied. However, Beutler et al. (1985; 1986) have provided interesting results in macrophages. Their work revealed that TNF gene expression is regulated both at transcriptional and at translational levels which allow massive induction of the TNF protein in response to bacterial lipopolysaccharide (Beutler *et al.* 1986). The structure of the TNF-α promoter has been well studied by Jongeneel et al. (1990) (fig 6). Their findings indicate that NF-κB plays a crucial role in the LPS-mediated transcriptional activation of the TNF-α gene in macrophages, and that more than one NF-κB binding site is required for activation. Pharmacologic agents including glucocorticoids (such as Dexamethasone), phosphodiesterase inhibitors (such as pentoxifylline) or cAMP analogues, and protein kinase inhibitors are drugs that effectively inhibit macrophage TNF-α biosynthesis in response to LPS (Beutler *et al.* 1992).

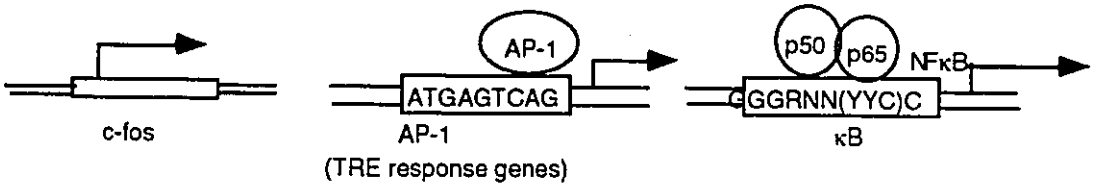

Fig. 6. Promoter region of TNF-α. Adapted from Heller et al. (1994).

Two TNF-α receptors (p60 and p80) which are members of the nerve growth factor receptor family have been found expressed on most nucleated cell types (Smith *et al.* 1990), and due to the co-expression of both forms on most cells it has been difficult to compare their signal transduction capacities. TNF-α and TNF-β bind with high affinity and can cross compete to bind to both receptors. Soluble forms of both receptors have been found in the human serum and urine of cancer patients (Engelmann *et al.* 1990; Gatanaga *et al.* 1990) which suggests that the shedding of these receptors may regulate some of the activities of TNF-α.

1.2.2 TNF-α and Inflammation

Nearly all inflammatory processes result in the activation of tissue macrophages and infiltration of blood monocytes. The production of cytokines such as TNF-α, IL-1 and

IL-6 are known to be the result of such cell activation and these cytokines can exert multiple systemic effects on the host, including the induction of fever, induction of acute phase proteins, and differentiation or activation of other cells (Titus *et al.* 1991).

TNF-α is a multifunctional cytokine that has effects on inflammation, sepsis, lipid and protein metabolism, hematopoiesis, angiogenesis and host resistance to parasites and malignancy (Old, 1985; Derynck *et al.* 1986; Beutler and Cerami, 1988a). It is produced by a broad number of cell types including monocytes, macrophages, lymphocytes (Cuturi *et al.* 1987) eosinophils (Finotto *et al.* 1994b), neutrophils (Djeu *et al.* 1990) and mast cells (Gordon and Galli, 1990). The presence of TNF-α in different diseases including septic shock (Beutler *et al.* 1985; Tracey *et al.*), listeriosis (Havell, 1987; Nakane *et al.* 1988), malaria (Butcher and Clark, 1990), leprosy and tuberculosis (Selmaj and Raine, 1988; Moreno *et al.* 1989), and in human ovarian cancer (Malik *et al.* 1990), has been well documented over the years. However, we have to remember that the presence of a given cytokine during the disease process does not necessary imply a pathological role. A probable beneficial role for TNF-α is the one described in mice infected with *Mycobacterium bovis* where a granuloma is formed by monocytic cells harboring the bacteria. TNF-α mRNA and protein accumulate in the granuloma as the bacteria expand and levels decline as the bacteria regress (Kindler *et al.* 1989).

The role of TNF-α in IBD and asthma has been suggested since administration of TNF-α induces bowel necrosis in animals (Beutler and Cerami, 1988b). TNF-α gene expression in intestinal eosinophils and macrophages is increased in patients with IBD (Tan *et al.* 1993). Enhanced production of TNF-α is observed in the PBMC and BAL of

asthmatic subjects and inhalation of TNF-α has been shown to induce bronchial constriction. Moreover, TNF-α production by eosinophils has been observed in upper airways inflammation (nasal polyposis) (Finotto *et al.* 1994b). In this thesis we present data related to how TNF-α mast cell production can be modulated by prostanoids. These results lead us to the hypothesis that mast cell activation in an acute allergic response may induce PGE production by surrounding cells. Such localized prostanoid production can then suppress TNF-α synthesis from a number of cells including mast cells and macrophages. This may be one event involved in minimizing the inflammatory process*(see chapter 3).*

During inflammation TNF-α plays important roles: it upregulates the expression of adhesion molecules on the vascular endothelium (ICAM-1, VCAM-1) helping to recruit cells to the site of inflammation (Dustin *et al.* 1986). It is chemotactic for monocytes (Ming *et al.* 1987). TNF-α can induce the production of PGE_2 and toxic oxygen metabolites by surrounding cells (Slungaard *et al.* 1990). Some actions of TNF-α may be important to host defence since through its cytotoxic effect against a number of cell targets, TNF-α could induce necrosis of certain tumors (Sugarman *et al.* 1985) and may play a role in the elimination of virally infected cells. Wegner et al. (Wegner *et al.* 1990) showed in a primate model of asthma that TNF-α can augment the intracellular leukocyte-adhesion molecule (ICAM-1) expression on the surface of vascular endothelial cells.

1.2.3 TNF-α and Mast cells

Gordon et al. (1990) have observed that both *in vitro* derived and freshly purified mouse mast cells constitutively store significant amounts of bioactive TNF-α. The same group (Gordon and Galli, 1991) showed that mast cell activation via $Fc_{\varepsilon}RI$ induced both the rapid and sustained release of TNF-α. These results are of interest because other cells contain little or no detectable TNF-α unless they are appropriately stimulated (Derynck *et al.* 1986; Cuturi *et al.* 1987). Human purified skin mast cells were first demonstrated to synthesize and release TNF-α in response to IgE-dependent activation (Benyon *et al.* 1991). TNF-α has also been localized in human lung mast cells (Ohkawara *et al.* 1992), and the proportion of mast cells expressing this cytokine is increased in asthma (Bradding *et al.* 1994).

The research groups of Galli and Kitamura (1989; 1990) have shown that W/W^v mice with local and selective repair of their mast-cell deficiency are a good animal model for studying the expression of biologic responses *in vivo* in tissues that differ in their mast cell content. Wershil et al. (1991) used this model where intradermal injection of cultured mast cells derived from the congenic normal (+/+) mice were used to repair the mast cell deficiency W/W^v mice. This paper demonstrated that the neutrophil infiltration during IgE-dependent cutaneous late phase responses is mast-cell-dependent. Evidence that TNF-α produced by mast cells was responsible for 50% of the observed cell infiltration was also observed with the use of a rabbit-anti-rmTNF-α antiserum. Although the other 50% of the leukocyte infiltration may have been TNF-α independent, mouse mast cells are potential sources of other mediators

capable of promoting cell infiltration (Gordon *et al.* 1990; Galli *et al.* 1991; Wershil *et al.* 1991).

Bissonnette et al. (1993) have recently reported in rodent mast cells, soluble and membrane-associated forms of TNF-α. The soluble form mediates a range of inflammatory and cellular immune responses (Beutler, 1992), while the membrane associated TNF is able to kill target cells through direct cell contact (Liu *et al.* 1989) and provide a co-stimulatory signal for B-cells (Aversa *et al.* 1993).

Studies presented in this thesis did not include the examination of IL-1 production by mast cells, however both mRNA and bioactivity of IL-1 had been already described in rodent mast cells (Burd *et al.* 1989; Costa, 1993). IL-1 is a cytokine that is worthwhile to be consider. This cytokine is primarily produced by macrophages and monocytes (Dinarello, 1989). Two forms of IL-1, IL-1α and IL-1β recognize the same receptor and exert similar and broad biological effects, including host immune defense reactions (Durum *et al.* 1985), synergism with other cytokines (Dinarello, 1989), and pro-inflammatory activity (Nakamura *et al.* 1992; Morteau *et al.* 1993). IL-1 is also known to augment histamine release (Otsuka *et al.* 1992) and the synthesis of arachidonic metabolites (Salari and Chan-Yeung, 1989) in mast cells. Recent studies have shown that IL-1 production is enhanced in animal models of intestinal inflammation (Morteau *et al.* 1993), in tissue specimens and cell preparations from patients with IBD (Nakamura *et al.* 1992) and in parasitic diseases (Dinarello, 1984). Animal studies have shown that IL-1 contributes to the pathogenesis of the acute immune complex-mediated colitis. Blocking the action of IL-1 with IL-1Ra (receptor antagonist) reduces the severity of acute inflammation and decreases eicosanoid

concentrations in affected bowel (Dinarello, 1993). In septicemia, bacterial infection stimulates the synthesis and release of large amounts of IL-1 leading to an induction of prostaglandins and nitric oxide (Dinarello, 1993). Patients with sepsis show plasma levels of IL-1β in the range of 250-500pg/ml, however, plasma concentrations of IL-1Ra of 20-30 μg/ml sustained over 3 days in humans with septic shock syndrome improved survival. In some models, the production of IL-6 appears to be controlled by IL-1, for example, mice treated with turpentine to induce an inflammatory response, failed to produce IL-6 when pretreated with anti-IL-1 receptor antibodies (Gershenwald *et al.* 1990). Moreover, the biological properties of TNF-α share remarkable similarities to those of IL-1.

1.3 IL-6

1.3.1 General Information

IL-6 is another multifunctional cytokine with a molecular weight heterogeneity of 20-30 kDa. While the human IL-6 shows N-glycosylation sites, murine IL-6 has several O-glycosylation sites (Van Snick *et al.* 1988; May *et al.* 1989). As with TNF-α, an IL-6 precursor must be cleaved to produce the mature form of IL-6 (Yasukawa *et al.* 1987). Rat and mouse IL-6 show a high homology at the amino acid level (93%) whereas human IL-6 is more homologous to porcine (60%) and bovine (65%) than to rodent derived cytokines (42%).

IL-6 is considered a pleiotropic cytokine because it is produced by a variety of cells and can act on a wide range of tissues. IL-6 has been shown to be produced by fibroblasts (Weissenbach *et al.* 1980), endothelial cells (Corbel and Melchers, 1984)

keratinocytes (Baumann *et al.* 1984), monocytes/macrophages (Aarden *et al.* 1987), T-cell lines (Hirano *et al.* 1985), mast cells (Plaut *et al.* 1989), T and B lymphocytes (Hirano *et al.* 1988) and a variety of tumor cell lines (Hirano *et al.* 1986). Unlike TNF-α, IL-6 is not a preformed cytokine. Rapid synthesis of protein and secretion occurs upon cell stimulation. A broad range of stimuli can induce the expression of IL-6 such as viral infections (Cayphas *et al.* 1987; Sehgal *et al.* 1988), LPS (Nordan, 1986), cytokines including IL-1 (Shalaby *et al.* 1989), TNF-α alone (Van Damme *et al.* 1987) or in combination with IFN-γ (Shalaby *et al.* 1989), PGDF (Kohase *et al.* 1987), PGE_1 and PGE_2 (Zhang, 1988; Leal-Berumen, 1995), and cholera toxin (our studies). The existence of multiple mechanisms to induce IL-6 production is consistent with the presence of several transcriptional enhancer elements in the 5' flanking region of the IL-6 gene: nuclear factors NF-IL6 α and β (also known as CCAAT element binding protein C/EBPβ and γ); nuclear factor for Kappa light chain in B cells (NF-*k*B); activator protein-1 (AP-1); cAMP response element binding protein (CREB); and glucocorticoid receptor (GR) (Ray *et al.* 1990; Chen-Kiang *et al.* 1993) of the IL-6 gene which has been extensively studied (fig 7).

IL-6 acts at a specific receptor as a monomeric entity, although IL-6 can be bound to serum/plasma proteins without modifying its bioactivity. IL-6 receptor (IL-6R) (80 kDa) has been extensively studied by several groups (Kishimoto *et al.* 1994) and belongs to the "cytokine receptor family" containing 4 conserved cysteine residues as well as an immunoglobulin-like domain. This receptor contains a major extracellular domain and studies have shown that its cytoplasmic domain is not necessary for signal transduction (Taga *et al.* 1989). However, the binding of IL-6

needs the participation of another membrane glycoprotein, gp130 (Taga *et al.* 1989; Hibi *et al.* 1990) that displays a large cytoplasmic domain which initiates IL-6 signal transduction. The gp130 molecule by itself does not bind IL-6, but IL-6R together with gp130 form a high affinity IL-6 binding site (Hirano *et al.* 1994). gp130 plays a role in other cytokine receptors (OSM, LIF, CNTF, and IL-11) (Taga *et al.* 1992; Yin *et al.* 1993) and interestingly, these cytokines have shown redundancy in some of their activities, which may be explained in part by sharing a receptor subunit.

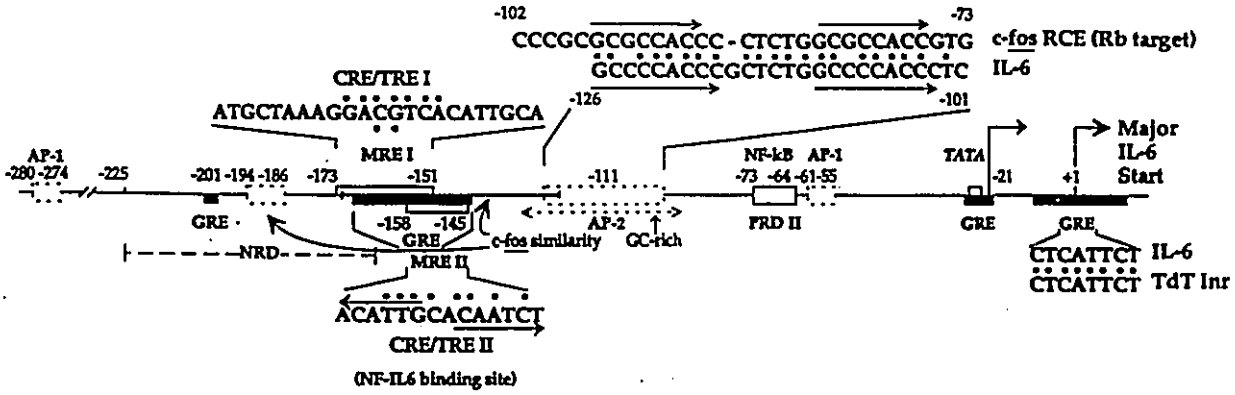

Fig. 7. IL-6 gene structure showing the promoter region. Adapted from Dendorfer et al. (1994)

As with other cytokine receptors such as soluble TNF-α I and II, soluble IL-6R has been found in serum of patients with juvenile rheumatoid arthritis (Debenedetti, 1994). The shedding of soluble IL-6R can occur due to proteolytic cleavage of the membrane bound receptor (Rose-John and Heinrich, 1994), although other studies suggest that a differentially spliced mRNA could encode for IL-6R with no transmembrane region (Lust *et al.* 1992). Moreover, the soluble complex IL-6/IL-6R can bind to cells expressing the gp130 and induce signal transduction and biological responses *in vitro* (Hibi *et al.* 1990).

1.3.2 IL-6 and Inflammation

IL-6 is a cytokine which plays an important role in the regulation of many immune and inflammatory processes. One of the major roles of IL-6 is to induce the terminal differentiation of B cells into plasma cells (Kishimoto and Hirano, 1988), which has important implications in the production of immunoglobulins (Bazin and Lemieux, 1989). The growth requirements of plasmacytomas suggest that IL-6 may act as a tumor growth factor *in vivo* (Van Snick *et al.* 1987), in fact, patients with fulminant myeloma showed increased levels of IL-6 in their bone marrow (Klein *et al.* 1989a) and IL-6 serum titers correlate with disease activity (Bataille *et al.* 1989). Moreover this property of IL-6 has served to aid the development of sensitive bioassays to detect IL-6 bioactivity (Aarden *et al.* 1987). In our studies we used the B9 hybridoma proliferation assay, which seemed to be specific for IL-6. B9 cells only grow in the presence of IL-6, however, recent studies show that these cells also grow in the presence of IL-11 (Burger and Gramatzki, 1993). Production of IL-11 by rodent

mast cells has not been described yet and we confirmed the presence of IL-6 in our samples by using neutralizing antibodies against IL-6 (see *appendix 2)*. IL-6 enhances some T-cell functions including proliferation of peripheral and thymic T cells (Lotz *et al.* 1988; Uyttenhove *et al.* 1988), and the development of cytolytic T cells (Takai *et al.* 1988; Uyttenhove *et al.* 1988). Although, IL-6 has been implicated in other biological activities such as hematopoiesis (Okano *et al.* 1989), and induction of neurite outgrowth (Van Snick, 1990), one of the primary roles of IL-6 is its participation in the hepatic acute phase response which is characterized by an increase of plasma liver-derived proteins (Kushner, 1982; Koj and Gordon, 1985). The macrophage or monocyte is thought to be the primary cell that initiates this acute phase response cascade (Koj and Gordon, 1985; Gauldie, 1991) by releasing a broad spectrum of cytokines such as IL-1, TNF-α and IL-6 which are known to contribute to the tissue response at the site of injury. IL-6 has been recognized as the major regulator of induction of type-2 hepatic acute phase proteins (Baumann and Gauldie, 1990) which include the three chains of fibrinogen, haptoglobin, and at least one of the following anti-proteases: α_1-anti-chymotrypsin, α_1-anti-trypsin, thiostatin or α_2-macroglobulin.

IL-6 can act on other cells to modulate certain aspects of inflammation. For example, IL-6 induces the tissue inhibitor of metalloproteinases (TIMP-1) in fibroblasts and chondrocytes (Sato *et al.* 1990; Lotz and Guerne, 1991) and it induces the production of α_1-anti-trypsin, an inhibitor of neutrophil elastase, by alveolar macrophages (Barbey-Morel *et al.* 1987; Perlmutter, 1989) probably to help regulate the enzymatic degradation of tissue. Other groups have reported *in vitro* experiments which suggest that IL-6 can inhibit the production of other cytokines such

as IL-1 and TNF-α (Aderka *et al.* 1989). Ulich et al. (Ulich *et al.* 1991; Ulich *et al.* 1992) found *in vivo* that administration of IL-6 inhibits LPS-induced neutrophil accumulation in rat lungs. In humans with atopic disease, both IL-1 and IL-6 have been identified in skin blister fluid after allergen challenge (Bochner *et al.* 1990; Lee *et al.* 1992). Mican et al. (1990) observed increased levels of IL-6 mRNA as well as immunoreactive IL-6 protein at sites of passive cutaneous anaphylaxis in mouse skin. These results demonstrate that IL-6 should be considered one of the important regulator in inflammation that follows allergic reactions and although IL-6 is enhanced along side pro-inflammatory molecules, it could also serve to decrease the ongoing inflammatory responses.

1.4 Lipopolysaccharide (LPS)

1.4.1 General Information

Lipopolysaccharide is a family of endotoxins derived from the cell wall of gram-negative and some gram-positive bacteria (Morrison and Ulevitch, 1978). LPS is a complex molecule consisting of three main regions: Lipid A, core polysaccharide, and O-antigenic side chains (fig 8). LPS serves as a selective permeability barrier to the bacteria, and helps the interaction of the bacterium with its environment (Leive, 1965). Years of studies have lead us to understand that the lipid A domain of LPS contains the structural information that triggers cell activation (Raetz, 1990). Different cell types are responsive to LPS including monocyte/macrophage, polymorphonuclear leukocytes (PMNs), endothelial and epithelial cells (Ulevitch and Tobias, 1994) and mast cells (Leal-Berumen *et al.* 1994). The interaction between

LPS and the macrophage has been extensively studied and such interaction requires LPS to interact with lipopolysaccharide-binding protein (LBP) (Tobias *et al.* 1986). LBP is a 60 kDa glycoprotein synthesized in hepatocytes that is found in human and rabbit serum. It binds with high affinity to the lipid A moiety of LPS, and it appears that LBP catalyses the LPS-mediated effects (Tobias *et al.* 1986; Tobias *et al.* 1988). Studies have shown that LBP plasma concentration rises during the acute phase response 10-fold (Schumann *et al.* 1990). Specific receptors on mononuclear cells for the LPS-LBP complex exist in a membrane-bound form designated mCD14 that is attached to the cell membrane via a glycosylphosphatidyl inositol (GPI) (Wright *et al.* 1990; Couturier *et al.* 1991; Tobias *et al.* 1993), or in a soluble form designated sCD14, which lacks the GPI anchor (Bazil *et al.* 1986). The expression of CD14 can be modulated by cytokines: it is upregulated by IL-1 and TNF-α, while IL-4 downregulates its expression (Ziegler-Heitbrock and Ulevitch, 1993). CD14 requires the presence of LPS to bind LBP (Wright *et al.* 1989). LBP is not the only binding protein for LPS, binding of LPS to other proteins could also have a profound effect. One of these proteins include the bactericidal/permeability-increasing protein (BPI) (Elsbach *et al.* 1979) which is a constituent of PMN primary granules and is released from PMNs during degranulation (Lynn and Golenbock, 1992). Despite the similarities in primary structure and ability to bind LPS via lipid A, LBP and BPI differ markedly. BPI neutralizes the ability of isolated LPS to stimulate cells and it is directly bactericidal, whereas LBP enhances the effects of LPS on cells and functions as an opsonin if bound to bacteria (Raetz *et al.* 1991). Thus, the LPS-LBP/CD14 pathway appears to be the primary pathway whereby the non-immune host recognizes LPS and mounts an inflammatory response either to

eliminate the invading bacteria or to develop systemic responses to LPS. This same LPS-LBP/CD14 complex has been shown to mediate TNF-α production in macrophages (Wright *et al.* 1990). CD11/CD18 is another cell surface-associated LPS-binding protein on leukocytes (Wright and Jong, 1986; Wright *et al.* 1989).

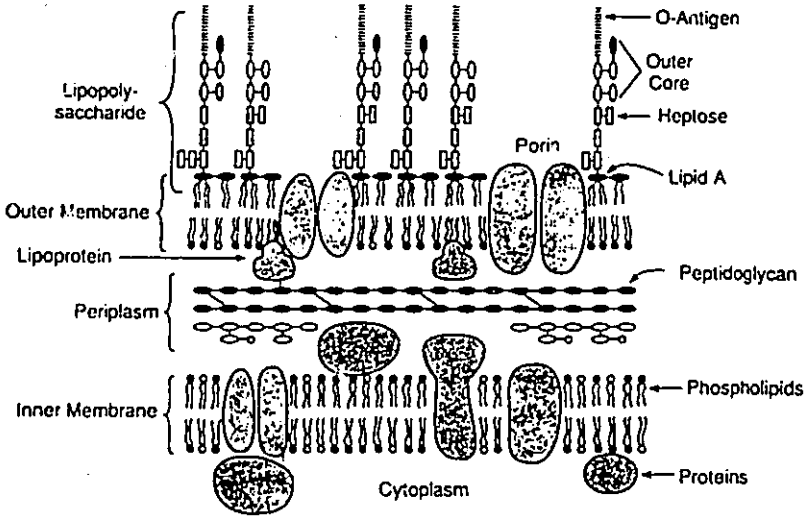

Fig. 8. The structure of the cell wall of a gram negative bacteria showing the localization of LPS. Adapted from Raetz et al. (1990). o-Antigen= o-specific side chain which is highly strain specific.

1.4.2 LPS in Inflammation

LPS stimulates a number of cell types to release a vast variety of proteins, free radicals (O_2^-, H_2O_2, $^{\bullet}OH$ and single oxygen), a number of arachidonic acid metabolites, including prostaglandins and leukotrienes, and the proinflammatory cytokines (TNF-α, IL-1, IL-6 and IL-8) that are involved in the inflammatory response that enhance host defense against bacteria (Table 3). These inflammatory reactions are highly synergistic with one another, and interrupting the effects of one mediator may profoundly alter the biological activities of others (Bone, 1991). Moreover, LPS promotes adherence of PMNs and monocytes to endothelial cells and phagocytes, and indirectly, through the release of cytokines and other inflammatory mediators (Pohlman *et al.* 1986; Morrison and Ryan, 1987). Therefore, whenever there is a gram-negative infection, where LPS concentration may be extremely high, the recruitment of cells may be mediated in this manner. However, small amounts of LPS into the blood stream may cause overstimulation of immune cells and cell recruitment into uninfected distant tissues leading to malfunctions of the organism (Watson *et al.* 1994) such as hypotension, multi-organ failure, disseminated intravascular coagulation and septic shock syndrome. This clinical syndrome of gram-negative bacterial septicemia has a high incidence, and a mortality rate of 40-70% (Schumann *et al.* 1994).

Other anti-LPS strategies include chemical breakdown of the LPS molecule with methyl formaldehyde derivatives, the use of CD14 inhibitors (Watson *et al.* 1994), and antibiotics such as polymyxin B that inhibit the biological effects of LPS through its high affinity to the lipid A region (Tracey and Lowry, 1990) but its high toxicity limits its use (Craig *et al.* 1974). TNF-α which is one of the most potent products released in

response to LPS and appears early after LPS injection in animals as well as in humans (Beutler *et al.* 1985). Passive immunization by injection of antibody against TNF-α prevented animals from later secretion of IL-1 and IL-6 during sepsis, and reduced mortality in the lethal LPS shock model (Fong *et al.* 1989). A phase I trial using anti-TNF in patients has been carried out, however the mortality rates did not differ significantly from those of control subjects (Exley *et al.* 1990). Despite the diversity of anti-LPS therapies currently available, results in patients have been singularly disappointing. It is now clear that random blockade of LPS is not consistently protective in septic shock (Watson *et al.* 1994). The contribution of the mast cell to the cytokine production that occurs during gram negative septicaemia or local bacterial infection has not previously been considered. In our studies we have observed how bacterial products (such LPS and cholera toxin) can affect mast cell cytokine production (see *chapters 2 and 4*).

Table 3. Effect of LPS on septic shock syndrome.

Biological target	Response to endotoxin
Monocyte/macrophage	Cytokine release: TNF-α, IL-1α, IL-1β, IL-6, IL-8 TGF-β. Release of IL-1 receptor antagonist, lipid inflammatory mediators, increase adherence to endothelium.
Neutrophils	Increased surface expression and adhesive capacity of integrins. Synthesis of cytokines (IL-1).
B-cells	B-cell mitogenesis, release of colony stimulating factors.
Epithelial cells	Generation of PMN chemotactic factors.

Adapted from Lynn et al. (1992)

1.5 Prostanoids

1.5.1 General information

The family of prostaglandins, leukotrienes, and related compounds are known as eicosanoids because they are derived from 20-carbon essential polyunsaturated fatty acids. Prostaglandins contain a cyclopentane ring structure (fig 9) and as we mentioned previously, they are produced by the action of the enzyme cyclooxygenase on arachidonic acid liberated from membrane phospholipid (Phipps *et al.* 1991) (see fig 5). Prostanoids of the E series (PGE_1 and PGE_2) have been extensively studied and they are produced by a wide variety of cells including macrophages, follicular dendritic cells, and fibroblasts (Frey *et al.* 1986; Heinen *et al.* 1986; Kurland and Bockman, 1978; Phipps *et al.* 1988) . Prostanoids are not stored by cells, but rather are synthesized in response to different stimuli including IL-1, TNF-α, the cross-linking of Fc receptors, LPS, and components of the complement cascade (Phipps *et al.* 1991). After prostanoids exit the cell they can act on parent cell/or neighboring cells in an autocrine/or paracrine fashion through specific G-protein-linked prostanoid receptors to further change the levels of a second messenger or intracellular ion (Smith, 1989). Plasma concentrations of eicosanoids are less than 10^{-9} M which normally are unable to elicit a cell response (Dunn *et al.* 1978; FitzGerald *et al.* 1981). However, in patients with Hodgkin's disease high PGE levels are closely associated with increased serum IgE levels (Waldmann *et al.* 1974). *In vitro* studies have shown that eicosanoids can induce a cell response at concentrations as low as $\leq 1 \times 10^{-8}$ M (Smith, 1989; Leal-Berumen, 1995).

Many functions of PGEs are known to be mediated by cAMP (Pelus and Strausser, 1977) such as the enhancing effect of PGE_2 on the production of IgE and IgG_1 (Phipps *et al.* 1990; Roper *et al.* 1990), and its effect on decreasing the production of Th1 subset cytokines. Forskolin, dibutyryl-8-bromo-cAMP and cholera toxin are compounds that specifically stimulate adenylate cyclase and have been used as tools to study the events where cAMP plays a critical role as a second messenger (Piquet-Pellorce, 1991).

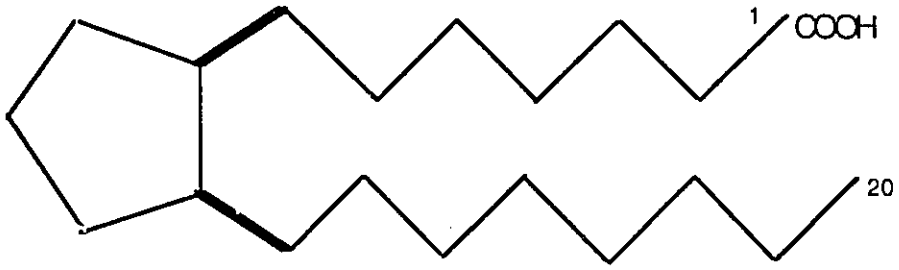

Fig.9. General model of prostanoid structure. Adapted from Smith W.L (1989).

Coleman et al. (1994) have reviewed extensively the classification of prostanoid receptors. EP receptors are specific for prostanoids of the E series and there are, so far, four subtypes termed arbitrarily EP_1 to EP_4. EP_1 is coupled to a G-protein which is insensitive to pertussis toxin and cholera toxin, and a characteristc increase in

intracellular calcium is observed when this receptor is occupied. EP_2 shows increase levels of cAMP via a Gs-protein, whereas EP_3 decreases cAMP levels through a Gi-protein. EP_4 has recently been described but it also increases cAMP levels, however its mechanism is less understood. EP_1 receptors do not appear to have a wide distribution. EP_2 receptors are widely distributed in smooth muscle where they mediate relaxation, on epithelial cells, and on inflammatory cells such as mast cells and basophils, where they mediate inhibition of mediator release (Coleman, 1990). However, EP_3 seems to be the most ubiquitous (smooth muscle of gastrointestinal, uterine, and vascular origin, in adiposites, gastric mucosal cells, and renal medulla).

1.5.2 Prostanoids in Inflammation: pro-inflammatory and anti-inflammatory effects

Prostaglandins are released by a host in response to a mechanical, thermal, chemical, bacterial, or other insults, and they contribute to the genesis of the signs and symptoms of inflammation (Samuelsson, 1983; Davies *et al.* 1984). PGE_2 markedly enhances edema formation and leukocyte infiltration and it also potentiates the pain-producing activity of bradykinin.

PGE_2 is a bronchodilator in normal and asthmatic subjects (Kawakami *et al.* 1973), but it also has a bronchoconstriction effects (Alam *et al.* 1992; Schwartz, 1994). The effects of inhaled PGE_2 on subsequent airway responses to bronchoconstrictor agents appears to be time-dependent (Walters *et al.* 1982). The bronchoconstriction effect of inhaled histamine in normal subjects can be significantly

antagonized by the initial bronchodilator action of PGE_2, however, if the bronchoconstrictor challenges are performed after the bronchodilator response to PGE_2 an enhancement of histamine effects are seen (Smith, 1973; Mathe and Hedqvist, 1975; Sladek, 1990). Moreover, PGEs act synergistically with other inflammatory mediators (Williams, 1983; Richards and Agro, 1994). Therefore, PGE_2 may be protective in the acute phase of a bronchoconstrictor challenge, however, in a chronic inflammatory condition it is not clear if PGE_2 has such protective action. Although PGEs are produced by inflammatory cells and they can influence the outcome of inflammation, there are situations where prostaglandins can act as anti-inflammatory mediators. For example, PGEs are known to inhibit the participation of lymphocytes in delayed hypersensitivity reactions by reducing their chemotactic response (Rivkin *et al.* 1975) and they inhibit the release of hydrolases and lysosomal enzymes from human neutrophils as well as from mouse peritoneal macrophages (Zurier *et al.* 1974). PGEs also prevent IgE-induced histamine release from lung fragments and basophils (Orange *et al.* 1971).

Kunkel et al. (1981) found that oral administration of an analog of PGE_1 can suppress both chronic adjuvant-induced polyarthritis and acute immune complex-induced vasculitis in a rat model. Others groups have reported a "cytoprotective" ability of prostglandins of the E series because their ability to reduce the severity of injury in the gastrointestinal tract (Hawkey and Rampton, 1985; Katakami *et al.* 1988). Such studies confirm the potential role of certain prostaglandins as anti-inflammatory agents.

1.5.3 Effect of prostanoids on cytokine production

During an inflammatory response several cell factors interact on inflammatory cells which may respond differently than if such mediators were presented separately. Prostaglandins have been shown to modulate cytokine production by different cells, for example, PGEs can act directly on B cells to enhance IL-4-induced production of IgE and IgG_1 (Phipps *et al.* 1990; Roper *et al.* 1990). Interestingly, PGEs have been shown to decrease production of Th_1 cytokines such as IL-2. IFN-γ in small amounts has an inhibitory effect on IgE synthesis, and IL-2 which contributes to the production of IgE by mixtures of human cells (Quill *et al.* 1989; Gajewski *et al.* 1990; Munoz *et al.* 1990b). PGEs also enhance the production of the Th_2 cytokine IL-5 (Munoz *et al.* 1990a; Betz and Fox, 1991) which promotes human B-cell IgE production (Pene *et al.* 1988). Furthermore, PGEs upregulate the expression of IL-1 receptors on fibroblasts and on PMNC (Akahoshi *et al.* 1988) and since the binding of IL-1 to its receptor promotes PGEs production, this feedback mechanism may favor the Th_2 subset (IL-4 and IL-5 production).

TNF-α and PGEs are thought to play an important role in mediating many of the detrimental and beneficial effects of the host response to trauma and sepsis (de Groote *et al.* 1989). TNF-α has been shown to increase in burned and severely injured patients which is followed by an increase in monocyte and macrophage PGE production (Latter *et al.* 1987). Thus, PGE may be important for initiating a negative feedback loop to regulate TNF-α. We and others (Kunkel *et al.* 1988; Dong *et al.* 1993; Leal-Berumen, 1995) have found that PGE can decrease TNF-α production in response to different stimuli, such as LPS, which suggests that PGE may be a major regulator of TNF-α

production. This effect of PGE could be considered to be beneficial since excessive TNF-α synthesis in response to endotoxin challenge can result in an increased mortality (Tracey *et al.*). IL-6 is another cytokine which is increased during inflammation and sepsis (Cavaillon *et al.* 1992) and it seems that prostaglandins can modulate its production. Holladay et al. (1993) recently reported that prostacyclin (PGI_2) can enhance IL-6 production from rat peritoneal macrophages. In this thesis we have examined the ability of PGEs to increase IL-6 production and to suppress TNF-α synthesis from rat peritoneal mast cells. Therefore, the benefit of prostaglandins during inflammation or sepsis could be the induction of IL-6 and the subsequent synthesis of acute phase mediators, and the downregulation of TNF-α.

Previous reports have confirmed that PBMNC from patients with atopic dermatitis (AD) produce low levels of IFN-γ (Reinhold *et al.* 1990; Renz *et al.* 1992). Chan et al. (1993) reproduced these results, however they found that when mononuclear leukocytes were removed the T-cell supernatants from the same AD patients had increased levels of IFN-γ suggesting the presence of an inhibitor of IFN-γ production which was removed upon the separation of mononuclear leukocytes. The authors suggest PGE_2 as a regulatory mediator between monocytes and T-cells in AD that modulates IFN-γ production because they found significantly higher levels of PGE_2 in the mononuclear leukocytes culture supernatants in AD than in normal controls. These studies are important to understand the regulatory interaction between cells and that extracellular messengers between them may modulate their cytokine production.

1.6 Cholera Toxin (CT)

1.6.1 General Information

Cholera toxin (CT) is a member of a family of enterotoxins that are produced by different strains of enteropathogenic bacteria. CT is a protein produced by *Vibrio cholera* which is responsible for the fluid secretion by intestinal epithelial cells seen in clinical cholera (Betley *et al.* 1986). The molecular structure of CT consists of two subunits: a 28,000 kD A subunit (CT-A) which has ADP-ribosylating enzymatic activity and an 11,500 kD B subunit (CT-B) that is known as the binding portion of the holotoxin (Elson, 1989). The A subunit is cleaved into A1 and A2 peptides and A1 appears to mediate the activation of the adenylate cyclase enzyme (Elson, 1989). CT binds to a GM1 ganglioside receptor, a constituent of the cell surface of virtually all nucleated cells (Cuatrecasas, 1973). In recent years, the interaction of CT-B and the GM1 receptor has been shown to have an effect on the proliferation of various cell types (Woogen *et al.* 1987; Spiegel and Fishman, 1987), which is important to consider because the biological effects of CT have often been attributed solely to the A subunit. CT has the ability to act as an oral adjuvant helping some antigens to stimulate secretory IgA (Elson and Ealding, 1984). Some of this adjuvant activity may be due to selective cytokine modulation.

1.6.2 Effect of CT on different cells

Bromander et al. (1991; 1993) have shown that CT potentiates antigen presentation by macrophages and intestinal epithelial cells by enhancing co-stimulation, i.e. increasing IL-1 and IL-6 production. Snider et al. (1994) described an *in vivo* model of immediate hypersensitivity reaction to intestinal Ag, where CT induced the

production of IgE Ab after oral immunization of mice with protein Ags such as HEL and OVA. These results are of interest to the use of CT as an adjuvant in oral vaccines. All this information related to CT indicates its importance as immunoregulator, however the mechanisms that are most important to the adjuvant function *in vivo* is still unclear. Actions of CT on mast cells could be of particular importance because of the number of mast cells within the intestinal mucosa. CT stimulates the IEC-6 intestinal epithelial cell line to produce IL-6 which can be enhanced by simultaneous presence of other cytokines, such as TNF-α, IL-1 and TGF-β (McGee *et al.* 1993). Our studies have examined the effects of CT on IL-6 and TNF-α production by rat peritoneal mast cells. Macrophage cytokine production such as IL-1 is also modulated by CT (Lycke and Strober, 1989).

G proteins act as signal transducers in a wide variety of cell types (Gilman, 1987), including some that lead increased production of cAMP through activation of adenylate cyclase. These G proteins can be identified by using bacterial toxins such as CT which uses these G proteins as substrates for ADP-ribosylation (Gill and Meren, 1978). cAMP itself has multiple effects on different cells. Lerner et al. (1988) found that CT reduces both the inositol phosphate (IP_3) production and the rise in intracellular Ca++ in Con A-stimulated T-cells which seems to be secondary to intracellular elevation of cAMP, since similar effects were observed with other agents that rise cAMP levels such as forskolin and PGE_2. Moreover, Munoz et al. (1990b) reported that CT inhibits c-*fos* and c-*myc* mRNA expression, IL-2 production and proliferation on Th_1 cells in response to TCR-mediated triggering. However, the same CT doses did not block IL-4 production or proliferation of Th_2 cells. These results suggest

that CT has a selective inhibitory effect on Th_1 vs Th_2 cells. More recently Elson et al. (1995) provided new insight into the potential mechanism of immunogenicity and adjuvanticity of CT in the gut. The authors showed that T-cell activation *in vitro* and *in vivo* was significantly inhibited by CT and CT-B, and although both CD8+ and CD4+ T-cells were inhibited, CD8+ intraepithelial lymphocytes were more sensitive to the CT effects. A single activity is unlikely to explain the special mucosal properties of CT, CT must have a direct effect on other cells or an indirect one through cytokines induced by CT.

1.7 The rat peritoneal mast cell model

In our studies, we have used a rat model which is based on isolating fresh peritoneal mast cells (CTMC) from Brown Norway rats that have endogenously high IgE levels. This model has several advantages:

- We can mimic the activation of cells observed in allergic responses by using an antibody against IgE (anti-IgE).
- A large number of CTMC can be isolated from the rat peritoneum (1-2x10^6 MC/rat) which then can be highly purified by using a 30%/80% Percoll gradient (98-100% purity).
- There have been differences observed between the cytokines released from mast cell lines and those from freshly isolated mast cells. We believe that the results obtained using freshly isolated mast cells may more closely represent what is happening *in vivo*.

We are aware that this system has certain limitations, for example, all our data comes from CTMC type and not from MMC which is the type of cell that one expects to participate in inflammatory disorders such as asthma and IBD. Therefore, it will be very interesting to examine if MMC cytokine production respond similarly to CTMC. We also realize that isolated cells may behave differently than cells *in vivo* which obviously are exposed to other cell types and other mediators. Moreover, a rat model may not be directly applicable to human. Another limitation we have found is that rat cytokine reagents are not always available, such is the case for rat IL-6. However, the rat model we use remains valid to examine some aspects of mast cell behavior which should then be confirmed in other systems as reagents and technology for isolating cells improves.

1.8 Rationale of this study

Mast cells are known to play an important role in the immediate response to allergen challenge by releasing stored mediators, such as histamine, which are indicators of mast cell granule release. Rodent mast cells produce a wide variety of cytokines in response to agents that trigger histamine release, such as anti-IgE (Befus, 1989; Burd *et al.* 1989; Galli *et al.* 1991). Such studies suggested a close relationship between mast cell histamine release and cytokine production. On the other hand, we know that IL-6 can be produced by a number of cell types including mast cells and macrophages in response to other forms of activation. Macrophages which have an important role during bacterial infection can synthesize large amounts of IL-6 in response to LPS. Since mast cells are localized to sites readily in contact with bacteria,

we decided to study the response of mast cells to LPS in terms of cytokine production (IL-6) and histamine release with other more traditional mast cell activating agents used as controls (A23187 and IgE) (*see Chapter 2*). Our results documented in *chapter 2* clearly changed our view of the potential of mast cells to produce cytokines and lead us to consider these cells as participants not only in an immediate response but also in late and chronic responses. Moreover, we should consider these cells having an important potential role during bacterial infection.

In view of our observations in this study, we became interested in other mediators present during inflammation which might modulate mast cell cytokine production. Prostanoids of the E family are known to participate in inflammatory processes, such as asthma and IBD, and substantial information on the effect of prostanoids on cytokine production from different cell types has been described. For example, PGE_2 has a synergistic effect with IL-1β on fibroblasts IL-6 production, or its suppressive effect on LPS-induced TNF-α by macrophages and anti-IgE-induced mast cell TNF-α. These data lead us to examine the hypothesis that prostanoids would modulate production of two different cytokines which are produced by the peritoneal mast cell: IL-6 and TNF-α (*see chapter 3*). Our results strengthened our findings from *chapter 2*, because prostanoids from the E series selectively modulated IL-6 production and suppressed endogenous mast cell TNF-α production without causing histamine release.

Increased levels of intracellular cAMP are a fundamental signalling event in many cell types. There are several agents that can induce such an enhancement of intracellular cAMP, including PGE_2, CT and pertussis toxin. Studies have shown that PGE_2 and CT exert similar cell responses due to their effect on cAMP. Moreover, CT has

been shown to induce IL-6 production by intestinal epithelial cells (Bromander *et al.* 1993). These results along with our findings from *chapter 3* and the fact that mast cells may play an important role in IBD lead us to examine the hypothesis that CT would modulate cytokine production by rat peritoneal mast cells through a cAMP dependent mechanism. Our results showed certain similarities with our previous results described in *chapter 3*: CT did not induced histamine release from PMCs, however, it enhanced IL-6 production while TNF-α was suppressed. To examine whether such an effect of CT on mast cell cytokine was due to an increase in cAMP, we used forskolin which is known to induce an increase in intracellular cAMP, and our results showed that forskolin, at optimal concentration, can mimic the effect of CT (*see chapter 4*). However, it will be interesting to confirm that the effect of PGE_2 and CT on mast cell IL-6 production occurs through increase $[cAMP]_i$. This could be approach by measuring the intracellular cAMP in mast cells with and without PGE_2, CT, forskolin (as a positive control), or 1-9, di-forskolin (as a negative control). Forskolin increases $[cAMP]_i$ levels through the stimulatory G-protein of adenylate cyclase.

Calcium plays an integral role in the regulation of numerous cellular responses. For example, increase levels in intracellular calcium ($[Ca^{++}]_i$) in mast cells is associated with histamine release and IgE mediated release. Such $[Ca^{++}]_i$ release is generally characterized by two phases: an initial rapid spike followed by a sustained, elevated phase. The initial phase is the result of Ca^{++} release from intracellular stores mediated by inositol 1,4,5-triphosphate (IP_3), whereas the second phase plateau is mainly caused by Ca^{++} influx from the extracellular medium (Amellal and Landry,

1983; Katakami *et al.* 1984). Moreover, the elevated cytosol calcium concentration may act through the activation of calmodulin since inhibition of mast cell histamine release has been observed with calmodulin antagonists (Weiss *et al.* 1984; Park *et al.* 1992). The effect of increased or decreased level of $[Ca^{++}]_i$ on mast cell cytokine production has not been extensively studied. Wodnar-Filipowicz et al. (1991) have shown that calcium ionophore A23187-mediated IL-3 mRNA accumulation in PB-3c mastocytes is the result of stabilization of the normally labile mRNA. In contrast, we found that A23187 had almost an inhibitory effect on mast cell IL-6 production which suggests that in this case high levels of $[Ca^{++}]_i$ were not sufficient to induce IL-6. However, our results demonstrate that calcium plays a critical role in mast cell IL-6 production in response to LPS, anti-IgE, or CT.

In our discussion we include the limitations of our studies and most important we hope you can realize the importance of our findings. And, although, we used an *in vitro* model we believe that by studying the mechanisms by which mast cells regulate their cytokine production and how mast cell cytokines can interact with other cells and their products, we will understand better the potential role of these cells in normal situations as well as in different inflammatory disorders.

CHAPTER TWO

IL-6 Production by Rat Peritoneal Mast Cells Is Not Necessarily Preceded by Histamine Release and Can Be Induced by Bacterial Lipopolysaccharide[1]

Irene Leal-Berumen, Peter Conlon, and Jean S. Marshall[2]

Molecular Virology and Immunology Program, Department of Pathology, McMaster University, Hamilton, Ontario, Canada

Mast cells produce a number of cytokines including IL-6. In view of the large amounts of de novo synthesis induced by the activation of rat peritoneal mast cells and previous observations of expression of this cytokine by human lung mast cells, we have studied the regulation of IL-6 production. We examined the hypothesis that mast cell IL-6 production is not related to previous histamine release. Highly purified rat peritoneal mast cells were activated with anti-IgE, calcium ionophore A23187, or LPS. Histamine was used as a marker of preformed mediator release and IL-6 production was assessed by using the B9 hybridoma growth factor bioassay. Anti-IgE activation of rat peritoneal mast cells induced IL-6 production and histamine release. In contrast, LPS activation induced substantial, serum-dependent, IL-6 production without a significant level of histamine release. No preformed IL-6 was detected in the cells. Calcium ionophore induced histamine release from mast cells to a greater extent than did anti-IgE, but no A23187-induced IL-6 production was observed. A23187-treated cells retained high viability and produced a significant amount of TNF-α. To further examine the concordance of IL-6 production and histamine release we used mast cell stabilizing drugs. Dexamethasone and nedocromil significantly inhibited IL-6 production in response to anti-IgE. Our results demonstrate that there is not a direct relationship between mast cell degranulation and IL-6 production. Our observations are important for understanding the role of mast cells in inflammation and for developing strategies to modulate mast cell function in disease. *Journal of Immunology*, 1994, 152: 5468.

Chronic inflammatory changes are the hallmark of severe allergic disorders such as asthma. The mast cell has been implicated as an initiating cell in the immediate responses to allergen challenge, but its role in the late phase response to and in the exacerbation of inflammation is less well understood. In most studies that have examined the role of mast cells in disease processes and the actions of anti-allergic drugs, researchers have relied on the measurement of release of preformed mast cell mediators, such as histamine or tryptase, to provide an indication of mast cell activation.

In recent years we have come to recognize that mast cells produce a number of cytokine mediators that are not necessarily found preformed within the cells. In the mouse, mast cell lines have been demonstrated to produce a wide variety of cytokines. These include IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12, TNF-α, GM-CSF[3], IFN-γ, TGF-β, MIP-1α, MIP-1β, JE, and TCA-3 (1–3). In the rat, freshly isolated PMCs have been shown to produce IL-6, TNF-α, and leukemia-inhibitory factor (4), whereas in the human, there is some evidence for IL-4, IL-5, IL-6, IL-8, and TNF-α production by mast cells (5–7).

IL-6 is a multifunctional regulator of immune and inflammatory processes that has a range of biologic activities, including important roles in the development of plasma cells and stimulation of the production of acute phase response protein by hepatocytes (8). IL-6 is produced by a number of different cell types, including fibroblasts, macrophages, mast cells, and T and B lymphocytes, after exposure to a number of extracellular stimuli (9). IL-6 has not been demonstrated in large amounts in vivo under normal circumstances. However, after tissue injury

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[1] This work is supported by the Medical Research Council of Canada.

[2] Address correspondence and reprint requests to Dr. Jean S. Marshall, Department of Pathology, McMaster University Health Sciences Centre, Room 4H17,1200 Main Street West, Hamilton, Ontario, L8N 3Z5, Canada.

[3] Abbreviations used in this paper: GM-CSF, granulocyte-macrophage colony-stimulating factor; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium); PMC, peritoneal mast cell; SBTI, soybean trypsin inhibitor; PKC, protein kinase C; MIP, macrophage inhibitory protein.

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or infection of the host, IL-6 is rapidly expressed and participates in host defense. Other cytokines, such as IL-1, TNF-α, platelet-derived growth factor, and IL-6 itself, stimulate IL-6 production (10, 11). Therefore, IL-6 is an integral part of the cytokine network and participates in both inflammatory and immune responses.

Several research teams have demonstrated that stimulation through the FcεRI induces mast cells to accumulate increased levels of mRNA for cytokines and to release cytokine protein (1–3). Other mast cell-activating agents that increase intracellular calcium concentrations, such as A23187 and ionomycin, also induce mast cell cytokine production. Wodnar-Filipowicz et al. (12, 13) have demonstrated accumulation of IL-3 and GM-CSF mRNAs after treating a murine mast cell line with A23187. Plaut et al. (14) also showed the production of cytokines by several murine mast cell lines previously stimulated with ionomycin (IL-3 and IL-6). In each of these cases, the production of cytokines occurred in the context of mast cell activation by agents known to induce histamine release.

We chose to examine the regulation of IL-6 production by mast cells in view of the wide range of biologic activities of IL-6 that are relevant to the initiation and progression of inflammation and because it been shown to be produced by human lung mast cells (5). In the mouse, this cytokine is produced in the skin after passive cutaneous anaphylaxis reactions and by several mast cell lines in response to a variety of stimuli (14, 15). The results of work that used mouse mast cell lines have suggested that IL-6 may be regulated differently than the cytokines IL-1, IL-3, TCA-3, JE, MIP-1α, or MIP-1β, because IL-6 production can be induced by PMA. In addition, IL-6 production in response to FcεRI-mediated activation was only partially inhibited in the mouse mast cell line Cl MC/9 by cyclosporin A, whereas the production of mRNA for other cytokines was completely abolished (14). In rat mast cell lines, we have not observed production of IL-6 at either the mRNA or protein level (4), although freshly isolated PMCs produce substantial quantities of this cytokine.

In the present study, we have examined the ability of highly purified mast cells obtained directly ex vivo to produce IL-6 in response to different degranulating agents, as well as the effects of LPS in the presence of FCS. We have focused on the relationship between the release of the preformed mast cell mediator histamine and IL-6 production. Our results demonstrate that mast cell production of IL-6 does not necessarily occur after histamine release and that mast cell cytokine production can be inhibited by corticosteroid treatment without causing a reduction in histamine release. We also show that mast cells may be induced to produce IL-6 in response to LPS without having substantial histamine release. These studies have important implications for our understanding of the potential role of mast cells in immunoregulation and inflammation.

Materials and Methods

Mast cell sources and activation

PMCs were obtained by peritoneal lavage, with the use of 15 ml 0.1% BSA in PBS, from female Brown Norway rats weighing 150 to 200 g (Harlan Sprague-Dawley, Inc., Indianapolis, IN). These animals were housed in the central animal facility at McMaster University, Hamilton, Ontario, Canada, in filter-hooded cages and were allowed food and water ad libitum. All experimental procedures were approved by the Animal Research Ethics Board of McMaster University. PMCs were purified on a 30%/80% discontinuous Percoll gradient (Pharmacia LKB, Uppsala, Sweden) and those preparations with a range of 95 to 100% purity were used for later studies. One million PMCs/ml were suspended in RPMI 1640 without phenol red (GIBCO BRL, Grand Island, NY), which was supplemented with 5% FCS(v/v), penicillin (50 U/ml), streptomycin (50 μg/ml) (1% Pen-Strep; GIBCO BRL), 1.4 mM Ca^{2+}, and 100 μg/ml soybean trypsin inhibitor (SBTI; Sigma, St. Louis, MO). These cells were initially degranulated for 10 min at 37°C by using the following: A23187, at either 1×10^{-6} M or 5×10^{-7} M (Boehringer Mannheim, Mannheim, Germany); a mouse monoclonal IgG_1 anti-IgE (MARE-1; University of Louvain, Belgium) at 10, 5, or 2.5 μg/ml; a mouse IgG_1 myeloma protein at 5 μg/ml (Sigma), as negative control; or LPS (*Escherichia coli* serotype 055-B5; Sigma) at 5.0, 0.5, and 0.05 μg/ml. After activation, the same cells were washed and resuspended in fresh media containing the secretagogue or control and were usually incubated for 6 h at 37°C. For time course experiments, a range of time points was examined between 10 min and 24 h. In some experiments, 0.1 mM nedocromil (Fisons Pharmaceuticals, Leics, UK) or 1 μM dexamethasone (SABEX, Boucherville, Quebec, Canada) were added to both the initial 10-min degranulation of the cells and the later 6-h incubation stage at the same time as the anti-IgE secretagogue.

Histamine release

A fluorometric assay was used to determine the extent of histamine release by PMCs as described previously (16). Briefly, after 10-min incubation at 37°C in either the presence or absence of secretagogues, PMCs were centrifuged at 800 rpm for 10 min at 4°C. The pellet was resuspended in the original volume to give a cell concentration of 1×10^6/ml. Samples of both the supernatant and pellet were diluted 1/50 (v/v) in HEPES-Tyrodes buffer and boiled for 5 min to inactivate histaminase. The composition of the buffer (in mM) was as follows: NaCl, 137; glucose, 5.6; KCl, 2.7, NaH_2PO_4, 0.4; $CaCl_2$, 1; HEPES, 10; and (supplemented with) BSA (0.1%), pH 7.3. After TCA precipitation of proteins, histamine levels were meesured by using a Shimadzu CR-15 fluorescence spectrometer. Histamine release was expressed as a percentage of the total cellular histamine content calculated by the following formula: (histamine in supernatant/histamine in supernatant + histamine in pellet) × 100. In some experiments, the histamine contents of the supernatant and pellet were also assessed after a 6-h incubation, which occurred after the initial degranulation and resuspension of cells.

B-9 bioassay

IL-6 bioactivity was measured by using a previously described B-9 hybridoma proliferation assay (17). Briefly, B-9 cells were cultured in MEM F-11 (GIBCO BRL), supplemented with 5% FCS, 2-ME, 1% Pen-Strep solution, and a supernatant source of IL-6. The IL-6 assay was performed in triplicate for each sample or standard in microtiter plates (Nunclon Inter-Med, Nunc, Roskilde, Denmark). B-9 cells (initially 2500/well) were cultured in the described medium in either the presence or absence of putative IL-6-containing samples. After 72-h culture of cells with samples or standard, 10 μl/well 0.5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; Sigma) was added, the plates were incubated for 4 h at 37°C, then 50 μl/well 10% Triton-HCl was added, and a further 18- to 24-h incubation in the dark was conducted. The colored reaction product was assessed at 570 nm by an ELISA reader. Both supernatant and pellet samples from mast cells were stored at −20°C for analysis. Immediately before analysis, pellets were sonicated for 5 min in a water bath sonicator to ensure complete liberation of soluble material. The samples were compared with a standard IL-6 supernatant at several dilutions to assess IL-6 content. Results are expressed as units of IL-6 per ml on the basis of the amount of sample

required to induce a half-maximal response. One unit is equivalent to approximately 0.45 pg IL-6. None of the known mast cell-derived cytokines have been shown to induce proliferation of this cell line under similar assay conditions.

Samples were compared with IL-6 standard curves run in the presence of the appropriate secretagogue because anti-IgE at 10 μg/ml and A23187 1 μM were found to cause a minor shift in B9 cell responsiveness to standard IL-6. The lower concentrations of these secretagogues had no significant effect on IL-6 detection as assessed by changes in the response of the cells to standard IL-6 preparations. Sonicated pellets of unstimulated mast cells added to the standard curve, for pellet IL-6 determination, also had no effect on IL-6 detection. Mast cell supernatants treated with either LPS or anti-IgE were preincubated with a polyclonal rabbit anti rat IL-6 serum generated in New Zealand rabbits by i.p. injection of a recombinant adenovirus, which contained rat IL-6 cDNA (Ad5E3:IL-6) (kindly provided by Dr. J. Gauldie, McMaster University, Hamilton, Ontario, Canada). The Ab completely abrogated the proliferation activity of a rat IL-6 control, as well as that of the supernatant from anti-IgE-treated mast cells (97.5% ± 0.5 inhibition and 90% ± 2, respectively; $n = 2$), whereas activity in the B9 assay in supernatants from LPS-treated mast cells were inhibited by 55.5% ± 4.5. These results demonstrate that LPS stimulates IL-6 production from rat PMCs, however, the presence of other unknown factors, which also induce B-9 cells proliferation, cannot be excluded completely.

TNF-α bioactivity assay

TNF-α was measured by a cytotoxicity bioassay with the use of a TNF-α-sensitive, human rhabdomyosarcoma clone A673/6 (New York University Medical Center, New York, NY). The method used was a modification of that described by Aderka et al. (18). Briefly, 50 μl/well of 5×10^5 A673/6 cells/ml in α-MEM medium (GIBCO BRL), supplemented with 5% FCS and 1% Pen-Strep, were plated into flat bottom Nunclon plates and incubated for 18 h at 37°C. The medium was discarded by suction and fresh medium was added, which contained 20 μg/ml cycloheximide (Sigma) and 100 μg/ml SBTI (Sigma). Human rTNF-α (Genentech, Inc., So. San Francisco, CA) was diluted in the same medium and used as a standard. This was examined in seven 10-fold serial dilutions from 20,000 pg/ml. We added 50 μl/well of either standard or samples in duplicates to the wells then plates were incubated at 37°C. After 18-h incubation, 10 μl/well MTT (5 mg/ml) was added and a further 4-h incubation was conducted. Then, 50 μl/well of a solution made with 50% *N,N*-dimethylformamide (Caledon Laboratories LTD, Edmonton, Ontario, Canada), 20% SDS (Bio-Rad, Mississauga, Ontario, Canada), pH 7.4, was added to dissolve the MTT. After an 18-h incubation at 37°C, the plates were read at 570 nm. Results were read off the standard curve to obtain the concentrations of TNF-α present in the samples. Media samples and supernatants from unactivated cells served as negative controls. Preincubation of mast cell supernatants with a specific rabbit anti-murine TNF-α Ab (Genentech) completely abrogated their cytotoxic activity. The addition of A23187 or anti-IgE at the concentrations used did not significantly alter the TNF-α standard curve.

Signal transduction

We studied some of the possible mechanisms involved in IL-6 production by rat PMCs ($n = 4$). After an initial release, PMCs (1×10^6/ml) were cultured for 6 h at 37°C with several agents, which are known to block specific signaling pathways, in the presence or absence of either LPS (5 μg/ml) or anti-IgE (5 μg/ml). To examine the calcium dependence in IL-6 production we used the same supplemented RPMI 1640 from GIBCO BRL as in the above experiments, but without the addition of Ca^{2+} and in the presence of EGTA (1 mM; Sigma) (19), to chelate any possible extracellular Ca^{2+} that might be released during the 6-h culture. To examine protein tyrosine kinase dependence, Genistein (Sigma) was added to the medium at a concentration of 37 mM (10 μg/ml) (20). To study the importance of Go and Gi proteins during IL-6 production, we used pertussis toxin (Sigma) at a concentration of 0.1 μg/ml (21). For protein kinase C (PKC) we used a similar approach to that described by Wodnar-Filipowicz et al. (12) to examine mast cell GM-CSF regulation. PMCs were incubated with their parallel controls in the presence of PMA (0.01 μg/ml; Sigma) for 18 h to deplete PKC, then the cells were washed and further incubated for 6 h at 37°C in the presence of either LPS or anti-IgE.

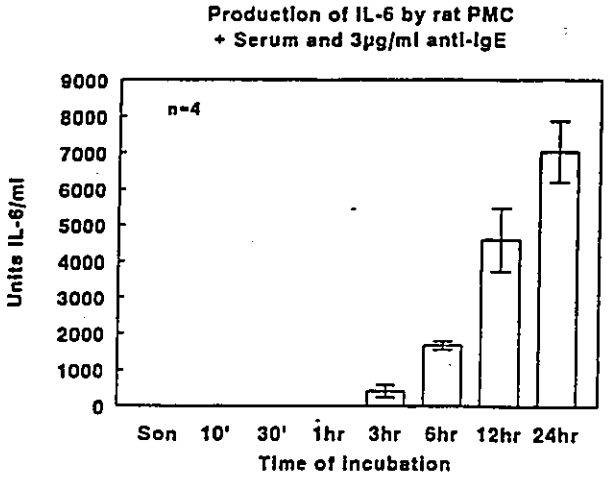

FIGURE 1. Time course IL-6 production by rat PMCs. Cells were stimulated with anti-IgE (3 μg/ml) in the presence of 5% FCS, washed, and resuspended in fresh anti-IgE-containing media after 10 min. IL-6 in the supernatants was first observed at 3 h and continued to increase up to 24 h. "Son" refers to freshly isolated PMCs that were frozen and thawed before sonication (5 min) to examine preformed IL-6 content. Bars represent mean values ± SE of the mean.

Viability assessment

The viability of PMCs after 6-h incubation with different secretagogues or controls was examined by using trypan blue exclusion according to standard protocols. The proportion of cells that were permeable to the trypan blue dye was assessed by counting cells with the use of a hemocytometer at ×400 magnification. Respiratory metabolism from parallel samples was examined by culturing them with MTT (0.5 mg/ml), which is metabolized only in active mitochondria. Freshly purified PMCs at a concentration of 0.25×10^4/ml and PMCs treated for 6 h with secretagogues (or diluent as control) were plated in triplicate into a 96-well, flat bottom plate. 10 μl MTT was added and the cells were incubated for 1 h at 37°C. After 1 h, we added 50 μl Triton-HCl and a further 1-h room temperature incubation was performed to dissolve the MTT. According to a previous report (22), the extent of respiratory metabolism in the cells is directly proportional to the OD reading obtained at 570 nm when examined on an ELISA reader. Trypan blue exclusion was also performed after a 6-h incubation of PMCs in media containing any of the agents used for the signaling studies.

Statistical analysis

The responses of aliquots of the same initial cell preparations to different secretagogues were compared by using a paired Students *t*-test. In some cases, in view of the data distribution, data were compared by using the nonparametric Mann-Whitney U statistic test or the Wilcoxon signed-ranks test (as stated in *Results*).

Results

Time course

In initial experiments, we examined the time course of IL-6 production by PMCs (see Fig. 1). Mast cells were activated with anti-IgE (3 μg/ml) in the presence of 5% FCS. Such anti-IgE treatment induced a mean histamine release of 32% after 10 min. The sonicated cell pellet, at the initiation of the experiment, and the degranulation products obtained after 10 min did not contain detectable

IL-6 bioactivity. This finding suggested that IL-6 was not preformed within these cells. The first detectable IL-6 was observed in the supernatants after 3-h incubation with anti-IgE. The levels of IL-6 continued to rise over a 24-h time course. In view of the results of this experiment, we chose 6 h as a suitable time point at which to examine IL-6 production by PMCs in later experiments. Examination of cell pellets at each time point did not reveal detectable levels of IL-6 bioactivity, suggesting that newly synthesized IL-6 is rapidly released from the cells. Sonication of a standard IL-6-containing supernatant did not reduce the amount of bioactivity detected.

Anti-IgE and A23187 activation of PMCs

Histamine release is a classical marker of PMC degranulation. One million pure PMCs/ml were initially stimulated for 10 min with A23187 or monoclonal mouse anti-IgE at a range of concentrations. These secretagogues are known to induce histamine release from PMCs without having a significant cytotoxic effect at the levels used. IgG_1 and diluent were used as negative controls. Under our experimental conditions, A23187 induced substantial of histamine release at both concentrations, anti-IgE induced histamine release, although to a lesser extent, and IgG_1 and diluent did not (see Fig. 2, *top*). An examination of the histamine contents of the supernatants obtained from the resuspended cells after 6-h incubation in the presence or absence of a secretagogue demonstrated a similar profile of ongoing histamine production, with A23187- and anti-IgE-treated cells exhibiting a highly significant ($p < 0.02$ for both secretagogues), greater histamine production than the control cells (data not shown).

Examination of the IL-6 content of the supernatants obtained after 6 h revealed that the cells treated with A23187, which exhibited the greatest histamine release, did not produce detectable levels of IL-6. In contrast, parallel anti-IgE-treated cells from the same animals produced substantial quantities of IL-6 that was significantly greater than that produced by IgG_1-treated and diluent control cells (Fig. 2, *bottom*).

Given these data, we considered the possibility that A23187 treatment was damaging the mast cells. Therefore, we confirmed the viability of the PMC after 6-h incubation with ionophore, anti-IgE, or medium. We found that 85 ± 3.5% of the cells treated with A23187 were still viable, as assessed by trypan blue exclusion after 6 h. This proportion was not significantly different from the viability of cells treated with anti-IgE (92.5 ± 2.6%) or of control cells (87 ± 3.35%). Respiratory metabolism was also examined by using MTT. There was no significant difference in this measure of metabolism among anti-IgE-treated cells, ionophore-treated cells, and controls. As a further measure of the ability of ionophore-treated cells to secrete protein, we examined the production of TNF-α bioactivity by PMCs treated with anti-IgE (5 μg/ml), control medium,

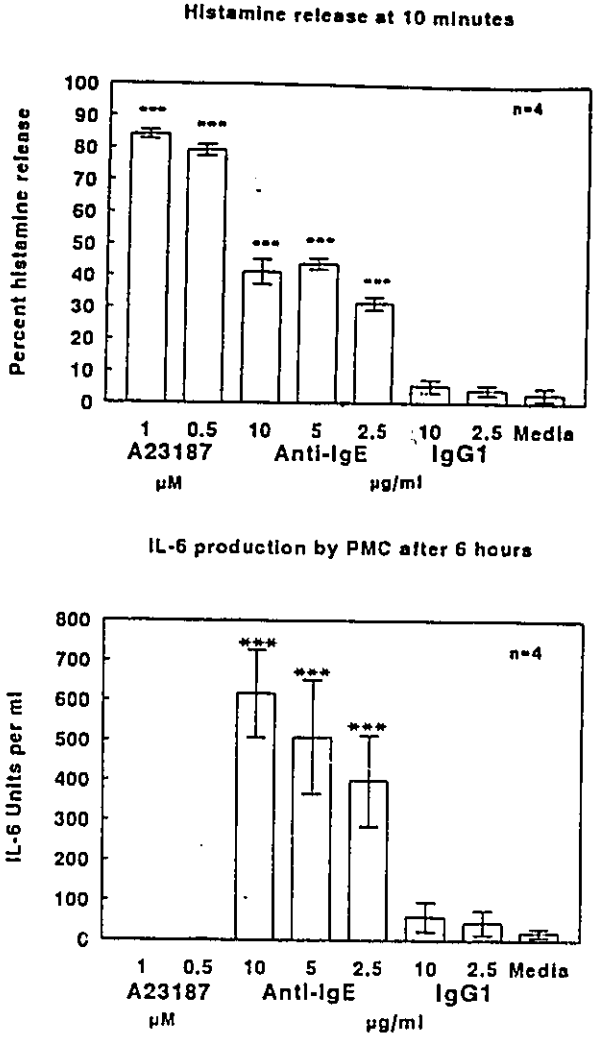

FIGURE 2. *Top panel*: Histamine release on initial degranulation induced by 10-min incubation of PMCs with different secretagogues. A23187 induced a significant amount of histamine release compared with media control ($p < 0.01$). Similarly, although of a lesser magnitude, anti-IgE released histamine to a significantly higher level than the IgG1 controls ($p < 0.01$). Results are expressed as mean percentage of histamine release ± SE of the mean. *Bottom panel*: IL-6 production, by PMCs in response to secretagogues. The cells were initially degranulated, washed, resuspended in fresh media containing 5% FCS, with or without secretagogues, and incubated for 6 h. A23187 at either concentration did not induce IL-6 production, whereas anti-IgE induced a substantial amount of IL-6 production that was significantly higher than the amount produced by cells incubated with control IgG1 ($p < 0.01$). Bars represent mean values ± SE of the mean. No IL-6 was detected that was associated with the cell pellet.

or A23187 (1 μM). After the initial 10-min incubation, resuspension in fresh media, and 6-h incubation, 132 ± 47 pg/ml TNF-α could be detected in the supernatants of the A23187-treated cells at a significantly greater level than was observed in supernatants from the control cells (21 ±

11 pg/ml). Anti-IgE-treated cells produced 48 ± 25 pg/ml bioactive TNF-α over the same time course.

To examine whether mast cell-derived protease enzyme activity interfered with accurate assessment of the IL-6 produced by A23187-treated cells, we performed an experiment in which the supernatant obtained from purified PMCs after 6-h incubation with A23187 or control medium was incubated with the IL-6 standard at a range of concentrations for 3 h at 37°C. No significant shift was observed between the standard curve mixed with medium and the one treated with the 6-h supernatant. IL-6 detected after incubation in PMC supernatant was equivalent to a mean of 98% of the IL-6 detected after incubation with medium alone ($n = 4$). These data strongly suggest that the blocking of protease degradation of IL-6 with SBTI was effective even in the presence of supernatants obtained from ionophore-activated PMCs.

To examine the possibility that mast cells activated with A23187 may produce detectable IL-6 at a later time point than 6 h, we incubated the mast cells with A23187 for longer periods of time (18 and 24 h). Our results show no detectable IL-6 at either of these time points (0 ± 0), which suggests that A23187 at the concentration of 1×10^{-6} M is not capable of inducing IL-6 production even after 24-h incubation of PMCs.

LPS activation of mast cells

LPS is known to induce IL-6 production by macrophages in a serum-dependent manner. In view of this, we examined the ability of mast cells to produce IL-6 when activated by LPS in both the presence and absence of serum. Three doses of LPS were examined in this experiment (5.0, 0.5, and 0.05 μg/ml), a serum concentration of 5% was used, and an equivalent protein content of BSA was maintained in the serum-free conditions. LPS did not induce a significant level of histamine release during the initial 10-min degranulation (see Fig. 3, *top*), although cells from the same animals exhibited substantial histamine release in response to A23187. The presence of serum did not significantly alter the degree of histamine release obtained. The PMCs treated with LPS in the presence of serum (see Fig. 3, *bottom*) produced a substantial amount of IL-6 during the 6-h incubation period and the longer time points (18–24 h) (1473 ± 655 U/ml, and 995 ± 405 U/ml, respectively) ($p < 0.02$, by Mann-Whitney U analysis), whereas there was no significant cytokine production by mast cells treated with LPS under serum-free conditions. Even after 18 to 24 h of incubation in the absence of serum there was no significant amount of IL-6 (34 ± 34 and 11 ± 11 U/ml, respectively). The ionophore-treated cells in this experiment demonstrated no IL-6 production in either the presence or absence of serum.

Despite the high purity of our mast cell preparations (95 to 100%) we considered the possibility that contaminating cells within our PMC preparations were responsible for

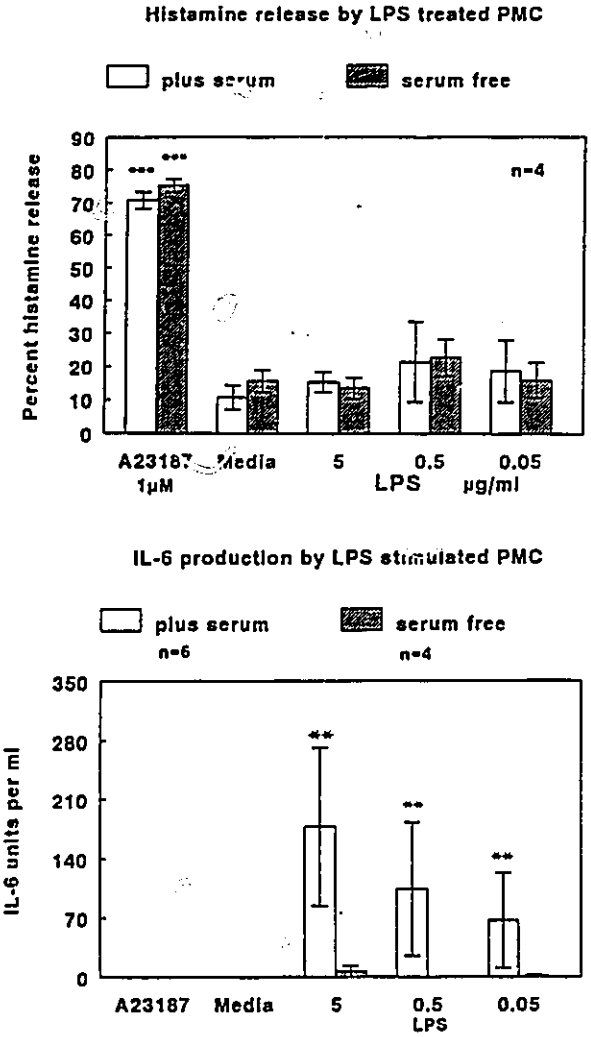

FIGURE 3. *Top panel*: Histamine release by LPS-treated PMCs. Both in the presence and absence of serum, a very similar histamine release profile was observed with each of the secretagogues we used. No significant release of histamine was observed at any of the three concentrations of LPS used. Results are expressed as mean values ± SE of the mean. *Bottom panel*: IL-6 production by LPS-stimulated PMCs. PMCs were cultured for a further 6 h in the presence or absence of LPS or A23187 as shown. Under serum-containing conditions, LPS was shown to induce IL-6 production. No IL-6 production was observed under serum-free conditions or by A23187-stimulated cells in either the presence or absence of serum. Results are expressed as mean values ± SE of the mean.

the observed IL-6 production. An examination of stained PMC preparations revealed that the predominant contaminating cell types were eosinophils and neutrophils, with few, if any, macrophages being observed. Furthermore, there was no significant correlation between the proportion of contaminating cells and the amount of IL-6 observed in the 6-h supernatant. In two cell preparations we obtained 100% PMCs with no detectable contamination by other

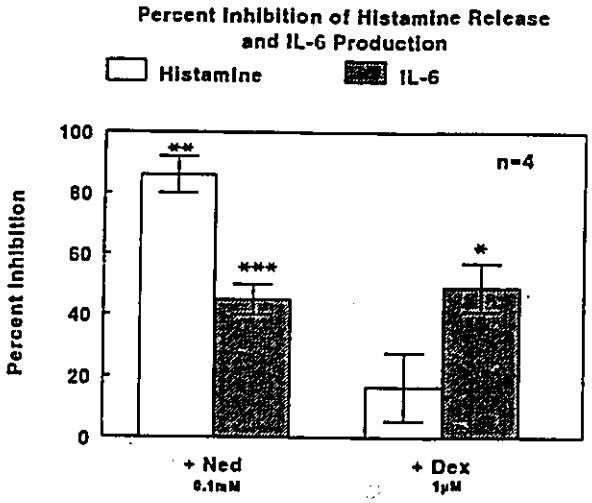

FIGURE 4. The effect of dexamethasone and nedocromil in IL-6 production and histamine release by PMCs. PMCs were activated in media containing 5% FCS with anti-IgE in either the presence or absence of nedocromil (0.1 mM) or dexamethasone (1 μM) for both the initial 10-min release and the later 6-h incubation. Nedocromil significantly inhibited histamine release from these cells, but dexamethasone did not. Both anti-allergic drugs were capable of effectively inhibiting IL-6 production by these cells. Values are expressed as mean percentage of inhibition compared with individual controls run in the presence of anti-IgE alone.

cell types; the supernatants after 6-h incubation from these cells at 5 μg/ml concentration of LPS contained 80 U/ml and 153 U/ml of IL-6, respectively. These data very strongly suggest that the PMC is the source of the detected IL-6. Inasmuch as no IL-6 was detectable in the cells at the initiation of culture, the IL-6 content of the supernatants cannot be explained in terms of earlier mast cell uptake of this cytokine from other cell types.

The effect of anti-allergic drugs

To gain further insight into the regulation of IL-6 production by the mast cell and the relationship of mast cell degranulation to IL-6 production, we examined the abilities of nedocromil (a mast cell stabilizing agent) and dexamethasone (a corticosteroid) to alter IL-6 production by anti-IgE-activated PMCs (see Fig. 4). Under the conditions we used, nedocromil induced significant inhibition of 10-min histamine release, but dexamethasone had no significant effect. In contrast, both dexamethasone ($p < 0.05$) and nedocromil ($p < 0.02$) significantly inhibited the amount of IL-6 produced by the PMCs during the 6-h incubation (Fig. 4). The effect of dexamethasone treatment was of particular interest, because, in this case, the degree of histamine release of the cell and its ability to produce IL-6 were differentially modulated. Cyclosporin A has been shown to only slightly reduce the IL-6 mRNA expression induced by a PCA response in mice and to have

Table I. *Percent inhibition of IL-6 response to anti-IgE or LPS after 6 h compared with parallel control*

Inhibitor	Stimulus	
	LPS (5 μg/ml)	Anti-IgE (5μg/ml)
Ca^{++}-free media[a] (1 mM EGTA)	100 ± 0[b]	100 ± 0[b]
Genistein (10 μg/ml)	39 ± 15	30 ± 17
Pertussis toxin (0.01 μg/ml)	30 ± 9[b]	61 ± 14[b]
PMA pretreatment[c] (0.01 μg/ml)	24.5 ± 24.5	100 ± 0[b]

[a] Supplemented RPMI 1640 (see *Materials and Methods*), no calcium added, 1 mM EGTA.
[b] ($p \leq 0.05$) Nonparametric Wilcoxon signed-rank test.
[c] PMC were incubated with media in the presence or absence of 0.01 μg/ml PMA during 18 h with the previous initial release (10 min). After long incubation, the cells were washed and resuspended in media with either LPS or anti-IgE, or with media as control for 6-h further.

no effect on the IgE-mediated induction of IL-6 in mouse mast cell line. However, under our experimental conditions cyclosporin A (1–10 μg/ml), did not significantly stabilize the rat anti-IgE- or A23187-induced histamine release from PMCs (2 ± 2% or 7 ± 1% mean inhibition, respectively, at 10 μg/ml) and also had no significant effect on IL-6 production. The histamine release experiments were run in parallel with mouse PMCs, which were stimulated with A23187 (1 μM/ml) in the presence or absence of cyclosporine A. We observed a 25 ± 2% inhibition of histamine release from the mouse mast cells cultured in the presence of cyclosporine A, which showed the bioactivity of the cyclosporine A that we used. These results suggest that cyclosporine A is not a good stabilizing agent for Brown Norway rat PMCs.

Signal transduction

To understand the possible mechanism(s) involved during IL-6 production by PMCs, we used several agents that are known to interfere with different signaling pathways. The results in Table I express the percent inhibition of IL-6 production after 6-h incubation in response to either LPS or anti-IgE. Response is defined as the difference in IL-6 production between cells in the presence of media, LPS, or anti-IgE alone compared with such LPS- or anti-IgE-induced products in the presence of any of the blocking agents. Our results show that, at 6 h, IL-6 production by rat PMCs in response to either LPS or anti-IgE is highly Ca^{2+}-dependent, because there was a 100% inhibition in IL-6 response in the absence of Ca^{2+}. However, when Genistein was used, inhibition of IL-6 production in response to LPS or anti-IgE was not significant, which suggests that PMC IL-6 produced during 6-h incubation may not require protein tyrosine kinase activation. However, when cells were treated with pertussis toxin, a significant inhibition of the 6-h IL-6 response to either LPS or anti-IgE was observed, which indicates a possible role of these

G-protein subtypes during IL-6 production by rat PMCs. When PKC was depleted from PMCs by a previous 18-h incubation with PMA only, the IL-6 response to anti-IgE was significantly inhibited ($P \leq 0.05$), whereas the IL-6 response to LPS not significantly altered. We also observed that PMA alone significantly induced IL-6 production (2960 ± 991 U/ml IL-6 at 6 h, $n = 4$) compared with media-incubated control cells (812 ± 555 U/ml IL-6, $n = 4$) Therefore, it seems that the mechanisms by which PMCs are capable of inducing IL-6 production are complex and may involve more than one pathway.

Discussion

The role of mast cells in a number of disease processes is poorly understood. Mast cells can be observed in increased numbers in the context of many chronic inflammatory conditions, including rheumatoid arthritis (23, 24), scleroderma (25), and inflammatory bowel disease (26–28). Our recent understanding of the potential for mast cells to produce cytokines has lead us to question whether the assessment of preformed mediator release is always an effective measure of the major biologic impact of these cells. In the current study, we have examined the relationship between the degranulation of mast cells as assessed by histamine release and the production of the pluripotent cytokine IL-6.

Our observation that A23187 can induce histamine release without IL-6 production or release demonstrates that it is possible for the mast cell to selectively produce histamine, and not IL-6, when appropriately stimulated. This finding also suggests that calcium influx induced by calcium ionophore (29) is not sufficient to induce IL-6 production by mast cells. However, PMC IL-6 synthesis is calcium dependent in response to both anti-IgE and LPS as demonstrated by our experiments under calcium-free conditions. The observation that LPS can induce IL-6 production by mast cells in a serum-dependent manner, confirms that IL-6 production and histamine release are independently regulated. The presence of serum had no significant effect on histamine release, but was necessary for IL-6 production. Serum response elements within the IL-6 gene have been well described in other cell types (17, 30, 31), such as fibroblasts and macrophages. In the context of LPS, the mast cell seems to mimic the IL-6-producing behavior of such cells. The observation of LPS-induced mast cell IL-6 production is important to our understanding of the potential of this cell as a source of cytokines under conditions in which classical mast cell mediator release is not observed.

During bacterial infection it is possible that the mast cell could act as a major source of IL-6 and other cytokines without classical degranulation occurring. The mast cell is found at high levels at those sites, such as the skin, trachea, and gastrointestinal tract, which interface directly with the external environment. Mast cells are also found to be associated with blood vessels. This anatomical arrangement would place them in the first line of defense against bacterial infection. Because some of our PMC preparations, which contained no detectable contaminating cells, produced a similar level of IL-6 to that observed in cell preparations containing up to 5% other cell types, we are convinced that the source of this cytokine in our PMC preparations is the mast cell. The ability of the cell to release IL-6 efficiently into the supernatant in the absence of histamine release suggests that the mast cell is capable of regulating the transport of proteins into the extracellular environment independent of classical degranulation. We hypothesize that the mast cell produces and secretes IL-6 in a manner similar to that observed in other cell types, such as macrophages and monocytes.

When cell-bound IgE is cross-linked by anti-IgE or specific Ag, the release of preformed mediators and the production of lipid mediators occurs (32, 33). A number of signal transduction pathways are initiated through such IgER mediated mast cell activation (34). As has been demonstrated in mouse mast cells, IgE-mediated activation of rat PMCs induces IL-6 production and also histamine release. It is possible that IL-6 is induced directly through one of the known signaling pathways or that the production of another cytokine, such as TNF-α or IL-1β, by the mast cell acts in an autocrine fashion to induce IL-6 production in vitro.

By using specific agents known to alter either histamine release or IL-6 production in other cell systems, we examined the relationship between degranulation and IL-6 production in immunologically activated PMCs. Nedocromil is a potent inhibitor of mast cell degranulation that is widely used in clinical settings. This agent, which is thought to act in preventing the initiation of degranulation (35, 36), also significantly inhibited PMC IL-6 production in response to anti-IgE. Thus, prevention of IgE-mediated degranulation may, in some cases, lead to a prevention of IL-6 production. However, prevention of preformed mediator release is not necessary to prevent cytokine production. Dexamethasone, a corticosteroid which has no significant effect on histamine release from PMCs under our experimental conditions, was capable of inhibiting the production of IL-6. In other cell systems, IL-6 production has been shown to be extremely steroid sensitive, having a defined steroid-response element characterized for this cytokine (37, 38). Our observations would indicate that mast cell IL-6 production can be regulated in a manner similar to that of other cells. Plaut et al. have observed production of several cytokines in mast cell lines and mouse PMCs after stimulation with either calcium ionophores or by FcεRI or FcεRII cross-linking (14, 39). In each of these studies, in contrast to our observations, cytokine production by mast cells has been related to mast cell degranulation. By using mouse mast cell lines, Wodnar-Filipowicz et al. have demonstrated that a large amount of IL-3 and GM-CSF mRNA accumulates very rapidly after exposure of cells to calcium ionophore A23187 (12, 13), which is

associated with later production of these cytokines at the protein level. The mRNA for these cytokines has been demonstrated to be continuously synthesized and degraded within the cell. The reduction in mRNA degradation after calcium influx allows for a very rapid increase in cytokine mRNA to be achieved by the cell upon activation. Currently, there is little additional information that concerns other mechanisms of cytokine gene induction in mast cells. Prolonged receptor cross-linking and extracellular calcium are thought to be required, on the basis of the ability of hapten DNP-lysine and calcium chelator EDTA to inhibit Ag-induced IL-3 and IL-4 production (40). Depletion of PKC by long term PMA exposure was not found to inhibit induction of either IL-3 or GM-CSF in response to A23187 (12). However, by using a similar approach we found significant inhibition of IL-6 production only when PMCs were activated with anti-IgE, not when they were activated with LPS. Our data on IL-6 production by freshly isolated mast cells would strongly suggest that IL-6 production in cells activated with A23187 is regulated through a different mechanism than the Ca^{2+}-dependent, post-transcriptional mechanism described for IL-3 and GM-CSF (12, 13). Interestingly, we found that IL-6 production is Ca^{2+} dependent when PMCs are activated with either anti-IgE or LPS. Our data also suggest that IL-6 production by PMCs does not involve protein tyrosine kinase. However, some G proteins may participate in anti-IgE- or LPS-induced IL-6 production, because, when pertussis toxin was used, we observed significant inhibition of IL-6 for both cases, although it was greater in the presence of LPS than of anti-IgE.

The regulation of IL-6 production has not been previously examined in detail in mast cells and has only been reported after activation of mast cells with agents known to induce histamine release. However, information is available concerning the regulation of IL-6 production by other cell types. Bacterial LPS has been shown to up-regulate IL-6 mRNA in monocytes and to induce IL-6 protein production in a serum-dependent manner (30, 31, 41). Similarly, viral infection has been shown to induce IL-6 production (42–44). Endothelial cells, fibroblasts, and monocytes/macrophages have traditionally been considered the major sources of IL-6 during systemic inflammation (45). However, not all cells respond to such factors in the same way, for example, IL-1 is the most potent inducer of IL-6 in fibroblasts, but induces little IL-6 in bone marrow, which, in turn, responds well to IL-3 and GM-CSF (46). The relative contribution of the mast cell to the IL-6 production observed during chronic inflammation or after different types of infection is yet to be determined. However, our data demonstrate that IL-6 can be produced by mast cells in substantial quantities within a few hours of activation. As such, mast cells could be of particular importance in the initiation of the inflammatory response. The ability of the mast cell to produce IL-6 in response to stimuli that do not induce degranulation further demonstrates the complex regulation and versatility of the mast cell response.

This work is, to our knowledge, the first report of cytokine production by mast cells in response to LPS and it suggests a potential role for mast cells in response to infection, which is independent of their release of preformed mediators. Our observations concerning the ability of dexamethasone to modulate IL-6 production without altering the degree of histamine release and the inability of A23187 to induce IL-6 production at dosages which induce histamine release further demonstrate the complexity of IL-6 regulation in the mast cell. Preliminary findings from our examination of signaling mechanisms also demonstrate that IL-6 production by PMCs may involve several signal transduction pathways that are dependent on the activating stimulus. Overall, these studies emphasize the importance of examining the mast cell and its production of cytokines in a variety of disease processes, including those in which the mast cell has not previously been considered to play a role because no preformed mediator release was observed. Additional studies should be aimed at increasing our understanding of the role and regulation of mast cell-derived cytokines in disease processes with a view to appropriate pharmacologic modulation of the mast cell response.

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CHAPTER THREE

Prostanoid Enhancement of Interleukin-6 Production by Rat Peritoneal Mast Cells[1]

Irene Leal-Berumen,* Paul O'Byrne,† Abha Gupta,* Carl. D. Richards,* and Jean S. Marshall[2]*

*Molecular Virology and Immunology Programme, Department of Pathology, and **Asthma Research Group, McMaster University, Hamilton, Ontario, Canada

Mast cells are traditionally associated with an acute response involving the short-term release of mediators such as histamine. We have shown previously that mast cells can produce IL-6 without prior histamine release. In this study we examined the hypothesis that mast cell IL-6 production can be selectively regulated by PGs. Highly purified rat peritoneal mast cells were cultured in the presence of PGE_1, PGE_2, or PGD_2 alone or in combination with anti-IgE or bacterial LPS. Histamine release was assessed after 10 min; IL-6 and TNF-α production was measured in supernatants after 18 h. Mast cell IL-6 production was induced by PGE_1 and PGE_2 to a similar level to that observed in anti-IgE-activated cells. In contrast, constitutive production of TNF-α was inhibited by PGE_1 and PGE_2, but not by PGD_2. PGE_2 had a synergistic effect, inducing IL-6 in the presence of LPS, whereas an additive effect was observed in the presence of anti-IgE. None of the prostanoids alone induced significant histamine release at the 10-min time point. However, PGE_2 significantly increased histamine release when added concurrently with anti-IgE. Flurbiprofen in the context of anti-IgE or LPS activation did not alter mast cell IL-6 or TNF-α production. IL-6 production in response to each of the stimuli was significantly inhibited by the corticosteroid dexamethasone. These observations of selective modulation of mast cell cytokine production are important to understand the mechanisms by which mast cells interact with other cells during an inflammatory process involving prostanoid synthesis. *The Journal of Immunology,* 1995, 154: 4759–4767.

PGE_1 and PGE_2 are products of arachidonic acid metabolism and can be synthesized by a wide range of cell types, including macrophages, neutrophils, fibroblasts, and follicular dendritic cells (1–4). Connective tissue-type mast cells in the rat produce mainly PGD_2 and low levels of PGE_2 and PGE_1 on IgE Ag-mediated activation (5, 6). However, mast cell products, such as histamine, have been shown to induce PGE_2 production by other cells (7, 8). PGE_1 and PGE_2 have been widely studied in the context of inflammation. They have been regarded generally as proinflammatory mediators, because they are potent vasodilators and can act synergistically with other inflammatory mediators (9). For example, oncostatin M alone does not induce IL-6 from lung or synovial fibroblasts, whereas in the presence of PGE_2 a synergistic effect on fibroblast IL-6 production is observed (10). However, there are other circumstances when these PGs may have an antiinflammatory action. Pretreatment of allergic subjects with inhaled PGE_2 has been shown to ablate both the early and late asthmatic response to airways challenge in sensitized subjects (1). Zurier et al. (11) also have suggested an antiinflammatory role for PGE_1 and demonstrated suppression of adjuvant arthritis in rats by PGE_1. In vitro, under appropriate conditions, PGE_1 and PGE_2 can inhibit the release of preformed inflammatory mediators from granulocytes (12–15), mast cells (16–18), and lung tissue (19–21).

There is evidence that PGs of the E series can enhance the production of IgE and IgG1 in the context of other stimuli. Although PGs alone do not enhance IgE or IgG1 production, mouse splenocytes stimulated with LPS and PGs synergize with IL-4 to stimulate such Igs, while simultaneously diminishing IgM and IgG3 synthesis (22, 23). PGE_2 has been shown to enhance IL-6 production in vitro from lung- or synovial tissue-derived fibroblasts (10). In addition, LPS-induced TNF-α production by activated

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[1] This work is supported by the Medical Research Council of Canada.

[2] Address correspondence and reprint requests to Dr. Jean S. Marshall, Department of Pathology, McMaster University, Health Sciences Centre, Room 4H17, 1200 Main St. West, Hamilton, Ontario, L8N 3Z5 Canada.

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macrophages and anti-IgE-induced mast cell TNF-α-mediated cytotoxicity have been shown to be suppressed by PGE_2 (24–27). Rodent mast cells are found to release preformed mediators such as histamine, proteases, proteoglycans, and TNF-α, and newly formed ones such as IL-6, IL-4, leukotrienes, and PGs after immunologic activation (28–31). Human mast cells are also known to store preformed TNF-α and to produce IL-6 among other cytokines (32). However, the production of some mast cell cytokines can occur without preformed mast cell mediator release (33).

In this study we have examined the ability of the prostanoids PGE_1, PGE_2, and PGD_2 to modulate mast cell IL-6 production in the context of anti-IgE or LPS challenge. We provide evidence that PGE_2 and PGE_1, without causing histamine release, are selective inducers of IL-6 production, while concurrently inhibiting endogenous TNF-α production. PGE_1 and PGE_2 induced a similar amount of IL-6 production as that resulting from anti-IgE activation. We also have observed that PGE_2, but not PGE_1, can synergize with LPS to enhance mast cell IL-6 production. Mast cells simultaneously incubated with PGE_2 in the presence of anti-IgE demonstrated enhanced histamine release. Neither PGE_1 nor PGE_2 synergized with anti-IgE in inducing IL-6 production. Because levels of PGs are markedly raised at sites of inflammation, these results are important to understanding the interaction of mast cells with other cells during an inflammatory process.

Materials and Methods

Mast cell source and activation

Brown Norway (high-IgE-producing rats) (Harlan Sprague-Dawley, Inc., Indianapolis, IN) weighing 150 to 200 g were used to obtain peritoneal mast cells (PMCs)[3] by peritoneal lavage. The animals were housed in the Central Animal Facility at McMaster University in filter-hooded cages and were allowed food and water ad libitum. All experimental procedures were approved by the Animal Research Ethics Board of McMaster University. PMCs were purified on a 30%/80% discontinuous Percoll gradient (Pharmacia LKB, Uppsala, Sweden) with a range of 95% to 100% purity. The predominant contaminating cells were neutrophils; macrophage contamination was not observed in any experiment using the Diff-Quik stain set (Baxter, McGaw Park, IL). One million purified PMCs per ml were suspended in RPMI 1640 without phenol red (GIBCO BRL, Grand Island, NY), supplemented with 5% FCS (v/v), penicillin (50 U/ml), streptomycin (50 μg/ml; 1% Pen-Strep; GIBCO BRL), 1.4 mM Ca^{2+}, and 100 μg/ml soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO). The cells were initially degranulated for 10 min at 37°C by using the following activating agents: a mouse monoclonal IgG1 anti-IgE (MARE-1; University of Louvain, Louvain, Belgium) at 5 μg/ml; LPS (*Escherichia coli* serotype 055-B5; Sigma Chemical Co.) at 5 μg/ml; PGE_1 (CAYMEN Chemical, Ann Arbor, MI), PGD_2 (Sigma Chemical Co.) at 1, 0.1, or 0.01 μM; or PGE_2 (Sigma Chemical Co.) from 0.001 to 1 μM. After activation, the same cells were washed and resuspended in fresh media containing the activating agents and were usually incubated for 18 h at 37°C. For the time course experiment, a range of times were examined between 10 min and 18 h. In some experiments 1 μM dexamethasone (SABEX, Boucherville, Quebec, Canada) or 1 μM Flurbiprofen (Sigma Chemical Co.) were added to both the initial 10-min degranulation of the cells and the later 18-h incubation concurrent with the prostanoid, anti-IgE, or LPS-activating agent.

Mediator release

Histamine release by PMCs was assessed by a fluorometric method as described previously (34). Briefly, after 10 min of initial degranulation at 37°C in the presence or absence of secretagogues, PMCs were centrifuged at 800 rpm for 10 min at 4°C. The pellet was resuspended in the original volume to give a cell concentration of 1×10^6/ml. Samples of pellet and supernatant were diluted 1/50 (v/v) in HEPES-Tyrodes buffer and boiled for 5 min to inactivate histaminase. The buffer composition (in mM) was: NaCl, 137; glucose, 5.6; KCl, 2.7; NaH_2PO_4, 0.4; $CaCl_2$, 1; and HEPES, 10, supplemented with BSA (0.1%), pH 7.3. After precipitation of proteins using TCA (25%), fluorescent products from *o*-phthalaldehyde reaction were measured by using a Shimadzu (Columbia, MD) CR-15 fluorescence spectrometer. Histamine release was expressed as percentage of the total cellular histamine content (histamine in supernatant/histamine in supernatant + histamine in pellet) × 100. Histamine release was also measured in supernatants and cell pellets after 18 h of incubation in some experiments.

PGD_2 release by mast cells was measured after 30 min in response to either anti-IgE or LPS alone or in the presence of Flurbiprofen (1 μM) by using a PGD_2 ^3H assay system (Amersham, International plc, Buckinghamshire, UK).

B-9 bioassay

The bioactivity of IL-6 was measured by a previously described B-9 hybridoma proliferation assay (35). Briefly, B-9 cells were cultured in MEM F-11 (GIBCO BRL), supplemented with 5% FCS, 2-ME (5×10^{-5} M), 1% Pen-Strep solution, and a supernatant source of IL-6 (normal human lung fibroblast stimulated with IL-1β for 24 h in MEM F-11 media containing 10% FCS and 1% Pen-Strep). The IL-6 assay was performed in triplicate for each sample or standard in microtiter plates (Nunc, Roskilde, Denmark). B-9 cells (initially 2500 per well) were cultured in the described medium in either the presence or absence of putative IL-6-containing samples. After 72 h of culture of cells with samples or standard, 10 μl/well 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT; Sigma Chemical Co.) were added, and the plates were incubated for 4 h at 37°C. Fifty microliters/well 10% Triton-HCl were then added, and a further 18-h incubation in the dark at room temperature was conducted. The colored reaction product was assessed at 570 nm by an ELISA reader. Supernatant samples from mast cells were stored at −20°C for analysis. The samples were compared with a standard IL-6 supernatant prepared from human lung fibroblasts, which had been standardized by comparison with recombinant human IL-6 at several dilutions to assess IL-6 content. Results are expressed as units of IL-6 per ml, on the basis that 1 U is equivalent to the amount of sample required to induce a half-maximal response. One unit is equivalent to approximately 0.45 pg of IL-6 according to the National Biologic Standard Board.

None of the known mast cell-derived cytokines have been shown to induce proliferation of this cell line under similar assay conditions. IL-6 standard curves were run in the presence of each prostanoid used, Flurbiprofen, or dexamethasone (at the highest concentration of 1 μM). None of these results significantly changed the response of the B-9 cells to standard IL-6 preparations.

TNF-α bioactivity assay

TNF-α was measured by a cytotoxicity bioassay with the use of the TNF-α-sensitive L929 (mouse fibroblast) cell line, using a modification of the method previously described (30, 33). Briefly, 50 μl/well 1×10^6 L929 cells/ml in RPMI media, supplemented with 5% FCS and 1% Pen-Strep, were plated into flat-bottom Nunclon plates and incubated overnight at 37°C. Media were discarded by suction and replaced with fresh media containing 20 μg/ml cycloheximide and 100 μg/ml soy bean trypsin inhibitor (Sigma Chemical Co.). Mouse rTNF-α (Genentech, Inc., San Francisco, CA) was diluted in the same media and used as a standard. Seven 10-fold, serial dilutions starting from 20,000 pg/ml were used to establish the standard curve. Fifty microliters per well of standard or samples in duplicates were added, then the plates were incubated at 37°C.

[3] Abbreviations used in this paper: PMC, peritoneal mast cell; MTT, (3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium); NRS, normal rabbit serum; GM-CSF, granulocyte-macrophage CSF; PGs, prostaglandins.

After an 18-h incubation, the live cell number was assessed. Ten microliters per well of MTT (5 mg/ml) were added, and a further 4-h incubation was conducted. Then 50 μl/well of a solution of 50% *N,N*-dimethylformamide (Caledon Laboratories, Ltd., Edmonton, Ontario, Canada), 20% SDS (Bio-Rad, Mississauga, Ontario, Canada), pH 7.4, were added to dissolve the MTT. After an 18-h incubation at 37°C, the plates were read at 570 nm. Results were read off the standard curve to obtain the concentration of TNF-α present in the samples. Media samples and supernatants from unactivated cells served as negative controls. The addition of anti-IgE, LPS, or prostanoids did not significantly alter the TNF-α standard curve.

Statistical analysis

The response of aliquots of the same initial preparations to different secretagogues were compared by using a paired Student's *t*-test for histamine release. However, for IL-6 and TNF-α experiments, in view of the data distribution, data were compared by using the nonparametric Wilcoxon signed-rank test.

Results

Effects of PGE_1 and PGE_2 on histamine release and IL-6 production

We initially examined the effect of prostanoids on histamine release and IL-6 production from highly purified rat peritoneal mast cells. Mast cells were activated with anti-IgE (5 μg/ml) or media as controls, and PGE_1 or PGE_2 as activating agents at different doses. PGE_1 or PGE_2 alone had no significant effect, at any dose, on histamine release compared with the anti-IgE-positive control (Fig. 1*A*). After the 10-min histamine release, the cells were washed and cultured in fresh media containing the same secretagogues for a further 18 h. The cell-free supernatants were then analyzed for IL-6 production. Our results show that PGE_1 or PGE_2 at concentrations of 0.1 or 1 μM significantly ($p < 0.05$) increased IL-6 production (Fig. 1*B*). A dose-response experiment was also performed with PGD_2 (from 10–0.001 μM); however, it did not induce a significant amount of IL-6 production or histamine release at any dose (data not shown).

Mast cell purity

To confirm that PGE_1- and PGE_2-induced IL-6 production originated from mast cells and not from contaminating cells, such as macrophages, we analyzed our data by comparing the IL-6 production in prostanoid-stimulated cultures of mast cell preparations with different degrees of purity in three groups: 1) 100% pure mast cells; 2) less than 100% pure; and 3) 96% or less pure. Our results showed no statistically significant difference in IL-6 production between these groups, as previously described for anti-IgE- and LPS-activated cells (33). Mast cell preparations in which no contaminating cells could be detected (100% pure) produced a mean of 3439 ± 1529 U/ml IL-6 ($n = 5$) in response to PGE_1 and a mean of 4775 ± 1607 U/ml IL-6 in response to PGE_2 ($n = 9$). The low numbers of contaminating cells in other preparations were identified as mainly neutrophils based on morphology. No macrophage-like cells were ever observed in our PMC preparations after Diff-Quik staining.

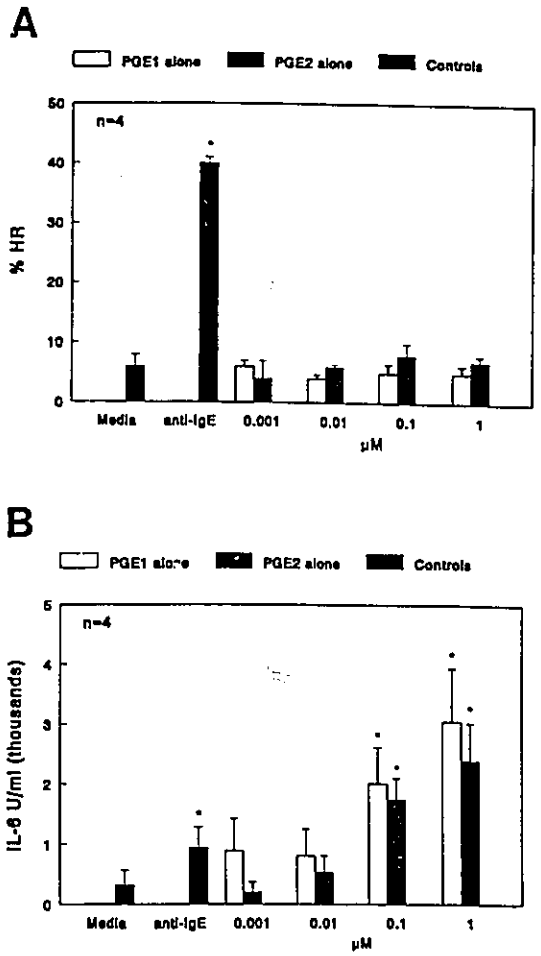

FIGURE 1. PGE_2 and PGE_1 dose response. *A*) Histamine release at 10 min. Rat PMCs were stimulated with anti-IgE or media as controls for 10 min or with different doses of PGE_2 or PGE_1. *B*) The same cells were washed and resuspended in fresh media containing the same activating agents and controls and allowed to further incubate for 18 h. After 18 h of culture, the cells were spun down, and the supernatants were diluted before the B-9 hybridoma assay for IL-6. Bars represent mean values ± SEM. Open bars denote PGE_1 alone; crossed bars represent PGE_2 alone; and filled bars represent controls. *, Significant ($p < 0.05$) difference compared with media control.

Comparison of PGE_1, PGE_2, and PGD_2

To directly compare the effects of PGE_2, PGE_1, and PGD_2, mast cells were initially incubated for 10 min in the presence or absence of the prostanoids at a concentration of 0.1 μM in parallel. No significant effect was observed on the initial 10-min histamine release by any of the prostanoids (Fig. 2*A*). Histamine release at 18 h showed a similar profile (media = 11 ± 1.5%; anti-IgE = 20.4 ± 2.6%; PGE_2 = 13.2 ± 2%; PGE_1 = 16.9 ± 2.4%; and PGD_2 = 11 ± 1%). Anti-IgE (positive control) significantly increased histamine release at 18 h ($p \leq 0.002$). PGE_2 and PGD_2

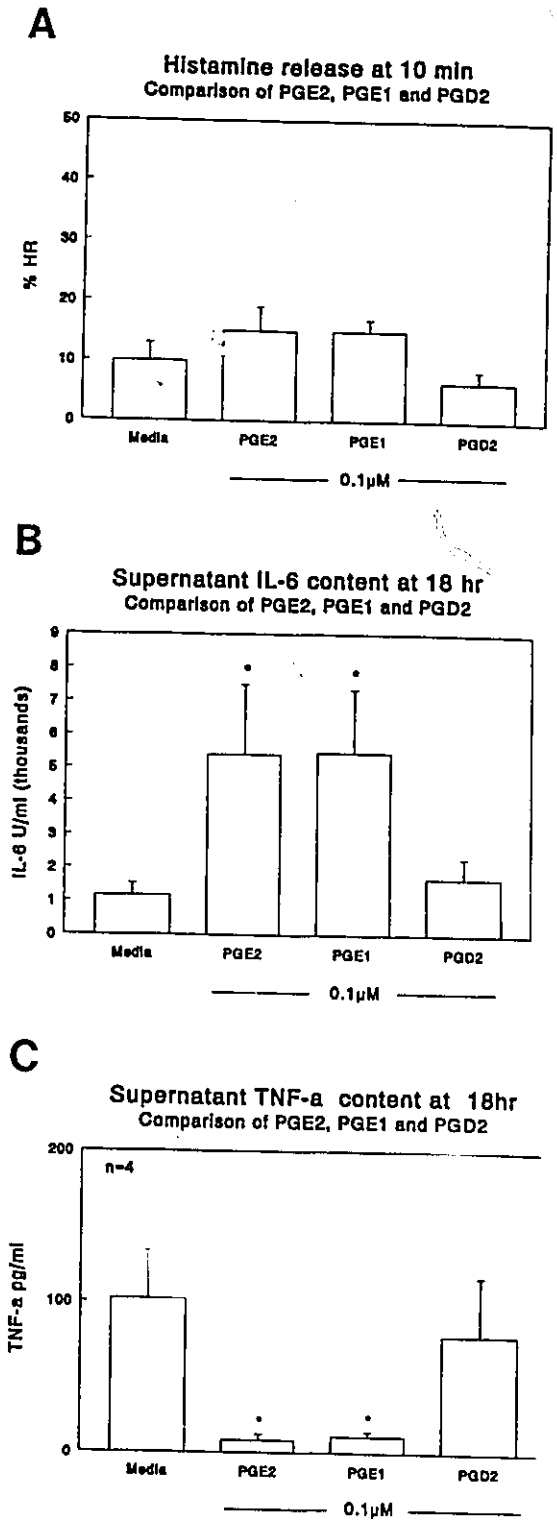

FIGURE 2. Effect of PGE_2, PGE_1, and PGD_2. *A*) Histamine release after 10 min of incubation, comparing the effects of PGE_2 (0.1 μM), PGE_1 (0.1 μM), and PGD_2 (0.1 μM). *B*) The same cells were washed and resuspended in media containing prostanoid or control. A further 18-h incubation was performed to assess IL-6 production, which was not significantly increased in response to PGD_2, whereas PGE_2 and PGE_1 significantly ($p < 0.05$) induced IL-6 production. *C*) TNF-α production after 18 h of incubation showed a significant ($p < 0.05$) decrease in the presence of PGE_2 or PGE_1 but not PGD_2. Note that $n = 4$ is represented in all experiments, except (*A*) and (*B*), where $n = 7$ for PGD_2 studies. Bars represent mean values ± SEM.

had no significant effect on histamine release compared with the media control, whereas cells treated with PGE_1 showed a marginal (5.9%) increase in histamine release at this time point ($p \leq 0.04$). IL-6 production from the same cells after an 18-h incubation demonstrated a significant ($p < 0.05$) increase in IL-6 production in the presence of PGE_1 or PGE_2, whereas PGD_2 did not induce a statistically significant increase in the IL-6 production at this dose (Fig. 2*B*). In contrast, as has been previously reported, baseline TNF-α production was significantly inhibited by PGE_1 or PGE_2, but no effect was observed with PGD_2 at this dose (Fig. 2*C*).

We further studied the effect of PGE_1 or PGE_2 on anti-IgE- or LPS-induced IL-6 production. We observed that PGE_2 induced a small but significant increase in histamine release in the presence of anti-IgE ($p < 0.01$ by t-test; $p < 0.05$ by Wilcoxon signed-rank test). In contrast, PGE_1 did not affect anti-IgE-mediated release, and neither PGE_2 nor PGE_1 increased histamine release in the presence of LPS (Fig. 3*A*). To further confirm the effect of PGE_2 on anti-IgE-induced histamine release, in a separate experiment, we incubated anti-IgE with different doses of PGE_2, and our results also showed a small but significant increase in histamine release at a lower PGE_2 concentration of 0.01 μM (percent histamine release in the presence of anti-IgE + PGE_2 = 50% ± 2, compared with anti-IgE alone, 40% ± 1). A significant increase in IL-6 production was observed under all conditions in the presence of PGE_1 or PGE_2 at a 0.1 μM concentration (Fig. 3*B*). Interestingly, a synergistic effect of PGE_2 on IL-6 production was observed in the context of LPS.

PGE_2 time course

We examined the time course of IL-6 production, over 18 h, by PMCs in response to anti-IgE or LPS in the presence or absence of PGE_2 (0.1 μM), where cells incubated with PGE_2 alone or media were used as parallel controls (data not shown). No IL-6 production was observed after 10 min in response to any of the agents. A significant ($p < 0.05$) increase in IL-6 production was observed compared with baseline, from a 3- to 18-h culture with PGE_2. We again observed that after a 12- or 18-h incubation, 0.1 μM PGE_2 had a synergistic effect in enhancing IL-6 production induced by LPS. Anti-IgE in combination with PGE_2, again, had an additive effect on PMC IL-6 synthesis. These results showed that under our experimental conditions, PGE_2 and PGE_1 had different effects on histamine

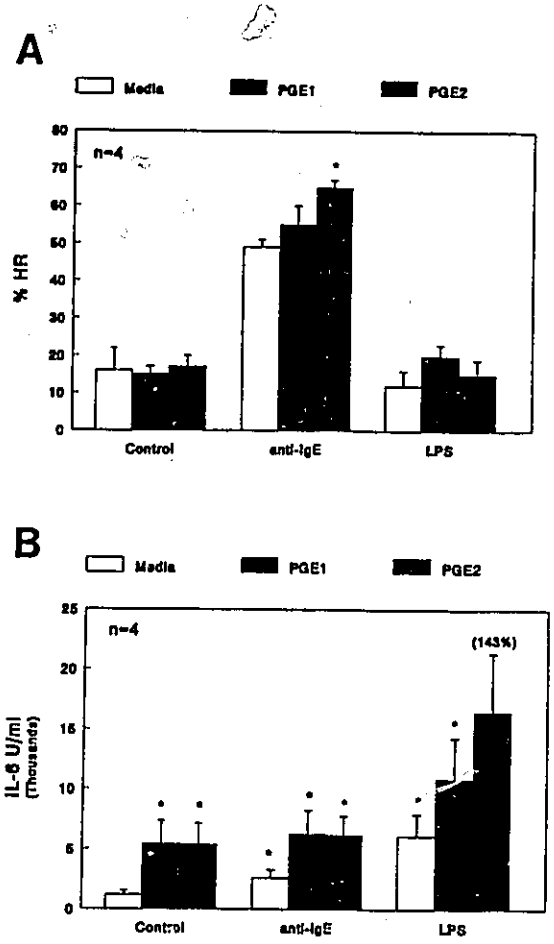

FIGURE 3. Effect of PGE_1 vs PGE_2. *A*) Histamine release (10 min) to compare the effect of PGE_2 (0.1 μM) and PGE_1 (0.1 μM) in combination with anti-IgE (5 μg/ml) or LPS (5 μg/ml). Only PGE_2 induced a significant ($p < 0.05$) increase in histamine released in the presence of anti-IgE compared with anti-IgE alone. *B*) After 18 h of incubation, supernatants from the same cells (previously washed and resuspended in media containing similar activating agent or a control) were assayed for IL-6 production, showing a significant ($p < 0.05$) enhancement in the presence of PGE_1 or PGE_2. A synergistic effect was observed in those cells incubated with PGE_1 or PGE_2 concurrently with LPS compared with LPS, PGE_1, or PGE_2 alone. Figures in brackets denote the combined response expressed as a percentage of the predicted additive response. Bars represent mean values ± SEM.

release in the presence of anti-IgE; nevertheless, similar effects were observed on IL-6 production.

Effect of Flurbiprofen on mast cells

It has been described that activated mast cells produce mainly PGD_2 and very little PGE_2 or PGE_1 (36, 37). Our observations of PGE_2 effects on mast cell cytokine production lead us to consider the possibility that cyclooxygenase products could function in an autocrine fashion during mast cell activation. Therefore, we decided to examine the effect of Flurbiprofen, which is a specific inhibitor of cyclooxygenase (38), on mast cell IL-6 production during anti-IgE or LPS activation. Flurbiprofen (1 μM) had no significant effect on IL-6 production, either alone or in the presence of anti-IgE or LPS (LPS = 4921 ± 1487 U/ml; LPS + Flurbiprofen = 5232 ± 1847 U/ml; anti-IgE = 1402 ± 342 U/ml; anti-IgE + Flurbiprofen = 1205 ± 194 U/ml; media and Flurbiprofen controls were subtracted respectively). These results suggest that prostanoids do not regulate mast cell IL-6 production in an autocrine manner. However, to confirm that the Flurbiprofen we used was active on mast cells in our system, we examined the effect of Flurbiprofen (1 μM) on mast cell PGD_2 production in response to anti-IgE or LPS after 30 min using an RIA for PGD_2. Our results cleraly showed a significant inhibition ($p < 0.03$) of PGD_2 in response to anti-IgE in the presence of Flurbiprofen (75.5 ± 3.4% inhibition), however, we did not observe mast cell PGD_2 production in response to LPS. Flurbiprofen alone did not alter the PGD_2 assay system.

The effect of blocking IL-6 from PGE_2-treated mast cells on TNF-α production

Aderka et al. (39) have described that IL-6 may play an antiinflammatory role in LPS-induced human monocytes and LPS-injected mice systems by suppressing TNF-α production. Therefore, we examined the possibility that PGE_2-induced mast cell IL-6 production was involved in the inhibition of mast cell TNF-α production. Purified peritoneal mast cells were incubated with normal rabbit serum (NRS; Dimension, Laboratories, Inc., Mississauga, Ontario, Canada) or with a polyclonal rabbit anti-rat IL-6 serum (Ad5ErIL-6; kindly provided by Dr. J. Gauldie, McMaster University) alone or in the presence of PGE_2 (0.1 μM). Anti-IL-6 inhibited the bioactivity of PGE_2-induced IL-6 in the B-9 bioassay to below detection levels, whereas the NRS control did not (8295 ± 2900 U/ml). The inhibitory effect of PGE_2 on TNF-α production by PMC at 18 h was observed in the presence of an NRS control (64 ± 15% inhibition) or rabbit anti-IL-6 (64 ± 16% inhibition).

The effect of dexamethasone in PGE_2- or PGE_1-induced IL-6

It is already known that short-term treatment with dexamethasone does not prevent histamine release from mast cells in response to anti-IgE (40). Moreover, we have described that dexamethasone significantly inhibits mast cell IL-6 production in response to anti-IgE. Therefore, we examined the effect of dexamethasone on IL-6 mast cell production in response to anti-IgE, LPS, or PGE_2. There was no significant effect on 10-min histamine release with any of the secretagogues in the presence of dexamethsone (Table I). However, a significant inhibition ($p < 0.05$) of IL-6

Table I. *Effect of dexamethasone on histamine release and IL-6 production*[a]

	% Histamine Release		% Inhibition IL-6 Production
	Media	Dexamethasone	Dexamethasone
Anti-IgE	49 ± 2	51 ± 2	92 ± 3
LPS	8 ± 4	15 ± 4	95 ± 3
PGE_2	17 ± 3.5	16 ± 2	98 ± 1
PGE_1	17 ± 4	19 ± 4	97 ± 3

[a] Effect of dexamethsone on histamine release and IL-6 production by rat PMCs in response to anti-IgE, LPS, PGE_2, or PGE_1. Histamine release is expressed as percentage of release, and no significant difference was observed between media control group vs the group treated with dexamethsone concurrently with activation. Mean media control histamine release for anti-IgE and LPS was 10 ± 7%. PGE_1 and PGE_2 results were obtained from a separate experiment in which media control release was 16 ± 5%. IL-6 production was significantly inhibitied by dexamethsone under all different conditions. These results are expressed as percent inhibition of the response to the activating agent when compared with media in the presence or absence of dexamethasone (mean values ± SEM).

production was observed in all cases (Table I). This demonstrates that dexamethasone can inhibit IL-6 production by mast cells induced by a range of stimuli.

Discussion

The presence of mast cells has been noted in a number of inflammatory diseases, such as asthma (41–43), rheumatoid arthritis (44, 45), and inflammatory bowel disease (46, 47). However, their role in such situations is poorly understood. Some studies have provided evidence that ongoing activation of mast cells occurs in such disease states, whereas others have suggested that these cells do not release substantial amounts of preformed mediators in situ (48). Recognition of the potential of mast cells to produce cytokines independent of preformed mediator release (33) allows us to reconsider the concept that mast cells may further play regulatory roles in the inflammatory process. The products of arachidonate oxygenation, in particular prostanoids, are known to play a central role in inflammation, for example, they are potent vasodilators and can act synergistically with other inflammatory mediators (9, 10). The current study examines the ability of such mediators to specifically regulate mast cell IL-6 production.

Our observations demonstrate that PGE_2 and PGE_1 modulate the production of IL-6 and TNF-α by freshly isolated mast cells. Both PGs alone did not have an effect on histamine release but significantly enhanced IL-6 production, whereas TNF-α production was significantly inhibited. To our knowledge, this is the first report of mast cell cytokine induction by prostanoids and of a discriminatory cytokine response of mast cells in response to a stimulus that does not induce mast cell degranulation. It has been reported previously that PGE_2 can decrease TNF-α-mediated mast cell cytotoxicity (27); however the production of other cytokines had not been examined.

We also have observed that PGE_2 synergises with LPS but not anti-IgE to enhance IL-6 production by PMCs, whereas PGE_1 did not show such synergism with either LPS or anti-IgE. These results add further support to the concept that mast cells use different signaling pathways to produce an IL-6 response depending on the activating stimulus (33). Moreover, we observed that both PGE_1 and PGE_2 can induce IL-6 production with a similar or greater potency than that observed with an optimal concentration of anti-IgE. The ability of PGE_1 and PGE_2 to regulate the production of inflammatory cytokines may be an example of a natural feedback mechanism for controlling inflammation, in which mast cells participate as local regulators. The induction of an acute phase response by PGE_1 or PGE_2 via IL-6 may play a role in initiating the healing-repair process. Because preformed mast cell mediators have been demonstrated to induce PGE_2 production, local mast cell degranulation could initiate this process (7, 8). The synergistic actions of LPS and PGE_2 may suggest that during a bacterial infection mast cells could be a major source of IL-6. This observation is of particular interest in light of recent reports of phagocytosis of bacteria by mast cells (49).

Our observations show that PGE_2 in the presence of anti-IgE enhanced histamine release from mast cells compared with anti-IgE alone. These results have some similarities to the studies by Piquet-Pellorce et al. (50), who showed that PGE_2 added concurrently with granulocyte-macrophage CSF (GM-CSF) potentiates histamine synthesis promoted by GM-CSF in partially purified histamine-producing cells derived from mouse bone marrow compared with GM-CSF alone (50). In contrast, others have reported that PGE_2 inhibits histamine release in response to an Ag in several models (51, 52). However, in such experiments, the cells were preincubated with PGE_2 before the Ag challenge, whereas in the current study, PGE_2 and anti-IgE are added simultaneously.

Our results suggest that IL-6 production by anti-IgE- or LPS-activated mast cells is not dependent on autocrine regulation by prostanoids, because significant inhibition of IL-6 production was not observed in the presence of Flurbiprofen. This may not be surprising, given that PGD_2 is the main prostanoid produced by rat peritoneal mast cells, and this mediator did not have a significant effect on IL-6 and TNF-α production from PMCs. However, in a microenvironment in which mast cells are close to other cells, another cell source of prostanoids may influence mast cell IL-6 production through a paracrine pathway. We also examined the concept that TNF-α production by mast cells was modulated by IL-6, through a potential cytokine autocrine regulatory circuit, which has been shown in other cell types, including human monocytes and U937 cells (39). These results suggest that in rat peritoneal mast cells, PGE_2-induced suppression of TNF-α production is not dependent on autocrine regulation by IL-6. Selvan et al. (53) have recently suggested that the gene regulation of particular chemokines (MCP-1 and I-309) that are produced by

mast cells and other cell types may be different. Our observations are in agreement with this hypothesis. The current study also demonstrates that IL-6 production by mast cells activated with anti-IgE, LPS, PGE_1, or PGE_2 in the presence of dexamethasone were significantly inhibited. These results are of interest, because although dexamethasone is not a potent mast cell stabilizer, it effectively inhibits mast cell IL-6 production regardless of the type of activation used to induce cytokine production.

Our results have some similarities to observations of rat peritoneal macrophage cytokine production, which may be regulated by PGE_1, PGE_2, or PGE_3 (54). These three prostanoids significantly inhibited TNF-α, IFNγ, and IL-1, whereas IL-6 production was significantly increased. It has been shown that PGE_2 production by monocytes and macrophages is increased in burned and severely injured patients (55, 56); therefore, it may play a beneficial role in modulating TNF-α production and subsequent responses (57). PGE_2 exerts a profound enhancing effect on the production of IgE and IgG1 (21, 22, 58, 59). IL-6 is known to play an important role in plasma cell development (60); one can speculate that the local mast cell production of IL-6 induced by prostanoids or other stimuli may enhance plasma cell development and local Ig production, potentially leading to dysregulation, as has been described in allergic or inflammatory diseases such as inflammatory bowel disease (61, 62) or in advanced Hodgkin's lymphoma (63, 64). In contrast, a possible antiinflammatory role for IL-6 has been described in a number of in vitro and in vivo studies (37, 65, 66).

Preformed mast cell products such as histamine are recognized as potent inducers of PGE_1 and PGE_2 production by surrounding tissue cells (7, 8). Thus, we can envisage a regulatory pathway whereby activation of a subpopulation of mast cells in an acute allergic response would induce PGE production by surrounding cells. This local prostanoid production then could help limit the inflammatory process by modulation of further mast cell and macrophage TNF-α production while enhancing IL-6 production, which could act to induce an acute phase response and enhance the development of plasma cells. Because mast cells are found in large numbers at body sites that interface directly with the environment, they are well suited to both initiate the acute inflammatory process and through interactions with other tissue cells continue to modulate later responses and plasma cell development. Dysregulation of such a response could be an important aspect of chronic inflammatory disease.

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CHAPTER FOUR

CHOLERA TOXIN INCREASES IL-6 SYNTHESIS AND DECREASES TNF-α PRODUCTION BY RAT PERITONEAL MAST CELLS

Irene Leal-Berumen*, Denis P. Snider**, Carlos Barajas-Lopez**,
and Jean S. Marshall**

*Molecular Virology and Immunology Programme (MVIP) and **Intestinal Disease Research Programme, McMaster University, Hamilton, Ontario CANADA.

Running title: MODULATION OF MAST CELL CYTOKINE PRODUCTION BY CHOLERA TOXIN.

Footnotes

Address for correspondence:

Dr. Jean. S. Marshall, Department of Pathology

McMaster University, Health Science Centre, Room 4H17

1200 Main St. West, Hamilton, Ontario, L8N 3Z5 CANADA

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Abbreviations used:

CT - Intact cholera toxin (A + B subunit)

CTB - B subunit of cholera toxin

cAMP- 3':5'-cyclic adenosine monophosphate

MTT - (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium)

PMC - Peritoneal mast cell

SBTI - Soy bean trypsin inhibitor

ABSTRACT

Mast cells have been traditionally associated with an acute allergic response. However, their role in regulating chronic inflammatory processes must also be considered in view of evidence that mast cells synthesize and release a number of cytokines. In this study, we have examined the effect of cholera toxin (CT) on peritoneal mast cell IL-6 and TNF-α production. Highly purified, freshly isolated, rat peritoneal mast cells from Brown Norway rats were cultured in the presence of CT or its B subunit (CTB) alone or in combination with anti-IgE or bacterial lipopolysaccharide (LPS). Histamine release was measured after 10 min, IL-6 and TNF-α production was assessed in supernatants after 18 h. We found that CT or CTB alone did not affect histamine release, however, mast cell IL-6 production was significantly enhanced by CT but not by CTB. In contrast, constitutive production of TNF-α was inhibited by CT. The effects of CT were similar to our previous observations of the actions of Prostaglandin E_2 on mast cells. We also examined the effects of CT in combination with other mast cell activating agents. CT had no significant effect on anti-IgE induced histamine release. An additive effect on IL-6 production was observed in the context of LPS. Forskolin, an agent known to increase intracellular cAMP levels also induced a significant increase in IL-6 production while TNF-α production was decreased. These data have important implications for our understanding of the regulation of mast cell cytokine production and the effects of CT on local cytokine production.

INTRODUCTION

Cholera toxin (CT) is the *Vibrio cholera* enterotoxin which exerts its effect on the small intestine *in vivo* through activation of an adenylate cyclase regulatory G protein. This event is triggered by the A subunit of cholera toxin resulting in increased cyclic AMP (cAMP) levels that eventually will cause the diarrhea and fluid loss that is characteristic of disease in patients with cholera (1-3). The B subunit of cholera (CTB) has five identical noncovalent-associated subunits which serve as the carrier for the A subunit and binding moiety for the receptor GM1-ganglioside (2,3). Besides its pathological effects, CT has been used as an effective mucosal adjuvant due to the strong antibody responses evoked when given orally (secretory IgA, plasma IgG, and IgE) and the long-term immunological memory that is observed in mucosal sites following immunization containing CT (4-7). McGee et al. (8) have described the ability of CT to induce a rapid increase in IL-6 secretion by the intestinal epithelial cell line (IEC-6), which may be mediated through an elevation of intracellular cAMP levels.

The participation of mast cells in a variety of pathological processes including asthma and inflammatory bowel disease, has been traditionally related to the release of preformed mediators such as histamine and mast cell proteases or production of newly formed mediators, such as PGD_2 and platelet activator factor, after immunological activation. More recently, we have come to recognise that mast cells also produce a number of cytokines which may be found preformed or may be produced only following cell activation (9). The release of cytokines, such as IL-6, can be observed under some circumstances without inducing mast cell degranulation (10). It has been suggested that GTP binding proteins (G-proteins) may play an important role in mediating cytokine production by other cell types (11) but their role in regulating mast cell cytokine expression has not been examined.

We chose to examine the regulation of IL-6 and TNF-α production by mast cells in view of the important biological activities of these cytokines during inflammation and the increased expression of these cytokines associated with diseases such as atopic asthma and inflammatory

bowel disease (12,13). Mast cell degranulation and secretion have been linked to mucosal injury (14-16). Intestinal inflammation is accompanied by a characteristic systemic response that corresponds with the effects of IL-1, IL-6 and TNF-α which include fever, anorexia, leukocytosis, and a hepatic acute phase response (17). IL-6 plays an important role in the development of plasma cells (18,19), induction of an acute phase response (20,21) and it has also been demonstrated to induce neurite outgrowth in cell culture systems (22). This cytokine may have an important regulatory role in inhibiting TNF-α production (23). TNF-α is a multifunctional cytokine which has a pro-inflammatory role. TNF-α is a monocyte chemoattractant, with a number of important effects including induction of endothelial expression of adhesion molecules ICAM-1 and VCAM-1 (24,25). High doses of TNF-α produce shock, diarrhea, necrosis of epithelial and endothelial cells in the colon and small intestine, and inhibit gastric motility (17). In the skin, mast cell derived TNF-α has been shown to be critical for neutrophil migration in allergic inflammation (26). However, mast cell derived TNF-α may also have useful functions as it has been suggested to mediate mast cell cytotoxicity against tumour cell targets (27).

The anatomical arrangement of mast cells place them in the first line of defense against injury and infection. Mast cells are particularly frequent in the skin, airways and gastrointestinal tract, sites that interface directly with the external environment. Mast cells are well suited to both initiate an acute inflammatory process and through interactions with other tissue cells continue to modulate later responses. The regulation of inflammatory cytokines in the mast cell is of particular interest in view of the potential role of these cells during chronic inflammatory diseases. We have previously demonstrated that the mast cell may use different signalling pathways to induce production of the same cytokine (IL-6) depending on the activating agent that is used (10). We have also recently observed that PGE_2 and PGE_1 treatment of PMCs upregulates IL-6 production while it downregulates TNF-α synthesis and release (28). In the current study, we have examined the effect of CT on IL-6 and TNF-α production in rat PMCs.

Our findings suggest that CT mimics some, but not all, of the effects of PGE_1 and PGE_2 on PMCs. We believe this is the first report to demonstrate the ability of CT to differentially modulate cytokine production in a single cell type.

MATERIALS AND METHODS

Mast Cell Source and Activation

Brown Norway rats (high IgE producers) (Harlan Sprague-Dawley, Inc. Indianapolis, IN) weighing 150-200 g were used to obtain PMCs by peritoneal lavage. The animals were housed in the Central Animal Facility at McMaster University, Hamilton, Ontario, Canada, in filter-hooded cages and were allowed food and water *ad libitum*. All experiments were approved by the Animal Research Ethics Board of McMaster University. PMCs were purified on a 30%/80% discontinuous Percoll gradient (Pharmacia LKB, Uppsala, Sweden) with a range of 95-100% purity. The predominant contaminating cells were neutrophils, macrophage contamination was not observed in any experiment using the Diff-QuiK stain set (Baxter, McGaw, Park, IL). One million purified PMCs were suspended in RPMI 1640 without phenol red (GIBCO BRL, Grand Island, NY), supplemented with 5% FCS (v/v), penicillin (50U/ml), streptomycin (50μg/ml), (1%Pen-Strep; GIBCO BRL), 1.4mM Ca^{2+}, and 100μg/ml soybean trypsin inhibitor (SBTI; Sigma, St. Louis, MO). The cells were initially activated for 10 min at 37°C by using the following activating agents: a mouse monoclonal IgG_1 anti-IgE (MARE-1; University of Louvain, Belgium) at 5μg/ml; LPS (*Escherichia coli* serotype 055-B5; Sigma) at 5μg/ml; PGE_2 (Sigma) at 0.1μM; A23187 at 1μM (Boehringer Mannheim, Mannheim, Germany); CT (List Biological Laboratories, Inc. Cambell, CA) from 0.0001-0.1μg/ml; CTB (List) from 0.0001-0.1μg/ml. After activation the same cells were washed and resuspended in fresh media containing the previous activating agents and were usually incubated for 18 h at 37°C. For the time course experiment, a range of times were examined between 10 min and 24 h. In some experiments a Ca^{2+} free RPMI 1640 (GIBCO BRL) media in the presence of EGTA (1mM; Sigma) was used. All reagents except CT when initially obtained from the supplier, contained less than 0.016 ng/ml (final concentration) of endotoxin when tested by the E-Toxate kit (Sigma). This minimum level of endotoxin was below that known to induce either IL-6 or TNF-α production from rat PMC. CT on receipt from the supplier had > 32 ng/ml.

Therefore CT was preabsorbed on a column of polymyxin B (Bio-Rad Laboratories, Harbour Way South, Richmond, CA) resin and was then confirmed to have below 0.002 ng/ml before use in experiments. Forskolin (Sigma) was used in some experiments at 1, and 10 μM to increase intracellular levels of cAMP .

Histamine Release Assay

Histamine release by highly purified PMCs was assessed by a fluorometric method as described previously (29). Briefly, after 10 min initial degranulation at 37°C in the presence or absence of secretagogues, PMCs were centrifuged at 800 rpm for 10 min at 4°C. The pellet was resuspended in the original volume to give a cell concentration of $1x10^6$/ml. Samples of pellet and supernatant were diluted 1/50 (v/v) in HEPES-Tyrodes buffer and boiled for 5 min to inactivate histaminase. The buffer composition (in mM) was: NaCl, 137; glucose, 5.6; KCl, 2.7; NaH_2PO_4, 0.4; $CaCl_2$, 1; HEPES 10; and (supplemented with) BSA (0.1%), pH 7.3. After precipitation of proteins using Trichloroacetic acid (25%), fluorescent products from o-phthalaldehyde (OPT) reaction were measured by using Shimadzu CR-15 fluorescence spectrometer. Histamine release was expressed as percentage of the total cellular histamine content (histamine in supernatant/ histamine in supernatant + histamine in pellet) X 100.

B-9 Bioassay for IL-6

The bioactivity of IL-6 was measured by a previously described B-9 hybridoma proliferation assay (30). Briefly, B-9 cells were cultured in MEM F-11 (GIBCO BRL), supplemented with 5%FCS, 2-ME($5x10^{-5}$M), 1%Pen-Strep solution, and supernatant source of IL-6 (normal human lung fibroblast stimulated with IL-1ß for 24 h in MEM F-11 media containing 10%FCS and 1%Pen-Strep). IL-6 assay was performed in triplicate for each sample or standard in microtiter plates (Nunc, Roskilde, Denmark). B-9 cells (initially 2500/well) were cultured in the described medium in either the presence or absence of putative IL-6 containing samples. After 72 h

culture, 10μl/well 5mg/ml MTT (3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; Sigma) was added and the plates were incubated for 4 h at 37°C. Fifty μl/well 10% Triton-HCl was then added, and a further 18h incubation in the dark at room temperature was conducted. The colored reaction product was assessed at 570 nm by an ELISA reader. Supernatant samples from mast cells were stored at -20°C for analysis. The samples were compared with a standard IL-6 supernatant prepared from human lung fibroblasts which had been standardised by comparison with recombinant human IL-6 at several dilutions to assess IL-6 content. Results were expressed as units of IL-6 per ml, on the basis that 1 unit is equivalent to the amount of sample required to induce a half-maximal response. One unit is equivalent to approximately 0.45 pg of IL-6 according to the National Biological Standard Board. Limit of detection for IL-6 was 10 U/ml.

None of the known mast cell-derived cytokines have been shown to induce proliferation of this cell line under similar assay conditions. IL-6 standard curves were run in the presence of each activating agent used including CT, CTB, or forskolin and none of these agents significantly changed the response of the B-9 cells to standard IL-6 preparations.

TNF-α Bioactivity Assay

TNF-α was measured by a cytotoxicity bioassay employing the TNF-α sensitive L929 (mouse fibroblast) cell line, using a modification of the method previously described (31). Briefly, 50μl/well of 1 x 10^6 L929 cells/ml in RPMI media, supplemented with 5%FCS and 1%Pen-Strep were plated into flat bottom Nunclon plates and incubated overnight at 37°C. Media was discarded by suction and replace with fresh media containing 20μg/ml of cycloheximide (Sigma) and 100 μg/ml of SBTI (Sigma). Mouse recombinant TNF-α (Genetech, Inc., San Francisco, CA) was diluted in the same media and used as a standard. Seven, 10 fold, serial dilutions starting from 20,000pg/ml were used to established the standard curve. 50 μl/well of standard or samples in duplicates were added, then the plates were incubated at 37°C.

After an 18h incubation 10μl/well MTT (5mg/ml) was added and a further 4h incubation was conducted. Then 50μl/well of a solution of 50% *N,N*-dimethylformamide (Caledon Laboratories, LTD, Edmonton, Ontario, Canada), 20% SDS (Bio-Rad, Mississauga, Ontario, Canada), pH 7.4, was added to dissolve the MTT. After an 18h incubation at 37°C, the plates were read at 570 nm. Results were read off a standard curve constructed using recombinant murine TNF-α to obtain the concentration of TNF-α present in the samples. Media samples served as negative controls. The addition of activating agents including CT and CTB did not significantly alter the TNF-α standard curve. The limit of detection for TNF-α was 0.25 pg/ml.

Measurement of cyclic-AMP

Mast cells at a concentration of 1 million cells per ml were preincubated for 10 min with 1mM isobutylmethylxanthine (Sigma) separate aliquots were then further treated with either media alone or media containing Forskolin (10μM) or CT (0.01μg/ml) for 1 min, 5 min, 10 min or 30 min. The reaction was stopped by the addition of 2 volumes of ice cold ethanol. The cyclic-AMP content of the cells was detemined in duplicate samples from each cell preparation. Using a commercially available cyclic-AMP assay kit (Biotrak enzyme immunoassay, Amersham, Oakville, Canada).

Statistical Analysis

The response of aliquots of the same initial preparations to different secretagogues were compared by using a paired Students *t*-test for histamine release. However, for IL-6 and TNF-α experiments, in view of the data distribution, data were compared by using the non-parametric Wilcoxon signed-rank test.

RESULTS

Effect of CT and CTB on Histamine Release, IL-6 and TNF-α Production

We initially examined the effect of CT and CTB alone on histamine release and IL-6 or TNF-α production from highly purified rat PMCs. Mast cells were activated with A23187 (1 μM) as a positive control, CT, or CTB as activating agents at different doses, and treated with media alone. CT or CTB alone had no significant effect, at any dose, on histamine release CT (0.1 μg/ml) = 1 ± 0.4% release of histamine, CTB (0.1 μg/ml) = 3 ± 1%) compared to a media control (3 ± 1%) and A23187 (69 ± 5.5%). Similarly there was no significant effect of CT on the short term release of TNF-α (7 ± 5 ng/ml media control, 6 ± 3 ng/ml CT, n=6 and no IL-6 release was observed following 10 min of activation with anti-IgE in the presence or absence of CT. After the 10 min histamine release, the cells were washed and cultured in fresh media containing the same secretagogues for further 18 h. The cell free supernatants were then analyzed for IL-6 and TNF-α production. CT at concentrations from 0.0001 to 0.1 μg/ml significantly ($p < 0.05$) enhanced IL-6 production compared to media control. CTB showed a small increase only at a high dose (0.1μg/ml) (fig 1A). Given the recognised difficulties in obtaining completely pure CTB, CTB contamination with low levels of the whole CT subunit might be responsible for this observation alternatively this may represent a direct effect of CTR. We also observed that TNF-α was significantly ($p < 0.05$) inhibited by either CT or CTB (from 0.0001 to 0.1 μg/ml) (fig 1B) although the magnitude of inhibition induced by CT was much greater than that induced by CTB. In a separate series of experiments we observed that the ability of CTB to inhibit TNF-α production was only observed at concentrations greater than 0.01 ng/ml. (Mean percentage inhibition compared with PMC incubated with media alone 27 ± 7% 0.01 ng/ml, 6 ± 5% 0.001 ng/ml).

We examined the time course of IL-6 and TNF-α production, over 24 h in response to CT (0.01 μg/ml), where cells incubated with media alone were used as parallel controls. The time course was examined in view of reports that the enhancement of IL-6 production in response to

CT (0.1 μ/ml) in epithelial cells (IEC-6) occurs around 50 h of culture (8). In contrast, we have previously shown that PMCs IL-6 production in response to different stimuli (LPS, anti-IgE and PGE_2) occurs at an early stage (around 3-6 h of culture) (10,28). We also wanted to examine if there was a temporal relationship between increased IL-6 and decreased TNF-α production. A significant ($p < 0.05$) increase in PMC IL-6 production was observed compared with controls, from 3 to 9 h of culture in the presence of CT (fig 2A), whereas constitutive TNF-α production was significantly ($p < 0.05$) inhibited at all time points from 3 to 24 h (fig 2B).

Effect of CT on anti-IgE induced histamine release and IL-6 production from LPS-activated mast cells

We have recently observed that histamine release was significantly increased when anti-IgE was used as an activating agent in the presence of PGE_2 and the synergistic induction of IL-6 production by LPS in the context of PGE_2 (28). We therefore examined the ability of CT to alter cytokine production and histamine release in combination with anti-IgE and LPS. As mentioned previously our results demonstrated no significant effect of CT on anti-IgE induced histamine release or TNF-α production at 10 min CT + LPS had an additive effect on IL-6 production and did not show the synergism which is observed when LPS and PGE_2 are simultaneously added in this system. IL-6 production after 18h incubation with LPS alone = 3600 $\pm$ 1100 U/ml; CT alone = 5500 $\pm$ 2200 U/ml; LPS + CT = 13,600 $\pm$ 5400 U/ml.

Ca^{2+} Dependence of CT-induced IL-6 Production

We have previously demonstrated that IL-6 production from PMCs in response to either LPS or anti-IgE is highly Ca^{2+} dependent (10). We examined the Ca^{2+} dependence of 18 h IL-6 production from PMCs in response to CT, PGE_2 and anti-IgE (as control) that were incubated in the presence or absence of Ca^{2+} for such a period of time. Our results show that IL-6 production in response to all of these different stimuli is a highly Ca^{2+} dependent process

because in the absence of Ca^{2+} there was a significant inhibition in stimulus induced IL-6 production (fig 3). As previously reported (10) the endogenous production of IL-6 by PMC was also inhibited in the absence of calcium. The presence or absence of Ca^{2+} had no significant effect on the ability of CTX to inhibit endogenous TNF-α production.

Effect of Forskolin on PMCs IL-6 production

Since CT might be expected to increase IL-6 secretion via enhancing intracellular cAMP levels, we examined the effect of forskolin, which is known to increase intracellular cAMP on IL-6 and TNF-α production. Forskolin alone had no effect on mast cell histamine release at any concentration used (data not shown). Forskolin induced a significant increase ($p<0.01$) in IL-6 production with a maximal effect at 10μM (fig 4A). However, the maximum production of IL-6 by forskolin was significantly lower ($p<0.01$) than that observed with CT. In a separate experiment, forskolin was used at its optimal concentration for induction of IL-6 (10 μM) to examine TNF-α, this induced a significant decrease ($p<0.05$) in spontaneous TNF-α production after 18h incubation of PMC when compared with parallel controls (fig 4B).

Effect of CT and forskolin on PMC cyclic-AMP production

Examination of the elevation of cyclic AMP levels in mast cells following activation with CT or forskolin demonstrated a significant rise ($P<0.05$) in activated cells but not in the media treated control cells. Both responses were maximal at 10 min post activation and declined at 30 min (maximal responses after subtraction of t=0 values were CT 62.72 $\pm$ 22, forskolin 125 $\pm$ 70, media 0 $\pm$ 16, fmol/10^5 cells n=3 per group). There was no significant difference in the magnitude of the responses observed.

DISCUSSION

In this study, we used rat peritoneal mast cells to examine the effect of CT on their cytokine production. We found that CT augments IL-6 production while inhibiting TNF-α production from rat PMCs.

The effect of CT on other cell types has already been documented by other groups. CT has been shown to inhibit T cell receptor-mediated IL-2 production and proliferation of Th1 cells, while CT failed to block IL-4 production and proliferation in Th2 cells (32). The authors found similar results with forskolin suggesting that while the intracellular increase in cAMP may be inhibitory in Th1 cells, it could be important for activation and proliferation in Th2 cells (32). Other studies have shown that CT potentiates the activity of IL-4 during isotype switching of IgG_1 and IgE by B cells (33,34). Bromander et al reported that CT can enhance antigen presentation by macrophages (35) and intestinal epithelial cells (36) in vitro probably by increased co-stimulation with cytokines such as IL-1, however, no modification of class II MHC expression was observed. Thus the *in vivo* adjuvant effects of oral CT may be due to actions on macrophages, epithelial cells, B cells, T cells and also potentially mast cells.

Some G-proteins can be altered with bacterial toxins affecting their functional properties through ADP ribosylation of their G_α subunit. For example, CT activates the α subunit ($G_{\alpha s}$) that stimulates adenyl cyclase, whereas pertussis toxin inactivates the ADP ribosylation ($G_{\alpha i}$) (37). Elevation of intracellular cAMP is known to be an important event in many cell types (38,39). PGE_2, CT and pertussis toxin are examples of agents which induce such a cAMP increase (40-42) . Several investigators have shown that anti-IgE cross-linking on mast cells induces a transient increase in intracellular cAMP (43,44), however, the precise role of cAMP as a second messenger in this process is still controversial (45-47). Katakami et al. (39) studied the role of cAMP in the regulation of LPS-induced TNF-α production by mouse peritoneal macrophages. They showed that LPS did not alter intracellular levels of cAMP, however, PGE_2 and CT, which are known to increase cAMP, inhibited LPS-induced TNF-α. Our data

demonstrates that CT reduces the constitutive as well as anti-IgE induced TNF-α production of cultured peritoneal mast cells. Our findings in rat PMC are in contrast with those of Narasimhan et al (48) examining rat basophilic leukemia cells. They observed an enhancement of IgE mediated mediator release by CT and an increase in calcium influx. This may reflect another aspect of mast cell heterogeneity. It is however, possible that enhanced calcium signals may contribute to the elevation of IL-6 production since production of this cytokine is highly calcium dependent.

Our studies with forskolin suggest that CT induction of IL-6 production by rat PMCs may have resulted from an elevation of intracellular cAMP levels, however the effect of optimal forskolin concentrations on IL-6 enhancement was less than that observed with CT. Therefore, we believe that other factor(s) produced by CT-stimulated PMC may act in an autocrine fashion to further enhance IL-6 production. McGee et al (8) have shown that intestinal epithelial cells are capable of secreting high levels of IL-6 after encountering CT, and they suggest that this effect could be enhanced in the presence of inflammatory cytokines, such as IL-1ß. The induction of IL-6 gene expression and IL-6 production has been linked to a cAMP-dependent pathway in fibroblasts (49). Dendorfer et al (50) have reported that cAMP and prostaglandins act through multiple regulatory elements to induce IL-6 expression in monocytic cells and, although nuclear events may overlap partially with the LPS response some distinctive pathways are involved. Our observation that forskolin (10μM) significantly decreased TNF-α production suggests that an intracellular increase in cAMP may down regulate the production of other cytokines such as TNF-α by mast cells.

Our results with CT had certain similarities with the previously reported effects of prostaglandins of the E family on mast cell IL-6 and TNF-α production (28). While none of these agents induced histamine release from PMCs, PGE_2 and CT enhanced PMCs IL-6 production while both downregulated TNF-α. PGE_2 and CT have other common biological effects and both agents are known to increase intracellular levels of cAMP (40,41). Both PGE_2

(51,52) and CT (7) have shown to favour the Th2 like response, to induce an increase in specific IgE and IgG_1, and may act in concert with other inflammatory mediators. Mast cells are at body sites, such as skin, airways and intestinal mucosa, which place them as the initiators of inflammatory and immune responses. Thus, cytokine production by these cells could play a major role in determining the local response to challenge and the nature of the induced immune response. The production of IL-6 and potentially other cytokines by mast cells in response to CT could be an important component of the action of CT as a mucosal adjuvant. Ramsay et al (53) have shown that in mice with targeted disruption of the gene that encodes for IL-6 there were greatly reduced numbers of IgA-producing cells at the mucosae as well as a deficient local antibody response to either OVA or vaccinia virus. Their findings demonstrate the critical role for IL-6 in vivo in the development of local IgA antibody response.

We still have a very poor understanding of the role of the mast cell in inflammation and immunoregulation. However, by increasing our understanding of the mechanisms which regulate mast cell cytokine responses we can begin to predict the effects of certain stimuli. Our evidence (10,28) and that of others (54,55) suggests that mast cells may be a major source of cytokines in the tissues and could provide a potential target for local manipulation of immune and inflammatory responses. The effects of CT on mast cell cytokine production *in vivo* and the relative importance of mast cells as a source of TNF-α and IL-6 during inflammatory and immune responses are important areas for future studies. Our results demonstrate that freshly isolated mast cells can selectively modulate their cytokine response to CT and produce substantial amounts of IL-6 following CT activation without preformed mediators release. To our knowledge this is the first report of such selective regulation of mediator production by CT in a single cell type.

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FIGURE LEGENDS

FIGURE 1. Effect of CT and CTB (dose response). Rat PMCs were stimulated with different doses of CT or CTB or media as a control. **A.** After the initial 10 min release the same cells were washed and resuspended in fresh media containing the same activating agents or control and allowed to further incubate for 18 h. After 18 h culture the cells were spun down and the supernatants were examined using a B9 hybridoma assay for IL-6. **B.** An aliquot from 18 h supernatants was examined using an L929 cytotoxicity assay for TNF-α. Bars represent mean values $\pm$ SE of the mean. Open bars denote CT alone, and crossed bars represent CTB alone.

* denotes significant $p<0.05$ difference compared to media control.

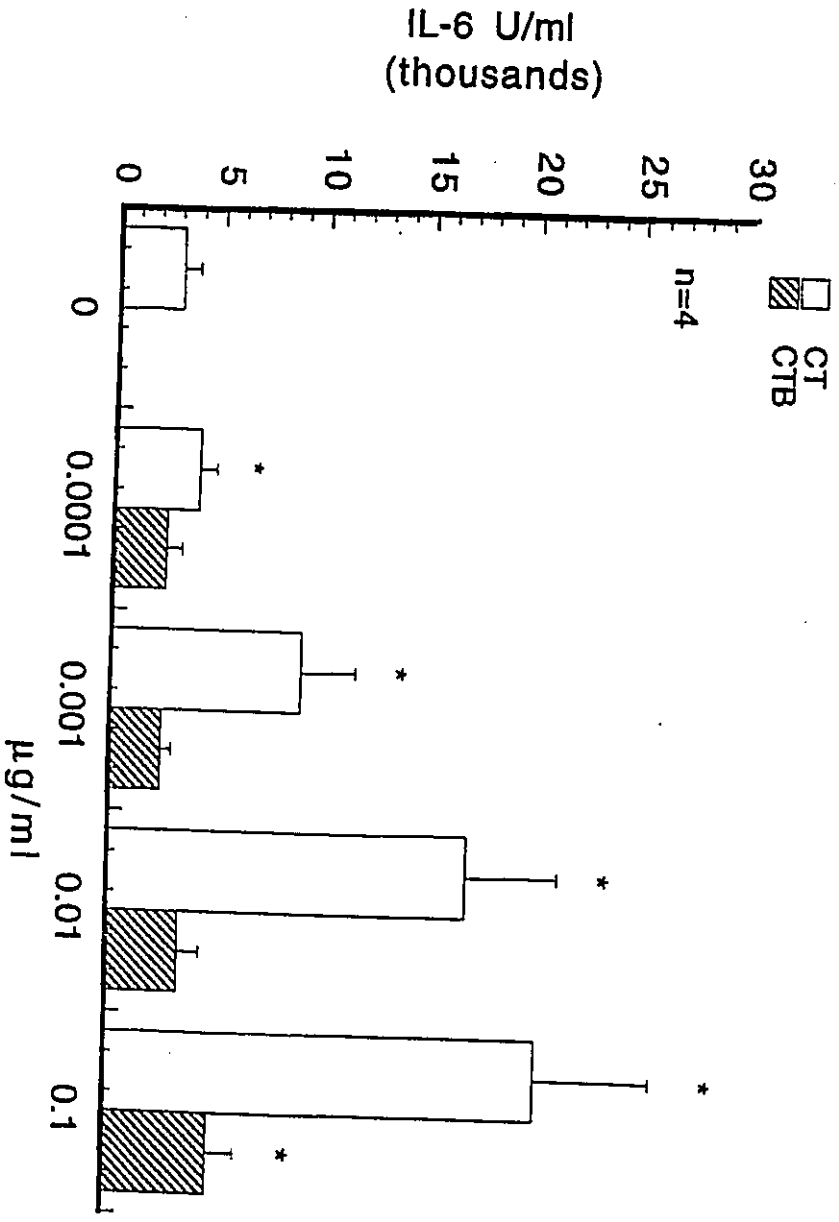
FIGURE 2. Effect of CT on PMC IL-6 and TNF-α time course. PMCs were incubated alone or in the presence of CT (0.01 μg/ml) for 10 min, then the same cells were washed and resuspended in fresh media containing the same activating agent and further incubated for different times. A supernatant aliquot was taken at each time point. **A.** IL-6 content of supernatants. **B.** TNF-α content of supernatants.

Bars represent mean values $\pm$ SE of the mean.

FIGURE 3. Calcium dependence of PMC IL-6 production in response to CT.

Highly purified PMCs were incubated 18 h alone or in the presence of CT (0.01 μg/ml), or PGE_2 (0.1 μM), or anti-IgE (5 μg/ml) in normal supplemented RPMI 1640 (see material and methods) or RPMI without calcium added, plus 1mM EGTA. IL-6 production was significantly inhibited when calcium was absent regardless the stimuli used. These results are expressed as percent inhibition of response to activating agent when compared with media controls in the presence or absence of calcium (mean values $\pm$ SE of the mean, n=4).

FIGURE 4. Effect of Forskolin (F) on PMCs IL-6 production. **A.** Different doses of forskolin were used to examine their effect on IL-6 production at 18 h. Media and CT alone were controls. Forskolin at 10μM concentration induced the maximum increase in IL-6 production by PMCs. **B.** Separate experiments using forskolin (10μM) were performed to assess TNF-α production by PMCs after 18 h incubation. Forskolin significantly decreased TNF-α production. These results suggest that the effect of CT on IL-6 and TNF-α production by PMCs may in part be due to an intracellular increase in cAMP. Bars represent mean values $\pm$ SE of the mean, n=4. In fig 4A n=7 for media, CT and forskolin at 10μM.


IL-6 U/ml
(thousands)
0
5
10
15
20
25
30
n=4
CT
CTB
μg/ml
0
0.0001
0.001
0.01
0.1
*
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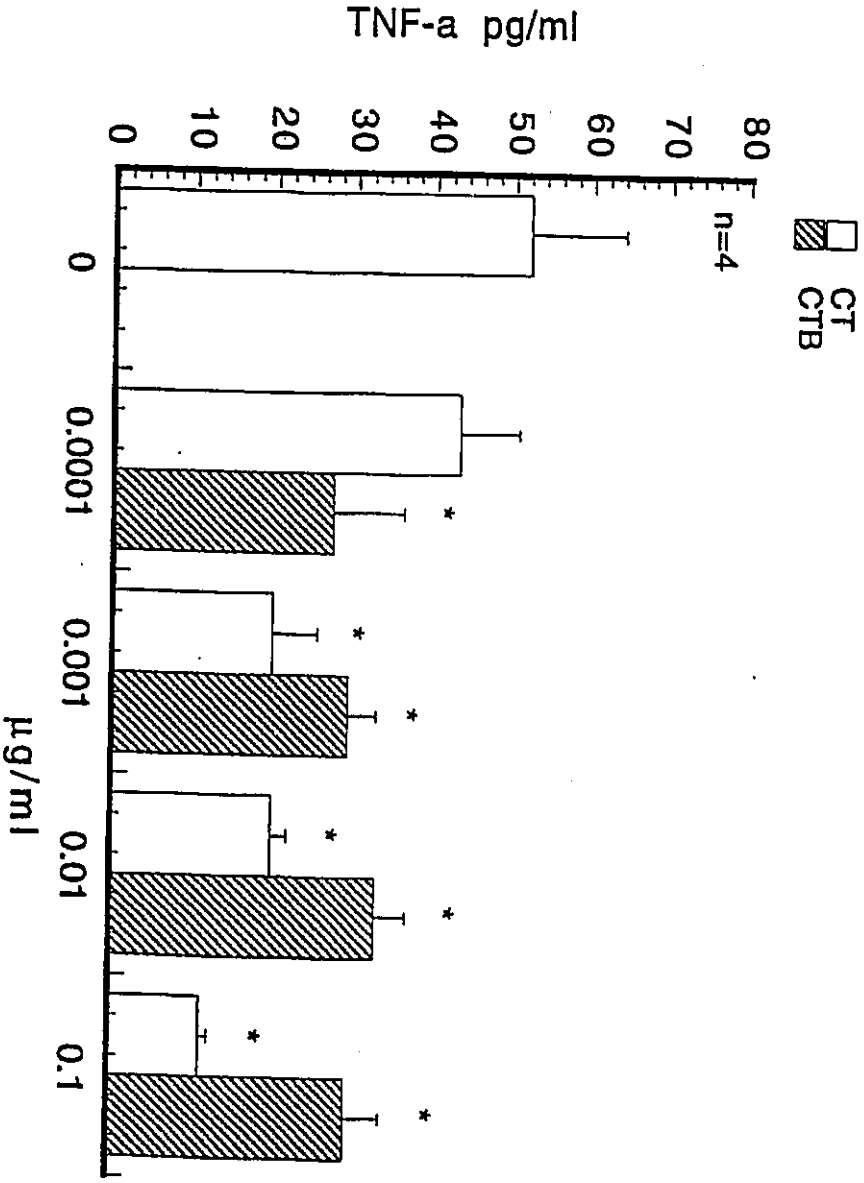
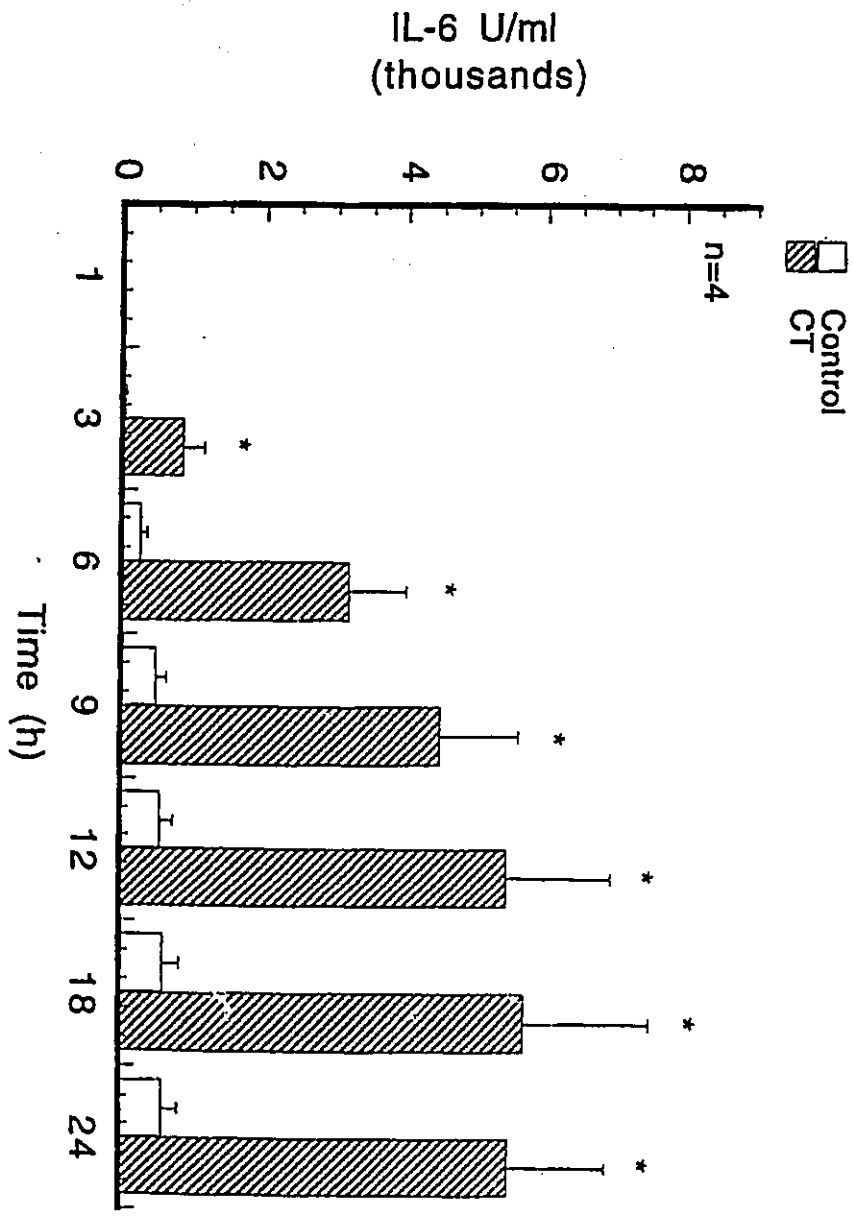
*
*
*


TNF-a pg/ml
0
10
20
30
40
50
60
70
80
n=4
CT
CTB
0
0.0001
0.001
0.01
0.1
μg/ml
*
*
*
*
*


IL-6 U/ml
(thousands)
0
2
4
6
8
n=4
Control
CT
Time (h)
1
3
6
9
12
18
24
*
*
*
*
*
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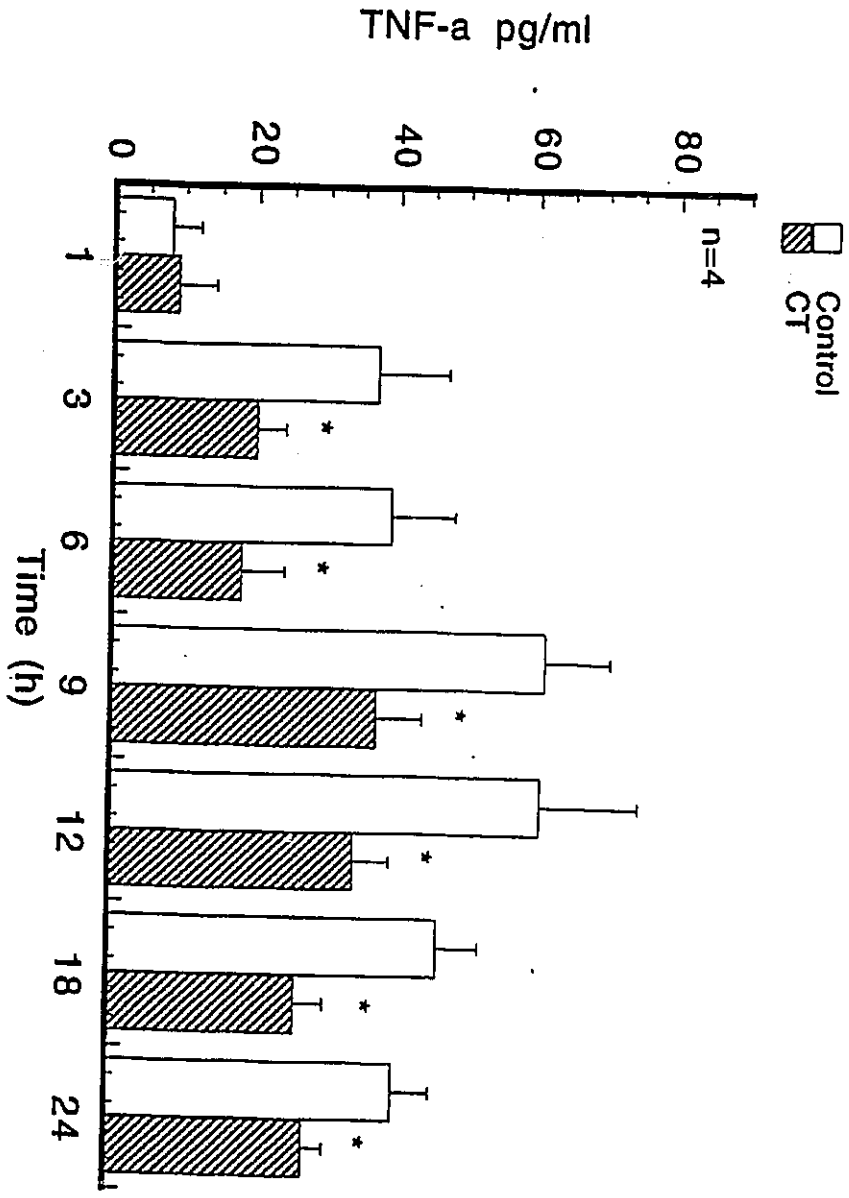
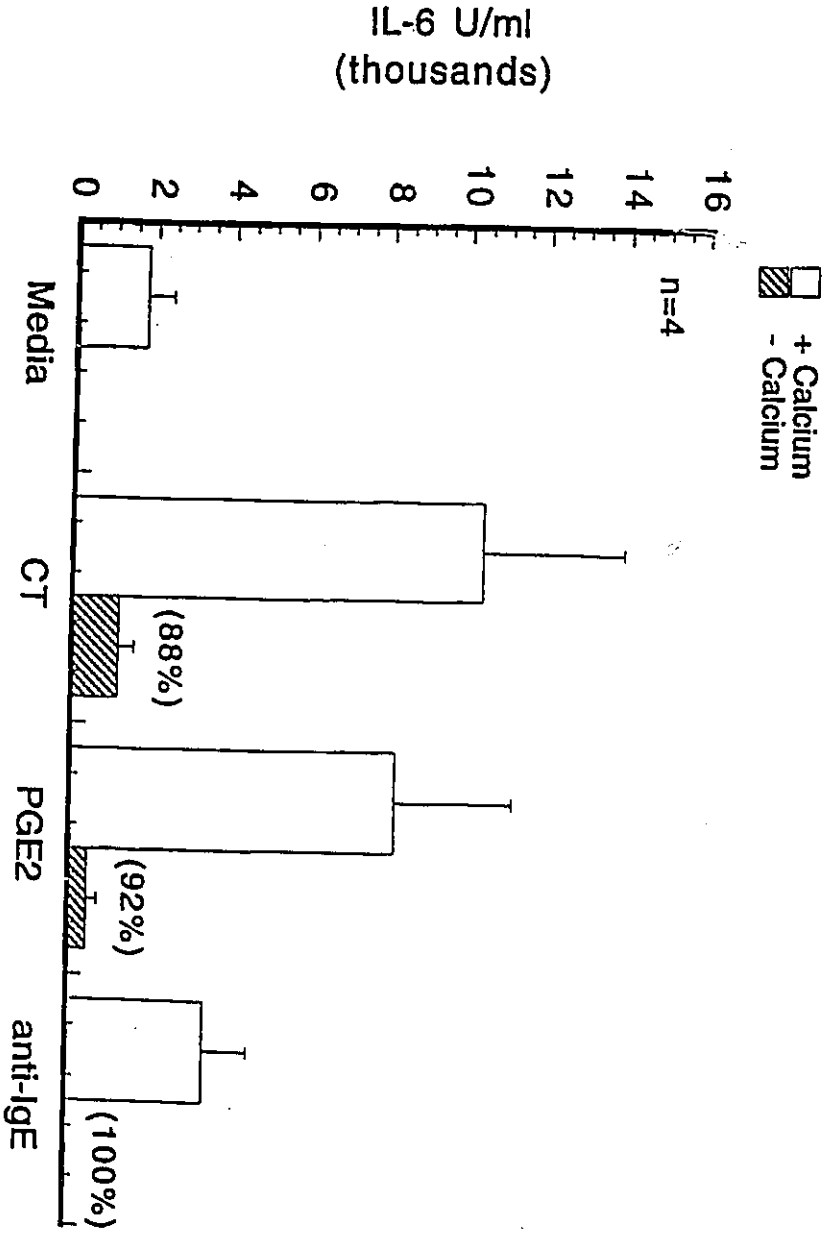
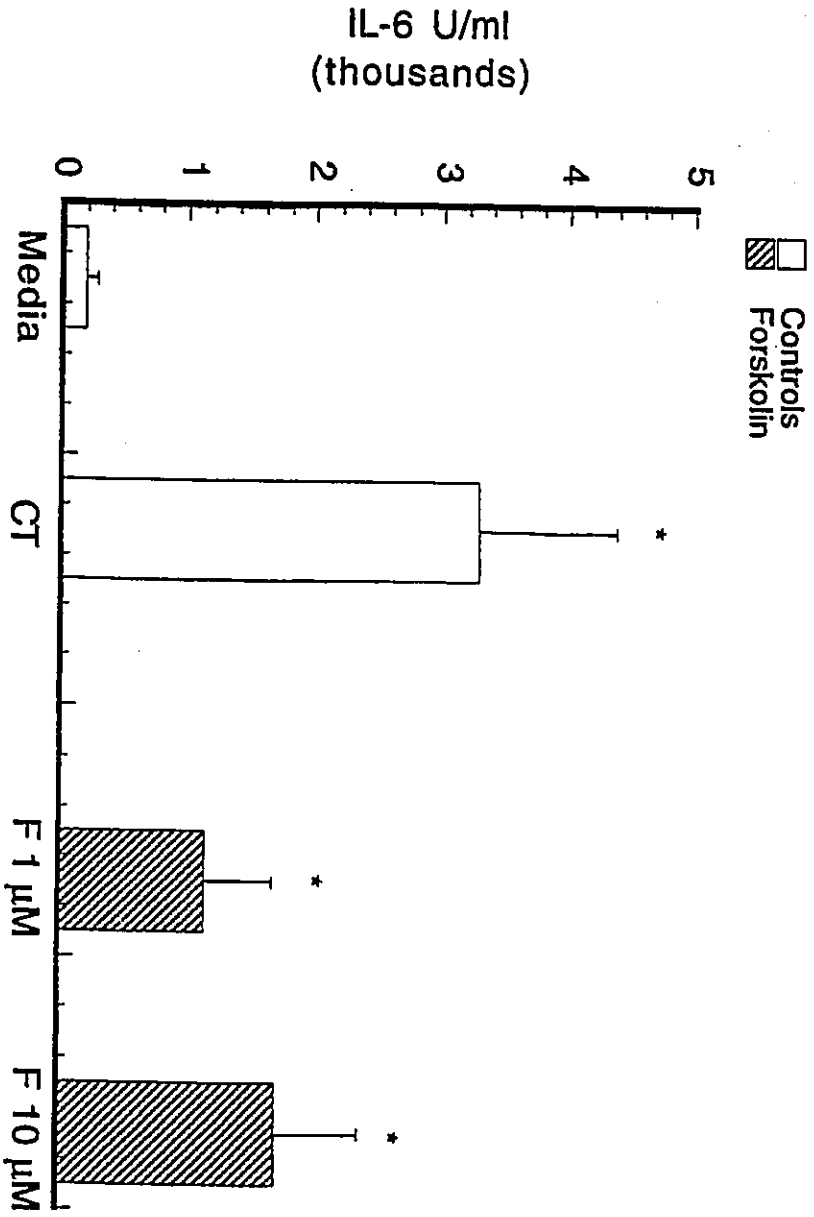
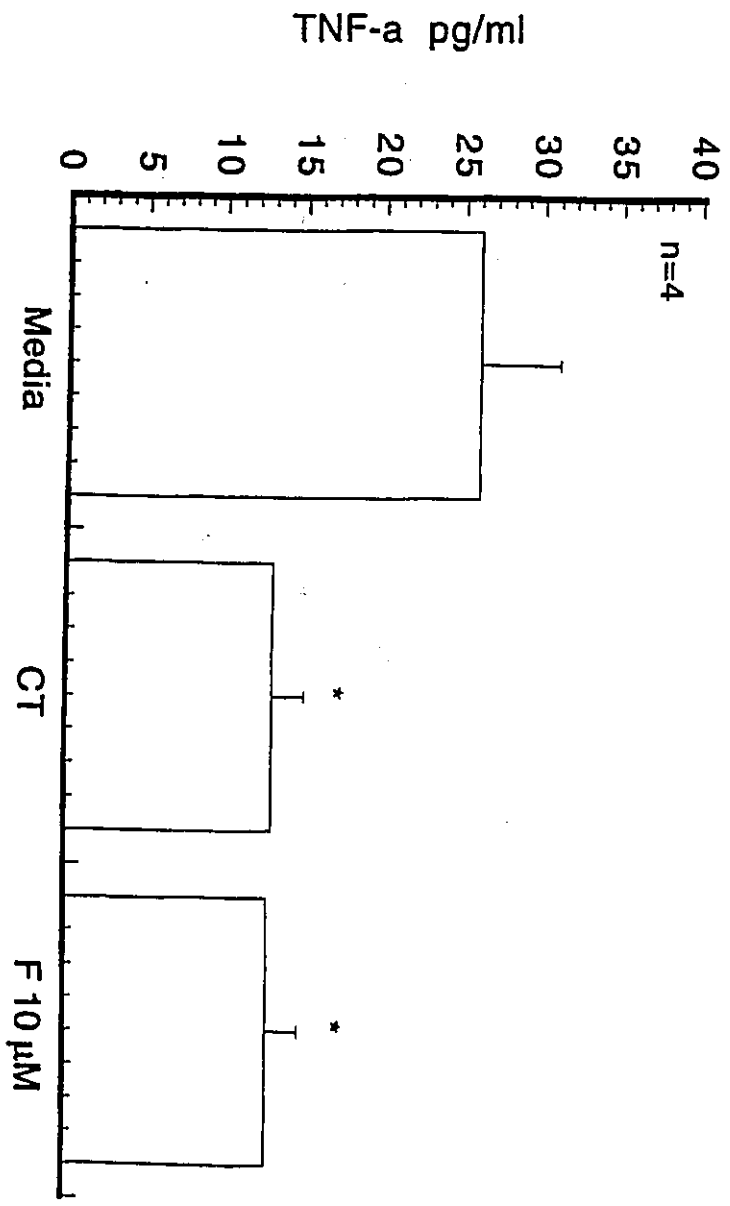

TNF-a pg/ml
0
20
40
60
80
n=4
Control
CT
1
3
6
9
12
18
24
Time (h)
*


IL-6 U/ml
(thousands)
0
2
4
6
8
10
12
14
16
n=4
+ Calcium
- Calcium
Media
CT
(88%)
PGE2
(92%)
anti-IgE
(100%)


IL-6 U/ml
(thousands)
0
1
2
3
4
5
Media
CT
F 1 µM
F 10 µM
Controls
Forskolin
*
*
*


TNF-a pg/ml
0
5
10
15
20
25
30
35
40
n=4
Media
CT
F 10 µM
*
*

CHAPTER FIVE

DISCUSSION

The work described in this thesis has lead to some advances in our understanding of mast cell biology in several areas. Of particular interest, we have obtained new information concerning the ability of mast cells to respond to bacterial products. We have also obtained further insight into the role of mast cells in inflammatory response through interactions with prostanoids and other mediators induced during inflammation and increased our awareness of the signalling pathways involved in inducing mast cell IL-6 and TNF-α production. In this chapter we will discuss each of these aspects, the limitations of our model, as well as the relevance of our findings to clinical disease.

Limitations

a) Conceptual.

In our system we have used rat PMCs because of the high purity that can be obtained after they pass through a Percoll gradient. However, mast cells found predominantly in the intestine and the lung are of the MMC type. Several studies have reported interesting results using MMC, as a source of cytokines but the purity obtained with these cells is not very high and we have to be aware that contaminating cells could interfere with final results. For example, intestinal epithelial cell, a common contaminant in MMC isolation in the rat, also produce IL-6 (Bromander *et al.* 1993). MMC lines, such as RBL cells might be considered as a possible alternative to freshly isolated cells, however, they did not produce detectable IL-6 in response to any of the stimuli we used (A23187, LPS, CT, or anti-IgE) in pilot studies. We believe that it will be important in future studies to optimize the purification conditions for MMC and examine if freshly isolated MMC cytokine production is similar to that observed in PMCs

to help assess the relevance of our studies to disease in the mucosae. Befus et al. (1982) described a purification procedure to isolate MMC with a purity close to 90% which might be increased if the contaminating cells are removed by newer technologies such as a MACS (Magnetic-activated Cell Sorting) column with antibodies directed against surface markers of the major contaminating cells.

A large number of cells participate in inflammatory responses. *In vitro* studies such as those described in this thesis are limited by the fact that we examined a single cell type in isolation. Normal conditions in the tissue as well as the context of inflammation provide special microenvironments where several factors may modulate cytokine production. Other limitations include the fact that results from animal experiments can not always be extrapolated to humans. However, they are essential since they provide experimental opportunities to carry out studies using rodent mast cells that can not be performed in humans. Further *in vivo* or *ex-vivo* studies using human mast cells are necessary to understand better the participation of mast cells during human inflammation and to confirm the clinical relevance of the observations we have made concerning the regulation of mast cell IL-6 and TNF-α production.

b) Methods.

We have some limitations in our study system of rat PMCs in view of the lack of rat recombinant cytokines available. In our TNF-α assays we used mouse recombinant TNF-α because it has been shown to share bioactivity with rat TNF-α (Chin *et al.* 1990). In the case of IL-6 bioassays we used human IL-6 as a standard which may have lead to an underestimate of IL-6 activity. However, if a standarized recombinant rat IL-

6 becomes available it would be worthwhile to run both standards in parallel to estimate the error given by human IL-6. Recently, it has been shown that the B9 IL-6 bioassay can respond to IL-11 (Burger and Gramatzki, 1993). However, IL-11 production by mast cells has not been described and IL-6 activity of our supernatants could be blocked by a neutralizing rabbit anti-rat IL-6 antibody (*see appendix 2).*

Interaction between mast cells and their products with other mediators in inflammation.

The inflammatory response involves the participation of a number of cell types and their products that establish a network of communication between cells. For example, studies have shown that TNF-α is a potent stimulus of IL-6 production in fibroblasts and other cells (Kohase *et al.* 1986; Defilippi *et al.* 1987). In other studies, IL-6 has been shown to inhibit TNF-α production (Aderka *et al.* 1989; Fattori *et al.* 1994). However, we examined the effect of recombinant mouse TNF-α on mast cell IL-6 production and we found no significant effect on IL-6 production (media control= 2127U/ml ± 561, 100pg/ml of rmTNF-α = 1830U/ml ± 557). Similarly, IL-6 had no effect on TNF-α production by rat PMCs which showed no difference compared to media control (media = 25 pg/ml ± 9, rhIL-6 = 22 pg/ml ± 9). These results suggest that IL-6 and TNF-α production by mast cells may be regulated differently from the production of these cytokines by other cells.

A number of cytokines have been shown by others to enhance or induce activation of mast cells. These include stem cell factor, IL-1, IL-3 and IL-4 for which the mast cell is known to have receptors. It would be interesting in future work to examine the

effect of these cytokines which are present in allergic inflammation, on mast cell IL-6 and TNF-α production.

Mast cells can interact with other mediators such as prostanoids. In this thesis we showed that in contrast to what other groups had reported, PGE_2 significantly enhanced histamine release in response to anti-IgE. These results indicate the possible role of PGE_2 on initiating or sustaining the early acute inflammatory response where the hallmark mediator is histamine.

We reported in this study that prostanoids of the E series have a selective modulatory effect on mast cell cytokine production, as we observed long term IL-6 production was enhanced while TNF-α synthesis was suppressed. We also observed that PGE_2 can act synergistically with LPS and this may extend to other mediators as described in other cell types (Richards and Agro, 1994). During an inflammatory response such as that which occurs in RA, IBD, and asthma, PGE_2 production can be enhanced. Some of this increase in PGE_2 may result from the interaction of mast cell products, such as histamine and TNF-α, with surrounding cells (epithelial cells, macrophages, fibroblasts) that can produce PGE_2 in response to such stimulant. We can envisage that the localized production of PGE_2 may induce IL-6 production from a number of local cell types including epithelial cells, fibroblasts, macrophages, and mast cells, and suppress TNF-α production from macrophages and mast cells. Moreover, if a bacterial product such as LPS is present in the context of PGE_2 a synergistic effect on IL-6 production may be seen (fig D1).

Our observations concerning the effects of prostanoids on mast cells may have particular relevance to intestinal inflammation. We mentioned previously that IBD is associated with increased levels of PGE_2, and that high doses of the cyclooxygenase inhibitor indomethacin induce intestinal inflammation in rats. Models of inflammation such as *Nippostrongylus brasiliensis* infection, a well characterized *in vivo* rat model of intestinal inflammation, could also be used to study the effect of prostanoids on intestinal mast cell cytokine production. Flurbiprofen, a more specific inhibitor of the cyclooxygenase pathway, may be preferable for *in vivo* instead of indomethacin. Mast cell cytokine expression could be assessed at an immunohistochemical level or by *in situ* hybridization in human samples from tissues undergoing inflammation where mast cells may be involved such as in the intestine (ulcerative colitis or Crohn's disease), lung, skin, and breast tissues obtained due to the presence of carcinoma. In some cases however, the normal control tissues for comparison may be difficult to obtain. It would be especially interesting to examine the baseline cytokine production, such as IL-6 and TNF-α, from inflamed and control tissues and their response to prostaglandins of the E series.

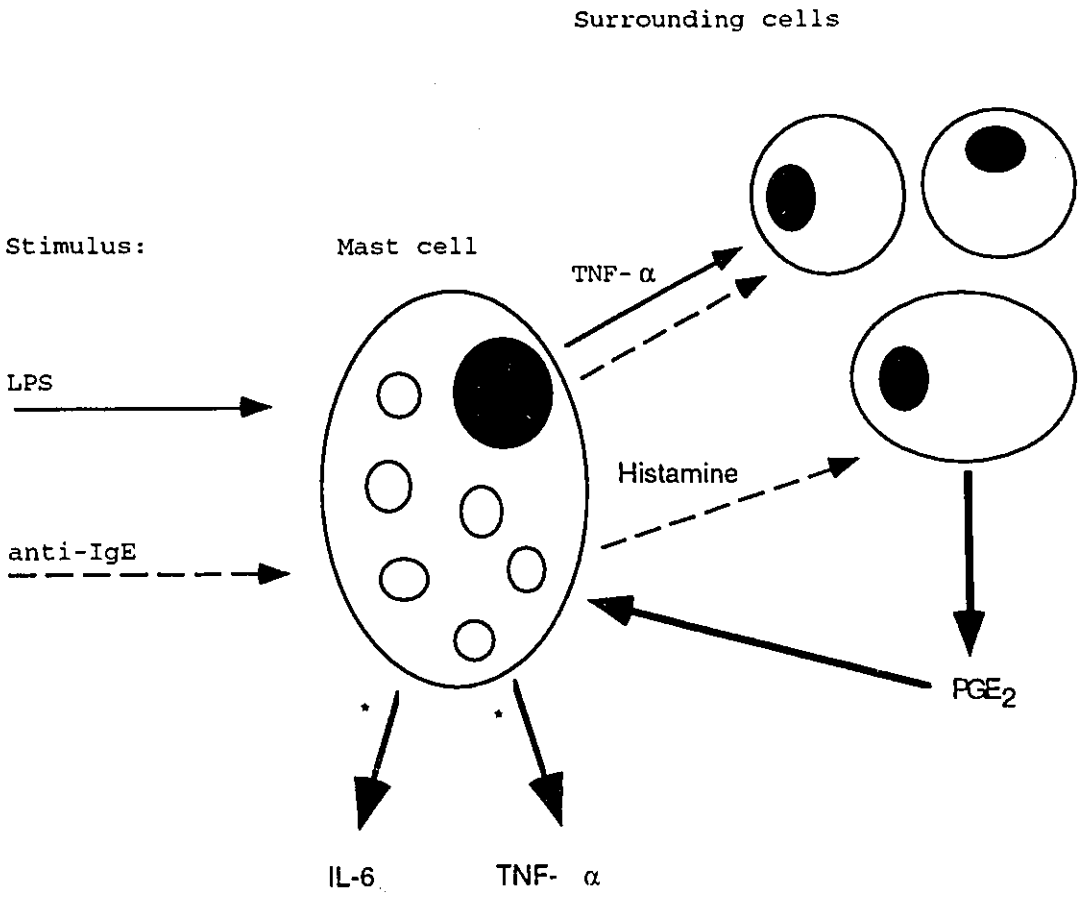


Fig. D1. Model of mast cell IL-6 and TNF-α production during inflammation. LPS (narrow solid line) and anti-IgE (dashed line) are probable stimulus which may activate mast cell cytokine production directly (solid lines with *) or indirectly by activation of surrounding cells. These cells may release other mediators such as PGE_2 (wide solid lines) which may also regulate mast cell cytokines release (increasing IL-6 or decresaing TNF-α).

Figure D1 essentially incorporates our findings into a story, however, we are aware that other factors and cells may be included. Such it is the case of IL-1, which plays an important role during inflammation. We believe that further studies on the modulation of mast cell IL-1 production may help us to complete our hypothetical

diagram. It is also important to realize that other cells may be capable of producing higher amounts of TNF-α or IL-6 compared to mast cells. Table D1 shows the amount of these two cytokines produced by $1x10^6$ mast cells/ml after 18 hr incubation.

Table D1. An estimation of TNF-α and IL-6 mast cell production.

Response to:	IL-6 U/1x10⁶MC/ml	TNF-α pg/1x10⁶MC/ml
Control (media alone)	1000 ± 300	50 ± 10
LPS (5μg/ml)	6000 ± 2000	250 ± 50
anti-IgE (5μg/ml)	2500 ± 500	200 ± 70
PGE_2 (0.1μM)	6500 ± 2300	10 ± 2
CT (0.01μg/ml)	10,000 ± 3500	12 ± 5

Note: 1U/ml of IL-6 = 0.45pg/ml. (studies from this thesis)(n=6).

There is evidence suggesting that other cell types may release greater amounts of TNF-α or IL-6 than mast cells. For example, Xing et al. (1992) reported approximately 10,000 U/ml IL-6 production in $1x10^6$ alveolar macrophages/ml after 24h incubation with no stimuli, whereas in the presence of LPS, under similar culture conditions, they reported 20,000 - 27,000 U/ml. However, they used higher LPS concentrations than we did. Schandene et al. (1992) reported TNF-α production by

$1x10^6$ monocytes/ml after 24h culture (150 pg/ml) and by T cells (50 pg/ml). The same authors showed PBMNC TNF-α production in response to LPS (1300 pg/ml) and media control (100 pg/ml). However, in this case they used a different assay to measure TNF-α production and the LPS concentration was different as well. We are aware that during an inflammatory process other cell types may be a better source for TNF-α or IL-6 production. However, further experiments should be performed comparing cytokine production by numerous cell types.

Mast cell response to bacterial products.

The role of mast cells in the pathophysiology of IgE-mediated allergic reactions has been well documented, however, their participation in bacterial infections has had little recognition. The particular localization of mast cells in the skin, mucosal surfaces and around blood and lymphatic vessels (Schwartz, 1987; Wasserman, 1989) as well as their ability to release such a wide variety of inflammatory mediators suggest that mast cells may be one of the first inflammatory cells to act against microbial infection. Recently Malaviya et al. (1994) reported that mast cells have the capacity to phagocytose and to destroy bacteria similarly to traditional phagocytic cells (neutrophils and macrophages). Previous studies have demonstrated that mast cells can be activated through complement receptors and our results presented in this thesis also suggest the potential response of mast cells to bacterial products such as LPS and CT. We found that bacterial toxins do not induce mast cell histamine release, contrary to what it is observed during an acute allergic response. However, both bacterial toxins had an effect on mast cell cytokine production enhancing IL-6 production. Cholera toxin

decreased TNF-α production over 18 hours, LPS, on the other hand, slightly increases TNF-α production (Marshall et al. personal communication). Therefore, our observations suggest that during a bacterial infection mast cells may be source of IL-6, and probably TNF-α, without undergoing classical degranulation. Other cytokines may also be selectively modulated dependent upon the nature of the infection and other environmental factors. IL-6 has a wide range of biological activities which could influence the initiation and progression of inflammation. For example, the ability of IL-6 to induce plasma cell development is essential to the local production of antibody of all classes. IL-6 has been shown to induce neuronal development which may be important in nerve remodelling that occurs in the context of inflammation (Nakafuku *et al.* 1992). Mast cells seem to produce IL-6 quite rapidly and in larger amounts than reported for other cells. Such mast cell cytokine production can persist for many hours after initial activation and could play an important role in long term responses to bacterial infection. However, it would be interesting to probe the role of the mast cell as a source of IL-6 compared with other cells by performing parallel studies with other IL-6 producing cells (macrophages, fibroblasts, intestinal epithelial cells) where such cells will be exposed to same stimuli and examined at a range of time points. The ability of mast cell deficient animals to respond to bacterial infection would also be an interesting area for future work.

Mast cell signalling pathways

Studies concerning the regulation of mast cell cytokine production have been performed by several groups mainly using rodent systems. Cytokine production is

regulated by complex mechanisms that may vary depending on the stimulus used to activate mast cells and the cytokine under examination. Immunological activation of mast cells with IgE and antigen results in Ca++ mobilization and PKC activation, among other processes. These signals are known to induce the production of certain cytokines (Fewtrel, 1989; Kinet, 1989b). For example, bone marrow derived mast cells treated with PMA, which activates PKC, showed accumulation of high mRNA levels of several cytokines such as TNF-α (Gordon and Galli, 1990). Evidence that $[Ca^{++}]_i$ controls IL-3 expression in mast cells by regulating stability of mRNA has been reported, whereas the expression of GM-CSF mRNA is controlled by $[Ca^{++}]_i$ and PKC pathways at both the transcriptional and the post-transcriptional level (Wodnar-Filipowicz *et al.* 1989; Hahn *et al.* 1991).

Our observations presented in this thesis clearly demonstrate that mast cells can respond to different stimuli to produce IL-6, in some cases this event takes place after mast cell degranulation, but in other circumstances the degranulation process does not occur. In *appendix 1* we will discuss preliminary observations at the electronic microscopy (EM) level which supports, in a different angle, these observations. Briefly, LPS mast cell activation which did not cause degranulation, showed no difference to media control at the EM level, whereas with anti-IgE we were able to obtain granulated and degranulated mast cells. Morphological differences between these two fractions were observed as well as their ability to induce IL-6 production.

In *chapter 2* we examined possible mechanisms involved in mast cell IL-6 production by using agents that are known to interfere with different signalling pathways, in the presence or absence of either LPS or anti-IgE. Our findings showed

that IL-6 production from both anti-IgE or LPS activated mast cells are calcium dependent, and both seem to involve G-proteins. In contrast, PKC seemed to participate only in anti-IgE activated mast cell IL-6 production, whereas tyrosine kinases seemed not to play an important role. These results suggest that mast cells may employ a number of signal transduction pathways to regulate IL-6 production. It is important to mention that the signalling studies reported *in chapter 2* were performed after 6 h culture, however, the same experiments were repeated in separate studies after 18 h culture. Although generally similar results were observed, Genistein, a tyrosine kinase inhibitor, seemed only to reduce LPS-activated mast cell IL-6 production (data not shown) and had no significant effect on anti-IgE induced IL-6. Thus the dependence of cytokine production on different pathways may alter mast cells depending on the timing at which we assess cytokine production and so the data presented should be interpreted with caution.

We also demonstrated that agents that increase intracellular cAMP in a number of cell types, including PGE_2, CT, and forskolin, significantly increased IL-6 mast cell production while TNF-α production was suppressed. Similar results were obtained with high doses of di-butyryl-cAMP (data not shown). Other groups have suggested $[cAMP]_i$ as an important second messenger that may regulate cytokine production from a number of cell types (Katakami *et al.* 1988; Zhang, 1988). Elevated cAMP levels are thought to act to enhance cytokine gene transcription through modulation of nuclear factor activity (such as AP-1, NF-IL6, and NF-κB) on the IL-6 gene (Dendorfer *et al.* 1994). Recently, Panettieri et al. (1995) examined whether an increase in $[cAMP]_i$ modulates cytokine-induced expression of cell adhesion molecules in human airway smooth muscle

cells (ASM). The authors used forskolin, PGE_2, and CT in this study and observed that cAMP-dependent pathways decreased TNF-α-induced ICAM-1 and VCAM-1 which further inhibited the adhesion of activated T cells to TNF-α-stimulated ASM cells. cAMP-dependent mechanisms also have been implicated in the regulation of TNF-α-induced cell adhesion molecules (CAM) expression on endothelial cells (Mattila *et al.* 1992; Pober *et al.* 1993). These results are of interest because inflammatory cells may modulate inflammatory responses either by release of pro-inflammatory cytokines or by regulating cell migration to a particular site by mechanisms that require CAMs. By understanding the mechanisms that regulate these cell responses we will be able to develop better therapeutic agents to control diseases characterized by inflammation.

We also have examined the effect of some anti-allergic drugs on IL-6 mast cell production. *In chapter 2* we showed that although nedocromil and dexamethasone had different effects on mast cell histamine release, both drugs were able to inhibit mast cell IL-6 production. The ability of drugs to modulate some aspects of mast cell function must be considered even if they do not alter mast cell preformed mediator release. β agonists are therapeutic agents used in asthma because they provoke bronchodilation through receptors that are coupled to Gs, which in turn activates adenylyl cyclase and increases $[cAMP]_i$ (Rothlein *et al.* 1986; Panettieri *et al.* 1990). Given our data concerning the effects of elevated cAMP, we would predict that β-agonists may elevate mast cell IL-6 production and inhibit TNF-α production. Other well known agents with actions on mast cell include Sodium Cromoglycate, Salbutamol, Cyclosporin A, and Budesonide. However, in our hands, Budesonide (a corticosteroid) was the only one that significantly inhibited (99% inhibition) IL-6 production by PMCs. The lack of inhibition of IL-6 production

by Cyclosporin A suggests that mast cell IL-6 production does not depend upon a calcineurin mediated signalling pathway. Further studies such as these could aid the design of new therapeutic approaches to control inflammatory responses where mast cell cytokine production is implicated.

Clinical relevance

The information presented in this thesis together with recent work from other groups examining mast cell cytokine production adds support to the concept that in addition to their traditional role in promoting inflammation in type I hypersensitivity responses and immunity to nematode parasites, mast cells may limit the inflammatory process. Our data demonstrating long term cytokine production without the necessity for degranulation, strengthens the concept that mast cells act as active participants in chronic inflammatory diseases and responses to bacterial infections. The ability of mast cells to phagocytose bacteria and their expression of MHC class II (Suzumura and Ohasi, 1991) raises the possibility that mast cells could process and present antigen to immune cells of the host.

The sustained production of TNF-α by mast cells may promote the inflammatory process by maintaining the upregulation of adhesion molecules on the vascular endothelium necessary for cell recruitment at inflammatory sites. The expression of both VCAM-1 and ICAM-1 can be upregulated by TNF-α. The former adhesion molecule may be of particular interest since it is the ligand for VLA-4 which is known to be expressed on activated eosinophils (Weg *et al.* 1993; Sriramarao *et al.* 1994) the recruitment of which is characteristic of allergic inflammation and exacerbations of

diseases such as asthma. Additionally, other mast cell mediators including histamine and PAF may act to further enhance cell recruitment through direct and indirect mechanisms. Therefore, mast cells in concert with other local cell populations may form an important part of pro-inflammatory pathways in which a complex cytokine and mediators network occurs.

Treatment of allergic and inflammatory diseases have relied on drugs such as corticosteroids which may act by controlling TNF-α and IL-6 production by mast cells as well as inhibiting T-cell function and cytokine production by other cell types. Similarly, drugs that stabilize mast cell degranulation such as Nedocromil may also inhibit long term mast cell cytokine production in response to certain stimuli. Further studies will be necessary to determine the ability of such drugs to modulate mast cell cytokine production in the tissue microenvironment in disease. PGE_2 has been shown to inhibit the enhancement of T cell adhesion to airway smooth muscle cells induced by TNF-α presumably through its effect on $[cAMP]_i$ (Panettieri, 1995). Inhaled PGE_2 pretreatment in allergic subjects has been shown to ablate both the early and late phase response to airways challenge in sensitized subjects. Through induction of prostanoids such as PGE_2, via histamine and TNF-α action (Platshon and Kaliner, 1978; Slungaard *et al.* 1990), mast cells may act to limit the range of a normal inflammatory response. IL-6 induced by PGE_2 or other local stimuli could help to decrease ongoing inflammation. IL-6 is also capable of decreasing TNF-α production in cells other than mast cells (Aderka *et al.* 1989; Fattori *et al.* 1994) that may provide a regulatory feedback mechanism. The effect of IL-6 on the final stage of B cell differentiation is important for immunoglobulin production. With a mucosal microenvironment the induction of IgA

synthesis may also contribute in limiting complement fixation and other IgG dependent immune events.

Given this information we should not assume that mast cell cytokine production is necessarily pro-inflammatory and we should consider selective modulation of mast cell cytokine production as an optimal approach to mast cell directed therapy. Information gained concerning the regulation of mast cell cytokine production may also be useful in understanding the regulation of cytokine production by other cell types under certain circumstances. Finally, *in vivo* experiments suggest that the levels of TNF-α and IL-6 that we report in our studies may not be that far from reality. For example, intradermal injection of 40 U/ml (10pg/ml) of recombinant murine TNF-α elicited neutrophil infiltration similar in magnitude and kinetics to that observed after IgE-dependent mast cell degranulation (Wershil *et al.* 1991). Regarding IL-6, serum IL-6 levels from children and adolescents with Crohn's disease (4.6 U/ml) have been shown to be significantly higher ($p < 0.0001$) than control serum samples (Hyams *et al.* 1993).

CONCLUSIONS

The results presented in this thesis demonstrated that rat freshly isolated and highly purified peritoneal mast cells can release IL-6 without any necessity for histamine release. These observations were determined with the use of bacterial products such as LPS and CT which significantly enhanced IL-6 production in our system. Further support for the concept of mast cell cytokine production without degranulation was confirmed by using agents such as Nedocromil and Dexamethasone which had different effects on histamine release, however, both significantly inhibited IL-6 production.

We have demonstrated that other mediators which may participate during inflammation such as PGE_1, PGE_2, PGD_2, CT and CTB alone had no significant effect on histamine release. However, PGE_1, PGE_2, and CT selectively modulated IL-6 production (significantly enhanced) while TNF-α was significantly inhibited. In contrast to other groups findings, we found that PGE_2 significantly increased histamine release in response to anti-IgE. A synergistic effect on IL-6 production in the context of PGE_2 + LPS was also observed. These findings illustrate the complexity of cell-cell and cell-mediator interaction likely to occur in inflammation.

Regarding mast cell signalling pathways, our findings indicated that IL-6 production seemed to be Ca^{++} dependent regardless of the type of stimulus used. However, mechanisms to induce IL-6 production seemed complex and may involve more than one pathway, some of which may be stimulus specific. For example, PKC was involved in a response to anti-IgE but not LPS, whereas G-proteins may participate in both LPS and anti-IgE response. Moreover, we also demonstrated that agents that increase $[cAMP]_i$

enhanced IL-6 production and decreased TNF-α synthesis. Such decrease on TNF-α could not be ascribed to a direct effect of IL-6 on rat PMC, suggesting a complex mast cell cytokine regulation.

APPENDIX ONE

GRANULATED AND DEGRANULATED MAST CELL POPULATIONS: RESPONSE TO ANTI-IgE AND LPS

Rationale

Our data from *chapter 2* demonstrated that mast cell degranulation and mast cell cytokine production are independent events. Our findings showed that A23187 significantly induced histamine release (close to 85% release), however, no detectable IL-6 was observed; anti-IgE induced around 40% release of histamine and significant amounts of IL-6; whereas LPS which did not had an effect on histamine release induced large amounts of IL-6. Our rationale was:

- A23187 induced degranulation to 85% of mast cells, therefore only 15% of the cells contained intact granules.
- Anti-IgE induced degranulation to 40% of mast cells, leaving 60% of the cells with granules.
- LPS had no effect on histamine (less than 10% histamine release) and most of the cells (around 90%) contained granules.

These results suggested that the proportion of granulated mast cells may be important in determining the extent of IL-6 production.

Methodology

In order to examine the potential of G vs DG mast cell IL-6 production, we purified PMCs, as previously described in *chapter2*, and activated initially with A23187 (in some experiments), anti-IgE, LPS or media. The cells were washed and passed through a second percoll gradient (40%/80%) to separate the granulated and degranulated fractions (fig A1). G and DG mast cells were adjusted to $1x10^6$ cells/ml

and cultured for 18hr with fresh media containing the same secretagogues used during the initial activation. After 18hr incubation IL-6 bioactivity was assessed in the supernatants using the B9 proliferation assay already described.

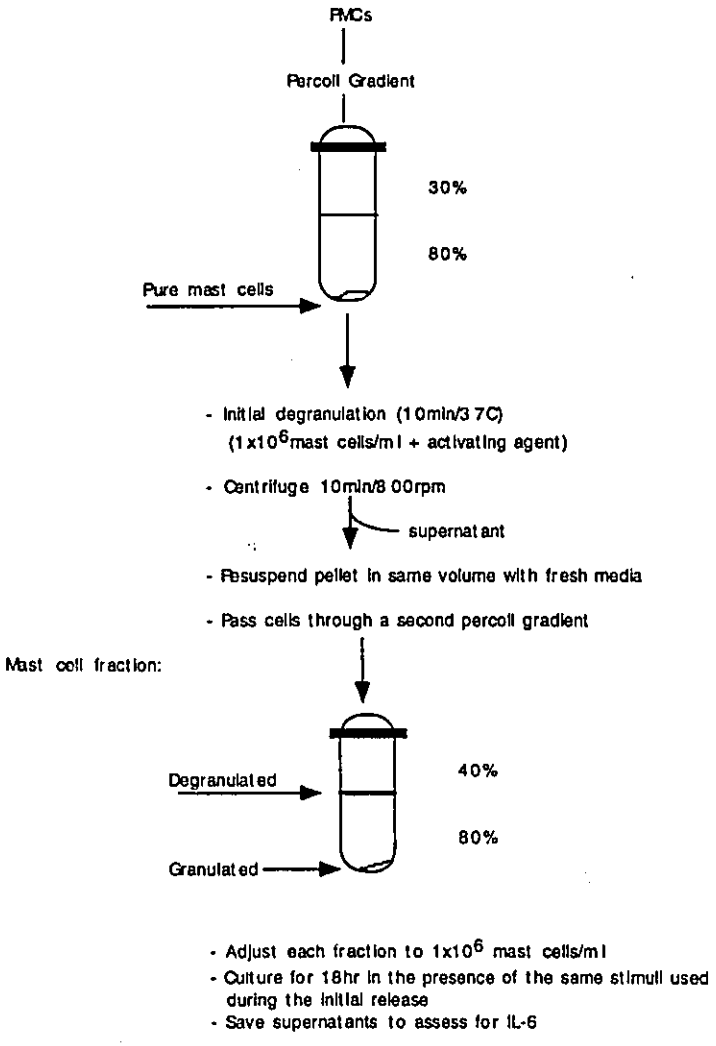


Fig. A1. Methodology to separate G and DG mast cells.

Morphological studies

In order to assess the presence or absence of granules in the two different mast cell populations, after isolation of granulated and degranulated cells we prepared cytospins ($1x10^6$MC/ml) to stain with Toluidine Blue (TB). Briefly, cytospins were fixed in Carnoys (60% ETOH, 30% Chloroform, and 10% Glacial acetic acid) for 10 min, another 10 min in 66% ETOH, one minute in 0.5% Acetic acid, 6 minutes in TB pH 0.5, and finally rinsed with tap water (fig A2). A similar aliquot was saved to be examined by electron microscopy. Briefly, an aliquot of each mast cell population ($1x10^6$/ml) was fixed in 2% glutaraldehyde in 0.1M Cacodylate buffer pH 7.4 for 2 hrs, followed by 1% osmium tetroxide in 0.1M Cacodylate buffer for 1 hr. The pellets were embedded in Spurr's resin and the ultra-thin sections stained with uranyl acetate and lead citrate (fig A3).

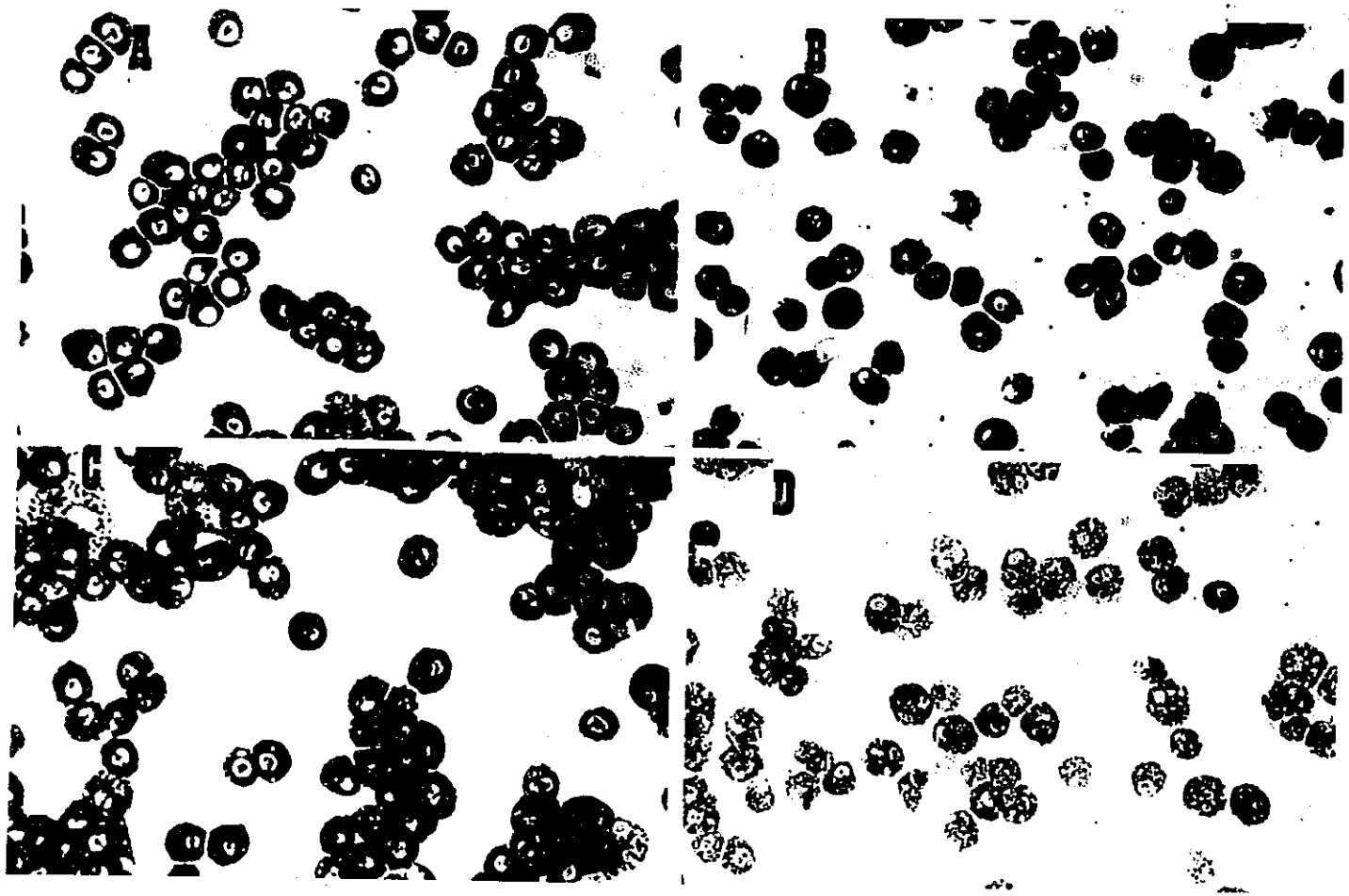
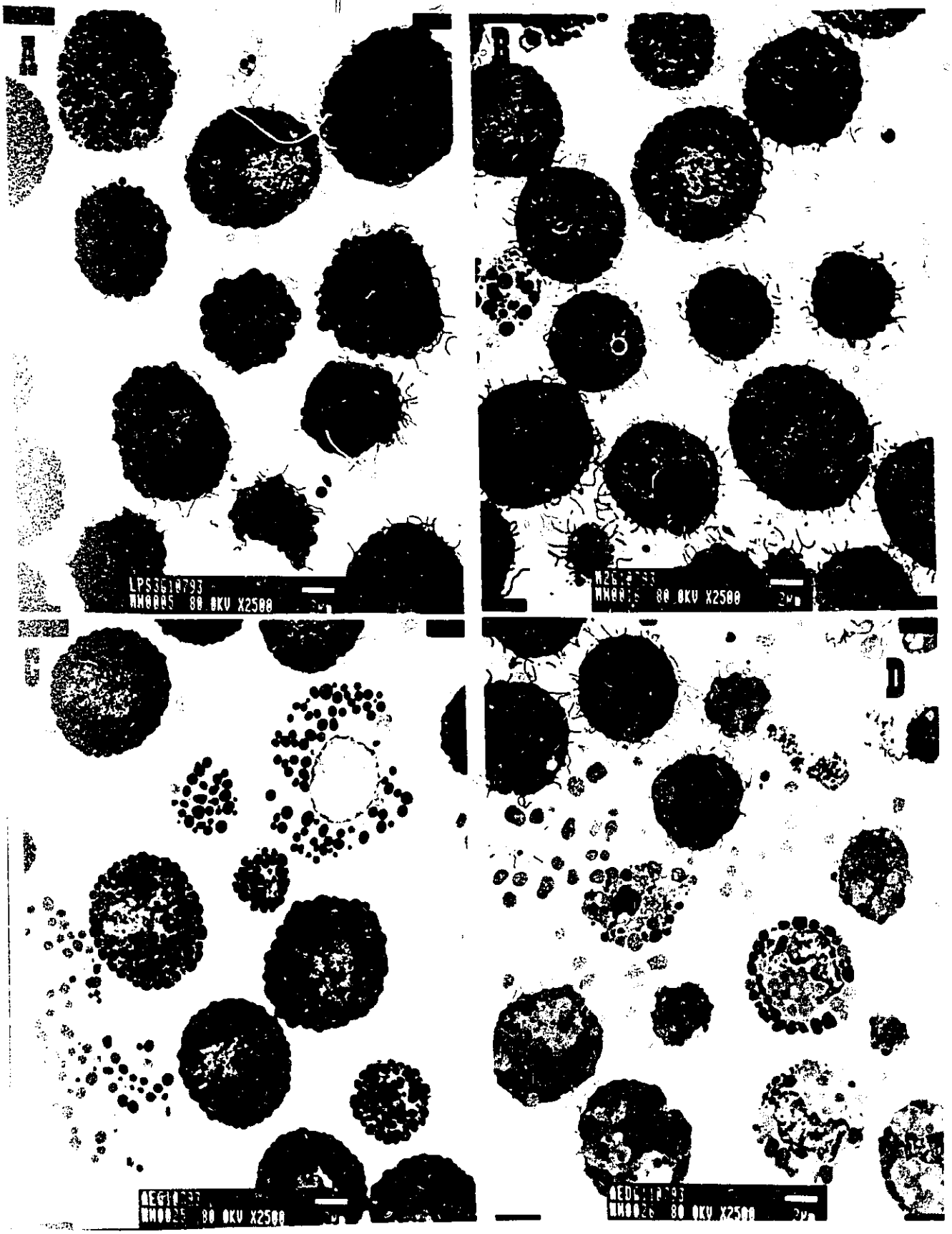


Fig. A2. Toluidine blue staining of G and DG mast cell populations following treatment with different stimuli: anti-IgE (5μg/ml), LPS (5μg/ml), or control. **Note:** we obtained both, G and DG mast cell fractions only from anti-IgE activated mast cells. A= Control G, B= LPS G, C= anti-IgE G, and D= anti-IgE DG. Observed at = X400.



.3. Electron microscopy (EM) of G and DG mast cell populations following treatment with different stimuli: anti-IgE (5µg/ml), LPS (5µg/ml), or control. **Note:** we obtained both, G and DG mast cell fractions only from anti-IgE activated mast cells. A= Control G, B= LPS G, C= anti-IgE G, and D= anti-IgE DG. Observed at = X2500.

Results

We used histamine release as a marker for mast cell degranulation using ionophore A23187 as a positive control (data not shown). With anti-IgE we obtained a similar proportion of both G and DG fractions. In contrast, LPS gave a greater amount of G cells and almost no DG cells. We examined the production of IL-6 from both fractions: G and DG PMCs in response to anti-IgE and LPS. Our results (fig A4) showed that, in response to anti-IgE, both mast cell populations are capable of producing significant levels of IL-6, however, degranulated cells produce a greater amount of IL-6 than granulated cells.

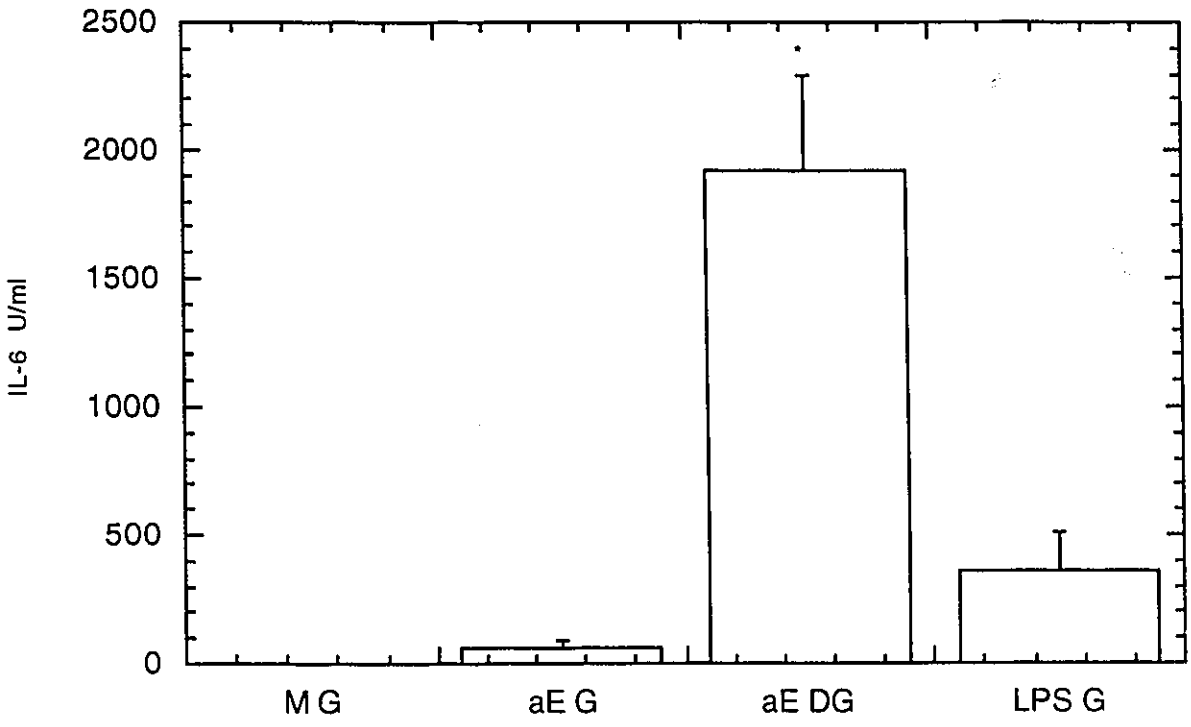


Fig. A4. IL-6 production by granulated and degranulated enriched mast cell population in response to different stimuli: media control (n=9), anti-IgE (n=9), and LPS (n=7). We obtained only a purified G fraction following incubation with media or LPS, whereas following anti-IgE activation we obtained both G and DG cells. Both G and DG mast cells are capable of inducing IL-6 production. However, the DG fraction of anti-IgE activated cells produced a greater ($p<0.05$) amount of IL-6 compared to the G anti-IgE activated fraction. MG= granulated mast cells after treated with media; aEG= granulated mast cells after anti-IgE treatment; aEDG= degranulated mast cells after anti-IgE treatment; and LPSG= granulated mast cell after LPS incubation.

Discussion

Our recent understanding of the potential for mast cells to produce cytokines has lead us to question whether preformed mediator release is always an effective measure of the biological impact of these cells. Our findings so far, suggests that mast cells may participate in a number of other biological processes by the production of cytokines, such as IL-6 which does not depend upon mast cell degranulation. The differences between A23187, anti-IgE or LPS induced PMC IL-6 production lead us to postulate that the proportion of granulated mast cells may be important in determining the extent of IL-6 production, since following A23187 activation we had a very small population of granulated mast cells and no IL-6 production is observed (*see chapter2*) compared to cells activated with anti-IgE or LPS which showed a greater proportion of granulated mast cells and a significant amount of IL-6 is produced. Although our results showed the potential of both cell populations to produce IL-6 in response to anti-IgE, IL-6 production was significantly greater in the DG fraction compared to the G one. These results demonstrate that degranulated mast cells activated with anti-IgE retain the capacity to produce IL-6 in contrast to A23187 activated degranulated cells.

APPENDIX TWO

VERIFICATION OF IL-6 AND TNF-α ASSAY SYSTEM

We measure IL-6 bioactivity with a B9 hybridoma proliferative bioassay, whereas TNF-α was measured by a cytotoxicity bioassay using TNF-α-sensitive L929 cell line (mouse fibroblast). We also confirmed the sensitivity of both assays by using specific polyclonal antibodies: rabbit anti-rat IL-6 serum, and rabbit anti-murine TNF-α. Our results showed that mast cells supernatant in response to anti-IgE, LPS, and media control in the presence of anti-murine TNF-α completely abrogated their cytotoxic activity. Anti-rat IL-6 completely blocked the proliferation activity of rat IL-6 control, as well as supernatants from anti-IgE and PGE_2-treated mast cells, whereas, IL-6 activity on the B9 assay from LPS-treated mast cells supernatants were inhibited only 55.5%. This result suggests the presence of other unknown factors capable of inducing B9 cells proliferation. We could think of IL-11, however, we do not know if rat mast cells produce this cytokine. Therefore, we should be aware that the B9 assay may not be that specific for IL-6 as we now believe.

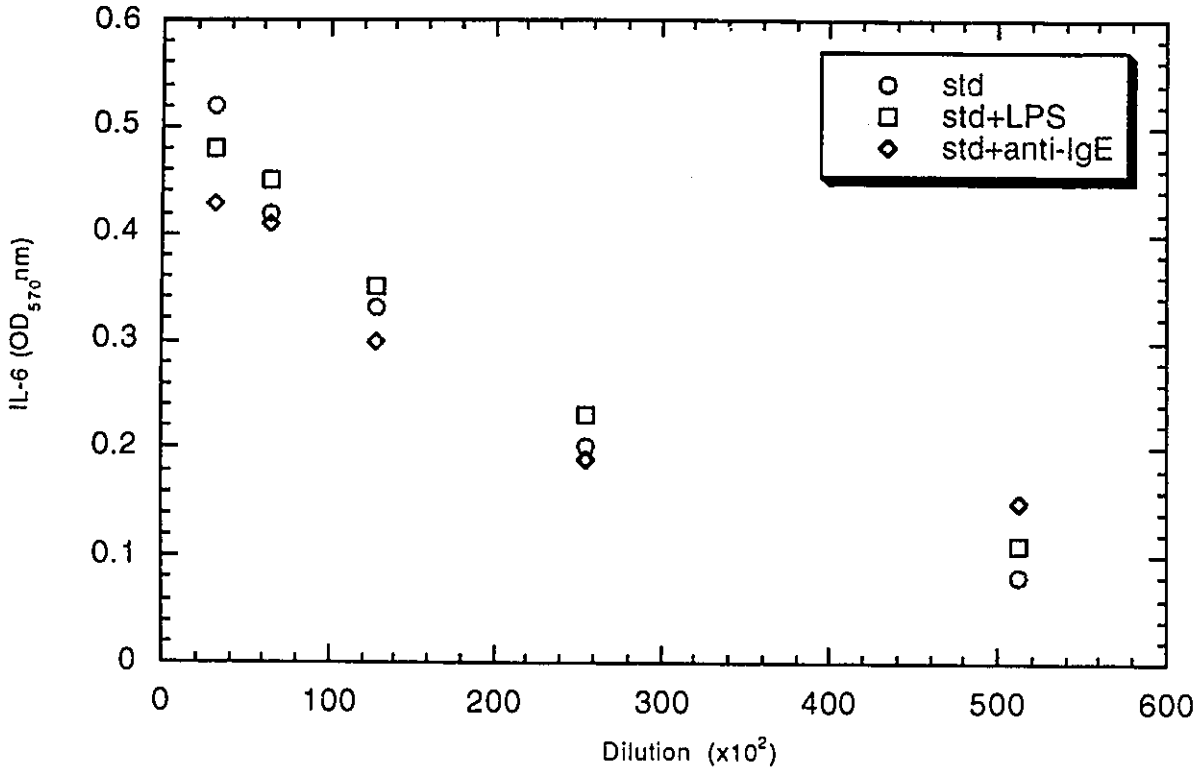
Because of the use of different agents in our experiments we verified their effect on the IL-6 and TNF-α bioassays by running a normal standard in parallel to a standard in the presence of the "x" agent which had the same final concentration as the one used in the experiment. The OD_{570nm} results were plot in a graph and a statistical analysis (t-test) was used to find out if such an agent does or does not interfere in either bioassay. Graph 1 shows an example of the plotted results obtained from both standard curves, whereas Table A1 shows the ODs obtained as half maximum values from standard alone and standard in the presence of the "x" agent.

Table A1. Half maximum values from IL-6 or TNF-α standard curve alone or in the context of a stimulating agent. Values are expressed as OD_{570}nm.

		IL-6		TNF-α	
Stimuli	final conc.	Std. alone	Std. + stimuli	Std. alone	Std. + stimuli
LPS	5 µg/ml	0.33	0.37	0.433	0.425
A23187	1 µM	0.42	0.41	0.283	0.276
anti-IgE	5 µg/ml	0.33	0.30	0.302	0.290
IgG_1	5 µg/ml	0.48	0.43	*	*
PMA	1 µM	0.30	0.27	0.283	0.299
Genistein	10 µg/ml	0.30	0.28	*	*
Nedocromil	0.1mM	0.41	0.35	*	*
Dexametha.	1 µM	0.41	0.38	*	*
Cholera tx.	0.01 µg/ml	0.41	0.37	0.112	0.113
Pertussis tx.	0.01 µg/ml	0.30	0.25	0.190	0.180
PGE_1	0.1 µM	0.36	0.39	0.130	0.128
PGE_2	0.1 µM	0.55	0.55	0.130	0.120
PGD_2	0.1 µM	0.39	0.39	0.142	0.148
Forskolin	10 µM	0.48	0.51	0.162	0.171
DbcAMP	10 µM	0.48	0.48	0.162	0.154
mTNF-α	100pg/ml	0.29	0.34	*	*

*not performed

Graph A1. OD_{570}nm values of IL-6 standard alone or in the context of LPS (5µg/ml) or anti-IgE (5µg/ml).



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