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Modulation of mast cell responses by prostaglandin E_2

by

Kaede Gomi

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

at

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For Kenji, you gave me wings so I could fly.

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Abstract

Prostaglandin E_2 (PGE_2) is overproduced in chronic inflammatory disorders, and acts on virtually every cell of the immune system. Although mast cells are implicated in the pathogenesis of inflammatory disorders, surprisingly little is known of the effects of PGE_2 on mast cells.

We investigated the effects of PGE_2 on mast cell degranulation and production of cytokines relevant to allergic disease. Mouse bone marrow-derived mast cells (BMMC) were treated with PGE_2 alone or in the context of IgE-mediated activation. PGE_2 treatment alone specifically enhanced interleukin (IL)-6 production, yet failed to modulate the production of a number of other mast cell cytokines. However in the context of IgE-mediated activation, PGE_2 potentiated mast cell degranulation and selectively enhanced the production of IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), and tumour necrosis factor-α (TNF-α). PGE_2 generally inhibits TNF-α production by many cell types through the elevation of intracellular cAMP. cAMP-elevating agents, forskolin and pentoxifylline, failed to similarly enhance IgE-mediated TNF-α production, arguing against a role for cAMP. PGE_2 acts by binding to one of four prostaglandin E receptor subtypes (EP_1-EP_4). RT-PCR analysis of resting MC/9 cells revealed the expression of EP_1, EP_3, and EP_4 prostaglandin E receptor subtypes, including a novel variant form of the mouse EP_1 receptor. Pharmacological studies using EP subtype-selective agonists indicated that PGE_2 differentially regulated mast cell IL-6 and TNF-α production through the activation of EP_1 and/or EP_3 receptors, with a possible role for intracellular calcium.

Taken together, our data indicate that PGE_2 may profoundly alter the nature of mast cell responses at sites of allergic inflammation. Furthermore, we have shown that PGE_2 acts through EP receptor subtypes not normally associated with the regulation of the immune responses, providing another glimpse into the complexity of the immunomodulatory effects of PGE_2.

Abbreviations

Ab	antibody
ADCC	antibody-dependent cell-mediated cytotoxicity
Ag	antigen
APC	antigen presenting cell
BAL	bronchoalveolar lavage
BMMC	bone marrow-derived mast cell
BSA	bovine serum albumin
cAMP	cyclic AMP
CBMC	cord blood-derived mast cell
CD	cluster determinant
cGMP	cyclic GMP
Con A	concanavalin A
COX	cyclooxygenase
CRE	cAMP response element
CSF	colony-stimulating factor
CTL	cytotoxic T lymphocyte
CTMC	connective tissue mast cell
DAG	diacylglycerol
DC	dendritic cell
DMSO	dimethylsulfoxide
DNaseI	deoxyribonuclease I
DNP-HSA	dinitrophenyl-human serum albumin;
DP	prostaglandin D receptor
DTH	delayed type hypersensitivity
ELISA	enzyme-linked immunosorbent assay
EP	prostaglandin E receptor
FACS	fluorescence-activated cell sorter
$Fc_\varepsilon RI$	high affinity IgE receptor
FcR	Fc receptor
FcRα	FcRα subunit
FcRβ	FcRβ subunit
FcRγ	FcRγ subunit
FCS	fetal bovine serum
GM-CSF	granulocyte macrophage colony-stimulating factor
h	hour
HEPES	*N*-2-hydroxyethylpiperazine-*N*-2ethanesulfonic acid
HLMC	human lung mast cell
ICAM	intercellular adhesion molecule
IFN-γ	interferon-gamma
Ig	immunoglobulin
IgE	immunoglobulin E
IgG	immunoglobulin G

IgM	immunoglobulin M
IL	interleukin
IFN-γ	interferon-γ
IMMC	intestinal mucosal-type mast cell
i.p.	intraperitoneal
IP3	inositol 1,4,5-trisphosphate
i.v.	intravenous
ITAM	immunoreceptor tyrosine-based activation motif
KO	knock out
LPR	late phase response
LPS	lipopolysaccharide
LT	leukotriene
mAb	monoclonal antibody
MAP-K	mitogen-activated protein kinase
2-ME	2-mercaptoethanol
MC_T	human mast cell containing tryptase
MC_{TC}	human mast cell containing both tryptase and chymase
MHC	major histocompatibility complex
min	minute
MLR	mixed lymphocyte reaction
MMC	mucosal mast cell
MMCP	murine mast cell protease
mRNA	messenger ribonucleic acid
NFAT	nuclear factor of activated T lymphocyte
NGF	nerve growth factor
NK cell	natural killer cell
NO	nitric oxide
PAF	platelet-activating factor
PBL	peripheral blood lymphocyte
PBS	phosphate buffered saline
PCA	passive cutaneous anaphylaxis
PG	prostaglandin
PGE_1	prostaglandin E_1
$PGE_{1/2}$	prostaglandin E_1/ prostaglandin E_2
PGE_2	prostaglandin E_2
PHA	phytohemagglutinin
PI3-K	phosphatidylinositol-3-kinase
PI3P	phosphatidylinositol-3,4,5-triphosphate
PKA	protein kinase A
PKC	protein kinase C
PL	phospholipase
PLCγ1	phospholipase C-γ1
PMA	phorbol myristate acetate
PMC	peritoneal mast cell
PTK	protein tyrosine kinase

RBC	red blood cells
rIL	recombinant interleukin
RT-PCR	reverse transcription-polymerase chain reaction
s.c.	subcutaneous
SE	staphylococcal enterotoxin
SEM	standard error of the mean
SH2	src homology-2 domain
STAT	signal transducers and activators of transcription
TCR	T cell receptor
Tec	Tec family of tyrosine kinases
Th	T helper cell
ThN	naïve Th cell
TNF-α	tumor necrosis factor-α
TNP-BSA	trinitrophenyl-bovine serum albumin
U	Unit
VCAM	vascular cell adhesion molecule

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thanks to you, for showing me the possibilities of love, and to God, for being the source of this Love.

Chapter 1: INTRODUCTION

1.1 Mast Cells

Mast cells are bone marrow-derived immune cells resident in nearly every tissue in the body (Metcalfe *et al.*, 1997). They are found in abundance at sites that directly interface the external environment such as the skin, respiratory and gastrointestinal tract. Mast cells are also clustered around blood vessels and in close proximity to peripheral nerves. There is evidence that mast cells are directly involved in angiogenesis as well as in nerve growth, activation and survival (Meininger and Zetter, 1992; Matsuda *et al.*, 1989; Marshall *et al.*, 1999). Moreover, their anatomical localization has helped earn these cells the title of sentinel cells (Galli *et al.*, 1999). Mast cells have been demonstrated to be critical in host defence against bacteria in two mouse models of peritoneal infection and mast cell-derived TNF-α was identified as a critical component for mouse survival (Echtenbacher *et al.*, 1996; Malaviya *et al.*, 1996). This beneficial role is sharply contrasted with the well-recognized involvement of mast cells in the pathogenesis of allergic diseases such as asthma and atopic dermatitis (Dvorak *et al.*, 1976; Galli, 1997). Indeed, mast cells are truly complex immune cells with multifaceted functions in both health and disease.

Mast cells arise from CD34+ hematopoietic progenitor cells that leave the bone marrow as mast cell precursors and mature in peripheral tissues (Nilsson *et al.*, 1999). They are found extensively in connective and mucosal tissues and represent a heterogeneous group of cells that differ in mediator content, appearance, and also in terms of activation and function.

1.2 Mast cell heterogeneity

1.2.1 Classification of mast cell subtypes

The existence of heterogeneity in rodent and human mast cell populations has long been appreciated (Schwartz and Huff, 1998). Mast cells are classified into subclasses based on their granule protease expression. In rodents, mast cells are classified under the headings of mucosal mast cell (MMC) and connective tissue mast cell (CTMC). MMC and CTMC were initially distinguished on the basis of proteoglycan content, i.e., chondroitin sulfate and heparin in MMC and CTMC, respectively. It soon became apparent that immature CTMC often failed to stain properly for heparin, leading to their erroneous identification as MMC.

A better classification system was developed based on neutral protease composition (Gibson and Miller, 1986), where rat MMC were identified by chymase II content in their granules, and CTMC by chymase I and carboxypeptidase A. In the mouse system, MMC and CTMC are distinguished by differences in their expression of multiple chymase and tryptase proteins (Schwartz and Huff, 1998). Mouse MMC contain chymase murine mast cell proteases (MMCPs) MMCP-1, -2, and -4, whereas CTMC contain chymases MMCP-3, -4, and -5, and tryptases MMCP-6 and -7.

In humans, mast cells are similarly classified into two subclasses; however, these cells are distinguished according to their neutral protease content, and are denoted MC_T and MC_{TC} depending on whether they contain only tryptase or both tryptase and chymase in their granules, respectively (Irani *et al.*, 1986; Schwartz, 1993). The MC_{TC} granules also contain the neutral proteases carboxypeptidase and cathespin G (Schwartz, 1993).

1.2.2 Distribution of mast cell subtypes

MC_T are mainly found at mucosal sites, particularly in the alveolar septa of the lung and the mucosa of the small intestine (Schwartz, 1993). Conversely, MC_{TC} predominate at connective tissue sites such as the skin, intestinal submucosa, conjunctiva, synovium, and blood vessels. Similar to MC_T, rodent MMC are largely distributed at mucosal sites, especially in the intestinal lamina propria. CTMC are more analogous to MC_{TC} in their distribution, and are the main subtype found in connective tissues such as the peritoneal cavity and skin (Schwartz and Huff, 1998). Although a particular mast cell subclass may predominate at a given anatomic site, both subclasses are usually present to some degree; therefore, tissue localization should not be employed as the sole discriminating factor. Furthermore, there is evidence that mast cell subtype profiles in various tissues are altered in pathological conditions, such as fibrosis (Schwartz and Huff, 1998).

1.3 $Fc_\varepsilon RI$ receptor signaling

Immunological activation of mast cells is mediated by high affinity $Fc_\varepsilon RI$ receptors (Kinet, 1999). These receptors bind the Fc portion of circulating IgE, leaving their Fab regions free to bind specific antigen. Binding of multivalent antigens by surface-bound IgE induces cross-linking of $Fc_\varepsilon RI$ receptors into dimers or larger aggregates, and the act of receptor cross-linking is the signal for mast cell activation (Segal *et al.*, 1977). $Fc_\varepsilon RI$ receptor-mediated activation leads to mast cell degranulation, lipid mediator generation, and cytokine production.

1.3.1 Structure of the $Fc_{\varepsilon}RI$ receptor

The $Fc_{\varepsilon}RI$ receptor is a tetrameric structure consisting of single α- and β-chains, and a pair of disulfide-bound γ-chains (Blank *et al.*, 1989; Ra *et al.*, 1989b) (Figure 1). The FcRα-subunit is the subunit responsible for binding IgE, and consists of a large extracellular domain, a single transmembrane domain, and a short cytoplasmic domain. The extracellular domain is heavily glycosylated and contains two immunoglobulin-related domains. The FcRβ-subunit possesses four transmembrane domains, and both the N- and C-terminal tails are located in the cytoplasm. The FcRγ-subunit is responsible for the signaling activity of the $Fc_{\varepsilon}RI$ while the FcRβ-subunit serves to amplify FcRγ-mediated signaling (Kinet, 1999). The FcRγ-subunit is not restricted to the $Fc_{\varepsilon}RI$ receptor complex. Rather, it is a common component of a number of receptors including macrophage FcγRII receptors (Ra *et al.*, 1989a).

1.3.2 Signaling through the $Fc_{\varepsilon}RI$ receptor

The earliest signaling events occur within seconds of $Fc_{\varepsilon}RI$ cross-linking (Metcalfe *et al.*, 1997) (Figure 2). Aggregation of the receptor complexes triggers the Src family related protein tyrosine kinase (PTK), Lyn, normally associated with FcRβ under resting conditions, to phosphorylate tyrosine residues in the FcRβ and FcRγ subunits (Jouvin *et al.*, 1994). The targeted tyrosines are found in specialized regions known as immunoreceptor tyrosine-based activation motifs (ITAMs) (Paolini *et al.*, 1991). The phosphorylated ITAMs function as docking sites for Src homology 2 (SH2) domain-containing downstream signaling molecules. One such molecule is Syk (Jouvin *et al.*,1994). This 72-kDa protein is recruited by phosphorylated ITAMs on FcRγ. It is subsequently phosphorylated by Lyn

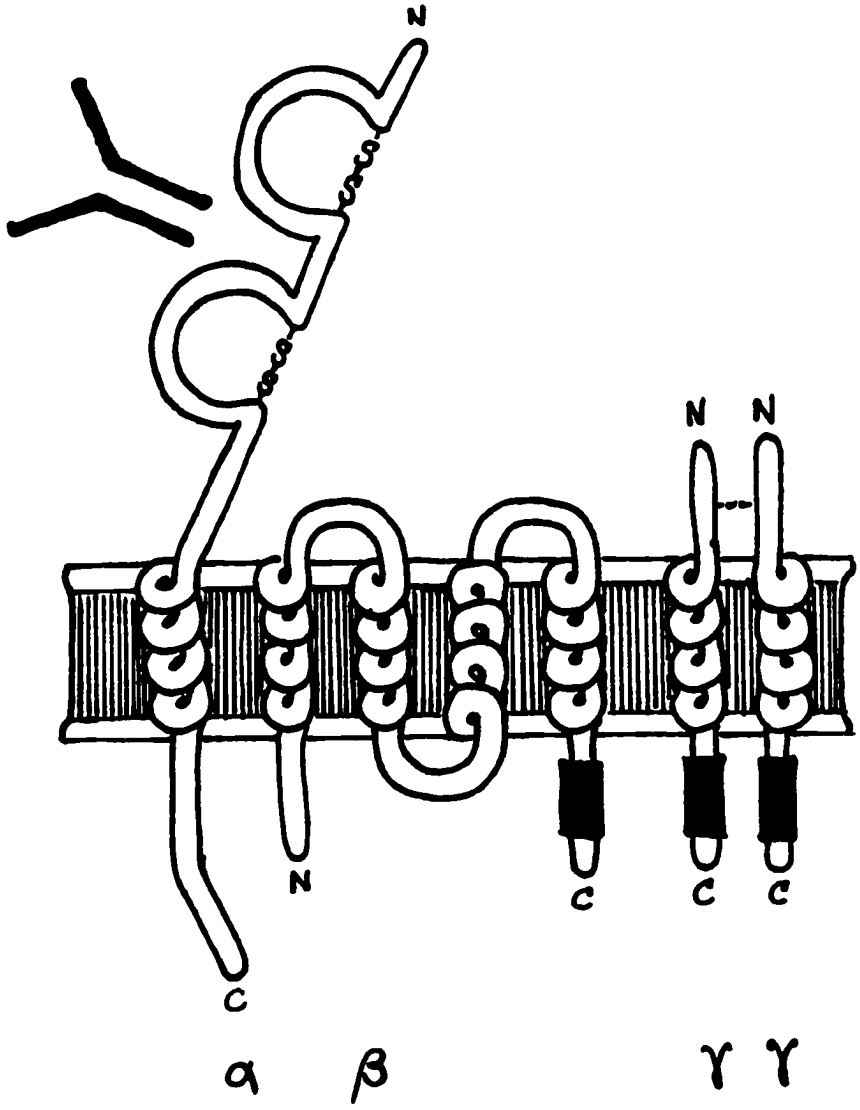

Figure 1 **Schematic of the $Fc_{\varepsilon}RI$ receptor (Abbas *et al.*, 2000).**

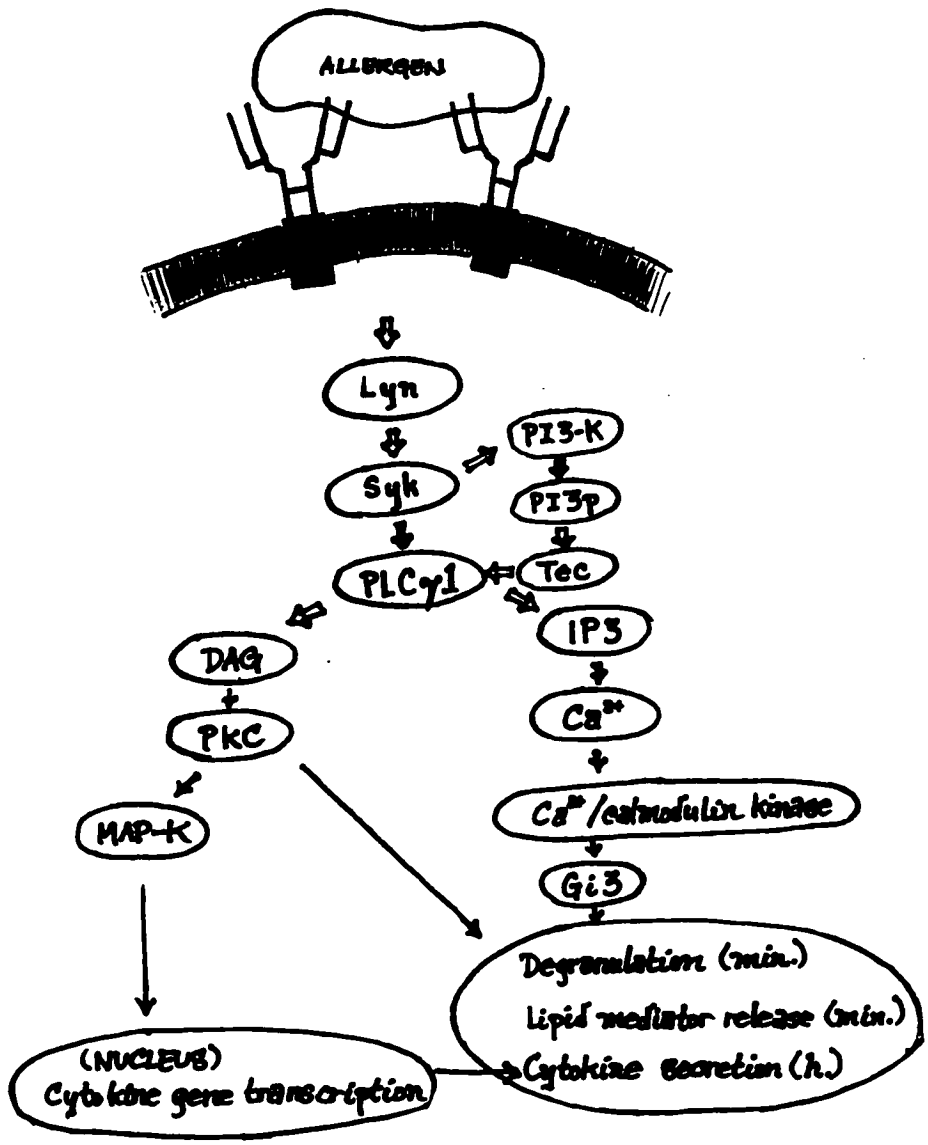

Figure 2. **Signaling through the FcERI receptor (Metcalfe *et al.*, 1997; Zhu, 1999; Kinet, 1999)**

which initiates autophosphorylation of further tyrosines on Syk, resulting in its activation. Once activated, Syk phosphorylates and activates newly recruited Syk molecules in addition to phosphorylating many other signaling molecules which include phosphatidylinositol-3-kinase (PI3-K) (Kinet, 1999). PI3-K is activated by Syk-induced phosphorylation and is responsible for the synthesis of phosphatidylinositol-3,4,5-triphosphate (PI3P). This second messenger functions as a docking site for Tec kinases via the pleckstrin-homology (PH) domain of the latter molecules. Membrane-recruited Tec kinases, most notably the Bruton tyrosine kinase, are activated via phosphorylation events. Tec kinases activate phospholipase C-γ (PLC-γ) which in turn generates the second messengers, diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP3). IP3 brings about a sustained influx of calcium, and DAG activates the serine/threonine protein kinase C (PKC). PKC and IP3-directed enhanced calcium signal synergize to initiate the mast cell's exocytotic machinery (Metcalfe *et al.*, 1997).

1.4 Mast cells are a source of pre-formed and newly-generated mediators

1.4.1 Pre-formed mediators

Mast cells, and their circulating counterpart, basophils, are easily distinguished by their numerous, electron-dense granules occupying much of the cytoplasmic space (Galli, 1990). These granules serve as the storehouses of the mast cell's pre-formed mediators. Of these mediators, the most well-recognized is histamine. This biogenic amine has many physiological effects including increased vascular permeability, and smooth muscle constriction of the airways and gastrointestinal tract (Nilsson *et al.*, 1999). Other pre-

formed mediators include neutral proteases (tryptase, chymase), proteoglycans (chondroitin sulfates, heparin), and acid hydrolases (β-hexosaminidase). These granule-associated mediators, particularly the neutral proteases, are believed to mediate physiologically important effects, e.g. the activation of various latent extracellular proteases and mitogenic effects on neighbouring cells.

1.4.2 Newly-formed mediators

Mast cells also newly synthesize mediators in response to IgE-mediated activation, and these include lipid-derived mediators, nitric oxide (NO), and cytokines/chemokines (Metcalfe *et al.*, 1997; Coleman, 2000). Lipid-derived mediators are formed and released from the cell within minutes. These are generally generated from arachidonic acid and consist of prostanoids, leukotrienes, and platelet activating factor (PAF). The major mast cell prostanoid and leukotriene products are PGD_2 and LTC_4, respectively (Ennis *et al.*, 1984; Schwartz and Huff, 1998; Church *et al.*, 1998). PGD_2 blocks human platelet aggregation, and is a chemoattractant for eosinophils (Smith *et al.*, 1974; Monneret *et al.*, 2001). The effects of LTC_4 include bronchoconstriction and induced vascular permeability (Barnes *et al.*, 1984; Soter *et al.*, 1983).

In addition to lipid-derived mediators, there is evidence that mast cells produce NO. Resting PMC have been reported to secrete NO (Bissonnette *et al.*, 1991). However, there is some controversy over whether the observed NO is actually mast cell-derived or released by contaminating cells in the mast cell preparation (Coleman, 2000). It is noteworthy that NO has been documented to inhibit mast cell degranulation (deSchoolmeester *et al.*, 1999).

Mast cell cytokines

Mast cells are a source of a wide array of cytokines and chemokines (Metcalfe *et al.*, 1997). These cytokines include interleukin (IL)-1, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, IL-16, interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and granulocyte macrophage-colony stimulating factor (GM-CSF) (Plaut *et al.*, 1989; Burd *et al.*, 1989; Wodnar-Filipowicz *et al.*, 1989; Selvan *et al.*, 1994). Moreover, mast cells elaborate a number of chemokines, such as RANTES, MIP-1α, MIP-1β, and MIP-3α (Burd *et al.*, 1989; Selvan *et al.*, 1994; personal communication).

A brief description of a few cytokines of interest in our studies is presented below.

TNF-α

The TNF-α gene is situated within the major histocompatibility complex (MHC) locus and mapped to chromosome 6 and 17 in humans and mice, respectively (Rink and Kirchner, 1996; Blank and Varin-Blank, 2000). TNF-α is a pleiotropic protein of 17 kDa that is first synthesized as a transmembrane protein and subsequently cleaved and secreted into the extracellular matrix where it functions as a homotrimer. TNF-α mediates its effects through binding to its specific receptors, TNFR1 and TNFR2 (Papadakis and Targan, 2000). TNFR1 is expressed by many cell types, whereas TNFR2 expression is more limited and found predominantly on immune cells and endothelial cells.

TNF-α is produced early during inflammatory reactions and exerts numerous pro-inflammatory actions (Papadakis and Targan, 2000). TNF-α aids in the recruitment of leukocytes to tissue sites of inflammation through both direct chemotactic effects and by

inducing endothelial cells to express adhesion molecules such as E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule¥1 (VCAM-1) (Rothlein *et al.*, 1988; Walsh *et al.*, 1991). TNF-α also enhances the activity of leukocytes. For instance, TNF-α increases the cytotoxicity of monocytes and eosinophils, and the phagocytic activity of neutrophils (Shalaby *et al.*, 1985; Philip and Epstein, 1986; Silberstein and David, 1986).

Mast cells are recognized as an important cellular source of TNF-α (Okuno *et al*, 1986; Plaut *et al*, 1989; Gordon and Galli, 1990). In addition to *de novo* synthesis of TNF-α, studies revealed that mast cells contained pre-formed TNF-α in their granules (Gordon and Galli, 1990; Gordon and Galli, 1991). These studies identified the presence of pre-formed TNF-α in a panel of mucosal type mast cell lines and in mouse peritoneal mast cells (PMC). Moreover, pre-formed TNF-α was released in parallel with another pre-formed mediator, serotonin, during mast cell degranulation. These findings indicate that mast cells are an immediate source of TNF-α upon activation.

IL-6

IL-6 is produced by many different cell types, the major producers are monocytes/macrophages, lymphocytes, keratinocytes, and endothelial cells (Akira *et al.*, 1990). This cytokine belongs to a family of cytokines that include oncostatin M, leukemia inhibitory factor, and IL-11. The IL-6 gene promoter contains a number of regulatory elements that permit its expression by diverse stimuli including IL-1 and TNF-α (Wolvekamp and Marquet, 1990).

Originally described as a pro-inflammatory cytokine, there is growing evidence that IL-6 exerts important anti-inflammatory actions both *in vivo* and *in vitro* (Tilg *et al.*, 1997). For instance, endotoxemia-induced circulating levels of pro-inflammatory cytokines TNF-α, MIP-2, IFN-γ, and GM-CSF were higher in IL-6 gene knock-out mice than in wild-type littermates (Xing *et al.*, 1998). Moreover, in humans, recombinant IL-6 administration upregulated production of natural antagonists for the pro-inflammatory cytokines, IL-1 and TNF-α (Tilg *et al.*, 1997).

GM-CSF

GM-CSF is a 127-amino acid monomeric protein that serves as a potent growth factor for granulocytes and macrophages, and enhances the activity of neutrophils and eosinophils (Clark and Kamen, 1987; Hill *et al.*, 1995). GM-CSF is also important for the development of dendritic cells. This growth factor is produced by a variety of cell types including T cells, stromal cells, and mast cells (Clark and Kamen, 1987; Wodnar-Filipowicz *et al.*, 1989).

IL-4

IL-4 is the prototypical cytokine of type 2 immune responses (Boulay and Paul, 1992). In addition to promoting the development of type 2 responses, IL-4 is a critical switch factor for IgE production. IL-4 acts on B cells to induce switching of the Ig heavy chain to the IgE isotype, and concomitantly inhibits the production of IgG2a and IgG2b antibodies (Paul, 1991).

1.5 Physiological role of mast cells

Mast cells are believed to participate in the pathogenesis of a number of pathological processes. Some key processes are described below.

1.5.1 Mast cells and asthma

Asthma is a debilitating disease characterized by reversible airways obstruction, airways hyperresponsiveness, and chronic airways inflammation (Galli, 1997). Over the last decade, new emphasis has been placed on the study of airways inflammation because it is becoming evident that the leukocyte infiltration and the resulting remodeling of lung architecture is largely responsible for the chronicity and increasing morbidity of asthma. Airways inflammation is the result of complex interactions occurring among recruited leukocytes, resident tissue cells, and secreted inflammatory mediators (Wasserman, 1994). Studies of allergic asthma have implicated an important role for mast cells in the development of both the acute and chronic phases of the inflammatory response.

Mast cells are strategically positioned in the lung to rapidly respond to inhaled allergens (Bingham and Austen, 2000). Evidence for mast cell involvement is provided by studies examining biopsies obtained from patient's bronchi which revealed changes in mast cell morphology consistent with mast cell degranulation. Total mast cell numbers are not significantly elevated in asthmatic lung with respect to controls (Djukanovic *et al.*, 1990; Bradley *et al.*, 1991). However, increases may be observed at particular respiratory sites which can be detected in sputum and BAL samples of asthmatics (Tomioka *et al.*, 1984; Pin *et al.*, 1992).

In atopic asthmatics, the appropriate allergen will bind and induce cross-linking of

membrane-bound IgE molecules triggering mast cell activation (Kinet, 1999). Activated mast cells release mediators that serve as powerful bronchoconstrictors such as histamine, sulfidopeptide leukotrienes (LTC_4, LTD_4, LTE_4), PAF, and PGD_2 (Williams and Galli, 2000; Bingham and Austen, 2000). Histamine is known to induce some of the hallmarks of asthma including mucus secretion and bronchoconstriction, and also to cause vasodilation and edema formation. The sulfidopeptide leukotrienes are approximately 1000-fold more potent than histamine as mediators of mucus secretion and bronchoconstriction (Drazen, 1998). PAF, a lipid mediator, has been associated with leukocyte recruitment (Okada *et al.*, 1997) and airways hyperresponsiveness (Chung *et al.*, 1986; Longphre *et al.*, 1996).

PGD_2 has recently been established as a crucial mediator in the development of allergic asthma (Matsuoka *et al.*, 2000). Experiments conducted in mice containing a null mutation for the PGD_2 receptor (DP-/- mice) resulted in diminished recruitment of eosinophils and lymphocytes, and downregulated airways hyperreactivity in OVA-challenged mice. Moreover, BAL levels of cytokines important in the pathogenesis of chronic inflammation in allergic settings (IL-4, IL-5, and IL-13) were also reduced in DP/- mice (Matsuoka *et al.*, 2000).

1.5.2 Mast cells and innate immunity

The presence of large numbers of mast cells at common entry points for pathogens (e.g. skin and gastrointestinal tract) lends credence to their assignment as sentinel cells. The role of mast cells in innate immunity is only beginning to be explored. Recently, two research groups published landmark findings in *Nature* investigating the role of mast cells in combating bacterial invasion (Echtenacher *et al.*, 1996; Malaviya *et al.*, 1996). Both studies

employed mouse models of acute bacterial infection, and demonstrated that mast cells were essential for host survival to bacterial peritonitis. Mast cell-derived TNF-α was identified as the critical factor for early recruitment of neutrophils to the site of infection.

These studies convincingly demonstrated that mast cells are required for effective resistance to microbes. What is less clear, though, are the initial interactions between the offending bacteria and mast cells leading to mast cell degranulation and TNF-α release. Mast cell activation is believed to involve a combination of both direct and indirect interactions with the pathogen (Galli *et al.*, 1999). Examples of the former include direct activation via endotoxin and bacterial fimbriae, while activation by components of the complement system has been shown to be an important means of indirect stimulation (Malaviya *et al.*, 1996; Galli *et al.*, 1999; Prodeus *et al.*, 1997). The discovery of Toll-like receptors has added a new dimension to our understanding of innate immunity (Brown, 2001), and will greatly help to elucidate the mechanisms of mast cell activation by different pathogens.

1.5.3 Mast cells and parasitic infection

Mast cell activation and proliferation are characteristic of parasite infections (Ruitenberg and Elgersma, 1976; Mayrhofer, 1979; Woodbury *et al.*, 1984). Infected rodents exhibit increased IgE levels, localized mast cell hyperplasia, and elevated recruitment of eosinophils. Mast cell-derived mediators enter into the systemic circulation during infection (Jarrett and Miller, 1982). Such observations, coupled to the association of mast cells with the expulsion of some parasites, led researchers to speculate that mast cells protected the host from parasites. They speculated that this protective effect was achieved through: 1)

damage to the integrity of the parasite by mast cell mediators (Henderson *et al.*, 1986); 2) mast cell mediator-directed eosinophil recruitment (Jarrett and Miller, 1982); and 3) enhanced peristalsis and mucous secretion in the intestine resulting in a more rapid expulsion of intestinal parasites (Nutman, 1993).

At first glance, one might easily conclude that mast cells are a beneficial presence in host defense against parasites. However, controversy exists regarding the role of mast cells in parasitic infections. Arguments for a beneficial role have largely been obtained from studies using mast-cell deficient mice (Oku *et al.*, 1984; Abe and Nawa, 1988). These mice showed a delayed response time for parasite expulsion which, in certain models, could be restored following reconstitution of the mice with mast cells. For instance, the expulsion of both the nematode *Strongyloides ratti* and the tick, *Haemaphysalis longicornis*, was impaired in mice with reduced numbers of mast cells; this impaired response was restored with multiple IL-3 injections which increased the number of intestinal mast cells, or by reconstitution of W/W^v mast cell-deficient mice with mast cells, respectively (Abe and Nawa, 1988; Reed *et al.*, 1984).

Conversely, many studies have yielded data that appeared to discredit or at least, downplay the role of mast cells in parasite expulsion. An illustrative example is provided by experiments investigating *Nippostrongylus brasiliensis* infection of mast cell-deficient mice (Crowle, 1983). These mice lack the usual manifestation of mast cell hyperplasia and activation, yet are capable of expelling helminthic parasites. Reconstitution of these mice with bone marrow corrected the mast cell defect; however, it did not alter the kinetics of parasite rejection. Moreover, although mast cells are critical for effective expulsion of *H. longicornis* ticks, this does not appear to be a general mechanism for tick expulsion as mast

cells were not required for immunity against another tick species (denHollander and Allen, 1985).

In summary, mast cells react vigorously to the presence of parasites, and mast cell proliferation and mediator release are common responses to parasitic infection (Jarrett and Miller, 1982; Metcalfe *et al.*, 1997). Mast cells appear to promote the expulsion of certain parasites and not others. The precise role of mast cells is unclear and there is ample room for furthering our understanding of mast cell function in parasite function.

1.6 Prostaglandin E_2 synthesis

Prostaglandin E_2 (PGE_2) is a C-20 fatty acid-derived, oxygenated autocoid that belongs to a family of cyclooxygenase products collectively termed prostanoids (Coleman *et al.*, 1994; Narumiya *et al.*, 1999) (Figure 3). Prostanoids consist of the prostaglandins (PGs) and thromboxanes (TXs). Prostaglandins are characterized by a cyclopentane ring and two side chains attached to C-8 and C-12 of the cyclopentane ring. Individual prostaglandins are classified A-J and are distinguished by the pattern of substitution on the ring structure. Thromboxanes have an oxane ring, and consist of only two members (TXA and its stable decomposition product, TXB). Prostanoids are further categorized by the number of double bonds they carry in their side chains. Series 1 prostanoids contain a single trans double bond at C-13; series 2 prostanoids have a cis-5 and trans-13; and series 3 prostanoids carry cis-5, trans-13, and cis-17 double bonds, and are synthesized from γ-homolinolenic acid, arachidonic acid, and timodonic acid, respectively. Arachidonic acid is the most abundant of these precursors and as a result, the series 2 prostanoids predominate in most mammals, including humans (Coleman *et al.*, 1990).

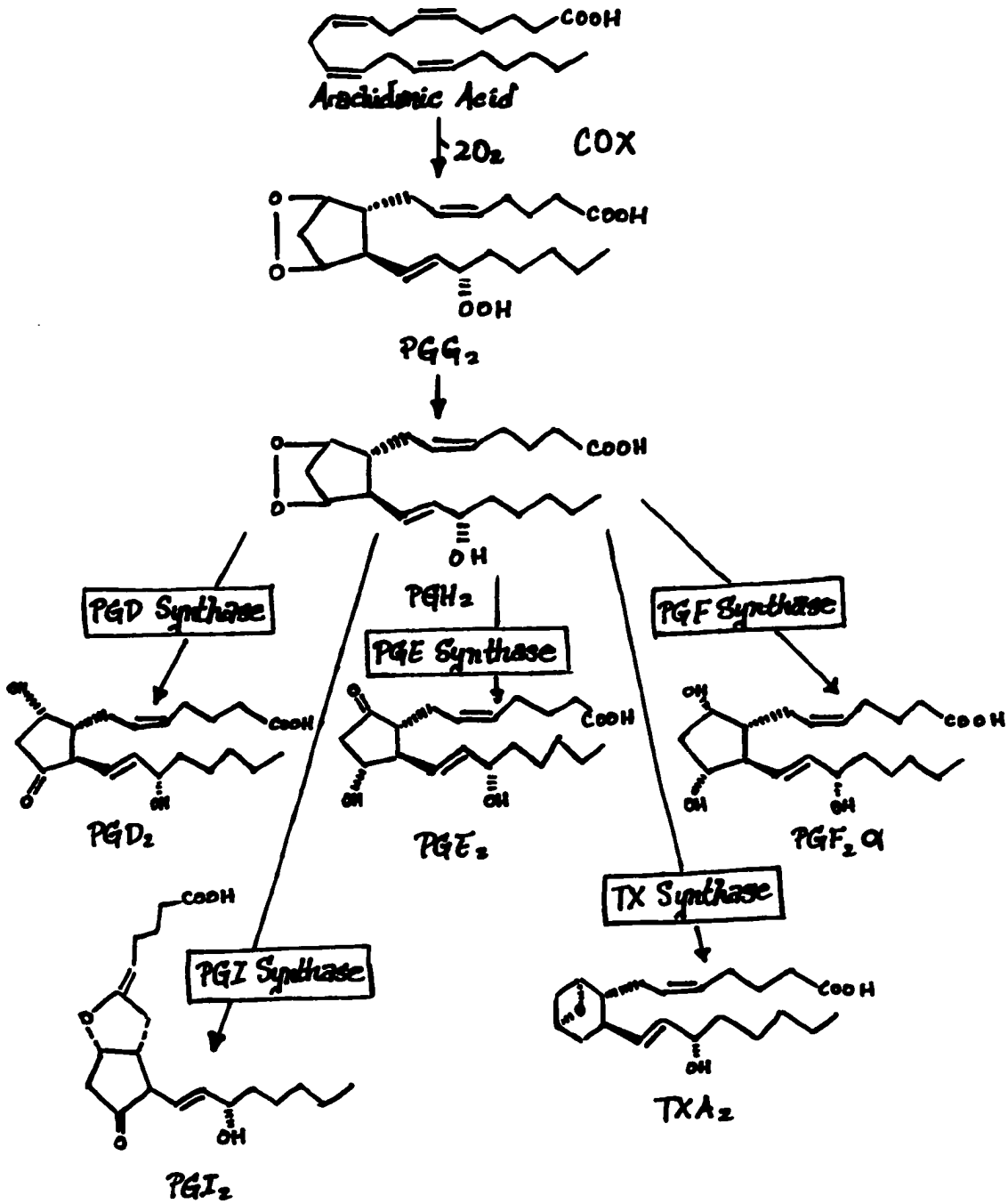

Figure 3. **Pathway for prostanoid synthesis (Narumiya *et al.*, 1999)**

PGE_2 is synthesized *de novo* following membrane perturbation by any of a large number of physiological, mechanical, and pathological stimuli (Narumiya *et al.*, 1999; Phipps *et al.*, 1991). Arachidonic acid is released from membrane phospholipids through the action of phospholipase A_2, and converted into PGE_2 through the actions of cyclooxygenase (COX) and PGE synthase. Newly formed PGE_2 is then released from the cell where it conducts autocrine or paracrine effects.

PGE_2 directs an impressive array of physiological effects (Coleman *et al.*, 1994; Funk, 2001). Among its diverse effects, PGE_2 functions in the cardiovascular system to help regulate vasomoter tone; in the gastrointestinal tract, to mediate peristalsis and maintain mucosal lining integrity; in the kidney, to regulate electrolyte and water transport; and in the hypothalamus to induce fever (Coleman *et al.*, 1994; Narumiya *et al.*, 1999).

1.7 Prostaglandin E receptors

The ability of PGE_2 to mediate such a wide range of effects is explained by the existence of multiple PGE receptors, denoted EP_1-EP_4 (Breyer *et al.*, 2001) (Figure 4). These receptors are seven transmembrane-spanning and G-protein coupled receptors, and each receptor activates particular signaling pathways.

1.7.1 EP_1 receptors

This receptor has been cloned in a number of species including humans and mice where it encodes a 402 and 405 amino acid polypeptide, respectively (Funk *et al.*, 1993;Watabe *et al.*, 1993). Tissue distribution of EP_1 is fairly limited and varies among species (Coleman *et al.*, 1994). For instance, in humans, EP_1 is found expressed in the myometrium. EP_1 is also

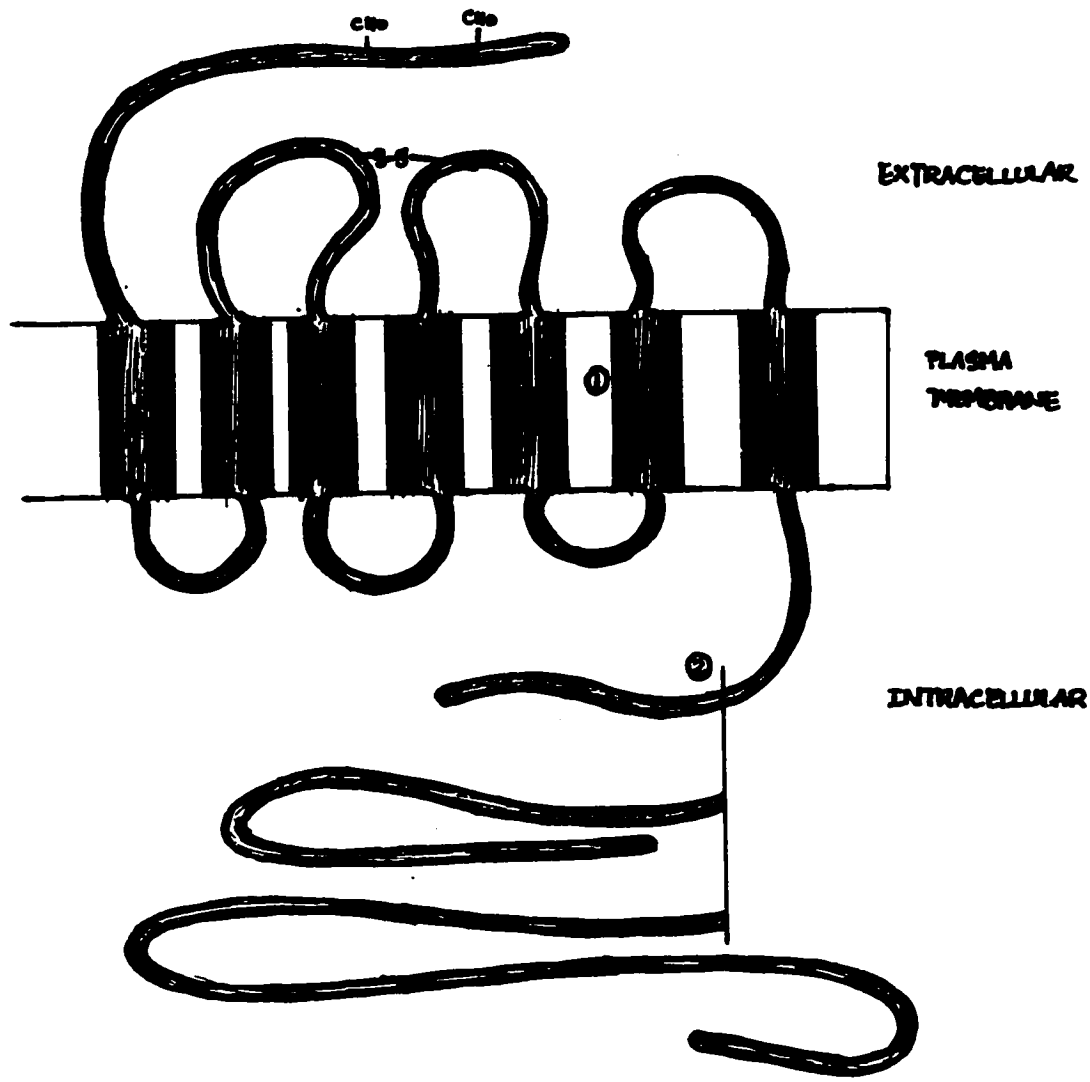

Figure 4. **General structure of prostanoid receptors. Location of splicing events for EP1 and EP3 are marked (1) and (2), respectively (Narumiya *et al.*, 1999)**

expressed in mouse kidney and lung (Watabe *et al.*, 1993). In the rabbit kidney, EP_1 regulates sodium transport whereas in guinea pig trachea, EP_1 mediates smooth muscle contraction (Coleman *et al.*, 1994; Guan *et al.*, 1998).

Functional studies with EP_1 have largely involved the use of selective agonists and antagonists. Among the most selective agonists are 17-phenyl-trinor-ω-PGE_2, iloprost, and sulprostone (Narumiya *et al.*, 1999; Breyer *et al.*, 2001). These agonists possess a similar degree of binding affinity for EP_1 as PGE_2. (Kiriyama *et al.*, 1997). EP_1 antagonists include SC-19220 and AH-6809. Neither antagonist is ideal due to their weak antagonistic activity (Coleman *et al.*, 1994). Moreover, AH-6809 also binds human DP receptors (Breyer *et al.*, 2001) and both SC-19220 and AH-6809 lack binding affinity to mouse EP_1 receptors (Kiriyama *et al.*, 1997).

It is well-recognized that activation of the EP_1 receptor results in elevated intracellular calcium levels (Narumiya *et al.*, 1999; Breyer *et al.*, 2001). Understanding the mechanism responsible for this increase has proved more difficult. It was believed that EP_1 was coupled to Gq G-proteins leading to phosphatidyl inositol hydrolysis and generation of the second messenger, IP3, which in turn, would act to release calcium from intracellular stores and initiate the entry of extracellular calcium (Breyer *et al.*, 2001). However, the increase in IP3 induced by EP_1 activation was often weak, making it unlikely that this mechanism represented the sole means by which calcium was elevated. These observations prompted investigators to suggest that the increase in calcium occurred through a Gq G-protein-independent means (Narumiya *et al.*, 1999). More recently, it was shown in bovine adrenal medullary cells that EP_1 receptors released calcium through a caffeine-sensitive receptor, providing evidence for the existence of alternative pathway(s) leading to increased

intracellular calcium (Shibuya *et al.*, 1999).

1.7.2 EP_2 receptors

The human and mouse EP_2 receptor have both been cloned (Regan *et al.*, 1994; Katsuyama *et al.*, 1995). EP_2 receptors are the least widely expressed of the four EP receptor subtypes (Narumiya *et al.*, 1999). EP_2 mRNA is expressed mainly in the uterus, lung and spleen (Breyer *et al.*, 2001). Very little EP_2 expression is observed in the kidney. Studies in a macrophage cell line reveal IFN-γ-sensitive upregulation of EP_2 expression in response to LPS stimulation (Katsuyama *et al.*, 1998). EP_2 receptors induce increases in intracellular cAMP through coupling of Gs G-proteins (Narumiya *et al.*, 1999). This increase in cAMP mediates relaxation of smooth muscle and inhibition of mast cell mediator release (Coleman *et al.*, 1994).

The presence of EP_2 receptors is best determined using the EP agonist, butaprost (Breyer *et al.*, 2001). The methyl ester form of this agonist is highly specific for EP_2, lacking affinity for the other EP subtypes as well as for any of the other prostaglandin receptors in both the mouse and human systems (Kiriyama *et al.*, 1997; Breyer *et al.*, 2001). Other ligands that are less selective yet possess relatively high affinity for EP_2 are in common use. These agonists include PGE_1, 16, 16-dimethyl-PGE_2, 11-deoxy-PGE_1, and misoprostol (Narumiya *et al.*, 1999; Breyer *et al.*, 2001).

1.7.3 EP_3 receptors

The EP_3 receptors were the first of the EP receptors to be cloned (Sugimoto *et al.*, 1992). They were isolated employing PCR, with primers based on the sequence of the human TXA_2 receptor followed by low-stringency cross-hybridization screening. The human EP_3 receptor consists of 365 amino acid residues (Adam *et al.*, 1994).

EP_3 receptors were long believed to function solely through inhibition of cAMP via receptor coupling of pertussis toxin-sensitive Gi G-alpha proteins. In 1993, Namba *et al.* reported the existence of alternative splicing of the EP_3 receptor in bovine adrenal medulla cells. Four splice variants were detected. Each variant differed only in the carboxyl terminus with the splice junction appearing ten amino acids following the putative seventh transmembrane domain. These variants were called EP_{3A}, EP_{3B}, EP_{3C}, and EP_{3D}. EP_{3A} and EP_{3D} inhibited cAMP production; EP_{3B} and EP_{3C} enhanced intracellular cAMP levels. Moreover, EP_{3D}, and to an extent EP_{3A}, also enhanced calcium levels.

In the mouse, three splice variants for EP_3 have been described. These splice variants were termed: $EP_{3\alpha}$, $EP_{3\beta}$, and $EP_{3\gamma}$ (Coleman *et al.*, 1994). The first two variants couple exclusively to Gi and inhibit cAMP with differing affinities. $EP_{3\gamma}$ is coupled to both Gi and Gs and may either increase or inhibit cAMP production depending on the conditions of activation. In the human, at least eight splice variants exist (Narumiya *et al.*, 1999). At least six of the variants are coupled to Gi and inhibit cAMP accumulation; some of the splice variants have also been documented to increase intracellular calcium (Pierce *et al.*, 1995).

1.7.4 EP_4 receptors

Similar to EP_2 receptors, EP_4 receptors are coupled to Gs and elevate intracellular cAMP (Bastien *et al.*, 1994). EP_4 receptors also bind to many of the EP_2 agonists and this lead to the initial erroneous classification of these receptors as "EP_2". These two receptors are differentiated using butaprost, which selectively binds EP_2 receptors with no affinity for EP_4 receptors, and the EP_4 antagonist, AH23848B.

EP_4 receptors have been cloned in the human, mouse, rat, cow and rabbit (Breyer *et al.*, 2001). It is found expressed in many more tissues than EP_2. Northern blot analysis revealed its expression in the gastrointestinal tract, kidney, spleen, lung, adrenal glands, and thymus. EP_4 receptor activation results in smooth muscle relaxation, and has also been shown to mediate a variety of immunological activities such as B cell differentiation and downregulation of TNF-α production (Fedyk and Phipps, 1996; Yamane *et al.*, 2000).

EP_4 receptors possess a long carboxy terminal tail of 156 amino acid residues, 38 of which are potential phosphorylation sites for protein kinase A (PKA) and C (PKC) (Breyer *et al.*, 2001). Phosphorylation of these site(s) has been suggested to mediate receptor desensitization (Nishigaki *et al.*, 1996). This is in contrast with the EP_2 receptor which has a much shorter tail and is less readily desensitized upon ligand binding.

1.8 Effects of PGE_2 in the immune system

1.8.1 Cellular source of PGE_2

In the context of the immune system, PGE_2 is produced mainly by cells of the monocyte/macrophage lineage (Fedyk *et al.*, 1996). It is released upon activation by such

stimuli as zymosan and endotoxin (Humes *et al.*, 1977), IL-1 and TNF-α, and the act of phagocytosis (Pouliot *et al.*, 1997). Recently, human neutrophils were demonstrated to release significant amounts of PGE_2 within minutes following stimulation with the toxin, pneumolysin (Cockeran *et al.*, 2001), and human eosinophils were shown to elaborate sub-nanomolar levels of PGE_2 in response to PAF activation (Tenor *et al.*, 1996).

In addition, PGE_2 may also be released by resident tissue cells at inflamed tissue sites. Human fibroblasts and synovial cells secrete PGE_2 in response to IL-1 and TNF-α stimulation (Dayer *et al.*, 1985; Dayer *et al.*, 1986). IL-1 also induces PGE_2 production by rheumatoid synovial cells (Dayer *et al.*, 1987). Other studies reported that keratinocytes release PGE_2 when incubated in the presence of *S. mansoni*. Mast cells have also been shown to be a source of PGE_2 under certain conditions (Marshall *et al.*, 1999). Rat serosal mast cells and human lung mast cells constitutively secrete low levels of PGE_2 and these levels are not consistently enhanced by anti-IgE stimulation (Lewis *et al.*, 1982). Both mouse BMMC and rat PMC have also been shown to release PGE_2 following activation with NGF (Marshall *et al*, 1999).

1.8.2 PGE_2 as a downregulator of the immune system

Beginning in the early 1970s, researchers began to examine the effects of PGE_2 on various immune functions. Smith *et al.* (1971) demonstrated that PGE_2 inhibited proliferation of PHA-stimulated human lymphocytes. Shortly thereafter, a number of other inhibitory effects were ascribed to PGE_2, including inhibition of cytolytic activity by murine lymphocytes (Henney *et al.*, 1972), migration inhibitory factor activity on guinea pig macrophages (Koopman *et al.*, 1973), and leukocyte inhibitory factor synthesis by mitogen-

stimulated human lymphocytes (Lomnitzer *et al.*, 1976). Based on these findings, Bourne *et al.* (1974) proposed that the E series prostaglandins may be a physiological mediator of immune responses.

Goodwin *et al.* (1977) made the interesting observation that although the growth of lymphocytes stimulated with phytohemagglutinin (PHA) and Concanavalin A (Con A) was potently suppressed by PGE_2, pokeweed mitogen (PWM)-stimulated lymphocytes were much more resistant to the inhibitory effects of PGE_2. PWM, unlike PHA and Con A, readily activates B lymphocytes in addition to T lymphocytes. Since the lymphocyte fraction being tested consisted of both T and B lymphocytes, the authors conjectured that T lymphocytes represented the responding population and that B lymphocytes were more refractive to growth inhibition by PGE_2.

Later work by Phipps *et al.* (1989) confirmed and extended these observations. A panel of B lymphomas were treated with PGE_2 and the degree of cell growth was assessed after 1-3 days of incubation. The lymphomas exhibited different sensitivities to PGE_2. For instance, growth of one cell line was strongly suppressed by 10^{-8} M PGE_2 while the growth of another was completely resistant to the effects of PGE_2 except at the highest dose employed (10^{-4} M). Several hypotheses were put forth to explain these observations. The two more likely hypotheses proposed that the heterogeneity in responses to PGE_2 reflected: 1) the stage of B lymphocyte maturity represented by a given lymphoma; and 2) the expression profile of PGE_2 receptors on the lymphoma. Regarding the first hypothesis, it is noteworthy that the resistant lymphoma possessed a more mature lymphocyte phenotype, whereas the lymphoma that was highly sensitive to growth inhibition was of a more immature phenotype (Phipps *et al.*, 1989).

Prostaglandins of the E series were also shown to downregulate class II MHC expression by murine peritoneal macrophages (Snyder *et al.*, 1982). PGE_2 blocked biosynthesis of class II MHC molecules at concentrations as low as 10^{-9} M. This effect was specific for class II MHC expression since surface levels of H-2K and Fc receptors were not altered by the same doses of PGE_2. In these experiments, the macrophages were cultured for up to six days following isolation from the peritoneum, and were incubated with various doses of PGE_2 during the last two days of culture. Timing of PGE_2 addition proved to be important. When PGE_2 was added only for the initial two days of the culture period, expression of class II MHC molecules was delayed, yet rapidly appeared following removal of PGE_2. Moreover, the maximal levels of class II MHC expression achieved remained unchanged relative to controls. Since class II MHC molecules allow macrophages to serve as APC to T lymphocytes, downregulation of expression of these surface proteins would be expected to translate into reduced APC activity by macrophages. Indeed, the authors found that pretreatment with PGE_2 caused macrophages to be less efficient at antigen presentation.

It soon became apparent that PGE_2 exerted additional effects on the immune system, namely, on the regulation of cytokine production. Kunkel *et al.* (1986b) were the first to report such a function for PGE_2. They demonstrated that PGE_2 suppressed the production of IL-1 and fibroblast growth factor by LPS-activated macrophages. Since LPS and IL-1 stimulation induces PGE_2 production by mononuclear phagocytes (Humes *et al.*, 1982; Kurland and Bockman, 1978; Dinarello *et al.*, 1983), the authors (Kunkel *et al.*, 1986b) suggested that PGE_2 participated in an autoregulatory, negative feedback loop whereby the magnitude of the LPS-induced IL-1 response was controlled by PGE_2. An immunosuppressive role for PGE_2 was similarly described for IL-2 and TNF-α production

(Walker *et al.*, 1983; Kunkel *et al.*, 1986a).

1.8.3 PGE_2 is more than just an immunosuppressive agent

Up until this point, PGE_2 had been largely regarded as an immunosuppressive agent whose main function was to dampen ongoing immune responses. This view changed dramatically in the early 1990s with the advent of publications that drew attention to the differential effects of PGE_2 on Th1 and Th2 T lymphocyte function. The seminal study by Betz and Fox (1991) showed that PGE_2 downregulates type 1 cytokines (IL-2 and IFN-γ) and either upregulates or is without effect on type 2 cytokines (IL-4, IL-5). Again, as was demonstrated with many of the above effects of PGE_2, cAMP was implicated in these actions. The cAMP elevating agent, forskolin, also suppressed IL-2 production while slightly increasing IL-5 and having no effect on IL-2 production. To rule against the possibility that PGE_2 did not activate Th2 cells, intracellular cAMP was measured in these cells following PGE_2 stimulation. PGE_2 induced an almost identical enhancement of cAMP in Th2 cells as in Th1 cells. This observation coupled with the observation that forskolin reproduces the effects of PGE_2, suggested that PGE_2's divergent modulation of type 1 and 2 cytokines was not due to a lack of responsiveness of Th2 cells to PGE_2.

The findings of Betz and Fox (1991) were significant for two reasons: 1) they demonstrated that PGE_2 is not solely immunosuppressive towards T lymphocyte function; and 2) they revealed a differential regulation by PGE_2 of type 1 and 2 cytokines. It should be mentioned, however, that this study was not the first to report an enhancing effect of PGE_2. This title belongs to Quill *et al.* (1989) who demonstrated that in certain murine Th clones, PGE_2 could act in combination with IL-2 to increase GM-CSF production.

Interestingly, PGE_2 suppressed GM-CSF production induced by specific antigen or anti-CD3 antibody. cAMP-increasing agents, forskolin and cholera toxin, similarly enhanced IL-2-directed GM-CSF production in PGE_2-responsive Th clones while inhibiting proliferation, implicating a central role for cAMP in mediating the effects of PGE_2 on Th cells (Quill *et al.*, 1989).

The elucidation of divergent effects of PGE_2 on type 1 and 2 cytokine production in murine Th clones and short term T cell lines was quickly followed up in the human system. Snijdewint *et al.* (1993) conducted comparable experiments using human peripheral blood lymphocytes (PBL) and CD4+ T cell clones. Cytokine production by PBL and the T cell clones following mitogenic activation was similarly skewed towards the type 2 phenotype. Production of type 1 cytokines, IL-2 and IFN-γ, was suppressed whereas that of type 2 cytokines, IL-4 and IL-5, was unaffected or enhanced when low PGE_2 concentrations were employed. However, PGE_2 at higher concentrations (i.e., greater than or equal to 10^{-6} M) inhibited IL-4 production by a number of human Th0 and Th2 cell lines. IL-4 production by human PBL was even more sensitive to such inhibitory effects (Snijdewint *et al.*, 1993).

1.8.4 PGE_2 as a promoter of type 2 responses

The landmark publications by Betz and Fox (1991) and Snijdewint *et al* (1993) caused a paradigm shift in how researchers viewed the role of PGE_2 in immune responses. No longer was PGE_2 viewed simply as an immunosuppressive agent. An appropriately more complex view replaced this simplistic image, one incorporating the differential effects of PGE_2 on type 1 and 2 cytokine production.

Since then, a number of subsequent studies have corroborated the view of PGE_2 as a promoter of type 2 immune responses. For instance, PGE_2 was shown to be powerful inhibitor of IL-12 production (van der Pouw Kraan *et al.*, 1995). Levels of this potent type 1 cytokine in LPS-activated human whole blood cultures were suppressed by PGE_2 in a dose dependent manner, first apparent at concentrations of PGE_2 as low as 10^{-10} M. PGE_2 also downregulated IL-6 production, albeit to a lower extent than IL-12 production. Conversely, PGE_2 enhanced IL-10 production. IL-10 similarly suppresses IL-6 and IL-12 production raising the possibility that PGE_2 was mediating its effects through endogenous IL-10 synthesis. The addition of neutralizing antibodies to block IL-10 activity abrogated PGE_2-directed IL-6 suppression, yet failed to reverse IL-12 inhibition. Therefore, PGE_2 downregulation of IL-6 production was mediated by IL-10 whereas IL-12 inhibition occurred through an IL-10 independent manner (van der Pouw Kraan *et al.*, 1995). PGE_2 has also been demonstrated to inhibit human peripheral blood mononuclear cell responsiveness to IL-12 at least in part through a downregulation of IL-12 receptor expression (Wu *et al.*, 1998).

In what appeared to be at odds with an inhibitory role for PGE_2 in IL-12 production, Rieser *et al.* (1997) reported that PGE_2 acted in concert with TNF-α to synergistically enhance IL-12 production. Kalinski *et al.* (2001) examined this discrepancy and confirmed these results by showing that a combination of PGE_2 and TNF-α increased expression by immature DC of the IL-12 p40 subunit. However, there was no corresponding increase in bioactive IL-12 (IL-12p70) which consists of the p40 subunit as well as a p35 subunit (Gately *et al.*, 1998). IL-12 p40 has been documented to assemble into homodimers which are subsequently released by APC (Gately *et al.*, 1998). These homodimers, and to a degree

the IL-12 p40 monomer, function as IL-12 receptor antagonists (Mattner *et al.*, 1993; Ling *et al.*, 1995; Gillessen *et al.*, 1995) and have been documented to attenuate IL-12-mediated immune responses (Gately *et al.*, 1996; Kato *et al.*, 1996; Heinzel *et al.*, 1997: Chen *et al.*, 1997; Mattner *et al.*, 1997; Schmidt *et al.*, 1998). Hence, PGE_2-directed potentiation of IL-12p40, by inhibiting IL-12 function, might actually serve to further promote type 2 responses (Kalinski *et al.*, 2001).

PGE_2 acts, in combination with LPS, to potentiate IL-4-mediated IgE production by enhancing isotype class switching to the epsilon heavy chain locus (Fedyk *et al.*, 1996). PGE_2 enhances both antigen-specific and non-specific IgE production. These effects are reproduced by the cAMP analogue, dibutryl cAMP, and the cAMP-elevating agent, forskolin. Therefore, PGE_2 may act to raise serum IgE levels in two ways: directly by acting on the isotype class switch mechanism, and indirectly by promoting production of IL-4, a cytokine required for IgE induction. Enhanced IgE levels would be expected to maintain, and perhaps exacerbate, allergic inflammation.

1.8.5 PGE_2 as a potential type 2-driving factor

The role of PGE_2 in promoting the development of type 2 responses was recently taken one step further by a series of seminal papers indicating that PGE_2 might act as a tissue-derived signal that induces a type 2 primary immune response. Initiation of primary immune responses depends on antigen-specific activation of naïve Th cells (ThN cells) by dendritic cells (Cella *et al.*, 1997). Immature DC, located in peripheral tissue sites, pick up antigen from their surroundings, process the antigen and present it to naïve T cells in the context of class II MHC. It had previously been observed that DC are capable of inducing both type 1

and 2 primary immune responses (Kapsenberg *et al.*, 1999). The polarizing actions of human DC were initially postulated to be a function of distinct DC lineages, a proposal hotly contested by some due to discrepancies with findings from mouse models and the inflexibility implicit in such a mechanism (Vieira *et al.*, 2000). Evidence is now emerging to suggest an alternative explanation. Specifically, immature DC may polarize the immune response towards either phenotype, the outcome depending on stimuli encountered in the periphery, at the site of initial DC activation (Vieira *et al.*, 2000).

Encouraged by the growing body of data indicating that PGE_2 promoted type 2 immune responses, Kalinski *et al.* (1997) examined how PGE_2 influenced the APC properties of developing human DC. DC were generated from monocytes, isolated from peripheral blood by incubating for six days with GM-CSF and IL-4 in the presence (PGE_2-DC) or absence (control-DC) of varying doses of PGE_2. PGE_2 treatment did not affect DC morphology or the expression of a number of surface molecules (e.g. CD80 and CD87); however, a downregulation of CD1a and concurrent upregulation of CD14 expression was observed. The most striking findings were revealed when control-DC and PGE_2-DC were compared in terms of their cytokine-secreting profile and ThN-stimulating activity. Control-DC secreted significant levels of IL-12 and very little IL-10, and promoted the induction of Th cells with a Th0-like phenotype. In sharp contrast, PGE_2-DC released abundant amounts of IL-10 and no detectable IL-12. Complete attenuation of IL-12 production was seen in DC generated in the presence of PGE_2 at levels as low as 10^{-8} M. This cytokine profile was still intact 48 h following PGE_2 withdrawal. The significance of the altered cytokine production became apparent upon examination of the ability of PGE_2-DC to present antigen to naïve T cells. Stimulation by PGE_2-DC, not control-DC, induced

ThN cells to develop into Th2 cells that secreted high levels of IL-4 and IL-5 and reduced amounts of IFN-γ (Kalinski *et al.*, 1997).

Similar data were obtained when DC were only exposed to PGE_2 during a 2 day-final maturation step as opposed to being present from the start of the culture (Kalinski *et al.*, 1998; Vieira *et al.*, 2000). For instance, DC matured in the presence of IL-1β, TNF-α and PGE_2 similarly induced ThN cells to develop into Th2-like cells (Kalinski *et al.*, 1998). PGE_2 alone did not enhance DC maturation yet potentiated such effects by IL-1β and TNF-α. Interestingly, although immature DC were sensitive to the IL-12-downregulating effects of PGE_2, fully mature DC were resistant to PGE_2-mediated IL-12 downregulation (Kalinski *et al.*, 1998). These findings are relevant because they suggest that DC are sensitive to the modulating effects of PGE_2 in the local inflammatory milieu and that this sensitivity is lost following DC migration to draining lymph nodes and final maturation. Moreover, since PGE_2-mediated changes in DC cytokine production are long-lasting (Kalinski *et al.*, 1997; Kalinski *et al.*, 1998), they ensure that IL-12 production will continue to be inhibited in DC recruited to the lymph nodes (Kalinski *et al.*, 1998).

It is thought that tissue signals might exist that serve to inform the immune system of the nature of a local inflammatory response so that an appropriate immune response might be launched (Matzinger, 1994). PGE_2 has been proposed to deliver such a signal to naive Th cells and polarize their development towards the Th2 phenotype (Kalinski *et al.*, 1998; Vieira *et al.*, 2000). It is worth noting here that a reciprocal signal polarizing towards a type 1 immune response has recently been identified (i.e. IFN-γ) (Vieira *et al.*, 2000).

1.8.6 Expression of EP receptors by immune cells

The expression of EP receptor subtypes by immune cells is becoming elucidated. Macrophages, neutrophils, and lymphocytes have been reported to express the various EP receptor subtypes (Fedyk and Phipps, 1996; Zeng *et al.*, 1998; Katsuyama *et al.*, 1998; Yamane *et al.*, 2000). These findings will be discussed in more detail in the Discussion section. For now, it suffices to mention that in each of these cell types, PGE_2 directed its effects through the EP_2 and EP_4 receptors.

1.9 Effects of PGE_2 on mast cells

1.9.1 PGE_2 promotes mast cell induction

PGE_2 has been demonstrated to be critical for mast cell induction (Hu *et al.*, 1995; Saito *et al.*, 1996). Hu *et al.* (1995) discovered that mast cell induction from murine splenocytes required the presence of contaminating LPS. Further investigation revealed that LPS promoted mast cell growth through endogenous PGE_2 production. PGE_1 and PGE_2 both triggered mast cell development in a dose-dependent manner with maximal effects occurring at 10 ng/ml. Addition of the prostaglandin synthesis inhibitor, indomethacin, to cell cultures attenuated the LPS-directed mast cell induction (Hu *et al.*, 1995). Moreover, the inhibitory effects of indomethacin were overridden by exogenously added PGE_2. The prostanoid appeared to act on mast cell precursors as opposed to immature mast cells, since mast cell induction was observed only when PGE_2 was added within the first two days of culturing. The effects of PGE_2 were mimicked by agents that elevated intracellular cAMP such as cholera toxin and dibutyryl cAMP. Based on these data, the authors suggested that

PGE_2 may serve as a commitment factor or a late-acting, lineage-specific factor for mast cells (Hu *et al.* 1995).

A similar function for PGE_2 was reported for the induction of human mast cells from CBMC (Saito *et al.*, 1996). PGE_2, acting in concert with SCF and IL-6, induced mast cell development and also increased cellular concentrations of mast cell granule-associated mediators, i.e., histamine and tryptase. PGE_2 was speculated to mediate its effects through the inhibition of endogenous GM-CSF production. GM-CSF, released by accessory cells in the culture, enhances the development of cells belonging to the granulocyte/macrophage lineage. Several lines of evidence supported this hypothesis including the observations that blocking antibodies to GM-CSF enhanced the degree of mast cell induction in SCF- and IL-6-supplemented CBMC cultures, and that PGE_2 inhibited CBMC-derived GM-CSF production (Saito *et al.*, 1996). Moreover, freshly isolated CBMC spontaneously secrete GM-CSF whereas purified CD34+ cells (upon which PGE_2 lacks any mast cell-inducing properties) do not secrete any detectable GM-CSF during the first four days of culture. Recall that, in the murine system, PGE_2 was only effective in inducing mast cells from murine spleen cells when it was added within the first two days of culturing. If PGE_2 functions by keeping GM-CSF levels to a minimum and thus preventing overpopulation by the granulocyte-macrophage lineage during the initial culturing period, then the absence of GM-CSF in early cultures of purified CD34+ cells is in keeping with the failure of PGE_2 to induce mast cells from this population. It would be of interest to determine the amounts, if any, of GM-CSF produced by freshly isolated murine spleen cells. Production of GM-CSF by these cells would suggest the involvement of a similar mechanism in the rodent system.

1.9.2 PGE_2 modulates mast cell degranulation

Perhaps the best-studied effects of PGEs on mast cell function are with regards to their modulation of mast cell degranulation. The predominant sentiment is that PGE_2, via increases in intracellular cAMP, inhibits mast cell degranulation. One of the earliest studies to demonstrate this inhibitory effect was that by Kaliner and Austen (1974). Pretreatment of rat CTMC with PGE_1 (10^{-6} M) inhibited $Fc_\varepsilon RI$-mediated histamine release by approximately 20%, and the degree of inhibition was increased to 70% in the additional presence of the phosphodiesterase inhibitor, aminophylline. A positive correlation was observed between the inhibition of degranulation and net increases in intracellular cAMP. In 1988, Peachell *et al.* showed that the inhibitory effects of PGE_2 were not restricted to rodent mast cells; PGE_2 also inhibited histamine release from human lung mast cells, with parallel increases in intracellular cAMP. Moreover, PGE_2 suppressed degranulation by human basophils and induced a transient increase in cAMP in these cells (Peachell *et al.*, 1988).

In contrast to the above inhibitory role assigned to PGE_2, our laboratory has previously reported a stimulatory effect on mast cell degranulation (Leal-Berumen *et al.*, 1995). Leal-Berumen *et al.* (1995) showed that PGE_2 enhanced degranulation in anti-IgE-activated rat PMCs by approximately 15 %, an effect that was modest yet statistically significant. In another study, Nishigaki *et al.* (1993) reported that PGE_2 potentiated ionomycin-induced histamine release in a cultured murine mast cell line, BNu-2cl3, providing another instance of PGE_2 enhancing mast cell degranulation.

1.9.3 Effects of PGE_2 on mast cell cytokine production

PGE_2 has been shown to modulate mast cell cytokine production which is in line with its ability to affect cytokine production by other immune cells. What is surprising though is the relative paucity of literature in this area of research. Nevertheless, cytokine data acquired in the mast cell is in good agreement with the known effects of PGE_2 on cytokine regulation in other leukocytes and tissue cells. In our laboratory, PGE_1 and PGE_2, at the examined concentration of 10^{-7} M, potently suppress basal TNF-α production by rat PMCs (Leal-Berumen *et al.*, 1995). Hogaboam *et al.* (1993) similarly observed PGE_2-mediated inhibition of TNF-α bioactivity for rat PMC as judged by the WEHI-164 cytotoxicity assay.

In contrast, PGE_1 and PGE_2 dose-dependently stimulated PMC IL-6 production, reaching statistical significance at concentrations of 10^{-7} M (Leal-Berumen *et al.*, 1995). The PGEs also enhanced IL-6 production induced by anti-IgE and LPS (Leal-Berumen *et al.*, 1995). However, differences were apparent in the response of PGE_1 and PGE_2 to these two mast cell stimuli. Enhancement of anti-IgE-mediated IL-6 production by PGE_1 and PGE_2 occurred in an additive manner whereas LPS-stimulated IL-6 production was synergistically increased. The effects of PGE_1 and PGE_2 on mast cell IL-6 production, and on TNF-α production, were mimicked by cAMP-enhancing agents, cholera toxin and forskolin (Leal-Berumen *et al.*, 1996), implicating a role for cAMP in mediating these effects.

1.10 Effects of PGE_2 on asthma

The known bronchodilatory and immunomodulatory actions of PGE_2 make this prostanoid an appealing candidate for the clinician's ever-expanding arsenal of anti-asthma drugs. Interest in administering PGE_2 to prevent or ameliorate allergen-induced bronchoconstriction has led to a number of clinical trials and encouraging data in support of a protective role for PGE_2 (Pavord *et al.*, 1991; Pavord *et al.*, 1993; Melillo *et al.*, 1994; Sestini *et al.*, 1996; Gauvreau *et al.*, 1999; Hartert *et al.*, 2000). Investigators have shown that PGE_2 protects against bronchoconstriction and bronchial hyperresponsiveness induced by a wide array of stimuli including aspirin (Sestini *et al.*, 1996), allergen (Pavord *et al.*, 1993; Gauvreau *et al.*, 1999), sodium metabisulphite (Pavord *et al.*, 1991), and exercise (Melillo *et al.*, 1994). PGE_2 aerosols were well-tolerated by the test subjects in all the studies; common side effects were transient cough, retrosternal soreness, and mild airway secretion (Walters and Davies, 1982; Pavord *et al.*, 1991; Pavord *et al.*, 1993; Gauvreau *et al.*, 1999) and in one case, a short-lasting headache (Melillo *et al.*, 1994).

Of particular interest was the observation that PGE_2 ablated both the early and late responses induced by specific allergen (Pavord *et al.*, 1993). It has been suggested that PGE_2 inhibits the early response through transient bronchodilatory effects while anti-inflammatory effects were speculated to be responsible for the attenuation of the late response (Gauvreau *et al.*, 1999). The clinical significance of the bronchodilatory actions of PGE_2, however, is questionable for several reasons. First, the bronchodilatory effect is modest and transient, peaking at 25 min and subsiding within an hour (Pavord *et al.*, 1993). Second, the ability of PGE_2 to induce bronchodilation is not consistently observed, both in *in vitro* and *in vivo* settings (Gardiner and Collier, 1980; Mathe and Hedqvist, 1975). Third,

PGE_2 is without effect on directly-acting bronchoconstrictors such as histamine (Haye-Legrand *et al.*, 1986) and methacholine (Pavord *et al.*, 1991). An alternative mechanism has been proposed where PGE_2-induced attenuation of the early response is the result of a suppressive effect on mast cell mediator release (Pavord *et al.*,1993).

Recent evidence suggests that the bronchoprotective effects of PGE_2 might also be mediated, in part, through its inhibitory actions on leukotriene synthesis (Sestini *et al.*, 1996). Sulfidopeptide leukotrienes (LTC_4/LTD_4/LTE_4) levels are significantly elevated in the sputum of asthmatics (Pavord *et al.*, 1999); furthermore, sputum levels of sulfidopeptide leukotrienes were shown to increase during the first 24 h post-allergen inhalation (MacFarlane *et al.*, 2000). The putative involvement of leukotrienes in the etiology of asthma is underscored by the suppression of allergic bronchoconstriction by leukotriene receptor antagonists (Roquet *et al.*, 1997). Consequently, finding a means to reduce sulfidopeptide leukotriene levels in the lung is desirable. Sestini and colleagues (1996) showed that leukotriene production was inhibited by PGE_2 inhalation in aspirin-sensitive asthmatics challenged with lysine acetylsalicylate. The authors suggested that, in aspirin-induced asthma, the ability of PGE_2 to inhibit leukotriene production might be a key factor in explaining the bronchoprotective effects of PGE_2. Changes in sulfidopeptide leukotriene levels were monitored using urinary LTE_4 as a marker of leukotriene synthesis. Very little of the lung-associated leukotrienes are believed to make it into the urine. One study reported that a paltry 4-9 % of inhaled LTC_4 is eventually detected as urinary leukotriene (Dworski and Sheller,1998). It would, therefore, be of interest to repeat the above study measuring leukotriene levels, not in the urine, but in induced sputum. Sputum induction has been shown to be a non-invasive, reproducible, and highly sensitive method of

measuring eicosanoid concentrations in asthmatics (Pavord *et al.*, 1999; MacFarlane *et al.*, 2000).

PGE_2 has also been demonstrated to inhibit allergen-induced PGD_2 release (Hartert *et al.*, 2000). Subjects with mild atopic asthma were treated with PGE_2 or placebo prior to challenge with specific allergen. Those individuals receiving placebo showed increases in PGD_2 and other inflammatory mediators following allergen provocation. The cellular source of PGD_2 was believed to be the mast cell, and levels of this prostanoid were significantly suppressed by PGE_2 treatment. Curiously, PGE_2 was without effect on the levels of two other mast cell mediators: histamine and tryptase. The authors also investigated the modulatory effects of PGE_2 on the airway cellular response to allergen challenge. Total cell numbers in BAL fluids were not significantly changed by PGE_2 inhalation nor were the number of neutrophils affected. In contrast, a significant drop in eosinophil numbers was observed. PGE_2-induced inhibition of eosinophil accumulation was proposed to lead to the attenuation of the late allergic response (Hartert *et al.*, 2000). Taking into account the pivotal role of PGD_2 in eosinophil recruitment and airways hyperreactivity as described earlier (Matsuoka *et al.*, 2000), one could take this hypothesis one step further and suggest that PGE_2 inhibits asthmatic responses through its inhibition of mast cell-derived PGD_2 release which in turn suppresses eosinophil recruitment and minimizes airways damage following allergen provocation.

In a related study, Gauvreau *et al.*, (1999) asked whether the protective function of PGE_2 in the asthmatic lung was the outcome, at least in part, of a dampening effect on existing airway inflammation. To this end, they conducted a double-blind study to investigate the effects of PGE_2 on 1) the nature of the airway cellular infiltrate; and 2) the

degree of airway hyperresponsiveness to a non-specific stimulus (methacholine). Subjects with mild atopic asthma (n=8) inhaled PGE_2 or placebo prior to challenge with specific allergen, and sputum samples were collected at baseline, 7, and 24 h post-challenge. Sputum analysis showed that inhaled allergen induced an elevation of eosinophils, including activated eosinophils, and an increase in metachromatic cells (mast cells/basophils). These increases were all significantly suppressed by PGE_2 pretreatment. The inhibitory effects of PGE_2 were selective because an allergen-induced increase in neutrophils was not affected by PGE_2. These data corroborate nicely with the PGE_2-induced decrease in eosinophil numbers in BAL samples described above (Hartert *et al.*, 2000). In terms of airway hyperresponsiveness, inhaled allergen triggered hyperresponsiveness to later methacholine challenge, a phenomenon that was blocked by PGE_2 treatment. These data indicated that PGE_2 pretreatment effectively inhibits allergen-mediated airway hyperresponsiveness and inflammation.

In conclusion, these data show that PGE_2 targets multiple processes in the allergic lung to induce its bronchoprotective effects. Among the most important effects of PGE_2, appears to be the inhibition of mast cell-derived mediator release, particularly PGD_2. Considering the evolving view of asthma as a disease of chronic airways inflammation, these findings are exciting as they suggest that PGE_2 might modulate both the acute and chronic aspects of asthma.

1.11 Objectives

The importance of PGE_2 in shaping the immune response, particularly in shifting the response towards the type 2 paradigm, led us to question whether PGE_2 similarly modulated the cytokine profile of mast cells. Mast cells are key players in mediating the development of allergic responses (type 2 response), and, as such, would be an attractive target for PGE_2. We hypothesized that PGE_2 acted on mast cells to favor production of type 2 cytokines, while inhibiting type 1 cytokine production. In this way, mast cells would contribute to the chronicity of inflammation observed in many allergic disorders.

The preceding sections provide the reader with sufficient background to understand the results presented in the following sections. The results are presented in the form of two manuscripts followed by a general discussion section which ties in the findings from both manuscripts. The first manuscript entitled "PGE_2 selectively enhances the IgE-mediated production of IL-6 by mast cells through an EP_1/EP_3-dependent mechanism", was accepted by the Journal of Immunology (December 2000). It examines the effects of PGE_2 on the production of a number of mast cell cytokines with special emphasis on IL-4, IL-6, and GM-CSF production. The second manuscript, entitled "PGE_2 does not suppress $Fc\varepsilon RI$ cross-linking-induced TNF-α production by mast cells", will be submitted to the Journal of Immunology in the near future. This study focused on the surprising observation that PGE_2 enhances, rather than inhibits, TNF-α production by IgE/Ag-activated mast cells. The mechanisms behind this phenomenon were investigated.

Chapter 2: PGE_2 selectively enhances the IgE-mediated production of IL-6 by mast cells through an EP_1/EP_3-dependent mechanism

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Abstract

Prostaglandin E_2 (PGE_2) is an endogenously synthesized inflammatory mediator that is over-produced in chronic inflammatory disorders such as allergic asthma. In the present study, we investigated the regulatory effects of PGE_2 on mast cell degranulation and the production of cytokines relevant to allergic disease. Murine bone marrow-derived mast cells (BMMC) were treated with PGE_2 alone or in the context of IgE-mediated activation. PGE_2 treatment alone specifically enhanced IL-6 production, and neither induced nor inhibited degranulation and the release of other mast cell cytokines including IL-4, IL-10, IFN-γ, and granulocyte-macrophage colony stimulating factor (GM-CSF). IgE/Antigen-mediated activation of BMMC induced the secretion of IL-4, IL-6, and GM-CSF, and concurrent PGE_2 stimulation synergistically increased mast cell degranulation and IL-6 and GM-CSF, but not IL-4, production. A similar potentiation of degranulation and IL-6 production by PGE_2, in the context of IgE-directed activation, was observed in the well-established IL-3-dependent murine mast cell line, MC/9. RT-PCR analysis of unstimulated MC/9 cells revealed the expression of EP_1, EP_3, and EP_4 prostaglandin E receptor subtypes, including a novel splice variant of the EP_1 receptor. Pharmacological studies using prostaglandin E receptor subtype-selective analogs showed that the potentiation of IgE/Ag-induced degranulation and IL-6 production by PGE_2 is mediated through EP_1 and/or EP_3 receptors. Our results suggest that PGE_2 may profoundly alter the nature of the mast cell degranulation and cytokine responses at sites of allergic inflammation through an EP_1/EP_3-dependent mechanism.

Introduction

Prostaglandin E_2 (PGE_2), an arachidonic acid metabolite, is synthesized and secreted by diverse cell types in response to many physiologic and non-physiologic stimuli, and is increasingly becoming recognized as a potent regulator of immune responses (Fedyk *et al.*, 1996a). PGE_2 differentially modulates type 1- and type 2-associated cytokine production (Fedyk *et al.*, 1996a; Betz and Fox, 1991); strongly inhibiting the production of the type 1 cytokines IL-2 (Tilden and Balch, 1982), IL-12 (van der Pouw Kraan *et al.*, 1995), and IFN-γ (Halser *et al.*, 1983), and depending on the stimulation conditions, either having no effect or enhancing production of the type 2-associated cytokines such as IL-4 and IL-5 (Betz and Fox, 1991; Snijdewint *et al.*, 1993). The general consensus is that PGE_2 acts to shift the immune response towards a type 2 cytokine profile. Moreover, this lipid mediator also upregulates IgE production (Fedyk *et al.*, 1996a), and may consequently support the development of asthma and other type 2 cytokine-associated inflammatory disorders. There is, however, evidence for a bronchoprotective role for PGE_2 in asthma (Melillo *et al.*, 1994; Pavord *et al.*, 1993; Pavord *et al.*, 1991; Szczeklik *et al.*, 1996).

Mast cells are critical effector cells of hypersensitivity reactions and allergy. Their expression of cell surface receptors for PGE_2 (Nishigaki *et al.*, 1995; Nishigaki *et al.*, 1993; Chan and Lau, 1998) combined with their close proximity to PGE_2-secreting cells such as fibroblasts (Newcombe and Ishikawa, 1976) and macrophages (Sahu and Lynn, 1977), make mast cells potential targets for immunoregulation by PGE_2. PGE_2 has been reported to be important for mast cell development from murine spleen cell precursors (Hu *et al.*, 1995) and human umbilical cord blood mononuclear cells (Saito *et al.*, 1996). In addition, PGE_2

enhances IL-6 production by rat peritoneal mast cells (PMC) (Leal-Berumen *et al.*, 1995) and potently inhibits TNF-α production by these cells (Leal-Berumen *et al.*, 1995; Hogaboam *et al.*, 1993) and intestinal mucosal mast cells (Hogaboam *et al.*, 1993). Depending on the mast cell population and timing of prostanoid treatment, PGE_2 has been documented to either block the release of histamine and other inflammatory mediators from immunologically activated mast cells (Hogaboam *et al.*, 1993; Kaliner and Austen, 1974; Peachell *et al.*, 1988) or to potentiate such release (Nishigaki *et al.*, 1993; Leal-Berumen *et al.*, 1995).

PGE_2 mediates many of its effects by binding to a specific group of seven-transmembrane domain, G protein-coupled receptors, of which there are four subtypes, designated EP_1, EP_2, EP_3, and EP_4 (Coleman *et al.*, 1994). EP_2 and EP_4 receptors activate adenylate cyclase and lead to increased levels in intracellular cAMP. Activation of EP_1 receptors is associated with increases in intracellular Ca^{2+}, and EP_3 generally couples to G_i and inhibits intracellular cAMP levels. To date, little work has been performed to characterize EP receptor expression on mast cells. The presence of EP_3 and EP_4 receptors has been reported for the murine mucosal type mast cell lines, BNu-2c13 (Nishigaki *et al.*, 1993) and P815 (Nishigaki *et al.*, 1995), respectively. More recently, rat PMCs were demonstrated to carry PGE_2 receptors (Chan and Lau, 1998). However, no attempt to delineate the EP subtypes was made in this study.

Here, we sought to investigate the effects of PGE_2 on mast cell cytokine responses in the context of IgE-mediated activation. As a model system, we have chosen the well-characterized murine bone marrow-derived mast cells (BMMC) (Tertian *et al.*, 1981) and an

IL-3 dependent murine mast cell line, MC/9 (Galli *et al.*, 1983). These cells have been demonstrated to share a number of characteristics with the mast cells resident in the airways and other mucosal sites of rodents and humans. We have focused on the effects of PGE_2 on three cytokines, IL-4, IL-6, and GM-CSF, which are produced in physiologically relevant quantities during allergic disease and are enhanced in symptomatic asthma (Humbert *et al.*, 1996; Broide *et al.*, 1992). IL-4 was selected for study in view of its critical role in the development of type 2-type immune responses and IgE class switch (Fedyk *et al.*, 1996a); IL-6, for its role in inducing the acute phase response and downregulating inflammatory processes (Tilg *et al.*, 1997); and GM-CSF, for its involvement in the pathogenesis of allergic inflammation largely through its role as a development and survival factor for eosinophils (Clark and Kamen, 1987). EP receptor expression and usage by MC/9 cells was also examined in the present study.

Materials and Methods

Mice

C57BL/6 mice (The Jackson Laboratory, Bar Harbor, MN) were housed in sterilized, filter-hooded cages and provided food and water *ad libitum*. All experiments were approved by the Animal Research Ethics Boards of McMaster University and Dalhousie University.

Mast cells

MC/9 cells (ATCC CRL 8306) were routinely grown in modified Dulbecco's modified Eagle's medium (Canadian Life Technologies, Inc., Burlington, ON) containing 36 mg/ L-asparagine, 0.1 mM non essential amino acids, 5 x 10^{-5} M 2-mercaptoethanol, 10% FCS, and 3 ng/ml rmIL-3 (Pepro Tech, Inc., Rocky Hill, N.J.) at 37°C, 10 % CO_2. Bone marrow-derived mast cells (BMMC) were generated from bone marrow of C57BL/6 mice. Briefly, mice were sacrificed, and intact femurs and tibias were removed. Sterile endotoxin-free medium was repeatedly flushed through the bone shaft using a needle and syringe, and the bone marrow cells were passed through a sterile wire screen to remove any bone fragments. The cell suspension was centrifuged at 320 x g for 20 min at 4°C, and cultured at a concentration of 0.5 - 1 x 10^6 nucleated cells/ml in RPMI 1640 (Canadian Life Technologies, Inc.) supplemented with 10% FCS (SIGMA-Aldrich, ON, Canada), 10% v/v concentrated WEHI-3 conditioned medium as a source of IL-3, 1% penicillin/streptomycin (Canadian Life Technologies, Inc.), and 50 μM 2-ME (BMMC medium). Non-adherent cells were transferred to fresh BMMC medium at least once a week. After 4-6 weeks, mast cell purity

of > 95% was achieved as assessed by Alcian blue or Toluidine blue staining of fixed cytocentrifuge preparations.

Mast cell activation with various stimulating agents

Mast cells were resuspended in experimental medium consisting of RPMI-1640 (Canadian Life Technologies, Inc.), 10% FCS (SIGMA-Aldrich Canada Ltd.), 1% penicillin/streptomycin (Canadian Life Technologies, Inc.), 1% HEPES (Canadian Life Technologies, Inc.), and 100 μg/ml soybean trypsin inhibitor (SIGMA-Aldrich Canada Ltd.; reconstituted in saline). Mast cells were incubated at 1 x 10^6 cells/ml for up to 24h at 37°C with the following reagents either alone or in combination: PGE_2 (SIGMA-Aldrich Canada Ltd.); PGE_1, PGE_1 alcohol, 17-phenyl-ω-trinor-PGE_2, sulprostone, and misoprostol (purchased from Cayman Chemical Co., MI, USA). In other studies, mast cells were also activated with the cAMP-elevating agents, pentoxifylline, forskolin, and β-isoproterenol (purchased from SIGMA-Aldrich Canada Ltd.). In our laboratory, each of these cAMP-elevating agents were observed to increase intracellular levels of cAMP in MC/9 cells by >2.5 fold (baseline levels were approximately 1.7 ± 0.4 pmol/10^6 cells). All samples were stored at ≤ -20°C until assayed.

IgE-mediated mast cell activation

BMMC and MC/9 cells were incubated at 37°C for 18-30 h in their respective media with murine hybridoma supernatant containing anti-DNP IgE (gift from Dr. F.T. Lui) or anti-TNP IgE (ATCC TIB-141) as stated. Sensitized cells were washed three times by centrifugation

to remove unbound IgE and used immediately in experiments. For activation, cells were incubated with DNP- human serum albumin (DNP-HSA; SIGMA-Aldrich Canada Ltd) or TNP-bovine serum albumin (TNP-BSA; Biosearch Technologies, Inc.) at a predetermined optimal concentration of 10 ng/ml for 20 min to assess β-hexosaminidase release or for up to 24 hours to examine cytokine production in supernatant samples.

RT-PCR of murine EP receptors

MC/9 cells and BMMC were homogenized with Trizol Reagent (Canadian Life Technologies, Inc.) and total RNA was isolated according to manufacturer's instructions. cDNAs were generated by reverse transcription using random primers. Primers used for PCR amplification of the EP receptor subtypes were purchased from Research Genetics, Inc. (AL, USA) and sequences were as follows (Arakawa *et al.*, 1996): EP_1 5'-CGCAGGGTTCACGCACACGA-3' and 5'-CACTGTGCCGGGAACTACGC-3' (336 bp); EP_2 5'-AGGACTTCGATGGCAGAGGAGAC-3' and 5'-CAGCCCCTTACACTTCTCCAATG-3' (401 bp); EP_3 5'-CCGGGCACGTGGTGCTTCAT-3' and 5'-TAGCAGCAGATAAACCCAGG-3' (437 bp); EP_4 5'-TTCCGCTCGTGGTGCGAGTGTTC-3' and 5'-GAGGTGGTGTCTGCTTGGGTCAG-3' (423 bp). To further amplify resulting EP_1 PCR products, a second round of PCR was performed using the following "nesting" primers: 5'-TGGTGTCGTGCATCTGCTGG-3' and 5'-TCCCAGGCACTCTTGGTTAG-3' (249 bp). Splice variants exist for EP_3 ($EP_{3\alpha}$, $EP_{3\beta}$, and $EP_{3\gamma}$) and the primers used in this study recognized sequences shared by all three isoforms. PCR was performed in a 50 μl reaction mixture comprised of 1 μM of each

forward and reverse oligonucleotide primer, 3 mM $MgCl_2$, 0.5 mM of the four deoxynucleotide triphosphates, 5 µl cDNA preparation, and 0.02 U/µl Taq DNA polymerase. PCR conditions were as follows: 3 min at 94°C followed by 40 cycles of 1 min at 94°C, 1 min at 55°C (EP_3) or 60°C (EP_1, EP_2, and EP_4), and 2 min at 72°C; followed by 7 min at 72°C. For deoxyribonuclease I (DNase I) treatment of MC/9 RNA, total RNA was incubated with DNase I (Canadian Life Technologies, Inc.) for 15 min at room temperature, after which time DNase I activity was inactivated by the addition of 2 mM EDTA and heating between 60-65°C for 20 min.

B-9 Bioassay

IL-6 bioactivity was measured by the B-9 hybridoma proliferation assay (Aarden *et al.*, 1987). B-9 cells were maintained in MEM or RPMI medium (Canadian Life Technologies, Inc.) supplemented with 5% FCS, 1% penicillin/streptomycin, 5 µM 2-mercaptoethanol, and normal human lung fibroblast- or murine monocyte macrophage J774 cell line-conditioned medium supernatant as a source of IL-6. Briefly, serial dilutions of standards and samples were performed in triplicate in Nunc 96-well microtiter plates (Canadian Life Technologies, Inc.). B-9 cells were washed, resuspended at 5 x 10^4 cells/ml in B-9 medium and incubated with standards and samples, for 3 d at 37°C. 10 µl/well 0.5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; SIGMA-Aldrich Canada Ltd.) was added, and 4h later, 50 µl/well of 10% Triton-HCl was added and the plates were stored 18-24 h in the dark. The optical densities of the resulting reaction product were determined at 570 nm. IL-6 concentrations were reported as U/ml of bioactivity, where 1 unit equals approximately

0.45 pg of IL-6. The sensitivity of the B-9 assay has been determined to be 10 U/ml. None of the reagents used in the present study including PGE_2 at the highest concentration used in this study (1 μM) altered B-9 cell growth under these conditions. Moreover, other mast cell-derived cytokines including TNF-α, GM-CSF, and IL-4, do not cause proliferation of B-9 cells under these conditions (Leal-Berumen *et al.*, 1995).

ELISAs

Murine IL-4 and IL-10 were assayed using ELISA kits purchased from R&D Systems, Inc. (Minneapolis, MN). IL-5 and IL-12 ELISA kits were obtained from Amersham Life Science (ON, Canada) and Genzyme Diagnostics (Cambridge, MA), respectively. GM-CSF was assayed using ELISA kits purchased from both R&D Systems, Inc. and Amersham Life Science. Cyclic AMP was measured by enzyme immunoassay purchased from Amersham Pharmacia Biotech (Quebec, Canada).

Murine IFN-γ was measured by an "in house" sandwich ELISA with all incubations performed at room temperature. Briefly, Maxisorp ELISA plates (Nunc/Inter Med, ON, Canada) were coated for 18-24 h at 4°C with 50 μl/well of 2 μg/ml anti-mouse IFN-γ capture antibody (BD PharMingen, ON, Canada) diluted in either borate-buffered saline (pH 8.3) or freshly prepared 0.1M bicarbonate solution (in distilled water). The wells were aspirated, and incubated for 1 h with 100 μl/well blocking solution (10 mg BSA/ml PBS, pH 7.4). The blocking solution was decanted and the wells were washed four times with Tris-buffered saline (pH 7.4) containing 0.05% Tween 20. Wells were aspirated after the final wash to ensure complete removal of liquid. Standards and samples were added to the plate at 50

µl/well and incubated between 1.5 and 2 h. The wells were washed as described above, and secondary biotinylated anti-mouse IFN-γ antibody (BD PharMingen) at 0.5 µg/ml in blocking solution, was added at 50 µl/well. After 1 h, the wells were washed and 50 µl/well of streptavidin-alkaline phosphatase (Canadian Life Technologies, Inc.) prepared in blocking solution, was added to the plates for an hour. The wells were washed, and bound labelled IFN-γ was detected with the GIBCO ELISA Amplification System (Canadian Life Technologies, Inc.). The colored product was read at 492 nm.

β-hexosaminidase release assay

Briefly, 1 x 10^6 BMMC or MC/9 cells per ml were incubated for 15 min at 37°C in HEPES-Tyrodes buffer (137 mM NaCl, 5.6 mM glucose, 2.7 mM KCl, 0.5 mM NaH_2PO_4, 1 mM $CaCl_2$, 10 mM HEPES, 0.1% BSA, pH 7.3, ~300 mOsm/kg) in the presence of various stimulating agents. β-hexosaminidase release was stopped by pelleting the cells at 140 x g for 10 min at 4°C. Supernatants were collected and the pellets were resuspended in the original volume of HEPES-Tyrodes buffer lacking the stimulating agents. β-hexosaminidase content in the supernatant and pellet samples was determined using a previously reported method (Zhu *et al.*, 1998). 50 µl of samples were transferred to individual wells of a 96-well plate and incubated with 50 µl of 1 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (SIGMA-Aldrich Canada Ltd.) dissolved in 0.1 M citrate buffer, pH 5, for 1 h at 37°C. The reaction was stopped by the addition of 200 µl/well of 0.1 M carbonate buffer, pH 10.5. The resulting yellow reaction product was read at 405 nm in an ELISA reader, and net percent β-

hexosaminidase release was calculated as follows: β-hexosaminidase in supernatant/ (β-hexosaminidase in supernatant + β-hexosaminidase in pellet) x 100%.

Statistical Analysis

All data are expressed as the mean ± SEM. Statistical analysis was performed by one-way ANOVA. The effects of different treatments were compared using the Student-Newman-Keuls post test for comparison of individual groups and controls with the exception of IL-6 production data which, in view of the data distribution, were compared using the Bonferroni multiple comparisons test.

Results

PGE_2 induces enhancement of IL-6 production by BMMC

To assess the effects of PGE_2 activation alone on mast cell cytokine production. BMMC were activated with different doses of PGE_2 for up to 24 h and supernatants were assayed for cytokines of interest. As previously demonstrated in rat PMCs (Leal-Berumen *et al.*, 1995), IL-6 production in BMMC was enhanced by PGE_2 in a dose-dependent manner (baseline IL-6 production of 21.6 ± 5.5 U/ml was increased to 595 ± 72 U/ml ($p < 0.001$) and 280 ± 77 U/ml ($p < 0.01$) following stimulation with PGE_2 at 10^{-6} M and 10^{-8} M, respectively (n=8)). In contrast, PGE_2 lacked any significant effect on the production of IL-4, IL-5, IL-10, IFN-γ, and GM-CSF (data not shown) whereas BMMC were capable of producing each of these cytokines in response to FcεRI cross-linking alone (IL-4, IL-5, GM-CSF) (Plaut *et al.*, 1989; Wodnar-Filipowicz *et al.*, 1989; Seder *et al.*, 1991), IL-3 treatment (IL-10) (Marietta *et al.*, 1996), or IL-12 treatment (IFN-γ) (Gupta *et al.*, 1996).

PGE_2 synergistically increases IL-6 and GM-CSF responses in the context of IgE-mediated activation

Mast cells are known to be activated via cross-linking of their surface FcεRI by specific allergen. To examine the regulatory effects of PGE_2 in the context of IgE-mediated activation, BMMC were passively sensitized with anti-DNP IgE or anti-TNP IgE for 18-30 h, and subsequently incubated with respective antigen, DNP-HSA or TNP-BSA (at 10 ng/ml), in the presence or absence of PGE_2. IgE-mediated activation increased the BMMC production of IL-6, GM-CSF, and IL-4 over that of media-treated controls (Figs. 1A-C). Co-

stimulation of IgE-sensitized mast cells with DNP-HSA and PGE_2 resulted in increased IL-6 and GM-CSF production over IgE-mediated activation alone ($p < 0.01$ for IL-6; $p < 0.001$ for GM-CSF) (Figs. 1A and B). IgE-mediated IL-4 production, on the other hand, was not enhanced by PGE_2, and at higher concentrations, PGE_2 (≥10 nM) had suppressive effects on IL-4 production ($p < 0.01$) (Fig. 1C). We also investigated the modulation of IL-6 production by PGE_2 in an IL-3-dependent murine mast cell line, MC/9. IL-6 production by MC/9 cells was also potentiated by PGE_2 in the context of IgE-mediated activation; however, PGE_2 alone failed to consistently induce IL-6 production by a range of PGE_2 doses (10^{-8}, 10^{-7}, 10^{-6} M) (data not shown).

Time course of PGE_2 effects on cytokine production

Kinetic studies were performed investigating IL-6 and GM-CSF release in response to PGE_2 and IgE-mediated activation of BMMC. IgE-mediated IL-6 and GM-CSF production which was minimal or absent at 1 h was readily detected by 6 h and cytokine levels were maintained up to the 24h timepoint (Table I). PGE_2-mediated potentiation of IL-6 and GM-CSF production in IgE/Ag-activated cells was readily apparent by 6 h post-stimulation. IgE-mediated activation also induced significant IL-4 release by 6 h (98.7 ± 11.4 pg/ml; $p <$ 0.001 with respect to the media control value of 11.3 ± 4.7 pg/ml; n=2) and such secretion was not modulated at this timepoint by PGE_2 (102 ± 4 pg/ml for concurrent IgE/Ag and PGE_2 treatment; n = 3).

PGE_2 induces potentiation of mast cell degranulation

To examine the effects of PGE_2 on mast cell degranulation, BMMC and MC/9 cells were activated for 20 min with PGE_2 alone or in combination with IgE/Ag-activation, and the degree of β-hexosaminidase release was measured as a marker of degranulation. PGE_2 activation alone did not induce β-hexosaminidase release by BMMC (Fig. 2A) or MC/9 cells (Fig. 2B). IgE-mediated activation induced significant β-hexosaminidase release by both mast cell populations, and concurrent stimulation with PGE_2 consistently enhanced this release by at least 30% (Fig. 2).

Effects of PGE_2 on mast cell degranulation and IL-6 production are unlikely to be mediated by the second messenger, cAMP

Our findings of enhanced degranulation induced by PGE_2 in the context of IgE-mediated activation are in contrast to the inhibitory effects of this prostanoid on mast cell degranulation reported when mast cells were pre-incubated with PGE_2 prior to addition of other mast cell stimuli (Hogaboam *et al.*, 1993; Kaliner and Austen, 1974; Peachell *et al.*, 1988). In the latter studies, intracellular cAMP was implicated as the second messenger mediating the inhibitory effects. To investigate whether cAMP played a critical role in PGE_2-mediated enhancement of degranulation and IL-6 production in IgE/Ag-activated mast cells, BMMC and MC/9 were stimulated with cAMP-elevating agents. In contrast to the stimulatory effects observed with PGE_2, forskolin, a direct activator of adenylate cyclase, inhibited IgE-mediated β-hexosaminidase release in both BMMC and MC/9 cells (Fig. 2),

and failed to potentiate IL-6 production in IgE/Ag-activated MC/9 cells (Fig. 3). Two additional cAMP-elevating agents, β-isoproterenol and the phosphodiesterase inhibitor, pentoxifylline, also failed to potentiate IL-6 production in the context of IgE-mediated activation (Fig. 3).

Involvement of EP_1/ EP_3 receptors in the potentiation of β-hexosaminidase and IL-6 production in IgE/Ag-activated mast cells

PGE_2 acts by interacting with one of four receptor subtypes designated EP_1, EP_2, EP_3, and EP_4 (Coleman *et al.*, 1994). To examine whether PGE_2 receptor agonists could modulate IgE/Ag-induced β-hexosaminidase release and IL-6 production, MC/9 cells were stimulated with a panel of synthetic agonists that demonstrate preferential binding of one or more EP subtypes. The EP_1 agonist, 17-phenyl-ω-trinor PGE_2, and the EP_1/EP_3 selective agonist, sulprostone, potentiated β-hexosaminidase release (Fig. 4) and IL-6 production (Fig. 5 and Table II) by IgE/Ag-activated mast cells. PGE_1, a PGE_2 homologue which binds with comparable affinity as PGE_2 to EP_2, EP_3, and EP_4 yet more weakly to EP_1, strongly potentiated β-hexosaminidase release by MC/9 cells. However, PGE_1 induced IL-6 production to a substantially lower degree than PGE_2 (Fig. 5 and Table II). The EP_2/ EP_4-selective agonist, PGE_1 alcohol, failed to enhance β-hexosaminidase (Fig. 4) and IL-6 production (Fig. 5 and Table II) above IgE-mediated activation alone. These data implicate the involvement of the EP_1 and/or EP_3 receptors in β-hexosaminidase release and IL-6

production. Involvement of EP_3 in mediating β-hexosaminidase release was further suggested by the observation that β-hexosaminidase release by IgE/TNP-activated mast cells was potentiated by the EP_2/ EP_3/ EP_4 selective analogue, misoprostol (15.4 ± 1.5 % release (IgE/TNP-activation alone) vs 24.8 ± 2.0 % release (concurrent IgE/TNP and misoprostol treatment); $p < 0.001$; comparison of means of three independent experiments); whereas, such potentiation was not observed with the EP_2/ EP_4-selective agonist, PGE_1 alcohol as described above (Fig. 4).

Expression of EP receptor subtypes by MC/9 cells

We used RT-PCR to determine which prostaglandin E receptor subtypes were expressed by MC/9 cells. Quiescent MC/9 cells expressed mRNA for EP_1, EP_3, and EP_4 receptors (Fig. 6A). However, MC/9 cells failed to express mRNA encoding EP_2 in three independent RNA preparations whereas a signal for EP_2 of the expected size (401 bp) was observed in murine uterus (data not shown). For the EP_1 receptor, in addition to a weak signal for the expected PCR product (336 bp), a more intense band corresponding to a larger amplicon at approximately 750 bp was observed (Fig. 6A), and the latter PCR product may represent a splice variant similar to that described in the rat (37). To rule out the possibility of genomic contamination, RNA preparations were treated with DNase I to degrade any contaminating genomic DNA, and then subjected to PCR with or without prior reverse transcription. No PCR products were obtained for any of the EP receptors including EP_1 when reverse transcription was not performed (Fig. 6A). Nesting primers were employed to amplify the EP_1 receptor signal, and two PCR products of expected sizes (249 bp and 668 bp) were

obtained (Fig. 6B). Subsequent sequence analysis indicated that the putative EP_1-variant receptor contained an intron positioned within the 6th transmembrane domain (data not shown) and hence, as in the rat, the EP_1-variant receptor arose from the failure to use a splice site located within this domain (Okuda-Ashitaka *et al.*, 1996).

Figure 1. **PGE_2 effects on IgE- activated mast cells.** BMMC passively sensitized with anti-DNP IgE were incubated with DNP-HSA alone or in the presence of various doses of PGE_2. BMMC incubated with medium alone were used as controls. Following 24h incubation, supernatants were harvested and assayed for IL-6 (A), GM-CSF (B), and IL-4 (C) content. Bars represent mean values ± SEM. ***, denotes $p < 0.001$ compared with media controls. ##, denotes $p < 0.01$; ###, denotes $p < 0.001$ compared with IgE-mediated activation alone. n.d. = not detected in the assay (limit of detection for the GM-CSF and IL-4 ELISAs were 1 pg/ml and 2 pg/ml, respectively).

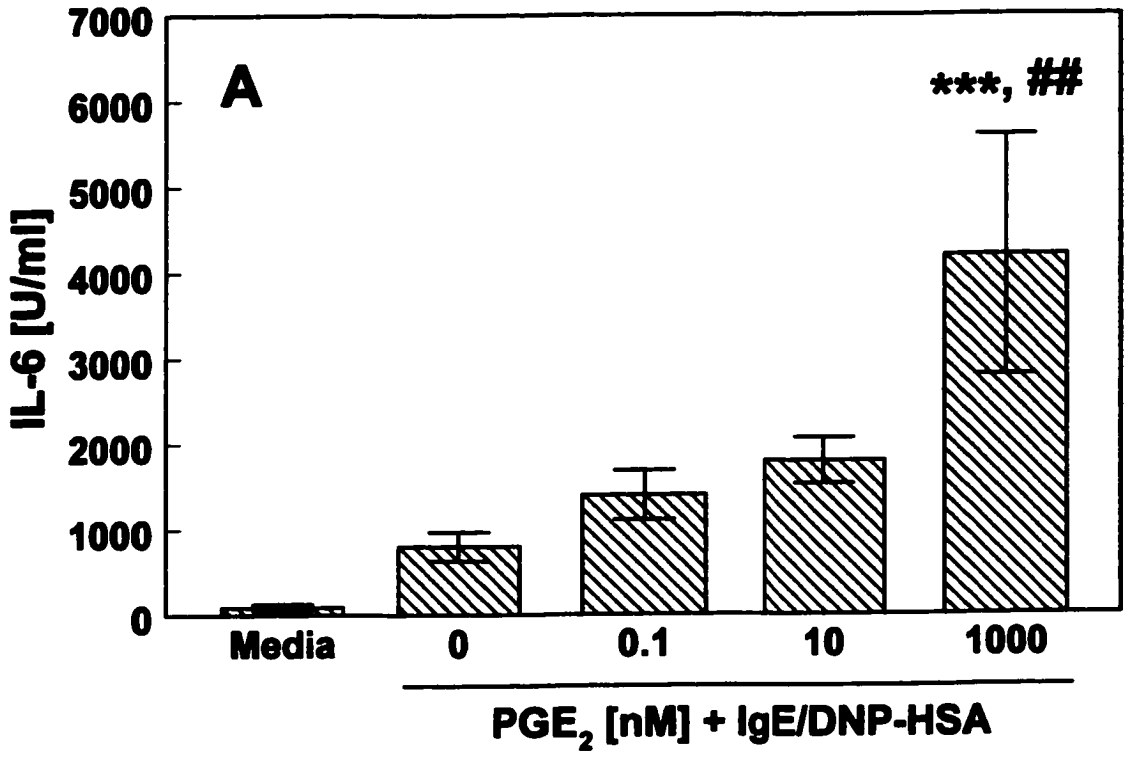
7000
6000
5000
4000
3000
2000
1000
0
IL-6 [U/ml]
A
***, ##
Media
0
0.1
10
1000
PGE2 [nM] + IgE/DNP-HSA

Figure 1A

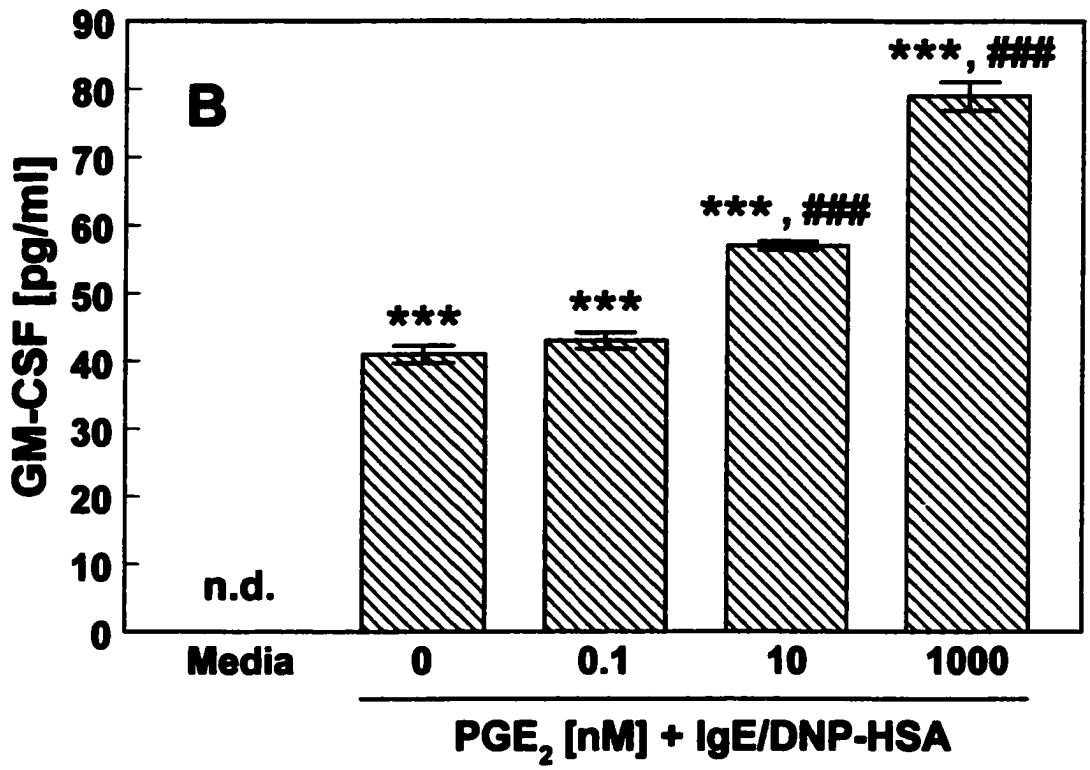
B
GM-CSF [pg/ml]
90
80
70
60
50
40
30
20
10
0
n.d.
***
***
***, ###
***, ###
Media
0
0.1
10
1000
PGE2 [nM] + IgE/DNP-HSA

Figure 1B

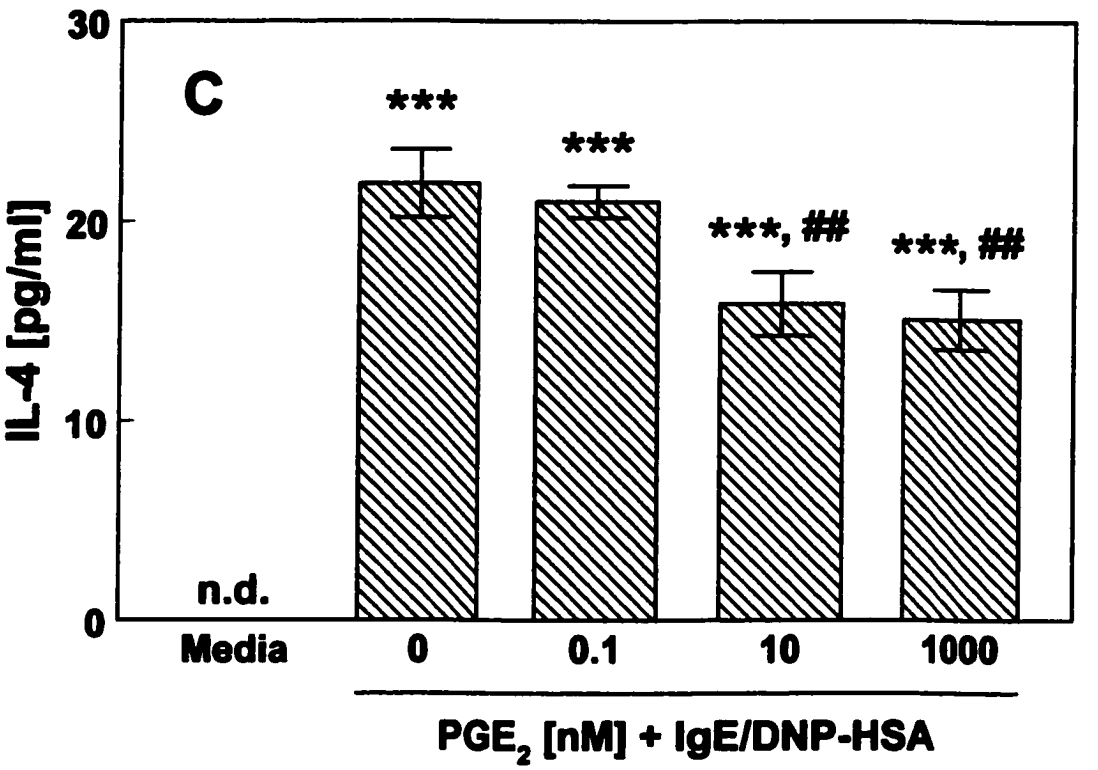

Figure 1C

Table I. **Kinetics of IL-6 and GM-CSF production by mast cells in response to PGE_2 and IgE/Ag-activation.** BMMC were passively sensitized with anti-DNP IgE, and incubated with DNP-HSA (10 ng/ml) in the presence or absence of PGE_2 (1μM). Supernatants were harvested at the indicated times and assayed for IL-6 and GM-CSF content. Figures represent mean values ± SEM. *, denotes $p < 0.05$; ***, denotes $p < 0.001$ compared with media controls. # #, denotes $p < 0.01$; and # # #, denotes $p < 0.001$ compared with IgE-mediated activation alone. n.d. = not detectable.

	IL-6 [U/ml]			GM-CSF [pg/ml]		
	1h	6h	24h	1h	6h	24h
Media	12.7 ± 1.3†	78.0 ± 15.5	116 ± 14	n.d.	n.d.	1.1 ± 0.1
PGE_2	10.0 ± 0.0	63.3 ± 9.3	263 ± 38	n.d.	n.d.	1.5 ± 0.2
IgE/DNP	12.7 ± 1.3	643 ± 20*	393 ± 87	1.1 ± 0.1	29.0 ± 1.5***	25.3 ± 2.2***
IgE/DNP + PGE_2	19.0 ± 2.5	1110 ± 283***	1190 ± 307***,##	n.d.	55.3 ± 1.3***,###	54.0 ± 2.1***,###

Table I

Figure 2. **β-hexosaminidase release by BMMC and MC/9 in response to PGE_2 and IgE-mediated activation.** BMMC (A) and MC/9 (B) were previously sensitized with anti-TNP IgE and incubated with TNP-BSA (10 ng/ml) in the presence or absence of PGE_2 (1 μM) for 15 min at 37°C. BMMC incubated in buffer alone served as a control for spontaneous β-hexosaminidase release. Bars represent mean values ± SEM. ***, denotes $p < 0.001$ compared with buffer controls. # # #, denotes $p < 0.001$ compared with IgE-mediated activation alone. A23 = A23187 (calcium ionophore; 1 μM); Forsk = Forskolin (10 μM). Data shown are representative of at least three independent experiments.

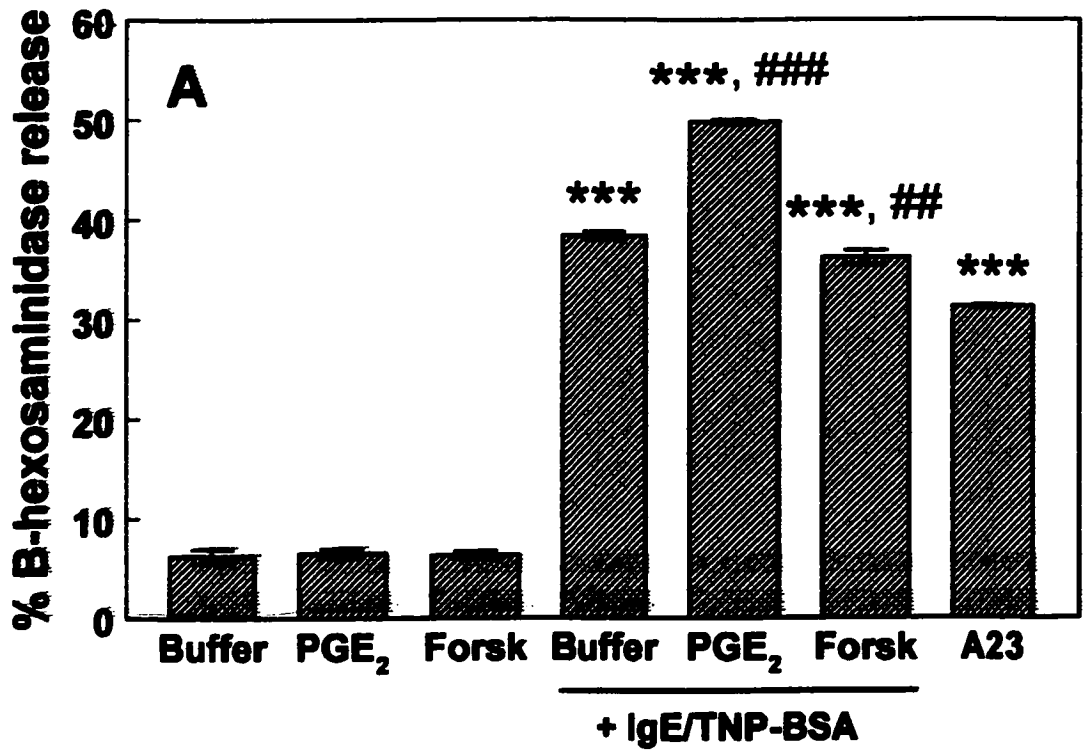
% B-hexosaminidase release
60
50
40
30
20
10
0
A
***, ###
***
***, ##
***
Buffer
PGE2
Forsk
Buffer
PGE2
Forsk
A23
+ IgE/TNP-BSA

Figure 2A

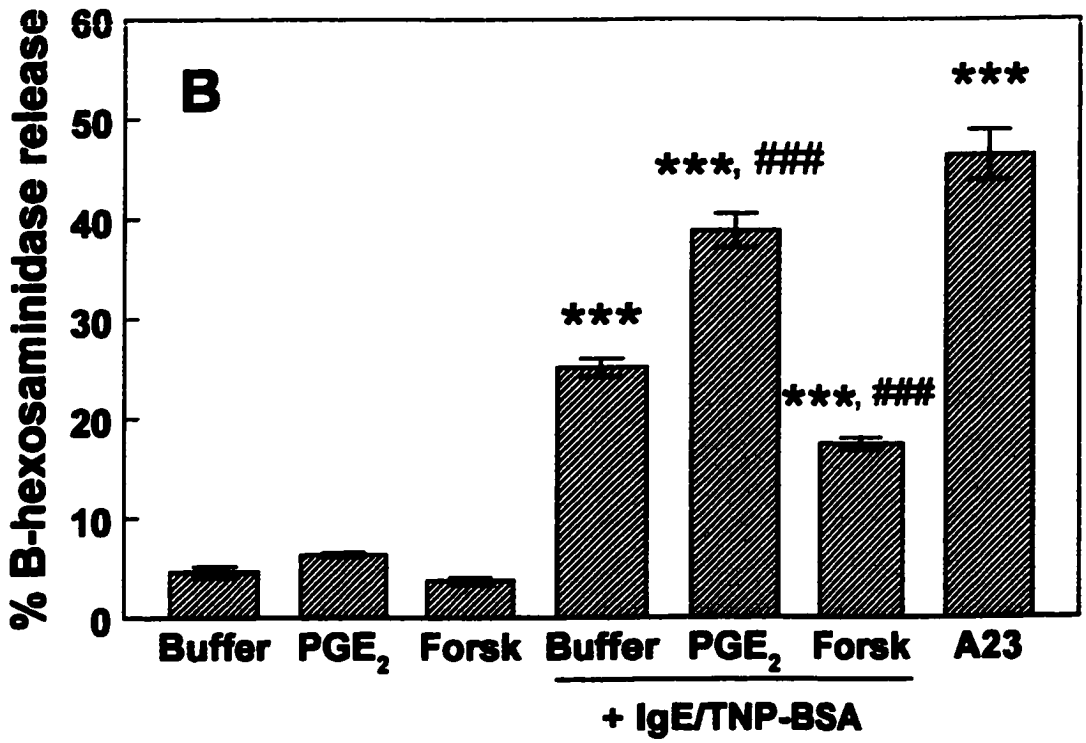
B
% B-hexosaminidase release
60
50
40
30
20
10
0
***
***, ###
***, ###
***
Buffer
PGE2
Forsk
Buffer
PGE2
Forsk
A23
+ IgE/TNP-BSA

Figure 2B

Figure 3. **Effect of cAMP-elevating agents on IL-6 production by IgE/Ag-activated mast cells.** MC/9 cells were passively sensitized with anti-TNP IgE and subsequently incubated for 24h with TNP-BSA (10 ng/ml) alone or in the presence of β-isoproterenol (10 μM), forskolin (10 μM), or pentoxifylline (1 mg/ml). MC/9 cells incubated with PGE_2 (1 μM) alone served as controls. Bars represent mean % change (± SEM) in IL-6 response with respect to IgE-mediated activation from at least two independent experiments.

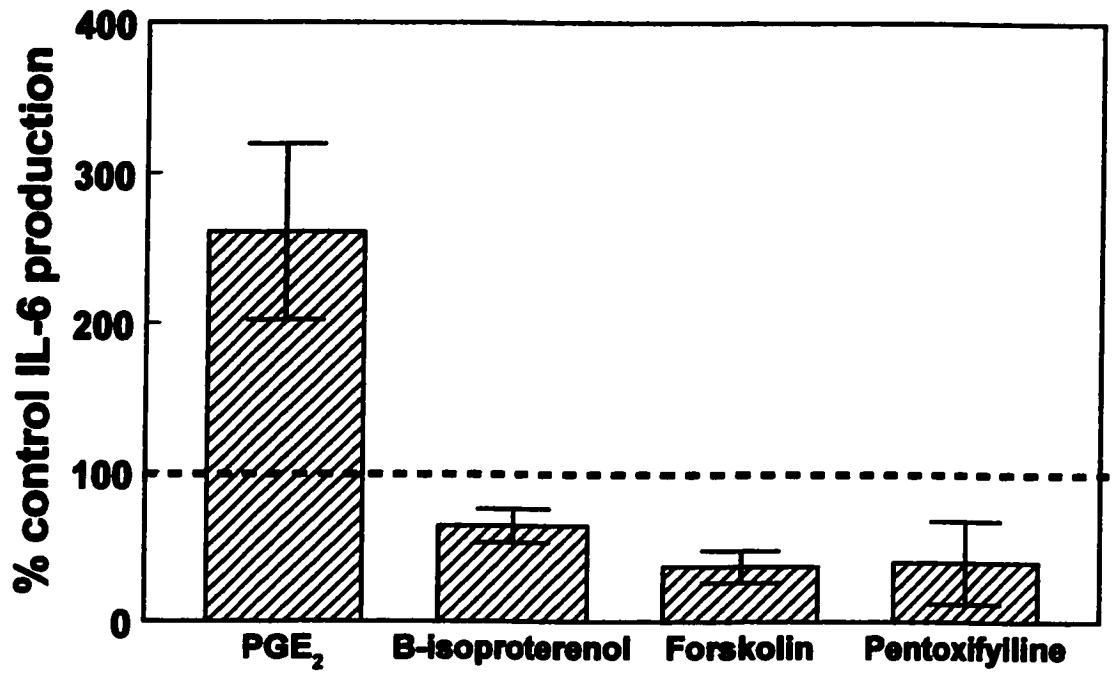
400
300
200
100
0
% control IL-6 production
PGE2
B-isoproterenol
Forskolin
Pentoxifylline

Figure 3

Figure 4. **Effect of EP-selective agonists on β-hexosaminidase release by IgE/Ag activated MC/9 cells.** MC/9 cells were passively sensitized with anti-TNP IgE and incubated with TNP-BSA (10 ng/ml) in the presence or absence of PGE_1, PGE_2, EP_1 agonist 17-phenyl-ω-trinor-PGE_2 (17-Ph), EP_1/ EP_3-selective agonist sulprostone (Sulp), or EP_2/ EP_4-selective agonist PGE_1 alcohol (PGE_1 Alc). PGE_1, PGE_2 and the EP analogues were used at a concentration of 1 μM. Following a 15 min incubation, cell supernatants and pellets were harvested and assayed for β-hexosaminidase. Bars represent mean % change ± SEM in β-hexosaminidase release with respect to IgE-mediated activation alone, from three independent experiments.

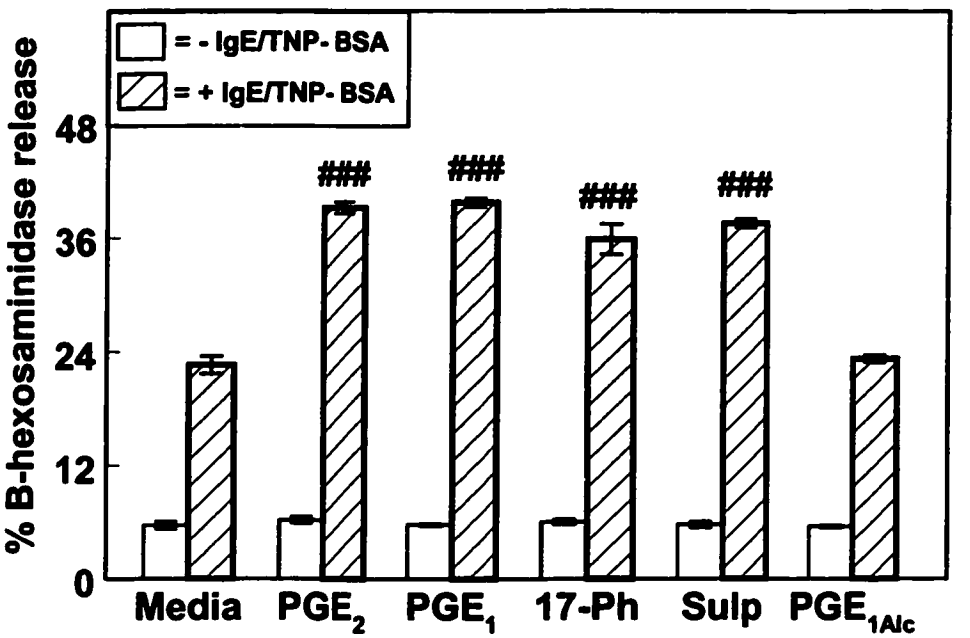
= - IgE/TNP- BSA
= + IgE/TNP- BSA
% B-hexosaminidase release
0
12
24
36
48
###
###
###
###
Media
PGE2
PGE1
17-Ph
Sulp
PGE1Alc

Figure 4

Figure 5. **Effect of EP-selective agonists on the IL-6 response by IgE/Ag-activated MC/9 cells.** MC/9 cells were passively sensitized with anti-TNP IgE and incubated with TNP-BSA (10 ng/ml) in the presence or absence of PGE_1, PGE_2, EP_1 agonist 17-phenyl-ω-trinor-PGE_2 (17-Ph), EP_1/ EP_3-selective agonist sulprostone (Sulp), or EP_2/ EP_4-selective agonist PGE_1 alcohol (PGE_1 Alc). PGE_1, PGE_2, and the EP agonists were used at a concentration of 1 μM. Following a 24 h incubation, supernatants were harvested and assayed for IL-6 by B9 bioassay. Bars represent mean % change ± SEM in IL-6 response with respect to IgE-mediated activation alone from three independent experiments.

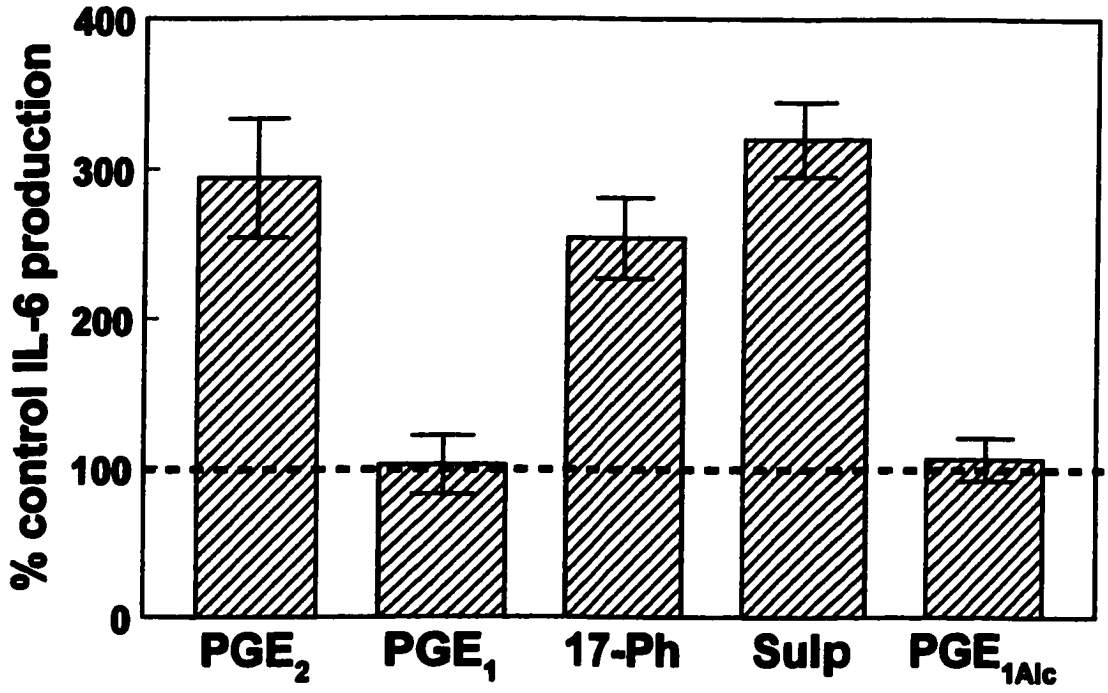
% control IL-6 production
400
300
200
100
0
PGE2
PGE1
17-Ph
Sulp
PGE1Alc

Figure 5

Table II. **Effects of EP-selective agonists on the IL-6 response by IgE/Ag-activated MC/9 cells.** Data are presented as mean percent change ± SEM in IL-6 response compared with IgE-mediated activation alone from three experiments for PGE_2 and two experiments for PGE_1 and the EP agonists. MC/9 cells were passively sensitized with anti-TNP IgE and concurrently incubated with TNP-BSA (10 ng/ml) and various doses of PGE_2, PGE_1, EP_1 agonist 17-phenyl-trinor-ω-PGE_2, $EP_{1/3}$-selective agonist sulprostone, and $EP_{2/4}$-selective agonist PGE_1 alcohol. IL-6 production was determined in cell-free supernatants following 24 h incubation. IL-6 production in response to IgE/TNP-activation alone was 480 ± 88 U/ml (presented as mean ± SEM of three independent experiments).

	Percentage change in IL-6 production by IgE/TNP-activated MC/9 cells following treatment with EP receptor agonists		
	0.01 μM	0.1 μM	1 μM
PGE_2	133 ± 5[†]	232 ± 27	294 ± 40
PGE_1	139 ± 6	176 ± 39	102 ± 20
17-Phenyl-trinor-PGE_2	115 ± 24	163 ± 23	254 ± 27
Sulprostone	163 ± 3	257 ± 38	320 ± 25
PGE_1 Alcohol	96.2 ± 8.4	102 ± 18	106 ± 14

Table II

Figure 6 **Resting MC/9 cells express mRNA for EP_1, EP_3, and EP_4 receptors.** Total RNA was isolated from MC/9 cells and DNase I-treated to remove genomic DNA contamination. (A) RT-PCR was performed with primers specific for mouse EP receptor subtypes. In lanes 2, 4, and 6, are EP_1, EP_3, and EP_4 PCR products, respectively. Lanes 1, 3, and 5, are PCR reactions where RNA samples were not reverse transcribed (as controls for genomic DNA contamination) for EP_1, EP_3, and EP_4, respectively. The 1 Kb Plus DNA Ladder (Canadian Life Technologies, Inc.) was employed for sizing the PCR products. (B) Ethidium bromide-stained gel electrophoresis of RT-PCR showing amplification of EP_1 PCR products shown in (A) using "nesting" primers as described in Methods. Molecular sizes are indicated in base pairs. Results are representative of three independent MC/9 RNA preparations.

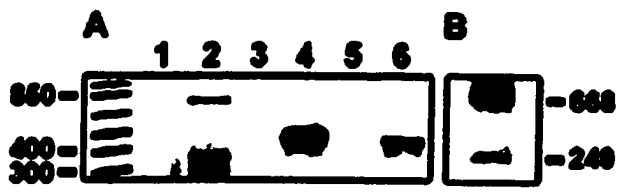

Figure 6

Discussion

Elevated numbers of mast cells and evidence of mast cell activation are observed in a variety of inflammatory disorders including asthma (Koshino *et al.*, 1996), rheumatoid arthritis (Mican and Metcalfe, 1990), and inflammatory bowel disease (Lloyd *et al.*, 1975). However, the full role of mast cells in the pathogenesis of such inflammatory disorders is largely unexplored. Mast cells are storehouses of pre-formed mediators including histamine and proteases, and are potent sources of a number of pro-inflammatory cytokines and chemokines. Levels of the lipid mediator, PGE_2, are also elevated in the context of many inflammatory conditions (Kuehl and Egan, 1980), and PGE_2 has been demonstrated to possess potent immunomodulatory actions and to shift the immune response towards a type 2 response through inhibition of type 1 cytokine production and either enhancing or having no effect on the production type 2 cytokines (Fedyk *et al.*, 1996a). Consequently, PGE_2 may support the induction and chronicity of certain types of inflammation.

Our current data show that PGE_2 alone selectively modulates cytokine production by murine mast cells, BMMC and MC/9, both of which are considered models of mucosal mast cells. In otherwise unactivated BMMC, PGE_2 enhanced IL-6 production and failed to alter the production of many other cytokines including IL-4, IL-5, IL-10, and GM-CSF, that are known to be produced by mast cells under alternate stimulation conditions. However, PGE_2 displayed a broader range of potent effects on cytokine production when used in conjunction with IgE/Ag stimulation. IgE-mediated activation alone induced significant release of IL-4, IL-6, and GM-CSF, and further addition of PGE_2 led to a synergistic increase in the production of both IL-6 and GM-CSF, but not IL-4, suggesting selectivity in the ability of

PGE_2 to interact with FcεRI-mediated cytokine induction.

The potentiation of IL-6 release by PGE_2 in the context of IgE-mediated activation was unlikely to be the result of increased secretion of stored cytokines as detectable levels of IL-6 were not observed in the cell pellets of unstimulated BMMC or PGE_2-stimulated BMMC; moreover, in IgE/Ag-activated BMMC, where low levels of IL-6 were recovered from cell pellets, concurrent PGE_2 treatment slightly increased these levels rather than decreasing them as one would expect if PGE_2 was acting by facilitating the release of stored cytokine (data not shown).

Originally described as a pro-inflammatory cytokine, there is growing evidence that IL-6 exerts important anti-inflammatory actions both *in vivo* and *in vitro* (Tilg *et al.*, 1997). For instance, endotoxemia-induced circulating levels of pro-inflammatory cytokines TNF-α, MIP-2, IFN-γ, and GM-CSF were higher in IL-6 gene knock-out mice than in wild-type littermates (Xing *et al.*, 1998), and in humans, recombinant IL-6 administration upregulated production of antagonists for the pro-inflammatory cytokines, IL-1 and TNF-α (Tilg *et al.*, 1994). Moreover, PGE_2 was recently reported to induce production of the anti-inflammatory agent, α_1-acid glycoprotein, in rat alveolar macrophages co-stimulated with dexamethasone (Fournier *et al.*, 1999). This acute phase protein possesses anticomplement activities and inhibits neutrophil activation, among other antiinflammatory effects which serve to reduce existing inflammation. In light of these data, the observed potentiation of IL-6 production by PGE_2 during IgE-mediated activation of mast cells may have *in vivo* significance by potentially facilitating the resolution of inflammation induced by earlier release of histamine and other pro-inflammatory mast cell-derived mediators.

GM-CSF is a potent growth factor for granulocytes and macrophages, and induces the differentiation of neutrophils, eosinophils and macrophages from myeloid progenitor cells (Clark and Kamen, 1987). GM-CSF also maintains the viability and enhances the activity of mature eosinophils and neutrophils. Our data indicates that GM-CSF production by mast cells is increased in the presence of PGE_2 and IgE-mediated activation, and such increased levels of secreted GM-CSF may partly explain the selective retention of granulocytes observed at sites of mast cell activation and PGE_2 production in chronic inflammation.

Previous studies examining the effects of PGE_2 on mast cell degranulation have led to conflicting findings. Several groups have reported an inhibitory effect of PGE_2 and PGE_1 on histamine release. Kaliner and Austen (1974) demonstrated that PGE_1 (1 μM) inhibited histamine release by rat mast cells in response to FcεRI cross-linking, and a similar inhibitory effect on degranulation was observed in human lung mast cells pre-incubated with PGE_2 (>1 μM) for 5 min prior to FcεRI cross-linking with anti-IgE (Peachell *et al.*, 1988). Hogaboam *et al.* (1993) reported that PGE_2 treatment inhibited histamine release in rat PMCs activated with calcium ionophore, A23187; however, PGE_2 was without effect on IgE-mediated histamine release by rat PMCs under the experimental conditions employed by this group. In contrast, PGE_2 has also been shown to potentiate histamine release by mast cells. Nishigaki *et al.* (1993) reported that PGE_2 potentiated ionomycin-mediated degranulation in the murine mast cell line, BNu-2cl3, and our group has previously demonstrated that although PGE_2 alone neither induced nor inhibited spontaneous histamine release by rat PMCs, PGE_2 enhanced such release from mast cells concurrently activated

with anti-IgE (Leal-Berumen *et al.*, 1995). Here, we further demonstrate PGE_2-mediated potentiation of degranulation in two different mast cell populations, BMMC and the IL-3-dependent mast cell line, MC/9. As observed in rat PMCs, PGE_2 treatment alone did not induce degranulation in either mast cell population yet strongly enhanced β-hexosaminidase release induced by IgE/Ag-activation.

The opposing stimulatory and inhibitory actions described for PGE_2 in the context of mast cell degranulation may reflect differences in the timing of PGE_2 treatment relative to the administration of other stimuli, and to possible differences in EP receptor subtype expression by the mast cell populations. In studies describing an inhibitory effect for PGE_2 on histamine release, mast cells were pre-incubated with PGE_2 for ≥ 5 min prior to the addition of the other stimuli (Hogaboam *et al.*, 1993; Peachell *et al.*, 1988); whereas in experiments where PGE_2 potentiated mast cell degranulation, concurrent activation with PGE_2 and the secretagogue was employed (Nishigaki *et al.*, 1993; Leal-Berumen *et al.*, 1995) (and the present study). Cyclic AMP has been implicated as the second messenger mediating PGE_2-directed inhibition of degranulation (Peachell *et al.*, 1988; Rossie and Miller, 1982; Soll and Toomey, 1989). Conversely, increased Ca^{2+} rather than cAMP was implicated in a study where degranulation was potentiated by PGE_2 (Nishigaki *et al.*, 1993), and these observations are not surprising considering the absolute requirement for increased intracellular Ca^{2+} in the induction of mast cell degranulation (Leal-Berumen *et al.*, 1996). The role of cAMP in mediating degranulation is less clear. Biphasic increases in cAMP are observed in IgE-mediated degranulation; however a causal link between increased cAMP and histamine release has not been established. Here, we have shown that cAMP-elevating

agents, forskolin, pentoxyfylline, and β-isoproterenol, fail to reproduce the enhancing effects of PGE_2 on both β-hexosaminidase release and IL-6 production, suggesting that the observed effects of PGE_2 are mediated by a cAMP-independent mechanism.

PGE_2 exerts its effects on target cells by interacting with specific G protein-coupled receptors, of which there are four subtypes (EP_1, EP_2, EP_3, and EP_4). EP_1 coupling elevates intracellular Ca^{2+} levels; signaling through EP_2 and EP_4 results in the activation of adenylate cyclase and subsequent increases in intracellular cAMP; and signaling through EP_3 is generally associated with diminished levels of intracellular cAMP although a number of splice variants of this receptor coupled to different G proteins have been described (Coleman *et al.*, 1994). Using RT-PCR, we have demonstrated that MC/9 cells express EP_1, EP_3, and EP_4, but not EP_2 receptors. The presence of EP_3 and EP_4 receptors has been reported for the mucosal type mast cells BNu-2cl3 (Nishigaki *et al.*, 1993) and P815 (Nishigaki *et al.*, 1995), respectively. EP_3 and EP_4 receptors are ubiquitously expressed in tissues (Narumiya *et al.*, 1996) and have been identified on murine macrophage-like cell line, RAW 264.7 cells (Arakawa *et al.*, 1996), primary and transformed murine B lymphocytes (Fedyk and Phipps, 1996; Fedyk *et al.*, 1996b), and human HSB.2 early T cells (Zeng *et al.*, 1998). EP_1 expression is somewhat more limited, and is most abundantly expressed in the kidney (Narumiya, 1996) where it is restricted to the collecting duct and regulates natriuretic actions of PGE_2 (Guan *et al.*, 1998). Using primers specific for EP_1, we observed two bands, a minor band of 336 bp corresponding to the expected PCR product and a stronger band of approximately 750 bp. Thus far, a splice variant for EP_1 receptors (EP_1-v) has only been described in the rat and arises from failure to use a potential splice site located in the 6^{th}

transmembrane domain (Okuda-Ashitaka *et al.*, 1996). In contrast to the EP_1 receptor, EP_1-v is devoid of a carboxy terminus and lacks signaling capacity. Experiments where CHO cells were co-transfected with EP_1 and EP_1-v showed that although the variant receptor alone was not coupled to Ca^{2+} mobilization, it inhibited Ca^{2+} mobilization mediated by EP_1 (Okuda-Ashitaka *et al.*, 1996) and hence, may serve as a sink for the EP_1 receptor (Pierce and Regan, 1998). The larger EP_1 PCR product observed in the present study is of the predicted size for a splice variant analogous to that observed in the rat, and did not arise from genomic DNA contamination in RNA samples. Sequence analysis confirmed that it contained the second intron as would be expected in the absence of splicing events occurring in the 6^{th} transmembrane domain during processing of primary RNA transcripts.

To identify the EP receptors mediating PGE_2-directed potentiation of degranulation and IL-6 production, MC/9 cells were stimulated with EP subtype-selective agonists in the presence of IgE/Ag-activation. Both the EP_1 agonist, 17-phenyl-ω-trinor-PGE_2, and the EP_1/EP_3-selective agonist, sulprostone, potentiated β-hexosaminidase release and IL-6 production in IgE/Ag-activated mast cells. Misoprostol, an EP_2/ EP_3/ EP_4-selective agonist also enhanced IgE-mediated degranulation. Such potentiation of degranulation or IL-6 production was not observed with the EP_2/ EP_4 -selective agonist, PGE_1 alcohol. PGE_1, a structural homologue of PGE_2 which binds EP_1 with weaker affinity than PGE_2 and binds with comparable affinity to EP_2, EP_3, and EP_4, enhanced IgE-mediated degranulation to a similar degree as PGE_2 but did not potentiate IL-6 production. Taken together, these findings strongly suggest the involvement of both EP_1 and/or EP_3 receptors in PGE_2-directed potentiation of degranulation and IL-6 production by IgE/Ag-activated mast cells.

The importance of EP_1 and/or EP_3 receptors in regulating mast cell function is intriguing in view of the fact that EP_2 and EP_4 receptors have generally been associated with immunological modulation. For instance, TNF-α inhibition in human blood monocytes (Meja *et al.*, 1997), B cell differentiation to IgE-secreting plasma cells (Fedyk and Phipps, 1996), and IL-8 production by human colonic epithelial cells (Yu and Chadee, 1998) have all been reported to be mediated by PGE_2 via EP_2 and/or EP_4 receptors. Moreover, in the human HSB.2 early T cell line, PGE_2 induced IL-6 production via EP_2/EP_4 receptors, and co-stimulation with Con A further enhanced IL-6 levels by upregulating EP_4 receptor expression and downregulating that of EP_2 and EP_3 (Zeng *et al.*, 1998). Interestingly, a study by Kozawa and colleagues (1998) investigating PGE_2-induced IL-6 synthesis in the murine osteoblast-like cell line, MC3T3, reported that both EP_1 and EP_2 receptors contributed to the production of IL-6. These data implicate the involvement of second messengers, Ca^{2+} and cAMP in IL-6 induction by osteoblasts, and a similar role for these two second messengers may be involved in IL-6 production by mast cells as rat PMC IL-6 production is both highly calcium dependent and is induced by the cAMP-elevating agent, cholera toxin (Leal-Berumen *et al.*, 1996). In the present study, EP_1 and/or EP_3 appear to play a substantial role in mast cell IL-6 production. Although activation of EP_3 receptors is generally associated with diminished intracellular cAMP levels, an isoform in the mouse has been shown, at higher agonist concentrations, to stimulate adenylate cyclase and increase intracellular cAMP levels (Coleman *et al.*, 1994). Coupling through EP_3 has also been linked with elevated Ca^{2+} in the murine mast cell line, BNu-2cl3 (Nishigaki *et al.*, 1993). Hence, stimulation of mast cells with PGE_2 alone may, through coupling to EP_1/ EP_3 receptors, elevate intracellular

Ca^{2+} and/or cAMP to levels exceeding the threshold required for IL-6 production, and it is possible that concurrent activation with PGE_2 and IgE/Ag results in synergism of such initial responses leading to potentiation of IL-6 production.

Taken overall, our results suggest a more complex role for PGE_2 in the modulation of allergic inflammation and disease than has been previously recognized. We have demonstrated that PGE_2 modulates IL-6 production in otherwise unstimulated bone marrow-derived mast cells with no changes in the production of many other cytokines or in the induction of mast cell degranulation. However, in the context of IgE-mediated activation, PGE_2 enhances preformed mediator release and selectively upregulates the production of IL-6 and GM-CSF, and these effects likely occur through coupling to EP_1 and/or EP_3 receptors. The residence of mast cells in the skin and mucosal linings positions them among our first line of defense against environmental insults, irritants, and pathogens. Mast cell mediators induce PGE_2 production by neighbouring tissue cells (Fournier *et al.*, 1997; Orehek *et al.*, 1975), and newly secreted PGE_2 may act to modulate cytokine production by mast cells and alter localized inflammatory reactions in an autocrine and paracrine manner. Understanding the mechanisms by which PGE_2 modulates cytokine production will undoubtedly be of prime importance if we are to harness the beneficial effects of prostanoids and related molecules in the treatment of inflammatory disease.

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Chapter 3: PGE_2 does not suppress $Fc_\varepsilon RI$ cross-linking-induced TNF-α production by mast cells

This manuscript will be submitted to the Journal of Immunology

Abstract

Tumour necrosis factor (TNF-α) is implicated in the pathogenesis of airways inflammation in asthma patients. Prostaglandin E_2 (PGE_2), an endogenously produced lipid mediator, has potent inhibitory effects on TNF-α production by a number of leukocytes. Here, we show that PGE_2 fails to suppress IgE-mediated TNF-α production by murine mast cells. Moreover, at concentrations $\geq 10^{-7}$ M, PGE_2 enhances the TNF-α response. PGE2-directed TNF-α enhancement was maximal when mast cells were activated concurrently with PGE_2 and IgE/Ag. PGE_2 mediates its effects by binding to one of four prostaglandin E receptor subtypes (EP_1-EP_4). PGE_2 inhibits TNF-α production by phagocytes through coupling of EP_2 and EP_4 receptors and elevating intracellular cAMP. In contrast, pharmacological studies employing selective EP agonists on a murine IL-3-dependent mast cell line (MC/9) indicated that PGE_2 enhanced mast cell TNF-α production through activation of $EP_1$and/or EP_3 receptors. Since coupling through EP_1 and EP_3 are associated with increases in Ca^{2+} and suppression of cAMP, respectively, these data suggested against a cAMP-dependent mechanism. Rather, these data implicated the possible involvement of intracellular calcium. PGE_2 was observed to induce transient increases in intracellular calcium in a dose-dependent manner, and co-activation with PGE_2 and $Fc_\varepsilon RI$-cross-linking resulted in greater initial peak rises in intracellular calcium and these levels were greatly sustained for prolonged periods. Our data indicate that PGE_2 can enhance TNF-α production, and might promote the release of this pro-inflammatory cytokine through an EP_2/EP_4- and cAMP-independent mechanism. These findings have important implications in the context of inflammatory states involving IgE-mediated mast cell activation.

Introduction

TNF-α is recognized as an important mediator in the pathogenesis of allergic disease (Kips *et al.*, 1993; Shah *et al.*, 1995). Levels of this pro-inflammatory cytokine are increased in the bronchoalveolar lavage (BAL) fluid of allergic asthmatics compared with non-asthmatics (Ying *et al.*, 1991). Moreover, TNF-α levels are significantly higher in asthmatics, manifesting a late phase response (LPR), than in those lacking such a response. TNF-α mediates a number of biological effects believed to contribute to the pathology of allergic inflammation (Papadakis and Targan, 2000). For instance, TNF-α aids in the recruitment of inflammatory cells through the upregulation of adhesion molecules such as E-selectin and ICAM-1 on endothelial cells (Rothlein *et al.*, 1988; Walsh *et al.*, 1991) as well as through direct chemotactic actions on leukocytes. TNF-α enhances production of granulocyte growth factors, and the IL-2 receptor (Leizer *et al.*, 1990; Hackett *et al.*, 1988), and potentiates the actions of the granulocyte growth factors, IL-3 and GM-CSF (Caux *et al.*, 1990). Furthermore, TNF-α enhances the cytotoxicity of monocytes and eosinophils (Philip and Epstein, 1986; Silberstein and David, 1986), and the phagocytic activity and cytotoxicity of neutrophils (Shalaby *et al.*, 1985). Consequently, TNF-α has been associated with the development of the cardinal features of allergic asthma, i.e., airways hyperresponsiveness, increased cellular infiltration into pulmonary tissues, and airways remodelling (Shah *et al.*, 1995).

TNF-α is produced by many cell types. Potential cellular sources in the airways include mononuclear phagocytes, neutrophils, epithelial cells, T lymphocytes, and mast cells (Kips *et al.*, 1993). Mast cells are unique in their ability to store pre-formed TNF-α in their cytoplasmic granules (Gordon and Galli, 1990; Beil *et al.*, 1994). Mast cells,

therefore, provide both an immediate and delayed source of TNF-α following allergen-induced activation (Gordon and Galli, 1990). They are critical for the recruitment of neutrophils in cutaneous LPR, with TNF-α identified as a key mediator in this response (Wershil *et al.*, 1991). Moreover, TNF-α mRNA and bioactivity have been localized to human lung mast cells (Ohkawara *et al.*, 1992; Bradding *et al.*, 1994), and TNF-α production was shown to be upregulated following IgE-mediated activation (Shah *et al.*, 1995).

TNF-α production by many cell types is regulated *in vivo* through the production of endogenous inhibitors. One of these is prostaglandin E_2 (PGE_2) (Eigler *et al.*, 1997). PGE_2 is a potent inhibitor of TNF-α production by murine macrophages (Kunkel *et al*, 1986; Kunkel *et al.*, 1988), neutrophils (Yamane *et al.*, 2000), and T cells (Ferreri *et al.*, 1992). PGE_2 has also been reported to inhibit constitutive TNF-α production by rat peritoneal mast cells (Hogaboam *et al.*, 1993; Leal-Berumen *et al.*, 1995). Due to the induction of PGE_2 by stromal cells and mononuclear phagocytes in response to TNF-α stimulation, PGE_2 is believed to function as part of an endogenous negative feedback loop for TNF-α production (Schultz *et al.*, 1978; Spengler *et al.*, 1989). PGE_2 binds to one of four G protein-coupled prostaglandin E receptors, denoted EP_1, EP_2, EP_3, and EP_4 (Coleman *et al.*, 1994). Coupling through EP_1 results in elevated intracellular calcium; EP_2 and EP_4 activate adenylate cyclase and increase intracellular cAMP; and EP_3 coupling is generally associated with an inhibition of cAMP, although activation of this receptor has also been linked with increased intracellular cAMP and calcium levels (Coleman *et al.*, 1994; Narumiya *et al.*, 1999). EP_2 and EP_4 receptors have been shown to mediate PGE_2-induced inhibition of TNF-α production both in macrophages (Ikegami

et al., 2001) and neutrophils (Yamane *et al.*, 2000); a role for cAMP was implicated in both cases. In support for the involvement of cAMP, similar suppressive effects on TNF-α production were induced by a number of cAMP-elevating agents including dbcAMP, 8-bromo-cAMP, forskolin, cholera toxin, and pentoxifylline (Strieter *et al.*, 1988; Taffet *et al.*, 1989).

Mast cell-derived TNF-α is clearly important in the pathogenesis of allergic inflammation (Wershil *et al.*, 1991), and production of this cytokine in mast cells is regulated differently than in other leukocytes (Blank and Varin-Blank, 2000). Nevertheless, little is known of the modulatory effects of PGE_2 on mast cell TNF-α production. In the present study, we investigated the actions of PGE_2 on TNF-α production by a murine IL-3-dependent mast cell line (MC/9) and primary cultures of mouse BMMC in the context of $Fc_\varepsilon RI$ cross-linking events.

Materials and Methods

Mast cells

MC/9 cells (American Type Culture Collection (ATCC); Manassas, VA) were routinely grown in modified Dulbecco's modified Eagle's medium (Invitrogen Canada Inc.; Burlington, ON) supplemented with 36 mg/ml L-aspartate, 0.1 mM non-essential amino acids, 5 x 10^{-5} M 2-ME, 10% FCS, and 10% v/v WEHI-3B-conditioned supernatant as a source of IL-3; or in RPMI medium (Invitrogen Canada Inc.) supplemented with 10% FCS, 1% penicillin/streptomycin, 0.1 mM non-essential amino acids, 5 x 10^{-5} M 2-mercaptoethanol, and 10% v/v WEHI-3B-conditioned supernatant. Bone marrow-derived mast cells (BMMC) were generated from the bone marrow of C57BL/6 mice. Briefly, mice were sacrificed, and intact femurs and tibias removed. Bone marrow was obtained by repeatedly flushing medium through the bone shaft and then passing the cells through a sterile wire screen to remove any bone fragments. Following centrifugation at 320 x g for 20 min at 4°C, cells were cultured at 0.5 - 1 x 10^6 nucleated cells/ml in RPMI medium (Invitrogen Canada Inc.) supplemented with 10% FCS (SIGMA-Aldrich Canada Ltd., Oakville, ON), 1% penicillin/streptomycin (Invitrogen Canada Inc.), 50 μM 2-ME, and 20% WEHI-3B-conditioned supernatant. At least once a week, cell cultures were replenished with fresh medium. Highly pure mast cell populations (> 95% mast cell purity as determined by Toluidine blue staining) were obtained after 4-6 weeks of culture.

IgE-mediated mast cell activation

BMMC and MC/9 cells were passively sensitized with anti-DNP IgE (Gift from Dr. F.T. Lui) or anti-TNP IgE (ATCC) for 18-30 h at 37°C, in their respective media. Mast cells were centrifuged several times to remove unbound IgE and then used immediately in experiments. Cross-linking of $Fc_{\varepsilon}RI$ was induced by incubating sensitized cells with DNP-human serum albumin (DNP-HSA; SIGMA-Aldrich Canada Ltd) or TNP- bovine serum albumin (TNP-BSA; Biosearch Technologies, Inc.; Novato, CA) at a concentration of 10 ng/ml for 15 min to examine pre-formed mediator release, or for up to 24 hours to investigate TNF-α production.

Mast cell activation with various stimulating agents

Mast cells were resuspended in experimental medium (RPMI-1640 (Invitrogen Canada Inc.), 10% FCS (SIGMA-Aldrich Canada Ltd.), 1% penicillin/streptomycin (Invitrogen Canada Inc.), 1% HEPES (Invitrogen Canada Inc.), 100 μg/ml soybean trypsin inhibitor (SIGMA-Aldrich Canada Ltd.; reconstituted in saline), and 1.5 ng/ml rmIL-3 (PeproTech, Inc., NJ)). Mast cells, at 1 x 10^6 cells/ml, were stimulated with the following reagents either alone or in the context of IgE-mediated activation: PGE_2 (SIGMA-Aldrich Canada Ltd.); pentoxifylline, forskolin, dbcAMP, and β-isoproterenol (purchased from SIGMA-Aldrich Canada Ltd.); and PGE_1, PGE_1 alcohol, 17-phenyl-ω-trinor-PGE_2, sulprostone, and misoprostol (purchased from Cayman Chemical Company; Ann Arbor, MI). Samples were stored at ≤-20°C until assayed for mediator content.

Determination of TNF-α levels

TNF-α levels were determined by ELISA using commercially available kits (R&D Systems Inc.; Minneapolis, MN) or by employing an in-house assay using the following antibody pair: goat anti-mouse TNF-α polyclonal (AF-410-NA; R&D Systems Inc.)/ biotinylated rat anti-mouse TNF-α monoclonal (MM-350D-B; Endogen, Inc.; Woburn, MA).

β-hexosaminidase release assay

Mast cells at 1 x 10^6 cells/ml were resuspended in HEPES-Tyrodes buffer (137 mM NaCl, 5.6 mM glucose, 2.7 mM KCl, 0.5 mM NaH_2PO_4, 1 mM $CaCl_2$, 10 mM HEPES, 0.1% BSA, pH 7.3, ~300 mOsm/kg) in the presence of various stimulating agents for 15 min at 37°C. β-hexosaminidase release was stopped by centrifugation (140 x g; 10 min; 4 °C). Supernatants were transferred to clean eppitubes. β-hexosaminidase remaining in the cell pellet fraction was released as follows: pellets were first resuspended with the original volume of HEPES-Tyrodes buffer lacking the stimulating agents; the cell suspension was freeze-thawed several times in quick succession to lyse the cells; and cellular debris was pelleted by centrifugation and supernatants assayed to determine β-hexosaminidase content in the pellets. β-hexosaminidase levels in the supernatant and pellet samples was determined as described previously (Zhu *et al.*, 1998). Briefly, 50 µl of samples were placed in individual wells of a 96-well plate followed by the addition of 50 µl/well of 1 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (SIGMA-Aldrich Canada Ltd.) dissolved in 0.1 M citrate buffer, pH 5. After incubating the plate for 1 h at 37°C, 200 µl/well of 0.1 M carbonate buffer (pH 10.5) was added. The intensity of the

resulting yellow reaction product was read at 405 nm in an ELISA reader, and net percent β-hexosaminidase release was calculated: β-hexosaminidase in supernatant/ (β-hexosaminidase in supernatant + β-hexosaminidase in pellet) x 100%.

Intracellular Ca^{2+} measurement

Mast cells were washed three times with HBSS (Invitrogen Canada Inc.) containing 0.1% BSA. The cells were then resuspended in HBSS supplemented with 10 mM HEPES (Invitrogen Canada Inc.). The cells were loaded at a cell concentration of 5-10 x 10^6 cells/ml with 4 μM fluo-4/AM (Molecular Probes, Eugene, OR) for 30 min at 37 °C. Mast cells were washed twice with 0.1% BSA/HBSS, and resuspended at a concentration of 1 x 10^6 cells/ml in HBSS supplemented with 10 mM HEPES and 1.5 mM $CaCl_2$. Mast cells were stimulated with the desired agent in the presence of continuous magnetic stirring, and fluorescence was measured in a RF-1501 spectrofluorophotometer (Shimadzu Scientific Instruments, Inc., Columbia, MD; excitation and emission wavelengths were 485 and 520 nm, respectively). Calcium signals were analyzed using PC-1501 Personal Fluorescence Software (Shimadzu Scientific Instruments, Inc.).

Statistical Analysis

All data are expressed as the mean ± SEM. Statistical analysis was performed by one-way ANOVA followed by the appropriate post-test. Due to variability in the TNF-α responses to IgE-mediated activation, the effects of additional stimuli were usually expressed as percent change where the levels induced by IgE/Ag-activation alone was normalized to 100%. Percent data values were first subjected to Log transformation and

then analyzed by Dunnett multiple comparison post test for determination of statistical significance.

Results

PGE_2 enhances IgE-mediated TNF-α production by mast cells

BMMC were passively sensitized with anti-TNP or anti-DNP IgE, and subsequently activated with specific antigen in the presence of various doses of PGE_2. IgE-mediated activation alone induced the production of significant amounts of TNF-α (313 ± 94 pg/ml; mean ± SEM calculated from seven independent experiments). In contrast to its known suppressive effects on TNF-α production by other cell types, PGE_2 did not inhibit IgE-mediated TNF-α production by BMMC; moreover, TNF-α production was enhanced by PGE_2 concentrations of $\geq 10^{-7}$ M and the degree of enhancement was significant at 10^{-6} M ($p < 0.01$) (Fig. 1). PGE_2 treatment alone at any of the concentrations tested did not induce significant TNF-α production.

Kinetics of the effect of PGE_2 on IgE-mediated TNF-α production

We next performed kinetic studies of the TNF-α-enhancing effect of PGE_2. PGE_2 increased IgE-mediated TNF-α production by BMMC within a few hours of mast cell activation, and was maximal 6 h post-stimulation (Fig. 2). BMMC treated with a combination of IgE/Ag and PGE_2 continued to release TNF-α levels in greater quantities at 24 h post-activation than cells activated by IgE/Ag alone (Fig. 2). There was some variation between experiments in the TNF-α response observed 1h post-activation but pooled results did not demonstrate any significant effect of PGE_2.

PGE_2 also enhances TNF-α production by the IL-3 -dependent mast cell line, MC/9

To confirm that the effects we observed in BMMC were not the due to contributions by low numbers of non-mast cells contaminating the culture, we carried out analogous studies in the IL-3 dependent murine mast cell line, MC/9. Comparable findings were obtained in studies with this cell line, i.e., PGE_2 similarly potentiated IgE/Ag-elicited TNF-α production (Table I). A few differences were noted, however. Although both BMMC and MC/9 cells released similar levels of TNF-α 1 h following co-activation with PGE_2 and IgE/Ag, PGE_2 enhanced IgE-mediated TNF-α production by at least 130% in the MC/9 (Table I) whereas no such enhancement was seen in the BMMC (92 ± 22 % of IgE/Ag response; mean from three independent experiments) at this early time point. Yet, by 6 h post-stimulation, PGE_2 greatly enhanced the TNF-α response in BMMC. It should be noted that in contrast to BMMC where PGE_2 continued to enhance IgE/Ag-elicited TNF-α for at least 24 h (Fig. 2), TNF-α levels in MC/9 activated via IgE/Ag in the presence or absence of PGE_2. The loss of the TNF-α signal may be a result of degradation by protease enzymes. Nevertheless, the phenomenon of TNF-α potentiation by PGE_2 was clearly evident in both cell types.

It was unlikely that TNF-α measured in the supernatants of IgE/Ag-activated MC/9 cells represented granule-associated, pre-formed TNF-α since cell pellets obtained from resting MC/9 (five different preparations of NaB-treated MC/9 were tested at concentrations of 2-10 x 10^6 cells/ml) contained TNF-α levels that fell below the level of detection of the ELISA (limit of detection = 20-30 pg/ml). Moreover, the amount of TNF-α measured in supernatants even 1 h following PGE_2 and IgE/Ag-stimulation far exceeded any baseline levels of TNF-α. Similarly, the amounts of TNF-α in cell pellets

obtained from three independent batches of BMMC (cell concentrations of 10–20 x 10^6 cells/ml) were also undetectable in the ELISA.

Involvement of EP_1 and/or EP_3 receptors

We next examined the prostaglandin E receptor(s) mediating the effects of PGE_2 on TNF-α production. Pharmacological studies showed that PGE_2 analogs which selectively bound to EP_1 and/or EP_3 receptors (17-phenyl-ω-trinor-PGE_2, sulprostone, misoprostol) mimicked PGE_2 and significantly enhanced TNF-α ($p < 0.01$) at the 10^{-6} M dose (Table II). In contrast, the $EP_{2/4}$-selective agonist, PGE_1 alcohol failed to enhance IgE-mediated TNF-α production. PGE_1 weakly enhanced IgE-mediated TNF-α elaboration; however, this effect was not significant at any dose examined (Table II).

Timing of PGE_2 addition

We investigated whether pretreatment with PGE_2 and various EP-selective agonists would modulate IgE-mediated TNF-α production differently than concurrent treatment. Pretreatment of mast cells with PGE_2 enhanced subsequent IgE-mediated TNF-α production, albeit to a lower degree than concurrent activation with PGE_2 and IgE/Ag (Fig. 3A). It was apparent though that pretreatment with PGE_2 and the EP-selective agonists generally resulted in a greater degree of variability regarding their effects on IgE-mediated TNF-α production (Fig. 3A). It was noteworthy, however, that pretreating mast cells with PGE_1 consistently resulted in significant inhibition of TNF-α ($p < 0.001$). These data are in contrast to the absence of TNF-α modulation by PGE_1 when added concurrently with the initiation of $Fc_\varepsilon RI$ cross-linking events (Fig. 3A).

The effects of pretreatment by PGE_2 and selected EP agonists on mast cell degranulation were also examined. It has been reported that pretreatment for 5 min with PGE_2 inhibits subsequent IgE-mediated mast cell degranulation (Kaliner and Austen, 1974; Peachell *et al.*, 1988). Pretreatment with PGE_2 for 30 min and 5 min prior to IgE/Ag-activation resulted in a lower degree of TNF-α enhancement than concurrent treatment; however, degranulation was not inhibited by either pretreatment protocol (Fig. 3B). Similar results were obtained with the $EP_{1/3}$-selective agonist, 17-phenyl-ω-trinor-PGE_2 (Fig. 3B). Pretreatment with the $EP_{2/4}$-selective agonist PGE_1 alcohol also did not suppress IgE/Ag-induced degranulation (Fig. 3B).

Putative involvement of calcium as a second messenger

Pharmacological data suggested that EP_1 and/or EP_3 receptors, both of which are expressed by MC/9 (Gomi *et al.*, 2000), were mediating the TNF-α enhancement effects of PGE_2 and IgE/Ag–activated cells (Table II). Both receptors have been documented to induce increases in intracellular calcium (Narumiya *et al.*, 1999). We therefore examined the ability of PGE_2 to enhance intracellular calcium levels in mast cells. Treatment of MC/9 with PGE_2 alone induced transient increases in calcium in a dose dependent manner (data not shown). Following PGE_2 treatment, mast cells were, at least temporarily, desensitized to further stimulation by PGE_2 (Fig. 4B). PGE_2 failed to increase intracellular calcium in the absence of exogenous calcium (data not shown), suggesting that PGE_2 was not activating the release of intracellular calcium stores.

IgE-mediated activation also induced transient increases in intracellular calcium (Fig. 4A), and co-activation of anti-TNP-IgE-sensitized cells with PGE_2 and TNP-BSA

resulted in a synergistic increase in calcium that was sustained for a prolonged period (> 5 min) (Fig. 4C and Table III).

cAMP-elevating agents fail to enhance IgE-mediated TNF-α production

The $EP_{2/4}$ selective agonist, PGE_1 alcohol, tested at the highest dose, did not significantly enhance TNF-α production by IgE/Ag-activated mast cells (Table II), suggesting the involvement of a cAMP-independent mechanism. To further study the role of cAMP, we examined the effects of two cAMP-elevating agents, forskolin and pentoxifylline, on TNF-α production by IgE/Ag-activated MC/9 cells. Treatment with forskolin or pentoxifylline alone did not alter baseline TNF-α levels (data not shown). Pentoxifylline at 1 mg/ml potently suppressed IgE-mediated TNF-α production (Fig. 5) whereas forskolin at 1 μM (data not shown) and 10 μM (Fig. 5) failed to significantly alter the TNF-α response. Overall, these data suggest that cAMP is unlikely to be mediating the enhancing effects of PGE_2.

Figure 1. **Effects of PGE_2 on TNF-α production by IgE/Ag-activated mast cells.** BMMC were passively sensitized with anti-DNP or anti-TNP IgE and were subsequently washed and incubated with the corresponding antigen alone, or in the presence of increasing doses of PGE_2. After 24 h incubation at 37 °C in a 5% CO_2 humidified atmosphere, supernatants were harvested and assayed for TNF-α content. Bars represent mean % change ± SEM in TNF-α production from that induced by IgE-mediated activation alone. Data was calculated from seven independent experiments. **, denotes $p < 0.01$ with respect to IgE-mediated activation alone.

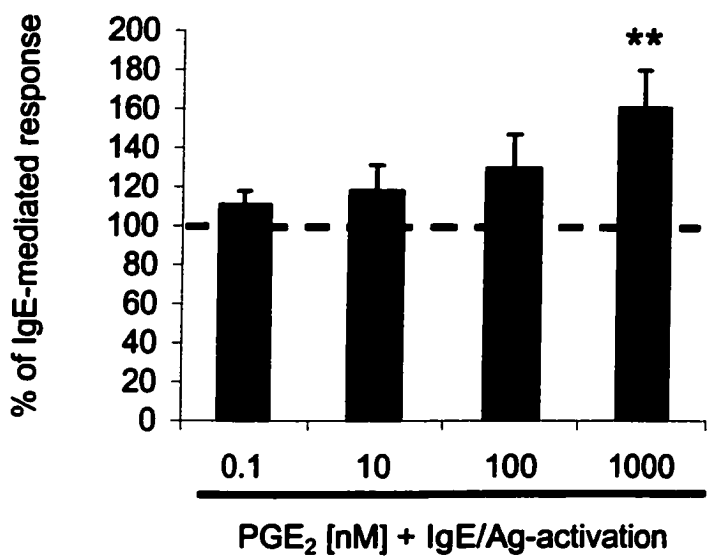
% of IgE-mediated response
200
180
160
140
120
100
80
60
40
20
0
**
0.1
10
100
1000
PGE2 [nM] + IgE/Ag-activation

Figure 1

Figure 2. **Kinetics of TNF-α production by PGE_2 and IgE/Ag-activated mast cells.** BMMC were passively sensitized with anti-TNP IgE, and activated with 10 ng/ml TNP-BSA in the presence or absence of 1 μM PGE_2. Supernatants were harvested following 1, 6, 12, and 24 h of incubation, and assayed for TNF-α content. A representative experiment from three independent experiments is shown.

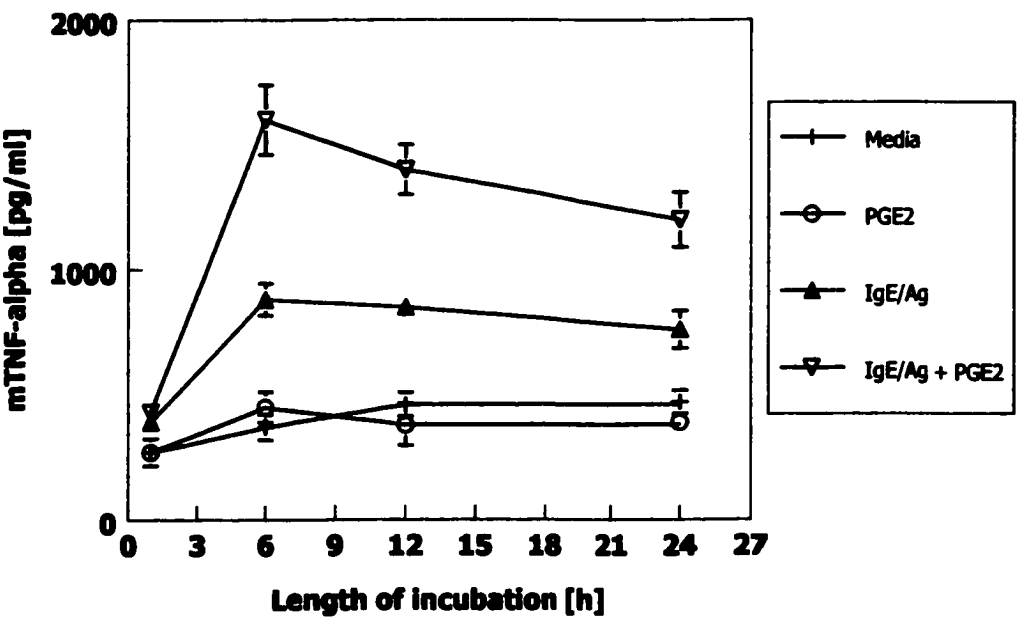
2000
1000
0
mTNF-alpha [pg/ml]
0
3
6
9
12
15
18
21
24
27
Length of incubation [h]
Media
PGE2
IgE/Ag
IgE/Ag + PGE2

Figure 2

Table I. **Kinetics of TNF-α production by MC/9 cells.** MC/9 were passively sensitized with anti-TNP IgE as described in *Materials and Methods*, and incubated with 10 ng/ml TNP-BSA in the presence ('IgE/Ag + PGE_2') or absence ('IgE/Ag') of PGE_2. Supernatants were collected at the indicated times and assayed for TNF-α content by ELISA.[b] Data are shown in pg/ml and represent the mean of triplicate samples for each condition.[c] '% of IgE/Ag response' presented in parentheses where the amount of TNF-α produced by IgE/Ag-mediated activation alone was normalized to 100%.

Experiment	1h		6h	
	IgE/Ag	IgE/Ag + PGE_2	IgE/Ag	IgE/Ag + PGE_2
#1	148[a]	192 (130%)[b]	128	229 (179%)
#2	72	187 (261%)	58	211(367%)

Table I

Table II. **Effects of EP-selective agonists on the TNF-α response by IgE/Ag-activated MC/9 cells.** Data is presented as mean % change in TNF-α response ± SEM from levels elicited by IgE-mediated activation alone (normalized to 100 %). MC/9 cells were passively sensitized with anti-TNP IgE and concurrently incubated with 10 ng/ml TNP-BSA and increasing doses of PGE_2, PGE_1, EP_1 agonist 17-phenyl-trinor-PGE_2, $EP_{1/3}$-selective agonist sulprostone, $EP_{2/3/4}$-selective agonist misoprostol, or $EP_{2/4}$-selective agonist PGE_1 alcohol. TNF-α production was determined in cell-free supernatants following 6 h incubation. **, denotes $p < 0.01$ with respect to IgE-mediated activation alone. Data shown are the mean of five independent experiments.

	Percentage change in TNF production by IgE/TNP-activated MC/9 cells following treatment with EP receptor agonists		
	0.1 nM	10 nM	1000 nM
PGE_2	99 ± 24[a]	110 ± 29	190 ± 44**
PGE_1	110 ± 24	120 ± 30	130 ± 41
17-Phenyl-trinor-PGE_2	86 ± 18	89 ± 20	180 ± 48**
Sulprostone	84 ± 14	130 ± 27	180 ± 47**
Misoprostol	99 ± 13	97 ± 17	180 ± 33**
PGE_1 Alcohol	96 ± 17	100 ± 16	110 ± 26

Table II

Figure 3. **Effects of timing of PGE_2 addition on IgE-mediated TNF-α production (A).** MC/9 were either pre-treated with PGE_2 or EP-selective agonists (1 μM) for 30 min prior to the addition of 10 ng/ml TNP-BSA, or were concurrently activated with both stimuli. **(B)** BMMC were pre-treated with PGE_2 or EP-selective agonists (1 μM) for 5 or 30 min prior to the addition of 10 ng/ml TNP-BSA. Alternatively, BMMC were activated concurrently with both stimuli. Supernatants were harvested 6h **(A)** or 15 min **(B)** following the initiation of IgE-mediated activation, and assayed for TNF-α or β-hexosaminidase content, respectively. In **(A)**, bars represent the mean % change (± SEM) from the IgE/Ag-induced response. Data was calculated from five independent experiments for PGE_2 and four independent experiments for the EP-selective agonists. In **(B)**, bars represent the mean % change (± SEM) from the IgE/Ag-induced response of three independent experiments. . **, denotes $p < 0.01$ with respect to IgE-mediated activation alone.

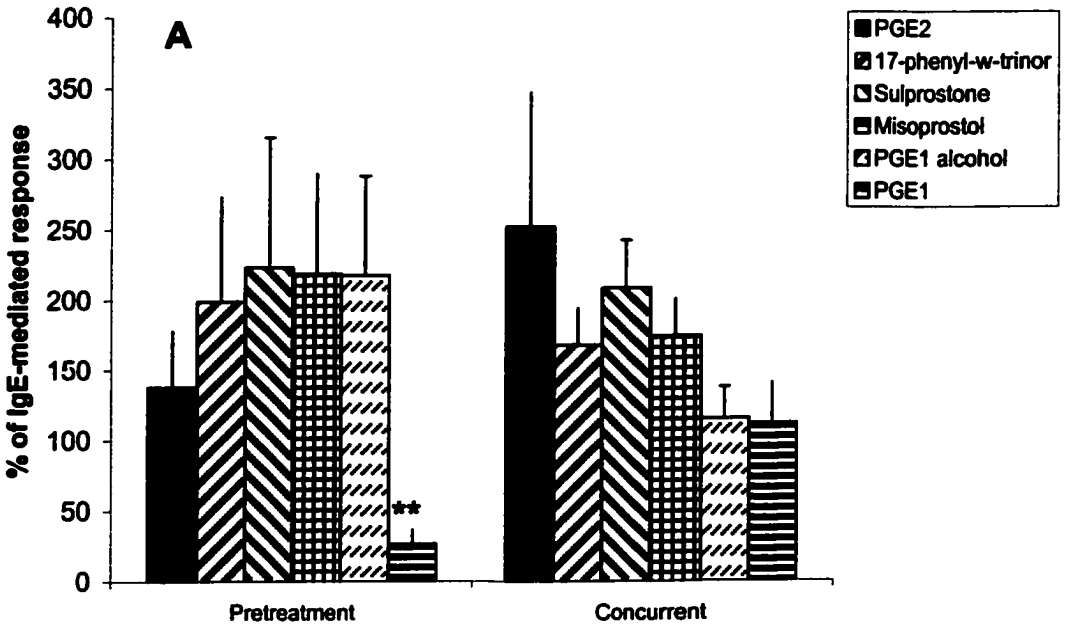
A
400
350
300
250
200
150
100
50
0
% of IgE-mediated response
**
Pretreatment
Concurrent
PGE2
17-phenyl-w-trinor
Sulprostone
Misoprostol
PGE1 alcohol
PGE1

Figure 3A

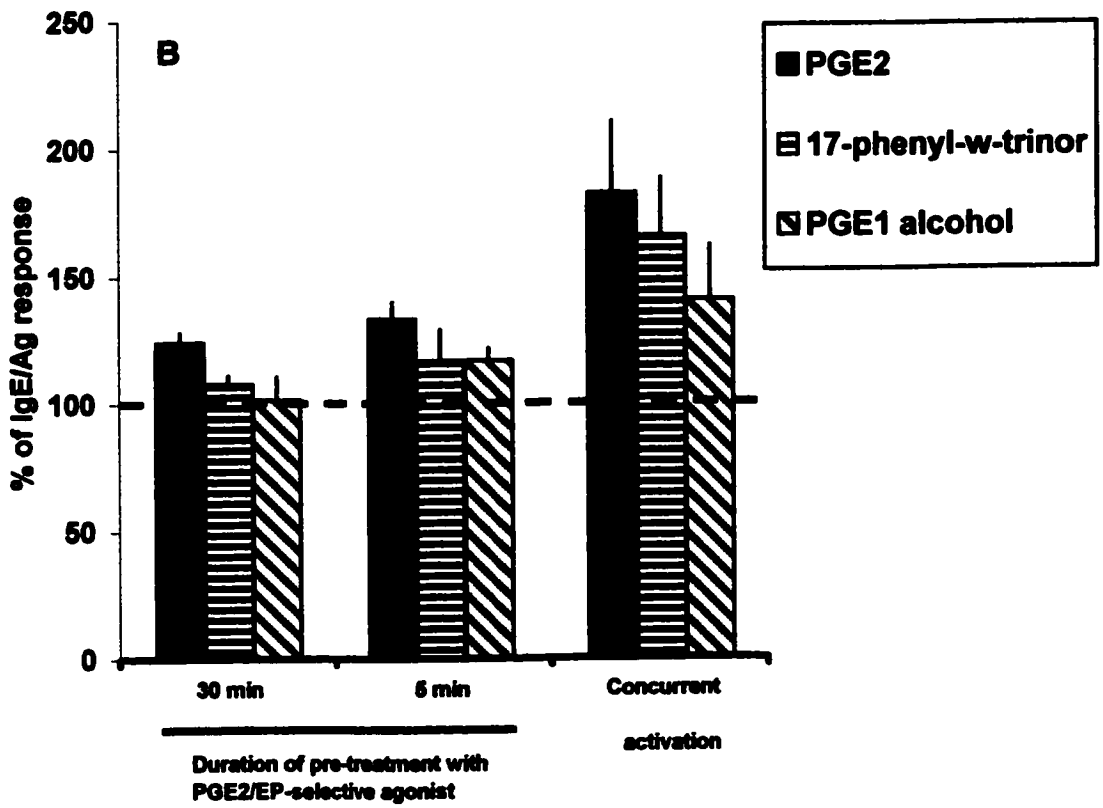
B
250
200
150
100
50
0
% of IgE/Ag response
PGE2
17-phenyl-w-trinor
PGE1 alcohol
30 min
5 min
Concurrent
activation
Duration of pre-treatment with
PGE2/EP-selective agonist

Figure 3B

Figure 4. **Increases in intracellular calcium elicited by PGE_2 and IgE-mediated activation of mast cells.** Following sensitization with anti-TNP IgE, MC/9 were loaded with 4 μM fluo-4/AM as described in *Materials and Methods*. Mast cells were activated, in the presence of 1.5 mM $CaCl_2$, with increasing doses of PGE_2. Changes in fluorescence were recorded by a fluorometer (λ_{ex} = 485 nm; λ_{em} = 520 nm). Mast cells were activated, in the presence of 1.5 mM $CaCl_2$, with **(A)** 10 μg/ml TNP-BSA, **(B)** 10 μM PGE_2, or **(C)** a combination of both TNP-BSA and PGE_2.

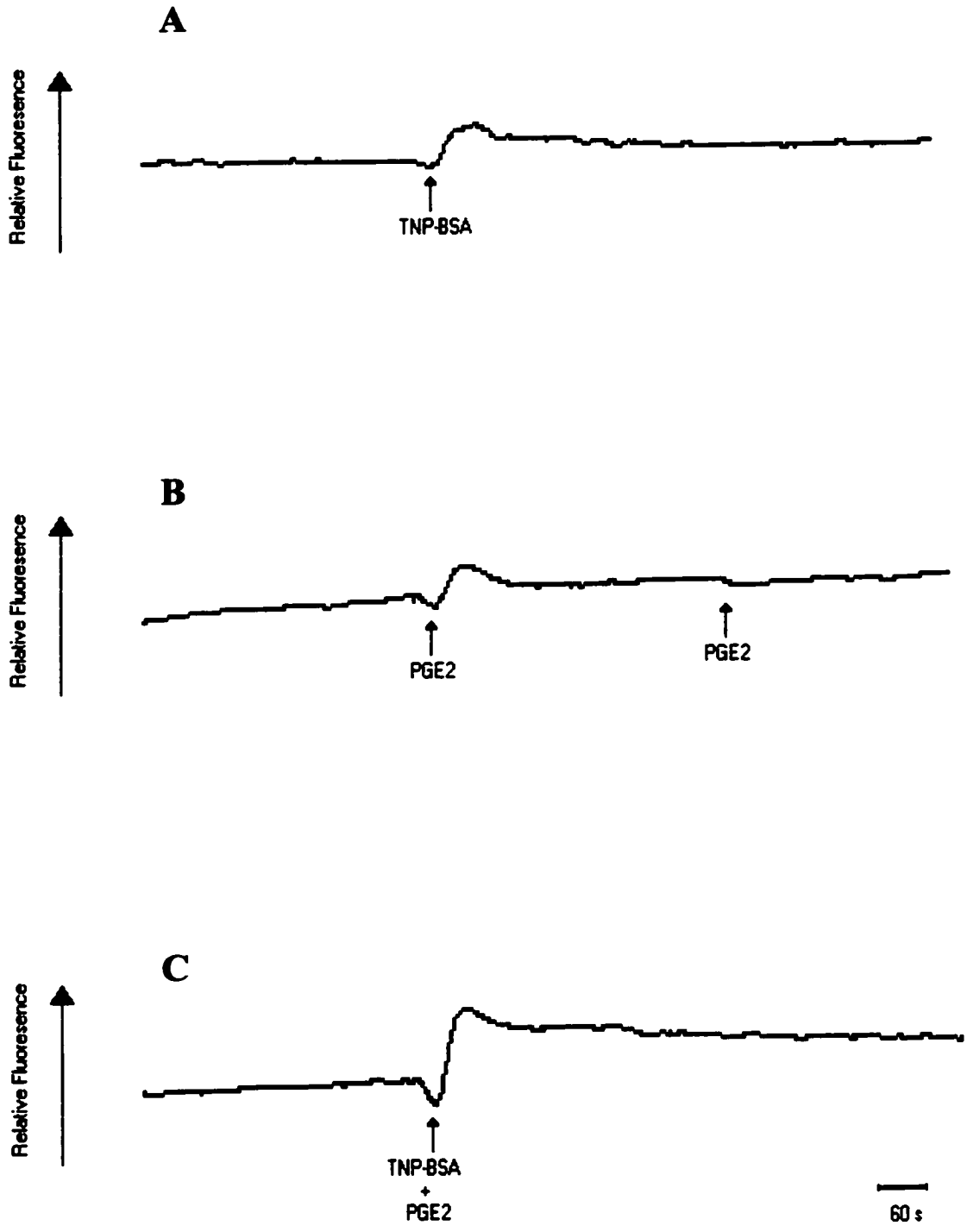
A
Relative Fluorescence
TNP-BSA
B
Relative Fluorescence
PGE2
PGE2
C
Relative Fluorescence
TNP-BSA
+
PGE2
60 s

Figure 4

Table III. **IgE/Ag-induced calcium influx is enhanced by PGE_2.** Mast cells were activated, in the presence of 1.5 mM $CaCl_2$, with 10 μg/ml TNP-BSA, 10 μM PGE_2, or a combination of both TNP-BSA and PGE_2. Changes in calcium influx for three independent experiments were tabulated in terms of maximum peak height and peak area under the curve for the first 300 s following stimulation with the given activator(s).

Experiment	Stimulation	Max. peak ht	Peak area
1	PGE_2	32.2	2270
	IgE/Ag	34.6	2990
	IgE/Ag + PGE_2	73.7	8660
2	PGE_2	14.3	1030
	IgE/Ag	25.8	2470
	IgE/Ag + PGE_2	35.3	4250
3	PGE_2	15.1	1550
	IgE/Ag	25.7	1800
	IgE/Ag + PGE_2	46.3	3890

Table III

Figure 5. **Effects of forskolin and pentoxifylline on IgE-mediated TNF-α production by mast cells.** Anti-TNP IgE-sensitized MC/9 were activated with 10 ng/ml TNP-BSA in the presence or absence of forskolin (10 μM), pentoxifylline (1 mM), or PGE_2 (1 μM). Following 6h incubation at 37°C in a 5% CO_2 humidified atmosphere, supernatants were harvested and assayed for TNF-α content. Bars represent the mean % change (± SEM) in TNF-α production from that elicited by IgE-mediated activation alone compiled from three independent experiments for PGE_2 and forskolin, and from two independent experiments for pentoxifylline treatment.

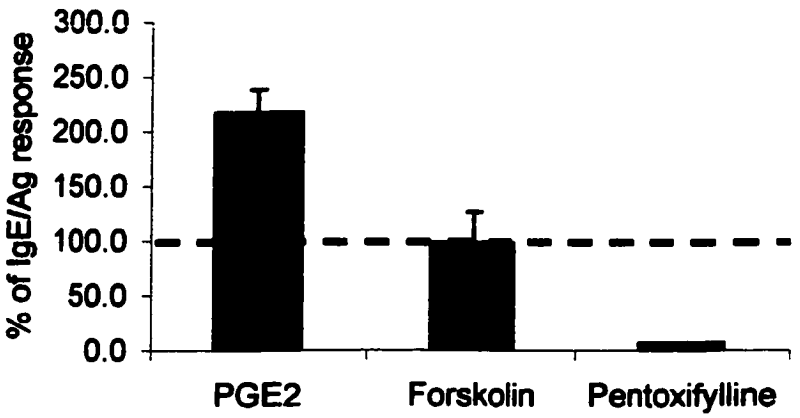
% of IgE/Ag response
300.0
250.0
200.0
150.0
100.0
50.0
0.0
PGE2
Forskolin
Pentoxifylline

Figure 5

Discussion

The inhibitory actions of PGE_2 on TNF-α production in various leukocytes are well-established. For instance, macrophages release large amounts of TNF-α in response to LPS stimulation. PGE_2 potently inhibits such TNF-α production (Kunkel *et al.*, 1986; Kunkel *et al.*, 1988). Moreover, PGE_2 inhibits TNF-α production by LPS-treated neutrophils (Yamane *et al.*, 2000) and mitogenically-activated T lymphocytes (Ferreri *et al.*, 1992). TNF-α is itself an inducer of PGE_2 production by macrophages and stromal cells; consequently, PGE_2 is speculated to participate in a negative feedback system for TNF-α production (Kunkel *et al.*, 1986). In the present study, we demonstrated that contrary to its inhibitory effects in other cell types, PGE_2 fails to suppress TNF-α production by IgE/Ag-activated mast cells. At concentrations $\geq 10^{-7}$ M, PGE_2 potentiated IgE-mediated TNF-α production. Levels of PGE_2 are elevated in a number of inflammatory disease processes including rheumatoid arthritis and dermatitis. Increased PGE2 levels have also been reported in the BAL of mice

Although many cell types produce TNF-α, mast cells are the only known cell type documented to store pre-formed TNF-α within their granules (Gordon and Galli, 1990). Therefore, mast cells provide an immediate source of TNF-α following $Fc_\varepsilon RI$ cross-linking events (Gordon and Galli, 1990). We have previously shown that PGE_2 potentiates mast cell degranulation in the context of IgE-mediated activation (Gomi *et al.*, 2000). Hence PGE_2 would concomitantly enhance the release of pre-formed TNF-α. Preformed TNF-α likely contributed little, if at all, to the enhanced production of TNF-α induced by PGE_2 in mouse BMMC and the MC/9 mast cell line. Both BMMC and MC/9

contained negligible amounts of pre-formed TNF-α. The absence of detectable pre-formed TNF-α in these cells, together with results from kinetic study, indicate that PGE_2 promoted *de novo* TNF-α production in immunogically activated mast cells.

Regarding our observations of PGE_2-directed potentiation of $Fc_\varepsilon RI$-induced degranulation, these data contradicted those reported by others who ascribed an inhibitory role to PGE_2 (Kaliner and Austen, 1974; Peachell *et al.*, 1988). It was possible that the discrepancy might be due to differences in timing the addition of PGE_2. Studies revealing an inhibitory effect involved pre-treating mast cells for five minutes prior to IgE/Ag-activation. In our studies, mast cells were activated concurrently with both stimuli. However, pretreatment of BMMC with PGE_2 for up to 30 minutes failed to inhibit degranulation although the degree to which PGE_2 potentiated subsequent IgE-mediated TNF-α enhancement was reduced. Timing of PGE_2 addition relative to $Fc_\varepsilon RI$ cross-linking events was, therefore, unlikely to be the determinant responsible for the paradoxical effects of PGE_2 on mast cell degranulation. This view is supported by a recent study conducted in rat PMC which reported that PGE_2 inhibited degranulation regardless of whether it was added 5 min prior to or concurrently with IgE-mediated activation of the cells (Ka-Ming Chang *et al.*, 2000).

PGE_2 binds to four prostaglandin E receptor subtypes. These are denoted EP_1-EP_4, and are coupled to specific signaling pathways (Coleman *et al.*, 1994; Narumiya *et al.*, 1999). The subtype(s) of prostaglandin E receptor expressed and activated by PGE_2 would largely dictate the modulatory effect of PGE_2 on TNF-α production. Not surprisingly, PGE_2 inhibits TNF-α production in mononuclear phagocytes and neutrophils through the activation of EP receptor subtypes known to increase intracellular

cAMP levels, i.e. EP_2 and EP_4 (Katsuyama *et al.*, 1998; Yamane *et al.*, 2000). The MC/9 murine mast cell line used in the present study expressed mRNA for EP_1, EP_3, and EP_4 (Gomi *et al.*, 2000). The inability of cAMP-elevating agents, forskolin and pentoxifylline, to mimic the TNF-α enhancing effects of PGE_2 in IgE/Ag-activated mast cells suggested that PGE_2 was probably not acting through EP_4. The $EP_{2/4}$-selective agonist, PGE_1 alcohol, failed to significantly increase IgE-mediated TNF-α production, providing further evidence that EP receptor subtypes other than EP_4 were involved. Accordingly, several EP_1 and/or EP_3-selective agonists (17-phenyl-ω-trinor-PGE_2, sulprostone, misoprostol) reproduced the potentiating activity of PGE_2 on TNF-α production. Because PGE_2 also mediated enhancement of IL-6 production by IgE/Ag-activated mast cells through EP_1, and possibly EP_3, receptors (Gomi *et al.*, 2000), these data suggest that EP_1 and/or EP_3-mediated enhancement of TNF-α production may represent a more general means by which PGE_2 modulates mast cell responses. It is interesting that PGE_2 exerts its diverse array of effects on most other leukocytes through activation of EP_2 and EP_4 receptor subtypes. For instance, EP_2 and EP_4 receptor subtypes have been implicated in PGE_2-directed augmentation of IgE production by B lymphocytes (Fedyk *et al.*, 1996), IL-6 production by early T cells (Zeng *et al.*, 1998), and differential modulation of TNF-α and IL-6 production by LPS-stimulated macrophages and neutrophils (Katsuyama *et al.*, 1998; Yamane *et al.*, 2000).

Activation of EP_1 receptors results in increased levels of intracellular calcium, whereas coupling of EP_3 receptors is typically linked to inhibition of cAMP, although increases in cAMP and intracellular calcium may occur through the activation of certain EP_3 splice variants (Narumiya *et al.*, 1999; Breyer *et al.*, 2001). The TNF-α gene

promoter region is positively regulated by calcium. In support of a role for calcium in mediating the effects of PGE_2 on mast cell TNF-α production, stimulation of MC/9 by PGE_2 alone induced dose-dependent transient spikes in intracellular calcium. Furthermore, PGE_2 greatly increased the calcium signal induced by $Fc_\varepsilon RI$ cross-linking, and enhanced levels of calcium were sustained over a prolonged period. It is plausible that such sustained increases in calcium would modulate TNF-α production at the transcriptional level, perhaps by permitting continued activity of NF-AT in the nucleus.

In addition to its modulation of TNF-α production, PGE_2 is known to inhibit the production of type 1 cytokines (e.g. IL-2, IL-12) while having no effect or enhancing type 2 cytokine production (e.g. IL-4, IL-5, IL-6) by Th cells and antigen-presenting cells (Fedyk *et al.*, 1996). PGE_2 also promotes the induction of IgE responses and simultaneously downregulates IgM and IgG1 synthesis. More recently, PGE_2 has been suggested to function as a tissue-derived signal that governs the development of primary immune responses with the Th2 phenotype (Kalinski *et al.*, 1998; Vieira *et al.*, 2000).

There is growing recognition of the participation of TNF-α in the pathogenesis of type 2 inflammatory disorders such as asthma (Shah *et al.*, 1995). Increased levels of TNF-α have been detected in asthmatics, especially in those exhibiting LPR (Ying *et al.*, 1991). TNF-α has been implicated in recruiting inflammatory cells, particularly neutrophils, into the airways, and also with mediating airways hyperreactivity and remodelling events (Shah *et al.*, 1995). Our data demonstrate that PGE_2 enhances mast cell TNF-α production induced by cross-linking of $Fc_\varepsilon RI$, and raises the provocative possibility that the resistance of IgE-mediated TNF-α production to inhibition by PGE_2 might be part of the underlying mechanism for the co-existence of increased levels of

both PGE_2 and TNF-α during certain inflammatory disorders including those characterizing asthma.

PGE_2-directed increases in IgE-mediated TNF-α production might provide a local stimulus for the production of further pro-inflammatory cytokines such as IL-1β, IL-6 and IFN-γ, in the context of largely type 2-driven diseases such as asthma and rheumatoid arthritis. Interestingly, elevated levels of each of these cytokines have been reported in asthma (Bradding *et al.*, 1994).

Overall, our data show that PGE_2 acts to enhance the production of the pro-inflammatory cytokine, TNF-α, by immunologically activated mast cells. These data are important in light of the interest in PGE_2 in treating asthma. Our findings suggest that modulation of the cytokine response by PGE_2 during airways inflammation that involves IgE-mediated mast cell activation may be complex and requires careful study.

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Chapter 4: GENERAL DISCUSSION

Modulation of inflammatory responses by PGE_2

PGE_2 is a powerful modulator of inflammation. It achieves its effects through a myriad of interactions with leukocytes and tissue cells in the inflammatory milieu. The majority of the studies investigating the immunomodulatory potential of PGE_2 have focused on its effects on monocyte/macrophage and lymphocyte function (Goetzl *et al.*, 1995; Fedyk *et al.*, 1996). Studies on mast cells have been few and far between, and these have mainly focused on the effects of PGE_2 on mast cell degranulation. In this thesis, we have shown that PGE_2 potentiates pre-formed mediator release by murine mast cells, and also potently and selectively modulates the production of a number of cytokines important in the development of allergic inflammation. Our data indicate that PGE_2 mediates its actions through one or both of a distinct pair of prostaglandin E receptor subtypes that are normally not active participants in the regulation of immune responses. We have also identified the existence of a variant form of the mouse EP_1 receptor, previously reported only in the rat (Okuda-Ashitaka *et al.*, 1996), raising the intriguing question of the nature of its physiological role.

4.1.1 Effects of PGE_2 on mast cell degranulation

Does PGE_2 enhance or inhibit mast cell degranulation?

It has long been believed that prostaglandins of the E series play an inhibitory role with regard to the release of pre-formed mediators by mast cells. Studies dating back to the

early 1970s indicate that PGE_1 employed at high concentrations effectively blocked degranulation by rat peritoneal mast cells (Loeffler *et al.*, 1971; Kaliner and Austen, 1974). Subsequently, Peachell and colleagues (1988) confirmed and extended these findings with PGE_2 to human lung mast cells and basophils. The observed inhibitory action of PGE_2 appeared to be the result of enhanced levels of intracellular cAMP since it was reproduced by agents that elevated cAMP (e.g., forskolin, β2-agonists). Furthermore, PGE_2 itself increased levels of cAMP in target cells (Kaliner and Austen, 1974; Peachell *et al.*, 1988). These data supported the established dogma of the 1970s and 1980s that the predominant role of PGE_2 was to dampen ongoing immune responses and induce its resolution.

In contrast to the above studies, our laboratory has previously demonstrated in rat PMC that prostaglandins of the E series (PGE_1 and PGE_2) failed to inhibit histamine release induced by anti-IgE activation (Leal-Berumen *et al.*, 1995). Moreover, co-stimulation with PGE_2 resulted in significant augmentation of degranulation ($p < 0.05$). We obtained comparable results in mouse BMMC and in the IL-3-dependent murine mast cell line, MC/9. PGE_2 enhanced $Fc_\varepsilon RI$-mediated pre-formed mediator release in these mast cell populations, yet stimulation with PGE_2 alone was without affect on mast cell degranulation (Figure 2, Chapter 2).

Nishigaki *et al.* (1993) examined the effects of PGE_2 on histamine release by the mucosal mast cell line, BNu-2C13. The authors reported that PGE_2 augmented degranulation in the presence of the calcium ionophore, ionomycin. Although supporting our observations that PGE_2 facilitated mast cell degranulation, the levels of degranulation reported by the group were strangely low. Moreover, it was surprising that ionophore

stimulation alone did not induce significant degranulation (Nishigaki *et al.*, 1993). In their study, concurrent activation of BNu-2cl3 with PGE_2 (1 μM) and ionophore (ionomycin; 1 μM) for 5 min resulted in 7 % histamine release (spontaneous release was 2 %). By comparison, we routinely observed > 25 % degranulation by BMMC and MC/9 cells stimulated with the calcium ionophore, A23187, and even greater release induced in similarly activated rat PMC (Leal-Berumen *et al.*, 1994).

Possible reasons for the observed discrepancy

Our data indicated that PGE_2 does not necessarily inhibit $Fc_\varepsilon RI$-induced degranulation. The reason for the observed discrepancy between our findings and those reported by others is unclear and was unlikely to be due to species differences of the mast cells. Our laboratory observed that PGE_1 failed to suppress histamine release by rat PMC (Leal-Berumen *et al.*, 1995), the same mast cell model used by those reporting inhibitory actions for this prostanoid (Loeffler *et al.*, 1971; Kaliner and Austen, 1974). However, there were differences in the strain of rats used. Our studies employed PMC obtained from the high IgE-producing Brown Norway rats, whereas the other groups used PMC from Sprague-Dawley and/or ACI rats. It is possible that strain-to-strain heterogeneity exists with respect to cell responsiveness to PGE_2. This may have, at least partially, influenced the overall effects of PGE_2 on mast cell degranulation.

We originally surmised that the opposing effects of PGE_2 on mast cell degranulation reflected a timing issue, i.e., differences in timing of PGE_2 addition, relative to initiation of $Fc_\varepsilon RI$-cross-linking events. Studies where PGE_2 suppressed mediator release employed a 5 min pre-incubation period with the prostanoid, prior to the

initiation of $Fc_{\varepsilon}RI$ cross-linking events (Kaliner and Austen, 1974; Peachell *et al.*, 1988). Conversely, concurrent activation with PGE_2 and either IgE/Ag or calcium ionophore was employed in studies reporting an enhancing effect of PGE_2 on pre-formed mediator release (Nishigaki *et al.*, 1993; Leal-Berumen *et al.*, 1995; Figure 2, Chapter 2). To investigate the importance of the timing of PGE_2 addition, we examined the effects of pre-incubating BMMC with PGE_2 prior to the initiation of IgE-mediated activation. Pre-treatment of murine mast cells for up to 30 min reduced the degree of potentiation evoked by PGE_2, yet did not inhibit B-hexosaminidase release (a marker of pre-formed mediator release) (Figure 3B, Chapter 3). Thus, the timing of PGE_2 addition is unlikely to be a major factor determining the effects of PGE_2 on pre-formed mediator release. This conclusion is corroborated by a recent study reporting that PGE_2 inhibited $Fc_{\varepsilon}RI$-mediated degranulation by rat PMC, regardless of whether PGE_2 was administered 5 min prior to or concurrently with anti-IgE addition (Chan *et al.*, 2000).

A provocative possibility is that PGE_2 suppresses histamine release through the synthesis of endogenous NO. NO is the product of nitric oxide synthase, of which an inducible form (iNOS) is associated with the large amounts of NO produced during inflammatory conditions (Kubes, 2000). NO is a known inhibitor of mast cell degranulation induced by $Fc_{\varepsilon}RI$ cross-linking events (Eastmond *et al.*, 1997; deSchoolmeester *et al.*, 1999). Close communication occurs between NO and PGE_2, as well as between iNOS and COX2 (Goodwin *et al.*, 1999; Weinberg, 2000). Moreover, production of iNOS and COX2 are triggered by many of the same inflammatory stimuli, such as immune complexes, microbial products, and cytokines (Weinberg, 2000). Activation through the EP_3 receptor has recently been demonstrated to induce iNOS in a

primary culture of neonatal porcine brain (Bhattacharya *et al.*, 1999). Should PGE_2 direct iNOS synthesis in tissue sites such as the lung and skin, resulting NO production could potentially suppress IgE-mediated mast cell degranulation. Although rat PMC have been reported to release NO (Bissonnette *et al.*, 1991), they are unlikely to produce NO at high enough concentrations to feedback inhibit degranulation (Coleman, 2000). A more likely source of any induced NO would be other tissue cells in close proximity to mast cells, including macrophages and epithelial cells (Coleman, 2000). To place the above information in context with the proposed hypothesis, one might speculate that PGE_2 suppressed $Fc_\varepsilon RI$-mediated degranulation in PMC indirectly through the production of NO by contaminating cells in the mast cell preparation, most likely macrophages. In instances where PGE_2 lacked inhibitory effects, such as in mouse BMMC and murine mast cell lines (MC/9, BNu-2cl3), the result may have, in part, been due to the absence of an endogenous source of NO. This hypothesis suggesting a role for NO in PGE_2-directed inhibition of mast cell degranulation, though highly speculative, is intriguing and merits further investigation.

Identification of the EP receptor subtypes receptors enhancing degranulation

To elucidate which prostaglandin E receptor subtypes were responsible for potentiating mast cell degranulation, we activated mast cells with a panel of EP-selective agonists that bind with different affinities to the various EP receptor subtypes (Narumiya *et al*, 1999). $EP_{1/3}$-selective agonists (sulprostone, 17-phenyl-ω-trinor-PGE_2) and the $EP_{3/4}$-selective agonist, misoprostol, potentiated IgE-mediated degranulation (Figure 4; Chapter 2). PGE_1, a structural homologue of PGE_2, also potentiated degranulation. This prostanoid

has an almost identical profile of EP binding affinities as PGE_2 with the notable exception that it binds with slightly lower affinity for EP_1 (Kiriyama *et al.*, 1997). In contrast to the effects of EP_1- and/or EP_3-selective agonists, the $EP_{2/4}$ agonist, PGE_1 alcohol, did not enhance degranulation by IgE/Ag-activated MC/9 cells. These data implicate the involvement of an EP_1 and/or EP_3-dependent mechanism in mediating the potentiating actions of PGE_2.

Two lines of evidence suggest that EP_3 may play a greater role than EP_1 in enhancing degranulation. First, our pharmacological data showed that both PGE_1 and the $EP_{3/4}$-selective agonist, misoprostol, enhanced degranulation. Second, the enhancement of ionomycin-induced degranulation in the BNu-2cl3 murine mast cell line was dictated by the EP_3 receptor subtype (Nishigaki *et al.*, 1993). The advent of mice deficient in each of the EP receptor subtypes will aid in clarifying the relative roles of EP_1 and EP_3 (Austin and Funk, 1999).

Chan and colleagues (2000) recently conducted an extensive study to determine the prostaglandin receptors responsible for inhibiting $Fc_\varepsilon RI$-mediated degranulation by rat PMC. They identified DP receptors as those predominantly expressed by rat PMC, and inferred that PGE_2 mediated its effects via the DP receptor (Chan *et al.*, 2000). To date, this is the only report examining the presence of DP receptors on mast cells (note: they did not look for DP expression at the mRNA level). Although DP expression by murine mast cells is currently unknown, ligand-binding studies have indicated that PGE_2 lacks binding affinity for the mouse DP receptor (Kiriyama *et al.*, 1997). Consequently, even if murine mast cells express DP receptors, PGE_2 is unlikely to mediate its effects via this receptor. The inability of PGE_2 to activate DP receptors provides another possible

explanation for the differential effects of PGE_2 on mast cell degranulation. PGE_2, in the absence of DP-binding capability in the mouse system, might act largely through EP_1 and/or EP_3 receptor subtypes leading to enhanced pre-formed mediator release in the context of IgE-mediated activation. Conversely, should human mast cells, similar to rat mast cells, express DP receptors which are responsive to PGE_2, these receptors might compete with EP receptors for PGE_2 binding, and consequently contribute to an overall inhibition of mast cell degranulation.

4.1.2 Modulation of IL-6 production by PGE_2

IL-6 is a pleiotropic cytokine whose production is upregulated in inflammation (Van Snick, 1990). Among the key effects of IL-6 are the induction of acute phase proteins by hepatocytes, downregulation of IL-1 and TNF-α production, and differentiation of B lymphocytes. Elevated levels of IL-6 are found in the synovial fluid of patients with rheumatoid arthritis and might contribute to the pathology of inflammatory disease (Hirano *et al.*, 1988).

PGE_2 can enhance IL-6 production

IL-6 production is increased by prostaglandins of the E series in many leukocytes and stromal cell types. $PGE_{1/2}$ treatment alone enhanced IL-6 production by rat peritoneal mast cells (Leal-Berumen *et al.*, 1995), mouse BMMC (Figure 1A, Chapter 2), a human fibroblast cell line (Zhang *et al.*, 1988), murine peritoneal macrophages (Hinson *et al.*, 1996), murine peritoneal neutrophils (Yamane *et al.*, 2000), and the human leukemic early T cell line, HSB.2 (Zeng *et al.*, 1998). In macrophages and T cells, PGE_2 acted to

increase IL-6 production at both the mRNA and protein levels (Hinson *et al.*, 1998; Zeng *et al.*, 1998).

PGE_2-directed enhancement of IL-6 has also been observed in the presence of additional stimulatory signals. $PGE_{1/2}$ augmented IgE-mediated IL-6 production in both rat and mouse mast cells (Leal-Berumen *et al.*, 1995; Figure 1A, Chapter 2). PGE_2 had an additive effect on anti-IgE-induced IL-6 production by rat PMC and a comparable degree of IL-6 enhancement was induced by PGE_1 (Leal-Berumen *et al.*, 1995). In contrast, PGE_2 synergistically increased IgE/Ag-induced IL-6 production by mouse BMMC in a dose dependent manner (Figure 1A, Chapter 2). PGE_2 similarly augmented IgE-mediated IL- 6 production in the murine IL-3-dependent mast cell line, MC/9 (Figure 3, Chapter 2). However, unlike its effects in BMMC, PGE_2 treatment alone failed to enhance IL-6 production in MC/9 cells.

PGE_2 enhances IL-6 production through EP_1 and/or EP_3 receptors in mast cells

We conducted pharmacological studies in MC/9 cells to characterize the prostaglandin E receptor subtypes responsible for enhancing IgE-mediated IL-6 production. The EP_1/EP_3-selective agonists, 17-phenyl-ω-trinor-PGE_2 and sulprostone, both reproduced the actions of PGE_2 (Figure 5, Chapter 2). Conversely, the EP_2/EP_4-selective agonist, PGE_1 alcohol, and PGE_1 failed to enhance IgE-mediated IL-6 production. These data suggested that PGE_2 induced its effects through binding of the EP_1 receptor although contribution of EP_3 could not be ruled out. However, the fact that PGE_1, which binds with weaker affinity for EP_1, did not reproduce the actions of PGE_2, further supports an EP_1-dominated response.

Activation of mast cells with bacterial LPS also stimulates IL-6 production (Leal-Berumen *et al.*, 1995). LPS-stimulated IL-6 production was enhanced by prostaglandins of the E series (Leal-Berumen *et al.*, 1995). However, a distinct difference was noted between the effects of PGE_1 and PGE_2. PGE_1, resembling its effects in anti-IgE-stimulated cells, increased LPS-mediated IL-6 production in an additive manner. In contrast, PGE_2 synergistically enhanced IL-6 production (Leal-Berumen *et al.*, 1995). The reason for this difference is unknown and might reflect differences in binding affinities to EP receptors or perhaps binding by PGE_2 or PGE_1 of additional stimulatory or inhibitory prostaglandin receptors. Binding affinity studies have not yet been conducted for rat EP receptors. However, judging by the high degree of homology of the rat and mouse EP receptors, it is likely that PGE_1 shows a similar profile of binding in the rat system. It is noteworthy that at higher concentrations, PGE_2 has been demonstrated to bind mouse FP receptors. These receptors are associated with increased intracellular Ca^{2+} (Coleman *et al.*, 1994). No such binding occurs with PGE_1. This potential increase in Ca^{2+} caused by FP receptor coupling could, in theory, promote further increases in IL-6, as calcium signals are known to be critical for mast cell IL-6 production (Leal-Berumen *et al.*, 1996). It could also explain the greater potency of PGE_2 at enhancing LPS-stimulated IL-6 release. At odds with this proposal is the fact that PGE_1 and PGE_2 stimulation alone induces comparable levels of IL-6 production (Leal-Berumen *et al.*, 1995).

PGE_2 enhances IL-6 production through EP_2 and/or EP_4 receptors in other immune cells

Yamane and colleagues (2000) reported that PGE_2 enhanced IL-6 production by murine peritoneal neutrophils. Neutrophils stimulated with LPS released even more IL-6 production than PGE_2, maximal levels being achieved by 8 h post-stimulation. Co-activation with both LPS and PGE_2 resulted in substantial augmentation of IL-6 production that was still increasing 12 h post-stimulation (Yamane *et al.*, 2000). The neutrophils used in the study expressed mRNA for EP_2 and EP_4 receptors, but lacked expression of the EP_1 and EP_3 receptors. EP_2 and EP_4 knock-out mice were employed to identify the receptor(s) responsible for enhancing IL-6 production by neutrophils. In EP_4-deficient mice, the EP_2-selective agonist, ONO-AE1-259, strongly enhanced LPS-stimulated IL-6 production. In contrast, the EP_4-selective agonist, ONO-AE1-329, proved to be a weak inducer of IL-6 in EP_2-deficient mice. These observations prompted the authors to conclude that EP_2 was the main receptor mediating the IL-6-enhancing effects of PGE_2 (Yamane *et al.*, 2000).

PGE_2 has also been observed to augment IL-6 production by resting and Con A-treated T cells. The human leukemic early T cell line, HSB.2, released modest amounts of IL-6 following treatment with either PGE_2 or Con A (Zeng *et al.*, 1998). PGE_2 increased the accumulation of IL-6 mRNA, whereas Con A-stimulation lacked an effect at this level. A closer look at Con A-induced activation of HSB.2 cells revealed that Con A downregulated levels of constitutively expressed EP_2 and EP_3 receptors. In contrast, Con A treatment enhanced EP_4 receptor expression. Whereas suppression of EP_2/EP_3 expression was most striking after 24 h treatment with Con A, upregulation of EP_4

expression occurred much more rapidly and maximal levels were observed within an hour of Con A stimulation. Furthermore, in the continued presence of Con A, EP_4 mRNA levels remained elevated for at least 24 h. The authors speculated that Con A-directed increases in EP_4 and concomitant inhibition of EP_2 and EP_3 receptors represented one mechanism whereby Con A enhanced PGE_2-mediated IL-6 production (Zeng *et al.*, 1998). Another possibility is that Con A treatment stabilized PGE_2-induced IL-6 transcripts. However, this potential mechanism was not addressed in the study.

PGE_2 can also inhibit IL-6 production

In contrast to the above studies, there have been reports in the literature of inhibitory effects induced by PGE_2 on IL-6 production. For instance, PGE_2 suppressed IL-6 production by rat Kupffer cells in regenerating liver (Goss *et al.*, 1993), LPS-activated murine peritoneal macrophages (Strassmann *et al.*, 1994), and human monocyte-derived macrophages (Zhong *et al.*, 1995). An unexpected observation in the study conducted by Zhong and colleagues (1995) was the inability of cholera toxin, an irreversible activator of the Gs protein, and of the cAMP analogue, 8-bromo-cAMP, to reproduce the IL-6-suppressive effects of PGE_2 in human macrophages. The authors suggested that PGE_2 could signal through both a cAMP-dependent and independent manner (Zhong *et al.*, 1995). In apparent conflict with such a notion, Kambayashi and co-workers (1995a) reported that the cAMP-elevating agent, rolipram, inhibited LPS-induced IL-6 production. Furthermore, a recent study reported that EP_2-selective and EP_4-selective agonists inhibited IL-1β-induced expression of IL-6 mRNA and protein by rat synovial

cells (Kurihara *et al.*, 2001). Since EP_2 and EP_4 receptors are positively coupled to adenylate cyclase, these data support a role for cAMP in the inhibition of IL-6 production. Hence, it appears that cAMP has the potential to inhibit or enhance IL-6 expression, and culture conditions and the cell type under study may be important in determining the effect of PGE_2 on IL-6 production.

Reconciling the paradoxical findings of PGE_2 on IL-6 production

Acknowledging the controversy regarding the nature of PGE_2's effects on IL-6 production, Hinson *et al.* (1996) stated that:

> "most of the confusion comes from trying to compare results obtained with different cell types, from comparing cell lines to primary cells, or from using different agents and conditions to stimulate cytokine and eicosanoid production."

One practical approach by which to make sense of this so-called "confusion" is to consider how PGE_2 modulates IL-6 production in the whole organism. Evidence that PGE_2 increases IL-6 production during inflammatory reactions *in vivo* was obtained by a study investigating the effects of a neutralizing antibody against PGE_2 (Portanova *et al.*, 1996). Antibody-induced suppression of PGE_2 resulted in decreased serum IL-6 levels in a rat model of chronic inflammation (i.e., adjuvant-induced arthritis), indicating that endogenous PGE_2 is at least partially responsible for the increase in IL-6 production. Anti-PGE_2 treatment also reduced the degree of local edema (Portanova *et al.*, 1996).

Two related studies also showed that PGE_2 acts to enhance IL-6 production in the whole animal. In one study, daily i.p. injections of Lewis rats with indomethacin lowered serum IL-6 levels and the degree of tissue edema (Leisten *et al.*, 1990). The second study, using the rat adjuvant arthritis model of chronic inflammation, demonstrated that

treatment with a specific COX-2 inhibitor (SC-58125) prevented the accumulation of IL-6 protein in the serum and IL-6 mRNA at the vicinity of tissue inflammation (Anderson *et al.*, 1996). Taken together, the data strongly suggested that PGE_2 functions *in vivo* to increase IL-6 production during inflammation. Incidentally, this is in agreement with the majority of the *in vitro* findings. More importantly, Portanova and colleagues (1996) demonstrated, through the use of neutralizing antibodies against PGE_2 and COX inhibitors, that endogenous PGE_2 is the primary prostanoid orchestrating the development of inflammation in the whole animal.

Possible role for endogenous IL-10 in determining the effects of PGE_2 on IL-6 production

Another aspect of the observed effects of PGE_2 on IL-6 is worth more careful study. In instances where PGE_2 inhibited IL-6 production by murine macrophages, the involvement of PGE_2-induced IL-10 was implicated (Strassmann *et al.*, 1994; Kambayashi *et al.*, 1995b). IL-10 is a potent inhibitor of IL-6 production (de Waal Malefyt *et al.*, 1991; Marshall *et al.*, 1996). It is conceivable that the potency of PGE_2 as an inducer of IL-10 production by a given cell type governs the resulting effects of PGE_2 on the IL-6 response. In support of this theory, van der Pouw Kraan *et al.* (1995) reported that PGE_2-directed IL-6 inhibition in LPS-treated human whole blood cultures was mediated through endogenous IL-10 production. Neutralization of endogenous IL-10 by specific mAb completely reversed the inhibitory effect of PGE_2 on IL-6 production.

As described above, PGE_2 enhanced IL-6 production by IgE/Ag-activated rat PMC (Leal-Berumen *et al.*, 1995) and murine BMMC and MC/9 cells (Figures 1A and 3,

Chapter 2). Although $Fc_{\varepsilon}RI$ cross-linking events elicited IL-10 production by BMMC, PGE_2 failed to further enhance such production and PGE_2 alone did not induce an IL-10 response. Furthermore, we have demonstrated that IL-10 dose-dependently inhibited IgE-mediated IL-6 production by rat PMC (Marshall *et al.*, 1996). These observations indicate that PGE_2 was unlikely to be mediating its effects through endogenous IL-10 production.

Production and release of IL-10 is delayed during inflammation; hence, the time point at which samples are assessed for IL-6 content might be a critical factor to consider. Samples taken early (i.e., within a few hours of PGE_2 stimulation) may contain greatly enhanced IL-6 levels relative to controls, whereas the degree of enhancement of IL-6 production at later time points ($\geq$ 18 h) may be far less impressive. In our studies, enhancement of IgE-mediated IL-6 production by PGE_2 was still evident 24 h post-activation. Hence, with respect to the theory, it is possible that endogenous IL-10 levels induced were not high enough to mediate IL-6 inhibition.

Production of IL-6, among other type 2 cytokines, is elevated in asthmatic airways and is believed to contribute to the pathology of the disease. Based upon the above *in vitro* observations, endogenously produced IL-10 would be expected to be beneficial by reducing levels of type 2 cytokines. It is therefore intriguing that a research group in Colorado reported diminished IL-10 production in the asthmatic lung and speculated that this phenomenon might contribute to the pathogenesis of asthma (Borish *et al.*, 1996; Mascali *et al.*, 1996).

4.1.3 Modulation of GM-CSF production by PGE_2

GM-CSF possesses a number of biological actions that might serve to prolong ongoing inflammatory reactions (Hill *et al.*, 1995). This hematopoietic cytokine is essential for the growth and development of granulocyte/monocyte progenitors. GM-CSF activates neutrophils and macrophages – enhancing their microcidal, phagocytic, and cytotoxicity functions. GM-CSF also serves as a chemoattractant for eosinophils and neutrophils, and induces the production of pro-inflammatory cytokines TNF-α and IL-1 by endothelial cells and fibroblasts.

PGE_2 augments GM-CSF production by Th2 cells

The modulatory actions of PGE_2 on GM-CSF production are not well-understood, and the mode of co-stimulation appears to be of particular importance (Quill *et al.*, 1989; Li and Fox, 1993). Nevertheless, a general trend is emerging that suggests that PGE_2 might serve to enhance the production of GM-CSF in the context of type 2 responses and conversely, suppress it during type 1 responses. It must be stressed that at this point, due to insufficient data, this proposal is highly speculative.

Li and Fox (1993) investigated the effects of PGE_2 on GM-CSF secretion by a small panel of murine Th1 and Th2 clones. The authors reported that PGE_2 suppressed GM-CSF induced by specific antigen/APC stimulation in 3/3 Th1 clones and enhanced it in 3/4 Th2 clones. The indicator cell line used in these studies did not distinguish between IL-3 and GM-CSF. Neutralizing antibodies against these cytokines subsequently showed that the majority of the response represented IL-3 production. Nevertheless, in the

limited number of clones tested with the blocking antibodies, the differential effects of PGE_2 on GM-CSF production were still apparent.

Such a pattern of T cell-specific regulation of cytokine production by PGE_2 has been reported previously. Two additional cytokines constitutively expressed by both T cell subsets, IL-3 and LT-β, were shown to be inhibited only in Th1 clones (Ferreri *et al.*, 1997). It is noteworthy, however, that TNF-α which is also produced by the two T cell subsets, albeit to a lesser degree in Th2 cells, was potently suppressed in both Th1 and Th2 cells (Ferreri *et al.*, 1997).

In another study, the effects of PGE_2 on GM-CSF production by the inducer T cell clone, BK2.43, was shown to depend on the mode of stimulation (Quill *et al.*, 1989). PGE_2 suppressed GM-CSF elicited by Ag/APC and anti-CD3/APC treatment, yet increased IL-2-induced GM-CSF. This potentiating effect on IL-2-mediated GM-CSF production was mimicked by forskolin and cholera toxin, suggesting that a cAMP-dependent mechanism was involved (Quill *et al.*, 1989). The observed augmentation of GM-CSF production induced by IL-2 and PGE_2 is interesting, considering that PGE_2 is an inhibitor of IL-2 production (Walker *et al.*, 1983; Chouaib *et al.*, 1985). These data provide one more glimpse into the complexity of the actions of PGE_2 in the immune system. The authors commented that they observed comparable PGE_2-directed increases in GM-CSF by two Th2 clones stimulated with a combination of IL-4 and IL-1. The latter observations strengthen the concept that PGE_2 enhances GM-CSF production in the context of type 2 responses.

Effects of PGE_2 on mast cell GM-CSF production

Mast cells are key players in the initiation of allergic inflammation, and cross-linking of their surface $Fc_\varepsilon RI$ triggers the production of GM-CSF (Figure 1B, Chapter 2). Local production of GM-CSF by mast cells would aid in the recruitment of eosinophils and neutrophils from the circulation into the inflammatory milieu, as well as promote their activation. We have demonstrated that PGE_2 enhances IgE-mediated GM-CSF production by mouse BMMC in a concentration-dependent manner (Figure 1B, Chapter 2). PGE_2 at 10^{-6} M effectively doubled the amount of GM-CSF elicited by IgE/Ag-elicited mast cells. Potentiation of IgE-mediated GM-CSF production by PGE_2, hence, serves as another means by which this prostanoid enhances type 2 responses.

4.5.2 Modulation of TNF-α production by PGE_2

TNF-α is a potent pro-inflammatory cytokine that is produced early during inflammatory responses (Papadakis *et al.*, 2000). Mast cells have been found to store pre-formed TNF-α in their granules that is released during IgE-mediated degranulation (Gordon and Galli, 1990; Gordon and Galli, 1991). Consequently, mast cells provide an early source of TNF-α during allergic reactions and contribute both qualitatively and quantitatively to the developing immune response. $Fc_\varepsilon RI$-cross-linking also stimulates substantial *de novo* TNF-α production (Gordon and Galli, 1991).

PGE_2 augments TNF-α production by IgE/Ag-activated mast cells

We have shown that PGE_2 can augment $Fc_\varepsilon RI$-induced TNF-α production (Figure 1, Chapter 3). These data were surprising, considering the universal view of PGE_2 as a powerful inhibitor of TNF-α production in other cell types, including mononuclear phagocytes (Kunkel *et al.*, 1986a), T lymphocytes (Ferreri *et al.*, 1992), and neutrophils (Yamane *et al.*, 2000). PGE_2 inhibits TNF-α in these cells through a combination of transcriptional and post-transcriptional means (Ferreri *et al.*, 1992; Kunkel *et al.*, 1988; Zhong *et al.*, 1995). Interestingly, as mentioned earlier, PGE_2 suppressed TNF-α mRNA and protein accumulation in both Th1 and Th2 helper CD4+ T cell clones whereas the production of two other cytokines elaborated by both Th subsets (LT-β and IL-3) was inhibited in Th1 clones only (Ferreri *et al.*, 1997).

In light of the well-accepted inhibitory actions of PGE_2 on TNF-α production, the lack of such suppression in $Fc_\varepsilon RI$-activated mast cells is of considerable interest, and these findings have important immunological and clinical implications that are discussed later.

Possible reasons for the ability of PGE_2 to enhance TNF-α production

Possible explanations for the lack of inhibitory effects of PGE_2 on TNF-α production by mast cells include: 1) nature of the co-stimulatory agent; 2) differences in prostaglandin E receptor subtype(s) employed and associated second messengers; and 3) ratio of cAMP to cGMP induced by PGE_2.

1) Nature of the co-stimulatory agent

In studies with T cells, TNF-α synthesis was elicited through the use of the polyclonal activators, anti-CD3 and Con A (Ferreri *et al.*, 1992; Ferreri *et al.*, 1997). Macrophages and neutrophils were activated by LPS (Kunkel *et al.*, 1988; Yamane *et al.*, 2000), a potent inducer of TNF-α. We have also examined the effects of PGE_2 on LPS-stimulated mast cells. BMMC activated with LPS (5 μg/ml) released significant levels of TNF-α that was dose-dependently suppressed by PGE_2 (data not shown). In light of our observations that PGE_2 inhibited LPS-induced TNF-α production by mast cells, the nature of the TNF-α-inducing stimulus appears to be an important determinant of the overall effect of PGE_2 treatment on TNF-α production.

2) differences in prostaglandin E receptor subtype(s) employed and associated second messengers

Suppression of LPS-induced TNF-α production by murine macrophages and neutrophils occurs through EP_2 and EP_4 receptors (Katsuyama *et al.*, 1998; Yamane *et al.*, 2000). Since these receptors are positively coupled to adenylate cyclase and their activation results in increased intracellular levels of cAMP (Coleman *et al.*, 1994); these data corroborate the large body of work showing that cAMP-elevating agents inhibit LPS-elicited TNF-α production (Katakami *et al.*, 1988; Taffet *et al.*, 1989).

In sharp contrast, PGE_2 enhanced IgE-mediated TNF-α production through the activation of EP_1 and/or EP_3 receptors (Table II, Chapter 3). Coupling through EP_1 triggers increases in intracellular Ca^{2+} whereas activation of EP_3 receptors is generally associated with suppression of cAMP (Narumiya *et al.*, 1999). However, EP_3 isoforms

have been characterized that elevate intracellular Ca^{2+}, and elevation of Ca^{2+} has been shown to be critical for induction of mast cell TNF-α (Blank and Varin-Blank, 2000). Conversely, cAMP negatively regulates TNF-α production (Kast, 2000).

Treatment of mast cells with PGE_2 transiently increased intracellular Ca^{2+} (Figure 4A, Chapter 3). Immediately following PGE_2 treatment, mast cells were refractory to further stimulation by PGE_2, indicating that receptor desensitization had occurred. Concurrent activation of mast cells by PGE_2 and $Fc_\varepsilon RI$ cross-linking resulted in larger and sustained increases in Ca^{2+} (Figure 4C, Chapter 3).

Taken together, these data suggest that activation of cAMP-signaling pathways via EP_2 and EP_4 inhibit TNF-α production, whereas induction of Ca^{2+}-signaling pathways through EP_1 and possibly EP_3 splice variant(s) promote TNF-α production. Experiments are currently underway to more closely delineate the relative participation of the EP_1 and EP_3 receptors in enhancing TNF-α production by IgE/Ag-activated mast cells.

3) ratio of cAMP to cGMP induced by PGE_2

Hidden among the abundant literature describing the inhibitory effects of PGE_2 on macrophage TNF-α production is one report of PGE_2-directed TNF-α augmentation. Renz *et al.* (1988) observed that PGE_2 at low concentrations (0.1-10 ng/ml) stimulated TNF-α production by rat resident peritoneal macrophages, and a role for guanosine 3', 5'-monophosphate (cyclic GMP or cGMP) was implicated. Higher concentrations, i.e. > 10 ng/ml, had an inhibitory effect on TNF-α production. We did not measure levels of intracellular cGMP induced by PGE_2 in our studies, and are unable to rule out the

involvement of cGMP in mediating the enhanced production of TNF-α. However, the fact that none of the EP receptor subtypes are known to increase intracellular cGMP suggests that in the study by Renz *et al.* (1988), PGE_2 is acting indirectly through the induction of another factor, possibly by contaminating cells, to elevate cGMP and enhance TNF-α production.

Implications of enhanced TNF-α levels in the context of allergic inflammation

TNF-α recruits leukocytes to sites of inflammation, in part, by upregulating adhesion molecules (e.g., E-selectin, ICAM-1, VCAM-1) on local endothelial cells and airway smooth muscle cells. PGE_2 has been demonstrated to block TNF-α-induced expression of ICAM-1 and VCAM-1 by human airway smooth muscle (Panettieri *et al.*, 1995). Based on our findings, the opposite may occur in the context of allergic reactions. The presence of PGE_2 in the inflammatory milieu may potentiate mast cell-derived TNF-α production that, in turn, would potentially result in greater degree of adhesion molecule upregulation and leukocyte recruitment. It is therefore of interest that PGE_2 acts on human colonic epithelial cells to evoke the production of IL-8, another potent neutrophil chemoattractant (Yu and Chadee, 1998). Moreover, unlike its inhibitory effects on IL-6 and TNF-α mRNA accumulation, PGE_2 is without effect on IL-8 production in human macrophages (Zhong *et al.*, 1995). There is another notable study in this area worthy of consideration. In this study, human colonic epithelial lines were treated with various inflammatory stimuli known to elicit an IL-8 response, and the effects of a PGE_2 analogue, enprostil, was investigated (Toshina *et al.*, 2000). Enprostil inhibited IL-1β and LPS-elicited IL-8 production, yet did not suppress IL-8 synthesis induced by TNF-α.

4.1.5 Modulation of IL-4 production by PGE_2

IL-4 is the prototypical type 2 cytokine, and is critical for the induction of the humoral branch of the immune system (Paul, 1991; Boulay and Paul, 1992). IL-4 induces the development of Th2 cells, potently suppresses the production of type 1 cytokines (IL-12 and IFN-γ), stimulates immunoglobulin class switching to IgE, and supports mast cell growth. Enhanced IL-4 levels are associated with allergic inflammatory conditions. For instance, augmented levels of IL-4 have been detected in bronchial mucosal biopsies of asthmatic patients compared with non-atopic, non-asthmatic subjects (Bradding *et al.*, 1994).

PGE_2 inhibits IgE-mediated IL-4 production by mast cells

Mast cells, a cellular source of IL-4, produced this cytokine in response to $Fc_\varepsilon RI$ cross-linking events (Figure 1C, Chapter 2). In our system, IgE/Ag-activated mouse BMMC produced significant levels of IL-4 following a 24-h incubation period. Unlike the enhancing effects of PGE_2 on IgE-mediated TNF-α, IL-6, and GM-CSF production, PGE_2 inhibited the IL-4 response (Figure 1C, Chapter 2). These data indicated that the actions of PGE_2 were selective, i.e., that PGE_2 was not simply inducing a general upregulation of IgE/Ag-elicited cytokine production.

Moreover, the lack of IL-4 enhancement by PGE_2 was consistent with what has been reported in the literature. PGE_2 was observed to neither enhance nor inhibit IL-4 production by antigenically and mitogenically activated murine T cells (Betz and Fox, 1991). Human PBL and CD4+ Th clones responded similarly to PGE_2 when low

concentrations ($<10^{-8}$ M) of the prostanoid were employed (Snijdewint *et al.*, 1993). In response to these findings, the accepted paradigm was that PGE_2 inhibited the production of type 1 cytokines while either having no effect (e.g. IL-4) or increasing (e.g. IL-5) type 2 cytokines (Fedyk *et al.*, 1996).

PGE_2 may similarly inhibit IL-4 production by T cells

The concept of PGE_2's absence of modulatory effects on IL-4 production by Th2 cells is an oversimplification, as there have been instances where inhibitory actions have been observed. Even in the above study conducted by Snijdewint and colleagues (1993), PGE_2, at higher concentrations ($\geq 10^{-7}$M human PBL; $\geq 10^{-6}$ M human T cell clones), significantly suppressed IL-4 augmentation. However, PGE_2 remained a much more potent suppressor of IL-2 and IFN-γ production (Snijdewint *et al.*, 1993).

Parker *et al.* (1995) reported that PGE_2 consistently abrogated IL-4 production by freshly isolated splenic mononuclear cells from DBA/2 mice. This suppressive action extended to six different strains/hybrids of mice indicating that it was not restricted to a particular mouse strain. PGE_2 was also observed to suppress IL-4 production by human Th2 cells (Khan, 1995). Surprisingly, known cAMP-increasing stimuli, forskolin, isoproterenol, and serotonin, failed to inhibit IL-4 secretion, implicating that cAMP was not mediating the effects of PGE_2.

IL-4 is a known inhibitor of PGE_2 production

The relative resistance of IL-4 to inhibition by PGE_2 may have physiological relevance. IL-4 has been demonstrated to inhibit both COX-2 and PGE_2 synthesis by human blood

monocytes and alveolar macrophages (Hart *et al.*, 1989; Dworski and Sheller, 1997). Hence, this cytokine might function as a natural inhibitor of PGE_2 induction. Suppression of IL-4 production, as was observed in our system, may represent a means by which PGE_2 avoids its own feedback inhibition. Rodent PMC and BMMC can be induced to secrete low levels of PGE_2 by certain stimuli such as NGF (Marshall *et al.*, 1999). Moreover, SCF together with IL-1β and IL-10 induced transient COX-2 expression in mouse BMMC (Murakami *et al.*, 1994) which could potentially lead to autocrine PGE_2 synthesis.

Although mast cells are poor producers of PGE_2, they are found in close proximity to the major cellular source of PGE_2, i.e., macrophages. It is intriguing that IL-4 was reported to be a much more powerful inhibitor of human blood monocyte PGE_2 production than alveolar macrophage responses (Dworski and Sheller, 1997). The decreased sensitivity of macrophages to the inhibitory effects of IL-4 would conceivably allow elevated levels of both IL-4 and PGE_2 at sites of type 2-dominant inflammation. Co-existence of these two mediators has important consequences, including providing the opportunity for synergistic interactions as described below.

PGE_2 synergizes with IL-4 to enhance type 2 responses

PGE_2 forms a unique partnership with IL-4 in the regulation of lymphocyte function (Roper *et al.*, 1990; Roper and Phipps, 1992; Fedyk and Phipps, 1996; Abe *et al.*, 1997). Most significantly, PGE_2 acts on murine B cells to inhibit their activation and to promote LPS and IL-4-mediated Ig class switching to IgE and IgG1 (Roper *et al.*, 1990). PGE_2 can induce both a general upregulation of IgE production as well as increase levels of

antigen-specific IgE (Fedyk *et al.*, 1996). IL-4-induced IgE production is similarly enhanced by factors that raise intracellular cAMP levels, and not surprisingly, PGE_2 mediates its actions on B cells through the activation of the EP_2 and EP_4 (Fedyk and Phipps, 1996).

4.2 Emerging picture of the role for PGE_2 in the development of type 2 responses

The last two decades have brought with it an enormous wealth of information describing the myriad actions of PGE_2 on leukocytes and their function, and underscoring the role of PGE_2 as a critical immunomodulator. This mountain of data might easily overwhelm the uninitiated reader - the diverse, and often seemingly unrelated range of PGE_2's effects may be simultaneously baffling and frustrating. Yet upon closer examination, it becomes apparent that much of this vast storehouse of information can be distilled into a beautifully simple paradigm: PGE_2 acts to promote and maintain type 2 immune responses (Fedyk *et al.*, 1996).

A detailed discussion of the type 2-promoting activity of PGE_2 is provided in the Introduction section (Chapter 1). However, key findings as well as an overall summary of these actions is presented below, and our observed effects of PGE_2 in mast cells will be placed in the context of this paradigm.

4.2.1 PGE_2 is the signal that instructs the development of type 2 responses

Dendritic cells (DC) are the only APC capable of presenting antigen to naïve Th cells (Banchereau and Steinman, 1998), and as such, these accessory cells determine the nature of primary immune responses (i.e., type 1 vs type 2 responses). In recent years,

Kapsenberg and colleagues have published a series of eloquent studies revealing that the microenvironment of immature DC supplies signals that ultimately induce the development of either type 1- or type 2-polarized effector DC (Kalinski *et al.*, 1997; Kalinski *et al.*, 1998; Viera *et al.*, 2000). These tissue-derived factors have been termed "signal 3", in suite of the well-known antigenic and co-stimulatory signals ("signal 1" and "signal 2", respectively) required for T cell differentiation into effector cells. The signal instructing the development of Th2-biased responses was identified as PGE_2 (Kalinski *et al.*, 1998), whereas the Th1-driving factor was determined to be IFN-γ (Vieira *et al.*, 2000). Following exposure to PGE_2 or IFN-γ, uncommitted, immature DC undergo maturation and acquire type 1- or type 2-driving phenotypes that are stable and unsusceptible to further modulation (Kalinski *et al.*, 1998; Vieira *et al.*, 2000). These data reveal that the type 2-promoting influence of PGE_2 is present at the earliest stages of immune response and may very well be the physiological factor governing the initiation of type 2 responses.

4.2.2 PGE_2 acts to maintain ongoing type 2-responses

Not only a key initiator of type 2 responses, PGE_2 continues to exert its type 2-promoting effects throughout the course of inflammatory responses. PGE_2 potently inhibits type 1 cytokine production (IL-2, IFN-γ) and is without effect, or enhances, production of type 2 cytokines (IL-4, IL-5) in both murine and human systems (Maca, 1987; Betz and Fox, 1991; Snijdewint *et al.*, 1993). Moreover, PGE_2 downregulates the expression of IL-2R (Rincon *et al.*, 1988; Anastassiou *et al.*, 1992) and IL-12R (Wu *et al.*, 1998). PGE_2 potentiates IL-4-directed IgE production by murine B lymphocytes (Roper *et al.*, 1990).

Evidence exists that a similar phenomenon occurs in the human system (Byron *et al.*, 1992).

In addition to its direct effects on lymphocytes, PGE_2 is also a powerful modulator of accessory cell function. For instance, PGE_2 acts on mononuclear phagocytes to suppress the production of the type 1-driving cytokine, IL-12, and enhances synthesis of the type 2-associated cytokine, IL-10 (van der Pouw Kraan *et al.*, 1995; Fedyk *et al.*, 1996). The involvement of PGE_2 in driving type 2 responses is corroborated by clinical studies of type 2-biased conditions that report elevated levels of PGE_2 and IgE/type 2 cytokines (de Vries, 1994; Leung *et al.*, 1988; Jakob *et al.*, 1990).

4.2.3 How do our data in mast cells fit into this paradigm?

We have demonstrated that PGE_2 greatly enhances many mast cell responses in the context of $Fc_\varepsilon RI$ cross-linking. PGE_2 potentiated IgE-mediated degranulation, and the production of IL-6, TNF-α, and GM-CSF. Although none of these mediators can be neatly classified under the headings of "type 1" or "type 2" factors, they contribute to the pathology of allergic inflammation, a predominantly type 2-biased condition. Moreover, mast cells are well-recognized participants in allergic inflammatory responses including asthma and other inflammatory conditions, such as rheumatoid arthritis (Schwartz and Huff, 1998).

Individually, each of the mast cell mediators whose release was enhanced by PGE_2 has the potential to aggravate existing inflammation. Increased degranulation, and accompanying histamine release, would be expected to enhance vasodilation, edema formation, and leukocyte infiltration (Nilsson *et al.*, 1999). Moreover, PGE_2 has been

shown to synergize with histamine to enhance edema formation during PCA reactions in rabbit skin (Hellewell *et al.*, 1992). In the context of asthma, increased histamine release would hypothetically lead to more severe episodes of reversible airways bronchoconstriction.

Augmentation of mast cell IL-6 production by PGE_2 would potentially support B cell growth and differentiation. It is noteworthy that elevated IL-6 levels have been documented in rheumatoid arthritis (Hirano *et al.*, 1988) and asthma (Broide *et al.*, 1992). PGE_2-potentiated GM-CSF production would likely act to recruit and support the growth and activation of more eosinophils and neutrophils, ensuring their continued presence at locally inflamed tissue sites.

PGE_2 potently inhibits TNF-α production by LPS-treated macrophages, neutrophils, and T lymphocytes (Kunkel *et al.*, 1988; Ferreri *et al.*, 1992; Yamane *et al.*, 2000). In contrast, PGE_2 augments TNF-α production in response to IgE-mediated activation (Figure 1, Chapter 3). The resistance of IgE-mediated TNF-α production to the normally inhibitory effects of PGE_2 would help reconcile the presence of increased levels of both PGE_2 and TNF-α in chronic inflammatory conditions such as rheumatoid arthritis. Moreover, the upregulation of TNF-α release would in turn regulate a number of cellular processes that take place during inflammation.

There exists also the potential for a positive feedback mechanism with respect to the induction of mast cells and EP receptors. TNF-α and IL-6 are both powerful inducers of mast cell development from murine spleen cells (Hu *et al.*, 1997). These cytokines appear to work through a mechanism that involves, in part, the induction of PGE_2 formation and the suppression of IL-4 levels. PGE_2 had already been demonstrated

previously to be critical for the selective development of mouse and human mast cells (Hu *et al.*, 1995; Saito *et al.*, 1996). Regarding feedback mechanisms for the promotion of EP receptor expression, TNF-α and IL-4 have been documented to increase the expression of the EP_1 receptor subtype in a human amnion cell line (Spaziani *et al.*, 1997; Spaziani *et al.*, 1998). It is interesting that PGE_2-treatment of IgE/Ag-activated mast cells enhances the release of mediators that not only exacerbate ongoing inflammation, but also potentially promote further mast cell induction, and activation through EP_1 receptors.

4.2.4 A few words on EP receptors in mast cells

Our studies revealed that PGE_2 mediated its effects on mast cells through the activation of EP_1/EP_3 receptors. Involvement of this pair of receptors was unexpected since PGE_2 generally modulates immune responses via EP_2/EP_4 receptors. Some examples of EP_2- and/or EP_4-mediated events include PGE_2-directed enhancement of IL-6 production by T cells (Zeng *et al.*, 1998), IL-4-induced IgE production by B cells (Fedyk and Phipps, 1996), LPS-mediated TNF-α and IL-6 release by macrophages and neutrophils (Katsuyama *et al.*, 1998; Yamane *et al.*, 2000), suppression of T cell proliferation (Nataraj *et al.*, 2001), and IL-8 synthesis by colonic epithelial cells (Yu and Chadee, 1998). The physiological significance of the sharp contrast between EP_2/EP_4 usage in the general regulation of immune responses, and the participation of EP_1 and/or EP_3 receptors in mast cells is unclear. Future studies will undoubtedly shed light on this matter.

We also identified in murine mast cells the presence of a splice variant of the EP_1 receptor (EP_1-v) (Figure 6; Chapter 2). This receptor had only been observed previously in the rat (Okuda-Ashitaka *et al.*, 1996). As in the rat, the mouse EP_1-v arose from failure

to use a splicing site in the region of the 6th transmembrane region, and the resulting receptor lacked a carboxyl terminal tail as determined by a hydrophobicity plot constructed according to the Kyte-Doolittle method. The rat EP_1-v was demonstrated to be devoid of signaling capability and downregulated signaling through other EP receptors (Okuda-Ashitaka *et al.*, 1996). It would be interesting to determine whether the mouse EP_1-v serves a similar inhibitory role in mast cells.

4.3 Clinical implications

The bronchodilatory and anti-inflammatory effects ascribed to PGE_2 and its endogenous production in inflamed airways made PGE_2 an attractive candidate for asthma treatment. Clinical studies testing such a potential for PGE_2 have provided largely encouraging data. For instance, PGE_2 has been reported to ablate the early and late responses to allergen (Pavord *et al.*, 1993), and more recent studies have shown that PGE_2 downregulates the inflammatory response (Gauvreau *et al.*, 1999). However, based on our findings, a caveat is in order. Our data suggests that, PGE_2 has the potential to upregulate mast cell production of a number of inflammatory mediators, most notably the potent pro-inflammatory protein, TNF-α.

In the above-mentioned clinical studies, PGE_2 was administered to asthmatic patients a few minutes prior to activation with specific antigen. We, therefore, investigated the effects of PGE_2 pre-treatment on subsequent IgE/Ag-induced TNF-α production. We incubated MC/9 cells with PGE_2 or EP-selective agonist for 30 min prior to mast cell activation via IgE/Ag-induced $Fc_\varepsilon RI$ cross-linking. Pre-treatment of mast cells with PGE_2 continued to enhance TNF-α production, albeit to a lower extent (Figure

3A, Chapter 3). However, the most consistent and noteworthy finding was the potent inhibitory effect of pre-treatment with PGE_1 on TNF-α production. These data contrasted sharply with the absence of a significant modulatory effect of PGE_1 when it was added concurrently with IgE-mediated activation.

This is not the first instance of a differential effect of PGE_1 and PGE_2 on mast cell function. We have demonstrated that PGE_2 is a more powerful potentiator of degranulation than PGE_1 in rat PMC (Leal-Berumen *et al.*, 1995). Furthermore, PGE_2, yet not PGE_1, potentiated IL-6 production by MC/9 cells (Figure 5, Chapter 2). These data are relevant because they suggest that despite their almost identical structures and EP binding affinities, PGE_1 and PGE_2 may induce substantially different immune responses. In terms of clinical significance, should prostaglandins of the E series be shown conclusively to be of therapeutic benefit as prophylactic agents in asthma treatment, PGE_1 may prove to be more potent than PGE_2, at least in the context of reducing TNF-α levels.

Regardless of the observed suppressive effects of PGE_1 pretreatment, our data suggests that exogenous PGE_2 may further exacerbate, rather than ameliorate, airways inflammation. One might argue that these effects were observed in the mouse, and are probably not relevant in the human or that the mast cell is a minor player in the cytokine milieu as a whole. This argument holds merit and we are currently conducting experiments in human cord blood-derived mast cells to address this issue. Nevertheless, it would be prudent for investigators to be wary of the potential pro-inflammatory effects of PGE_2 treatment on mast cells.

Lessons can be learned from the controversy currently surrounding the use of β2-agonists. β2-agonists are in widespread use for the treatment of asthma and make up an important part of the strategy of daily regimen for treating asthma. Despite their clear therapeutic benefits, the overuse of β2-agonists has been implicated in increased asthma morbidity and mortality (Green and Nelson, 1998). Fedyk *et al.* (1996) proposed that β-agonists, through their cAMP-elevating properties, enhanced type 2 cytokine as well as IgE production. The authors suggested that these immunological changes lead to chronic inflammation and may be a contributing factor for the rising morbidity associated with asthma. There are striking similarities between the known effects of β2-agonists and PGE_2. Both stimuli suppress Th1 cell development (Panina-Bordignon *et al.*, 1997; Kalinski *et al.*, 1997; Kalinski *et al.*, 1998), and enhance the production of type 2 cytokines and IgE (Fedyk *et al.*, 1996). Furthermore, they mediate their effects through increased intracellular cAMP (Fedyk *et al.*, 1996). These observations suggest that long-term use of PGE_2 might result in similar problems.

The potential aggravating effects of PGE_2 in allergic inflammation are by no means restricted to asthma. PGE_2 levels are elevated in a number of other pathological inflammatory conditions including rheumatoid arthritis and rhinitis, and the same potential exists for PGE_2 to polarize the immune response towards the type 2 phenotype in these diseases.

4.4 Major limitations/criticisms of our studies

4.4.1 Issues regarding PGE_2

Pharmacological vs physiological concentrations of PGE_2

In our hands, PGE_2 at doses of $\geq 10^{-8}$ M routinely modulated mast cell responses in the context of IgE/Ag-activation. Although these concentrations may initially seem high, they are within PGE_2 levels found *in vivo*. For instance, PGE_2 levels of greater than 5 x 10^{-8} M have been detected in synovial fluids taken from patients with rheumatoid arthritis (Higgs *et al.*, 1974), and PGE_2 concentrations exceeding 10^{-6} M have been detected in periodontal lesions (Offenbacher *et al.*, 1986). It is conceivable that even higher levels are present in the immediate microenvironment where tissue cells (e.g., fibroblasts, macrophages) might secrete PGE_2 directly to neighbouring mast cells. Therefore, the doses of PGE_2 required for eliciting mast cell responses reflect those reported in clinical studies of inflammatory states.

Possibility of autocrine production of PGE_2

The possibility arose that the mast cell mediators or PGE_2 itself might be inducing further production of PGE_2 which would contribute to our observed effects. PGE_2 has been observed to induce COX-2 in murine bone marrow-derived mast cells (Murakami *et al.*, 1994), and mast cells are capable of secreting low levels of PGE_2 in response to certain stimuli including NGF (Marshall *et al.*, 1999). Additionally, each of the mast cell mediators whose production was enhanced by PGE_2 (i.e., histamine, IL-6, TNF-α, GM-

CSF) is capable, in turn, of stimulating PGE_2 synthesis (Orehek *et al.*, 1975; Dinarello *et al.*, 1991; Bachwich *et al.*, 1986; Delemarre *et al.*, 1995).

It is unlikely that endogenous PGE_2 production contributed significantly, if at all, to the observed effects of PGE_2 treatment in mast cell population used in our studies. Mast cells are poor producers of PGE_2 (Marshall *et al.*, 1999). We investigated the PGE_2-inducing capabilities of various factors on mouse BMMC including PGE_2 itself, IL-6, TNF-α, and LPS. None of these mediators provoked significant PGE_2 production. Moreover, we have previously reported that the cyclooxygenase inhibitor, flurbiprofen, does not affect anti-IgE or LPS-induced IL-6 production by rat PMC (Leal-Berumen *et al.*, 1995).

Possible residual PGE_2 in BMMC cultures

Because mast cell induction required the presence of PGE_2 (Hu *et al.*, 1995), we cultured the mouse BMMC in the presence of relatively low levels of PGE_2 (approx. 10^{-7} M). To minimize the effects of BMMC exposure to PGE_2, mast cells were thoroughly washed and rested out of PGE_2 for at least a day prior to conducting our experiments. Nevertheless, the effects of pre-exposure of BMMC to PGE_2 and possible residual PGE_2 may have potentially altered the data. The fact that PGE_2 similarly augmented IL-6, TNF-α, and degranulation in MC/9 cells which are grown in the absence of PGE_2, argues against the significant immunomodulatory effects of residual PGE_2 on BMMC responses.

4.4.2 Pre-formed vs newly produced mast cell TNF-α

Mast cells are unique in their ability to store TNF-α in their granules (Gordon and Galli, 1990; Gordon and Galli, 1991). The kinetics of release of pre-formed TNF-α are identical to those of other granule-stored mediators (Gordon and Galli, 1991). This important characteristic of mast cells seems to be at odds with the lack of significant TNF-α release 1 h following IgE/Ag-activation. The apparent conflict is reconciled when one takes into account the great heterogeneity that exists in terms of pre-formed TNF-α content (Gordon and Galli, 1990). Such heterogeneity reflects differences observed between freshly isolated cells and cultured cell lines, mature and immature cells, and possibly, species differences. Murine PMC store abundant TNF-α in their granules; conversely, cultured mast cells contain minimal or undetectable levels of this cytokine (Gordon and Galli, 1990). Similarly, we find that the levels of pre-formed TNF-α present in BMMC and MC/9 fall below the limits of detection of our in-house ELISA (generally 20-40 pg/ml), and therefore, contribute little to the total TNF-α response.

4.4.3 Issue of macrophage contribution in the BMMC studies

The BMMC used in our studies were of high purity (>95% based on Toluidine blue staining), contaminating cells consisting mainly of macrophage-like cells. To rule out possible contribution by macrophages, we examined the effects of IgE/Ag and PGE_2 stimulation in mouse peritoneal macrophages and the murine macrophage-like cell line, J774. IgE/Ag-activation in the presence or absence of PGE_2 failed to enhance IL-6 or TNF-α production indicating the unlikelihood of macrophage involvement in our observed effects. The lack of significant contribution to the observed cytokine responses

is further supported by the reproduction of the effects of PGE_2 (in terms of degranulation and IL-6/ TNF-α production) in the mast cell line, MC/9.

4.5 Future Experiments/Directions

4.5.1 Experiments currently underway in our laboratory

One of the central themes of this thesis has been the paradoxical actions of PGE_2 on murine mast cell mediators and the same mediators produced by other immune cell types, or even different species of mast cells (e.g., degranulation and TNF-α production). The key underlying mechanism for these opposing effects is likely to be differential usage of prostaglandin E receptor subtypes. For this reason, we recognize the importance of determining whether our findings were the result of coupling EP_1 or EP_3 or both receptor subtypes. We are currently conducting experiments to address this question. Due to the general paucity of receptor antagonists on the market at the moment, we have decided to employ agents that suppress specific signaling pathways to approach this question. For instance, SQ 22536 (an adenylate cyclase inhibitor) and pertussis toxin will be used to differentiate between the EP_4 (EP_2) and EP_3 receptors, respectively. In the case of EP_1, we have in our possession an EP_1 antagonist, SC-51322, which has been demonstrated to effectively block activation through the EP_1 receptor (Kimura *et al.*, 2001). Initial experiments examining mast cell pre-formed mediator release proved inconclusive because of possible direct effects of these drugs on mast cell degranulation. We expect to resolve these issues in the near future.

A major drawback to the current pharmacological approach is the relative non-selectivity of the EP agonists. In addition, observed modulation of a given response by PGE_2 represents the sum of effects on all expressed EP receptor subtypes, and in some cases other prostaglandin receptors. Solutions to these limitations are forthcoming. Mice deficient for every major prostaglandin receptor through targeted gene disruption are now in existence (Austin and Funk, 1999; Ushikubi *et al.*, 2000), and hopefully, will soon be commercially available. Experiments conducted in mast cells derived from mice lacking in each of the EP subtypes will definitively elucidate the relative participation of EP_1 and EP_3 receptors, and confirm any lack of involvement of the EP_2 and EP_4 receptor subtypes.

4.5.2 Effects of mast cell activation on EP receptor expression

We have demonstrated that resting MC/9 cells express the EP_1, EP_3, and EP_4 receptor subtypes (Figure 6, Chapter 2). The EP_2 receptor was conspicuously absent. Recent literature reported that LPS treatment upregulated EP_2 receptor expression on mouse macrophages and neutrophils (Katsuyama *et al.*, 1998; Yamane *et al.*, 2000). It would be interesting to determine whether IgE/Ag- or LPS-activation of mast cells resulted in *de novo* expression of EP_2 receptors. It would also further our understanding of how PGE_2 mediates its effects on mast cells if we track changes in the expression of the other EP receptors, particularly that of EP_1-v, the receptor of unknown physiological function.

4.5.3 Investigating additional activities of PGE_2 in type 2 inflammation

As described in earlier sections, PGE_2 performs two functions that make it a pivotal player in the pathogenesis of allergic inflammation. First, PGE_2 functions as an

environmental signal that "instructs" DC to initiate primary immune responses with a type 2 phenotype (Kalinski *et al.*, 1997; Kalinski *et al.*, 1998; Vieira *et al.*, 2000). Second, PGE_2 potentiates IL-4-directed IgE production by B cells (Roper *et al.*, 1990; Roper and Phipps, 1992).

Does PGE_2 upregulate $Fc_\varepsilon RI$ expression by mast cells?

It would, consequently, be of interest to study the actions of PGE_2 on $Fc_\varepsilon RI$ expression by mast cells. Our laboratory has previously shown that surface expression of this high affinity IgE receptor on rat mast cells is upregulated in the presence of exogenous IgE (Shaikh *et al.*, 1997), indicating that $Fc_\varepsilon RI$ expression is sensitive to modulation by tissue-derived signals. Based on the augmentation of IgE production by PGE_2 and on our observations of the sensitivity of IgE-mediated responses to modulation by PGE_2, it is conceivable that $Fc_\varepsilon RI$ expression might be similarly enhanced by the presence of PGE_2. Possible enhancement of receptor expression by pretreatment with PGE_2 may be examined indirectly by monitoring levels of bound IgE by FACS analysis.

4.6 Summary and concluding remarks

The evidence presented in this dissertation is consistent with the type 2-promoting activity of PGE_2 in other immune cells. Mast cells activated through cross-linking of surface $Fc_\varepsilon RI$ responded to PGE_2 stimulation with augmented degranulation as well as increased production of IL-6, TNF-α, and GM-CSF. These enhancing effects were selective as IL-4 production was suppressed by PGE_2 treatment. Moreover, they occurred via a cAMP- and IL-10-independent mechanism. Mast cells expressed the EP_1, EP_3, and EP_4 prostaglandin E receptor subtypes; whereas, EP_2 expression was absent. Pharmacological studies indicated that PGE_2 modulated mast cell responses through coupling of the EP_1 and EP_3 receptors. PGE_2 stimulation evoked a transient Ca^{2+} response. Dual activation of mast cells with IgE/Ag and PGE_2 induced a larger and sustained Ca^{2+} signal, suggesting that Ca^{2+} may play a role in mediating the effects of the prostanoid. Activation through EP_1/EP_3 presented a novel means of immune cell regulation since PGE_2 mediates its immunomodulatory effects through the EP_2/EP_4 receptors in many other leukocytes including macrophages, neutrophils, and lymphocytes. In addition, a splice variant of EP_1 (EP_1-v) was identified in mast cells that had previously only been reported in the rat system. Similar to the rat EP_1-v, the mouse counterpart presumably lacked an intracellular carboxyl terminus and may be devoid of signaling capability. Elucidation of the physiological function of this receptor is eagerly anticipated.

PGE_2-directed increases in TNF-α production by IgE/Ag-activated mast cells contrasts sharply with its well-recognized inhibitory effects on TNF-α production by other cell types. This is likely a stimulus-dependent effect since PGE_2 inhibited TNF-α

production by mast cells in the context of LPS stimulation. Nevertheless, these data indicate that PGE_2 is not necessarily a universal inhibitor of TNF-α production, and helps reconcile the presence of elevated levels of both TNF-α and PGE_2 in inflammatory milieu. Furthermore, they suggest caution with respect to studies testing PGE_2's potential as an anti-inflammatory drug to add to the arsenal of therapeutic tools for treating asthma.

Overall, the data presented in this thesis provide new insight into the complexity of the actions of PGE_2 in immune response, particularly in terms of its proposed function as a switch factor for type 2-dominated immune responses. Our findings that mast cells are uniquely regulated by PGE_2 might underscore the importance of these cells in the pathogenesis of a number of allergic inflammatory diseases, and also opens up fruitful avenues for future in-depth research.

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APPENDIX

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January 14, 2002

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