ANTIBODY-ENHANCED DENGUE VIRUS INFECTION OF HUMAN MAST CELLS

By

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The most beautiful thing we can experience is the mysterious. It is the source of all true art and science.

Albert Einstein

The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them.

Sir William Bragg

Science is the search for truth - it is not a game in which one tries to beat his opponent, to do harm to others.

Linus Pauling

What can I wish to the youth of my country who devote themselves to science? ...Thirdly, passion. Remember that science demands from a man all his life. If you had two lives that would not be enough for you. Be passionate in your work and in your searching.

Ivan Pavlov

The important thing is not to stop questioning. Curiosity has its own reason for existing. One cannot help but be in awe when he contemplates the mysteries of eternity, of life, of the marvellous structure of reality. It is enough if one tries merely to comprehend a little of this mystery every day. Never lose a holy curiosity.

Albert Einstein

The most exciting phrase to hear in science, the one that heralds new discoveries, is not "Eureka!" (I found it!) but "That's funny ..."

Isaac Asimov

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Abstract

Dengue virus, family *Flaviviridae*, is thought to be the most important vector-borne disease as it is estimated that 100 million individuals are infected with dengue virus annually.

We investigated the ability of dengue virus to infect mast cells and the resultant response. We have shown that human mast cell lines, KU812 and HMC-1, are permissive to antibody-enhanced dengue virus infection and that infection is dependent on FcγRII-mediated binding. Antibody-enhanced dengue virus infection resulted in infectious virus production as well as induction of significant levels of IL-1β and IL-6. Moreover, elevated levels of secreted RANTES, MIP-1α, and MIP-1β, but not IL-8 or ENA-78, were observed following infection of KU812 or HMC-1 cells. In some cases a greater than 200 fold increase in RANTES production was observed in response to dengue virus infection. Primary cultures of cord blood derived human mast cells (CBMCs) treated with dengue virus in the presence of sub-neutralizing concentrations of dengue specific antibody also demonstrated significantly ($p<0.05$) increased RANTES production, under conditions which did not induce significant short-term degranulation. In addition, we have shown IFN-γ treated CBMCs expressed higher levels of FcγRI and FcγRIII and enhanced RANTES production. Chemokine responses were not observed when mast cells were treated with UV-inactivated dengue virus in the presence or absence of human dengue specific antibody suggesting the response is dependent on active infection.

The mast cell response to dengue virus appears to be highly selective and specific as neither antibody-enhanced dengue virus infection of the highly permissive U937 monocytic cell line, nor adenovirus infection of mast cells, induced RANTES, MIP-1α, or MIP-1β. These results suggest a role for mast cells in the initiation of chemokine-dependent host responses to viral infection.

Abbreviations

Ab	antibody
Ad	adenovirus
ADCC	antibody-dependent cell-mediated cytoxicity
ADE	antibody-dependent enhancement
Ag	antigen
APC	antigen presenting cell
BAL	bronchoalveolar lavage
BSA	bovine serum albumin
CBMC	cord blood-derived mast cell
C	C chemokine
CC	CC chemokine
CCR	CC chemokine receptor
CD	cluster determinant
CSF	colony stimulating factor
CTL	cytotoxic T lymphocyte
CTMC	connective tissue mast cell
CXC	CXC chemokine
CXCR	CXC chemokine receptor
DC	dendritic cell
Den	dengue virus
DF	dengue fever
DHF	dengue hemorraghic fever
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNFB	2,4-dinitrofluorobenz
DSS	dengue shock syndrome
ELISA	enzyme-linked immunosorbent assay
ENA-78	epithelial cell-derived neutrophil activating protein 78
FACS	fluorescence-activated cell sorter
FcR	Fc receptor
FcεRI	high affinity IgE receptor
FcγR	Fc gamma receptor
FcγRI	high affinity IgG receptor
FcγRII	
FcγRIII	
FCS	fetal bovine serum
fg	femtogram
GM-CSF	granulocyte macrophage colony-stimulating factor
hr	hour
HEPES	*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid
HIV	human immunodeficiency virus
HMC-1	human mast cell line

ICAM	intracellular adhesion molecule
IFN-γ	interferon-gamma
Ig	immunoglobulin
IgE	immunoglobulin E
IgG	immunoglobulin G
IgM	immunoglobulin M
IL	interleukin
IL-1β	interleukin 1 beta
IL-6	interleukin 6
IL-8	interleukin 8
IP	immunoprecipitation
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
kDa	kilodalton
KU812	human mast cell/basophil line
LIF	leukemia inhibitory factor
LPS	lipopolysaccharide
LT	leukotriene
mAb	monoclonal antibody
MC_T	human mast cell containing tryptase
MC_{TC}	human mast cell containing both tryptase and chymase
MFI	mean fluorescent intensity
MHC	major histocompatibility complex
MIP-1α	macrophage inflammatory protein 1 alpha
MIP-1β	macrophage inflammatory protein 1 beta
MIP-3α	macrophage inflammatory protein 3 alpha
ml	millilitre
mRNA	messenger ribonucleic acid
NaB	sodium butyrate
ng	nanogram
NHS	normal human serum
PAF	platelet activating factor
PBL	peripheral blood lymphocyte
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
p.i.	post-infection
pg	picogram
PGE_2	prostaglandin E_2
PHA	phytohemagglutinin
PMA	phorbol myristate acetate
rhIL	recombinant human interleukin
RANTES	regulated upon activation normal T cell expressed and (presumed) secreted
RNA	ribonucleic acid
RSV	respiratory syncytial virus
RT-PCR	reverse transcription-polymerase chain reaction

SCF	stem cell factor
SEM	standard error of the mean
SD	standard deviation
SDF-1	stromal cell derived factor 1
SDS-PAGE	sodium-diodecyle-sulphate polyacrylamide gel electrophoresis
TLR	toll-like receptor
Th	T helper cell
TNF-α	tumour necrosis factor alpha
μg	microgram
UV	ultraviolet
UV-Den	UV-inactivated dengue virus
UV-Ad	UV-inactivated adenovirus
VCAM	vascular adhesion molecule
VCP	vaccinia virus complement protein

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Chapter 1: Introduction

The following introduction will provide the reader with an overview of basic mast cell biology, mast cell interactions with viruses and dengue virus biology. The introduction should provide the reader with enough information so that the following sections of the thesis are understood.

1.1 Mast cells

Mast cells were first described by Paul Ehrlich in 1878 as a group of connective tissue-resident cells that stained purple with basic blue aniline dyes (Ehrlich, 1878). Since their discovery over a hundred years ago we have come a considerable way in investigating the nature and role of mast cells. However, despite considerable investigation, the complexity and multifunctional nature of mast cells remains a 'murky' area. Mast cells are a group of unique immune effector cells that exhibit complex heterogeneity between different species and tissue locations. For many years mast cells were viewed as harmful due to their role in allergic disease though there has been increasing evidence of a protective role for mast cells against bacterial infection (Echtenacher *et al.,* 1996; Malaviya *et al.,* 1996).

Mast cells are bone marrow derived immune effector cells resident in almost every major tissue and organ (Metcalfe *et al.,* 1997) and it is estimated that the total number of mast cells in the body can amount to the size of an individual spleen (Bradding, 1996). Most notably, mast cells are found in high numbers at sites that directly interface with the external environment including the skin, gastrointestinal and respiratory tract. It has been estimated that there are approximately 7,000 mast cells per mm3 in human skin (MIkhail & Miller-

Milinska, 1964). Mast cells have also been shown to be in close association with blood vessels (Selye, 1966) and nerves (Marshall & Waserman, 1995). Furthermore, mast cells have been recently described as sentinel cells (Galli *et al.,* 1999) due to their anatomical location and their ability to influence and direct specific immune responses.

1.2 Characteristics of mast cells

Mast cells arise from CD34+ hematopoietic progenitor cells that exit the bone marrow as immature mast cell precursors with maturation taking place in the peripheral tissues (Nilsson *et al.,* 1999). Mast cells range in size from 10 to 18 microns and may appear round, oval or elongated with many long, thin projections of the cell surface. The single, eccentric nucleus is generally round or oval, with occasional multiple lobules. Data suggest that each mast cell contains approximately 50 to 200 secretory granules varying in size from 0.2-0.5 μm in diameter (Weber *et al.,* 1995). Four types of granules are observed, by electron microscopy, in human mast cells based on granule structures including scroll, crystal, particle or a combination of all three (Holgate *et al.,* 1993). Granule structure varies with regard to tissue location as human lung and gut mast cells mainly contain scroll type granules and numerous lipid bodies while human skin mast cells contain predominately crystal type granules with few lipid bodies (Figure 1) (Dvorak, 1986). Mast cells also exist as heterogeneous populations that differ in mediator content as well as mode of activation and function.

1.3 Mast cell heterogeneity

1.3.1 Classification and distribution of human mast cell subtypes

Human mast cells have been classified into two subclasses based on neutral protease content and are denoted MC_T and MC_{TC} depending on whether they contain only tryptase or both tryptase and chymase (Irani *et al.,* 1986; Schwartz & Huff, 1993) (Table 1).

MC_{TC} are found mainly at connective tissue sites such as the skin, intestinal submucosa, conjunctiva, synovium and around blood vessels. In contrast, MC_T are most prevalent at mucosal sites including the alveolar septa of the lung and the mucosa of the small intestine (Irani *et al.*, 1986). The picture of mast cell distribution, however, is not that simple. Studies on mast cell subtype tissue-specific distribution have demonstrated that while a particular subclass is more abundant at certain anatomical locations, both subtypes can usually be found. Interestingly, the ratio of mast cell subtypes has also been found to alter in various tissues under certain pathological condition (Schwartz & Huff, 1998).

1.4 Mast cells - a source of multifunctional mediators

Upon activation, mast cells are capable of producing a wide range of mediators, including preformed granule associated products such as histamine, proteoglycans and protease enzymes, with substantial effects on the vasculature and immune system. In addition, mast cells can produce newly synthesized lipid mediators such as LTC_4, LTB_4, PAF, PGD_2 and PGE_2 as well as low levels of nitric oxide (Masini *et al.,* 1991). However, probably most important to the role of mast cells in regulating immune responses are the very wide range of cytokines and chemokines they can produce including IL-1β, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, IL-16, GM-CSF, TNF-α, LIF, PAF, IFN-γ, TRAIL, RANTES, MIP-1β, MIP-1α, MIP-3α, Eotaxin, ENA-78 and VEGF (Hogaboam *et al.,* 1998). While the majority

of cytokine and chemokines released by mast cells are newly synthesized, some cytokines like TNF-α (Beil *et al.,* 1995; Walsh *et al.,* 1991), IL-4 (Bradding *et al.,* 1992; Bradding *et al.,* 1993), IL-5 (Bradding *et al.,* 1993), IL-6 (Bradding *et al.,* 1993) and bFGF (Dvorak *et al.,* 2001) have been shown to be stored both in mast cell lipid bodies and/or granules.

1.4.1 Preformed mediators

Mast cells contain abundant cytoplasmic granules within which they store a variety of preformed mediators, including histamine, proteoglycans, neutral proteases and other enzymes. Release of preformed mediators usually occur within a few seconds upon activation either through cross-linking of surface Fc receptors, particularly, IgE-FcεRI cross-linking, or through elevation of intracellular Ca2+ for example as a result of calcium ionophore stimulation. Such activation induces mast cell degranulation, a process that is characterized by granules rapidly moving towards and fusing with the plasma membrane, resulting in release of granular contents into the microenviroment. The mediators released serve to modulate the inflammatory response and thus both the innate and specific immune responses.

Of the preformed mediators released by mast cells, histamine is probably the most widely recognized. Histamine is a biogenic amine synthesized from the precursor amino acid histidine and is stored in granules in ionic association with heparin (Riley & West, 1953). Histamine has many physiological effects including bronchospastic and vasodilator activity (Dale & Laidlaw, 1910) as well as increasing vascular permeability and induction of smooth muscle contraction of the gastrointestinal tract (Nilsson *et al.,* 1999).

Other preformed mediators include the neutral proteases, tryptase and chymase, on which human mast cell classification is based. Neutral proteases are a group of proteolytic enzymes that primarily act as degradative enzymes and have been shown to mediate several pathophysiological processes, including growth and development, tissue remodelling, and inflammation (Dery *et al.,* 1998; Schechter *et al.,* 1998). In addition proteoglycans, including heparin and chondrotin sulfate, are present at the central core of granules and serve as storage matrices for neutral protease and histamine (Metcalfe *et al.,* 1997). Recent evidence suggest that heparin is essential for proper granule formation and storage (Forsberg *et al.,* 1999; Humphries *et al.,* 1999). The final group of preformed mediators include acid hydrolases, of which the most well known is β-hexosaminidase. β-hexosaminidase is released in parallel with histamine during degranulation and is often the choice method to measure mast cell degranulation. As mentioned above, some cytokines are also stored within granules or lipid bodies.

1.4.2 Newly synthesized mediators

Mast cells synthesize mediators in response to activation by a variety of methods including IgE cross-linking, calcium ionophore, bacterial stimulation, and recently IgG-mediated activation. These newly synthesized mediators include lipid-derived mediators and cytokine and chemokines (Metcalfe *et al.,* 1997).

1.4.2.1 Lipid mediators

Lipid-derived mediators are generated from arachidonic acid and consist of prostaniods,

PGD_2 and PGE_2, and leukotrienes, LTC_4 and LTB_4. These *de novo* mediators are often formed and released from mast cells within minutes of activation and have important pathophysiological roles. The major lipid-derived mediators produced by human mast cells are PGD_2 and LTC_4 (Peters *et al.*, 1984). PGD_2 is a potent inhibitor of platelet aggregation, is chemokinetic for human neutrophils and it is has been found to enhance mast cell histamine release by antigen (Lewis & Austen, 1984). Leukotrienes, including LTC_4, have been demonstrated to, among others, stimulate prolonged bronchoconstriction and enhance vascular permeability with 10-1000 times more potency than histamine (Drazen & Austen, 1987). Leukotriene production by mast cells has also been shown to be very important in the pathogenesis of asthma by virtue of its bronchoconstriction activity. Platelet activation factor (PAF), another lipid-derived mediator, has been shown to aggregate and degranulate human platelets, induce wheal-and-flare reactions in human skin, and lead to systemic hypotension (Demopoulos *et al.*, 1979).

1.4.2.2 Cytokines and chemokines

Cytokines and chemokines are a group of proteins or glycoproteins synthesized and secreted by cells. They exhibit a wide range of biological activities including generation and maintenance of inflammatory responses and directed chemotaxis of immune effector cells. As described previously, mast cells are a good source of a variety of both cytokines and chemokines. Many of the cytokines and chemokines identified to date can be found in the list of mast cell mediators including the pro-inflammatory cytokines IL-1α and β, IL-6 and TNF-α (Benyon *et al.*, 1991; Bradding *et al.*, 1993; Gordon *et al.*, 1990; Grabbe *et al.*, 1994;

Moller *et al.*, 1998; Nilsson *et al.*, 1995), type 1 cytokines IL-2, IFN-γ and IL-12 (Gupta *et al.*, 1996; Oh *et al.*, 2001), as well as type 2 cytokines IL-4, IL-5, IL-13 (Kobayashi *et al.*, 1998; Plaut *et al.*, 1989; Tachimoto *et al.*, 2000). In addition GM-CSF, IL-3, IL-10, and IL-16 (Plaut *et al.*, 1989; Razin *et al.*, 1991; Rumsaeng *et al.*, 1997; Wodnar-Filipowicz *et al.*, 1989) are all produced by mast cells. The chemokines RANTES, MIP-1α, MIP-1β, MIP-3α, IL-8, SDF-1α, and MCP-1 (Lukacs *et al.*, 1998; Moller *et al.*, 1993; Rajakulasingam *et al.*, 1997; Selvan *et al.*, 1994; Tedla *et al.*, 1998; Teshima *et al.*, 2000; Yano *et al.*, 1997) have also been shown to be produced by mast cells.

1.4.2.2.1 Characteristics of chemokines

Chemokines are a superfamily consisting of over forty small (7 to 10 kDa) soluble proteins (Baggiolini *et al.*, 1997a; Taub, 1996). Amongst the structural similarity between chemokines, a motif consisting of four conserved cyteine residues is common to most chemokines. The chemokines containing the tetra-conserved-cysteine motif are subdivided into two groups depending on the arrangement of the amino terminal two conserved cysteines; CC (also termed β chemokines) and CXC, where X represents any amino acid residue. Recently, two new chemokines were identified that did not fit into the above divisions, and have introduced two new groups: CX_3C and C (Baggiolini *et al.*, 1997b; Pease & Murphy, 1998). Interestingly, the C chemokine only has two of four conserved cysteines, and the CX_3C chemokine can exist in both soluble and membrane bound forms, demonstrating some greater variability amongst chemokines. Throughout the text, I use common abbreviations for chemokines for which common names and new nomenclature as

well as receptors are provided. A partial list, including the chemokines I will discuss, can be found in Table 2. All tissue cells are capable of expressing chemokines, although the set of chemokines that are expressed may vary between cell types. Some chemokines have limited tissue specific expression. Furthermore, knock-out experiments suggest that some chemokines are essential while others appear to serve redundant roles (Olson & Ley, 2002).

1.4.2.2.2 Characteristics of chemokine receptors

Chemokines are soluble ligands for chemokine receptors expressed on a wide range of leukocytes and other cells. Over fifteen such cellular chemokine receptors have been identified and each receptor is capable of binding different sets of chemokines (Premack & Schall, 1996). As demonstrated in Table 2, each receptor is capable of binding different sets of chemokines. With exception of the duffy antigen, chemokine receptors recognize either CC or CXC chemokines but not both, and are therefore called CCR (CC receptors) or CXCR (CXC receptors) respectively. Expression of different chemokine receptors is cell-type specific, thereby providing each leukocyte type with a specificity for particular chemokines (Baggiolini *et al.*, 1997a; Taub, 1996). Regulation of chemokine receptor expression differs not only between cell-type, but also between different T-cell subsets (Th1 vs. Th2) and activation or differentiation states. The variability available to the chemokine system is important for providing distinct functions. The promiscuity and redundancy, however, may serve to amplify a response or increase its rapidity.

Chemokine receptors are serpentine (seven transmembrane spanning domains) heterotrimeric G- protein coupled receptors, which initiate signaling pathways upon binding

to their ligands. The amino acid identity of chemokine receptors varies between roughly 40-80%; however, they share several conserved structural motifs (Schluger & Rom, 1997). Receptor binding to ligand is mediated by the extracellular amino-terminus. The hydrophobic membrane spanning domains are highly conserved, as are certain intracellular loop regions that allow G-protein interactions and phosphorylation. Differences between chemokine receptors reflect their binding to distinct ligands, but also their ability to respond to binding with signaling events that lead to degranulation, lymphocyte homing, activation, hematopoiesis or chemotaxis (Campbell *et al.,* 1997; Foxman *et al.,* 1997; Quan *et al.,* 1996).

1.4.2.2.3 Chemokines and chemokine receptors - function

The most recognized function of chemokines is the chemokine-specific recruitment of distinct leukocyte populations to sites where an inflammatory or immunological response is desired (Baggiolini & Moser, 1997; Taub, 1996). Chemokines expressed at such sites promote rapid adhesion, activity and chemotaxis of leukocytes from the circulatory system into the tissue. Briefly, as leukocytes are rolling along the blood endothelium, they come in contact with chemokines held non-covalently, via glycosylaminoglycans, to activated endothelial cells. Binding of chemokines to the respective chemokine receptor on the leukocyte induces increased expression of integrins that allow firm adhesion to the endothelium. A 'hierarchy' and gradient of chemokines that provide directional cues mediate leukocyte extravasation through the endothelium and migration towards the site of inflammation (Foxman *et al.,* 1997). Not only do chemokines direct leukocytes to the

correct site, but differential chemokine release also ensures that the correct leukocyte types are recruited and that the requisite effector response is thereby provided. For example, altering of chemokine receptor expression by dendritic cells allows the recruitment of immature cells to the site of inflammation for antigen sampling followed by upregulation of chemokine receptors that guide the mature cells to lymph nodes for stimulation of antigen specific T-cells. The large collection of chemokines and chemokine receptors, along with the temporal and spatial variability of expression on different cell types provides the immune system with a method to direct specific effector functions depending on the circumstance.

Chemokines are presumed to be involved in hematopoiesis as well as the constitutive recirculation and homing of lymphocytes (Cook, 1996; VerFaillie, 1996). Studies have also demonstrated that chemokine-receptor interactions are necessary during the developmental stage of leukocytes, as well as in angiogenesis, the formation of new blood vessels (Arenberg *et al.*, 1997).

A brief description with respect to function of a few cytokines and chemokines of interest to our studies is presented below.

TNF-α

TNF-α is produced early during inflammatory reaction and exerts many biological effects (Papadakis & Targan, 2000). TNF-α is a pro-inflammatory cytokine that induces fever and plays a major role in sepsis (reviewed in Standiford & Strieter, 1992). In addition, TNF-α stimulates the production of IL-1 and IL-6 in what is known as the cytokine cascade, further

contributing to the inflammatory environment. Furthermore, TNF-α plays a role in generation of an immune response by aiding in the recruitment of leukocytes through both direct chemotactic effects and by upregulating adhesion molecules on the surface of endothelial cells. TNF-α has been demonstrated to upregulate both ICAM-1 and VCAM-1 on the surface of endothelial cells thus allowing for attachment of leukocytes to the endothelium thereby promoting extravasation through the tissues (Rothlein *et al.*, 1988; Walsh *et al.*, 1991). Interestingly, mast cells are the only known source of pre-formed TNF-α (Beil *et al.*, 1991).

IL-1β

IL-1β shares many pro-inflammatory properties with TNF-α including induction of fever (Atkins, 1960), inflammation and shock (Dinarello, 1996). IL-1β is synthesized as inactive pro-IL-1β that is cleaved by IL-1β converting enzyme (ICE) to generate active IL-1β (Wewers *et al.*, 1997). Specific blockade of IL-1β reduces morbidity and mortality in septic shock (Dinarello, 1991). IL-1β induces IL-6 production and activates endothelial cells (Pober, 1988) modulating the expression of the adhesion molecules ELAM-1 (Bevilacqua *et al.*, 1987; Pober *et al.*, 1986) and ICAM-1 (Pober *et al.*, 1987). Such enhanced adhesion molecule expression may lead to inflammatory cell activation and migration with consequent potential vasculitic damage. IL-1β alters endothelial cell morphology causing reorganization of monolayers (Pober *et al.*, 1987). This activation and reorganization most probably contributes to enhanced vascular permeability. In addition, IL-1β effectively induces antigen-specific serum IgG production and lymphocyte proliferation (Staats & Ennis, 1999).

IL-6

IL-6 is generally known as a pro-inflammatory cytokine but has been shown to exert anti-inflammatory effects as well (Tilg *et al.,* 1997; Zhu *et al.,* 1998). Inflammatory properties associated with IL-6 include induction of the acute phase response and promotion of the TNF-IL-1-IL-6 inflammatory cascade (Bluethmann *et al.,* 1994). IL-6 is an endogenous pyrogen (Helle *et al.,* 1988) known to mediate increased endothelial cell permeability (Maruo *et al.,* 1992). In rodent systems, mast cells have been shown to be a much more potent source of IL-6 than other cell types such as the macrophage (Leal-Berumen *et al.,* 1994). In addition, IL-6 promotes B and T cell growth and differentiation and induces maturation and activation of neutrophils, eosinophils and macrophages, thus playing an important role in host defense (Curfs *et al.,* 1997). Furthermore, IL-6 has been shown to increase mucous production and enhance B cell IgA class switching (Goodrich & McGee, 1999) and thus appears to be important in mucosal host defense. IL-6, in combination with SCF, has also been demonstrated to induce a high tryptase expressing mast cell like phenotype (Saito *et al.,* 1995).

GM-CSF

GM-CSF is a cytokine that serves as a potent growth factor for granulocytes, macrophages, and dendritic cells and enhances the activity of neutrophils and eosinophils (Clark & Kamen, 1987; Hill *et al.,* 1995). In addition, GM-CSF has been shown to be a critical mediator of dendritic cell generation and maturation *in vitro* (Herbst *et al.,* 1998; Lardon *et al.,* 1997) and mast cells are the most potent known cellular source of GM-CSF (Wodnar-Filipowicz *et*

al., 1989).

RANTES

RANTES (regulated upon activation normal T cell expressed and (presumed) secreted) was first discovered in1988 by Schall and colleagues (Schall *et al.*, 1988). Since then studies have demonstrated a variety of specific biological functions of RANTES. These include directing selective recruitment of monocytes, CD4+ T cells, eosinophils, basophils, CD4+/CD45RO+ memory T cells and mast cells (Baggiolini, 1998; Schall *et al.*, 1990; Baggiolini & Dahinden, 1994; Alam *et al.*, 1993). Cellular sources of RANTES include T cells, platelets, epithelial cells, endothelial cells, eosinophils, fibroblasts and mast cells (Graziano *et al.*, 1999). RANTES has been shown to increase adherence of monocytes to endothelial cells and selectively support the migration of monocytes and CD4+ memory helper T cells (Schall *et al.*, 1990). In addition, RANTES has been demonstrated to activate human eosinophils and basophils inducing histamine release (Alam *et al.*, 1993; Kuna *et al.*, 1992).

MIP-1α

MIP-1α (macrophage inflammatory protein 1 α) is a chemoattractant for a wide variety of immune cells (reviewed in Luster, 1998), though it has its greatest effect on CD8+ T cells (Taub *et al.*, 1993) and immature dendritic cells (Foti *et al.*, 1999). MIP-1α, along with RANTES, also facilitates the induction of a Th1 immune response (Schrum *et al.*, 1996). Finally, MIP-1α has been shown to improve CTL activity when combined with a plasmid

DNA vaccine for HIV (Lu *et al.,* 1999) and when used as an exogenous adjuvant in a hepatitis B vaccine (Flesch *et al.,* 2000). Furthermore, MIP-1α has been demonstrated both *in vitro* (Taub *et al.,* 1993) and more recently, *in vivo* (van Deventer *et al.,* 2002) to have a greater effect on CD8+ T cells than on CD4+ T cells. MIP-1α has also been shown to induce fever (Davatelis *et al.,* 1989) and inhibit hematopoietic progenitor cell growth (Bonnet *et al.,* 1995).

MIP-1β

In vitro studies suggest that MIP-1α and MIP-1ß preferentially attract CD8+ and CD4+ T cells, respectively (Taub *et al.,* 1993; Tanaka *et al.,* 1993; Schall *et al.,* 1993). Interestingly, Tedla and colleagues demonstrated mast cells as the predominant cellular source of MIP-1ß in the lymph nodes of DNFB-treated mice, a model of contact hypersensitivity (Tedla *et al.,* 1998). Previous *in vitro* experiments described the expression of MIP-1ß mRNA by mast cell lines (Burd *et al.,* 1989; Kulmburg *et al.,* 1992) though until this time mast cell MIP-1β protein expression had not be investigated *in vivo*. The authors suggest that the brisk appearance of mast cells in a distinctive location in the subcapsular and hilar regions of the nodes, in association with the decrease in their number in the affected skin shortly after repainting with DNFB, strongly suggests that mast cells travel from the skin via the afferent lymphatic system. Furthermore this study demonstrated that mast cells are not only a source of histamine, serotonin, and other vasoactive amines that are believed to control vascular tone and permeability but also act as a key early regulator of T cell recruitment into draining lymph nodes (Tedla *et al.,* 1998).

1.5 Human mast cell models

Much of the work investigating mast cell function has been accomplished using rodent mast cell models of which there are a wide supply. In addition to mast cell lines, primary mast cells can be generated from short term culture of bone marrow progenitors isolated from rats or mice and have been instrumental in contributing to our current understanding of mast cell biology. There are very few human mast cell models available for use, due in part, to the difficulty in obtaining and culturing mast cells from humans. Mast cell are resident tissue cells which makes isolation of primary cells extremely difficult. Many investigators have turned to the popular mast cell lines KU812 and HMC-1. In addition, primary mast cells have been generated by a few committed mast cell labs in the world by isolation and long term culture of either human CD34+ peripheral blood progenitors or cord blood mononuclear cells.

1.5.1 KU812 cells

The KU812 cell line (Table 3) was established from a patient with blastic crisis of chronic myelogenous leukemia in 1985 and has been widely used as a mast cell/basophil model (Kishi, 1985). KU812 cells are generally described as pre-basophilic; however, studies over the past several years have demonstrated that these cells have properties of both mast cell and basophils (Table 3) (Blom *et al.*, 1992) closely resembling MC_T type human mast cells (Figure 2) (Hartmann *et al.*, 1995). Kishi's original report demonstrated that KU812 cells could be stained positive with toluidine blue, had granules that stained positive with Astra blue and the cells contained a low level of histamine, approximately 5 fg/cell. KU812 cells

have been demonstrated to express mRNA for mast cell tryptase, mast cell carboxypeptidase and low level expression of major basic protein, a basophil marker (Blom *et al.*,1992). Furthermore, the KU812 cell line can be differentiated into a more mature mast cell with high tryptase content upon treatment with stem cell factor (SCF) and IL-6 (Saito *et al.*, 1995). Differentiation with SCF and IL-6 has been shown to increase the percentage of cells with metachromatic, tryptase positive and chymase positive granules. In addition, these differentiated KU812 cells expressed CD117 and lacked CD18, CD26 and CD31, markers not present on mast cells (Saito *et al.*, 1995). Differentiation of KU812 cells into more mature mast cells has also been demonstrated by treatment with sodium butyrate and TNF-α (Nilsson *et al.*, 1994) and IL-4 (Hara *et al.*, 1998). Differentiation with IL-4 upregulated surface FcεRI and resulted in IgE-mediated histamine release from these cells (Hara *et al.*,1998).

1.5.2 HMC-1 cells

The HMC-1 mast cell line was originally derived from a patient with mast cell leukemia in 1988 (Butterfield *et al.*, 1988). Butterfield and colleagues demonstrated that HMC-1 cells showed many characteristics (Table 4; Figure 3) of immature mast cells, including low levels of histamine that stained metchromatically by toluidine blue and they contain tryptase activity. Furthermore, they found that HMC-1 cells lacked T and B cell, as well as myeloid cell markers. Interestingly, while characterized as an immature mast cell, HMC-1 cells were found to lack IgE receptors (Butterfield, *et al.*,1988) meaning they are unable to bind IgE (Nilsson *et al.*, 1994). Subsequent work has demonstrated that HMC-1 cells express high

levels of c-Kit (CD117) on the surface of these cells as well as expression of chymase (Xia *et al.*, 1995). While HMC-1 cells express c-Kit, a gain-of-function mutation has been described (Mitsui *et al.*, 1993) making this cell line unresponsive to stem cell factor (SCF), a major growth factor for mast cells (Furitsu *et al.*, 1993; Welker *et al.*, 1995; Grabbe *et al.*, 1994). Finally, HMC-1 cells have been found to exhibit a number of phenotypic and functional properties typical of human tissue mast cells including, among others, production of IL-8 (Moller *et al.*, 1993) and IL-6 (Kruger-Krasagakes *et al.*, 1996).

1.5.3 Primary cultured mast cells

Tissue mast cells differentiate and mature *in situ* from committed progenitor cells (PrMCs), which arise in the bone marrow compartment from pluripotent hematopoietic progenitors (Kitamura *et al.*, 1978; Kirshenbaum *et al.*, 1991) and circulate as mononuclear leukocytes lacking characteristic secretory granules (Castells *et al.*, 1996; Rodewald *et al.*, 1996). Circulating PrMCs differ from monocytes by their surface expression of the receptor for stem cell factor (SCF), c-kit (Rodewald *et al.*, 1996), by their SCF-dependent proliferation *in vitro* in humans (Rottem *et al.*, 1994) which in mice is strongly augmented by IL-3 (Rodewald *et al.*, 1996), and by their lack of CD14 expression (Agis *et al.*, 1993). The normal development and survival of all tissue mast cell populations *in vivo* requires SCF. Additional factors that are derived from T lymphocytes, including IL-3 (Lantz *et al.*, 1998), are required for the development of the mast cell sub-population that normally resides in the intestinal epithelium in both mice and humans but not for normal mast cell development in the skin or connective tissues. However, mast cell hyperplasia at all sites following nematode parasite infection is

highly IL-3 dependent.

One of the best models for study of tissue mast cells are primary cultures of mast cells from stem cells found within the cord blood or bone marrow of humans. These cells have many of the characteristics, both morphological and functional, of tissue mast cells. There are several different methods available to generate primary cultured mast cells that employ different mast cell growth and differentiation factors.

Saito *et al.,* (1996) described the generation of primary mast cells from the culture of cord blood mononuclear cells (CBMC). Cord blood derived mast cells cultured in the presence of SCF, IL-6, and PGE2 for >10 wk were found to be 99% pure, and appeared to be functionally mature. These cells were found to have high histamine and tryptase content and, when sensitized with human IgE and then challenged with anti-human IgE or anti-FcεRI, the cells released a variety of mediators such as histamine, and an increase in intracellular Ca^{2+} was found in advance of the activation of membrane movement consistent with tissue mast cells. Furthermore, electron microscopic analysis revealed many of the cultured mast cells were morphologically mature as assessed by the presence of scroll and crystal granules (Saito *et al.,* 1996).

Our laboratory employs a modification of the method of Saito *et al.,* (Saito *et al.,* 1996) for the generation of primary mast cells from the culture of human cord blood mononuclear cells. These cells have been characterized as previously described (Lin *et al.,* 2000) and found to be consistently positive by flow cytometry for c-kit, FcεRI and CD13 but not CD14. Cells are cultured in the presence of IL-6, PGE_2 and SCF. This culture method

yields mast cells with characteristic morphology, including multiple metachromatic granules and appropriate nuclear morphology, and a very high purity.

K.F. Austen's group employs a modification of the method of Saito *et al.,* (1996). This group generates primary mast cells from cord blood mononuclear cells cultured in the presence of SCF, IL-6, and IL-10, as previously described for the development of mouse mast cells (Yuan *et al.,* 1998). Cultures are carried for up to 9 weeks and found to be morphologically and functionally similar to tissue mast cells.

The fourth major group to generate primary mast cell cultures uses CD34+ progenitor cells isolated from peripheral blood. CD34+ cells are cultured in the presence of SCF , IL-6 and IL-3 for 8-10 weeks (Rottem *et al.,* 1994). These mast cells are found to be morphologically similar to tissue mast cells as examination of the ultrastructural anatomy of cells by electron microscopy show immature mast cells containing predominantly tryptase-positive granules that were either homogeneous or contained lattice structures, partial scroll patterns, or central dense cores and mixtures of vesicles, fine granular material, and particles. By 6 weeks, CD34+-derived mast cells express Kit, FcεRI and FcγRII, and are negative for CD34 antigen (Rottem *et al.,*1994).

1.6 Fc receptors on mast cells

Human mast cells express the high affinity receptor for IgE (FcεRI) (reviewed in Daeron, 1997) and have recently been shown to express the high affinity receptor for IgG (FcγRI) upon treatment with IFN-γ (Okayama *et al.,* 2000) as well as the low affinity IgG receptor FcγRII (Okayama *et al.,* 2001). KU812 cells express FcεR1, the high affinity IgE receptor,

(Almlof *et al.,* 1988; Magnusson *et al.,* 1995), and FcγRII, (Kawata *et al.,* 1996). In contrast to KU812 cells, HMC-1 cells have been shown to express FcγRII (Wedi *et al.,* 1996) but do not consistently express FcεRI (Xia, *et al.,*1995).

1.7 Mast cells and host defence

1.7.1 Location and role of mast cells

Mast cells are resident tissue cells widely studied in the context of allergic disease. They are found throughout the body, but are particularly prevalent at sites which interface with the environment such as the skin, airways and gastrointestinal tract and have been viewed as sentinel cells in host defence (Galli *et al.,* 1999). Furthermore, mast cells have been shown to be essential mediators of specific immunity. Mast cells have been found in high numbers in lymph nodes in close association with T cells (Mekori & Metcalfe, 1999; Tedla *et al.,* 1998). Mast cells express and are able to present antigen via MHC Class II molecules to T cells (Frandji *et al.,* 1996). Mast cells have been shown to phagocytose antigen via IgG opsonization (Daeron *et al.,* 1993; 1994) and may play a role in promoting B cell class switch. In most locations, they are closely associated with blood vessels and nerves (Selye, 1966; Stead *et al.,* 1989) and mast cell mediators have been shown to alter vascular permeability.

1.7.2 Selective mast cell mediator production in response to pathogens and their products.

In early studies examining the production of mast cell mediators, it was largely assumed that

mast cells produced a full range of products upon activation. In models of allergic disease, the cross linking of IgE, bound to high affinity FcεRI receptors, by antigen or anti-IgE induces degranulation, lipid mediator production and the production of certain cytokines and chemokines. However, in studies examining mast cell responses to other stimuli such as bacterial and viral products it has become increasingly apparent that mast cells are capable of producing very different profiles of mediator production dependent upon the mechanism by which they are activated. For example, early studies examining the responses of rat peritoneal mast cells to LPS and PGE_2 demonstrated the production of IL-6 in the absence of significant degranulation determined by histamine release (Leal-Berumen *et al.,* 1994; 1995). Since that time, a variety of other stimuli and cell types such as LPS treatment of mouse bone marrow derived mast cells (BMMC) (McCurdy *et al.,* 2001) and cholera toxin (Leal-Berumen *et al.,* 1996) have been demonstrated to exhibit similar responses. This ability to selectively produce cytokines is not limited to IL-6 and isolated bacterial products. For example, treatment of rat peritoneal mast cells with IL-12 induced mast cell IFN-γ production (Gupta *et al.,* 1996) and treatment of mouse mast cells with CpG-containing oligonucleotides can induce TNF-α production without degranulation (Zhu & Marshall, 2001). Whole pathogens or viral infection can also lead to such degranulation-independent cytokine responses. Our studies have recently demonstrated that treatment of human cord blood derived mast cells with *P. aeruginosa* lead to GM-CSF and MIP-3α production (Lin and Marshall, personal communication). IgE-mediated activation of mast cells, although widely used as the "gold standard" for physiological mast cell activation, does not induce the full range of cytokines induced by some other stimuli. For example, neither IFN-γ nor

MIP-3α are induced following IgE mediated activation of mast cells while they can be induced by IL-12 or *P. aeruginosa* treatment, respectively.

A number of mast cell cytokine responses such as the TNF-α response to LPS challenge in mice and the MIP-3α response in humans occur very rapidly. In these cases, the mast cell may not be the prevalent cytokine source over the long term, but early production may be critical in mobilizing effective immunity against a rapidly growing infectious agent. Mast cells are, however, capable of long term ongoing production of several other cytokines such as IL-6, TGF-β, IL-10 and VEGF. In such cases, mast cell cytokine expression may contribute to tissue remodelling, and the resolution of local inflammation.

1.7.3 A critical role for mast cells in host defence and inflammation.

Mast cells have been shown to play a critical protective role in host defence against bacterial infections. In mouse models, of acute enterobacterial infection and septic peritonitis following caecal ligation and puncture (Echtenacher *et al.*, 1996; Malaviya *et al.*, 1996), mast cell-deficient mice were shown to have a higher death rate following infection than mast cell-containing mice. Such mast cell enhancement of effective immunity was dependent on mediator production, with TNF-α singled out as having a critical role, since antibody blockade of TNF-α ablated mast cell-dependent responses to bacteria *in vivo* (Echtenacher *et al.*, 1996). The mast cell-dependent recruitment of neutrophils is thought to provide a key effector mechanism for effective bacterial responses. It is well established that neutrophil recruitment can be enhanced by TNF-α and murine mast cells have both preformed, granule associated stores of this cytokine as well as the ability to rapidly generate and release newly

synthesised TNF-α (Gordon & Galli, 1990). However, it has still not formally been demonstrated that mast cells are the direct source of the majority of TNF-α in bacterial host defence. It remains likely that TNF-α is predominately produced by other cell types such as resident macrophages but plays a critical role in an early mast cell dependent effector cascade involving multiple mediators.

Leukotrienes have also been shown to be important mast cell mediators *in vivo* in the context of bacterial infection. Significant levels of LTB_4 have been reported to be produced by murine mast cells in response to *E .coli* and a leukotriene synthesis inhibitor has been demonstrated to block bacterial clearance and neutrophil influx in peritoneal cavities of mast cell-containing but not mast cell-deficient mice (Malaviya & Abraham, 2001). In more recent work examining the role of Janus Kinase 3 (JAK3), it was suggested that this kinase plays a key role in regulating mast cell mediated innate immunity against *E. coli* induced peritonitis (Malaviya *et al.,* 2001). In these studies, W/W^v mice reconstituted with $JAK3^{+/+}$ mast cells showed improved survival, bacterial clearance and neutrophil influx compared with similar mice reconstituted with $JAK3^{-/-}$ mast cells (Malaviya *et al.,* 2001).

Mast cells express a variety of receptors for complement products suggesting that mast cell function can be modulated at multiple levels by the complement system (Furdere *et al.,* 1995). There is considerable heterogeneity in the expression pattern of complement receptors and the responses to complement products by human and murine mast cells (Furdere *et al.,* 1995). Human skin mast cells but not lung mast cells expressed the receptor for C5a, CD88 and activation of these cells with C5a results in histamine release by human skin mast cells but not human lung mast cells (Shulman *et al.,* 1988; Kubota, 1992; el-Lati

et al., 1994). This is analogous to the murine system where cutaneous mast cells but not peritoneal mast cells release histamine in response to C5a (Lim *et al.,* 1991).

Mast cells also readily bind and phagocytose pathogens that have been opsonized by complement products. Complement coated *Salmonella typhimurium* or the helminth *Schistosoma mansoni* effectively binds to mast cells and can be blocked using anti-C3 antibodies suggesting the involvement of complement receptor 3 (CR3) (Sher, 1976; Sher & McIntyre, 1977; Sher *et al.,* 1979).

The long-lived resident nature of the mast cell within the tissues and the range of cytokines and chemokines these cells are known to produce suggests that the ongoing level and type of mast cell cytokine production within a given tissue could profoundly alter host responses. For example, the ability to generate either predominately type 1 or type 2 cytokine profiles in response to pathogen challenge could be modified substantially by the existing mast cell activity and response. It has been suggested, in several inflammatory diseases, including rheumatoid arthritis, asthma and ulcerative colitis, that mast cells are not only increased in number at sites of inflammation but are also activated, potentially as a result of interactions with pathogens or their products. Local selective activation of mast cells to produce specific cytokine profiles could provide a novel approach to the therapy of site specific inflammatory diseases or for the development of new adjuvant systems for vaccines.

1.8 Mast cell interactions with viruses

1.8.1 The role of mast cells in viral infection

It has long been recognized that some aspects of allergic disease are enhanced by the

presence of viral infection. This has been most widely studied in the context of allergic asthma, where upper respiratory tract infections are known to lead to asthma exacerbations (Corne & Holgate, 1997; Johnston, 1997). This type of response may involve mast cell activation, directly as a result of viral infection or viral products or indirectly as a consequence of other host responses. Recent work from a number of groups suggests that mast cells may have a broader role in responses to viral pathogens, outside the context of allergic disease. They have the potential to serve both as effector cells in host defence through mediator production, and as potential reservoirs for infection. There are relatively few studies directly examining mast cell-viral interactions. However, the location, range of mediator production and receptor repertoire of mast cells makes them ideal targets of infection for a number of viruses. Moreover, mast cell responses to infection or viral products could be key to host defence and/or disease pathogenesis. In many cases, mast cells as a source of cytokines in the context of viral infection has not been fully considered, although there is evidence of mast cell activation, that may likely lead to some level of cytokine secretion (see Table 5). Below, some of the known mast cell-viral interactions with an emphasis on the cytokine and chemokine responses that are involved are described.

1.8.2 Human immunodeficiency virus (HIV)/AIDS

HIV, family *Retroviridae*, is an enveloped, single-stranded, linear, diploid, positive sense, RNA virus capable of reverse transcription. Several studies have recently implicated mast cells in the pathogenesis of HIV infection and have demonstrated mast cell infection. Much of this work has been recently reviewed (Marone *et al.*, 2001)

The first indication that mast cells may play a role in HIV infection came with the demonstration of significantly increased serum IgE levels in HIV infected children (Vigano *et al.,* 1995) and adults (Lucey *et al.,* 1990) that positively correlated with disease progression (Israel-Biet *et al.,* 1992). Furthermore, there has been some speculation that there is a shift from a TH1-type immune response towards a TH2 type during HIV infection. In fact, CD4+ T cell clones infected with HIV demonstrate increased production of TH2 cytokines (Meyaard *et al.,* 1994a), though not all groups have confirmed this observation (Graziosi *et al.,* 1994; Maggi *et al.,* 1994). Increased mast cell density in the lymph node of HIV infected individuals has been observed (Paiva *et al.,* 1996) suggesting that mast cells are being recruited and activated during long term HIV infection *in vivo*.

Further evidence for mast cell involvement in HIV infection comes from work demonstrating that HIV envelope glycoprotein gp120 acts a viral superantigen (Karray & Zouali, 1997) and interacts with the variable region 3 of IgE causing the induction of IL-4 and IL-13 from human cells expressing FcεRI, including mast cells isolated from human lung parenchyma (Patella *et al.,* 2000). The authors suggest that FcεRI expressing cells, particularly within lymphoid organs, could then contribute to a TH1-TH2 shift and contribute to the elevation in serum IgE. Not only has gp120 been implicated in activating mast cells, secreted HIV Tat protein, shown to activate bystander cells, can also activate FcεRI expressing cells. De Paulis *et al.,* (2000), demonstrated that Tat is a β-chemokine homologue capable of chemoattracting human lung mast cells. Interestingly, Tat was also shown to up-regulate surface expression of CCR3 on these cells. This observation is intriguing in that HIV has been shown to utilize CCR3 as a co-receptor for entry (Choe *et al.,* 1996).

Up-regulation of CCR3 on the surface of mast cells may allow for viral infection of these cells leading to a substantial move forward in the field of HIV biology. HIV infection occurs at mucosal sites where mast cells are present in large numbers. Recent reports have demonstrated that a population of mast cells/basophils isolated from the circulation of allergic individuals are susceptible to M-tropic HIV infection *in vitro* by virtue of surface CD4, CCR5 and CXCR4 expression (McCurdy *et al.*, 2001). Subsequent work has demonstrated human cultured cord blood derived mast cells are permissive to M-tropic and dual-tropic HIV strains but not T-tropic strains (Bannert *et al.*, 2001) and that infection could be blocked by pre-treatment of cells with anti-CCR5 mAb. Furthermore, this infection resulted in an increasing amount of virus, as assessed by p24 antigen detection, for 21 days post-infection suggesting a potent reservoir for persistent HIV infection *in vivo*. It would be interesting to know whether such infection induces the production of large amounts of chemokines such as RANTES, which may limit the subsequent infection of neighbouring cells since infection with other viruses, such as dengue, have been shown to have this effect (King *et al.*, 2002).

1.8.3 Respiratory syncytial virus (RSV)

RSV is a member of the *Paramyxoviridae* family with a linear, non-segmented ,negative sense RNA genome. The virus is the most common respiratory pathogen and the most important cause of lower respiratory tract infections in infants and young children (Simoes, 1999). Furthermore, there has been some evidence that RSV infection in children is an important risk factor for the development of asthma (Welliver *et al.*, 1986; Welliver & Duffy,

1993). Despite the advances made in the study of RSV, the underlying pathological mechanisms are not fully understood. As early as 1970, researchers speculated as to the involvement of an IgE mediated type I hypersensitivity reaction in individuals who developed more severe illness (Meyaard *et al.,* 1994b). Since this time several groups have attempted to unravel the possible role of IgE in disease. Welliver *et al.,* (1980), demonstrated that some infants infected with RSV generate virus-specific IgE detectable in a cell-bound form in respiratory epithelium. A subsequent study also detected virus-specific IgE and increased histamine levels in nasopharyngeal secretions after infection (Welliver *et al.,* 1981). Furthermore, they observed a correlation between IgE levels and severity of disease. Taken together, the data suggests a role for mast cell activation, possibly through IgE, in RSV infections. However, not all subsequent studies have confirmed these initial findings. Everard *et al.,* (1995) found detectable levels of IgE in only two of seven BAL samples and mRNA for IgE in three of six BAL samples and samples obtained from the upper respiratory tract. The same study found significantly increased levels of tryptase in 11 of 12 BAL samples versus controls, but not in serum samples. These observations suggest mast cell activation in the lungs of RSV-infected infants. Despite significant increases in BAL tryptase, the levels observed were still much lower than those of childhood asthmatics (Ferguson *et al.,* 1992). IL-8 levels are also increased in the BAL of RSV infected individuals (Everard *et al.,* 1995) and mast cells could contribute to this local IL-8 production (Moller *et al.,* 1993). More recent studies (van Schaik *et al.,* 1999) have provided evidence of increased levels of cysteinyl leukotrienes (CysLTs) in both humans and rats infected with RSV. Van Schaik and colleagues measured the levels of LTC_4 in nasopharyngeal secretions

obtained from 129 infants with acute respiratory infection. Results indicated significantly higher quantities of LTC_4 in children with virus-induced wheezing as compared to control groups. In addition, IFN-γ levels were found to be elevated (van Schaik *et al.*, 1999). The authors suggest that the increased production of IFN-γ might lead to alterations in the function of resident mast cells, as has been reported in other systems (Bissonnette *et al.*, 1995; Coleman *et al.*, 1991) and to increased production of LTC_4 contributing to increased inflammation and smooth muscle spasm contributing to virus-induced wheezing. Notably, IFN-γ treatment of human mast cells has been shown to upregulate FcγRI expression, with consequent IgG-mediated mast cell activation (Okayama *et al.*, 2001) as well as enhancing mast cell-mediated antigen presentation (Tkaczyk *et al.*, 1999). Mast cells may not be the major source of LTC_4 in this system since *in vitro* studies have shown increased expression of 5-lipoxygenase (5-LO) in RSV-infected bronchial epithelial cells (Behera *et al.*, 1998) and eosinophils are also known to be a potent source of this lipid mediator. Wedde-Beer *et al.* (2002) also demonstrated increased concentrations of 5-lipoxygenase and cysteinyl leukotrienes in lung tissue of RSV-infected rats during acute infection. Interestingly, histopathological analysis of infected lung tissues showed a 7-fold increase in mast cell density with no eosinophils detected; the mechanisms regulating mast cell populations in the lung are not well understood, but mast cell activation has been shown to play a role in increasing numbers of mast cells at mucosal sites (Marshall, 1993; Marshall *et al.*, 1990). It is clear from the current literature on mast cell involvement in RSV infection that much works remains to be done in this area. Recent recognition that the F-protein from RSV can activate immune cells through a TLR4-dependent pathway may provide an explanation for

some of the evidence of mast cell activation in rodent models. In the human system, however, the mechanisms of mast cell activation, which may be direct or indirect, remain to be elucidated.

1.8.4 Vaccinia Virus

Vaccinia virus is a member of the *Poxviridae* family and has a large, linear, double-stranded DNA genome. Poxviruses are extremely complex and often encode more than 200 proteins, many of which are used to subvert the host's immune response ensuring survival and propagation of the virus. Vaccinia virus, while capable of causing rare infections in humans is used in vaccinations. The ability of vaccinia virus to directly induce mast cell activation has not been examined.

Vaccinia virus complement protein (VCP) has been demonstrated to inhibit both classical and alternative complement pathway activation (Kotwal *et al.,* 1990; McKenzie *et al.,* 1992; Sahu *et al.,* 1998) and to exhibit lysozyme-like heparin binding activity (Kotwal *et al.,*1990). Kotwal and colleagues (Reynolds *et al.,*1990) also demonstrated that VCP can be taken up by mast cells in HMC-1/endothelial cell cultures perhaps enabling mast cell to contribute to viral persistence in tissues *in vivo*. Based on their broad tissue distribution and long-lived nature, mast cells may provide a previously unrecognised site as a reservoir for viral persistance

1.8.5 Influenza A virus and Sendai Virus

Influenza A is a member of the *Orthomyxoviridae* and is characterized by a negative sense,

single stranded, segmented RNA genome. Influenza has a viral envelope comprised of viral hemagglutinin (HA) and neuraminidase (NA) glycoproteins. Sendai virus is a parainfluenza virus, family *Paramyxoviridae*, that has some similar properties to RSV (described above) with a viral hemagglutinin (HN) glycoprotein that sometimes has NA activity and a fusion (F) glycoprotein. Parinfluenza viruses are ubiquitous and cause severe respiratory tract disease in infants and young children while Influenza A is often associated with pandemics.

Both influenza and Sendai virus have been implicated to interact with mast cells in the lungs of infected individuals. This area of study provides some interesting preliminary evidence for mast cell-virus interaction during infection. Clementsen *et al.* (1989) found that influenza A virus stimulated significant increases in histamine in BAL samples obtained from 5 of 7 non-atopic, non asthmatic patients. Further studies are required to determine if mast cells are the source of the released histamine. A more recent study by Chen and colleagues (Chen *et al.*, 2000) supports the concept of mast cell-influenza interaction. They isolated a trypsin-like serine protease from pig lungs that was shown to be located in pig lung mast cells and has the ability to process influenza A HA perhaps contributing to infectivity. However, this group found that human mast cell tryptase β and rat mast cell tryptase failed to process influenza A HA, lending to speculation as to the relevance of these findings in the pig.

As early as 1977, mast cells were found to be responsive to Sendai virus. Using an *ex vivo* model, rat peritoneal mast cells were exposed to Sendai virus at varying doses and for various times. Sendai virus was found to induce significant short term histamine release (Sugiyama, 1977). Furthermore, in a rat model of Sendai virus-induced bronchiolitis there

are significantly increased numbers (100 fold) of bronchiolar rat mast cells and 2-3 fold greater densities of mast cells as compared to uninfected rats at 90 days post-infection (Castleman *et al.*, 1989). Increases in mast cells in response to Sendai virus infection in rats have been confirmed in subsequent studies. The mechanisms of these increases in mast cells is unclear. However, enhanced proliferation of local mast cell populations, mobilization of mast cell precursors from the bone marrow or recruitment of mast cells from adjacent tissues are all potential explanations. Increases in the number of bronchiolar mast cells following Sendai virus infection occur as early as 30 days and persists until at least 90 days after inoculation. Mast cell increases have also been associated with airway hyper responsiveness and heightened allergic airway inflammatory reaction (Castleman *et al.*, 1990). Furthermore, this same group found that rats are more sensitive to virus-induced lung injury and mast cell increases were also less efficient at viral clearance as evidenced by prolonged virus infection and 5-fold higher titres as compared to control rats (Sorden & Castleman, 1995a; Sorden & Castleman, 1995b). These studies included suggestions that infection might involve local mast cell proliferation and mobilisation of mast cell precursors from the bone marrow. As previously mentioned, increases in mast cells are observed in the context of other viral infections, and may involve an autocrine mechanism whereby mast cell activation enhances mast cell recruitment.

1.8.6 Mast cells, bacteria and viruses - a summary

There is very clear evidence from rodent models that mast cells play a critical role in host defence against bacteria infection. In the context of viral infection, the role of mast cell

derived cytokines and chemokines has only recently received attention. In the context of HIV-1 infection, however, there is substantial evidence of direct mast cell infection and mast cell cytokine and chemokine responses. Certainly, increased understanding of how the functions of this versatile and potent immune effector cell are regulated by pathogens will provide new opportunities for therapy in the context of inflammatory diseases, infection and vaccine development.

1.9 Flaviviruses

Dengue viruses are lipid-enveloped (Figure 4) positive sense RNA viruses of the family *Flaviviridae*. Four antigenically distinct serotypes, dengue 1 - 4, (Rice, 1996) are transmitted to humans via the mosquito vector *Aedes aegypti* (Halstead, 1988).

Flavivirus virions are spherical with a diameter of 40-60 nm and they include an envelope and spike protein which is usually glycosylated (Chambers *et al.*, 1990). The nucleocapsid (20-30 nm) is surrounded by a lipid bilayer and consists of core (C) protein and viral RNA. Embedded in the envelope bilayer are the envelope (E) protein and pre-membrane (prM) protein. Mature virions contain mostly processed membrane (M) protein while immature virions contain mainly unprocessed prM protein and are less infectious than mature virions (Chambers *et al.*, 1990).

1.9.1 Flavivirus genome

The flavivirus genome (Figure 5) contains a single-stranded RNA of approximately 11 kb with a type 1 cap at the 5' end and no poly A tail at the 3' terminus. It contains one long open

reading frame that encodes for a single polyprotein. The polyprotein is cotranslationally or posttranslationally cleaved at specific sites by viral and host cell proteases to produce the smaller viral polypeptides. The structural proteins are encoded at the 5' end of the genome followed by the non-structural proteins encoded towards the 3' end of the virus RNA. There are three structural proteins: capsid protein (C), pre-membrane protein (prM) and the envelope protein (E), and seven non-structural (NS) proteins: NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5 (Markoff *et al.*, 1997).

1.9.2 Flavivirus (Dengue) proteins

1.9.2.1 The C protein

The C protein is the first viral polypeptide synthesized during translation and has a molecular weight of about 13.5kDa. The protein is highly basic and it is this basic character that probably enables it to interact with viral RNA (Deubel *et al.*, 1988; Hahn *et al.*, 1988). The C protein lacks an N-terminal hydrophobic signal sequence suggesting that it is synthesized on non-membrane bound ribosomes. Other studies have suggested that the hydrophobic stretch of amino acids at the carboxy terminus acts as a transmembrane signal for the adjacent prM protein (Henchal & Putnak, 1990). Cleavage of C-prM is thought to be carried out by a host cell signalase at the N terminus of prM (Markoff, 1989; Nowak *et al.*, 1989). Several groups have shown the C protein of dengue virus to have three putative nuclear localization signals, one of which is a bipartite nuclear targeting sequence motif which has been found in all flavivirus core proteins sequenced to date (Burglin & De Robertis, 1987) (Dingwall & Laskey, 1991). Interestingly, Bulich and Aaskov (1992) demonstrated nuclear

localization of the dengue 2 C protein in infected cells. The function of C in the nucleus is not known.

1.9.2.2 The prM and M proteins

The prM is the precursor protein of the M protein. Specific proteolytic cleavage of prM (22kDa)during virus maturation results in formation of the 8kDa mature M protein (Deubel *et al.*,1988; Hahn *et al.*, 1988). The prM glycoprotein is cleaved to produce the mature M protein just before or at the time of release of virions from cells (Murray *et al.*, 1993) and the internal cleavage of prM has been found to be effected by the host signalase protease furin (Stadler *et al.*, 1997). Formation of M from prM appears to be a terminal event in virus maturation (Randolph *et al.*, 1990) and is directly related to virus infectivity (Wengler, 1989). The prM and E proteins form heterodimers in cell-associated virions (Wengler, 1989) to presumably prevent premature acid-induced conformation changes in the E protein during virion maturation in the trans-Golgi network and secretory vesicles (Heinz *et al.*, 1994).

1.9.2.3 The E protein

The E glycoprotein is the major virion envelope protein and is approximately 51-60 kDa. This protein appears as a homotrimer on the surface of mature virions (Wengler *et al.*, 1987). The E protein has been demonstrated to play an important role in virion assembly (Nowak *et al.*, 1989), receptor binding (Anderson *et al.*, 1992), and membrane fusion during virus entry and replication (Gollins & Porterfield, 1986; Kimura & Ohyama, 1988; Randolph *et al.*, 1990). The X-ray crystallographic structure of a soluble fragment of the E protein from

tick-borne encephalitis (TBE) virus has been determined (Rey *et al.,* 1995) and has demonstrated three non-overlapping antigenic domains. In addition, the E protein has two hydrophobic sequence clusters at the carboxy terminus and one is believed to act as a membrane anchoring domain. Evidence suggests that the regions might also be involved in prM-E association and modulation of fusion activity (Heinz *et al.,* 1995; Stuart & Gouet, 1995; Wang *et al.,* 1999). Furthermore, the E protein is also a major target for neutralizing antibodies (Della-Porta & Westaway, 1977; Peiris *et al.,* 1982; Hall *et al.,* 1996).

1.9.2.4 The NS1 and NS2a proteins

The flavivirus NS1 glycoprotein is a glycosylated protein of approximately 46 kDa (Smith & Wright, 1985). The NS1 protein is inserted into the endoplasmic reticulum (ER) by a signal sequence located at the C terminus of the E protein and is processed by a host signal peptidase (Bukowski *et al.,* 1989). Soon after synthesis, NS1 is cleaved from NS2a by an unknown ER-resident host proteinase (Falgout & Markoff, 1995). In the ER NS1 modifications take place and the modified NS1 dimerizes (Smith & Wright, 1985; Winkler *et al.,* 1989) and associates with membranes via a previously unknown mechanism. The NS1 protein has no putative membrane anchoring domains though very recently a landmark study demonstrated that the NS1 is a GPI-linked protein (Jacobs *et al.,* 2000). The authors demonstrated the NS1 uses a cellular glycosyl-phosphatidylinositol pathway to express a GPI-anchored form of NS1. Furthermore, they found that this form of NS1 is capable of signal transduction in response to binding of specific antibody. The signal for the NS1 GPI anchor was found to be a downstream hydrophobic domain in the NS2a protein.

Dimerization is a prerequisite for NS1 export along the secretory pathway to the plasma membrane (Pryor & Wright, 1993). The NS1 protein, through GPI-anchoring, is the only non-structural viral protein to be found on the surface of infected cells (Schlesinger *et al.*, 1990). In addition, NS1 is also secreted in a hexameric form (Flamand *et al.*, 1999) from infected cells and has been found in sera from infected patients (AbuBakar *et al.*, 1997). The function of NS1 is not well understood, though studies have suggested an essential role for NS1 in viral replication (Lindenbach & Rice, 1997; Mackenzie *et al.*, 1996; Muylaert *et al.*, 1996) including formation of the replication complex by association with the viral dsRNA replicative form (Mackenzie *et al.*, 1996).

1.9.2.5 The NS2b and NS3 proteins

Cleavage of the viral polyprotein occurs by activity of host proteases and a single viral encoded serine protease NS3 (Falgout *et al.*, 1991). The NS3 protease (69 kDa) has been found to be in complex with the viral activator protein NS2b (Chambers,*et al.*, 1993; Arias *et al.*, 1993) and it is this complex that catalyzes the cleavages at NSS2a-NS2b, NS2b-NS3, NS3-NS4a, and NS4b-NS5 sites in the polyprotein (Chambers *et al.*, 1990). Thus the biologically active viral protease is a heterodimeric complex of NS2b-NS3. Furthermore, studies have demonstrated that it is the conserved hydrophilic domain of NS2b that is necessary and sufficient for activation of the NS3 protease domain (Jan *et al.*, 1995). The three hydrophobic domains of NS2b are essential for cotranslational membrane insertion of full length NS2b and efficient activation of NS3 (Clum *et al.*, 1997). NS3 has also been demonstrated to have RNA helicase activity and has been implicated in unwinding of dsRNA

replicative intermediate as well as found to have RNA-stimulated NTP-ase activity thus suggesting NS3 is a trifunctional protein (Wengler, 1991; Li *et al.,* 1999). In addition, NS3 has also been found to interact with the viral RNA-dependent RNA polymerase, NS5, in the cytoplasm and thus probably facilitates genome replication (Kapoor *et al.,* 1995).

1.9.2.6 The NS5 protein

The NS5 protein (104kDa) is predicted to contain at least two distinct domains; the N-terminal domain has been predicted to be a S-adenosyl-methionine transferase domain while the C-terminus contains the RNA-dependent RNA polymerase activity (Koonin, 1991; 1993). Cui and colleagues demonstrated that the NTP-ase activity of NS3 is specifically stimulated by NS5 (Cui *et al.,* 1998). In addition, NS5 has been localized to the cytoplasm in conjunction with NS3 as well as in large amounts in the nucleus of infected cells. The NS5 in the nucleus is not associated with NS3 and until recently it was not understood how this occurred and why. Forwood *et al.* (1999) demonstrated that the NS5 protein contains a nuclear localization signal (NLS) that is capable of targeting NS5 to the nucleus and is recognized by nuclear import factors including importin-β. Furthermore, NS5 import into the nucleus can be inhibited by phosphorylation (Forwood *et al.,* 1999; Johansson *et al.,* 2001). The reason for NS5 localization into the nucleus remains a mystery. Perhaps movement into the nucleus and thus away from the replication complex serves to down-regulate replication when needed. It will be interesting to discover the function of nuclear NS5 perhaps providing previously unknown insights into flavivirus replication.

1.9.2.7 The NS4a and NS4b proteins

The NS4a and NS4b proteins are hydrophobic with molecular weights of approximately 16 and 27 kDa, respectively (Rice, 1996). The functions of these proteins remain unknown though it is postulated that they are involved in viral replication as the NS4a protein of hepatitis C virus facilitates protease activity of NS3 and increases cytoplasmic accumulation of NS3 (Vishnuvardhan *et al.,* 1997; Ishido *et al.,* 1997).

1.10 Forms of human disease

1.10.1 Classic dengue fever

Classic dengue fever develops 3 to 15 days after infection (Halstead *et al.,* 1969). Dengue fever is an acute viral illness characterized by fever, severe headache (often described as retro-ocular); myalgias and arthralgias that can be very severe; as well as nausea and vomiting. A rash may present at different stages of the illness, and appearance can be variable in that it may be maculopapular, petechial, or erythematous (Monath *et al.,* 1986; Henchal & Putnak, 1990). Patients may also report other symptoms such as itching and aberrations in the sense of taste. In addition, there have been reports of severe depression after the acute phase of the illness. The fever is usually biphasic, in that high fever and some of the symptoms reoccur after the first phase (Halstead, 1989). Fortunately, dengue fever is a self-limited disease and patients usually recover within 10-14 days (Halstead *et al.,* 1969).

1.10.2 Dengue hemorrhagic fever (DHF)

While most of the people who acquire dengue infection develop mild disease, a small

number of patients may develop hemorrhagic manifestations. The onset of disease is similar to that of classic dengue fever except that about 3-4 days after onset patients have signs of increased vascular permeability, thrombocytopenia, hypovolemia and hemorrhagic manifestations. These hemorrhagic manifestations are usually mild, such as skin hemorrhages including petechiae. Other hemorrhages have been described and include gingival or nasal bleeding, gastrointestinal bleeding (the severity of which may vary from very mild to very severe), hematuria, and increased menstrual flow (WHO, 1997; Pan American Health Organization, 1994; Nimmannitya *et al.,* 1987). In some cases, however, the hemorrhage may be frank and severe enough to cause shock from blood loss. If untreated, the case fatality rate can be as high as 40-50%; if treated, approximately 5% (Monath, 1994).

According to the World Health Organization, four criteria must be fulfilled to meet the case definition of DHF. These are: 1) Fever, or recent history of acute fever; 2) Hemorrhagic manifestations; 3) Low platelet count; and 4) Objective evidence of plasma leakage caused by increased vascular permeability, as reflected by one or more of the following: elevated hematocrit (defined as 20% or more over baseline, or a similar drop after volume replacement treatment), low protein, or pleural or other effusions (WHO, 1986; 1997).

Plasma leakage is the critical difference between dengue hemorrhagic fever and dengue fever and means that the patient requires fluids, sometimes large amounts of intravenous fluids.

In addition there are four grades of DHF. For all grades the four criteria (as discussed above) for DHF must be met. In Grade 1, fever and nonspecific constitutional symptoms are

present and the only hemorrhagic manifestation is provoked by a positive tourniquet test. In Grade 2, in addition to the Grade I manifestations, there is spontaneous bleeding. Grades 3 and 4 are Dengue Shock Syndrome (DSS). Grade 3 is incipient shock with signs of circulatory failure. In Grade 4, the patient has profound shock, with undetectable pulse and blood pressure.

1.10.3 Dengue shock syndrome (DSS)

The clinical case definition for dengue shock syndrome includes: 1) the four criteria for DHF; 2) evidence of circulatory failure (monitored by rapid and weak pulse); 3) narrow pulse pressure and cold, clammy skin; and 4) altered mental status. All three of these conditions must be met to indirectly demonstrate circulatory failure. Evidence of circulatory failure may also be manifested directly by frank shock (WHO, 1986; 1997).

1.11 Epidemiology

Dengue virus has emerged as one of the most important mosquito borne viruses (Figure 6). There are about 2.5 billion people in tropical areas of the world that are at risk for dengue infection. In the Americas, dengue hemorrhagic fever is on the rise: the mean annual number of reported cases in the Americas between 1989 and 1993 increased over 50-fold, as compared with the preceding 5-year period, from 1984 to 1988 (Organization of American States, Human Health in the Americas, 1996). In 1998, there were 700,000 cases of dengue infection in the Americas (CDC, 2001) (Table 5). With the widespread abundance of *Aedes aegypti* in the Americas, including the US, there is danger that this worsening trend will

continue. It is estimated that 100 million individuals are infected with dengue virus annually (Halstead, 1988), including an estimated 250,000-500,000 cases of DHF (Pinheiro & Corber, 1997; Rigau-Perez *et al.,* 1998; WHO, 1999; 2000).

Benjamin Rush reported the first epidemics of dengue fever in 1779-1780 in Asia, Africa, and North America (Rush, 1789). The near simultaneous occurrence of outbreaks on three continents indicates that these viruses and their mosquito vector have had a worldwide distribution in the tropics for more than 200 years. During most of this time, dengue fever was considered a benign, nonfatal disease of visitors to the tropics. Generally, there were long intervals (10-40 years) between major epidemics, most probably due to low population densities.

In Southeast Asia, dengue hemorrhagic fever has been recognized for approximately 40 years and the majority of the cases are reported from Thailand, Indonesia, and Vietnam. Statistics indicate that, per decade, the number of DHF cases has increased from a mean of 50,000 per year in the 1970s to 200,000 in the 1990s (CDC). In the Americas there is a similar alarming trend. In the 1980s, the total number of cases was 15,000, and by the mid-1990s, through 1999, there were approximately 56,000 cases. DHF is now endemic in many countries in the Americas (Gubler & Clark, 1995). Furthermore, in Texas there were 18 cases of locally acquired dengue in 1999. In Canada, the NML diagnoses approximately 40 cases of imported dengue infection in individuals every year. This is likely to be an underestimate of the actual number of cases occurring as most people who get dengue fever will not go to a doctor or submit a specimen for lab testing (personal communication, Dr. Harvey Artsob, NML).

1.12 Risk factors for DHF

There are several factors now believed to contribute to development of severe dengue disease (Figure 7). Dengue viruses differ genotypically and differences appear to be associated with a difference in virulence (Bielefeldt-Ohmann, 1997). Some virus strains, such as the Southeast Asia genotype of dengue 2, are associated with more severe disease and appearance coincides with emergence of DHF (Rico-Hesse, 1990; Rico-Hesse *et al.*, 1997; 1998). It has also been proposed that virus strain virulence contributes to DHF manifestation in primary infection with certain genetic strains of virus. Work is underway investigating the molecular determinants of virulence including analysis of 'virulent' amino acids in the E protein and the non-structural proteins NS1, NS2a, NS3 and NS5 (Leitmeyer *et al.*, 1999; Mangada & Igarashi, 1998; Soemanto *et al.*, 1999).

Analysis of the Cuban epidemic in 1981 suggests that host genetics seems to be a factor (Bravo *et al.*, 1987; Guzman *et al.*, 1987). The data from Cuba suggest that Caucasians may be at greater risk as they have a greater frequency of severe disease. Furthermore, age appears to influence susceptibility, as in Southeast Asia children are most affected, as is gender in that adult females are at higher risk than adult males.

One of the most fascinating risk factors for development of severe forms of dengue disease is the presence of pre-existing anti-dengue antibody, either caused by a previous infection or by maternal antibodies passed to infants. For individuals, there is a higher risk for DHF in secondary infections. There is also higher risk in locations of hyperendemic transmission where two or more serotypes are circulating simultaneously at high levels. The most widely accepted hypothesis for the increased risk of DHF in secondary infections is

called antibody-dependent enhancement of viral infection (Halstead & O'Rourke, 1977a).

1.13 Antibody-dependent enhancement (ADE)

In 1977, Halstead was the first to suggest that ADE was at least, in part, responsible for the pathogenesis of severe dengue disease. ADE is thought to facilitate greater viral infection and thus more severe disease. Symptoms of severe disease most often occur in individuals experiencing secondary dengue virus infections (reviewed in Rothman & Ennis, 1999). The presence of heterotypic sub-neutralizing antibodies to dengue virus as a result of primary infection has been shown to potentiate secondary infection via antibody-dependent enhancement (ADE)(Fagbami *et al.*, 1988; Fischer & Halstead, 1970; Halstead & O'Rourke, 1977a;1977b; Halstead, 1989). Non-neutralizing, cross-reactive antibodies markedly augment dengue virus infection of FcγR-bearing cells (Halstead, 1982; Halstead *et al.*, 1976; Halstead & O'Rourke, 1977a; Halstead *et al.*, 1980). Dengue virus-IgG antibody complexes are thought to bind to Fc receptors on the surface of monocytes or other immune cells by the Fc portion of the antibody, thereby enhancing virus binding and infection (Halstead, 1988; 1989 ; Kliks *et al.*, 1988; Morens, 1994). Subsequent studies have suggested that ADE is not dengue-antibody-FcR-specific but rather a result of antibody bringing a greater number of dengue viruses in close proximity to target cells (Mady *et al.*, 1991) as bispecific antibodies targeting dengue virus to β2-microglobulin, CD15 or CD33 on the surface of monocytes could mediate ADE. The authors suggest that cell surface molecules other than FcγRs can mediate ADE and that FcγR does not provide a unique signal necessary for enhanced infection (Mady *et al.*,1991).

Epidemiological and statistical studies in Thailand demonstrated that most primary dengue infections were relatively benign, while severe dengue disease occurred almost exclusively during secondary infection (Basaca-Sevilla & Halstead, 1966; Halstead, 1965; Johnson *et al.*, 1967). Age prevalence of severe dengue disease was noted as well. Halstead demonstrated that severe dengue disease most often occurs between the ages of 7 months to 3-5 years. In addition, infants with circulating maternal anti-dengue antibodies developed more severe disease during primary infection while school age children develop more severe disease during secondary infection (Kliks *et al.*, 1989; Halstead *et al.*, 1969; Papaevangelou & Halstead, 1977).

In vitro studies have demonstrated that dengue virus infection of peripheral blood monocytes could be enhanced in the presence of non-neutralizing or sub-neutralizing antibodies (Fagbami *et al.*, 1988; Halstead *et al.*, 1976; Marchette *et al.*, 1975; 1976). While much of the work demonstrating ADE of dengue virus infections have been carried out *in vitro*, *in vivo* demonstration of antibody-dependent enhancement of dengue virus infection was carried out using rhesus monkeys in the early 1970s. Studies with rhesus monkeys showed that sequential infection with dengue 4 and dengue 2 occasionally caused severe disease (Halstead *et al.*, 1973; Marchette *et al.*, 1973). Furthermore, administration of heterologous anti-dengue sera to monkeys caused a significant increase in viral load (Halstead, 1979).

The role of antibody-dependent enhancement in the pathogenesis of DHF was suggested by studies that examined pre-infection serum samples from subjects who subsequently developed DHF and those did not during secondary infection. Kliks and

colleagues (1989) found that pre-infection serum from the group that subsequently developed DHF enhanced infection of human monocytes *in vitro* while serum from the latter group had strong neutralizing activity at low dilutions. This same group also showed maternal antibody enhancement of infection in infants (Kliks *et al.,* 1988).

1.14 Pathogenesis

Increased viremia, vascular permeability, shock, and severe thrombocytopenia are associated with severe forms of dengue infection such as dengue hemorrhagic fever (DHF) and/or dengue shock syndrome (DSS) (Brinton *et al.,* 1998; Castleman *et al.,* 1989; Halstead, 1990; Kurane *et al.,* 1994). Substantial T cell activation is also observed in severe dengue disease (Kurane & Ennis, 1992). While the pathogenesis of severe dengue disease is not well understood, current evidence indicates a strong role for host immune responses in the pathogenesis of severe dengue disease.

1.14.1 Immune responses to dengue virus infection

1.14.1.1 Humoral immune responses

IgM and IgG are the major immunoglobulins detected after dengue virus infection (Russell *et al.,* 1969; Dittmar *et al.,* 1979). Primary infection results in initial production of dengue-specific IgM antibodies which are then followed by dengue specific IgG antibodies. Levels of dengue specific IgM antibodies have been found to persist for up to 4-6 months after infection (Gunasegaran *et al.,* 1986). Upon secondary dengue virus infection, IgG levels are significantly enhanced and maintained at high levels for a longer time (Jianmin *et*

al., 1995). Furthermore, when secondary infection occurs with a heterologous serotype, the antibody response is markedly different from that elicited by the primary infection (Halstead, 1988).

The majority of dengue-specific antibodies are generated against the major structural protein E. Studies have demonstrated NS3, NS5 and E-specific antibodies are generated both during the primary and secondary immune response, while NS1-specific antibodies are mainly generated during a secondary immune response (Churdboonchart *et al.*, 1991; Kuno *et al.*, 1990; Se-Thoe *et al.*, 1999; Valdes *et al.*, 2000).

Dengue-specific IgG antibodies mediate ADE (Halstead & O'Rourke, 1977b) and classical complement activation (Scott *et al.*, 1976). In addition, dengue-specific IgG can cross-react with heterologous serotypes while dengue-specific IgM does not generally cross-react (Figueiredo *et al.*, 1989; Holzmann *et al.*, 1996).

1.14.1.2 Cellular immune responses

Circulating dengue-specific CD4+ and CD8+ T cells have been detected in individuals after primary dengue virus infection (Bukowski *et al.*, 1989). Analysis of specificity suggested the predominant response was to the dengue serotype with which the individual was infected although cross-reactive responses to one or more heterologous serotypes were also detected. Furthermore, serotype cross-reactive T cells are found to more frequently recognize epitopes on more highly conserved non-structural proteins. The NS3 protein has been suggested to be a major target for CD4+ and CD8+ T cells (Kurane *et al.*, 1991; Mathew *et al.*, 1996). In addition, data suggest that following secondary dengue virus infections, the dengue virus

specific memory T cells are activated and the subsequent T cell response is composed predominantly of serotype cross-reactive T cells that recognize epitopes on non-structural proteins (Mathew *et al.,* 1998).

Contribution of T cell activation to the pathogenesis of severe dengue disease is postulated through the production of cytokines including IFN-γ, IL-2, and TNF-" (Gagnon *et al.,* 1999; Kurane *et al.,* 1989) that can upregulate expression of Fc receptors on the surface of target cells and also influence endothelial cell permeability. Dengue-specific T cell mediated lysis of dengue-infected immune cells, for example monocytes or macrophages, (Kurane *et al.,* 1991; Zivny *et al.,* 1995) may also contribute to pathogenesis in that lysed cells release chemical mediators which can enhance inflammatory responses and subsequently alter vascular permeability.

1.14.1.3 Complement

Several studies have suggested a role for complement activation in the pathogenesis of severe dengue virus infections, particularly in plasma leakage. Complement is activated during dengue virus infections. Decreased serum levels of C3, C4 and C5 , increased metabolism of C1q and C3 have been demonstrated in patients with DHF, with the most marked differences seen in Grade 3 and 4 disease (Bokisch *et al.,* 1973). In addition, another group demonstrated increased plasma levels of products of complement activation including C3a and C5a in patients with DHF. These increases also correlated with disease severity and were maximal around the time of defervescence and coincident with shock, suggesting that complement activation contributes to plasma leakage (Malasit, 1987).

The complement cascade is postulated to be activated by circulating dengue-antibody immune complexes. Such immune complexes have been detected in patient sera (Sobel *et al.,* 1975; Theofilopoulos *et al.,* 1976).

Finally, C3a and C5a are anaphylatoxins that can mediate mast cell histamine release (Johnson, 1975; Schulman *et al.,* 1988) and heparin sulfate shedding on endothelial cells (Ihrcke & Platt, 1996), respectively. Histamine mediation of vascular permeability in DHF/DSS has been suggested and is supported by the findings of increased urinary histamine in patients with DHF as compared to healthy controls. Histamine excretion was also found to parallel clinical severity of DHF (Tuchinda *et al.,* 1977).

1.15 Cellular targets of dengue virus infection

For several decades now monocytes have been recognized as important cell targets for dengue virus *in vitro* (Halstead *et al.,* 1977). In concert with this observation, dengue virus-infected patient PBMC analysis indicated that infectious virus was detected more frequently in the adherent PBMC population (Scott *et al.,* 1980). In addition, histological and autopsy studies have also detected dengue virus in Kupffer cells of the liver, mononuclear cells in the skin, adherent PBLs, and in several organs including liver, lymph node, bone marrow , heart and lung (Halstead, 1989).

Several groups have examined the role of monocytes/macrophages in the pathogenesis of severe dengue disease including the production of vasoactive cytokines which will be discussed later.

More recently, dendritic cells have been implicated in dengue virus infections. A

landmark study published in Nature in 2000 (Wu *et al.*, 2000) demonstrated that blood derived-dendritic cells were 10-fold more permissive to dengue virus infection than monocytes or macrophages and that infection occurred in the absence of antibody. Furthermore, the study demonstrated *ex vivo* confirmation, using human cadaveric skin explants, and *in vivo* confirmation of dengue virus infection of skin dendritic cells (Wu *et al.*, 2000). This study was one of the first to suggest and provide evidence for additional targets of dengue virus infection. The authors proposed that human skin Langerhans cells may be one of the initial cellular targets for dengue virus infection.

While the investigation of cellular targets has been underway for some time, most groups have focussed on monocytes and macrophages. The finding that dendritic cells are permissive is intriguing in that it provides an impetus to continue exploring other cellular targets for the virus. Such exploration will certainly contribute to our knowledge of the mechanisms of pathogenesis of severe dengue disease.

1.16 Circulating cytokines and chemokines during disease

A number of cytokines and chemokines are found to be elevated in DHF/DSS patients, including TNF-α, IL-1, IL-8 and IL-10 (Avirutnan *et al.*, 1998; Chaturvedi *et al.*, 2000; Green *et al.*, 1999a; Hober *et al.*, 1993; Kurane *et al.*, 1991; Kuno & Bailey, 1994). A prospective clinical study found that TNF-α, IL-1β, IL-1Ra but not IL-6 concentrations were elevated in the circulation during early stage of infection and at discharge (Suharti *et al.*, 2002). Another study demonstrated elevated levels of TNF-α and IL-6 but not IL-1β. Interestingly, the complexity of the disease, and the importance of timing of sample isolation,

as well as grade of disease was found to play a role in that the authors found variable or inconsistent levels of TNF-α and IL-6 dependent on these criteria (Hober *et al.*, 1993). Such information should alert individuals as to the complexity of the disease and most probably the pathogenesis. Not all studies have observed the same elevations in cytokine/chemokine levels in patients suffering severe disease. Perhaps this reflects timing and grade of disease.

Increased serum and plasma levels of IFN-γ and IL-2 have been observed in patients with DHF (Green *et al.*, 1999b; Kurane *et al.*, 1991). Kurane and colleagues (1991) have also demonstrated increased levels of soluble IL-2 receptor, soluble CD4 and CD8 in children with severe dengue disease. Another group has found elevated serum levels of IL-13 and IL-18 in patients with DHF as compared to uninfected controls. Furthermore, levels of IL-13 and IL-18 were found to be the highest in grade 4 DHF patients and the lowest in dengue fever, suggesting a role for these cytokines in pathogenesis of disease (Mustafa *et al.*, 2001). A recent study found that IL-12, a cytokine whose major function is to direct a TH1 immune response, was actually decreased in patients with DHF as compared to DF, suggesting that the lack of IL-12 may contribute to a TH2 type immunity and thus contribute to pathogenesis (Pacsa *et al.*, 2000).

1.17 *In vitro* dengue virus infection

Anderson *et al.* (1997) were the first to show that dengue-infected peripheral blood monocytes released vasoactive cytokines, particularly TNF-α, which activated adhesion molecule expression on vascular endothelial cells. Both dengue infection and TNF-α release were strongly dependent on the presence of dengue-specific antibody, thus implicating ADE

as an important cofactor in dengue-induced cytokine upregulation. Chaturvedi *et al.*(1999) demonstrated that *in vitro* dengue virus infection of PBMCs isolated from healthy individuals, who had circulating IgG anti-dengue antibodies, sequentially produced TH1-TH2 cytokines. By day 1 p.i significant levels of TNF-α, IL-2 and IL-6 were observed followed by IFN-γ by day 3 p.i. Interestingly by day 4 p.i the levels of these cytokines dropped substantially and were replaced by IL-10, IL-5 and IL-4, TH2 type cytokines. In addition, another group found that PBMC isolated from patients with DHF had greater expression, assessed by immunohistochemical staining, of IL-4 and TNF-α as compared to those cells isolated from patients with DF. Levels, however, were lower than expected and the authors suggested that cellular activation in the tissues may contribute to high serum cytokine levels in DHF (Gagnon *et al.*, 2002). Bosch and colleagues have demonstrated that *in vitro* primary human monocytes and human epithelial and endothelial cell lines produced significant levels of IL-8, as assessed by ELISA, when infected with dengue virus (Bosch *et al.*, 2002). Such production may contribute to the elevated levels observed in sera from patients with more severe disease manifestations (Juffrie *et al.*, 2000; Raghupathy *et al.*, 1998). Another group demonstrated IL-1 and TNF-α production by human monocytes by 4 hours p.i (Chang & Shaio, 1994). This group also found that there was no difference in IL-1 production by dengue infection of monocytes isolated from dengue fever patients versus healthy controls. This apparent 'discrepancy' is not surprising in that monocytes might not have the capacity to produce more IL-1 if taken from dengue patients versus healthy controls. The elevated IL-1 might rather reflect interactions between other immune effector cells and contribution by other cellular sources of IL-1. In addition, primary human Kupffer cells have

been shown to be permissive to dengue virus infection and produce significant levels of IFN-γ, IL-6 and TNF-α when compared to controls. Interestingly, this infection was shown to be non-productive in that no viral progeny were observed, while cytokine production was evident (Marianneau *et al.,* 1999). Increased production of IFN-γ and TNF-α by PBMC treated with inactivated dengue antigen were observed by PBMC isolated from hospitalized patients with DHF versus those isolated from non-hospitalized patients with dengue fever (Mangada *et al.,* 2002).

Chemokine involvement in dengue disease is less well studied. Lin *et al.* (2000) demonstrated increased levels of RANTES in the serum of patients with dengue virus infection as compared to healthy controls, as well as increased production of RANTES by dengue virus infected human liver cells *in vitro.* Avirutnan *et al.* (1998) demonstrated increased RANTES production, in response to active dengue infection, by ECV304 cells and primary HUVEC endothelial cells, though could not detect any difference in *in vivo* plasma RANTES concentration when healthy versus dengue infected patients were compared. Furthermore, MIP-1α was found to be produced by cord blood mononuclear cells (Murgue *et al.,* 1998) and both MIP-1-α and MIP-1β were produced in response to dengue virus infection of K562 cells and PBMC *in vitro* (Spain-Santana *et al.,* 2001). Spain-Santana *et al.* also indicated that MIP-1α and MIP-1β mRNA was detected in patients with dengue infection.

1.18 Rationale for study

Due to the lack of an animal model for DHF/DSS most of the work investigating primary

target cells and potential mechanisms of pathogenesis has been done *in vitro*. During antibody-enhanced dengue infection of monocytes (Brinton *et al.*, 1998) increased uptake of antibody-virus complexes occurs via Fc receptor mediated binding to cells (Peiris *et al.*, 1981). Mast cell expression of such receptors also makes them potential targets for infection. Dengue infection of monocytes stimulates cytokine production (particularly TNF-α) which perturbs endothelial cell function and contributes to vascular dysfunction and cell recruitment.

For many years there has been speculation as to the involvement of mast cells in dengue pathogenesis. Dengue patients exhibit increased levels of urinary histamine (a major granule product of mast cells) which correlates with disease severity (Tuchinda *et al.*, 1977). A large study from Thailand demonstrated that mast cells in the connective tissue around the thymus showed swelling, vacuolation of the cytoplasm as well as loss of granule integrity (Bhamarapravati et al., 1967) suggestive of mast cell activation. However, the potential role of mast cells has not yet been explored with regard to dengue pathogenesis.

1.19 Objectives

The objectives of the current study were two fold: first, to investigate the potential role of mast cells in contributing to the pathogenesis of severe dengue disease; second, to expand our limited understanding of mast cell function in host defence against viral pathogens.

The preceding sections should provide the reader with sufficient background to understand the results presented in the following sections. The results are presented in the form of three manuscripts followed by a general discussion section aimed at addressing both

objectives. The first manuscript entitled 'Release of Vasoactive Cytokines by Antibody-Enhanced Dengue Virus Infection of a Human Mast cell/Basophil Line' was published in the Journal of Virology August 2000, and provides evidence for mast cell involvement in pathogenesis of severe dengue disease through the production of vasoactive cytokines IL-1β and IL-6. It is also the first publication to demonstrate virus infection of human mast cells. The second manuscript entitled 'Dengue virus selectively induces human mast cell chemokine production' was published in the same journal in August 2002, and provides evidence for selective mast cell chemokine responses to dengue virus but not to RSV or adenovirus. The third manuscript entitled 'FcγRII-dependent dengue virus infection of human mast cells' will be submitted to the Journal of Leukocyte Biology in the near future pending further experiments. This study focussed on the mechanism of antibody-dependent dengue virus infection of human mast cells.

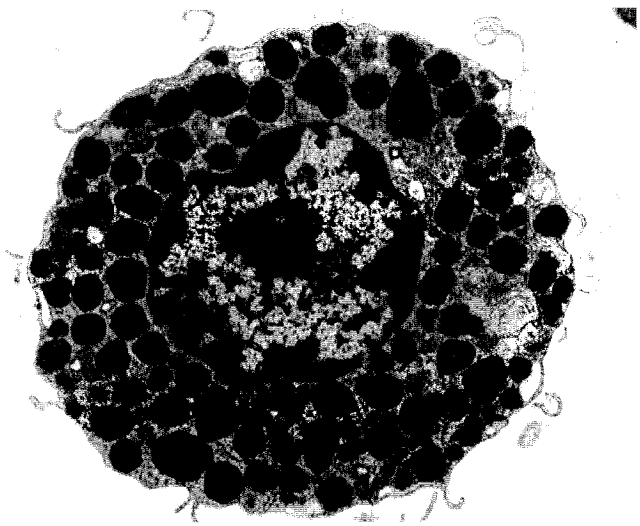


Figure 1. Electron microscopy of a human skin mast cell x11,000 (Dvorak, 1993).

Table 1. Characteristics of human MC_T and MC_{TC} mast cells

	MC_T	MC_{TC}
Main location	Intestinal mucosa Lung alveolar wall	Intestinal submucosa Skin
Protease	Tryptase	Tryptase & chymase Carboxypeptidase Cathepsin G
Development	T cell dependent	T cell independent
Major function Proposed	Th2 immune response Host defense	Angiogenesis Tissue remoldelling
Granule morphology	Scroll rich	Grating/lattice Scroll poor
Cytokine content	IL-4	IL-4, IL-5, IL-6

Comprised from (Irani and Schwartz, 1994; Schwartz and Huff, 1993; Church and Levi-Schaffer, 1997, Bradding *et al.*, 1995).

Table 2. Chemokines and chemokine receptors.

Common name	Systematic name	Receptor
ENA-78	CXCL5	CXCR2
IL-8	CXCL8	CXCR1, CXCR2
SDF-1 /	CXCL12	CXCR4
MCP-1	CCL2	CCR2, CCR11
MIP-1	CCL3	CCR1, CCR5
MIP-1	CCL4	CCR5
RANTES	CCL5	CCR1, CCR3, CCR5
MIP-3	CCL20	CCR6

Table 3. Major features of KU812 cells

Surface marker/contents	
Preformed mediators	Histamine, tryptase, chymase, heparin, chondroitin & -hexosominidase
Mast cell specific surface markers	CD117
Major surface markers	CD14 (-), MHC Class I (+), Class II (-)
Ig receptors	Fc RI (-), Fc RII (+), Fc RIII(-), Fc RI (+)
Complement receptors	CR1, CR3, CR4, & C5aR
Adhesion molecules	1 integrins: VLA-2, VLA-4, VLA-5 2 integrins: LFA-1, MAC-1, p150,95, ICAM-1, LFA-3
Cytokine/chemokine receptors	IL-2, 3, 4, TNF- , IFN- , - , - , GM-CSF, CXCR1 & CXCR4

Note: (+) denotes presence of the surface marker, (-) denotes absence of marker, all others in right column are present on KU812 cells.

References (Blom *et al.*, 1992; Fureder *et al.*, 1995; Nilsson *et al.*, 1994; Valent and Bettelheim, 1992; Ghammadan *et al.*, 2002)

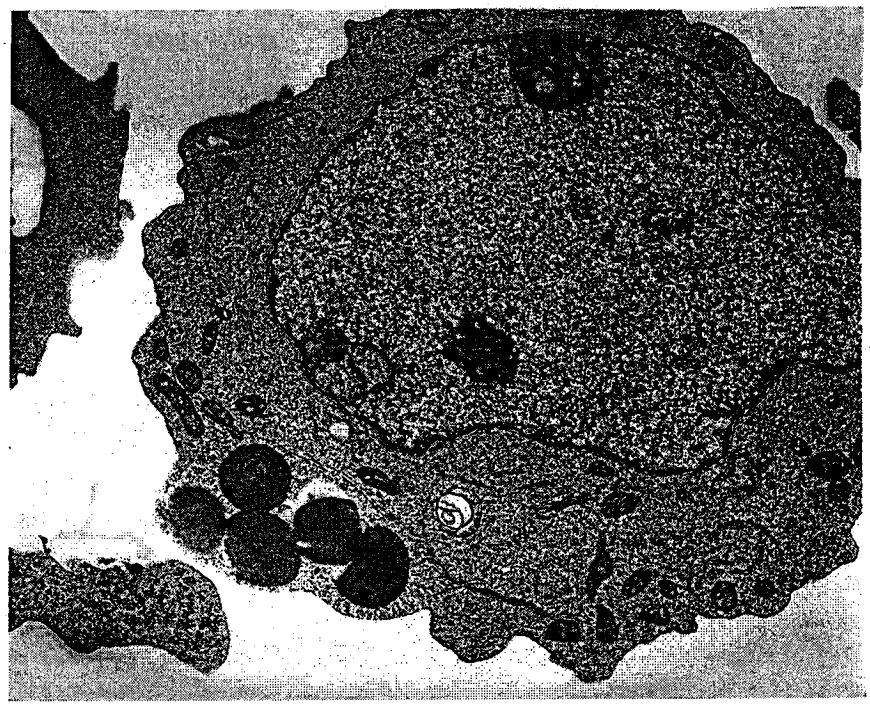


Figure 2. Electron microcopy of a KU812 cell 8200x.

Table 4. Comparison of KU812, HMC-1 and MC_T and MC_{TC} mast cells.

	KU812	HMC-1	MC_T and MC_{TC}
CD117	+ (subset)	+	+
Fc receptors	Fc RI, Fc RII	Fc RII	Fc RI, Fc RII
Histamine (pg/cell)	0.01-0.1	$< 1 \times 10^{-3}$	1-3
Tryptase (pg/cell)	0.01	0.1-0.6	10-35
Chymase (pg/cell)	0.4	0.4	0-5
Alcian blue	+ weak	+	+
Toluidine blue	+ weak	+	+
CR1, CR3, C5aR	+		+
Carboxypeptidase A	+	+	+

References (Fureder *et al.*, 1995; Nilsson *et al.*, 1994; Blom and Hellman, 1993; Valent and Bettelheim, 1992; Grabbe *et al.*, 1998; Saito *et al.*, 1995, Okayama *et al.*, 2000; Magnusson *et al.*, 1995; Almlof *et al.*, 1988; Kawata *et al.*, 1996; Wedi *et al.*, 1996; Xia *et al.*, 1995)

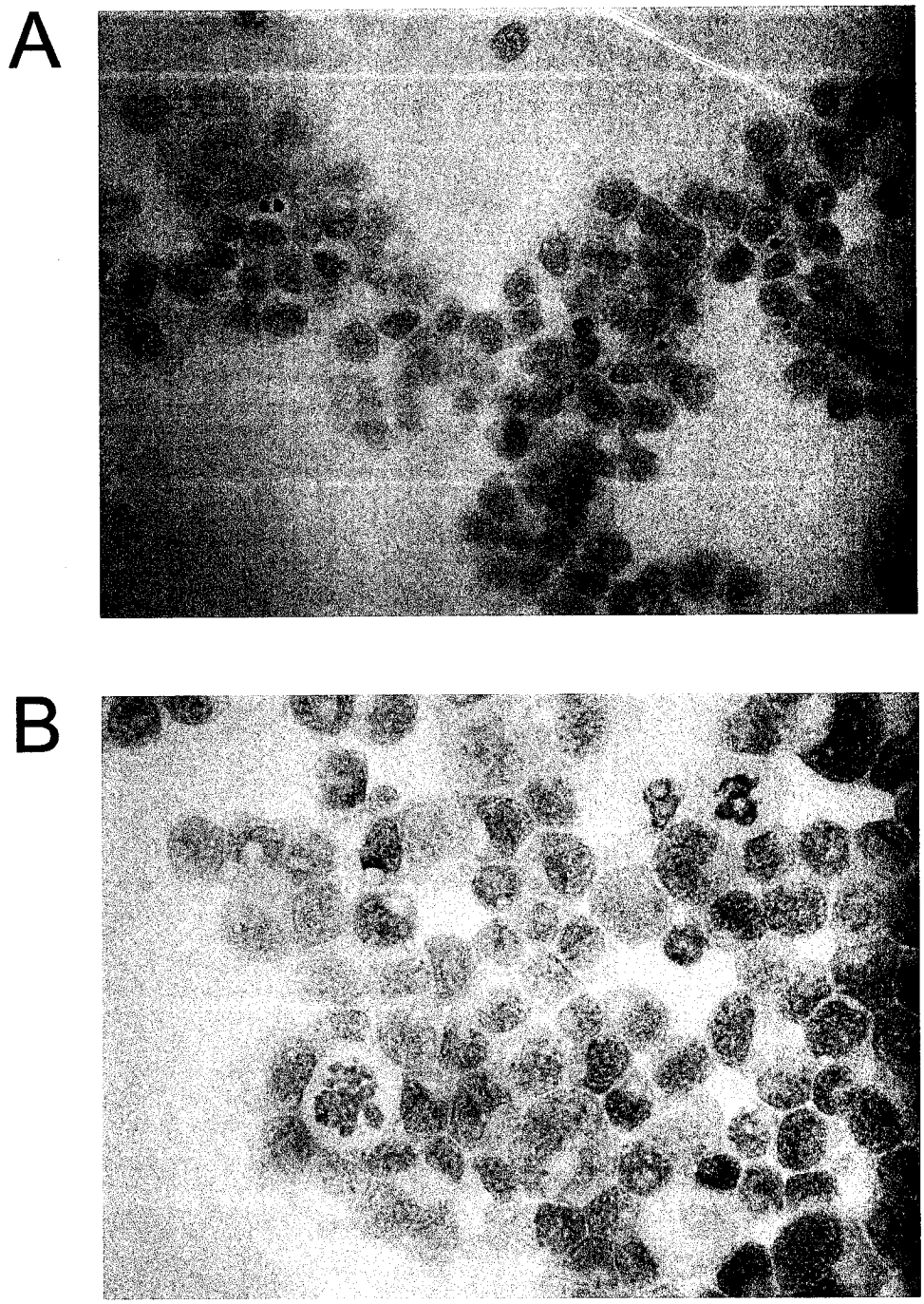
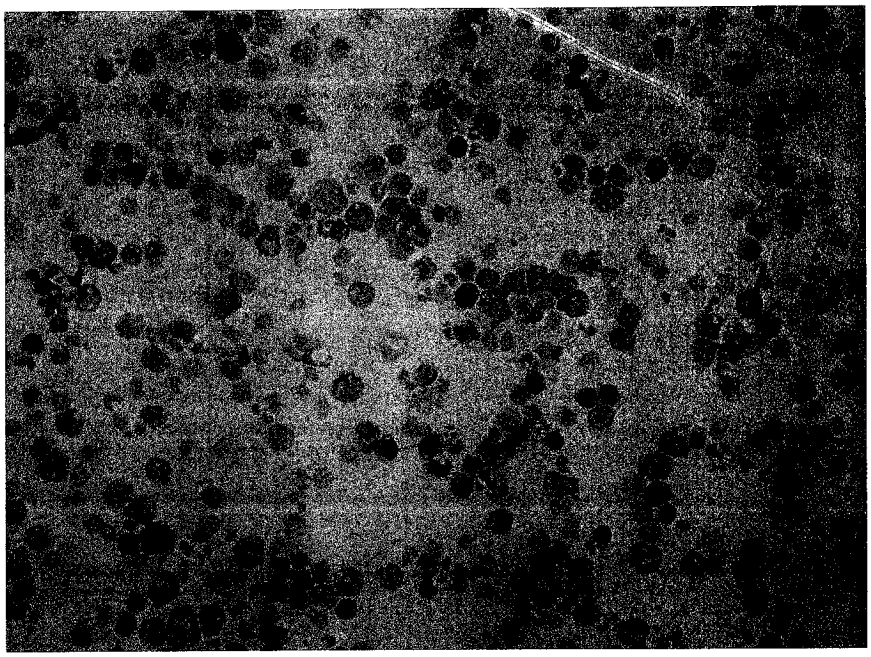


Figure 3. Toluidine blue staining of HMC-1 and cord blood derived mast cells (CBMCs). (A) HMC-1, 20x; (B) HMC-1, 40x

C



D

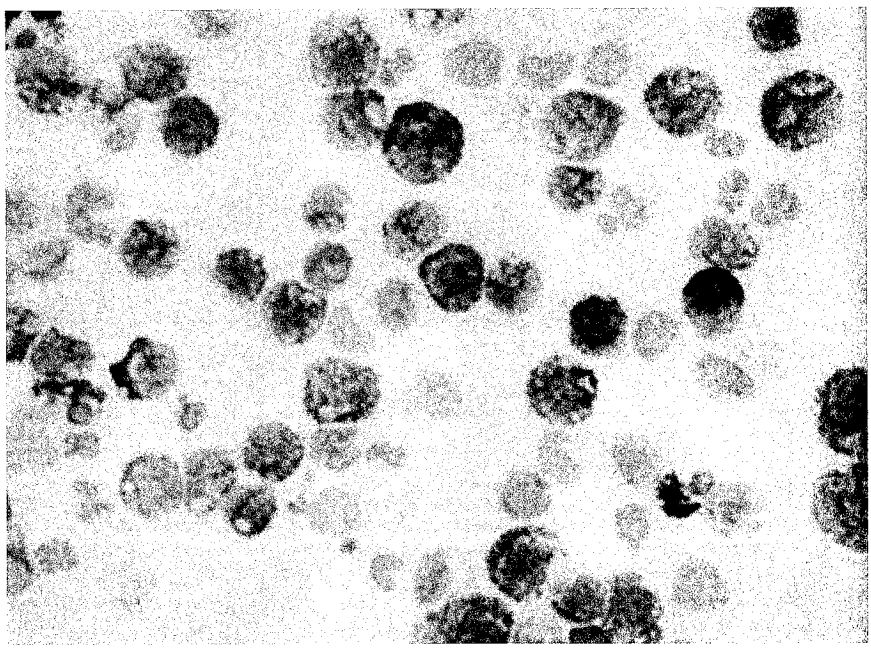


Figure 3. (C) CBMCs, 20x; (D) CBMCs, 40x.

Table 5. Mast cell responses to viruses or viral proteins.

Virus	Infection	*In vitro* mast cell/basophil mediator response	*In vivo* observations	Reference
RSV Intact virus	No	? (no RANTES)		King et al., 2000;2002
			↑IgE	
			↑CysLTs ↑mast cell density	Wedde-Beer et al., 2002; van Schaik et al., 1999
Influenza virus Intact virus	?	↑histamine release	?	Clementsen et al., 1989
Sendai virus Intact virus	?	degranulation histamine release		Sugiyama, 1977
			↑numbers of mast cells	Castleman et al., 1989;1990; Sorden and Castleman, 1995b
Adenovirus Intact virus	Yes	? (no RANTES, MIP-1α, MIP-1β)	?	King et al., 2002
Dengue virus Intact virus	Yes	IL-1β, IL-6		King et al., 2000
		RANTES, MIP-1α, MIP-1β		King et al., 2002
			↑urinary histamine	Tuchinda et al., 1977
			loss of cell integrity	Bhamarapravati et al., 1967
HIV Intact virus	Yes			Li et al., 2000; Bannert et al., 2001
gp120		IL-4, IL-13		Patella et al., 2000
Tat		↑CCR3 chemotaxis		De Paulis et al., 2000
			↑IgE ↑mast cell density	Vigano et al., 1995;Lucey et al., 1990 Paiva et al., 1996

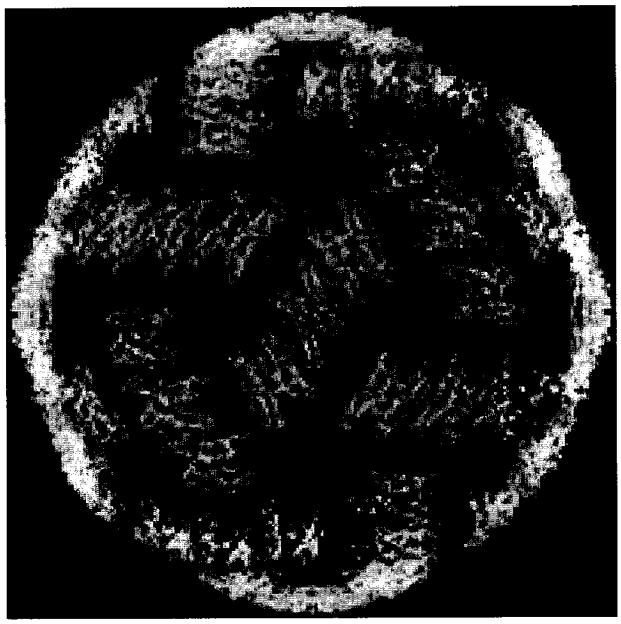


Figure 4. Dengue virus structure. The major protein in dengue virus, the envelope protein, is colour-coded red, yellow and blue, and represents the domains of the protein I, II, and III , respectively. The green represents the fusion peptide. The surface is unusually smooth, revealing an architectural structure different from any other virus that has been seen.

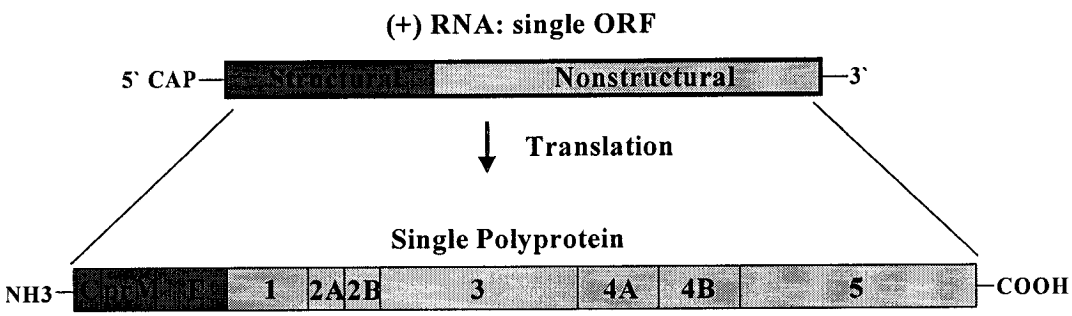


Figure 5. Flavivirus genome

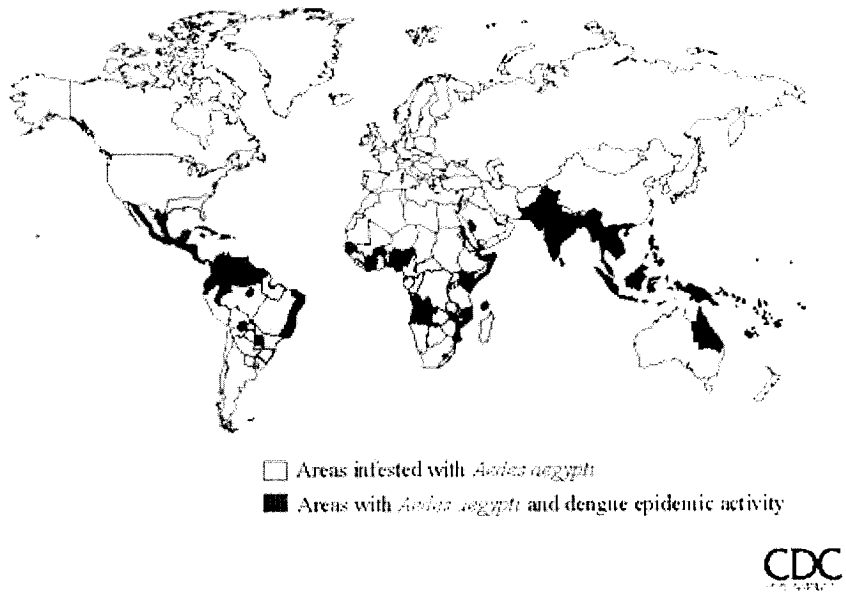


Figure 6. World distribution of dengue - 2000

Table 6. Total number DF and DHF cases reported to the WHO in 1996-1998

Region	1996	1997	1998
Asia (DF & DHF)	232832	287092	445186
Oceania (DF & DHF)	1121	1598	29210
The Americas (DF)	276691	389917	708146
The Americas (DHF)	4439	10309	12426

Modified from (Kurane and Takasaki, 2001)

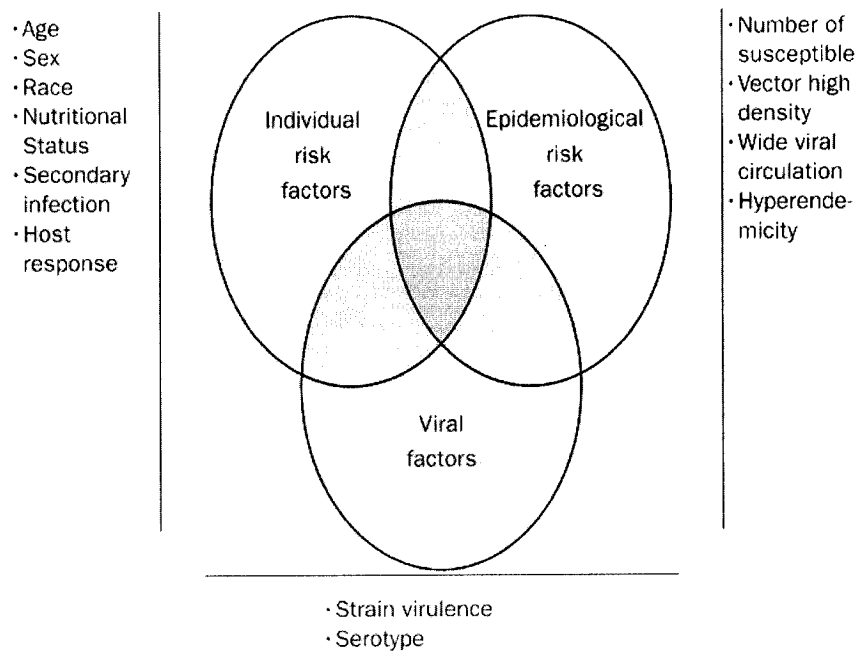


Figure 7. Risk factors for DHF/DSS: an integral hypothesis.
(Guzman and Kouri, 2002)

Chapter 2

RELEASE OF VASOACTIVE CYTOKINES BY ANTIBODY-ENHANCED DENGUE VIRUS INFECTION OF A HUMAN MAST CELL/BASOPHIL LINE.

2.1 Introduction to the chapter

We report here the first demonstration of dengue virus infection and vasoactive cytokine response of a cell of the mast cell/basophil lineage. Infection of KU812 cells was dependent on dengue-specific antibody and gave rise to infectious virions. This antibody-enhanced dengue virus infection triggered a 4-5 fold increase in the release of IL-1 and a modest increase in IL-6, but not an alternate cytokine GM-CSF. The results suggest a potential role for mast cells/basophils in the pathogenesis of dengue virus induced disease (King *et al.*, 2000).

2.2 Introduction

Dengue virus infection is associated with disease, ranging from dengue fever to dengue hemorrhagic fever (DHF) and /or dengue shock syndrome (DSS). Host immunological factors play a role in DHF/DSS, as severe disease occurs most often in individuals experiencing secondary dengue virus infections (Rothman & Ennis, 1999). Sub-neutralizing levels of dengue-specific antibodies have been shown to potentiate dengue virus infection via antibody-dependent enhancement (ADE) (Halstead, 1989). Antibody-enhanced dengue infection of monocytes (Halstead & O'Rourke, 1977; Halstead *et al.,* 1980) stimulates the production of cytokines such as TNF-α which act on endothelium (Anderson *et al.,* 1997). Specific cytokines are also found to be elevated in sera from DHF/DSS patients (Green *et al.,* 1999b; Green *et al.,* 1999a; Hober *et al.,* 1998; Hober *et al.,* 1993; Raghupathy *et al.,* 1998; Vitarana *et al.,* 1991; Yadav *et al.,* 1991).

The role of mast cells/basophils has not yet been explored with regard to dengue pathogenesis. Mast cells play an important role in inflammation (reviewed in Marshall & Bienenstock, 1994) and in host defense against foreign pathogens (Echtenacher *et al.,* 1996; Galli *et al.,* 1999; Malaviya *et al.,* 1996). These cells mediate immune responses by selective production and secretion of a variety of soluble mediators including chemokines, vasoactive cytokines such as IL-1, IL-6 and TNF-α (Benyon *et al.,* 1991; Bradding *et al.,* 1993; Grabbe *et al.,* 1994; Moller *et al.,* 1998; Moller *et al.,* 1993; Nilsson *et al.,* 1995), lipid mediators and granule associated products (Schwartz & Austen, 1984). Mast cells reside mainly in the

tissues and associate closely with blood vessels (Alving,1991; Anton *et al.,* 1998; Pesci *et al.,* 1996; Pulimood *et al.,* 1998; Selye, 1966; Selye *et al.,* 1968) and nerves (Olsson, 1971; Stead *et al.,* 1989; Wiesner-Menzel *et al.,* 1981) while basophils normally circulate in the blood. Mast cell activation is closely linked with local increases in vascular permeability in allergic disease.

Mast cells/basophils express both FcRI (the high affinity human IgE receptor) and some Fc (Guo *et al.,* 1992; Sperr *et al.,* 1994; Wedi *et al.,* 1996) receptors. As such, they are potential targets for antibody-enhanced virus infection as well as for the consequent induction of powerful vasoactive cytokines. We therefore sought to investigate the human mast cell/basophil KU812 cell line with respect to dengue virus susceptibility and concomitant vasoactive cytokine responses.

2.3 Materials and Methods, Results and Discussion

Human mast cell/basophil KU812 cells, maintained in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% FCS, 10 mM Hepes, 100 U/ml penicillin, 100 μg/ml streptomycin (Life Technologies) and, where noted, treated for 8 days with 0.3 mM sodium butyrate and 40 ng/ml IFN (R& D systems, Minneapolis, MN) and P815 mouse mastocytoma cells were mock inoculated or inoculated with either dengue virus or respiratory syncytial virus, (RSV) (an unrelated virus) in the presence or absence of corresponding human immune serum (1:1000 final dilution). Cultures were incubated at 37°C and radiolabeled with [^{35}S]-Methionine-cysteine (NEN, Mississauga, ON) from 24 hours postinfection for 3-4 hours followed by 12-14 hours chase. Cell supernatants were harvested, immunoprecipitated with dengue virus immune sera and protein A-bearing, formalin fixed *Staphylococcus aureus* as previously described (He *et al.*,1995). Immunoprecipitates were resolved by SDS-PAGE (Laemmli,1970) using 10% polyacrylamide gels. Gels were impregnated with 1M sodium salicylate and fluorographed by exposure to Kodak X-ray film at -70C. As shown in Fig 1A, both undifferentiated and sodium butyrate/IFN differentiated KU812 cells were permissive to dengue virus infection in the presence of human dengue immune sera (even though sodium butyrate/IFN- treatment reduced the level of infection, possibly due to the antiviral properties of residual IFN-γ. In contrast the mouse mastocytoma P815 cells were much less permissive to dengue virus infection with or without human dengue immune serum (Fig. 1A). P815 cells, have been previously shown to be susceptible to antibody-enhanced dengue virus infection (Legrand *et al.*, 1986). However, our data show clearly that KU812 cells are superior to P815 cells in their permissiveness to antibody-enhanced dengue virus infection.

No antibody-enhanced infection of RSV was observed. Dengue virus infected KU812 cells produced infectious virions by 24 hours postinfection which began to decline at 72 hours postinfection (Fig. 2). A requirement for human dengue immune serum (rather than normal human serum) in enhanced dengue infection of KU812 cells is shown in Fig 1B. As expected, Vero cells showed infection with dengue virus alone which was not subject to antibody enhancement (Fig. 1B).

Fluorescence microscopy was used to investigate the number of dengue infected cells. Vero cells inoculated with dengue virus and KU812 cells inoculated with dengue virus, dengue virus/normal human serum, dengue virus/human dengue immune serum combinations were harvested 24 hours post-infection and examined for virus antigen expression by fluorescence microscopy (Fig 3). Cells were fixed with 4% paraformaldehyde, washed, resuspended in 10% DMSO in PBS and frozen at -80C. Following permeabilization with 0.1% saponin for 1 hour at room temperature samples were washed and resuspended in 1% BSA/ 0.2% sodium azide in PBS. Mouse anti-dengue monoclonal 1B7 (Henchal *et al.*, 1985) or isotype matched mouse anti-IgG2a antibody (negative control) was employed as a primary antibody and incubated on ice for 1 hour. Subsequently, samples were washed and incubated with goat anti-mouse FITC or goat anti-mouse Texas Red labeled antibody for 1 hour on ice. Cytospins were made of each sample and viewed via fluorescence microscopy to assess number of infected cells. A total of 1000 cells was counted under UV illumination with the number of positive green (FITC) or red (Texas-Red) fluorescent cells recorded to give the percentage of cells infected with dengue virus. Vero cells inoculated with dengue virus alone

were 30% fluorescence positive at 24 hours (Fig 3A). KU812 cells showed no virus positive cells when inoculated with dengue virus either alone or in combination with normal human sera (Fig 3B). However, KU812 cells inoculated with dengue virus in combination with human dengue immune serum at a dilution of 1:1000 showed an infection rate of 0.6% - 11% (0.6%, 11.0%, and 5.5% in three experiments) (Fig 3C). KU812 cells inoculated with dengue and human dengue immune serum at a 1:10000 dilution showed an infection rate of 2%-14% (2.4%, 14.4%, and 10.5%) (Fig 3D). All isotype staining controls were negative.

To analyse cytokine production in response to antibody-enhanced dengue virus infection of KU812 cells 72 hour supernatants were harvested from KU812 cultures. IL-1β production was significantly enhanced in supernatants obtained from dengue infected cultures as analysed by ELISA using a matched antibody pair (IL-1β M-412B-E, M-420B-B; Endogen, Wuburn, MA) (Fig 4A). IL-6 was found to follow a similar, though less dramatic, pattern of enhanced production (Fig 4B). ELISA antibody pair employed (IL-6 M-620-E, M-621-B Endogen, Wuburn, MA). In contrast to both IL-1β and IL-6, GM-CSF production, analysed by ELISA as previously described (Zhu *et al.*, 1998), was not elevated in antibody-enhanced dengue infected KU812 cell supernatants as compared to controls (Fig 4C). No increased cytokine production, (IL-1β, IL-6, or GM-CSF), was detected in KU812 cells inoculated with UV-inactivated virus/human dengue immune serum combinations or dengue virus/normal human serum combinations (data not shown). These data thus indicate that KU812 cells are stimulated by antibody-enhanced dengue virus infection to produce selective vasoactive cytokines.

Two potentially important vasoactive factors, histamine and TNF-α, could not be measured in the dengue-KU812 cell system. KU812 cells are relatively poorly granulated and therefore contain little histamine (Zhu *et al.*,1998). They also release little TNF-α, upon appropriate stimulation (unpublished observations). Nevertheless our findings demonstrate that human KU812 mast cells/basophils undergo antibody-enhanced dengue virus infection and that antibody-enhanced dengue virus infection results in selective vasoactive IL-1β and IL-6 cytokine release. Selective production of the vasoactive cytokines IL-1β and IL-6, by antibody-enhanced dengue virus infection of KU812 cells, may provide additional insights into the pathogenic mechanisms of severe dengue disease. IL-6 is an endogenous pyrogen (Helle *et al.*, 1988) known to mediate increased endothelial cell permeability (Maruo *et al.*, 1992). In rodent systems, mast cells have been shown to be a much more potent source of IL-6 than other cell types such as the macrophage (Leal-Berumen *et al.*, 1994). Our finding of increased IL-1β production in antibody-enhanced dengue virus infected KU812 cells raises the possibility that mast cells/basophils may represent a local source of IL-1 in dengue infection. Local production of IL-1 by dengue infected tissue mast cells that reside in close proximity to blood vessels may then act directly on endothelial cells. IL-1β is recognized to induce fever (Atkins, 1960), inflammation, and shock (reviewed in Dinarello, 1996). IL-1β induces IL-6 and TNF-α production and activates endothelial cells (reviewed in Pober, 1988) modulating the expression of the adhesion molecules (Bevilacqua *et al.*, 1987; Pober, *et al.*, 1986; Pober *et al.*, 1987) as well as altering endothelial cell morphology (Pober *et al.*,1987). Such enhanced adhesion molecule expression may induce inflammatory cell activation and migration with consequent potential vasculitic damage.

Our findings that mast cell/basophil KU812 cells are permissive to dengue infection which leads to infectious virion production and vasoactive cytokine production support the hypothesis that mast cells/basophils may contribute to the vascular pathology seen in severe dengue disease. Mast cells are resident tissue cells and are present in large numbers in the skin (Marshall *et al.,* 1987) while basophils comprise approximately 1% of total circulating cells. Mast cells are therefore present at the site of dengue infection in the skin whereas basophils would be accessible to dengue virus in the circulation.

The observation that virus antibody-complexes are much more potent than virus alone in inducing mast cells to produce vasoactive cytokines is consistent with the known epidemiological evidence that pre-existing immunity is a risk factor for DHF/DSS in human dengue infections (Halstead, 1988). Our present study employed homotypic (dengue-2) convalescent sera, although antibody-dependent enhancement of dengue virus infection can be readily achieved with either homo- or heterotypic antibodies (Halstead, 1988). In attempting to extrapolate our *in vitro* results to clinical disease it will be important to determine the relative contributions of different cell types involved in virus amplification and in modulating hemostasis. So far, this has only been achieved for circulating monocytes (Rothman & Ennis, 1999). The contribution to pathogenesis of other cell types, including cells of the basophil/mast cell lineage, is unknown. Nevertheless, the results of the present study support further investigation to eventually identify the full spectrum of cell types which are infected by dengue virus *in vivo* and which contribute to perturbation of vascular function.

2.4 Figures

Figure 1. Antibody-enhanced dengue virus infection of KU812 cells. A) Cultures of Vero, P815 or KU812 cells, were inoculated with dengue 2 virus strain 16681 (Halstead & Simasthien, 1970) (MOI 0.1), RSV (MOI 0.1) or combinations of either virus with respective human immune serum (final dilution 1:1000). Virus infection was monitored by radiolabelling with ^{35}S-methionine-cysteine followed by immunoprecipitation and fluorographic SDS PAGE. B) Vero cells (positive control) and KU812 cells were inoculated with dengue virus alone (Den) (MOI 0.2), and normal human serum (NHS) (final dilution 1:1000) or dengue virus and human dengue immune sera (final dilutions of 1:1000 or 1:10000). The position of the radiolabelled $(NS1)_2$ and E proteins are indicated. Data are representative of 9 separate experiments. Human sera, described in (He *et al.,* 1995) were obtained from dengue virus type 2 convalescent patients, normal human AB sera and respiratory syncytial virus (RSV) positive sera were obtained from volunteer donors.

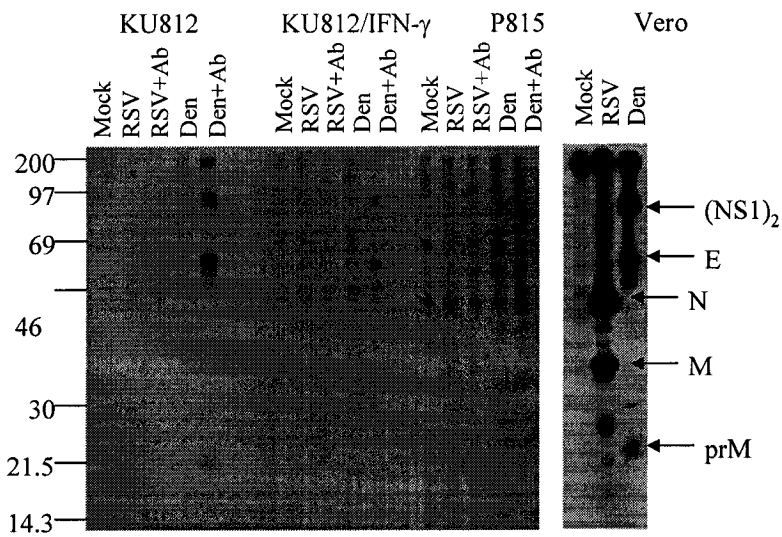

KU812
KU812/IFN-γ
P815
Vero
Mock
RSV
RSV+Ab
Den
Den+Ab
200
97
69
46
30
21.5
14.3
(NS1)2
E
N
M
prM

Figure 1A

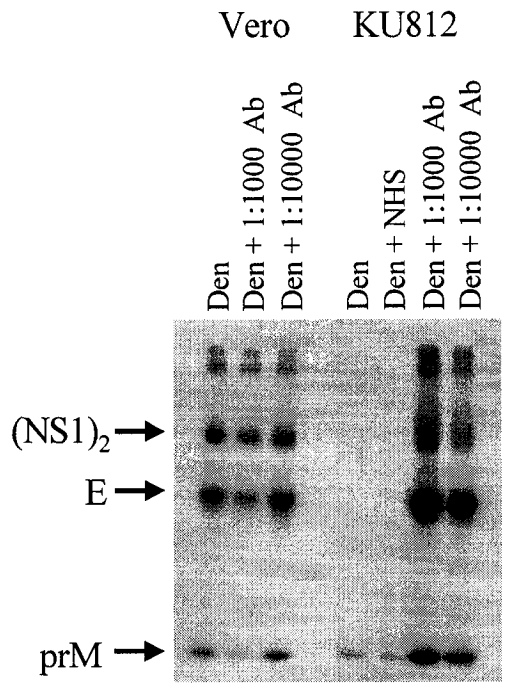

Vero
KU812
Den
Den + 1:1000 Ab
Den + 1:10000 Ab
Den
Den + NHS
Den + 1:1000 Ab
Den + 1:10000 Ab
(NS1)2
E
prM

Figure 1B

Figure 2. Infectious virion production in dengue virus infected KU812 and Vero cells. Infection conditions were performed as described in the legend to Fig 1 (MOI 0.1-0.3). Virion production was assessed by $TCID_{50}$ assay (Russell *et al.,* 1966) on Vero cells at 4, 24, 48 and 72 hours postinfection. Data are representative of three separate experiments and expressed as mean ± SEM.

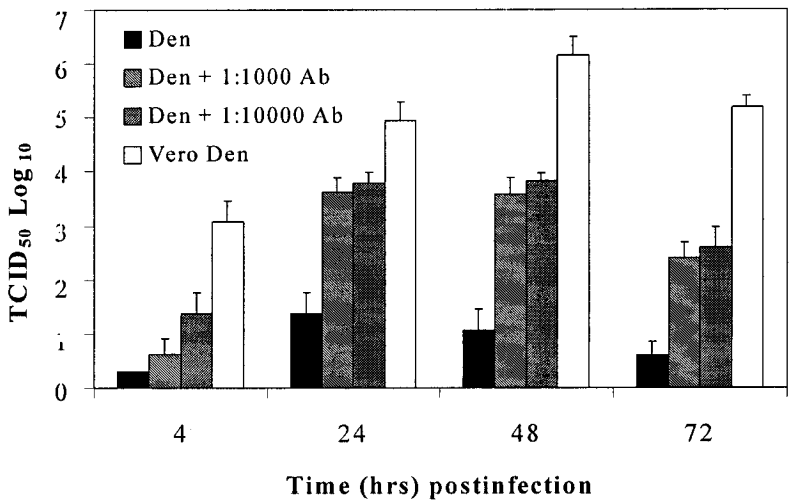

Den
Den + 1:1000 Ab
Den + 1:10000 Ab
Vero Den
TCID50 Log10
0
1
2
3
4
5
6
7
4
24
48
72
Time (hrs) postinfection

Figure 2

Figure 3. Immunofluorescence of dengue virus infected KU812 cells. Vero and KU812 cells inoculated with dengue virus (MOI 0.1-0.3) or dengue virus/antiserum combinations were harvested 24 hours postinfection. Dengue virus infected Vero cells (A), KU812 cells inoculated with dengue virus and normal human serum (1:000 final dilution) (B), antibody-enhanced dengue infected KU812 cells with 1:1000 final dilution of human dengue immune serum (C), and antibody-enhanced dengue infected KU812 cells with 1:10000 final dilution of human dengue immune serum (D). The data are representative of 3 separate experiments.

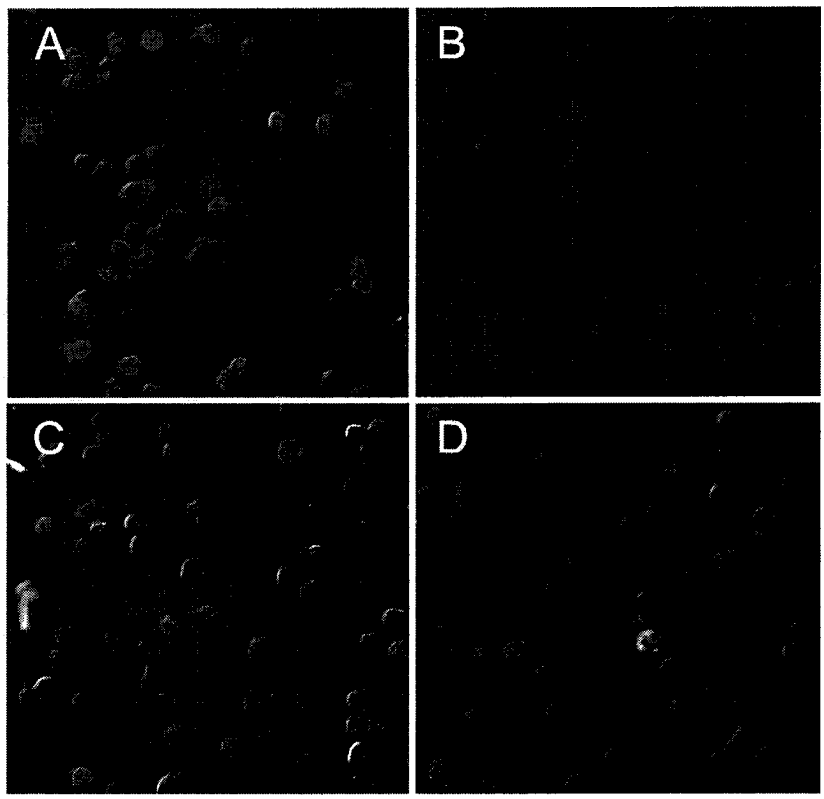


Figure 3

Figure 4. Cytokine responses in dengue virus infected KU812 cells. A) IL-1 production assayed at 72 hours postinfection by antibody-enhanced dengue virus infected KU812 cells. Infection of KU812 cells was carried out as described in the legend to Fig 1 (MOI 0.1-0.3). KU812 cells incubated with media alone (Media) indicates level of constitutive production, 25 ng/ml phorbol myristate acetate (PMA) and 5 x 10^{-7}M A23187 (calcium ionophore) treated KU812 cells were used as positive controls. For some experiments virus was inactivated with UV light (UV-Den) as previously described (Anderson *et al.*, 1997). The data are from six separate experiments with triplicate samples and using one dengue convalescent patient serum sample 7873. B) IL-6 production assayed at 72 hours postinfection by antibody enhanced dengue virus infected KU812 cells. The data are from five separate experiments with triplicate samples. C) GM-CSF production assayed at 72 hours postinfection by antibody enhanced dengue virus infected KU812 cells. The data are from three separate experiments with triplicate samples. Significant differences from the dengue alone samples are indicated by *($p<0.05$), and ** ($p<0.01$). Data are represented as mean ± SEM. Statistical significance was assessed using a nonparametric approach. GM-CSF and IL-1 were initially analyzed using Friedman's test for all data obtained, followed by examination of specific groups using Dunn's multiple comparison test. In view of the large differences between baseline response between individual experiments IL-6 data was analyzed using Freidman's test followed by Wilcoxon signed rank test to compare responses between specific groups. Sensitivity of the IL-6 and IL-1 assays were 1.95 pg/ml, that of GM-CSF was 3 pg/ml.

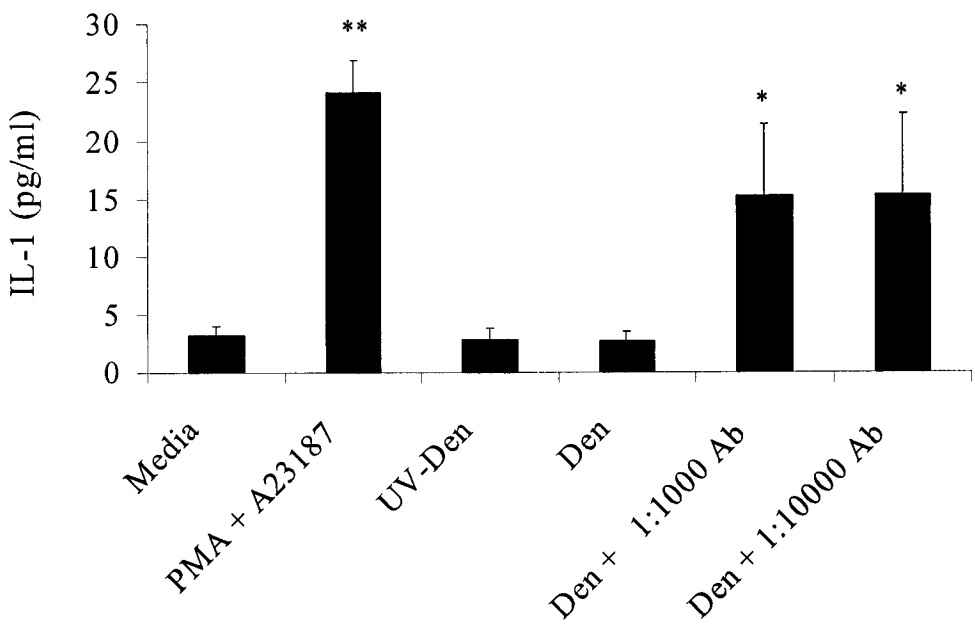

IL-1 (pg/ml)
30
25
20
15
10
5
0
**
*
*
Media
PMA + A23187
UV-Den
Den
Den + 1:1000 Ab
Den + 1:10000 Ab

Figure 4 A

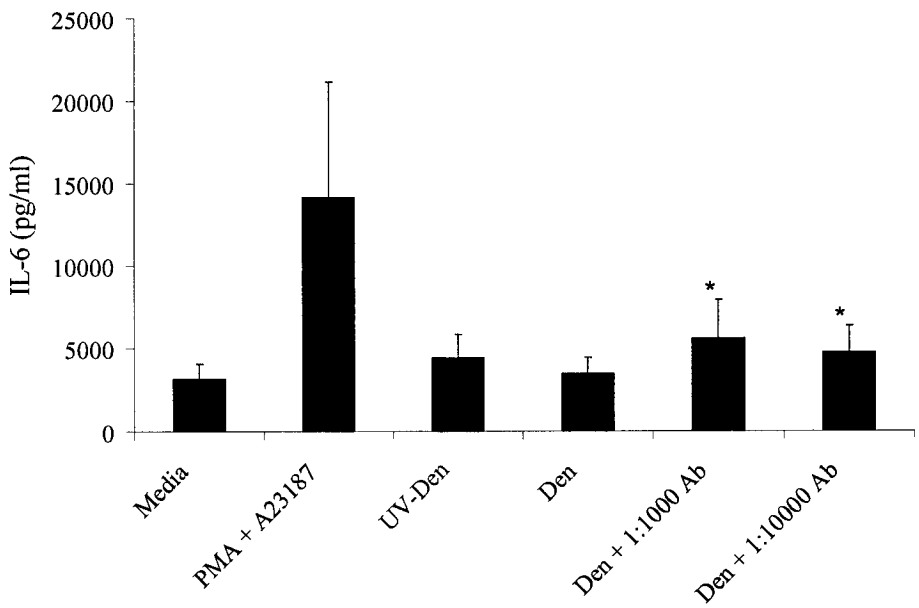

25000
20000
15000
10000
5000
0
IL-6 (pg/ml)
*
*
Media
PMA + A23187
UV-Den
Den
Den + 1:1000 Ab
Den + 1:10000 Ab

Figure 4B

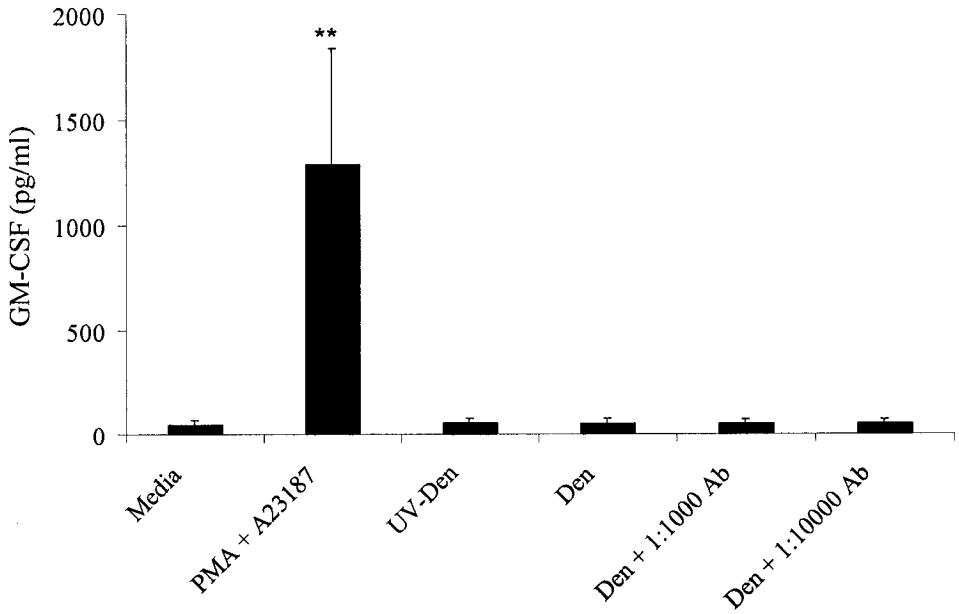

2000
1500
1000
500
0
GM-CSF (pg/ml)
**
Media
PMA + A23187
UV-Den
Den
Den + 1:1000 Ab
Den + 1:10000 Ab

Figure 4C

2.5 Conclusions

This chapter detailed the initial observations of antibody-enhanced dengue virus infection of KU812 cells. The following chapter will detail further observations and work as a result of this infection.

Chapter 3

DENGUE VIRUS SELECTIVELY INDUCES HUMAN MAST CELL CHEMOKINE PRODUCTION

3.1 Introduction to the chapter

Severe dengue virus infections usually occur in individuals who have pre-existing anti-dengue antibodies. Mast cells are known to play an important role in host defense against several pathogens but their role in viral infection has not yet been elucidated. The effects of dengue virus infection on the production of chemokines by human mast cells were examined. Elevated levels of secreted RANTES, MIP-1α, and MIP-1β, but not IL-8 or ENA-78, were observed following infection of KU812 or HMC-1 human mast cell/basophil lines. In some cases a greater than 200 fold increase in RANTES production was observed. Cord blood derived, cultured human mast cells treated with dengue virus in the presence of sub-neutralizing concentrations of dengue specific antibody also demonstrated significantly ($p<0.05$) increased RANTES production, under conditions which did not induce significant degranulation. Chemokine responses were not observed when mast cells were treated with UV-inactivated dengue virus in the presence or absence of human dengue specific antibody. Neither antibody-enhanced dengue virus infection of the highly permissive U937 monocytic cell line, nor adenovirus infection of mast cells, induced a RANTES, MIP-1α, or MIP-1β response, demonstrating a selective mast cell response to dengue virus. These results suggest a role for mast cells in the initiation of chemokine-dependent host responses to dengue virus infection (King *et al.,* 2002).

3.2 Introduction

Dengue viruses are lipid-enveloped RNA viruses that belong to the family Flaviviridae. Four antigenically distinct serotypes, dengue 1 - 4, are transmitted to humans via the mosquito vector *Aedes aegypti* (Halstead, 1988). It is estimated that up to100 million individuals are infected with dengue virus annually (Halstead, 1988). Increased vascular permeability, shock, and severe thrombocytopenia (Brinton *et al.,* 1998; Halstead, 1988; Kurane *et al.,* 1994; Halstead, 1989)are associated with severe forms of dengue infection such as dengue hemorrhagic fever (DHF) and/or dengue shock syndrome (DSS). Substantial T cell activation is also observed in severe dengue disease (Kurane & Ennis, 1992). The pathogenesis of severe forms of dengue virus infection is not completely understood. Symptoms of severe disease most often occur in individuals experiencing secondary dengue virus infections (Rothman & Ennis, 1999). A number of cytokines and chemokines are found to be elevated in DHF/DSS patients (Avirutnan *et al.,* 1998; Chaturvedi *et al.,* 2000; Green *et al.,* 1999; Hober *et al.,* 1993; Kurane *et al.,* 1991; Raghupathy *et al.,* 1998). The presence of heterotypic sub-neutralizing antibodies to dengue virus as a result of primary infection has been shown to potentiate secondary infection via antibody-dependent enhancement (ADE) (Halstead, 1989). Due to the lack of an animal model for DHF/DSS most of the work investigating primary target cells and potential mechanisms of pathogenesis has been done *in vitro.* During antibody-enhanced dengue infection of monocytes (Brinton *et al.,*1998), increased uptake of antibody-virus complexes occurs via Fc receptor mediated binding to cells (Peiris *et al.,* 1981). Such infection of monocytes, stimulates cytokine

production (particularly TNF-α) which perturbs endothelial cell function (Halstead, 1989). For many years there has been speculation as to the involvement of mast cells in dengue pathogenesis. Dengue patients exhibit increased levels of urinary histamine (a major granule product of mast cells) which correlates with disease severity (Tuchinda, *et al.,* 1977). However, the potential role of mast cells has not yet been explored with regard to dengue pathogenesis. Mast cells play an important role in a wide variety of inflammatory reactions and in host defense against bacterial pathogens (Echtenacher *et al.,* 1996; Malaviya *et al.,* 1996). These cells selectively produce and secrete a variety of mediators including chemokines, cytokines, lipid mediators and granule associated products. Production of a wide variety of cytokines and chemokines including TNFα (Benyon *et al.,* 1991) IL-6 (Bradding *et al.,* 1993), IL-1β (Moller *et al.,* 1998), IL-16 (Rumsaeng *et al.,* 1997), IL-8 Moller *et al.,* 1993), ENA-78 (Lukacs *et al.,* 1998), MIP-1α (Yano *et al.,* 1997), MIP-1β (Selvan *et al.,* 1994), and RANTES (Rajakulasingam *et al.,* 1997) have been demonstrated by human mast cells. Mast cells reside mainly in the tissues and have been shown to associate closely with blood vessels (Selye, 1966) and nerves (Wiesner-Menzel *et al.,* 1981). Human mast cells can express both FcεRI (Guo *et al.,* 1992; Sperr *et al.,* 1994) and some Fcγ receptors including FcγRI (Okayama *et al.,* 2001a; Okayama *et al.,* 2001b;Okayama *et al.,* 2000) and FcγRII, (Okayama*et al.,* 2001b; Wedi *et al.,* 1996) and contain FcγRIII mRNA (Okayama *et al.,* 2001b).

The mast cell is a potential target cell for dengue infection in view of its Fc receptor

expression. This laboratory previously reported that the human KU812 mast cell/basophil line is permissive to dengue virus infection (King *et al.,* 2000). In view of the critical role of chemokines in mobilizing effective immunity the current study sought to investigate the production of the key chemokines RANTES, MIP-1α, and MIP-1β by mast cells in response to viral infection. Human cord blood derived mast cells (CBMC) as well human mast cell lines were examined. The data obtained suggests that mast cells may act as an early and important source of such chemokines during dengue induced disease.

3.3 Materials and Methods

Dengue virus propagation

Dengue type 2 virus strain 16681 (Halstead & Simasthien, 1970) was propagated in African Green Monkey kidney Vero cell monolayers cultured in endotoxin free RPMI 1640 (Sigma, Oakville, ON) supplemented with 1% FCS (Life Technologies, Grand Island, NY). For some experiments, virus was inactivated by UV irradiation [254nm; 1,000 J/m^2] (Anderson *et al.,* 1997).

Adenovirus and respiratory syncytial virus (RSV) propagation

Adenovirus type 37 (Sheu,*et al.,* 1988; Wadell *et al.,* 1981) was propagated in human lung epithelial A549 cell monolayers cultured in endotoxin free RPMI 1640 medium (Sigma, Oakville, ON) supplemented with 1% FCS (Life Technologies, Grand Island, NY). RSV (Long strain) was propagated in HEp-2 cells using the same medium.

Sera

For antibody dependent enhancement assays of dengue virus infection, a serum pool was prepared using nine convalescent sera from patients recovering from a dengue serotype 2 infection. Anti-dengue IgG titers among the nine sera ranged from 207 to 465 EIA units per ml of serum. The individual patient sera and the dengue specific monoclonal antibody 1B7 were obtained from a collection provided by Dr Bruce Innis and described briefly in (He *et al.,* 1995). Normal human AB sera were obtained from volunteer donors.

Mast cell culture

Human pre-basophilic KU812 cells were maintained in RPMI 1640 (Life Technologies), supplemented with 10% FCS, 10 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies). HMC-1 cells were maintained in Iscove's Modified Dulbecco's Medium (Life Technologies) supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies). The human monocytic U937 cell line was maintained in RPMI 1640 (Life Technologies), supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies). All cell lines were passaged 2-3 times per week.

Primary human cultured cord blood derived mast cells

Primary mast cells were generated by culture of human cord blood mononuclear cells using a modification of the method of Saito et al.(Saito *et al.,* 1996) and characterized as previously described (Lin *et al.,* 2000). These cells were consistently positive by flow cytometry for c-kit and CD13 but not CD14. Cells were cultured at an initial concentration of 0.6 x 10^6/ml in RPMI supplemented with 20% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin (Life Technologies), 20% CCL-204 supernatant as a source of IL-6, 1 x 10^{-7}M PGE_2 (Sigma), and 50-100 ng/ml SCF (Peprotech, Rocky Hill, NJ). Media was changed once weekly for 5-10 weeks. Purity of each CBMC preparation was assessed by Toluidine blue (pH 1.0) staining of cytocentrifuge preparations and examination of cells for the presence of multiple metachromatic granules and appropriate nuclear morphology. Only those, mast cell

preparations, >95% pure were used for this study. The mean purity of cord blood mast cells employed was 97%.

Dengue virus infection

Primary cultured human mast cells, HMC-1 and U937 infection experiments were carried out as previously described (King *et al.,* 2000). Briefly, human KU812, HMC-1, and U937 were washed and resuspended at 1 x 10^6 cells/ml in appropriate media. Cells were adsorbed (90 min at 4°C) with aliquots of dengue virus (MOI 0.1-0.3), UV-inactivated dengue virus, combinations of UV-inactivated virus and human dengue immune serum (1:1000 or 1:10000 final dilution), combinations of virus and human dengue immune serum (1:1000 or 1:10000 final dilution) or virus and normal human sera (1:1000 final dilution) (premixed 4°C for 90 minutes). Two dilutions of human dengue immune serum were used for every experiment to control for variation in dengue virus titer from experiment to experiment since sub-neutralizing concentrations of antibody are necessary for ADE infection to occur. In some experiments KU812 cells were used to monitor effective dengue infection (King *et al.,* 2000). Cells were then washed twice with RPMI culture media (KU812, U937 and primary cultured human mast cells) or Iscove's culture media (HMC-1), resuspended at a concentration of 1 x 10^6 cells/ml in the appropriate medium and incubated at 37°C, 5%CO_2. CBMC were cultured in RPMI 1640 (Life Technologies), supplemented with 20% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin (Life Technologies), 20 ng/ml SCF (Peprotech) and 100 μg/ml soya bean trypsin inhibitor (STI), (Sigma).

Adenovirus and RSV infection

Human KU812, HMC-1, and U937 were washed once and resuspended at 1 x 10^6 cells/ml in the appropriate media supplemented as described above. Cells were adsorbed (3 hr at 37°C, 5% CO2) with aliquots of adenovirus (MOI 1), or UV-inactivated adenovirus, or RSV (MOI 1) in the absence or presence of human RSV-positive serum (1:1000 and 1:10,000 dilutions). Human lung epithelial A549 cell monolayers were used to monitor infection by adenovirus by immunoprecipitation. Cells were washed twice with the appropriate media supplemented as described above and incubated at 37°C, 5%CO_2. An aliquot was removed to assess infection by immunoprecipitation of radiolabeled viral proteins.

Radiolabeling of KU812, HMC-1 and U937 cells with [^{35}S] methionine-[^{35}S] cysteine.

At 24 hours post-infection, culture supernatants were removed and cells were resuspended in [^{35}S] methionine-[^{35}S] cysteine (NEN) in MCFM (Gibco, BRL, Burlington, ON) for 3-4 hours, with 12-14 hours chase at 37°C, 5%CO_2. Culture supernatants were harvested and immunoprecipitated with dengue virus immune sera as described (King *et al.*,2000) or adenovirus rabbit immune sera and fixed *Staphylococcus aureus* as previously described (He *et al.*, 1995).

Chemokine and cytokine analysis

RANTES, MIP-1α, MIP-1β, IL-8, and ENA-78 were examined in 72 hour post-infection supernatants harvested from primary cultured human mast cells, KU812, HMC-1 and U937 cells inoculated with dengue virus (MOI 0.1-0.3) alone, UV-inactivated dengue virus,

combinations of UV-inactivated virus and human dengue immune serum (1:1000 or 1:10000 final dilution), combinations of virus and human dengue immune serum (1:1000 or 1:10000 final dilution) or virus and normal human sera (1:1000 final dilution). Activation with 25 ng/ml PMA and 5 x 10^{-7}M A23187 was used as a positive control and media alone as a negative control. RANTES and IL-8 were analyzed via an "in house" enzyme-linked immunosorbent assay (ELISA). ELISA involved capture antibodies (RANTES P-230-E, Endogen, Wuburn, MA; IL-8 MAB 208, R&D Systems, Minneapolis, MN). Nonspecific binding was blocked using 1% BSA in PBS (for IL-8), or 1% BSA in Na_2HPO_4 (pH 8.3-8.5) (for RANTES) for 1 hour at 37°C. Matched biotinylated chemokine detection antibodies were (RANTES BAF 278; IL-8 BAF 208; R & D Systems). A commercial ELISA amplification system (Life Technologies) was used for detection. The sensitivities of both the RANTES and IL-8 assays were 7.8 pg/ml. MIP-1α and MIP-1β in culture supernatants was analyzed using commercial ELISA kits (Endogen).

Short-term mediator release and β-hexosaminidase assay

CBMC cells (5 x 10^4/ml) were incubated for 30 min at 37°C in the presence or absence 50 µl of dengue virus containing media alone, combinations of UV-inactivated virus and human dengue immune serum (1:1000 or 1:10000 final dilution), combinations of virus and human dengue immune serum (1:1000 or 1:10000 final dilution) or combinations of media and human dengue immune serum (1:1000 or 1:10000 final dilution). Activation with 5 x 10^{-7}M A23187 was used as a positive control and media alone as a negative control. The reaction was stopped with the addition of 450µl of cold HEPES-Tyrode's buffer. Cells were

centrifuged at 300 *g* for 10 min at 4°C. After collection of supernatant, the pellets were resuspended in the 500µl of the buffer and disrupted by sonication. The modified HEPES-Tyrode''sbuffer was prepared as follows (in mM): Na, 137; glucose, 5.6;KCl, 2.7; NaH_2PO_4, 0.5; $CaCl_2$, 1; HEPES, 10; plus 0.1% BSA, pH 7.3.

ß-Hexosaminidase assay was carried out using a previouslyreported method (Schwartz *et al.*, 1979). Briefly, 50 µL of supernatant and pellet samples, in duplicate, were incubated with 50 µLof 1 mM *p*-nitrophenyl-*N*-acetyl-ß-D-glucosaminide (Sigma)dissolved in 0.1 M citrate buffer, pH 4.5 in a 96-well microtiter plate at 37°C for 1 h. The reaction was stopped with 200 µL/well of 0.1 M carbonate buffer, pH 10.5. The plate was read at 405 nm in an ELISA reader. The net percent of ß-hexosaminidase release was calculated as follows: ((ß-hexosaminidase in supernatant)/(ß-hexosaminidase in supernatant + ß-hexosaminidase in pellet)) x 100.

Inflammatory cytokine stimulation of mast cells

KU812 cells (1 x 10^6 cells/ml) were treated for 24 hrs with various concentrations of recombinant human IL-6 (R&D Systems) or IL-1β (R&D Systems) in RPMI 1640 (Life Technologies), supplemented with 10% FCS, 10 mM HEPES, 100 U/ml penicillin, and 100 µg/ml streptomycin (Life Technologies). Cell free supernatants were harvested and analyzed for RANTES concentration by ELISA.

Fluorescence Microscopy

KU812, HMC-1 and U937 cells inoculated with dengue virus (MOI 0.1-0.3) or dengue virus/antiserum combinations were harvested 24 hours post-infection. Cells were fixed with 4% paraformaldehyde, washed, resuspended in 10% DMSO in PBS and frozen at -80°C. Following permeabilization with 0.1% saponin for 1 hour at room temperature, samples were washed and resuspended in 1%BSA/ 0.2% sodium azide solution. Mouse anti-dengue monoclonal 1B7 (Henchal *et al.*, 1985) or isotype matched mouse IgG2a antibody (negative control) were employed as primary antibodies and incubated on ice for 1 hour. Subsequently, samples were washed and incubated with Texas Red labeled anti-mouse IgG antibody (Molecular Probes, Eugene, Oregon) for 1 hour on ice. Cytospins were made of each sample and viewed via fluorescence microscopy to assess number of infected cells. A total of 1000 cells were counted under bright field with the number of positive red fluorescent cells (Texas-Red) recorded to give the percentage of cells infected with dengue virus.

Statistical Analysis

Statistical significance of changes in RANTES production by KU812 cells was assessed using a parametric approach. Repeated measures ANOVA was performed followed by examination of specific groups using the Bonferroni multiple comparison test. Statistical significance of RANTES, MIP-1α, and MIP-1β production by HMC-1 and U937 cells, as well as KU812 MIP-1α and MIP-1β, in response to dengue and adenovirus infection were assessed using a nonparametric approach. CBMC RANTES production in response to dengue virus was assessed in the same manner. These data were initially analyzed using Friedman's test for all data obtained, followed by examination of specific groups using

Wilcoxon signed ranks test.

3.4 Results

Antibody-enhanced infection of dengue virus in mast cells and monocytes

The human mast cell lines KU812 and HMC-1 and the monocytic U937 cell line were mock inoculated or inoculated with dengue virus in the presence or absence of human dengue immune serum (1:1000 and 1:10000 final dilution). Immunoprecipitation analysis (Fig 1A) demonstrated that U937 cells as well as the mast cell/basophil line KU812 and the mast cell line HMC-1 were permissive to dengue virus infection in the presence of human dengue immune sera. The degree of infection observed was less in HMC-1 cells as compared to either KU812 and U937 cells. KU812, HMC-1 and U937 cells inoculated with dengue virus in the presence of normal human serum (NHS) (1:1000 dilution), UV-inactivated dengue alone or in the presence of human dengue immune serum at either the 1:1000 or 1:10000 dilution showed no evidence of infection (data not shown).

Relative permissiveness of mast cells versus monocytes infected with dengue in the presence of antibody

In order to assess the proportion of mast cells/basophils or monocytes infected, fluorescence microscopy was employed. KU812, HMC-1 and U937 cells inoculated with dengue virus/NHS, dengue virus/human dengue immune serum combinations were harvested 24 hours post-infection and examined following staining with an antibody specific for the envelope protein of the virus (Fig 1B-E). All three cell lines showed no virus positive cells when inoculated with dengue virus in the presence of normal human serum. The percentages of KU812, HMC-1 and U937 dengue positive cells in three experiments is shown in Table

I. A range of between 3.10% and 14.19%, and 1.2% and 3.0% of the population of KU812 and HMC-1 cells, respectively, were infected with dengue virus while the proportion of infected U937 cells ranged from 3.18% and 6.38%. IgG2a isotype control antibody stained slides were less than 0.2 percent positive.

Chemokine production by antibody-enhanced dengue virus infected cells

To analyze chemokine production in response to antibody-enhanced dengue virus infection of KU812, HMC-1 and U937 cells 72 hour cell free supernatants were harvested from cultures and screened by ELISA. RANTES production was 260 fold greater in supernatants obtained from dengue infected KU812 cultures as compared to cells incubated with medium alone. KU812 cells treated with UV-inactivated dengue, or dengue in the presence of normal human serum (Fig 2A) did not demonstrate enhanced RANTES production. MIP-1α and MIP-1β production were also significantly enhanced in antibody-enhanced dengue infected KU812 cells (Fig 2 B & C). In contrast, neither IL-8 nor ENA-78 were elevated (data not shown). Analysis of HMC-1 chemokine production indicated that HMC-1 cells produce significantly increased amounts of RANTES (Fig 2D) and MIP-1α (Fig 2E) but not MIP-1β (Fig 2F) in response to antibody-enhanced dengue virus infection. However, U937 cells failed to produce RANTES (Fig 2G), MIP-1α (Fig 2H) or MIP-1β (Fig 2I) in response to any of the conditions employed.

Time course of chemokine production by antibody-enhanced dengue virus infected KU812 cells

KU812 cells were inoculated with dengue virus/normal human serum, dengue virus/human dengue immune serum combinations. Time course analyses of antibody-enhanced dengue virus infected KU812 cells were carried out in 4, 24, 48 and 72 hour cell free supernatants harvested from KU812 cultures. RANTES, MIP-1α, MIP-1β and IL-8 levels were assessed by ELISA. The majority of RANTES production occurred by 24 hours post-infection, levels persisted in supernatants for up to 72 hours (Fig 3A). A continuous increase in MIP-1α (Fig 3 B) and MIP-1β (Fig 3C) production by dengue virus infected cells was noted from 4 to 72 hours post-infection with the highest levels observed by 72 hours. Examination of IL-8 production revealed no modulation by any of the infection conditions at any time point (Fig 3D).

Concurrent infectious virion and RANTES production by antibody-enhanced dengue virus infected KU812 cells.

A more detailed time course examining the RANTES response in KU812 cells was undertaken to determine the temporal relationship between dengue virus/antibody treatment , RANTES production and infectious virion production as assessed by $TCID_{50}$ (King *et al.*, 2000). KU812 cells were inoculated with dengue virus/NHS (1:1000 final dilution), dengue virus/ human dengue immune serum combinations (1:1000 and 1:10000 final dilutions) or dengue alone. RANTES and infectious virion production was assessed at 4, 8, 16, 24, 48, and 72 hours post-infection. Analysis indicated that there was a large increase in RANTES production by antibody-enhanced dengue virus infected KU812 cells between 8 and 16 hours post-infection (Fig 4) with coincident virion production. RANTES levels continued to

increase up to 72 hours post-infection while robust infectious virion production persisted until 24 hours and began to decline by 48 hours post-infection.. The amount of pre-formed RANTES associated with unactivated KU812 cells was < 0.0026 fg/cell, n=2.

Cord blood derived human mast cell induction of chemokines by dengue virus

Under conditions which were effective in inducing dengue virus infection in KU812 cells, all five of the CBMC preparations examined, demonstrated increased RANTES production (Fig 5A) at one or both of the dengue virus/human dengue immune sera combinations. Statistical analysis demonstrated a significant increase in RANTES production (10-70 fold) when compared to treatment with UV-inactivated dengue virus at both concentrations of specific antibody, $p<0.05$. In addition, all four CBMC preparations examined had increased levels (1.5 -3.5 fold) of MIP-1β when treated with dengue virus in the presence of dengue specific antibody (Fig 5B). This increased chemokine production was restricted to only those conditions where live dengue virus/human dengue immune sera combinations were employed. UV inactivated virus alone (Fig 5A and B) or in combination with human dengue immune sera at both 1:1000 and 1:10000 final dilutions (Table II), as well as dengue virus/NHS conditions failed to modulate either mediator in CBMC.

Cross-linking of surface Fc receptors is not responsible for the chemokine response

To further investigate the mechanism of the mast cell response to dengue virus, specifically the importance of Fc receptor cross-linking, KU812 cells were inoculated with UV-inactivated dengue virus or, UV-inactivated virus/human dengue immune serum

combinations (1:1000 and 1:10000 final dilutions). These conditions would be expected to produce Fc receptor cross-linking but not active infection. As further controls dengue virus/NHS (1:1000 final dilution) or dengue virus/human dengue immune serum combinations (1:1000 or 1:10000 final dilution) were employed. The RANTES response was examined at 72 hour post-infection. Low levels of RANTES (Table II) were produced by KU812 cells treated with virus and antibody combinations that did not yield infection. The degree of RANTES production in response to Fc receptor cross-linking was a mean of 0.62% of the response to live virus plus antibody for KU812 cells (n=3) and a mean of 4.9% for CBMC (n=3).

Dengue virus alone or in combination with human dengue specific antibody does not induce mast cell degranulation

β-hexosaminidase release by CBMC incubated with live or inactivated virus in the presence or absence of human dengue specific antibody or media containing antibody alone was assessed to determine if surface cross-linking of Fc receptors upon binding of the virus/antibody complexes or antibody alone could activate mast cells. Data from two separate experiments indicated no significant β-hexosaminidase release in response to any of the conditions tested, UV-Den + 1:1000 Ab 8.79% ± 6.22%; Den + 1:1000 Ab 10.12% ± 7.16%; media + 1:1000 Ab 7.53% ± 5.32%, when compared to spontaneous levels 7.94% ± 5.62% observed. The positive control calcium ionophore gave 16.06% ± 11.36%. Data are expressed as mean ± SEM.

Inflammatory cytokines do not induce significant levels of RANTES by KU812 cells

KU812 cells were stimulated with 10, 50, 100 or 200 pg/ml rhIL-1β and 5, 10, or 20 ng/ml rhIL-6 for 24 hrs. Cell free supernatants were harvested and analyzed by ELISA for RANTES content. Data from four separate experiments indicated no significant increase in production of RANTES by KU812 cells for any of the conditions tested (rhIL-1β 10pg/ml 209.1 ± 61.4; rhIL-1β 50 pg/ml 258.7 ±69.3; rhIL-1β 100 pg/ml 366.5 ± 92.5; rhIL-1β 200 pg/ml 317.8 ± 74.2; rhIL-6 5 ng/ml 242.2 ± 62.7; rhIL-6 10 ng/ml 200.6 ± 82.6; rhIL-6 20 ng/ml 250.9 ± 97.1) as compared to media alone (204.2 ±60.7). PMA and A23187 were used as a positive control and gave significant levels RANTES (526.5 ± 71.9, $p<0.01$). Data are expressed as mean ± SEM.

Dengue virus, but not two unrelated viruses, activate the chemokine response in mast cell/basophils

To assess the specificity of the chemokine mast cell/basophil response to dengue virus infection two unrelated viruses were employed, adenovirus type 37 and respiratory syncytial virus (RSV). To assess mast cell permissiveness to adenovirus, the KU812 and HMC-1 cell lines, the monocytic U937 cell line and A549 human lung epithelial cells were mock inoculated with UV-inactivated adenovirus or with adenovirus alone. Supernatants were harvested and immunoprecipitated. KU812, HMC-1 and U937 cells were permissive to adenovirus infection (Fig. 6) with A549 cells as a positive control. 72 hour post-infection supernatants were analyzed for chemokines. Neither mast cells/basophils or U937 cells demonstrated enhanced production of RANTES, MIP-1α or MIP-1β in response to

adenovirus infection (Table III). Furthermore, similar experiments carried out using RSV, both in the presence and absence of RSV immune serum, indicated KU812 cells were not permissive to RSV infection and treatment did not result in RANTES production at 24, 48 or 72 hours post-treatment (data not shown).

3.5 Figures

Figure 1. Antibody-enhanced dengue virus infection of KU812, HMC-1 and U937 cells. A) Cultures of KU812, HMC-1 or U937 cells were inoculated with UV-inactivated dengue virus (MOI 0.1-0.3), or combinations of virus with normal human serum (final dilution 1:1000), or with human dengue immune serum (final dilution 1:1000 or 1:10000 to ensure sub-neutralizing concentrations) and dengue proteins immunoprecipitated. Exposure time for the KU812 and U937 cells was 2 days, HMC-1 was eight days. Data are representative of 4 separate experiments. B-E) Immunofluorescence of dengue virus inoculated KU812 and U937 cells. Dengue virus infected cells were visualized by fluorescent microscopy using Texas red labeled secondary antibody. KU812 cells inoculated with dengue virus and normal human serum (1:000 final dilution) B, antibody-enhanced dengue infected KU812 cells with 1:1000 final dilution of human dengue immune serum, C; U937 cells inoculated with dengue virus and normal human serum (1:000 final dilution), D; and antibody-enhanced dengue infected U937 cells with 1:1000 final dilution of human dengue immune serum, E. Data are representative of 3 separate experiments.

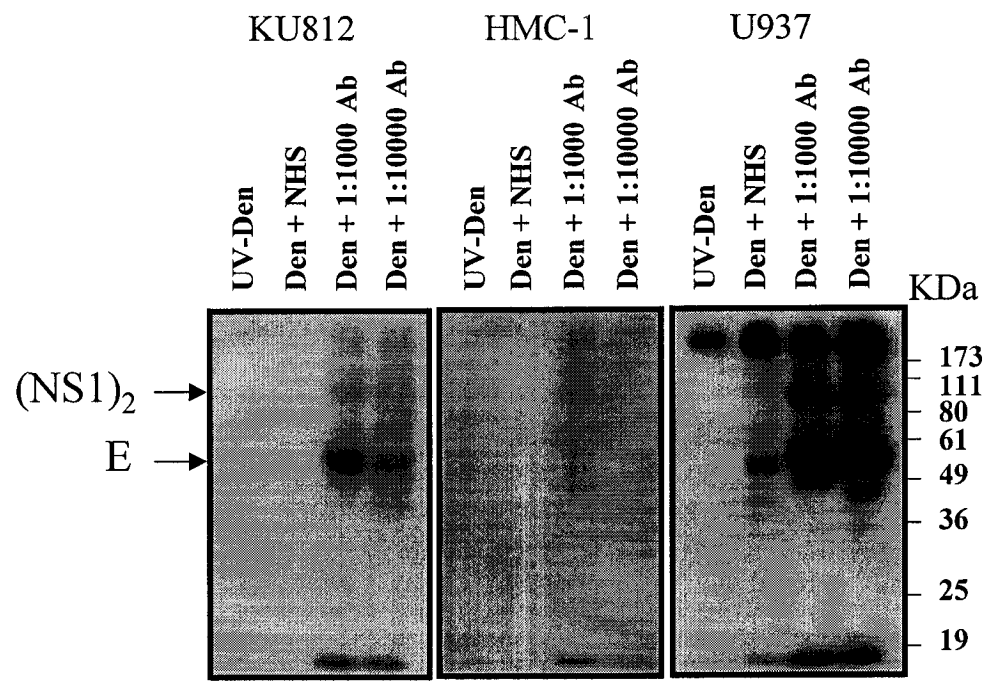

KU812
HMC-1
U937
UV-Den
Den + NHS
Den + 1:1000 Ab
Den + 1:10000 Ab
KDa
173
111
80
61
49
36
25
19
(NS1)2
E

Figure 1A

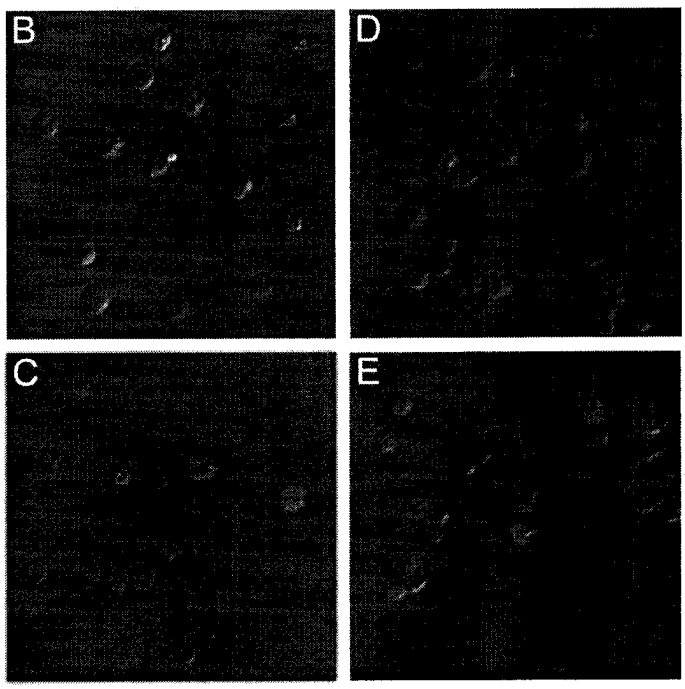


Figure 1 B-E

Table I. Percentage of dengue virus infected KU812, HMC-1 and U937 cells.

		Den + NHS		Den + 1:1000 Ab		Den + 1:10000 Ab	
		IgG2a[A]	1B7[B]	IgG2a	1B7	IgG2a	1B7
KU812	Expt 1	0	0	0.1	9.88	0.1	5.15
	Expt 2	0	0.1	0	8.35	0	3.10
	Expt 3	0	0	0	14.19	0	11.49
HMC-1	Expt 1	0	0	0	2.1	0	2.9
	Expt 2	0.2	0	0	3.0	0	1.2
U937	Expt 1	0	0	0	3.18	0	3.58
	Expt 2	0	0	0	6.66	0	6.38
	Expt 3	0	0	nd[C]	nd	0	5.89

[A] Isotype control
[B] Anti-dengue E protein antibody
[C] Not done

Figure 2. Chemokine production by KU812, HMC-1 and U937 cells following inoculation with dengue virus. KU812 chemokine production at 72 hours post-infection RANTES data (A), are from 9 separate experiments with duplicate samples, MIP-1α (B), and MIP-1β (C), data are from five separate experiments with duplicate samples; HMC-1 chemokine production at 72 hours post-infection, (D-F), the data are from five separate experiments with duplicate samples; U937 chemokine production at 72 hours post-infection, (G-I), the data are from four separate experiments with duplicate samples. Experiments used either pooled dengue convalescent patient serum or one patient serum 7873. Cells incubated with media alone indicate constitutive production, PMA and A23187 treated cells were used as positive controls. Culture supernatants were analyzed by ELISA. Significant differences from the media alone samples are indicated by *($p<0.05$), ** ($p<0.01$), *** ($p<0.001$). Please note y-axis differences. Data are represented as mean ± SEM. [a] > 5000 pg/ml, [b] > 7000 pg/ml

KU812

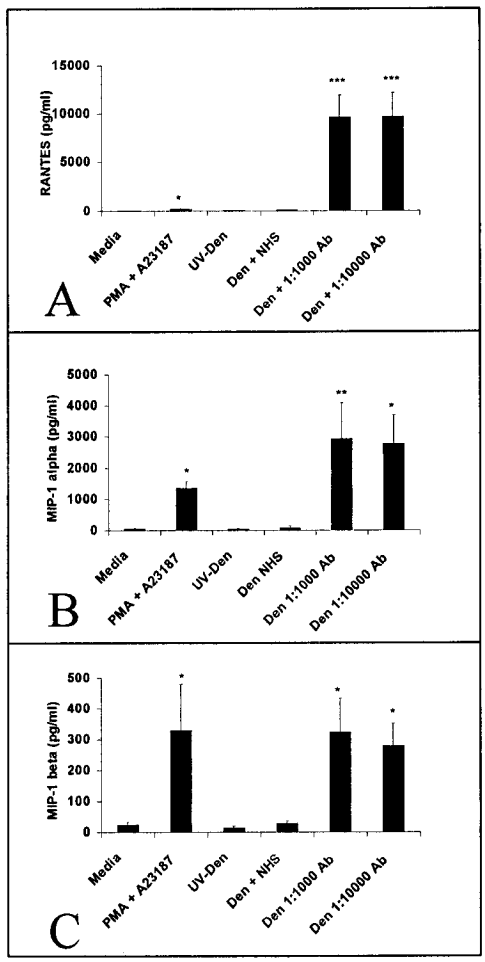


Figure 2 A-C

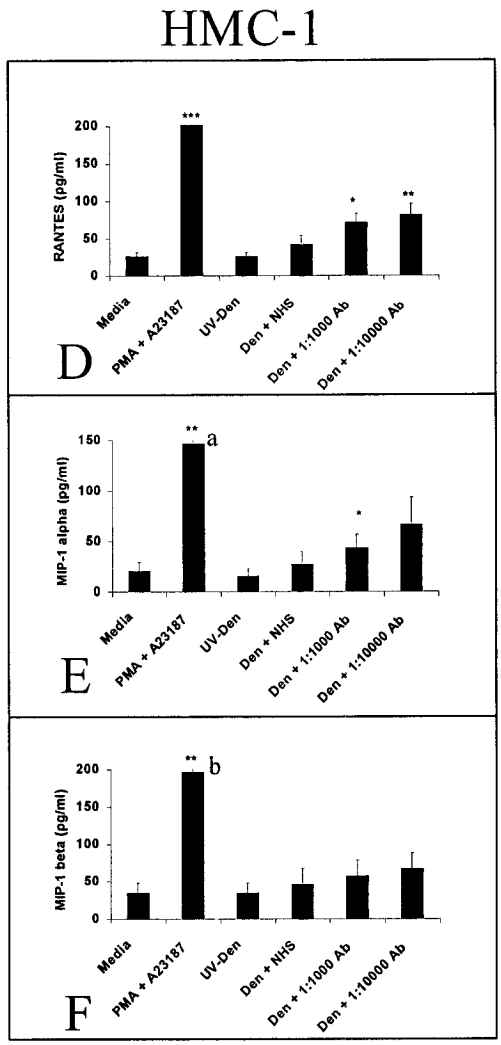

HMC-1
RANTES (pg/ml)
200
150
100
50
0
***
*
**
Media
PMA + A23187
UV-Den
Den + NHS
Den + 1:1000 Ab
Den + 1:10000 Ab
D
MIP-1 alpha (pg/ml)
150
100
50
0
** a
*
Media
PMA + A23187
UV-Den
Den + NHS
Den + 1:1000 Ab
Den + 1:10000 Ab
E
MIP-1 beta (pg/ml)
200
150
100
50
0
** b
Media
PMA + A23187
UV-Den
Den + NHS
Den + 1:1000 Ab
Den + 1:10000 Ab
F

Figure 2 D-F

U937

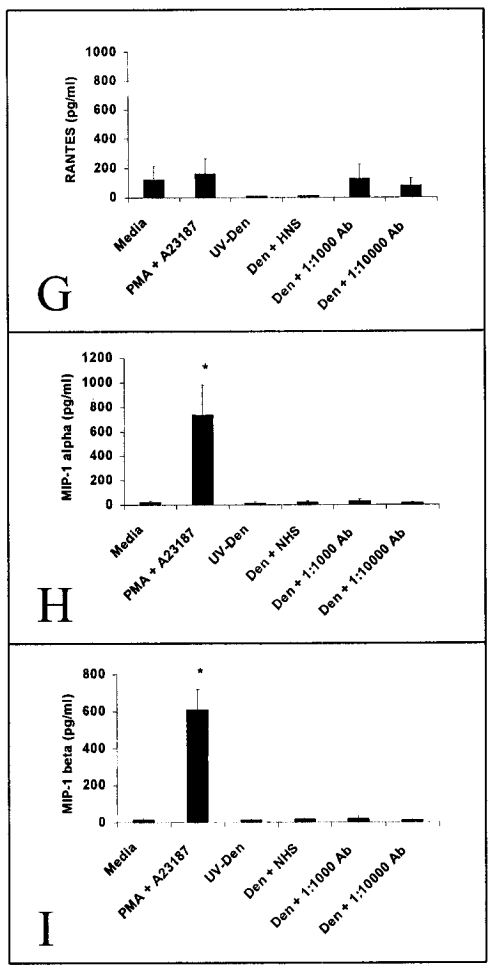


Figure 2 G-I

Table II. Fc receptor cross-linking by dengue virus/antibody complexes fails to induce appreciable levels of RANTES (pg/ml) in comparison to levels produced in response to active infection.

	Cord blood derived mast cells			KU812 cells		
Condition	**Subject 1**	**Subject 2**	**Subject 3**	**Expt 1**	**Expt 2**	**Expt 3**
Media	7.8	81.3	7.8	7.8	6.2	120.7
PMA + A23187	7.8	301.8	651	120.8	165.7	1004.5
UV-Den alone	7.8	43.1	7.8	498.6	131.1	59.4
UV-Den + 1:1000 Ab	7.8	7.8	36.4	739.6	205.1	36.1
Den + NHS	7.8	202.3	83.2	437.7	1343.9	266.8
Den + 1:1000 Ab	**546.3**	**1613.5**	**284.7**	**53242.4**	**100500.3**	**13502.6**

Figure 3. Time course of RANTES, MIP-1α, and MIP-1β production by antibody-enhanced dengue virus infected KU812 cells. RANTES, A; MIP-1α, B; MIP-1β, C; and IL-8, D; were measured in cell free supernatants at 4, 24 , 48 and 72 hours post-infection by ELISA. KU812 cells incubated with media alone indicate constitutive production. The data (mean ± SEM.) are from two separate experiments with duplicate samples and using pooled dengue convalescent patient serum.

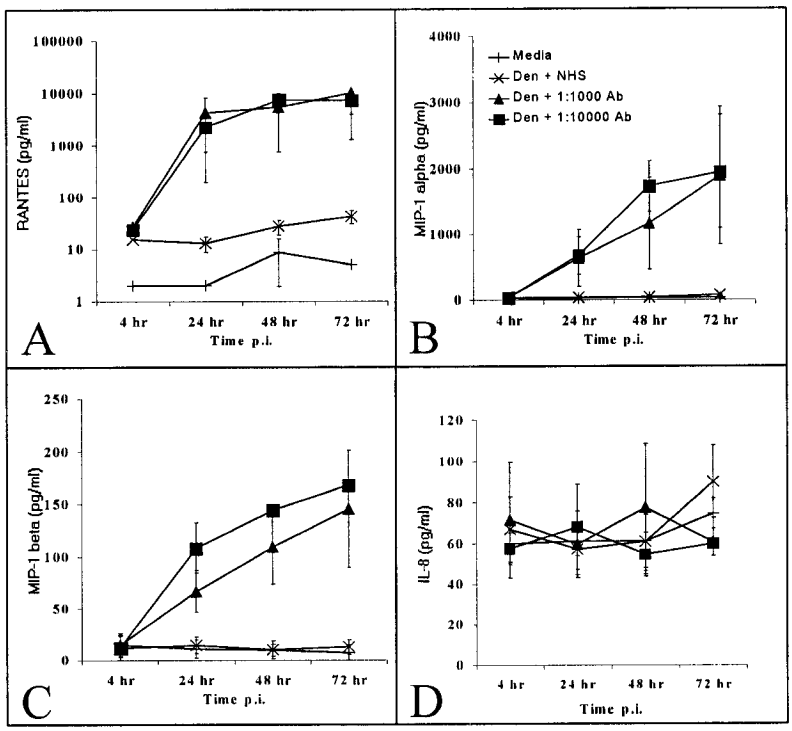

100000
10000
1000
100
10
1
RANTES (pg/ml)
4 hr
24 hr
48 hr
72 hr
Time p.i.
A
4000
3000
2000
1000
0
MIP-1 alpha (pg/ml)
Media
Den + NHS
Den + 1:1000 Ab
Den + 1:10000 Ab
4 hr
24 hr
48 hr
72 hr
Time p.i.
B
250
200
150
100
50
0
MIP-1 beta (pg/ml)
4 hr
24 hr
48 hr
72 hr
Time p.i.
C
120
100
80
60
40
20
0
IL-8 (pg/ml)
4 hr
24 hr
48 hr
72 hr
Time p.i.
D

Figure 3

Figure 4. Concurrent infectious virion and RANTES production by antibody-enhanced dengue virus infected KU812 cells. Both RANTES and virion production were assessed in cell free supernatants at 4, 8, 16, 24, 48, and 72 hours post-infection by ELISA and $TCID_{50}$, respectively. KU812 cells were inoculated with dengue alone (Den), dengue/normal human serum (NHS), or dengue/immune serum combinations (1:1000 and 1:10000 final dilutions). The data (mean ± SEM.) are from two separate experiments with duplicate samples and using pooled dengue convalescent patient serum.

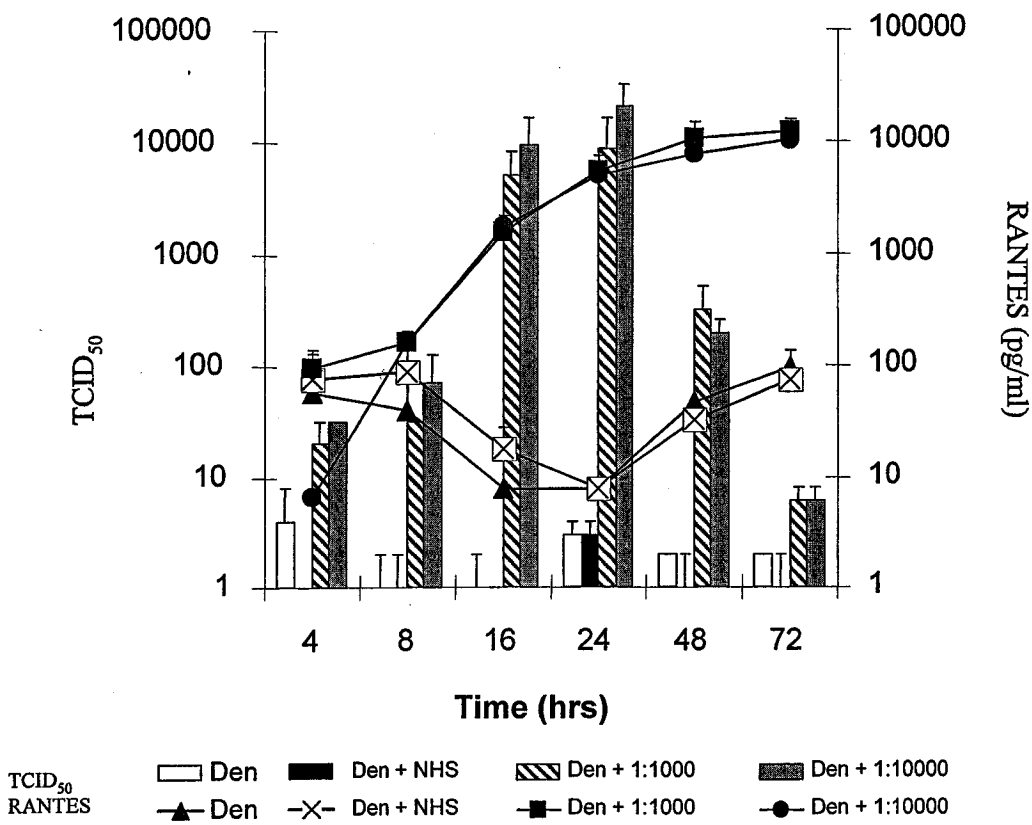

100000
10000
1000
100
10
1
TCID50
RANTES (pg/ml)
4
8
16
24
48
72
Time (hrs)
TCID50
RANTES
Den
Den + NHS
Den + 1:1000
Den + 1:10000
Den
Den + NHS
Den + 1:1000
Den + 1:10000

Figure 4

Figure 5. Cord blood derived mast cell production of RANTES and MIP-1β in response to dengue virus and antibody. RANTES, A; and MIP-1β, B; were measured in cell free supernatants at 72 hours post-infection by ELISA. Cord blood derived mast cells treated with media alone indicates constitutive production, PMA and A23187 treated cord blood derived mast cells were used as positive controls. These data are from five (RANTES) or four (MIP-1β) separate subjects with duplicate samples. Experiments used pooled dengue convalescent patient serum. [a] > 400 pg/ml. ND, not done. Statistical analysis of RANTES production indicated that dengue virus/dengue immune serum versus UV-inactivated dengue virus at both serum dilutions, induced significant increases, $p<0.05$.

A

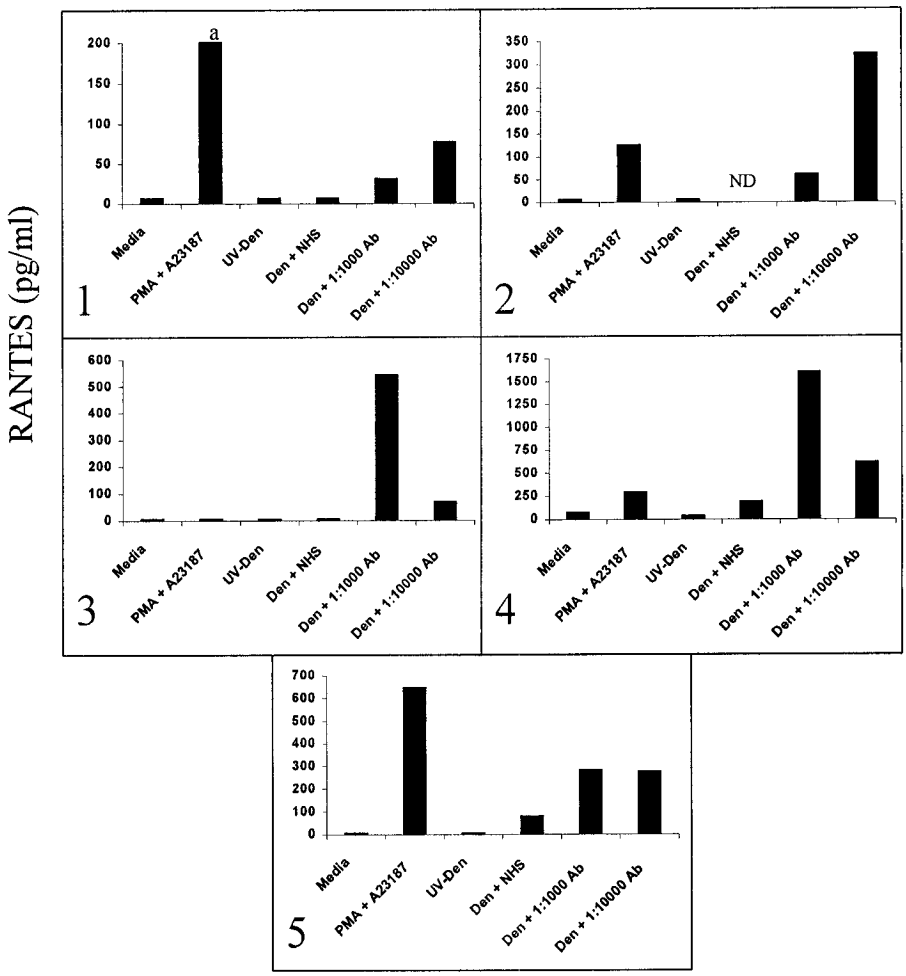

RANTES (pg/ml)
a
ND
Media
PMA + A23187
UV-Den
Den + NHS
Den + 1:1000 Ab
Den + 1:10000 Ab
1
2
3
4
5

Figure 5 A

B

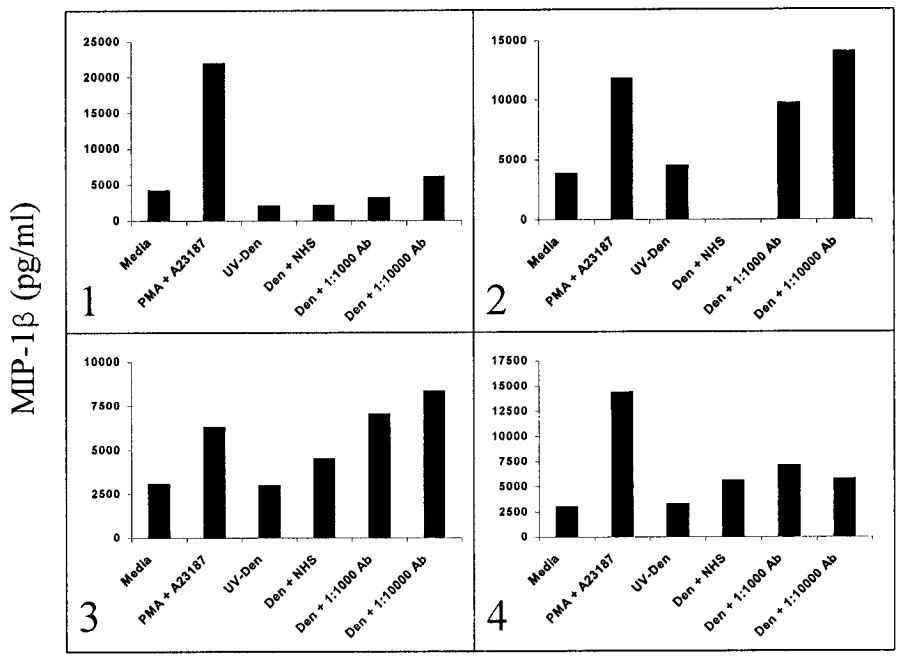

MIP-1β (pg/ml)
1
2
3
4
Media
PMA + A23187
UV-Den
Den + NHS
Den + 1:1000 Ab
Den + 1:10000 Ab

Figure 5 B

Figure 6. Adenovirus infection of A549, KU812, HMC-1 and U937 cells. Cultures of KU812, HMC-1 or U937 cells were inoculated with adenovirus or UV-inactivated adenovirus virus (MOI 1). Cultures were incubated at 37°C and radiolabeled with [^{35}S]-Methionine- [^{35}S] cysteine from 24 hours post-infection for 3-4 hours followed by 12-14 hours chase. Cell supernatants were harvested, immunoprecipitated and analyzed by SDS-PAGE fluorography. The positions of radiolabelled viral hexon, penton and fiber proteins are indicated. Exposure time for the A549 cells was 16 hours, KU812, HMC-1 and U937 cells were two days. Data are representative of 3 separate experiments.

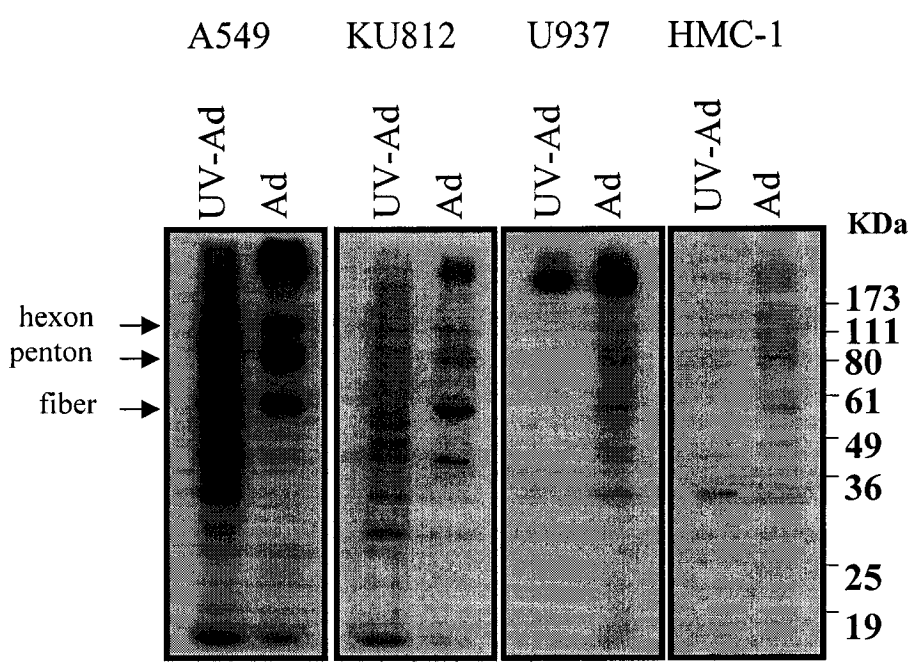

A549
KU812
U937
HMC-1
UV-Ad
Ad
KDa
hexon
penton
fiber
173
111
80
61
49
36
25
19

Figure 6

Table III. Adenovirus infection does not modulate chemokine production in KU812, HMC-1 and U937 cells.

	Condition	RANTES	MIP-1α	MIP-1β
		pg/ml	pg/ml	pg/ml
KU812	Media	50.1[A] ± 8.7[B]	27.0 ± 5.2	160.00 ± 59.3
	PMA + A23187[C]	212.2 ± 50.2	836.7 ± 285.0	> 600
	UV-Ad[D]	77.7 ± 18.8	130.8 ± 102.7	173.7 ± 49.3
	Ad[E]	66.7 ± 11.9	48.6 ± 31.1	105.6 ± 36.8
HMC-1	Media	10.1 ±4.2	15.4 ±8.0	40.0 ±22.4
	PMA + A23187	337.4 ±294.2	>2000	> 600
	UV-Ad	17.6 ±1.6	23.4 ±5.1	36.8 ±20.5
	Ad	12.7 ±2.4	21.7 ±5.5	32.6 ±17.1
U937	Media	8.7 ±1.7	5.2 ±2.0	25.5 ±16.2
	PMA + A23187	105.3 ±49.8	754.6 ±117.2	> 600
	UV-Ad	11.6 ±4.6	4.9 ±0.9	15.7 ±8.3
	Ad	10.8 ±3.8	4.0 ±0	11.6 ±5.2

[A] mean, n=3
[B] SEM
[C] positive control
[D] UV-inactivated adenovirus
[E] adenovirus

3.6 Discussion

These findings demonstrate that dengue virus plus dengue-specific antibody treatment results in selective production of the T cell chemoattractants RANTES, MIP-1α and MIP-1β by human mast cells. To our knowledge, this is the first report of a mast cell chemokine response to a viral pathogen.

Dengue virus infection of peripheral blood monocytes is increased dramatically in the presence of subneutralizing concentrations of virus-specific antibody (Brandt *et al.,* 1979; Brandt *et al.,* 1982; Daughaday *et al.,* 1981; Halstead & O'Rourke, 1977; Morens & Halstead, 1990), due to enhanced virus-cell attachment via surface Fcγ receptors (Peiris *et al.,* 1981). The current study investigates the mast cell production of essential chemokines, involved in T cell mobilization, in response to treatment with dengue virus and specific antibody. A number of complementary human mast cell/basophil models were employed in this study. The KU812 cell line has been documented to possess properties of both basophils (Kishi, 1985) and mast cells (Blom *et al.,* 1992). KU812 cells have been demonstrated to express mRNA for mast cell tryptase, mast cell carboxypeptidase with low level expression of major basic protein (Blom *et al.,* 1992). KU812 cells express FcεR1, the high affinity IgE receptor, (Almlof*et al.,* 1988), and FcγRII, (Kawata *et al.,* 1996). The HMC-1 mast cell line (Butterfield *et al.,* 1988) has been found to express chymase mRNA and higher levels of tryptase mRNA than KU812 cells (Xia *et al.,* 1995). In contrast to KU812 cells, HMC-1 cells have FcγRII (Wedi *et al.,* 1996) but do not consistently express FcεRI (Xia *et al.,* 1995).

We have previously demonstrated antibody-enhanced dengue virus infection of the mast cell/basophil KU812 cell line (King *et al.,* 2000). This infection resulted in the selective production of vasoactive cytokines IL-1β and IL-6. In the current study, antibody-enhanced dengue virus infection of a further mast cell line, HMC-1, was demonstrated. The level of infection was lower in HMC-1 cells than either KU812 or U937 cells (Fig 1A; Table I). Assessment of the number of dengue virus infected cells by immunofluorescence to detect the dengue envelope protein indicated that a greater proportion of KU812 cells were infected as compared to monocytic U937 cells, under the same infection conditions. However, immunoprecipitation indicated greater amounts of viral protein by 24 hours post-infection in U937 cells (Fig 1A) as compared to either KU812 cells or HMC-1 cells.

Antibody-enhanced dengue virus infection of KU812 and HMC-1 cells resulted in the induction of significant levels of RANTES , MIP-1α , and MIP-1β in both KU812 cells and HMC-1 cells. The levels of RANTES production observed from dengue virus infected KU812 cells were very substantial with a 260 fold increase over controls. In some cases, levels of RANTES detected exceeded 50 fg/cell and could not be accounted for by preformed RANTES pools of <0.003 fg/cell. Interestingly, lymphocytes stimulated with PMA or monocytes stimulated with LPS have been reported to produce only 1 to 5 fg/cell RANTES (Hariharan *et al.,* 2000). In contrast, production of the neutrophil chemoattractants, IL-8 (Fig. 3D) and ENA-78 (data not shown), were not enhanced in antibody-enhanced dengue infected KU812 cells as compared to controls. KU812 cells stimulated with vasoactive cytokines IL-6 and IL-1β, previously shown to be produced by dengue virus infected KU812

cells (King *et al.,* 2000), at concentrations observed by 72 hrs post-infection or greater did not induce the production of significant levels of RANTES suggesting the RANTES observed in our system is not a result of feedback from cytokines already present in our system. This suggests a highly selective mast cell response to infection. Both KU812 cells and HMC-1 cells have been demonstrated to produce IL-8 in response to treatment with stromal cell derived factor 1 α (SDF-1α) (Lin *et al.,* 2000) or PMA and ionophore (Moller *et al.,* 1993). Antibody-independent dengue virus infection of the human umbilical cord vein endothelial cell line (ECV304) has been shown to result in significant IL-8 production (10fg/cell) by 72 hours p.i. (Avirutnan *et al.,* 1998). Furthermore, influenza A and Sendai virus infection of human macrophages result in significant IL-8 production indicating that IL-8 can be induced by other viral infections (Matikainen *et al.,* 2000). While U937 cells are fully permissive to antibody-enhanced dengue virus infection they do not produce any of the chemokines examined suggesting differential responses to dengue virus infection in mast cells as compared to monocytes.

Significantly increased RANTES production was not detected following UV-inactivated virus/human dengue immune serum treatment of either KU812 or CBMC (Table II) derived from three separate individuals. These data suggest that Fc receptor cross-linking is not responsible for the majority of the observed increase in chemokine production. In addition, CBMC fail to degranulate in response to virus/antibody complexes or antibody alone, as measured by percent β-hexosaminidase, further supporting the concept that a virus specific rather than degranulation related signal is responsible for inducing chemokine production.

We have not directly addressed the role of IgE which may be involved in our system, however heat inactivation (56°C, 30 min) of serum does not influence infection (personal communication, R. Anderson) while it is known to prevent human IgE binding to FcεRI (Rousseaux-Prevost *et al.,* 1983) suggesting the role of IgE in dengue virus infection is minimal. Furthermore, no increase in chemokine production was observed when mast cells (KU812, HMC-1, CBMC) were treated with dengue virus/normal human serum combinations further demonstrating the critical importance of specific antibodies in enhancing dengue virus infection in mast cells.

Confirmation of the chemokine response observed with the cell lines was demonstrated using human CBMC (Saito, *et al.,* 1996). Exposure of CBMC to dengue virus in the presence of dengue specific antibodies resulted in a significant RANTES response by mast cells from five separate subjects (Fig.54A) and an increased MIP-1β (Fig. 5B) response by mast cells from four subjects examined. These data support the hypothesis that mast cells may contribute to the initiation of chemokine dependent host responses during dengue virus infections. The amount of RANTES produced by CBMC was significantly less that observed using KU812 cells and similar to data obtained using the HMC-1 cell line. Low levels of dengue virus infection may relate to the lower levels of RANTES produced. Perhaps due to the high levels of protease activity in CBMC we have been unable to demonstrate antibody-enhanced infection of these cells by immunoprecipitation.

To determine whether the observed mast cell chemokine response occurred in response to

infection with an alternate virus, adenovirus a non-enveloped DNA virus that does not undergo antibody-dependent enhancement, was employed. KU812 and HMC-1 mast cells were permissive to adenovirus infection. However, analysis of the mast cell chemokine response to infection did not demonstrate enhanced RANTES, MIP-1α, and MIP-1β production. *In vitro* adenovirus 21 infection of Hep-2 human respiratory epithelial cells, and WI-38, human embryonic lung fibroblasts, has been shown to result in significant (2-4 fg/cell) RANTES production (Bonville *et al.,* 1999). These observations provide further evidence for a selective response of mast cells to dengue virus though do not rule out the possibility of other virus activators of RANTES in these cells. Furthermore, RSV treatment of KU812 mast cells in the presence or absence of sub-neutralizing concentrations of human RSV immune serum also failed to induce a significant RANTES response (data not shown). It would be interesting to investigate mast cell response to another flavivirus to determine if the observed chemokine response occurs in other models of flavivirus infection.

Mast cells have been implicated in several viral diseases including dengue virus infection. Dengue virus infected patients exhibit increased levels of urinary histamine, a major granule product of mast cells. Increased histamine was also found to correlate with disease severity (Tuchinda *et al.,* 1977). A large study from Thailand that examined 100 dengue infected patients demonstrated that mast cells in the connective tissue around the thymus showed swelling, vacuolation of the cytoplasm as well as loss of granule integrity (Bhamarapravati *et al.,* 1967) suggestive of mast cell activation. However, if selective chemokine production by mast cells occurred *in vivo* in the absence of mast cell degranulation, as observed in our

in vitro studies, mast cells could be an important source of such mediators in the absence of classical degranulation.

Information regarding the mechanism of the RANTES response in antibody-enhanced dengue virus infected KU812 cells was obtained from examination of the early time course. Levels of RANTES production were low at 4 hour post-infection and rose rapidly between 8 and 16 hours post-infection (Fig 4). Concurrent infectious virion production, as assessed by $TCID_{50}$, suggests that the RANTES response is dependent on viral replication and is perhaps the result of a viral gene product. Dengue virus RNA replication in vertebrate cells has been demonstrated by 6 hours post-infection followed by increasing viral protein synthesis (Brinton, 1986). Taken together, this is consistent with expression of viral proteins in infected mast cells contributing to the observed RANTES response. β-chemokine induction, as a result of replicating virus, has been demonstrated in other viral systems, specifically epithelial cells infected with RSV (Harrison *et al.,* 1999). The lack of a very early RANTES response following viral treatment suggests that the RANTES response in mast cells was not the result of preformed RANTES release as a result of dengue virus/antibody complexes, but rather *de novo* synthesis. The finding that KU812 cells contain little preformed RANTES, <0.0026 fg/cell, supports this concept. The lack of a mast cell RANTES, MIP-1α or MIP-1β response to UV-inactivated virus/human dengue immune serum combinations suggests that viral attachment and entry were not sufficient signals for the mast cell RANTES production. Elucidation of the RANTES mechanism is currently underway in our laboratory.

While this is the first report of chemokine production in response to viral infection in mast cells, enhanced β-chemokine production following virus infection has been demonstrated in other cells undergoing viral infection including dengue virus. Antibody-independent dengue virus infection of ECV304 cells has been reported to result in significant RANTES elevation (Avirutnan *et al.,* 1998). In addition, *in vitro* infection of cord blood mononuclear cells by a clinical isolate of dengue 3 resulted in higher levels of MIP-1α (Murgue *et al.,* 1998). Furthermore, MIP-1α and MIP-1β were recently found to be induced by dengue 2 infection in the human K562 myelomonocytic cell line and PBMCs (Spain-Santana *et al.,* 2001). HIV infection of both activated and naive Th1 cells results in significant levels of RANTES, MIP-1α and MIP-1β by 4 to 6 days postinfection (Annunziato *et al.,* 2000). In addition, PBMCs obtained from individuals infected with human T lymphotropic virus Type II (HTLV-II) spontaneously secreted significant levels of MIP-1α and MIP-1β, and to a lesser extent RANTES as compared to uninfected controls (Lewis *et al.,* 2000). Both influenza A and Sendai virus infection of macrophages results in upregulation of, among others, RANTES, MIP-1α, and MIP-1β (Matikainen *et al.,* 2000). RSV infection of Hep-2, MRC-5 and WI-38 cells results in significant levels of MIP-1α and RANTES production (Bonville *et al.,* 1999). However, β-chemokine production is not enhanced in all models of viral infection as cytomegalovirus (CMV) and herpes simplex 1 (HSV-1) infection in those cell lines failed to modulate MIP-1α or RANTES (Bonville *et al.,* 1999).

Selective production of RANTES, MIP-1α, and MIP-1β by antibody-enhanced dengue virus

infection of mast cells, may provide additional insights into the pathogenic mechanisms of severe dengue disease. Our finding of increased RANTES, MIP-1α, and MIP-1β production in antibody-enhanced dengue virus infected mast cells raises the possibility that mast cells/basophils may represent either a systemic or local source of chemokines involved in mobilization of immune effector cells, including T lymphocytes, in dengue infection. Extravasation of T cells through the endothelium to the site of inflammation is dependent on both adhesion molecule expression and β-chemokine-receptor interaction on recruited cells (Butcher & Picker, 1996). Dengue virus-specific CD4+ and CD8+ T lymphocytes have been shown to be generated following primary dengue virus infection (Kurane *et al.,* 1991; Kurane, *et al.,* 1989; Kurane *et al.,* 1989). Dengue virus-specific memory CD4+ T cell have been detected in the PBMC isolated from patients following primary exposure. Furthermore, these memory T cells are serotype cross reactive (Kurane *et al.,* 1989). Cross-reactive CD8+ dengue specific T cells have also been isolated from PBMC obtained from a dengue infected individual (Bukowski *et al.,* 1989). The β-chemokine RANTES has been demonstrated to attract CD4+ memory T cells (Schall *et al.,* 1990) while MIP-1β has been shown to preferentially attract naive CD4+ T cells. MIP-1α is a more potent lymphocyte chemoattractant mainly attracting B cells and CD8+ T cells (Schall *et al.,* 1993). Interestingly, mast cells have been implicated in regulating T lymphocyte traffic into peripheral lymph nodes during development of an immune response. Mast cells accumulated in the lymph nodes of contact sensitized mice were found to be the predominant source of MIP-1β (Tedla *et al.,* 1998; Wang *et al.,* 1998). The finding that dengue virus induced significant RANTES, MIP-1α, and MIP-1β production by human mast cells suggests a

potential role for mast cells in both recruiting and activating T lymphocytes at the site of infection during secondary dengue virus infection.

The observation that dengue virus-antibody complexes are much more potent than dengue virus alone in inducing mast cells to produce these chemokines shows an interesting parallel with the known epidemiological evidence that pre-existing immunity is a risk factor for DHF/DSS in human infections (Halstead, 1988). The underlying mechanisms of hemorrhagic disease induced by a number of viruses are poorly understood, including the more severe forms of dengue virus infection, DHF and DSS. DHF and DSS patients are characterized by increased capillary permeability and abnormal homeostasis (Halstead, 1988). Our results strongly indicate that antibody-enhanced dengue virus infection of mast cells and basophils *in vitro* leads to the production of mediators which may contribute to cell recruitment during severe dengue virus infection *in vivo.* The location of mast cells in skin and in close association with blood vessels in most tissues (Selye, 1966) may enhance the effectiveness of this process. Mast cell responses could play a role in host defense or in enhancing the process of vascular damage.

In conclusion, we have demonstrated a selective and potent induction of chemokines by dengue virus in the presence of dengue specific antibody in both mast cell/basophil lines and CBMC. The consequences of this novel aspect of the mast cell response to viral infection requires further study.

3.7 Conclusions

This chapter detailed our observations regarding the chemokine response of mast cells when infected with dengue virus. The next chapter will focus on the receptor usage of dengue virus mast cell infection.

Chapter 4

FcγRII-DEPENDENT DENGUE VIRUS INFECTION OF HUMAN MAST CELLS

4.1 Introduction to the chapter

Dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) are severe complications in secondary dengue virus infections. They are believed, in part, to result from antibody-dependent enhancement (ADE) of infection. Fc receptors (FcR), including FcγRI and FcγRII, have been shown to be involved in ADE. Mast cells express a number of Fc receptors including Fcε receptors and Fcγ receptors and have been implicated in severe dengue disease. We sought to determine the class of FcR involved in mediating antibody-enhanced dengue virus infection in human mast cells. KU812, a mast cell/basophil cell line, and the HMC-1 mast cell line were found to express FcγRII by flow cytometry. Blocking FcγRII with mAb IV.3 was found to abrogate dengue binding and infection in KU812 cells as well as the RANTES response, while blocking FcεRI had no effect. Primary cultures of cord blood derived human mast cells (CBMC) were found to express all classes of Fcγ receptors and could be induced to express significantly higher levels of surface FcγRI and FcγRIII by differentiation with IFN-γ. Furthermore treatment of CBMCs with IFN-γ resulted in enhanced RANTES production in response to dengue virus/specific antibody inoculation.

4.2 Introduction

Fc receptors have long been known to have a major role in host defence. Circulating pathogens are opsonized by immunoglobulins which then bind to FcR-bearing cells triggering a range of effector functions. These functions include antibody-dependent cell-mediated cytotoxity (ADCC), complement fixation, regulation of antibody production, neutralization, endocytosis and antigen presentation (Metzger, 1992)

Human mast cells express the high affinity receptor for IgE (FcεRI) and have recently been shown to express the high affinity receptor for IgG (FcγRI) upon treatment with IFN-γ (Okayama *et al.*, 2000) as well as the low affinity IgG receptor FcγRII (Okayama *et al.*, 2001a). Until recently, activation of mast cells through IgE cross-linking of surface FcεRI was thought to be the principal method of mast cell FcR activation and the primary manner responsible for allergen-dependent allergic reaction. More recently it has been demonstrated that mast cell activation can occur via cross-linking of surface FcγRI on mast cells treated with IFN-γ (Okayama *et al.*, 2000). FcγRI aggregation induced mast cell degranulation and up-regulation of mRNAs for a number of cytokines and chemokines (Okayama *et al.*, 2000) as well as generation of arachidonic acid metabolites PGD_2 and LTC_4 (Okayama *et al.*, 2001). These responses were found to be qualitatively indistinguishable from those induced by FcεRI aggregation suggesting an additional, previously unknown, mechanism for mast cell involvement in inflammation. Interestingly the cytokines TNF-α, IL-1β and IL-6, among others, were found to be generated to a greater degree after FcγRI aggregation than following FcεRI-mediated activation suggesting a selective mast cell response dependent on the nature

of Fc receptor usage.

Three classes of receptors for IgG have been identified in humans (Unkeless *et al.,* 1988). FcγRI receptors bind monomeric IgG with high affinity, while FcγRII and FcγRIII primarily bind IgG-containing immune complexes. All three receptors have homologous Ig-like extracellular domains but different transmembrane and intracytoplasmic regions (Ravetch & Kinet, 1991) and exist in multiple isoforms and alleles (van de Winkel & Capel, 1993). Different Fcγ receptors can exhibit multiple functions and include FcRs capable of triggering cell activation and FcRs that may inhibit such activation. FcRs containing tyrosine-based intracytoplasmic activation motifs (ITAMs) or those that associate with other proteins/receptors containing ITAMs positively regulate cell activation while FcRs associating with or containing inhibitory motifs (ITIMS) negatively regulates cell activation (Ravetch & Bolland, 2001).

Coincident with the beneficial role FcRs play in host defence, FcRs have also been shown to be involved in detrimental responses including allergic reactions and viral manipulation. Both dengue and HIV (Homsy *et al.,* 1989) have evolved to utilize FcRs to gain access to cells. For dengue the presence of heterotypic sub-neutralizing antibodies to dengue virus as a result of primary infection has been shown to potentiate secondary infection of monocytes and macrophages via antibody-dependent enhancement (ADE) (Halstead, 1989). Furthermore, during antibody-enhanced dengue infection of monocytes (Brinton *et al.,* 1998) increased uptake of antibody-virus complexes occurs via Fc receptor-mediated binding to

cells (Peiris *et al.,* 1981).

The mast cell is a potential target cell for dengue infection in view of its Fc receptor expression. This laboratory previously reported that the human KU812 and HMC-1 cells were permissive to antibody-enhanced dengue virus infection (King *et al.,* 2000; 2002) and that KU812, HMC-1 and human cord blood derived mast cells respond specifically and selectively to dengue virus infection. In view of the expanding role of mast cells in host defense the current study sought to investigate dengue Fc receptor usage on human mast cells. Human cord blood derived mast cells were found to express significant baseline levels of FcγRI, FcγRII and FcγRIII. Expression of RI and RIII could be significantly upregulated by treatment with IFN-γ. The data obtained suggest that dengue utilizes FcγRII on mast cells for binding/entry but has the potential to employ both FcγRI and FcγRIII on mast cells exposed to an IFN-γ rich microenviroment. Furthermore IFN-γ treatment of mast cells resulted in an increase in RANTES production in response to dengue/antibody infection.

4.3 Materials and Methods

Dengue virus and sera

Dengue type 2 virus strain 16681 (Halstead & Simasthien, 1970) was propagated in African Green Monkey kidney Vero cell monolayers cultured in endotoxin free RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 1% fetal calf serum (FCS; Sigma, Oakville, ON). Briefly, frozen stock dengue virus containing supernatants were thawed and used to inoculate 95% confluent Vero monolayers in 24-well sterile flat bottom plates. Approximately 250µl of virus containing supernatants were adsorbed onto Vero monolayers at 4°C for 90 minutes. After adsorption 300µl of fresh 1% FCS RPMI was added to each well and plates were incubated at 37°C, 5% CO_2. The medium in each well was changed daily; virus was harvested for use on Day 3 or 4 post-infection based on titer. Virus was titrated by $TCID_{50}$ assay (Russell *et al.,* 1966) on Vero cells. For some experiments virus was inactivated with UV light (Anderson *et al.,* 1997). For antibody dependent enhancement assays of dengue virus infection, a serum pool was prepared using nine convalescent sera from patients recovering from a dengue serotype 2 infection. Anti-dengue IgG titers among the nine sera ranged from 207 to 465 EIA units per ml of serum. The individual patient sera and the dengue-specific monoclonal antibodies were obtained from a collection provided by Dr Bruce Innis and described briefly in (He *et al.,* 1995). Normal human AB sera were obtained from volunteer donors.

Mast cell/Basophil culture

The human mast cell/basophil cell line KU812 (Kishi, 1985; Blom *et al.,* 1992) was

maintained in RPMI 1640 supplemented with 10% FCS, 10 mM HEPES, 100 U/ml penicillin, and 100μg/ml streptomycin (Life Technologies). The human mast cell line, HMC-1 (Butterfield *et al.,* 1988) was maintained in Iscove's media (Life Technologies) supplemented with 10% FCS. Both the KU812 and HMC-1 cells were passaged 2-3 times per week. Differentiation procedures for both KU812 cells and HMC-1 cells followed previously reported conditions that lead to a more mature mast cell phenotype (Saito *et al.,* 1995). Cells were cultured in appropriate medium supplemented with 80 ng/ml human stem cell factor (SCF; Peprotech, Rocky Hill, NJ) alone or in combination with 50 ng/ml recombinant human interleukin 6 (IL-6; Peprotech) for a period of 8 days. Differentiation with sodium butyrate/IFN-γ involved addition of 0.3 mM sodium butyrate for the first 3 days and 40 ng/ml recombinant human IFNγ (rhIFN-γ; R & D Systems) in combination with sodium butyrate for the following 3 days. Differentiation with sodium butyrate/TNF-α (R & D Systems) followed a similar protocol with a final recombinant human TNF-α concentration of 40 ng/ml. Cells were also differentiated with rhIFN-γ alone at 15 ng/ml for 48 hours (Okayama *et al.,* 2000).

Primary human cultured cord blood derived mast cells

Primary mast cells were generated by culture of human cord blood mononuclear cells as previously described (King *et al.,* 2002) using a modification of the method of Saito *et al.,* (1996) and characterized as previously described (Lin *et al.,* 2000). These cells were consistently positive by flow cytometry for CD117 (c-kit) and CD13 but not CD14. Cells were cultured at an initial concentration of 0.6 x 10^6/ml in RPMI suplemented with 20%

FCS, 100 U/ml penicillin, 100μg/ml streptomycin (Life Technologies), 20% CCL-204 supernatant as a source of IL-6, 1 x 10^{-7}M PGE_2 (Sigma), and 50-100 ng/ml SCF (Peprotech). Media was changed once weekly for 5-10 weeks. Purity of each CBMC preparation was assessed by Toluidine blue (pH 1.0), staining of cytocentrifuge preparations and examination of cells for the presence of multiple metachromatic granules and appropriate nuclear morphology. Only those mast cell preparations, >95% pure were used for this study. The mean purity of cord blood mast cells employed was 98%.

Before use in experiments, CBMCs were either left undifferentiated or differentiated with the addition of rhIFN-γ at 15 ng/ml for 48 hours. Undifferentiated cells were cultured in the absence of PGE_2 in media supplemented with 10 ng/ml SCF for 24 hours before use while differentiated cells were cultured in he absence of PGE_2 for 72 hours with addition of rhIFN-γ for the final 48 hours. In addition, some CBMC cultures were cultured in absence of PGE_2 for 72 hours without addition of rhIFN-γ as a control.

Antibody-enhanced binding of dengue virus

Virus binding assays were carried out as previously described (Anderson *et al.*, 1992) with the following modifications. Radiolabelled dengue virus was prepared by incubating dengue infected Vero cells in the presence of [^{35}S] methionine-cysteine (NEN, Mississauga, ON) in MCFM (Gibco, BRL) with 1% FCS overnight. The following day, virus containing supernatants were harvested and virus was purified on a sucrose gradient (Wang *et al.*, 1999). Purified radiolabelled virus was incubated with 1:1000 or 1:10000 dilutions of dengue

sera or various dilutions of monoclonal mouse anti-dengue antibodies 3H5, 5F10, 9D12, 1B7, and JN120 at 4°C for 90 minutes. Dengue virus or virus-antisera mixtures were used to inoculate 1-2 x 10^6 mast cells. Following 90 min adsorption at 4°on ice, unbound virus was removed by washing 3 times with 1 ml 1% bovine serum albumin (BSA; Boehringer Mannheim, Germany) in PBS. Cell-bound radiolabelled proteins were detected in solubilized pellets via SDS-PAGE (Laemmli, 1970)and fluorography. Fc receptor blocking studies were carried out by pre-incubation of cells with either 1:4 dilution of ascites containing mAb IV.3 (FcγRII blocking Ab; subclass IgG2b; originally from ATCC, kindly provided by Dr. A Issekutz) or 10 μg/ml IgE (FcεRI blocking; Chemicon, Temecula, CA) for 1 hour on ice. Unbound antibody was removed by washing 2 times with RPMI 1640. Irrelevant isotype matched mAb subclass IgG2b (BD Parminigen, San Diego, CA) for IV.3 was used as controls at approximately the same concentration.

Dengue virus infection of mast cells

In order to investigate FcR enhanced dengue virus infection of human mast cells, 1 x 10^6 KU812 cells were washed once in RPMI 1640 media supplemented as described above and inoculated with aliquots of dengue virus (MOI 0.1- 0.3) alone, UV-inactivated dengue virus, combinations of UV-inactivated virus and human dengue immune serum (1:1000 or 1:10000 final dilution), combinations of virus and human dengue immune serum (1:1000 or 1:10000 final dilution) or virus and normal human sera (1:1000 final dilution). Cells were washed twice with RPMI culture media, resuspended at 1 x 10^6 cell/ml and incubated at 37°C, 5%CO_2. An aliquot of 200μl was removed from the culture and incubated in a separate tube

to assess infection.

Radiolabelling of KU812 cells with [^{35}S] methionine-[^{35}S] cysteine

Radiolabelling of KU812 cells was carried out as previously described (King *et al.,* 2000). Briefly, 24 hours post-infection, culture supernatants were removed and cells were resuspended in [^{35}S] methionine-[^{35}S] cysteine (NEN) in MCFM (Gibco) for 3-4 hours, with chase overnight at 37°C, 5%CO_2. Culture supernatants were harvested and immunoprecipitated with dengue virus immune sera and protein A-bearing, formalin fixed *Staphylococcus aureus* as previously described (He *et al.,* 1995). Immunoprecipitates were resolved by SDS-PAGE using 10% polyacrylamide gels. Gels were impregnated with 1M sodium salicylate and fluorographed by exposure to Kodak X-ray film at -70°C.

Abs and flow cytometric analysis

Analysis of FcR surface expression on human mast cells was evaluated with a panel of monoclonal antibodies using a FACScan flow cytometer (Becton Dickinson, San Jose, CA). The monoclonal antibodies used were mouse anti-human FcγRI (clone 10.1, subclass IgG1; BD Pharmingen, San Diego, CA), mouse anti-human FcγRII (clone C1KM5, subclass IgG1; Caltag, Burlingame, CA) and mouse anti-human FcγRIII (clone 3G8, subclass IgG1;Caltag) as well as the isotype matched mouse IgG1 control (BD Pharmingen) and were used at a final concentration of 5 μg/ml. Detection of FcγRII and FcγRIII was by addition of a rat anti-mouse FITC conjugated secondary antibody; the FcγRI antibody was FITC conjugated. To prevent non-specific binding 1µl of human IgG AB sera per 100µl final volume was added

during both the primary and secondary antibody incubation steps. Cell analysis was performed using CellQuest (Becton Dickinson) and FCS Express (De Novo Software, ON, Canada) software.

Flow cytometry results are expressed as median values of fluorescence intensity of mast cells and percent positive cells assessed according to the Overton method of histogram subtraction (Overton, 1988). CBMCs samples were analysed by gating on two sub-populations, differing in granularity, in the cultures and defined as sub-population 1 and sub-population 2. Data regarding percentage of sub-population 1 and 2 versus the entire sample as well as median flourescent intensity (MFI) and percent shift of both sub-populations and the entire sample are given for both control and experimental samples. Data are expressed as mean and standard deviation.

Chemokine analysis

RANTES production was examined in 24, 48 and 72 hour post-infection supernatants harvested from primary cultured human mast cells by ELISA as previously described (King *et al.*, 2002). CBMCs were inoculated with dengue virus (MOI 0.1-0.3) alone, UV-inactivated dengue virus, combinations of UV-inactivated virus and human dengue immune serum (1:1000 or 1:10000 final dilution), combinations of virus and human dengue immune serum (1:1000 or 1:10000 final dilution) or virus and normal human sera (1:1000 final dilution). Treatment with media alone was used to assess constitutive production of RANTES.

4.4 Results

Antibody-enhanced binding of dengue virus to human mast cells

To determine the optimal virus binding time for subsequent studies we assessed binding of dengue to the surface of KU812 cells at various adsorption times from10 to 90 minutes. Both live and UV-inactivated dengue virus-immune serum complexes displayed enhanced binding of virus to KU812 cells as early as 10 min post-adsorption with increased binding up to 90 min (data not shown). To confirm that binding of dengue virus was enhanced by the presence of dengue immune serum, KU812 and HMC-1 cells were adsorbed with ^{35}S-radiolabelled dengue virus on ice with or without dengue immune serum for 90 min. Samples were washed three times to ensure any unbound virus was removed and cell bound radiolabelled dengue virus was detected in solubilized pellets via SDS-PAGE and fluorography. As shown in Fig 1A dengue virus-immune serum at a dilution of 1:10000 demonstrated enhanced binding of virus to both KU812 and HMC-1 cells as indicated by markedly increased levels of radiolabelled E protein. There was little binding of dengue virus in the absence of antibody. Vero cells inoculated with the same virus or virus-antibody mixtures did not display this antibody-enhanced binding which is consistent with the lack of Fc receptors on Vero cells. Antibody-enhanced virus-cell binding, as well as requirement for the viral E protein, was demonstrated using E-specific mouse mAbs (Fig 1B) and suggested that the IgG portion of the dengue immune serum was, at least, in part, responsible for enhanced virus binding observed with the immune serum.

Surface expression of FcγR's on human mast cells

Flow cytometry analysis of surface Fcγ receptors was carried out to determine which class(es) of Fcγ receptors were present on the surface of human mast cells. KU812, HMC-1 and CBMCs were analysed for surface expression of FcγRI, FcγRII, and FcγRIII. Substantial expression of FcγRII was found on the surface of undifferentiated KU812 and HMC-1 cells (Fig 2B) with higher expression of FcγRII on the surface of KU812 cells (mean ± SD) (90.31% ± 1.62 of the population) versus HMC-1 cells (64.65% ± 14.97). In contrast, very low (<4), if any, FcγRI (Fig. 2A) or FcγRIII (Fig 2C) were expressed on KU812 cells; HMC-1 cells were found to express low FcγRIII but moderate levels of FcγRI (21.03% ± 1.93). Median fluorescent intensity of surface FcγRII on KU812 cells (25 ± 9) versus HMC-1 cells (7 ± 0) indicates that not only do KU812 cells have a greater percentage of the population expressing FcγRII but KU812 cells also have a greater number of FcγRII receptors on the surface. Forward and side scatter analyses of CBMCs demonstrated that the population could be divided into more or less granulated sub-populations, defined as sub-population 1 (less granulated) and 2 (more granulated) (Fig 2D). Sub-population 1 was found to comprise 55.23% ± 13.00 while sub-population 2 was found to be 30.67% ± 4.93 (Table 1). Both sub-populations (Fig 2E, Table 1) were analysed for surface expression of FcγRs and data suggested that sub-population 1 had the greatest percent positive for FcγRI (67.07% ± 2.78 vs 13.83% ± 1.61) and FcγRIII (45.61%± 17.42 vs 6.95% ± 0.14) while both sub-population 1 and 2 expressed similar levels of FcγRII (50.78% ± 7.18 vs 36.65% ± 2.47).

FcγRII mediates antibody-enhanced binding and infection in KU812 cells

We have previously demonstrated antibody-enhanced infection of KU812 cells (King *et al.*,

2000; 2002). In addition, we have shown significant FcγRII expression (Fig 2A) on the surface of KU812 cells and mast cells are known to express the high affinity FcεRI receptor for IgE. To determine the role of FcεRI in antibody-enhanced binding of dengue virus to the surface of KU812 cells, blocking studies were carried out. Binding studies using KU812 cells pre-incubated with either 10 ug/ml of human IgE, 1:4 dilution of ascites containing IV.3 or 10 ug/ml irrelevant IgG control antibody followed by adsorption with dengue virus and dengue immune sera at a final dilution of 1:1000 illustrated that antibody-enhanced binding of dengue virus was mediated predominately by FcγRII and not by FcεRI (Fig 3A). Blocking with IgE prior to adsorption resulted in characteristic levels of binding (compare lanes 5 and 6 with lanes 1 and 2) while blocking with IV.3 totally abrogated binding (lanes 3 and 4) to the surface of KU812 cells. Pre-incubation with control IgG had no effect on binding (lanes 1 and 2). Furthermore, blocking FcγRII with IV.3 impeded dengue virus infection (compare lanes 5 and 6 with lanes 3 and 4) of KU812 cells while pre-incubation with control mouse IgG had no effect (compare lanes 7 and 8 with lanes 3 and 4) (Fig 3B). Concommitant with blocking infection, pre-treatment of KU812 cells with IV.3 also abrogated the RANTES response to dengue virus and antibody treatment (compare lanes 2 and 3) (Fig 3C). Taken together, these data suggested antibody-enhanced virus-cell-binding and subsequent infection as well as the substantial RANTES response of KU812 cells previously documented (King *et al.*, 2002) was mediated primarily by FcγRII.

Upregulation of FcγRI and FcγRIII on CBMCs

To determine if differentiation of human mast cells, KU812, HMC-1 and CBMCs,

modulated surface FcγR expression, cells were treated with a variety of factors known to induce a more mature mast cell phenotype. Differentiation of KU812 (Fig 4A) and HMC-1 (Fig 4B) cells with factors including SCF which induces a mast cell like phenotype with enhanced FcεRI expression, a combination of IL-6 and SCF which induces a high tryptase expressing mast cell like phenotype (Saito *et al.,* 1995), as well as sodium butyrate/IFN-γ, sodium butyrate/TNF-α or IFN-γ alone failed to induce a significant shift in the percentage of the KU812 or HMC-1 population expressing each of the Fcγ receptors. Differentiation of CBMCs with IFN-γ for 48 hours resulted in no significant change in percentage of cells in sub-population 1 and 2 (Table 2). However, significant upregulation (Fig 4D) of the percentage of the cells expressing FcγRI and FcγRIII in both sub-population 1 cells (Table 3) and sub-population 2 cells (Table 4) was observed upon treatment with IFN-γ. In addition, significant increases in FcγRI expression per cell (MFI), in sub-population 1 cells, were observed when cells were differentiated with IFN-γ. No significant upregulation of FcγRII was observed following CBMC treatment with IFN-γ for either sub-population 1 or 2 from any of the three subjects.

Enhanced RANTES production by IFN-γ treated CBMCs

Having demonstrated the ability to upregulate surface FcγRs on CBMCs we sought to investigate the potential effect on CBMC RANTES production in response to dengue virus/antibody treatment. CBMCs from the same subject were either left untreated or differentiated with IFN-γ as discussed above. Cells were then mock inoculated or inoculated with dengue virus/normal human serum (1:1000 final dilution), UV-inactivated virus in the

presence or absence of human dengue immune serum (1:1000 final dilution) or live dengue virus in the presence or absence of human dengue immune serum (1:1000 and 1:10000 final dilution). Cell free supernatants were harvested at 24, 48 and 72 hours for one subject and 24 hours for two additional subjects and analysed for RANTES protein by ELISA. All three subjects produced higher levels of RANTES in response to dengue virus/immune serum inoculation when differentiated with IFN-γ (1.54; 4.21; 1.19 fold increase) as compared to undifferentiated cells (Table 5). Time course analysis indicated that while the highest levels of RANTES were observed 48 hours post-inoculation the greatest difference between undifferentiated (Fig 5A) and IFN-γ differentiated (Fig 5B) CBMCs RANTES production occurred 24 hours post-inoculation.

4.5 Figures

Figure 1. Antibody-enhanced binding of dengue virus to KU812 and HMC-1 cells. (A) Radiolabelled virus was incubated with KU812, HMC-1 and VERO cells for 90 min on ice in the presence human dengue immune serum. (B) Antibody-enhanced virus-cell binding was demonstrated using E-specific mouse mAbs. KU812 cells were incubated with radiolabelled virus in the presence of either polyclonal human immune serum or a range of mouse monoclonal antibodies specific for the E protein. Cultures were washed 3 times to remove any unbound virus and then solubilized and analyzed by SDS-PAGE fluorography. Virus binding is indicated by the presence of radiolabelled envelope (E) protein. The data are representative of two separate experiments.

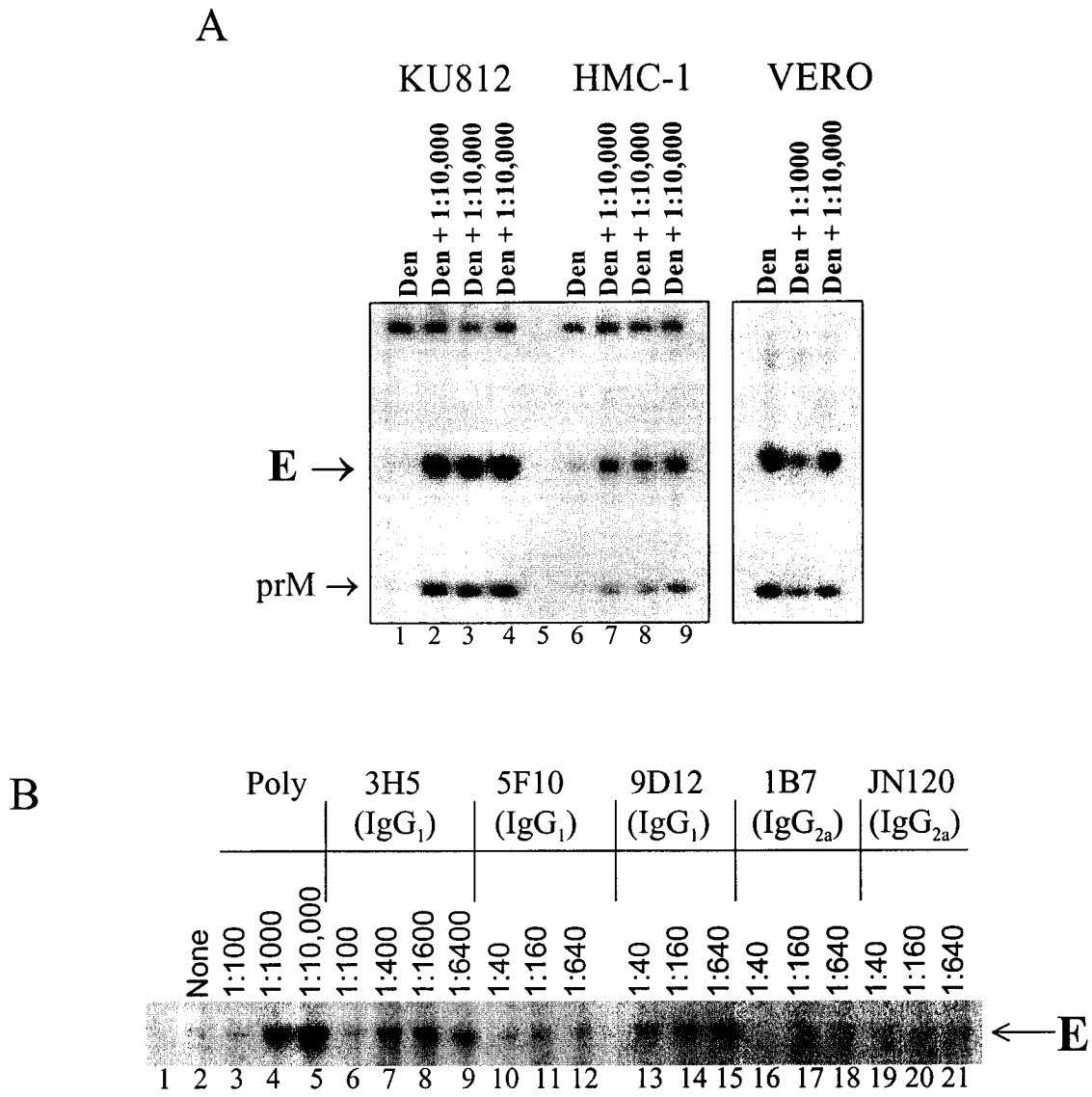


Figure 1 A and B

Figure 2. Surface expression of FcγRs on human mast cells. KU812 and HMC-1 cells were analyzed by FACS for surface expression of FcγRI (A), FcγRII (B), and FcγRIII (C). Data are representative of two separate experiments. Grey lines indicate isotype control; black lines indicate FcγR specific antibody. Cord blood derived mast cell samples were divided into more or less granulated sub-populations based on SSC (D). Populations were gated and designated sub-population 1 (red- less granulated) and sub-population 2 (blue- more granulated). Cord blood derived mast cells from two separate subjects were analyzed for surface FcγRI, FcγRII and FcγRIII (E). The less granulated sub-population is illustrated and exhibited lower levels of FcR expression at baselines. Fig 2E; red vs green - sub-population1 (isotype control) vs sub-population 1(specific antibody); Data are representative of two separate experiments. Data in tables are expressed as MFI and percentage shift indicating the percentage of the population expressing the receptor.

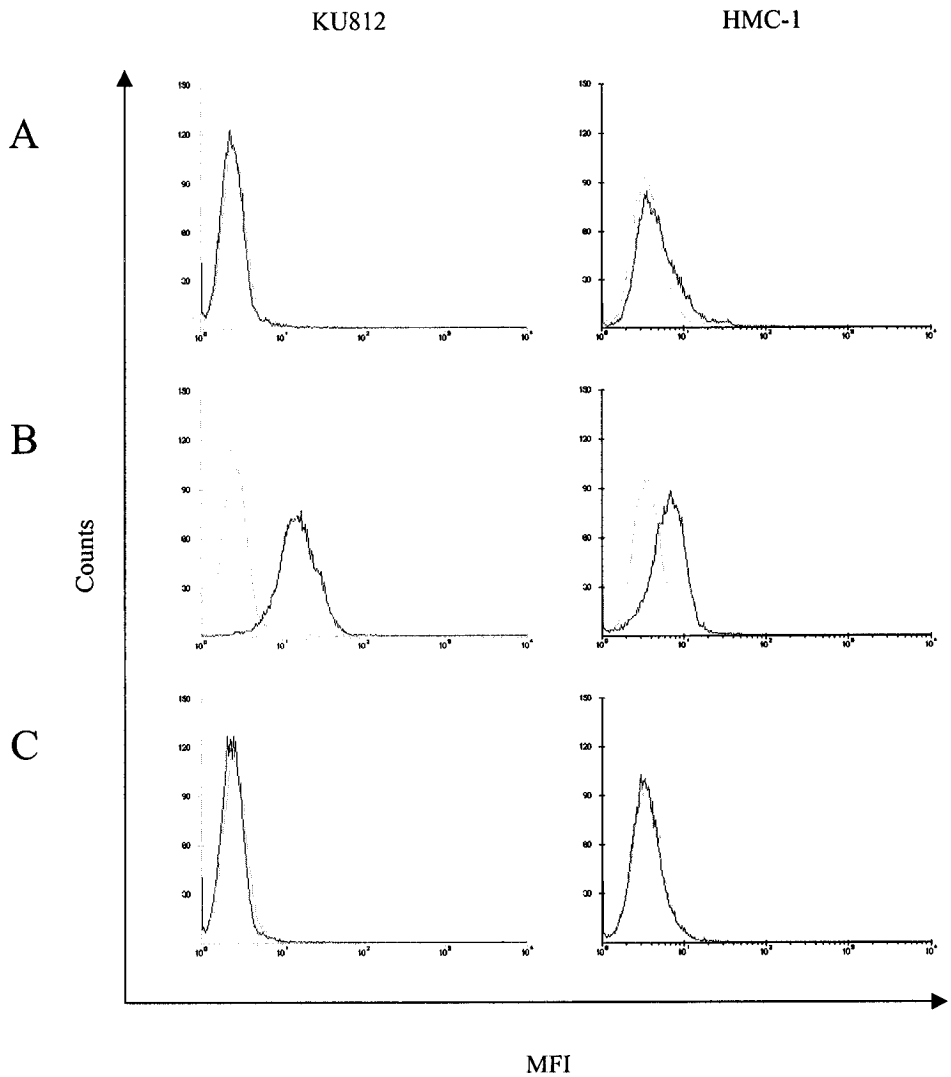


Figure 2 A - C

Baseline	KU812			HMC-1		
	FcγRI	FcγRII	FcγRIII	FcγRI	FcγRII	FcγRIII
Expt 1[a]						
MFI[b]	2	16	2	4	7	3
Percent Shift[c]	<4	91.92	<4	19.10	49.68	<4
Expt 2						
MFI	4	34	3	4	7	3
Percent Shift	<4	88.69	<4	22.95	79.62	<4

[a] Representative experiment

[b] Median fluorescent intensity of specific antibody

[c] Percent positive by Overton histogram subtraction

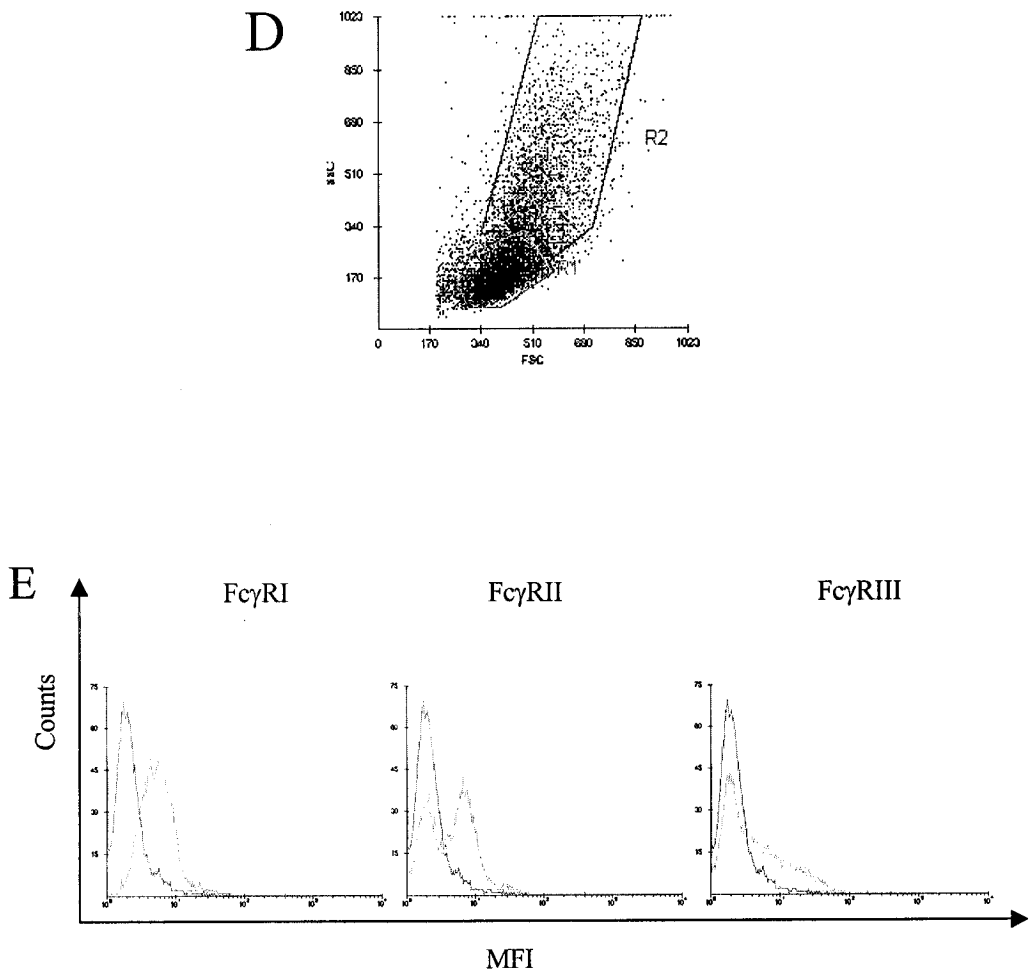


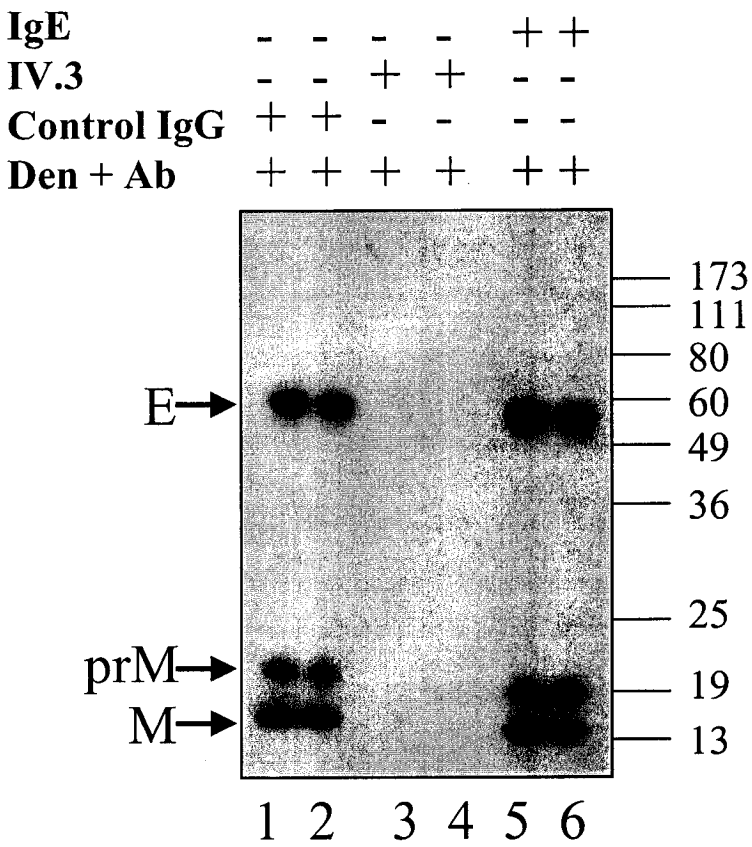
Figure 2D and E

Table 1. CBMC baseline total population, sub-population 1 and sub-population 2 percentages, MFI and analysed for FcγRI, FcγRII and FcγRIII.

	Subject 1			Subject 2		
	All[a]	Sub-pop 1[b]	Sub-pop 2[c]	All	Sub-pop 1	Sub-pop 2
# of events	10000	6442	2718	10000	4604	3415
% of total	100	64.42	27.18	100	46.04	34.15
MedianFI	3	2	9	3	1	5
Mean FI	4	2	12	3	2	5
MFI[d]						
FcγRI		2/5[f]	9/11		1/4	5/6
FcγRII		9/16	2/5		1/4	5/8
FcγRIII		2/3	9/10		1/4	5/5
% Shift[e]						
FcγRI		69.03	12.69		65.10	14.97
FcγRII		45.70	34.90		55.85	38.40
FcγRIII		33.29	6.85		57.92	7.05

[a] total population
[b] sub-population 1 (less granulated)
[c] sub-population 2 (more granulated)
[d] median fluorescent intensity
[e] percent positive by Overton histogram subtraction
[f] isotype vs specific antibody

Figure 3. FcγRII-mediated binding and infection of KU812 cells. KU812 cells were pre-treated with media, irrelevant control 10μg/ml IgG subclass IgG2b, 10μg/ml IgE or 1:4 dilution of ascites containing IV.3 for 1 hour on ice. Cells were then washed twice to remove any unbound antibody. (A) Radiolabelled dengue virus was incubated with KU812 cells for 90 min on ice in the presence human dengue immune serum (1:1000 final dilution).Cultures were washed 3 times to remove any unbound virus and then solubilized and analyzed by SDS-PAGE fluorography. Virus binding is indicated by the presence of radiolabelled envelope (E) protein.(B) Cultures of KU812 cells were inoculated with dengue virus (MOI 0.1-0.3) in the presence of human dengue immune serum (final dilution 1:1000) and dengue proteins immunoprecipitated at 24 hours post-infection. Infection is indicated by the presence of radiolabelled envelope (E) protein.(C) KU812 72 hour post-infection cell free supernatants were harvested and RANTES protein immunoprecipitated. * indicates the position of RANTES protein. Experiments used pooled dengue convalescent patient serum. The data are representative of two separate experiments.


IgE - - - - + +
IV.3 - - + + - -
Control IgG + + - - - -
Den + Ab + + + + + +
E
prM
M
173
111
80
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1 2 3 4 5 6

Figure 3 A

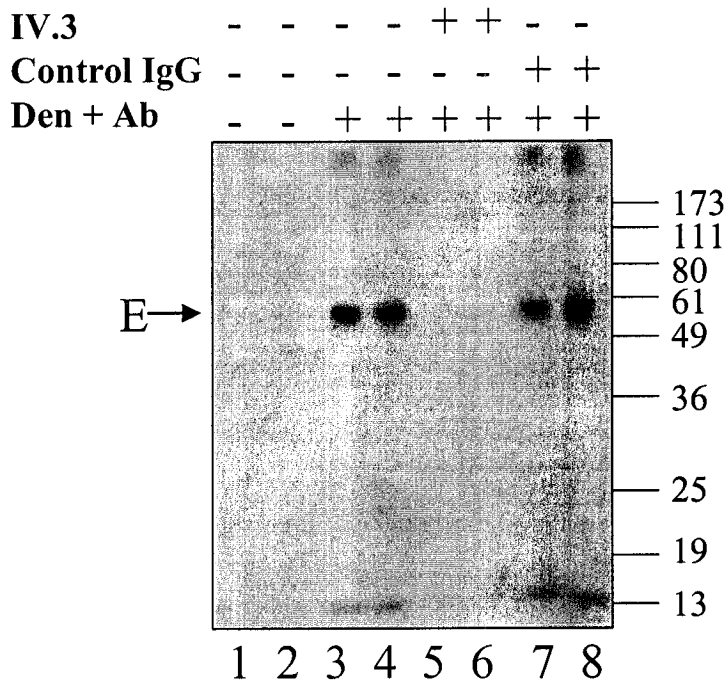


Figure 3B

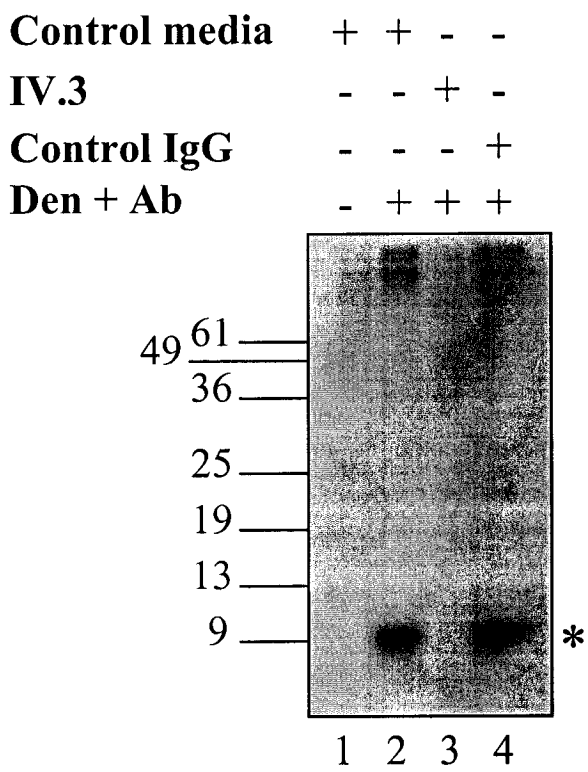
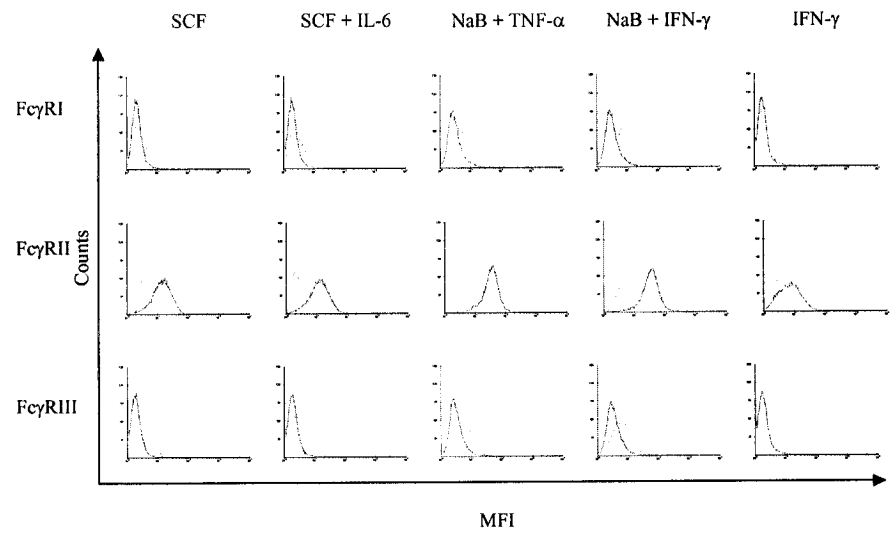


Figure 3 C

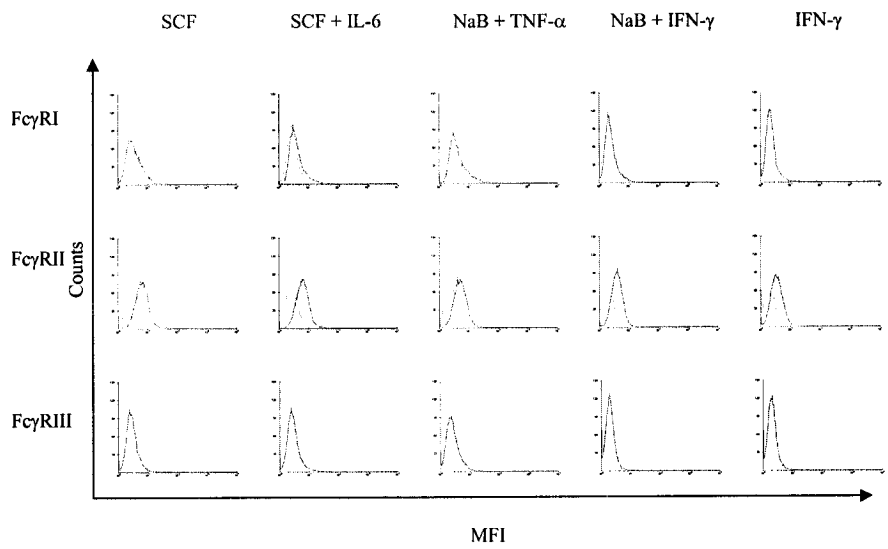
Figure 4. Surface FcγR expression on differentiated human mast cells. KU812 (A) and HMC-1 (B) cells were treated with a variety of mediators as discussed in the Material and Methods and analysed by FACS for FcγRI, FcγRII and FcγRIII expression. Grey lines indicate isotype control; black lines indicate FcγR specific antibody. (C) Cord blood derived mast cells from 3 separate subjects were analysed for surface FcγR expression with and without IFN-γ differentiation. Grey lines indicate isotype control; black lines indicate FcγR specific antibody for the total population. (D) CBMC sub-population surface FcγR expression with and without IFN-γ differentiation ; red vs green - sub-population1 (isotype control) vs sub-population 1(specific antibody); blue vs purple - sub-population 2 (isotype control) vs sub-population 2 (specific antibody). Data are representative on three separate experiments. Data in tables are expressed as MFI and percentage shift indicating the percentage of the population expressing the receptor.



KU812	MFI[a]			Percent Shift[b]		
	FcγRI	FcγRII	FcγRIII	FcγRI	FcγRII	FcγRIII
SCF	2	14	2	<4	72.56	<4
SCF + IL-6	2	13	2	<4	66.63	<4
Sodium Butyrate + TNF-α	3	34	3	<4	81.94	<4
Sodium Butyrate + IFN-γ	3	35	3	<4	84.03	<4
IFN-γ	2	7	2	<4	66.67	<4

[a] Median fluorescent intensity of specific antibody
[b] Percent positive by Overton histogram subtraction

Figure 4A



HMC-1	MFI[a]			Percent Shift[b]		
	FcγRI	FcγRII	FcγRIII	FcγRI	FcγRII	FcγRIII
SCF	4	6	3	23.79	62.58	<4
SCF + IL-6	4	6	3	29.73	64.56	<4
Sodium Butyrate + TNF-α	3	5	2	24.68	56.09	<4
Sodium Butyrate + IFN-γ	2	4	2	18.59	65.60	<4
IFN-γ	2	3	2	11.73	54.01	<4

[a] Median fluorescent intensity of specific antibody
[b] Percent positive by Overton histogram subtraction

Figure 4 B

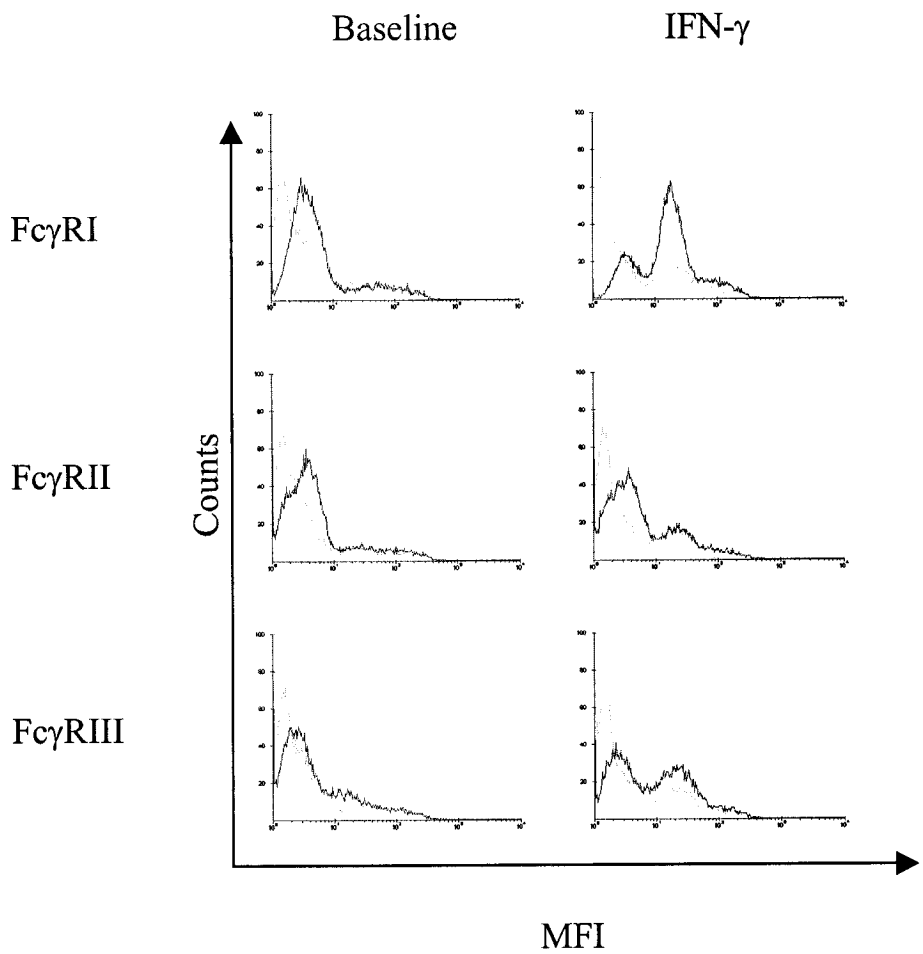


Figure 4 C

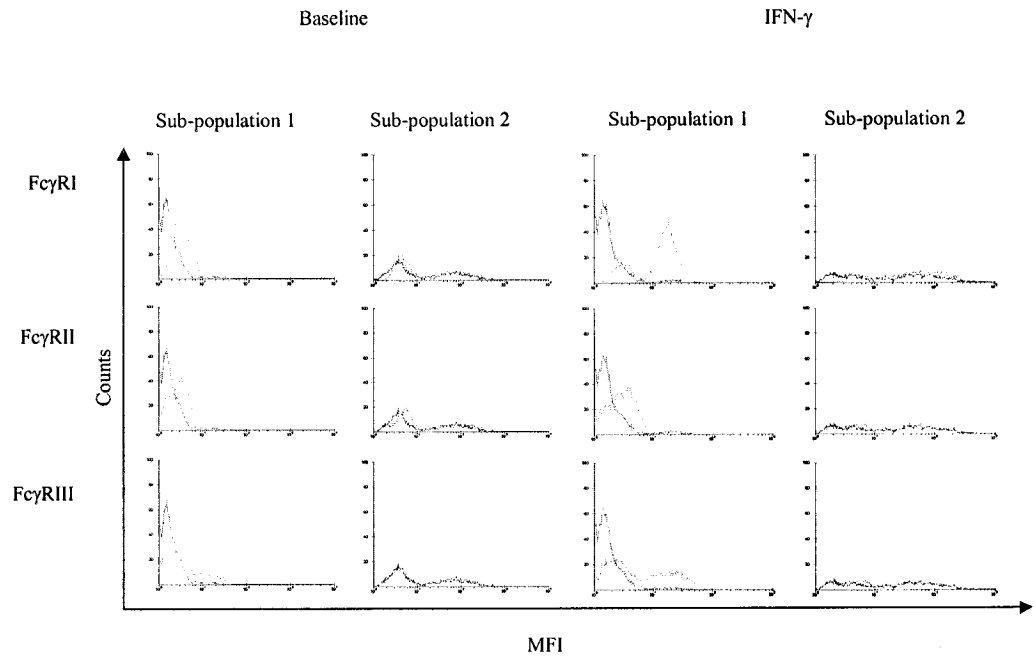

Baseline
IFN-γ
Sub-population 1
Sub-population 2
Sub-population 1
Sub-population 2
FcγRI
FcγRII
FcγRIII
Counts
MFI

Figure 4 D

Table 2. CBMC baseline and IFN-γ treatment total population, sub-population 1 and sub-population 2 percentages and MFI.

Subject	Sub-population	# of Events	% of Total	Median	Mean
1 (127) Baseline	None	10000	100	2	4
	1	6051	60.51	2	2
	2	2796	27.96	6	12
IFN-γ	None	10000	100	2	4
	1	5634	56.34	2	2
	2	2126	21.26	18	16
2 (128) Baseline	None	10000	100	4	4
	1	5331	53.31	2	2
	2	4125	41.25	8	9
IFN-γ	None	10000	100	3	4
	1	5532	55.32	2	2
	2	3851	38.51	8	9
3 (157B) Baseline	None	10000	100	15	15
	1	2365	23.65	5	5
	2	4929	49.25	20	21
IFN-γ	None	10000	100	15	15
	1	2307	23.07	5	5
	2	5386	53.86	20	21

Table 3. MFI values and percent positive of CBMCs in sub-population 1 analysed for FcγRI, FcγRII and FcγRIII with and without IFN-γ treatment.

Sub-population 1	MFI[a]			Percent Shift[b]		
	FcγRI	FcγRII	FcγRIII	FcγRI	FcγRII	FcγRIII
Subject 1 (127)						
Baseline	2/3[c]	2/3	2/3	56.62	41.73	33.03
IFN-γ	2/15	2/3	2/4	76.16	46.67	52.23
Fold increase	5	0	1.33	1.35	1.12	1.58
Subject 2 (128)						
Baseline	2/4	2/4	2/3	58.69	49.57	33.29
IFN-γ	2/9	2/4	2/3	71.88	54.07	39.2
Fold increase	2.25	0	0	1.22	1.09	1.18
Subject 3 (157B)						
Baseline	5/18	5/13	5/11	78.76	52.72	45.59
IFN-γ	5/41	5/14	5/16	85.28	53.05	56.62
Fold increase	2.28	1.08	1.45	1.08	1.01	1.24

[a] Median fluorescent intensity
[b] Percent positive by Overton histogram subtraction
[c] isotype vs specific antibody

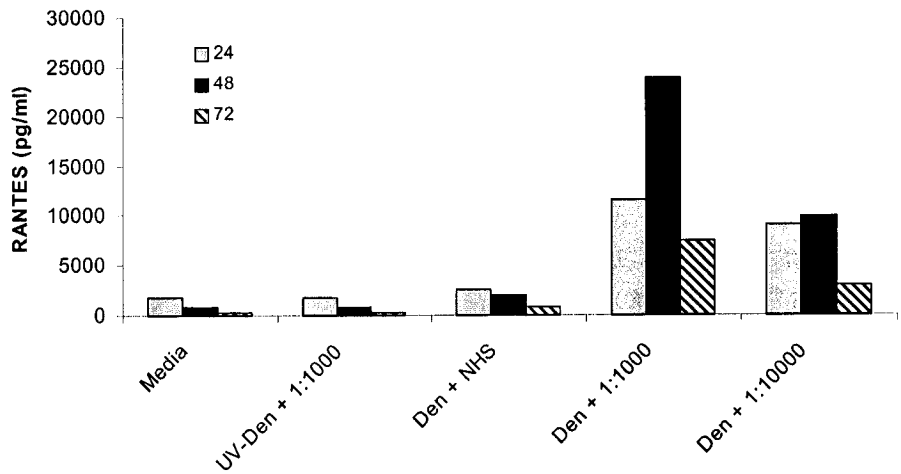
Table 4. MFI values and percent positive of CBMCs in sub-population 2 analysed for FcγRI, FcγRII and FcγRIII with and without IFN-γ treatment.

Sub-population 2	MFI[a]			Percent Shift[b]		
	FcγRI	FcγRII	FcγRIII	FcγRI	FcγRII	FcγRIII
Subject 1 (127)						
Baseline	6/8	6/7	6/6	14.14	14.91	1.29
IFN-γ	18/45	18/21	18/30	22.95	6.94	7.56
Fold increase	1.875	0	1.67	1.62	-2.14	5.86
Subject 2 (128)						
Baseline	8/9	8/12	8/9	5.81	30.26	2.71
IFN-γ	8/9	8/12	8/9	13.4	32.16	7.36
Fold increase	0	0	0	2.31	1.06	2.72
Subject 3 (157B)						
Baseline	20/22	20/30	20/19	5.39	29.68	0.71
IFN-γ	20/21	20/28	20/20	4.25	27.11	0.51
Fold increase	-1.05	-1.07	1.05	-1.27	-1.09	–1.39

[a] Median fluorescent intensity
[b] Percent positive by Overton histogram subtraction
[c] isotype vs specific antibody

Figure 5. Cord blood derived mast cell production of RANTES in response to dengue virus and antibody. RANTES was measured in cell free supernatants at 24, 48 and 72 hours post-infection by ELISA. (A) Undifferentiated CBMC (B) IFN-γ differentiated CBMC RANTES production. Cord blood derived mast cells treated with media alone indicates constitutive production. Experiments used pooled dengue convalescent patient sera.

A


30000
25000
20000
15000
10000
5000
0
RANTES (pg/ml)
24
48
72
Media
UV-Den + 1:1000
Den + NHS
Den + 1:1000
Den + 1:10000

B

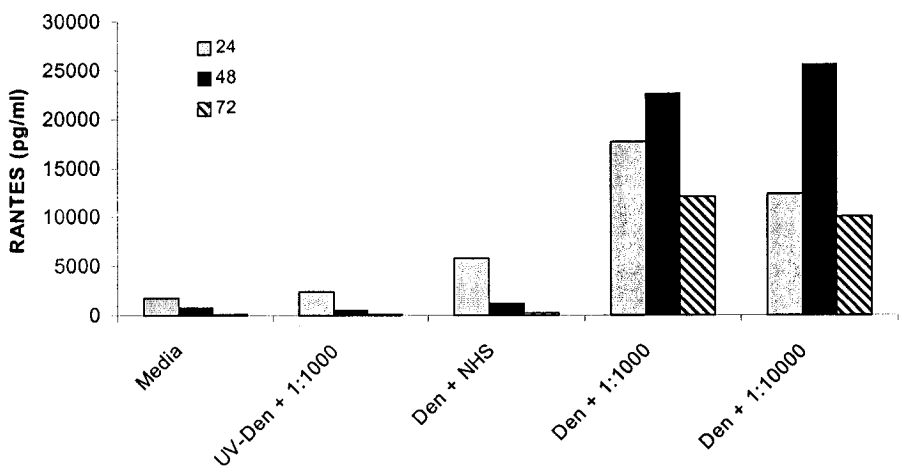

30000
25000
20000
15000
10000
5000
0
RANTES (pg/ml)
24
48
72
Media
UV-Den + 1:1000
Den + NHS
Den + 1:1000
Den + 1:10000

Figure 5

Table 5. IFN-γ differentiation of CBMCs results in enhanced RANTES production at 24 hours post-inoculation (MOI 0.1 - 0.3) with dengue and dengue immune serum.

	Undifferentiated	IFN-γ differentiated
Subject 1	11473.74	17690.66
Subject 2	1932.412	8135.752
Subject 3	8781.21	10429.95

4.6 Discussion

Our findings demonstrate that in the mast cell/basophil cell line, KU812, dengue virus utilizes primarily FcγRII during antibody-dependent infection. In addition, treatment of CBMCs with rhIFN-γ, which enhances FcγRI and FcγRIII expression, results in augmentation of RANTES production when cells are treated with dengue virus and antibody. Furthermore, human cord blood derived mast cells cultured under the conditions we describe express significant baseline levels of all three FcγRs including FcγRI, FcγRII and FcγRIII.

We have previously demonstrated antibody-enhanced dengue virus infection of human mast cells that resulted in the selective production of vasoactive cytokines IL-1β and IL-6 (King *et al.*, 2000) and potent T cell/monocyte chemoattractants RANTES, MIP-1α and MIP-1β (King *et al.*, 2002). The current study investigates virus-antibody-complexed Fc receptor usage during antibody-dependent enhancement of dengue virus infection in human mast cells. A number of complementary human mast cell/basophil models were employed in this study including KU812, HMC-1 and CBMCs. The KU812 cell line has been documented to possess properties of both basophils (Kishi, 1985) and mast cells (Blom *et al.*, 1992). KU812 cells have been demonstrated to express mRNA for mast cell tryptase, mast cell carboxypeptidase with low level expression of major basic protein (Blom *et al.*, 1992). KU812 cells express FcεR1, the high affinity IgE receptor (Almlof *et al.*, 1988), and FcγRII (Kawata *et al.*, 1996). The HMC-1 mast cell line (Butterfield *et al.*, 1988) has been found to express chymase mRNA and higher levels of tryptase mRNA than KU812 cells (Xia *et al.*, 1995). In contrast to KU812 cells, HMC-1 cells have FcγRII (Wedi *et al.*, 1996) but do not

consistently express FcεRI (Xia *et al.,* 1995). Both of these cell types will undergo ADE of dengue virus infection *in vitro*.

FACS analysis of surface FcγR expression on KU812, and HMC-1 cells indicated that substantial surface expression of FcγRII on undifferentiated KU812 and HMC-1 cells (Fig 2B) with higher expression of FcγRII on the surface of KU812 cells [(mean ± SD) 90.31% ± 1.62 versus 64.65% ± 14.97%] versus HMC-1 cells. In contrast, FcγRI (Fig. 2A) and FcγRIII (Fig 2C) were undetectable on KU812 cells, while HMC-1 cells expressed moderate levels of FcγRI (21.03% ± 1.93). Analysis of median fluorescent intensity of surface FcγRII on KU812 cells (25 ± 9) versus HMC-1 cells (7 ± 0) demonstrated that KU812 cells have a greater number of FcγRII receptors on the surface in addition to having a greater percentage of the population expressing FcγRII as compared to HMC-1 cells. Taken together, the significant difference in both percentage of the population expressing FcγRII and number of FcγRII, on the surface on a per cell basis, may explain previous published observations demonstrating a less effective dengue infection of HMC-1 cells in comparison to KU812 cells (King *et al.,* 2002).

Examination of CBMCs generated from two individuals included separate analysis of more and less complex subpopulations (Fig 2D). Differences in granularity are not uncommon in these cultures and have been demonstrated by toluidine blue staining. The less complex sub-population 1 was the focus of our studies and likely represents a less 'mature' subset of mast cells. All of our five CBMC preparations were found to be of high purity. Purity, based on

metachromatic staining, for all five CBMC preparations were found to be greater than 95% with an average purity of 98%. Thus it is highly unlikely that the findings of two populations are the result of contaminating cells of a different lineage but rather reflect granularity and/or maturity. The findings of two sub-populations that reflect granularity have also been described by another group. Ochi *et al.* (1999) found that cord blood derived mast cells at 6 weeks segregated into two sub-populations based on side angle light scatter (SSC). Their data demonstrated that both the less granulated (lower SSC) as well as the more granulated (higher SSC) cells expressed FcεRI, CD13 and c-Kit indicating a mast cell phenotype (Ochi *et al.*, 1999). Furthermore, both populations stained with toluidine blue and were positive for tryptase, chymase and chloroacetate esterase. Further culture of the less granulated cells was found to increase granularity reflecting further maturation (Ochi *et al.*, 1999).

FACS analysis demonstrated that CBMCs express all three FcγRs examined. Both sub-populations (Fig 2E) were analysed for surface expression of FcγRs and data suggested that sub-population 1 had the greatest percent positive for FcγRI (67.07% ± 2.78 vs 13.83% ± 1.61) and FcγRIII (45.61%± 17.42 vs 6.95% ± 0.14) while both sub-population 1 and 2 expressed similar levels of FcγRII (50.78% ± 7.18 vs 36.65% ± 2.47). MFI analysis also indicated that there were a greater number of surface FcγRII receptors/cell on CBMCs in sub-population 1. The findings that the less complex, perhaps more immature, mast cells express the greatest surface level of FcγRII and contain the greatest number of cells expressing FcγRI and FcγRIII is intriguing and may suggest differential receptor expression throughout maturation. To our knowledge this is the first report of human mast cell surface

FcγRIII expression though FcγRIII mRNA has been detected in human CD34+ cultured mast cells (Okayama *et al.*, 2001b) and surface expression has been observed on murine mast cells (Daeron *et al.*, 1992).

KU812 cells were found to express FcγRII by flow cytometric analysis consistent with previous observations (Kawata *et al.*, 1996) while HMC-1 cells were found to express both FcγRII and FcγRI. Previous work has reported exclusive expression of FcγRII on the surface of HMC-1 cells (Wedi *et al.*, 1996). The apparent difference in FcγR expression determined here may reflect differences in culture. Our findings that resting human CBMCs express significant levels of all three FcγRs are consistent with the suggestion (Okayama *et al.*, 2000) that mast cells may play an IgG-dependent role in inflammation.

Dengue virus has been demonstrated to employ FcγRI (Kontny *et al.*, 1988) and FcγRII (Littaua *et al.*, 1990) during the process of antibody enhanced infection of monocytes. Binding assays using both KU812 and HMC-1 mast cell lines indicated markedly enhanced binding of radiolabelled dengue virus in the presence of dengue specific antibody. The use of freshly passaged and harvested dengue virus allowed for high titers enabling radiolabelling of the virus. The method of generating continuos fresh virus stocks for experiments has been demonstrated to result in significantly higher titers of dengue virus as compared to other methods and these higher titers provides sufficient levels of dengue virus for radiolabelling and subsequent binding studies. This antibody-enhanced binding of dengue-antibody complexes was observed using polyclonal dengue immune sera (Fig 1A). Interestingly,

reduced virus-antibody binding was seen when HMC-1 cells were used as compared to the KU812 cell line (Fig 1A; compare lanes 2, 3 and 4 to 7, 8 and 9). FACS data demonstrating reduced FcγR expression on HMC-1 cells versus KU812 cells may contribute to reduced virus-antibody binding to HMC-1 cells. In agreement with this, we previously have demonstrated lower levels of antibody-enhanced virus infection of HMC-1 cells as compared to KU812 cells (King *et al.,* 2002). As expected, in contrast to mast cells, Vero cells exhibited no antibody-enhanced binding (Fig 1A; compare lane 10 to 11 and 12) consistent with the lack of Fc receptors on these cells (He *et al.,*1995).

Confirmation that dengue-specific IgG antibodies are required for the enhanced virus binding to mast cells was confirmed using a panel of 5 murine monoclonal antibodies specific for the E protein. MAb 3H5 is dengue-2-type specific (Henchal *et al.,* 1985) while the other mAbs recognize the E protein among the four serotypes of dengue as well as certain other flaviviruses (Kaufman *et al.,* 1987; Patarapotikul *et al.,* 1993). As shown in Fig 1B mAbs 3H5 and to a lesser extent, 9D12, were able to enhance virus binding to the surface of KU812 cells while mAbs 1B7, 5F10 and JN120 did not. The dengue-2-type specific mAb 3H5 exhibited the greatest ability to enhance binding when compared to binding using the polyclonal dengue immune sera (compare lanes 7, 8 and 9 to 4 and 5). ADE is generally the result of heterotypic antibodies though homotypic ADE has been previously demonstrated (Halstead, 1988). In addition, the data also suggest that binding was subclass specific in that only the IgG1 subclass enhanced binding. Taken together, these data suggest that ADE of human mast cells is a function of both IgG isotype and epitope specificity of the antibody.

The question of dependence on mast cell Fc receptors for antibody-enhanced dengue virus binding was further investigated using mAb IV.3 (which blocks binding to FcγRII) and human myeloma IgE (binds and occupies FcεRI). As shown in Fig. 3A pretreatment of KU812 cells with IV.3 completely abrogated virus binding suggesting that unoccupied/unblocked FcγRII on the surface of KU812 cells was required for antibody-enhanced binding. In contrast, pretreatment with IgE had no effect on binding of virus-antibody complexes (compare lanes 5 and 6 with 3 and 4). Furthermore, pretreatment of cells with a control antibody of the same subclass IgG2b as IV.3 had no effect on binding. The results therefore suggest that dengue virus utilizes FcγRII on the surface of KU812 cells for antibody-dependent virus binding. It is interesting that both FcγRI and FcγRII have been implicated in ADE of dengue virus infection of U937 monocytes and erythroleukemic K562 cells (Kontny *et al.*, 1990).

Intriguingly, dengue virus has also been shown to utilize alternate FcγRs on the surface of cells. COS cells transfected with the FcγRI were found to bind and internalize dengue-antibody complexes (Schlesinger & Chapman, 1999) thus providing an additional means of enhanced binding. Given the ability of dengue to utilize additional FcγRs we sought to investigate receptor usage on mast cells expressing more than one FcγR. KU812 and HMC-1 cells were differentiated with a variety of agents known to induce a more mature mast cell phenotype and analyzed by flow cytometry for upregulated/induced surface expression of FcγRI, FcγRII and FcγRIII. None of the agents tested were able to increase expression in either KU812 (Fig 4A) or HMC-1 (Fig 4B)cells. Other groups have also demonstrated an

inability to upregulate expression of FcγRI or FcγRIII on monocytes, neutrophils, eosinophils and mast cells (Liesveld *et al.,* 1988; Okayama *et al.,* 2000).

IFN-γ has long been known to enhance phagocytic function and augment host defence by virtue of its wide spectrum of activity on a variety of immune cells. Not only does IFN-γ affect conventional cellular components of the immune system it also acts on non-professional host defence cells including, among others, endothelial cells, epithelial cells, and platelets (Gallin *et al.,* 1995). IFN-γ has been demonstrated to influence both innate and adaptive immunity (Boehm *et al.,* 1997). It has been found to stimulate innate cell-mediated immunity through activation of natural killer (NK) cells; it stimulates specific cytotoxic immunity through upregulation of major histocompatibility complex (MHC) Class I and II proteins on the surface of cells; and it activates antiviral and microbiocidal activity of macrophages (Bach *et al.,* 1997; Boehm *et al.,* 1997). Furthermore, IFN-γ has also been shown to induce differentiation of CD4+ T cells of the Th0 phenotype into a Th1 phenotype (Gallin *et al.,* 1995). IFN-γ also causes a 5-10 fold increase in the number of FcγRI receptors on monocytes, monocyte derived macrophages, alveolar macrophages, peritoneal macrophages and neutrophils (Arend *et al.,* 1987; Girard *et al.,* 1987; Rossman *et al.,* 1986; Guyre *et al.,* 1993; 1988). IFN-γ is produced mainly by NK, CD4+ Th1 cells and CD8+ T cells in response to a variety of stimuli, including viral and bacterial infection, and production is stimulated by the cytokines IL-12 and IL-18 (Fantuzzi *et al.,* 1999). During severe dengue virus infections many cytokines and chemokines have been found to be elevated in comparison to both healthy controls and patients experiencing less severe forms

of disease. Of interest are the findings of increased serum and plasma levels of IFN-γ and IL-18 observed in patients with DHF suggesting a role for these cytokines in pathogenesis of disease (Mustafa *et al.,* 2001; Green *et al.,* 1999 ; Kurane *et al.,* 1991). While elevated levels of IFN-γ and IL-18 during severe dengue virus infection are not surprising they are perhaps interesting in light of the recent observations that IFN-γ has a profound effect on mast cells (Okayama *et al.,* 2000).

Recently, resting human CD34+ peripheral blood derived mast cells were shown to express minimal mRNA for the high affinity receptor for IgG (FcγRIa) and differentiation with IFN-γ increased both FcγRIa mRNA and protein levels (2.2% to 43.4%) (Okayama *et al.,* 2000). In addition, resting cultured human mast cells were found to exhibit mRNA for FcγRIIA, FcγRIIb1, FcγRIIb2 and FcγRIII but not FcγRIIC. FACS analysis of Fcγ receptors showed no expression of FcγRIII with expression of FcγRII to be approximately 40-44% (Okayama *et al.,* 2001a; 2001b; 2000) and expression was not influenced by IFN-γ treatment. As shown in Figure 2E we have demonstrated a significant portion of our CBMC population expressed all three FcγRs in contrast to CD34+ peripheral blood derived mast cells (Okayama *et al.,* 2000). This difference may relate to differences in culture conditions since there is substantial heterogeneity in human mast cells dependent upon their microenviroment (Irani *et al.,* 1986; Irani & Schwartz, 1994).

FcγRI is mainly expressed on mononuclear phagocytes including monocytes and macrophages. Normal peripheral blood monocytes have been demonstrated to have a range

of 15000 to 40000 FcγRI sites per cell, while macrophages have more than 50000 sites per cell (Fries *et al.*, 1983; Jones *et al.*, 1985; Rossman *et al.*, 1986). In contrast, freshly isolated granulocytes have less than 1000 FcγRI sites per cell (Perussia *et al.*, 1983; Petroni *et al.*, 1988) though this number can be upregulated by treatment with IFN-γ (Petroni *et al.*, 1988). FcγRII has been shown to be expressed on the surface of virtually all hematopoietic cells, including platelets, but not erythrocytes (Fanger *et al.*, 1989; Nichols *et al.*, 1985). FcγRIII has been demonstrated on the surface of human neutrophils in extremely high levels, 100000-200000 sites per cell (Fleit *et al.*, 1982; Petroni *et al.*, 1988). In contrast, human monocytes have low levels (1000-5000) of FcγRIII though human macrophages have higher levels (40000-100000) (Clarkson & Ory, 1988).

We have demonstrated that CBMCs treated with IFN-γ underwent significant upregulation of surface FcγRI and FcγRIII (Fig 4C). Differentiation of CBMCs with IFN-γ for 48 hours resulted in no significant change in percentage of cells in sub-population 1 and 2 (Table 2) indicating that treatment did not induce changes in granularity/complexity. However, significant upregulation (Fig 4D) of the percentage of the cells expressing FcγRI and FcγRIII was observed upon treatment with IFN-γ. In addition, significant increases in FcγRI expression per cell (MFI), in sub-population 1 cells, were observed when cells were differentiated with IFN-γ. Taken together, IFN-γ was demonstrated to both increase the number of mast cells expressing FcγRI and FcγRIII and increase the number of FcγRI sites on the surface of a proportion of the total population of mast cells. No significant upregulation of FcγRII was observed by treatment with IFN-γ for either sub-population 1

or 2 from any of the three subjects. Furthermore, treatment of CBMCs with rhIFN-γ, followed by inoculation with dengue virus/dengue immune serum combinations, resulted in enhanced RANTES production at 24 hours post-inoculation (Table 5). We have previously reported enhanced RANTES production by CBMCs inoculated with dengue virus/dengue immune serum combinations (King *et al.,* 2002). Here we demonstrate that treatment with IFN-γ results in upregulation of surface FcγRs and further augments RANTES production by these cells in response to dengue infection. The observed response was also found to be dependent on live virus-complexed to sub-neutralizing concentrations of dengue specific antibody as UV-inactivated virus alone or in the presence of dengue specific antibody as well as inoculation with dengue virus/normal human non-immune sera failed to induce significant RANTES production in CBMC. The higher levels of RANTES protein observed in this study, when CBMCs were treated with IFN-γ, suggest that upregulation of surface FcγRs may allow for greater virus-antibody complex-cell binding or may reflect priming of the cells by IFN-γ. It is likely that enhanced virus binding would result in a greater number of infected cells and in turn facilitate greater RANTES production.

An alternate explanation for enhanced RANTES production by IFN-γ treated mast cells builds on the observation that the RANTES promoter contains an interferon response element (ISRE) that can be stimulated by type 1 interferons (Cremer *et al.,* 2002) and IFN-γ has been shown to activate the RANTES promoter through interferon regulatory factors (IRFs) (Lee *et al.,* 2000). In addition, a question remains as to the identity of the FcγR responsible for the enhanced RANTES, if a product of enhanced infection, as both FcγRI

and FcγRIII were found to be upregulated by IFN-γ treatment. Additional blocking studies, including the use of mAb IV.3 to block FcγRII as well as blocking FcγRI and FcγRIII on CBMCs, will need to be done in order to determine this. These data suggest that upregulation of FcγRs on CBMCs may be responsible for the enhanced RANTES response although we are unsure of the actual receptor as FcγRI and/or FcγRIII may contribute. Increased surface expression of FcγRIII may contribute to the enhanced RANTES response as FcγRIII has a higher affinity for IgG-immune complexes than FcγRI. Dengue virus has been shown to utilize FcγRI on monocytes (Kontny *et al.,* 1988) and thus the possibility exists that the enhanced RANTES production by IFN-γ treated mast cells is due to enhanced virus binding mediated through FcγRI on the surface of CBMCs. Enhanced cytokine and chemokine production has been observed following aggregation of FcγRI (Okayama *et al.,* 2001a; 2000) but not FcγRII (Okayama *et al.,* 2001b) on the surface of human IFN-γ treated CD34+ progenitor cell derived mast cells.

Furthermore, we have not examined the method of infection through FcγRII. FcRs bind opsonized antigen and can mediate clearance by phagocytosis. The possibility exists that dengue virus could be entering mast cells by phagocyosis rather than the traditional receptor-mediated endocytosis. Several studies have examined phagocytosis of viruses by macrophages and granulocytes and have found that phagocytosis of foot-and-mouth disease virus (Rigden *et al.,* 2002) in the absence of antibody, Fc-receptor-mediated-phagocytosis of influenza virus (Huber *et al.*, 2001; Yamamoto *et al.,* 1997), as well as complement or surfactant protein A mediated-phagocytosis of herpes simplex virus (Van Strijp *et al.,* 1989;

Benne *et al.,* 1997) mediates clearance of virus rather than a productive infection. Therefore it is unlikely that FcγRII-mediated binding and infection of human mast cells occurs through phagocytosis but rather is likely to be the result of receptor-mediated endocytosis. The primary receptor for dengue virus binding and entry is unknown. Several studies have attempted to elucidate this receptor using Vero, CHO, mosquito cell lines and human macrophages. To date several candidates have been identified based on affinity chromatography, immunofluorescence, virus overlay protein-binding assays and Western blotting. These include heparin sulfate (Germi *et al.,* 2002), CD14 (Chen *et al.,* 1999), as well as several unknown proteins of molecular masses of 27, 45, 67 and 87 kDa on the surface of macrophages (Moreno-Altamirano *et al.,* 2002). In addition, on mosquito cell lines, unknown proteins with molecular masses of 45, 67 and 80 kDa (Munoz *et al.,* 1998; Mendoza *et al.,* 2002) have been identified to mediate binding of dengue virus.

The finding that human mast cells support antibody-dependent dengue virus-cell binding supports our previous finding of antibody-enhanced infection in these cells. On KU812 cells we have demonstrated FcγRII dependent binding and infection as well as the subsequent RANTES response. However our data suggest that in primary cultured human mast cells surface FcγR expression encompasses all three classes and IFN-γ treatment can enhance both FcγRI and FcγRIII expression. Such enhanced expression may lead to enhanced binding of virus-antibody immune complexes to the surface of mast cells and facilitate both greater infection and the subsequent RANTES response. Reports that IFN-γ, and the inducer cytokine IL-18, are significantly elevated during severe dengue disease (Green *et al.,* 1999;

Kurane*et al.*, 1991; Mustafa *et al.*, 2001) suggest that mast cells may be exposed to an IFN-γ rich microenviroment. This microenviroment may then augment the mast cell response to dengue virus. The data presented here also provide support for an IgG-mediated mast cell involvement in inflammation recently put forth by another group (Okayama *et al.*, 2000).

4.7 Conclusions

The work presented here details our evidence that FcγRII mediates dengue virus infection of mast cells. Though it should be noted that further experiments must be completed.

Chapter 5 : General Discussion

We have demonstrated antibody-enhanced dengue virus infection of human mast cells. The results were presented in the form of three chapters. The first chapter entitled 'Release of Vasoactive Cytokines by Antibody-Enhanced Dengue Virus Infection of a Human Mast cell/Basophil Line' provided evidence for possible mast cell involvement in pathogenesis of severe dengue disease through production of the vasoactive cytokines IL-1β and IL-6. It was also the first publication to demonstrate infection of human mast cells by any virus. The second chapter entitled 'Dengue virus selectively induces human mast cell chemokine production' provided evidence for selective mast cell chemokine responses to dengue virus but not to RSV or adenovirus. The third chapter entitled 'FcγRII-dependent dengue virus infection of human mast cells' focused on the mechanism of antibody-dependent dengue virus infection of human mast cells and demonstrated that antibody-enhanced dengue virus infection of human mast cells was dependent on FcγRII.

Chapters 2 through 4 provide detailed conclusions and discussions, pertinent to the results presented, that will not be restated in this section. Instead I will provide a discussion aimed at addressing both of our overall scientific objectives. The objectives of the current study were two fold: first, to investigate the potential role of mast cells in contributing to the pathogenesis of severe dengue disease; second, to expand our limited understanding of mast cell function in host defence against viral pathogens.

5.1 Objective 1 - Mast cell involvement in pathogenesis of severe dengue disease

Although many investigators have attempted to elucidate the mechanisms contributing to

severe forms of dengue virus infection the pathogenesis is not completely understood. A number of cytokines and chemokines are found to be elevated in DHF/DSS patients including TNF-α, IL-1, IL-8 and IL-10 (Avirutnan *et al.,* 1998; Chaturvedi *et al.,* 2000; Green *et al.,* 1999; Hober *et al.,* 1993; Kurane *et al.,* 1991) and mast cells are a potential contributing source of all of these cytokines (Benyon *et al.,* 1991; Bradding *et al.,* 1993; Grabbe *et al.,* 1994; Moller *et al.,* 1998; Nilsson *et al.,* 1995; Gordon & Galli, 1990; Plaut, Pierce *et al.,* 1989; Razin *et al.,* 1991; Rumsaeng *et al.,* 1997;Wodnar-Filipowicz *et al.,* 1989). Symptoms of severe disease most often occur in individuals experiencing secondary dengue virus infections (reviewed in Rothman & Ennis, 1999). The presence of heterotypic sub-neutralizing antibodies to dengue virus as a result of primary infection has been shown to potentiate secondary infection via antibody-dependent enhancement (ADE) (Halstead, 1989). Due to the lack of an animal model for DHF/DSS most of the work investigating primary target cells and potential mechanisms of pathogenesis has been done *in vitro.* During antibody-enhanced dengue infection of monocytes (Brinton *et al.,*1998) increased uptake of antibody-virus complexes occurs via Fc receptor mediated binding to cells (Peiris, Gordon *et al.,* 1981), mast cell expression of such receptors also makes them potential targets for infection.

For many years there has been speculation as to the involvement of mast cells in dengue pathogenesis. Dengue patients exhibit increased levels of urinary histamine (a major granule product of mast cells) which correlates with disease severity (Tuchinda *et al.,* 1977). A large study from Thailand, demonstrated that mast cells in the connective tissue around the thymus showed swelling, vacuolation of the cytoplasm as well as loss of granule integrity

(Bhamarapravati *et al.,* 1967) suggestive of mast cell activation. However, the potential role of mast cells had not yet been explored with regard to dengue pathogenesis.

Our laboratory has reported that the human KU812 mast cell/basophil line and the HMC-1 mast cell line are permissive to antibody-enhanced dengue virus infection (King *et al.,* 2000). In addition, we have demonstrated that FcγRII mediated antibody-enhanced dengue virus infection in KU812 cells. Furthermore, antibody-enhanced dengue virus infection results in infectious virus production as well as induction of significant levels of IL-1β and IL-6. In view of the critical role of chemokines in mobilizing effective immunity we extended our work to investigate the production of the key chemokines RANTES, MIP-1α, and MIP-1β by mast cells in response to viral infection. Elevated levels of secreted RANTES, MIP-1α, and MIP-1β, but not IL-8 or ENA-78, were observed following infection of KU812 or HMC-1 cells (King *et al.,* 2002). Surprisingly, in some cases a greater than 200 fold increase in RANTES production was observed in response to dengue virus infection. To extend the relevance of our results we used cord blood derived, cultured human mast cells (CBMCs) as a closer model for *in vivo* human mast cells. In concert with our cell line data, CBMCs treated with dengue virus in the presence of sub-neutralizing concentrations of dengue specific antibody also demonstrated significantly ($p<0.05$) increased RANTES and MIP-1β production, under conditions which did not induce significant short-term degranulation. Furthermore, chemokine responses were not observed when mast cells were treated with UV-inactivated dengue virus in the presence or absence of human dengue specific antibody suggesting the response is dependent on active infection.

5.1.1 Hypothesis and Model

The observation that dengue virus-antibody complexes are much more potent than dengue virus alone in inducing mast cells to produce these chemokines shows an interesting parallel with the known epidemiological evidence that pre-existing immunity is a risk factor for DHF/DSS in human infections (Halstead, 1988). The underlying mechanisms of hemorrhagic disease induced by a number of viruses are poorly understood, including the more severe forms of dengue virus infection, DHF and DSS. DHF and DSS patients are characterized by increased capillary permeability and abnormal homeostasis (Halstead, 1989). Our results strongly indicate that antibody-enhanced dengue virus infection of mast cells and basophils *in vitro* leads to the production of mediators which may contribute to cell recruitment during severe dengue virus infection *in vivo*. The location of mast cells in skin and in close association with blood vessels in most tissues (Selye, 1966) may enhance the effectiveness of this process. Mast cell responses could play a role in host defense or in enhancing the process of vascular damage (see Figure 1). In addition to the well established role of monocytes/macrophages in the pathogenesis of severe forms of dengue disease we postulate that mast cells may become infected with dengue virus. This infection may lead to the production of vasoactive cytokines (IL-1β and IL-6) which are able to directly influence the endothelium and promote perturbation of vascular permeability. Furthermore, mast cell infection may result in substantial chemokine production (RANTES and MIP-1β) that would contribute to the generation of a dengue virus-specific immune response through the recruitment of T cells and monocytes (see Figure 1). This recruitment may also contribute to dissemination of dengue by attracting cells believed to be the primary

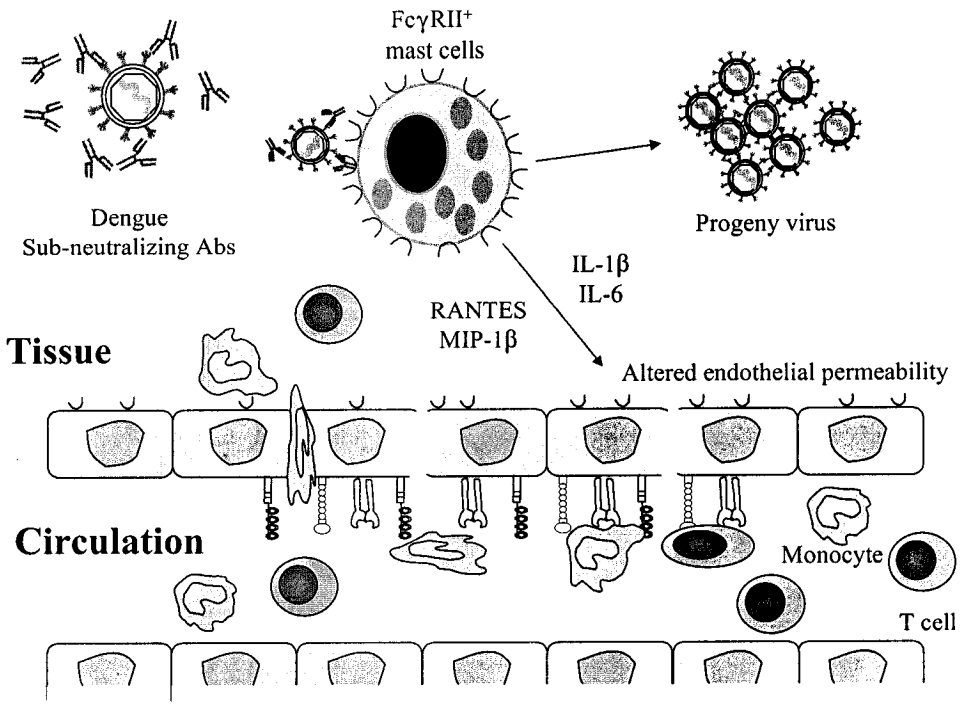


Figure 1. Model of mast cell involvement in pathogenesis of severe dengue virus disease

target of the virus (monocytes/macrophages) to the site of infection thus providing a means for further infection. The location of mast cells in tissues close to blood vessels may enhance the effectiveness of this process.

5.2 Objective 2 - Mast cells in host defence against viral pathogens

Mast cells have been most widely studied in the context of allergic disease but also play a critical role in host defence against bacterial infection, most elegantly demonstrated in studies using mast cell deficient w/w^v mice. There is less data available concerning the role of mast cells in defence against viral pathogens; however, mast cells have been demonstrated to be a potential reservoir of infection for several pathogens, such as HIV-1 and dengue, and capable of producing mediators following challenge with a number of viral products. Traditional mast cell mediators such as histamine, protease enzymes and leukotrienes are important for effective host responses. The cytokines and chemokines produced by mast cells in response to pathogens are known to profoundly alter the nature of the innate immune response and its effectiveness in eliminating infection. Cytokine and chemokine production by mast cells is closely regulated and may occur independently of classical mast cell degranulation. Depending upon the nature of the stimulus or type of infection, a unique profile of cytokines is induced.

There is very clear evidence from rodent models that mast cells play a critical role in host defence against bacterial infection. There are multiple potential mechanisms for mast cell activation and subsequent mediator production. Since the production of cytokines and chemokines in response to bacteria and their products can occur through both degranulation-

dependent and degranulation-independent mechanisms, the lack of overt mast cell degranulation, or lack of modulation by classical mast cell stabilizing agents cannot be taken as an indication that the mast cell does not play an important role in responses to any given organism. Certain cytokines produced by mast cells, such as TNF-α, have received extensive attention in the context of host defence, but others including GM-CSF, IL-1, IL-8 and IL-6 that are produced by human mast cells in response to several bacteria may have equally important roles in host defence and disease.

In the context of viral infection, the role of mast cell derived cytokines and chemokines has only recently received attention. In the context of both HIV-1 and dengue virus infection, however, there is substantial evidence of direct mast cell infection and mast cell cytokine and chemokine responses. For example, the mast cell response to dengue virus appears to be highly selective and specific as neither antibody-enhanced dengue virus infection of the highly permissive U937 monocytic cell line, nor adenovirus infection of mast cells, induced RANTES, MIP-1α, or MIP-1β. These results suggest a role for mast cells in the initiation of chemokine-dependent host responses to viral infection. In addition, if selective chemokine production by mast cells occurred *in vivo* in the absence of mast cell degranulation, as observed in our *in vitro* studies, mast cells could be an important source of such mediators in the absence of classical degranulation. Furthermore, the production of cytokines, such as IFN-γ by T-cells in the context of viral infection may profoundly alter the role of mast cells through modulation of Fc receptor expression and antigen presenting functions as well as mediator release. We have shown IFN-γ treatment of human cord blood derived mast cells upregulated surface expression of FcγRI and FcγRIII and lead to

enhanced RANTES production in response to dengue virus. The role of chemokines such as RANTES produced by mast cells in response to viral infection is currently unclear. In some cases a greater than 200 fold increase in RANTES production was observed in response to dengue virus infection. It is possible such chemokine expression aids in preventing viral entry dependent upon chemokine co-receptors or assists in the recruitment and activation of effector T-cells.

Both bacterial- and viral-induced cytokine and chemokine responses are likely to enhance inflammation at the site of infection. Such an inflammatory response, driven by resident mast cells, may be critical to the pathogenesis of disease associated with chronic or repeated infection such as the chronic lung inflammation associated with *P. aeruginosa* in cystic fibrosis patients or the vascular damage and T-cell activation observed in dengue shock syndrome. There needs to be substantial further work to elucidate the mast cells contribution in such processes. Given the substantial cytokine responses induced and the long lived resident nature of the mast cell as well as their increased numbers at sites of chronic inflammation, such as the synovium in rheumatoid arthritis, we should also consider the possibility that mast cell-pathogen interactions could play a wider role in chronic site-specific inflammatory disease.

5.3 Mast cells: good guys or bad guys

The question remains as to whether mast cells play an important role in the pathogenesis of severe dengue virus infections in comparison to the monocyte/macrophage. Mast cells are found in various concentrations throughout all major tissues and organs. This highly

specialized compartmentalization provides clues as to the role they play *in vivo*. Dengue virus is transmitted to humans by the bite of a mosquito, thereby delivering dengue to the dermal layer of the skin. This area of the skin has an enormous concentration of mast cells and Langerhan's cells, with fewer macrophages. It is possible that, at this site, mast cells, in the presence of sub-neutralizing concentrations of antibody, become infected with dengue. Our studies indicate such infection can result in mediators that greatly influence the endothelium, specifically by promoting activation and increased permeability. It is likely that, in this case, the mast cell is an important contributor to the pathology observed. However, in the circulation, mast cells likely play a less important role in comparison to monocytes which have been shown to support active infection and produce vascular altering mediators. Mast cells do not circulate, though they have been shown to migrate to the lymph node in response to chemotactic signals, while monocytes are one of the major immune cells in circulation. Furthermore, mast cells are known to 'react' to activation, whether by IgE-cross-linking or pathogen interaction, in a extremely rapid manner when compared to other cells. What might take other effector cells 24 hours to make and secrete, takes the mast cell considerably less time. For example, RANTES production and release occurs within 8 hours of dengue infection. Similarly, TNF-α, as well as a range of other mediators, are stored within the granules of mast cells and can be released within 30 minutes of activation, and lipid mediators, such as LTC_4 and PAF, are produced within 20 minutes. This immediate release of mediators from mast cells makes them powerful players.

The production of chemokines by mast cells, that direct the recruitment and activation of both T cells and monocytes, can be considered a double-edged paradigm. The recruitment

of T cells and other effector cells to the site of infection is critical in generating an appropriate and effective immune response. In this way, mast cells are important contributors to generating an effective host defence. Moreover, if mast cells are permissive to other virus infections, as in the case of HIV, and produce such large amounts of mediators like RANTES in response, mast cells might contribute to altering the dynamics of virus infection *in vivo*. To clarify, in the case of HIV, increased RANTES may aid in preventing subsequent virus infection by occupying one of the co-receptors for HIV. In such a case, mast cells would certainly be an important contributor to an effective host defence. Conversely, in the case of secondary dengue infection, recruitment of monocytes/macrophages to the site of infection provides the virus with a large concentration of highly permissive host cells thus enabling enhanced propagation. Increased infection of host cells would lead to more severe infection and likely more severe disease.

Mast cells play both positive roles and damaging roles in the pathogenesis of dengue and host defence against viral pathogens. The overall outcome is dependent on the site, nature of infection and immune status of the host as well as other factors.

5.4 Major limitations/criticisms of the current studies

5.4.1 Inability to show CBMC infection

We have demonstrated antibody-enhanced dengue virus infection of two mast cells lines by both immunoprecipitation and $TCID_{50}$. We have been unable to demonstrate infection in CBMCs though our data suggests a CBMC-dengue-specific response similar to that observed with the cell lines. CBMCs are highly granulated (see Introduction Figure 3) and contain

numerous proteases including tryptase, chymase, and carboxypeptidase. Mast cells continually secrete low levels of proteases into culture media. These proteases make detection of mast cell-produced proteins difficult and may contribute to our inability to detect dengue virus protein by immunoprecipitation. Furthermore, we attempted to use immunofluorescence to detect dengue virus infected CBMCs. This technique worked well for both cell lines but due to high levels of autofluorescence in CBMCs we were unable to detect virus infected cells. Background levels of fluorescence made detection of positives extremely difficult and unreliable. Immunohistochemistry studies performed in Thailand at AFRIMS on CBMCs (data not shown) detected low levels of infection in CBMCs inoculated with dengue and specific antibody. These preliminary data supported our hypothesis that the inability to detect infection may reflect the low levels of dengue virus infection in these cells. We have inoculated our mast cells with a MOI of 0.1-0.3 and this low MOI, coupled with less permissive primary cultured mast cells, would contribute to lower levels of infected cells and thus increased difficulty in detection. Immunohistochemistry studies on CBMCs inoculated with a higher MOI should be undertaken in future studies.

5.4.2 Lack of *in vivo* demonstration

In order to strengthen our hypothesis we should provide evidence for *in vivo* involvement of mast cells. Immunohistochemistry of tissue sections obtained from dengue-infected patients would enable us to investigate if mast cells are infected with dengue virus *in vivo* during the course of infection. We could stain to detect dengue virus proteins to determine if mast cells in these sections are infected with dengue. This might prove more difficult than expected for

a number of reasons including difficulty in obtaining tissue samples, lack of large supply of tissue samples and lack of control tissue.

In addition, a mouse model for dengue virus infection has recently been described (An *et al.,* 1999) which may be useful for studying various aspects of pathogenesis. The authors used severe combined immunodeficient (SCID) mice transplanted with human HepG2 cells, previously shown to be permissive to dengue virus (Marianneau *et al.,* 1996) (Marianneau *et al.,* 1997). The HepG2-grafted mice were infected with dengue 2 virus and the authors demonstrated that the mice developed many features of DHF including thrombocytopenia and increased haematocrit. Virus inoculated i.p. was detected in the serum, liver and brain (An *et al.,* 1999). This mouse model could be employed to study the effects of dengue virus infected mast cells *in vivo* by transplanting dengue virus infected human mast cells or by transplanting human mast cells followed by inoculation with dengue and sub-neutralizing concentrations of dengue specific antibody.

5.4.3 Macrophage/monocyte contribution in CBMC studies

Primary cultured mast cells were generated by culture of human cord blood mononuclear cells using a modification of the method of Saito et al. (Saito *et al.,* 1996) and characterized as previously described (Lin *et al.,* 2000). These cells were consistently positive by flow cytometry for c-kit and CD13 but not CD14, a major monocyte/macrophage surface marker. Purity of each CBMC preparation was assessed by toluidine blue (pH 1.0) staining of cytocentrifuge preparations and examination of cells for the presence of multiple metachromatic granules and appropriate nuclear morphology for every experiment. Only

those mast cell preparations, >95% pure were used for this study. The mean purity of our mast cell preparations used throughout our experiments was approximately 97%. The contaminating cells were assessed by microscopy and found to resemble neutrophils with multi-lobed nuclei and fewer cytoplasmic granules. The high purity of our preparations coupled with the similar observations made using two mast cells lines, KU812 and HMC-1, suggest that the observed responses we have documented were due to mast cell dengue virus infection not as a result of infection of any contaminating cells.

5.5 Future experiments/directions

5.5.1 Apoptosis

During the course of our studies we determined that KU812 cells in cultures were dying post-infection. During the course of infection experiments cell viability was assessed via trypan blue exclusion. The data indicated that in conditions where KU812 cells are infected with dengue virus there was significant death at 48 and 72 hours post-infection as compared to the uninfected and exposed controls; n=6 $p<0.05$ (see Appendix Figure 1). To determine if the death occurring during infection was due to apoptosis, DNA laddering was carried out by Dr. Yan Huang. She has demonstrated quite clearly DNA fragmentation in KU812 cells infected with dengue in the presence of the patient anti-sera; n=1 by 48 hours post-infection (personal communication, Dr. Yan Huang). Confirmation of apoptosis using AnnexinV/propidium iodide double staining techniques and FACS analysis should be carried out in the future. In addition, multi-colored FACS analysis should be carried out to determine if the infected or uninfected cells are undergoing apoptosis. We do not know if the apoptosis is a result of

infection or a response to infection, i.e. are the virus infected cells dying or the uninfected cells dying. Characterization of this event and its specific mechanism would provide useful insights into both dengue virus pathogenic and mast cell responses to viral pathogens.

5.5.2 Source of RANTES

In our system we have two populations of cells, infected and non-infected. It is of interest to determine which population is responsible for the large RANTES chemokine response we have observed. Many avenues are open for the investigation of the source of the RANTES. Initial experiments undertaken involved pulsing CBMC and KU812 cells with supernatants obtained from dengue infected KU812 cells, along with controls, for 2-5 hours. 24 hour supernatants were harvested and examined for RANTES chemokine production. The data from these preliminary experiments were inconclusive (data not shown). Supernatant stimulation experiments are difficult in that RANTES is already present in those supernatants making detection of solely newly synthesized RANTES difficult. Future studies, employing this method, should consider RANTES depletion of original supernatants before stimulation.

Another method to assist in determining if the uninfected or infected cells are responsible for the RANTES protein is the use of Brefeldin A. BFA is a protein-secretion inhibitor that acts on Golgi-mediated vesicular transport. Dr. Marshall's lab has previously demonstrated that BFA has no toxic effects on KU812 cells (Zhu *et al.*, 1998). Pretreatment of mast cells with BFA followed by infection with dengue virus would prevent both virus and any other protein secretion. By RT-PCR viral mRNA and RANTES mRNA could be assessed 8-24 hrs p.i. In theory BFA should not interfere with viral replication and protein synthesis

though assembly and release would be inhibited. If a soluble factor, acting on bystander cells, derived from infected cells is responsible for the RANTES response we should see no upregulation in RANTES expression. If the infected cells were the source of the RANTES then we would expect to see upregulation in RANTES message.

Perhaps the most reliable method involves intracellular double staining of ADE dengue virus infected mast cells detecting both infection using mAb 1B7 specific for the dengue envelope protein and RANTES. This technique would help elucidate if the RANTES response is from the infected cells directly or rather, a result of a unknown 'factor' stimulating bystander uninfected cells. Preliminary experiments carried out using solubilized dengue virus infected and uninfected KU812 cells suggested that both infected and uninfected KU812 cells were the source of the RANTES protein (see Appendix figure 2, Table 1). The data suggests that a soluble factor(s) produced by infected cells is contributing to uninfected/bystander KU812 cell RANTES production. In addition, the findings that infected KU812 cells are also producing RANTES suggests that a viral factor, either directly or indirectly, is stimulating the RANTES response in these cells.

5.5.3 Mechanism of RANTES production

1) The bystander cells are the source of the RANTES

The data suggests that a soluble factor is being released from virus-infected cells and acting on bystander cells to induce RANTES. Characterization of this factor, which may be either a viral product or a product of the host cell, could begin with blocking experiments to a number of factors. Perhaps the RANTES response is due to host cell-bystander cell

interactions, or a cytokine/chemokine produced by the host cell that stimulates RANTES.

NS1, a non-structural protein of dengue virus, is only produced during active infection and is secreted by infected cells. The NS1 protein, through GPI-anchoring, is the only non-structural viral protein to be found on the surface of infected cells (Schlesinger *et al.,* 1990). Furthermore, this form of NS1 is capable of signal transduction in response to binding of specific antibody (Jacobs *et al.,* 2000). In addition, NS1 is also secreted in a hexameric form (Flamand *et al.,* 1999) from infected cells. Perhaps this protein is acting on bystander cells to induce the RANTES response? In addition, blocking of known inducers of RANTES including IL-1β and IL-6, as described in Chapter 3, both of which are produced in our infected cultures (King *et al.,* 2002) suggests that these cytokines are not responsible for the RANTES response.

2) The infected cells are the source of the RANTES

Preliminary data suggests that dengue virus-infected cells are a source of the RANTES response. It would be interesting to determine the viral factors involved in this response. Dengue virus encodes 3 structural proteins and 7 non-structural proteins, the functions of which have not been fully characterized. Two of these proteins, the C (core) and NS5 (RdRp) have been shown to localize to the nucleus where their function is unknown (Bulich & Aaskov, 1992). Forwood *et al.,* (Forwood *et al.,* 1999) demonstrated that the NS5 protein contains a nuclear localization signal (NLS) that is capable of targeting NS5 to the nucleus and is recognized by nucleus import factors including importin-β. The reason for NS5

localization into the nucleus remains a mystery though modulation of host gene expression would not be unheard of. These nucleus-localizing proteins are strong candidates for a role in modulating host gene expression and would be a good place to start.

There are two approaches we could take: A. Many infectious cDNA clones of dengue are available, including (Kinney *et al.,* 1997), and by using a clone we would be able to examine what viral protein is responsible for the observed effect. Deletion of individual genes would allow us to examine their role in the RANTES response. This would be advantageous in that it is possible that more than one gene is acting to increase RANTES. B. Alternately cloning and expression of individual viral proteins could be undertaken. All ten viral genes could be obtained by RT-PCR amplification from purified dengue viral RNA. The dengue virus genome contains only one translation initiation site but individual proteins can be cloned and expressed if the necessary ATG codon is added. There are many cases when the two or more proteins act cooperatively the corresponding genes could be amplified in tandem. We could begin by expressing those proteins that localize to the nucleus, individually or in combination. Transient expression in KU812 cells could be used to assess activation of RANTES. Transfected cells would be assayed for viral protein expression by IP and RANTES secretion in culture supernatants.

Conversely, the possibility exists that a viral protein is not directly responsible for the observed increase in RANTES but rather acts through an indirect method. Perhaps a viral protein(s) act on another factor upstream of the RANTES gene to influence expression. To investigate we could begin by examining the regulation of RANTES expression by first using broad inhibitors of signaling pathways/transcription and followed by more specific inhibitors.

This would provide a general idea of the pathway involved in our system. Preliminary experiments were undertaken to examine the calcium dependence of RANTES production in viral-infected cells. Cells were incubated with RPMI without addition of Ca++ and in the presence of EDTA (to chelate any extracellular Ca++ released during culture). To examine the dependence on PKC, cells were depleted of PKC by incubation with PMA for 18 hr pre-infection. The use of a G-protein coupled receptors was assessed by pre-treatment and post-treatment with Pertussis Toxin and dependence on a protein tyrosine kinase was investigated using cells pre-treated and post-treated with Genistein. The preliminary data suggests that the response is PT sensitive and Ca++ dependent, n=1 (data not shown). In addition, IL-8 was used as a control as it is not modulated by any condition (King *et al.*, 2002) but constitutively produced by KU812 cells. Little or no modulation was observed in IL-8 with any of these conditions suggesting that the cells were alive and capable of making chemokines. This must be repeated to confirm the observation. In parallel, confirmation of dengue virus infection of KU812 cultures must be carried out to exclude the possibility that the lack of the RANTES response observed was not due to lack of infection but rather specific inhibition. The possibility exists that the treatments influenced infection and that is why we saw a modulation in RANTES. If the observation is confirmed a more in-depth analysis of the signaling events should be undertaken.

5.5.4 Additional mediators/responses of mast cells to dengue virus infection

We have demonstrated interesting mast cell mediator responses to dengue virus. Our studies focussed on obvious cytokines and chemokines that could be involved in pathogenesis of

severe dengue disease though the full scope of mast cell responses to activation are many. It would be interesting to delve deeper into the mast cell response by looking at a broader number of mediators. For example, DNA array is an extremely useful tool for examining upregulation of gene expression. This method provides tremendous amounts of data and could be used for screening purposes. Comparing uninfected and dengue-infected mast cells by DNA array would provide a substantial amount of information. The RANTES response we have observed is quite striking with a more than 200 fold increase in response to infection. The possibility exists that, in comparison to another response, this striking increase is actually quite small.

Investigation of leukotriene responses, that have been shown to be very important in the pathogenesis of asthma, may provide additional evidence for a mast cell role in pathogenesis of severe dengue virus disease. LTC_4 is known to enhance vascular permeability with 10-1000 times more potency than histamine (Drazen & Austen, 1987) and could contribute to the enhanced vascular permeability observed during disease. In addition, platelet activation factor (PAF), another mast cell lipid-derived mediator, has been shown to aggregate and degranulate human platelets and lead to systemic hypotension (Demopoulos *et al.,* 1979).

5.6 Concluding remarks

We have demonstrated that mast cells are permissive to dengue virus infection and that such infection results in the production of specific cytokines and chemokines. These cytokines and chemokines have the potential to influence endothelial cell permeability and effector/host cell

migration to the site of infection. The mechanisms of pathogenesis of severe dengue virus infections remains incomplete. Our work suggest a potential role for cells, in addition to monocytes/macrophages, of the hemotopoeitic lineage in this pathogenesis. The work presented here should stress the need for additional studies on cells and systems other than the well described monocyte and macrophage. The complexity of dengue virus infections and resulting pathogenesis underlies the importance of a broadening of the field of study.

Furthermore, mast cells are truly unique cells and our work supports the newly emerging field addressing the role for mast cells in host defence, not only against bacterial pathogens, but also viral pathogens. Much work remains to be done to understand the full scope of the mast cell's varied and essential involvement in innate immunity.

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Appendix

Figure 1. Increased cell death in antibody-enhanced dengue virus infected KU812 cells by 48 hours post-infection. KU812 cells were inoculated with virus/antibody combinations, cultured in media alone, or stimulated with PMA and A23187. Cell viability was assessed by trypan blue exclusion at 24 , 48 and 72 hours post-infection. Data are expressed as Mean ± SEM, n=6. Statistical analysis consisted of Anova followed by students paired t test. * $p< 0.05$, *** $p<0.001$.

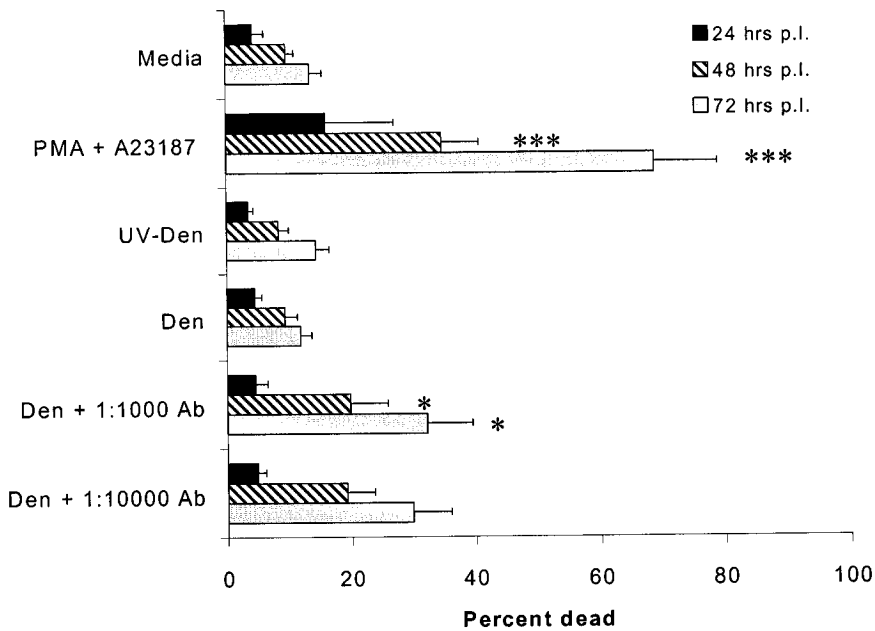
24 hrs p.l.
48 hrs p.l.
72 hrs p.l.
Media
PMA + A23187
UV-Den
Den
Den + 1:1000 Ab
Den + 1:10000 Ab
***
***
*
*
0
20
40
60
80
100
Percent dead

Figure 1

Figure 2. Bystander and infected KU812 cell RANTES production. KU812 cells were mock inoculated or inoculated with dengue virus immune serum combinations and cultured for 24 hrs. 24 hrs p.i. cultures were harvested, fixed and permeabilized with saponin for 1 hr. Intracellular FACS analysis was performed using mouse anti-dengue mAb 1B7 specific for the dengue envelope protein and goat anti- RANTES biotinylated mAb BFA 278 specific for RANTES protein. Secondary antibodies used for detection included rat anti-mouse Ig-FITC conjugated for detection of dengue positive cells, and streptavidin-APC conjugated for detection of RANTES.

Figure 2

Table 1. Bystander and infected KU812 cell RANTES production.

		Percent positive		
Expt	Condition	RANTES	Dengue	Double
1	Den + NHS	1.29	0.54	0.10
	Den + 1:1000 Ab	17.17	3.83	2.23
	Den + 1:10000 Ab	13.17	3.09	1.31
2	Den + NHS	0.79	0.43	0.29
	Den + 1:1000 Ab	13.74	6.95	3.60
	Den + 1:10000 Ab	8.79	7.49	2.33
3	Den + NHS	3.61	0.55	0.48
	Den + 1:1000 Ab	10.90	9.62	3.77
	Den + 1:10000 Ab	9.03	10.77	4.17